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Review

Isolation of Novel Strains of *Lactobacillus gasseri* EJL and *Bifidobacterium breve* JTL from Breast Milk and Infant Feces: A Longitudinal Study of a Mother-infant Pair

Heetae Lee[1], Chong-Kil Lee[2], and Kyungjae Kim[1]*

[1]*College of Pharmacy, Sahmyook University, Seoul 01795, Republic of Korea*
[2]*College of Pharmacy, Chungbuk National University, Cheongju 28644, Republic of Korea*

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Human breast milk is a potential source of bacteria for the development of the intestinal microbiota of infants. Several species within the genera *Lactobacillus* and *Bifidobacterium* were demonstrated to shape the gut microbiota of infants. In this study, the bacterial diversity was investigated in the breast milk and feces of a mother-infant pair, and probiotic candidates were identified. Importantly, the novel *L. gasseri* EJL and *B. breve* JTL strains were isolated from breast milk and infant feces samples, respectively; their completed genome was resolved using *de novo* sequencing. In addition, the bacterial composition in the infant's feces at 1 week revealed the prevalence of *Bifidobacterium* and *Streptococcus*; a higher diversity was observed after 3 weeks. In particular, the abundance of *Akkermansia* was sharply increased at 7 weeks, further increasing thereafter, up to 15 weeks. Our results suggest that human breast milk and infant's feces are a source of probiotic candidates.

Keywords: **Human breast milk, *Lactobacillus gasseri*, *Bifidobacterium breve*, gut microbiota, probiotics**

Introduction

Probiotics are defined as 'live micro-organisms which when administered in adequate amounts confer a health benefit on the host' [1]. An increasing number of studies have supported the health benefits of probiotics and highlighted the significant role of probiotics in the modulation of gut microbiota composition [2]. Dysbiosis of gut microbiota can lead to several diseases associated with host energy metabolism and immune system. Recent studies have highlighted the function of probiotics for health improvement [3, 4].

Human breast milk is the ultimate source of nutrition for newborns, and provides not only essential nutrition but also the micro-organisms for the development of initial intestinal microbiota [5, 6]. Several isolated bacterial species belonging to *Lactobacillus*, *Bifidobacterium*, and *Streptococcus* genera are potential probiotics with health benefits [7]. Microbiota in human breast milk is reported to be originated from the gut microbiota of mother's intestine, and thus breastfeeding can have an important role in mother-to-infant microbial transmission [8, 9]. Moreover, breastfeeding has a protective effect against the development of various allergic diseases, including asthma and atopic dermatitis, in children via shaping of the gut microbiota [8], due to which probiotics of breast milk could be considered as a potential therapy for these diseases. In addition, recent studies have reported that certain *Lactobacillus* and *Bifidobacterium* strains isolated from breast milk and infant feces show protective effects against metabolic disorders, including obesity

***Corresponding author**
Tel: +82-2-3399-1601, Fax: +82-2-3399-1617
E-mail: kimkj@syu.ac.kr

and hyperglycemia [10, 11].

Human breast milk and infant feces contain a variety of bacterial species. However, the potential candidates for use as beneficial probiotics have not been fully identified. This study aims to 1) identify the isolated potential probiotic candidates of *Lactobacillus* and *Bifidobacterium* strains, 2) investigate the bacterial diversity and vertical transfer of bacteria from breast milk to infant feces in one mother-to-infant pair.

Materials and Methods

Subjects and sample collection

One mother-baby pair was recruited in this longitudinal study. The mother was a 35-year-old Korean (BMI: 22.4) and had no underlying diseases. Male infant was delivered at 39 weeks' gestation by spontaneous vaginal delivery. Mother was administered a probiotic formulation (Duolac-Gold, Cell Biotech Co. Ltd.) daily 20 weeks before delivery and the administration was discontinued after delivery. Probiotics formulation included *Bifidobacterium latics* (KCTC 11904BP), *Bifidobacterium longum* (KCTC 12200BP), *Bifidobacterium bifidum* (KCTC 12199BP), *Lactobacillus acidophilus* (KCTC 11906BP), *Lactobacillus rhamnosus* (KCTC 12202BP), and *Streptococcus thermophilus* (KCTC 11870BP). Mother and infant had no medical history and no antibiotics were used during the sampling period after birth. The infant was alternately fed breast milk and powdered formula without probiotics supplement.

Fecal samples and milk were collected from mother-infant pair at 1, 3, 5, 7, 9, 11, and 15 weeks. Prior to obtaining the milk samples using breast pump (after UV sterilization), the nipple and areola of the mother were cleaned with 70% isopropyl alcohol swabs. Fecal samples were collected in sterile containers and all samples were stored at -20℃ until shifting them to -70℃ in the laboratory. The study was approved by the Sahmyook University Institutional Review Board (2-104781-AB-N-012016115BR).

Isolation of *Lactobacillus* and *Bifidobacterium*

Lactobacillus was isolated from milk (1 week) and *Bifidobacterium* was isolated from infant feces (15 weeks), respectively. Milk and fecal samples were centrifuged at 5,000 g for 1 min. The supernatants were inoculated on MRS agar supplemented with 0.05% cysteine and incubated for 24–48 h at 37℃ under anaerobic conditions. Positive colonies, determined by PCR using primers for amplification of *Lactobacillus* (LacF-forward: AGCAGTAGGGAATCTTCCA, LacR-reverse: CACCGCTACACATGGAG) and *Bifidobacterium* (Bif164-forward: GGGTGGTAATGCCGGATG, Bif662-reverse: CCACCGTTACACCGGGAA), were sub-cultured in MRS broth supplemented with 0.05% cysteine. Bacterial glycerol stocks (20% v/v) were stored at -70℃ [12, 13].

Identification of isolates by 16S rDNA sequencing

Bacterial total DNA was extracted using Exgene™ Cell SV mini (GeneAll Biotechnology Co., Ltd.), according to manufacturer's instructions. 16S rRNA gene was amplified using 27f/1492r primer set (forward: 5′-AGAGTTTGATCCTGGCTCAG-3′, reverse: 5′-GGTTACCTTGTTACGACTT-3′). Each 25 μl PCR reaction mixture contained 2.5 μl 10X IP-*Taq* buffer I, 0.2 μl forward primer (50 uM), 0.2 μl reverse primer (50 uM), 0.5 μl IP - Taq DNA polymerase (2.5 u/ul), 1 μl dNTPs (each 2.5 mM), 1 μl DNA template, and 19.6 μl distilled water. PCR was performed in a T100™ thermal cycler (Bio-Rad Laboratories, Inc.) using the following cycling conditions: initial denaturation at 95℃ for 5 min, 35 cycles of denaturation at 95℃ for 30 s, annealing at 50℃ for 30 s, and extension at 72℃ for 30 s. A final extension was performed at 72℃ for 10 min. Amplified PCR products were separated by electrophoresis on a 1% agarose gel, extracted using FavorPrep™ GEL/PCR Purification Mini Kit (Favorgen Biotech Co.). Sequence analysis of the products was contracted out to a Cosmo Genetech, South Korea. The sequences were compared to those in the GenBank database using the NCBI BLAST search program. Phylogenetic analysis was performed to confirm the taxonomy of the isolated genus *Lactobacillus* and *Bifidobacterium* using the neighbor-joining method [14]. The nucleotide sequences were analyzed against the reference sequences of *Lactobacillus* and *Bifidobacterium,* using MEGA X software.

De novo whole genome sequencing

Library preparation and sequencing were conducted by Theragen Bio Itex, South Korea. Briefly, sequences of quality control-passed genomic DNA from isolates were produced by PacBio RS II sequencing system. Whole

genome *de novo* assembly was performed using Canu (ver. 1.7). Prokka (ver. 1.13) was used for gene prediction, and gene annotation was performed using UniProt, RefSeq, and Pfam database.

Bacterial diversity of breast milk and feces

Total bacterial DNA was extracted from milk and fecal samples using a PowerSoil DNA Isolation Kit (MO BIO Laboratories Inc.), according to manufacturer's instruction. 16S rDNA genes were amplified using the 341F/785R primer set targeting the V3-V4 region (forward primer: 5′- TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG-3′; reverse primer: 5′ GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC-3′). Sequencing of partial bacterial 16S rRNA genes was performed using

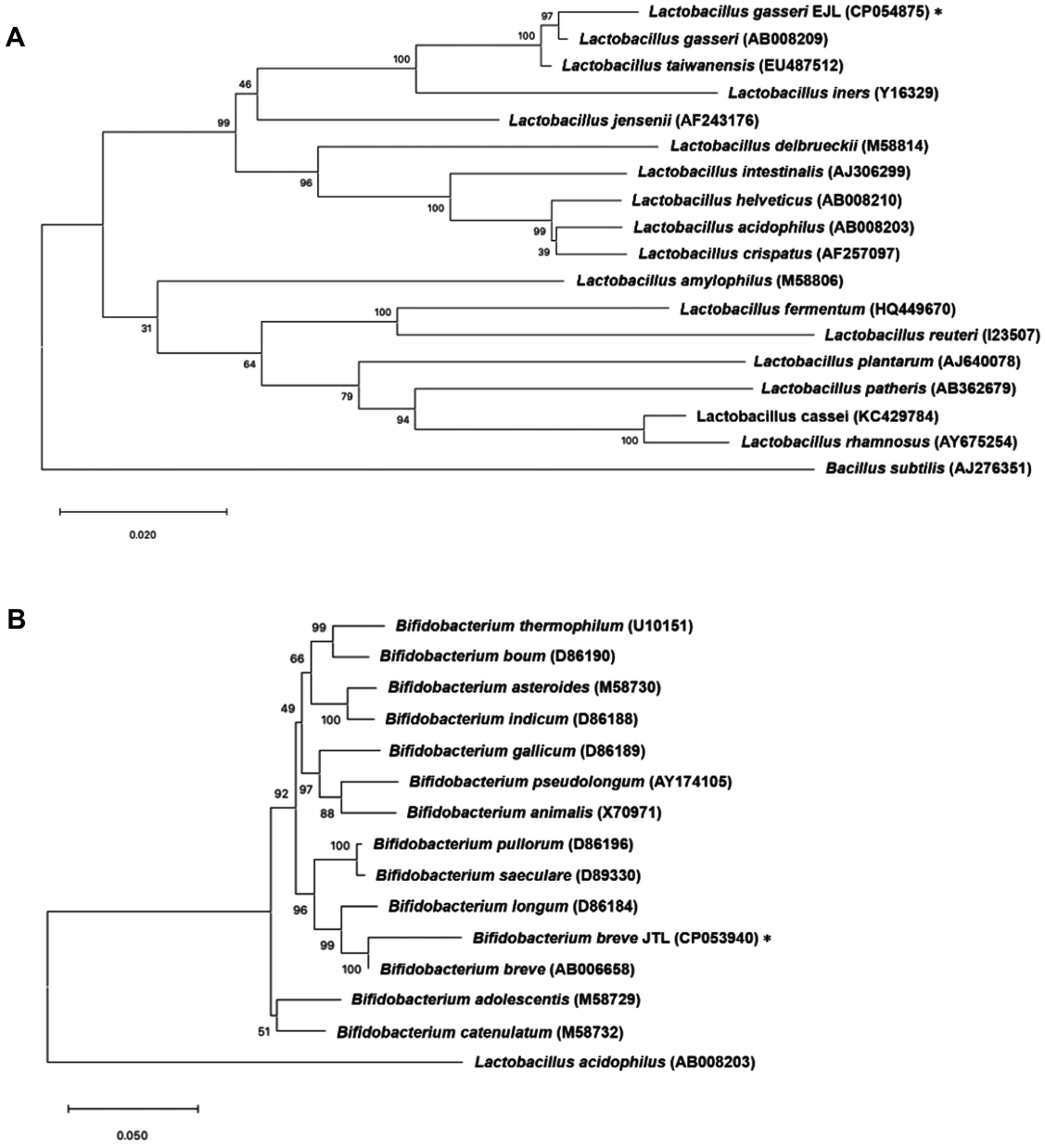

Fig. 1. Phylogenetic tree of bacterial species of genus *Lactobacillus* (A) and *Bifidobacterium* (B). The tree was constructed using the MEGA X software with neighbor-joining method. *Bacillus subtilis* and *Lactobacillus acidophilus* were used as the outgroup microorganisms. The GenBank accession numbers are shown in parenthesis. *: Isolates in this study.

the MiSeq Reagent Kit V3 (600 cycles) and MiSeq platform (Illumina) at Theragen Etex, Korea. After preprocessing (adaptor trimming, merge paired-end reads, quality control, and removal of chimeric sequence), sequences were assigned to operational taxonomic units (genus level, OTUs, 97% identity) using RDP classifier v.2.11. Additionally, taxonomy profiles of the bacterial species were identified using Cd-hit v.4.6 by comparing with NCBI Megablast database (identify > 99%, coverage > 80%).

Results

Phylogenetic analysis of isolated *Lactobacillus* and *Bifidobacterium* strains

Amplified PCR products of 16S rRNA gene from milk and infant feces were identified as novel strains of *L. gasseri* and *B. breve,* respectively, using the NCBI BLAST search program. In the phylogenetic analysis, the isolates from milk and infant feces were compared to 16 representative *Lactobacillus* strains and 13 *Bifidobacterium* strains, and were confirmed to be *L. gasseri* and *B. breve*, respectively (Figs. 1A and 1B).

Whole-genome sequencing of *L. gasseri* EJL and *B. breve* JTL

The genomic attributes of *L. gasseri* EJL and *B. breve* JTL are provided in Table 1. The final assembly of *L. gasseri* EJL was composed of total 2,289,039 bp, with a mean G + C content of 35.0% from three contigs, and contained 2,325 coding genes (CDS), 14 of rRNA genes, and 65 of tRNA genes. In *B. breve* JTL, total 2,289,549 of bp, with a mean G + C content of 58.5% from one contig were identified, and contained 1,874 coding genes (CDS), 6 of rRNA genes, and 54 of tRNA genes. The nucleic acid sequences of novel *L. gasseri* EJL and *B. breve* JTL strains were deposited into GenBank and assigned GenBank accession numbers CP054875 and CP053940, respectively.

Table 1. Genome features of *L. gasseri* EJL and *B. breve* JTL.

Attributes	Strains	
	L. gasseri EJL	*B. breve* JTL
No. of bases	2,289,039	2,289,549
G + C content (%)	35.0	58.5
No. of contigs	3	1
Coding genes (CDS)	2,325	1,874
rRNA	14	6
tRNA	65	54

Bacterial community profiles of the fecal and breast milk microbiota from mother and infant

Infant's fecal samples (n = 7) at 1, 3, 5, 7, 9, 11, and 15 weeks and mother's fecal (n = 2) and milk (n = 2) samples at 1 and 15 weeks were analyzed for bacterial community. A total of 1,168,314 sequence reads were generated from 11 samples. An average of 97,360 ± 33,681 reads were covered per sample. The lowest Shannon diversity index was observed in infant's feces at 1 week, which increased after 3 weeks. The Shannon diversity index of mother's feces and milk were higher than that of infant's feces (Fig. S1).

Composition of the fecal and milk microbiota obtained from mother and infant

In mother's fecal samples, the most abundant bacterial phyla at 1 and 15 weeks were *Bacteroidetes* (56.9% and 59.1%), followed by *Firmicutes* (39.8% and 33.7%). In contrast, in breast milk, *Firmicutes* (61.1% and 41.5%) was the most abundant bacterial phylum, while *Bacteroidetes* (0.9% and 8.1%) was relatively low, instead *Proteobacteria* was 14.1% and 30.1%, *Actinobacteria* was 22.3% and 15.7% at 1 and 15 weeks, respectively. In infant fecal samples, *Firmicutes* (58.1% and 27.1%) was most abundant, while *Bacteroidetes* was under 1% at both 1 and 15 weeks. In contrast, *Proteobacteria* was 0.2% and 15.1%, *Actinobacteria* was 41.6% and 20.4% at 1 and 15 weeks, respectively. Interestingly, *Verrucomicrobia* was hardly detected at 1 week, but increased to 37.3% at 15 weeks. The relative abundances of the bacterial phyla are described in Figure S2.

At the genus level, *Bacteroides* (43.5%), *Gemmiger* (11.1%), and *Megamonas* (8.0%) were most abundant at 1 week, while *Bacteroides* (51.1%), *Megamonas* (10.5%), and *Parabacteroides* (3.7%) were most abundant at 15 weeks in the mother's fecal samples. In breast milk, *Streptococcus* (26.8%), *Rothia* (16.1%), and *Veillonella* (14.8%) were most abundant at 1 week, and *Streptococcus* (20.3%), *Haemophilus* (14.3%), and *Rothia* (12.3%) were most abundant at 15 weeks. The bacterial composition in infant feces at 1 week included genus *Bifidobacterium*

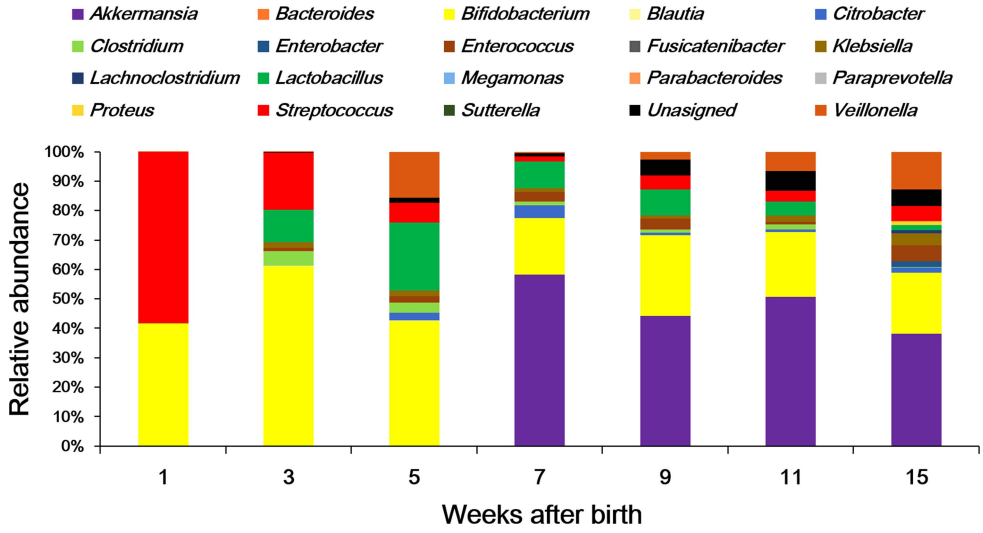

Fig. 2. Relative abundance of bacteria at genus level in infant feces from 1 to 15 weeks after birth. Top 20 abundant bacterial genera are shown. Sequencing of partial bacterial 16S rRNA genes targeting to V3-V4 region was performed using the MiSeq Reagent Kit V3 (600 cycles) and MiSeq platform (Illumina), and sequences were assigned to operational taxonomic units (genus level, OTUs, 97% identity) using RDP classifier v.2.11. Bacterial composition in infant feces at 1 week were mostly comprised of genus *Bifidobacterium* and *Streptococcus*, and a higher diversity was observed after 3 weeks. Abundances of *Lactobacillus*, *Veillonella*, *Klebsiella*, and *Enterococcus* increased after birth and replaced the abundance of *Bifidobacterium* and *Streptococcus*. In particular, the abundance of *Akkermansia* was sharply increased at 7 weeks, continued until 15 weeks.

(41.4%) and *Streptococcus* (57.8%), and a higher diversity was observed after 3 weeks. Relative abundances of *Bifidobacterium* and *Streptococcus* gradually decreased to 20.3% and 5.1%, respectively, at 15 weeks, and were replaced by *Lactobacillus*, *Veillonella*, *Klebsiella*, and *Enterococcus*. In particular, a sharp increase in the abundance of *Akkermansia* was seen at 7 weeks (51.6%) and continued until 15 weeks (37.3%). The relative abundances of the bacterial genera are described in Figs. 2 and S3.

Relative abundance of *Lactobacillus* and *Bifidobacterium* strains in feces and breast milk

The relative abundance of genus *Lactobacillus* and *Bifidobacterium* were 0.4% and 0.05% at 1 week and 1.1% and 0.6% at 15 weeks, respectively, in fecal samples, and 6.3% and 0.04% at 1 week, and 1.3% and 0.06% at 15 weeks in breast milk. In infant fecal samples, *Lactobacillus* was hardly detected at 1 week and was 1.6% at 15 weeks, whereas *Bifidobacterium* was 41.4% and 20.3% at 1 and 15 weeks, respectively. In the mother's fecal samples, four *Lactobacillus* species and one *Bifidobacterium* species were identified at 1 week and 15 weeks, respectively. In breast milk, 12 *Lactobacillus* species and 3 *Bifidobacterium* species and 12 *Lactobacillus* species and 4 *Bifidobacterium* species were identified at 1 week and 15 weeks, respectively. In infant fecal samples, *B. longum* (41.4%) was only identified at 1 week, which was the same as the species found in the probiotics formulation. In addition, *B. breve* (17.5%) and *B. stellenboschense* (0.005%) were identified at 15 weeks. *L. rhamnosus*, the same species as in the probiotics formulation, was identified in breast milk (0.002%) and infant fecal samples (0.03%) at 15 weeks. In addition, *L. gasseri* (0.3% and 1.5%), and *L. johnsonii* (0.01% and 0.02%) were identified at 15 weeks in both milk and infant fecal samples. The relative abundances of the bacterial genera are described in Table S1.

Discussion

Breast milk was identified as a source of abundant bacteria, using culture-independent genome sequencing. Two novel strains, namely *L. gasseri* EJL and *B. breve* JTL, were isolated in breast milk and infant feces, respectively, and their complete genomes were constructed using *de novo* sequencing. In addition, as breastfeeding has an impact on the construction of intestinal

microbial community in infants, the changes in fecal microbiota composition of infant was investigated for 15 weeks after birth.

Breast milk is the potential source of bacteria for infant intestinal track (estimated as 1×10^5 to 1×10^7 bacteria daily in 800 ml/day of breast milk) [15]. Specific bacterial strains such as *Bifidobacterium, Lactobacillus,* and *Staphylococcus* are transferred from mother to infant, and thus mother and infant share similar bacterial strains through breast-feeding [16, 17]. Among them, *Lactobacillus* strains such as *L. fermentum L. rhamnosus, and L. gasseri* have demonstrated potential probiotic properties, and were reported to have beneficial health effects, including anti-inflammation and metabolic improvements [18, 19]. In this study, a novel *Lactobacillus* strain, *L. gasseri* EJL, was isolated in breast milk and its complete genome was identified. This strain would be further evaluated for potential clinical applications in future studies.

Moreover, the composition of microbiota in breast milk varies within individuals and at different time periods, which in turn may be affected by the type of birth and maternal clinical factors [20, 21]. A recent study reported that *Lactobacillus* strains were not detected in colostrum [22]. Interestingly, in this study, *Lactobacillus* strains were more abundant in colostrum (6.3%) when compared to that in mature breast milk (0.8%). In addition, while several *Lactobacillus* strains, including *L. gasseri*, *L. salivarius*, *L. fermentum*, and *L. reuteri,* are known components of breast milk [23], this study identified additional *Lactobacillus* strains, such as *L. johnsonii* and *L. oris* from breast milk. Recently, the probiotic properties and health benefits of these strains have been reported [24, 25]. Thus, breast milk can be considered as a source of potential probiotics. Although there is a consensus that breast milk is the gold standard for infant nutrition, the effect of compositional differences of breast milk on the infant's health is not clear. Therefore, to better understand the importance of breast milk, the role of the breast milk-associated microbiota should be investigated in future studies.

In this study, *B. breve* JTL was another isolate identified in the feces of the infant after receiving breast milk. Low concentrations of *Bifidobacterium* strains comprised the microbiota of breast milk, whereas *Bifidobacterium* was the major component of the microbiota in infant feces. Moreover, *B. longum* was the only *Bifidobacterium* strain identified in infant at 1 week and the diversity of *Bifidobacterium* strains increased through breastfeeding. In particular, the abundance of *B. breve* was more dominant than *B. longum* at 15 weeks. Recent studies have reported that the transfer of *B. breve* to infant occurs through breast milk [26, 27], and human milk oligosaccharides (HMOs), which are the major components of breast milk, promote the dominance of *Bifidobacterium* sp. in early infancy [28]. In this study, *B. breve* JTL may have been transferred from mother to infant through breastfeeding. In addition, the supplementation of *Bifidobacterium* to infant had an impact on the fecal microbiota and metabolome [29]. Settled *Bifidobacterium* strains, such as *B. longum* and *B. breve.* in infant gut may have beneficial effects in the development of immune system and metabolism. For example, early gut colonization by *Bifidobacterium* had protective effects on allergic diseases [30, 31], and abundance of these strains in early infancy have been related with the vaccine response [32].

Among the bacterial strains in probiotics formulation that the mother was administered during pregnancy, *B. longum*, *L. rhamnosus*, and *S. thermophilus* were detected in mother's feces, breast milk, and infant feces, whereas *B. bifidum* was detected only in breast milk. Since the coincidence of these sequences was not confirmed, it is not reasonable to consider the strains in infant feces the same as those in the probiotics formulation. Nevertheless, it could be used as a reference while selecting a probiotics formulation for pregnancy and breastfeeding because the microbial composition of breast milk is suggested to be originated from the gut microbiota of the mother [9].

In conclusion, novel strains of *L. gasseri* EJL and *B. breve* JTL were isolated from breast milk and infant feces, respectively, and their completed genomes were constructed using *de novo* sequencing. Diverse probiotics candidates were identified in breast milk and infant feces in this longitudinal study of one mother-infant pair. *L. gasseri* EJL and *B. breve* JTL may have been transferred from mother to infant through breastfeeding. The potential probiotic properties for clinical application will be evaluated in the future.

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Conflict of Interest

The authors have no financial conflicts of interest to declare.

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