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
lactose was subjected to an enzymatic hydrolysis by  $\beta$-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 *vivo*. 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 *Enterobacteriaceae*. Since the results showed that TOS was a suitable carbohydrate for growth of bifidobacteria in *vivo*, 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 $H_2$ 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 per m$^2$ of body surface area. Breath hydrogen test was performed by the methods of Nose et al. (14).

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

**Group I**: TOS feeding (3 g or 10 g per day), 5 subjects.
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Table 1. Utilization of TOS by Bifidobacterium and other strict or facultative anaerobes

<table>
<thead>
<tr>
<th>Bifidobacterium</th>
<th>B. bifidum</th>
<th>E 319</th>
<th>++</th>
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<tr>
<td>B. infantis</td>
<td>S12</td>
<td>#</td>
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</tr>
<tr>
<td>B. lacteis</td>
<td>659</td>
<td>#</td>
<td></td>
</tr>
<tr>
<td>B. longum</td>
<td>AS50</td>
<td>#</td>
<td></td>
</tr>
<tr>
<td>B. adolescentis</td>
<td>E194b</td>
<td>#</td>
<td></td>
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<tr>
<td>E194a</td>
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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.

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

Group II: B. breve feeding (3 × 10⁹ per day), 6 subjects.

Group III: Combined feeding of B. breve (3 × 10⁹ 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

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Table 2. Experimental schedule

<table>
<thead>
<tr>
<th>Test group</th>
<th>No. of subject (25–35 years, male)</th>
<th>Feeding schedule a</th>
</tr>
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<tbody>
<tr>
<td>I TOS</td>
<td>5</td>
<td>1W 1W 1W</td>
</tr>
<tr>
<td></td>
<td>C* T T</td>
<td>3 g 10 g</td>
</tr>
<tr>
<td></td>
<td>No. of specimen</td>
<td>3 3 3</td>
</tr>
<tr>
<td>II B. breve 4006</td>
<td>6</td>
<td>1W 1W 1W 1W</td>
</tr>
<tr>
<td></td>
<td>C B B B</td>
<td></td>
</tr>
<tr>
<td></td>
<td>No. of specimen</td>
<td>3 3 3</td>
</tr>
<tr>
<td>III + TOS</td>
<td>5</td>
<td>1W 1W 1W 1W</td>
</tr>
<tr>
<td></td>
<td>C B B+T C</td>
<td>3 g 10 g</td>
</tr>
<tr>
<td></td>
<td>No. of specimen</td>
<td>3 3 3</td>
</tr>
</tbody>
</table>

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.

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
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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 significant 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. 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).
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), (\(p<0.05\)).

Slight differences 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
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.
References


