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Research Article

Synbiotic promotion of epithelial proliferation by orally ingested encapsulated *Bifidobacterium breve* and raffinose in the small intestine of rats

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We evaluated the effects of *Bifidobacterium breve* JCM1192^T and/or raffinose on epithelial proliferation in the rat small and large intestines. WKAH/Hkm Slc rats (4 wk old) were fed a control diet, a diet supplemented with either encapsulated *B. breve* (30 g/kg diet, 1.5×10^7 colony-forming unit/g capsule) or raffinose (30 g/kg diet), or a diet supplemented with both encapsulated *B. breve* and raffinose, for 3 wk. Epithelial proliferation in the small intestine, as assessed by bromodeoxyuridine immunohistochemistry, was increased only in the *B. breve* plus raffinose-fed group. We determined the number of bifidobacteria in cecal contents using fluorescence *in situ* hybridization and confirmed the presence of ingested *B. breve* only in the *B. breve* plus raffinose-fed group. This suggests that the ingested *B. breve* cells used raffinose and were activated in the small intestine, where they subsequently influenced epithelial proliferation. In conclusion, we found a prominent synbiotic effect of encapsulated *B. breve* in combination with raffinose on epithelial proliferation in rat small intestine but not in large intestine. To our knowledge, this is the first report of a synbiotic that affects epithelial proliferation.

Keywords: *Bifidobacterium breve* / 5-Bromo-2′-deoxyuridine / Intestinal epithelial proliferation / Raffinose / Rats

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1 Introduction

The oligosaccharide raffinose (β-D-fructofuranosyl-*O*-α-D-galactopyranosyl-(1,6)-α-D-glucopyranoside) is found naturally in many fruits and vegetables [1]. Raffinose administration has been shown to increase the bifidobacterial population in the rat cecum [2] and human intestine [3, 4]. Dietary raffinose has also been shown to influence host immune status, by reducing allergic airway eosinophilia in Brown Norway rats [5, 6] and suppressing T-helper type 2 immunity in BALB/c mice [7], although the mechanisms remain unclear.

In our previous study [2], we showed that dietary raffinose supported the growth of ingested *Bifidobacterium breve* JCM1192^T in the cecal microbiota in rats. In that study [2], we used encapsulated *B. breve*, which allows the *B. breve* to survive the acidic conditions of the stomach. Ingested *B. breve* was found in the rat cecal contents only when encapsulated *B. breve* was fed together with raffinose. Although the rat cecal microbiota comprises a relatively large indigenous population of *B. animalis*, *B. breve* is normally not present and thus is considered a suitable marker for investigating whether exogenous bifidobacteria can reach the intestine [2]. We demonstrated the successful proliferation of ingested *B. breve*, but the physiological contribution of the *B. breve* population to host health has not yet been studied.

The luminal content influences the proliferation of epithelial cells. For example, short-chain fatty acids (SCFAs) produced by intestinal bacteria promote epithelial proliferation [8]. Dietary intervention has been shown to modify

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Abbreviations: BrdU, 5-bromo-2′-deoxyuridine; **CFU,** colony forming unit; **DAPI,** 4′,6-diamidino-2-phenylindole dihydrochloride *n*-hydrate; **FISH**, fluorescence *in situ* hybridization

Table 1. Composition of the experimental diets

Component	Control (g/kg diet)	Bb (g/kg diet)	Raf (g/kg diet)	Bbraf (g/kg diet)
Sucrose	602.5	572.5	572.5	542.5
Casein[a)]	250	250	250	250
Microcrystalline cellulose[b)]	50	50	50	50
Corn oil	50	50	50	50
Mineral mixture[c)]	35	35	35	35
Vitamin mixture[d)]	10	10	10	10
Choline bitartrate	2.5	2.5	2.5	2.5
B. breve-containing capsule[e)]	–	30	–	30
Raffinose[f)]	–	–	30	30

a) NZMP Lactic Casein (Fonterra, Auckland, New Zealand).
b) Ceolus (PH-102, Asahi Kasei Chemicals Corporation, Tokyo, Japan).
c) Prepared according to the AIN-93G mineral mixture formulation [33].
d) Prepared according to the AIN-93 vitamin mixture formulation [33].
e) *B. breve* JCM1192^T (1.5×10^7 CFU/g capsule).
f) Provided by Nippon Beet Sugar Manufacturing (Tokyo, Japan).

SCFA production in the lumen of the large intestine. In our previous study [2], we also found that ingestion of raffinose increased the acetate concentration in rat cecal content, suggesting enhanced epithelial proliferation in the cecum. In some cases [9, 10], the direct injection of a SCFA solution into rat ileum stimulated crypt cell production in the jejunum as well, indicating that the effect was not restricted to the site of injection.

The intestinal interface between the luminal content and host tissue is covered with a monolayer of epithelial cells. The epithelium acts as the first line of defense against invasion by luminal pathogens. The epithelial compartment is well characterized in terms of its proliferation status [11], with epithelial stem cells located toward the bottom of the intestinal crypts and differentiated epithelial cells moving up to the crypt mouth over a couple of days. Epithelial atrophy allows pathogens to invade the epithelium [12]. Thus, maintenance of epithelial proliferation is essential for host health.

In this study, we investigated the possible synbiotic effects in rats of encapsulated *B. breve* and/or raffinose, focusing on epithelial proliferation in the small and large intestines.

2 Materials and methods

2.1 Animals and diets

This study was approved by the animal use committee at our institute. The animals were maintained under the guidelines for the care and use of laboratory animals, Hokkaido University.

Male WKAH/Hkm Slc rats ($n = 24$; 4 wk old, Japan SLC, Hamamatsu, Japan) were housed in individual cages in a temperature-controlled room under a 12 h photoperiod (light: 800 – 2000 h). Rats were allowed free access to food and water over the experimental period.

After an acclimation period with the control diet (Table 1), the rats were divided into four dietary groups, and each group was fed one of the following: A control diet (control), a diet supplemented with encapsulated *B. breve* JCM1192^T (30 g/kg diet) (Bb) as 1.5×10^7 colony-forming unit (CFU)/g capsule (provided by Morishita Jintan, Osaka, Japan), a diet supplemented with raffinose (30 g/kg diet; Nippon Beet Sugar Manufacturing, Tokyo, Japan) (Raf), or a diet supplemented with the *B. breve* capsule (30 g/kg diet) and raffinose (30 g/kg diet) (Bbraf), at the expense of sucrose in the control diet. The coating materials of the *B. breve* capsule consisted of gelatin, pectin, and hydrogenated oil, all of which are digestible in the small intestinal lumen. The gelatin content (175 g/kg capsule) in the diet containing the *B. breve* capsules was 5.25 g/kg diet. After 3 wk feeding with the test diets, the rats were subcutaneously injected with 5-bromo-2′-deoxyuridine (BrdU, 15 mg/kg body weight) and 5-fluoro-2′-deoxyuridine (1.5 mg/kg body weight) in PBS 1 h before euthanization, for immunohistochemistry [13].

2.2 Sample preparation

The rats were sacrificed (1000 – 1300 h) by decapitation under diethylether anaesthesia, and the small intestine, cecum, and distal colon were removed. The whole cecum was weighed and the contents were immediately collected for pH measurement and fluorescence *in situ* hybridization (FISH). The cecal tissue was washed with saline and weighed again for the calculation of cecal content weight. For immunohistochemistry, segments of the intestine (small intestine, cecum, and distal colon) were flushed with saline, fixed with 4% formalin in PBS, embedded in optimal cutting temperature compound (Sakura Finetek Japan, Tokyo, Japan) in liquid nitrogen, and frozen at -80°C.

2.3 Preparation of cecal contents for pH measurement and fluorescence *in situ* hybridization (FISH)

For pH measurement, the cecal contents were diluted 1:10 with deionized water and homogenized using a Teflon homogenizer. The samples were centrifuged (15 000 × *g*, 10 min, 4°C), and the pH of each supernatant was measured. For FISH analysis, the cecal samples for microscopic analysis were prepared as previously described [2]. Briefly, the cecal contents were suspended in ice-cold PBS, followed by low-speed centrifugation to remove large fecal debris. This step was repeated three times, and then bacterial cells were separated from the pooled supernatants by high-speed centrifugation. The pellets were washed with PBS three times to remove materials inhibitory to the FISH reaction. The washed bacterial cells were fixed and stored in 50% v/v ethanol in PBS at −20°C until use. Aliquots of fixed bacterial cells were applied to Teflon-printed glass slides (ADCELL, 12 wells, 5 mm in diameter, Erie Scientific Company, Portsmouth, NH, USA) and dehydrated. The bacterial cells were hybridized by the addition of a hybridization buffer (0.9 M NaCl, 0.01% SDS, 20 mM Tris-HCl, 20% deionized formamide, pH 7.2) and a Cy3-labeled oligonucleotide probe (Espec Oligo Service, Tsukuba, Japan), followed by washing with washing buffer. The oligonucleotide probes (Bif164m for *Bifidobacteria*, PBR2 for *B. breve*, and Bani449 for *B. animalis*) were described in our previous report [2]. The specificities of the oligonucleotide probes have been assessed in previous studies, using a variety of reference microorganisms [2, 14]. The PBR2 and Bani449 probes required individual helpers to raise accessibility for the probes [2]. The washed slides were stained with 4′,6-diamidino-2-phenylindole dihydrochloride *n*-hydrate (DAPI) solution (Wako Pure Chemical Industries, Osaka, Japan) at room temperature to visualize total bacteria. The dried slides were mounted with Vectashield (Vector Laboratories, Burlingame, CA, USA) and examined under an Olympus BX50 epifluorescence microscope (Olympus Corporation, Tokyo, Japan). The DAPI and Cy3 signals were captured in 10–15 random microscopic fields *per* slide. The hybridization results from the CCD camera were analyzed using an IP Lab-spectrum image analysis system (BD Biosciences Bioimaging, Rockville, MD, USA) and were colored and retouched using Adobe Photoshop 5.5, for manual counting. Specific signals from the probes were represented as the average percentage of total bacterial cells visualized by DAPI signals in the same microscopic field.

2.4 Bromodeoxyuridine immunohistochemistry

Frozen sections from the small intestine (the mid-part of the small intestine, about 30 cm from the ligaments of Treitz), cecum, and distal colon were prepared and stained with an anti-BrdU mAb (NA20, Calbiochem, EMD Chemicals, Darmstadt, Germany) [13]. Briefly, sections were fixed in 10% v/v formalin in PBS, immersed in 3% v/v hydrogen peroxide in methanol to block endogenous peroxidase activity, and treated with pepsin solution (0.4 mg/mL in 0.1 M HCl) and normal rabbit serum to reduce nonspecific binding. After incubation with the primary antibody, as described above, the sections were incubated with biotinylated rabbit antimouse IgG + A + M (H + L; Zymed Laboratories, San Francisco, CA, USA), followed by incubation with peroxidase-conjugated streptavidin (Zymed). 3,3′-Diaminobenzidine tetrahydrochloride was used as the chromogen. After BrdU staining, the sections were counterstained with hematoxylin. To verify the specificity of the signals, sections were processed according to the above procedure, except that incubation with the primary antibody was omitted as a negative control. The number of BrdU-incorporating cells among the epithelial layer cells in the well-shaped crypt sections was scored for 50 half-crypts in each part of the intestine. For the small intestine in particular, we scored the epithelial cells located within cell position 20 from the bottom of the crypt. We then calculated the number of BrdU-incorporating cells *per* 100 epithelial layer cells.

Table 2. Body weight (BW) and food intake in rats fed the control diet (control), or a diet containing encapsulated *B. breve* (Bb) or raffinose (Raf) or *B. breve* plus raffinose (Bbraf), for 3 wk

Diet	Initial BW (g)	Food intake (g)	Final BW (g)	BW gain (g)
Control	129.0 ± 3.6	352.4 ± 6.8	248.9 ± 4.8	119.9 ± 3.3
Bb	131.1 ± 3.2	358.9 ± 6.8	260.6 ± 5.5	129.5 ± 4.1
Raf	130.8 ± 3.1	349.0 ± 10.0	258.0 ± 5.9	127.3 ± 3.3
Bbraf	129.4 ± 2.5	350.2 ± 7.4	254.1 ± 5.9	124.8 ± 4.5

Values are expressed as means ± SEM ($n = 4–6$). No significant differences were observed among the diets.

2.5 Statistical analysis

All results are expressed as means ± SEMs. Statistical comparisons among dietary groups were analyzed by the Tukey–Kramer test. Differences were considered statistically significant for p values <0.05.

3 Results

There was no significant difference in food intake, final body weight, or body weight gain among the groups throughout the experiment (Table 2). Although no statistically significant change was found in the total bacterial count *per* gram of cecal contents among the treatments, ingestion of raffinose resulted in a prominent increase in

Table 3. Identification of bifidobacteria in the cecal contents in rats fed the control diet (control), or a diet containing encapsulated *B. breve* (Bb) or raffinose (Raf) or *B. breve* plus raffinose (Bbraf), for 3 wk

Diet	Total bacteria ($\times 10^{10}$/g contents)	% of total bacteria		
		Bifidobacteria	*B. breve*	*B. animalis*
Control	1.37 ± 0.14	0.5 ± 0.2 [c]	ND [b]	0.4 ± 0.1 [c]
Bb	1.65 ± 0.21	0.4 ± 0.2 [c]	ND [b]	0.2 ± 0.1 [c]
Raf	1.64 ± 0.06	16.7 ± 1.0 [b]	ND [b]	16.8 ± 1.0 [b]
Bbraf	1.75 ± 0.07	33.4 ± 9.3 [a]	2.7 ± 1.4 [a]	29.7 ± 8.1 [a]

Values are expressed as means ± SEM ($n = 4-6$). Values not sharing the same letter in a column are significantly different. ND, not detected.

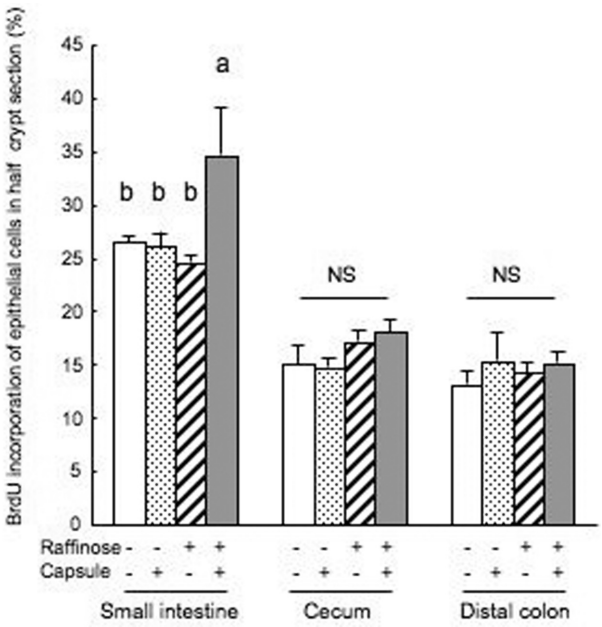

Figure 1. BrdU incorporation in crypt epithelial cells of the small intestine, cecum, and distal colon in rats fed the control diet (control, open bar), or a diet containing encapsulated *B. breve* (Bb, dotted bar) or raffinose (Raf, slashed bar) or *B. breve* plus raffinose (Bbraf, closed bar), for 3 wk. SEMs are shown as error bars ($n = 4-6$). Values not sharing the same letter are significantly different among the values in the small intestine.

the population of bifidobacteria in the cecal contents of Raf- and Bbraf-fed rats, to 16.7 and 33.4%, respectively, whereas the bifidobacterial population was only 0.5% of the total bacteria in control-fed rats (Table 3). Ingested *B. breve* was detected only in the cecal contents of the Bbraf-fed group and failed to increase in the Bb-fed rats. The *B. breve* population in the Bbraf-fed group represented about 2.7% of the total bacterial population. This value was lower than that obtained in a previous study (6.3%) [2], probably owing to the lower viable cell count in the present study compared with that in the previous study (1.5×10^7 *vs.* 5.7×10^7 CFU/*g* capsule, respectively). Almost all of the bifidobacteria population in Raf- and Bbraf-fed rats appeared to be *B. animalis* (Table 3).

Table 4. Cecal tissue weight (wt), cecal contents wt, and pH of cecal contents in rats fed the control diet (control), or a diet containing encapsulated *B. breve* (Bb) or raffinose (Raf) or *B. breve* plus raffinose (Bbraf), for 3 wk

Diet	Cecal tissue wt (g)	Cecal contents wt (g)	Cecal contents pH
Control	0.65 ± 0.04 [b]	1.25 ± 0.15 [b]	7.9 ± 0.1 [a]
Bb	0.68 ± 0.07 [b]	1.05 ± 0.08 [b]	7.7 ± 0.1 [ab]
Raf	0.96 ± 0.04 [a]	1.91 ± 0.17 [a]	7.4 ± 0.1 [b]
Bbraf	1.02 ± 0.11 [a]	2.13 ± 0.06 [a]	6.9 ± 0.2 [c]

Values are expressed as means ± SEM ($n = 4-6$). Values not sharing the same letter in a column are significantly different.

Table 5. Number of total epithelial cells in the half-crypt section of the cecum and distal colon in rats fed the control diet (control), or a diet containing encapsulated *B. breve* (Bb) or raffinose (Raf) or *B. breve* with raffinose (Bbraf), for 3 wk

Diet	No. of total epithelial cells in the half-crypt section	
	Cecum	Distal colon
Control	26.3 ± 0.8	26.1 ± 0.7
Bb	27.0 ± 0.9	26.7 ± 0.6
Raf	28.9 ± 1.0	28.4 ± 0.5
Bbraf	28.4 ± 1.3	28.4 ± 1.3

Values are expressed as means ± SEM ($n = 4-6$). No significant differences were observed.

As shown in Table 4, dietary raffinose increased the weight of the cecal contents and the tissue, but no significant influence of ingested *B. breve* was observed. Ingestion of encapsulated *B. breve* lowered the luminal pH of the cecal contents only when combined with raffinose.

Figure 1 shows the proliferation of epithelial cells in the crypts of the small intestine, cecum, and distal colon in rats fed encapsulated *B. breve* and/or raffinose. A prominent synbiotic effect on epithelial proliferation was found only in the small intestine of Bbraf-fed rats; no statistically significant effect of diet was observed on epithelial proliferation in the cecum or distal colon. The values of BrdU incorporation were lower in the large intestine than in the small intestine, because of the large number of differentiated epithelial cells in the large intestinal crypts as compared with that in the small intestinal crypts. Therefore, the number of epithelial cells in a half-crypt section was determined in the cecum and distal colon (Table 5). Dietary intervention did not significantly change the number of epithelial cells in a half-crypt section in either the cecum or distal colon.

4 Discussion

Since the concept of a synbiotic was introduced by Gibson and Roberfroid [15], many studies on synbiotic effects have been conducted in relation to lipid metabolism [16, 17], colon carcinogenesis [18–21], intestinal inflammation [22–27], natural killer cell activity [28], and allergies [29]. In terms of intestinal epithelial proliferation, some studies have focused on the large intestine [20, 21], but there appears to have been no study on the small intestine. In our previous study [2], we found a synergistic increase in the donated *B. breve* in the cecal bacterial population in combination with raffinose. Thus, in this study, we investigated the synbiotic effect on epithelial proliferation in the whole intestine under the same experimental conditions.

A promotion of epithelial proliferation was seen only in the small intestine, and no influence of *B. breve* or raffinose alone was observed (Fig. 1). In our previous study [2], we showed that raffinose was an excellent energy source for the growth of *B. breve* JCM1192^T in *in vitro* growth experiments and found an increase in the acetate concentration in cecal contents of rats fed raffinose-containing diets (Raf and Bbraf). It is possible that *B. breve* cells were released from the capsules soon after entering the small intestine and were immediately activated by raffinose in the environment. The results from the BrdU immunohistochemistry suggested that ingested *B. breve* cells present in the midpart of the small intestine were energized by raffinose and were producing organic acids or other molecules, leading to the promotion of epithelial proliferation. However, further analysis of the bacterial population and organic acids in the luminal content of the small intestine is required to clearly understand this synbiotic effect.

The cecal tissue weights of the Raf- and Bbraf-fed rats were significantly higher than those of the control- and Bb-fed rats (Table 4). In line with these observations, a tendency, although not significant, for enhanced epithelial cell proliferation was also seen in the cecum of rats fed raffinose-containing diets (Raf and Bbraf; Fig. 1). These effects of raffinose were considered to be brought about, at least in part, by the increased acetic acid concentration in the lumen [2] associated with the increased bifidobacterial population (Table 3), which consisted largely of *B. animalis*. We were not able to see a significant synbiotic effect of epithelial cell proliferation in the cecum. This was probably because of the denser population of microbiota in the cecum than in the small intestine, resulting in already higher concentrations of SCFAs in the cecum and thereby making it difficult to see any additional effect. In fact, the number of total anaerobes in the small intestine has been reported to be three to four orders of magnitude lower than that in the large intestine in rats [30]. Therefore, ingested *B. breve* cells might easily proliferate, aided by raffinose, and influence the small intestine in the host. Other probiotics that produce SCFAs may also be candidates to promote epithelial proliferation in the host, when introduced together with suitable energy sources.

Bifidobacteria are not believed to tolerate the acidic environment in the stomach. Thus, we used encapsulation to protect *B. breve* cells from gastric acids. The gelatin and other materials used to encapsulate *B. breve* cells appeared to have no effect on epithelial proliferation, as there was no enhancement of BrdU incorporation in epithelial cells in Bb-fed rats.

Ichikawa *et al*. [31] reported the probiotic effects of *Lactobacillus casei* and *Clostridium butyricum* on epithelial proliferation in the jejunum, ileum, cecum, and distal colon. They administered either *L. casei* or *C. butyricum* (10^7 CFU/day) intragastrically for 7 days and found that both treatments promoted epithelial proliferation. Hooper *et al*. [32] reported that colonization of *Bacteroides thetaiotaomicron* into germ-free mice enhanced the expression of genes involved in the maturation of the ileum, clarifying interactions of commensal bacteria and the small intestine in the host. These studies demonstrated that mucosal functions can be modified in response to the bacteria in the luminal environment. Our simple dietary intervention study revealed a significant synbiotic effect on epithelial proliferation in the rat small intestine.

In conclusion, we found a prominent synbiotic effect of exogenous *B. breve* and raffinose on epithelial proliferation in the small intestine but not the large intestine. To our knowledge, this is the first report showing that promotion of epithelial proliferation can be attained by an appropriate synbiotic treatment. Finally, we would like to emphasize that the upper gastrointestinal tract may be an ideal target for synbiotics, because of the relatively small population of resident bacteria.

The authors have declared on conflict of interest.

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