Population Dynamics of *Bifidobacterium* Species in Human Feces during Raffinose Administration Monitored by FISH-Flow Cytometry

Running title: BIFIDOBACTERIAL POPULATION DURING RAFFINOSE TREATMENT

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ABSTRACT

Population dynamics of bifidobacteria in human feces during raffinose administration were investigated at the species level using fluorescence in situ hybridization (FISH) coupled with flow cytometry (FCM) analysis. Although double-staining FISH-FCM using both fluorescein isothiocyanate (FITC) and indodicarbocyanine (Cy5) as labeling dyes of fecal samples has been reported, the analysis was interfered by strong autofluorescence at FITC fluorescence region because of the presence of autofluorescence particles/debris in the fecal samples. We circumvented this problem by using only Cy5 fluorescent dye in the FISH-FCM analysis. Thirteen subjects received 2 g of raffinose twice a day for 4 weeks. Fecal samples were collected, and the bifidobacterial populations were monitored using the established FISH-FCM method. The results showed an increase of bifidobacteria from about 12.5% of total bacteria in the pre-feeding period to about 28.7 and 37.2% after 2-week and 4-week feeding periods, respectively. *Bifidobacterium adolescentis, B. catenulatum* group, and *B. longum* were major species in that order at the pre-feeding period, and these bacteria were found to increase nearly in parallel during the raffinose administration. During feeding periods, indigenous bifidobacterial populations became more diverse, such that minor species in human adults, such as *B. breve, B. bifidum*, *B. dentium*, and *B. angulatum*, proliferated. Four weeks after stopping raffinose administration, the proportion of each major bifidobacterial species, as well as that of total bifidobacteria, returned to approximately the original values of the pre-feeding period, whereas each minor species appeared to vary considerably from its original value. To the best of our knowledge, these results provide the first clear demonstration

of population dynamics of indigenous bifidobacteria at the species level in response to raffinose administration.

Raffinose (β-D-fructofuranosyl-O-α-D-galactopyranosyl-(1,6)-α-D-glucopyranoside) is a non-digestible oligosaccharide that is widely distributed in many plants, such as sugar beet, cane, cabbage, potato, grape, wheat, barley, corn, and seeds of many legumes (20, 21). In Hokkaido, Japan, raffinose is an important agricultural product that is extracted from sugar beets as a byproduct of sugar processing. Several prebiotic effects in humans have been reported for this oligosaccharide, including reduction of fecal ammonia and indole (18), improvement of defecation frequency (18), and increased cell numbers of indigenous bifidobacteria (5). Increase in bifidobacterial population in rat (8), and the suppression of T-helper 2 cell-mediated immune response in mice (19) have also been reported.

To obtain a scientific basis for the use of raffinose as a prebiotic, precise determination of its effects on microbiotic composition in the human intestine is required, especially on the bifidobacterial population at the species level. Because the majority of microbiota in the human intestine is not yet cultured (26), it is difficult to obtain reliable and quantitative results at the species level using culture-dependent methods. For this purpose, molecular ecological methods are required to be applied to investigate the population dynamics of bifidobacteria.

Among the many molecular ecological methods for analyzing microbiota, fluorescence in situ hybridization (FISH) is a widely used method for monitoring microorganisms in complex ecosystems (9, 11, 14). In FISH analysis, sets of group-, genus-, or species-specific ribosomal RNA-targeted oligonucleotide probes are applied for the identification and quantification of microorganisms. In our previous study (8), we successfully applied FISH analysis to evaluate the effect of raffinose administration on modulation of rat cecal microbiota and demonstrated a significant increase in the

population of *Bifidobacterium animalis*, an indigenous bifidobacterial species in rats, up to 20.5% of the total bacterial population, as compared to 0.2% observed in the basal diet group. Although FISH analysis is considered suitable for the enumeration of bacterial cells in complex microbial communities, the manual counting of detected bacteria is time-consuming and laborious. Several attempts have therefore been made to minimize the counting effort by combining the analysis with a flow cytometry technique (FCM) for high throughput analysis. Double staining FISH-FCM analysis using both fluorescein isothiocyanate (FITC) and indodicarbocyanine (Cy5) as labeling dyes has been successfully applied to enumerate bacterial populations in human fecal samples collected in European countries (12, 13, 17, 22, 23, 24, 30). In our case, however, this approach was not directly applicable because of strong autofluorescence materials found in the feces of volunteers (mostly Asian people living in Japan). We therefore modified the FISH-FCM procedure to solve this problem, and applied this modified method to investigate the population dynamics of bifidobacteria at the species level in the human intestine upon the administration of raffinose.

MATERIALS AND METHODS

Bacterial strain and medium. *Bifidobacterium breve* JCM 1192^T was obtained from the Japan Collection of Microorganisms (JCM, Wako, Japan). This bacterium was cultured in GAM Broth (Nissui Pharmaceutical Co., Ltd., Tokyo, Japan) at 37°C for 12 h under anaerobic conditions using mixed gas N_2:CO_2:H_2 (8:1:1).

Design of human trial. Fecal samples were collected from 13 healthy adults (11 males and 2 females; 23–57 years old) who originated from Japan (11 people), Indonesia (1 person), and Brazil (1 person). All subjects had lived in Japan for at least 6

months before the trial and they consumed their usual diet without restrictions on daily food consumption. Two grams of raffinose (Nippon Beet Sugar Manufacturing Co., Ltd., Tokyo, Japan) were introduced twice per day (total 4 g/day) to all subjects for 4 weeks. Fecal samplings were conducted at 1 week before raffinose consumption (0W), at 14^{th} day (2W) and 28^{th} day (4W) of raffinose intake, and 4 weeks after stopping raffinose intake (8W). This study was approved by the Ethics Committee of the Research Faculty of Agriculture, Hokkaido University.

Sample collection and preparation. Fecal samples were collected in sterile falcon tubes and stored at 4°C under anaerobic conditions using an anaerobic pouch (Mitsubishi Gas Chemical Company, Inc., Tokyo, Japan) for a maximum of 4 h before processing. Sample preparations were conducted as reported previously (8). About 0.5 g of fecal sample was suspended in ice-cold phosphate-buffered saline (PBS; 130 mM NaCl, 10 mM sodium phosphate buffer, pH 7.2) and centrifuged at 200 x*g* (low speed) for 5 min to remove large fecal particle/debris. This step was repeated three times and the supernatants were pooled. Fecal bacteria were then pelleted from the pooled supernatant using high-speed centrifugation at 9,000 x*g* for 2 min, and washed with PBS three times to remove inhibitory materials of the FISH reaction. Cultured *B. breve* JCM 1192^{T} cells were collected by centrifugation at 9,000 x*g* for 2 min and washed twice with PBS. Fecal samples and cultured bacterial cells were fixed with 4% (wt/vol) paraformaldehyde in PBS for 24 h. Following fixation, the cells were washed with PBS and stored in a known volume of 50% (vol/vol) ethanol/PBS at –20°C until use.

FISH-FCM analysis. For each hybridization, 50 µL of fixed cells was centrifuged for 2 min at 20,600 x*g* in a 1.5-mL eppendorf tube and resuspended in a

mixture of 40 µL of hybridization buffer (0.9 M NaCl, 0.01% SDS, 20 mM Tris-HCl, pH 7.2) and 5 µL of oligonucleotide probe (25 ng/µL, Tsukuba Oligo Service Co., Ltd., Tsukuba, Japan). Formamide was added to the mixture of hybridization buffer for probes Non338, Eub338, Bif164m, and PBR2 at indicated concentrations (Table 1). In the case of probe PBR2, the unlabeled oligonucleotides (helpers; Table 1) were added to the hybridization mixture at the same concentration as PBR2 to improve the accessibility of the probe (8). After hybridization at 46°C for 16 h, 150 µL of washing buffer (225 mM NaCl, 0.01% SDS, 20 mM Tris-HCl, pH 7.2) was added, and cells were collected by centrifugation for 2 min at 20,600 x*g*. Cells were then resuspended in 300 µL of washing buffer and incubated at 48°C for 20 min to remove nonspecifically-bound probes. Finally, hybridized cells were centrifuged and resuspended in 1 mL of PBS for FCM analysis. Analyses of FCM were conducted using BD FACSCanto^{TM} flow cytometer (BD Biosciences, San Jose, CA) equipped with a 20 mW solid-state blue laser (488 nm) and a 17 mW helium–neon (He-Ne) red laser (633 nm). The 488 nm laser was used to measure the forward angle scatter (FSC, the photodiode with 488/10 nm band-pass filter), the side angle scatter (SSC, the photomultiplier tube [PMT] with 488/10 nm band-pass filter), and the green fluorescence intensity (the PMT with 530/30 nm band-pass filter) conferred by FITC-labeled probe. The He-Ne red laser was used to detect the red fluorescence conferred by Cy5-labeled probes (the PMT with 660/20 nm band-pass filter). The system threshold was set on FSC signals and all bacterial analyses were performed at low-flow-rate settings (10 µL/min). A total of 100,000 events were stored in list mode files and data were analyzed using BD FACSDiva^{TM} Software (BD Biosciences, San Jose, CA). The entire hybridization and counting analysis was performed three times for each probe and each fecal sample.

To select suitable fluorochrome for the analysis of fecal samples by FISH-FCM, the background fluorescence of pure culture of *B. breve* JCM 1192^T and a representative fecal sample were evaluated using FISH-FCM, using negative probes (Non338-FITC and Non338-Cy5; Fig. 2). In the measurements of microbial populations in the raffinose trials, fecal samples were hybridized with oligonucleotide probes labeled with a single fluorochrome (Cy5) for FISH-FCM analysis. Gating of the bacterial cells/particles was conducted, and counting was performed until 100,000 events were reached within the gated area. The proportion of target cells hybridized with Cy5-labeled probe (Table 1) having fluorescence intensities >200 (Fig. 3, right side of the vertical line in each histogram) was calculated against 100,000 events. This proportion was then corrected by subtracting the background proportion measured using Non338-Cy5 (Fig. 3B) to obtain the precise value in the fecal sample. The percentage of target *Bifidobacterium* (genus or species level) was recalculated taking the proportion of Eub338 obtained in this manner as total bacteria (100%).

FISH-microscopy analysis. FISH-microscopy analyses were conducted as described previously (8) for the analysis of autofluorescence particles in fecal samples and the validation of FISH-FCM results. The total bacterial count was conducted by DAPI (4', 6-diamidino-2-phenylindole dihydrochloride n-hydrate) staining. Aliquots of 3 μL of fixed cells applied on Teflon printed glass slides (ADCELL, 12 wells, 5 mm in diameter; Erie Scientific Company, Portsmouth, NH) were hybridized by the addition of 8 μL of hybridization buffer with 1 μL of oligonucleotide probe (25 ng/μL) in a moist chamber at 46°C for 16 h. Washing of hybridized cells was conducted in pre-warmed washing buffer for 20 min at 48°C. After drying, bacterial cells on the glass slides were stained with DAPI and the dried slides were mounted with VECTASHIELD® Mounting

Medium (Vector Laboratories, Inc., Burlingame, CA). To evaluate the occurrence of autofluorescence from debris/particles in feces, a representative fecal sample was analyzed without probe or after hybridization with Eub338 labeled with appropriate fluorochromes. For the validation of FISH-FCM results, several 16S rRNA-targeted oligonucleotide probes labeled with Cy3 and the helpers (Table 1) were used to enumerate the bifidobacterial populations at genus and species levels. Bacterial cells were monitored using an Olympus BX50 epifluorescence microscope (Olympus Corporation, Tokyo, Japan) equipped with an Olympus DP30BW CCD camera (Olympus Corporation) operated by MetaMorph® Imaging System software (Molecular Devices Corporation, Sunnyvale, CA). DAPI and Cy3 signals were captured for at least 10 microscopic fields of each well and counted manually using Adobe Photoshop version 7.0 software (Adobe Systems Incorporated, San Jose, CA).

Measurement of fecal pH. The pH of each fecal sample was measured by inserting the electrode of an ISFET pH meter KS701 (Shindengen Electric Manufacturing Co., Ltd., Tokyo, Japan) into the feces.

Data analyses. The changes in bacterial proportions and pH values of fecal samples were analyzed statistically using SPSS software version 10.0.1 (SPSS, Inc., Chicago, IL). Bonferroni tests were performed for pair-wise multiple comparisons of the mean values for the control week (0W) and for the rest of the weeks (2W, 4W and 8W).

RESULTS

Feasibility test of FISH-FCM method for enumeration of bifidobacterial population in fecal samples. In the preliminary experiments for the enumeration of

bacterial populations both in pure culture of *B. breve* JCM 1192^T and in fecal samples, we applied a previously reported FISH-FCM method in which FITC and Cy5 were used as fluorescent dyes to double-stain the bacterial cells (30). Although satisfactory results were obtained for pure culture samples (data not shown), measurements of the fecal samples were not successful because of interference from high background fluorescence observed when Non338-FITC was used as a negative probe. We assumed these phenomena were caused by the presence of autofluorescence materials in the fecal samples. We checked the fecal samples using epifluorescence microscopy to confirm this assumption, and found that autofluorescence particles could be seen in fecal samples even without hybridization probes (data not shown) when the detection filter for FITC was selected, but not when the other filters (for Cy3 and Cy5) were selected. In addition, the autofluorescence fecal particles were found to be similar in size to bacterial cells (Fig. 1A) when the fecal sample was observed after hybridization with Eub338-FITC. In contrast, only bacterial cells were detected when the sample was hybridized with Eub338-Cy5 (Fig. 1B). From these results, Cy5 was expected to be a suitable fluorochrome for FISH-FCM analysis of fecal samples. To confirm this, FISH-FCM analysis of a representative fecal sample was conducted by hybridization with negative probes (Non338-FITC or Non338-Cy5). As expected, very high background fluorescence was observed when the fecal sample was hybridized with Non338-FITC (Fig. 2A), whereas relatively low background fluorescence was observed when Non338-Cy5 was used as a negative probe (Fig. 2B). In contrast, very low background fluorescence was detected when pure culture of *B. breve* JCM 1192^T was hybridized with either Non338-FITC (Fig. 2C) or Non338-Cy5 (Fig. 2D), indicating autofluorescence particles were derived from fecal materials. Based on these results,

Cy5 was selected for labeling all the oligonucleotide probes for FISH-FCM analysis of bacterial populations in fecal samples. When we applied this single-staining procedure to fecal samples, the results of bifidobacterial enumeration were very similar to those obtained by FISH-manual counting analysis (examples shown in Table 3). A practical FISH-FCM method using Cy5 as a single fluorochrome was therefore successfully established for monitoring bifidobacterial populations in fecal samples having high-autofluorescence particles.

Effect of raffinose administration on bacterial population and pH values in human feces. i) Total bacterial count by DAPI staining and fecal pH. Total DAPI counts did not change significantly during and after the raffinose administration relative to the initial values (0W). Total counts (mean ± standard errors of mean) at 0W were $1.4 \times 10^{11} \pm 0.2 \times 10^{11}$ cells/g wet feces. The average counts were reduced slightly to $1.2 \times 10^{11} \pm 0.2 \times 10^{11}$ and $1.0 \times 10^{11} \pm 0.1 \times 10^{11}$ cells/g wet feces after 2W and 4W, respectively. At 8W, the total count recovered to $1.2 \times 10^{11} \pm 0.1 \times 10^{11}$ cells/g wet feces. The pH of fecal samples (mean ± standard errors of mean) at 0W, 2W, 4W, and 8W was 7.0 ± 0.2, 6.6 ± 0.2, 6.6 ± 0.2, and 6.9 ± 0.2, respectively. Although there was a tendency for a decrease in pH during raffinose administration, these differences were not statistically significant because of the high variation in pH among subjects.

ii) Population dynamics of bifidobacteria. The newly developed FISH-FCM method described above was used for the analysis of fecal samples to enumerate the proliferation of bifidobacteria in response to raffinose administration. Typical histograms of FISH-FCM analysis of a representative subject are shown in Fig. 3, and the results of the average total and species-level bifidobacterial populations obtained from the 13 volunteers are summarized in Table 2. At 0W, the average total

bifidobacteria (Bif164m) accounted for 12.5% of the total bacteria (Eub338). During raffinose administration, the averages of total bifidobacteria dramatically increased to 28.7 and 37.2% of total bacteria at 2W and 4W, respectively. At 8W, the population of bifidobacteria decreased to 16.1% of total bacteria. A similar tendency was confirmed by enumeration using FISH-microscopy analysis (examples shown in Table 3), in which the stimulation effect of raffinose on bifidobacterial growth was clearly demonstrated (Fig. 4). In the species-level analysis, three bifidobacterial species, *B. adolescentis, B. catenulatum* group, and *B. longum*, predominated at 0W, and accounted on average for 4.3, 1.8, and 1.6% of total bacteria, respectively, whereas *B. breve* and *B. bifidum* were detected at low levels of 0.4 and 0.2% of total bacteria, respectively. The species *B. dentium* and *B. angulatum* were not detected at 0W. During raffinose administration, populations of all the *Bifidobacterium* species, including *B. dentium* and *B. angulatum*, increased at 2W. However, at 4W, only *B. adolescentis, B. catenulatum* group, and *B. longum,* the predominant species, continued to proliferate, whereas the populations of minor species (*B. breve, B. bifidum, B. dentium*, and *B. angulatum*) decreased. At 8W, the populations of all *Bifidobacterium* species were reduced. Although populations of the major group (*B. adolescentis, B. catenulatum* group, and *B. longum*) returned to approximately initial 0W values, populations of the minor group were quite variable. For instance, *B. breve*, which was detected at 0.4% at 0W and proliferated up to 1.7% at 2W, was reduced to 0.04% at 8W, whereas the previously undetected *B. angulatum* and *B. dentium* appeared to persist at the considerable level of 0.5% and 0.05%, respectively at 8W. These results not only confirm the previously reported growth stimulation effect of raffinose on indigenous bifidobacteria, but also for the first time, clarify the population dynamics of bifidobacteria at the species level.

Discrepancy between total bifidobacteria and sum of bifidobacterial species in FISH-FCM analysis. We found discrepancies between the proportion of total bifidobacteria (Bif164m) and sum of the proportions of each bifidobacterial species detected using species-specific probes. On average, the species-specific probes used in this study detected 49–66% of the bifidobacterial population in fecal samples (Table 2). The undetected percentage varied depending on individuals and the time of sampling. Almost no discrepancy was found in subject 1 at 0W, 2W, and 4W, whereas subject 2 showed lower proportions of detected *Bifidobacterium* species throughout the experiments (Table 3). To further examine these discrepancies, we enumerated fecal samples from both subjects using FISH-microscopy. The results were quite similar to those obtained by FISH-FCM (Table 3), suggesting that the discrepancy described above was not caused by the FCM methodology.

DISCUSSION

We established a practical high-throughput FISH-FCM method for monitoring bacterial populations of fecal samples, particularly those containing autofluorescence particles. The double staining method using FITC and Cy5 was not applicable because of interference from significant background fluorescence originating from autofluorescence materials contained in the fecal samples collected in our study. Biological molecules such as NADH, riboflavin, and flavin coenzymes contained in plant and animal cells are responsible for the autofluorescence in FCM analysis, where the peak of the autofluorescence emission heavily overlaps with the FITC fluorescence region (2, 6). It seems that the autofluorescence particles could be residues of plant fibers because we frequently observed fiber-shaped highly autofluorescent particles

under epifluorescence microscopy when the detection filter for FITC was selected (data not shown). It has also been noticed that autofluorescence arises in plant tissue from chlorophylls, alkaloids, and flavonoids in addition to the aforementioned fluorescent molecules (2). It is important to note that interference by autofluorescence in FISH-FCM analysis using FITC dye has also been experienced for fecal samples collected in European countries (E. E. Vaughan, Wageningen University, Netherlands, personal communication). However, judging from the relatively low background signals observed in FISH-FCM using unhybridized fecal samples (30), and the fact that dual staining using both FITC and Cy5 as fluorochromes has been successfully applied to the analysis of fecal samples (12, 17, 22), we assumed that fecal samples from European countries generally contained less autofluorescence particles than those from Japan.

For the most part, FISH-FCM has been applied to monitor bacterial populations at the genus or group level. The application of FISH-FCM in the analysis of fecal bacteria at the species level is still limited to *Bacteroides* species (23) and *Clostridium* species (13). We reported, for the first time, the FISH-FCM analysis of fecal bifidobacterial populations at the species level to clarify the effects of raffinose administration. The average proportion of bifidobacterial populations in human feces accounted for 12.5% of total bacteria at 0W (Table 2). This was higher than the average for people living in European countries, where it is generally about 3.5% of total bacteria (12, 17, 22). The difference in bifidobacterial proportions between these studies may be caused by differences in the diet of each subject, as well as differences in common food materials between Japan and European countries. At the species level analysis using FISH-FCM, the majority of bifidobacteria was composed of *B. adolescentis*, *B. catenulatum* group, and *B. longum,* in that order (Table 2). This result

is in agreement with that reported by Matsuki et al. (15), where the same order has been found using real-time PCR analysis for bifidobacterial cell numbers in fecal samples of human adults in Japan.

With the consumption of 4 g of raffinose per day, the average proportion of bifidobacteria in feces increased from 12.5% (0W) to 37.2% (4W) of total bacteria (Table 2). This result represents the first precise clarification of the effect of raffinose on the growth of indigenous bifidobacteria in the human intestine by molecular approach. We have not conducted crossover and/or parallel study, since at least the increases of bifidobacterial population by administration of raffinose have been established both in rat experiment (8) and in human study (5). A higher proportion of bifidobacteria (58–80% of total culturable bacteria) in response to raffinose administration than we found has been reported previously (5). This may be because of the higher raffinose intake (15 g/day) and/or the application of a culture-dependent method for the enumeration of bacterial populations. In many cases, results obtained using culture-dependent methods for the evaluation of bacterial proportions in complex ecosystems tend to underestimate or overestimate.

Species-level analysis during and after raffinose intake revealed many interesting features of population dynamics of bifidobacteria. The proportions of each species of the major group (*B. adolescentis*, *B. catenulatum* group, and *B. longum*) continued to increase until the end of the administration period (4W), reaching two to three times the original (0W) levels, then returned almost to the 0W proportion at 8W. Moreover, the order of predominancy at the species level was almost the same throughout the experimentation period (0W–8W). These results may reflect the established niches of the predominant bifidobacteria in the human intestine. In contrast,

the proportions of the minor members were not stable. Although members of the minor group appeared to be boosted dramatically at 2W, they generally failed to establish niches at 4W, and their populations became more variable after stopping raffinose administration (8W). For example, although the proportion of *B. breve* increased four times from 0W to 2W, it then decreased to 1/10 of 0W at 8W. In contrast, *B. angulatum* and *B. dentium*, which were not detected at all at 0W, persisted considerably at 8W. *B. dentium* was originaly isolated from dental caries (25) and its presence has also been demonstrated in human intestine at about 10^7 cells/g feces by real-time PCR (15). Although relatively high proportion of this bacterium was detected in the present study as much as 0.6% of the total bacteria (Table 2) (which may correspond to about 10^{10} cells/g feces), its impacts on health of human host are generally not well understood. The reason for instabilities in population dynamics of the minor members of bifidobacteria is not clear. However, these results at least indicate that the administration of raffinose not only increases the diversity of bifidobacterial populations, but also possibly establishes different bifidobacterial compositions in the human intestine after stopping the administration.

A discrepancy was found between the total bifidobacterial proportion (Bif164m) and the sum of the proportions of each species of bifidobacteria in fecal samples (Table 2). The presence of other known bifidobacteria associated with fecal samples, such as *B. lactis* and *B. gallicum*, may be one reason for this discrepancy. Whereas we did not monitor these two species, the presence of these species in human feces is relatively rare (3, 16). The presence of other unidentified bifidobacteria in the feces was also considered a possibility. In another report (10), 16S rDNA clone library derived from human fecal samples showed bacterial clones affiliated with uncultured

Bifidobacterium species. Similarly, many uncultured *Bifidobacterium*–related 16S rDNA clones have been found in fecal samples of adults and distinguished among live and dead cell fractions using FCM with a sorting system (4). Our results suggest that there may still be unidentified bifidobacteria that have not yet been characterized in fecal samples. To test this suggestion, we conducted a matching analysis of probe sequences to 16S rDNA sequences stored in the Ribosomal Database Project (RDP-II) (7). It appeared that about 9% of the total *Bifidobacterium*-related 16S rRNA sequences were categorized into "unidentified uncultured bifidobacteria" (Table 4) by matching their sequences (>1200 bp) to seven 16S rRNA sequences of *Bifidobacterium* species-specific oligonucleotide probes (Table 1). Based on our study and previous works (4, 10), the occurrence of certain unidentified *Bifidobacterium* species may be expected. This possibility must be analyzed in future to clarify the more detailed population structure of bifidobacterial species.

In conclusion, we established a feasible method of FISH-FCM for the high-throughput analysis of microbiota in a wide variety of fecal samples. Using this method, we demonstrated the effect of raffinose administration on the growth of indigenous bifidobacteria by showing their population dynamics not only at genus level, but also at the species level. Although we cannot obtain the general conclusion by human trial with participation of only 13 volunteers, monitoring the population dynamics at the species level revealed many interesting features regarding the differences in growth responses to raffinose between major and minor groups of bifidobacteria. These findings contribute not only to the scientific characterization of raffinose effects, but also to a more comprehensive understanding of bifidobacterial ecology in the human gastrointestinal tract.

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FIG 1. Microscopic images of a fecal sample hybridized with (A) Eub338-FITC and (B) Eub338-Cy5, monitored using epifluorescence microscopy. Bacterial cells (c) and fecal particle/debris (d) are indicated with arrows.

FIG 2. FCM dot plots of fecal samples hybridized with (A) Non338-FITC and (B) Non338-Cy5; and plots of *B. breve* cells hybridized with (C) Non338-FITC and (D) Non338-Cy5, monitored using FCM. Fluorescence intensity is indicated on the vertical axis and forward scatter (FSC) intensity is indicated on the horizontal axis. The area above the intensity of 200 in FITC and Cy5 (solid line above the horizontal axis of each figure) was evaluated as the occurrence of autofluorescence.

FIG 3. FCM histograms obtained from FISH analyses of fecal samples of one representative subject in the raffinose-administration trial. Histograms are ordered vertically as samples that hybridized with (A) total bacterial probe/Eub338-Cy5, (B) negative probe/Non338-Cy5, or (C) bifidobacteria-specific probe/Bif164m-Cy5, and are ordered horizontally following the time period of raffinose administration; pre-feeding period (0W), 2-week feeding period (2W), 4-week feeding period (4W), and post-feeding period (8W).

FIG 4. Population dynamics of the bifidobacteria of one representative subject in the raffinose-administration trial as determined using FISH-microscopy analysis. Bacterial cells from fecal samples were stained with DAPI (green) and hybridized with a *Bifidobacterium* genus-specific oligonucleotide probe (Bif164m) (red) in FISH

analyses. Images are for each sampling time period: pre-feeding (0W), 2 weeks (2W), 4 weeks (4W), and post-feeding (8W).

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

Probe name	Probe sequence from 5′ to 3′	Target organism	Target site [a]	Formamide (%)	Source [b]
Labeled oligonucleotide probes:					
Non338	ACATCCTACGGGAGGC	Negative probe	-	20	22, 29
Eub338	GCTGCCTCCCGTAGGAGT	Bacteria	338	20	1
Bif164m	CATCCGGYATTACCACCC	Genus *Bifidobacterium*	164	20	8
Bado434	GCTCCCAGTCAAAAGCG	*B. adolescentis*	434	0	28
Bang198	AATCTTTCCCAGACCACC	*B. angulatum*	198	0	28
Bbif186	CCACAATCACATGCGATCATG	*B. bifidum*	186	0	28
Bcat187	ACACCCCATGCGAGGAGT	*B. catenulatum* group	187	0	28
Bden82	ACTCTCACCCGGAGGCGAA	*B. dentium*	82	0	28
Blon1004	AGCCGTATCTCTACGACCGT	*B. longum*	1004	0	28
PBR2	CCATGCGGTGTGATGGAGC	*B. breve*	182	20	27
Non labeled oligonucleotides (Helpers):					
PBR2-1st Upper helper	ATCCGGCATTACCACCCGT	*B. breve*	163	20	8
PBR2-1st Lower helper	CAAAGGCTTTCCCAACACA	*B. breve*	201	20	8
PBR2-2nd Upper helper	TTCCAGGAGCTATTCCGGT	*B. breve*	144	20	8
PBR2-2nd Lower helper	GCGACCCCATCCCATGCCG	*B. breve*	220	20	8

[a] Positions of target sites are indicated based on the *E. coli* 16S rRNA sequence.

[b] Reference number.

TABLE 2. The average genus and species-level bifidobacterial populations in human feces monitored using FISH-FCM analysis.

Microorganisms	Probe	% of microbiota in fecal samples[b]			
		Pre-feeding (0W)	Feeding (2W)	Feeding (4W)	Post-feeding (8W)
Total bacterial cells [a]	Eub338	100	100	100	100
Bifidobacterium genus	Bif164m	12.5 (1.8)	28.7 (4.5)*	37.2 (4.2)*	16.1 (2.6)
B. adolescentis	Bado434	4.3 (1.1)	6.9 (1.9)	9.7 (2.6)	3.7 (1.2)
B. catenulatum group	Bcat187	1.8 (0.6)	3.8 (1.5)	4.7 (1.7)	1.7 (0.7)
B. longum	Blon1004	1.6 (0.7)	3.3 (0.8)	5.3 (1.0)*	1.8 (0.6)
B. breve	PBR2	0.4 (0.3)	1.7 (0.4)*	0.5 (0.2)	0.04 (0.03)
B. bifidum	Bbif186	0.2 (0.1)	0.7 (0.3)	0.3 (0.2)	0.1 (0.0)
B. dentium	Bden82	ND [c]	0.6 (0.2)*	0.2 (0.1)	0.05 (0.03)
B. angulatum	Bang198	ND	1.0 (0.2)*	0.7 (0.2)*	0.5 (0.2)
Sum of detected species		8.3	18.0	21.4	7.9

[a] Total bacterial cells are represented as total cells hybridized with probe Eub338.

[b] Percentages of bifidobacterial cells in total bacterial cells in fecal samples were enumerated using FISH-FCM analysis. The values are means (standard errors of means) (n = 13).

[c] ND, not detected.

*Statistically significant difference ($p < 0.05$) compared with the pre-feeding period (0W).

TABLE 3. *Bifidobacterium* populations in fecal samples of two subjects determined using FISH-FCM and FISH-microscopy [a].

Subject	Microorganisms	FISH-FCM				FISH-microscopy			
		Pre-feeding (0W)	Feeding (2W)	Feeding (4W)	Post-feeding (8W)	Pre-feeding (0W)	Feeding (2W)	Feeding (4W)	Post-feeding (8W)
1	Total bacterial cells [b]	100	100	100	100	100	100	100	100
	Bifidobacterium genus	16.7 (1.7)	38.5 (3.5)	42.7 (0.5)	32.6 (4.9)	19.9 (3.9)	40.8 (5.1)	45.5 (5.2)	26.9 (5.7)
	B. adolescentis	11.6 (1.3)	22.5 (1.2)	27.5 (1.3)	12.3 (5.3)	10.7 (4.0)	21.4 (3.3)	35.6 (1.4)	14.3 (3.1)
	B. catenulatum group	2.7 (0.7)	3.5 (1.5)	1.1 (0.0)	2.2 (1.6)	2.5 (0.7)	5.5 (1.4)	2.6 (0.5)	2.6 (0.9)
	B. longum	8.5 (1.8)	10.5 (2.9)	9.1 (0.2)	ND [c]	5.7 (1.8)	11.6 (3.1)	6.6 (0.6)	0.5 (0.5)
	B. breve	ND	0.6 (0.9	ND	ND	ND	0.1 (0.1)	ND	ND
	B. bifidum	ND	3.9 (0.1)	0.3 (0.3)	ND	ND	2.4 (1.4)	0.2 (0.5)	ND
	B. dentium	ND	1.9 (0.6)	ND	ND	ND	1.3 (1.2)	0.0 (0.1)	ND
	B. angulatum	ND	2.8 (1.8)	0.7 (0.4)	ND	ND	1.0 (0.7)	0.6 (0.6)	ND
	Sum of detected species	22.8	45.7	38.7	14.5	18.9	43.3	45.6	17.4
2	Total bacterial cells	100	100	100	100	100	100	100	100
	Bifidobacterium genus	6.7 (0.9)	32.0 (1.2)	36.5 (2.1)	26.0 (0.6)	6.9 (0.7)	24.5 (7.5)	31.4 (0.9)	18.2 (1.3)
	B. adolescentis	ND	0.7 (0.2)	2.6 (1.5)	1.9 (1.0)	ND	0.7 (0.6)	2.5 (1.2)	1.2 (1.1)
	B. catenulatum group	1.7 (0.3)	11.0 (0.9	12.5 (0.6)	7.2 (0.2)	1.4 (0.7)	8.7 (2.7)	9.6 (3.2)	5.2 (3.3)
	B. longum	2.3 (0.1)	2.5 (0.2)	6.4 (0.3)	6.8 (0.3)	3.0 (0.9)	3.2 (1.0)	5.5 (1.1)	5.4 (1.8)
	B. breve	ND	1.9 (1.2)	1.0 (0.1)	0.1 (0.2)	ND	1.3 (1.2)	0.8 (0.7)	ND
	B. bifidum	ND	0.2 (0.1)	ND	ND	ND	0.2 (0.4)	ND	ND
	B. dentium	ND	0.7 (0.1)	0.1 (0.1)	0.1 (0.1)	ND	0.4 (0.8)	ND	ND
	B. angulatum	ND	0.2 (0.2)	0.7 (0.4)	1.3 (0.6)	ND	0.2 (0.6)	0.4 (0.5)	0.5 (0.7)
	Sum of detected species	4.0	17.2	23.3	17.4	4.4	14.7	18.8	12.3

[a] The values are means (standard deviations) representing the proportion of bifidobacterial cells against total bacterial cells (Eub338). In FISH-FCM, the measurements were conducted in triplicate (three independent hybridization), while ten microscopic fields were counted in FISH-microscopy monitoring.

[b] Total bacterial cells as represented by total cells hybridized with probe Eub338.

[c] ND, not detected.

TABLE 4. Computer analysis of identification of uncultured bacterial 16S rRNA sequences[a] using oligonucleotide probe sequences based on the Ribosomal Database Project II Release 9[b].

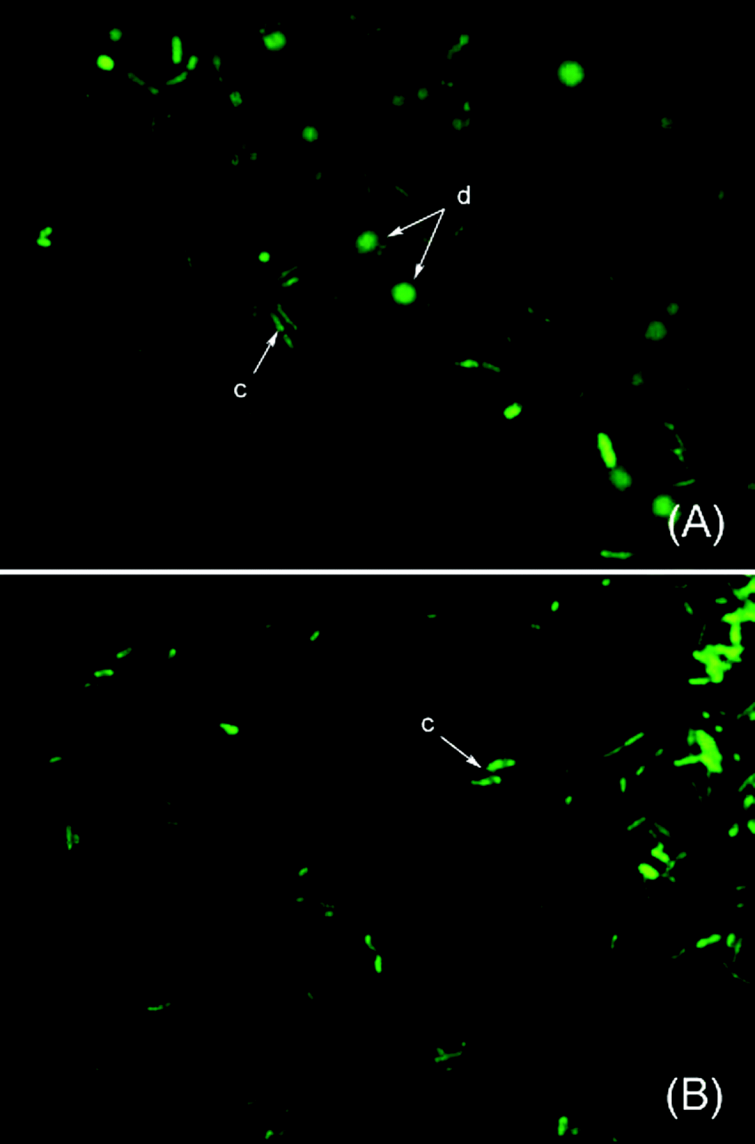
	No. sequence	Rate of sequences/total entry (%)
Bifidobacteria [c]	145	100
Isolates	98	68
Uncultured bifidobacteria	47	32
Identified [d]	34	23
Unidentified	13	9

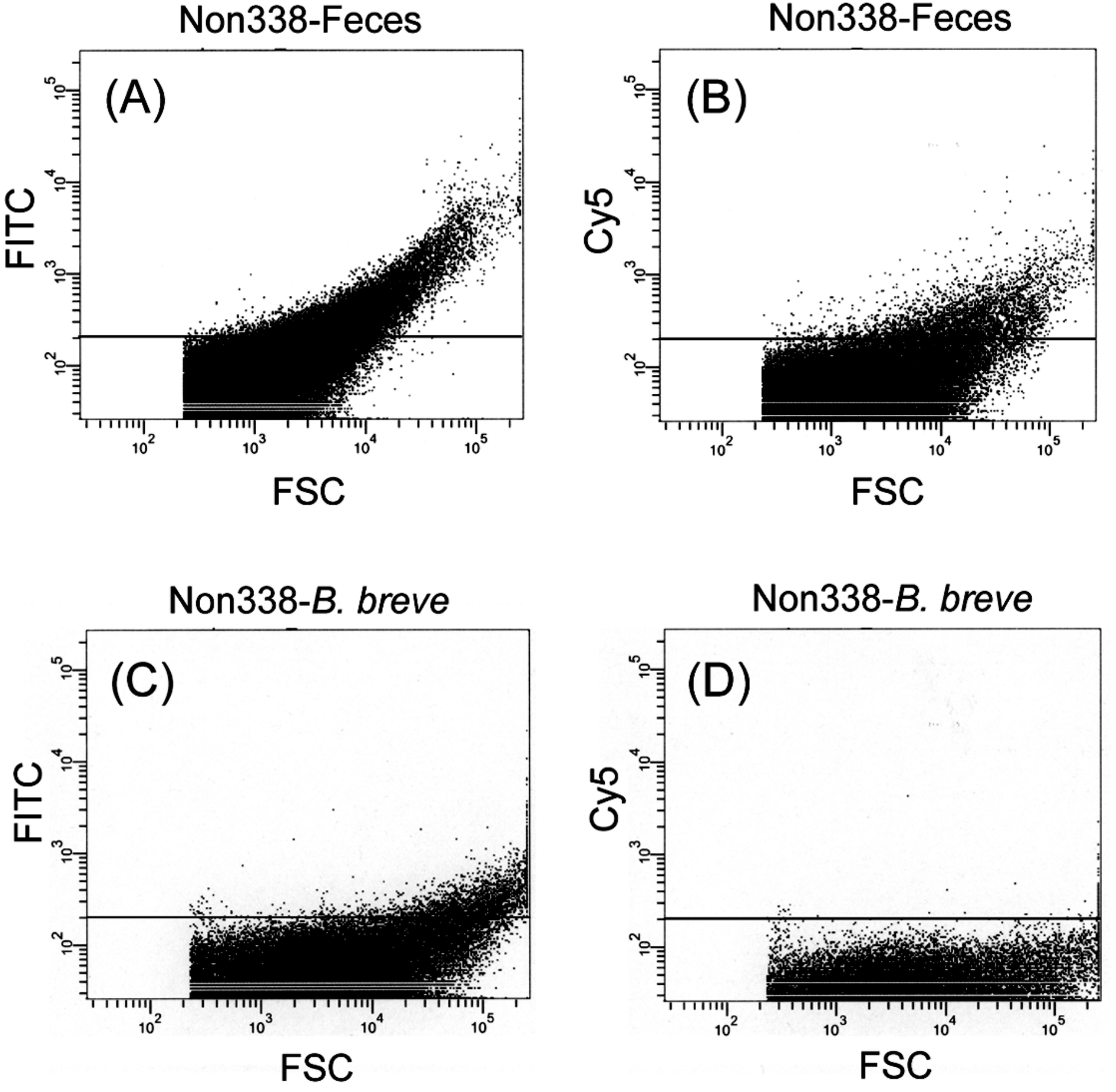
[a] Sequences used in the analysis are ≥ 1200 bp in size.

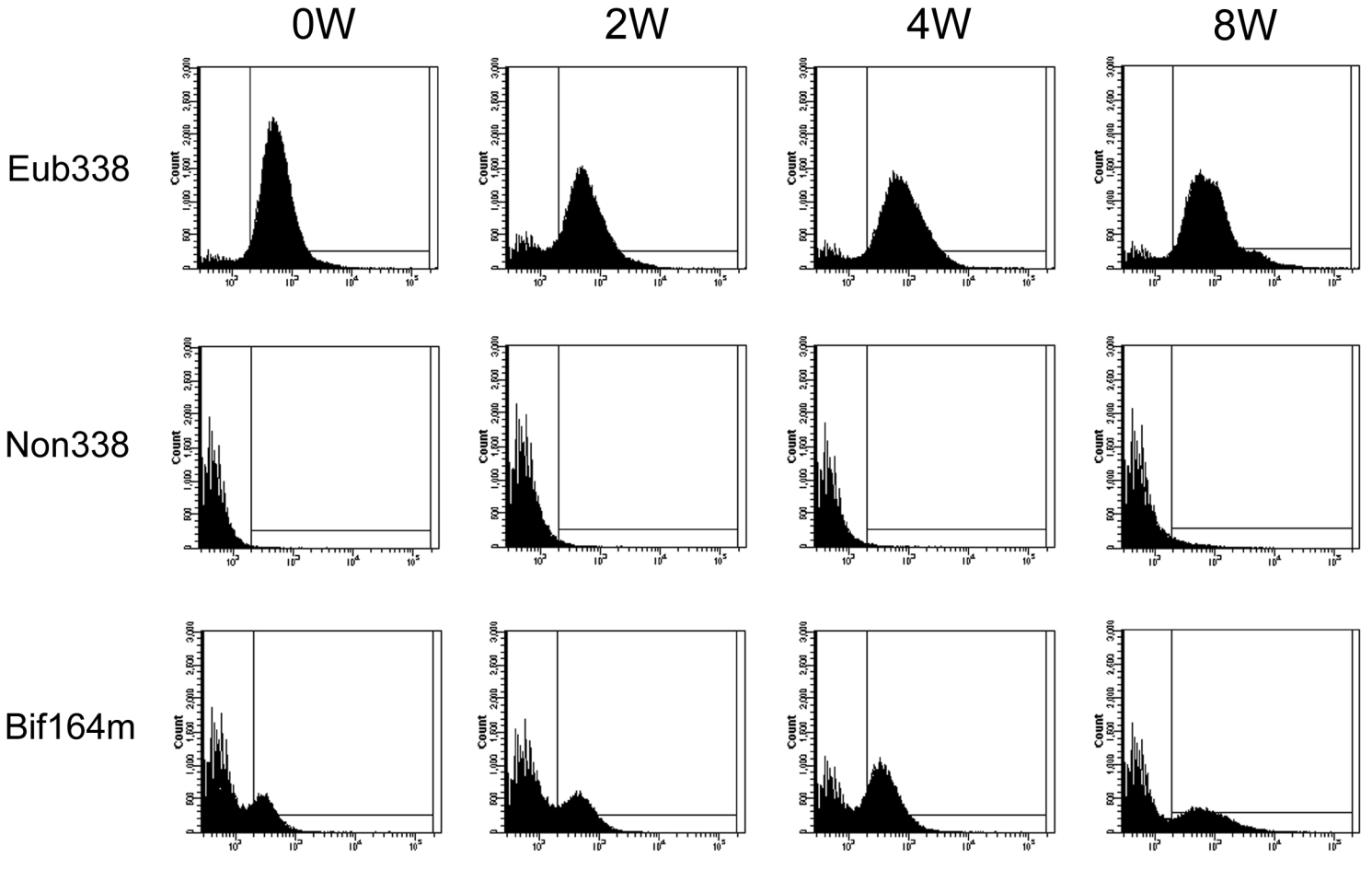
[b] Data collection was conducted in March 2006.

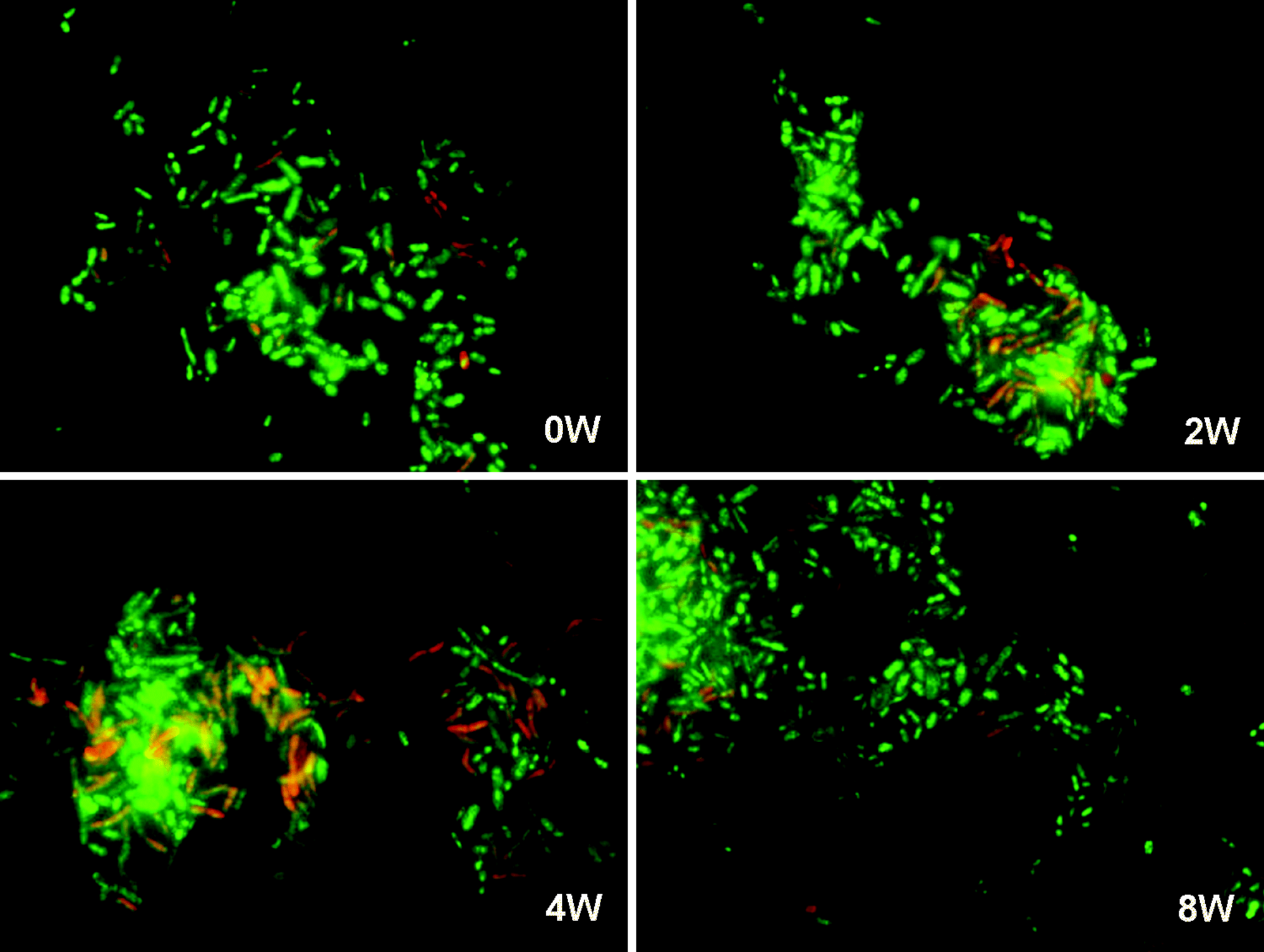
[c] Sequences that match the probe Bif164m.

[d] Identification was conducted by matching the sequences of seven 16S rRNA-targeted oligonucleotide probes (Bado434, Bang198, Bbif186, PBR2, Bcat187, Bden82, and Blon1004) to the sequences that match the probe Bif164m stored in the Ribosomal Database Project II Release 9.


d
c
(A)
c
(B)


Non338-Feces
(A)
FITC
FSC
Non338-Feces
(B)
Cy5
FSC
Non338-B. breve
(C)
FITC
FSC
Non338-B. breve
(D)
Cy5
FSC


0W
2W
4W
8W
Eub338
Non338
Bif164m
Count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