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Effect of lactulose on short-chain fatty acids and lactate production and on the growth of faecal flora, with special reference to *Clostridium difficile*

Y. ITO, H. MORIWAKI, Y. MUTO, N. KATO*, K. WATANABE* and K. UENO*†

First Department of Internal Medicine and *Institute of Anaerobic Bacteriology, Gifu University School of Medicine, 40 Tsukasa-machi, Gifu 500, Japan

Lactulose exerts a beneficial effect on hepatic encephalopathy by decreasing toxic short-chain (iC4–nC6) fatty acid (isobutyrate, butyrate, isovalerate, valerate, isocaproate and caproate) production. However, the precise mechanism by which lactulose exerts this effect remains uncertain. This study investigated the effect of lactulose on faecal flora, particularly *Clostridium difficile*, which produces mostly iC4–nC6 fatty acids. An in-vitro faecal incubation system was used to estimate how lactulose influences production of short-chain (C2–nC6) fatty acids and lactate. Faecal specimens were collected from patients with liver cirrhosis, who carried *C. difficile* in the colon. Supplementation of lactulose along with blood in faecal specimens decreased iC4–nC6 fatty acids production and increased acetate and lactate production, resulting in increased faecal acidity. These changes were statistically significant when compared with supplementation by blood alone. Quantitative faecal culture demonstrated that lactulose supplementation suppressed the growth of *C. difficile* and *Bacteroides* spp. (*B. fragilis* group), iC4–nC6 fatty acids-producing organisms. These results suggest that decreased faecal levels of iC4–nC6 fatty acids after lactulose supplementation may be related to suppression of iC4–nC6 fatty acids-producing faecal organisms, especially *C. difficile*.

Introduction

Cerebrotoxic substances derived from the gut such as ammonia, short-chain fatty acids (SCFAs), mercaptans and γ -aminobutyric acids are thought to be responsible for hepatic encephalopathy (HE). While ammonia is most widely studied among these toxic substances, hyperammonaemia is not always associated with HE. Increased blood levels of isobutyrate (iC4), butyrate (nC4), isovalerate (iC5), valerate (nC5), isocaproate (iC6) and caproate (nC6) in patients with HE [1] and induction of HE by these SCFAs in animals [2] have been reported.

Lactulose is a disaccharide (β 1-4 galacto-fructose) which has been used extensively since 1966 in the treatment of HE [3]. Taken orally, lactulose passes unchanged into the colon where it is hydrolysed by bacterial action to organic acids, principally acetate

and lactate. Although the precise mode of action of lactulose remains unclear, proposed mechanisms are: (i) lowering colonic pH, thereby decreasing the production of ammonia by bacteria [4] and the absorption of non-ionised ammonia [5]; (ii) serving as substrate to increase the incorporation of ammonia into bacterial protein [6]; and (iii) decreasing the intestinal transit time available for production and absorption of ammonia because of its cathartic effect [7]. Mortensen *et al.* [8] showed that lactulose decreased the degradation of amino acids, albumin and blood to iC4–nC6 fatty acids by its acidifying effect.

The majority of SCFAs are produced by anaerobic bacteria that ferment dietary carbohydrates to SCFAs in the colon [9]. These bacteria include *Clostridium* spp., *Bacteroides* spp. (*B. fragilis* group), *Fusobacterium* spp. and *Megasphaera* spp. Among them, the *Bacteroides* spp. are one of the major components of intestinal flora, contributing to ammonia production but producing only a small amount of iC5. In contrast, *C. perfringens* produces a large amount of nC4 and *C. difficile* produces large amounts of iC4–nC6 fatty acids [10, 11].

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Corresponding author: Dr Y. Ito.

†Present address: Gifu College of Medical Technology, Seki City, Gifu 501-32, Japan.

A previous study showed an overgrowth of *C. difficile* and *C. perfringens* in the faecal flora of patients with HE, which was induced by administration of antimicrobial agents [12]. The rate of carriage of *C. difficile* was *c.* 20% in patients with liver cirrhosis (unpublished observations) and 7% in healthy elderly adults [13]. Although these observations indicate that *Clostridium* spp. are of clinical importance in the development of HE, the effect of lactulose on *C. perfringens* has been examined in only a few studies [14, 15] and no report has focused on *C. difficile*.

The aim of this study was to evaluate the effect of lactulose on the faecal levels of SCFAs and lactate as well as on the growth of intestinal flora, especially *C. difficile*, with an in-vitro faecal incubation system, where faecal specimens containing *C. difficile* were employed.

Materials and methods

Patients

Six inpatients with liver cirrhosis, who carried *C. difficile*, provided stool specimens. No antimicrobial agents had been prescribed during the 4 weeks prior to the study. Clinical characteristics and laboratory data on admission are shown in Table 1. Two patients had mild diarrhoea while others had normal faeces.

Preparation of samples

The in-vitro faecal incubation system was similar to that of Mortensen *et al.* [16]. Freshly passed faeces were homogenised with five volumes of 100 mM NaCl and 50 mM KCl. Ten ml of faecal homogenates were mixed with one of the following; blood (0.5 ml/10 ml faecal homogenate), lactulose (25 mmol/L) and blood (0.5 ml/10 ml faecal homogenate) or no addition. Each faecal sample was then incubated anaerobically at 37°C for 24 h in an atmosphere of N₂ 80%, H₂ 10% and CO₂ 10%.

Bacteriological examination

One ml of the incubated faecal sample was homogenised with 9 ml of anaerobic diluent, and serial 10-fold dilutions were made to give a final dilution of 10⁷. Each odd number of logarithmic dilution was inoculated quantitatively onto the media shown in Table 2. Aerobic and anaerobic cultures were incubated at 37°C for 72 h. The number of organisms was expressed as log₁₀ cfu/ml of incubated faecal sample. Isolates were identified to genus level according to standard methods [10, 11, 17].

Determination of SCFAs and lactate in incubated faecal samples

One ml of the incubated faecal sample was mixed with 0.1 ml of H₂SO₄ 50% and 1 ml of diethyl ether, and centrifuged briefly to make an ether extract for the determination of SCFAs – acetate (C2), propionate (C3), iC4, nC4, iC5, nC5, iC6 and nC6 [10]. For the determination of lactate, a mixture of 1 ml of the incubated faecal sample, 0.1 ml of H₂SO₄ 50% and 1 ml of (CH₃OH)₂BF₃ was heated at 100°C for 5 min

Table 2. Media for the isolation of aerobic and anaerobic bacteria from faecal samples

Medium	Selectivity
Anaerobic	
Modified GAM agar*	Non-selective
CCMA agar*	<i>C. difficile</i>
Kanamycin CW egg-yolk agar*	<i>C. perfringens</i>
Modified FM agar*	<i>Fusobacterium</i> spp.
BL blood agar*	<i>Bifidobacterium</i> spp.
LBS agar†	<i>Lactobacillus</i> spp.
BBE agar‡	<i>Bacteroides</i> spp.
PMS blood agar§	<i>Megasphaera</i> spp.
Aerobic	
TSA blood agar*	Non-selective
DHL agar*	Enterobacteriaceae

*Nissui Pharmaceutical Co. Ltd.

†BBL Microbiology Systems.

‡Kyokuto Pharmaceutical Industries Co. Ltd.

§EG agar (Nissui) supplemented with PMS 1% solution (70 mg crystal violet and 50 mg achromycin were dissolved in distilled water to make 100 ml of PMS solution).

Table 1. Clinical characteristics and laboratory data of six patients studied

Parameter	Normal range	Case 1	Case 2	Case 3	Case 4	Case 5	Case 6
Age	...	64	65	67	61	78	64
Sex	...	Male	Male	Male	Male	Female	Male
TP	(63–82 g/Q)	72	94	75	56	52	55
Albumin	(37–50 g/Q)	26	22	21	21	25	21
TB	(6.8–20.5 µmol/Q)	23.9	99.2	128.3	58.1	44.5	37.6
GOT	(0.08–0.58 µkat/Q)	1.70	3.08	1.88	7.83	0.75	1.58
GPT	(0.08–0.58 µkat/Q)	0.98	1.70	0.88	2.87	0.35	1.25
PT	(10.0–14.0 s)	15.0	21.9	22.1	15.0	17.7	14.2
BUN	(3.0–7.5 mmol/Q)	19.1	32.9	7.2	15.1	4.3	11.5
Creatinine	(35–97 µmol/Q)	141	283	53	177	53	80
Ammonia	(<44 µmol/Q)	21	39	48	10	64	58
HBsAg	...	–	–	–	–	–	–
HCVAb	...	+	–	+	+	+	+
Defaecation/day	...	4	3	1	3	2	4
Form of stool	...	Soft	Normal	Normal	Soft	Normal	Normal

TP, total protein; TB, total bilirubin; GOT, glutamate oxaloacetate transaminase; GPT, glutamate pyruvate transaminase; PT, prothrombin time; BUN, blood urea nitrogen; HBsAg, hepatitis B surface antigen; HCVAb, hepatitis C antibody; +, positive; –, negative. All cases were diagnosed as liver cirrhosis with hepatocellular carcinoma.

and centrifuged briefly with 1 ml of chloroform to make a chloroform extract. These extracts were subjected to gas-liquid chromatography (Shimadzu GC-14A, Shimadzu Corp., Kyoto, Japan) with a 1.6 m × 3.2-mm column packed with Reoplex 400 10% on Chromosorb W(AW-PMCS) (80-100 mesh). Gas flows were 50 ml/min for N₂, 50 ml/min for H₂ and 1000 ml/min for air. Temperatures were kept at 150°C for the column and 220°C for the injector and the flame-ionisation detector.

Production of SCFAs and lactate by isolates from faeces

A standard loopful of isolate was inoculated into Gifu Anaerobic Medium (GAM) broth [18] (Nissui Pharmaceutical Co., Ltd) and incubated anaerobically at 37°C for 3–4 days. SCFAs and lactate production were determined as described above.

pH analysis

The pH of incubated faecal samples was measured with pH test paper (Advantec[®], Toyo Roshi Co. Ltd, Tokyo, Japan).

Statistical methods

All comparisons were performed by paired *t* test with Statview software (Abacus Concepts Inc., Berkeley, CA, USA).

Results

SCFAs and lactate levels in incubated faecal samples

Following incubation, the faecal samples of all six individuals showed production of C2–nC6 fatty acids. The addition of blood increased acetate, iC4–nC6 and lactate production while addition of lactulose extinguished the effect of blood on iC4–nC6 fatty acids production and increased faecal levels of acetate and lactate (Table 3). A marked decrease in faecal pH was observed following the addition of lactulose.

Changes in faecal flora

The growth of *C. difficile*, *Bacteroides* spp., *Bifidobacterium* spp. and Enterobacteriaceae was significantly suppressed by the addition of lactulose, while growth of *C. perfringens* was not suppressed (Table 4).

Table 3. Effect of lactulose and blood on production of short-chain fatty acids and lactate, and pH values in six incubated faecal samples

Supplement to faecal specimen	Mean (SD) pH value	Mean (SD) concentration (mmol/L)		
		acetate	iC4–nC6	lactate
None	6.2 (0.2)	24.55 (13.77)	7.22 (5.79)	ND
Blood	6.3 (0.2)	32.62 (12.30)*	13.23 (7.61)*	1.70 (1.72)
Blood + lactulose	3.9 (0.3) [†]	75.20 (42.61) [†]	2.53 (1.18) [†]	109.58 (74.20) [†]

iC4–nC6 = sum of iC4, nC4, iC5, nC5, iC6 and nC6. iC4, isobutyrate; nC4, butyrate; iC5, isovalerate; nC5, valerate; iC6, isocaproate; nC6, caproate. N.D., not detectable.

**p* < 0.05 versus medium without supplement.

[†]*p* < 0.05 versus blood.

**p* < 0.001 versus blood.

Table 4. Effect of lactulose and blood on the composition of faecal flora

Organism	Mean (SD) log ₁₀ cfu/ml and frequency of occurrence () in faecal specimen supplemented with		
	No supplement	Blood	Blood + lactulose
Total anaerobes	9.3 SD 0.5 (6/6)	9.4 SD 0.4 (6/6)	8.8 SD 1.2 (6/6)
<i>C. difficile</i>	5.5 SD 1.8 (6/6)	6.1 SD 1.3 (6/6)	3.2 SD 0.8 [‡] (5/6)
<i>C. perfringens</i>	5.2 SD 1.8 (5/6)	5.2 SD 2.0 (5/6)	5.0 SD 1.9 (5/6)
<i>B. fragilis</i> group	8.6 SD 0.8 (6/6)	8.8 SD 0.5 (6/6)	3.7 SD 2.0 [†] (4/6)
<i>Fusobacterium</i> spp.	2.5 SD 1.2 (1/6)	2.5 SD 1.2 (1/6)	ND*
<i>Megasphaera</i> spp.	ND*	ND*	ND*
<i>Bifidobacterium</i> spp.	7.7 SD 2.9 (5/6)	7.7 SD 2.9 (5/6)	ND [†]
Total aerobes	8.6 SD 0.4 (6/6)	8.9 SD 0.5 (6/6)	8.5 SD 0.5 (6/6)
<i>Lactobacillus</i> spp.	7.7 SD 1.0 (6/6)	7.8 SD 1.1 (6/6)	8.3 SD 0.7 (6/6)
Enterobacteriaceae	6.7 SD 2.1 (6/6)	7.0 SD 1.9 (6/6)	2.3 SD 0.7 [†] (1/6)

Figures in parentheses are frequency of occurrence (number of subjects yielding the organisms/number of subjects examined).

*ND (not detectable) is calculated as 2.0.

[†]*p* < 0.01 versus blood.

[‡]*p* < 0.001 versus blood.

Lactobacillus spp. became the major component after lactulose was added to faecal samples.

SCFAs and lactate production by faecal isolates

Bacteroides isolates produced iC5, *Fusobacterium* and *C. perfringens* isolates produced nC4, while *C. difficile* isolates produced iC4–nC6 fatty acids.

Discussion

Although the precise mode of action of lactulose has not been clarified, one of the speculated mechanisms is the acidification of the colon contents by fermented products of lactulose. Mortensen *et al.* [16] showed that iC4–nC6 fatty acid production by faecal flora was increased by the addition of blood, albumin and amino acids but decreased by adding lactulose *in vitro*. They initially suggested that this effect was the result of substrate competition, i.e., colonic bacteria preferred lactulose to blood when both were present. Later it was reported that the acidifying effect of lactulose was more important in decreasing iC4–nC6 fatty acids production [8].

The present study confirmed that the addition of lactulose decreased faecal levels of iC4–nC6 fatty acids, increased acetate production and reduced faecal pH. It further demonstrated that lactulose caused an increase in lactate production in the faecal incubation system, which contributed to an increase in faecal acidity. Of particular importance was the finding that the growth of both *C. difficile* (mostly iC4–nC6 fatty acid-producing) and *Bacteroides* spp. (iC5-producing) was suppressed by lactulose.

The growth of *C. difficile* is particularly susceptible to changes in colonic acidity [19]. Rolfe *et al.* [20] showed that, *in vitro*, *Lactobacillus* spp. and group D enterococci produced large amounts of lactate that inhibited the growth of *C. difficile*. They also suggested that SCFAs played an important role in the induction of colonisation resistance against *C. difficile* in a conventional hamster model [21]. The *in vitro* study by May *et al.* [22] suggested that acidification with SCFAs produced by fermentation of dietary fibre suppressed *C. difficile*.

The study data can be interpreted as follows: acidification with acetate and lactate produced by fermentation of lactulose suppressed faecal anaerobes, especially *C. difficile*, resulting in a decrease of iC4–nC6 fatty acids production.

Others have reported the effect of oral lactulose on human faecal flora *in vivo*. An increase in *Lactobacillus* and *Bifidobacterium* spp. and a decrease in *Bacteroides* spp. and coliform organisms (Enterobacteriaceae) were noted [15, 23]. These *in vivo* findings

are similar to the *in vitro* results of the present study except for *Bifidobacterium* spp. The conflicting *Bifidobacterium* spp. data seem to result from a difference in environmental pH. The pH in the right colon decreases to 4.85 after ingestion of lactulose [24], whereas the pH was below 4 in the *in vitro* faecal incubation system. As reported by Vince *et al.* [6], when the pH was uncontrolled and acidic conditions developed, *Bifidobacterium* spp. were suppressed by the addition of lactulose to samples. In any event, the action of lactulose in increasing the number of acid (lactate and/or acetate)-producing bacteria such as *L. acidophilus*, *Enterococcus* spp. or *Bifidobacterium* spp. seems to be important. Administration of these bacteria was shown to have an effect on HE [25–27].

Furthermore, a poorly absorbed disaccharide, lactitol, which was shown to improve HE, induces colonic acidification to a similar degree to lactulose [28]. Oligosaccharides taken orally significantly increased the number of *Bifidobacterium* spp. in human faecal flora [29]. Dietary fibre, which escapes from host digestion and is fermented to acetate, propionate and butyrate in the colon [9], was shown to increase the number of acid-producing bacteria but decrease the number of *C. difficile* and resultant toxin [22]. A vegetable protein diet supplemented with dietary fibre was also effective in HE [30]. In addition to lactulose, these substances may also exert a beneficial effect on HE by suppressing the growth of the iC4–nC6 fatty acids-producing organisms, *C. difficile* and *Bacteroides* spp., through colonic acidification. Further *in vivo* studies are needed to establish the mechanisms of action of these substances.

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