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Bacterial, Archaeal, and Eukaryal Diversity in the Intestines of Korean People

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The bacterial, archaeal, and eukaryal diversity in fecal samples from ten Koreans were analyzed and compared by using the PCR-fingerprinting method, denaturing gradient gel electrophoresis (DGGE). The bacteria all belonged to the *Firmicutes* and *Bacteroidetes* phyla, which were known to be the dominant bacterial species in the human intestine. Most of the archaeal sequences belonged to the methane-producing archaea but several halophilic archarea-related sequences were also detected unexpectedly. While a small number of eukaryal sequences were also detected upon DGGE analysis, these sequences were related to fungi and stramenopiles (*Blastocystis hominis*). With regard to the bacterial and archaeal DGGE analysis, all ten samples had one and two prominent bands, respectively, but many individual-specific bands were also observed. However, only five of the ten samples had small eukaryal DGGE bands and none of these bands was observed in all five samples. Unweighted pair group method and arithmetic averages clustering algorithm (UPGMA) clustering analysis revealed that the archaeal and bacterial communities in the ten samples had relatively higher relatedness (the average Dice coefficient values were 68.9 and 59.2% for archaea and bacteria, respectively) but the eukaryal community showed low relatedness (39.6%).

***Keywords*:** human intestinal microbes, Bacteria, Archaea, Eukaryote, DGGE, UPGMA

Starting with Louis Pasteur's postulation that human health is dependent on gut-resident microbiota (Pasteur, 1885), our knowledge of intestinal microbes has expanded considerably. In particular, the molecular PCR-based techniques examining bacterial 16S rRNA gene sequences that have been employed over the past two decades have provided a great deal of information about intestinal microbes (Zoetendal *et al.*, 1998; Backhed *et al.*, 2004; Eckburg *et al.*, 2005). Consequently, it is now known that the human intestine is an extremely complex microbial ecosystem (Moore and Holdeman, 1974) in which microbes from the three domains of life (Bacteria, Archaea, and Eukarya) reside (Rajilic-Stojanovic *et al.*, 2007). This highly diverse ecosystem harbors more than 100 trillion microorganisms (Backhed *et al.*, 2005) (i.e. ten-fold higher than the total number of host cells in the human body) and 1,000 species (Simon and Gorbach, 1984). Moreover, it has been shown that these intestinal microbes interact with various nutrients, host cells, and each other, and play an important role in human health and disease (Hooper *et al.*, 2001).

Most human intestinal bacteria belong to the *Bacteroidetes*, *Firmicutes*, and *Proteobacteria* phyla (Eckburg *et al.*, 2005). In particular, the former two phyla have been shown to play very important roles in human health. For example, butyrate-producing bacteria that belong to the class *Clostridia* (*Firmicutes* phylum) have been shown to contribute to the maintenance and protection of normal colonic epithelium (Pryde *et al.*, 2002), while *Bacteroides thetaiotaomicron* (*Bacteroidetes* phylum) play beneficial roles in nutrient absorption and epithelial cell maturation and maintenance (Hooper *et al.*, 2001). With regard to the intestinal archaea, hydrogen-consuming methanogens are the predominant archaea in the intestinal tracts of many invertebrate and vertebrate species (Hackstein and Van Alen, 1996; Morvan *et al.*, 1996; Lin and Miller, 1998). In particular, *Methanobacter smithii* can be comprised up to 10% of all anaerobes in the colons of healthy human adults (Miller and Wolin, 1986). While *Methanosphaera stadmanae* and *Crenarchaeotes* also have been detected, they are only minor members (Rieu-Lesme *et al.*, 2005). Small eukaryotes, including fungi, yeast, protozoa, and stramenopiles, also exist in our body in small numbers (Rajilic-Stojanovic *et al.*, 2007) but unlike the bacterial and archaeal species, most of those in the intestinal tract appear to be opportunistic or severe pathogens (Tawfik *et al.*, 1989; Stenzel and Boreham, 1996; Noverr *et al.*, 2002).

While the introduction of molecular techniques such as 16S rRNA gene PCR and cloning (Hooper *et al.*, 2001) has greatly improved our understanding of the intestinal microbes, the human gastrointestinal ecosystem remains incompletely characterized (Hooper *et al.*, 2001). It is known that human intestines harbor hundreds of bacterial species that are unique to individuals along with a few species that are common to all individuals (Zoetendal *et al.*, 1998; Suau *et al.*, 1999). However, much less is known about the archaeal and eu-

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Table 1. Characteristics of the individuals participating in this study

Sample ID	Age	Sex	Diet	Obesity	Disease
a	37	female	vegetarian	normal	gastritis
b	64	male	polyphagous	normal	gastritis
c	63	female	polyphagous	normal	gastritis
d	63	female	vegetarian	overweight	gastritis
e	41	female	polyphagous	normal	normal
f	66	female	polyphagous	obese	normal
g	64	female	polyphagous	overweight	normal
h	64	female	vegetarian	overweight	IBD (irritable bowel disease)
i	60	male	vegetarian	obese	gastritis
j	68	male	polyphagous	obese	gastritis

karyal communities in the human intestine and microbial community of Korean fecal sample is not well investigated with molecular techniques Thus, the in-depth inspection of untouched human fecal samples is likely to further improve our understanding of intestinal microbial diversity. Moreover, molecular ecological analysis of the diversity of the three domains in the same people, which has not been performed previously, will indicate the overall shape of microbial community in the human intestine. Here, we assessed the microbial diversity and overall shape of communities in the three kingdoms in the intestines of Korean people by PCR-denaturing gradient gel electrophoresis (DGGE) analysis of the 16S rRNA and 18S rRNA genes. The composition of the three domains in each individual was then characterized by unweighted pair group method and arithmetic averages clustering algorithm (UPGMA) clustering analysis of the DGGE banding patterns and tree comparison.

Materials and Methods

Fecal sample collection and extraction of metagenomic DNA

Fecal samples were collected from ten unrelated individuals (denoted a to j) who differed in terms of their geographical location within South Korea, dietary preferences, age, and sex (Table 1). Six subjects had gastritis and one had irritable bowel disease (IBD) at the time of sampling. Fecal samples were taken immediately after defecation into sterile plastic containers and stored at -80°C until use.

The bulk community DNAs were isolated from the fecal samples by using the previously described bead-beating method (Yeates *et al.*, 1998). All DNA samples were treated with RNase A (Sigma, USA), purified by ethanol precipitation, and purified further by using an UltraClean Microbial DNA Isolation Kit (Mo Bio Laboratories, CA) with the following modifications: the bead-beating and MD2 steps were not performed and the bulk DNAs were added to solution MD1 instead of the MicroBead solution. The DNA concentrations were determined in triplicate by using a spectrophotometer (Nanodrop Technologies, USA).

PCR amplification

The DNAs from each sample were subjected to PCR amplification with particular primer sets (Table 2), followed by DGGE. All primers that were used in this study are indicated in Table 2. To analyze bacterial diversity, the primer set GC338f-518r (Muyzer *et al.*, 1993) was used as described previously (Henckel *et al.*, 1999). Nested PCR amplification of archaeal 16S rRNA genes was performed by using the arch20f-958r primer set (DeLong, 1992; DeLong *et al.*, 1999)

Table 2. List of PCR primers used in this study

	Designation	Sequence (5'→3')
Bacteria-specific primers	GC338f	ACGGGGGGACTCCTACGGGAGGCAGCAG
	518r	ATTACCGCGGCTGCTGG
Archaea-specific primers	Arch20f	TTCCGGTTGATCCYGCCGGA
	Arch958r	YCCGGCGTTGAMTCCAATT
	GCarch340f	GGCACGGGCCCTACGGGGYGCASCAG
	Arch519r	TTACCGCGGCKGCTG
Eukarya-specific primers	EukA	AACCTGGTTGATCCTGCCAGT
	EukB	TGATCCTTCTGCAGGTTCACCTAC
	GCEuk1209f	CAGGTCTGTGATGCCC
	Uni1392r	ACGGGCGGTGTGTRC

A GC clamp (5'-CGCCCGCCGCGCGGCGGGCGGGGCGGGGGC-3') was added to primers 338f, arch340f, and Euk1209f for DGGE analysis.

Table 3. Identification of the bacterial 16S rRNA gene sequences of the DGGE bands obtained from the fecal samples of ten individuals. The position of each band in the DGGE gel is shown in Fig. 1

Clone	Nearest sequences	Identity	Nearest isolates	Identity	Phylogenetic affiliation
FB1	Uncultured bacterium clone MD19_aaa03a07 (EU506500)	98.30	*Eubacterium tenue* ATCC 25553^T (M59118)	98.29	*Clostridia*
FB3	Uncultured bacterium clone SJTU_C_08_88 (EF404360)	95.90	*Gracilibacter thermotolerans* DSM 17427^T (DQ117465)	91.74	*Clostridia*
FB4	Uncultured Firmicutes bacterium clone MS193A1_A02 (EF709001)	97.50	*Gracilibacter thermotolerans* DSM 17427^T (DQ117465)	90.98	*Clostridia*
FB5	Uncultured bacterium clone SJTU_G_02_82 (EF405367)	98.60	*Dialister propionicifaciens* CIP 108336^T (AY850119)	89.26	*Clostridia*
FB6	Uncultured bacterium clone LM0ACA9ZD02FM1 (EU064141)	96.10	*Porphyromonas endodontalis* ATCC 35406^T (L16491)	87.30	*Bacteroidetes*
FB7	Uncultured bacterium clone RL184_aao68f11 (DQ810157)	98.70	*Catenibacterium mitsuokai* JCM 10609^T (AB030224)	96.53	*Clostridia*
FB8	Uncultured bacterium clone RL176_aah44f12 (DQ793879)	97.50	*Bacteroides capillosus* ATCC 29799^T (AY136666)	92.74	*Bacteroidetes*
FB9	Uncultured bacterium clone RL308_aal82d01 (DQ809144)	100.00	*Faecalibacterium prausnitzii* ATCC 27768^T (AJ413954)	96.75	*Clostridia*
FB10	Uncultured Lachnospiraceae bacterium clone MS149A1_H05 (EF705135)	96.40	*Anaerofilum pentosovorans* DSM 7168^T (X97852)	88.46	*Clostridia*
FB11	Uncultured bacterium clone RL307_aam06a02 (DQ807392)	99.20	*Subdoligranulum variabile* DSM 15176^T (AJ518869)	97.52	*Clostridia*
FB12	Uncultured bacterium clone SJTU_G_06_62 (EF405503)	94.60	*Phascolarctobacterium faecium* ACM 3679^T (X72865)	94.59	*Clostridia*
FB13	Uncultured Mollicutes bacterium clone MS030A1_H11 (EF706155)	100.00	*Clostridium innocuum* ATCC 14501^T (M23732)	99.32	*Clostridia*
FB14	*Clostridium populeti* ATCC 35295^T (X71853)	99.18	*Clostridium populeti* ATCC 35295^T (X71853)	99.18	*Clostridia*
FB15	*Faecalibacterium prausnitzii* ATCC 27768^T (AJ413954)	97.56	*Faecalibacterium prausnitzii* ATCC 27768^T (AJ413954)	97.56	*Clostridia*
FB17	Uncultured Lachnospiraceae bacterium clone MS146A1_F12 (EF706771)	99.30	*Ruminococcus torques* ATCC 27756^T (L76604)	99.20	*Clostridia*
FB18	*Ruminococcus lactaris* ATCC 29176^T (L76602)	99.17	*Ruminococcus lactaris* ATCC 29176^T (L76602)	99.17	*Clostridia*
FB19	Uncultured Lachnospiraceae bacterium clone MS033A1_D01 (EF701395)	98.40	*Clostridium herbivorans* ATCC 49925^T (L34418)	98.39	*Clostridia*
FB20	Uncultured Lachnospiraceae bacterium clone MS195A1_B05 (EF709160)	98.30	*Ruminococcus gnavus* ATCC 29149^T (X94967)	96.67	*Clostridia*
FB21	*Eubacterium eligens* 27750^T (L34420)	99.19	*Eubacterium eligens* ATCC 27750^T (L34420)	99.19	*Clostridia*
FB22	Uncultured bacterium clone E721 (DQ327191)	98.20	*Dorea longicatena* 111-35^T (AJ132842)	84.13	*Clostridia*
FB24	*Ruminococcus lactaris* ATCC 29176^T (L76602)	95.76	*Ruminococcus lactaris* ATCC 29176^T (L76602)	95.76	*Clostridia*
FB25	*Parasporobacterium paucivorans* DSM 15970^T (AJ272036)	99.19	*Parasporobacterium paucivorans* DSM 15970^T (AJ272036)	99.19	*Clostridia*
FB26	Uncultured bacterium clone X578 (AY980581)	100.00	*Faecalibacterium prausnitzii* ATCC 27768^T (AJ413954)	99.18	*Clostridia*
FB27	Uncultured Gram-positive bacterium clone NS2A2 (AB064851)	97.40	*Staphylococcus vitulinus* ATCC 51145^T (AB009946)	91.30	*Clostridia*
FB28	Uncultured bacterium clone SJTU_G_10_90 (EF405491)	96.60	*Bacteroides plebeius* JCM 12973^T (AB200217)	95.97	*Bacteroidetes*
FB29	Uncultured bacterium clone RL308_aal82d01 (DQ809144)	98.30	*Faecalibacterium prausnitzii* ATCC 27768^T (AJ413954)	94.87	*Clostridia*
FB30	Uncultured Clostridiales bacterium clone D0-2 (AB331486)	96.80	*Clostridium proteolyticum* ATCC 49002^T (X73448)	90.91	*Clostridia*
FB31	Uncultured Bacteroidales bacterium clone MS146A1_B01 (EF706714)	99.20	*Bacteroides vulgatus* ATCC 8482^T (M58762)	96.15	*Bacteroidetes*
FB32	*Clostridium herbivorans* ATCC 49925^T (L34418)	99.18	*Clostridium herbivorans* ATCC 49925^T (L34418)	99.18	*Clostridia*
FB33	Uncultured Bacteroidales bacterium clone MS146A1 (EF706779)	97.70	*Bacteroides dorei* DSM 17855^T (AB242142)	93.53	*Bacteroidetes*
FB34	Uncultured bacterium clone RL243_aai86b02 (DQ808847)	97.50	*Ruminococcus lactaris* ATCC 29176^T (L76602)	97.41	*Clostridia*
FB35	Uncultured Lachnospiraceae bacterium clone MS216A1_H02 (EF705700)	98.50	*Clostridium herbivorans* ATCC 49925^T (L34418)	97.54	*Clostridia*

Table 4. Identification of the archaeal 16S rRNA gene sequences of the DGGE bands obtained from the fecal samples of ten individuals. The position of each band in the DGGE gel is shown in Fig. 1

Clone	Nearest sequences	Identity	Phylogenetic affiliation
FA1	*Methanosphaera stadtmanae* DSM 3091 (AY196684)	98.2	*Methanobacteria*
FA2	*Methanosphaera stadtmanae* DSM 3091 (AY196684)	98.2	*Methanobacteria*
FA3	Uncultured archaeon clone EcoP2-14F (AY911637)	92.1	*Methanobacteria*
FA4	*Methanobrevibacter smithii* ATCC 35061 (CP000678)	97.3	*Methanobacteria*
FA5	*Methanobrevibacter smithii* ATCC 35061 (CP000678)	97.3	*Methanobacteria*
FA6	*Methanobrevibacter smithii* ATCC 35061 (CP000678)	98.2	*Methanobacteria*
FA7	*Methanobrevibacter smithii* ATCC 35061 (CP000678)	98.2	*Methanobacteria*
FA8	*Methanobrevibacter smithii* ATCC 35061 (CP000678)	98.2	*Methanobacteria*
FA9	*Halorubrum koreense* strain B6 (EF077636)	99.1	*Halobacteria*
FA11	*Methanobrevibacter smithii* ATCC 35061 (CP000678)	98.2	*Methanobacteria*
FA12	*Methanobrevibacter smithii* ATCC 35061 (CP000678)	94.5	*Methanobacteria*
FA14	Methanogen MEm2 (AB026171)	92.0	*Methanobacteria*
FA16	Uncultured archaeon clone k5f-43aF (AY907218)	93.1	*Methanobacteria*
FA17	*Halorubrum alimentarium* strain B43 (EF077641)	93.0	*Halobacteria*
FA18	*Halococcus morrhuae* NRC 16008 (D11106)	99.1	*Halobacteria*
FA20	*Methanosphaera stadtmanae* DSM 3091 (AY196684)	96.3	*Methanobacteria*
FA24	*Methanobrevibacter smithii* ATCC 35061 (CP000678)	98.2	*Methanobacteria*
FA27	*Methanobrevibacter smithii* ATCC 35061 (CP000678)	98.2	*Methanobacteria*
FA29	*Halorubrum saccharovorum* isolate ARC2GR (EU491515)	93.8	*Halobacteria*
FA30	*Methanosphaera stadtmanae* DSM 3091 (AY196684)	93.8	*Methanobacteria*
FA31	*Methanosphaera stadtmanae* DSM 3091 (AY196684)	98.2	*Methanobacteria*
FA32	*Methanobrevibacter smithii* ATCC 35061 (CP000678)	93.6	*Methanobacteria*
FA33	*Methanobrevibacter smithii* ATCC 35061 (CP000678)	98.2	*Methanobacteria*
FA34	*Halorubrum koreense* strain B6 (EF077636)	99.1	*Halobacteria*

for the first round of amplification and the archGC340f-519r set (Ovreas *et al.*, 1997) for the second round of amplification as described previously (Ferris *et al*., 1996). To analyze the eukaryotic diversity in the fecal samples, the 18S rRNA genes were subjected to PCR amplification as described previously (Diez *et al.*, 2001), namely, with an initial amplification using the eukaryotic universal primers EukA-EukB set (Medlin *et al*., 1988) that was followed by nested PCR using the primers GCEuk1209f-Uni1392r set (Lane *et al.*, 1985; Giovannoni *et al*., 1988). All PCR amplifications were performed in a final volume of 50 μl that contained 1 μl (50 ng/μl) template, 10 mM Tris HCl (pH 9.0), 40 mM KCl, 250 μM deoxynucleoside triphosphates (dNTPs), 2.5 U *Taq* polymerase, 1.5 mM $MgCl_2$ and 20 pmole of each primer. The reactions were performed in a PTC-220 DNA Engine Dyad MJ Research thermalcycler (PharmaTech, Korea).

DGGE and sequence analysis

To separate the PCR products, DGGE was performed by using the D-Code universal mutation system (Bio-Rad, USA). PCR products were applied directly onto 8% (wt/vol) polyacrylamide gels in a running buffer containing 1× TAE [20 mM Tris, 10 mM acetate, 0.5 mM EDTA (pH 8.3)] and a denaturing gradient of 30 to 60% (for bacteria and yeast) or 35 to 55% (for archaea) urea and formamide (where 100% denaturant contains 7 M urea and 40% formamide). Electrophoresis was performed at 80 V for 14 h at a constant temperature of 60°C. After electrophoresis, the gels were stained by using the SYBR Green I nucleic acid stain (Bioneer, Korea) and photographed under UV transillumination. Sterile blades were used to excise bands from the gels, which were then mixed with 20 μl of 0.1× TE buffer solution and incubated overnight at 4°C. One microliter of each solution was then PCR-reamplified with the appropriate primer set and directly sequenced. The sequences were taxonomically assigned by using BLAST on the GenBank nucleotide database [National Center for Biotechnology Information (NCBI)] (Altschul *et al*., 1990) and the database of type strains of valid prokaryotic names at EzTaxon server 2.0. (Chun *et al*., 2007)

Analysis of DGGE profiles

The DGGE profiles were subjected to cluster analyses by using BioNumerics software (BioSystematica, UK). For DGGE profile analysis, each lane was examined separately and the common bands were selected as positions for normalization. The lanes were normalized to compensate for

Table 5. Identification of the eukaryal 18S rRNA gene sequences of the DGGE bands obtained from the fecal samples of ten individuals. The position of each band in the DGGE gel is shown in Fig. 1

Clone	Nearest sequences	Identity	Nearest isolates	Identity	Phylogenetic affiliation
FE1	Uncultured organism clone MC0409c2 (AY897854)	98.9	*Pelargonium alchemilloides* (DQ317026)	98.4	*Viridiplantae*
FE2	Uncultured organism clone MC0409c2 (AY897854)	98.9	*Pelargonium alchemilloides* (DQ317026)	98.4	*Viridiplantae*
FE4	*Physocarpus opulifolius* (DQ886373)	99.5	*Physocarpus opulifolius* (DQ886373)	99.5	*Viridiplantae*
FE5	Uncultured organism clone MC0409c2 (AY897854)	98.9	*Parmentiera cereifera* (L49291)	98.4	*Viridiplantae*
FE6	*Candida vinaria* (AB018135)	97.3	*Candida vinaria* (AB018135)	97.3	*Fungi*
FE7	*Candida vinaria* (AB018135)	98.4	*Candida vinaria* (AB018135)	98.4	*Fungi*
FE8	*Candida vinaria* (AB018135)	97.8	*Candida vinaria* (AB018135)	97.8	*Fungi*
FE9	*Blastocystis hominis* HJ96A-26 (AB091235)	98.3	*Blastocystis hominis* HJ96A-26 (AB091235)	98.3	*Stramenopiles*
FE10	*Blastocystis hominis* HJ96A-26 (AB091235)	98.8	*Blastocystis hominis* HJ96A-26 (AB091235)	98.8	*Stramenopiles*
FE11	*Blastocystis hominis* HJ96A-26 (AB091235)	98.9	*Blastocystis hominis* HJ96A-26 (AB091235)	98.9	*Stramenopiles*
FE12	*Blastocystis hominis* HJ96A-26 (AB091235)	98.8	*Blastocystis hominis* HJ96A-26 (AB091235)	98.8	*Stramenopiles*
FE13	*Blastocystis hominis* HJ96A-26 (AB091235)	100	*Blastocystis hominis* HJ96A-26 (AB091235)	100	*Stramenopiles*
FE14	*Blastocystis hominis* HJ96A-26 (AB091235)	98.2	*Blastocystis hominis* HJ96A-26 (AB091235)	98.2	*Stramenopiles*
FE15	Uncultured organism clone MC0409c2 (AY897854)	98.9	*Rubus idaeus* (DQ886380)	98.4	*Viridiplantae*
FE16	Uncultured organism clone MC0409c2 (AY897854)	99.5	*Rubus idaeus* (DQ886380)	98.9	*Viridiplantae*
FE17	*Pelargonium alchemilloides* (DQ317026)	98.9	*Pelargonium alchemilloides* (DQ317026)	98.9	*Viridiplantae*
FE18	*Rubus idaeus* (DQ886380)	98.9	*Rubus idaeus* (DQ886380)	98.9	*Viridiplantae*
FE19	Uncultured organism clone MC0409c2 (AY897854)	99.4	*Ziziphus obtusifolia* (AY929374)	98.8	*Viridiplantae*
FE23	*Sus scrofa* (AK236845)	99.5	*Sus scrofa* (AK236845)	99.5	*Metazoa*
FE24	*Sus scrofa* (AK236845)	99.3	*Sus scrofa* (AK236845)	99.3	*Metazoa*
FE25	*Candida edaphicus* (AB247500)	99.4	*Candida edaphicus* (AB247500)	99.4	*Fungi*
FE26	*Candida edaphicus* (AB247500)	98.9	*Candida edaphicus* (AB247500)	98.9	*Fungi*
FE27	*Candida edaphicus* (AB247500)	98.8	*Candida edaphicus* (AB247500)	98.8	*Fungi*
FE28	*Candida edaphicus* (AB247500)	99.4	*Candida edaphicus* (AB247500)	99.4	*Fungi*
FE30	*Hypseocharis pimpinellifolia* (DQ317014)	97.7	*Hypseocharis pimpinellifolia* (DQ317014)	97.7	*Viridiplantae*
FE31	*Hypseocharis pimpinellifolia* (DQ317014)	98.9	*Hypseocharis pimpinellifolia* (DQ317014)	98.9	*Viridiplantae*
FE32	*Saccharomyces cerevisiae* strain CICC1862 (AY790536)	99.4	*Saccharomyces cerevisiae* strain CICC1862 (AY790536)	99.4	*Fungi*
FE33	*Desmaria mutabilis* (EF464465)	97.3	*Desmaria mutabilis* (EF464465)	97.3	*Viridiplantae*
FE34	*Rubus idaeus* (DQ886380)	98.8	*Rubus idaeus* (DQ886380)	98.8	*Viridiplantae*
FE35	Uncultured organism clone MC0409c2 (AY897854)	98.9	*Pela*rgonium alchemilloides (DQ317026)	97.2	*Viridiplantae*
FE36	*Saccharomyces servazzii* strain ATCC 58439 (AY251643)	100	*Saccharomyces servazzii* strain ATCC 58439 (AY251643)	100	*Fungi*
FE37	*Saccharomyces servazzii* strain ATCC 58439 (AY251643)	98.9	*Saccharomyces servazzii* strain ATCC 58439 (AY251643)	98.9	*Fungi*
FE38	*Saccharomyces servazzii* strain ATCC 58439 (AY251643)	100	*Saccharomyces servazzii* strain ATCC 58439 (AY251643)	100	*Fungi*
FE40	*Sus scrofa* (AK236845)	98.7	*Sus scrofa* (AK236845)	98.7	*Metazoa*
FE41	*Hypseocharis pimpinellifolia* (DQ317014)	97.8	*Hypseocharis pimpinellifolia* (DQ317014)	97.8	*Viridiplantae*
FE42	*Hypseocharis pimpinellifolia* (DQ317014)	98.9	*Hypseocharis pimpinellifolia* (DQ317014)	98.9	*Viridiplantae*
FE43	*Hypseocharis pimpinellifolia* (DQ317014)	99.4	*Hypseocharis pimpinellifolia* (DQ317014)	99.4	*Viridiplantae*

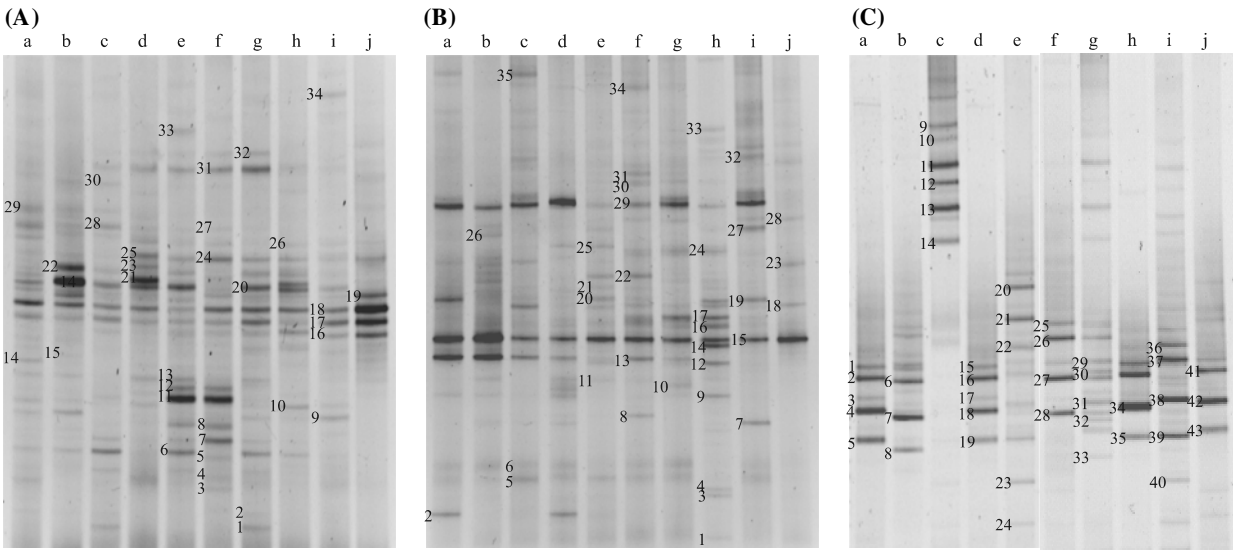

Fig. 1. DGGE analysis of PCR-amplified 16S and 18S rRNA fragments from the archaeal (A), bacterial (B), and eukaryotic (C) communities in human fecal samples from ten different individuals.

differences in migration distance due to gel heterogeneity. Dendrograms were calculated by using the Dice product moment correlation coefficient with the unweighted pair group method and arithmetic averages clustering algorithm (UPGMA). Principal component analysis (PCA) was also used to investigate compare DGGE profiles (presence/absence of bands) of microbial communities among the 10 human fecal samples by using BioNumerics software (Bio-Systematica, UK).

Results

Phylogenetic analysis of DGGE band clones

To gain insights into the diversity of the archaeal, bacterial, and eukaryal communities in human intestines, the 16S (for bacteria and archaea) and 18S (for eukaryote) rRNA genes represented by the dominant DGGE bands were re-amplified and subjected to nucleotide sequencing. Bands that were located at the same position in the gel and showed identical nucleotide sequences after repeated excision and sequencing were named, counted and indicated as one representative. Since some full-length bacterial sequences could not be determined due to chimerism or co-migration of DGGE fragments (Qiu *et al*., 2001), incomplete sequences were not used for analysis (Tables 3, 4, and 5). In conclusion, a total of 93 sequences were obtained. Of these, 32, 24, and 37 were from bacterial, archaeal, and eukaryal DGGE bands, respectively (Fig. 1). However, with regard to the eukaryal diversity, *Viridiplantae*- and metazoan (animal)-related sequences, which accounted for 20 of the 37 eukaryal sequences, were excluded from diversity analysis because these sequences were thought to come from foodstuffs (Table 5).

Comparison of the 16S rRNA gene sequences from the DGGE bands to those in the GenBank database revealed that all sequenced bacterial clone bands clustered into the *Firmicutes* and *Bacteroidetes* phyla (Table 3). Twelve of the 32 bacterial sequences (37.5%) fell within the *Clostridium coccoides* group (Gram-positive cluster XIVa), six (18.75%) were closely related to the *C. leptum* group (cluster IV), eight (25%) fell into the other *Clostridium* groups (group II, III, XI, IX, and XVI), five (15.62%) were related to *Bacteroidetes*, and one was related to *Bacillus* (*Firmicutes* phylum). Fourteen of the 32 sequences closely resembled previously isolated type strains (over 97% similarity), namely, *Subdoligranulum variabile* DSM 15176^T (97.5%), *Faecalibacterium prausnitzii* ATCC 27768^T (two sequences with 97.6~99.2% similarity), *Clostridium populeti* ATCC 35295^T (99.2%), *Ruminococcus torques* ATCC 27756^T (99.2%), *Ruminococcus lactaris* ATCC 29176^T (two sequences with 97.4~99.2% similarity), *Clostridium herbivorans* ATCC 49925^T (three sequences with 97.5~99.2% similarity), *Eubacterium eligens* ATCC 27750^T (99.2%), *Parasporobacterium paucivorans* DSM 15970^T (99.2%), *Clostridium innocuum* ATCC 14501^T (99.3%), and *Eubacterium tenue* ATCC 25553^T (98.3%).

The sequences of the 24 archaeal DGGE bands were also compared to sequences in the GenBank database (Table 4). This analysis revealed that 19 (79.16%) of the 24 sequences were related to methane-producing archaea that belonged to the class *Methanobacteria* and the remaining five (20.8%) were related to halophilic archaea that belonged to the class *Halobacteria*. Of the 24 sequences, 15 were closely related to isolated organisms (over 97% similarity), namely, *Methanosphaera stadtmanae* DSM 3091^T (three sequences with 98.2% similarity), *Methanobrevibacter smithii* ATCC 35061^T (nine sequences with 97.3 to 98.2% similarity), *Halorubrum koreense* strain B6 (two sequences with 99.1% similarity), and *Halococcus morrhuae* NRC 16008 (one sequence with 99.1% similarity).

In the case of eukaryal diversity, apart from the 20 *Viridiplantae*- and metazoan (animals)-related sequences, all 17 sequences retrieved from the DGGE bands belonged to

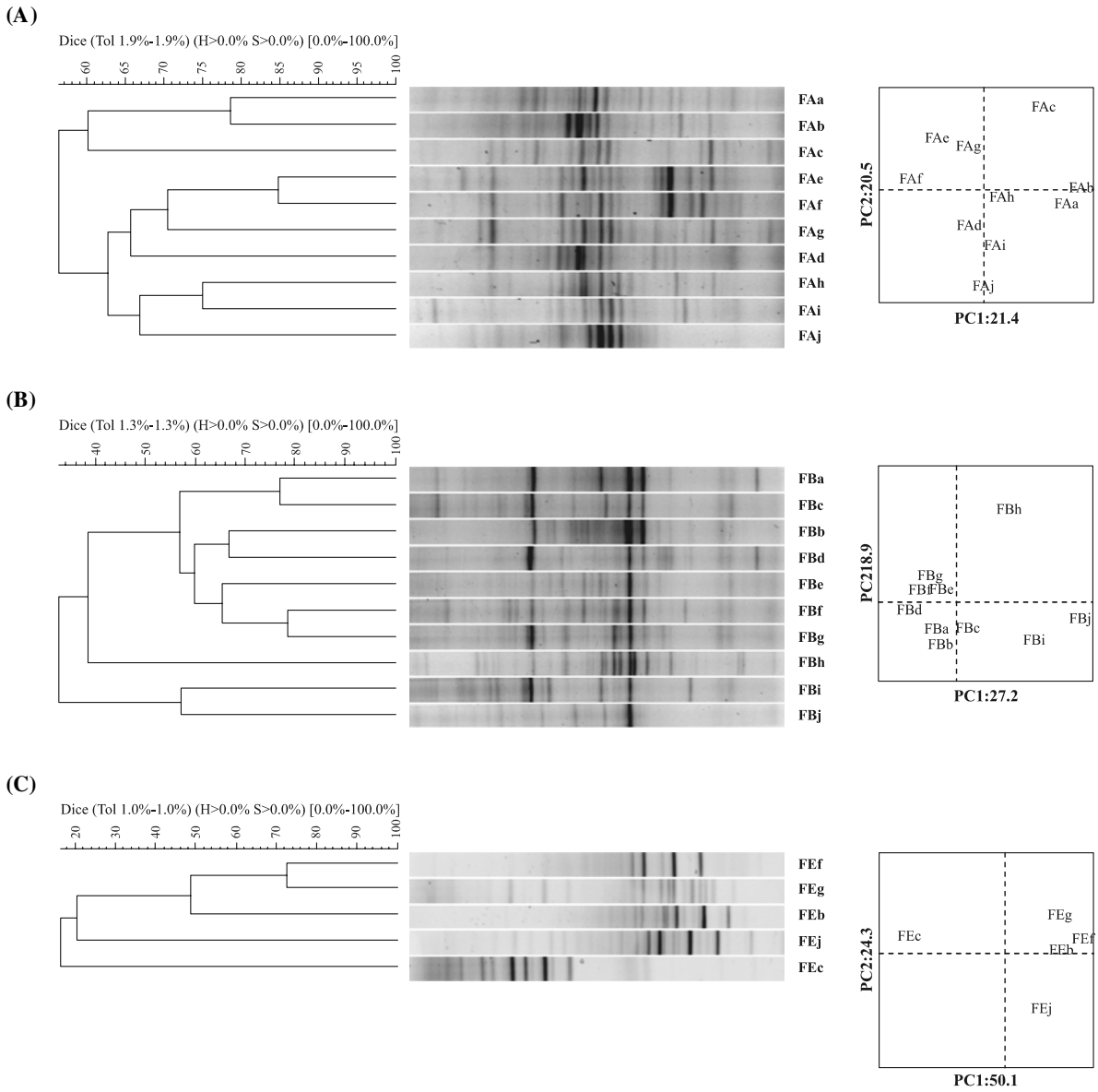

Fig. 2. Dendrogams constructed by using Dice correlations and the UPGMA clustering method and 2D scatter grams of each DGGE banding patterns of archaeal (A), bacterial (B), and eukaryal (C) communities in human fecal samples from ten different individuals. These dendrograms were produced by BioNumerics software (BioSystematica, UK).

fungi (11 sequences) and stramenopiles (six sequences). All of these small eukaryal sequences showed more than 97% sequence similarity to previously isolated organisms, namely, *Candida vinaria* JCM 1813 (three sequences with 97.3 to 98.4% similarity), *Candida edaphicus* CE1-01 (four sequences with 98.8 to 99.4% similarity), *Saccharomyces cerevisiae* CICC1862^T (one sequence with 99.4% similarity), *Saccharomyces servazzii* ATCC^T (three sequences with 98.9 to 100% similarity), and *Blastocystis hominis* HJ96A-26 (six sequences with 98.2 to 100% similarity).

DGGE pattern analysis of the three domains

The fecal microbial communities of ten Korean people were profiled by performing DGGE of amplified 16S and 18S rRNA gene fragments (Fig. 1). With regard to the bacterial DGGE band patterns, only one prominent band (FB 15) was found in all ten samples. There were also six bands (FB 5, 6, 13, 15, 18, and 29) that were widely distributed (found in more than seven samples); these bands were located in various denaturing positions (Fig. 1). Six specific bands (FB 1, 3, 4, 10, 14, and 26) were observed in the same three samples (b, g, and h) but most bands (20 bands) were found in only two to six individuals. With regard to the archaeal DGGE pattern, two prominent bands (FA17 and 18) were observed in all samples and nine thick bands (FA 6, 9, 10, 17, 18, 19, 20, 26, and 31) were found in more than seven samples. Seven bands (FA 3, 5, 15, 23, 25, 33, and 34) were found in only one individual and 16 bands

were found in only two to six individuals. In the case of the eukaryal DGGE analysis, only five samples had small eukaryotes. None of the bands were observed in all five samples.

To determine the shapes of the microbial communities in the human intestinal tract more clearly, we subjected the DGGE profiles to UPGMA cluster analysis (Fig. 2). The different lanes were compared numerically in terms of their entire spectra of bands by calculating Dice product moment correlation coefficients (r). When r equalled 100, this indicated that the dominant species in the samples being compared was identically distributed

The Dice coefficients (i.e. similarity values) of the comparisons of the bacterial DGGE bands patterns ranged from 33.2 to 78.6% with an average value of 59.2%. Similarity values for the comparisons of the archaeal patterns ranged from 56.3 to 84.9% with an average value of 68.9%. Unlike the bacterial and archaeal DGGE patterns, only the five samples with small eukaryotes were subjected to clustering analysis. During this analysis process, bands identified as the sequences related to small eukaryotes were manually selected and subjected to UPGMA clustering analysis. None of the eukaryotic bands were found in all five samples. Moreover, with Dice coefficients ranging from 16.5 to 72.7% with an average value of 39.6%, the eukaryal communities of the individuals were much less similar than the archaeal and bacterial communities.

With regard to the bacterial communities of the ten individuals, the individuals could be divided into two main groups, namely, the 'a-g' group that had bacterial community similarities exceeding 56.9%, and the 'h-j' group that had similarities exceeding 33.2% (Fig. 2). The 'a-g' group was subsequently subgrouped into the 'a and c' subgroup, where the similarities were 76.9%, and the 'b and d-g' subgroup, where the similarities exceeded 59.7%. The ten individuals could also be divided into two main groups according to their archaeal communities (Fig. 2), namely, the 'a-c' group, where the similarities exceeded 60.1%, and the 'd-j' group, where the similarities exceeded 62.1%. The latter group was then sub-grouped into the 'h-j' and 'd-g' subgroups, where the similarities were 66.8% and 65.3%, respectively. The five small eukaryote-positive individuals were also grouped according to their eukaryotic communities into the 'c' with similarities of 16.5% and the 'f and g' group with similarities of 72.7%. Apart from individual f and g, none of the samples had similarities exceeding 50% in terms of the eukaryal community.

By comparing the UPGMA trees of the three domains, we observed several relationships between the communities of the different domains. First, individuals 'a and c', and 'd, f, and g' and 'i and j' could be grouped together in terms of their archaeal and bacterial communities, although the similarities between these communities were relatively low (>57.2%). Eukaryotic community analysis revealed that individuals 'f and g' could also be grouped together into a high similarity (72.7%) group. Interestingly, the e, f and g individuals did not have gastrointestinal tract disease, whereas the other individuals had weak or severe gut illness at the time of sampling.

Scattering analysis (PCA) also showed that sample e and f were well related together than other samples in bacterial, archaeal, and eukaryal DGGE profiles.

Discussion

Several studies have shown that the DGGE and TGGE techniques are effective tools for investigating the diversity of the intestinal ecosystem (Zoetendal *et al.*, 1998; Vaughan *et al.*, 2000). These studies also revealed that the gastrointestinal microbiota of each human individual are unique. However, these studies only focused on the bacterial community and did not examine the archaeal, and eukaryotic communities. To redress this imbalance, in the present study, we analyzed the bacterial, archaeal, and eukaryal communities in fecal samples from ten unrelated Korean people by a culture-independent approach that examines 16S and 18S rRNA gene sequence variability.

The human gastrointestinal tract is a very complex environment that is populated by diverse species of bacteria, archaea and small eukaryotes (Konig, 2006). The density of the bacteria in this environment is higher than in any other ecosystem on earth (Whitman *et al.*, 1998). However, the diversity of the human intestinal tract at the bacterial phylum level is very low (Hugenholtz *et al.*, 1998) as only eight of 55 known bacterial phyla have been identified in the human intestinal tract to date (Backhed *et al.*, 2005), with most of the bacterial species falling into one of three phyla (*Bacteroides*, *Firmicutes*, and *Proteobacteria*) (Eckburg *et al.*, 2005). In our study, however, we did not detect any *Proteobacteria*-elated sequences.

Butyrate-producing bacteria are widely distributed across several *Clostridium* clusters (clusters I, IV, XIVa, XV, and XVI). In particular, most belong to the *Clostridium coccoides-Eubacterium rectale* cluster (Clostridium cluster XIVa) and *Faecalibacterium prausnitzii* cluster (Clostridium cluster IV) (Suau *et al*., 2001; Hold *et al*., 2003). Significant differences in bacterial communities with regard to these clusters could therefore influence butyrate production. In our study, we found while *F. prausnitzii* was found in all samples, 12 bacterial sequences related to *Clostridium* cluster XIVab showed a sample-specific distribution. That the intestinal tract has a high frequency (56.3%) of these butyrate-producing bacteria is understandable because these organisms are known to participate in the maintenance and protection of normal colonic epithelium (Pryde *et al.*, 2002). In two fecal samples (samples 'd and e'), we also detected sequences related to *Parasporobacterium paucivorans* DSM 15970^T, which is a bacterium that degrades methoxylated aromatic compounds in sulfide-rich conditions. This bacterium was known to be an important player in the global sulfur cycle (Lomans *et al*., 2001) but has not been observed in the human intestinal tract previously. The sulfur supply of the human body is based mainly on the uptake of meat, which suggests that the existence of *Parasporobacterium paucivorans* in the intestine could be related to diet. However, individuals d and e have different diet styles (polyphagous and vegetarian, respectively).

At present, the domain archaea is divided into four phyla: *Euryarchaeota*, *Crenarchaeota*, *Koarchaeota*, and *Nanoarchaeota* (Forterre *et al.,* 2002; Huber *et al.,* 2002). Within the human

body, however, only a few *Euryarchaeota* that belong to the methane-producing archaea have been described. Methanogens have been isolated from the human oral cavity, gut, and vagina (Miller *et al.*, 1982; Belay *et al.*, 1990; Kulik *et al*., 2001). These archaea use H_2 to reduce CO_2 to CH_4. In particular, *Methanobrevibacter smithii* is responsible for almost all of the CH_4 produced in the human intestine (Miller and Wolin, 1982). Moreover, when Eckberg *et al*. (2005) performed large scale sequencing analysis of microbial 16S rRNA genes from human intestines and feces, they found that all 1524 archaeal sequences belonged to the *Methanobrevibacter smithii* phylotype (Eckburg *et al.*, 2005). However, in 2005, Rieu-Lesme *et al.* (2005) recovered sequences belonging to *Crenarchaea* phylotypes and quantitatively analyzed the total gene copy numbers of these phylotypes in human fecal samples (Rieu-Lesme *et al.*, 2005). Apart from these two type of archaea (methanogens and crenarchaea), other phylotypes of archaea have not yet been observed in human samples. In our study of human fecal samples, however, we recovered sequences that were related not only to methanogens but also to halophilic archaea. The *Halobacterium*-related sequences showed greatest identity with the sequences of *Halorubrum alimentarium* strain B43 (93% similarity), *Halorubrum koreense* strain B6 (99.1% similarity), *Halorubrum saccharovorum* isolate ARC2GR (93.8% similarity), and *Halococcus morrhuae* NRC 16008^T (99.1% similarity). In particular, sequences related to *H. koreense* strain B6 were found in all samples. This may be related to the Korean cuisine since *H. alimentarium* strain B43 and *H. koreense* strain B6 have been isolated from salt-fermented seafood made from tiny shrimps that are very popular in Korea. This salt-fermented food is also added to kimchi, which is a traditional food in Korea.

The fungal diversity in the human intestine has mainly been assessed previously by using culturing approaches. While seventeen *Candida*, *Aspergillus*, and *Penicilium* species have been isolated from human intestinal samples to date, none of these apart from *Candida albicans* and *C. rugosa* appear to be widely distributed or abundant in the human intestinal tract (Rajilic-Stojanovic *et al*., 2007). Protozoans are also found in the human intestinal tracts. Many of these organisms are believed to have a pathogenic role in the human body. In particular, *Blastocystis hominis* is a unicellular anaerobic organism that inhabits the human intestinal tract and is thought to be a pathogen that causes intestinal disease (Stenzel and Boreham, 1996). In this study, only four types of fungi and one stramenopiles were detected and none of these small eukaryal band sequences were found in all samples. *Blastocystis hominis*-related sequences were observed in only one sample, in which other eukaryotic sequences were not detected. Thus, small eukaryal diversity in the human intestinal tract seems to be much smaller than the bacterial and archaeal diversity.

In this study, we characterized the diversity of the bacterial, archaeal, and small-eukaryal communities in the fecal samples of ten Korean people by PCR-DGGE analysis and asked whether these compositions had similar patterns. In terms of the bacterial and archaeal DGGE patterns, only one and two bands were found in all samples, respectively, while the remaining bands were present in some but not all individuals (Fig. 1). These patterns suggest that each individual has a specific archaeal and bacterial community in their intestinal tract, although a few dominant microbial species may be present in all individuals. Moreover, the eukaryotic DGGE patterns revealed that only five samples had small eukaryotes, that the diversity of this eukaryal community was small, and that bands common to all five samples could not be detected. Thus, the eukaryotic community is more host-specific than the archaeal and bacterial communities.

These tendencies were confirmed by UPGMA cluster analysis and tree comparison. The average Dice coefficient values of the archaeal, bacterial, and eukaryal communities were 68.9, 59.2, and 39.57%, respectively. When clustering is performed at a relative similarity of 50% or higher, it can be regarded as being similar (Minamida *et al*., 2004). Thus, the Dice coefficient values of the three domains indicate that the archaeal communities of the various individuals are relatively more similar than their bacterial or eukaryal communities, although individual-specific archeal species are also present. The relatively low average Dice coefficient value for the eukaryotes (39.6%) suggests that the eukaryote communities of human indivduals are less similar than their archeal or bacterial communities. Indeed, the eukaryal communities of the various individuals had little in common.

By comparing the UPGMA clustering trees of the three domains and principal component analysis (PCA), several relationships between the three domains communities were detected. Tree comparisons of the archaeal and bacterial communities showed that the ‘a and c’ samples, the ‘d, f, and g’ samples, and the ‘i and j’ samples could grouped together because of similarities that exceeded 57.15%. Eukaryotic community analysis also showed that individuals f and g could be grouped together because of high similarity (72.73%). Thus, the clustering analysis of the three domains revealed that the communities of individuals f and g were always grouped together with similarities exceeding 70.50%. principal component analysis (PCA) of DGGE banding patterns in three domains also showed high relationship of sample f and g. When we examined the characteristics of the individuals (Table 1), we found that the f and g individuals both lacked intestinal disease at the time of sampling, unlike the other individuals, all of whom had gastritis or weak gastritis symptoms at the time of sampling (Table 1). Moreover, in the case of the eukaryal community, small eukaryotes were only detected in individuals that had a polyphagous diet.

In summary, in this study, we characterized the overall archaeal, bacterial, and small-eukaryal diversity in the human intestine by using a molecular method. We found some unexpected sequences belonging to class *Haloarchaea* and *Parasporobacterium paucivorans* that have not been detected previously in the human intestinal tract. Clustering analysis of the DGGE band patterns of the three domains revealed that the archaeal communities among individuals are much similar than the bacterial or eukaryal community. Moreover, eukaryal diversity is much smaller and community composition is more host dependent than other two domains. This study has thus extended our understanding of the microbial diversity and overall shape of these three domains of life in

the human gut.

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