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Distribution of Dominant Bifidobacteria in the Intestinal Microflora of Korean Adults and Seniors, Identified by SDS-PAGE of Whole Cell Proteins and 16S rDNA Sequence Analysis

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Abstract In order to investigate the distribution of dominant *Bifidobacterium* species in intestinal microflora of Korean adults and seniors, SDS-PAGE profiles of whole cell proteins were used for the identification of bifidobacteria. To confirm the reliability of SDS-PAGE, the *Bifidobacterium* species identified by SDS-PAGE of whole cell proteins were validated by using 16S rDNA sequencing analysis. The results of SDS-PAGE corresponded well with those determined by the analysis of 16S rDNA sequencing. Based on the analysis of SDS-PAGE patterns on unidentified fecal strains which showed positive in fructose-6-phosphate phosphoketolase activity, *B. adolescentis*, *B. longum*, and *B. bifidum* were identified in the feces of adults, and *B. adolescentis*, *B. longum*, *B. bifidum*, *B. breve*, and *B. dentium* were identified in those of seniors. In most of the fecal samples tested, the predominant *Bifidobacterium* species consisted of only a few species, and differences in the distribution and numbers of *Bifidobacterium* species were observed between adults and seniors. *B. adolescentis* and *B. longum* were found to be the most common species in feces of adults, but not in seniors. Accordingly, the distribution and abundance of bifidobacteria in the human intestinal microflora varied depending on the age of hosts.

Key words: Bifidobacteria, SDS-PAGE, 16S rDNA sequence

The genus *Bifidobacterium* has been reported as one of the predominant bacteria in the human intestinal microflora and is considered to be important in maintaining human health such as balancing of intestinal microflora, suppressing colonization of intestinal pathogens, and prevention of diarrhea [5, 8, 19, 22]. To increase the number of bifidobacteria in the human intestine, many attempts on the administration of certain bifidobacteria naturally found in the human intestinal tract have been carried out. Recently, these bacteria have largely been used in food and pharmaceutical industries as food additives or probiotics [3, 9, 21]. For the isolation and identification of bifidobacteria, several methods have been used. However, it is very difficult to exactly and rapidly identify bifidobacteria, because of their phenotypic and genetic similarity. Traditional identification methods take a great deal of effort and time, and the result may not lead to definitive identification. Previous report showed that some strains which belonged to *Bifidobacterium animalis* on the basis of sugar fermentation were reidentified as *Bifidobacterium longum* by DNA homology [29]. In order to overcome these limitations associated with traditional identification methods, molecular biological methods are increasingly being applied to identify the bifidobacteria isolated from humans [24, 27, 28]. Nowadays, several methods, based on amplification and comparisons of 16S rDNA sequence, have been developed [4, 14, 18]: There is no doubt that it is one of the best available methods for the identification of bifidobacteria. However, they can not differentiate easily and simultaneously all bifidobacteria isolated from humans. Some specific primers, based on 16S rDNA sequence, would not recognize specific bifidobacteria, because of the high similarity of the 16S rDNA sequence among bifidobacteria strains. Phylogenic analysis, based on 16S rDNA sequence, showed close relatedness among *B. longum*, *B. infantis*, and *B. suis* [17], and Sakata *et al.* [23] suggested that these three strains should be unified into a single species on the basis of DNA-DNA hybridization. Another way of utilizing the rDNA sequence heterogeneity is to analyze the fragment of ribosomal DNA (rDNA) obtained by PCR, using temperature gradient gel electrophoresis (TGGE) or denaturing gradient gel electrophoresis (DGGE) in a sequence specific manner [24, 31]. Although this technique is useful for detection of bifidobacteria in human fecal samples, it is not easy to handle in a routine manner.

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SDS-PAGE of whole cell proteins is useful to quickly identify large numbers of strains at the species or subspecies level without performing any preidentification experiments using database, and has attracted attention as a reliable method for identification of lactic acid bacteria [7, 12, 13]. However, to the best of our knowledge, the identifications of bifidobacteria isolated from human feces have hardly been performed, based exclusively on whole cell proteins [2, 30]. Although there have been many reports that the *Bifidobacterium* species typically found in adults are different from those in infants [16, 18], little is known on the distribution of *Bifidobacterium* species in seniors.

The objective of this study was to investigate the distribution of dominant *Bifidobacterium* species in feces of Korean adults and seniors by using SDS-PAGE of whole cell proteins.

MATERIALS AND METHODS

Bacterial Strains Used and Isolation of *Bifidobacterium* Species

The reference *Bifidobacterium* strains were obtained from the Korean Collection for Type Cultures (KCTC, Daejon, Korea) and German Collection of Microorganisms and Cell Cultures (DSM, Braunschweig, Germany) (Table 1) and cultured in an MRS (deMan, Rogosa, and Sharpe) medium (Difco, Detroit, U.S.A.) containing 0.05% L-cysteine under anaerobic condition at 37°C for 48 h. Fecal samples were collected from five adults (2 women, 3 men, 25-30 years old) and six seniors (3 women, 3 men, older than 65 years of age). The collected fecal samples were directly processed or kept in sterilized water containing 0.05% L-cysteine at 4°C and processed within 5 h. To obtain bacterial isolates, fecal samples were resuspended and diluted in sterilized water containing 0.05% L-cysteine and then spread onto the surface of an MRS agar plate. Agar plates were incubated under anaerobic condition for 2-3 days at 37°C to allow colonies to develop. Colonies obtained from appropriately diluted fecal samples were randomly selected and were then examined by fructose-6-phosphate phosphoketolase activity.

Table 1. List of reference strains used in this study.

Species	Strain	Lane No.[a]
Bifidobacterium adolescentis	KCTC 3459	1
Bifidobacterium breve	KCTC 3220	2
Bifidobacterium breve	KCTC 3419	3
Bifidobacterium infantis	KCTC 3249	4
Bifidobacterium infantis	KCTC 3460	5
Bifidobacterium bifidum	KCTC 3418	6
Bifidobacterium bifidum	KCTC 3440	7
Bifidobacterium longum	KCTC 3421	8
Bifidobacterium longum	KCTC 3466	9
Bifidobacterium animalis	KCTC 3126	10
Bifidobacterium catenulatum	KCTC 3358	11
Bifidobacterium pseudocatenulatum	KCTC 3223	12
Bifidobacterium gallicum	KCTC 3277	13
Bifidobacterium dentium	KCTC 3361	14
Bifidobacterium suis	KCTC 3375	15
Bifidobacterium pullorum	KCTC 3370	16
Bifidobacterium indicum	KCTC 3367	17
Bifidobacterium thermophilum	KCTC 3470	18
Bifidobacterium angulatum	KCTC 3236	19
Bifidobacterium lactis	DSM 10140	20

[a]Numbers correspond to the lane numbers in Fig. 1.

Fructose-6-Phosphate Phosphoketolase Activity Assay

Fructose-6-phosphate phosphoketolase (F-6-PPK) activity was assayed by a modification of the method described in *Bergey's Manual of Systematic Bacteriology* [25]. The assay was downscaled to a volume suitable for 1.5-ml Eppendorf tubes [28]. Cells harvested from 1.5 ml MRS broth containing 0.05% L-cysteine were washed twice with 0.05 M-phosphate buffer (pH 6.5) containing L-cysteine 500 mg/l and resuspended in 0.1 ml of the same buffer. The cells were lysed by glass bead at 4°C. Following vortex for 30 sec, cells were held on ice to avoid heating. This step was repeated 3 times, and 0.025 ml of NaF (6 mg/ml) solution containing Na iodoacetate (10 mg/ml) and fructose-6-phosphate (80 mg/ml in water) were then added to the lysate. After incubation at 37°C for 30 min, the reaction was stopped with 0.15 ml of hydroxylamine HCl (13.9 g/100 ml of water, freshly neutralized with NaOH to pH 6.5). After 10 min at room temperature, 0.1 ml of reagent trichloroacetic acid [15% (w/v) in water] and 4 M HCl were added. Finally, 0.1ml of $FeCl_3{\cdot}6H_2O$ [5% (w/v) in 0.1 M HCl] was added and inverted for developing of color. Any reddish violet color that immediately developed was taken as a positive result.

SDS-PAGE of Whole Cell Proteins

SDS-PAGE of whole cell proteins was carried out as described by Kim *et al.* [12]. Strains were incubated overnight at 37°C in 5 ml of MRS broth containing 0.05% L-cysteine and centrifuged at 12,000 ×*g* for 3 min at 4°C. The pellet of sample was washed twice with deionized water and suspended in 50 µl of 50 mM Tris-HCl buffer (pH 8.0). Fifty mg of glass beads (diameter, 425 to 600 microns; Sigma, St. Louis, U.S.A.) were added to the tubes, and the bacteria were vortexed for 5 min. The pellet of sample was resuspended in an equal volume of sample buffer [2× SDS sample buffer; 25 ml of 4× Tris-HCl/SDS (pH 6.8), 20 ml of glycerol, 4 g of sodium dodecyl sulfate, 2 ml of 2-mercaptoethanol, 1 mg of bromophenol blue, and H_2O added to 100 ml]. For protein denaturation, samples were heated for 5 min at 95°C. The cell debris was

removed by centrifuge, and the supernatants were collected for analysis by gradient (10–15%) SDS-PAGE. SDS-PAGE was preformed on vertical slab gels. After electrophoresis, the gel was stained for 2 h with 0.05% Coomassie brilliant blue R-250 (Bio-Rad Laboratories, Richmond, U.S.A.), and destained with 10% acetic acid and 30% methanol solution for 2 h. The destained gels were scanned for further analysis. For each strain, a data record was constructed in which each band of particular molecular weight was represented as either being present (1) or not (0). Correlation coefficients between the electrophoretic patterns were obtained and used to construct a dendrogram to relate the strains by using the program of NTSYS-pc (numerical taxonomy system by using multivariate statistical programs, version 2.02j). The dendrogram was obtained by UPGMA (unweighted pair group method by using average linkage) cluster analysis [26].

DNA Isolation for PCR Amplification of 16S rDNA

The chromosomal DNA was isolated by using a modification of the method of Ausubel *et al.* [1]. Five ml of the culture was harvested by centrifugation. The cells were then washed in a TE buffer (50 mM Tris-HCl, 50 mM EDTA, pH 8.0) and resuspended in 0.5 ml of a TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0). Lysis was initiated by the addition of 50 µl of 10 mg/ml lysozyme. After incubation, 30 µl of 10% (w/v) SDS and 3 µl of 20 mg/ml proteinase K (Sigma) were added, gently mixed, and further incubated for 1 h at 37°C. Next, the samples were mixed with 100 µl of 5 M NaCl and 80 µl of CTAB/NaCl solution, and the mixture was incubated for 10 min at 65°C. Treatment of the above mixture with an equal volume of a phenol-chloroform removed the protein. After centrifugation at 12,000 ×*g* for 5 min, the supernatant was added to 0.6 vol of isopropanol, gently mixed, and spun down at 12,000 ×*g* for 10 min. The pellet was then washed twice in ice-cold ethanol and resuspended in ultrapure water.

16S rDNA Sequence Analysis

The 16S rDNA was amplified by using a universal primer pair [10]. The sequences of the 16f primer (forward) and 16r primer (reverse) were 5'-GAGTTTGATCCTGGCTCAG-3' (16S rDNA position 9–27 of *E. coli*) and 5'-AGAAA-GGAGGTGATCCAGCC-3' (16S rDNA position 1525–1544 of *E. coli*), respectively. The PCR products, purified with a QIAquick gel extraction kit (Qiagen, Valencia, U.S.A.), were ligated into a pGEM-T easy vector (Promega, Madison, U.S.A.), and then transformed into *Escherichia coli* DH5α competent cells. The recombinant plasmids were purified by using a plasmid purification kit (Nucleogen, Seoul, Korea) and digested with *Eco*RI to confirm the insert. The nucleotide sequences of the plasmids were determined by using an ABI PRISM Dye Terminator sequencing kit and ABI PRISM 377 sequencer (Perkin-Elmer, Norwalk, U.S.A.), according to the manufacturer's instructions. The T7 (forward) and SP6 (reverse) primers were used as the sequencing primers.

RESULTS AND DISCUSSION

Isolation of the Genus *Bifidobacterium* from Feces of Adults and Seniors

Fecal samples were collected from 5 adults (3 men and 2 women, 25–30 years old) and 6 seniors (3 men and 3 women, older than 65 years old). Intestinal microflora colonies obtained from appropriately diluted fecal samples on 0.05% L-cysteine containing MRS plate were randomly selected. To confirm that the isolates were of the genus *Bifidobacterium*, F-6-PPK activity assay was performed

Table 2. Distribution of *Bifidobacterium* species isolated from feces of adults and seniors.

Subject[a]		No. of *Bifidobacterium* species						No. of positive strains (%)[b]	Total no. of isolates
		B. adolescentis	*B. longum*	*B. dentium*	*B. breve*	*B. bifidum*	Unknown		
Adults	AW	23	13				2	38 (95%)	40
	BM		33					33 (82.5%)	40
	CM	30	9					39 (98%)	40
	DW	31	3				1	35 (88%)	40
	EM	25				7		32 (80%)	40
Seniors	FW				1		25	26 (65%)	40
	GM	12	3		2		21	38 (95%)	40
	HW			2	1		15	18 (45%)	40
	IW	3						3 (8%)	40
	JM	6	9				20	35 (88%)	40
	KM	3					36	39 (98%)	40
	LW						7	7 (18%)	40

[a]W, M indicate woman and man, respectively.
[b]Number is strain number that showed positive result in fructose-6-phosphate phosphoketolase activity.

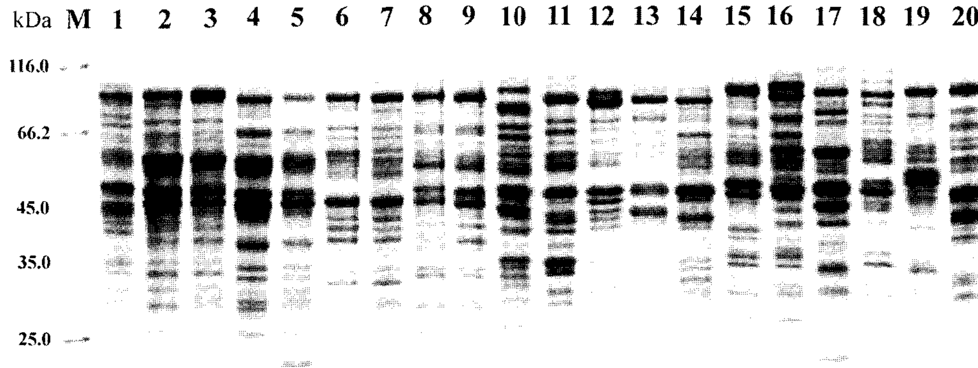

Fig. 1. SDS-PAGE profiles of whole cell proteins from reference *Bifidobacterium* species.

Lanes: M, Protein molecular weight markers (kDa); 1, *Bifidobacterium adolescentis* KCTC 3459; 2, *B. breve* KCTC 3220; 3 *B. breve* KCTC 3419; 4, *B. infantis* KCTC 3249; 5, *B. infantis* KCTC 3460; 6, *B. bifidum* KCTC 3418; 7, *B. bifidum* KCTC 3440; 8, *B. longum* KCTC 3421; 9, *B. longum* KCTC 3466; 10, *B. animalis* KCTC 3126; 11, *B. catenulatum* KCTC 3358; 12, *B. pseudocatenulatum* KCTC 3223; 13 *B. gallicum* KCTC 3277; 14, *B. dentium* KCTC 3361; 15, *B. suis* KCTC 3375; 16, *B. pullorum* KCTC 3370; 17, *B. indicum* KCTC 3367; 18, *B. thermophilum* KCTC 3470; 19, *B. angulatum* KCTC 3236; 20, *B. lactis* DSM 10140.

on the colonies selected above: The genus *Bifidobacterium* has unique glucose metabolism that is often referred to as the bifidus pathway. The characteristic key enzyme of the bifidus pathway is F-6-PPK, which is usually used to identify the genus *Bifidobacterium*, and the demonstration of F-6-PPK in cellular extracts is known as the most direct and reliable characteristic method to assign an organism to the genus *Bifidobacterium* [25]. A scaledown to a volume for 1.5-ml Eppendorf tube was enough to detect *Bifidobacterium* species. In adults, 177 strains of the 200 colonies isolated on MRS plate were shown to contain the activity of F-6-PPK (mean 88.5%). In seniors, however, the component ratio of *Bifidobacterium* species to isolates was lower than that of adults (mean 59.3%), and a large deviation was observed, depending on individuals (Table 2). Also, the total number of endogenous bifidobacteria detected in seniors was less than that of adults (data not shown). Considerable differences in the component ratio to intestinal microflora and total number of bifidobacteria were observed between adults and seniors.

Identification of *Bifidobacterium* Species by SDS-PAGE of Whole Cell Proteins

The identification of *Bifidobacterium* species isolated from different human feces was performed by analysis of whole cell proteins, using SDS-PAGE. SDS-PAGE patterns of soluble cell proteins in bacterial cell have been used for identification at the species level and also to group strains within a species according to their similarities [7, 11, 12, 13] Although SDS-PAGE of whole cell proteins requires an extensive database covering all known target species, this technique is usually accepted as a simple and convenient method for identification of microorganisms.

First, the whole cell proteins profiles of reference bifidobacteria were analyzed by SDS-PAGE for identification of *Bifidobacterium* species isolated from human feces (Fig. 1). The result of the analysis proved that it was able to clearly discriminate almost all of these species: None of the reference strains had protein profiles similar to those of the species of *B. longum*, *B. adolescentis*, *B. breve*, *B. infantis*, *B. bifidum*, *B. animalis*, *B. catenulatum*, *B. pseudocatenulatum*, *B. gallicum*, *B. dentium*, *B. suis*, *B. pullorum*, *B. indicum*, *B. thermophilum*, *B. angulatum*, and *B. lactis*. As shown in Fig. 2, a matrix of simple matching coefficients of whole-cell proteins patterns was used to construct the dendrogram. Reference strains formed a separate cluster among each other with correlation level of below 85% (r×100). The cluster could be identified at the species level: The highest correlation values were obtained

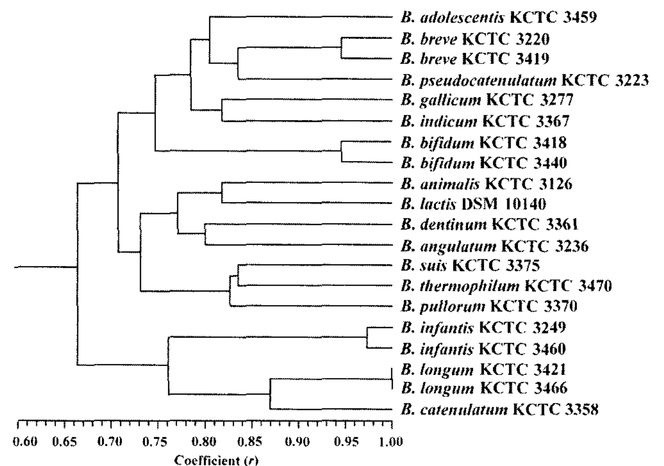

Fig. 2. Dendrogram showing correlation coefficients among *Bifidobacterium* species, as determined by analysis of gel electrophoretic patterns.

Similarity was analyzed by UPGMA clustering.

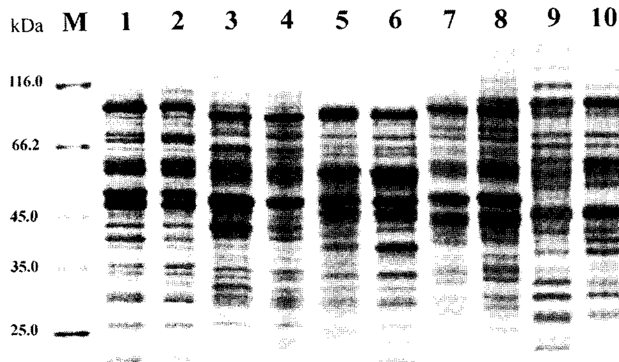

Fig. 3. Comparison of SDS-PAGE profiles of whole cell proteins between reference strains and isolates from human feces.
Lanes: M, Protein molecular weight markers (kDa); 1, *Bifidobacterium longum* KCTC 3466; 2, *B. longum* isolated from human feces; 3, *B. dentium* KCTC 3361; 4, *B. dentium* isolated from human feces; 5, *B. breve* KCTC 3419; 6, *B. breve* isolated from human feces; 7, *B. adolescentis* KCTC 3459; 8, *B. adolescentis* isolated from human feces; 9, *B. bifidum* KCTC 3418; 10 *B. bifidum* isolated from human feces.

only between the same species, but there was a clear overall visual difference in the protein patterns among different species. The 343 strains detected as the genus *Bifidobacterium* by F-6-PPK activity were analyzed by using SDS-PAGE patterns of whole cell protein, and protein profiles of bifidobacteria isolated from human feces were compared with those of the respective reference strains. Of the 343 isolates from the adults and seniors, 133 isolates were *B. adolescentis*, 70 isolates were *B. longum*, 7 isolates were *B. bifidum*, 4 isolates were *B. breve*, and 2 isolates were identified as *B. dentium* (Table 2). However, 127 isolates could not be assigned to any known bifidobacteria used as reference strains in this study, although these isolates showed positive F-6-PPK activity. Therefore, these strains remain unidentified. Although there were some minor differences in SDS-PAGE patterns, the identified isolates showed protein patterns almost similar to the patterns obtained from reference bifidobacteria strains (Fig. 3).

This distinction might have resulted from the differences in the environment of the habitat that could affect phenotypic characteristics, even though they have the same genotype.

Analysis of 16S rDNA Sequencing of *Bifidobacterium* Species Identified by SDS-PAGE of Whole Cell Proteins

To confirm the reliability of SDS-PAGE, the *Bifidobacterium* species identified by SDS-PAGE of whole cell proteins, including *B. adolescentis* GM-28, *B. longum* AW-10, *B. dentium* HW-34, *B. breve* GM-8, and *B. bifidum* EM-7, were subjected to the analysis of 16S rDNA sequencing. The 16S rDNA sequences of the above 5 *Bifidobacterium* species were obtained with universal primers 16f and 16r of the 16S rDNA gene position of *E. coli*. The amplified 16S rDNA genes were cloned into vectors, and clones of each gene were sequenced. The comparative analysis with sequences available in GenBank showed significant homology with 16S rDNA sequences of bifidobacteria present in the database (Table 3). The results of SDS-PAGE corresponded well with those determined by the analysis of 16S rDNA sequencing, thus confirming its reliability. Therefore, SDS-PAGE analysis was found to be a useful means for identifying *Bifidobacterium* species isolated from human feces. Overall, the SDS-PAGE assay described here is a simple and reliable method that could be used to differentiate *Bifidobacterium* species in human fecal samples.

Table 3. Percentages of 16S rDNA sequence homology of bifidobacteria isolated in this study to that of bifidobacteria in the GenBank database.

Isolated strains[a]	Bifidobacteria strains from the database (GenBank accession No.)	Homology (%)
GM-28	*B. adolescentis* (AF275882)	98
AW-10	*B. longum* (AE014756)	99
HW-34	*B. dentium* (D86183)	99
GM-8	*B. breve* (AB006658)	98
EM-7	*B. bifidum* (U25952)	98

[a]A, G, and H indicate subject and W, M indicate woman and man, respectively.

The Distribution of *Bifidobacterium* Species in Feces of Adults and Seniors

Table 2 summarizes the distribution of *Bifidobacterium* species in the subjects studied. *B. adolescentis*, *B. longum*, and *B. bifidum* were identified in the feces of adults, and *B. adolescentis*, *B. longum*, *B. bifidum*, *B. breve*, and *B. dentium* were identified in those of seniors.

B. adolescentis and *B. longum* were the predominant species in feces of the adults group, but not in the seniors group. In most adults and seniors, the predominant *Bifidobacterium* species consisted of only a few species, and individuals showed a host-specific population of *Bifidobacterium* species. Many studies on the distribution of *Bifidobacterium* species have been reported. According to Malinen *et al.* [14], the most common bifidobacteria of adult volunteers (mean age of 32 years) was the *B. longum* that was found in all subjects, followed by *B. adolescentis* (82.1% of subjects) and *B. bifidum* (42.9% of subjects), and typically two or three species were detected in the subjects studied in the present study. In the study of Matsuki *et al.* [15], the *B. adolescentis*, *B. catenulatum*, and *B. longum* groups were found to be the three predominant species in intestinal microflora of adults (average 37±9 years). As compared to the results obtained by Malinen *et al.* [14] and Matsuki *et al.* [15], our result was similar to those in the case of *B. adolescentis* and *B. longum*, whereas a low incidence was shown in other bifidobacteria. In this study, differences in the distribution and number of *Bifidobacterium* species were observed between adults and

seniors. It has been well established that the composition and levels of bifidobacteria tend to change or decrease upon aging, and this is generally considered to be an undesirable change for the elderly and may negatively affect their health. Some results indicated that reduced adhesive properties of *Bifidobacterium* flora to intestinal mucus might explain the decrease of bifidobacteria levels in the intestinal microflora of aging people [6, 20].

In conclusion, the distribution and abundance of bifidobacteria in the human intestinal microflora seemed to vary according to the age of the host, and it might provide important clues for the understanding of the relationship between bifidobacteria and health in Korean adults and seniors.

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