The Effects of Di-d-fructofuranose-1,2': 2,3'-Dianhydride (DFA III) Administration on Human Intestinal Microbiota

KIMIKO MINAMIDA,[1] I NENGAH SUJAYA,[4] AKIKO TAMURA,[2] NORIHIRO SHIGEMATSU,[2] TERUO SONE,[3] ATSUSHI YOKOTA,[4] KOZO ASANO,[3] YOSHIMI BENNO,[5] AND FUSAO TOMITA[3*§]

Northern Advancement Center for Science and Technology, Nishi 12, Kita 21, Kita-ku, Sapporo, Hokkaido 001-0021, Japan,[1] Fancl Corporation, 12-13 Kamishinano, Totsuka-ku, Yokohama, Kanagawa 244-0806, Japan,[2] Laboratory of Applied Microbiology, Graduate School of Agriculture Hokkaido University, Kita 9, Nishi 9, Kita-ku, Sapporo, Hokkaido 060-8589, Japan,[3] Laboratory of Microbial Resources and Ecology, Graduate School of Agriculture Hokkaido University, Kita 9, Nishi 9, Kita-ku, Sapporo, Hokkaido 060-8589, Japan,[4] and Japan Collection of Microorganisms, RIKEN, Wako, Saitama 351-0198, Japan[5]

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*Corresponding author. e-mail: ftomita@chem.agr.hokudai.ac.jp

phone: +81-(0)11-736-6318 fax: +81-(0)11-736-6319

Present address: §The University of the Air, Hokkaido Study Center, Kita 17, Nishi 8, Kita-ku, Sapporo, Hokkaido 060-0817, Japan.

ABSTRACT

Di-d-fructofuranose-1,2': 2,3'-dianhydride (DFA III) was shown to enhance Ca absorption in rat and human intestine. The effects of DFA III administration (9 g per day for 4 weeks that corresponded to 3-fold the optimal dosage of DFA III) on human intestinal microbiota were studied using denaturing gradient gel electrophoresis (DGGE). The major groups of human intestinal microbiota reported previously: the *Bacteroides*, the *Clostridium coccoides* group (*Clostridium* cluster XIVa), the *Clostridium leptum* group (*Clostridium* cluster IV), and the *Bifidobacterium* group were detected. The similarity of thirty DGGE profiles based on the V3 region (before and after administration to the 15 subjects) of the 16S rDNA were calculated using Pearson's correlation based on numbers, positions and intensity of bands, and then a dendrogram of DGGE profiles was constructed by the unweighted pair group method using arithmetic average (UPGMA) clustering method. By these analyses, no difference in DGGE profiles after DFA III administration was observed in healthy subjects, while two subjects with chronic constipation showed different profiles, namely on numbers, positions and the intensity of some bands. Their stools were softer and stool frequencies increased and they obtained relief from constipation.

INTRODUCTION

A small amount of di-d-fructofuranose-1,2': 2,3'-dianhydride (DFA III) exists in chicory tubers, with the natural occurrence in caramel and roasted chicory root being shown to be about 2%. This amount is not sufficient to meet the high demands both for scientific and industrial purposes. DFA III was produced in large quantities with high purity from inulin using inulase II from *Arthrobacter* sp. H65-7 (1). This sugar has half the sweetness of sucrose, is stable in acid and at high temperature, and absorbs less moisture. *In vivo* studies on rats revealed that DFA III was not digested in the upper part of the intestinal tract, and reached the colon without degradation (2). In the colon, DFA III was fermented and significant amounts of short chain fatty acids (SCFAs) were produced, which enhanced calcium solubility and led to an increase of its absorption (2, 3). DFA III directly affects epithelial tissue and opens the tight junctions which are located on the luminal side adjacent to epithelial cells (4). This showed the unique properties of DFA III in terms of calcium transport.

DFA III is a candidate as a food supplement to improve calcium insufficiency, and its safety has been demonstrated by acute and sub-acute toxicity tests. It is not assimilated by such bacteria as bifidobacteria, lactobacilli, the *Bacteroides, Streptococcus mutans*, *Clostridium butyricum* and *Escherichia coli*, however the

number of lecithinase-negative clostridia increased in the cecum of rats fed DFA III (2). Moreover, an increase in organic acids and lowering of pH was observed in the rat contents (2). These changes were probably attributable to the fermentation of bacteria in the rat cecum. Therefore, we investigated the changes in human intestinal microbiota following DFA III administration.

Recent publications showed that only 15% of human intestinal microbiota could be recovered using culture-dependent methods (5, 6) and application of a culture-independent approach gives a more realistic view of intestinal microbiota. DNA/RNA-based methods have been introduced and have been validated as accurate methods for analyzing the microbial component of certain diverse communities such as soil, compost and intestinal microbiota. Among these methods, clone libraries (7), fluorescence *in situ* hybridization (FISH) (8), *in situ* PCR (9), terminal restriction fragment length polymorphism (T-RFLP) (10), temperature gradient gel electrophoresis (TGGE) (11), and denaturing gradient gel electrophoresis (DGGE) (12, 13) have been widely used. FISH, *in situ* PCR and T-RFLP are used to investigate known bacteria. On the other hand, the DGGE/TGGE methods are commonly used in the rapid monitoring of population dynamics of unknown constituents of a microbial community. The aim of this study was to identify the changes in human intestinal microbiota following DFA III

administration. We applied DGGE techniques to detect both cultivable and uncultivable bacteria. Additionally, the mainly saccharolytic *Bacteroides* group was reported to account for approximately 20% of the normal fecal flora and this group is present at about 10^{10} cells per g of dry feces as determined by conventional culture and FISH techniques (14, 15). On the other hand, this group was not identified in TGGE profiles using the bacterial universal primers (11). Thus, we performed DGGE using specific primers for the *Bacteroides*.

MATERIALS AND METHODS

Subjects Fifteen healthy subjects (eight men and seven women), 24 to 45 years old participated in this study. The subjects were administered 9 g/d (3 g x 3 times) of DFA III for 4 weeks. This dose was chosen in separate experiments on the promotion of Ca absorption and changes in stool conditions by DFA III in which 23 healthy Japanese persons were administered different amounts of DFA III (3, 6, 9, 12 and 15 g). It was found that their stool conditions did not change and the effective amount promoting Ca absorption was determined to be 3 g/person/d (Tamura *et al.,* in preparation). The subjects in the present study ate their usual diet and recorded their food intake, frequency of defecation and the condition of their stools. This study was conducted

based on the principles of the Declaration of Helsinki (Ethical Principles for Medical Research Involving Human Subjects). Informed consent was obtained from each subject before this study.

Fecal samples Fecal samples were collected from the middle part of stools before and after 4 weeks of administration. Samples were immediately frozen at -20°C after defecation and stored at -80°C until further use.

DNA extraction A 0.1-g (wet weight) amount of fecal sample was washed 4 times with 1 ml of TE buffer (pH 8), then suspended in 1ml of the same buffer, to which lysozyme (Wako Pure Chemical Industries, Osaka) (final concentration 2.5 mg/ml), labiase (Seikagaku, Tokyo) (final concentration 2.5 mg/ml), and *N*-acetylmuramidase (Seikagaku) (final concentration 30 μg/ml) were added. The suspension was incubated at 37°C for 15 min. The DNA was isolated using the UltraClean™ Soil DNA Kit (Mo Bio Laboratories, Salana Beach, CA, USA) according to the instructions of the manufacturer with some modifications (16), *i. e.*, spun for 20 s at 4 m/s on FastPrept™ FP120 (Qbiogene, Carlsbad, CA, USA).

PCR amplification Primers 338f-gc (5' CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG GAC TCC TAC GGG AGG CAG CAG 3') and 518r (5' ATT ACC GCG GCT GCT GG 3') (17) were used to amplify the V3 regions of the bacterial 16S rDNA. The reaction mixture (50 μl) contained 50 pmol amounts of each primer, 5 μl of dNTP mixture (2.5 mM each), 1 x PCR reaction buffer, 3.5 μl of $MgCl_2$ solution (25 mM), 2.5 U of AmpliTaq Gold (Applied Biosystems, Foster City, CA, USA), and 3 μl of DNA solution (100-200 ng). Amplification was performed on a GeneAmp® PCR System 9700 (Applied Biosystems). The amplification program was 95°C for 5 min; 2 cycles of 80°C for 1 min, 65°C for 1 min and 72°C for 3 min; 18 cycles of 94°C for 1 min, 64°C for 1 min and 72°C for 3 min; 11 cycles of 94°C for 1 min, 55°C for 1 min and 72°C for 3 min; and a final extension at 72°C for 7 min. The amplification was checked by electrophoresis by running 5 μl of the amplicons on a 1.5% agarose gel, staining it with ethidium bromide and visualizing the DNA with a UV transilluminator.

DGGE analysis The PCR products (100 μl PCR reaction mixtures) were concentrated by ethanol precipitation and dissolved in 10 μl of sterilized water. Prior to DGGE, samples were heated at 95°C for 5 min and 65°C for 60 min, and then were left

at 37°C overnight. The DGGE was performed on the DCode™ system (Bio-Rad Laboratories, Hercules, CA, USA) at 65 V, 60°C in 1 x TAE for 14 h, on 10% polyacrylamide gels containing a 30% to 60% gradient of urea-formamide, where 100% was defined as 7 M urea and 40% (vol/vol) formamide (17). The gel was stained with SYBR® Green I (Cambrex Bio Science Rockland, Rockland, ME, USA) for 45 min. The bands were excised with a razor blade and were washed twice using sterilized water, and then were stored at -20°C until use.

PCR amplification and DGGE analysis for the *Bacteroides* group The specific primers for the *Bacteroides* group; g-Bact-F (5' ATA GCC TTT CGA AAG RAA GAT 3') corresponding to numbering positions 148-169 of *E. coli* and g-Bact-R (5' CCA GTA TCA ACT GCA ATT TTA 3') (18) corresponding to numbering positions 646-626 of *E. coli* were used to amplify 501 bp of the bacterial 16S rDNA. The reaction mixture was the same as described above. PCR conditions were: pre-denaturation for 5 min at 94°C, followed by 30 cycle of denaturation for 30 s at 95°C, annealing for 30 s at 50°C, and extension for 1.5 min at 72°C. A final extension of 5 min at 72°C was added. Long DNA fragments of approximately 500 bp were not suitable for DGGE. The secondary PCR was performed by using the first PCR products as templates and the universal

primers for the bacterial V3 region, and then DGGE was performed as described above.

Sequencing of DGGE fragments The DGGE fragments (gel pieces) were directly subjected to a PCR reaction. The PCR was performed as described in the DGGE-PCR protocol with some modifications. The PCR was in a 50-μl reaction mixture containing: small pieces of gels as the DNA template (gel volume approximately 2 μl); 50 pmol of each primer (338f without incorporation of a gc clamp and 518r); 1 x PCR buffer; 5 μl of dNTP mixture (2.5 mM each); 3.0 μl of $MgCl_2$ solution (25 mM); and 2.5 U of AmpliTaq Gold. PCR conditions were the same as mentioned in the PCR amplification and DGGE analysis for the *Bacteroides* group except for the annealing condition for 30 s at 53°C. PCR products were purified using SUPREC^{TM} PCR (Takara Bio, Otsu), and then were sequenced using the BigDye Primer Cycle Sequencing FS Ready Reaction Kit (Applied Biosystems). Sequences were automatically analyzed with a 3100 Genetic Analyzer (Applied Biosystems).

Analysis of the sequence Homology searches were performed in the GenBank database with the BLAST search program. Some 16S rDNA sequences were retrieved from the DDBJ, EMBL and GenBank databases for comparison in the phylogenetic

analysis. Sequences data were aligned with the CLUSTAL X package program and corrected by manual inspection. Nucleotide substitution rates were calculated, and the phylogenetic tree was constructed using the neighbor-joining method. Nucleotide sequence data reported are available in the DDBJ/EMBL/GenBank databases under the accession numbers from AB112961 to AB113007, AB160863 to AB160864 and AB174887.

A dendrogram of DGGE profiles was constructed using Pearson's curve-based correlation and the unweighted pair group method using arithmetic average (UPGMA) clustering method (BioNumerics™ software ver. 2.5, Applied Maths BVBA, Keistraat, Sint-Martens-Latem, Belgium). Three pieces of DGGE gel were arranged by the position of the closest relative of five amplicons as shown in Fig. 4. Arrows 1, 2, 3, 4, and 5 show uncultured *Clostridium* sp. (AB064860), *Fusobacterium prausnitzii* (AJ413954), *Ruminococcus obeum* (L76601), *Bifidobacterium catenulatum* (AF432082), and *B. adolescentis* (AF275882).

Stool frequencies and stool conditions The stool frequencies and softness were recorded daily by completing the questionnaire given to the volunteers throughout the experiments.

RESULTS AND DISCUSSION

The stability of intestinal microbiota Before initiating DFAIII administration, the intestinal microbiota in one of the volunteers was examined as a control. Fecal samples from a healthy woman eating a normal diet were monitored by DGGE for 4 weeks at intervals of a few days (Fig. 1). The numbers in Fig. 1 are amplicon numbers. The DGGE profiles of days 0 to 28 were very similar, except for some changes on days 14 and 20 that gave slightly different profiles from the others, namely amplicons no. 2, 3 and 4 were very weak on days 14 and 20, and amplicon no. 8 on day 20 became stronger than on the other days. However, the profile of day 21 was very similar to the others and, thus, it could be concluded that the microbiota was stable in the healthy subject.

These results coincided well with previous reports that the dominant microbiota was found to be stable when human fecal samples from two individuals were monitored over a period of 6 months using TGGE analysis (11). It was also reported that human intestinal microbiota was stable under various daily diets, except when probiotics and/or symbiotics were administered during which bacteria transiently colonize the intestine (12, 13, 19). Furthermore, the TGGE/DGGE profile reflected the predominant bacteria

in fecal samples (11-13).

Changes in intestinal microbiota after DFA III administration The effects of DFAIII administration on intestinal microbiota were examined in 15 healthy subjects. Fecal samples from 15 healthy subjects administered DFA III（9 g per day for 4 weeks） were monitored by DGGE. To correlate amplicons with closely related bacteria, each visible amplicon (a total of 300 gel pieces) was excised, sequenced and then a BLAST search was done as described in Materials and Methods. The base sequence of approximately 70% of 300 amplicons could be determined. Each amplicon that was in the same position on the gel correlated with the same closest relative. All amplicons of all 15 subjects showed that there was no increased or decreased amplicons in general (data not shown). As typical examples, the DGGE profiles of subjects A, B and C are shown in Fig. 2. There were differences in terms of numbers, positions and intensities of amplicons among the subjects, although almost amplicons as shown in the previous report (11) was detected. Thus, it was concluded that effects of DFA III administration could not be studied using this method.

The detection of the *Bacteroides* group As shown in Fig. 2, we also could not

detect the *Bacteroides* group with bacterial universal V3 region primers. Thus, we employed the specific primers for the *Bacteroides* (18). The PCR resulted in the amplification of 500-bp fragments from all the samples (data not shown). DGGE profiles of the *Bacteroides* group are shown in Fig. 3 with subjects A, B, C, D and E showing the presence of eleven amplicons specific to the *Bacteroides* group. Subjects A, B and C correspond to subjects A, B and C in Fig. 2. However, as outlined in the above section, there were differences in terms of numbers, positions and intensities of amplicons among the subjects, although all amplicons corresponding to the *Bacteroides* group was detected. Thus, it was concluded that the effects of DFA III administration also could not be studied using this method.

Analysis of entire DGGE profiles As shown in the above two sections, simple comparisons of the DGGE profiles did not give a clear understanding of microbiota changes following the administration of DFAIII. This could be due to the fact that the DNA extracted from feces was not quantitated. The Pearson's correlation analysis as shown in the Methods section was applied to examine DGGE profiles. The similarities among thirty DGGE profiles based on the V3 region (before and after administration to 15 subjects) were calculated based on the position and intensity of bands, and then a

dendrogram of DGGE profiles was constructed by the UPGMA clustering method (Fig. 4). DGGE profiles appeared to be best clustered at the relative similarity of 48% to describe intestinal microbiota of subjects before and after DFA III administration. The intestinal microbiota of healthy subjects before and after DFA III administration was not significantly changed as their DGGE profiles of before and after DFA III administration remained in the same cluster. In contrast, there was a clear change in the microbiota profile of constipated subjects before and after DFA III administration (*e.g.* subjects D and E in Fig. 4 showing two distinct clusters before and after administration). When clustering was performed at a relative similarity of 50% or higher, no distinct microbiota changes were observed. Thus, DFA III did not change indigenous microbiota in healthy subjects, but did so in subjects with constipation.

Furthermore, a phylogenetic tree was constructed based on the sequence data of the *Bacteroides* amplicons (Fig. 3) and that in Fig. 4 as summarized in Fig. 5. This showed that most of the reported groups in human intestinal microbiota (6, 7) were detected, *i.e.*, the *Bacteroides*, the *Clostridium coccoides* group (*Clostridium* cluster XIVa) (20), the *Clostridium leptum* group (*Clostridium* cluster IV), the *Bifidobacterium* group, the *Clostridium* cluster IX, the *Clostridium* cluster XVIII, and the *Collinsella*.

Effects of DFAIII administration on stool condition and frequency The relationship between the relative similarity of DGGE profiles (Fig. 4) and the stool condition from questionnaires is shown in Table 1. It was found that the lower the value of the relative similarity, the greater the changes in stool frequency and condition. Two among the three subjects with a relative similarity <48% (Fig. 4) had chronic constipation. Moreover, two subjects with chronic constipation obtained relief from the following symptoms, namely slight stool bulking, hard stool and the feeling of incomplete elimination despite higher stool frequency. The stool frequency of subject D increased from 7 times/ week to 8 times/ week, and the stool became softer, changing from pellets to a more normal consistency similar to that of banana or soft ice cream. The stool frequency of subject E increased from 13 times/ week to 16 times/ week in the first week of DFA III administration, and the stool became softer as in the case of subject D.

In general, constipation is the state resulting from the confusing of the normal rhythm of defecation whereby the stool remains in the colon for a long time and the subject experiences a feeling of discomfort, hard stools, the feeling of incomplete elimination and decrease of stool discharge. Relief from these symptoms can occur in two ways: to stimulate defecation by increasing fecal bulking and softening of stools,

and to move the bowels. The first way can achieved by ingesting fermentable fibers, inulin and oligosaccharides, which are fermentable to a large extent by anaerobic bacteria and result in an increase in bacterial biomass, an increase in fecal mass and production of SCFAs (21- 23). The contractile response may have stimulated by SCFAs in the rat terminal ileum and could be resulted from an acid-sensitive calcium-dependent myogenic mechanism (24). SCFAs, especially acetate, are involved in the defecation-improving effect of dietary fiber by promoting colon motility (25).

DFA III was not digested by the intestinal enzymes of rat, while it was fermented by microorganisms in the rat cecum (2). The increase in stool frequency and softness in chronically constipated subjects following DFA III administration can be considered to be due to the increase in bacterial biomass and the promotion of colon motility by SCFAs owing to DFA III-assimilating bacteria. Investigation of the effects of DFAIII administration on a large number of chronically constipated subjects is currently ongoing.

In conclusion, DFA III administration (9 g per day for 4 weeks corresponding to 3-fold the optimal dosage of DFA III) resulted in no difference in DGGE profiles as shown in the dendrogram (Fig. 4) based on the V3 region. However, two subjects with

chronic constipation showed different profiles, namely in the numbers, positions and intensities of some amplicons (Fig. 4). The stools of these subjects became softer and stool frequency increased following DFA III administration (Table 1) and they obtained relief from the symptoms of constipation.

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FIG. 1. The profile of human intestinal microbiota determined using DGGE. The intestinal microbiota of a healthy woman eating a normal diet was monitored for 28 d. The picture is the negative image of the DGGE gel stained using SYBR® Green I nucleic acid gel stain. The experiment is described in detail in the text. The numbers in Fig. 1 are amplicon numbers.

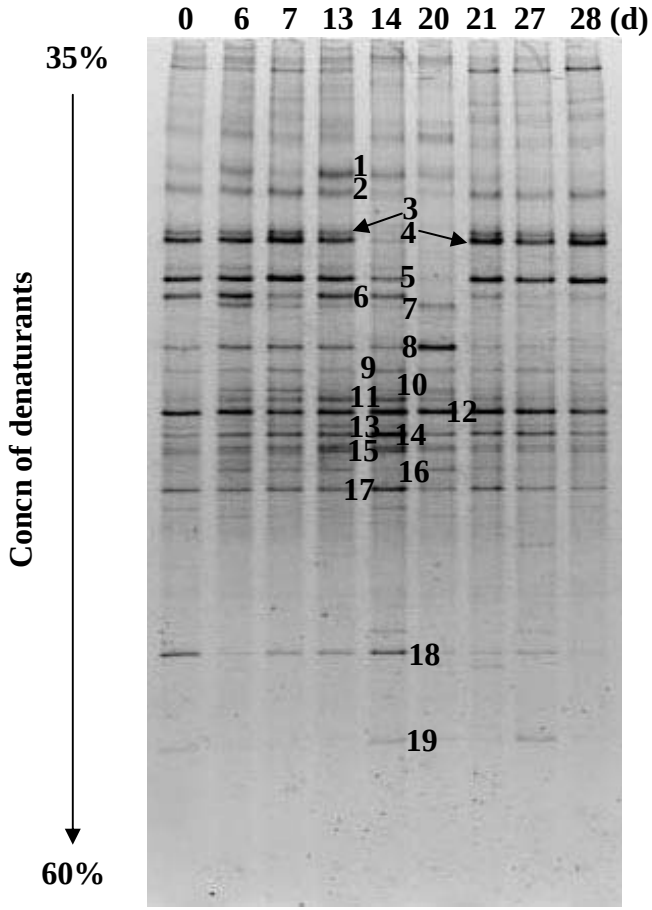
FIG. 2. DGGE profiles of intestinal microbiota obtained from representative subjects (A, B and C) before (pre) and after (post) DFAIII administration. The correlation of amplicons with closely related bacteria is given in the table on the right. The picture is the negative image of a DGGE gel stained using SYBR® Green I nucleic acid gel stain.

FIG. 3. DGGE profiles of the *Bacteroides* obtained from representative subjects (A, B, C, D and E) before (pre) and after (post) DFAIII administration. The correlation of amplicons with closely related bacteria is given in the table on the right. The picture is the negative image of a DGGE gel stained using SYBR® Green I nucleic acid gel stain. Subjects A, B and C correspond to subjects A, B and C in Fig. 2.

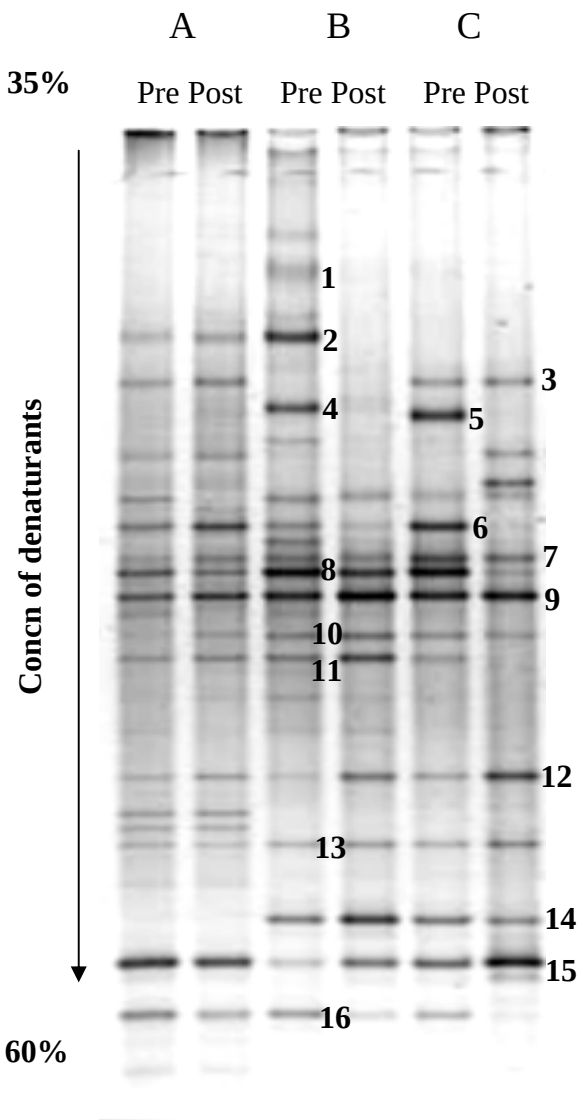
FIG. 4. Dendrogram of human intestinal microbiota before and after DFAIII administration constructed using Pearson's curve-based correlation and the UPGMA clustering method (BioNumerics™ software ver. 2.5, Applied Maths BVBA). Three pieces of DGGE gel were arranged by the position of the closest relative correlating to five amplicons: uncultured *Clostridium* sp. (AB064860), *Fusobacterium prausnitzii* (AJ413954), *Ruminococcus obeum* (L76601), *Bifidobacterium catenulatum* (AF432082), and *B. adolescentis* (AF275882) are shown by arrows 1, 2, 3, 4, and 5. The letters shown at the right side of the negative image of the gel correspond to the code of subjects involved in this study, while "0" and "1" correspond to "before and after" DFAIII administration, respectively. Subjects A, B, C, D and E correspond to subjects A, B, C, D and E in Fig. 3.

FIG. 5. Phylogenetic relationship of intestinal microbiota of human subjects from the partial sequences of 16S rDNA from DGGE amplicons determined in this study with their closely related bacteria retrieved from DDBJ/EMBL/GenBank databases. The sequences of type strains were used for the comparison. The

phylogenetic tree was constructed using the Cluster X package program as described in Materials and Methods. Amplicons from DGGE are shown by each character (B, subject B; F, subject F). The scale bar represents 0.05 substitutions per nucleotide position. Bootstrap values (expressed as percentages of 100 replications) greater than 50% are shown at branch points. Subjects B and F correspond to subjects B and F in Fig. 4.



Minamida *et al*. Fig. 1



Amplicon no.	Closest relative (accession number)	Similarity (%)
1	Uncultured *Clostridium* sp. (AB064860)	100
2	*Ruminococcus bromii* (X85099)	99
3	*Ruminococcus* sp. CO47 (AB064904)	99
4	Human intestinal firmicute CB15 (AB064931)	97
5	Human intestinal firmicute CB15 (AB064931)	97
6	*Butyrivibrio fibrisolvens* (AF125217)	100
	Eubacterium rectale (L34627)	100
7	Uncultured bacterium clone p-30-a5 (AF371665)	100
8	*Fusobacterium prausnitzii* (AJ413954)	100
9	*Ruminococcus obeum* (L76601)	100
10	*Coprococcus catus* (AB038359)	97
	Ruminococcus sp. CO27 (AB064900)	97
11	Uncultured firmicute gene (AB064801)	100
12	*Bifidobacterium longum* (M58739)	100
	Bifidobacterium infantis (AF528507)	100
13	*Bifidobacterium bifidum* (U25952)	97
14	*Bifidobacterium catenulatum* (AF432082)	100
	Bifidobacterium pseudocatenulatum (D86187)	100
15	*Bifidobacterium adolescentis* (AF275882)	100
16	*Collinsella aerofaciens* (AJ245920)	100
	Coriobacterium sp. (AJ131150)	100

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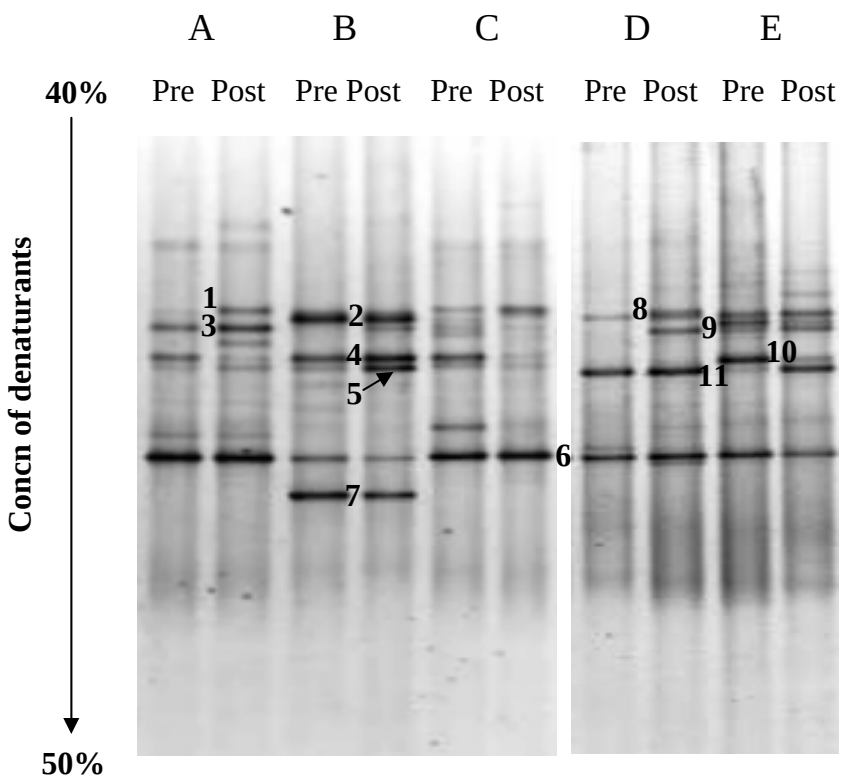

Amplicon no.	Closest relative (accession number)	Similarity (%)
1	*Bacteroides ovatus* (AB050108)	97
2	*Bacteroides* sp. CS3 (AB064916)	98
3	*Bacteroides* sp. CS2 (AB064920)	100
4	*Bacteroides vulgatus* (AB050111)	100
5	*Bacteroides uniformis* (AB050110)	97
6	*Bacteroides uniformis* (AB050110)	100
7	*Bacteroides* sp. AR20 (AF139524)	100
8	*Bacteroides thetaiotaomicron* (M58763)	98
9	*Bacteroides ovatus* (X83952)	97
10	Uncultured *Bacteroides* sp. (AB064826)	100
11	*Bacteroides caccae* (X83951)	99

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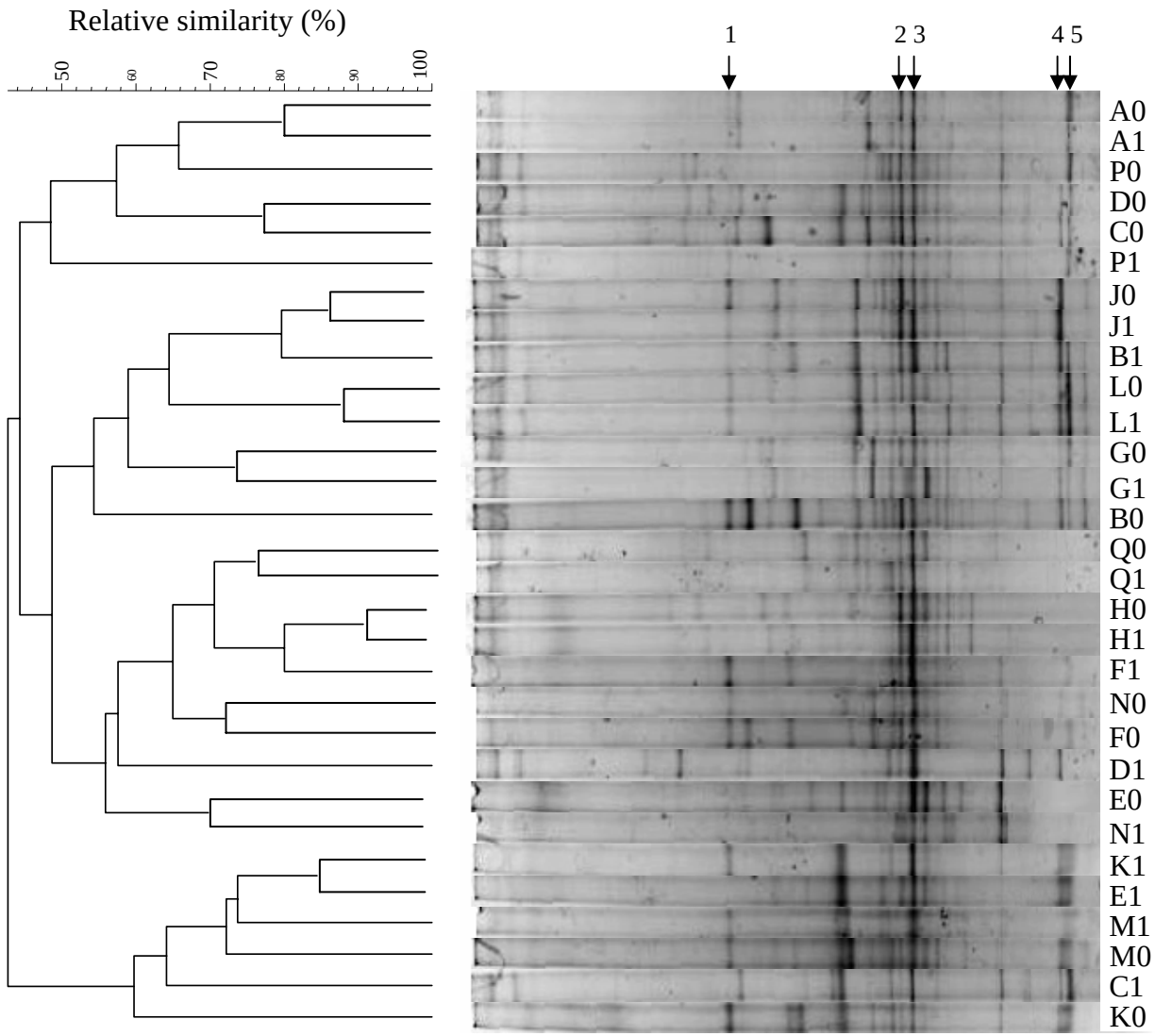
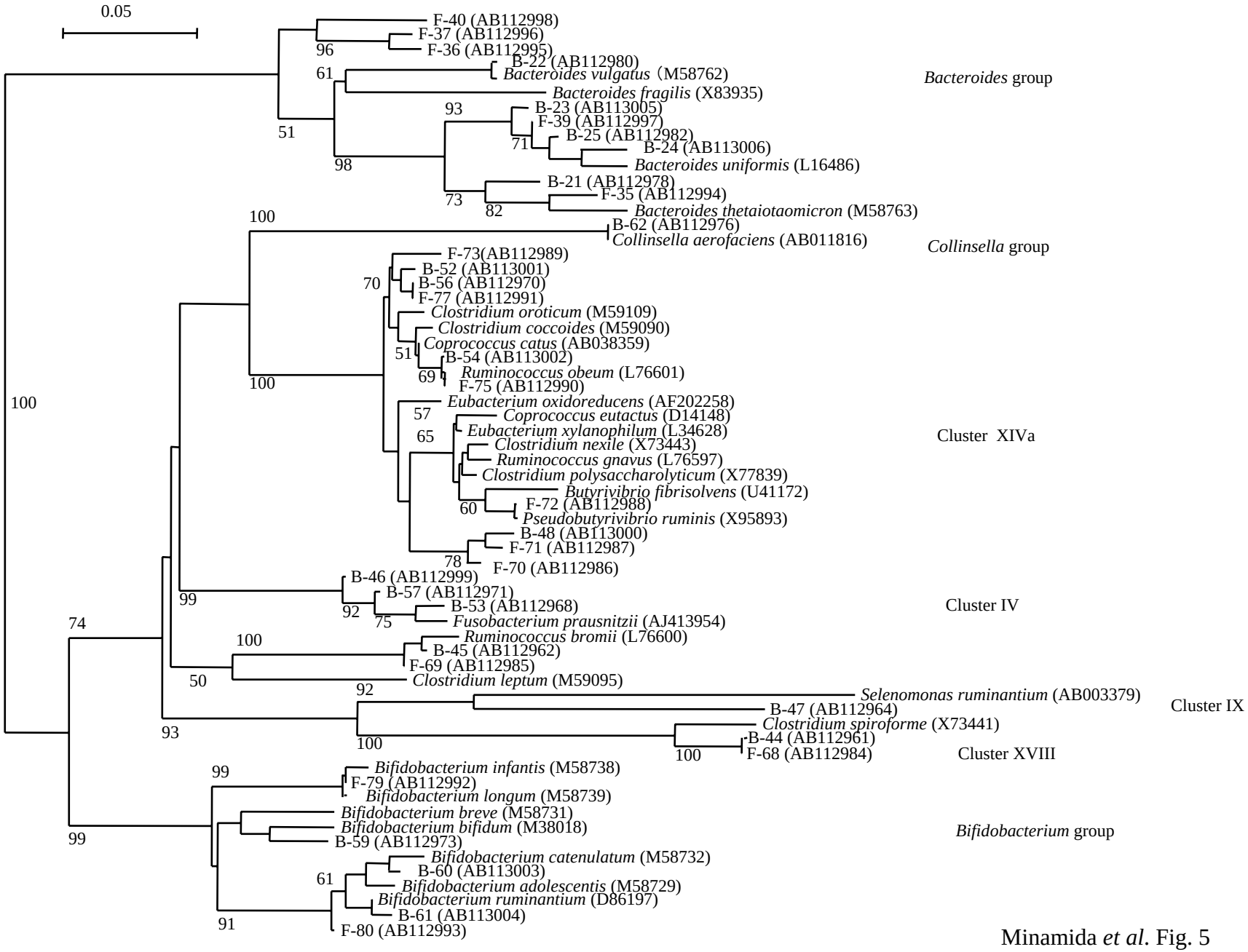


Minamida *et al*. Fig. 4



Minamida *et al*. Fig. 5

TABLE 1. The relationship between the relative similarity of DGGE profiles and the stool condition

Subject	Relative similarity (%)	The number of subjects (rate, %)				
		Change			No change	Total
		Softer stool	Increased stool frequency	Softer stool and increased stool frequency		
A, B, F, G, H, J, K, L, M, N, P and Q	>48	3 (25)	2 (17)	1 (8)	6 (50)	12 (100)
C, D and E	<48	0	1 (33)	2 (67)	0	3 (100)

TABLE 1. The relationship between the relative similarity of DGGE profiles and the stool condition

Subject	Relative similarity (%)	The number of subjects (rate, %)				
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C, D and E	<48	0	1 (33)	2 (67)	0	3 (100)