Screening of Antioxidative Activity of *Bifidobacterium* Species Isolated from Korean Infant Feces and Their Identification

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Abstract Among 59 Korean isolated, 20 were confirmed as members of the genus *Bifidobacterium* species based on gram staining, microscopic examination of cell morphology and the TLC method. The oxygen tolerance and antioxidative activities of these 20 *Bifidobacterium* strains and 5 standard *Bifidobacterium* strains were tested. All the strains demonstrated antioxidative activities as regards inhibiting linoleic acid peroxidation. The antioxidative activities of isolated and standard strains were found to range from 10.7-46.4% and from 10.7-22.2%, respectively. In addition, all tested strains exhibited a scavenging ability on DPPH free radicals, range from 15-41% for the isolated strains and 8.3-22% for the standard strain. Accordingly, the isolated *Bifidobacterium* strains demonstrated higher antioxidative activities than the 5 standard *Bifidobacterium* strains. On the base of grades for each test, HJL 7511 was identified as the best strain, followed by HJL 7501. 2 strains were identified with Polymerase Chain Reaction (PCR) assay using group-specific primers designed from the nucleotide sequences of the 16S rRNA and internal transcribed spacer (ITS) regions of the Bifidobacteria. Based on the sequencing results, HJL 7511 and HJL 7501 were identified as *Bifidobacterium infantis*.

Keywords: antioxidative activity, isolated bifidobacteria, 16S rRNA- ITS primers, PCR

INTRODUCTION

Bifidobacterium species is Gram-positive anaerobes, fermentative rods, often Y-shaped or clubbed at the ends, and asporogenous bacteria [1]. It is also recognized that *Bifidobacterium* species constitute one of the major organisms in the colonic flora of breast-feed infants and healthy adults [2]. The establishment of high numbers of bifidobacteria has been reported to provide biological barriers against the proliferation of exogenous pathogens [3, 4]. In addition, humans suffering from lactose malabsorption would can benefit from ingesting β-galactosidase-producing bifidobacteria cells and from the reduced lactose content in bifidobacteria-fermented milk [5]. Bifidobacteria are considered beneficial for all age groups because as they have been found to prevent carcinogenesis, improve the protein metabolism, reinforcing immune functions, and prevent intestinal infections in the host [6, 7]. The antioxidative effect of lactic acid bacteria has been reported only recently, while previous studies on the antioxidative effect of bifidobacteria are rare. During the biological oxidation process for the production of energy form fuels, the generated oxidative stress can damage some biological molecules. It has been well established that a wide variety of oxygen-centered free radicals and other reactive oxygen species (ROS) are continuously produced in the human body and food system [8,9]. Including aging, it is well known that the oxidative damage plays an important pathological role in cancer, emphysema, cirrhosis, atherosclerosis, and arthritis [10]. Recently, the molecular biological methods have been used for the identification of microorganisms and phylogenetic study [11,12]. The conventional method for the analysis of bifidobacteria in intestinal microflora requires labor-intensive and time-consuming techniques, including the single colony isolation of candidate from a specific selective medium, and testing for multiple physiological and biochemical traits. Moreover, these tests do not always provide clear-cut results, and are sometimes unreliable. In order to develop a high value-added bifidobacteria having the antioxidative ability, we isolated bifidobacteria species from Korean infant feces and identified those bifidobacteria by amplification and sequencing of 16S-23S rDNA ITS region.

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MATERIALS AND METHODS

Strains Sampling and Cultivation

Bifidobacterium strains were isolated from healthy Korean infant feces in Incheon, Seoul and Gyeonggi-do.

One gram of a fecal sample from each of the 59 volunteers was suspended in 0.85% NaCl solution (pH 7.0) to give a final concentration of 10% (w/v). These fecal solutions were agitated by a laboratory blender Stomacher (Seward Co., UK) and filtered to remove any large particles, and then serially diluted and plated on the TPY (trypticase-proteose peptone-yeast extract) agar, and then incubated in anaerobic jar filled with CO_2 gas for 16-24 h at 37°C. Also, 5 type of *bifidobacterium* strains from human origin, *B. longum* KCTC 3128, *B. breve* KCTC 3220, *B. bifidum* KCTC 3202, *B. infantis* KCTC 3127, and *B. adolescentis* KCTC 3216, were cultured in a TPY broth supplemented with 0.5% glucose and 0.05% L-cysteine HCl and incubated for 16-24 h at 37 °C [1].

Isolation and Identification of *Bifidobacterium* Strains from Korean Infant Feces

The colonies representing different morphological type were picked up from the TPY agar plates, and then Gram-stained, and examined under a microscope [13]. A tentative identification of the genus *Bifidobacterium* from the isolated bacterial stains was made on the basis of a TLC determination of the short chain fatty acid metabolites in the culture broth [14,15]. Development of the chromatograms was allowed to proceed in the solvent system of acetone:water:chloroform:ethanol:ammonium (60 : 2 : 6 : 10 : 22). A standard solution containing 10% lactic acid, acetic acid, propionic acid, butyric acid, succinic acid and citric acid in water was spotted on Merck silica gel $60F_{254}$ with culture broth sample. The chromatograms of the organic acids were sprayed with an indicator solution of 0.25 g methyl red-0.25 g bromophenol blue in 100 mL of 70% methanol, and the color was developed by brief heating (1 to 3 min) in 180 °C.

Preparation of Intact cells and Supernatant

The bifidobacteria cells were harvested by centrifugation at 8,000 rpm for 10 min after incubation 18 h at 37°C, and each supernatant was then collected into a new tube. For the preparation of intact cells, the harvested cells were washed three times with PBS solution (0.02 M K_2HPO_4 adjusted to pH 7.4 with 0.02 M KH_2PO_4) and resuspended in PBS. Total cell numbers were adjusted to 10^9 CFU/mL.

Antioxidative Activities of Isolated *Bifidobacterium* Strains

The measurement of antioxidative activity of isolated *bifidobacterium* strains was performed by the thiobarbituric acid (TBA) method, based on the monitoring of inhibition of linoleic acid peroxidation by intact cells [9, 16] The linoleic acid peroxidation inhibition percentage of intact cells was defined as follows: $[1-(A_{532}(\text{sample})/A_{532}(\text{control}))] \times 100\%$.

Scavenging Effect on DPPH Free Radical of Isolated *Bifidobacterium* Strains

The scavenging effect on DPPH free radical of isolated bifidobacteria was analyzed by modification of method utilized by Shimada *et al.* [17]. A 0.4 mL of supernatant and 1 mL of freshly prepared 0.2 mM DPPH solution (in ethanol) were mixed and allowed to react for 30 min. The control sample contained a TPY medium instead of sample. The absorbance of the mixture was measured at 517 nm, and DPPH radical scavenging ability (%) was defined as follows: $[1-(A_{517}(\text{sample})/A_{517}(\text{control}))] \times 100\%$.

Oxygen Tolerance of Isolated *Bifidobacterium* Strains

1 mL of the sub-cultured strains was inoculated into 4 mL of a TPY broth with and without 1,000 ppm of oxygen peroxide(H_2O_2) and incubated at 37 °C for 10 and 20 min. Next, 0.2 mL of the cultured strains was inoculated into 10 mL of TPY broth and incubated at 37 °C for 18 h. The bacterial growth was measured spectrometrically at 600 nm.

Identification of Screened *Bifidobacterium* Strains Using PCR

The Group-specific primer pair, Bifido-16S-1f (5'-TCCAGGGCTTCACGCATGC-3') and Bifido-ITS-1r (5'-TCCAGTTCTCAAACCACCAC-3'), was designed using the multi-alignment program for PCR. A QIAamp DNA stool mini kit (QIAGEN, USA) was used for genomic DNA preparation from the colonies that grew on the TPY agar plates. The PCR amplification was carried out in a 50 μL reaction mixture for the PCR of the 16S rRNA and ITS region consisted of a reaction buffer (*Taq*, $MgCl_2$, dNTP, each primer, BSA, DNA, and ddH_2O). Each reaction was carried out for 35 cycles consisting of 95°C (5 min) for preheating, 94°C (1 min) for denaturation, 50°C (1 min) for annealing, and 72°C (2 min). And final extension was carries out 8 min at 72°C. The PCR products were electrophoresed in 1% agarose gels, followed by ethidium bromide staining [11]. To extract the PCR fragments, a Gel Extraction Kit-Spin (NucleoGen, Korea) was used. The sequences of the final products were analyzed by Bionics (Bionics Inc., Korea), and homology was presented by NCBI gene bank.

RESULTS AND DISCUSSION

Identification of *Bifidobacterium* Strains by TLC Method

After the incubation and centrifugation of these isolated strains, the supernatants were analyzed by TLC method. The Fig. 1(a) shows the separation of six organic acids and lactic-acetic acid mixture on TLC plates, respectively. The chromatogram of lactic, succinic and citric acid appeared as yellow spots, while acetic, propionic and butylic acid appeared as blue spots. Also, the R_f values of the tested organic acids are differ each other (data not shown). In the case of the lactic-acetic acid mixture, an area of acetic acid appeared between two areas of lactic acid, other time an area of acetic appeared

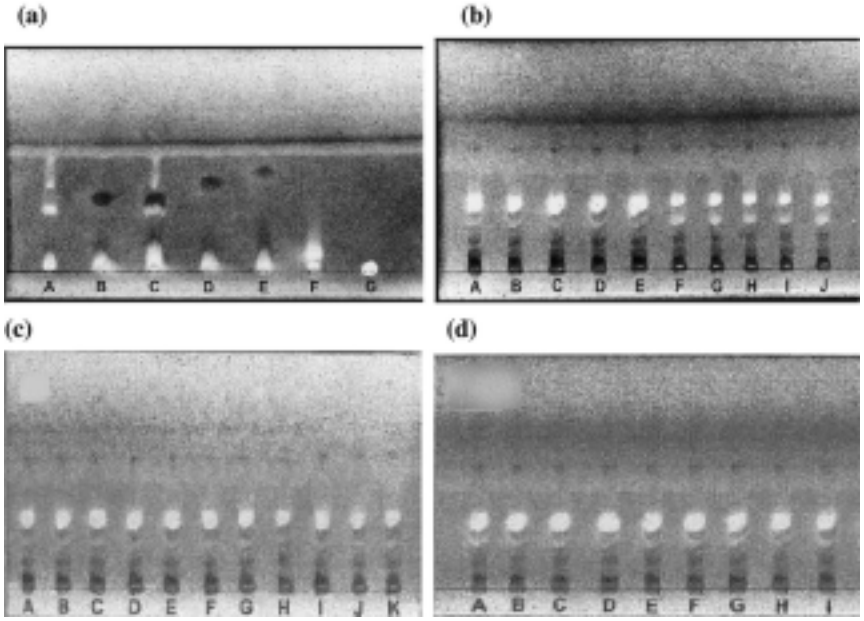

Fig. 1. Isolation of the *bifidobacterium* strains from Korean infants feces by TLC method. (a) Separation of standard organic acids. A; Lactic acid, B; Acetic acid, C; lactic-acetic acid mixture, D; Propionic acid, E; Butyric acid, F; Succinic acid, G; Citric acid. (b) The organic acids from the culture broth of several lactic acid bacteria. A; *B. longum*, B: *B. breve*, C: *B. infantis*, D: *B. bifidum*, E: *B. adolescentis*, F: *L. casei*, G: *L. bulgaricus*, H: *L. acidophilus*, I: *S. thermophilus*, J: *S. lactis.* (c) Screened *Bifidobacterium* strains. A-K;HJL 7501, 7509, 75011, 7519, 7521, 7523, 7526, 7527, 7528, 7529, 7530. (d) Screened *Bifidobacterium* strains. A-I;HJL 7532,7533,7534,7537,7539,7540, 7541,7542,7543,7544,7545.

below an area of lactic acid. Fig. 1(b) shows the analysis of organic acids from the culture broths of lactic acid bacteria, including bifidobacteria. lactic and acetic acid were both detected on all the TLC plates form the bifidobacteria culture broths. However, only lactic acid was detected in the other bacterial culture broths, indicating that 20 strains were members of the genus *Bifidobacterium* according to the TLC methods (Fig. 1(c) and (d)).

Antioxidative Activity of Isolated *Bifidobacterium* Strains

20 isolated *Bifidobacterium* strains and 5 standard strains exhibited an inhibiting effect on linoleic acid peroxidation (Fig. 2). The antioxidative activities of intact cell ranged from 10.7% to 46.4% and from 10.7% to 22.2% linoleic acid peroxidaion inhibition for 10^9 cells of 20 isolated and 5 standard *Bifidobacterium* strains, respectively, indicating that the antioxidative activities of the isolated *Bifidobacterium* strains were stronger than those of the standard strains. The most effective strain was HJL 7501(46.4%), followed by HJL 7519 (43.5%) and HLJ 7511 (43.3%).

Scavenging Effect of Isolated *Bifidobacterium* Strains on DPPH Radical

The scavenging effects of the supernatant (10^9 CFU/mL) of the 20 isolated *Bifidobacterium* strains and 5 standard strains on DPPH radical are shown in Fig. 3. The scavenged DPPH radical was monitored by measuring absorbance at 517 nm. The isolated *Bifidobacterium* strains

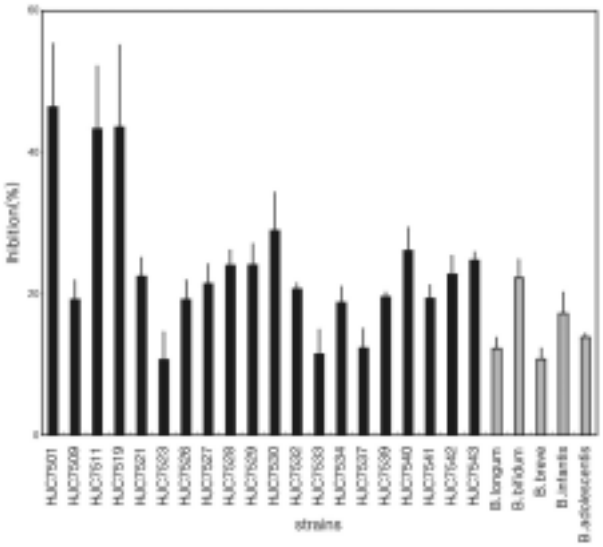

Fig. 2. Antioxidative activity of isolated *bifidobacterium* strains by TBA method on the inhibition of linoleic acid peroxidation. Total cell numbers were adjusted to 10^9 CFU/mL. A 0.4 mL intact cells were mixed and incubated at 37°C for 24 h. The control was with PBS solution instead of intact cells. Inhibition(%) of linoleic acid peroxidation was defined as follows: [1-A_{532}(sample)/A_{532}(control)]×100%. Results are expressed as the mean of three experiments.

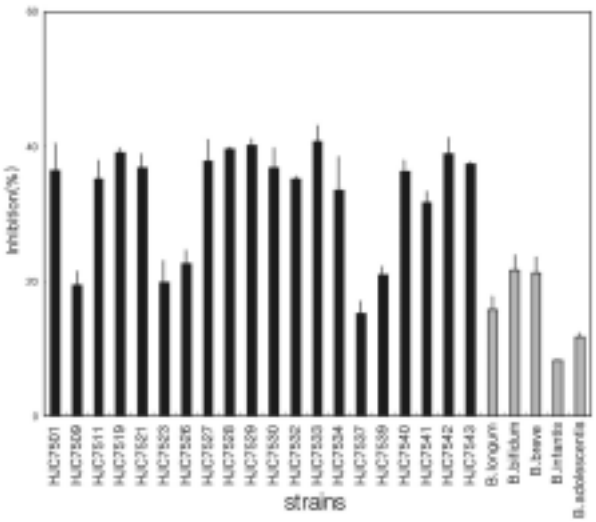

Fig. 3. Scavenging effect of isolated *Bifidobacterium* strains on DPPH free radical. Total cell numbers were adjusted to 10^9 CFU/mL. A 0.4 mL of supernatant and 1 mL of prepared DPPH solution (in ethanol) were mixed and allowed to react for 30 min. The scavenging ability was defined as follows : [1-A(sample)/A(blank)] × 100%. Results are expressed as the mean of three experiments.

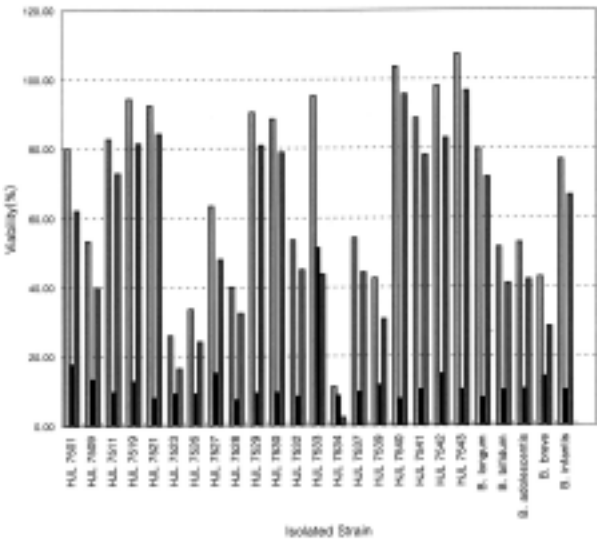

Fig. 4. Effect of oxygen tolerance of isolated *bifidobacterium* strains. Subcultured strains was inoculated into TPY broth containing 1,000ppm H_2O_2 and incubated at 37°C for 30 min. The bacterial growth was measured spectrometrically at 600 nm : The percentage of bacterial growth after exposure to H_2O_2 for 10 min, : The percentage of bacterial growth after exposure to H_2O_2 for 20 min, : Difference of percentage of bacterial growth after exposure to H_2O_2 for 10min and 20 min.

demonstrated a higher scavenging ability (15-41%) than the 5 standard *Bifidobacterium* strains (8.3-22%). The highest strain was HJL 7533, followed by HJL 7501, HJL 7511, HJL 7519, HJL 7528 and HJL 7529.

Oxygen Tolerance of *Bifidobacterium* Strains

On the basis that antioxidative ability is related the oxygen tolerance, oxygen tolerance of isolated and standard bifidobacteria was tested (Fig. 4). The resulting data did not exactly correspond with the result for antioxidative activity inhibiting linoleic acid peroxidationy. However in the case of DPPH radical scavenging, the oxygen tolerant bifidobacteria exhibited more antioxidative activity.

Superiority Among Isolated *Bifidobacterium* Strains

The stronger antioxidative bifidobacteria among the isolated strains was determined based on 6 grades: below 20%, 20-25%, 25-30%, 30-35%, 35-40% and over 40% in the DPPH test. Plus, 6 grades were assigned for the TBA test based on division between 10 and 30%, as with the DPPH test. In the case of oxygen tolerance, bacterial growth after exposure hydrogen peroxide relative to a control was divided into one hundred parts, over 90%, from 70 to 90%, from 50 to 70%, from 30 to 50%, and below 30% giving 5 grades. The strain with the best overall grade was HJL 7511 with 1.33, followed by HJL 7501 with 1.50. All the standard strains had a lower grade than the isolated strains and ranged from 4.67 to 3.17. Therefore, HJL 7511 and 7501 were selected as the superior *Bifidobacterium* strains.

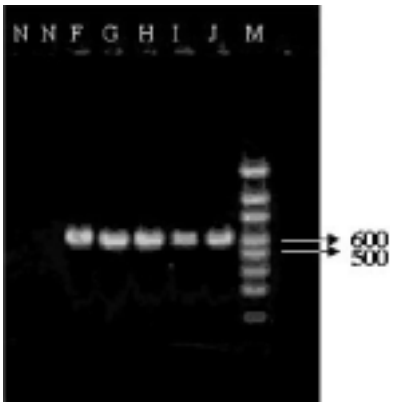

Fig. 5. Group-specific amplification of bifidobacteria 16S rRNA and flanking ITS by designed primers Bifido-16s-1f and Bifido-ITS-1r. DNA fragments were clearly amplified almost 600 bp, as expected. M: 1kb ladder maker, N: negative control, F; HJL 7501, G; HJL 7511, H; HJL 7519, I; HJL 7529, J; HJL 7530.

Identification of *Bifidobacterium* Strains Using PCR

The 16S rRNA and ITS region sequence of the *Bifidobacterium* species from human intestinal microflora were obtained from the Genebank. The sequence identification between the designed primers and the bifidobacteria 16S rRNA and ITS region was confirmed with a BLAST search (http://www.ncbi.nih.gov/) using the DNA space program developed by Toshiba. Five strains, HJL 7511,

Fig. 6. 16S-ITS rDNA sequence of HJL 7511 aligned with that of *Bifidobacterium infantis* 16S-23S internal transcribed spacer (ITS), (Genebank no. gi 6572114).

HJL 7501, HJL 7519, HJL 7529 and HJL 7530 exhibiting high antioxidative activity and oxygen tolerance, were amplified using the primers Bifido-16S-1f and Bifido-ITS-1r for detecting *Bifidobacterium* species (Fig. 5). The PCR product size was adjusted 570 bp. As expected, 5 strains respected *Bifidobacterium* species, and HJL 7511 and HJL 7501 were identified as *Bifidobacterium infantis* based on 99% and 98% homological results, respectively (Fig. 6).

CONCLUSION

Bifidobacterium species are known for their health promoting effects in both human and animal intestinal tracts, and certain numbers of these bacteria are lyses and release their products into the gut. For the determinate antioxidative activity of *Bifidobacterium* strains isolated from Korean infant feces, inhibition of lipid peroxidation and scavenging ability were examined. 20 these strains were members of the genus *Bifidobacterium* according to the TLC methods. The antioxidative activities of 20 isolated *Bifidobacterium* strains ranged from 10.7% to 46.4% linoleic acid peroxidaion inhibition and from 15% to 41% scavenging activity on DPPH radical. The screened strains having high antioxidative ability strains were identified on basis of the 16S rRNA and ITS regions of bifidobacteria using PCR assay. Two strains, HJL 7511 and HJL 7501, were identified as *Bifidobacterium infantis* based on 99% and 98% homological results, respectively. The results of this study demonstrate the antioxidative potential of intestinal bifidobacteria, and suggest these strains are useful for functional fermented milk and probiotics.

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