Analysis of the Composition of Lactobacilli in Humans

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We collected fecal samples twice from 8 subjects and obtained 160 isolates of lactobacilli. The isolates were genetically fingerprinted and identified by pulsed-field gel electrophoresis (PFGE) and 16S rDNA sequence analysis, respectively. The numbers of lactobacilli detected in fecal samples varied greatly among the subjects. The isolates were divided into 37 strains by PFGE. No common strain was detected in the feces of different subjects. Except for one subject, at least one strain, unique to each individual, was detected in both fecal samples. The strains detected in both fecal samples were identified as Lactobacillus amylovorus, L. gasseri, L. fermentum, L. delbrueckii, L. crispatus, L. vaginalis and L. ruminis. They may be the indigenous Lactobacillus species in Japanese adults.

Key words: lactobacilli; Lactobacillus; composition; identification; PFGE

Members of the genus Lactobacillus are gram-positive organisms that belong to the general category of lactic acid bacteria. They inhabit a wide variety of habitats, including foods, plants and the gastrointestinal tracts of humans and animals. Some Lactobacillus strains are used in the manufacture of fermented foods. Recently, they are also used in functional foods as probiotics which influence the composition of intestinal microflora and benefit the well-being of the consumer (7). It is important to know the ecology of lactobacilli in humans for the development of probiotics. There are several reports about the composition of Lactobacillus species in the gastrointestinal tract of humans (1, 6). However, the taxonomy of Lactobacillus has recently been changed, and the taxonomic rearrangement and a proposal of new Lactobacillus species have been reported (4, 5). The genus Lactobacillus includes over 100 species described to date (3). In the present study, we analyzed the strain and species composition of numerically predominant lactobacilli in the gastrointestinal tract of humans to understand the precise ecology of intestinal lactobacilli.

Two fecal samples were collected one week apart from eight healthy Japanese adults who were male and between 27 and 48 years of age (mean age: 33.1). No subject included yogurt or milk products and pickled vegetables containing lactic acid bacteria in their diet. The samples were collected in plastic bags and immediately taken to the laboratory, where a 1-g portion of feces was removed and introduced into an anaerobic chamber (gas mixture: 5% CO2, 10% H2, and 85% N2). Then 1 g of the sample was used to make a fecal homogenate in 9 ml of Trypticase soy broth without dextrose (BBL, Cockeysville, MD). A dilution series (10−1 to 10−7) was made in the same medium, and 100-μl aliquots of each dilution were spread on Rogosa SL agar (Difco, Sparks, MD), selective medium for lactobacilli, to isolate Lactobacillus strains. The plates were incubated anaerobically for 2 days at 37 °C. Following incubation, colony counts were made of the dilution plates of the selective medium that contained discrete colonies (30 to 300), and the total population of lactobacilli was calculated as CFU per gram (wet weight) of feces. For each sample, 10 Lactobacillus colonies were randomly selected from the plate used for enumeration and subcultured onto Lactobacilli MRS agar (Difco, Sparks, MD). The number of isolates examined per sample was chosen on the basis of the publication of McCartney et al. (9), who reported that 10 randomly selected colonies gave good coverage of the numerically predominant strains.

The pulsed-field gel electrophoresis (PFGE) method of McCartney (9) was used to differentiate the strains among the randomly selected isolates obtained from each fecal sample. The DNA of lactobacilli was digested with a restriction endonuclease Apa I (Roche Applied Science, Penzberg, Germany). The restriction fragments were separated by PFGE for 17 hr using the CHEF-DRII Pulsed Field Electrophoresis Systems (Bio-Rad Laboratories, Hercules, CA). An initial pulse time of 1 sec and a final pulse time of 12 sec were used, and the gel was run at 5 V/cm with the buffer maintained at 14°C. Gels were stained with SYBR Green I (200 μg/ml) and examined by UV transillumination.

Provisional identification of genus was conducted on the basis of Gram stain reactions, cell morphology, and the catalase test, in conjunction with the ability of the...
isolate to grow on the selective medium. Identification of the isolates of lactobacilli to the species level was conducted by 16S rDNA sequence analysis. Bacterial DNA was extracted from cultures of each isolate using a NucleoSpin Tissue Kit (Macheret-Nagel, Düren, Germany) according to the manufacturer’s instructions. Partial 16S rDNA (around 500 bp from the 5’ end) was amplified using universal primers 27F (5’-AGAGTTTGATCCTGGCTCAG-3’) and 520R (5’-ACCAGCMGGCTGCTGCG-3’). The reaction mixture (20 µl) was composed of 0.1 µl of TaKaRa Ex Taq (Takara Bio, Shiga, Japan), 2 µl of 10× Ex Taq Buffer (Takara Bio), 1.6 µl of dNTP mixture (2.5 mM each, Takara Bio), 0.2 µl of each primer (100 µM), 1 µl of template DNA, and 14.9 µl of distilled water. The amplification program consisted of 1 cycle of 95°C for 3 min, followed by 30 cycles of 95°C for 30 sec, 50°C for 30 sec, and 72°C for 1 min, and a final cycle of 72°C for 10 min. The PCR products were purified using GFX PCR DNA and Gel Band Purification Kit (Amersham Biosciences, Piscataway, NJ) according to the manufacturer’s instructions. DNA sequence analysis of the purified DNA was performed by the dideoxy chain termination method using the 27F primer, ABI PRIZM BigDye Terminator Cycle Sequencing Kit (Applied Biosystems, Foster, CA), and ABI PRIZM 3100 Genetic Analyzer (Applied Biosystems). The sequences were compared with reference strains deposited in the DNA Data Bank of Japan (DDBJ) using the BLAST search. A similarity of more than 99% to 16S rDNA sequences of type strains was used as the criterion for identification. The identification of some Lactobacillus species such as L. amylovorus, L. gasseri and L. plantarum which showed high levels of sequence relatedness to closely related species was further confirmed by 16S-23S rDNA sequence analysis or multiplex PCR assay with recA gene-derived primers according to the methods of Tannock et al. (13) and Torriani et al. (14), respectively.

We collected fecal samples twice from 8 subjects and obtained 160 isolates of lactobacilli. The numbers of lactobacilli detected in fecal samples varied greatly among the subjects (Table 1). The isolates were genetically fingerprinted and identified. They were divided into 37 strains by PFGE (Table 2). PFGE has been shown to be the most powerful method for the differentiation of strains (2, 8, 15). No common strain was detected in the feces of different subjects. Each subject harbored a unique collection of lactobacilli in the gastrointestinal tract. Except for subject 7, at least one strain, unique to each individual, was able to be detected in both fecal samples (Table 2). Thirteen Lactobacillus species (L. amylovorus, L. mucosae, L. plantarum, L. gasseri, L. fermentum, L. delbrueckii, L. salivarius, L. crispatus, L. vaginalis, L. brevis, L. casei, L. acidophilus and L. ruminis) were detected in the feces of Japanese adults. We obtained fecal samples twice from each subject to investigate the indigenous Lactobacillus flora of the gastrointestinal tract. The strains detected in both fecal samples were identified as L. amylovorus, L. gasseri, L. fermentum, L. delbrueckii, L. crispatus, L. vaginalis and L. ruminis. They may be indigenous Lactobacillus strains, not the transient strains ingested with food. The dominant species in Japanese adults were L. gasseri and L. fermentum (Table 3).

McCartney et al. (9) examined the strain composition of Lactobacillus of fecal samples collected from two humans and found that one Lactobacillus strain, unique to each individual, was numerically predominant throughout a 12-month period in the two human subjects. In our previous study, we examined the strain composition of Lactobacillus of fecal samples collected from 10 human subjects (nine New Zealanders and one Japanese). The results showed that each subject harbored a unique collection of Lactobacillus strains. These results were comparable to those of the present study using the fecal samples of Japanese adults.

In the present study, L. amylovorus, L. mucosae, L. plantarum, L. gasseri, L. fermentum, L. delbrueckii, L. salivarius, L. crispatus, L. vaginalis, L. brevis, L. casei, L. acidophilus and L. ruminis were detected in feces of Japanese adults. L. gasseri and L. fermentum were most commonly detected among these species. It is difficult to identify some Lactobacillus species by physiological characteristics. We were able to identify all isolates at the species level by 16S rDNA sequence analysis, 16S–23S rDNA sequence analysis, and multiplex PCR assay with recA gene-derived primers. L. acidophilus was recently divided into 6 species (L. acidophilus, L. crispatus, L. amylovorus, L. gallinarum, L. gasseri, L. johnsonii) by

### Table 1. Populations of lactobacilli in fecal samples

| Subject | Lactobacillus populations (log$_{10}$ cfu/g) |
|---------|-------------------------------------------|
|         | Sample 1 | Sample 2 |
| 1       | 6.1      | 6.0      |
| 2       | 5.1      | 4.7      |
| 3       | 6.2      | 7.6      |
| 4       | 6.7      | 6.1      |
| 5       | 6.0      | 6.8      |
| 6       | 4.2      | 4.2      |
| 7       | 5.8      | 5.9      |
| 8       | 8.7      | 9.5      |
Table 2. Composition of lactobacilli detected in fecal samples obtained from 8 human subjects

| Subject | PFGE pattern | Numbers of *Lactobacillus* isolates | Species                  |
|---------|--------------|------------------------------------|--------------------------|
|         |              | Sample 1  | Sample 2  |                          |
| 1       | 1            | 7        | 6         | *Lactobacillus amylovorus* |
| 2       | 2            | 3        | 0         | *Lactobacillus amylovorus* |
|         | 3            | 0        | 2         | *Lactobacillus mucosae*   |
| 4       | 4            | 0        | 2         | *Lactobacillus plantarum* |
| 5       | 5            | 3        | 1         | *Lactobacillus gasseri*   |
| 6       | 6            | 3        | 0         | *Lactobacillus gasseri*   |
| 7       | 7            | 4        | 9         | *Lactobacillus fermentum* |
| 8       | 8            | 7        | 1         | *Lactobacillus delbrueckii* |
| 9       | 9            | 3        | 0         | *Lactobacillus fermentum* |
| 10      | 10           | 0        | 7         | *Lactobacillus salivarius* |
| 11      | 11           | 0        | 1         | *Lactobacillus gasseri*   |
| 12      | 12           | 0        | 1         | *Lactobacillus gasseri*   |
| 4       | 13           | 1        | 0         | *Lactobacillus gasseri*   |
| 14      | 14           | 3        | 1         | *Lactobacillus crispatus* |
| 15      | 15           | 0        | 1         | *Lactobacillus crispatus* |
| 16      | 16           | 0        | 1         | *Lactobacillus mucosae*   |
| 17      | 17           | 3        | 2         | *Lactobacillus vaginalis* |
| 18      | 18           | 3        | 4         | *Lactobacillus vaginalis* |
| 19      | 19           | 0        | 1         | *Lactobacillus gasseri*   |
| 5       | 20           | 10       | 3         | *Lactobacillus gasseri*   |
| 21      | 21           | 0        | 4         | *Lactobacillus fermentum* |
| 22      | 22           | 0        | 3         | *Lactobacillus fermentum* |
| 6       | 23           | 7        | 6         | *Lactobacillus gasseri*   |
| 24      | 24           | 1        | 4         | *Lactobacillus gasseri*   |
| 25      | 25           | 1        | 0         | *Lactobacillus amylovorus* |
| 26      | 26           | 1        | 0         | *Lactobacillus fermentum* |
| 7       | 27           | 6        | 0         | *Lactobacillus gasseri*   |
| 28      | 28           | 4        | 0         | *Lactobacillus fermentum* |
| 29      | 29           | 0        | 4         | *Lactobacillus brevis*    |
| 30      | 30           | 0        | 2         | *Lactobacillus vaginalis* |
| 31      | 31           | 0        | 1         | *Lactobacillus plantarum* |
| 32      | 32           | 0        | 1         | *Lactobacillus plantarum* |
| 33      | 33           | 0        | 1         | *Lactobacillus casei*     |
| 34      | 34           | 0        | 1         | *Lactobacillus acidophilus* |
| 8       | 35           | 9        | 8         | *Lactobacillus ruminis*   |
| 36      | 36           | 1        | 0         | *Lactobacillus mucosae*   |
| 37      | 37           | 0        | 2         | *Lactobacillus plantarum* |

Table 3. Frequency of occurrence of *Lactobacillus* species in fecal samples

| Species              | Frequency of occurrence (%) |
|----------------------|-----------------------------|
| *L. amylovorus*      | 25.0                        |
| *L. mucosae*         | 37.5                        |
| *L. plantarum*       | 37.5                        |
| *L. gasseri*         | 75.0                        |
| *L. fermentum*       | 62.5                        |
| *L. delbrueckii*     | 12.5                        |
| *L. salivarius*      | 12.5                        |
| *L. crispatus*       | 12.5                        |
| *L. vaginalis*       | 25.0                        |
| *L. brevis*          | 12.5                        |
| *L. casei*           | 12.5                        |
| *L. acidophilus*     | 12.5                        |
| *L. ruminis*         | 12.5                        |
DNA-DNA homology (5). L. gasseri was the most prevalent species among these 6 species, and L. gasseri and L. fermentum were the dominant lactobacillus species in the feces of Japanese women. Mitsuoka (10) reported that L. crispatus, L. gasseri, L. salivarius and L. reuteri were the major lactobacillus species. Reuter (11) found that L. gasseri, L. reuteri, L. ruminis and L. salivarius were the indigenous lactobacillus species. These findings are comparable to the present study with the exception of L. reuteri. On the other hand, L. rhamnosus and L. casei/paracasei (16), L. plantarum and L. rhamnosus (1) were mainly isolated from feces and rectal mucosa of adults, respectively. The difference of these results may be due to the difference in race, diet, and so forth.

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