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Research Article

RESEARCH ARTICLE

Investigating bifidobacteria and human milk oligosaccharide composition of lactating mothers

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One sentence summary: In the current study, we quantified the abundance of members of the Bifidobacterium genus in 163 human milk samples using a combination of metagenomic and flow cytometry approaches.

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ABSTRACT

Human milk is known to carry its own microbiota, of which the precise origin remains obscure. Breastfeeding allows mother-to-baby transmission of microorganisms as well as the transfer of many other milk components, such as human milk oligosaccharides (HMOs), which act as metabolizable substrates for particular bacteria, such as bifidobacteria, residing in infant intestinal tract. In the current study, we report the HMO composition of 249 human milk samples, in 163 of which we quantified the abundance of members of the *Bifidobacterium* genus using a combination of metagenomic and flow cytometric approaches. Metagenomic data allowed us to identify four clusters dominated by *Bifidobacterium adolescentis* and *Bifidobacterium pseudolongum*, *Bifidobacterium crudilactis* or *Bifidobacterium dentium*, as well as a cluster represented by a heterogeneous mix of bifidobacterial species such as *Bifidobacterium breve* and *Bifidobacterium longum*. Furthermore, *in vitro* growth assays on HMOs coupled with *in silico* glycobiome analyses allowed us to elucidate that members of the *Bifidobacterium bifidum* and *B. breve* species exhibit the greatest ability to degrade and grow on HMOs. Altogether, these findings indicate that the bifidobacterial component of the human milk microbiota is not strictly correlated with their ability to metabolize HMOs.

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Keywords: *Bifidobacterium*; microbiota; human milk; HMOs

INTRODUCTION

Human milk has long been considered sterile (Andreas, Kampmann and Mehring Le-Doare 2015). However, recent studies have revealed that human milk does contain a complex bacterial community that impacts on the development of the gut microbiota of the corresponding nursling (Castanys-Munoz, Martin and Vazquez 2016; Milani *et al.* 2017). In order to explain the origin of milk microbiota, two routes have been proposed, encompassing the entero-mammary translocation of the maternal gut microbiota and inoculation by the infant's oral microbiota (Rodriguez 2014; Ruiz *et al.* 2019). Specifically, the presence of bacteria in colostrum collected before the first infant feeding supports the entero-mammary route, while the observation that pumped breastmilk has a different microbiota composition than that of directly breastfeeding mothers supports the hypothesis of retrograde inoculation by (elements of) the infant oral microbiota (Rodriguez 2014; Moossavi *et al.* 2019; Ruiz *et al.* 2019).

Human milk is the sole or main source of nutrients for many infants and, in addition to sugars and fats that fulfill the energy demands for infant development, it contains a range of components that provide complete nourishment and protection for the baby (Andreas, Kampmann and Mehring Le-Doare 2015). The latter include bioactive molecules such as immunoregulatory proteins, miRNA contained in extracellular vesicles, hormones, antimicrobial peptides, milk fat globule membranes, micronutrients and human milk oligosaccharides (HMOs). In human milk, HMOs are the third most abundant group of compounds after lactose and fat, at levels that vary from 5 to 23 g/L, with very substantial compositional variations being observed between women and across the lactation period (Ballard and Morrow 2013; Hong *et al.* 2014). HMOs are a structurally diverse set of oligosaccharides, which are produced in the mammary glands. Specifically, HMOs consist of a lactose backbone that is elongated into a variety of oligomeric structures by the addition of β1-3- or β1-6-linked lacto-*N*-biose (Galβ1-3GlcNAc-, type 1 chain) or *N*-acetyllactosamine (Galβ1-4GlcNAc-, type 2 chain) (Bode 2012; Plaza-Diaz, Fontana and Gil 2018). Moreover, HMOs are classified as neutral non-fucosylated [e.g. lacto-*N*-tetraose (LNT) and lacto-*N*-neotetraose (LNnT)] or fucosylated [e.g. 2′-fucosyllactose (2′FL) and 3-fucosyllactose (3'FL)], and acidic non-fucosylated [e.g. 3′-sialyllactose (3′SL) and 6′-sialyllactose (6′SL)] or fucosylated [e.g. FS-LNH, which corresponds to one Fuc and one NeuAc on a lacto-N-hexaose core] (Bode 2012; Plaza-Diaz, Fontana and Gil 2018).

Notably, humans lack the necessary enzymes, such as fucosidases and sialidases, to metabolize HMOs. Therefore, HMOs are digested by bacteria present in the intestinal microbiota, but only those which possess and express the required genetic repertoire for its metabolism, thus facilitating selection of specific gastrointestinal colonizers. Altogether these findings represent a prime example of host-microbe co-evolution in humans. In this context, bifidobacteria are among the first colonizers of the human gut and generally represent more than 50% (in many cases, more than 70%) of the total bacterial population in the gut of healthy, breast-fed infants (Gotoh *et al.* 2018). The notion that bifidobacteria represent key members of the infant gut microbiota has recently been supported by several studies that revealed the ability of particular bifidobacterial species, such as *Bifidobacterium bifidum*, *Bifidobacterium breve* and *Bifidobacterium longum* subsp. *infantis*, to metabolize (certain) HMOs (Sela *et al.* 2008; Turroni *et al.* 2010; James *et al.* 2016)G23.

Despite the importance of human milk as a microbial vector that drives colonization of the infant gut, the precise composition of milk microbiota, especially the bifidobacterial component, is not particularly well defined. For this reason, the aim of this study was to assess the bifidobacterial composition of human milk samples using the internal transcribed spacer (ITS) bifidobacterial profiling approach coupled with a flow cytometry approach, which allowed assessment of the absolute abundance of each bifidobacterial taxon. Altogether these analyses unveil the relationship between members of the *Bifidobacterium* genus and the different categories of HMOs. Furthermore, *in vitro* bifidobacterial growth assays on HMOs coupled with *in silico* glycobiome analyses elucidated the genetic adaptation of various members of the human milk bifidobacterial microbiome that enable them to metabolize various HMOs.

MATERIALS AND METHODS

Sample collection and HMOs classification

Each mother (Supplementary Table S2) provided one to three samples of milk at 1, 6 and/or 12 months postpartum in a sterile milk container (Larsson *et al.* 2019). Samples were stored at −80°C until analysis. HMOs were previously detected and measured in milk samples by means of high performance liquid chromatography with online coupled fluorescence detection and offline mass spectrometry (Larsson *et al.* 2019). This analysis allowed absolute quantification of 19 distinct HMOs that account for >90% of the estimated HMO within a given sample, i.e. 2′FL, 3FL, 3′SL, 6′SL, LNT, LNnT, lacto-*N*-fucopentaose (LNFPI, LNFPII and LNFPIII), sialyl-LNT (LSTb and LSTc), difucosyl-LNT (DFLNT), disialyl-LNT (DSLNT), fucosyl-lacto-*N*-hexaose (FLNH), difucosyl-lacto-*N*-hexaose (DFLNH), fucosyl-disialyl-lacto-*N*-hexaose (FDSLNH) and disialyl-lacto-*N*-hexaose (DSLNH), difucosyllactose (DFLac) and lacto-*N*-hexaose (LNH). Furthermore, the relative abundance of each HMO and the abundance of HMO-bound fucose and HMO-bound sialic acid were calculated (Supplementary Table S1).

Bacterial DNA extraction from human milk

Bacterial DNA was extracted from 1.8 mL breast milk using DNeasy PowerFood Microbial Kit following the manufacturer's protocol (QIAGEN, Hilden, Germany). Briefly, milk samples were centrifuged (13 000 x *g* for 1 min), fat was removed using a sterile swab and the supernatant was discarded using a pipet tip. Subsequently, pellets were resuspended in 450 µL of MBL buffer solution. The cells were resuspended in a Power Bead tube and were lysed by shaking the mix on a BioSpec homogenizer at 4°C for 2 min (maximum setting) three times. The mixture was then centrifuged at 13 000 x *g* for 1 min and the supernatant was transferred into a clean 2 ml tube. Next, 100 µL of IRS solution were added to samples and incubated at 2–8°C for 5 min. After incubation, the samples were centrifuged at 13 000 x *g* for 1 min and the supernatant was transferred into a new collection tube. At this point, 900 µL of MR solution was added. The entire volume of samples were loaded into a MB spin column. The DNA was purified using PW solution and ethanol solution

and finally 100 μL of water was added to elute the DNA. MBL, IRS, MR and PW buffers, as well as the MB spin column, were provided by QIAGEN as described in the DNeasy PowerFood Microbial Kit Handbook (10/2017).

16S rRNA gene-based microbial profiling

Partial 16S rRNA gene sequences were amplified from extracted DNA using the primer pair Probio_Uni/Probio_Rev, targeting the V3 region of the 16S rRNA gene sequence (Milani *et al.* 2013). 16S rRNA gene amplification and amplicon checks were carried out as previously described (Milani *et al.* 2013). A bacterial mock community was prepared, consisting of a pool of 25 different bacterial strains of known concentration prepared by combining equal volumes (20 μL) of DNA. 16S rRNA gene sequencing was performed using a MiSeq (Illumina, San Diego, CA, USA) at the DNA sequencing facility of GenProbio srl (www.genprobio.com) according to a previously reported protocol (Milani *et al.* 2013). Following sequencing, the .fastq files were processed using a custom script based on the QIIME software suite (Caporaso *et al.* 2010). Paired-end read pairs were assembled to reconstruct the complete Probio_Uni/Probio_Rev amplicons. Quality control retained sequences with a length of between 140 and 400 bp and mean sequence quality score >20 while sequences with homopolymers >7 bp and mismatched primers were omitted. To calculate downstream diversity measures (alpha and beta diversity indices, unifrac analysis), 16S rRNA operational taxonomic units (OTUs) were defined at 100% sequence homology using DADA2 (Callahan *et al.* 2016); OTUs not encompassing at least two sequences of the same sample were removed. Notably, this approach allows highly distinctive taxonomic classification at single nucleotide accuracy (Callahan *et al.* 2016). All reads were classified to the lowest possible taxonomic rank using QIIME2 (Caporaso *et al.* 2010; Bokulich *et al.* 2018) and a reference dataset from the SILVA database (Quast *et al.* 2013).

ITS microbial profiling analysis

Partial ITS sequences, the spacer regions between the 16S rRNA and the 23S rRNA genes within the ribosomal RNA (rRNA) locus, were amplified from extracted DNA using the primer pair Probio-bif_Uni/Probio-bif_Rev (Milani *et al.* 2014). Illumina adapter overhang nucleotide sequences were added to the generated ITS amplicons of 200 bp, which were further processed using the 16S Metagenomic Sequencing Library Preparation Protocol (Part No. 15 044 223 Rev. B-Illumina). Amplifications were carried out using a Verity Thermocycler (Applied Biosystems, Foster City, CA, USA). DNA products obtained following PCR-mediated amplification of the ITS sequences were purified by a magnetic purification step involving the Agencourt AMPure XP DNA purification beads (Beckman Coulter Genomics, Bernried, Germany) to remove primer dimers. DNA concentration of the amplified sequence library was determined by a fluorimetric Qubit quantification system (Life Technologies, Carlsbad, CA, USA). Amplicons were diluted to a concentration of 4 nM and 5 μL quantities of each diluted DNA amplicon sample were mixed to prepare the pooled final library. A bifidobacterial mock community was also prepared, consisting of a pool of 11 different *Bifidobacterium* strains of known concentration prepared by combining equal volumes (20 μL) of DNA. ITS sequencing was performed using an Illumina MiSeq sequencer with MiSeq Reagent Kit v3 chemicals.

Following sequencing, the .fastq files were processed using a custom script based on the QIIME software suite (Caporaso *et al.* 2010). Paired-end read pairs were assembled to reconstruct the complete Probio-bif_Uni/Probio-bif_Rev amplicons. Quality control retained sequences with a length of between 100 and 400 bp and mean sequence quality score of >20, while sequences with homopolymers >7 bp in length and mismatched primers were removed. To calculate downstream diversity measures (alpha and beta diversity indices, unifrac analysis), ITS OTUs were defined at 100% sequence homology using uclust (Edgar 2010). All reads were classified to the lowest possible taxonomic rank using QIIME2 (Caporaso *et al.* 2010; Bokulich *et al.* 2018) and a reference dataset, consisting of an updated version of the bifidobacterial ITS database (Milani *et al.* 2014; Milani *et al.* 2017).

Evaluation of the cell density by flow cytometry assay

For cell counting, 1 ml aliquots of human milk were diluted 10 times in physiological solution (phosphate-buffered saline). To remove the fat layer, the diluted samples were centrifuged at 6200 x *g* rpm for 15 min. After centrifugation, the supernatant was discarded and the pellet was resuspended in 1 mL of sterile water. Subsequently, bacterial cells were stained with 10 μL mL^{-1} SYBR®Green I (1:100 dilution in DMSO; Molecular Probes, Eugene, OR, USA) and incubated in the dark for at least 15 min before measurement. Where necessary, samples were diluted just before measurement in filtered (0.22 μm; Millex®-GP, Millipore, Burlington, MA, USA) bottled mineral water. All count experiments were performed using an Attune NxT flow cytometer (ThermoFisher Scientific, Waltham, MA, USA) equipped with a blue laser set at 50 mW and tuned to an excitation wavelength of 488 nm. Multiparametric analyses were performed on both scattering signals, i.e. forward scatter (FSC) and side scatter (SSC) and SYBR Green I fluorescence was detected on the FL1 channel. Cell debris was excluded from acquisition analysis by a sample-specific FL1 threshold. All data were statistically analyzed with Attune NxT flow cytometer software.

Fermentation profiles on milk-glycans

Prior to carrying out growth profile assays, *Bifidobacterium* cultures were grown from stock in Difco™ De Man, Rogosa and Sharpe (MRS) agar (Becton Dickinson, Italy), and sub-cultured twice in the same medium. Subsequently, 135 μL of freshly prepared modified MRS (mMRS) medium [peptone from casein, 10 g/L; meat extract, 10 g/L and yeast extract, 5 g/L (purchased from Becton Dickinson); K_2HPO_4, 3 g/L; KH_2PO_4, 3 g/L; pyruvic acid, 0.2 g/L; polysorbate 80, 1 mL/L; tri-ammonium citrate, 2 g/L; $MgSO_4.7H_2O$, 0.575 g/L; $MnSO_4.4H_2O$, 0.12 g/L; cysteine-HCl, 0.3 g/L; and $FeSO_4.7H_2O$, 0.034 g/L], supplemented with 0.05% cysteine hydrochloride and 0.5% of particular carbohydrate, was inoculated with 15 μl (1%) of a stationary phase culture. All carbohydrates (lactose, 3′-sialyllactose, 2′-fucosyllactose and lacto-N-tetraose) were dissolved in water and then sterilized by filtration using 0.2 micron filter size and then added to mMRS after autoclaving. Inoculated mMRS medium supplemented with 2% of glucose was used as a positive control. By contrast, uninoculated mMRS medium was used as a negative control. Cultures were incubated under anaerobic conditions in a modular atmosphere-controlled system (Davidson and Hardy, Belfast, Ireland) at 37°C for 24 h and the optical density at 600 nm was determined using a PowerWave 340 Microplate Spectrophotometer (BioTek, Winooski, VT, USA). Growth assays were carried out in triplicate, with standard deviation calculated for error values.

Statistical analyses and hierarchical clustering

Co-variance and ANOVA statistical analyses, coupled with Bonferroni and Tukey Post Hoc Multiple Comparisons, were performed with SPSS software (IBM SPSS Statistics for Windows, version 22; IBM, Armonk, NY, USA). Hierarchical clustering was performed using MeV v. 4.9.0 software (http://mev.tm4.org/) applying the Pearson correlation distance metric.

Data deposition

Raw sequences retrieved by 16S rRNA microbial profiling and bifidobacterial ITS profiling are accessible through SRA BioProject PRJNA558904.

RESULTS AND DISCUSSION

HMO classification among human milk samples

Milk samples belonging to 157 breastfeeding mothers were collected and classified based on the mothers' lactation stage (Supplementary Fig. S1). Milk samples of every mother were gathered in the first month following delivery (T0), while further samples were collected after 6 and 12 months (T1 and T2), together representing a total of 249 milk samples (Supplementary Tables S1 and S2). To investigate the differences in the composition of complex sugars present in these milk samples, we analyzed data collected for these samples using high performance liquid chromatography followed by mass spectrometry analysis, as previously described (Supplementary Table S1) (Larsson *et al.* 2019), resulting in the quantitative analysis of 19 distinct HMO components. This information allowed us to explore HMO variability among samples, resulting in the identification of three major clusters inferred by means of hierarchical clustering as based on relative HMO abundances (Fig. 1A). The smaller cluster, represented by 23 samples, included milk samples from non-secretor mothers, based on the absence of 2′FL and DFLac, yet showing a higher abundance of LNFPII (Fig. 1A and B). Altogether, this cluster exhibits higher diversity in terms of HMO variability when compared with the other two larger clusters. In this context, differences between the other two clusters, based on milk samples from secretor mothers, encompassed minor changes in the relative abundance of 2′FL, DFLNT and LNFP (Fig. 1A and B. These findings corroborate the notion that the main differences in HMO composition are strictly correlated with the secretor status of the mothers, which are linked to the capability of producing α1-2-fucosylated HMOs (Castanys-Munoz, Martin and Prieto 2013; Thurl *et al.* 2017). The observed severely reduced capability of non-secretor mothers to produce α1-2-fucosylated HMOs was also reflected in the profiling of the absolute abundance of HMOs (Fig. 1C). In this context, the level of HMOs per volume of non-secretor mothers was shown to be much lower than that observed for secretor mothers (Fig. 1C), which is also supported by T-test analysis (a Student t test *P*-value of <0.001).

Microbial composition of the milk samples

To determine the microbiota composition of human milk, we subjected 163 of the 249 collected milk samples to 16S rRNA gene-based microbial profiling through Illumina sequencing, resulting in a total of 6080 390 sequenced reads, with an average of 37 303 reads per sample (Supplementary Table S3). As reported in Supplementary Table S3, the analyzed milk samples encompass different time points of the same mother, allowing evaluation of bacterial biodiversity at various time points during breastfeeding.

To identify the presence of possible milk types (MTs) within the collected samples, hierarchical clustering was performed based on the pan-microbiota, i.e. 66 bacterial families which showed a relative abundance higher than 1% in at least one sample. This analysis allowed the identification of three major clusters, representing the dominant key players of the milk microbiota (Fig. 2A and B). As shown in Fig. 2, the most representative cluster shared among 63 milk samples was predominantly composed of *Ruminococcaceae* (with an average relative abundance of 19.9% among samples) and *Lachnospiraceae* (19.2%) (MT-A), followed by two equally distributed groups showing a prevalence in *Streptococcaceae* (54.7%) (MT-B), and *Staphylococcaceae* (27.4%) together with *Bifidobacteriaceae* (18.5%) (MT-C). Looking at the microbiota complexity of each group, a higher alpha diversity in the MT-A cluster was observed when compared with MT-B and MT-C, highlighting a milk microbiota rich in bacterial diversity. Interestingly, none of the samples collected at the 12th month (T2) showed a bacterial composition related to the MT-A cluster.

To evaluate the overall microbial abundance within milk samples, we followed a quantitative microbiome profiling approach employing flow cytometric enumeration of microbial cells present in each sample. This analysis allowed the identification of the microbial load of a subset of 87 samples encompassing all three MT clusters at different time points. Using these cell counts to normalize the sequencing data into absolute abundance of each profiled taxa, we were able to perform quantitative microbiome profiling employing a previously described method (Vandeputte *et al.* 2017). Thus, quantitative microbiome profiling, deduced from complementing sequencing with microbial cell counts, unveiled that microbial numbers varied up to a 1000-fold among samples (Supplementary Fig. S2). To reveal possible associations between microbial loads and particular MT clusters at different time points, non-parametric ANOVA was performed using data of the normalized samples. Box and whisker plots revealed higher cell loads in samples belonging to the MT-A cluster with an apparent drop in cell number at T2 (Fig. 2C and D), although statistical analyses did not show this to be significant when comparing the total number of microbial cells and the abovementioned groups. Instead, as shown in Fig. 2, only four milk samples were shown to contain a total number of bacterial cells that was statistically different from the average (Supplementary Table S4). The same analysis performed on samples of secretor and non-secretor mothers did not reveal any statistically significant difference. Thus, based on the obtained results, it seems that additional genetic and environmental factors related to the mothers are involved in the definition of the milk microbiota.

When we compared our results with the milk microbiota profiles described in three recently published studies (Sakwinska *et al.* 2016; Urbaniak *et al.* 2016; Tuominen *et al.* 2018), and considering only those bacterial families that were present at an abundance of higher than 1%, it appears that no conserved core milk microbiota exists. Collected data were reanalyzed using the parameters employed in this study, unveiling the prevalence of *Streptococcaceae* and *Moraxellaceae* families in more than 25% of samples belonging to each study (Supplementary Table S5). Thus, analysis of milk samples collected in geographically distinct sites suggests that multiple genetic and environmental factors define the human milk microbiota.

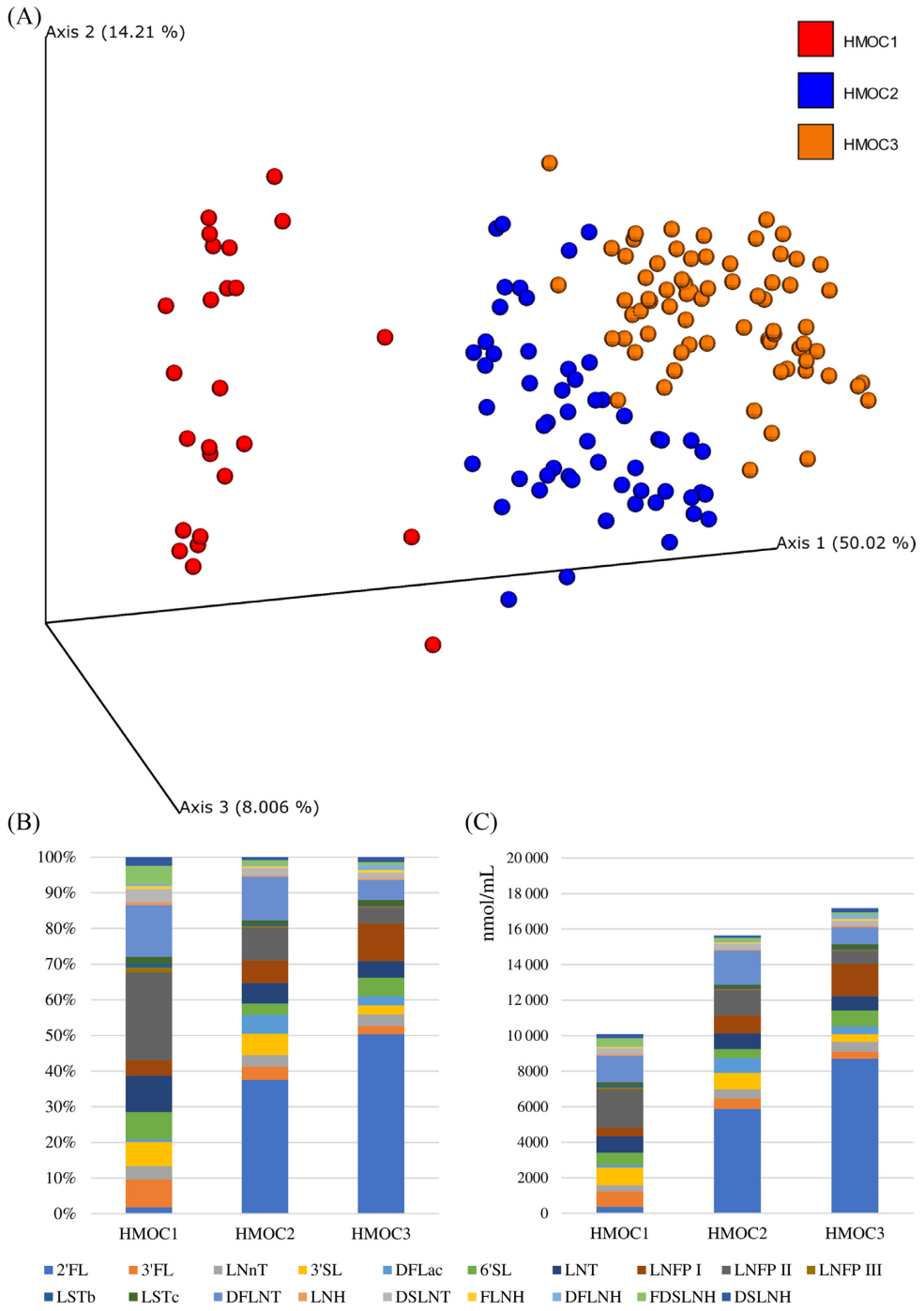

Figure 1. Human milk oligosaccharide composition. (**A**) A PCoA plot of the beta-diversity analysis performed for HMO data using the Bray–Curtis index. (**B**) The relative abundances of HMO clusters as derived from the analyzed human milk samples, while (**C**) shows the absolute abundance of the same samples. Cluster HMOC1 encompasses milk samples of non-secretor mothers, while clusters HMOC2 and HMOC3 are those of secretor mothers. HMOs are abbreviated as follows: 2′-fucosyllactose (2′FL), 3′-fucosyllactose (3′FL), 3′-sialyllactose (3′SL), 6′-sialyllactose (6′SL), lacto-*N*-tetraose (LNT), lacto-*N*-neotetraose (LNnT), lacto-*N*-fucopentaose (LNFPI, LNFPII and LNFPIII), sialyl-LNT (LSTb and LSTc), difucosyl-LNT (DFLNT), disialyl-LNT (DSLNT), fucosyl-lacto-*N*-hexaose (FLNH), difucosyl-lacto-*N*-hexaose (DFLNH), fucosyl-disialyl-lacto-*N*-hexaose (FDSLNH) and disialyl-lacto-*N*-hexaose (DSLNH), difucosyllactose (DFLac) and lacto-*N*-hexaose (LNH).

Elucidating the bifidobacterial population in human milk samples

The bifidobacterial populations present in 163 milk samples, representing 107 different breastfeeding mothers, were evaluated by means of bifidobacterial ITS profiling (Milani *et al.* 2014), resulting in 5001 444 sequenced reads with an average of 30 684 reads per sample (Supplementary Table S6). These bifidobacterial profiling efforts revealed that OTUs corresponding to five bifidobacterial species were identified in more than half of the analyzed samples, corresponding to *Bifidobacterium adolescentis*, *B. breve*, *Bifidobacterium dentium*, *Bifidobacterium longum* and *Bifidobacterium pseudolongum* (Fig. 3A). Interestingly, beside these species that are typical inhabitants of the gastrointestinal tract

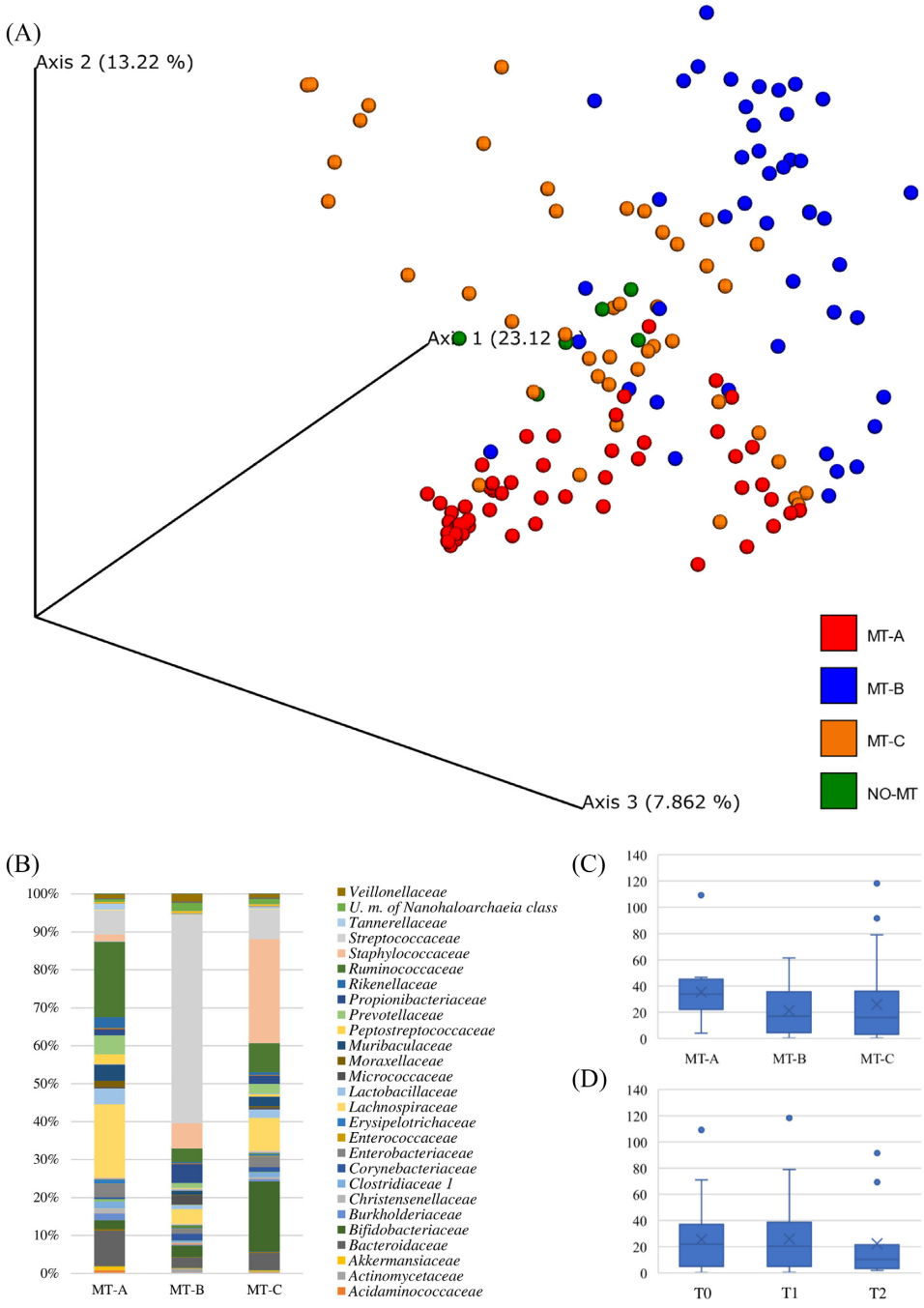

Figure 2. Bacterial profiling of human milk. (**A**) A PCoA plot of the beta-diversity analysis performed for 16S rRNA gene-based data at family level using the Bray–Curtis index. Clusters are abbreviated as follows: *Ruminococcaceae/Lachnospiraceae* (MT-A), *Streptococcaceae* (MT-B) and *Staphylococcaceae/Bifidobacteriacea* (MT-C). (**B**) The relative abundance of bacterial clusters retrieved from the analyzed human milk samples. The 17 most abundant bacterial families are reported on the right-hand side of the chart. (**C**) and (**D**) Whisker plots based on bacterial clusters and collected time points, respectively. Dots reflect the distribution of a dataset, while the boxes represent 50% of the dataset, distributed between the 1st and 3rd quartiles. The median divides the boxes into the interquartile range, while the X represents the mean. The lines extending vertically outside the boxes show the outlier range.

of humans (Milani *et al.* 2017), we identified, in 48% of milk samples, the presence of members of *Bifidobacterium crudilactis*, a species that was previously identified in bovine milk (Delcenserie *et al.* 2013; Milani *et al.* 2015).

Bifidobacterial distribution and the possible existence of bifidobacterial MTs (BMTs) were further investigated by means of hierarchical clustering based on the bifidobacterial community retrieved from ITS profiling. Altogether, a cluster of 78 analyzed samples was dominated by *B. adolescentis/B. pseudolongum* (BMT-A), 18 samples shared a high abundance of *B. crudilactis* (BMT-B), 49 samples were enriched in *B. dentium* (MTB-C), while 16 samples represented a heterogeneous cluster composed of a mix of bifidobacterial species without any being prevalent (MTB-D) (Fig. 3B). In this context, BMT-A was shown to be the most represented BMT at both T0 and T1, and at 57% and 43% of the analyzed samples, respectively. Remarkably, BMT-B seems to be

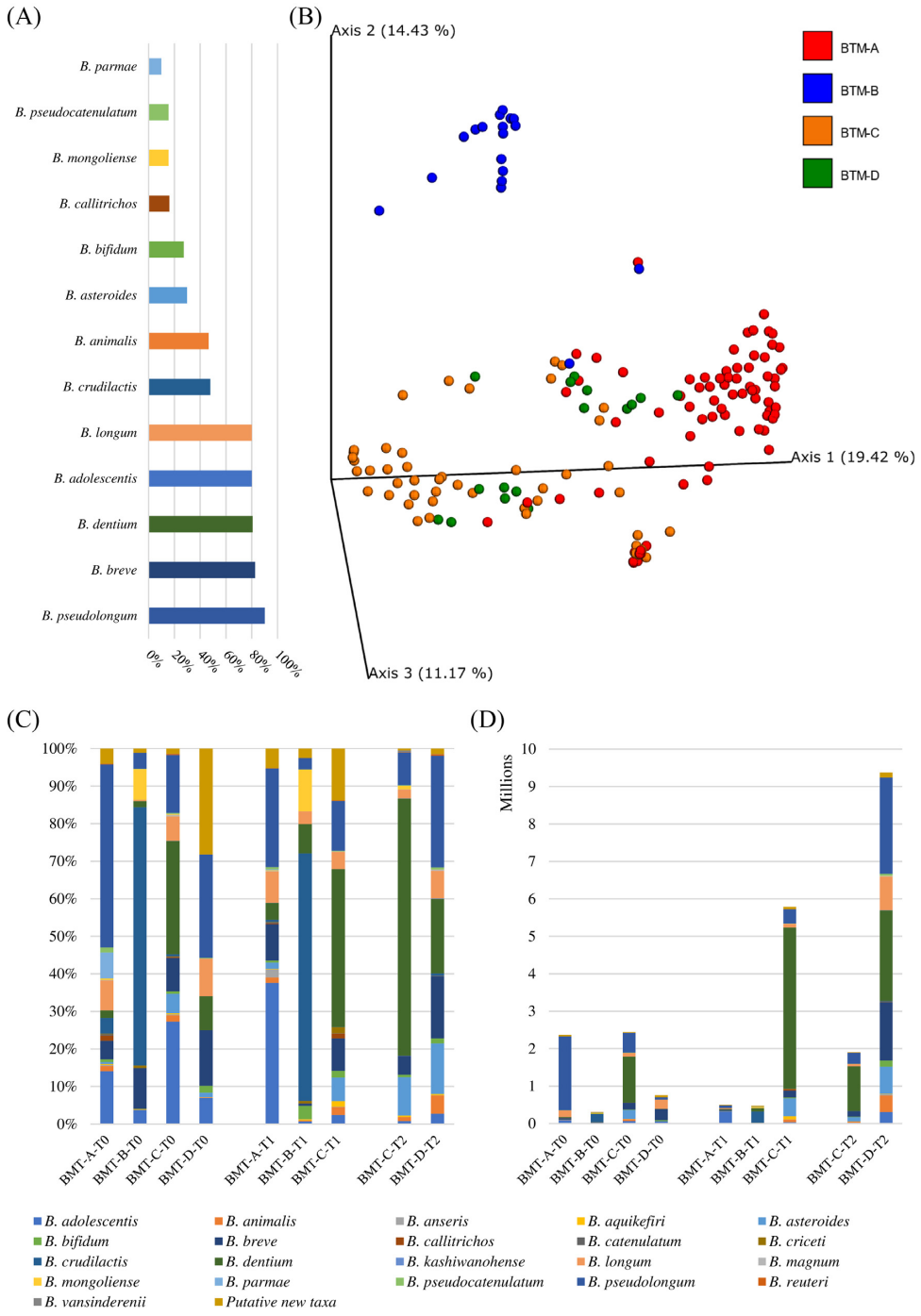

Figure 3. Bifidobacterial profiling of human milk samples. (**A**) The bifidobacterial prevalence among analyzed milk samples. (**B**) A PCoA representation of the beta-diversity analysis performed for bifidobacterial ITS data at species level using the Bray–Curtis index. Clusters are abbreviated as follows: *B. adolescentis*/*B. pseudolongum* (BMT-A), *B. crudilactis* (BMT-B), *B. dentium* (BMT-C) and *B. breve*/*B. longum* (BMT-D). (**C**) The relative abundances of bifidobacterial species distributed among clusters identified at each time point; (**D**) the absolute abundance of the same data.

absent in the samples collected at the 12th month, while BMT-C was the most represented at this time point (50%), highlighting a shift in the bifidobacterial composition of milk (Fig. 3C). Interestingly, the observed distribution of BMTs during the breast-feeding period reflects the transition from a milk-only to a milk-plus-solids based diet and coincides with the teething in babies.

Flow cytometric enumeration of microbial cells also facilitated the conversion of ITS profiling data into absolute bifidobacterial abundance, thereby enabling quantitative bifidobacterial profiling similar to that performed for the 16S rRNA gene-based analysis. The bifidobacterial cell counts were shown to be highly variable between samples, irrespective of collection time points or secretor status of the mother (Supplementary Fig. S3). Furthermore, milk samples obtained from the same mother showed comparable abundances (Supplementary Fig. S3). Interestingly, the quantitative abundance of bifidobacterial taxa in each sample allowed us to depict the actual bifidobacterial distribution among BMTs. The composition of BMT-A was shown to be

(A)	beta-galactosidase	beta-hexosaminidase	lacto-N-biose phosphorylase	alpha-L-fucosidase	sialidase
B. adolescentis	100%	0%	2%	2%	0%
B. animalis	100%	8%	15%	3%	0%
B. bifidum	100%	99%	100%	100%	99%
B. breve	100%	99%	97%	7%	99%
B. dentium	100%	0%	0%	96%	29%
B. longum	100%	100%	99%	8%	13%
B. pseudolongum	100%	2%	25%	32%	7%

(B)	MRS	Lactose	LNT	2'FL	3'SL
B. adolescentis	0.94	0.33	0.22	0.20	0.19
B. animalis	0.88	0.57	0.27	0.25	0.23
B. bifidum	0.67	0.46	0.57	0.30	0.49
B. breve	0.62	0.55	0.52	0.29	0.27
B. dentium	0.55	0.20	0.18	0.24	0.25
B. longum	0.57	0.50	0.39	0.25	0.23
B. pseudolongum	0.65	0.48	0.34	0.24	0.23

Figure 4. Bifidobacterial glycobiome profiling. (**A**) The percentages related to enzymes involved in the degradation of HMOs identified in 749 analyzed bifidobacterial genomes. (**B**) The ability of 86 bifidobacterial strains to grow on HMOs. HMOs are abbreviated as follows: lacto-*N*-tetraose (LNT), 2′-fucosyllactose (2′FL) and 3′-sialyllactose (3′SL).

statistically different at T0 when compared with T1 ($P < 0.01$ for *B. adolescentis* and *B. pseudolongum* species) (Fig. 3D). Initially, the latter BMT was mainly represented by *B. pseudolongum* (1971 200 out of 2362 508 bifidobacterial counts), which seems to disappear at T1 with *B. adolescentis* taking its place (with 323 594 out of 497 526 bifidobacterial counts). Looking at T0 and T1, BMT-B was associated with a lower abundance of bifidobacterial cells, while BMT-C was the BMT with the highest bifidobacterial cell count. Interestingly, we observed a high abundance of *B. dentium* cells at T2 (1196 159 out of 1905 368 bifidobacterial counts). Since the presence of *B. dentium* strains was previously associated with the oral microbiota of children together with its genetic adaptation to the human oral cavity (Mantzourani *et al.* 2009; Ventura *et al.* 2009), our findings suggest that the developing oral microbiota of the infant impacts on the milk microbiota of its corresponding mother. Finally, BMT-D at T0 showed *B. breve* and *B. longum* as the most abundant species (296 364 and 246 609 out of 756 614 bifidobacterial counts, respectively), which at T2 were paired with bifidobacterial species already identified in the other bifidotypes (Fig. 3D).

Results of ITS profiling normalized for cell counts when coupled with HMO profiling allowed us to evaluate the co-occurrence and co-exclusion between the distribution of bifidobacterial species and HMOs (Supplementary Table S7). This statistical analysis revealed the near absence of significant correlations between bifidobacterial species and specific HMOs (Supplementary Table S7). In a similar fashion, analyses based on samples collected from secretor and non-secretor mothers were not significant. Remarkably, based on these results, we assume that the persistence time of the identified microorganisms in the mammary gland is too short to have an effect on their growth.

Insights into the glycobiome of key bifidobacterial species of milk samples

Based on the seven prevalent species of the genus *Bifidobacterium* identified through the above-mentioned ITS bifidobacterial profiling, we performed a screening of the enzymes responsible for HMO degradation (Sela *et al.* 2008). To accomplish this, 749 sequenced bifidobacterial genomes were employed, encompassing *B. adolescentis*, *B. animalis*, *B. bifidum*, *B. breve*, *B. dentium*, *B. longum* and *B. pseudolongum* species (Fig. 4). While putative β-galactosidase enzymes were identified among all analyzed genomes, each bifidobacterial strain was shown to possess a different composition of additional putative enzymes capable of breaking down HMO molecules, i.e. β-hexosaminidase, lacto-*N*-biose phosphorylase, α-L-fucosidase and sialidase (Fig. 4). Members of the *B. adolescentis* and *B. animalis* speciespossess little or no capacity to encode these additional enzymes (2% and 15% exhibited the presence of a predicted lacto-*N*-biase-encoding gene in their genomes, respectively), followed by *B. pseudolongum* strains, of which 25% and 32% displayed the presence of genes encoding a putative lacto-*N*-biase and α-L-fucosidase in their genomes, respectively. By contrast, members of the *B. bifidum* species were predicted to encode a complete gene set for HMO breakdown (Fig. 4). Notably, previous studies have demonstrated that the extracellular hydrolytic activity of *B. bifidum* towards HMOs and other human glycoproteins, such as mucins, supports growth of other bifidobacterial species, such as *B. breve*, through cross-feeding on released carbohydrates (Egan *et al.* 2014; Duranti *et al.* 2019). In this context, members of the *B. breve* species were predicted to encode genes for β-hexosaminidase, lacto-*N*-biose phosphorylase, sialidase, and, though not for all strains, α-L-fucosidase, thus clustering together with members of the *B. bifidum* taxon, the species with the widest predicted genetic makeup for the degradation of HMOs (Fig. 4). Besides, members of *B. longum* and *B. dentium* species were predicted to encode genes for β-hexosaminidase and lacto-*N*-biose phosphorylase, and α-L-fucosidase, respectively. This indicates that these species participate in HMO metabolism but probably cannot access such carbohydrates without syntrophic support from other taxa. In addition, several strains of these two species possessed genes predicted to encode sialidases (13% and 7%, respectively), but it does not seem to be a characteristic of the whole species.

To corroborate the glycobiome screening of the 749 sequenced bifidobacterial genomes, 86 bifidobacterial taxa belonging to the same species as assessed above were cultivated using HMOs as the unique carbon source. In this context, bifidobacteria were cultivated in media containing LNT, 2′FL and 3′SL. As shown in Supplementary Table S8, the obtained growth data confirmed the *in silico* glycobiome analysis (Fig. 4). Members of the *B. adolescentis* and *B. animalis* species were not able to grow in any of the used HMOs media, nor were members of the *B. dentium* species. Interestingly, previous published data had indicated that members of the latter species are able to remove the fucose at the terminal position of HMOs, although they are not able to grow on this sugar (Duranti *et al.* 2017). As expected from the *in silico* analysis, members of the *B. bifidum* and *B. breve* species were shown to exhibit the highest ability to degrade and grow on HMOs in each tested medium (Supplementary Table S8), while the presence of a putative lacto-*N*-biose phosphorylase in each analyzed *B. longum* genome may reflect their ability to grow on LNT. Instead, eight members of the *B. pseudolongum* species were able to grow on LNT, displaying an ability to degrade and grow on HMOs. These findings suggest that within this bifidobacterial species, the ability to metabolize HMOs is strictly strain-dependent. The peculiar profile of the latter species was also observed in the *in silico* glycobiome analysis, reflecting the huge genomic/genetic diversity previously mapped among members of the *B. pseudolongum* species (Lugli *et al.* 2019). Altogether, the combination of *in silico* and *in vitro* analyses highlights that just a small number of species belonging to the genus *Bifidobacterium* are able to grow on HMOs. Furthermore, based on the absolute bifidobacterial abundance profiles, achieved through normalization by cytometric assay, we were able to identify that the most abundant species in milk samples is not the same species that possesses the highest HMO-degrading performance, e.g. *B. dentium* and *B. pseudolongum* species (Fig. 3). By contrast, bifidobacterial species showing the most prominent ability to degrade and grow on HMOs are usually those species that are dominant in the gut of infants, i.e. *B. breve* and *B. bifidum*. Based on these data, it may be argued that the persistence time of the identified bifidobacteria in the mammary gland is not sufficiently long in order to take any advantage of HMOs, and that these oligosaccharides do not represent the main selective force driving the composition of the human milk microbiome. Instead, HMOs will be utilized by bifidobacteria in the infant gut, where these microbes are present at an abundance which exceeds that of any other genus of the gut microbiota at any other host age (Milani *et al.* 2017; Turroni *et al.* 2018).

CONCLUSION

During lactation, mothers transfer microorganisms and nutrients, such as bifidobacteria and HMOs, to their newborn. Therefore, the complex community of bacteria and nutrients that are transmitted by a mother's milk influence the development of the infant gut microbiota. The availability of human milk samples obtained from more than 100 breastfeeding mothers and corresponding to multiple time points of collection enabled an in-depth investigation of the HMO composition as well as a detailed bifidobacteria-specific profiling across the milk samples.

Performing 16S rRNA microbial profiling of 163 human milk samples, we identified three major clusters, predominantly composed of *Ruminococcaceae* and *Lachnospiraceae* (MT-A), *Streptococcaceae* (MT-B) and *Staphylococcaceae* together with *Bifidobacteriaceae* (MT-C). Nonetheless, comparison with milk samples collected from different studies revealed the absence of a defined core milk microbiota, reinforcing the notion that multiple genetic and environmental factors are involved in defining this community.

Using a metagenomic approach, i.e. the ITS bifidobacterial profiling, combined with a flow cytometry approach, allowed the assessment of the absolute abundance of each bifidobacterial taxon and consequently provided a precise view of the actual number of various bifidobacterial (sub)species present in the investigated samples. Statistical analyses allowed the identification of four BMT clusters, three of which were dominated by *B. adolescentis*/*B. pseudolongum* (BMT-A), *B. crudilactis* (BMT-B) or *B. dentium* (MTB-C), while the fourth cluster constituted a heterogeneous mix of bifidobacterial species such as *B. breve* and *B. longum* (MTB-D). Notably, these bifidobacterial patterns did not correspond to those that are shown to be genetically adapted to grow on HMOs.

One limitation of this study is that we did not assess the corresponding infant fecal samples, which may have been informative to assess the impact of the microorganisms present in a mother's milk on the gut microbiota composition of the respective babies. Accordingly, it would be of great interest, in a follow-up project, to collect infant fecal samples together with milk samples in order to evaluate any possible species-specific prebiotic activity of HMOs towards the infant gut microbiota. Another limitation of this study is that we did not perform an analysis of the mammary gland biology. The inclusion of such information may have allowed validation of our hypothesis that the length of bifidobacterial persistence in the mammary gland is too short to promote/stimulate their growth. Currently our data suggest that the composition of the human milk microbiota is not directly and majorly influenced by HMOs, but that human milk merely represents a vector for transmission of a very variable and complex microbiota. This human milk microbiota may only become subject to selective pressures once it is introduced in the infant gut, where the simultaneously present HMOs are believed to become strong influencers of the infant gut microbiota, in which bifidobacteria are highly abundant.

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SUPPLEMENTARY DATA

Supplementary data are available at *FEMSEC* online.

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