Journal of Developmental Origins of Health and Disease (2016), 7(1), 54–60.

doi:10.1017/S2040174415001397
Themed Issue: The Gut Microbiome and Immunity: How it is Shaped by Early Life

ORIGINAL ARTICLE

Impact of mode of delivery on the milk microbiota composition of healthy women

R. Cabrera-Rubio[1], L. Mira-Pascual[1], A. Mira[2] and M. C. Collado[1]*

[1]*Department of Biotechnology. Av. Agustin Escardino 7, Institute of Agrochemistry and Food Technology, Spanish National Research Council (IATA-CSIC), Valencia, Spain*
[2]*Department of Health and Genomics. Av. Cataluña 21, FISABIO Foundation, Center for Advanced Research in Public Health, Valencia, Spain*

Breast milk constitutes one of the most important sources of postnatal microbes. However, the influence of perinatal factors on the milk microbiome is still poorly understood. The aim of our study was to assess the impact of mode of delivery on the microbiome composition and diversity present in breast milk of healthy mothers.Mature milk samples ($n = 10$) were taken from mothers after 1 month of exclusively breastfeeding. Microbiomes from milk samples were analyzed with 16S ribosomal RNA gene pyrosequencing and targeted quantitative polymerase chain reaction (PCR).Despite inter-individual variability in bacterial composition, The Principal Coordinates Analysis clearly separated milk microbiome from mothers with vaginal delivery ($n = 6$) from those who undergo C-section ($n = 4$). In addition, higher bacterial diversity and richness was found in milk samples from vaginal deliveries. Quantitative PCR data showed that higher levels of *Bifidobacterium* spp. were related significantly to lower levels of *Staphylococcus* spp. Despite the low sample size, our data suggest that mode of delivery has an important impact on milk microbiome composition. Further studies with larger sample sizes are needed to confirm these results and to understand the biological effects of C-section associated microbes on infant's health.

Received 27 May 2015; Received 13 July 2015; Accepted 16 July 2015; First published online 19 August 2015

Key words: 16S pyrosequencing, C-section, diversity, milk microbiota, qPCR, vaginal delivery

Introduction

The human microbiome development is a complex process that has been traditionally assumed to start at birth when the infant is exposed to the maternal microbiota and it is followed by diet mainly breast milk. Moreover, changes in this colonization pattern have been shown to increase the risk of disease later in life.[1–3] To what extent the maternal milk microbiota composition influences that of their newborns is not yet fully clear.

It has been reported that a microbial contact during pregnancy may occur, but the massive microbial transfer occurs during birth. Differences in microbiota composition depending on birth mode affects the infant microbial colonization, which is altered in infants delivered by C-section compared with vaginally born infants[4–6] impacting in their health.[7–9] After birth, the transfer of microbiota continues during breastfeeding, playing a key role during the 1st months after birth. Differences in gut microbiota between exclusively breastfed and formula fed infants have been widely reported.[5,10,11]Together with these two factors, mode of delivery and breastfeeding, there are other perinatal factors known to influence the infant microbial exposition as maternal health, host genetics, gestational age, sanitation, hospitalization, environment, and also, antibiotic treatment.[2,6,12] However, little is known about the effects of those perinatal factors in maternal microbiota including breast milk microbes, which is important because they will be transfered to the offspring.

Breast milk is the most relevant postnatal element for the metabolic and immunological programming of the infant's health.[13] Breast milk contains a high diversity of microbes which drives the infant's microbial colonization.[14–18] It has been reported that breast milk microbiota from allergic mothers showed lower *Bifidobacterium* group levels than those observed in healthy mothers.[19] Furthermore, specific shifts in milk microbiota composition were associated with maternal factors such as body mass index (BMI), weight, weight gain over pregnancy and also, mode of delivery,[14] lactational stage and gestational age,[20] maternal health during lactation[21,22] and also, antibiotherapy.[23] In addition, transmission of specific intestinal microbes as *Bifidobacterium* strains and also, *Staphylococcus* spp. from mothers to infants have been recently reported[24–26] suggesting that each mother–infant pair might have unique family-specific strains. These results imply a potential role of the factors that transfer aberrancies in the composition and activity of microbiota to the infant influencing their early and later health.

In this context, the aim of our study was to assess whether perinatal factors such as delivery mode may exert an influence on the microbiome composition present in the breast milk of healthy mothers after 1 month of exclusive breastfeeding. If we understand which perinatal factors are influencing the milk microbiome, we may develop new tools in the future to modulate this microbiome in order to improve and impact infant microbial colonization and health.

*Address for correspondence: M. C. Collado – IATA-CSIC, Av. Agustin Escardino 7, 49860 Paterna, Valencia, Spain.
(Email mcolam@iata.csic.es)

Experimental procedures

Subjects and sampling

A total of 10 healthy Spanish mothers participated in the study and provided a sample of breast milk (BM) after 1 month with exclusive breast feeding practices. Details of delivery and gestational age were collected after birth. Written informed consent was obtained from the participants and the study protocol was approved by the Ethics Committee of the CSIC (Spanish National Research Council).

All mothers were healthy, normal weight (BMI < 25 kg/m^2) and 30.60 ± 6.6 year old. The gestational age was 36.6 ± 4.2 weeks. Vaginal delivery was present in 60% of the participants (6/10) while C-section was 40% (4/10). All mothers provided exclusive breastfeeding to their offspring during the 1st month of life. No antibiotics or other medication consumption, including probiotics, were reported.

Before sample collection, the mothers were given oral and written instructions for standardized collection of samples. The milk samples were collected in a sterile tube manually. Previously, nipples and mammary areola were cleaned with soap and sterile water and soaked in chlorhexidine to reduce bacteria residing on the skin. The first drops (500 µl approximately) were discarded. All of the samples were kept frozen −20°C until delivery to the laboratory.

DNA extraction

Milk samples were thawed and centrifuged at 10,000 g for 10 min to separate cells and fat, concentrating the sample. Thereafter, total DNA was isolated from the pellets using the QIAamp DNA Stool Mini Kit (QIAgen, Hilden, Germany) following the manufacturer's instructions.

Pyrosequencing of tagged 16S rRNA gene amplicons

The first 500 bp of the 16S rRNA genes covering V1–V3 region were amplified with the universal eubacterial primers 27F and 533R using the high-fidelity AB-Gene DNA polymerase (Thermo Scientific) with an annealing temperature of 52°C and 20 cycles to minimize polymerase chain reaction (PCR) biases, as described previously.[14] Specificity and amplicon size were verified by gel electrophoresis and the final DNA concentration per sample was measured by picogreen fluorescence in a Modulus 9200 fluorimeter from Turner Biosystems. Purified PCR products were pooled in equimolar amounts, as described by 454 Roche protocols, and were pyrosequenced from the forward primer end only using a GS-FLX sequencer with Titanium chemistry (Roche) at the Center for Public Health Research (CSISP-FISABIO) in Valencia, Spain.

Bioinformatics and statistical analysis

The raw data set, were filtered by quality and length sequence. An end-trimming was performed by removing low quality nucleotides at the 3′ end through windows of 20 nt of average quality values lower than 20, and a second filtering was performed by removing those reads with an average quality value lower than 20 and with less than 200 nt of length. These high-quality sequences were assigned to each sample by the 8-bp barcode present in the forward primers, All these steps were performed through the galaxy server (http;//getgalaxy.org/). These sequences were further filtered to remove chimeric sequences with the Usearch program,[27] and taxonomically classified by the Ribosomal Database Project classifier, where each read was assigned a phylum, class, family and genus, as long as the taxonomic assignment was unambiguous within an 80% confidence threshold (Table S1). Rarefaction curves were calculated at the species-level operational taxonomic units (OTU). Sequences were normalized for the same number of sequences and clustered at 97% sequence identity employing CD-HIT software.[28] The resulting clusters were then used to generate a predictive rarefaction model using Analytic Rarefaction 1.3 software.[29] Principal Coordinates Analyses (PCoA) were performed with Fast UniFrac[30] using clustering at 97% sequence identity with the weighted normalized option. The Unifrac analysis compares the 16S-estimated diversity with a phylogenetic approach that takes into account both taxonomically assigned and unassigned reads.

Quantitative PCR with 16S rRNA-gene-targeted bacterial groups

PCR primers targeted to total bacteria count, *Bifidobacterium*, *Lactobacillus*, *Enterococcus*, *Staphylococcus* and *Streptococcus* groups. These oligonucleotides were purchased from the Isogen (Isogen Life Science, De Meern, the Netherlands). qPCRs were conducted as previously described.[31] qPCR amplification and detection were performed in a LightCycler® 480 Real-Time PCR System (Roche). Each reaction mixture of 10 µl was composed of SYBR® Green PCR Master Mix (Roche), 0.5 µl of each of the specific primers at a concentration of 0.25 µM, and 1 µl of template DNA. The fluorescent products were detected in the last step of each cycle. A melting curve analysis was made after amplification to distinguish the targeted from the non-targeted PCR product. The bacterial concentration in each sample was calculated comparing the C_t values obtained from standard curves. These were created using serial 10-fold dilution of pure culture-specific DNA fragments corresponding to 10–10^9 number of specific fragment gene copies/ml.

For statistical analysis, IBM®-SPSS® 22.0 software was used. Due to non-normal distribution of microbial data, they were expressed as medians with interquartile ranges and non-parametric tests were performed. Mann–Whitney U-test was used for comparisons between two groups. The χ^2 test was applied to establish differences in bacterial prevalence between the studied groups. A P-value < 0.05 was considered statistically significant. Spearman's rank test allowed the study of the correlation between variables and significance was established at a coefficient of 0.5%.

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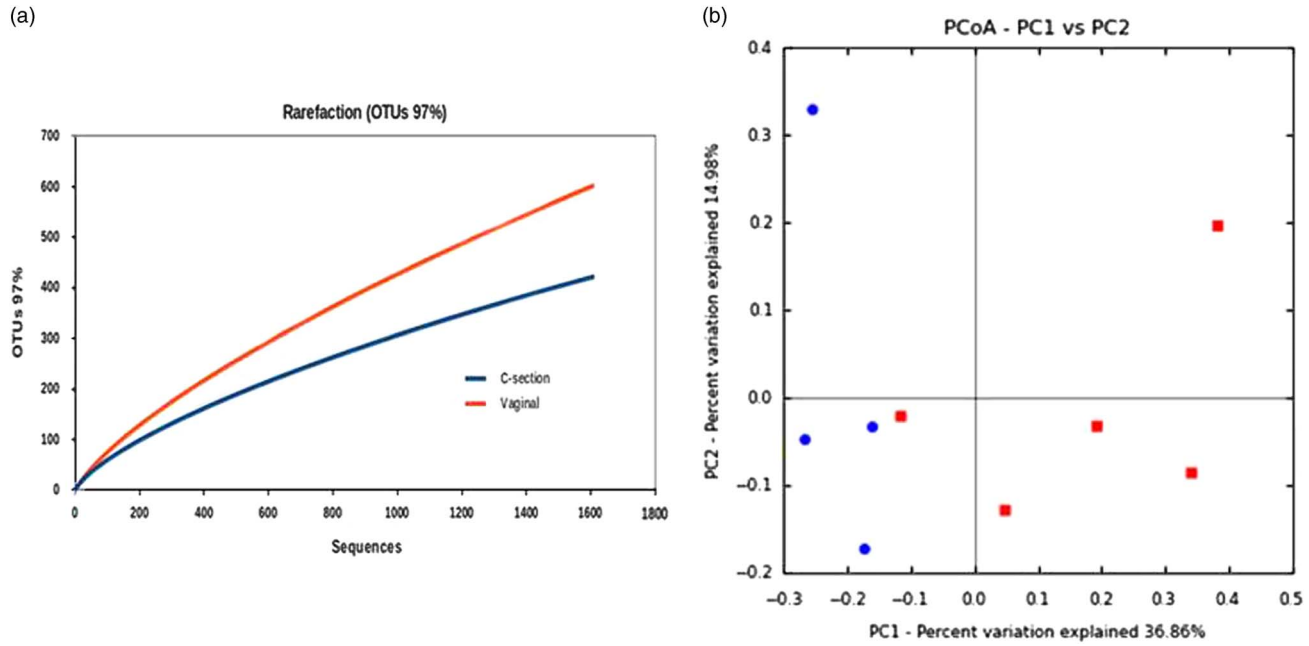

Fig. 1. Diversity of the Spanish mature milk microbiome. (*a*) Graphs show rarefaction curves relating the sequencing effect with an estimate of the number of bacterial species, as inferred by the number of observed operational taxonomic units (OTUs). An OTU was a cluster of 16S rRNA sequences that were >97% identical, the established estimate for the boundary between species. (*b*) Principal Coordinate Analysis (PCoA) on the basis of the bacterial composition of human breast milk of vaginal (red colour) and C-section (blue colour) deliveries.

Results

Pyrosequencing of tagged 16S rRNA gene amplicons

We found higher diversity in milk samples from vaginal deliveries compared with those from C-sections (Fig. 1a). This is confirmed by the Chao richness index, which produced an estimated number of 500 species-level OTUs in breast milk from mothers giving birth by vaginal delivery but only 250 OTUs in mothers giving birth by C-section (Fig. 2). When samples were plotted in a PCoA according to their species-level bacterial composition (Fig. 1b), they appeared to have a high inter-individual variability. However, the principal component of the PCoA plot clearly separated milk microbiome from mothers with vaginal delivery (positive values on the PC1 axis) from those who undergo C-section (negative values along the axis). We found different milk microbial profiles between the two groups (Fig. 3). Higher relative abundance of *Staphylococcus* ($P = 0.085$) and lower of *Streptococcus* ($P = 0.306$) were found in C-section compared with vaginal samples although no significant difference was found.

The taxonomic assignment showed that the milk microbiota composition is dominated by Firmicutes followed by Proteobacteria (Fig. 4a). We observed a high microbial variability and also, differential microbial profiles between mothers (Fig. 4b). Among Firmicutes, the most predominant microbial genera were *Streptococcus* and *Staphylococcus* spp. We found lactic acid bacterial families such as *Streptococcocaceae*, *Leuconostocaceae* and *Lactobacillaceae* but also typical inhabitants of the oral cavity such as *Veillonellaceae* and *Flavobacteriaceae*.

Quantitative PCR with 16S rRNA-gene-targeted bacterial groups

Higher levels of *Staphylococcus* spp. group ($P = 0.052$) and lower levels of *Enterococcus* spp. ($P = 0.085$) were detected in breast milk of mothers giving birth by C-section compared with vaginal deliveries (Table 1). *Bifidobacterium* group was also detected more frequently in vaginal than in C-section deliveries but the difference was not statistically significant (6/6, 100% *v.* 3/4, 75%, $P > 0.05$). Taking all samples, we found that higher levels of *Bifidobacterium* spp. were related to lower levels of *Staphylococcus* spp. group (Partial correlation controlled by gestational age and delivery mode, $R = -0.832$ and $P = 0.040$).

Discussion

Our study reports on the milk microbiota composition in healthy mothers after 1 month of exclusively breastfeeding practices, demonstrating the potential impact of the mode of delivery in milk microbes.

Our results are in agreement with the previous data describing that the most abundant genera in milk were *Streptococcus* and *Staphylococcus* spp.[15–18] Other study showed higher

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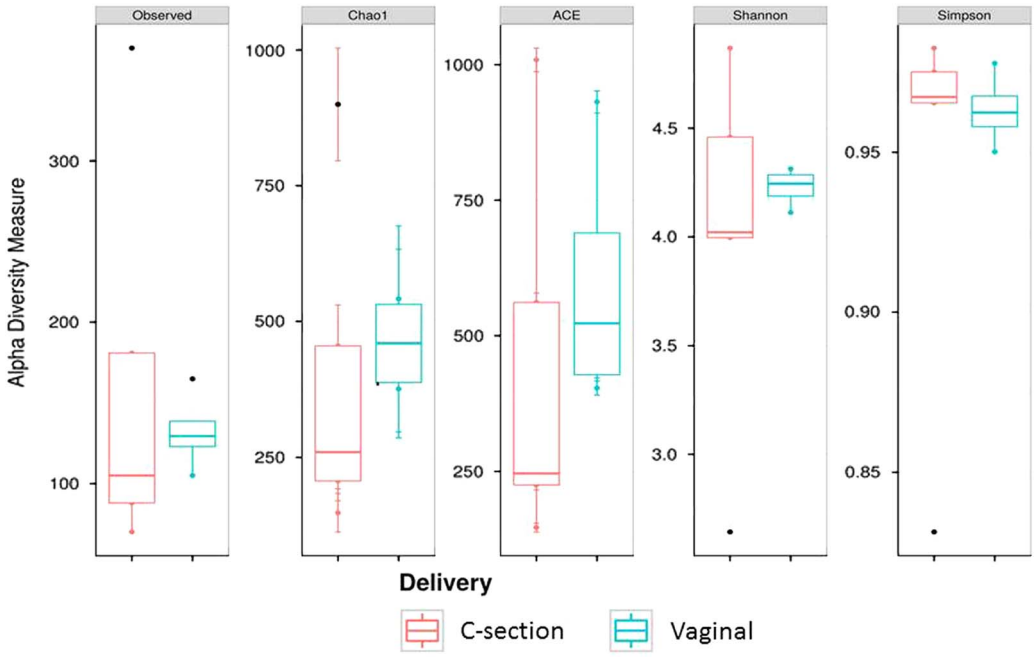

Fig. 2. Comparison of diversity between milk samples from C-section ($n = 4$) and vaginal ($n = 6$) deliveries using different measures of alpha diversity.

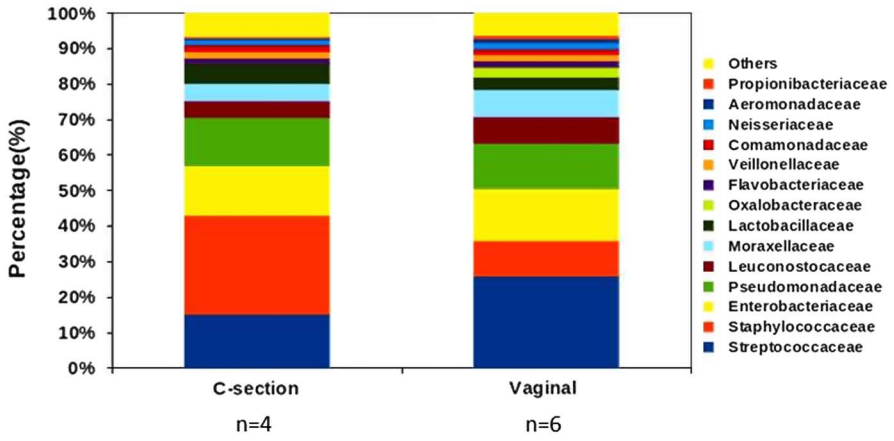

Fig. 3. Bacterial taxonomic composition of human breast milk according to the mode of delivery (vaginal and C-section). The graphs show the proportion of bacterial families as inferred by polymerase chain reaction amplification and pyrosequencing of the 16S rRNA. Data show mean values of 10 healthy mothers.

abundance of lactic acid bacteria followed by *Streptococcus* and *Staphylococcus* spp.[14] We found higher levels of Firmicutes followed by Proteobacteria in our samples and within Firmicutes, *Streptococcaceae* and *Staphylococcaceae* were the predominant families. A recent milk metagenome study[15] reported that among the bacterial sequences, the predominant phyla were Proteobacteria, Firmicutes and Bacteroidetes. The same study showed that the healthy core microbiome included *Staphylococcus* and *Streptococcus* spp. and at the species level, a high degree of inter-individual variability was observed among healthy women. We also observed a high variability in the milk samples analysed. Another milk metagenome study[33] showed that the predominat phyla were Proteobacteria (65%) and Firmicutes (34%), and the genera *Pseudomonas* (61.1%) and *Staphylococcus* (33.4%). All the differences found in the published reports would suggest geographic, genetic or diet-related factors that could drive the observed differences and that should be further studied.

Early microbiota colonization provides the infant with vital stimuli that guide the immune maturation and this microbial establishment is influenced by pre and postnatal factors, including genetic and environmental variables (gestational age,

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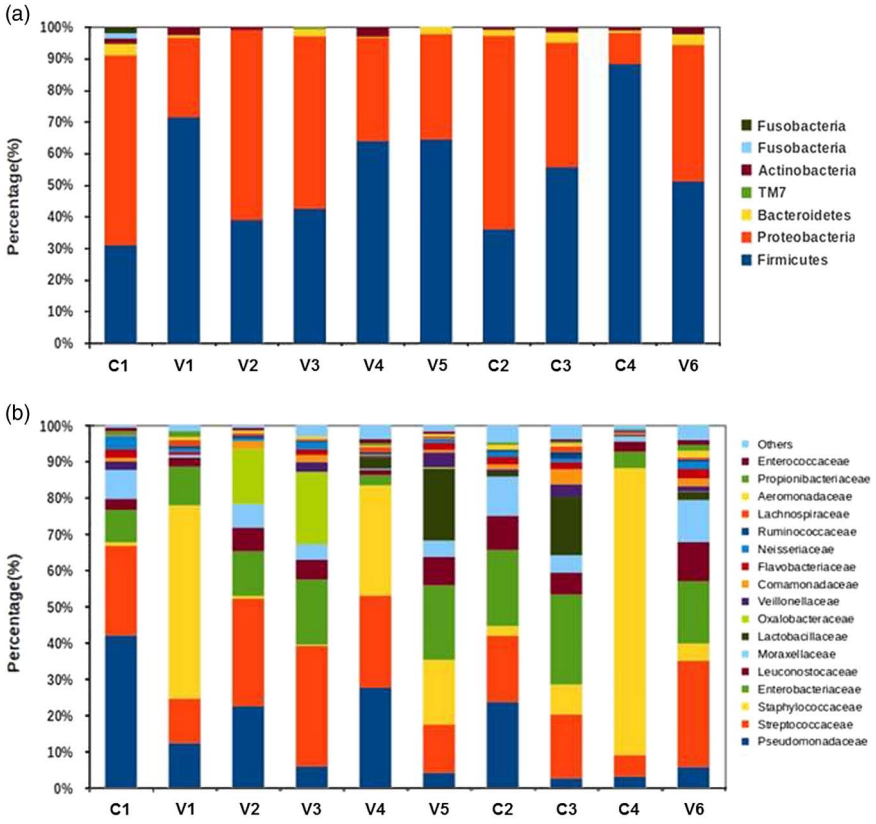

Fig. 4. Bacterial taxonomic composition of human breast milk (V = vaginal and C = C-section deliveries). The graphs show the proportion of bacterial phyla (*a*) and families (*b*) in samples from 10 individuals, as inferred by polymerase chain reaction amplification and pyrosequencing of the 16S rRNA.

Table 1. *Microbiota composition present in breast milk samples by qPCR*

	Log bacterial group (fragment gene copies/ml)				Mann–Whitney U-test
	Pr	Vaginal ($n = 6$)	Pr	C-section ($n = 4$)	*P*-value†
Total bacteria	6	5.11 (4.09–7.20)	4	4.53 (3.33–7.00)	0.670
Bifidobacterium spp.	6	2.32 (2.00–2.60)	3	2.06 (2.02–2.21)	0.831
Lactobacillus group	6	4.14 (3.84–4.42)	4	4.34 (3.92–4.56)	0.394
Staphylococcus spp.	6	3.09 (3.00–3.34)	4	3.50 (2.56–4.60)	0.052
Streptococcus spp.	5	3.68 (3.15–3.86)	3	3.49 (3.42–3.95)	0.655
Enterococcus spp.	6	4.18 (3.65–4.56)	4	3.80 (3.03–4.25)	0.085

Data are shown as median and interquartile range (IQR). Statistical analysis was calculated by Mann–Whitney U- test. For prevalences (Pr) study (positive samples) statistical analysis was calculated by χ^2 test. †$P < 0.05$ was considered significant.

mode of delivery, hospitalization, and nutrition). Western countries are facing with a progressive increase of non-communicable diseases.[34] This increase may be related to a parallel increase in the rates of deliveries by cesarean section which has exponentially increased far beyond 15% recommended by the World Health Organization. Indeed, an increased risk for atopic diseases, asthma, celiac disease, and obesity among others was reported in children born by Cesarean delivery.[7–9,35] However, scarce information is available on the impact of these factors on the microbial transference from mother to infants.

The mode of delivery is known to influence the neonatal gut microbiota composition. It has been shown that the gut microbiota of infants delivered by C-section showed significantly less resemblance to their mothers.[6] We show that breast milk from mothers who had given birth by C-section delivery showed a different microbial profile compared with those who delivered vaginally. Our results are in agreement

https://doi.org/10.1017/S2040174415001397 Published online by Cambridge University Press

with previous findings reporting a skewed microbiota composition in breask milk from mothers giving birth by elective or C-section.[14,20] We also observed a lower bacterial diversity in C-section samples compared with vaginal ones. Thus, although our data are based on a very limited number of samples, the trend appears to be repeated in different studies. Given that lower bacterial diversity has been associated to children developing different kinds of allergic conditions,[36,37] future work should study the effect of vaginal delivery on infant microbiota development in larger cohorts.

Modification of the microbiota by probiotics-prebiotics and even antibiotics early in life has also attracted scientific interest, particularly during the 1st months of life when the establishment of the intestinal microbiota and maturation of the immune system are not yet completed, as it would envisage an important opportunity for health programming. Interestingly, nutrition counselling and probiotic intervention have been demonstrated to have a distinct effect on gestational diabetes, whereby probiotics reduce the risk of the disorder while dietary counselling reduces the risk of fetal overgrowth associated with it.[38] A recent meta-analysis showed a significant reduction in the incidence of atopic eczema in children aged 2–7 years whose mothers received probiotics during pregnancy.[39] In line with these observations, a recent finding has shown that perinatal probiotic intervention moderates excessive weight gain during childhood, the impact being most pronounced at the age of 4 years.[40] Fetal and placental immune physiology may be modulated by maternal dietary intervention using specific probiotics and also, probiotics consumption can modulate the composition of immune molecules such as transforming growth factor (TGF)-β2 present in breast milk.[41] In addition, probiotic bacteria consumed by the lactating mother have been detected in breast milk and infant feces.[42]

Based on the present data, we hypothesize that perinatal factors also affect microbial transference from mother to infant via breast milk. Bacterial transfer from the mother during the perinatal period may offer a novel target for devising dietary strategies aiming to modulate the microbes in order to reduce the incidence of non-communicable diseases risk. Furthermore, our data might help to identify potential targets to improve an adequate colonization with major effects on early health mainly in those cases where microbial exposition is not optimal. We are increasing our knowledge on milk microbiota, although further studies with larger sample sizes and from different geographical regions are needed in order to understand the biological effects of these microbes on infant's health.

Acknowledgments

All authors participated in preparation of the manuscript. None of the authors have conflict of interests. Finally, the authors would like to thank all the volunteers who gave their samples for this study.

Supplementary material

To view supplementary material for this article, please visit http://dx.doi.org/10.1017/S2040174415001397

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