(54) Titre : BACTERIES CONSOMMANT DE L'ACIDE LACTIQUE ET LEUR UTILISATION THERAPEUTIQUE
(54) Title: LACTIC ACID UTILISING BACTERIA AND THEIR THERAPEUTIC USE

(57) Abrégé/Abstract:
There is provided a method of isolating novel lactic acid utilising bacteria from human faeces, as well as novel strains so obtained. The use of the novel lactic acid utilising bacteria in therapy, including prophylactic therapy, is described and is of particular relevance for lactic-acidosis, short bowel syndrome and inflammatory bowel disorders such as Crohn's disease and ulcerative colitis. A probiotic comprising the live lactic acid utilising bacteria is also described.
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Abstract: There is provided a method of isolating novel lactic acid utilising bacteria from human faeces, as well as novel strains so obtained. The use of the novel lactic acid utilising bacteria in therapy, including prophylactic therapy, is described and is of particular relevance for lactic-acidosis, short bowel syndrome and inflammatory bowel disorders such as Crohn’s disease and ulcerative colitis. A probiotic comprising the live lactic acid utilising bacteria is also described.
Information about Correction:

see PCT Gazette No. 49/2004 of 2 December 2004, Section II

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.
Lactic Acid Utilising Bacteria and
their Therapeutic Use

This invention relates to improvements in health and
nutrition for both animals and humans following the
ingestion of specific bacteria capable of utilising
lactic acid.

Under normal conditions the concentration of lactic acid
(lactate) in the mammalian gut is very low despite the
fact that many bacterial species, such as lactobacilli,
streptococci, enterococci and bifidobacteria that reside
in the intestine produce this acid in large quantities as
a fermentation end product. Lactic acid is also produced
by host tissues.

It has been hypothesised that the accumulation of lactic
acid is normally prevented by the ability of certain
other bacteria that inhabit the gut to consume lactic
acid and to use it as a source of energy. The identity
of the micro-organisms that are postulated to conduct
this metabolic process in the mammalian large intestine
has largely not previously been elucidated, Bourriaud et
al (2002). Kanauchi et al (1999) revealed that a strain of *Bifidobacterium longum* was co-incubated with a strain of *Eubacterium limosum* on germinated barley feedstuff for three days there was a marked increase in acetate formed and a small increase (less than 3 mM) in butyrate formed when compared to the incubations with *E. limosum* alone.

In the rumen of cattle and sheep the species *Selenomonas ruminantium, Veillonella parvula* and *Megasphaera elsdenii* are regarded as the most numerous utilisers of lactate (Gilmour et al., 1994; Wiryawan and Brooker, 1995). The contribution of *Megasphaera elsdenii* appears to be particularly significant in the rumen, based on the high proportion of carbon flow from lactic acid to propionic acid and this species employs the acrylate pathway for this purpose (Counotte et al., 1981). *Megasphaera elsdenii* produces a variety of end products including propionate, butyrate, caproate and branched chain fatty acids from lactate — see Ushida et al (2002), Kung and Hession, (1995). This probably reflects the ability of this species to use lactate despite the presence of other carbon sources such as sugars, whereas *Selenomonas* uses lactic acid only in the absence of other energy sources. This has led to interest in the use of *Megasphaera* as a probiotic organism that might be added to animal (Kung and Hession, 1995; Ouwerkerk et al., 2002), or even human diets to prevent the harmful accumulation of lactic acid. In ruminant animals (cattle and sheep) accumulation of lactic acid occurs when a large amount of readily fermentable substrate (such as starch and sugars) enters the rumen. Rapid fermentation, particularly by organisms such as *Streptococcus bovis*, drives down the pH, creating
more favourable conditions for the proliferation of
lactic acid producing bacteria such as lactobacilli, and
S. bovis itself. Normal populations of bacteria capable
of utilising lactate (lactate utilisers) are unable to
cope with the greatly increased production of lactic
acid. Unaided, lactic acid may accumulate to levels that
can cause acute toxicity, laminitis and death (Nocek,
1997; Russell and Rychlik, 2001).

Similar events occurring in the large intestine can also
cause severe digestive and health problems in other
animals, for example in the horse where high lactate
levels and colic can result from feeding certain diets.

In humans lactic acid accumulation is associated with
surgical removal of portions of the small and large
intestine, and with gut disorders such as ulcerative
colitis and short bowel syndrome (Day and Abbott, 1999).
High concentrations of lactic acid in the bloodstream can
cause toxicity (Hove et al., 1994), including
neurological symptoms (Chan et al., 1994). Much of this
lactic acid is assumed to derive from bacterial
fermentation, particularly by bifidobacteria and by
lactobacilli and enterococci. Lactic acid can also be
produced by host tissues, but the relative contributions
of bacterial and host sources are at present unclear.

Conversely, the formation of other acid products, in
particular butyric acid (butyrate), is considered to be
beneficial as butyric acid provides a preferred energy
source for the cells lining the large intestine and has
anti-inflammatory effects (Inan et al., 2001, Pryde et
al., 2002). Butyrate also helps to protect against
colorectal cancer and colitis (Archer et al., 1998;
Csordas, 1996).

We have now established a method of isolating novel
bacteria that are remarkably active in consuming lactic
acid. The bacteria have been isolated from human faeces.
Preferably the method allows isolation of bacteria which
convert the lactic acid to butyric acid. According to
this method several new bacteria that are remarkably
active in converting lactic acid to butyric acid have
been isolated.

One group of these bacteria is from the newly described
genus Anaerostipes caccae (Schwertz et al., 2002).
Although some main characteristics of A. caccae are
described in this publication, its ability to use lactate
was not reported and has only recently been recognised as
described herein.

The invention relates to a method for selecting a strain
of lactic acid-utilising bacteria, which method comprises
the steps of:

a) providing (for example isolating) a bacterial
culture from a human faecal sample;

b) selecting a single colony of bacteria;

c) growing said colony in a suitable medium
containing lactic acid; and

d) selecting a strain of bacteria consuming
relatively large amounts of lactic acid, all of
the above steps being conducted under anaerobic
conditions.
In the above method, the reference to "relatively large amounts of lactic acid" is defined as meaning the bacteria used at least 10 mM of D, L or DL lactic acid during growth into stationary phase, per 24 hours at 37°C in YCFALG or YCFAL medium.

Preferably the strain of lactic acid utilising bacteria also produces high level of butyric acid and the method of the invention may therefore comprise an additional step of:

e) selecting a strain of bacteria producing relatively large quantities of butyric acid.

In the above step the reference to "relatively large quantities of butyric acid" is defined as meaning the bacteria produces at least 10 mM of butyric acid during growth into stationary phase, per 24 hours at 37°C in YCFALG or YCFAL medium.

Preferably the strain of lactic acid utilising bacterium must be capable of converting lactate produced by another gut bacterium from dietary components such as resistant starch.

Preferably the lactic acid used in step c) is both D- and L- isomers of lactic acid.

Preferably the suitable medium to grow bacteria is nutritionally rich medium in anaerobic Hungate tubes.

Preferably the selected strain of bacteria is re-purified using nutritionally rich medium in anaerobic roll tubes.
A further aspect of the invention is a bacterial strain that produces butyric acid as its sole or predominant fermentation product from lactate and which has been isolated according to the method of the invention described above. Such novel bacterial strains include:

the bacteria *Anaerostipes caccae* strain L1-92 deposited at NCIMB (National Collections of Industrial, Marine and Food Bacteria in Aberdeen, United Kingdom) under No 13801\(^T\) on 4 November 2002 and at DSM under No 14662 on 4 November 2002.

the *Clostridium indolis* bacterial strain Ss2/1 deposited at NCIMB under No 41156 on 13 February 2003;

the bacteria strain SM 6/1 of *Eubacterium hallii* deposited at NCIMB under No. 41155 on 13 February 2003.

Another aspect of the invention is a strain of bacteria having a 16S rRNA gene sequence which has at least 95% homology to one of the sequences shown in Figure 1, preferably 97% homology (i.e. differs at less than 3% of residues out of approximately 1400 from one of the sequences shown in Figure 1).

Another aspect of the invention is the use of at least one of the above-mentioned bacterial strains in a medicament or foodstuff.

Another aspect of the invention is a method to promote butyric acid formation in the intestine of a mammal, said method comprising the administration of a therapeutically
effective dose of at least one of the above described
strains of live butyric acid producing bacteria. The
bacterial strain may be administered by means of a
foodstuff or suppository or any other suitable method.

Another aspect of the invention is a method for treating
diseases associated with a high dosage of lactic acid
such as lactic-acidosis, short bowel syndrome and
inflammatory bowel disease, including ulcerative colitis
and Crohn's disease, which method comprises the
administration of a therapeutically effective dose of
Anaerostipes caccae or at least one above-mentioned
strains of live lactic acid utilising bacteria.
Advantageously the strain selected may also produce a
high level of butyric acid.

Further, another aspect of the invention is a
prophylactic method to reduce the incidence or severity
of colorectal cancer or colitis in mammals caused in part
by high lactic acid and low butyric acid concentrations,
which method comprises the administration of a
therapeutically effective dose of at least one above
identified strains of live lactic acid utilising bacteria
and/or butyric acid producing bacteria mentioned above or
of Anaerostipes caccae.

Another aspect of the invention is the use of live
Anaerostipes caccae or at least one of the above
mentioned lactic acid utilising bacteria as a medicament.
Advantageously the strain chosen may produce butyric acid
as its sole or predominant fermentation product from
lactate. Preferably the bacteria are used in the
treatment of diseases associated with high levels of
lactic acid such as lactic acidosis, short bowel syndrome
and inflammatory bowel disease including ulcerative
colitis and Crohn's disease.

According to another aspect of the invention at least one
lactate-utilising strain of bacteria as mentioned above
or Anaerostipes caccae are used in combination with
lactic acid producing bacteria including those such as
Lactobacillus spp. and Bifidobacterium spp. or other
additives or growth enhancing supplement currently used
as probiotics.

The combination of strains would potentially enhance the
health-promoting benefits of the lactic acid bacterium by
converting its fermentation products (lactic acid alone
or lactic acid plus acetic acid) into butyrate. Indeed
it is possible that certain health-promoting properties
currently ascribed to lactic acid bacteria might actually
be due to stimulation of other species such as lactate-
consumers in vivo, particularly where probiotic
approaches (see below) are used to boost native
populations in the gut. Furthermore the presence of the
lactic acid producing bacteria in a combined inoculum
could help to protect the lactate consumer against oxygen
prior to ingestion.

The growth and activity of the novel bacteria may be
promoted by means of providing certain growth
requirements, required for optimal growth and enzyme
eexpression to the bacteria, present in the animal or
human gastrointestinal tract. These bacterial growth
enhancing nutrients are often referred to as prebiotics
or synbiotics. Thus the invention provides methods to promote the growth
and enzyme expression of the micro-organism and hence
removal of lactate and production of butyrate in vivo,
for example, via a prebiotic or symbiotic approach
(Collins and Gibson, 1999).

Another aspect of the invention is a method for treating
acidosis and colic in animals, particularly in ruminants
and horses or other farm animals, by administration of a
therapeutically effective dose of Anaerostipes caccae or
at least one of the lactate utilising bacteria mentioned
above. Advantageously the bacteria can be administrated
as feed additives.

For the use, prevention or treatment of conditions
described herein, the bacteria or prebiotic(s) or
symbiotic(s) are preferentially delivered to the site of
action in the gastro-intestinal tract by oral or rectal
administration in any appropriate formulae or carrier or
excipient or diluent or stabiliser. Such modes of
delivery may be of any formulation included but not
limited to solid formulations such as tablets or
capsules; liquid solutions such as yoghurts or drinks or
suspensions. Ideally, the delivery mechanism delivers
the bacteria or prebiotic or symbiotic without harm
through the acid environment of the stomach and through
the rumen to the site of action within the gastro-
intestinal tract.
Another aspect of the invention is the use of at least one bacterial strain mentioned above or *Anaerostipes caccae* in a method to produce butyric acid from lactate and acetate. The method includes the fermentation of the above described microorganism selected for both their lactic acid utilising and butyric acid producing abilities in a medium rich in lactate and acetate. The method can be used in industrial processes for the production of butyrate on a large scale.

**Brief description of the Figure**

**Figure 1:** Sequence information of 16S rRNA for five lactic acid utilising strains.

**Figure 2:** Co-culture experiment. Concentration of SCFA are shown after 24 hours growth in YCFA medium with 0.2% starch as energy source (values for acetate, initially present in the medium, are shown on a 10 fold reduced scale). Butyrate production by *A. caccae* L1-92, and by *E. hallii* L2-7 and SM 6/1, is stimulated by co-culture with *B. adoloscentis* L2-32, while L-lactate disappears from the co-cultures.

**Figure 3:** SCFA formation and lactate utilisation for new and existing isolates. Acids produced or consumed during anaerobic growth are shown for strains incubated for 24 hours: a) YCFA medium containing 35mM DL lactate (YCFAL); b) YCFA medium containing 10mM glucose and 35mM DL lactate (YCFALG); c) YCFA medium with no addition. Carbon recoveries (%) for growth on lactate, and lactate plus glucose, respectively, were as follows: SM 6/1
(94.6, 76.4); SL 6/1/1 (100.2, 78.7); L1-92 (96.2, 97.9);
SS2/1 (92.1, 90.1); SSC/2 (104.4, 96.9); SR1/1 (103,
93.8). This suggests that there may be unidentified
fermentation products in the case of SM 6/1, SL 6/1/1 and
SS3/4 when grown on glucose plus lactate.

Figure 4: Time course of SCFA formation and growth in
batch culture of E. hallii L2-7 on media containing DL
lactate, glucose, or DL lactate plus glucose.

Figure 5: Time course of SCFA formation and growth in
batch culture of strain SS2/1 on media containing DL
lactate, glucose, or DL lactate plus glucose.

DETAILED DESCRIPTION

The experimental work performed shows the following:

1. Certain human colonic anaerobic bacteria, including
   A. caccae strains, are strong and efficient
   utilisers of lactic acid.

2. Certain human colonic anaerobic bacteria, including
   A. caccae strains, are strong and efficient
   producers of butyric acid.

3. Certain human colonic anaerobic bacteria, including
   A. caccae strains, convert lactic acid to butyric
   acid.
Example 1: Isolation and characterisation of bacteria

A faecal sample was obtained from a healthy adult female volunteer that had not received antibiotics in the previous 6 months. Whole stools were collected, and 1g was mixed in 9ml anaerobic M2 diluent. Decimal serial anaerobic dilutions were prepared and 0.5ml inoculated into roll tubes by the Hungate technique, under 100% CO₂ (Byrant, 1972).

Bacterial strains were isolated by selection as single colonies from the nutritionally rich medium in anaerobic roll tubes as described by Barcenilla et al. (2000). The isolates were grown in M2GSC broth and the fermentation end products determined. Butyrate producing bacteria were re-purified using roll tubes as described above. Strains L1-92, S D8/3, S D7/11, A2-165, A2-181, A2-183, L2-50 and L2-7 were all isolated using this medium.

Omitting rumen fluid and/or replacing the sugars with one additional carbon source such as DL lactate increased the selectivity of the roll tube medium and this medium was used to isolate strain S D6 1L/1. Strains G 2M/1 and SM 6/1 were isolated from medium where DL-lactate was replaced with mannitol (0.5%). Separately, non-rumen fluid based media routinely used for isolating Selenomonas sp., namely Ss and Sr medium (Atlas, 1997) was used to isolate other strains. Inoculating Sr medium roll tubes with dilutions of faecal samples resulted in the isolation of strain Sr1/1 while the Ss medium resulted in the isolation of strains Ss2/1, Ss3/4 and Ssc/2.
Example 2: *A. caccae* and other human colonic bacterial isolates consumes lactic acid and acetic acid and produces butyric acid when grown in rumen fluid

Table 1 summarises the fermentation products formed by twelve strains of anaerobic bacteria when grown under 100% CO₂ in a rumen fluid-containing medium containing 0.5% lactate (M2L) or 0.5% lactate, 0.2% starch, 0.2% cellobiose and 0.2% glucose (M2GSCL) as the energy sources. Ten of these strains were isolated from human faeces as described above in Example 1. Strains 2221 and NCIMB8052 are stock collection isolates not from the human gut and are included for comparison. Table 1 demonstrates that three strains, L1-92 (*A. caccae*), SD6 1L/1 and SD 6M/1 (both *E. hallii*-related) all consumed large amounts of lactate (>20mM) on both media examined, M2L and M2GSCL, and produced large quantities of butyric acid. *A. caccae* L1-92 in particular consumed large amounts of lactate and produced large amounts of butyrate. Acetate is also consumed by all three strains. The other 9 butyrate producing bacteria tested either consumed relatively small amounts of lactate, or consumed no lactate, on this medium. L-lactate concentrations were determined enzymatically and glucose concentrations were determined by the glucose oxidase method (Trinder, 1969). Analyses were conducted in a robotic clinical analyser (Kone analyser, Konelab Corporation, Finland).
Table 1. Comparison of human faecal isolates for the ability to utilise (negative values) or produce (positive values) lactate on a rumen fluid based medium (M2) supplemented with lactate (M2L) and lactate plus glucose, cellobiose and soluble starch (0.2% each) (M2GSC).

<table>
<thead>
<tr>
<th>Strain ID</th>
<th>Closest relative</th>
<th>Medium</th>
<th>Formate</th>
<th>Acetate</th>
<th>Butyrate</th>
<th>Lactate</th>
</tr>
</thead>
<tbody>
<tr>
<td>S D8/3</td>
<td>Adhufec 406*+</td>
<td>M2L</td>
<td>1.15</td>
<td>0.97</td>
<td></td>
<td>-3.94</td>
</tr>
<tr>
<td>S D8/3</td>
<td></td>
<td>M2GSC</td>
<td>21.66</td>
<td>0.77</td>
<td>10.88</td>
<td>6.43</td>
</tr>
<tr>
<td>SL 6/1/1</td>
<td>E. hallii</td>
<td>M2L</td>
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<td></td>
<td>35.48</td>
<td>-32.41</td>
</tr>
<tr>
<td>SL 6/1/1</td>
<td></td>
<td>M2GSC</td>
<td>-9.78</td>
<td></td>
<td>22.58</td>
<td>-21.85</td>
</tr>
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<td>E. hallii</td>
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<td>-23.72</td>
</tr>
<tr>
<td>SM 6/1</td>
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<td>-5.06</td>
<td>22.77</td>
<td>-28.42</td>
</tr>
<tr>
<td>G 2M/1</td>
<td>HucA19*</td>
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<td></td>
<td>7.97</td>
<td>23.66</td>
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<tr>
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<td></td>
<td>12.94</td>
<td>9.52</td>
</tr>
<tr>
<td>S D7 11/1</td>
<td>ND*</td>
<td>M2L</td>
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<td></td>
<td>0.08</td>
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<td>2221</td>
<td>But. Fibrisolvens</td>
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<td>8052</td>
<td>Cl. acetobutylicum</td>
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<td>Butyrate</td>
<td>Lactate</td>
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<tr>
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<tr>
<td>A2-165</td>
<td>F. prausnitzii</td>
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<tr>
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<td>-12.70</td>
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<tr>
<td>A2-181</td>
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<td>M2GSCL</td>
<td>0.33</td>
<td>-11.05</td>
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<tr>
<td>L2-50</td>
<td>Coprococcus sp.</td>
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<td>1.06</td>
<td>2.32</td>
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</table>

* clone library sequence, uncultured (Hold et al., 2002)
+ clone library sequence, uncultured (Suau et al., 1999)
* ND not determined
Example 3: A. caccae and other human colonic bacterial isolates consumes lactic acid and acetic acid and produces butyric acid when grown in rumen fluid free medium.

Table 2a shows the utilisation and production of formate, acetate, butyrate, succinate and lactate, on this occasion performed using the rumen fluid-free medium YCFA (Duncan et al. 2002) containing no added energy source, or with 32 mM lactate (YCFAL) or lactate plus 23 mM glucose (YCFALG) as added energy sources. Separately Table 2b reveals the levels of the two isomers of lactate (D and L) remaining at the end of the incubations and the concentration of glucose metabolised during the incubations. Five additional new lactate-utilising isolates were discovered using the semi-selective medium as described earlier and are included in Tables 2a and 2b, although one of these (Ss 3/4) proved to consume a relatively small amount of lactate only on the YCFAL medium (Table 2a). Analysis of the consumption of the D and L isomers reveals that three strains (Ss2/1, Ssc/2 and Sr1/1) preferentially consumed D lactate. Partial repression of lactate consumption by glucose was observed on this medium with A. caccae L1-92, and almost complete repression for SL 6/1/1 and Ss 3/4. The previously isolated E. hallii strain L2-7 (Barcenilla et al., 2000) behaved in a similar manner to SL 6/1/1. The higher glucose concentration in this medium compared with M2GSL is likely to explain the difference in behaviour of A. caccae compared with Table 1. The remaining five strains showed no evidence of repression of lactate utilisation in the presence of glucose although it is possible they
use the glucose before switching to lactate. Butyrate levels exceeding 30mM were obtained for four strains on YCFALG medium.

Results: The three E. hallii-related strains (L-27, SL 6/1/1, SM 6/1) and the two A. caccae strains (L1-92 and P2) were able to use both the D and L isomers of lactate during growth either on DL lactate or DL lactate plus glucose (Fig. 3). The four remaining new isolates SR1/1, SSC/2, SS2/1 and SS3/4 however showed a strong preference for using D-lactate. In most strains, except SS3/4 and L2-7, some utilisation of lactate was detectable following 24 hours incubation even when glucose was initially present in the medium (Fig. 3).

Table 2a. Fermentation products formed or utilised (U as indicated by minus values) by human gut isolates incubated on yeast extract-casitone-fatty acids medium (YCFA); YCFA supplemented with lactate (YCFA-L); and YCFA supplemented with glucose and lactate (YCFA-LG). The initial concentration of glucose added to the medium was 23 mM and 32 mM lactate was added that contained 15.5 mM L-lactate.

Strain identity is based on 16S rRNA sequence information (% identical residues with closest relative is shown). See Figure 1 for sequence information.
All strains except 2221 and 8052 (Table 1) were isolated as described in Example 1.

Table 2a

<table>
<thead>
<tr>
<th>Strain ID</th>
<th>Closest relative(^a)</th>
<th>Isolation Medium</th>
<th>Medium</th>
<th>Formate</th>
<th>Acetate P/U</th>
<th>Butyrate</th>
<th>Succin</th>
<th>Lactate P/U</th>
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<td>Ss2/1</td>
<td>Cl. indolis (95%)</td>
<td>Selenomonas selective</td>
<td>YCFA</td>
<td>0.02±0.04</td>
<td>-4.25±4.68</td>
<td>2.24±0.26</td>
<td></td>
<td>0.39±0.03</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>YCFAL</td>
<td>0.18±0.02</td>
<td>-12.51±1.27</td>
<td>12.98±0.19</td>
<td>-15.27±2.53</td>
<td></td>
</tr>
<tr>
<td>Sr 1/1</td>
<td>Ruminococcus obeum</td>
<td>Selenomonas ruminantium</td>
<td>YCFA</td>
<td>-5.42±1.77</td>
<td>-5.42±1.77</td>
<td>2.33±0.03</td>
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<td>0.36±0.12</td>
</tr>
<tr>
<td></td>
<td>HucB 12*</td>
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<td>YCFAL</td>
<td>0.76±0.19</td>
<td>-13.35±2.27</td>
<td>14.15±0.17</td>
<td>-15.04±0.89</td>
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</tr>
<tr>
<td></td>
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<td></td>
<td>YCFALG</td>
<td>9.53±2.03</td>
<td>-22.47±1.40</td>
<td>35.77±1.50</td>
<td>-13.71±0.40</td>
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</tr>
<tr>
<td>SL 6/1/1</td>
<td>E. hallii</td>
<td>M2 + 0.5% lactate</td>
<td>YCFA</td>
<td>-4.96±3.26</td>
<td>-4.96±3.26</td>
<td>1.42±0.23</td>
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<tr>
<td></td>
<td>HucA 15*</td>
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<td>YCFAL</td>
<td>-18.51±0.96</td>
<td>-18.51±0.96</td>
<td>21.06±1.06</td>
<td>-29.93±0.60</td>
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</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>YCFALG</td>
<td>-9.22±2.52</td>
<td>-9.22±2.52</td>
<td>20.78±1.52</td>
<td>-2.43±0.70</td>
<td></td>
</tr>
<tr>
<td>SM 6/1</td>
<td>E. hallii (98%)</td>
<td>M2 + 0.5% mannitol</td>
<td>YCFA</td>
<td>0.09±0.03</td>
<td>-2.61±2.36</td>
<td>1.42±0.05</td>
<td></td>
<td>-6.27±1.27</td>
</tr>
<tr>
<td></td>
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<td>YCFAL</td>
<td>0.21±0.1</td>
<td>-7.20±2.08</td>
<td>6.54±0.43</td>
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</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>YCFALG</td>
<td>20.68±</td>
<td>-10.95±</td>
<td>29.2±</td>
<td>-25.82±</td>
<td></td>
</tr>
<tr>
<td>Ss 3/4</td>
<td>Ruminococcus gnavus</td>
<td>Selenomonas selective</td>
<td>YCFA</td>
<td>4.75±2.20</td>
<td>4.75±2.20</td>
<td>6.10±0.27</td>
<td></td>
<td>1.09±0.47</td>
</tr>
<tr>
<td></td>
<td>HucA19*</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Strain ID</td>
<td>Closest relative*</td>
<td>Isolation Medium</td>
<td>Medium</td>
<td>Formate</td>
<td>Acetate P/U</td>
<td>Butyrate</td>
<td>Succin</td>
<td>Lactate P/U</td>
</tr>
<tr>
<td>----------</td>
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<td>--------</td>
<td>---------</td>
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<td>----------</td>
<td>--------</td>
<td>------------</td>
</tr>
<tr>
<td>Ss 3/4</td>
<td><em>Ruminococcus gnarus</em>&lt;br&gt;HucA19*</td>
<td>Selenomonas selective</td>
<td>YCFA</td>
<td></td>
<td>4.75±2.20</td>
<td>6.10±0.27</td>
<td></td>
<td>1.09±0.47</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>YCFAL</td>
<td></td>
<td>6.68±2.09</td>
<td>6.19±0.34</td>
<td></td>
<td>-9.78±2.56</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>YCFALG</td>
<td>0.54±0.13</td>
<td>5.06±4.28</td>
<td>8.66±0.53</td>
<td></td>
<td>3.86±1.09</td>
</tr>
<tr>
<td>Ssc/2</td>
<td><em>Cl. indolis (95%)</em></td>
<td>Selenomonas selective</td>
<td>YCFA</td>
<td>0.25±0.04</td>
<td>-0.16±1.32</td>
<td>2.37±0.09</td>
<td></td>
<td>0.48±0.03</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>YCFAL</td>
<td>0.36</td>
<td>-12.12</td>
<td>13.49</td>
<td></td>
<td>-13.78</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>YCFALG</td>
<td>10.98±1.27</td>
<td>-25.35±2.87</td>
<td>36.10±0.49</td>
<td></td>
<td>-13.34±1.28</td>
</tr>
<tr>
<td>L1-92</td>
<td><em>A. caccae</em>&lt;br&gt;(type strain)</td>
<td>M2GSC</td>
<td>YCFA</td>
<td>0.00±0.08</td>
<td>-2.35±2.03</td>
<td>1.99±0.09</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>YCFAL</td>
<td>-0.05±0.10</td>
<td>-21.98±2.45</td>
<td>23.35±1.16</td>
<td></td>
<td>-28.92±0.54</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>YCFALG</td>
<td>1.49±0.13</td>
<td>-26.83±0.58</td>
<td>36.81±3.61</td>
<td></td>
<td>-12.0±1.32</td>
</tr>
<tr>
<td>L2-7</td>
<td><em>E. hallii</em></td>
<td>M2GSC</td>
<td>YCFA</td>
<td>0.02±0.01</td>
<td>-1.58±1.73</td>
<td>0.63±0.03</td>
<td></td>
<td>0.00±0.00</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>YCFAL</td>
<td>1.09±1.55</td>
<td>-14.77±0.93</td>
<td>22.58±0.76</td>
<td></td>
<td>-30.47±0.00</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>YCFALG</td>
<td>3.93±3.38</td>
<td>12.78±0.94</td>
<td>5.80±0.97</td>
<td></td>
<td>1.67±0.47</td>
</tr>
</tbody>
</table>

* clone library sequences, uncultured (Hold et al., 2002)
**Table 2b.** Total lactate (mM) remaining in the tubes at the end of the 24 h incubation period and separately the concentration of the two forms D and L. Total glucose (gluc) metabolised during growth also recorded (mM).

<table>
<thead>
<tr>
<th>Strain number</th>
<th>Closest relative</th>
<th>Medium</th>
<th>Total lact.</th>
<th>L-lact</th>
<th>D-lact</th>
<th>Gluc used</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ss2/1</td>
<td><em>Cl. indolis</em> (95%)</td>
<td>YCFA</td>
<td>0.84±0.02</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>YCFAL</td>
<td>17.08±2.53</td>
<td>16.07±0.40</td>
<td>1.01±2.15</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>YCFALG</td>
<td>18.40±2.70</td>
<td>15.90±1.06</td>
<td>2.50±3.30</td>
<td>22.1±0.0</td>
</tr>
<tr>
<td>Sr 1/1</td>
<td>Huc B12*</td>
<td>YCFA</td>
<td>0.81±0.12</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>YCFAL</td>
<td>17.31±0.89</td>
<td>15.05±0.34</td>
<td>2.26±0.68</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>YCFALG</td>
<td>18.64±0.40</td>
<td>16.37±0.79</td>
<td>2.27±0.71</td>
<td>22.0±0.2</td>
</tr>
<tr>
<td>SL 6/1/1</td>
<td><em>E. hallii</em></td>
<td>YCFA</td>
<td>0.00±0.00</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Huc A15*</td>
<td>YCFAL</td>
<td>2.42±0.60</td>
<td>0.21±0.10</td>
<td>2.21±0.51</td>
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<tr>
<td></td>
<td></td>
<td>YCFALG</td>
<td>29.92±0.07</td>
<td>10.65±0.69</td>
<td>19.27±0.79</td>
<td>22.1±0.1</td>
</tr>
<tr>
<td>SM 6/1</td>
<td><em>E. hallii</em> (98%)</td>
<td>YCFA</td>
<td>0.00±0.00</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>YCFAL</td>
<td>26.08±1.27</td>
<td>9.94±0.50</td>
<td>16.14±1.06</td>
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<tr>
<td></td>
<td></td>
<td>YCFALG</td>
<td>6.57±0.16</td>
<td>4.02±2.26</td>
<td>2.55±2.32</td>
<td>22.1±0.1</td>
</tr>
<tr>
<td>Ss 3/4</td>
<td>HucA19* (new species to be named)</td>
<td>YCFA</td>
<td>1.54±0.47</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>YCFAL</td>
<td>22.58±2.55</td>
<td>16.56±0.12</td>
<td>6.02±2.65</td>
<td></td>
</tr>
<tr>
<td>Strain number</td>
<td>Closest relative</td>
<td>Medium</td>
<td>Total incl.</td>
<td>Glue used</td>
<td></td>
<td></td>
</tr>
<tr>
<td>--------------</td>
<td>------------------</td>
<td>--------</td>
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<td>-----------</td>
<td></td>
<td></td>
</tr>
<tr>
<td>L1-92</td>
<td><em>A. caccae</em> (type strain)</td>
<td>YCFALG</td>
<td>22.39±0.63</td>
<td>1.54±0.47</td>
<td></td>
<td></td>
</tr>
<tr>
<td>L2-7</td>
<td><em>E. halii</em></td>
<td>YCFALG</td>
<td>20.34±1.32</td>
<td>0.00±0.00</td>
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<td></td>
</tr>
<tr>
<td></td>
<td>n/a</td>
<td>YCFALG</td>
<td>31.95±0.47</td>
<td>0.00±0.00</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

- Clone library sequence, uncultured (Hold et al., 2002)
Example 4: 16S rRNA sequencing of new isolates and phylogenetic relationships

Cell pellets from 1 ml cultures grown on M2GSC medium (24 h) that were resuspended in 50 μl of sterile dH2O served as templates for PCR reactions (0.5 μl per 50 μl of PCR reaction). 16S rRNA sequences were amplified with a universal primer set, corresponding to positions 8-27 (27f, forward primer, AGAGTTTGATCMTGGCTCAG) and 1491-1511 (rP2, reverse primer ACGGCTACCTTGTTAGACTT) of the Escherichia coli numbering system (Brosius, 1978; Weisberg, 1991) with a MgCl2 concentration of 1.5 mM. PCR amplifications were performed using the following conditions: initial denaturation (5 min at 94°C), then 30 cycles of denaturation (30 s at 94°C), annealing (30 s at 51°C), and elongation (2 min at 72°C), and a final extension (10 min at 72°C). The amplified PCR products were purified using QIA quick columns (Qiagen GmbH, Germany) according to manufacturer’s instructions and directly sequenced using a capillary sequencer (Beckman) with primers 27f, rP2, 519f (CAGCMGCCGCGTAAWTG) and 519r (GWATTACCGCGGTGCTG) (corresponding to positions 518-535 of the E. coli numbering system) and 926f (AACTCAAAGAAATTGACGG) and 926r (CCGTCATTCMTTTRAGTTT) corresponding to positions 906-925). Two independent PCR products were sequenced per strain.

Similarity of the 16S rRNA sequences (minimum 1444 bases) of the isolates with other organisms was compared with all sequence data in GenBank using the BLAST algorithm (Altschul, 1990).
Example 5 : Co-culture of lactate utilisers with
*Bifidobacterium adolescentis*

Three lactate utilising strains, *Anaerostipes caccae* L1-92 and two strains of *Bacterium hallii* (SM 6/1 and L2-7) were incubated alone and in co-culture with *B. adolescentis* L2-32 on YCFA medium modified to contain reduced casitone (0.1%) and 0.2% soluble starch as an added energy source. The inoculated tubes were incubated for 24 h at 37°C. *B. adolescentis* L2-32 was enumerated on Mann Ragosa Sharpe (MRS) medium containing 2.0% agar with a final concentration of 0.5% propionate and the three butyrate producing strains, were enumerated on M2 medium containing 0.5% DL lactate.

Results: In most human diets, resistant starch is considered to be the most important energy source for microbial growth in the large intestine (Topping, 2001). The major amylolytic species in the human colon are generally considered to be *Bacteroides* and *Bifidobacterium* spp. (MacFarlane, 1986; Salyers, 1977). Bifidobacteria produce acetate and lactate from carbohydrate substrates, typically in the molar ratio of 3:2. Since the lactate utilisers isolated here either do not utilise starch or utilised it weakly, as a growth substrate in pure culture, it was of interest to co-culture them with a starch-degrading *Bifidobacterium* strain in order to establish whether they could remove the lactate formed. The recently isolated, actively amylolytic *B. adolescentis* strain L2-32 was used for these experiments. As shown in Tables 3a, 3b and Fig. 2, co-culture with any one of three lactate utilisers
tested, with starch as the growth substrate, resulted in complete conversion of the L-lactate, and some of the acetate, formed by *B. adolescentis* L2-32 into butyric acid. This corresponded with greatly increased growth of the lactate utilisers in the presence of the *B. adolescentis* L2-32, as determined by selective plating. Viable counts (cfu ml\(^{-1}\)) after 24 hours growth for L1-92, SM 6/1 and L2-7 were, respectively, 2.4 \(x\) \(10^8\), 1.0 \(x\) \(10^7\) and 8.0 \(x\) \(10^6\), in the absence of *B. adolescentis*, and 1.7 \(x\) \(10^9\), 6.8 \(x\) \(10^8\) and 5.4 \(x\) \(10^9\), in the presence of *B. adolescentis* L2-32. Growth of *B. adolescentis* L2-32 was unaffected by co-culture (mean 4.3 \(x\) \(10^8\) cfu ml\(^{-1}\)). There may have been some contribution of starch hydrolysis products that escape uptake by the *B. adolescentis* L2-32, in addition to lactate and acetate, to the growth of the lactate utilisers. This might account for the apparent effectiveness of *B. hallii* SM 6/1 in co-culture, even though this strain used rather little in pure culture when supplied with lactate alone.
Table 3a. Fermentation profiles for *Bifidobacterium adolescentis* L2-32 and three lactate utilisers when incubated alone or in co-culture for 24 hours at 37°C on modified YCFA medium (modified to contain 0.1% casitone) containing 0.2% soluble starch.

<table>
<thead>
<tr>
<th>Culture/co-culture</th>
<th>Formate</th>
<th>Acetate</th>
<th>Butyrate</th>
<th>Total Lactate</th>
<th>L-Lactate</th>
</tr>
</thead>
<tbody>
<tr>
<td>L2-32</td>
<td>4.29±0.92</td>
<td>51.04±5.44</td>
<td>0</td>
<td>5.00±0.09</td>
<td>5.16±0.45</td>
</tr>
<tr>
<td>L1-92</td>
<td>0.01±0.01</td>
<td>34.99±0.93</td>
<td>1.57±0.26</td>
<td>0.40±0.69</td>
<td>0</td>
</tr>
<tr>
<td>SM 6/1</td>
<td>0</td>
<td>35.25±2.15</td>
<td>0.75±0.06</td>
<td>0.27±0.27</td>
<td>0</td>
</tr>
<tr>
<td>L2-7</td>
<td>0.04±0.06</td>
<td>35.70±0.44</td>
<td>0.83±0.02</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>L2-32+L1-92</td>
<td>4.29±0.04</td>
<td>44.82±1.13</td>
<td>7.62±0.66</td>
<td>0.61±0.53</td>
<td>0</td>
</tr>
<tr>
<td>L2-32+SM 6/1</td>
<td>4.81±1.08</td>
<td>48.17±6.47</td>
<td>6.23±1.15</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>L2-32+L2-7</td>
<td>5.16±1.37</td>
<td>43.88±3.74</td>
<td>7.35±0.27</td>
<td>0.36±0.01</td>
<td>0</td>
</tr>
</tbody>
</table>
Table 3b. Total viable counts (cfu per ml) of Bifidobacterium adolescentis L2-32 and three lactate utilisers following 24 hours at 37°C in monoculture and co-culture. Bifidobacterium adolescentis L2-32 was selected for on MRS + 0.25% propionate roll tubes and the butyrate producing/lactate utilisers were selected for on M2 + 0.5% lactate roll tubes following incubation for 24 hours at 37°C.

<table>
<thead>
<tr>
<th>Culture / Co-culture</th>
<th>B. adolescentis L2-32</th>
<th>Butyrate producer / lactate utiliser</th>
</tr>
</thead>
<tbody>
<tr>
<td>L2-32</td>
<td>3.8 x 10⁸</td>
<td></td>
</tr>
<tr>
<td>L1-92</td>
<td></td>
<td>2.4 x 10⁸</td>
</tr>
<tr>
<td>S M6/1</td>
<td></td>
<td>1.0 x 10⁷</td>
</tr>
<tr>
<td>L2-7</td>
<td></td>
<td>8.0 x 10⁶</td>
</tr>
<tr>
<td>L2-32+L1-92</td>
<td>6.4 x 10⁸</td>
<td>1.7 x 10³</td>
</tr>
<tr>
<td>L2-32+SM 6/1</td>
<td>3.8 x 10⁸</td>
<td>6.8 x 10⁸</td>
</tr>
<tr>
<td>L2-32+L2-7</td>
<td>3.2 x 10⁸</td>
<td>5.4 x 10³</td>
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</tbody>
</table>
Example 6: Time Course of Lactate Utilisation

Time courses were followed in batch culture for growth on glucose, lactate or glucose and lactate (Figs. 4, 5). E. hallii L2-7 when grown with DL-lactate used all of the added lactate together with some acetate, producing more than 20 mM butyrate (Fig. 4). Less butyrate, but significant formate, was produced during growth on glucose, or on glucose plus lactate, and lactate utilisation was almost abolished by the presence of glucose. Hydrogen production in 24 hours was 12 μmol ml⁻¹ for growth on glucose, 15.5 μmol ml⁻¹ for growth on lactate and 10.9 μmol ml⁻¹ for growth on glucose plus lactate. A. cacaoe L1-92 similarly produce larger quantities of butyrate when grown on lactate compared with growth on glucose, when formate was also a product. This strain was able to use lactate once glucose had been exhausted, following inoculation into glucose plus lactate medium.

Strain SS2/1 is likely to represent a new species, since its closest relative (95% identity in 16S rRNA sequence) is the non-butyrate producing Clostridium indolis. This strain was able to use D-, but not L-, lactate following glucose exhaustion in lactate plus glucose medium (Fig. 5). Again formate was not a significant product when lactate was the sole energy source but 4.7 μmol ml⁻¹ hydrogen was formed.
Summary

A. c cacque strain L1-92 was able to consume up to 30mM DL lactate, along with 20-30 mM acetate during batch culture incubation for 24 hours at 37°C with the production of >20mM, and up to 45mM butyrate; this occurred also when glucose was added as an alternative energy source (Table 1). Lactate or lactate plus glucose thus resulted in very much higher production of butyrate than observed with 23mM glucose alone, when only <15mM butyrate was formed. Furthermore none of the 74 strains screened previously by Barcenilla et al. (2000) produced more than 25mM butyrate when tested in M2GSC medium. Lactate consumption is not a general characteristic of butyrate-producers, and six of the strains screened in Table 1 failed to consume lactate in M2GSCL medium.

Six further strains that are highly active lactate utilisers (defined for example as net consumption of at least 10mM of lactate during growth to stationary phase or for 24 hours in YCFALG or YCFAL medium at 37°C - see Table 2a) were obtained following deliberate screening of new human faecal isolates for lactate utilisation. At least two of these (SL 6/1/1 and SM 6/1 - Tables 1, 2) are related to Eubacterium hallii. (Table 2a), based on determination of their 16S rDNA sequences. These isolates again consume large quantities of lactate and produce high levels of butyrate in vitro. With one exception where considerable glucose repression occurred (strain SL 6/1/1), significant lactate utilization occurred in the presence of glucose (Table 2). Three strains (Ss 2/1, Sr 1/1 and Ssc/2) showed preferential
utilization of D-lactate, whereas the two *E. hallii*-related strains SM 6/1, SL 6/1/1 and *A. caccae* L1-92 utilise both isomers (Table 2b). The two stereoisomers differ in their toxicity in the human body, with the D-isomer being regarded as the more toxic (Chan et al., 1994, Hove et al., 1995). The present invention thus provides a means of utilising both D and L lactate isomers or preferentially utilising D-lactate in preference to L-lactate.

*A. caccae* and newly isolated bacteria related to *E. hallii* and *Ct. indolis* were shown to consume up to 30mM DL, D or L lactate, along with 20-30 mM acetate during batch culture incubation and convert this energy in to production of at least 20mM, and up to 45mM butyrate. Furthermore, these strains were shown to convert all of the L-lactate produced by a starch-degrading strain of *Bifidobacterium adolescentis* into butyrate when grown in culture. This is the first documentation demonstrating the conversion of lactate to butyrate by human colonic bacteria, some of which are likely to be new species.
References


bacterium *Selenomonas ruminantium*. Microbiol., 1440, 2077-2084.


CLAIMS

1. A method of selecting a strain of lactic acid-utilising bacteria, which method comprises the steps of:

   a) providing a bacterial culture from a human faecal sample;
   b) selecting a single colony of bacteria;
   c) growing said colony in a suitable medium containing lactic acid; and
   d) selecting a strain of bacteria consuming relatively large amounts of lactic acid,

   all of the above steps being conducted under anaerobic conditions.

2. The method as claimed in Claim 1 wherein at least 10 mM of lactic acid is consumed during growth into the stationary phase per 24 hours at 37°C in YCFALG or YCFAL medium.

3. The method as claimed in either one of Claims 1 and 2 wherein said method comprises the additional step of:

   e) selecting a strain of bacteria producing relatively large quantities of butyric acid.

4. The method as claimed in Claim 3 wherein at least 10 mM butyric acid is produced during
bacterial growth into the stationary phase per
24 hours at 37°C in YCFALG or YCFAL medium.

5. The method as claimed in any one of Claims 1 to
4 wherein said lactic acid is a mixture of D
and L isomers of lactic acid.

6. Anaerostipes caccae strain L1-92 deposited at
NCIMB under No. 13801T.

7. Clostridium indolis bacterial strain Ss2/1
deposited at NCIMB under No. 41156.

8. Eubacterium hallii strain SM 6/1 deposited at
NCIMB under No. 41155.

9. A lactic acid utilising bacterium having a 16S
rRNA gene sequence with at least 95% homology
to one of the sequences shown in Fig. 1.

10. A bacterial strain as claimed in any one of
Claims 6 to 9 for use as a medicament to treat
lactic acid induced disorders or as a
foodstuff.

11. A bacterial strain as claimed in Claim 10 for
use as a medicament to treat lactic-acidosis,
short bowel syndrome or inflammatory bowel
disease.

12. A method to promote butyric acid formation in
the intestine of a mammal, said method
comprising the administration of a therapeutically effective dose of at least one of the strains of bacteria as claimed in any one of Claims 6 to 9.

13. The method of Claim 12 wherein said bacteria is administered as a foodstuff or as a suppository.

14. A method for treating a disease associated with a high dosage of lactic acid, which method comprises the administration of a therapeutically effective dose of at least one strain of live lactic acid utilising bacteria as claimed in any one of Claims 6 to 9.

15. The method of Claim 14 wherein said disease is lactic-acidosis, short bowel syndrome or inflammatory bowel disease.

16. The method of either one of Claims 14 and 15 wherein said bacteria is Anaerostipes caccae.

17. A prophylactic method to reduce the incidence or severity of colorectal cancer or colitis in mammals caused in part by high lactic acid and low butyric acid concentrations, which method comprises the administration of a therapeutically effective dose of at least one strain of live lactic acid utilising bacteria as claimed in any one of Claims 6 to 9.
18. The method of Claim 17 wherein said bacteria is
Anaerostipes caccae.

19. A probiotic composition comprising a live
bacterial strain as claimed in any one of
Claims 6 to 9, in combination with live lactic
acid producing bacteria.

20. The composition as claimed in Claim 19 wherein
said lactate acid producing bacteria is
Lactobacillus spp, Bifidobacterium spp or a
mixture thereof.

21. The method of either one of Claims 19 and 20
wherein said bacteria is Anaerostipes caccae.

22. The composition as claimed in any one of Claims
19 to 21 further containing other additives or
growth enhancing supplements.
Figure 1

Sequence information for five of the lactate utilising strains.

S D6 1L/1

GATGAACGCGTGGCGGCTGCTAATAACACTGCAAAGTCGGAACACCTTTAACCCTGATTCTTTCCGGATGAA
GGTGCGGTGACGTAGTGCGAACGGACGGTACGGGTCCTGAACTGCAATGCTTATCCTCTGGCGGTCTTTG
GTGGAAAGCGCTGCTAATACCGCATAAGCGGACGAGGACATCCTTCTTGCTGGGAAACCTGGGTG
GTACAGGAAGGGGCGCGCTCTGAGTTAGCTGCTGCTGCGCAAGGCTAAGGCGCGCGGGTCAAGTCGTA
GCCGGTCTCGAGGAAGGAGGAACGCCACATTTGGAACCTGAACTGGCTACAACTGCAAACCGAGG
TGCGGAAATATTCACAATGGGGAAGAAACGCTGATGCGCAAGCGCGCGCGGCGGAGTGAAGGAAGTAATCTTG
ATGTAAGCTCTCTACGAGGGAAGATATAGAGCAGCCATTGATTCACTTGGAACTGGGCTTACAACTGCTGG
CAGCAGTCGCTGAGTAAGAAGCAGGCGCGAGGTCGGCTCAACCAGGAGCTGCTATGGAAACTCGCATAGCTAG
AGTACAGGAAGGGGCGCGCTCTGAGTTAGCTGCTGCTGCGCAAGGCTAAGGCGCGCGGGTCAAGTCGTA
TGCCGCAAGGCGCGCTCGCTGGACAGTTACTGCACTAGGCGCCAGCAAGGGGAAGGCGACACCGGTCG
GATACCCCTGAGTGGCTGACAAGGCTGTTAAACGATGAATACTAGGTGTGGCGGCCTATGGCATTCTGC
TGCGAAGGCGCGCTCTGAGGAAGGAGGAACGCCACATTTGGAACCTGAACTGGCTACAACTGCAAACCGAGG

SM 6/1

GATGAACGCGTGGCGGCTGCTAATAACACTGCAAAGTCGGAACACCTTTAACCCTGATTCTTTCCGGATGAA
TCGTCGACGCTGCTGCGAACGGACGGTACGGGTCCTGAACTGCAATGCTTATCCTCTGGCGGTCTTTG
GTGGAAAGCGCTGCTAATACCGCATAAGCGGACGAGGACATCCTTCTTGCTGGGAAACCTGGGTG
GTACAGGAAGGGGCGCGCTCTGAGTTAGCTGCTGCTGCGCAAGGCTAAGGCGCGCGGGTCAAGTCGTA
GCCGGTCTCGAGGAAGGAGGAACGCCACATTTGGAACCTGAACTGGCTACAACTGCAAACCGAGG
TGCGGAAATATTCACAATGGGGAAGAAACGCTGATGCGCAAGCGCGCGCGGCGGAGTGAAGGAAGTAATCTTG
ATGTAAGCTCTCTACGAGGGAAGATATAGAGCAGGCGCGAGGTCGGCTCAACCAGGAGCTGCTATGGAAACTCGCATAGCTAG
AGTACAGGAAGGGGCGCGCTCTGAGTTAGCTGCTGCTGCGCAAGGCTAAGGCGCGCGGGTCAAGTCGTA
TGCCGCAAGGCGCGCTCGCTGGACAGTTACTGCACTAGGCGCCAGCAAGGGGAAGGCGACACCGGTCG
GATACCCCTGAGTGGCTGACAAGGCTGTTAAACGATGAATACTAGGTGTGGCGGCCTATGGCATTCTGC
TGCGAAGGCGCGCTCTGAGGAAGGAGGAACGCCACATTTGGAACCTGAACTGGCTACAACTGCAAACCGAGG
GCCAGCAAGCGCCTAAATAGATTATAGGAGCAAGCAGGTTTACCGAATTCTGGGTGTAAGGATGTCGATTG
CGTGAGTGTAGTCAAGAGTATGAAAGGCCGCCCGGCTCAACCCGGACACGCTCATTTGAAACTGCTWYRGCT
AGAAGTACGGAGAGGCGCCGGATTCTCTAGTGGAGTGGGAAATCTGATAGATAGGAAACCAAC
AGTGCGRAAGGCGCCCTCTGCGACTGTTACTGACACTGGGCAAAGCGGATGGGGGAGCGAAACAGGAT
TAGATACCTGGTACGGCCTAATTACGAGAAGAAGAGAAGGCTGGTTACTGCTGGTT
GAGATCCACGCTGTAGTGCTAGGTAACATCGCAGAAAAGACGCGGAGCCACCCCCACTTACGAGTCA
CAGCAGGTAAGCGCTGACCTCGAGAGAGAGGCGGAGTTAATCTGAGGAAAGGCTGGGAGACGCTG
AAATACGTAGTCTTGGCCTATCTTGGGAGCAAGAGGAGGAGAGGAGGAGGAGGAGGAGGAGGAGGAGG
CCGCGAGGGGGAGCGAAAAACACAAAAAGGGGCGCTCCAGTnCGGCACGTATGCTGCAACCGGACACTACA
GAAGCTGGAATCTGCTGACTAATCCGGAGAGGCTGGTGAATTCGGGTAATGTCGCCGAGTTCTGGGTTCAACA
CCGCCGTCACACCACTTGGGAGGAAATGCCCCGAGCAGGCTGACCCACCTTTATAGAGAGAGCCmG
TCCAAGGTGAAACCGGTATTCAGGGGmTT

17

Ss3/4

18

GAGTTTGGATCTTGGTACGGATGAAAGGCTGGGAGCCTGCTAACTACGATGCGAAAGCGTGT
19

ATATGGATCTGCTGGGATGAAAAATCTAGGCTAGAATGGGCGAGGGCTGGGAGGAAACGCGGG
20

TAAACTGACTCTAGAGAGGAGAAGGATGAAGATGACTGCAATAACTCCGTCACACGACAGAGGCGGA
21

GTACCAGTGCTGAGCTGCTGAAATACGCTCTTGGTGAGTGGGAGAGAGGAGGAGGAGGAGGAGG
22

TTGAGGCTGAGCTGCTGAAATACGCTCTTGGTGAGTGGGAGAGAGGAGGAGGAGGAGGAGGAGG
23

GGAGAAGAAATACGCTGAGCTCTTGGTGAGTGGGAGAGAGGAGGAGGAGGAGGAGGAGGAGGAGG
24

ATACTGAGGAGGAGAAGGCTGCTGAAATACGCTCTTGGTGAGTGGGAGAGAGGAGGAGGAGGAGG
25

GAGTTTGGATCTTGGTACGGATGAAAGGCTGGGAGCCTGCTAACTACGATGCGAAAGCGTGT
26

ATATGGATCTGCTGGGATGAAAAATCTAGGCTAGAATGGGCGAGGGCTGGGAGGAAACGCGGG
27

GAGTTTGGATCTTGGTACGGATGAAAGGCTGGGAGCCTGCTAACTACGATGCGAAAGCGTGT
28

ATATGGATCTGCTGGGATGAAAAATCTAGGCTAGAATGGGCGAGGGCTGGGAGGAAACGCGGG
29

GAGTTTGGATCTTGGTACGGATGAAAGGCTGGGAGCCTGCTAACTACGATGCGAAAGCGTGT
30

ATATGGATCTGCTGGGATGAAAAATCTAGGCTAGAATGGGCGAGGGCTGGGAGGAAACGCGGG
31

GAGTTTGGATCTTGGTACGGATGAAAGGCTGGGAGCCTGCTAACTACGATGCGAAAGCGTGT
32

ATATGGATCTGCTGGGATGAAAAATCTAGGCTAGAATGGGCGAGGGCTGGGAGGAAACGCGGG
33

GAGTTTGGATCTTGGTACGGATGAAAGGCTGGGAGCCTGCTAACTACGATGCGAAAGCGTGT
34

ATATGGATCTGCTGGGATGAAAAATCTAGGCTAGAATGGGCGAGGGCTGGGAGGAAACGCGGG
35

GAGTTTGGATCTTGGTACGGATGAAAGGCTGGGAGCCTGCTAACTACGATGCGAAAGCGTGT
36

ATATGGATCTGCTGGGATGAAAAATCTAGGCTAGAATGGGCGAGGGCTGGGAGGAAACGCGGG
37

GAGTTTGGATCTTGGTACGGATGAAAGGCTGGGAGCCTGCTAACTACGATGCGAAAGCGTGT
38
SS2/2 and Ssc/2

AGAGTTGATCTGCTGAGTGAACGCTGCGGGGCTTTAACACATCGAAGTGAACGAAA

CACCTTTGATTTTTGTTGCAACTGGAATGGGTATTTGTTGATGGATTGCGGACGGTGAACG

CTGTTGTTGACCGTGTAACCGCGCTTCCCTGACGAGCTTACAGCTTTGTGGTTGAGAAGAG

ACACCGGCGCCGATGCGGACGGGTAATTTGCTGCGCCTCAGGGACAGCAGAGTGAGGAGG

GTTGTACTCCGAGGGTAACTCGATCTGCGTACAACTCGAAGACAGGATGGCAGCAGAGG

GCTCCAGAAGAAGAAACGCTGTTTCTCCTCAGGGGCTTTAATCGCGACGGGTAATCGTG

TGACGAGAGTTAAGCGAATTCTCAGTGTACGCGTTAAATCGTGATAGTATAGGAGAAGCA

AGTGGCGAGGCTCGCTTCGGGTAACAGCGTAAATCGGAGGCTGAGGACCGAAGCGGAGG

CATTATGGCTGCGAACCGTATCGGTCCAATCGGCTTATGCTGAGGACTCTTATCGGGGCA

AAGGAACTTCGCGGAACCGGCAACAGGCGGCTTATGCTGAGGACTCTTATCGGGGCA

ACCTTACTGCTGCTTACTGACATTCGCCTTCGACAGGCTTTTACCCGAGACCTCTTTTCGAC

TGACGAGGTTGTCATGTTGTGCTGACAGTCTGAGATGTTGTTGTTAGTACCCCGGCAACGA

GCCCAACCCCTATCTTTAGTCGCTGACGCTCAAAATATCGGGAACCTCGTGAAGGATGAAAA

ACCTGGAGGAAGGTGGAGGGACGTAATTTATCTGCAAGAATTCGAGGCTACACACCTGT

CTACATCTGCGGAACAGGAAGGAGGACGACGGTCAAGATCGAATATTTTTGAGAATACCAAA

CAGTTGGATTTGATGTCGCAACTCAGTATACTGAGTGTGAAATCGTCAACTGCAACTGAGT

AAGTGGCGACGTTTACGCTTGCCGCTGCCCTCTGTAACGCCCGTCACAAGATGAGACGGAGG

ACGCCAGAAGTTTAGCTACGGCAATTTCATGGAGGAGCTGCGGAGGACCGGACACGATAACTCG

W = A or T
Y = T or C
R = G or A
N = Unknown
Fig. 4
L2-7 (Glucose plus DL lactate)

Fig. 4 continued
L2-7 (DL lactate)

Fig. 4 continued
SS2/I (Glucose plus DL lactate)

Fig. 5 continued