Distribution of the *Lactobacillus acidophilus* Complex in Human Fecal Specimens Examined by Restriction Fragment Length Polymorphism of the 16S rRNA Genes

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Received May 31, 2002; Accepted for publication, April 15, 2003

Fresh fecal samples from healthy volunteers were examined for their content of the *Lactobacillus acidophilus* complex (LAC). A two-step isolation method for fecal lactobacilli was developed and employed in this study. Isolates of lactobacilli were identified according to their restriction fragment length polymorphism (RFLP) of genes coding for ribosomal RNA. Our results suggested that all samples contain lactobacilli and the most dominant species of human fecal lactobacilli is *L. paracasei*. In addition, several strains of LAC were recovered from specimens of volunteers. Among six LAC species, namely, A1: *L. acidophilus*, A2: *L. crispatus*, A3: *L. amylovorus*, A4: *L. gallinarum*, B1: *L. gasseri*, and B2: *L. johnsonii*, isolates classified into cluster B were recovered from 6 out of 15 volunteers; however isolates of cluster A were recovered from 3 of 15 volunteers. Four of 15 volunteers had strains of *L. gasseri*, 3 of them had strains of *L. johnsonii*, and strains of *L. amylovorus* were also isolated from specimens of 3 of 15 volunteers. These findings suggest that group B of LAC is the predominant cluster of LAC found in human feces and *L. amylovorus* was the only species of cluster A found in this study. No volunteer with blood type O had LAC in the fecal *Lactobacillus* flora.

Key words: PCR-RFLP; Isolation of fecal *Lactobacillus*; Distribution of LAC

INTRODUCTION

Bibel (2) reviewed the findings of Metchnikoff who had suggested in 1901 that the lactic acid bacteria inhabiting the gastrointestinal tract might prevent human diseases. Previously, Sandine (28) had reported that *Lactobacillus acidophilus* was a lactic acid-producing inhabitant of the human intestines. Numerous studies have claimed that *Lactobacillus acidophilus* preparations and milk fermented by *Lactobacillus acidophilus* have prophylactic and therapeutic properties (3, 6–9, 12, 13). The interest in this idea has increased along with a growing awareness of the importance of a healthy (balanced) microflora. Traditionally, a beneficial role has been assigned to *L. acidophilus*. However, Katelaris et al. (20) reported that *L. acidophilus* strain LA, like *L. fermentum* strain KLD, did not have any protective effect against diarrhea. This controversy still remains to be solved. In 1992, Fujisawa et al. (11) reported that the *Lactobacillus* strains identified as *Lactobacillus acidophilus* according to their biochemical characteristics, including carbohydrate fermentation pattern and isomer of lactic acid, could be divided into two major clusters which were in turn subdivided into six smaller

clusters (*Lactobacillus acidophilus* complex: LAC) which were given species status as *L. acidophilus* (A1), *L. crispatus* (A2), *L. amylovorus* (A3), *L. gallinarum* (A4), *L. gasseri* (B1), and *L. johnsonii* (B2). This may, at least partly, clarify the confusion over the anti-infectious ability of *L. acidophilus*.

There is very limited knowledge about the species of LAC responsible for the beneficial role in the human gastrointestinal tract. Colonization is an important property of beneficial bacteria entering the gastrointestinal tract. It is important that bacteria adhere, at least transiently, to the intestinal epithelium to colonize the intestine. If the bacteria do not reach and adhere to the intestine, they are not likely to influence the intestinal balance. Thus we focused our interest on the difference regarding colonization abilities, among six species of LAC. It is likely that the intrinsic capacity for survival and colonization differs among the species of LAC; however, there is very little information available at present in this respect. In order to achieve our objectives, we have to survey the population of human fecal *Lactobacillus* flora as the first step.

From the results of reexaminations of *Lactobacillus acidophilus* stock cultures of human fecal origin which had been identified phenotypically, *L. gasseri* was the predominant species (11, 18, 19, 22). However, in general, there are two major difficulties in the isolation of fecal lactobacilli. One is the suppression of lactobacilli

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growth on selective media. The other refers to the selection, or isolation, of lactobacilli grown on non-selective media used for fecal flora analysis (24). LBS agar is a well-known selective medium for isolating lactobacilli; however, lactobacilli growth on the plates is often suppressed. In addition, Gilliland et al. (12) reported that most colonies formed on original LBS agar under anaerobic conditions (GasPak system, BBL, Cockeysville, MD: (10% H₂, 10% CO₂, and 80% N₂)) had characteristics of *Bifidobacterium* species. It is questionable whether the library of *Lactobacillus* strains which has been constructed from isolates from the medium reflects the proportion of original fecal *Lactobacillus* flora. Moreover, BL agar is often used to isolate fecal bacteria including lactobacilli. However, isolation of lactobacilli from the medium is quite difficult because of the growth of a huge number of strains of other major anaerobic species. Therefore, the library of *Lactobacillus* strains constructed by isolates from these media seems to be inappropriate for the examination of fecal *Lactobacillus* flora. In this article, we developed a two-step lactobacilli isolation method to construct a stock library.

The aim of the present study was to determine which are the dominant species of *Lactobacillus acidophilus* complex in human fecal samples.

MATERIALS AND METHODS

Examination of the culture system for isolating lactobacilli from fecal specimens. Four commercially available media, namely BL agar (Nissui Pharmaceuticals Co., Ltd., Tokyo, Japan), Lactobacilli MRS agar (DIFCO, Detroit, MI), LBS agar (Becton Dickinson, Cockeysville) and LBS broth (Becton Dickinson), were examined to establish a method for isolating lactobacilli from fecal samples. Three types of CO₂ generators, AnaeroPack CO₂ (Mitsubishi Gas Chemical Co., Inc., Tokyo: a limited aerobic (15% O₂, 5% CO₂, and 80% N₂)), AnaeroPack Campylo (Mitsubishi Gas Chemical Co., Inc.: a limited anaerobic (10% O₂, 10% CO₂, and 80% N₂)), and AnaeroPack Anaero (Mitsubishi Gas Chemical Co., Inc.: strict anaerobic (20% CO₂ and 80% N₂)), were evaluated. To establish the optimal lactobacilli isolation process, isolation was performed using a combination of these media and gas generators. Furthermore, combinations of isolation steps were examined to optimize the isolation process.

Internal standard LAC strains used to evaluate recovery from fecal samples. Streptomycin-rifampicin resistant LAC strains were selected and used to estimate the recovery of LAC strains from fecal samples.

L. gasseri SBT2055SR, *L. acidophilus* SBT2062SR, *L. crispatus* SBT1264SR, *L. amylovorus* SBT1762SR, *L. gallinarum* SBT1739SR, and *L. johnsonii* SBT2050SR were used as internal standard strains to evaluate the recovery.

Volunteers. Fifteen healthy volunteers participated in this study. All volunteers, between 23 and 48 years of age, were researchers or research assistants of Snow Brand Milk Products Co., Ltd. They did not have a history of gastrointestinal disorders or constipation. None of them had eaten fermented food such as fermented milk, pickled vegetables or "natto," a Japanese traditional food, nor had they taken any antibiotics during four weeks previous to the sampling of feces. All the volunteers agreed to participate in this study after being informed of the details of the study.

Established isolation protocol of lactobacilli. One gram of fresh fecal samples was homogenized using a Teflon homogenizer with an anaerobic diluter (19) and serially diluted 10-fold. A 0.1 ml aliquot of each diluted suspension was plated on MRS agar plates and incubated under limited anaerobic conditions at 37°C for 2 days to avoid the growth of anaerobic bacteria including *Bifidobacterium*. Bacterial colonies grown on the MRS agar plates were transferred onto optimized LBS agar with a piece of sterilized velvet or with sterilized tooth picks and incubated under limited anaerobic conditions for 2 days at 37°C. In this way, facultative cocci contaminating lactobacilli colonies on MRS plates were eliminated. The resulting bacterial colonies were isolated and cultured in optimized LBS broth. Most of the bacterial strains grown in the optimized LBS broth were lactobacilli. Light microscopy and conventional staining were used to confirm that the isolates were rods, Gram positive and non-motile, and therefore most likely to be lactobacilli.

Identification of isolated lactobacilli strains. The resulting lactobacilli strains isolated from fresh human fecal samples were identified by a genetic method employing restriction patterns of the enzymatically amplified 16S rRNA gene (14, 25, 26) as described by Joho and Hashiba (18). Briefly, the lactobacillus strains freshly isolated from fecal specimens of human volunteers were cultured in 1 ml of MRS broth at 37°C for 1 day. Bacterial cells were harvested and washed twice in STM buffer (50 mM Tris-maleate containing 1 M sucrose, pH 6.5) by centrifuging them at 7000 × g for 10 min. Washed cells were stored at -80°C until use.

The frozen cells were resuspended in 0.5 ml of STM buffer, and treated with *N*-acetylmuramidase-SG (Seikagaku Corporation, Tokyo) at a final concentra-

tion of 10 µg/ml at 37°C for 30 min. The bacterial cell lysates thus obtained were treated with SDS at a final concentration of 1 %. Proteins were eliminated by extraction with an equal volume of phenol/chloroform/isoamyl alcohol (25:24:1). The aqueous layer was washed with an equal volume of chloroform, then 20 ml of 0.5 % Proteinase-K (Sigma Chemicals, St. Louis, MO) was added. Proteinase-K treatment was carried out at 37°C for 20 min. The DNA fraction was recovered by cold ethanol precipitation; then it was dried and resuspended in sterilized water and stored at -20°C until use.

Polymerase chain reaction (PCR) for amplification of the 16S rRNA gene was done according to the procedure described by Joho and Hashiba (18). The sequences of the forward and the reverse primers used were 5'-AGAGTTTGATCCTGGCTCAG-3' and 5'-AAGGAGGTGATCCAGCCGCA-3', respectively. The 16S rRNA gene was selectively amplified by PCR in total volume of 100 µl, with 2.5 units of Taq DNA polymerase (Perkin Elmer, Cetus, Sweden), 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 1.5 mM MgCl₂ 0.01% (w/v) gelatin, 100 pmol of each oligonucleotide primer and 200 µM dNTP. The mixture was subjected to 36 cycles of the following incubations: denaturation at 94°C for 1 min, annealing at 62°C for 2 min and chain extension at 72°C for 3 min.

Amplified DNA was extracted with phenol/chloroform/isoamyl alcohol (25:24:1), precipitated with 6 volumes of cold ethanol and 1/5 volume of 3 M KCl, and then rinsed with 70% ethanol. Precipitated DNA was dried and dissolved in 10 ml of sterilized water.

An aliquot of 0.1–0.5 µg DNA in 10 µl of the appropriate buffer was digested overnight at 37°C with 5 units of a restriction endonuclease. Digestion with *Dra* I and *Hinf* I was performed independently; the restriction fragments of each reaction were loaded on separate lanes, and then electrophoresed using a 4–20% polyacrylamide gradient gel in Tris-borate buffer (18 mA, 100 min). Gels were stained with Silver Stain Kit (Bio-Rad, Hercules, CA) and photographed. λDNA fragments digested with *Eco* RI and *Hind* III and pBR329 fragments digested with *Hae* III were used as molecular size markers.

Six other species of *Lactobacillus*, namely *L. paracasei*, *L. salivarius*, *L. reuteri*, *L. brevis* and *L. plantarum*, are also considered to be identifiable with the PFLP method described above. However, isolates classified to these species by this method were re-identified with the DNA-DNA hybridization method. Isolates with other fragment patterns with the RFLP

method were defined as unidentified strains here. The unidentified strains with the same alphabetical code showed the same DNA fragment patterns.

Statistics. Statistical analyses were done with StatView ver 4 (SAS institute Inc., Cary, NC), with χ -square test for homogeneity of LAC distribution. The level of significance was set at $p < 0.05$.

RESULTS

Establishment of Lactobacilli-Isolation Protocol from Fecal Specimens

The effects of culture media and oxygen concentration in the culture systems on the selection of LAC strains and strains of major species in fecal anaerobic microflora were tested. The use of MRS agar and AnaeroPack Campylo did not affect the growth of the strains of 6 LAC species tested, while LBS agar prepared according to the manufacturer's instructions diminished the viable number of lactobacilli in fecal samples. The number of colonies of the LAC strains tested (2 strains per each species) grown on original LBS agar, was up to 10 times lower than the count obtained with MRS/Campylo. The declines were not LAC strain- or species-specific (Fig. 1). The count of viable LAC strains obtained with MRS agar under limited anaerobic conditions (MRS/Campylo culture) was comparable to that obtained with the conventionally strict anaerobic culture method (that is, with BL agar and steel wool jars (Hirayama Manufacturing Co., Ltd., Tokyo): data not shown). In addition, the MRS/Campylo culture did not allow the growth of other strains of major anaerobic species of fecal origin (Fig. 2).

To eliminate the small number of colonies of cocci concomitantly grown in MRS/Campylo culture, we employed optimized LBS agar which was prepared by adding a half volume of glacial acetic acid according to the manufacturer's instructions (0.66 ml/l). The colonies grown on MRS agar were transferred to the optimized LBS agar; then the transferred plates were incubated under limited anaerobic conditions for another 2 days at 37°C. Among the colonies isolated from fecal samples on MRS plates, 70 out of 261 colonies transferred from MRS to the optimized LBS could grow under limited anaerobic conditions (10% CO₂, 10% O₂ and 80% N₂). Of them, 62 were lactobacilli (identified on the basis of bacterial cell morphology after Gram staining).

Table 1 shows the recovery of streptomycin- and rifampicin-resistant strains of *Lactobacillus acidophilus* complex which had been premixed in human fecal

Fig. 1. Differences in recovery of strains of LAC with three different culture methods. MRS/Campylo: cultures were performed using MRS agar plates and AnaeroPack Campylo. LBS/Campylo: cultures were done using original LBS agar plates and AnaeroPack Campylo. BL/Anaero: BL agar plates and AnaeroPack Anaero were used for cultivation. Each column and bar represents the mean $\pm$ SD ($n = 5$).

Fig. 2. Recovery of strains of anaerobes often found in fecal specimens using three different culture methods. MRS/Campylo: the culture conditions were the same as described in the legend to Fig. 1. BL/Campylo: cultures were done using BL agar plates and AnaeroPack Campylo. BL/Anaero: the culture conditions were the same as described in the legend to Fig. 1. Each column and bar represents the mean $\pm$ SD ($n = 5$).

specimens. All of them were recovered from fecal specimens without significant losses. These results showed that the optimized LBS agar did not affect the number of colonies of lactobacilli formed. The combination of two culture systems proved an excellent strategy for

the isolation of lactobacilli from feces.

The established protocol for the isolation of lactobacilli is summarized in Fig. 3. In brief, fresh fecal samples were weighed and homogenized using a Teflon homogenizer with an anaerobic diluter. The resulting suspen-

Table 1. Recovery of streptomycin- and rifampicin-resistant variants of strains of *Lactobacillus acidophilus* complex using two different isolation methods from fecal specimens in which each variant strain had been premixed.

Strains premixed in fecal sample	log number of bacteria added per g-feces	Recovery method	
		MRS-SR/Campylo ^{a)}	MRS-SR/Campylo + optimized LBS-SR/Campylo ^{b)}
<i>L. acidophilus</i> SBT2062SR	7.05	7.108 ± 0.285 ^{c)}	7.029 ± 0.206
<i>L. crispatus</i> SBT1264SR	7.10	7.061 ± 0.197	7.031 ± 0.235
<i>L. amylovorus</i> SBT1762SR	7.15	7.203 ± 0.417	7.047 ± 0.214
<i>L. gallinarum</i> SBT1739SR	7.00	7.050 ± 0.132	6.904 ± 0.287
<i>L. gasseri</i> SBT2055SR	6.95	7.013 ± 0.231	6.857 ± 0.227
<i>L. johnsonii</i> SBT2050SR	6.96	6.981 ± 0.175	6.978 ± 0.138

^{a)} MRS-SR agar plates were employed as a selection agar and cultured under a limited anaerobic atmosphere using AnaeroPack Campylo, at 37°C for 2 days. The culture conditions were the same as those described in Materials and Methods, except that MRS-SR agar (MRS agar containing 1 mg/g of streptomycin and 10 µg/g of rifampicin) was used instead of MRS agar.

^{b)} After MRS-SR/Campylo isolation, colonies grown on the medium were transferred to optimized LBS-SR plates and then cultured under the limited anaerobic atmosphere at 37°C for 2 days. Variant strains premixed in fecal sample were cultured using the MRS-SR/Campylo system and the colonies that grew on MRS-SR agar plates were transferred to optimized LBS-SR agar plates (optimized LBS agar containing 1 mg/g of streptomycin and 10 µg/g of rifampicin). The transferred colonies were cultured again under limited anaerobic conditions at 37°C for 2 more days.

^{c)} Data are expressed as the mean ± SD of 5 separate examinations.

- 1) Isolated on MRS agar under a specific microaerobic atmosphere
- 2) Transfer to optimized LBS agar
- 3) Amplification of 16S rRNA gene by PCR
- 4) PCR-RFLPs of amplified 16S rRNA gene

Fig. 3. Established protocol for the isolation and classification of fecal lactobacilli.

sion was serially diluted ten-fold with the anaerobic diluter. Aliquots of the dilutions were cultured. In the final step of isolation, 30 isolates, which were randomly chosen from the plates of one sample, were cultured in optimized LBS broth and the bacterial pellets were suspended in 30% glycerol in the broth and stored at -80 °C until identified.

Distribution of *Lactobacillus Acidophilus* Complex in Human Feces

Typical RFLP of LAC are shown in Table 2. Results of identification based on the RFLP of fecal LAC isolates were exactly the same as those obtained by DNA-

DNA hybridization method.

The distribution of LAC in fecal samples from volunteers is presented in Fig. 4. All the volunteers had at least one species of *Lactobacillus* in their feces. Five species of *Lactobacillus* other than four unidentified species were found in fecal *Lactobacillus* microflora; the most dominant species of *Lactobacillus* found in volunteers of this study was *L. paracasei*. This species was isolated from 12 out of 15 fecal samples. The average logarithmic number of *L. paracasei* in fecal specimens of volunteers was 6.780 ± 0.702 (per g; mean ± SD, $n = 12$). LAC followed *L. paracasei* in terms of frequency of occurrence. While LAC strains were isolated from 6 out of 15 volunteers in this study, LAC was dominant among isolated lactobacilli of LAC positive volunteers except for one volunteer.

Isolates of *L. gasseri* and isolates of *L. johnsonii* were recovered from 5 and 3 fecal specimens, respectively. *L. amylovorus* was the only species of cluster A LAC found in human fecal specimens in this study. Three out of 15 specimens contained *L. amylovorus*.

Lactobacillus flora of volunteers of blood type O seemed to be quite simple, with only 3 species other than LAC species (Fig. 4c: $p = 0.0440$) being found in the fecal specimens; the major component was *L. paracasei*. On the other hand, *Lactobacillus* flora in fecal samples of volunteers of other blood types was respectively complex (Fig. 4b). Besides, LAC was the dominant *Lactobacillus* species in fecal samples of the volunteers whose blood was type A, B or AB.

Table 2. Restriction fragment length polymorphic DNA fragment profiles of *Dra* I- and *Hinf* I-digested 16S rRNA-DNA of *Lactobacillus acidophilus* complex.

<i>L. acidophilus</i> complex												
	<i>L. acidophilus</i> (A1)		<i>L. crispatus</i> (A2)		<i>L. amylovorus</i> (A3)		<i>L. gallinarum</i> (A4)		<i>L. gasseri</i> (B1)		<i>L. johnsonii</i> (B2)	
Restriction fragment length (bp)												
Restriction enzyme	<i>Dra</i> I	<i>Hinf</i> I	<i>Dra</i> I	<i>Hinf</i> I	<i>Dra</i> I	<i>Hinf</i> I	<i>Dra</i> I	<i>Hinf</i> I	<i>Dra</i> I	<i>Hinf</i> I	<i>Dra</i> I	<i>Hinf</i> I
Type patterns	a	I	a	II	b	II	a	III	b	IV	a	IV
	912	948	912	948		948	912	948		948	912	948
		840		840		840		840		840		840
	596		596		731	596	596		731	596	596	
				398		398		412		412		412
		374										
				339		339		339		339		339
		306										
					214				214			
								191				
		124		124		124						
										84		84
		80		80		80				80		80
		60		60		60		60				
										39		39

Restriction fragment length polymorphisms (RFLPs) of *Dra* I- and *Hinf* I-digested 16S rRNA gene are examined respectively on gradient gels (4–20% polyacrylamide).

Fig. 4. Distribution of fecal *Lactobacillus* species in human fecal specimens.

Overall: data from all fecal specimens. A/B/AB: data from fecal samples of volunteers who were of blood type A, B and AB. O: data from fecal specimens of volunteers who were of blood type O.

Fig. 5. Population of *Lactobacillus* species in human fecal specimens.

Overall: data from all fecal specimens. A/B/AB: data from fecal samples of volunteers who were of blood type A, B and AB. O: data from fecal specimens of volunteers who were of blood type O.

Statistical analyses showed the distribution of *Lactobacillus* species in fecal samples was not homogenous in this study (Fig. 4: $p < 0.0001$). Cluster B LAC occurred much more frequently than cluster A LAC ($p = 0.0226$). In addition, *L. gasseri* seemed to be the dominant species of LAC in fecal specimens of volunteers.

From the point of view of population, the number of viable cells of each *Lactobacillus* species in feces is shown in Fig. 5. The log number of fecal *Lactobacillus* was 7.133 ± 0.723 (per g; mean $\pm$ SD, $n = 15$), while that of LAC species in feces of volunteers who had LAC strains varied from 4.30 to 7.72 (per g; $n = 6$).

DISCUSSION

This study focuses on the composition of the *Lactobacillus* in feces of humans. It has been reported that *L. acidophilus* species is part of the human intestinal *Lactobacillus* flora (21). However, the *L. acidophilus* species is a very heterogenous group (10, 17). The study of Johnson et al. (16) revealed the genetic heterogeneity of the strains designated as *L. acidophilus*, on the basis of biochemical characteristics. The purpose of this study was to develop a simple protocol to isolate strains of *Lactobacillus* from human fecal specimens and to examine the fecal *Lactobacillus* flora of 15 volunteers using this newly developed method.

In evaluating the composition of *Lactobacillus* flora in human feces, the selection method for strains of *Lactobacillus* is of great importance. However, there has been no suitable isolation or selection method for lactobacilli because of reduction of their original number due to selective pressure. It is well known that LBS agar is a very good selective medium for lactobacilli. However, it is also well known that the conditions under which LBS agar is incubated greatly influence the recovery of LAC (12). Furthermore, Rogosa and Sharp (27) indicated that high concentrations of acetate caused by evaporation may inhibit some lactobacilli. In addition, incubation under strictly anaerobic conditions enables anaerobic *Bifidobacterium* species to grow and completely prevents the detection of LAC (12).

In order to reflect the original composition of the *Lactobacillus* population in feces, we improved the isolation method of lactobacilli from human fecal samples. In the first step, strict anaerobes including bifidobacteria could be eliminated by culture in an atmosphere of 10% CO₂, 10% O₂, and 80% N₂. Not all organisms detected on MRS/Campylo culture in this step were classified as typical *Lactobacillus*. Therefore, in the following step, acid resistant and facultative anaerobic rods were selected by replication from MRS agar plates to optimized LBS agar plates and cultured under limited anaerobic conditions. This step was important to elimi-

nate facultative cocci that may have grown on MRS agar plates in the first step. The number of LAC colonies from a single LAC strain culture subsequently cultured on optimized LBS plates was almost the same as that of LAC grown on non-selective BL plates under strict anaerobic conditions.

In order to identify the isolates, an RFLP method for LAC identification, developed by Joho and Hashiba (18), was used to identify 12 species of fecal *Lactobacillus*. To date, several methods have been used to identify *Lactobacillus* strains present in the gastrointestinal microbiota (1, 15). The identification method is quite simple and rapid enough for the analysis to be completed within 2 days. In addition, results obtained with the RFLP method corresponded with the groupings obtained by DNA-DNA hybridization.

Using the two-step lactobacilli isolation protocol and RFLP identification, an appropriate analysis of human fecal *Lactobacillus* flora could be performed. This is a convenient and reliable method to analyze human fecal LAC flora. It could be applied to other analyses of *Lactobacillus* flora in samples of human origin, for example, that in vaginal smears.

Joho and Hashiba (18) reported that 44 out of 68 strains of LAC of human fecal origin were *L. gasseri*, 7 were *L. acidophilus*, 8 were *L. crispatus*, 3 were *L. amylovorus*, 1 was *L. gallinarum*, and 5 were *L. johnsonii*. Kuroshima and Kodaira (22) reported that 15 out of 17 LAC strains were classified into *L. gasseri*, and the remaining 2 were classified into *L. acidophilus*. Fujisawa et al. (11) also surveyed the LAC composition of stock cultures and reported that 6 of 9 strains were *L. gasseri*, 2 were *L. crispatus*, and 1 was *L. amylovorus*. However, there is no evidence that the strains found in LAC culture stocks are completely independent of the others. The frequency of occurrence of *L. gasseri* in human fecal *Lactobacillus* flora followed that of *L. paracasei*. Our results basically agreed with those reported by other researchers who re-examined the composition of stock libraries of *L. acidophilus* of human fecal origin (11, 18, 19, 22).

It is interesting to note that no LAC species were isolated from fecal specimens of volunteers with blood type O at all. The distribution of isolates from volunteers with other blood types was highly complex. In volunteers with blood type O, however, the cell counts of *Lactobacillus* species were lower and more homogeneously distributed in the fecal specimens, indicating that the influence of blood type variation may be significant. Do blood group active antigens relate to the capability of LAC to colonize the human intestinal mucosa?

In animals, small intestinal glycolipid patterns including blood type glycosphingolipids vary among individuals as well as among species (4). At this point, further examination will be needed to clarify the mechanism(s) which underlie the phenomenon observed.

It can be concluded that LAC strains of human origin are mainly classified into cluster B; in particular, the frequency of isolation of *L. gasseri* from fresh fecal specimens of volunteers was higher than that of other LAC species. Thus, it appears that care must be taken in selecting strains of LAC for use in dietary preparations intended as a source of lactobacilli for establishment in the intestinal tract.

Carlsson and Gotherfors (5) reported that, in the human vagina, species of LAC account for most of the *Lactobacillus* flora. They also indicated that transmission of LAC from mother to child occurred at birth. More recently, Lachlak et al. (23) clarified that the dominant species of vaginal *Lactobacillus* of humans was *L. gasseri*. We found *L. gasseri* was the most frequently isolated species of LAC from human feces in this study. These facts may suggest that the development of intestinal *Lactobacillus* flora and the delivery of babies are closely related. Now we are interested in the composition of those of babies born by caesarean section.

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