Mother-to-Infant Transmission of Intestinal Bifidobacterial Strains Has an Impact on the Early Development of Vaginally Delivered Infant’s Microbiota

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Abstract

Objectives: *Bifidobacterium* species are one of the major components of the infant’s intestine microbiota. Colonization with bifidobacteria in early infancy is suggested to be important for health in later life. However, information remains limited regarding the source of these microbes. Here, we investigated whether specific strains of bifidobacteria in the maternal intestinal flora are transmitted to their infant’s intestine.

Materials and Methods: Fecal samples were collected from healthy 17 mother and infant pairs (Vaginal delivery: 12; Cesarean section delivery: 5). Mother’s feces were collected twice before delivery. Infant’s feces were collected at 0 (meconium), 3, 7, 30, 90 days after birth. Bifidobacteria isolated from feces were genotyped by multilocus sequencing typing, and the transitions of bifidobacteria counts in infant’s feces were analyzed by quantitative real-time PCR.

Results: Stains belonging to *Bifidobacterium adolescentis*, *Bifidobacterium bifidum*, *Bifidobacterium catenulatum*, *Bifidobacterium longum* subsp. *longum*, and *Bifidobacterium pseudocatenulatum* were identified to be monophyletic between mother’s and infant’s intestine. Eleven out of 12 vaginal delivered infants carried at least one monophyletic strain. The bifidobacterial counts of the species to which the monophyletic strains belong, increased predominantly in the infant’s intestine within 3 days after birth. Among infants delivered by C-section, monophyletic strains were not observed. Moreover, the bifidobacterial counts were significantly lower than the vaginal delivered infants until 7 days of age.

Conclusions: Among infants born vaginally, several *Bifidobacterium* strains transmit from the mother and colonize the infant’s intestine shortly after birth. Our data suggest that the mother’s intestine is an important source for the vaginal delivered infant’s intestinal microbiota.

Introduction

The intestinal microbiota is a complex ecosystem with extensive metabolic activity, accounting for 10¹¹ to 10²² bacteria per gram of feces and comprising more than 1000 bacterial species [1–3]. It has been recognized to play an important role in the health and disease of the host [4–8]. The gastrointestinal tract of newborns is suggested to rapidly become colonized with bacteria while in the disease of the host [9–12]. The gastrointestinal tract of newborns has been recognized to play an important role in the health and metabolic activity, accounting for 10¹¹ to 10¹² bacteria per gram of feces and comprising more than 1000 bacterial species [1–3].

In a previous study, it was demonstrated that strains originated from the maternal gut were transmitted to the infant’s intestine [21]. The transmitted strains were found in the first feces (meconium) of the infant and at days 3, 7, 30, and 90 after birth, indicating that these organisms stably colonize the infant’s intestine soon after birth. This mother-to-infant transmission study was focused on one species, *Bifidobacterium longum* subsp. *longum*, one of the predominant bifidobacterial species in the crucial role in protecting the host against pathogenic bacteria, contributing to the priming of the mucosal immune system and consequently protecting against the susceptibility to diverse diseases later in life [15–17]. However, little information is available regarding the source of these intestinal microbes, although they are hypothesized to be derived from the maternal intestinal microbiota, diet, and the environment [18–20].
intestine of adults and infants [22]. Moreover, the investigated subjects were all mother and infant pairs who gave birth by vaginal delivery, and infants delivered by cesarean section (C-section) were not included in the study. Although cesarean delivered infants has been suggested to be initially colonized by bacteria from the environment, hospital staff and/or other neonates [23], the influence on the occurrence of mother-to-infant transmission among the differences in mode of delivery was not examined.

Because little is known about the vertical transmission of bifidobacteria from mothers to their newborn infants, the aim of this study was to verify whether mother-to-infant transmission is occurring among several different *Bifidobacterium* species in the infant’s gut microbiota. Furthermore, we have undertaken to investigate whether the difference in mode of delivery influences the mother-to-infant transmission. For this purpose, we have collected fecal samples at different times from vaginal delivered and cesarean delivered mother and infant pairs, isolated *Bifidobacterium* strains from the fecal samples and identified the strains similarity within mother and infant, and analyzed the transition of the numbers of bifidobacteria.

**Materials and Methods**

**Collection of the samples**

Samples of feces were collected aseptically from 17 mother and infant pairs (vaginal delivery: 12 pairs; cesarean delivery: 5 pairs) (see table S1). Fecal samples were taken from mothers twice (at least 1 week apart) before delivery (see table S2), and those from infants were collected at 0 (meconium), 3, 7, 30, and 90 days of age. All mothers were healthy and had full-term pregnancies and all of the infants were exclusively breast-fed for at least 2 months. These subjects belong to a larger observational study conducted in Belgium (protocol number ISRCTN66704989).

Participants were given a sterile glass tube containing 6 mL of anaerobic transport medium [content in 1 L: 0.225 g KH$_2$PO$_4$, 0.225 g K$_2$HPO$_4$, 0.45 g NaCl; 0.225 g (NH$_4$)$_2$SO$_4$, 0.0225 g CaCl$_2$, 0.0225 g MgSO$_4$, 0.5 g L-cysteine hydrochloride, 0.001 g resazurin, 0.5 g agar, 10 g Lab-Lemco powder (Oxoid Ltd., Basingstoke, England), 100 mL glycerol; and 2.1 mL 8% Na$_2$CO$_3$] [24], and an empty tube for the qPCR reaction. They were placed a spoonful of fecal sample (approximately 0.5 g) into each collection tube immediately after defecating. Samples were kept at 4°C until delivery to the laboratory, which occurred within 72 h after collection.

This study was conducted in accordance with ethical principles that originated from the Declaration of Helsinki, guidelines of good clinical practice, and applicable regulatory requirements. The ethics committee of the hospital network of Antwerp (Ziekenhuisnetwerk Antwerpen) approved the study, and informed written consent was obtained from the mothers.

**Isolation and identification of *Bifidobacterium* species**

Fecal samples in anaerobic transport medium were homogenized by vortex mixer to create fecal suspensions, which were serially diluted with phosphate-buffered saline (pH 7.2), inoculated onto TOS propionate agar (Yakult Pharmaceutical Industry Co., Ltd., Tokyo, Japan) containing 50 µg/mL mupirocin (TOS-M agar), and incubated anaerobically at 37°C for 72 h. From each sample, 2 to 6 colonies showing different morphologies on the agar, and incubated anaerobically at 37°C for 72 h. From each sample, 2 to 6 colonies showing different morphologies on the medium were isolated for subsequent analyses.

DNA was extracted from all pure cultures of the colonies sampled from the agar plates as described [25], with slight modification. Briefly, all isolated strains were cultured anaerobically in TOS-M broth for 48 h at 37°C. Cell pellets were collected from 1.0 mL of each culture by centrifugation (20,000 x g, 5 min, 4°C). Pellets were resuspended in 250 µL extraction buffer (100 mM Tris-HCl, 40 mM EDTA; pH 9.0). Glass beads (diameter, 0.1 mm; 700 mg), 500 µL phenol, and 50 µL 10% sodium dodecyl sulfate were added to each resuspension, and the mixture was vortexed vigorously for 30 s by using a FastPrep-24 (M.P. Biomedicals, Irvine, CA) at power level of 6.5. After vortexing, 150 µL 3 M sodium acetate was added to each sample, which then was cooled on ice for 5 min. After centrifugation (20,000 x g, 5 min, 4°C), the supernatant was collected, and DNA was precipitated with isopropanol. Finally, each DNA pellet was diluted in 100 µL TE buffer (10 mM Tris-HCl, 1 mM EDTA; pH 8.0).

Isolates were identified at the species level through polymerase chain reaction (PCR) sequencing of the 16S rRNA gene by using the universal primers 8F (5‘-AGAGTTTGATCCTGTCGCTAG-3’) and 1492R (5‘-ACGGGTACCTTGTATATCA-3’) [26]. PCR was carried out in 25-µL reaction, containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl$_2$, 200 µM each dNTP, 0.5 U Taq DNA polymerase (Takara, Shiga, Japan), 0.4 µM of each respective primer, and 10 ng DNA template. The PCR amplification program consisted of an initial heating step at 94°C for 2 min; 32 cycles of 94°C for 20 s, 55°C for 20 s, and 72°C for 20 s; and a final extension step at 72°C for 3 min. All amplifications were performed on a C1000™ Thermal Cycler (Bio-Rad, Hercules, CA). Amplicons were purified by using ExoSAP-IT (USB, Cleveland, OH) and sequenced by using the primers 8F and 520R (5’-ACGGGTACCTTGTATATCA-3’) [27], and BigDye Terminator v1.1 chemistry (Life Technologies, Carlsbad, CA) on a 3130xl Genetic Analyzer (Life Technologies). The resulting sequences were used to search sequences deposited in the EMBL database by using the BLAST algorithm (http://blast.ncbi.nlm.nih.gov/BLAST). The identities of the isolates were determined on the basis of the highest scores. Isolated strains that belonged to *Bifidobacterium adolescentis*, *Bifidobacterium bifidum*, *B. catenulatum*, and *B. pseudocatenulatum* continuum were further identified by PCR sequencing using primers BClpC-F (5’-ATCGCGGARACBATYGAAG-3’) and BClpC-R (5’-ATATGCGGCTTGGTCG-3’) [28] for the ClpC ATPase (*clpC*) gene, and primers B11 (5’-GTCGARGCGCTTSAAGAG-3’) and B12 (5’-CCRTGCGTCGCGG-GACCGTCA-3’) [29] for the heat-shock protein 60 (*hsp60*) gene (see figure S1 for the identification of *B. catenulatum*/*B. pseudocatenulatum* continuum), according to the methodology described above.

**Multilocus sequencing typing (MLST) analysis**

For the species belonging to *Bifidobacterium adolescentis*, *Bifidobacterium bifidum*, *B. catenulatum*, and *B. pseudocatenulatum* isolates, multilocus sequencing typing (MLST) analysis was performed using the seven housekeeping genes (*clpC*, *fusA*, *gybB*, *ileX*, *purF*, *rplB*, *rpoB*) previously described in the MLST protocol by Deletéole et al. [30], with slight modification. Isolates that belonged to *B. longum* subsp. *longum* were analyzed by using these seven housekeeping genes (*clpC*, *dnaG*, *dnuJ*, *fusA*, *gybB*, *purF*, *rpoB*) [21]. Each 25-µL reaction contained 1 µL PCR buffer, 200 µM dNTPs, 2 mM MgCl$_2$, 0.4 µM of each primer (Table 1), 10 µL GC-RICH solution, 2 U Fast Taq polymerase (Roche, Basel, Switzerland), and 10 ng template DNA. The PCR amplification program consisted of an initial heating step at 95°C for 5 min; 30 cycles of 95°C for 30 s, 57°C for 30 s, and 72°C for 1 min; and a final extension step at 72°C for 10 min. For compensating unavailable nucleotide sequence for *rplB* gene, the following primer set was used: forward: 5’TGGTGCTGCTAGGCCTGATATCA-3’, reverse:...
Products were sequenced as described above. BioNumerics software version 6.6 (Applied-Maths, Sint-Martens-Latem, Belgium) was used to perform all phylogenetic analyses. For MLST data, the sequences obtained for the seven housekeeping genes were aligned and compared. Each distinct gene sequence was assigned an allele number, and each unique combination of seven allele numbers was assigned a sequence type (ST). Based on allelic profiles, cluster analysis of the categorical coefficient was performed using the unweighted pair group method with arithmetic means (UPGMA) algorithm implemented in BioNumerics. This categorical parameter implies that the same weight is given to any multistate character at each locus, whatever the repeat number is [31]. Nucleotide sequences of MLST loci were deposited in GenBank/EMBL/DDBJ under accession numbers AB845863 to AB845966 and AB845704 (B. adolescentis), AB845967 to AB846141 (B. bifidum), AB846142 to AB846190 (B. catenulatum), AB846233 to AB846582 (B. longum subsp. longum), and AB846191 to AB846232 (B. pseudocatenulatum).

Quantitative real-time PCR (qPCR) assays
Quantitative real-time PCR (qPCR) was used to determine the population sizes of the predominant and subdominant bifidobacteria groups, as described by Matsuki et al. [32]. First, fecal samples (20 mg) stored in the empty tubes were washed 3 times by suspending them in 1 mL of phosphate-buffered saline (PBS) (Nissui Pharmaceutical Co. Ltd., Tokyo, Japan) to extract DNA. The thawed sample was mixed with 250 μL of extraction buffer and 50 μL of 10% sodium dodecyl sulfate. Three hundred mg of glass beads (diameter, 0.1 mm) and 500 μL of TE-saturated phenol (Sigma-Aldrich, St. Louis, MO) were added to the suspension, and the mixture was vortexed vigorously for 30 s using a FastPrep-24 at a power level of 5.0. Phenol-chloroform extractions were performed, and 250 μL of the supernatant was subjected to isopropanol precipitation. Finally, the DNA was suspended in 1 mL of TE buffer.

PCR amplification and detection were performed with a 7900HT Fast Real-Time PCR System and SDS software (version 4.0) (Life Technologies). The reaction mixture (10 μL) was composed of 10 mM Tris-HCl, pH 8.3; 50 mM KCl; 1.5 mM MgCl2; 0.2 mM of each dNTP; 0.2 μM of each primer; and 1X SYBR green master mix (Life Technologies). The primers were designed as follows:

Table 1. Primer sets for MLST analysis.

| Target species       | Gene     | Primer         | Sequence (5’–3’)                          | Amplicon size (bp) |
|----------------------|----------|----------------|-------------------------------------------|--------------------|
| B. adolescentis     | clpC     | ClpC-uni       | GATACCCGGAAGTAGATCGAG                   | 600                |
| B. bifidum          | ClpC     | ClpC-rev       | CAGCTCATCGTGGCAGAGGCAGGAC               |                    |
| B. catenulatum      | fusA     | fusAB3         | ATCGGGCATCTAGGCCYCATGAG                 | 666                |
| B. pseudocatenulatum| gyrB     | gyrBB3         | AGTGGACGCGAGCTGGTCTGGGTTGCTG            | 627                |
|                      | ileS     | ileSB3         | ATCCCGCGCTACAGAGGATG                   | 489                |
|                      | purF     | PurF-uni       | CATGCAGTGATGAGGGATG                   | 591                |
|                      | purF     | PurF-rev       | GTGGGGTAGCTGGCACGTG                   |                    |
|                      | rlpB     | rlpBB3         | GGAAAGAGCCCGCGGCTGGGCGGCGG             | 357                |
|                      | rpoB     | rpoBB3         | GCGGCTGATGACAGAAAGACC                 | 501                |
| B. longum subsp. longum | clpC    | ClpC-F         | CCTGAGAGGCTGTCTGAAAGG                 | 563                |
|                      | dnaG     | dnaG-F         | GGTGGCGATAGTGGCTGGTG                   | 449                |
|                      | dnaJ     | dnaJ-F         | GCTGACGAGAAGAGAGAGCTG                 | 421                |
|                      | dnaJ     | dnaJ-R         | AGTGCTGGTCCAGGCTGCAG                 |                    |
|                      | fusA     | fusA-F         | ACCATCTAGAGGAGAGCTG                   | 536                |
|                      | glyB     | glyB-R         | AGTGGACGCGAGCTGGGCTG                   | 473                |
|                      | purF     | purF-R         | GTGGCGAGCTGGGCTG                   | 501                |
|                      | rpoB     | rpoB-R         | ACGAGGCGATCCGAGAGAGCG                 | 575                |

Primer targeting species belonging to B. adolescentis, B. bifidum, B. catenulatum, B. pseudocatenulatum were designed by Deletéole et al. [30], and B. longum subsp. longum primers were designed by Makino et al. [21].

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5’-TAAGCGCCATCCTTGGCG-3’. Products were sequenced as described above.

BioNumerics software version 6.6 (Applied-Maths, Sint-Martens-Latem, Belgium) was used to perform all phylogenetic analyses. For MLST data, the sequences obtained for the seven housekeeping genes were aligned and compared. Each distinct gene sequence was assigned an allele number, and each unique combination of seven allele numbers was assigned a sequence type (ST). Based on allelic profiles, cluster analysis of the categorical coefficient was performed using the unweighted pair group method with arithmetic means (UPGMA) algorithm implemented in BioNumerics. This categorical parameter implies that the same weight is given to any multistate character at each locus, whatever the repeat number is [31]. Nucleotide sequences of MLST loci were deposited in GenBank/EMBL/DDJ under accession numbers AB845863 to AB845966 and AB845704 (B. adolescentis), AB845967 to AB846141 (B. bifidum), AB846142 to AB846190 (B. catenulatum), AB846233 to AB846582 (B. longum subsp. longum), and AB846191 to AB846232 (B. pseudocatenulatum).
Bifidobacterial population between vaginal delivered infants and value. Statistical analysis was performed to compare the total of feces, "detection limit/2^m" [34] was used as a complement value. The bacterial count was estimated from the slope of the standard curve, generated by using the following type strains: B. adolescentis ATCC 15703^T (for B. adolescentis), B. bifidum ATCC 29521^T (for B. bifidum), B. breve ATCC 15706^T (for B. breve), B. dentium ATCC 27534^T (for B. dentium), B. longum subsp. infantis ATCC 15697^T (for B. longum subsp. infantis), B. longum subsp. longum ATCC 15707^T (for B. longum subsp. longum), B. pseudocatenulatum JCM 12001^T (for B. catenulatum group). The total bifidobacteria was expressed as the sum of the counts of the measured 7 predominant bifidobacterial species. For values less than the qPCR detection limit (10^6 cells/g of feces), “detection limit/2^m” [34] was used as a complement value. Statistical analysis was performed to compare the total bifidobacterial population between vaginal delivered infants and cesarean delivered infants, with the Wilcoxon rank-sum test (SAS version 0.02, SAS Institute Inc., Cary, NC).

Results

Identification of Bifidobacterium species obtained from the feces of mothers and infants
In total, 273 bifidobacterial isolates were obtained from 17 mother-infant pairs (vaginal delivered pairs: 211 isolates; cesarean delivered pairs: 62 isolates). Among the 12 mother-infant pairs who gave birth by vaginal delivery, B. adolescentis, B. bifidum, B. catenulatum, B. longum subsp. longum, and B. pseudocatenulatum were the five Bifidobacterium species that were obtained from the feces of both mothers and infants (see table S5). B. adolescentis was isolated from both mother and infant from 3 families. Likewise, B. bifidum was isolated from 7 families (including one family which gave birth to twins), B. catenulatum from 2 families, B. longum subsp. longum from 5 families, and B. pseudocatenulatum from 2 families.

On the other hand, B. longum subsp. longum, was the only species that was obtained from the feces of both mothers and infants among the 5 mother-infant pairs who gave birth by C-section.

Comparison of MLST profiles of Bifidobacterium strains isolated from both members of a mother-infant pair
The 273 isolates (B. longum subsp. longum, 153 isolates; B. bifidum, 64 isolates; B. adolescentis, 32 isolates; B. catenulatum, 15 isolates; B. pseudocatenulatum, 9 isolates) were determined by the sequences of the seven loci. Isolates that had the same STs, and were obtained from the same source and at the same sampling point were defined as identical strains; otherwise, they were identified as individual strains. Consequently, among the 273 Bifidobacterium isolates, 103 individual strains were identified (B. longum subsp. longum, 50 strains; B. bifidum, 25 strains; B. adolescentis, 15 strains; B. catenulatum, 7 strains; B. pseudocatenulatum, 6 strains). An unweighted pair group method with arithmetic mean (UPGMA) dendrogram was generated based on the MLST profiles for all 103 individual strains.

(i) Vaginal delivered mother-infant pair
Among the 12 vaginal delivered mother-infant pairs, four strains of B. adolescentis (STs: ADO-C, ADO-D, ADO-G, ADO-H; Figure 1A), seven of B. bifidum (STs: BIF-A to BIF-G; Figure 1B), one of B. catenulatum (ST: CAT-A; Figure 1C), seven of B. longum subsp. longum (STs: LON-A, LON-B, LON-C, LON-D, LON-E, LON-F, LON-I; Figure 1D), and two of B. pseudocatenulatum (STs: PSE-A and PSE-C; Figure 1E) were identified as monophyletic between mothers and infants. These mother-infant monophyletic strains were isolated from the mother’s feces before delivery at different times, and also from the infant’s feces at different time. The mother-infant monophyletic strains such as STs BIF-D, BIF-G, LON-A, LON-D, and LON-I, were isolated not only from infants at age day 3 but also from day 90 and/or 90 (Figure 1B and 1D).

Table 2. 16S rRNA gene targeted primer sets used for qPCR.

| Target | Primer | Reference |
|--------|--------|-----------|
| Bifidobacterium adolescentis group^a | BIADOg-1a | CTCAGTTGAGTAGCATGTC | [32] |
| | BIADOg-1b | TCCAGTTGAGCGCATGTT |
| | BIADO-2 | CGAAGGCTGTGCTCCAGT |
| Bifidobacterium bifidum | BIBIF-1 | CCACATGATGAGCATGATTG | [33] |
| | BIBIF-2 | CCGAGAGGCTGTGCTCCAAA |
| Bifidobacterium breve | BIBRE-1 | CCGGATGCTTCATCACAC | [33] |
| | BIBRE-2 | ACAAGATGCTGCTCCACTCT |
| Bifidobacterium catenulatum group^b | BICAgt-1 | CGGATGCTGCGACCTCT | [33] |
| | BICAgt-2 | CGAAGGCTGTGCTCCAGAT |
| Bifidobacterium dentium | BIDEN-1 | ATCCCCGGGGGTCGCCCT | [22] |
| | BIDEN-2 | GAAGGCTGTGCTCCAGA |
| Bifidobacterium longum subsp. infantis | BIINF-1 | TCCAGTTGATGCGATGTCGTC | [22] |
| | BIINF-2 | GGAAGCCTACCTTCGGGAT |
| Bifidobacterium longum subsp. longum | BILON-1 | TCCAGTTGATGCGATGTCGTC | [22] |
| | BILON-2 | GGAAGCCTACCTTCGGGAT |

^aThe B. adolescentis group consists of B. adolescentis genotypes A and B.
^bThe B. catenulatum group consists of B. catenulatum and B. pseudocatenulatum.

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Figure 1. Comparison of MLST profiles of strains obtained from 12 vaginal delivered mother-infant pairs. Dendrogram of (A) 15 individual strains of *B. adolescentis* (ADO), (B) 25 individual *B. bifidum* strains (BIF), (C) 7 individual *B. catenulatum* strains (CAT), (D) 28 individual *B. longum* subsp. *longum* strains (LON), (E) 6 individual *B. pseudocatenulatum* strains (PSE) were all generated by a multi-scale setting for comparison and UPGMA for clustering. Isolates from both members of a mother–infant pair and showing the same MLST profile within a given cluster.

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Family no. 5, which gave birth to twins, an identical mother-infant monophyletic B. bifidum strain was found from both of the twins. The monophyletic strain (ST: BIF-B; Figure 1B) was isolated from the mother’s feces 17 and 10 days before delivery and also on days 3 and 7 from both of the twin infants.

(ii) Cesarean delivered mother-infant pair
Among the 5 mother-infant pairs which gave delivery by C-section, none of the strains were identified as monophyletic between mothers and infants (Figure 2). Two strains were monophyletic within the same mother, collected at different sampling period (STs: LON-e and LON-m), and one strain was monophyletic only within the same infant (ST: LON-d).

Diversity among the mother-infant monophyletic Bifidobacterium species
Mother-infant monophyletic strains were found among all five Bifidobacterium species, which were obtained from fecal samples of both mother and infant (Table 3). These strains were obtained only from families which gave birth by vaginal delivery. Eleven out of 12 mother-infant pairs yielded at least one mother-infant monophyletic Bifidobacterium strain. Moreover, strains from two different species were monophyletic between 7 mothers and their children (nos. 3, 5, 6, 7, 9, 10, 11).

Transitions of the bifidobacteria population within the infant’s intestine
The population sizes of the total bifidobacteria and the predominant/subdominant Bifidobacterium species were analyzed by qPCR. For the vaginally delivered infants, the total bifidobacterial counts showed an increase from about 10^5 cells/g of feces, which is the complement value for values less than the qPCR detection limit (10^6 cells/g of feces), to about 10^10 cells/g of feces during the 7 days after birth (Figure 3). On the other hand, the bifidobacteria count of the fecal samples from for the infants delivered by C-section was below 10^5 cells/g of feces at all the time points.

Confirming the transition and stability of bifidobacteria in infants individually, vaginal delivered infant such as no. 2, the B. bifidum counts increased from below the detection limit to 10^{11} cells/g of feces from 0 to 7 days of age. This bacterial count showed equivalent numbers with the total bifidobacteria, indicating that this species was the most and the only predominant bifidobacteria during early infancy (Figure 4A). The monophyletic B. bifidum strains were found to within the no. 2 mother-infant pair (Table 3). Likewise, the two most predominant species for vaginal delivered infant no. 5B (B. adolescentis, B. bifidum) and no. 6 (B. bifidum, B. catenulatum) were the species which were transmitted from each of their mother (Figure 4, Table 3). Such increase in numbers of the bifidobacterial species, to which the monophyletic
strains belong, was confirmed among 11 out of 12 vaginally born infants. On the other hand, infant delivered by C-section such as no. 15, bifidobacteria was rarely detected during the first weeks of life, as the total bifidobacteria was below the detection limit until 3 days of age and increased only up to $10^{6.5}$ cells/g at 7 days after birth (Figure 4D). The other four infants delivered by C-section have also showed a similar transition as bifidobacteria being rarely detected during the early infancy.

**Discussion**

Among the five Bifidobacterium species (B. adolescentis, B. bifidum, B. catenulatum, B. longum subsp. longum, B. pseudocatenulatum), we identified four strains of B. adolescentis, seven of B. bifidum, one of B. catenulatum, seven of B. longum subsp. longum, and two of B. pseudocatenulatum that were monophyletic between mothers and infants (Figure 1A–1E, Table 3). These findings confirm that mother-to-infant transmission occurs among several Bifidobacterium species. Moreover, two strains from different species were monophyletic in several families (Table 3), confirming that mother-to-infant transmission occurs parallel among several Bifidobacterium species within a single family. These results suggest that other commensal bacterial species might also transmit from the mother and colonize the intestine of the infant soon after birth.

Table 3. Detection of mother-infant monophyletic Bifidobacterium strains among 16 families.

| Mode of delivery | Family No. | B. adolescentis | B. bifidum | B. catenulatum | B. longum subsp. longum | B. pseudocatenulatum |
|------------------|------------|----------------|------------|----------------|------------------------|----------------------|
| Vaginal delivery | 1          | *              |            |                |                        |                      |
|                  | 2          | *              |            |                |                        |                      |
|                  | 3          | *              |            |                |                        |                      |
|                  | 4          |                |            |                |                        |                      |
|                  | 5 (A)      |                |            |                |                        |                      |
|                  | 5 (B)      |                |            |                |                        |                      |
|                  | 6          |                |            |                | *                      |                      |
|                  | 7          |                |            |                | *                      |                      |
|                  | 8          |                |            |                | *                      |                      |
|                  | 9          |                |            | *              |                        |                      |
|                  | 10         |                |            |                | *                      |                      |
|                  | 11         |                |            |                |                        |                      |
| Cesarean delivery| 12         |                |            |                |                        |                      |
|                  | 13         |                |            |                |                        |                      |
|                  | 14         |                |            |                |                        |                      |
|                  | 15         |                |            |                |                        |                      |
|                  | 16         |                |            |                |                        |                      |

*" represents that at least one mother-infant monophyletic Bifidobacterium strain was isolated from the indicated family. Mother no. 5 gave birth to twins (A, B). doi:10.1371/journal.pone.0078331.t003

Figure 3. The total bifidobacteria detected in 17 infant’s feces from 0 to 7 days of age. Numbers of vaginal delivered (VD) infants were 12, infants delivered by C-section (CS) were 5. Bars represent the median values respectively. For values less than the qPCR detection limit ($10^6$ cells/g of feces), “detection limit/2” [34] was used as a complement value. Stars indicate significant differences between VD infants and CD infants (*P<0.05; **P<0.01). doi:10.1371/journal.pone.0078331.g003
The bifidobacterial counts of the species to which the monophyletic strains belong, increased predominantly in the vaginal delivered infant’s intestine within 3 days after birth (Figure 4A–4C). On the other hand, among infants that were born by C-section, the number of the total bifidobacteria was significantly lower than that in the vaginally delivered infants until 7 days of age (Figure 3; Day 3: $P = 0.0223$, Day 7: $P = 0.0042$). It has been proposed that the intestinal colonization of bifidobacteria starts faster among vaginal delivered infants than infants delivered by C-section [35,36]. Our results suggest that among infants born vaginally, the bifidobacterial increase within few days after birth may have a correlation with the occurrence of the mother-to-infant transmission. Moreover, since mother-infant monophyletic strains were not observed in the infants delivered by C-section, the maternal strains might have been transmitted during the transit through the birth canal. Our results confirmed that strains in the pregnant mother’s intestine transmit to their infant’s intestine. However, the relationship of the bacterial strains between the vagina and the intestine was not analyzed. In some studies, it has been shown that there is a certain degree of correspondence between the rectal and the vaginal microbiota, suggesting that the intestine can be a source for the vaginal microbiota [37,38]. Breast milk and oral samples were also not obtained for this study. Further strain-level investigation among these sources will aim to clarify the route of these mother-to-infant transmission strains.

The environment such as hospital staff or other neonates are also suggested to be one of the sources of the infant’s microbiota; strains being transmitted horizontally to the newborn’s intestine after birth [23]. In our study, nine infants (nos. 1, 2, 3, 4, 6, 9, 12, 13, 15) were born in the same hospital (see table S1). Among these infants, monophyletic strains among different infants were not observed. Likewise, none of the strains were identified as monophyletic among the other infants (nos. 5A, 5B, 8, 10; 7, 14) which were born in the same hospital. Thus, we did not find evidences which can show the occurrence of horizontal transmission for bifidobacteria.

Several studies suggest that in addition to environment, diet, and delivery mode, host genetic may also be a key factor that influences the composition of the intestinal microbiota [39,40]. In a previous study, mother-infant monophyletic B. longum subsp. longum strains were specific to each family and were not found in the clusters derived from other families [21]. Similarly, in this study, mother-infant monophyletic strains among all five of the

![Figure 4](https://example.com/figure4.png)

**Figure 4.** The numbers of bifidobacteria in infants from 0 day to 7 days after birth. Infant no. 2, 5B, 6 were born vaginally, and infant no. 15 was born by cesarean delivery. a The B. adolescentis group consists of B. adolescentis genotypes A and B. b The B. catenulatum group consists of B. catenulatum and B. pseudocatenulatum.

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Bifidobacterium species that we evaluated formed an individual cluster for each family, suggesting that each family has its own unique bifidobacterial group, which is transmitted from mother to infant. Furthermore, the vertical transfer concept is strengthened by the fact that Bifidobacterium strains from the mother (family 5) can seed the intestine of both twin progeny (Figure 1B, Table 3). This is in agreement with previous studies that each family harbors his or her own set of Lactobacillus and Bifidobacterium species [41].

Interestingly, not all mother-infant monophyletic Bifidobacterium strains were isolated throughout the sampling period. Mother-infant monophyletic B. bifidum and B. longum subsp. longum strains were isolated from the infant fecal samples up to 90 days after birth (Figure 1B and 1D). The bacterial count also showed that these species were predominates from day 3 until day 90 of age (see figure S2). On the other hand, monophyletic strains belonging to B. adolescentis and B. catenulatum were not found in the infant’s fecal samples after 7 days of age (Figure 1A and 1C). Moreover, although several vaginal delivered infants harbored these species as high as $10^{10}$ cells/g of feces, these species were basically not the dominant species in the vaginal delivered infant’s intestine during the early infancy as the median value showing below $10^{6.4}$ cells/g of feces (see figure S2). These results suggest that some bifidobacterial species are predominantly proliferating and colonizing in the vaginal delivered infant’s gut. All infants in our study were exclusively breastfed for at least 2 months. It is widely known that human milk contains a wide variety of complex oligosaccharides (HMO) that selectively stimulate the growth of specific bifidobacterial species [42,43]. Typical infant species such as B. bifidum and B. longum are known to effectively degrade HMOs [44–48], while adult type bifidobacteria such as B. adolescentis are less efficient in degrading HMOs [46,48]. Therefore, breastfeeding and the HMOs present may be one of the key factors to explain why, while infants acquire a wide spectrum of bifidobacterial species from the mother, species that are able to degrade HMOs become one of the predominant colonizers in the infant’s gut.

B. breve is known to be one of the predominant species in the infant’s intestinal microbiota, and is able to utilize HMOs [47,49]. It has also been suggested that B. breve strains might be transferred from mother to infant [49,50]. While it was found to be common in infant’s microbiota, we could not isolate any B. breve strains from the mother, due to low prevalence of this bifidobacterial species in the maternal intestinal microbiota as previously described [49]. Because of this limitation of our study using cultivation technique, we were not able to assess the mother-to-infant transmission of this species, and therefore dismissing any such transmission would be questionable. Although cultivation technique cannot accurately assess the occurrence of mother-to-infant transmission, we are convinced that our results show, at a strain-specific level, that several species are transmitted from mother to infant and provide genetic data to support the hypothesis that bacteria are transmitted from mother to infant soon after birth. New techniques, such as single cell cultivation, that allows for bacterial cells isolation and characterization are rapidly developing and will allow us to expand our knowledge.

In conclusion, our results show that mothers who gave birth vaginally transmit their unique family-specific strains to their infant’s intestine during early infancy. Factors like nutrition may influence the persistence of family-specific bifidobacteria strains in infants throughout the sampling period, where they become one of the predominant bifidobacteria groups during early infancy.

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