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

TAKAKO YAMAMOTO-OSAKI, SHIGERU KAMIYA, SADA AKI SAWAMURA, MASANORI KAI and ATSUSHI OZAWA*

Department of Infectious Diseases, Division of Host Defense Mechanism, School of Medicine, Tokai University, Bohseidai, Isehara, Japan

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.^{1–4} Wilson *et al.*² 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.⁴

Bacterial interference between *C. difficile* and other faecal bacteria, such as *Escherichia coli*,⁵ non-toxigenic *C. difficile*^{6–8} and lactobacilli⁹ 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.^{10–14} 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.¹⁵

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

sample of these specimens was spread on the surface of a cycloserine-cefoxitin-fructose agar (CCFA) plate. The CCFA plate modified by Nakamura *et al.*¹⁶ contained cycloserine 300 µg/ml, cefoxitin 10 µg/ml and sodium taurocholate 1 mg/ml (Difco) in *C. difficile* Agar Base (Oxoid) supplemented with egg yolk 5%. The inoculated plate was incubated at 37°C for 3 days in an anaerobic chamber containing an atmosphere of H₂ 10%, CO₂ 10% and N₂ 80%. Isolates were subcultured on to Gifu Anaerobic Medium (GAM) agar (Nissui Chemical Co., Tokyo, Japan) and the pure cultures obtained were assayed for biochemical characteristics. They were identified according to morphology by Gram's staining, biochemical characteristics and fermentation products determined by gas chromatography. Cytotoxicity of *C. difficile* isolates was assayed according to the method described previously.¹⁶

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 Strep 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

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 obligative 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— aspartic 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 ($c. 10^{8-9}$ cfu/ml) continued during the experimental period. However, *C. difficile* did not grow and was washed out. The number of *C. difficile* was 10^2 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 $10^{5.5}$ and $10^{7.6}$ cfu/ml.

When a *C. difficile*-negative faecal specimen was inoculated, it appeared that the total counts of faecal flora reached $> 10^9$ 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*

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

Isolation of <i>C. difficile</i> from faecal sample	Faecal sample no.	Mean (SD) number of <i>C. difficile</i> in CF culture (log ₁₀ cfu/ml)*
Positive†	1	4.8 (1.1)
	2	6.5 (0.6)
	3	6.7 (1.0)
Negative‡	1	3.9 (0.6)
	2	3.2 (1.1)
	3	2.4 (1.1)
	4	6.8 (0.4)

* 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 *Can. 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

Table II. Intestinal flora of *C. difficile*-positive or -negative faecal samples of infants during anaerobic CF culture

<i>C. difficile</i> -negative faeces			<i>C. difficile</i> -positive faeces		
Species	Viable count (log ₁₀ /ml)		Species	Viable count (log ₁₀ /ml)	
	days after inoculation Day 0	17		days after inoculation Day 0	17
<i>Ent. avium</i>	5.0	9.6	<i>Ent. avium</i>	6.0	9.0
<i>E. coli</i>	4.9	8.3	<i>Ent. faecium</i>	5.0	8.6
<i>K. pneumoniae</i>	4.0	9.3	<i>Citrobacter freundii</i>	0.8	7.8
<i>S. capitis</i>	1.8	< 1.0	<i>K. pneumoniae</i>	0.7	7.5
<i>Ent. faecium</i>	< 1.0	9.7			
<i>Providencia rettgeri</i>	< 1.0	8.0			
<i>Ent. faecalis</i>	< 1.0	7.2			
<i>Lactococcus lactis</i>	< 1.0	7.0			
<i>Can. albicans</i>	1.0	< 1.0	<i>Can. albicans</i>	1.2	4.8
<i>B. distasonis</i>	7.3	8.3	<i>Eu. lentum</i>	7.5	8.8
<i>Bif. adolescentis</i>	7.0	8.9	<i>B. distasonis</i>	7.5	8.0
<i>Pstr. productus</i>	6.9	8.9	<i>Bif. adolescentis</i>	7.1	8.7
<i>Eu. lentum</i>	6.7	9.3	<i>L. brevis</i>	7.0	< 1.0
<i>B. vulgatus</i>	6.5	9.5	<i>B. fragilis</i>	6.9	< 1.0
<i>L. acidophilus</i>	6.4	5.0	<i>C. paraputrificum</i>	6.0	< 1.0
<i>C. ramosum</i>	5.7	8.5	<i>L. fermentum</i>	5.8	< 1.0
<i>C. paraputrificum</i>	5.3	< 1.0	<i>Str. intermedius</i>	5.0	< 1.0
<i>L. plantarum</i>	5.3	< 1.0	<i>C. aminovalericum</i>	3.3	< 1.0
<i>Veillonella parvula</i>	5.0	8.0			
<i>Bif. longum</i>	3.8	< 1.0	<i>V. parvula</i>	1.6	9.5
<i>Fusobacterium nucleatum</i>	2.4	< 1.0	<i>C. difficile</i>	3.2	(8.5)
<i>C. perfringens</i>	2.3	9.4			
<i>Str. intermedius</i>	< 1.0	8.5			
<i>L. casei</i> subsp. <i>rhamnosus</i>	< 1.0	8.3			
<i>C. difficile</i>		(2.0)*			

* The number in parentheses is the number of *C. difficile* strain N8 that were inoculated 10 days after inoculation of faecal sample in CF culture to give an initial count of 10⁵ cfu/ml.

Table III. Effect of mixed CF culture with various bacterial strains on the growth of *C. difficile* strain N8

Mixed CF culture	Mean (SD) number of <i>C. difficile</i> harvested 2–7 days after inoculation (log ₁₀ cfu/ml)	pH of the culture	
		Day 0	7 days
None*	8.1 (0.2)	6.6	6.3
<i>C. perfringens</i>	7.5 (0.3)	6.4	6.3
<i>Ent. avium</i> , <i>E. coli</i>	7.4 (0.3)	7.3	6.6
<i>Ent. avium</i> , <i>K. pneumoniae</i> , <i>C. perfringens</i>	7.1 (0.3)	6.3	6.6
<i>Ent. avium</i> , <i>E. coli</i> , <i>C. perfringens</i>	6.9 (0.2)	7.1	6.5
<i>Ent. avium</i> , <i>E. coli</i>	6.2 (0.6)	5.4	5.4
<i>B. distasonis</i> , <i>Eu. lentum</i>			
<i>C. ramosum</i> , <i>C. perfringens</i>			
<i>Ent. avium</i> , <i>K. pneumoniae</i>	5.8 (0.7)	5.5	5.9
<i>B. distasonis</i> , <i>Eu. lentum</i>			
<i>C. ramosum</i> , <i>C. perfringens</i>			

* *C. difficile* strain N8 was incubated in CF culture for 7 days.

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-toxicogenic *C. difficile* than in the CF culture of toxicogenic *C. difficile*. In the mixed CF culture of the toxicogenic strain T-C-9, 1–7 days after inoculation, neither *C. difficile* nor cytotoxic activity was detected. In the case of the toxicogenic 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*

Strain no.		Mean (SD) number of <i>C. difficile</i> (log ₁₀ cfu/ml)		Maximum cytotoxin titre (log ₂)	
		Single CF culture	Mixed CF culture	Single CF culture	Mixed CF culture
Non-toxicogenic	N8	8.2 (0.2)	4.9 (0.3)	ND	ND
	N13	8.1 (0.1)	6.4 (0.3)	ND	ND
Toxicogenic	T-C-2	8.1 (0.2)	2.6 (0.6)	13	4
	T-C-9	8.0 (0.2)	ND	9	ND

ND, not detected.

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

Mixed CF culture with	Concentration* of VFA (μmol/ml)									
	Acetic acid		Propionic acid		Butyric acid		<i>i</i> -Caproic acid		Total VFAs	
	B	A	B	A	B	A	B	A	B	A
<i>C. perfringens</i>	12.4	23.5	0.3	0.4	19.3	23.6	ND	17.2	32.0	64.7
<i>Ent. avium</i> , <i>E. coli</i>	18.6	35.5	0.5	0.6	0.2	16.3	ND	18.8	19.3	71.2
<i>Ent. avium</i> , <i>K. pneumoniae</i> , <i>C. perfringens</i>	24.2	42.9	0.7	0.8	18.7	20.9	ND	19.6	43.6	84.2
<i>Ent. avium</i> , <i>E. coli</i> , <i>C. perfringens</i>	19.6	40.0	0.6	0.4	11.9	20.6	ND	18.0	32.0	79.1
<i>Ent. avium</i> , <i>E. coli</i> , <i>B. distasonis</i> , <i>Eu. lentum</i> <i>C. ramosum</i> , <i>C. perfringens</i>	36.0	35.2	1.3	2.5	21.6	23.2	ND	16.4	58.9	77.3
<i>Ent. avium</i> , <i>K. pneumoniae</i> , <i>B. distasonis</i> , <i>Eu. lentum</i> <i>C. ramosum</i> , <i>C. perfringens</i>	30.1	44.6	1.5	6.3	24.4	23.4	ND	12.9	55.9	87.1

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-toxicogenic 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

Amino acid	None (A)	Relative concentration of amino acid* after co-cultivation of <i>C. difficile</i> with					
		<i>C. perfringens</i> <i>Ent. avium</i> <i>E. coli</i>		<i>C. perfringens</i> <i>C. ramosum</i> <i>Eu. lentum</i> <i>B. distasonis</i> <i>Ent. avium</i> <i>E. coli</i>		<i>C. perfringens</i> <i>C. ramosum</i> <i>Eu. lentum</i> <i>B. distasonis</i> <i>Ent. avium</i> <i>K. pneumoniae</i>	
		B	A	B	A	B	A
Asp	39	8†	9	98	0	7	14
Cit	0	0	0	265	343	181	197
Ile	45	105	16	99	53	110	52
Leu	10	95	8	88	33	96	38
Pro	0	0	0	155	34	62	26
Ser	0	0	0	2	3	3	4
Thr	0	0	0	16	8	8	9
Arg	111	53	65	0	0	0	0
Asn	87	0	0	56	0	0	0
Glu	127	77	86	70	50	12	15
Gly	118	73	87	136	110	158	131
His	149	119	127	116	158	133	156
Lys	0	13	68	16	2	105	118
Met	76	94	55	101	98	112	113
Phe	81	92	71	77	82	81	85
Tyr	123	0	0	76	0	11	12
Val	90	117	36	120	94	139	95
Ala	198	132	60	123	141	133	144
Orn	186	130	102	1456	1812	1275	1534
Trp	185	53	103	96	222	187	160

* 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

Medium	Viable count of <i>C. difficile</i> (log ₁₀ cfu/ml)		pH		
			0 h	24 h	48 h
	24 h	48 h			
GAM broth	7.1	6.8	6.9	6.1	6.4
Dialysed culture filtrate†	2.0	2.1	6.3	6.2	6.1
+ aspartic acid	2.1	2.3	6.3	6.3	6.3
+ serine	2.0	2.1	6.3	6.1	6.2
+ threonine	2.0	2.0	6.3	6.3	6.3
+ arginine	2.1	2.1	6.3	6.5	6.4
+ asparagine	2.1	2.0	6.2	6.4	6.4
+ mixture of five amino acids	2.1	6.0	6.3	6.3	7.4

* *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.^{4, 10-13} There have been many reports of the antagonistic effect of micro-organisms isolated from faeces on *C. difficile*.¹⁷⁻¹⁹ 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 Freter⁵ took advantage of CF culture, which can serve as a model of the ecology of large intestinal flora,²⁰ 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 to 0.0002%, whereas the suppression was only 10% when dissociated CF culture between *E. coli* and *C. difficile* was performed.

In the present study, the effects of both faecal flora and some combinations of faecal isolates on the growth of *C. difficile* were examined in CF culture. The growth of *C. difficile* strain N8 was inhibited more strongly in the CF culture with *C. difficile*-negative faeces than with *C. difficile*-positive faeces. Analysis of the faecal flora showed more intestinal species in *C. difficile*-negative infant faeces than in *C. difficile*-positive faeces. The total bacterial counts and counts of the predominant bacterial species such as *Ent. avium*, *B. distasonis* and *Eu. lentum* in the cultures of *C. difficile*-negative faeces were almost the same as those in the cultures of *C. difficile*-positive faeces. In addition to the effect of faecal flora, some combinations of faecal isolates were investigated for their inhibitory effect on the growth of *C. difficile*. Inhibition of growth was demonstrated in mixed CF culture with intestinal strains of *Ent. avium*, *B. distasonis*, *Eu. lentum*, *C. ramosum*, *C. perfringens* and either *E. coli* or

K. pneumoniae. The pH of the mixed CF culture was markedly decreased. Borriello and Barclay⁷ stated that, in most cases so far examined, the antagonism noted between various components of the faecal flora and *C. difficile* is attributable to the acidic conditions produced. However, it seems that mechanisms other than decreased pH might exist in the CF culture as there was no significant decrease in pH in the mixed CF culture with faecal flora (figs. 1 and 2).

In the CF cultures of *C. difficile* with the faecal flora of infants, the concentration of butyric acid and total VFAs of 10-day cultures after inoculation of *C. difficile*-negative faeces was higher than that after inoculation of *C. difficile*-positive faeces (data not shown). In the mixed CF culture of *C. difficile* with combinations of faecal isolates, inhibition of growth of *C. difficile* was correlated with the concentrations of acetic acid, propionic acid and total VFA in the CF culture before inoculation with *C. difficile*, but not with that of butyric acid. Rolfe²¹ 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.*²² showed that VFA could not inhibit intestinal colonisation by *C. difficile* in gnotobiotic mice associated with hamster intestinal flora. Similarly, Borriello and Barclay⁴ 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.²³ It is reported that intestinal strains compete for various nutrients in CF culture, resulting in inhibition of growth of *C. difficile* as described previously.^{8, 24} Furthermore, the ecological mechanism of the suppressive effect of intestinal flora in cytotoxin production by *C. difficile* has been discussed.²⁴

Haslam *et al.*²⁵ 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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