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1 Modulation of Rat Cecal Microbiota by the Administration of Raffinose and

2 Encapsulated *Bifidobacterium breve*

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4 Running Title: MODULATION OF RAT MICROBIOTA BY RAFFINOSE AND *B. BREVE*

5 Byline: DINOTO ET AL.

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1 **ABSTRACT**

2 To investigate the effects of raffinose and encapsulated *Bifidobacterium breve* JCM
3 1192^{T} cells administration on rat cecal microbiota, as a preclinical synbiotic study, groups of
4 male WKAH/Hkm Slc rats were fed for three weeks with four different test diets: basal diet
5 (BD), BD supplemented with raffinose (RAF), BD supplemented with encapsulated *B. breve*
6 (CB), and BD supplemented with both raffinose and encapsulated *B. breve* (RCB). The
7 bacterial populations of cecal samples were determined by fluorescence *in situ* hybridization
8 (FISH) and terminal restriction fragment length polymorphism (T-RFLP). *B. breve* cells were
9 detected only in the RCB diet group and represented about 6.3% of the total cells as
10 determined by FISH analysis. The presence of *B. breve* was also detected only in the RCB
11 group by T-RFLP analysis. This was in contrast to the CB group in which no *B. breve* signals
12 were detected by either FISH or T-RFLP. Increase of the population of *Bifidobacterium*
13 *animalis*, an indigenous *Bifidobacterium* of the rat, was observed in the RAF and RCB groups.
14 Principal component analysis of T-RFLP results revealed significant alterations in the
15 bacterial populations of rats in the RAF and RCB groups; the population in the CB group was
16 similar to that of the control (BD) group. To the best of our knowledge, these results provide
17 the first clear picture of the changes of rat cecal microbiota in response to the synbiotic
18 administration.

The human gastrointestinal tract harbors various kinds of bacteria that may affect positively or negatively on the health conditions of the host (9). There is an increasing interest in the idea that diet can promote or maintain beneficial colonic bacteria that improve immunopotentiation (25), prevent the invasion of pathogenic bacteria (45), and provide metabolic energy for the host (17). This concept has led to the terms 'probiotic' (15), 'prebiotic,' (17) and 'synbiotic' (17).

Synbiotic was first defined as a mixture of probiotics and prebiotics that beneficially affects the host by improving the survival and implantation of live microbial dietary supplements in the gastrointestinal tract by selectively stimulating the growth and/or by activating the metabolism of one or a limited number of health promoting bacteria and thus improving host welfare (17). Synbiotics are believed to increase the persistence of the probiotic bacteria in the gastrointestinal tract. Although concept of synbiotics has been introduced, clear demonstration of synbiotic effects seems not available in the literature due to unclear experimental design or unreliable methods for microbiota analysis. Molecular ecological methods, which reveal the entire range of bacterial diversity and more accurately detect population changes, are now considered more appropriate for evaluating synbiotic effects.

In this study, alteration of rat cecal microbiota upon administration of raffinose in combination with *Bifidobacterium breve* JCM 1192^T was analyzed. *B. breve* JCM 1192^T, was employed in gelatin-encapsulated form to protect the bacterial cells from the acidic environment of the stomach and to increase bacterial access to the intestine. This strain has recently been reported to accumulate large amounts of cholic acid, a primary bile acid in humans, in an energy-dependent manner (26). Thus, we intended to amplify this strain in rat cecum as a preclinical study to design rational human trial to see the effects on lipid metabolism and on intestinal bile acids composition. In *in vitro* growth experiments, *B. breve*

JCM 1192^T grew better on raffinose than on the other fermentable sugars tested. Therefore, raffinose was chosen as suitable carbon source in combination with encapsulated *B. breve* JCM 1192^T. Although this mixture, i.e. raffinose and encapsulated *B. breve* JCM 1192^T, has not yet been demonstrated as a synbiotic in accord with Gibson and Roberfroid's definition (17), we sometimes call this mixture "synbiotic" for simplicity and clarity in the text. Similarly, encapsulated *B. breve* JCM 1192^T is tentatively referred to as "probiotic". Rat experiment was designed in four groups (control, prebiotic, probiotic and synbiotic). Two molecular techniques, fluorescence *in situ* hybridization (FISH) (4) and terminal restriction fragment length polymorphism (T-RFLP) analyses (31, 29) were applied for the first time to monitor rat microbiota alteration in synbiotic experiment. As the result, proliferation of *B. breve* JCM 1192^T was successfully observed only in synbiotic group. Furthermore, we have analyzed other members of rat microbiota and determined organic acids content in cecum to obtain more comprehensive picture of the changes of rat intestinal microbiota. We believe these results can provide the first clear picture of microbiota changes in rat cecum upon administration of synbiotic, and can add fundamental knowledge to rat intestinal microbiology.

MATERIALS AND METHODS

Bacterial strain and media. *B. breve* JCM 1192^T was obtained from the Japan Collection of Microorganisms (JCM, Wako, Japan). Cultures were conducted in MRS medium (11) prepared at half strength (1/2 MRS) containing filter-sterilized L-cysteine.HCl at a concentration of 0.25 g/l unless otherwise stated. TOS Propionate Agar (Yakult Pharmaceutical Ind. Co., Ltd., Tokyo, Japan) was used for the isolation of bifidobacteria from rat cecal samples.

1 **Measurement of the growth rate.** Seed culture for the growth experiments of *B. breve* JCM 1192^T was conducted overnight in 1/2 MRS. The seed culture was transferred to the same medium at the optical density at 660 nm (OD_{660}) of 0.05, but containing one of the following five carbohydrates (10 g/l): glucose, sucrose, raffinose (Sigma-Aldrich, St. Louis, Mo.), kestose and nystose (both donated from the Hokuren Federation of Agricultural Cooperatives, Sapporo, Japan). Bacterial growth was periodically monitored by measuring OD_{660} of the culture broth. All the cultures were carried out at 37 °C under anaerobic conditions using mixed gas ($N_2/CO_2/H_2$; 8:1:1). Specific growth rates (μ, h^{-1}) were calculated during logarithmic growth phase using the equation: $\mu = (\ln x_{t2} - \ln x_{t1})/(t2-t1)$, where x_{t2} and x_{t1} are OD_{660} at time t2 and time t1, respectively.

11 **Animals and diets.** Male WKAH/Hkm Slc rats (n = 24, Japan SLC, Hamamatsu, Japan), aged 4 weeks old, were acclimatized with basal diet for 7 days in individual rat cages. Twenty four rats were divided equally into 4 groups and fed test diets for three weeks. The control group (BD) received basal diet, which contained (g/kg diet): casein, 250; corn oil, 50; AIN-93G mineral mixture, 35; AIN-93 vitamin mixture, 10; choline bitartrate, 2.5; and sucrose, 602.5; supplemented with crystalline cellulose (Avicel, Asahi Kasei Corporation, Osaka, Japan), 50 g/kg diet (35). Diet group 2 (RAF) received BD supplemented with raffinose (Nippon Beet Sugar Manufacturing Co., Ltd., Tokyo, Japan), 30 g/kg diet. Diet group 3 (CB) was fed BD supplemented with gelatin-encapsulated *B. breve* JCM 1192^T (provided by Morishita Jintan Co., Ltd., Osaka, Japan) with a viable cell count of about 5.7 x 10^7 CFU/g capsule), 30 g/kg diet. The gelatin content of the capsule was 17.5% (w/w). Thus, CB group received 5.25 g gelatin/kg diet. For the preparation of the capsule, *B. breve* JCM 1192^T was cultured in a jar fermentor until late exponential growth phase using 1/2 MRS medium containing both 40 g/l raffinose as the carbon source and 0.5 g/l L-cysteine.HCl. The culture was conducted at 37 °C by stirring at 200 rpm under anaerobic conditions with CO_2

1 gas into the headspace of the fermentor. The pH of the medium was controlled at 6.5 with
2 NaOH. The cells were harvested by centrifugation, washed twice with 150 mM NaCl,
3 resuspended in 10% (w/v) skim milk, and then freeze-dried before encapsulation. Diet group
4 4 (RCB) received BD supplemented with raffinose and encapsulated *B. breve* JCM 1192^T in
5 the same amounts as in the RAF and CB diets, respectively. Rats were maintained and
6 handled according to the recommendations of our Institutional Ethics Committee.

7 **Sample preparation.** Individual rat cecal contents were immediately weighed after
8 sacrificing rats. One portion of the contents was used for pH and organic acid measurements
9 (see measurement of organic acids). The remainder (about 0.5 g) was washed three times
10 with ice-cold phosphate-buffered saline (PBS; 130 mM NaCl, 10 mM sodium phosphate
11 buffer, pH 7.2) with low-speed centrifugation at 200 x *g* for 5 min. Bacteria were pelleted
12 from the pooled supernatant by high-speed centrifugation three times at 9000 x *g* for 2 min
13 each time. The bacterial cell pellet was resuspended in PBS and divided into two parts. The
14 first part was used for bacterial cell fixation for FISH analysis (see FISH analysis). As
15 sample amounts were limited, the remaining parts were pooled according to test diet group
16 for genomic DNA extraction for T-RFLP analysis (see T-RFLP analysis).

17 **Measurement of organic acids.** The concentrations of organic acids (succinate,
18 lactate, propionate, butyrate, isovalerate, and valerate) in the rat cecal samples were measured
19 by HPLC (SCL-10AVP, Shimadzu Corporation, Tokyo, Japan) according to the method
20 described previously (18). Briefly, the cecal samples were added to sodium hydroxide
21 aqueous solution containing crotonic acid as an internal standard. After centrifugation, the
22 fat-soluble substances in the supernatant were removed by extraction with chloroform. The
23 aqueous phase was passed through a membrane filter and subjected to HPLC.

24 **FISH analysis.** Individual washed samples were fixed in 4% (w/v) para-
25 formaldehyde in PBS (pH 7.2) for 24 h. Fixed samples were washed once in PBS and stored

in a known volume of 50% (v/v) ethanol/PBS at –20°C until use. Aliquots of 3 µl of fixed cells were applied to Teflon printed glass slides (ADCELL, 12 wells, 5 mm in diameter, Erie Scientific Company, Portsmouth, N.H.) and air-dried. The cells were then dehydrated through a series of 50%, 80%, and 99.5% ethanol for 3 min each. The cells fixed on the glass slides were hybridized by addition of 8 µl of hybridization buffer (0.9 M NaCl, 0.01% SDS, 20 mM Tris-HCl, 20% deionized formamide, pH 7.2) with 1 µl of Cy3-labeled oligonucleotide probe at 25 ng/µl (Tsukuba Oligo Service Co., Ltd., Tsukuba, Japan). The slides were hybridized at 46°C for 16 h in a moist chamber. After hybridization, the slides were rinsed with warm hybridization buffer at 48°C and washed in pre-warmed washing buffer (225 mM NaCl, 0.01% SDS, 20 mM Tris-HCl, pH 7.2) for 20 min at 48°C. The washed slides were stained with DAPI (4', 6-diamidino-2-phenylindole dihydrochloride n-hydrate) solution for 5 min at room temperature to stain the chromosomes as a control signal. The slides were washed with distilled water for 5 min at room temperature and air-dried in the dark. The dried slides were mounted with Vectashield (Vector Laboratories Inc., Burlingame, Calif.) and examined under an Olympus BX50 epifluorescence microscope (Olympus Corporation, Tokyo, Japan) equipped with a SenSys CCD camera (Photometrics Ltd., Tucson, Ariz.) operated by the IPLab software (Scanalytics, Inc., Fairfax, Va.). DAPI and Cy3 signals were captured in pairs of ten to fifteen random microscopic fields (about 500 cells per microscopic field). Hybridization images were manually counted and were colorized when necessary using Adobe Photoshop 5.5 (Adobe Systems Incorporated, San Jose, Calif.). Specific signals from the probes are represented as average percentages of the total cells visualized by DAPI signals in the same microscopic field.

Oligonucleotide probes. The oligonucleotide probes and their target microorganisms used in this study are shown in Table 1; the target region alignment of PBR2 and Bani449 (newly created in this study) is shown in Fig. 1. The computer alignment of 16S rRNA genes

from *Bifidobacterium* revealed that *B. animalis* and some other bifidobacteria are not targets of the probe Bif164 because the 16S rRNA sequence of these bacteria showed a single-base mismatch with Bif164 (27). The mismatched nucleotide (C or T) was replaced with Y to create a new probe designated Bif164m. No cross hybridizations was observed against non-target bacterial strains that include strains from *Lactobacillus, Clostridium, Bacteroides, Streptococcus* and *Enterococcus* (data not shown). In a preliminary experiment to visualize the secondary structures of the 16S rRNA of *B. breve* and *B. animalis* by computer simulation (GENETYX-Win, Software Development, Tokyo, Japan), it was found that the target regions for PBR2 and Bani449 were difficult to be accessed due to RNA-RNA interactions. Therefore, we used unlabeled oligonucleotides (helper probes), which were complimentary to the up- and downstream regions neighboring the probe target site on the 16S rRNA, to increase the target accessibility for the probes as suggested by Fuchs et al. (14). The sequences of these helper probes are also shown in Table 1. Indeed the hybridization efficiencies of PBR2 and Bani449 were significantly increased in both target strains by the application of these helper probes (data not shown).

T-RFLP analysis. To establish standard T-RF peak-sets for the identification of bacterial strains present in the cecal samples by T-RFLP analysis, T-RF lengths were determined for *B. breve* JCM 1192^T and an indigenous rat strain *B. animalis* isolated from rat cecal samples using TOS Propionate Agar. Bacterial genomic DNA was extracted from pure cultures of these microorganisms and from pooled cecal samples of the four experimental groups (BD, RAF, CB, and RCB) using an Isoplant DNA Extraction Kit (Nippon Gene, Co., Ltd., Tokyo, Japan) according to the manufacturer's instructions. Two replications of each sample were separately amplified by PCR and digested by restriction enzymes as described below. A PCR mixture was made from each sample using a GeneAmp PCR System 2400 (Applied Biosystems, Foster City, Calif.) with the 6-carboxyfluorescein (6-FAM)-labeled

primer 46F (5'-GCYTAACACATGCAAGTCGA-3') (23), which was synthesized by Applied Biosystems Japan, and the unlabeled primer 1080R (5'-CCCAACATCTCACGAC-3') (33). PCR conditions were based on the method described by Kaplan et al. (23) with modifications as follows: reaction mixture volume, 100 µl; template DNA, 200 ng; 1 x Gold buffer (Applied Biosystems); deoxynucleoside triphosphates, 0.6 mM; bovine serum albumin, 0.8 µg/l; $MgCl_2$, 3.5 mM; 6-FAM-labeled 46F primer, 0.2 µM; 1080R primer, 0.2 µM; and AmpliTaq Gold DNA polymerase, 4 U (Applied Biosystems). Reaction temperatures and the thermal cycling program for the fecal samples were as follows: 94°C for 2 min; 35 cycles of 94°C for 2 min, 48.5°C for 1 min, and 72°C for 1 min; with a final cycle of 72°C for 10 min. Amplified fragments were purified by SUPREC PCR (Takara Bio Inc., Otsu, Japan), and the purified fragments from three replications were pooled. Aliquots of about 1 µg of DNA in purified amplicon were digested with 10 U of each of *Hae*III, *Hha*I, and *Msp*I (Takara Bio Inc.) at 37°C for 24 h. The digestion reaction was stopped by incubation at 65°C in a water bath for 20 min followed by immediate cooling in an ice bath. The restriction digest products were subjected to ethanol precipitation and vacuum-dried. Fifty µl of distilled water was added to dissolve each dried sample. Aliquots of 1 µl of dissolved samples were then added to 10 µl mixture of formamide and size standard (GS 500 ROX, Applied Biosystems) (100:5, v/v). Each sample was denatured at 95°C for 2 min and then immediately placed on ice. The fluorescently labeled terminal restriction fragments (T-RFs) were analyzed by electrophoresis on an automatic sequence analyzer (ABI PRISM 3100, Applied Biosystems) in gene-scan mode, and the lengths of the T-RFs were determined by comparison with size standards using Genescan 3.7 software (Applied Biosystems).

Data analyses. The effects of diet on cecal organic acids and microbiota were analyzed separately by two-way ANOVA at $P < 0.05$ using JMP software (SAS Institute Inc., Cary, N.C.). The data obtained in T-RFLP analysis was processed according to Kaplan et al.

1 (23). The T-RF peaks with areas less than the threshold value were excluded. The remaining
2 T-RF peaks from three restriction enzymes were combined and examined by principal
3 component analysis (PCA) using SPSS 10 software (SPSS Inc., Chicago, Ill.)

4

5 ## RESULTS

6 **Carbon source preference of *B. breve* JCM 1192^T.** Prior to *in vivo* experiments, we
7 have conducted growth experiments of *B. breve* JCM 1192^T to characterize this bacterium in
8 terms of carbon source preference. The results showed that *B. breve* JCM 1192^T grew better
9 on raffinose than other carbon sources tested as assessed by the μ value in MRS medium.
10 The μ values measured (mean±SD, where n=2) on carbon sources tested were 0.45±0.02
11 (glucose), 0.65±0.02 (raffinose), and 0.64±0.08 (kestose), while sucrose and nystose were not
12 at all assimilated by this strain. Therefore, raffinose was chosen as a prebiotic for this
13 bacterium.

14 **Animal experiments.** All rats were healthy and alive until the day on which they
15 were sacrificed. The pH value of the cecal contents of rats in the BD group (control) was
16 significantly higher (8.1 ± 0.1) than those of rats in the RAF and RCB groups (6.8 ± 0.1 and
17 7.0 ± 0.2, respectively); the pH value of the cecal contents of CB rats was not different from
18 that of BD rats (Table 2). Cecal samples from RAF and RCB rats were yellow; BD and CB
19 cecal samples were brown-green. In addition, the cecal content weight of rats in the RAF and
20 RCB groups were significantly greater than those of rats in the BD and CB groups (Table 2).
21 To confirm the reproducibility of the results, we have conducted the second trial and obtained
22 the similar results (data not shown).

23 As shown in Table 2, the amounts of lactic and acetic acids in RAF and RCB cecal
24 samples were significantly higher than those in BD and CB cecal samples. However, the
25 amounts of butyric, isovaleric, and valeric acids in the cecal samples from the RAF and RCB

1 groups were significantly lower than those in control samples due to the effect of raffinose in
2 the diets. The amounts of propionic and succinic acids were significantly higher in CB and
3 RCB, respectively, than those in BD.

4 **FISH analysis of microbiota in the cecal samples.** FISH was performed to
5 enumerate the total and the target bacterial populations (Table 3). The total bacterial
6 populations of each group were slightly different (ca. 1.74 – 2.19 x 10^{10} cells/g wet weight,
7 DAPI signal), and the ratio of the total bacteria (probe Eub338) to the total cells (DAPI) was
8 decreased from about 90% in the BD and CB groups to about 85% in the RAF and RCB
9 groups. The cell morphologies of the predominant bacteria were different among the samples
10 (Fig. 2). Long curved rods were the major forms in the BD and CB groups, while RAF and
11 RCB samples contained short rods. The second trial gave the similar results (data not shown).

12 As shown in Table 3, at 23.9% of the total cell count, the *Clostridium coccoides* and
13 *Eubacterium rectale* group (probe Erec482) represented the major proportion of the bacterial
14 population in BD rats. The *Bacteroides* group (probe Bac303) made up 3.6% of the total
15 population. As compared with BD (control) rats, RAF rats had a significantly lower number
16 of targets for *Clostridium coccoides* and *Eubacterium rectale* group (12.9%). The
17 *Lactobacillus* group (probe Lacb722) and bifidobacteria (probe Bif164m) accounted for 7.2%
18 and 19.5%, respectively, of the total population. These proportions were significantly higher
19 than those in BD rats (0.2% and 0.4%, respectively). Signals from *Clostridium histolyticum*
20 group (probe Chis150) were slightly higher in RAF samples (1.2%) than in control samples
21 (0.3%). RAF rats showed the highest proportion of streptococci and lactococci (probe
22 Strc493) among all diet groups, although these bacteria did not account for more than 1.5% of
23 the total population. CB rats showed no significant population differences from control rats,
24 although the proportions of *Clostridium coccoides* and *Eubacterium rectale* group were
25 slightly increased (29.7%). No *B. breve* (probe PBR2) signals were detected in CB rats even

1 though the target strain was contained in the CB diet. RCB rats showed nearly the same
2 proportion of *Clostridium coccoides* and *Eubacterium rectale* group (23.2%) and *Bacteroides*
3 group (2.5%) as BD rats. The proportion of *Lactobacillus* group was higher in RCB rats
4 (2.8%) than in BD rats (0.2%), but lower than in RAF rats (7.2%). A striking feature of the
5 RCB group was the large proportion of bifidobacteria, which at 25.4% of the total DAPI-
6 stained cells (corresponding to 29.4% of the total cell count using the probe Eub338) was the
7 highest value of all the samples. *B. breve*, the administered bacterial strain, represented 6.3%
8 of the total DAPI-stained cells (7.3% of total cell count using the probe Eub338) as detected
9 using the PBR2 probe with its helpers. By the isolation of bifidobacteria using TOS
10 Propionate Agar and 16S rRNA gene sequencing (data not shown), the bifidobacteria found in
11 RAF rats and present in RCB rats along with *B. breve* JCM 1192^T were shown to be
12 *Bifidobacterium animalis*. In addition, a newly designed *B. animalis*-specific probe (probe
13 Bani449) was used in this study. This species represented a significant proportion of the
14 population, accounting 20.5% and 18.5% of the total populations in RAF and RCB rats,
15 respectively. The second trial also gave the similar changes in the population of total
16 bifidobacteria, *B. breve* and *B. animalis* confirming the reproducibility of the bifidobacterial
17 responces (data not shown).

18 The increase in number of cells of the administered strain was strictly dependent on
19 raffinose (Table 3). These results strongly support the synbiotic concept. Two-way
20 ANOVA suggested that most of the alterations in the bacterial populations, particularly the
21 increases in lactobacilli and bifidobacteria, were significantly influenced by the inclusion of
22 raffinose in the diets.

23 **TRF analysis of cecal samples.** T-RF lengths of *B. animalis* isolated on TOS
24 propionate agar from rat cecal samples and *B. breve* JCM 1192^T were observed using three
25 restriction enzymes as the standard T-RF peak-sets. *B. animalis* isolate resulted the observed

1 T-RF lengths of 28, 220 (*Hae*III); 335 (*Hha*I); 38, 41, 95 (*Msp*I), showing a slight difference
2 from predicted values: 36, 220 (*Hae*III); 334, 337 (*Hha*I); 45, 98 (*Msp*I). The observed T-RF
3 lengths of *B. breve* JCM 1192^T were 218 (*Hae*III); 332 (*Hha*I); 93(*Msp*I), whereas the
4 predicted values were 219 (*Hae*III); 333 (*Hha*I); 97 (*Msp*I). Most of the experimentally
5 determined T-RF lengths were within 4 bp of the predicted lengths, which was near to the
6 shortest size of DNA standard used (GS 500 ROX). Similar phenomena have been reported
7 (23, 36). The observed T-RF lengths of these strains contributed to the identification and the
8 interpretation of the T-RF patterns from rat cecal samples.
9 By comparison with the T-RF peak-sets of the reference strains, presence of *B.*
10 *animalis* was detected in the RAF and the RCB groups (Fig. 3). The T-RFLP
11 electropherogram revealed that *B. breve* was present only in the RCB group and that it
12 accounted for about 3.3 to 6.0% of the total peak area, as shown in Fig. 3. Although peak
13 areas do not represent the actual population proportions of bacteria of interest, these data
14 confirmed the existence of *B. breve* in the expected cecal samples.
15 According to the alignment of the T-RF peak-set, seven peak-sets were common to the
16 four groups, although in different proportions. Approximately 29 peak-sets present in the
17 control (BD) group were not found in the RAF or RCB groups; two of the indigenous peak-
18 sets had relatively smaller areas in the BD group than in the RAF and RCB groups (data not
19 shown). On the other hand, about 16 peak-sets were newly detected in the RAF and RCB
20 groups, and two indigenous peak-sets had relatively greater areas than the corresponding
21 peak-sets in the BD group (data not shown).
22 Then, we performed PCA, an advanced statistical technique, to reveal the variance-
23 covariance structure of the T-RF patterns among all samples (Fig. 4). A total of 209
24 fragments from three restriction enzyme digests (*Hae*III, 54 fragments; *Hha*I, 82 fragments;
25 and *Msp*I, 73 fragments) were included in the analysis. We found that the correlation of

1 variance was comprised of two principal components and the yield cumulative sum of squared
2 loading (variance) was 97.3%. The spots representing the RAF and RCB groups were
3 separated from the spots for the BD and CB groups on the PC1 axis (82.8%). This suggested
4 that the first principal component (PC1) separates samples based on the presence of raffinose
5 in the diet and constitutes a major factor in bacterial population alterations (Fig. 4). Spot of
6 the CB group was close to that of the BD group, while those of the RAF and RCB groups
7 were significantly separated from each other on the PC2 axis (14.5%). The second principal
8 component (PC2) appeared to separate samples based on the presence of *B. breve* in RCB
9 group.
10
11 **DISCUSSION**
12 Rat/mouse intestinal microbiota consists of complex bacterial communities, which is
13 confirmed by culture methods and 16S rRNA gene clone library analyses (24, 37, 46). In
14 FISH analysis, we focused on *Bacteroides*, *Bifidobacterium*, *Clostridium*, *Eubacterium*,
15 *Lactobacillus*, and *Streptococcus*, which are reported as the member of rat/mouse intestinal
16 microbiota (37, 38, 46). Six sets of genus/group-specific oligonucleotide probes identified
17 about 29-55% of DAPI-stained cells and about 32-64% of active bacterial cells (Eub338) in
18 the rat cecal communities (Table 3). The relatively high proportions of "unidentified
19 bacteria" (about 45-71%) in Table 3 seemed to be due to the presence of large population of
20 uncharacterized bacterial group called "fusiform-shaped anaerobic bacteria" in murine
21 microbiota (10, 39). This group of bacteria is morphologically rather unique as being long
22 curved rods with sharp thin ends (39), and can be seen among microbial population in BD and
23 CB groups (Fig. 2ac).
24 The bifidobacteria were found to dominate the cecal microbial community in the RCB
25 (synbiotic) group (Table 3). The presence of *B. breve* in this group was clearly demonstrated

1 by FISH analysis (Fig. 2d, Table 3), and was also confirmed by T-RFLP analysis (Fig. 3).
2 Successful proliferation of administrated *B. breve* JCM 1192^T observed in RCB group seems
3 to be attributable to the availability of raffinose for *B. breve* JCM 1192^T cells, because
4 raffinose is not digested in rat intestine (17) and reach directly to cecum as the most
5 preferable carbon source for this bacterium. The substantial increase of *B. breve* JCM 1192^T
6 cell number in the RCB group strongly demonstrated the effectiveness of the combination of
7 raffinose and *B. breve* JCM 1192^T.

8 Raffinose administration alone (RAF) appeared to induce a marked increase in
9 indigenous bifidobacteria and lactobacilli (Table 3). This bifidobacteria was identified as *B.*
10 *animalis* by FISH and T-RFLP analyses. Although the presence of *B. animalis* in rat intestine
11 has been reported long time ago (40), the population of this bacterium in rat intestine has not
12 been reported precisely. This study showed for the first time using molecular ecological
13 methods that *B. animalis* exists as a minor member in rat cecum (0.2%) and can be boosted up
14 to about 20% of the microbiota on raffinose administration. The increases in *Bifidobacterium*
15 and *Lactobacillus* populations in RAF and RCB groups corresponded to the increased
16 production of acetic and lactic acids and the decreased pH values of the cecal contents (Table
17 2).

18 Interestingly no proliferation of *B. breve* JCM 1192^T was observed in CB group in
19 contrast to RCB group (Table 3). This may be explained by the fact that this bacterium lacks
20 the ability to utilize sucrose that is contained in large amounts in rat basal diet (602.5g/kg
21 diet). On the other hand, *B. breve* can utilize glucose and fructose (data not shown), which
22 are the hydrolysate products of sucrose. Thus, we can also expect that there was a good
23 chance for *B. breve* to be fed by sucrose hydrolysate (glucose and fructose) in rat intestine.
24 Since, we did not observe any proliferation of *B. breve* cells in CB group, our results (*B. breve*

1 proliferation observed only in RCB group) can illustrate the dependence of this strain upon
2 administered prebiotic all the more clear owing to the inability of this strain to utilize sucrose.
3 FISH analysis can provide information on bacterial populations at the genus, group,
4 and even species levels in terms of identification and enumeration (4). However, limited
5 range of oligonucleotide probe set prevents us from obtaining whole picture of microbial
6 population in rat cecum. Therefore, we performed PCA to distinguish the bacterial
7 communities in the rat cecal contents based on T-RFs profiles (Fig. 4). The purpose of PCA
8 is data reduction, which allows data interpretation through a few linear combinations of the
9 original variables (treatments). PCA confirmed the differences of rat microbiota in CB and
10 RCB based on T-RFLP profile. Composition of microbiota in CB was more similar to BD,
11 indicating that probiotic administration only was not enough to modulate the bacterial
12 population. When synbiotic was applied, the significant changes of bacterial population were
13 observed along with the proliferation of *B. breve* as also confirmed by FISH analysis. These
14 analyses led to the conclusion that application of synbiotic is necessary for the target strain, *B.*
15 *breve* JCM 1192^T, to proliferate in rat cecum.
16 Introduction of synbiotics concept by Gibson and Roberfroid (17) promoted the
17 application of potential non-indigenous probiotic strain and its preferable carbon source to
18 proliferate the administered strain in intestine. Several works published on the synbiotic
19 application include modulation of intestinal microbiota (1, 7, 8, 32, 44), reduction of colon
20 carcinogenesis (12, 16, 28) and protection from pathogen infection (6). Recently synbiotic
21 therapy for the improvement of intestinal function (19, 20) or reduction of pathogenic bacteria
22 (21, 22) has also been reported.
23 However, in many cases, demonstration of synbiotic effects seems not sufficient from
24 microbiological point of view. One of the most important criteria for the evaluation of
25 synbiotic effects is to monitor the proliferation of the administered probiotic bacteria at the

1 species level, which seems possible only by the application of molecular ecological methods
2 for microbiota analysis. In most cases, however, culture-based methods were applied using
3 selective-agar medium for counting the numbers of bacteria in fecal and cecal samples (6, 8,
4 28, 32, 44). Selective medium is sometimes not selective or too selective for target bacteria
5 in microbiota analysis (5). Thus, the culture-based methods are not suitable to distinguish the
6 administered bacteria from similar group of bacteria, thereby allowing only group-level
7 analysis. In addition, it has been shown that only about 20-30% of microorganisms in human
8 intestines are culturable (42). Hence, the reliability of the culture-based methods is judged to
9 be rather low. Besides these technological obstacles, many studies lack control experiments
10 such as probiotic or prebiotic alone (7, 19, 20, 21, 22, 32, 44), making the interpretation of the
11 synbiotic results not conclusive.

12 In contrast to these previous reports, our work may provide the first clear
13 demonstration of the microbiological aspects of the synbiotic effects in rat intestinal
14 microbiota. First of all, we have quantitatively monitored the proliferation of administered
15 strain at the species level and analyzed the other group of bacteria using FISH method. In
16 addition, T-RFLP technique was applied to confirm FISH results and to compare whole
17 microbiota between the experimental groups. These two molecular methods were applied for
18 the first time to microbiota analysis in synbiotic experiment. As a potential probiotic we have
19 employed *B. breve* JCM 1192^T the carbon source utilization of which is well characterized.
20 Analysis of complete set of treatment groups (BD, RAF, CB and RCB) was conducted.
21 Organic acids as the indicator of the microbial activity were also analyzed. All these
22 experimental design made precise analysis and interpretation of the obtained data possible.

23 Although complex nature of human diet, availability of raffinose in human intestine
24 seems relatively low. Raffinose is found in food legumes at relatively high content (up to
25 about 2%), but this amount was reduced significantly during food processing (34). Therefore,

1 supplementation of raffinose would be necessary to support the proliferation of administered

2 *B. breve* JCM 1192^{T} also in human intestine, which will be evaluated in human trial.

3
4

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REFERENCES

1 **Alander, M., J. Mättö, W. Kneifel, M. Johansson, B. Kögler, R. Crittenden, T. Mattila-Sandholm, and M. Saarela.** 2001. Effect of galacto-oligosaccharide supplementation on human faecal microflora and on survival and persistence of *Bifidobacterium lactis* Bb-12 in the gastrointestinal tract. Int. Dairy J. **11**:817-825.

2. **Alm, E. W., D. B. Oerther, N. Larsen, D. A. Stahl, and L. Raskin.** 1996. The oligonucleotide probe database. Appl. Environ. Microbiol. **62:**3557-3559.

3. **Amann, R. I., B. J. Binder, R. J. Olson, S. W. Chisholm, R. Devereux, and D. A. Stahl.** 1990. Combination of 16S rRNA-targeted oligonucleotide probes with flow cytometry for analyzing mixed microbial populations. Appl. Environ. Microbiol. **56:**1919-1925.

4. **Amann, R. I., W. Ludwig, and K. H. Schleifer.** 1995. Phylogenetic identification and *in situ* detection of individual microbial cells without cultivation. Microbiol. Rev. **59:**143-169.

5. **Apajalahti, J. H. A., A. Kettunen, P. H. Nurminen, H. Jatila, and W. E. Holben.** 2003. Selective plating underestimates abundance and shows differential recovery of bifidobacterial species from human feces. Appl. Environ. Microbiol. **69**:5731-5735.

6. **Asahara, T., K. Nomoto, K. Shimizu, M. Watanuki, and R. Tanaka.** 2001. Increased resistance of mice to *Salmonella enterica serovar Typhimurium* infection by synbiotic administration of bifidobacteria and transgalactosylated oligosaccharides. J. Appl. Microbiol. **91:**985-996**.**

7. **Bartosch, S., E. J. Woodmansey, J. C. M. Paterson, M. E. T. McMurdo, and G. T. Macfarlane.** 2005. Microbiological effects of consuming a synbiotic containing *Bifidobacterium bifidum*, *Bifidobacterium lactis*, and oligofructose in elderly persons,

1 determined by real-time polymerase chain reaction and counting of viable bacteria. Clin.
2 Infect. Dis. **40**:28-37.
3 8. **Bielecka, M., E. Biedrzycka, and A. Majkowska.** 2002. Selection of probiotics and
4 prebiotics for synbiotics and confirmation of their *in vivo* effectiveness. Food Res. Int.
5 **35**:125-131.
6 9. **Crittenden, R. G.** 1999. Prebiotics, p. 141-156. *In* G. W. Tannock (ed.), Probiotics: a
7 critical review. Horizon Scientific Press, Wymondham, England.
8 10. **Dewhirst, F. E., C. C. Chien, B. J. Paster, R. L. Ericson, R. P. Orcutt, D. B. Schauer,**
9 **and J. G. Fox.** 1999. Phylogeny of the defined murine microbiota: altered Schaedler
10 flora. Appl. Environ. Microbiol. **65:**3287-3292.
11 11. **de Man, J. C., M. Rogosa, and M. E. Sharpe**. 1960. A medium for the cultivation of
12 lactobacilli. J. Appl. Bacteriol. **23**:130-135.
13 12. **Femia, A. P., C. Luceri, P. Dolara, A. Giannini, A. Biggeri, M. Salvadori, Y. Clune,**
14 **K. J. Collins, M. Paglierani, and G. Caderni.** 2002. Antitumorigenic activity of the
15 prebiotic inulin enriched with oligofructose in combination with the probiotics
16 *Lactobacillus rhamnosus* and *Bifidobaterium lactis* on azoxymethane-induced colon
17 carcinogenesis in rats. Carcinogenesis **23**:1953-1960.
18 13. **Franks, A. H., H. J. M. Harmsen, G. C. Raangs, G. J. Jansen, F. Schut, and G. W.**
19 **Welling.** 1998. Variations of bacterial populations in human feces measured by
20 fluorescent *in situ* hybridization with group-specific 16S rRNA-targeted oligonucleotide
21 probes. Appl. Environ. Microbiol. **64:**3336-3345.
22 14. **Fuchs, B. M., F. O. Glöckner, J. Wulf, and R. Amann.** 2000. Unlabeled
23 oligonucleotides increase the *in situ* accessibility to 16S rRNA of fluorescently labeled
24 oligonucleotide probes. Appl. Environ. Microbiol. **66:**3603-3607.
25 15. **Fuller, R.** 1989. Probiotics in man and animals. J. Appl. Bacteriol. **66:**365-378.

1 16. **Gallaher, D. D., and J. Khil.** 1999. The effect of synbiotics on colon carcinogenesis in
2 rats. J. Nutr. **129:**1483S-1487S.
3 17. **Gibson, G. R., and M. B. Roberfroid.** 1995. Dietary modulation of the human colonic
4 microbiota: introducing the concept of prebiotics. J. Nutr. **125:**1401-1412.
5 18. **Hoshi, S., T. Sakata, K. Mikuni, H. Hashimoto, and S. Kimura.** 1994
6 Galactosylsucrose and xylosylfructoside alter digestive tract size and concentrations of
7 cecal organic acids in rats fed diets containing cholesterol and cholic acid. J. Nutr.
8 **124:**52-60.
9 19. **Kanamori, Y., K. Hashizume, M. Sugiyama, M. Morotomi, and N. Yuki.** 2001.
10 Combination therapy with *Bifidobacterium breve, Lactobacillus casei,* and
11 galactooligosaccharides dramatically improved the intestinal function in a girl with short
12 bowel syndrome. Dig. Dis. Sci. **46**:2010-2016.
13 20. **Kanamori, Y., K. Hashizume, M. Sugiyama, M. Morotomi, N. Yuki, and R. Tanaka.**
14 2002. A novel synbiotic therapy dramatically improved the intestinal function of a
15 pediatric patient with laryngotracheo-esophageal cleft (LTEC) in the intensive care unit.
16 Clin. Nutr. **21**:527-530.
17 21. **Kanamori, Y., K. Hashizume, Y. Kitano, Y. Tanaka, M. Morotomi, N. Yuki, and R.**
18 **Tanaka.** 2003. Anaerobic dominant flora was reconstructed by synbiotics in an infant
19 with MRSA enteritis. Pediatr. Int. **45**:359-362.
20 22. **Kanamori, Y., M. Sugiyama, K. Hashizume, N. Yuki, M. Morotomi, and R. Tanaka.**
21 2004. Experience of long-term synbiotic therapy in seven short bowel patients with
22 refractory enterocolitis. J. Pediatr. Surg. **39**:1686-1692.
23 23. **Kaplan, C. W., J. C. Astaire, M. E. Sanders, B. S. Reddy, and C. L. Kitts.** 2001. 16S
24 ribosomal DNA terminal restriction fragment pattern analysis of bacterial communities in

1 feces of rats fed *Lactobacillus acidophilus* NCFM. Appl. Environ. Microbiol. **67:**1935-
2 1939.
3 24**. Kibe, R., M. Sakamoto, H. Hayashi, H. Yokota, and Y. Benno.** 2004. Maturation of
4 the murine cecal microbiota as revealed by terminal restriction fragment length
5 polymorphism and 16S rRNA gene clone libraries. FEMS Microbiol. Lett. **235**:139-146.
6 25. **Kimura, K., A. L. McCartney, M. A. McConnell, and G. W. Tannock.** 1997. Analysis
7 of fecal populations of bifidobacteria and lactobacilli and investigation of the
8 immunological responses of their human hosts to the predominant strains. Appl. Environ.
9 Microbiol. **63:**3394-3398.
10 26. **Kurdi, P., H. Tanaka, H. W. van Veen, K. Asano, F. Tomita, and A. Yokota.** 2003.
11 Cholic acid accumulation and its diminution by short-chain fatty acids in bifidobacteria.
12 Microbiology **149:**2031-2037.
13 27. **Langendijk, P. S., F. Schut, G. J. Jansen, G. C. Raangs, G. R. Kamphuis, M. H. F.
14 Wilkinson, and G. W. Welling.** 1995. Quantitative fluorescence *in situ* hybridization of
15 *Bifidobacterium* spp. with genus-specific 16S rRNA-targeted probes and its application in
16 fecal samples. Appl. Environ. Microbiol. **61:**3069-3075.
17 28. **Le Leu, R. K., I. L. Brown, Y. Hu, A. R. Bird, M. Jackson, A. Esterman, and G. P.
18 Young**. 2005. Synbiotic combination of resistant starch and *Bifidobaterium lactis*
19 facilitates apoptotic deletion of carcinogen-damaged cells in rat colon. J. Nutr. **135**:996-
20 1001.
21 29. **Liu, W. T., T. L. Marsh, H. Cheng, and L. J. Forney.** 1997. Characterization of
22 microbial diversity by determining terminal restriction fragment length polymorphisms of
23 genes encoding 16S rRNA. Appl. Environ. Microbiol. **63**:4516-4522.
24 30. **Manz, W., R. Amann, W. Ludwig, M. Vancanneyt, and K. H. Schleifer.** 1996.
25 Application of a suite of 16S rRNA-specific oligonucleotide probes designed to

1 investigate bacteria of the phylum cytophaga-flavobacter-bacteroides in the natural
2 environment. Microbiology **142:**1097-1106.

3 31. **Marsh, T. L.** 1999. Terminal restriction fragment length polymorphism (T-RFLP): an
4 emerging method for characterizing diversity among homologous populations of
5 amplification products. Curr. Opin. Microbiol. **2:**323-327.

6 32. **Morelli, L., D. Zonenschain, M. L. Callegari, E. Grossi, F. Maisano, and M. Fusillo.**
7 2003. Assessment of a new synbiotic preparation in healthy volunteers: survival,
8 persistence of probiotic strains and its effect on the indigenous flora. Nutr. J. **2**:11-16.

9 33. **Mori, K., K. Yamazaki, T. Ishiyama, M. Katsumata, K. Kobayashi, Y. Kawai, N.**
10 **Inoue, and H. Shinano.** 1997. Comparative sequence analyses of the genes coding for
11 16S rRNA of *Lactobacillus casei*-related taxa. Int. J. Syst. Bacteriol. **47:**54-57.

12 34. **Reddy, N. R., S. K. Sathe, and D. K. Salunkhe.** 1989. Carbohydrates, p. 51-74. *In* D. K.
13 Salunkhe and S. S. Kadam (ed.), CRC handbook of world food legumes: nutritional
14 chemistry, processing technology, and utilization, vol. 1. CRC Press, Inc., Boca Raton,
15 Florida.

16 35. **Reeves, P. G., F. H. Nielsen, and G. C. Fahey Jr.** 1993. AIN-93 purified diets for
17 laboratory rodents: final report of the American Institute of Nutrition ad hoc writing
18 committee on the reformulation of the AIN-76A rodent diet. J. Nutr. **123:**1939-1951.

19 36. **Sakamoto, M., Y. Takeuchi, M. Umeda, I. Ishikawa, and Y. Benno.** 2003. Application
20 of terminal RFLP analysis to characterize oral bacterial flora in saliva of healthy subjects
21 and patients with periodontitis. J. Med. Microbiol. **52:**79-89.

22 37. **Salzman N. H., H. de Jong, Y. Paterson, H. J. M. Harmsen, G. W. Welling, and N. A.**
23 **Bos.** 2002. Analysis of 16S libraries of mouse gastrointestinal microflora reveals a large
24 new group of mouse intestinal bacteria. Microbiology **148:**3651-3660.

1 38. **Savage, D. C**. 1977. Microbial ecology of the gastrointestinal tract. Ann. Rev. Microbiol.
2 **31**:107-133.
3 39**. Savage, D. C., J. S. McAllister, and C. P. Davis.** 1971. Anaerobic bacteria on the
4 mucosal epithelium of the murine large bowel. Infect. Immun. **4**:492-502.
5 40. **Scardovi, V., and L. D. Trovatelli.** 1974. *Bifidobacterium animalis* (Mitsuoka) comb.
6 nov. and the *"minimum"* and *"subtile"* groups of new bifidobacteria found in sewage. Int.
7 J. Syst. Bacteriol. **24**:21-28.
8 41. **Sghir, A., D. Antonopoulos, and R. I. Mackie.** 1998. Design and evaluation of a
9 *Lactobacillus* group-specific ribosomal RNA-targeted hybridization probe and its
10 application to study of intestinal microecology in pigs. System. Appl. Microbiol. **21:**291-
11 296.
12 42. **Suau, A., R. Bonnet, M. Sutren, J. J. Godon, G. R. Gibson, M. D. Collins, and J.
13 Doré.** 1999. Direct analysis of genes encoding 16S rRNA from complex communities
14 reveals many novel molecular species within the human gut. Appl. Environ. Microbiol.
15 **65:**4799-4807.
16 43. **Suksomcheep, A., I N. Sujaya, K. Saito, A. Yokota, K. Asano, and F. Tomita.** 2001.
17 Development of 16S rDNA targeted probes for detection of some human health
18 beneficial strains of intestinal microorganisms. Biotechnol. Sust. Util. Biol. Resour.
19 Tropics **15:**111-125.
20 44. **Tortuero, F., E. Fernández, P. Ruperéz, and M. Moreno.** 1997. Raffinose and lactic
21 acid bacteria influence caecal fermentation and serum cholesterol in rats. Nutr. Res. **17:**
22 41-49.
23 45. **Vollaard, E. J., and H. A. L. Clasener.** 1994. Colonization resistance. Antimicrob.
24 Agents Chemother. **38:**409-414.

1 46. **Yanabe, M., M. Shibuya, T. Gonda, H. Asai, T. Tanaka, K. Sudou, T. Narita, and K.
2 Itoh.** 2001. Establishment of specific pathogen-free (SPF) rat colonies using gnotobiotic
3 techniques. Exp. Anim. **50:**293-298.

1 FIG. 1. Alignment of probe sequences, their target sites, and the sequences of the
2 corresponding sites in reference microorganisms of the large intestine (Type strains). Probe
3 names are in accordance with the Oligonucleotide Probe Database nomenclature (2). N is an
4 A/T/C/G wobble nucleotide.
5
6 FIG. 2. Epifluorescence images of bacterial cells from the four groups of rat cecal samples
7 stained with DAPI (green) and hybridized with a *Bifidobacterium breve* species-specific
8 oligonucleotide probe (PBR2) (red) in FISH analysis.
9
10 FIG. 3. Partial electropherogram of three restriction enzymes, *Hae*III, *Hha*I, *Msp*I-derived
11 TRF profile from four rat cecal samples and two bifidobacteria. The size of each T-RF is
12 indicated in base pairs along with the horizontal scale at the top the GeneScan results display.
13
14 FIG. 4. PCA of T-RFLP profiles from four groups of rat cecal samples. The percent
15 variation accounted for each principal component is indicated in parentheses in the axis titles
16 below and to the left, along with the principal component loading values.

TABLE 1. 16S rRNA-targeted oligonucleotide probes used for the molecular analysis of rat cecal samples.

Probe name (systematic name)	Probe sequence from 5′ to 3′	Target organism	Target site[a]	Source[b]
Eub338 (S-D-Bact-0338-a-A-18)	GCTGCCTCCCGTAGGAGT	Bacteria	338	3
Genus/group specific probe				
Erec482 (S-*-Erec-0482-a-A-19)	GCTTCTTAGTCARGTACCG	*Clostridium coccoides* and *Eubacterium rectale* group	482	13
Chis150(S-*-Clos-0150-a-A-23)	TTATGCGGTATTAATCTYCCTTT	*Clostridium histolyticum* group	150	13
Bac303 (S-G-Bac-0303-a-A-17)	CCAATGTGGGGGACCTT	*Bacteroides* group	303	30
Lacb722 (S-G-Lacb-0722-a-A-25)	YCACCGCTACACATGRAGTTCCACT	*Lactobacillus* group	722	41
Strc493 (S-*-Strc-0493-a-A-19)	GTTAGCCGTCCCTTTCTGG	Streptococci and lactococci	493	13
Bif164m (S-G-Bif-0164-b-A-18)	CATCCGGYATTACCACCC	*Bifidobacterium* spp.	164	This work[c]
Species/strain specific probe				
PBR2 (S-S-Pbr2-0182-a-A-19)	CCATGCGGTGTGATGGAGC	*Bifidobacterium breve*	182	43
Bani433 (S-S-Bani-0449-a-A-21)	CACTCAACACGGCCCGAAGCC	*Bifidobacterium animalis* JCM 1190^T	449	This work[d]
Non labeled oligonucleotides (helpers)				
PBR2-1st-Upper helper	ATCCGGCATTACCACCCGT	*Bifidobacterium breve*	163	This work
PBR2-1st-Lower helper	CAAAGGCTTTCCCAACACA	*Bifidobacterium breve*	201	This work
PBR2-2nd-Upper helper	TTCCAGGAGCTATTCCGGT	*Bifidobacterium breve*	144	This work
PBR2-2nd -Lower helper	GCGACCCCATCCCATGCCG	*Bifidobacterium breve*	220	This work
Bani433-1st-Upper helper	GTGCCTTGCCCTTGAACAAAA	*Bifidobacterium animalis* JCM 1190^T	428	This work
Bani433-1st-Lower helper	CCGGTGCTTATTCGAACAATC	*Bifidobacterium animalis* JCM 1190^T	470	This work

[a] Positions in *E. coli* 16S rRNA.

[b] Reference number.

[c] Bif164m was modified from Bif164 reported by Langendijk et al. (27) by changing the eighth 5′-nucleotide from C to Y.

[d] Newly designed in this investigation.

TABLE 2. Characterization of rat cecal samples: organic acids produced, cecal contents weight, and pH value and two-way ANOVA.

	Amount of organic acids [a] (μmol/g cecal content)				Two-way ANOVA		
	1. BD	2. RAF	3. CB	4. RCB	Raffinose	*B. breve*	Raffinose x *B. breve*
Lactate	ND[b]	16.8 (5.8)	1.4 (0.9)	4.7 (2.1)	*	NS[c]	NS
Acetate	42.1 (5.7)	54.3 (6.7)	35.7 (2.9)	53.3 (8.3)	*	NS	NS
Propionate	9.4 (1.0)	11.1 (0.7)	12.8 (0.8)	11.9 (1.3)	NS	*	NS
Butyrate	9.3 (2.2)	3.4 (1.0)	8.9 (0.9)	6.4 (2.3)	*	NS	NS
Succinate	26.7 (5.1)	21.2 (6.1)	5.4 (2.0)	31.1 (7.9)	NS	NS	*
Isovalerate	1.0 (0.3)	0.1 (0.1)	1.0 (0.1)	ND	*	NS	NS
Valerate	1.6 (0.1)	0.4 (0.2)	1.9 (0.2)	0.7 (0.2)	*	NS	NS
Cecal contents weight (g)	1.21 (0.09)	2.13 (0.09)	0.94 (0.05)	2.29 (0.24)	*	NS	NS
Cecal contents pH	8.1 (0.1)	6.8 (0.1)	8.0 (0.1)	7.0 (0.2)	*	NS	NS

[a] Values are expressed as means (SEM), n = 6. BD represents basal diet with crystalline cellulose (50 g/kg diet), RAF represents BD supplemented with raffinose (30 g/kg diet), CB represents BD supplemented with gelatin-encapsulated *B. breve* (30 g/kg diet), and RCB represents BD supplemented with raffinose (30 g/kg diet) and encapsulated *B. breve* (30 g/kg diet).

[b] ND, not detected.

[c] NS, not significant.

[*] Significantly different (p<0.05).

.

TABLE 3. Characterization of rat cecal microbiota based on FISH analysis: Number of bacterial cells per gram, percentage of total microbiota, and two-way ANOVA.

Population	Stain or probe	1. BD (control)	2. RAF	3. CB	4. RCB	Two-way ANOVA		
		% Microbiota[a] of cecal sample from 4 test diets				Raffinose	*B. breve*	Raffinose x *B. breve*
Total cells	DAPI	100	100	100	100			
Total bacteria	Eub338	89.6 (1.5)	84.4 (1.4)	90.4 (0.8)	86.3 (1.3)	*	NS[c]	NS
Clostridium coccoides and *Eubacterium rectale* group	Erec482	23.9 (2.7)	12.9 (3.3)	29.7 (4.0)	23.2 (5.8)	*	*	NS
Clostridium histolyticum group	Chis150	0.3 (0.1)	1.2 (0.4)	0.1 (0.0)	1.2 (0.4)	*	*	*
Bacteroides group	Bac303	3.6 (1.4)	2.1 (0.6)	0.9 (0.2)	2.5 (1.1)	NS	NS	*
Lactobacillus group	Lacb722	0.2 (0.0)	7.2 (1.1)	0.8 (0.2)	2.8 (0.6)	*	*	*
Streptococci and lactococci	Strc493	0.5 (0.2)	1.5 (0.9)	0.8 (0.4)	0.3 (0.2)	NS	NS	NS
Bifidobacterium spp.	Bif164m	0.4 (0.2)	19.5 (4.8)	0.3 (0.1)	25.4 (4.6)	*	NS	NS
Unidentified bacteria	none	71.1	55.6	67.4	44.6			
Bifidobacterium breve	PBR2	ND[b]	ND	ND	6.3 (1.9)	*	*	*
Bifidobacterium animalis	Bani449	0.2 (0.1)	20.5 (2.2)	0.1 (0.0)	18.5 (2.7)	*	NS	NS
Number of cells/g cecal contents[d]		$1.90 (0.9)\times10^{10}$	$1.74 (0.5)\times10^{10}$	$2.06 (0.4)\times10^{10}$	$2.19 (0.3)\times10^{10}$			

[a] Average percentage of the total cells visualized by DAPI signals in the same microscopic field. Values are expressed as means (SEM), n = 6.

[b] ND, not detected.

[c] NS, not significant.

[d] Wet weight value are expressed as means (SEM), n=6.

[*] Significantly different ($p<0.05$).

PBR2

(S-S-Pbr2-0182-a-A-19)	3′CGAGGTAGTGTGGCGTACC 5′
Target	5′GCTCCATCACACCGCATGG 3′
Bifidobacterium breve	....
Bifidobacterium longum	.T....GTTG.T.......
Bifidobacterium infantis	.T....GTTG.T.......
Bifidobacterium adolescentis	......GTTGGAT.....T
Bacteroides distasonis	.AAG..GGGAT..CGCAT.
Bacteroides ovatus	AGCAT.CG.ANANCGCAT.
Lactobacillus acidophilus	AAGAA.G..G.T......A
Lactobacillus salivarius	ATCT.TAAGG.T......A
Clostridium innocuum	AGGTATA.GG.G......C
Enterococcus faecalis	AACAGT.T.TG........
Eubacterium aerofaciens	A.C..GGGGTG........
Ruminococcus productus	AAG.GCA..GGA.CGCAT.

Bani449

(S-S-Bani-0449-a-A-21)	3′CCGAAGCCCGGCACAACTCAC 5′
Target	5′GGCTTCGGGCCGTGTTGAGTG 3′
Bifidobacterium animalis	....
Bifidobacterium pseudolongum	C.G..TC..............
Bifidobacterium lactis	C.G..TC..............
Bifidobacterium adolescentis	..G.A.....G.NC.......
Bifidobacterium breve	CAAGG.ACTTT..........
Bacteroides vulgatus	T.A.A.T..ATA.C.......
Bacteroides distasonis	..GACGT.T....T...TA..
Lactobacillus acidophilus	.AGG.A.TAA.TG.CCTTTAT
Lactobacillus salivarius	.TGAGA.TAA.TGT.CATTC.
Clostridium clostridiiforme	ACGG.ACCTGAC.AAGA..CC
Clostridium cocleatum	.ATGGA..AAATGACATCCGA
Enterococcus faecalis	ACG..A.TAA.TGAAC.TCCC
Ruminococcus productus	..TAC.T.A.TAA.AA.CCCC

FIG 1. Dinoto et al

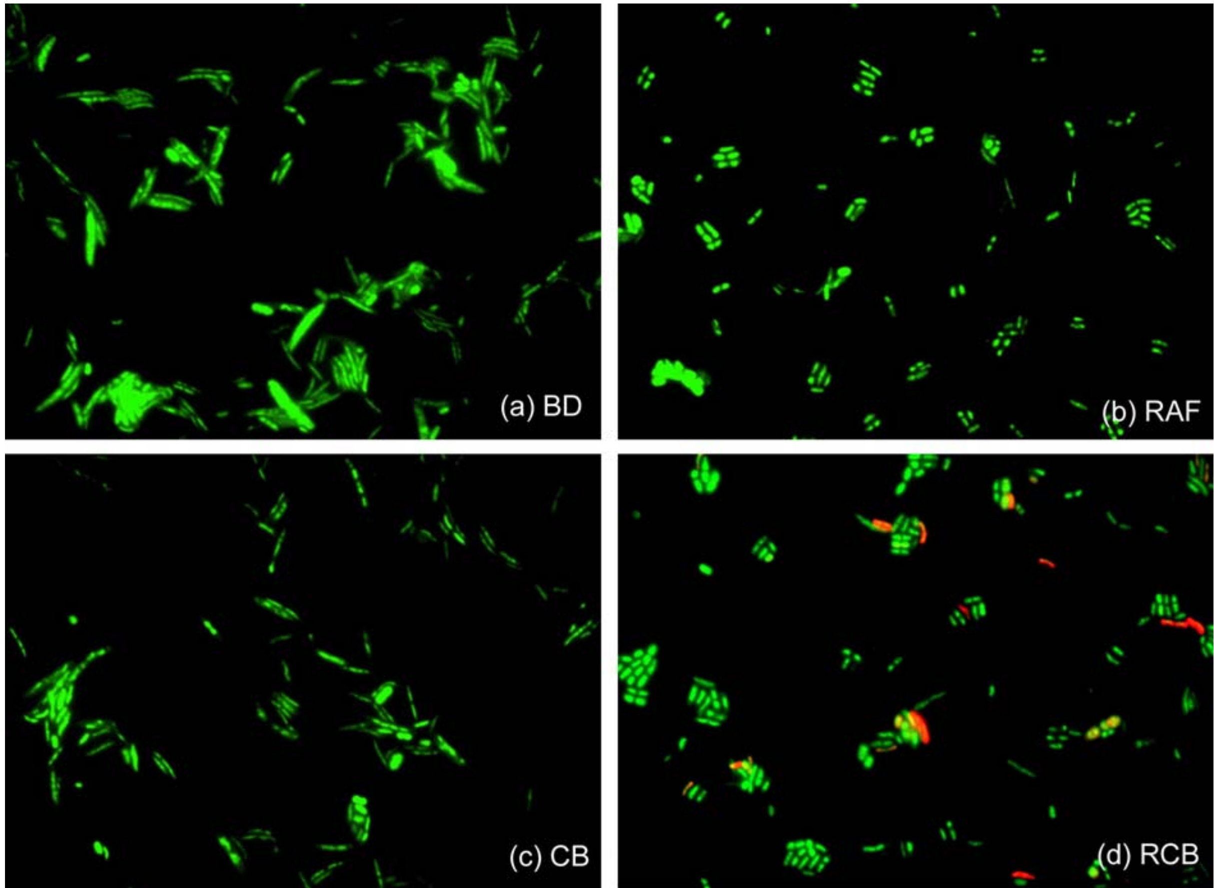

FIG 2. Dinoto et al

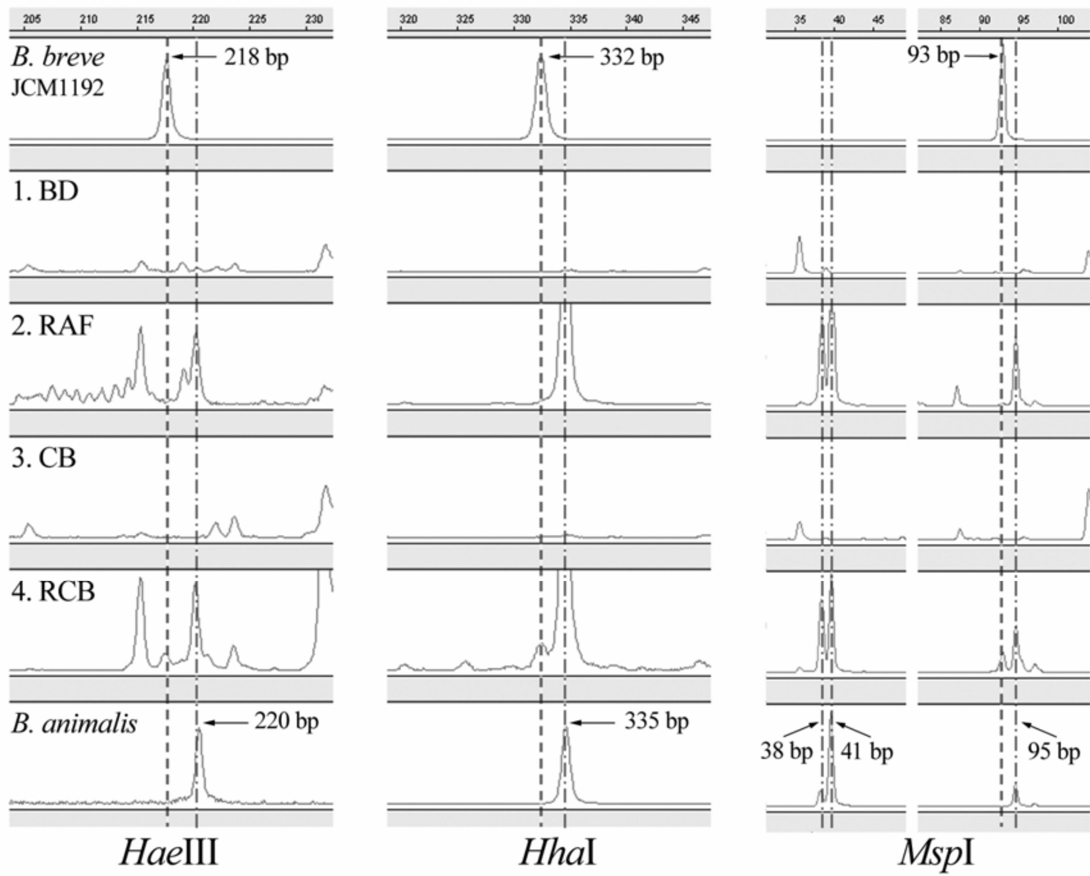

FIG 3. Dinoto et al

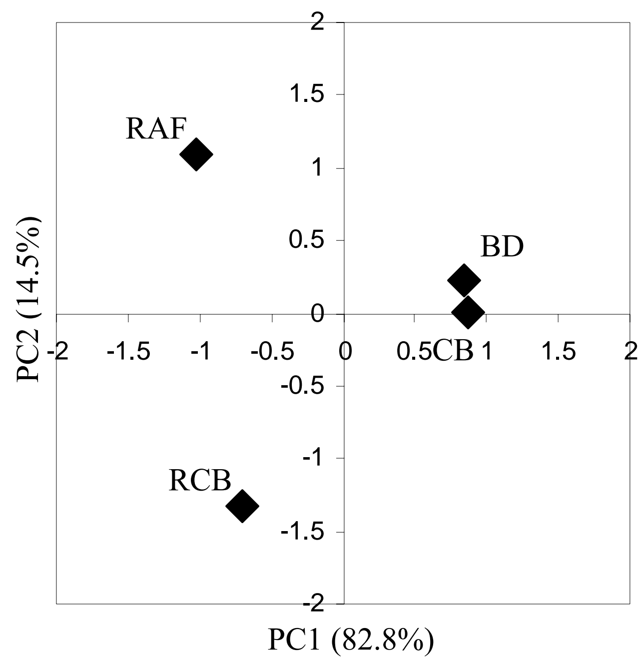

FIG 4. Dinoto et al