Growth inhibition of *Clostridium difficile* by intestinal flora of infant faeces in continuous flow culture

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**Summary.** Growth of *Clostridium difficile* was inhibited more strongly in continuous flow (CF) culture with *C. difficile*-negative faeces of infants than with *C. difficile*-positive faeces. Culture of faecal flora of infants yielded a greater variety of bacterial species in *C. difficile*-negative than in *C. difficile*-positive faeces. In the mixed CF culture of *C. difficile* with *Enterococcus avium*, *Bacteroides distasonis*, *Eubacterium lentum*, *C. ramosum*, *C. perfringens* and either *Escherichia coli* or *Klebsiella pneumoniae* isolated from *C. difficile*-negative faeces, inhibition of growth of *C. difficile* was demonstrated when the pH of the culture medium was decreased. Amino-acid analysis of CF cultures showed considerable utilisation of aspartic acid, serine, threonine, arginine and asparagine. A marked increase in concentrations of citrulline and ornithine was found in the culture that inhibited growth of *C. difficile*. The addition of citrulline and ornithine into a Gifu anaerobic medium (GAM) broth produced no inhibition of growth of *C. difficile*. The addition of the mixture of the depleted amino acids (aspartic acid, serine, threonine, arginine and asparagine) to the culture filtrate or adjustment of the pH of the culture filtrate induced considerable growth of *C. difficile*. These results suggest that the inhibition of growth of *C. difficile* may be due to consumption of amino acids by intestinal flora, and not to the presence of inhibitors produced by the intestinal flora.

**Introduction**

The normal flora of the intestinal tract forms a barrier against colonisation by microbial pathogens. When the intestinal flora is disturbed by antimicrobial agents, colonisation or overgrowth of bacteria resistant to the antimicrobial agents occurs. It is accepted generally that pseudomembranous colitis (PMC) associated with *Clostridium difficile* develops in this manner. Wilson et al. reported that the administration of normal caecal homogenates reduced counts of viable *C. difficile* and prevented caecitis in hamsters given antibiotics. In an in-vitro model of colonisation resistance to *C. difficile* infection, growth of *C. difficile* and cytotoxin production were inhibited when faecal emulsions from healthy adults were added.

Bacterial interference between *C. difficile* and other faecal bacteria, such as *Escherichia coli*, non-toxigenic *C. difficile* and lactobacilli was investigated in either continuous flow (CF) culture or an animal model with gnotobiotic mice and hamsters.

Colonisation of *C. difficile* is common in infants but very rare in adults. The mechanism by which *C. difficile* colonises infants without causing symptoms, despite the presence of both toxin A (enterotoxin) and toxin B (cytotoxin) in the faeces, is still unclear. The higher infant colonisation rate is probably due to differences in the mucous membrane and its microflora at this age.

To clarify the role of the normal intestinal flora in preventing colonisation by *C. difficile*, the effect of faecal flora of infants on the growth of *C. difficile* was studied in CF culture. The correlation between the production of volatile fatty acids and utilisation of amino acids in the culture with growth of *C. difficile* was also analysed.

**Materials and methods**

Isolation and identification of *C. difficile* from faecal samples

Faecal samples were collected from healthy infants under 3 years of age who lived in Azabu Infants' Home. Faeces (0·5 g) was suspended in 4·5 ml of anaerobic diluent (H₂PO₄ 4·5 g, Na₂HPO₄ 6·0 g, L-cysteine hydrochloride 0·5 g, Tween 80 0·5 g and agar 0·5 g in 1 L of distilled water), homogenised, and diluted in serial 10-fold steps with the diluent. A 0·1-ml
counts and determination of cytotoxic activity. A culture was sampled at an appropriate time for viable fermentation products and amino acids on days 0 and 10 after the inoculation of C. difficile. One ml of the culture was inoculated in the same manner 10 days after the inoculation of faecal flora as described above. In the CF culture of the C. difficile-negative faecal sample, the C. difficile strain N8 isolated from one faecal sample was inoculated in the same manner as described above. In the CF culture of the C. difficile-positive faecal sample, a C. difficile strain that had been isolated from the same faecal sample and pre-cultured in another CF culture for 3 days was inoculated 10 days after the inoculation of faecal flora. One ml of 10²-fold diluted C. difficile culture, which had been pre-cultured in GAM broth at 37°C for 18 h, was inoculated into the CF culture, resulting in an initial count of 10⁵ cfu/ml. For the CF culture of the C. difficile-negative faecal sample, C. difficile strain N8 isolated from one faecal sample was inoculated in the same manner 10 days after the inoculation of faecal flora as described above. One ml of the culture was sampled at an appropriate time after the inoculation for viable counts of C. difficile and other bacteria. On days 0, 10 and 17, 5 ml of the culture were collected to analyse fermentation products and identify the bacterial flora.

Continuous flow (CF) culture

Faecal specimens (0.5–1.0 g) for CF culture were suspended in five times their weight of GAM broth and kept at −80°C until use. Three C. difficile-positive faecal samples and four C. difficile-negative faecal samples were used in this study. One ml of the faecal suspension was inoculated into the culture vessel containing 100 ml of GAM broth under anaerobic conditions at 37°C. The dilution rate was 0.05/h. For the CF culture of the C. difficile-positive faecal sample, a C. difficile strain that had been isolated from the same faecal sample and pre-cultured in another CF culture for 3 days was inoculated 10 days after the inoculation of faecal flora. One ml of 10³-fold diluted C. difficile culture, which had been pre-cultured in GAM broth at 37°C for 18 h, was inoculated into the CF culture, resulting in an initial count of 10⁵ cfu/ml. For the CF culture of the C. difficile-negative faecal sample, C. difficile strain N8 isolated from one faecal sample was inoculated in the same manner 10 days after the inoculation of faecal flora as described above. One ml of the culture was sampled at an appropriate time after the inoculation for viable counts of C. difficile and other bacteria. On days 0, 10 and 17, 5 ml of the culture were collected to analyse fermentation products and identify the bacterial flora.

CF cultures of several bacterial strains were performed in the same manner as described above. In the mixed culture of anaerobes and aerobes, the aerobes were inoculated 1 day after the inoculation of the anaerobes. Toxigenic (C. difficile T-C-2 and T-C-9) or non-toxigenic (C. difficile N8 and N13) strains that had been isolated from faecal samples and pre-cultured in another CF culture for 3 days, were inoculated 10 days after the inoculation of the aerobes. Samples (5 ml) of the culture were collected to analyse both fermentation products and amino acids on days 0 and 7 after the inoculation of C. difficile. One ml of the culture was sampled at an appropriate time for viable counts and determination of cytotoxic activity.

Quantitative study of bacterial flora and C. difficile

A 0.1 ml sample of the culture diluted 10-fold serially with the anaerobic diluent was spread on the surfaces of various selective media. Deoxycholate-Hydrogen sulphide-Lactose (DHL) Agar (Nissui Chemical Co.) and Phenylethyl alcohol (PEA) Agar (Difco) supplemented with horse blood 5% were used for the selection of aerobes. Candida GS Agar (Nissui Chemical Co.) was used for the selection of Candida spp. Cultures were incubated aerobically at 37°C for 2 days. GAM agar supplemented with gentamicin, PEA agar, Veillonella Agar (Difco) supplemented with both kanamycin and neomycin, Fusobacterium (FM) Agar (Nissui Chemical Co.), Eugon Agar (Difco) supplemented with either kanamycin and vancomycin with horse blood 5% or rifampicin with horse blood 5%, were used for the isolation of anaerobic intestinal flora. Cultures were incubated anaerobically at 37°C for 3 days. The colonies were counted and expressed as cfu/g of faeces or cfu/ml. Anaerobes were identified according to the method described above. Aerobes were identified by API 20E, API Staph, API Streap and API 20C Aux (API system S.A., Montaneu Vercieu, France).

Gas chromatography of the fermentation products

Cultures were stored at −80°C for analysis of volatile fatty acids (VFAs) by gas-liquid chromatography (model 263-70, Hitachi Ltd, Tokyo, Japan): 0.2 ml of H₂SO₄ 50%, NaCl 0.4 g, 1 ml of ethylether and 0.1 ml of 5 mM 2 methyl-n-valeric acid (Tokyo kasei, Tokyo, Japan) used for the internal standard, were added to 1 ml of the culture. The ethylether and the culture were mixed in a culture tube by inverting the tube 100 times. The mixture was centrifuged at 3000 rpm for 3 min. The ether layer was pipetted into another test tube to which anhydrous CaCl₂ (c. 0.5 g) was added and the test tube was left to stand for 5 min. The ether extract was then discarded into another culture tube and placed at −80°C until injection. The extract was injected on to a gas chromatography column (2 m × 5 mm) packed with PolyesterNF 15% on Neosorb NS 60/80 (Chromato Research Ltd, Kanagawa, Japan). The flow rate of carrier gas (N₂) was 30 ml/min. The injection temperature was 200°C and the temperatures of the column and detector were 150°C and 200°C, respectively. The VFA concentration of each peak was quantified by comparison with the peaks of standard VFA solution and was expressed as micro-equivalents/ml of culture.

Amino-acid analysis

The CF culture medium, GAM broth and the culture harvested at various times after inoculation were treated with the same volume of sulphosalicylic acid dihydrate 5% solution to remove protein. After the treatment, the compositions of amino acids in
C. difficile inhibition by infant faecal flora

Fig. 1. Growth of C. difficile strain N8 in anaerobic CF culture with a C. difficile-positive faecal specimen of an infant: viable counts of C. difficile (○), enterococci (●), enterobacteriaceae (△), total obligate anaerobes (▲) and Can. albicans (□) were determined; redox potential (Eh) (----) and pH (——) were also measured.

these samples were analysed by a rapid amino acid analyser (Model 835, Hitachi).

Preparation of dialysed culture filtrate of faecal bacterial strains

Enterococcus avium, Escherichia coli, Bacteroides distasonis, Eubacterium lentum, C. ramosum and C. perfringens were inoculated into 80 ml of GAM broth in a cellophane dialysis tube suspended in 120 ml of GAM broth; 7 days after inoculation, the outer culture filtrate (120 ml) was collected. Five amino acids—aspatic acid, serine, threonine, arginine and asparagine—were added to the dialysed culture filtrate to give the same concentration of the amino acids as GAM broth, which was quantified by amino acid analysis.

Results

Incidence of C. difficile in the faecal samples

Faecal samples from 62 healthy infants were tested for C. difficile. Five infants from whom C. difficile was isolated were examined again 1 year after the first investigation. Two of the five infants had C. difficile-positive faeces again and three infants gave negative results. A high isolation frequency of C. difficile (65%) was observed in babies <1 year old (20 faecal samples); 23 and 33% of faecal samples from 1–2-year-old (35 samples) and 2–3-year-old (12 samples) children respectively, were positive for C. difficile. C. difficile was isolated from 25 (37%) samples out of a total of 67 faecal samples examined. Of 17 strains of C. difficile tested for toxigenicity, 11 strains were cytotoxic.

Growth of C. difficile in the anaerobic CF culture with faecal flora

When a C. difficile-positive faecal specimen was inoculated into CF culture, considerable growth of faecal flora was observed (fig. 1). The stable growth of faecal flora (c. 10⁶ cfu/ml) continued during the experimental period. However, C. difficile did not grow and was washed out. The number of C. difficile was 10⁵ cfu/ml at 3 days after the inoculation of faeces and no C. difficile was subsequently detected 4 days after inoculation. When the N8 strain of C. difficile that had been isolated from the same faecal specimen was inoculated again into the CF culture of faecal flora 10 days after inoculation of the faeces, the number of C. difficile 2–7 days after the second inoculation was found to be between 10⁵ and 10⁷ cfu/ml.

When a C. difficile-negative faecal specimen was inoculated, it appeared that the total counts of faecal flora reached > 10⁹ cfu/ml after 1 day (fig. 2). When the C. difficile N8 strain was inoculated into the CF culture of faecal flora 10 days after inoculation of the faeces, the N8 strain did not grow and its viable count gradually decreased.

The CF cultures of C. difficile-positive or -negative faecal samples were examined for growth of C. difficile.
Days after inoculation

Fig. 2. Growth of *C. difficile* strain N8 in anaerobic CF culture with a *C. difficile*-negative faecal specimen from an infant: viable counts of *C. difficile* (○), enterococci (●), enterobacteriaceae (▲) and total obligative anaerobes (◀) were determined; redox potential (Eh) (-----) and pH (----) were also measured.

Table I. Numbers of *C. difficile* harvested 2–7 days after inoculation of *C. difficile* into CF cultures with various faecal samples

<table>
<thead>
<tr>
<th>Isolation of <em>C. difficile</em> from faecal sample</th>
<th>Faecal sample no.</th>
<th>Mean (SD) number of <em>C. difficile</em> in CF culture (log₁₀ cfu/ml)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive†</td>
<td>1</td>
<td>4.8 (1.1)</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>6.5 (0.6)</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>6.7 (1.0)</td>
</tr>
<tr>
<td>Negative‡</td>
<td>1</td>
<td>3.9 (0.6)</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>3.2 (1.1)</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>2.4 (1.1)</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>3.8 (0.4)</td>
</tr>
</tbody>
</table>

* One ml of CF culture was harvested every day and used for quantification of *C. difficile*.
† The *C. difficile* strain isolated from the faecal sample was inoculated again 10 days after the inoculation of faecal samples in CF culture.
‡ *C. difficile* strain N8 was inoculated 10 days after the inoculation of faecal samples in CF culture.

2–7 days after the second inoculation with *C. difficile* (table I). Generally, much higher counts of *C. difficile* were detected in the CF culture with *C. difficile*-positive faeces than with *C. difficile*-negative faeces.

Growth of *C. difficile* in the anaerobic CF culture with several intestinal strains

The intestinal flora of the *C. difficile*-positive or -negative faeces was analysed (table II). Four *C. difficile*-negative samples had faecal flora consisting of 16–17 anaerobic species, 8–10 aerobic species and *Candida albicans*. In contrast, three *C. difficile*-positive samples had faecal flora consisting of 7–11 anaerobic species, 4–5 aerobic species and *C. albicans*.

The effect of a mixed culture of faecal bacterial strains such as *C. perfringens*, *E. coli*, *Ent. avium*, *Klebsiella pneumoniae*, *C. ramosum*, *B. distasonis* and *Eu. lentum* on the growth of *C. difficile* strain N8 was examined (table III). These bacterial strains were all isolated from the *C. difficile*-negative faecal samples. In the mixed CF culture of *C. difficile* with *Ent. avium*, *B. distasonis*, *Eu. lentum*, *C. ramosum*, *C. perfringens*, and either *E. coli* or *K. pneumoniae*, considerable inhibition of growth with a decreased pH of culture medium was demonstrated (statistically significant at p < 0.01).

In several CF cultures of toxigenic or non-toxigenic *C. difficile* strains with *Ent. avium*, *B. distasonis*, *Eu. lentum*, *C. ramosum*, *C. perfringens* and either *E. coli* or
K. pneumoniae, growth of C. difficile 2–7 days after inoculation with C. difficile was also examined (table IV). Greater viable counts of C. difficile were detected in the CF culture of non-toxigenic C. difficile than in the CF culture of toxigenic C. difficile. In the mixed CF culture of the toxigenic strain T-C-9, 1–7 days after inoculation, neither C. difficile nor cytotoxic activity was detected. In the case of the toxigenic strain T-C-2, lower cytotoxin production with considerable inhibition of growth of C. difficile was detected in the culture.

Volatile fatty acids (VFAs) in mixed CF culture

VFAs in the six serial CF cultures before and after inoculation of C. difficile strain N8 were studied.
Table IV. Inhibition of growth of *C. difficile* by co-cultivation with *C. perfringens*, *C. ramosum*, *Eu. lentum*, *B. distasonis*, *Ent. avium* and *E. coli*

<table>
<thead>
<tr>
<th>Strain no.</th>
<th>Mean (SD) number of <em>C. difficile</em> (log&lt;sub&gt;10&lt;/sub&gt; CFU/ml)</th>
<th>Maximum cytotoxin titre (log&lt;sub&gt;10&lt;/sub&gt;)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Single CF culture</td>
<td>Mixed CF culture</td>
</tr>
<tr>
<td>Non-toxigenic</td>
<td></td>
<td></td>
</tr>
<tr>
<td>N8</td>
<td>8.2 (0.2)</td>
<td>4.9 (0.3)</td>
</tr>
<tr>
<td>N13</td>
<td>8.1 (0.1)</td>
<td>6.4 (0.3)</td>
</tr>
<tr>
<td>Toxigenic</td>
<td></td>
<td></td>
</tr>
<tr>
<td>T-C-2</td>
<td>8.1 (0.2)</td>
<td>2.6 (0.6)</td>
</tr>
<tr>
<td>T-C-9</td>
<td>8.0 (0.2)</td>
<td>ND</td>
</tr>
</tbody>
</table>

ND, not detected.

Table V. Concentration of VFAs in CF culture in faecal bacterial strains before and after inoculation with *C. difficile* strain N8

<table>
<thead>
<tr>
<th>Mixed CF culture with</th>
<th>Concentration* of VFA (µmol/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Acetic acid</td>
</tr>
<tr>
<td></td>
<td>B</td>
</tr>
<tr>
<td><em>C. perfringens</em></td>
<td>12.4</td>
</tr>
<tr>
<td><em>Ent. avium, E. coli</em></td>
<td>18.6</td>
</tr>
<tr>
<td><em>Ent. avium, K. pneumoniae, C. perfringens</em></td>
<td>24.2</td>
</tr>
<tr>
<td><em>Ent. avium, E. coli, C. perfringens</em></td>
<td>19.6</td>
</tr>
<tr>
<td><em>Ent. avium, E. coli, B. distasonis, Eu. lentum C. ramosum, C. perfringens</em></td>
<td>36.0</td>
</tr>
<tr>
<td><em>Ent. avium, K. pneumoniae B. distasonis, Eu. lentum C. ramosum, C. perfringens</em></td>
<td>30.1</td>
</tr>
</tbody>
</table>

Concentration* of VFA in CF culture harvested before (B) and 7 days after (A) inoculation with *C. difficile* strain N8. ND, not detected.

Quantitatively (table V). In most samples harvested before inoculation, acetic acid was the major product, with butyric acid in second place. Statistically, the increased concentrations of acetic acid, propionic acid and total VFAs before *C. difficile* inoculation were inversely proportional to the average number of *C. difficile* harvested 2-7 days after inoculation in the six serial CF cultures. There was a significant inverse correlation between the concentrations of acetic acid, propionic acid and total VFAs before inoculation with *C. difficile* and the inhibitory effect on growth of *C. difficile*. However, no direct correlation between the concentrations of VFAs in the samples harvested after inoculation with *C. difficile* and the inhibition of growth of *C. difficile* was observed, although the concentration of iso-caproic acid was parallel to the viable count of *C. difficile*. Similar results were obtained when the toxigenic strain T-C-9 was inoculated (data not shown).

In static culture of *C. difficile*, the addition of either acetic acid or propionic acid to the high concentration detected in the mixed CF culture, did not inhibit growth of *C. difficile* at all (data not shown). However, addition of all four volatile acids (acetic, propionic, butyric and iso-caproic acids) inhibited the growth of *C. difficile* with a marked decrease in pH (pH 4.8) (data not shown).

Analysis of amino acids in mixed CF culture

Concentrations of amino acids in both single CF culture of *C. difficile* and mixed culture of *C. difficile* with various faecal flora were analysed. Aspartic acid, citrulline, leucine, isoleucine, proline, serine and threonine were utilised well by *C. difficile*, including two toxigenic and two non-toxigenic strains, in single culture. In the mixed culture of *C. difficile* strain N8 with *C. perfringens*, *C. ramosum*, *Eu. lentum*, *B. distasonis*, *Ent. avium* and either *E. coli* or *K. pneumoniae*, complete or almost complete consumption of aspartic acid, serine, threonine, arginine and asparagine and marked increase of concentrations of citrulline and ornithine were shown before and after inoculation with *C. difficile* (table VI). Neither cit-
Table VI. Amino-acid analysis of mixed CF culture of C. difficile with various bacterial strains

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>None (A)</th>
<th>C. perfringens</th>
<th>C. perfringens</th>
<th>C. perfringens</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Ent. avium</td>
<td>Ent. avium</td>
<td>Ent. avium</td>
</tr>
<tr>
<td></td>
<td></td>
<td>E. coli</td>
<td>E. coli</td>
<td>E. coli</td>
</tr>
<tr>
<td></td>
<td></td>
<td>B</td>
<td>A</td>
<td>B</td>
</tr>
<tr>
<td>Asp</td>
<td>39</td>
<td>8†</td>
<td>9</td>
<td>98</td>
</tr>
<tr>
<td>Cit</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>265</td>
</tr>
<tr>
<td>Ile</td>
<td>45</td>
<td>105</td>
<td>16</td>
<td>99</td>
</tr>
<tr>
<td>Leu</td>
<td>10</td>
<td>95</td>
<td>8</td>
<td>88</td>
</tr>
<tr>
<td>Pro</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>155</td>
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<td>Ser</td>
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<td>Thr</td>
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<td>0</td>
<td>0</td>
<td>16</td>
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<td>Arg</td>
<td>111</td>
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<td>65</td>
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</tr>
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<td>Asn</td>
<td>87</td>
<td>0</td>
<td>0</td>
<td>56</td>
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<tr>
<td>Glu</td>
<td>127</td>
<td>77</td>
<td>86</td>
<td>70</td>
</tr>
<tr>
<td>Gly</td>
<td>118</td>
<td>73</td>
<td>87</td>
<td>136</td>
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<td>His</td>
<td>149</td>
<td>119</td>
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<td>Lys</td>
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<td>13</td>
<td>68</td>
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<td>Met</td>
<td>76</td>
<td>94</td>
<td>55</td>
<td>101</td>
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<td>Phe</td>
<td>81</td>
<td>92</td>
<td>71</td>
<td>77</td>
</tr>
<tr>
<td>Tyr</td>
<td>123</td>
<td>0</td>
<td>0</td>
<td>76</td>
</tr>
<tr>
<td>Val</td>
<td>90</td>
<td>117</td>
<td>36</td>
<td>120</td>
</tr>
<tr>
<td>Ala</td>
<td>198</td>
<td>132</td>
<td>60</td>
<td>123</td>
</tr>
<tr>
<td>Orn</td>
<td>186</td>
<td>130</td>
<td>102</td>
<td>1456</td>
</tr>
<tr>
<td>Trp</td>
<td>185</td>
<td>53</td>
<td>103</td>
<td>96</td>
</tr>
</tbody>
</table>

* The concentration of each amino acid in fresh GAM broth was expressed as 100.
† The relative concentrations of each amino acid in the culture were assayed before (B) and 7 days after (A) inoculation with C. difficile strain N8.

Table VII. Effect of addition of amino acid into the dialysed culture filtrate harvested after incubation of intestinal bacteria* on the growth of C. difficile strain N8

<table>
<thead>
<tr>
<th>Medium</th>
<th>Viable count of C. difficile (log₁₀ cfu/ml)</th>
<th>pH</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0 h</td>
<td>24 h</td>
</tr>
<tr>
<td>GAM broth</td>
<td>7.1</td>
<td>6.9</td>
</tr>
<tr>
<td>Dialysed culture filtrate†</td>
<td>2.0</td>
<td>2.1</td>
</tr>
<tr>
<td>+ aspartic acid</td>
<td>2.1</td>
<td>2.3</td>
</tr>
<tr>
<td>+ serine</td>
<td>2.0</td>
<td>2.1</td>
</tr>
<tr>
<td>+ threonine</td>
<td>2.0</td>
<td>2.0</td>
</tr>
<tr>
<td>+ arginine</td>
<td>2.1</td>
<td>2.1</td>
</tr>
<tr>
<td>+ asparagine</td>
<td>2.1</td>
<td>2.0</td>
</tr>
<tr>
<td>+ mixture of five amino acids</td>
<td>2.1</td>
<td>6.0</td>
</tr>
</tbody>
</table>

* E. coli, Ent. avium, C. perfringens, C. ramosum, B. distasonis and Eu. lentum were inoculated into a dialysis bag containing 80 ml of GAM broth. The dialysed culture filtrate (120 ml) was collected 7 days after inoculation.
† Each amino acid was added into the dialysed culture filtrate to the same concentration as that in fresh GAM broth.

rulline nor ornithine affected the growth of C. difficile at all when added to GAM broth to the concentration detected in the mixed CF culture (data not shown). The concentrations of amino acids in mixed CF culture of either Ent. avium and E. coli, or C. perfringens, K. pneumoniae and Ent. avium were not significantly different from those of the mixed structure of C. perfringens, Ent. avium and E. coli. Similar results were obtained when toxigenic C. difficile strain T-C-2 was used.
Growth of C. difficile in the dialysed culture filtrate of faecal bacterial strains

To examine the effect of adding the deprived amino acids to the mixed CF culture (table VI), each amino acid (aspartic acid, serine, threonine, arginine and asparagine) was added to the outer dialysed culture filtrate. Although the addition of each amino acid individually did not restore the growth of C. difficile, the addition of all five amino acids resulted in a considerable growth of C. difficile after incubation for 48 h (table VII). Adjustment of the pH of the dialysed culture filtrate to 6.9 resulted in rapid growth of C. difficile after incubation for 24 h as did the use of fresh GAM broth (data not shown).

Discussion

C. difficile is frequently isolated from healthy infants. In this study the isolation rate was c. 65% in babies <1 year old, which was similar to those in previous reports. There have been many reports of the antagonistic effect of micro-organisms isolated from faeces on C. difficile. In these reports, the antagonistic aerobes included Pseudomonas aeruginosa, Staphylococcus aureus, Group D enterococci, Ent. faecalis, Ent. faecium, Streptococcus mitis and Streptococcus sp., and the antagonistic anaerobes included C. beijerinckii, Bacteroides sp., Bifidobacterium adolescentis, Bif. infantis, Bif. longum and Lactobacillus sp. However, these organisms were shown to be antagonistic to C. difficile in vitro when grown together on the surface of agar media. Wilson and Freter took advantage of CF culture, which can serve as a model of the ecology of large intestinal flora, for an analysis of the interaction of C. difficile with hamster microflora. The entire faecal flora of the hamster suppressed the growth of C. difficile in the CF culture before inoculation with C. difficile, but not with that of butyric acid. Rolfe reported that there was a direct correlation between the in-vitro inhibitory activity of the VFAs and the susceptibility of 4-day-old or older hamsters to intestinal colonisation of C. difficile when mixtures of VFA were prepared at concentrations equal to those present in the caeca of hamsters. In contrast, Su et al. showed that VFA could not inhibit intestinal colonisation by C. difficile in gnotobiotic mice associated with hamster intestinal flora. Similarly, Borriello and Barclay showed no correlation between the qualitative VFA analysis of faecal emulsion and the ability to inhibit growth of C. difficile in the in-vitro model. The results of this study show no significant correlation between the concentrations of VFAs and the inhibition of growth of C. difficile after inoculation of C. difficile into CF cultures. The limited growth of intestinal flora in the colonic ecosystem due to a lack of a carbon source has also been reported. It is reported that intestinal strains compete for various nutrients in CF culture, resulting in inhibition of growth of C. difficile as described previously. Furthermore, the ecological mechanism of the suppressive effect of intestinal flora in cytotoxin production by C. difficile has been discussed.

Haslam et al. studied the ability of several strains of C. difficile to grow in defined culture media and showed that proline, valine, leucine, iso-leucine and tryptophane were essential for growth of C. difficile. In this study, aspartic acid, citrulline, iso-leucine, leucine, proline, serine and threonine were shown to be utilised well by C. difficile strains. In the CF cultures that showed inhibition of growth of C. difficile, complete or almost complete consumption of several amino acids and significant increase of citrulline and ornithine were detected. It is possible that these changes in amino acid concentrations are associated with the inhibition of growth of C. difficile. Although the separate addition of each amino acid (aspartic acid, serine, threonine, arginine or asparagine) into the dialysed culture filtrate induced no increase in growth of C. difficile, either the...
addition of the mixture of the five amino acids or the
adjustment of the pH of the filtrate to 6.9 did (table
VII). This also implies that there is no dialysable
substance that is inhibitory to the growth of C. difficile.
It is possible that culture conditions, including pH and
the presence or absence of amino acids as nutrients,
are associated with the inhibition of growth of C.
difficile by faecal bacterial strains.

Interestingly, growth inhibition in the mixed CF
culture of toxigenic C. difficile strains was much
stronger than that in the mixed CF culture of non-
toxigenic strains. It was reported that toxigenic C.
difficile was suppressed when a non-toxigenic strain
was established first in the animal models.4,6 Seal et
al.26 administered a non-toxigenic strain of C. difficile
to treat a case of relapse and to prevent further relapse
in another patient, in both cases successfully. It is
possible that the growth characteristics of C. difficile
may differ in toxigenic and non-toxigenic strains.

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