

MICROBIAL ECOLOGY

# Influence of intestinal anaerobes and organic acids on the growth of enterohaemorrhagic *Escherichia coli* O157:H7

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**A suspension of human faeces (FS) and its anaerobic culture (FC), bacterial metabolic products and organic acids were examined for inhibitory effects on growth and verotoxin 2 (VT2) production of *Escherichia coli* O157:H7 *in vitro*. FS and FC showed a marked inhibitory activity to growth and production of VT2 by *E. coli* O157:H7 under anaerobic conditions. They may have both bacteriostatic and bactericidal effects on *E. coli* O157. The growth of *E. coli* O157 was markedly suppressed by acetic, propionic and butyric acids compared with hydrochloric acid and lactic acid at concentrations between 25 mM and 40 mM, being proportional to the pH values. At pH 5.5, 40 mM of short-chain fatty acids (SCFAs) almost completely inhibited the growth of *E. coli* O157. SCFAs markedly inhibited the growth of *E. coli* O157 at pH 6.0 rather than pH 7.0. Propionic acid is likely to be more suppressive to *E. coli* than acetic and butyric acids. The production of VT2 was approximately proportional to the growth of *E. coli* O157. However, incubation for 24 h *in vitro* showed that the growth and VT2 production of *E. coli* O157 decreased but were not completely inhibited at pH 6.5 and 7.0 in a mixture of acetic, propionic and butyric acids at a physiological concentration (110 mM, 60:25:25, respectively, in molar ratio). It is probable that the colonic microflora could contribute to a reduction of *E. coli* O157:H7 infections *via* the activation of intestinal fermentation by dietary manipulation or something similar to give pH 6.0 or <6.0 and that factors such as age, chemical therapy and body condition, which have effects on the balance of the intestinal microflora, would be associated with the incidence rates of *E. coli* O157 infections.**

## Introduction

In 1996, large outbreaks of enterohaemorrhagic *Escherichia coli* O157:H7 disease occurred in Japan. According to the Annual Report of Food Poisoning Outbreaks in 1996 (Welfare Ministry of Japan), the prevalence of *E. coli* O157:H7 infections in five school outbreaks ranged from 15.7 to 50.3% in children who ate school lunches. The prevalence rates in two homes for the elderly were 11.5% and 18.5%. However, in the same year, the prevalence rate for *E. coli* O157 infection in a company outbreak was only 1.5% in the staff who ate canteen food. Adults, excluding elderly individuals, are considered to be more resistant to *E. coli* O157 infection than children. Other reported

risk factors, apart from being young or elderly, include recent antimicrobial therapy and previous gastrectomy [1]. These factors are considered to be associated with changes in the composition of the intestinal microflora, or with reduced activity of the microflora, which provides protection against bacterial infections of the intestine. The factors associated with this protective activity are pH, redox potential and fermentation products such as acetic, propionic, butyric and lactic acids [2–5]. The present study was undertaken to examine the effect of human faecal mixed cultures and organic acids on the production of verocytotoxin 2 (VT2) and the growth of *E. coli* O157:H7 *in vitro*.

## Materials and methods

### *Bacterial strains and culture media*

*E. coli* O157:H7 strain HYM, a human isolate from Hiroshima, Japan, was kindly provided by Dr K.

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Tamura, Department of Bacteriology, National Institute of Infectious Diseases, Japan. Non-pathogenic *E. coli* strain 128 was obtained from the Laboratory of Animal Cell Systems, Institute of Chemical and Physical Research (RIKEN), Japan. *E. coli* strains were grown aerobically in Trypticase Soy Broth (TSB; BBL, Becton Dickinson Microbiology Systems, Cockeysville, MD, USA).

Fresh faecal suspension (FS) was obtained by homogenising 1 g of fresh faeces from a healthy human adult in 9 ml of Gifu Anaerobic Medium (GAM) broth (Nissui Seiyaku, Tokyo, Japan), and an anaerobic culture of FS (FC) was obtained by 24-h anaerobic cultivation of five-fold dilutions of FS in GAM broth. Anaerobic incubation was performed at 37°C by the steel wool method in an anaerobic jar as described previously [6, 7].

### Organic acids

Stock solutions of acetic, propionic, butyric and lactic acids were sterilised by membrane filtration and diluted in distilled water to yield appropriate concentrations of acids before adding to TSB. The chemicals were purchased from Wako Chemical Co. Ltd (Tokyo, Japan). The pH of TSB with added acids was adjusted with HCl and NaOH.

### Examination for inhibition of *E. coli* by acids

An 18-h culture in TSB of *E. coli* strains, pathogenic and non-pathogenic, was diluted with phosphate buffer solution (1:300). The resulting suspension (0.1 ml) was inoculated at  $1 \times 10^5$  *E. coli* cells/ml into fresh TSB in duplicate with a single acid or mixture of acids and no acid, and incubated aerobically and anaerobically for 6 and 24 h at 37°C without shaking in the anaerobic jar with reduced copper [6]. Acid mixtures at concentrations of 40, 75 and 110 mM were composed of acetic, propionic and butyric acids at a molar ratio of 60:25:25, respectively. The physiological level of acid in the upper part of the human colon is *c.* 110 mM [8]. After incubation, the turbidity of cultures was determined at 650 nm with a spectrophotometer (Bio Spec-1600, Shimadzu, Tokyo, Japan).

### Mixed culture of *E. coli* O157 with faecal suspension (FS) and anaerobic culture of FS (FC)

The TSB culture of *E. coli* O157 was added to 6 ml of GAM broth in 10-ml tubes in duplicate to give a final cell concentration of *c.*  $1 \times 10^5$ /ml, and then inoculated with 0.6 ml of FS (final concentration *c.*  $5 \times 10^8$ /ml) and 0.2 ml of FC (*c.*  $5 \times 10^8$ /ml). The inoculated media were incubated anaerobically for 6 h and 24 h at 37°C. After incubation, cultures were serially diluted in phosphate buffer for enumeration of *E. coli* O157, and 50- $\mu$ l samples of suitable dilutions

were spread in duplicate on Trypticase Soy Agar (TSA; BBL) and incubated aerobically overnight at 37°C [6, 7]. The pH of the cultures was determined with a pH meter. Colonies of *E. coli* grown on TSA in the mixed cultures of FS and FC were examined for VT2 production to identify *E. coli* O157.

### VT 2 assays

To assay VT 2 in broth cultures, each culture was centrifuged at 12 000 *g* for 5 min at 20°C, and then serial two-fold dilutions of supernates (25  $\mu$ l) were examined for agglutination with a VTEC-RPLA (Verocytotoxin-producing *E. coli*-reversed passive latex agglutination) kit (Denka Seiken Co., Tokyo, Japan) according to the manufacturer's instructions. Titres were expressed as the highest dilution that gave agglutination after 24 h at room temperature. The sensitivity of this kit was 1 ng of VT2/ml.

## Results

### Effect of pH on *E. coli* O157 growth under aerobic and anaerobic conditions

Fig. 1 shows the effect of pH on growth of *E. coli* O157 in pH-adjusted TSB under aerobic and anaerobic conditions. The growth of *E. coli* O157 was approximately proportional to the pH value after incubation for 24 h. Anaerobic incubation resulted in less *E. coli* O157 growth than aerobic incubation, with the exception of cultures at pH 4.2 at which *E. coli* O157 did not grow aerobically, but grew slightly in anaerobic conditions.

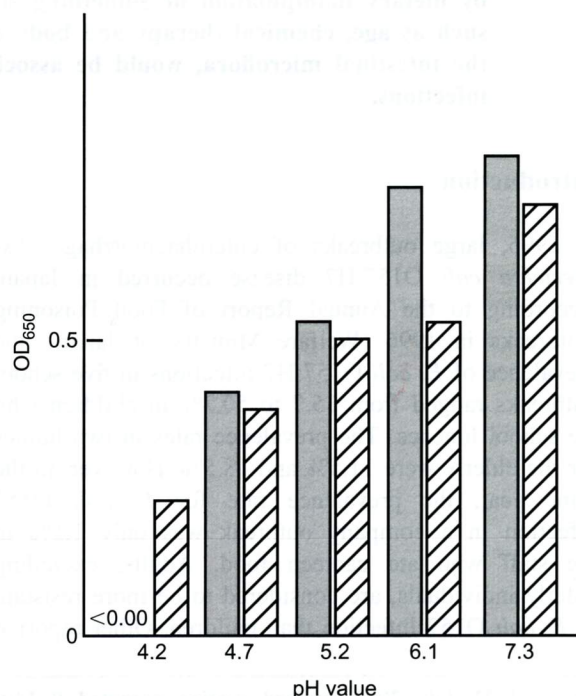


Fig. 1. Effect of pH on growth of *E. coli* O157 in aerobic (□) and anaerobic (▨) cultivation. Bacteria were grown in TSB with the pH adjusted by HCl.

### Effect of organic acids on anaerobic growth and VT2 production of *E. coli* O157

Table 1 shows growth and VT2 titre of *E. coli* O157 at different pH values in acid concentrations of 40 mM under anaerobic conditions. Short-chain fatty acids (SCFAs), such as acetic, propionic and butyric acids, at 40 mM concentration showed marked inhibition of *E. coli* O157 at pH 5.5, both at 6 h and 24 h, and at pH 6.0 at 6 h, whereas hydrochloric and lactic acids showed only slight inhibition of *E. coli* O157 at pH 5.5 compared with pH 6.0 and 7.0. Lactic acid showed slightly higher inhibitory activity than hydrochloric acid at the three pH values after incubation for 6 h but much lower inhibitory activity than SCFAs. VT2 titres were proportional to the amount of growth of *E. coli* O157. With incubation for 6 h at pH 7.0 all SCFAs inhibited production of VT2 and there was slower growth of *E. coli* O157 compared with lactic and hydrochloric acids after incubation for 6 h at pH 7.0. Among SCFAs, propionic acid showed the greatest inhibition of *E. coli* O157 growth.

Table 2 shows the effect of different concentrations (25 mM and 40 mM) of all acids on the growth of *E. coli* O157 and non-pathogenic *E. coli* 128 at pH 6.0 and pH 7.0 under anaerobic conditions. At concentrations of 25 and 40 mM acetic, propionic and butyric acids markedly delayed growth of both strains at pH 6.0 compared with lactic and hydrochloric acids. Propionic acid was the most suppressive for both *E. coli* strains, with markedly reduced growth after 6 h but not 24 h.

### Effect of mixture of SCFA on growth and VT2 production of *E. coli* O157

Table 3 shows the growth and VT2 titre of *E. coli* O157 in media with SCFA mixtures of acetic, propionic and butyric acids at a molar ratio of 60:25:25 under aerobic and anaerobic conditions. Acid mixtures of 40, 75 and 110 mM strongly inhibited growth and VT2 production after incubation for 6 h at pH 6.0, 6.5 and 7.0. After incubation for 24 h, acid mixtures of 75 and 110 mM showed strong inhibition of growth and VT2 production at pH 6.0, while only moderate growth and VT2 production were found at

pH 6.5 and 7.0 at the acid concentrations of 75 and 110 mM. Growth and VT2 production of *E. coli* O157 tended to be greater under aerobic than anaerobic conditions.

### Inhibition of *E. coli* O157 growth and VT2 production by a faecal suspension (FS) and 24-h culture of FS (FC)

The viable count of *E. coli* O157 increased from  $1 \times 10^5$  cfu/ml to  $8 \times 10^5$  cfu/ml after incubation for 6 h compared with that in pure culture ( $7 \times 10^8$  cfu/ml), followed by decreased numbers of *E. coli* O157 after 24 h (Fig. 2a). No VT2 production was detected from the mixed culture after 6 h and 24 h, while higher titres of VT2 were detected in the pure cultures of *E. coli* O157 after culture for 24 h than at 6 h. The pH value of the mixed culture increased from 5.6 after 6 h of cultivation to 6.5 after 24 h. FC also inhibited *E. coli* O157 growth and VT2 production, which was similar to the findings for FS. The pH value of mixed cultures was higher after 24 h (6.3) than after 6 h (5.8) in FC (Fig. 2b). The pH values were higher in both mixed cultures than in the *E. coli* O157 pure culture.

## Discussion

This study indicates that SCFAs are clearly more suppressive to the growth of *E. coli* O157 and the non-pathogenic strain at both lower pH and under anaerobic conditions. SCFAs were much more inhibitory to the growth of *E. coli* than lactic and hydrochloric acids. These findings agree with previous studies on the growth of *Salmonella* and *Shigella* spp. at low pH under anaerobic conditions [2–5]. Furthermore, they suggest that propionic acid is likely to be more inhibitory to the growth of *E. coli* O157 and non-pathogenic strain 128 than acetic and butyric acids during the early period of incubation (6 h).

SCFAs completely inhibited the growth of *E. coli* O157 and production of VT2 at pH 5.5 at a concentration of 40 mM, which is much less than physiological concentrations in the colon. However, in media with a higher pH (pH 6.5–7.0), the mixture of acids did not

**Table 1.** Effects of SCFAs and pH on growth and VT2 production of *E. coli* O157:H7 in anaerobic conditions

Organic acid	OD <sub>650</sub> (log <sub>2</sub> VT2 titre)* at					
	pH 5.5		pH 6.0		pH 7.0	
	6 h	24 h	6 h	24 h	6 h	24 h
Acetic	0.00 (NT)	0.02 (ND)	0.02 (NT)	0.37 (4)	0.09 (ND)	0.49 (5)
Propionic	0.00 (NT)	0.00 (ND)	0.00 (NT)	0.35 (4)	0.04 (ND)	0.41 (5)
Butyric	0.00 (NT)	0.00 (ND)	0.00 (NT)	0.34 (4)	0.07 (ND)	0.47 (5)
Lactic	0.37 (NT)	0.40 (2)	0.37 (NT)	0.46 (5)	0.39 (2)	0.50 (7)
HCl	0.40 (NT)	0.42 (2)	0.42 (NT)	0.49 (6)	0.42 (4)	0.52 (7)

ND, not detected; NT, not tested.

\*Average of duplicate findings.

**Table 2.** Effects of SCFAs and pH on growth of *E. coli* O157:H7 and non-pathogenic *E. coli* in anaerobic conditions

Strain and organic acid	OD <sub>650</sub> * with 25 mM SCFAs at				OD <sub>650</sub> with 40 mM SCFAs at			
	pH 6.0		pH 7.0		pH 6.0		pH 7.0	
	6 h	24 h	6 h	24 h	6 h	24 h	6 h	24 h
O157								
Acetic	0.04*	0.47	0.35	0.70	0.02	0.39	0.26	0.57
Propionic	0.02	0.53	0.26	0.62	0.00	0.38	0.12	0.46
Butyric	0.03	0.47	0.40	0.61	0.01	0.36	0.28	0.52
Lactic	0.35	0.55	0.49	0.66	0.33	0.43	0.41	0.53
HCl	0.35	0.58	0.55	0.67	0.36	0.47	0.43	0.55
Non-pathogenic strain								
Acetic	0.04	0.51	0.84	0.72	0.03	0.51	0.76	0.72
Propionic	0.01	0.52	0.16	0.82	0.01	0.46	0.09	0.72
Butyric	0.01	0.44	0.76	0.79	0.01	0.35	0.63	0.74
Lactic	0.68	0.58	0.86	0.75	0.57	0.59	0.86	0.73
HCl	0.57	0.61	0.89	0.75	0.65	0.66	0.87	0.75

\*Average of duplicate findings.

inhibit growth and VT2 production of *E. coli* O157 even at high concentrations of 75 and 110 mM after incubation for 24 h. These findings suggest that the colon contents probably should be kept at pH 6.0 or lower for prevention of *E. coli* O157 growth and VT2 production for a 24-h incubation period even at physiological concentrations (*c.* 110 mM) of SCFA in the colon. The upper part of the colon is, on average, at *c.* pH 6.0 or slightly less in healthy individuals [8]. Therefore, the normal colon should inhibit *E. coli* O157 for at least 24 h after ingestion, although there are individual differences in the colonic pH. However, people who have a colon pH of  $\geq 6.5$  could be susceptible to *E. coli* O157 infections. Therefore, it may be important to ingest food supplements such as non-digestible oligosaccharides and polysaccharides favourable for bacterial fermentation as prebiotics in the colon with the intention of preventing infection or growth of *E. coli* O157 [8–12].

FS and FC may inhibit growth and VT2 production of *E. coli* O157 in two stages. *E. coli* O157 showed only slight growth after incubation for 6 h and then the viable count decreased even when the pH increased after 24 h. During the early stage of incubation, SCFAs and the low pH of the mixed culture with FS or FC

probably inhibit the *E. coli* O157 growth and VT2 production bacteriostatically. During later stages, some bactericidal substances that are produced by the faecal microflora may decrease the number of *E. coli* O157 under neutral conditions. These findings suggest the presence of not only SCFAs but also anti-*E. coli* O157 substances produced by anaerobic bacteria in the colon. It is not clear what anti-*E. coli* O157 substances are produced *in vitro*. However, anti-*E. coli* O157 substances may act under neutral conditions, which suggests that they may be a combination of hydro-sulphide and alkaline substances, such as ammonia and amines, produced in the later stages of cultivation and after exhaustion of sugar under anaerobic conditions. Hydrogen sulphide has been reported to be inhibitory to the growth of *E. coli* at neutral pH [13]. Furthermore, the present observations are similar to the finding that the population of *E. coli* O157 in bovine faeces increased and then decreased with increased pH during an incubation period of 24 h at 37°C [14]. However, stimulation of hydrogen sulphide production in the colon is unlikely to be recommended because it is toxic.

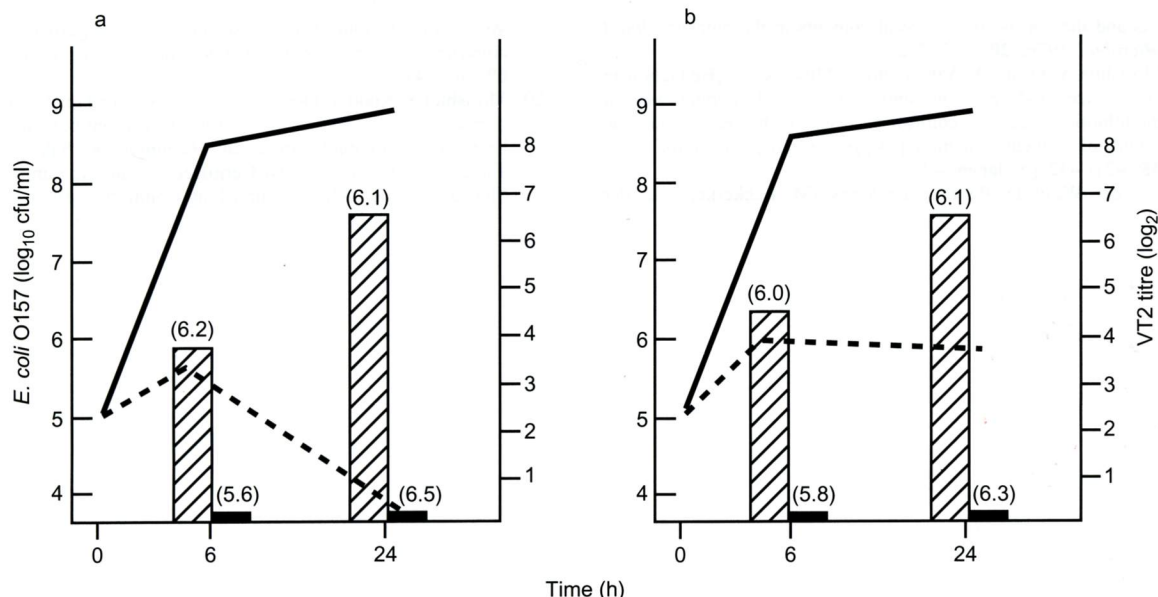
The population of non-pathogenic *E. coli* in the large bowel decreases with colonisation of anaerobic bacteria

**Table 3.** Effects of mixtures of SCFAs on growth and VT2 production of *E. coli* O157 in aerobic and anaerobic conditions

Concentration (mM)	Incubation time (h)	OD <sub>650</sub> (log <sub>2</sub> VT2 titre) at					
		pH 6.0		pH 6.5		pH 7.0	
		Aerobic	Anaerobic	Aerobic	Anaerobic	Aerobic	Anaerobic
0	6	0.38 (3)*	0.23 (2)	0.43 (4)	0.26 (2)	0.47 (5)	0.27 (3)
	24	0.77 (5)	0.54 (4)	0.81 (8)	0.57 (7)	0.78 (8)	0.60 (7)
40	6	0 (ND)	0.00 (ND)	0.06 (ND)	0.04 (ND)	0.21 (1)	0.09 (ND)
	24	0.43 (NT)	0.41 (NT)	0.61 (NT)	0.48 (NT)	0.75 (NT)	0.58 (NT)
75	6	0.00 (ND)	0.00 (ND)	0.02 (ND)	0.02 (ND)	0.10 (ND)	0.06 (ND)
	24	0.06 (ND)	0.06 (1)	0.56 (4)	0.46 (4)	0.68 (6)	0.52 (4)
110	6	0.00 (ND)	0.00 (ND)	0.01 (ND)	0.01 (ND)	0.05 (ND)	0.03 (ND)
	24	0.00 (ND)	0.01 (ND)	0.48 (3)	0.44 (3)	0.63 (5)	0.52 (4)

ND, not detected; NT, not tested.

\*Average of duplicate findings.



**Fig. 2.** Inhibition of growth and VT2 production of *E. coli* O157 strain HYM in mixed cultures under anaerobic conditions by (a) faecal suspension (FS) and (b) 24-h culture of FS (FC). Lines indicate growth of *E. coli* O157 (—, pure culture; ---, mixed culture), bars express the titre of VT2 (▨, *E. coli* pure culture; ■, mixed culture) and the pH of cultures is shown in parentheses.

in the intestine of animals and man [15–18]. This is associated with colonisation resistance flora (CRF) and chloroform-resistant bacteria (CRB) that are important for inhibiting *E. coli* growth [17, 19, 20]. CRB include colonic clostridia and they contribute not only to decreasing the population of *E. coli* but also to inhibiting *E. coli* translocation from the intestinal tract [20]. CRB are probably the more important part of the CRF. Therefore, it is understandable that FS and FC should inhibit growth and VT2 production of *E. coli* O157, as FS and FC contain anaerobic bacteria including CRB.

*E. coli* O157 infections may be associated with changes in the activity of the colonic microflora. The present findings suggest that SCFAs, lower pH and certain bactericidal substances produced by intestinal anaerobes in the colon may be important for reducing the incidence of *E. coli* O157 infections. Stimulation of acid fermentation may also be important for prevention of *E. coli* O157 infections. For that purpose ingestion of food and food supplements containing non-digestible carbohydrates is recommended.

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Fig. 1. Relationship between cecal size and bacterial populations in mice. The x-axis represents cecal size (mm) and the y-axis represents bacterial populations (log CFU/g). The solid line shows the relationship in conventional mice, and the dashed line shows the relationship in gnotobiotic mice.

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