Effects of Administration of TOS and *Bifidobacterium breve* 4006 on the Human Fecal Flora

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We studied the effects of administration of TOS, a new growth factor derived from lactose for *Bifidobacterium*, and *Bifidobacterium breve* 4006 on the fecal flora of normal subjects. All of the *Bifidobacterium* species tested, eight reference strains and *B. breve* 4006 were capable of fermenting TOS *in vitro*, while others, 2 *Bacteroides* strains and 4 *Lactobacillus* and *Enterobacteriaceae* strains, showed an appreciable growth among 55 cultures tested. It was evident that TOS is not intestinally absorbed by the recipient subjects, from hydrogen breath test. *In vivo*, TOS (3 g or 10 g/day) was observed to promote the growth of both administered *B. breve* 4006 and resident *Bifidobacterium* strains. Simultaneous administration of *B. breve* 4006 and TOS caused the suppression of gram negative anaerobes and aerobes, *Bacteroidaceae* and *Enterobacteriaceae*, and the reduction of fecal ammonia and urinary indican excretion. It is concluded that TOS is a typical bifidus factor.

Key words: Bifidus-growth factor; TOS (Transgalactosylated Oligosaccharide); *Bifidobacterium breve* 4006; fecal flora; ammonia and indican excretion

It has been recognized that breast-feeding prevents infants from a variety of diarrheal and respiratory infections which are major causes for infantile morbidity and mortality (5, 6). Extensive studies have shown the significance of anti-infectious factors in breast milk, notably the maternal immune factors and the bifidus factor (13, 23).

Fecal microbiological examination shows that *Bifidobacterium* (10^{10} – 10^{11} /g of feces) is predominant in the intestine of the breast-fed infants, whereas the bottle-fed infants colonize *Bacteroides*, *Peptostreptococcus*, *Enterobacteriaceae* and *Streptococcus* to a similar proportion with *Bifidobacterium* (2, 16, 23). It is evident that acetic and lactic acid in the colon, produced by the bifidus flora of breast-fed infants, are involved in defense mechanisms against gastroenteritis (3).

Much effort has been made to stimulate

the propagation of bifidobacteria in bottle-fed infants either by adding bifidus-growth factors or the direct implantation of bifidobacteria. Bifidus-growth factors were reported such as N-acetyl-D-glucosamine (8), lactulose (7, 15), panthetine (17), bovine casein hydrolysates (12) and oligosaccharides (25). However most of the bifidus-growth factors, reported so far, were found to be effective *in vitro* only, while the *in vivo* activity of these factors remained obscure or unsatisfactory (4, 9).

The present study was undertaken to determine the effects of the administration of transgalactosylated oligosaccharides (TOS), the new bifidus factor, or *Bifidobacterium breve* 4006 on ecology or metabolism of the fecal flora. The TOS was isolated by the present authors during incidental transfer reactions to a hydrolysis reaction when

	Gal— $\overset{X}{\text{---}}$ —(Gal) $_n$ — $\overset{Y}{\text{---}}$ —Glc
Saccharide	Gal: Galactose Glc: Glucose
	$n=1-4$
Linkage	$X=\beta-1,6>\beta-1,4, \beta-1,3$ $Y=\beta-1,4>\beta-1,3, \beta-1,6$
Composition	Trisaccharides : $55\pm 2\%$ Tetra- : $33\pm 2\%$ Penta- & Hexa- : $12\pm 2\%$

Fig. 1. Structure of TOS (Transgalactosylated Oligosaccharide).

lactose was subjected to an enzymatic hydrolysis by β -galactosidase produced by *Aspergillus oryzae*. The molecular formula for the effective constituent of TOS is Gal-(Gal) $_n$ -Glu, wherein Gal, Glu and n denote a galactose residue, glucose residue and an integer of 1 to 4 respectively (Fig. 1). The methods for preparation and purification of TOS was described in the Japanese Patent Publications Nos. (104885, 1980). The *Bifidobacterium breve* 4006 was the predominant species in breast-fed infants and was isolated from them by the present authors.

1. Materials and Methods

Subjects: Sixteen healthy men, ranging from 25 to 35 years of age, were employed in the study. None received medication within 3 weeks before or during the study. Written consent was previously obtained from each subject.

Utilization of TOS *in vitro* and *in vivo*: Prior to the feeding experiment with *Bifidobacterium breve* 4006 and/or TOS, we tested the hypothesis that TOS is exclusively utilized by bifidobacteria *in vitro*. The ability of *Bifidobacterium* and the other strict or facultative anaerobes to ferment TOS in 1% solution was tabulated in Table 1. All of the *Bifidobacterium* strains tested, eight reference strains and *B. breve* 4006 expressed an active growth in TOS medium within 24 hr. On the contrary, none of 55 cultures of other genera showed growth except 2 strains of *Bacteroides* and 4 strains of *Lactobacillus* and *Enterobac-*

Fig. 2. Maximum increases of breath H₂ excretion after oral administration of TOS (0.5 g/kg) in 5 normal subjects. Increases in H₂ is expressed in terms of ml/min per m² body surface area. Breath hydrogen test was performed by the methods of Nose et al. (14).

teriaceae. Since the results showed that TOS was a suitable carbohydrate for growth of bifidobacteria *in vitro*, we attempted to confirm the non-absorbability of TOS *in vivo* by using the hydrogen breath test (14). The mean hydrogen response-time curves of the 5 healthy subjects, arbitrarily selected, were presented in Fig. 2. After the ingestion of 0.5 g of TOS per kilogram body weight by the fasting subjects, the H₂ concentrations in the breath samples were determined for the following period of 4 hr by means of gas chromatography. The values of maximum increases in breath hydrogen concentrations above the basal level in fasting subjects were higher than 0.05 ml/min/m² of body surface in all 5 subjects. These results are comparable with the observation on the lactulose-hydrogen breath test by Nose et al. (14). Therefore, it is concluded that TOS is metabolized by intestinal bacteria with the production of hydrogen while it is absorbed very limitedly by the recipient subjects, which is analogous to the case of lactulose.

The feeding experiment: The three feeds studies were conducted as follows.

Group I; TOS feeding (3 g or 10 g per day), 5 subjects.

Table 1. Utilization of TOS by *Bifidobacterium* and other strict or facultative anaerobes^a

<i>Bifidobacterium</i> $\frac{8}{8}$			<i>Clostridium</i> $\frac{1}{8}$		<i>Streptococcus</i> $\frac{1}{4}$	
<i>B. bifidum</i> E 319	+++ ^b		<i>C. sporogenes</i>	—	<i>S. faecium</i>	
<i>B. infantis</i> S12	+++		<i>C. perfringens</i>		ATCC 19434	+
<i>B. lactentis</i> 659	+++		Type A PB6K	—	H 129	—
<i>B. liberorum</i> S76C	+++		<i>C. perfringens</i>		H 31	+
<i>B. breve</i> S1	+++		Type B NCTC 4969	—	<i>S. faecalis</i>	
<i>B. breve</i> AS50	+++		<i>C. butyricum</i>	—	ATCC 19433	—
<i>B. longum</i> E194b	+++		<i>C. bifementans</i>		H 543	—
<i>B. adolescentis</i>			NCTC 506	—	<i>S. bovis</i>	
E194a	+++		<i>C. sordelli</i> CN 4707	—	ATCC 9809	—
<i>Bacteroides</i> $\frac{8}{8}$			<i>C. difficile</i>		H 324	—
<i>B. fragilis</i>			ATCC 9689	+	<i>S. salivarius</i>	
ATCC 12522	+++		<i>C. paraputrificum</i>		ATCC 9759	—
<i>B. fragilis</i>			ATCC 25780	—	H 153	++
NCTC 9343	+++		<i>Propionibacterium acnes</i>		<i>S. avium</i>	
<i>B. ovatus</i>			ATCC 6919	—	H 455	—
VPI 10649	+		<i>Lactobacillus</i> $\frac{8}{14}$		H 456	—
<i>B. vulgatus</i>			<i>L. plantarum</i>		<i>S. equinus</i>	
ATCC 8482	+		YIT 0160	+++	H 311	—
<i>B. distasonis</i>			YIT 0166	++	<i>S. mitis</i>	
VPI 4243	+		YIT 0172	++	H 515	++
<i>Fusobacterium</i> $\frac{8}{8}$			<i>L. casei</i>		<i>S. durans</i>	
<i>F. russi</i>			YIT 0153	+++	H 36	++
VPI 0307	—		<i>L. fermentum</i>		<i>Enterobacteriaceae</i> $\frac{8}{8}$	
<i>F. varium</i>			YIT 0151	—	<i>E. coli</i>	
ATCC 8501	—		YIT 0156	++	IFO 12734	++
<i>F. necrophorum</i>			YIT 0159	++	H-UWR-1	+++
ATCC 25286	—		<i>L. acidophilus</i>		H-UWR-2	+++
<i>Eubacterium</i> $\frac{8}{8}$			YIT 0173	—	<i>Klebsiella pneumoniae</i>	
<i>E. aerofaciens</i>			ATCC 11506	+++	ND-11	+++
VPI 1003	—		ATCC 19992	—	<i>Citrobacter freundii</i>	
<i>E. contortum</i>			<i>L. salivarius</i>		UWR-10	+++
VPI 8700	—		ATCC 11741	+++	<i>Enterobacter cloacae</i>	
<i>E. alactolyticum</i>			<i>L. helveticus</i>		11-3	—
VPI 8615b	—		ATCC 15009	—	<i>Staphylococcus aureus</i>	
<i>E. cylindroides</i>			<i>L. bulgaricus</i>		FDA 209p	—
VPI 8072	—		ATCC 11842	—		
<i>E. limosum</i>			<i>L. brevis</i>			
VPI 6684	—		ATCC 14869	—		
<i>E. elingens</i>						
VPI C1548	—					

^a Bacterial cultures, which had been grown for 24 to 48 hr in VL-G broth (I), were washed with anaerobic diluents twice and 0.5 ml of the washed suspension were inoculated in 5 ml of test media with or without 1% TOS. After incubation at 37°C for 5 days, color change of B.C.P. (bromcresol purple) in the test media was determined for criteria of bacterial growth.

^b Acid from TOS; +: within 1 day, ++: 2-3 days, +: 4-5 days, -: negative.

Group II; *B. breve* 4006 feeding (3×10^9 per day), 6 subjects.

Group III; Combined feeding of *B. breve* 4006 (3×10^9 per day) and TOS (3 g or 10 g per day), 5 subjects.

Details of the dietary manipulations and fecal collection schedules are described in Table 2.

Bacteriology: One g portion (wet weight) of freshly voided fecal sample were subjected to the test. Medium preparation, dilution

Table 2. Experimental schedule

Test group	No. of subject (25-35 years, male)	Feeding schedule ^a				
		1W C ^b	1W T	1W T	1W T	1W T
I TOS	5					
			3 g	10 g		
	No. of specimen	3	3	3		
II <i>B. breve</i> 4006	6					
		1W C	1W B	1W B	1W B	1W B
	No. of specimen	3	3	3	3	3
III <i>B. breve</i> 4006 + TOS	5					
		1W C	1W B	1W B+T	1W B+T	1W C
				3 g	10 g	
	No. of specimen	3	3	3	3	3

^a Subjects were not fed milk or milk products containing viable *Lactobacillus* or *Bifidobacterium* during the study. Serial assay of fecal bacteria, on 3 stool samples per individuals in a week, was performed during the study.

^b C: Control, B: *B. breve* 4006, 2×10^9 – 5×10^9 /day, T: TOS, 3 g or 10 g/day.

and inoculation were anaerobically carried out in order to enumerate the strict anaerobes according to the modified Hungate roll tube method (1). VL-G (1), kanamycin (80 µg/ml)–vancomycin (1 µg/ml) VL-G, MPN (18), kanamycin-egg yolk (Nissui, Tokyo, Japan) roll tube agars were used for counting total bacteria, *Bacteroidaceae*, *Bifidobacterium* and *Clostridium*, respectively. MacConkey (Nissui), KMN (20), modified LBS (BBL) and *Staphylococcus* No. 110 (Nissui) were also used for assay of aerobic and facultative bacteria. The MPN medium, containing streptomycin (3,000 µg/ml) and neomycin (100 µg/ml), was used as a selective medium for administered *B. breve* 4006.

Biochemical analysis of fecal and urinary samples: The fecal ammonia and urinary indican were determined as indices of putrefactive metabolites of intestinal bacteria (19).

2. Results and Discussion

Bifidobacterium: Effects of *B. breve* 4006 and/or TOS administration on the number of fecal bifidobacteria are illustrated in Fig. 3. The number of administered *B. breve* 4006 was increased significantly after the

introduction of TOS in group III. In addition, increase in counts of administered *B. breve* 4006 were accompanied by a definite decrease in the numbers of resident bifidobacteria. Resident bifidobacterial counts were raised in 4 of 5 subjects after ingesting TOS in group I. However, the numbers of resident and administered bifidobacteria remained unchanged in group II who ingested *B. breve* 4006 only for 3 weeks. It is therefore, concluded that TOS promoted the growth of both resident and administered *Bifidobacterium* strains *in vivo*. The growth stimulation for administered *B. breve* 4006 was more convincing than that for resident *Bifidobacterium* strains (*B. adolescentis*, *B. longum* and *B. bifidum*) when TOS was fed. It was quite compatible with the authors' separate experimental results, showing that the β-galactosidase of administered *B. breve* 4006 was more active than that of resident *Bifidobacterium* strains in hydrolyzing TOS *in vitro* (data not shown).

Bacteroidaceae and Enterobacteriaceae: The changes in the counts of the fecal *Bacteroidaceae* and *Enterobacteriaceae* are shown in Fig. 4 and 5, respectively. In group I, TOS (10 g/day) caused a significant decrease in the

Fig. 3. Effect of TOS and/or *Bifidobacterium breve* 4006 administration on fecal *Bifidobacterium* in normal subjects. C: Control, T: TOS, B: *B. breve* 4006. ●—●: *B. breve* 4006, ○—○: resident bifidobacteria. Significance (paired-*t* test) (i) *B. breve* 4006 B+T (3 g, 10 g) > C (before), B, ($p < 0.05$). (ii) Resident bifidobacteria B+T (3 g, 10 g,) < C (before), ($p < 0.05$). C (before) > C (after), ($p < 0.05$).

number of *Bacteroidaceae*. The significant decreases in the fecal *Bacteroidaceae* counts were also observed in group II and III after 2 weeks' ingestion of *B. breve* 4006 and/or TOS. *Enterobacteriaceae* count was also decreased in group III who were ingested with *B. breve* 4006 and TOS. It is conceivable that gram negative anaerobes and aerobes, *Bacteroidaceae* and *Enterobacteriaceae* are suppressed by the administration of *B. breve* 4006 and/or TOS. However, the reason for the decreases in these gram negative bacterial counts remains to be solved with respect to the antagonistic bacterial interactions in the intestinal microbial ecosystem.

Other intestinal bacteria: Administration of *B. breve* 4006 with TOS caused a sig-

nificant decrease in the total bacterial counts in group III. This was probably due to the reduction of both *Bacteroidaceae* and *Bifidobacterium* counts. In contrast, TOS (10 g/day) caused a significant increase of *Lactobacillus* counts in group I. The counts of clostridia, streptococci and staphylococci were not or only limitedly changed in all groups tested.

Fecal ammonia and urinary indican excretion: The fecal ammonia level was lowered markedly after the introduction of TOS in 4 of 5 subjects of group III. No apparent changes were observed in the other two groups. The ingestion of TOS also caused significant decreases in the urinary indican excretion in group III, but no apparent dif-

Fig. 4. Effect of TOS and/or *Bifidobacterium breve* 4006 administration on fecal *Bacteroidaceae* in normal subjects. C: Control, T: TOS, B: *B. breve* 4006.

Significance (paired-*t* test) (i) T (10 g) > C, ($p < 0.05$). (ii) B (2 w, 3 w) > C, ($p < 0.05$).

(iii) B+T (3 g, 10 g) > C (before), ($p < 0.05$). C (before) > C (after), ($p < 0.05$).

Fig. 5. Effect of TOS and/or *Bifidobacterium breve* 4006 administration on fecal *Enterobacteriaceae* in normal subjects. C: Control, T: TOS, B: *B. breve* 4006.

Significance (paired-*t* test) B+T (3 g, 10 g) > C (before), ($p < 0.05$).

ferences were found in the remaining two groups. The reduction in fecal ammonia and urinary indican excretion are a reflection of the decreases in the rate of ammonia and indole production by the intestinal bacteria from both urea or unabsorbed amino acids and tryptophane, respectively. It has been shown that the members of *Bacteroides* group and *Enterobacteriaceae* are responsible

for the production of ammonia and indole *in vitro* (11, 22). It seems that the reduction of both fecal ammonia and urinary indican excretion were caused by decreased counts of *Bacteroidaceae* and *Enterobacteriaceae* due to the administration of *B. breve* 4006 and TOS. The present results were in accordance with our previous animal studies in which the administration of *B. breve* 4006 and TOS

Fig. 6. Effect of TOS and/or *Bifidobacterium breve* 4006 administration on fecal ammonia excretion. C: Control, T: TOS, B: *B. breve* 4006.

Fig. 7. Effect of TOS and/or *Bifidobacterium breve* 4006 administration on urinary indican excretion. C: Control, T: TOS, B: *B. breve* 4006.

FMU: Total volume of the first urine voided after rinsing.

Significance (paired-*t* test) B+T (3 g, 10 g) > C (before), ($p < 0.05$).

effected a significant suppression of gram negative bacteria and their putrefactive metabolites in human flora rats (19). It has been suggested that diet might influence the composition and/or activity of the intestinal bacterial flora, but rather less information is available on this aspect (10). The present data provide the clear-cut evidence that feeding *Bifidobacterium breve* 4006 with TOS alter the composition and/or activity of the

intestinal bacterial flora to a more favorable one for human health. It might be probable that the present new bifidus factor, TOS, elevates bifidobacteria level in the intestine of bottle-fed infant.

Further work is now in progress to assess the therapeutic application of the co-administration of *B. breve* 4006 and TOS for constipation (24) and portal-systemic encephalopathy (4, 21) on the analogy of lactulose therapy.

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