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Use of 16S rRNA Gene-Targeted Group-Specific Primers for Real-Time PCR Analysis of Predominant Bacteria in Human Feces

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16S rRNA gene-targeted group-specific primers were designed and validated for specific detection and quantification of the *Clostridium leptum* subgroup and the *Atopobium* cluster. To monitor the predominant bacteria in human feces by real-time PCR, we used these specific primers together with four sets of group-specific primers for the *Clostridium coccoides* group, the *Bacteroides fragilis* group, *Bifidobacterium*, and *Prevotella* developed in a previous study (T. Matsuki, K. Watanabe, J. Fujimoto, Y. Miyamoto, T. Takada, K. Matsumoto, H. Oyaizu, and R. Tanaka, Appl. Environ. Microbiol. 68:5445–5451, 2002). Examination of DNA extracted from the feces of 46 healthy adults showed that the *C. coccoides* group was present in the greatest numbers ($\log_{10}$ 10.3 ± 0.3 cells per g [wet weight] [average ± standard deviation]), followed by the *C. leptum* subgroup ($\log_{10}$ 9.9 ± 0.7 cells per g [wet weight]), the *B. fragilis* group ($\log_{10}$ 9.9 ± 0.3 cells per g [wet weight]), *Bifidobacterium* ($\log_{10}$ 9.4 ± 0.7 cells per g [wet weight]), and the *Atopobium* cluster ($\log_{10}$ 9.3 ± 0.7 cells per g [wet weight]). These five bacterial groups were detected in all 46 volunteers. *Prevotella* was found in only 46% of the subjects at a level of $\log_{10}$ 9.7 ± 0.8 cells per g (wet weight). Examination of changes in the population and the composition of the intestinal flora for six healthy adults over an 8-month period revealed that the composition of the flora of each volunteer remained stable throughout the test period.

The human intestinal tract harbors a large, active, and complex community of microbes. The intestinal microflora plays significant roles in the digestion of food, the metabolism of endogenous and exogenous compounds, immunopotentiation, and the prevention of colonization of the gastrointestinal tract by pathogens and hence is involved in maintaining human health (6, 31). To understand the relationship between the intestinal flora and human health, it is important to develop an accurate method to analyze the various microbial populations.

The microflora of the gut has been monitored in great detail by cultivation-based techniques (3, 20), but limitations inherent in these techniques and the development of more sensitive and accurate molecular detection methods have brought new insights to the field (35). In complex mixed populations, 16S rRNA-targeted oligonucleotide probes have been used with fluorescent in situ hybridization (FISH) as a culture-independent method (5, 7, 13). Techniques such as the clone library method and temperature gradient gel electrophoresis allow analysis of predominant bacteria that are difficult to culture (9, 10, 28, 36). Currently, PCR with 16S rRNA-based specific primers has been applied to flora analysis as the most sensitive and rapid method. So far, specific oligonucleotide primers have been designed for many bacterial species known to be present in the intestinal tract (12, 18, 19, 27, 33, 34). Although conventional PCR does not permit quantitative detection of target bacteria, real-time PCR with species-specific primers can provide precise quantification through measurement of SYBR Green I fluorescence to determine the amounts of PCR products in each cycle (11, 14, 16, 17, 24, 29).

For analysis of the human gut microflora, it is useful to prepare specific primers not only for species but also for major genera and groups due to the complexity of this ecosystem. To date, group-specific primers for the *Clostridium coccoides* group, the *Bacteroides fragilis* group, *Bifidobacterium*, and *Prevotella* have been developed and applied to the analysis of the human intestinal microflora (18).

In the present study, we developed new sets of primers to detect the *Clostridium leptum* subgroup and the *Atopobium* cluster. After validation of their specificity, these primers were used with previously developed group-specific primers to quantify populations in fecal samples from 46 healthy individuals by real-time PCR analysis. The changes in the intestinal floras of six healthy adults during an 8-month period were also examined.

MATERIALS AND METHODS

Development of 16S rRNA gene-targeted group-specific primers. Through the use of 16S rRNA sequences obtained from the DDBJ/GenBank/EMBL database, multiple alignments of the target groups and reference organisms were constructed by using the program Clustal X (32). After comparison of sequences unique to groups with large numbers of reference strains, potential target sites for specific detection were identified (Table 1). The specificity of the primers shown in Table 2 was then checked with the database by submitting the sequences to the Check Probe program of the Ribosomal Database Project (www.cme.msu.edu/RDP) (15).

Reference strains and culture conditions. Seven strains of the *C. leptum* subgroup, five strains of the *Atopobium* cluster, seven strains of *Bacteroides*, nine strains of *Bifidobacterium*, eight strains of the *C. coccoides* group, seven species of *Prevotella*, and 18 species of a disparate cluster were used in this study (Table 3). These organisms were obtained from the Culture Collection of the Yakult Central Institute (Tokyo, Japan) (YIT). The strains were cultured anaerobically

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TABLE 1. Partial 16S rRNA gene sequences of reference organisms with group-specific primers

Organism or site[a]	Forward primer		Reverse primer	
	Designation	Sequence[b]	Designation	Sequence[b]
C. leptum subgroup-specific primers	sg-Clept-F	5′ GCACAAGCAGTGGAGT 3′	sg-Clept-R3	3′ AACTGTTTTGCCTCCTTC 5′
Target site		5′ GCACAAGCAGTGGAGT 3′		5′ TTGACAAAACGGAGGAAG 3′
Clostridium cellulosi[c]		.....		....T.....
Clostridium leptum[c]		.....		.....
Clostridium orbiscindens[c]		.....G.....		.....
Clostridium sporosphaeroides[c]		.....N.....		.....
Clostridium viride[c]		.....G.....		.....
Eubacterium desmolans[c]		.....		.....
Eubacterium siraeum[c]		.....		.....
Faecalibacterium prausnitzii[c]		.....		.....
Ruminococcus albus[c]		.....		.....
Ruminococcus bromii[c]		.....		.....
Ruminococcus callidus[c]		.....		.....
Ruminococcus flavefaciens[c]		.....		.....
Clostridium coccoides		.....G.....C		.GG..TN.CC.....
Bifidobacterium longum		.....G.C.....C		.GG.TT..CT.....
Collinsella aerofaciens		.....C.....C		.CC.T...GG-.....G.
Bacteroides vulgatus		.....G.A.....C		..C.TA.G.TGT.....
Atopobium cluster-specific primers	c-Atopo-F	5′ GGGTTGAGAGACCGACC 3′	c-Atopo-R	3′ GGACGTCTTCTTCGRGGC 5′
Target site		5′ GGGTTGAGAGACCGACC 3′		5′ CCTGCAGAAGAAGCYCCG 3′
Atopobium minutum[c]		.....		.....
Atopobium fossor[c]		.....		.....
Atopobium rimae[c]		.....		.....
Atopobium parvulum[c]		.....		.....
Collinsella aerofaciens[c]		.....		.....
Collinsella intestinalis[c]		.....		.....
Collinsella stercoris[c]		.....		.....
Coriobacterium sp. strain EKSO3[c]		.....		.....
Coriobacterium glomerans[c]		.....A		.....
Eggerthella lenta[c]		.....		.....
Cryptobacterium curtum[c]		.....		.....
Slackia heliotrin reducens		...C.....G.T...T		.....
Denitrobacterium detoxificans		..AC.....GT....T		.....
Bifidobacterium longum		..CC.....GG.....		..C.TT...T....A...
Bacteroides vulgatus		.TTC.....GAA..GT		.T.TAT...T...GAT..
Clostridium coccoides		..CC.....GGT...A		....ACTN.....
Faecalibacterium prausnitzii		..AC.....GTT...A		.TCAACA.G....TGA..

[a] The positions of the target sites for the primers are as follows (numbering based on the *Escherichia coli* 16S rRNA gene sequence): sg-Clept-F, nucleotides 933 to 948; sg-Clept-R3, nucleotides 1164 to 1181; c-Atopo-F, nucleotides 292 to 308; and g-Atopo-R, nucleotides 488 to 505.
[b] Only nucleotides that differ from nucleotides in the target sequences are shown.
[c] Targeted organism.

in GAM broth (Nissui Seiyaku, Tokyo, Japan) supplemented with 1% glucose at 37°C for 12 to 48 h. The numbers of cells of the strains listed in Table 4 were counted microscopically by the DAPI (4′,6′-diamidino-2-phenylindole) staining method as described previously (17). Serial 10-fold dilutions of the cultures were also plated on nonselective GAM agar (Nissui Seiyaku). The plates were subsequently incubated at 37°C for 3 to 5 days in an anaerobic chamber (Coy Laboratory Products Inc., Grass Lake, Mich.), and cultural counts (in CFU) were determined in triplicate.

Collection and preparation of fecal samples. Forty-six healthy volunteers from our institute staff (41 males and 5 females; ages, 25 to 59 years [average, 37 ± 9 years]) who subsisted primarily on a Japanese diet provided fresh fecal samples. Forty volunteers provided samples only once (subjects Y-1 to Y-40), while six volunteers provided samples once a month for an 8-month period (subjects A to F). No subject had received antibiotics, probiotics, or prebiotics during the 2 weeks prior to the sampling, although subject A had received antibiotics up until 2 weeks prior to the first sampling date. The samples were collected in sterile plastic bags, refrigerated under anaerobic conditions, and immediately taken to the laboratory.

Enumeration and identification of predominant bacteria by the culture method. Fecal specimens were collected from six healthy volunteers (subjects A to F) at the eighth month, and cultural counts (in CFU) of total anaerobes were determined by using medium 10 agar as described previously (18). All colonies that appeared at the first- and second-highest dilutions were transferred with a sterile toothpick to 50 μl of TE buffer (10 mM Tris-HCl, 1 mM EDTA; pH 8.0). Each suspension was boiled for 15 min to lyse the cells that were to be used for template DNA, and each isolate was identified by using group-specific PCR primers as described previously (Table 1) (18). The number of CFU of each group was calculated from the number of colonies and the results of the identification procedure.

Enumeration of predominant bacteria in the fecal samples by FISH. FISH analyses with 16S rRNA-targeted group-specific oligonucleotide probes were carried out by using the procedure described by Franks et al. (5). The following probes were used to enumerate the target bacterial groups in the fecal samples: Bact338 (5′-GCT GCC TCC CGT AGG AGT-3′) for *Bacteria*, ATO291 (5′-GGT CGG TCT CTC AAC CC-3′) for the *Atopobium* cluster (8), Bac281 (5′-CTA CCT ATC CCC AAG ACT C-3′) for the *B. fragilis* group and *Bacteroides distasonis*, Bif153 (5′-ACC ACC CGT TTC CAG GAG-3′) for *Bifidobacterium* (30), Clept1240 (5′-GTT TTR TCA ACG GCA GTC-3′) for the *C. leptum* subgroup (26), and Erec482 (5′-TCC ATG RAC TGA TTC TTC G-3′) for the *C. coccoides* group (5). Fecal samples were applied to glass slides and were hybridized with the probes or stained with DAPI. The fluorescent cells in the samples were counted with a Leica Q550FW system (Leica, Wetzlar, Germany) by using the Image-Pro Plus image analysis software (version 4; Media-Cybernetics, Silver Spring, Md.). Microscopic counts were determined from 10 images, and a minimum of 50 cells per image were counted.

TABLE 2. 16S rRNA gene-targeted group-specific primers used in this study

Target bacterial group	Primer	Sequence	Size (bp)[a]	Annealing temp (°C)	Reference
Clostridium coccoides group	g-Ccoc-F	AAATGACGGTACCTGACTAA	440	50	18
	g-Ccoc-R	CTTTGAGTTTCATTCTTGCGAA			
Clostridium leptum subgroup	sg-Clept-F	GCACAAGCAGTGGAGT	239	50	This study
	sg-Clept-R3	CTTCCTCCGTTTTGTCAA			
Bacteroides fragilis group	g-Bfra-F	ATAGCCTTTCGAAAGRAAGAT	495	50	18
	g-Bfra-R	CCAGTATCAACTGCAATTTTA			
Bifidobacterium	g-Bifid-F	CTCCTGGAAACGGGTGG	550	55	18
	g-Bifid-R	GGTGTTCTTCCCGATATCTACA			
Atopobium cluster	c-Atopo-F	GGGTTGAGAGACCGACC	190	55	This study
	c-Atopo-R	CGGRGCTTCTTCTGCAGG			
Prevotella	g-Prevo-F	CACRGTAAACGATGGATGCC	513	55	18
	g-Prevo-R	GGTCGGGTTGCAGACC	513	55	18

[a] DNAs extracted from *Ruminococcus productus* YIT 6141^T, *Faecalibacterium prausnitzii* YIT 6174, *Bacteroides vulgatus* YIT 6159^T, *Bifidobacterium longum* YIT 4021^T, *Collinsella aerofaciens* ATCC 25986^T, and *Prevotella melaninogenica* YIT 6039^T were used as real-time PCR controls.

DNA extraction from fecal samples. Fecal samples (20 mg) were washed three times by suspending them in 1.0 ml of phosphate-buffered saline and centrifuging each preparation at 14,000 × *g* in order to remove possible PCR inhibitors. The fecal pellets were resuspended in a solution containing 450 μl of extraction buffer (100 mM Tris-HCl, 40 mM EDTA; pH 9.0) and 50 μl of 10% sodium dodecyl sulfate. Three hundred milligrams of glass beads (diameter, 0.1 mm) and 500 μl of buffer-saturated phenol were added to the suspension, and the mixture was vortexed vigorously for 30 s by using a FastPrep FP 120 (BIO 101, Vista, Calif.) at a power level of 5.0. After centrifugation at 14,000 × *g* for 5 min, 400 μl of the supernatant was collected. Subsequently, phenol-chloroform extractions were performed, and 250 μl of the supernatant was subjected to isopropanol precipitation. Finally, the DNA was suspended in 1 ml of Tris-EDTA buffer.

Real-time PCR. PCR amplification and detection were performed with an ABI PRISM 7900HT sequence detection system (Applied Biosystems, Foster City, Calif.). Each reaction mixture (10 μl) was composed of 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, each deoxynucleoside triphosphate at a concentration of 200 μM, a 1:75,000 dilution of SYBR Green I (Molecular Probes, Eugene, Oreg.), 11 ng of TaqStart antibody (ClonTech, Palo Alto, Calif.) per μl, 0.05 U of *Taq* DNA polymerase (Takara, Tokyo, Japan) per μl, each of the specific primers at a concentration of 0.25 μM, and 1 μl of 1×-, 10×-, or 100×-diluted template DNA. The amplification program consisted of one cycle of 94°C for 5 min and then 40 cycles of 94°C for 20 s, 55 or 50°C for 20 s (Table 2), and 72°C for 50 s and finally one cycle of 94°C for 15 s. The fluorescent products were detected at the last step of each cycle. A melting curve analysis was done after amplification to distinguish the targeted PCR product from the nontargeted PCR product (23). The melting curves were obtained by slow heating at temperatures from 60 to 95°C at a rate of 0.2°C/s, with continuous fluorescence collection. DNAs extracted from *Ruminococcus productus* YIT 6141^T, *Faecalibacterium prausnitzii* YIT 6174, *Bacteroides vulgatus* YIT 6159^T, *Bifidobacterium longum* YIT 4021^T, *Collinsella aerofaciens* ATCC 25986^T, and *Prevotella melaninogenica* YIT 6039^T were used as real-time PCR controls for the group-specific g-Ccoc, sg-Clept, g-Bfra, g-Bifid, c-Atopo, and g-Prevo primers, respectively.

RESULTS

Primer specificity. To analyze the predominant bacteria in human feces, we designed and evaluated two group-specific primers. Primers sg-Clept-F and sg-Clept-R3 were developed to detect the *C. leptum* subgroup, including certain members of the genera *Clostridium*, *Ruminococcus*, *Eubacterium*, and *Faecalibacterium*, which belong to *Clostridium* cluster IV (2). Primers c-Atopo-F and c-Atopo-R are specific for the *Atopobium* cluster (8), including members of the genera *Atopobium*, *Collinsella*, *Eggerthella*, and *Coriobacterium*. The specificity of the group-specific primers was then tested by using DNA extracts from strains representing 61 different bacterial species (Table 3). Melting temperature analysis was used to evaluate the PCR

TABLE 3. Specificity test of group-specific primers for the *C. leptum* subgroup and the *Atopobium* cluster

Species[a]	Strain	Other designation	PCR results[b]	
			sg-Clept	c-Atopo
Clostridium leptum	YIT 6169^T	DSM 753^T	+	−
Clostridium viride	YIT 10050^T	DSM 6836^T	+	−
Eubacterium siraeum	YIT 10049^T	DSM 3996^T	+	−
Faecalibacterium prausnitzii	YIT 6174	ATCC 27766	+	−
Ruminococcus albus	YIT 6083^T	ATCC 27210^T	+	−
Ruminococcus bromii	YIT 6078^T	ATCC27255^T	+	−
Ruminococcus callidus	YIT 6175^T	ATCC 27760^T	+	−
Atopobium parvulum	YIT 10055^T	JCM 10300^T	−	+
Collinsella aerofaciens	ATCC 25986^T	ATCC 25986^T	−	+
Collinsella intestinalis	YIT 10051^T	JCM 10643^T	−	+
Collinsella stercoris	YIT 10052^T	JCM 10641^T	−	+
Eggerthella lenta	YIT 6077^T	ATCC 25559^T	−	+

[a] In addition, negative PCR results with primers sg-Clept and g-Atopo were obtained for the following bacterial species: *Bacteroides fragilis* YIT 6158^T (= ATCC 25285^T), *Bacteroides ovatus* YIT 6161^T (= JCM 5824^T); *Bacteroides thetaiotaomicron* YIT 6163^T (= JCM 5827^T), *Bacteroides uniformis* YIT 6164^T (= JCM 5828^T), *Bacteroides vulgatus* YIT 6159^T (= ATCC 8424^T), *Bacteroides caccae* ATCC 43185^T, *Bacteroides eggerthii* DSM 20697^T, *Clostridium clostridiiforme* YIT 6051^T (= JCM 1291^T), *Clostridium coccoides* YIT 6035^T (= JCM 1395^T), *Clostridium nexile* YIT 6170^T (= ATCC 27757^T), *Clostridium oroticum* YIT 6037^T (= JCM 1429^T), *Clostridium sphenoides* YIT 6059^T (= JCM 1415^T), *Eubacterium rectale* YIT 6082^T (= ATCC 33656^T), *Ruminococcus gnavus* YIT 6176^T (= ATCC 29149^T), *Ruminococcus productus* YIT 6141^T (= ATCC 27340^T), *Prevotella corporis* YIT 6132^T (= JCM 8529^T), *Prevotella denticola* YIT 6131 (= JCM 8528), *Prevotella melaninogenica* YIT 6039^T (= ATCC 25845^T), *Prevotella veroralis* YIT 6126^T (= JCM 6290^T), *Prevotella buccae* DSM 20615, *Prevotella intermedia* YIT 6130^T (= JCM 7365^T), *Prevotella oralis* YIT 6127 (= JCM 6330), *Bifidobacterium adolescentis* YIT 4011^T (= ATCC 15703^T), *Bifidobacterium angulatum* YIT 4012^T (= ATCC 27535^T), *Bifidobacterium bifidum* YIT 4039^T (= ATCC 29521^T), *Bifidobacterium breve* YIT 4014^T (= ATCC 15700^T), *Bifidobacterium catenulatum* YIT 4016^T (= ATCC 27539^T), *Bifidobacterium pseudocatenulatum* YIT 4072^T (= JCM 1200^T), *Bifidobacterium longum* YIT 4021^T (= ATCC 15707^T), *Bifidobacterium infantis* YIT 4018^T (= ATCC 15697^T), *Bifidobacterium dentium* YIT 4017^T (= ATCC 27534^T), *Clostridium baratii* YIT 6052^T (= JCM 1385^T), *Clostridium celatum*, YIT 6056^T (= JCM 1394^T), *Clostridium perfringens* YIT 6050^T (= JCM 1290^T), *Clostridium sporogenes* YIT 6060^T (= JCM 1416^T), *Clostridium limosum* YIT 6061^T (= JCM 1427^T), *Clostridium bifermentans* YIT 6053^T (= JCM 1386^T), *Clostridium glycolicum* YIT 6058^T (= JCM 1401^T), *Clostridium sordelii* YIT 6065^T (= JCM 3814^T), *Fusobacterium varium* ATCC 8501^T, *Fusobacterium gonidiaformans* YIT 6079^T (= ATCC 25563^T), *Escherichia coli* YIT 6044^T (= JCM 1649^T), *Enterococcus faecalis* YIT 2031^T (= ATCC 19433^T), *Enterococcus faecium* YIT 2032^T (= ATCC 19434^T), *Porphyromonas gingivalis* YIT 6030 (= JCM 8525), *Porphyromonas asaccharolytica* ATCC 25260^T, *Propionibacterium acnes* YIT 6118 (= ATCC 6919^T), *Lactobacillus acidophilus* YIT 0070^T (= ATCC 4356^T), and *Peptostreptococcus prevotii* YIT 10027^T (= ATCC 9321^T).

[b] +, positive; −, negative.

TABLE 4. Comparison of bacterial populations in GAM broth determined by real-time quantitative PCR, direct counting with DAPI staining, and the culture method[a]

Primer	Species	Strain	Log_{10} bacteria/ml as determined by:		
			qPCR[b]	DAPI	Culture
g-Ccoc	*Clostridium clostridiiforme*	YIT 6051^T	9.5 ± 0.1	9.5 ± 0.1	9.1 ± 0.1
	Clostridium coccoides	YIT 6035^T	9.3 ± 0.2	9.4 ± 0.3	8.7 ± 0.1
	Clostridium nexile	YIT 6170^T	8.9 ± 0.1	8.7 ± 0.1	7.4 ± 0.3
	Clostridium oroticum	YIT 6037^T	8.6 ± 0.1	8.5 ± 0.1	8.3 ± 0.1
	Clostridium sphenoides	YIT 6059^T	9.1 ± 0.2	9.2 ± 0.2	9.3 ± 0.1
	Eubacterium rectale	YIT 6082^T	8.9 ± 0.1	8.9 ± 0.1	<7.0
	Ruminococcus gnavus	YIT 6176^T	8.7 ± 0.2	8.7 ± 0.2	8.4 ± 0.1
	Ruminococcus productus	YIT 6141^T	9.5 ± 0.2	9.3 ± 0.2	9.1 ± 0.1
sg-Clept	*Faecalibacterium prausnitzii*	YIT 6174	8.9 ± 0.1	8.9 ± 0.2	<7.0
	Eubacterium siraeum	YIT 10049^T	9.1 ± 0.1	8.9 ± 0.2	<7.0
	Ruminococcus bromii	YIT 6078^T	9.3 ± 0.1	9.2 ± 0.1	<7.0
	Ruminococcus callidus	YIT 6175^T	8.5 ± 0.1	8.8 ± 0.2	<7.0
	Ruminococcus albus	YIT 6083^T	8.3 ± 0.1	9.0 ± 0.1	<7.0
	Clostridium viride	YIT 10050^T	8.0 ± 0.1	8.9 ± 0.1	<7.0
	Clostridium leptum	YIT 6169^T	8.1 ± 0.2	9.1 ± 0.1	<7.0
g-Bfra	*Bacteroides caccae*	ATCC 43185^T	9.4 ± 0.1	9.6 ± 0.1	8.8 ± 0.2
	Bacteroides eggerthii	DSM 20697^T	9.0 ± 0.1	8.8 ± 0.1	9.4 ± 0.1
	Bacteroides fragilis	YIT 6158^T	9.4 ± 0.1	9.4 ± 0.2	8.3 ± 0.3
	Bacteroides ovatus	YIT 6161^T	9.1 ± 0.1	9.4 ± 0.2	8.5 ± 0.1
	Bacteroides thetaiotaomicron	YIT 6163^T	8.8 ± 0.1	9.0 ± 0.2	8.8 ± 0.2
	Bacteroides uniformis	YIT 6164^T	8.8 ± 0.1	8.8 ± 0.1	8.6 ± 0.1
	Bacteroides vulgatus	YIT 6159^T	8.7 ± 0.1	8.7 ± 0.1	8.6 ± 0.1
c-Atopo	*Atopobium parvulum*	YIT 10055^T	8.8 ± 0.2	8.7 ± 0.1	9.3 ± 0.1
	Collinsella aerofaciens	ATCC 25986^T	9.3 ± 0.1	9.1 ± 0.1	8.9 ± 0.2
	Collinsella intestinalis	YIT 10051^T	9.2 ± 0.1	9.3 ± 0.1	10.0 ± 0.1
	Collinsella stercoris	YIT 10052^T	8.8 ± 0.2	9.0 ± 0.2	8.7 ± 0.2
	Eggerthella lenta	YIT 6077^T	8.1 ± 0.1	8.7 ± 0.2	8.2 ± 0.1
g-Prevo	*Prevotella corporis*	YIT 6132^T	9.0 ± 0.1	8.8 ± 0.2	8.7 ± 0.1
	Prevotella denticola	YIT 6131	8.8 ± 0.1	8.9 ± 0.3	9.4 ± 0.1
	Prevotella melaninogenica	YIT 6039^T	9.4 ± 0.1	9.2 ± 0.2	9.5 ± 0.1
	Prevotella veroralis	YIT 6126^T	8.8 ± 0.1	8.8 ± 0.1	8.9 ± 0.1

[a] A comparison of bifidobacterial species has been reported previously (17).
[b] PCR, real-time quantitative PCR.

results, as the shape and position of a melting curve can be used to differentiate target PCR products from nontarget products (data not shown) (23). Each specific primer gave positive PCR results for the corresponding target bacteria and negative PCR results for nontarget microorganisms.

Real-time PCR detection. DNA extracted from a known amount of *C. aerofaciens* ATCC 25986^T was added in serial dilutions from 10^6 to 0 cells to a series of PCR mixtures with *Atopobium* cluster-specific primers, and fluorescence was monitored throughout the reactions. As a result, the number of starting cells and the cycle number at which the product fluorescence surpassed a defined threshold were found to be linear over the range of DNA concentrations from 10^6 to 10 cells per PCR mixture ($r^2 = 0.99$) (data not shown). This indicates that the linear range for the procedures used in this study is 10^{11} to 10^6 cells per g of feces. Virtually identical results were obtained for *R. productus*, *F. prausnitzii*, *B. vulgatus*, and *P. melaninogenica* with their group-specific primers (data not shown).

Quantification of the target bacteria in the culture medium. The number of bacteria in GAM broth was quantified by real-time PCR by using group-specific primers for the *C. coccoides* group, the *C. leptum* subgroup, the *B. fragilis* group, the *Atopobium* cluster, and *Prevotella*. The results were compared with those obtained by using the DAPI staining method and the culture method (Table 4). Compared to the DAPI staining method, the quantitative PCR method gave similar counts for most members of the *C. coccoides* group, the *B. fragilis* group, and the *Atopobium* cluster except *Eggerthella lenta*, with a difference of less than $\log_{10}$ 0.2 cell per g. However, quantitative PCR gave estimates for *E. lenta* and a member of the *C. leptum* subgroup that were lower than the corresponding counts obtained by the DAPI staining method. With culture methods, the levels of members of the *C. leptum* subgroup and the *C. coccoides* group were underestimated compared to the results obtained by real-time PCR and the DAPI counting method ($P < 0.05$, as determined by a paired t test). This may have been due to biases inherent in the media used.

Quantification of the six predominant bacterial groups in fecal samples. Real-time PCR analyses were performed to quantify individual bacterial groups in fecal samples collected from six subjects. The results were then compared to the results obtained by the FISH and culture methods (Table 5). Overall, the populations of the bacterial groups determined by real-time PCR and FISH methods were similar. However, the

TABLE 5. Comparison of quantitative PCR, FISH, and the culture method for detection and quantification of predominant bacteria in fecal samples[a]

Bacteria	Log_{10} cells/g (wet wt) for[a]:																	
	Subject A			Subject B			Subject C			Subject D			Subject E			Subject F		
	qPCR[b]	FISH	Culture	qPCR	FISH	Culture	qPCR	FISH	Culture	qPCR	FISH	Culture	qPCR	FISH	Culture	qPCR	FISH	Culture
C. coccoides group	10.4	10.4	10.4	10.7	10.4	10.3	10.4	10.2	9.9	10.5	10.5	10.3	10.3	10.3	10.2	9.5	9.4	ND[c]
C. leptum subgroup	10.1	10.3	10.1	10.7	10.2	9.9	10.7	10.0	10.4	10.8	10.4	10.2	10.5	9.8	10.1	6.5	ND	ND
B. fragilis group	10.5	10.3	10.7	10.1	10.4	10.3	10.1	10.3	10.3	9.4	9.7	9.4	10.0	10.0	10.2	9.7	8.5	9.7
Bifidobacterium	9.9	9.7	9.2	10.3	10.3	9.9	10.3	9.9	10.0	9.7	9.3	9.4	9.8	9.4	9.4	6.5	7.8	ND
Atopobium cluster	9.6	9.7	9.9	9.6	9.9	9.9	10.3	9.6	10.2	9.9	9.7	10.1	9.9	9.1	9.8	6.8	ND	ND
Prevotella	ND	NT[d]	ND	ND	NT	ND	10.2	NT	9.8	10.7	NT	10.4	ND	NT	ND	ND	NT	ND
Sum of six groups	10.9	10.9	11.0	11.1	11.0	10.8	11.2	10.8	10.9	11.2	10.8	10.9	10.9	10.6	10.7	9.9	9.5	9.7
Total bacteria	NT	11.0[e]	11.1[f]	NT	11.1	10.9	NT	10.9	11.0	NT	10.9	11.0	NT	10.8	10.7	NT	9.7	9.9
Total cells (DAPI)		11.2			11.3			11.2			11.1			11.1			10.3	

[a] Fecal samples collected in the eighth month were used for comparison.
[b] qPCR, quantitative PCR.
[c] ND, not detected.
[d] NT, not tested.
[e] Total number of bacteria determined by hybridization with probe Bact338. In the present study, 66, 63, 46, 60, 53, and 28% (average ± standard deviation, 53% ± 14%) of the total cells were detected with the Bact338 probe in samples from subjects A, B, C, D, E, and F, respectively.
[f] Total cultivated bacteria with medium 10.

real-time PCR method gave higher values than the FISH method for certain samples (e.g., the *C. leptum* subgroup in subjects B, C, and E; the *B. fragilis* group in subject F; and the *Atopobium* cluster in subjects C and E). The populations of *Bifidobacterium* determined by the culture method were smaller than the populations determined by the real-time PCR ($P < 0.05$, as determined by a paired t test). In addition, culture-based methods tended to give lower values than real-time PCR for the *C. leptum* subgroup and the *C. coccoides* group. Detection of *Prevotella* by FISH was not performed, since a group-specific probe for this genus has not been established.

Distribution of the six bacterial groups in intestinal flora. Table 6 shows the distribution of bacterial groups in the intestinal tracts of 46 healthy adult volunteers. The total bacterial count as determined by DAPI staining was $\log_{10}$ 10.9 ± 0.2 cells per g (wet weight). We found larger populations of the *C. coccoides* group ($\log_{10}$ 10.3 ± 0.3 cells per g or 29% ± 12% of the total cell count) than of the other five groups of bacteria ($P < 0.01$, as determined by a paired t test). The *C. leptum* subgroup ($\log_{10}$ 9.9 ± 0.7 cells per g or 15% ± 10% of the total cell count) and the *B. fragilis* group ($\log_{10}$ 9.9 ± 0.3 cells per g or 11% ± 7.8% of the total cell count) were present at nearly equal levels. These two groups were present at higher levels than *Bifidobacterium* ($\log_{10}$ 9.4 ± 0.8 cells per g or 6.0% ± 6.4% of the total cell count) and the *Atopobium* cluster ($\log_{10}$ 9.3 ± 0.7 cells per g or 4.9% ± 4.2% of the total cell count) ($P < 0.01$, as determined by a paired t test). The variations in the populations of the *C. coccoides* group and the *B. fragilis* group (SD values, $\log_{10}$ 0.3 cell per g) were less marked than those of the *C. leptum* subgroup, *Bifidobacterium* (SD values, $\log_{10}$ 0.7 cell per g), or the *Atopobium* cluster (standard deviation, $\log_{10}$ 0.7 cells per g) (Table 7). Except for *Prevotella*, the five bacterial groups were detected in all volunteers. *Prevotella* was found in only 21 of 46 subjects (46%) at a level of $\log_{10}$ 9.7 ± 0.8 cells per g (4.4% ± 4.9% of the total cell count). The total population of these six bacterial groups was $\log_{10}$ 10.8 ± 0.3 cells per g or 71% ± 22% of the total cell count.

Temporal fluctuations in the six bacterial groups. Fecal samples were collected monthly from six healthy adults over an 8-month period to assess changes in the level of each bacterial group (Fig. 1). Table 7 provides a summary of the longitudinal variations (SD_{long}) and the interindividual variations (SD_{inter}) of these bacteria and shows that there were smaller longitudinal temporal variations than interindividual variations for all bacterial groups. The interindividual and longitudinal variations of the *C. coccoides* group and the *B. fragilis* groups were less than those of the other bacterial groups (Table 7). Although the SD_{inter} values of the *C. leptum* subgroup and the *Atopobium* cluster are relatively large, the longitudinal shifts were moderate. On the other hand, bifidobacteria showed significant variations in SD_{inter} and SD_{long} values.

DISCUSSION

In order to clarify the population structure of the predominant phylogenetic groups in the human intestinal flora, newly developed group-specific primers for the *C. leptum* subgroup and the *Atopobium* cluster were used in a real-time PCR analysis with primers for the *C. coccoides* group, the *B. fragilis* group, *Bifidobacterium*, and *Prevotella*. Since the quantitative PCR method targets extracted DNAs, the number of 16S rRNA genes in the genome, differences in DNA extraction efficiency, and point mutations in the target region of a primer may influence the measurements. However, the cell counts determined by the quantitative PCR method were similar to those determined by the DAPI counting method for members of the *C. coccoides* group, the *B. fragilis* group, and the *Atopobium* cluster (Table 4). This indicates that the sensitivity of quantitative PCR assays may not vary greatly, depending on the species or strains. On the other hand, the real-time quantitative PCR method gave lower values for certain members of

TABLE 6. Mean counts of six major bacterial groups in fecal samples collected from 46 health Japanese volunteers as determined by real-time PCR

Subject	Log_{10} cells/g (wet wt)							
	With genus- and species-specific primers[a]						Sum of six groups	Total cells (DAPI count)
	g-Ccoc	sg-Clept	g-Bfra	g-Bifid	c-Atopo	g-Prevo		
A	10.4	10.1	10.5	10.0	9.6	ND	10.9	11.2
B	10.7	10.7	10.1	10.3	9.6	ND	11.1	11.3
C	10.4	10.7	10.1	10.6	10.3	10.2	11.2	11.2
D	10.5	10.8	9.4	10.4	9.9	10.7	11.3	11.1
E	10.3	10.5	10.0	9.3	9.9	ND	10.8	11.1
F	9.5	6.5	9.7	6.9	6.8	ND	9.9	10.3
Y-1	10.3	9.7	9.7	9.6	9.5	ND	10.5	10.9
Y-2	10.1	9.8	9.9	10.1	10.0	ND	10.7	11.0
Y-3	10.5	10.2	9.7	9.2	9.7	9.9	10.8	11.0
Y-4	10.1	10.0	9.7	8.2	7.7	ND	10.4	10.6
Y-5	10.2	10.2	9.2	8.4	9.6	10.3	10.7	10.9
Y-6	10.4	9.9	10.0	9.5	9.7	ND	10.7	10.9
Y-7	10.3	9.8	9.6	9.1	8.8	ND	10.5	10.6
Y-8	10.4	10.3	9.8	9.6	9.8	10.4	10.9	10.8
Y-9	10.6	10.5	10.1	9.4	8.6	10.0	11.0	11.1
Y-10	10.6	10.5	10.0	9.6	9.2	ND	10.9	11.0
Y-11	11.0	10.6	9.9	10.2	9.9	10.2	11.3	11.3
Y-12	10.4	9.9	9.6	9.4	9.9	ND	10.7	10.6
Y-13	10.0	9.7	9.5	10.0	9.7	ND	10.5	11.1
Y-14	10.4	10.2	9.6	10.1	10.1	10.4	11.0	11.0
Y-15	10.0	9.9	9.4	8.9	9.6	10.1	10.6	10.7
Y-16	10.6	10.2	10.3	8.4	9.1	ND	10.9	11.0
Y-17	10.8	10.4	10.3	9.0	9.8	9.6	11.1	11.1
Y-18	10.5	10.0	10.2	9.7	9.5	ND	10.8	11.0
Y-19	10.4	10.3	9.9	9.6	10.1	9.9	10.9	11.0
Y-20	10.2	9.6	9.8	9.3	9.0	ND	10.5	10.9
Y-21	10.5	10.5	10.2	9.3	9.3	9.9	11.0	11.1
Y-22	10.3	9.5	9.7	10.1	9.9	8.4	10.7	10.8
Y-23	10.5	10.1	10.3	7.5	9.6	9.5	10.8	11.0
Y-24	10.3	10.3	10.3	9.6	9.4	ND	10.8	10.9
Y-25	10.7	10.5	10.2	10.1	9.9	ND	11.1	11.1
Y-26	9.9	8.8	9.4	9.0	9.1	9.9	10.3	10.6
Y-27	10.4	9.9	9.5	9.1	9.5	ND	10.6	10.9
Y-28	10.6	10.4	10.0	8.9	7.3	8.3	10.8	11.0
Y-29	10.4	9.8	10.1	9.7	8.2	ND	10.7	10.7
Y-30	10.6	10.1	10.2	9.4	9.6	ND	10.8	11.0
Y-31	10.0	8.4	10.2	10.0	8.1	9.4	10.6	10.9
Y-32	10.4	10.3	10.0	9.2	8.8	10.1	10.8	11.0
Y-33	10.5	10.1	9.5	9.4	10.0	9.5	10.8	11.1
Y-34	9.7	9.2	10.0	9.5	9.7	ND	10.4	10.8
Y-35	10.4	9.8	9.9	10.1	10.0	ND	10.8	11.0
Y-36	10.7	10.1	10.0	9.8	9.8	9.0	11.0	11.3
Y-37	9.7	9.1	10.0	10.2	8.9	ND	10.5	10.6
Y-38	10.2	9.9	9.9	8.6	9.3	ND	10.5	10.8
Y-39	10.3	9.7	9.3	9.6	9.6	7.5	10.6	10.7
Y-40	9.9	8.5	10.0	9.6	8.9	ND	10.3	10.5
Mean ± SD	10.3 ± 0.3	9.9 ± 0.7	9.9 ± 0.3	9.4 ± 0.7	9.3 ± 0.7	9.7 ± 0.8	10.8 ± 0.3	10.9 ± 0.2

[a] Primers g-Ccoc, g-Bfra, sg-Clept, g-Bifid, and c-Atopo are specific for the *C. coccoides* group, the *B. fragilis* group, the *C. leptum* subgroup, *Bifidobacterium*, and the *Atopobium* cluster, respectively.
[b] ND, not detected ($<\log_{10}$ 6 cells/g).

the *C. leptum* subgroup, such as *Ruminococcus albus*, *Clostridium viride*, and *C. leptum*, compared to DAPI counting methods (Table 4). This suggests that the population of the *C. leptum* subgroup may have been underestimated when these species were the predominant species of the *C. leptum* subgroup. Therefore, real-time PCR detection of the *C. leptum* subgroup from fecal DNA should be improved further.

Although the populations of each bacterial group in six healthy volunteers enumerated by the three methods were similar, the real-time PCR method sometimes gave higher values than the FISH method (Table 5). This variation may be explained in terms of the different targets of the methods used. Since real-time PCR targets extracted DNA, PCR methods can detect all bacterial cells present in a specimen. While the FISH method targets rRNA, it is difficult to detect bacterial cells that have little rRNA but are present in feces. Probe permeation of the gram-positive cell wall has been reported to be problematic in FISH experiments (5). In this study, 34, 37,

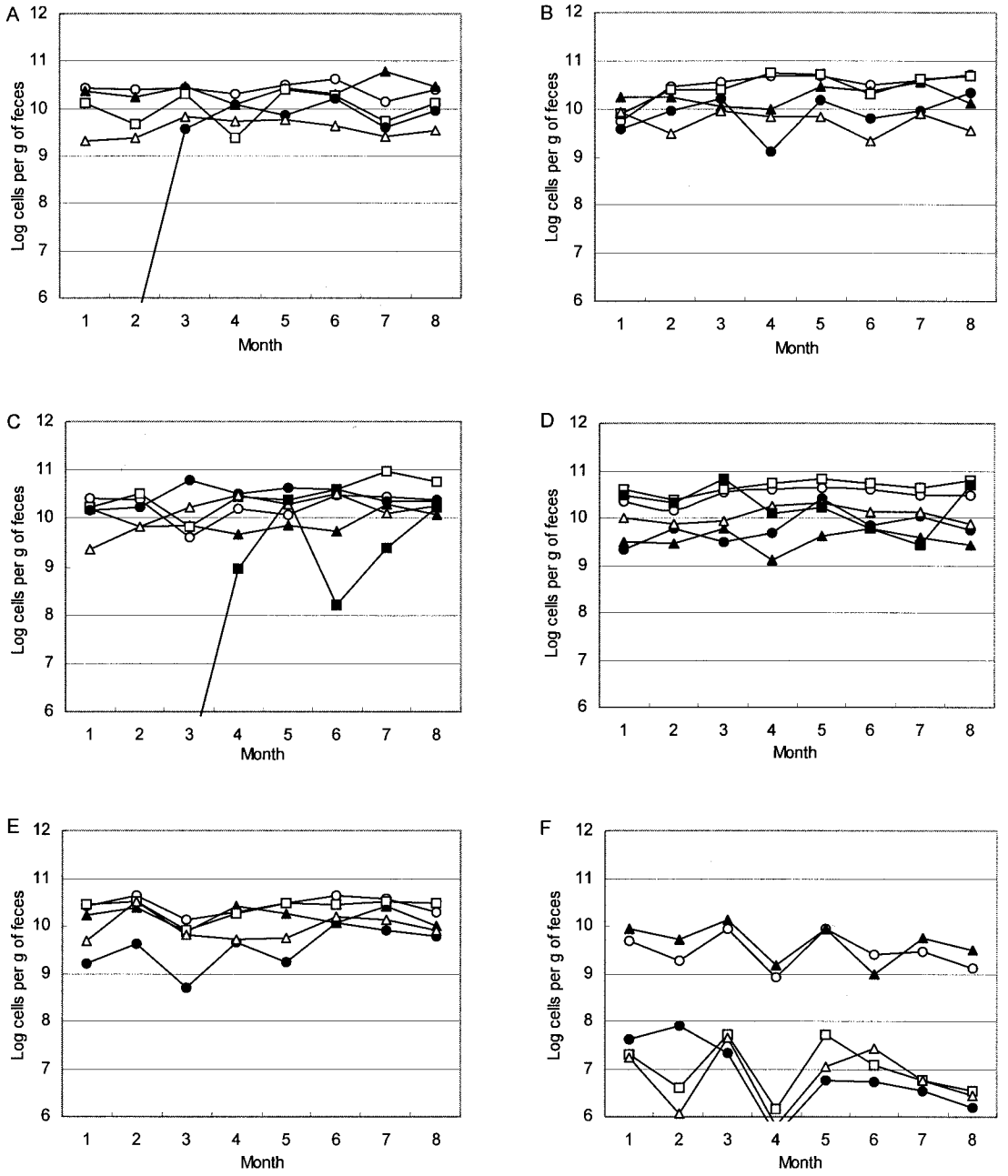

FIG. 1. Populations of predominant bacteria in fecal samples collected from six human subjects over an 8-month period. (A) Subject A; (B)(A) subject B; (C) subject C; (D) subject D; (E) subject D; (F) subject F. Symbols: ○, *C. coccoides* group; □, *C. leptum* subgroup; ▲, *B. fragilis* group; ●, *Bifidobacterium*; △, *Atopobium* cluster; ■, *Prevotella*.

54, 40, 47, and 72% of DAPI-counted cells were not detected by FISH with the Bact338 probe in subjects A, B, C, D, E, and F, respectively (Table 5). Such undetected bacteria have been reported to account for approximately 30 to 40% of the total cell counts (7, 28). Therefore, the presence of these bacteria may explain the differences in the results of the real-time PCR and FISH methods observed in this study. Extensive efforts have been made in the past to cultivate the bacteria found in human feces, with the result that the human intestinal flora is one of the most successfully studied natural communities of bacteria (1, 3, 4, 20, 21). However, the culture method tended to give lower population values than the quantitative PCR method, probably due to the presence of bacterial cells lacking colony-forming capacity, cell aggregation, and the selection bias of the medium.

When the results for 46 healthy adults were compared to current knowledge, the total bacterial count determined by the DAPI staining method ($\log_{10}$ 10.9 ± 0.2 cells per g [Table 6]) was in good agreement with values obtained by other investigators (7). The populations of the *C. leptum* subgroup, the *B. fragilis* group, *Bifidobacterium*, and the *Atopobium* cluster determined in this study were in general agreement with results

TABLE 7. Variations in fecal flora populations between volunteers (SD_{inter}) and within each volunteer over time (SD_{long})

Organism or group	Variation[a]			
	Mean for total	SD for total	SD_{inter}[b]	SD_{long}[c]
C. coccoides group	10.25	0.44	0.45	0.22
B. fragilis group	9.99	0.40	0.39	0.22
C. leptum subgroup	9.83	1.34	1.43	0.30
Bifidobacterium	9.21	1.48	1.55	0.65
Atopobium cluster	9.38	1.23	1.29	0.30
Prevotella[d]	9.18	1.72	1.50	1.11

[a] Means and standard deviations were calculated by using all 48 fecal samples collected from subjects A to F over an 8-month period.
[b] SD_{inter} represents the difference in bacterial count between volunteers.
[c] SD_{long} represents change in bacterial counts within individual volunteers over time.
[d] The values were calculated from serial samples C and D.

obtained by molecule-based methods, such as FISH (5, 7, 8), quantitative hybridization (26), and the cloning library method (9, 10, 28). On the other hand, the populations and proportions of the *C. coccoides* group obtained by real-time PCR tended to be greater than the populations and proportions obtained by the FISH method (7). However, the proportions for this group were in good agreement with those obtained by the cloning library method (9, 10, 28). The considerable variation may be explained in terms of the different targets of the methods used (rRNA gene versus rRNA molecule), as discussed above.

Although *Prevotella* has been reported to be one of the major components of the oral flora (25), there have been few reports of detection of this bacterial group in the human intestinal flora (18, 28). *Bacteroides* and *Prevotella* have not been distinguished in the previous studies by the FISH method (7). Therefore, the present quantitative PCR analysis likely represents the first report of a comprehensive analysis of the distribution of the genus *Prevotella* in the intestinal flora. As shown in Table 6, *Prevotella* was detected in only 21 of 46 subjects (46%), but the mean count in those individuals was $\log_{10} 9.7 \pm 0.8$ cells per g. This indicates that *Prevotella* was one of the predominant bacterial groups in these subjects. The count for *Prevotella* in the remaining 25 subjects did not exceed 10^6 cells per g of feces, suggesting that there were considerable differences in this bacterial population among individuals. Since most *Prevotella* species are reportedly sensitive to bile acids (22), it should be of interest to investigate the species of *Prevotella* in the intestine, their resistance to bile acids, and factors in the intestinal environment that may influence colonization by this bacterial group.

In the present 8-month analysis, changes in bacterial counts over time within individual volunteers (SD_{long}) were compared to differences in bacterial counts between volunteers (SD_{inter}) (Table 7). The SD_{long} values were found to be smaller than the SD_{inter} values, suggesting that the variations in the flora over time within one individual tended to be less marked than the differences among volunteers. Although considerable changes were observed in the population of *Bifidobacterium* in subject A, it should be noted that subject A had received antibiotics 2 weeks prior to the first sampling date, treatment that may have resulted in significant changes in the *Bifidobacterium* population (17). We also found interesting the significant change in the *Prevotella* population observed in serial sample C (Fig. 1). The cause of the considerable change requires further investigation, involving, for example, a change in bile acid secretion caused by dietary variations.

Conclusions. In this study, we developed a quantitative PCR detection method to investigate the composition of human intestinal flora by using DNA extracted from fecal samples. The advantages of this method are higher sensitivity, easy sample handling, and simple procedures. Therefore, the group-specific PCR method shows promise as an effective tool for investigating the effects of probiotics or prebiotics, the side effects of antibiotics, and the relationship between the microflora and digestive diseases, such as inflammatory bowel disease, infectious diseases, and colon cancer. The next challenge for real-time PCR is to develop group- and species-specific primers to cover most of human intestinal bacteria. The wide-ranging PCR primers should make the real-time PCR technique a more advanced method for the study of the human intestinal microflora.

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