Exploring vertical transmission of bifidobacteria from mother to child

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Exploring Vertical Transmission of Bifidobacteria from Mother to Child

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Passage through the birth canal and consequent exposure to the mother's microbiota is considered to represent the initiating event for microbial colonization of the gastrointestinal tract of the newborn. However, a precise evaluation of such suspected vertical microbiota transmission has yet to be performed. Here, we evaluated the microbiomes of four sample sets, each consisting of a mother's fecal and milk samples and the corresponding infant's fecal sample, by means of amplicon-based profiling supported by shotgun metagenomics data for two key samples. Notably, targeted genome reconstruction from microbiome data revealed vertical transmission of a *Bifidobacterium breve* strain and a *Bifidobacterium longum* subsp. *longum* strain from mother to infant, a notion confirmed by strain isolation and genome sequencing. Furthermore, PCR analyses targeting unique genes from these two strains highlighted their persistence in the infant gut at 6 months. Thus, this study demonstrates the existence of specific bifidobacterial strains that are common to mother and child and thus indicative of vertical transmission and that are maintained in the infant for at least relatively short time spans.

Human beings may be considered superorganisms that consist of both eukaryotic and prokaryotic cells (1). It is generally accepted, not only that the nuclear and mitochondrial genomes are transferred to the next generation, but that such transfer may also include certain members and associated genomes of the symbiotic community, whose microbial genes outnumber those of the eukaryotic host by over 100-fold (2). Passage through the birth canal, together with breastfeeding, represents a very important opportunity for transfer of symbionts from one generation to the next. Notably, this transmission is facilitated by the maternal holobiont, i.e., the organism, together with its associated microbial communities, and the mother is both actively and passively engaged in providing a symbiotic and perhaps long-lasting microbial community to her offspring. However, each individual develops a specific microbial community by adulthood, suggesting that stochastic colonization is more important than direct vertical transmission. In fact, while individual strains may be directly transmitted, the majority of the long-lasting community is probably not. In mammals, it is generally accepted that the fetus develops in an essentially sterile environment within the amnion and that bacterial colonization of the fetus is made possible as soon as the amnion breaks prior to its delivery through the birth canal (3). Bifidobacteria are among the first bacterial colonizers of the human gut and have been subject to extensive scientific scrutiny (4, 5). It has become clear that certain species of the genus *Bifidobacterium*, e.g., *Bifidobacterium breve* and *Bifidobacterium bifidum*, are genetically adapted to colonize the infant gut (for a review, see reference 6). Such bacteria have evolved genetic strategies that allow them to metabolize particular glycans present in human milk (7, 8, 9). However, human milk not only represents a reservoir of glycan compounds that significantly impact the composition of the infant gut microbiota, but also appears to act as a repository of bacteria for vertical transmission from mother to infant. In fact, the isolation of bifidobacteria from human milk has been reported (9, 10), even though it is currently not known how bifidobacteria reach this human bodily fluid (11). In addition, PCR-based approaches have provided suggestive evidence for the occurrence of direct transmission of bifidobacterial strains and other gut commensals from mother to newborn (12–14).

In this study, we investigated this notion of vertical transmission of bifidobacteria from mother to newborn by assessing the gut and milk microbiomes of four mother-child sample sets, revealing the existence of shared bifidobacterial strains.

MATERIALS AND METHODS

Subject recruitment and sample collection. The study is a pilot for a larger infant gut investigation protocol approved by the Ethical Committee of the University of Trento, and informed written consent was obtained from the participating mothers. Four mother-infant pairs were enrolled in the study; on the same day, stool samples were collected from both 3-month-old infants and mothers, while a fresh breast milk sample was also collected. At the time of the first sampling, breastfeeding was exclusive for mother-infant pairs 2 and 4, whereas 70% and 30% supplement of formula milk was given to the infant for pairs 1 and 3, respectively.

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Sampling of stool and milk samples was repeated following weaning 6 months after the first sample collection. At the time of the second sampling, the infant of pair 4 was still receiving a 20% dietary supplementation of breast milk from the mother (calculated as a percentage of total calories). All subjects were healthy, which was established based on self-reporting, and had not received any antibiotic or probiotic in the previous month. Stool samples consisted of 6 to 10 g of fresh fecal material, which was immediately frozen upon collection at −80°C until it was processed for DNA extraction. Human milk samples were collected as previously described (9) and were immediately subjected to DNA extraction.

Recovery of bifidobacteria on selective media. Fecal samples were pour plated onto bifidobacterium selective agar medium (BSM) for selective outgrowth of bifidobacteria. The BSM was prepared by the addition to MRS agar (Scharlau Chemie, Barcelona, Spain) of 0.05% (wt/vol) L-cysteine hydrochloride and 50 mg mupirocin (Delchimica, Italy) per liter of MRS, as described previously (15). The agar plates were incubated in an anaerobic atmosphere (2.99% H_2, 17.01% CO_2, and 80% N_2) in a chamber (Concept 400; Ruskin) at 37°C for 24 h. Ten colonies were taken as an adequate representation of the major bacterial strains cultured on the selective medium. DNA was extracted from each isolate through rapid mechanical cell lysis, as described previously (15), and subjected to (sub)-species identification through internal transcribed spacer (ITS) amplification and DNA sequencing.

ITS and 16S rRNA gene amplification. Partial ITS sequences were amplified from extracted DNA using the primer pair Probio-bif_Uni/Probio-bif_Rev (16). Partial 16S rRNA gene sequences were amplified from extracted DNA using the primer pair Probio_Uni/Probio_Rev, which targets the V3 region of the 16S rRNA gene sequence (17). These primers were designed in order to include, at their 5′ ends, one of the two adaptor sequences used in the Ion Torrent sequencing library preparation protocol linking a unique tag barcode of 10 bases to identify different samples. The PCR conditions used were 5 min at 95°C and 35 cycles of 30 s at 94°C, 30 s at 55°C, and 90 s at 72°C, followed by 10 min at 72°C. Amplification was carried out using a Veriti Thermocycler (Applied Biosystems). The integrity of the PCR amplicons was analyzed by electrophoresis on an Experion workstation (Bio-Rad, United Kingdom).

AQ: A **Ion Torrent PGM sequencing of ITS and 16S rRNA gene-based amplicons.** PCR products obtained following amplification of the ITS and 16S rRNA gene sequences were purified by electrophoretic separation on a 2% agarose gel and the use of a Wizard SV Gen PCR Clean-Up system (Promega), followed by a further purification step involving Agencourt AMPure XP DNA purification beads (Beckman Coulter Genomics GmbH, Bernried, Germany) in order to remove primer dimers. The DNA concentration of the amplified sequence library was estimated through the MultiTape system (Agilent). From the concentration and the average size of each amplicon library, the amounts of these DNA fragments per microliter were calculated, and libraries for each run were diluted to 3E9 DNA molecules prior to clonal amplification. Emulsion PCR was carried out using the Ion OneTouch 200 Template kit v2 DL (Life Technologies) according to the manufacturer's instructions. Sequencing of the amplicon libraries was carried out on a 316 chip using the Ion Torrent Personal Genome Machine (PGM) system and employing the Ion Sequencing 200 kit (Life Technologies), according to the supplier's instructions, at GenProbio srl (Parma, Italy). After sequencing, the individual sequence reads were filtered by PGM-provided software to remove low-quality and polyclonal sequences. Sequences matching the PGM 3′ adaptor were also automatically trimmed. All PGM quality-approved, trimmed, and filtered
AQ: B data sets were exported as BAM files.

ITS-based microbiota analysis. Fastq files obtained by sequencing of the ITS amplicons were analyzed using a custom script, named bif_ITS_analysis.sh script (available at http://probiogenomics.unipr.it/sw/bif_ITS_analysis.zip). This script requires QIIME (18) to be installed (or works in a QIIME virtual machine) and accepts .bam or .fastq input files containing sequencing reads. Input data were processed as previously described (16). The trees were saved in Phylip format, and each sequence was named, including the name of the operational taxonomic unit (OTU) and the number of sequences it represents. The script is easily modifiable in order to obtain a profile based on a different marker sequence, as long as a reliable database is available for such a marker sequence.

Evaluation of *Bifidobacterium longum* subsp. *longum* BLOI2 and *B. breve* BBRI4 persistence. DNA extracted from stool or milk samples was submitted to PCR amplification using the specific primers BLOI2_0636 fw (5′-GAACTTGAAGGGCTGCTGGA-3′) and BLOI2_0636 rev (5′-CTCGGTCTTGAACTGTTCGA-3′) or BBR14_0962 fw (5′-GTCTCCTCTACCCGAACCT) and BLOI2_0636 rev (5′-TCCTCGTTGATCCAATCCTC-3′). Each PCR mixture (25 μl) contained 1.5 mM $MgCl_2$, 20 mM Tris-HCl, 50 mM KCl, 200 μM each deoxynucleoside triphosphate, 25 pmol of each of the two primers, 1 U of *Taq* DNA polymerase (*Taq* PCR master mix kit; Qiagen, United Kingdom), and 50 ng of DNA template. Each PCR cycling program consisted of an initial denaturation step of 3 min at 94°C, followed by amplification for 35 cycles as follows: denaturation (30 s at 94°C), annealing (30 s at 56.5°C), and extension (1 min at 72°C). The PCR was completed with a single elongation step (10 min at 72°C). The resulting amplicons were separated on a 0.8% agarose gel, followed by ethidium bromide staining.

16S rRNA gene-based microbiota analysis. The fastq files were processed using QIIME (18) as previously described (17). Quality control retained sequences with a length between 140 and 400 bp and a mean sequence quality score of >25, with truncation of a sequence at the first base if a low-quality rolling 10-bp window was found. Sequences with mismatched primers were omitted.

Shotgun metagenomics. Sequencing libraries for samples SS2_Infant and SS4_Infant were prepared starting from the extracted DNA, using the Illumina Nextera-XT DNA kit (Illumina Inc., San Diego, CA, USA) according to the manufacturer's instructions; pooled; and sequenced on the Illumina HiSeq-2000 platform (100-bp paired end). We generated a total of 673,922 raw paired-end reads for the SS2_Infant samples and 4,950,848 raw paired-end reads for the SS4_Infant samples.

Analysis of metagenomic data sets. The fastq outputs were filtered for reads with quality of <25 and presence of human DNA, as well as reads of <80 bp. Bases were also removed from the ends of the reads until the average quality in a window of 5 bp was >25. Taxonomic classification of SS2_Infant and SS4_Infant reads was obtained using RapSearch2 software (19) for sequence homology in the National Center for Biotechnology Information (NCBI) nr database, followed by data processing using MEGAN5 software (20).

Genome reconstruction of bacterial strains from shotgun metagenomic data. Fastq files of quality- and alien DNA-filtered metagenomics data sets were used for metagenomic assembly with the spades assembler (21). The contigs obtained were subjected to open reading frame (ORF) prediction using Prodigal software (22) and to taxonomic classification using RapSearch2 software (19) for homology searches in the NCBI nr database, allowing identification and collection of contigs including at AQ: C least 40% of the ORFs attributed to a species of interest. These contigs were used as a backbone for mapping of the metagenomic reads. Reads that successfully mapped were collected in a fastq file and used as input for assembly with MIRA software (23). Generated contigs encompassing at least 40% of the ORFs attributed to a species of interest and with coverage diverging less than 33% from the average were then ordered based on a reference genome. Protein-encoding ORFs were predicted using Prodigal (22), and assignment of protein function to predicted coding regions was performed using a custom script based on RapSearch2 software (19), the PFAM database (24), and the nonredundant protein database provided by the National Center for Biotechnology Information. Additional bioinformatics analyses included tRNA gene identification using tRNAscan-SE (25), rRNA gene detection using RNAmmer (http://www.cbs.dtu.dk/services/RNAmmer/), and insertion sequence (IS) family detection using AQ: D ISFinder (https://www-is.biotoul.fr/). Attribution of ORFs to a specific AQ: E cluster of orthologous genes (COG) family was performed by searching against the EggNog database (26). Prediction of ORFs putatively involved

in horizontal gene transfer (HGT) events was performed with Colombo software with the sensitivity value set at 0.9 in order to ensure maximum sensitivity.

Targeted genome sequencing and bioinformatics analyses. DNA extracted from the bifidobacterial isolates was subjected to whole-genome sequencing (WGS) using MiSeq (Illumina, United Kingdom) at GenProbio srl (Parma, Italy) following the supplier's protocol (Illumina, United Kingdom). Fastq files obtained from targeted genome sequencing of the isolated strains were used as input for assembly with MIRA software (23). Protein-encoding ORFs were predicted using Prodigal (22), and assignment of protein function to predicted coding regions was performed using a custom script based on RapSearch2 software (19), the PFAM database (24), and the nonredundant protein database provided by the National Center for Biotechnology Information. Whole-genome alignments between contigs obtained from assembly of metagenomics and isolated data sets were obtained using MAUVE software (27) with the metagenomic genome as a reference. Average nucleotide identity (ANI) values based on BLAST and mummer software were calculated using JSpecies (28).

Pangenome and extraction of shared and unique genes. For all *B. breve* and *B. longum* genomes used in this study, a pangenome calculation was performed using the PGAP pipeline (29). The ORF content of all genomes was organized in functional gene clusters using the GF (gene family) method involving comparison of each protein against all other proteins using BLAST analysis (cutoff, an E value of 1×10^{-4} and 50% identity over at least 50% of both protein sequences), followed by clustering into protein families using MCL (a graph theory-based Markov clustering algorithm) (30). Pangenome profiles were built using all possible BLAST combinations for each genome that was sequentially added. Following this, the unique protein families for each of the analyzed bifidobacterial genomes were classified. Protein families shared between all genomes were defined by selecting the families that contained at least one single protein member for each genome. Each set of orthologous proteins constituting core COGs with one member per genome were aligned using MAFFT (31), and phylogenetic trees were constructed using the neighbor-joining method in Clustal W version 2.1 (32). The supertree was built using FigTree (http://tree.bio.ed.ac.uk/software/figtree/).

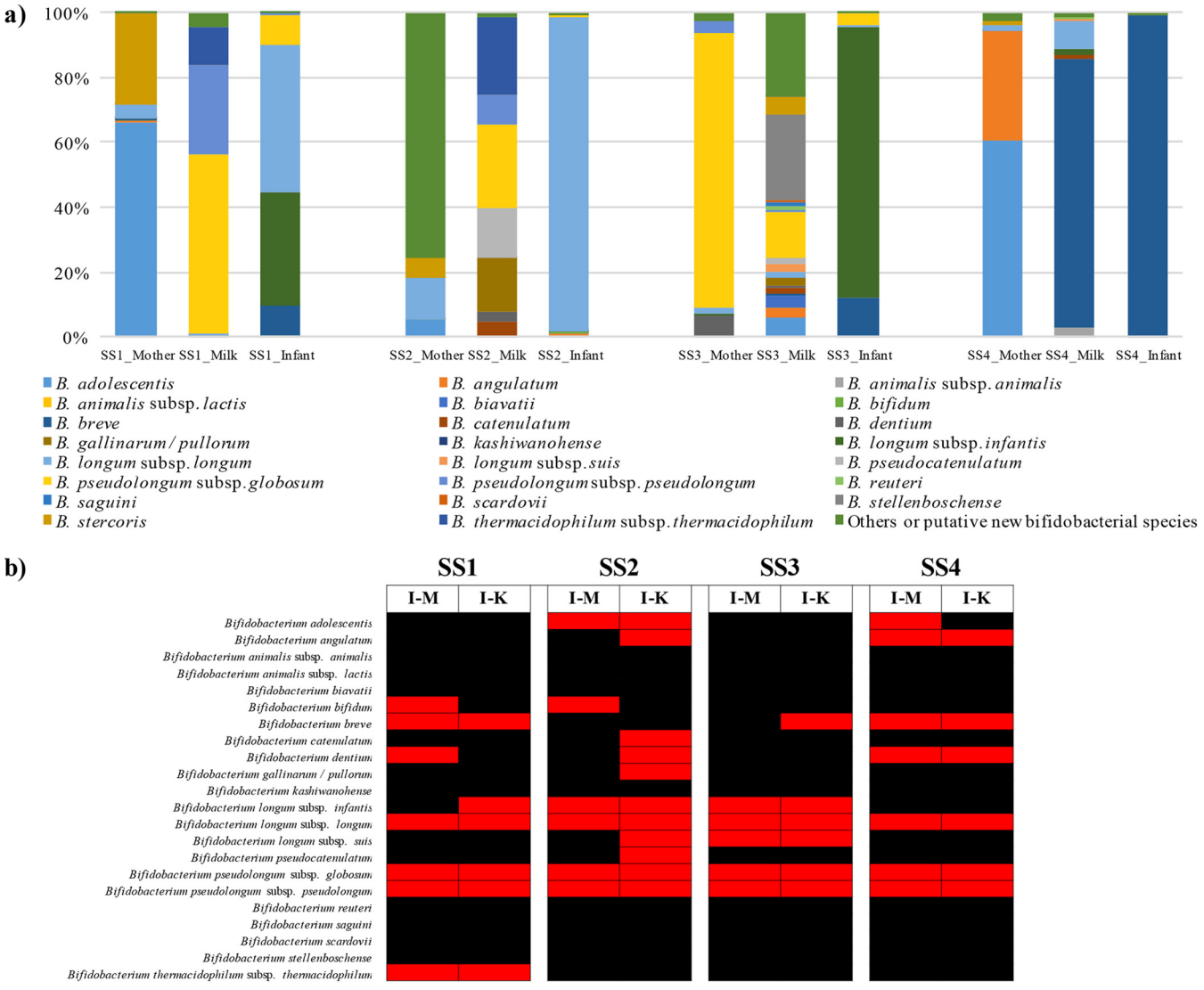
Nucleotide sequence accession numbers. The assembled *B. breve* BBRI4 and *B. longum* subsp. *longum* BLOI2 genomes were deposited in GenBank under accession numbers LFII00000000 and LFDU00000000, respectively. WGS data for SS2_Infant and SS4_Infant, as well as bifidobacterial ITS-profiling and 16S rRNA gene-profiling data for SS1_T6m, SS2_T6m, SS3_T6m, and SS4_T6m sample sets, are accessible through Sequence Read Archive (SRA) study accession number SRP059631.

RESULTS AND DISCUSSION

Characteristics of the subjects included in the study. The work described here represents a pilot study to determine the optimal experimental conditions for a much larger metagenomic survey, which is aimed at exploring the gut microbial biodiversity in infants and the corresponding mothers. The current study enrolled four mother-infant pairs, which were selected based on the following criteria: the subjects were in good health, had not been prescribed antibiotics, and were not taking any probiotics. Furthermore, since we were interested in evaluating the compositions of bifidobacterial populations occurring in the guts of mother-child pairs, we included only mothers who were exclusively or in large part breastfeeding their newborns and babies that had been vaginally delivered, conditions that were previously shown to be associated with the highest densities of bifidobacteria in infants (4).

Evaluation of the bifidobacterial population in the gut/milk microbiota of mother-newborn pairs. We assessed the composition of the fecal/milk microbiota of four corresponding mother-infant sets, SS1 to SS4, using a previously described bifidobacterial ITS-profiling approach (16). As outlined in Fig. 1, bifidobacterial ITS profiling of fecal samples from the mother and the corresponding infant revealed the presence of representatives of the genus *Bifidobacterium*. Notably, members of the species *Bifidobacterium adolescentis*, *Bifidobacterium angulatum*, *B. bifidum*, *B. breve*, *Bifidobacterium dentium*, *B. longum*, *Bifidobacterium pseudolongum*, and *Bifidobacterium thermacidophilum* were commonly detected in fecal samples from both mothers and infants (Fig. 1). As previously suggested, human milk may represent a medium for bacterial transmission from mother to newborn (33). Thus, the bifidobacterial microbiota of human milk samples retrieved from each mother was also evaluated by ITS-profiling analyses. As shown in Fig. 1, we observed the presence of members of the genus *Bifidobacterium* in the bacterial communities residing in these milk samples, while comparing the data with those obtained from the mother and infant fecal samples revealed the common presence of *B. adolescentis*, *B. angulatum*, *B. breve*, *B. dentium*, *B. longum*, *B. pseudolongum*, and *B. thermacidophilum*.

In order to identify the possible presence of the same or a similar strain in a mother and/or milk sample and a corresponding infant fecal sample, for each ITS data set, we compared the reference sequences of all predicted ITS OTUs, i.e., clusters of identical sequences generated by the ITS-profiling analysis protocol (16). Since the ITS sequence is highly variable, the retrieval from different samples of ITS sequences displaying 100% identity would be indicative of identical or a very closely related strains being present in such samples (16). Interestingly, OTUs shared by an entire sample set ranged from 63 in SS3 to 4 in SS4, for a total of 126 OTUs, as shown in Fig. 2. These OTUs encompass members of the species *B. adolescentis*, *B. angulatum*, *B. breve*, *B. longum*, and *B. pseudolongum*, as well as a putative novel bifidobacterial species (Fig. 2), suggesting a crucial role of these bacterial species in microbiota transmission and colonization of the newborn gut. Additionally, of these 126 shared OTUs, 28 are present in high relative abundance (among the 100 most represented OTUs) in at least one sample set and thus are relevant in defining the overall bifidobacterial community (Fig. 2). Furthermore, we observed that in infant fecal samples from SS2 and SS4, the bifidobacterial population appears to predominantly (97.1 and 99.4%, respectively) consist of OTUs that belong to *B. longum* subsp. *longum* and *B. breve*, respectively (Fig. 1). Interestingly, the OTU corresponding to the most represented *B. longum* subsp. *longum* strain in infant sample SS2, named SS2_Infant_515, was also identified in the corresponding fecal and milk samples from the mother (Fig. 2). The OTU corresponding to the most represented *B. breve* strain in infant sample SS4, named SS4_Infant_617, was also identified in the mother's milk sample, but not in the mother's fecal sample, possibly due to its low abundance, which fell below the bifidobacterial ITS-profiling limit of detection of 10E4 CFU/g (16) (Fig. 2). These results indicate that infant fecal samples from SS2 and SS4 may be good candidates for targeted bifidobacterial genome reconstruction (see below). Despite the existence of common bifidobacterial OTUs between the investigated mother-child pairs, there were also various bifidobacterial OTUs that appear to be uniquely present in the data set of the infant and not in that of the corresponding mother (e.g., SS1 and SS3 data sets). This may be due to the acquisition of such bacteria from the environment (e.g., during birth or from siblings) or due to the presence of these microorganisms below the limit of detection in the fecal samples from the mother.



COLOR

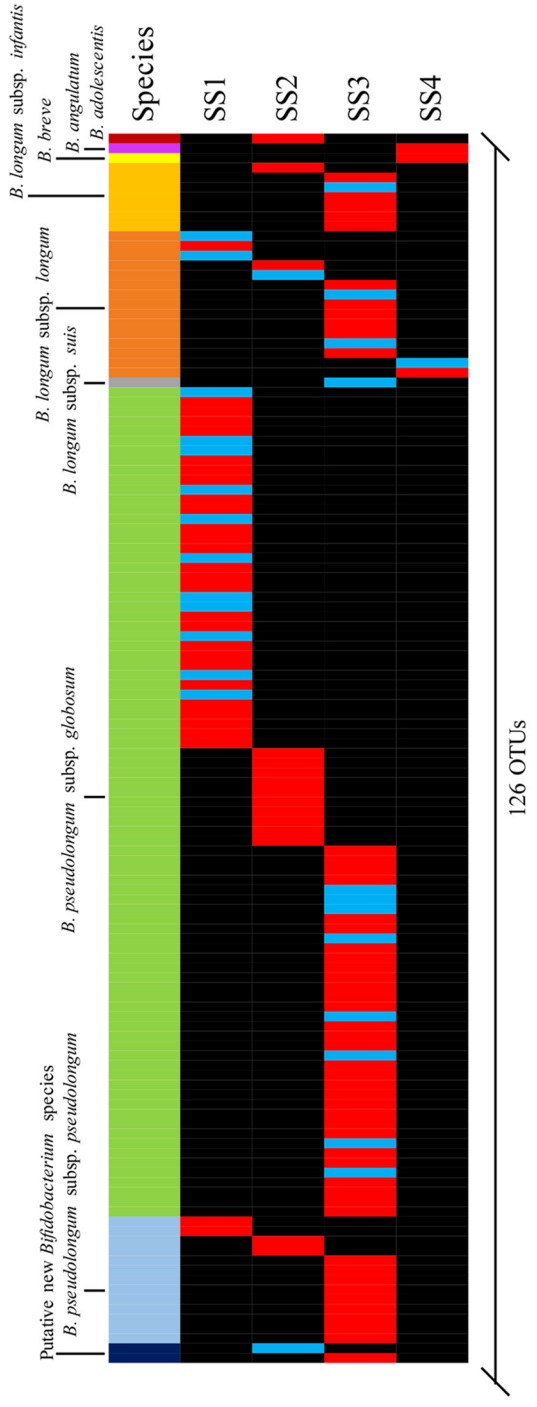
FIG 1 Bifidobacterial ITS profiling of four sample sets, each encompassing the fecal and milk samples from a mother and a fecal sample from the corresponding infant. (a) Bar plot of the identified bifidobacterial populations in the 12 analyzed samples. (b) Heat map of bifidobacterial species that appeared to be present in both the infant's fecal sample and the mother's fecal sample (I-M) and/or present in the infant's fecal sample and the mother's milk sample (I-K). Red shading represents presence, and black shading indicates absence.

AQ: N

Notably, comparison of the OTUs identified in the data sets analyzed (SS1, SS2, SS3, and SS4) revealed the presence of identical ITS OTUs in different sample sets. This observation implies that these identical ITS sequences correspond to very closely related strains that are present in different mother-infant pairs or that the ITS profiling is unable to accurately distinguish isolates below the species/subspecies level. Thus, these findings need to be confirmed through the use of a more robust method to distinguish intraspecies relationships, such as the use of multilocus sequencing or shotgun metagenomics.

Genome reconstruction of bacterial strains from shotgun metagenomic data. In order to validate and precisely map the occurrence of potentially identical bifidobacterial strains in the microbiome of a mother's fecal sample and that of a corresponding sample from infant feces and/or from breast milk, an in-depth shotgun metagenomics analysis was applied to the SS4 and SS2 infant samples. Taxonomic assignment (based on publicly available genomic data) of shotgun metagenomic reads obtained from the SS4 infant fecal sample showed that 62.92% of these reads were classified as bifidobacterial DNA, 69% of which was taxonomically assigned to the species *B. breve*, confirming what had previously been observed by means of 16S rRNA gene profiling (16) (Fig. 3). These data are in good agreement with the ITS-profiling F3 results, which indicate high prevalence of a putative strain in the fecal sample corresponding to *B. breve* OTU SS4_Infant_617, representing 66.8% of the total collected ITS sequences, as well as 67.2% of the reads constituting OTUs clustering together with the *B. breve* reference ITS sequence (see Fig. S1 in the supplemental material). Thus, the SS4 infant metagenomic data set was exploited in order to develop an optimized bioinformatics protocol for the reconstruction of a bacterial genome from shotgun metagenomics data (see the text in the supplemental material). Such a AQ: J

a)



b)

	SS1_Mother	SS1_Milk	SS1_Infant	
SS1_Infant_249		SS1_Milk_114	SS1_Infant_249	G
SS1_Infant_419			SS1_Infant_419	G
SS1_Infant_674	SS1_Mother_3131	SS1_Milk_296	SS1_Infant_674	H
SS1_Infant_832			SS1_Infant_832	C
SS1_Infant_1559	SS1_Mother_2064	SS1_Milk_654	SS1_Infant_1559	H
SS1_Milk_209		SS1_Milk_209	SS1_Infant_1113	L
SS1_Milk_495	SS1_Mother_8601	SS1_Milk_495	SS1_Infant_1520	L
SS1_Milk_651	SS1_Mother_4254	SS1_Milk_651	SS1_Infant_1558	L
SS1_Milk_812		SS1_Milk_812		L
SS1_Milk_1153	SS1_Mother_3930	SS1_Milk_1153	SS1_Infant_1304	L
SS1_Mother_324	SS1_Mother_324			P
SS1_Mother_633	SS1_Mother_633			P
SS1_Mother_1733	SS1_Mother_1733	SS1_Milk_1141		A
SS1_Mother_2423	SS1_Mother_2423	SS1_Milk_306		A
SS1_Mother_7363	SS1_Mother_7363			A

SS1

	SS2_Mother	SS2_Milk	SS2_Infant	
SS2_Infant_502	SS2_Mother_1116		SS2_Infant_502	H
SS2_Infant_515	SS2_Mother_2967	SS2_Milk_3273	SS2_Infant_515	H
SS2_Infant_744	SS2_Mother_1648		SS2_Infant_744	H
SS2_Infant_1201	SS2_Mother_2607		SS2_Infant_1201	H
SS2_Infant_1218	SS2_Mother_2644		SS2_Infant_1218	H
SS2_Milk_1107		SS2_Milk_1107		N
SS2_Milk_1184		SS2_Milk_1184		I
SS2_Milk_1987		SS2_Milk_1987		N
SS2_Milk_2462		SS2_Milk_2462		F
SS2_Milk_3241		SS2_Milk_3241		I
SS2_Mother_401	SS2_Mother_401		SS2_Infant_166	O
SS2_Mother_915	SS2_Mother_915		SS2_Infant_398	A
SS2_Mother_995	SS2_Mother_995	SS2_Milk_1082	SS2_Infant_438	O
SS2_Mother_1993	SS2_Mother_1993		SS2_Infant_271	O
SS2_Mother_2468	SS2_Mother_2468	SS2_Milk_2844	SS2_Infant_1144	A

SS2

	SS3_Mother	SS3_Milk	S4_Infant	
SS3_Infant_110	SS3_Mother_1380	SS3_Milk_1188	SS3_Infant_110	G
SS3_Infant_192	SS3_Mother_1425		SS3_Infant_192	G
SS3_Infant_703	SS3_Mother_2327	SS3_Milk_2029	SS3_Infant_703	G
SS3_Infant_809		SS3_Milk_1519	SS3_Infant_809	C
SS3_Infant_1080		SS3_Milk_2008	SS3_Infant_1080	C
SS3_Milk_71	SS3_Mother_91	SS3_Milk_71		L
SS3_Milk_138	SS3_Mother_178	SS3_Milk_138		A
SS3_Milk_187		SS3_Milk_187		M
SS3_Milk_281		SS3_Milk_281		D
SS3_Milk_1073		SS3_Milk_1073		A
SS3_Mother_534	SS3_Mother_534	SS3_Milk_443	SS3_Infant_919	L
SS3_Mother_570	SS3_Mother_570	SS3_Milk_474		E
SS3_Mother_1457	SS3_Mother_1457	SS3_Milk_1251	SS3_Infant_265	L
SS3_Mother_1716	SS3_Mother_1716	SS3_Milk_1215	SS3_Infant_816	L
SS3_Mother_1731	SS3_Mother_1731	SS3_Milk_1492	SS3_Infant_129	L

SS3

	SS4_Mother	SS4_Milk	SS4_Infant	
SS4_Infant_177		SS4_Milk_382	SS4_Infant_177	C
SS4_Infant_548		SS4_Milk_11	SS4_Infant_548	C
SS4_Infant_617		SS4_Milk_563	SS4_Infant_617	C
SS4_Infant_754		SS4_Milk_453	SS4_Infant_754	C
SS4_Infant_805		SS4_Milk_552	SS4_Infant_805	C
SS4_Milk_11		SS4_Milk_11	SS4_Infant_548	C
SS4_Milk_281	SS4_Mother_2277	SS4_Milk_281		H
SS4_Milk_453		SS4_Milk_453	SS4_Infant_754	C
SS4_Milk_552		SS4_Milk_552	SS4_Infant_805	C
SS4_Milk_563		SS4_Milk_563	SS4_Infant_617	C
SS4_Mother_260	SS4_Mother_260		SS4_Infant_846	A
SS4_Mother_1570	SS4_Mother_1570	SS4_Milk_604	SS4_Infant_830	B
SS4_Mother_2277	SS4_Mother_2277	SS4_Milk_281		H
SS4_Mother_2972	SS4_Mother_2972			A
SS4_Mother_3694	SS4_Mother_3694	SS4_Milk_796		A

SS4

A *B. adolescentis*
B *B. angulatum*
C *B. breve*
D *B. callitrichos*
E *B. dentium*
F *B. gallinarum* / *B. pullorum*
G *B. longum* subsp. *infantis*
H *B. longum* subsp. *longum*
I *B. pseudocatenulatum*
L *B. pseudolongum* subsp. *globosum*
M *B. stellenboschense*
N *B. thermacidophilum* subsp. *thermacidophilum*
O Putative new *Bifidobacterium* species
P Unknown *Bifidobacteriaceae* member

COLOR

protocol allows the reconstruction of a final consensus genome sequence that can be entirely attributed to the *B. breve* strain being present in higher abundance in the shotgun metagenomics data set (see the supplemental material for details). The genome obtained, corresponding to OTU SS4_Infant_617, was named *B. breve* BBRI4. Notably, the ITS sequence of *B. breve* BBRI4 showed 100% identity to ITS OTU SS4_Infant_617.

The developed pipeline (see the text in the supplemental material) was also applied to the shotgun-sequencing reads achieved from the *Bifidobacterium*-rich SS2 infant fecal sample. Taxonomic classification of this data set determined that 57.4% of the microbiome reads obtained were assignable to bifidobacterial DNA, 69.8% of which were taxonomically annotated as a *B. longum* species, as previously noted at the genus level by 16S rRNA gene profiling (16) (Fig. 3). Furthermore, ITS profiling demonstrated high prevalence of OTU SS2_Infant_515, corresponding to a putative *B. longum* subsp. *longum* strain named BLOI2 (see Fig. S1 in the supplemental material), which represents 69.9% of the total bifidobacterial community and 72.1% of the predicted *B. longum* subsp. *longum* population in this particular sample.

The final assembly of the *B. breve* BBRI4 genome consisted of 14 contigs with a total length of 2,411,812 bp, while chromosome reconstruction based on shotgun metagenomics data for *B. longum* subsp. *longum* BLOI2 allowed the retrieval of 72 contigs, corresponding to a chromosome of 2,417,590 bp. In both cases, the final number of contigs generated was comparable to that achieved when attempting targeted genome sequencing (34–36).

Comparative genomics of *B. breve* BBRI4 with 16 publicly available *B. breve* genomes led to prediction of a *B. breve* pangenome encompassing 3,889 *B. breve*-specific COGs (Bb-COGs), 1,203 of which are shared by all the analyzed *B. breve* strains, thus constituting the core genome of the species. These results are consistent with observations from a previous study encompassing 13 *B. breve* genomes (37). In addition, 82 Bb-COGs represented the truly unique genes (TUG) of *B. breve* BBRI4 (see Fig. S2 in the supplemental material). Similarly, 30 genomes, encompassing all publicly available *B. longum* genome sequences, were used in order to identify the *B. longum* pangenome, which consists of 6,077 *B. longum*-specific COGs (Bl-COGs), 827 of which appear to be shared by all 31 genomes analyzed, thus representing the core genome. The TUG of *B. longum* subsp. *longum* BLOI2 comprises 121 Bl-COGs (see Fig. S3 in the supplemental material). Additional information regarding general genome features, as well as comparative genomics data, for *B. breve* BBRI4 and *B. longum* subsp. *longum* BLOI2 can be found in the supplemental material.

The chromosomes of *B. breve* BBRI4 and *B. longum* subsp. *longum* BLOI2 were screened for genes putatively acquired through HGT events by employing Colombo software (38) set at the maximum sensitivity and low specificity. This *in silico* analysis predicted that 76.3% of the *B. breve* BBRI4 ORFeome and 72.8% of the *B. longum* subsp. *longum* BLOI2 ORFeome was not acquired by HGT events. Notably, among the BBRI4 genes that had not been predicted to have been acquired by HGT events, we identified 23 ORFs, which are also encompassed by the TUG of *B. breve* strain BBRI4. These ORFs represent optimal marker genes for *B. breve* strain BBRI4. Furthermore, of the 1,469 *B. longum* subsp. *longum* BLOI2 ORFs putatively not involved in HGT events, 34 were observed to be unique to the BLOI2 strain by comparative genomic analyses, thus representing optimal marker genes for identification of the strain.

Isolation and genome sequencing of *B. breve* BBRI4 and *B. longum* subsp. *longum* BLOI2 strains. In order to validate the reconstruction of *B. breve* BBRI4 and *B. longum* subsp. *longum* BLOI2 genomic data from microbiomic data sets, we decided to isolate these strains from fecal samples of the SS4 and SS2 infants, respectively, and to subject the isolated strain to targeted genome sequencing. The *B. breve* BBRI4 and *B. longum* subsp. *longum* BLOI2 strains were isolated using a selective bifidobacterial medium (15). Selected colonies were identified by PCR amplification and subsequent sequencing of the amplicons corresponding to the ITS and marker genes specific to *B. breve* BBRI4 and *B. longum* subsp. *longum* BLOI2 (see above). Two positively verified isolates (i.e., one isolate of *B. breve* BBRI4 and one of *B. longum* subsp. *longum* BLOI2) were then subjected to whole-genome sequencing employing the Illumina MiSeq platform. Analysis of the assembled genome sequences was performed utilizing the identical pipeline that had been applied for genome reconstruction using the shotgun metagenomics data sets (see above). The genome sequencing of the *B. breve* BBRI4 isolate allowed the identification of 96 contigs with an average coverage of 37, including 1,935 ORFs, while the chromosome of the *B. longum* subsp. *longum* BLOI2 isolate encompasses 77 contigs with an average coverage of 51.86, including 2,047 ORFs. These genomes were used to evaluate the quality of the corresponding assembly obtained from shotgun metagenomics reads. Alignment of the *B. breve* BBRI4 and *B. longum* subsp. *longum* BLOI2 chromosomes achieved by targeted genome sequencing with those obtained from reconstruction by shotgun metagenomics data sets revealed a syntenic structure and very limited differences (see below), most likely attributable to DNA-sequencing mistakes (see Fig. S4 in the supplemental material). Moreover, the average nucleotide identity based on mummer alignments (ANIm) between the genome sequences achieved by targeted genome sequencing or by microbiomic data sets for *B. breve* BBRI4 and *B. longum* subsp. *longum* BLOI2 revealed identities ranging from 99.91% to 99.92% (99.96% and 100.00% in the case of BLAST-based ANI), respectively. These data indicate the reliability of the metagenomic assembly protocol for accurate reconstruction of targeted bacterial genomes. In order to further confirm the reliability of the genomic data reconstructed from microbiomic data sets, we compared the predicted marker genes for *B. breve* BBRI4 and *B. longum* subsp. *longum* BLOI2 using genome sequencing data for both strains. Notably, the predicted marker genes of these isolates show an identity value at the nucle-

FIG 2 Distribution of computed ITS OTUs in the four analyzed sample sets. (a) The 126 ITS OTUs identified as being present in an entire sample set (e.g., present in the mother's fecal and milk samples, as well as in the infant's fecal sample). Red indicates the presence of a specific OTU in the three samples of a given set; light blue is used to highlight those OTUs that were among the 100 most represented in at least two members of a sample set. OTUs are also clustered by taxonomic assignment and colored accordingly in the leftmost column of the heat map. (b) Heat map illustrating the presence of the five most abundant OTUs of each analyzed sample and their presence or absence in the other two samples of the corresponding set, accompanied by their taxonomic assignments. The most represented OTUs in the infant samples of SS2 and SS4 are highlighted in blue and green, respectively.

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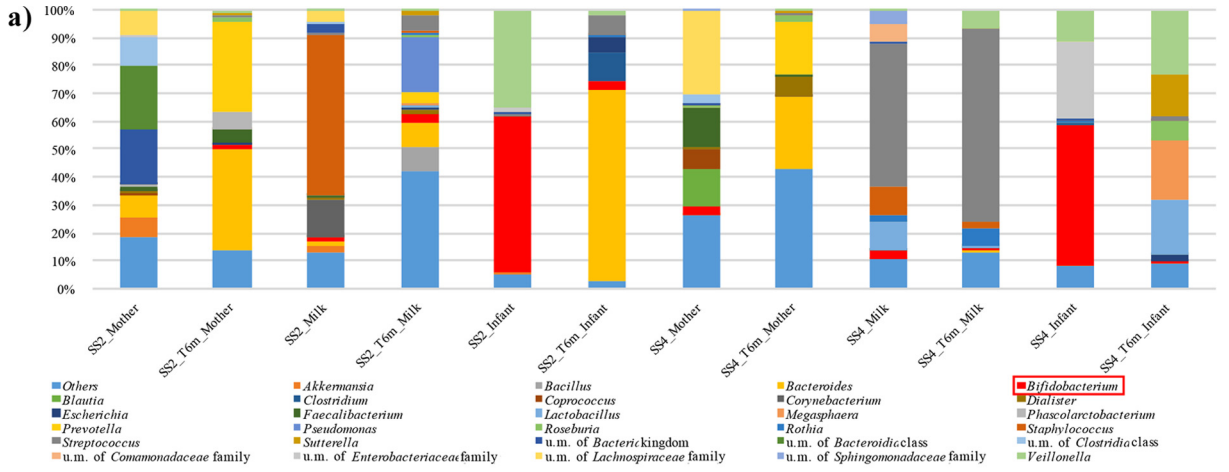

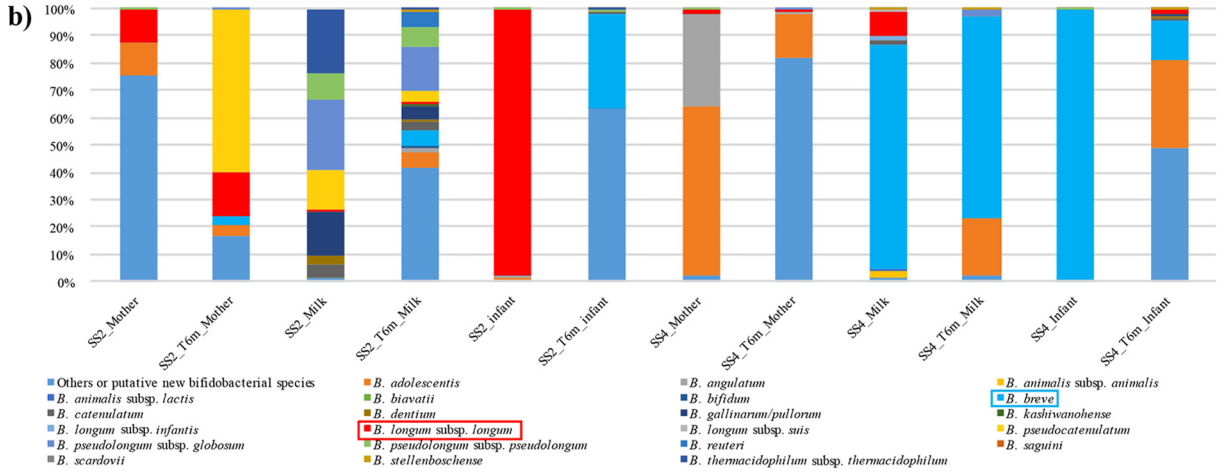

c)

SS2			SS2_T6m			
M2	K2	I2	M2_T6m	K2_T6m	I2_T6m	
SS2_Infant_96	SS2_Milk_241	SS2_Infant_96		SS2_T6m_Milk_210	SS2_T6m_Infant_256	A
SS2_Infant_316	SS2_Mother_731	SS2_Infant_316				D
SS2_Infant_408	SS2_Mother_718	SS2_Infant_408				D
SS2_Infant_502	SS2_Mother_1116	SS2_Infant_502				D
SS2_Infant_515	SS2_Mother_2967	SS2_Milk_3273	SS2_Infant_515	SS2_T6m_Mother_2457		D
SS2_Infant_590	SS2_Mother_1318		SS2_Infant_590			D
SS2_Infant_595	SS2_Milk_1436	SS2_Infant_595		SS2_T6m_Milk_198		B
SS2_Infant_656	SS2_Mother_1458	SS2_Infant_656				D
SS2_Infant_744	SS2_Mother_1648	SS2_Infant_744				D
SS2_Infant_879	SS2_Mother_1938	SS2_Infant_879				D
SS2_Infant_1096	SS2_Mother_2375	SS2_Infant_1096				D
SS2_Infant_1201	SS2_Mother_2607	SS2_Infant_1201				D
SS2_Infant_1218	SS2_Mother_2644	SS2_Infant_1218	SS2_T6m_Mother_2218			D
SS2_Infant_1344	SS2_Mother_924	SS2_Infant_1344				D
SS2_Infant_1506	SS2_Mother_3280	SS2_Infant_1506				D

SS4			SS4_T6m			
M4	K4	I4	M4_T6m	K4_T6m	I4_T6m	
SS4_Infant_177	SS4_Milk_382	SS4_Infant_177		SS4_T6m_Milk_121	SS4_T6m_Infant_346	C
SS4_Infant_533	SS4_Milk_1065	SS4_Infant_533		SS4_T6m_Milk_341	SS4_T6m_Infant_1881	C
SS4_Infant_548	SS4_Milk_11	SS4_Infant_548		SS4_T6m_Milk_3	SS4_T6m_Infant_1013	C
SS4_Infant_550	SS4_Milk_16	SS4_Infant_550		SS4_T6m_Milk_5	SS4_T6m_Infant_1017	C
SS4_Infant_617	SS4_Milk_563	SS4_Infant_617		SS4_T6m_Milk_183	SS4_T6m_Infant_2452	C
SS4_Infant_660	SS4_Milk_246	SS4_Infant_660			SS4_T6m_Infant_1205	C
SS4_Infant_754	SS4_Milk_453	SS4_Infant_754		SS4_T6m_Milk_146	SS4_T6m_Infant_1381	C
SS4_Infant_805	SS4_Milk_552	SS4_Infant_805		SS4_T6m_Milk_175	SS4_T6m_Infant_1066	C
SS4_Infant_980	SS4_Milk_710	SS4_Infant_980		SS4_T6m_Milk_235	SS4_T6m_Infant_1818	C
SS4_Infant_1101	SS4_Milk_53	SS4_Infant_1101		SS4_T6m_Milk_17	SS4_T6m_Infant_2034	C
SS4_Infant_1242	SS4_Milk_346	SS4_Infant_1242		SS4_T6m_Milk_110	SS4_T6m_Infant_2270	C
SS4_Infant_1244	SS4_Milk_350	SS4_Infant_1244		SS4_T6m_Milk_112	SS4_T6m_Infant_1894	C
SS4_Infant_1343	SS4_Milk_556	SS4_Infant_1343		SS4_T6m_Milk_177	SS4_T6m_Infant_2447	C
SS4_Infant_1900	SS4_Milk_598	SS4_Infant_1900		SS4_T6m_Milk_196	SS4_T6m_Infant_1226	C
SS4_Infant_2061	SS4_Milk_923	SS4_Infant_2061		SS4_T6m_Milk_23	SS4_T6m_Infant_3794	C

A *B. adolescentis* B *B. angulatum* C *B. breve* D *B. longum* subsp. *longum*

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otide level of 100% between the gene sets retrieved from targeted genome attempts and microbiomic data sets, thus confirming the precise identification of *B. breve* BBRI4 and *B. longum* subsp. *longum* BLOI2.

Assessing the gut persistence of *B. breve* BBRI4 and *B. longum* subsp. *longum* BLOI2 during the life spans of infants. In order to assess whether the strains *B. breve* BBRI4 and *B. longum* subsp. *longum* BLOI2 were maintained in the guts of the SS4 and SS2 infants, respectively, at later stages of life, we collected additional fecal samples from these infants 6 months (SS2_T6m and SS4_T6m) after the initial sample collection, along with fecal and milk samples from the mothers. These samples were assayed by bifidobacterial ITS profiling (16) (Fig. 3). Interestingly, comparison of the results from the 16S rRNA gene profiling of SS2_T6m and SS4_T6m with taxonomy profiles of SS2 and SS4 (16) highlighted a marked decrease in the relative abundances of phylotypes belonging to the genus *Bifidobacterium*, especially in the infant fecal samples, with reduction from 56.4% in SS2 to 2.9% in SS2_T6m and reduction from 50.8% in SS4 to 0.5% in SS4_T6m (Fig. 3). This observation is indicative of microbiota evolution toward that typical of adult human beings as a result of dietary changes (39). Furthermore, bifidobacterial ITS profiling allowed the identification of nine bifidobacterial species that appear to be shared by the three samples constituting SS2_T6m and 5 bifidobacterial species shared by the three samples constituting SS4_T6m. Notably, *B. adolescentis*, *B. longum* subsp. *longum*, and *B. pseudolongum*, which are found in these data sets, were also shared by all samples constituting SS2 and SS4 (Fig. 1 and 3). These findings suggest high persistence of these human gut-adapted bifidobacterial species in the guts of the infants and the corresponding mothers, as well as in mother's milk. In addition, a manual search in the T6m sample sets for the presence of the 15 most abundant OTUs in SS2_Infant and SS4_Infant samples showed that 1 and 15 OTUs were still present in SS2_T6m and in SS4_T6m, respectively (Fig. 3). Interestingly, ITS OTU SS2_Infant_515, corresponding to *B. longum* subsp. *longum* BLOI2, was detected in the SS2_T6m_Mother sample but not in the SS2_T6m_Milk and SS2_T6m_Infant data sets (Fig. 3). This observation was verified by PCR using strain-specific primer pairs based on a predicted marker gene of *B. longum* subsp. *longum* BLOI2 encoding an ATPase domain-containing protein (BLOI2_0636) (see Fig. S1 in the supplemental material). The primers were tested for specificity using the NCBI tool Primer-BLAST and the NCBI nt database containing all the bacterial genomes sequenced so far. Notably, these results allowed the identification of this strain in all samples constituting both SS2 and SS2_T6m (see Fig. S5 in the supplemental material), suggesting that the prevalence of the BLOI2 strain was below 10E4 CFU/g, which is the limit of detection previously estimated by the bifidobacterial ITS-profiling approach in the infant fecal and milk samples of SS2_T6m (16). Notably, ITS OTU SS4_Infant_617, corresponding to *B. breve* BBRI4, was still present in the mother's milk and infant fecal sample of SS4_T6m. Validation through PCR using strain-specific primer pairs based on a predicted marker gene of *B. breve* BBRI4, encoding a putative solute-binding component of a dipeptide ABC transporter (BBRI4_0962), confirmed the persistence after 6 months of this strain in these two samples but its absence (or presence below detection level) in the mother's gut (see Fig. S5 in the supplemental material). These observations underline the importance of initial colonization of the newborn gut by bacterial strains that may be maintained during the infant's life span. However, due to the lack of stool samples collected at later time points, we cannot rule out the possibility that persistence of these bifidobacterial strains extends beyond 6 months or that it is restricted to relatively short time spans.

Furthermore, the mother's fecal microbiota was shown to have changed significantly at the 6-month follow-up time point, an observation confirming previous studies that had found high variability in the compositions of fecal microbiota during pregnancy and breastfeeding (40).

Conclusions. Vertical transmission of gut bacteria from mothers to their offspring is considered a pivotal route for microbiota establishment in newborns, although an in-depth evaluation of this process has not been performed. Here, we collected and analyzed four sample sets, each encompassing a mother's fecal and milk samples combined with a corresponding infant fecal sample. Bifidobacterial ITS profiling coupled with shotgun metagenomics analyses allowed the identification of a common bifidobacterial profile in the mother-infant pairs and revealed the presence of identical strains shared by these hosts. Such data suggest the existence of a microbiota transfer process that drives the acquisition and subsequent persistence of specific bacterial strains in the infant gut, which appears to be influenced by the mother's gut and breast milk microbiota. Bifidobacteria represent the dominant members of the gut microbiota of infants (4), and thus, it is not surprising to identify their occurrence in high numbers in the analyzed infant stool samples. However, it is interesting that even though bifidobacteria are generally poorly detectable in adult fecal samples (4, 15), despite the fact that they have been found to persist at high levels in some adult individuals (41), we were able to trace the occurrence of a specific strain in the guts of both mother and infant. One may argue that bifidobacteria, even when they decrease in relative abundance following weaning, persist following their initial transfer to the infant gut. The particular route(s) used for such a transfer is still unknown, although it has been speculated that transfer is facilitated by a human milk route (42, 43) and/or by contamination of fecal and vaginal samples in the partum canal (44). Very recently, the possibility of fetal colonization of the infant gut through the placenta has been put forward (45). Due to the small cohort of subjects investigated in this study, we cannot reach any statistically robust conclusions about the incidence of vertical transmission of the gut microbiota from mother to child in the human population. Nonetheless, our findings represent the first important molecular evidence of the existence of this route for the generation of the gut microbiota in the earlier stages of life.

FIG 3 Evaluation of gut microbiota persistence in SS2 and SS4. (a) Bar plot representing results from 16S rRNA gene profiling of SS2, SS2_T6m, SS4, and SS4_T6m at the genus level. Only taxa with relative abundances of >5% in at least one sample are shown. u.m., unclassified members. (b) The bifidobacterial taxonomic profiles in SS2, SS2_T6m, SS4, and SS4_T6m obtained through bifidobacterial ITS profiling. (c) Heat map showing persistence of bifidobacterial OTUs (putative bifidobacterial strains) in SS2 and SS4 after 6 months. The most represented OTUs in the infant samples of SS2 and SS4 are highlighted in blue and green, respectively.

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