

Maternal Diet Shapes the Breast Milk Microbiota Composition and Diversity: Impact of Mode of Delivery and Antibiotic Exposure

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ABSTRACT

Background: Breast milk is a complex biofluid that provides nutrients and bioactive agents, including bacteria, for the development of the infant gut microbiota. However, the impact of maternal diet and other factors, such as mode of delivery and antibiotic exposure, on the breast milk microbiota has yet to be understood.

Objectives: This study aimed to examine the association between maternal diet and breast milk microbiota and to ascertain the potential role of mode of delivery and antibiotic exposure.

Methods: In a cross-sectional study of the MAMI cohort, breast milk microbiota profiling was assessed in 120 samples from healthy mothers by 16S rRNA gene sequencing. Maternal dietary information was recorded through an FFQ, and clinical characteristics, including mode of delivery, antibiotic exposure, and exclusive breastfeeding, were collected.

Results: Maternal diet was grouped into 2 clusters: Cluster I (high intake of plant protein, fiber, and carbohydrates), and Cluster II (high intake of animal protein and lipids). Breast milk microbiota was shaped by maternal dietary clusters. *Staphylococcus* and *Bifidobacterium* were associated with carbohydrate intake whereas the *Streptococcus* genus was associated with intakes of the n-3 PUFAs [EPA and docosapentaenoic acid (22:5 ω -3)]. Mode of delivery and antibiotic exposure influenced breast milk microbiota in a diet cluster-dependent manner. Differences between/among the maternal dietary clusters were found in the milk microbiota of the cesarean-section (C-section)/antibiotic group, whereas no differences were observed in vaginal births. Lower abundances of *Lactobacillus*, *Bacteroides*, and *Sediminibacterium* genera were observed in Cluster II/C-section/antibiotic exposure compared with the other groups.

Conclusions: Maternal diet shapes the composition and diversity of breast milk microbiota, with the most important contributions coming from dietary fiber and both plant and animal protein intakes. The relation between the maternal diet and the milk microbiota needs further research because it has a key impact on infant microbiota development and contributes to infant health outcomes in the short and long term. This trial was registered at clinicaltrials.gov as NCT03552939. *J Nutr* 2021;151:330–340.

Keywords: maternal diet, breast milk, microbiota, plant protein, animal protein

Introduction

Breast milk is the best food for infants during early life (1, 2). It represents the most relevant postnatal link between mothers and newborns and it drives the microbial, metabolic, and immunological programming of infants' health (3, 4). Breastfed infants harbor higher abundances of *Bifidobacterium* and *Lactobacillus* in the gut than formula-fed infants (5), contributing to a distinct immune system maturation, protection against infections, and also to reduction in the risk of disease, such as allergies, obesity, and other disorders later in life (3, 6, 7).

Breast milk provides an optimal source of nutrients and beyond; it contains bioactive compounds, including immunoglobulins, lysozymes, growth factors, oligosaccharides, and beneficial microorganisms (4, 8–10). Breast milk microbiota composition is diverse and commonly characterized by *Staphylococcus* and *Streptococcus* spp. followed by other genera, such as *Veillonella*, *Propionibacterium*, lactic acid bacteria, and *Bifidobacterium* spp. (11). Furthermore, there is a large inter- and intraindividual variability in breast milk composition, and the contribution of these variations to infant development is

not yet completely understood. Maternal and infant factors, including lactational stage, maternal pregestational BMI, weight gain over the pregnancy, mode of delivery, geographical location, antibiotics, parity, infant gender, and also the method of breastfeeding all shape the breast milk microbiota composition (12–16).

Limited data are available on the impact of maternal diet on breast milk microbiota. We hypothesized that breast milk microbiota composition and diversity would be shaped by perinatal factors in a diet-dependent manner. Thus, this study aimed to assess whether maternal diet and specific nutrients during pregnancy would shape breast milk microbiota, and to ascertain the potential influence of other perinatal factors, including mode of delivery and antibiotic exposure, on breast milk microbiota composition according to maternal diet.

Methods

Study design and volunteers

A total of 120 healthy mothers from the observational study MAMI (MAternal MIcrobes) cohort were included in this cross-sectional study according to the full availability of biological samples and clinical and dietary data (Supplemental Figure 1). The MAMI cohort is a prospective mother-infant cohort in the Spanish Mediterranean area, as described elsewhere (17).

Nutritional, anthropometrical, and clinical parameters were collected including maternal antibiotic exposure, pregestational BMI, weight gain during pregnancy, mode of delivery, and for newborns gestational age, infant feeding, and weight and length at birth, 7, and 15 d. BMI (kg/m^2) was stratified according to the Sociedad Española para el Estudio de la Obesidad criteria: normal weight (18.5–25.0); overweight (25.1–29.9); and obese (≥ 30.0) (18). None of the participating volunteers were diagnosed with any disease; none were receiving drug treatment or pro- and prebiotic administration, with the exception of antibiotic exposure during pregnancy and/or at delivery.

All participants received oral and written information about the study and written consent was obtained. The study was approved by the hospital ethics committees (Hospital Clínico Universitario de Valencia and Hospital Universitario y Politécnico La Fe). The study was registered on clinicaltrials.gov with registration number NCT03552939.

Maternal nutritional assessment

The maternal diet was estimated with the use of an FFQ (19). The FFQ questionnaire included 140 items, covering usual foods and recipes, as well as the frequency of consumption (daily, weekly, monthly, or during the end of pregnancy and birth time), the number of times the participant consumed a particular food item, the median portion

(in household measures—grams and milliliters), and the size of each participant's portion. All the food surveys were conducted by trained nutritionists. FFQ information was analyzed using data from the Food Composition Tables developed by the Centro de Enseñanza Superior de Nutrición Humana y Dietética (20). The intake of soluble and insoluble fiber types was determined by using the Marlett food composition tables (21). Polyphenol content was obtained from the Phenol-Explorer database (22). Data were standardized by 2500 kcal/d energy intake to control for overconsumption or underconsumption and to reduce the potential measurement error in the dietary assessment (23, 24). The FFQ data have been validated elsewhere for collecting information on diet during pregnancy (19), and we have also validated our results' data in a randomly selected subsample ($n = 20$) through the use of a 3-d recall food record questionnaire for the intake of dietary nutrients (19) (Supplemental Table 1).

Maternal dietary profile determination

Maternal diet clustering was performed as described elsewhere (25). Briefly, Jensen–Shannon distance and partitioning around medoid clustering were used. The optimal number of clusters was calculated by the Calinski–Harabasz index (CHI). The clusters were generated using the phyloseq (26), cluster (27), MASS (28), clusterSim (29), and ade4 (30) R packages (R Foundation). Clustering was carried out by using specific dietary information, such as intake of animal protein, plant protein, soluble and insoluble fiber, SFAs, total MUFAs, total PUFAs and *trans* fatty acids, and carbohydrates.

Influence of mode of birth and antibiotics on breast milk microbiota

In our cohort, intrapartum antibiotic exposure was mainly associated with cesarean section (C-section) births, although in our study, some vaginal deliveries were also exposed to antibiotics ($n = 3$, representing 3.84%). Due to the low number of samples exposed to antibiotics during vaginal delivery, we removed them from the analysis to make homogeneous groups: 1) vaginal birth and no antibiotic exposure; and 2) C-section birth and antibiotic exposure.

To identify the impact of mode of birth/antibiotics on milk microbiota, depending on maternal diet, 4 groups were distinguished, according to the mode of delivery and maternal diet (Cluster I_C-section, Cluster I_VAG, Cluster II_C-section, and Cluster II_VAG) (Supplemental Figure 1).

Breast milk samples

Breast milk samples were collected during 7–15 d after birth, following a standardized protocol. In brief, breast skin was cleansed with soap or 0.5% chlorhexidine solution and the first drops were discarded. Morning collection was recommended. Then, breast milk was collected from 1 breast with the use of a sterile pump and stored in sterile bottles to normalize the milk collection protocol. Finally, breast milk samples were sent to a biobank and then aliquoted and stored at -80°C until further analysis.

DNA extraction and total bacterial levels

Total DNA was isolated from 1.5–2.0-mL breast milk samples using the Master-Pure DNA Extraction Kit (Epicentre) according to the manufacturer's instructions; modifications included physical and enzymatic treatments as previously detailed (31). DNA purification was performed using a MagSi-NGS Plus kit (AMSBIO) and quantification was assessed by Qubit 2.0 Fluorometer (Life Technologies). Breast milk is a low-microbial biomass sample that would be affected by potential contaminants from the environment and DNA extraction kit reagents (“kitome”). Controls during DNA extraction and PCR amplification were also included and sequenced.

Total bacterial quantification was performed using targeted qPCR in a LightCycler 480 real-time PCR system (Roche Technologies). Each reaction mixture of 10 μL was composed of SYBR Green PCR Master Mix (Roche), 0.25 μL of each of the specific primers 789R (5'-GCGTGGACTACCAGGGTATCT) and 515F (5'-GTGCCAGCMGCCGCGGTAA) (32, 33) at a concentration of 10 μM ,

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Data described in the manuscript will be made available upon request.

Supplemental Figures 1–3 and Supplemental Tables 1 and 2 are available from the “Supplementary data” link in the online posting of the article and from the same link in the online table of contents at <https://academic.oup.com/jn/>.

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Abbreviations used: CHI, Calinski–Harabasz index; C-section, cesarean section; DPA, docosapentaenoic acid (22:5 ω -3); LEfSe, linear discriminant analysis effect size; PERMANOVA, permutational multivariate analysis of variance; RDA, redundancy discriminant analysis.

and 1 μ L of template DNA, with an annealing temperature of 62°C.

16S rRNA amplicon sequencing

Breast milk microbiota composition was determined by V3–V4 variable regions of the 16S rRNA gene sequencing, following Illumina protocols. A Nextera XT Index Kit (Illumina) was used for the multiplexing step, and a Bioanalyzer DNA 1000 chip (Agilent Technologies) for checking the PCR product quality. Libraries were sequenced using a 2 \times 300 bp paired-end run (MiSeq Reagent Kit v3) on a MiSeq-Illumina platform (FISABIO sequencing service), according to the manufacturer's instructions (Illumina) (34).

Sequences and data analysis

A DADA2 pipeline was used to achieve quality filtering, sequence joining, and chimera removal (35). Using the SILVA v132 database (36), taxonomic assignment was performed also including the species-level classification. Samples with a relative abundance <0.01% and those present <3 times in \geq 20% of the samples were filtered. Furthermore, the decontam package (37) was used to determine the presence of potential contaminant-related sequences, resulting in 48 amplicon sequence variants classified as possible contaminants, which were also filtered. Sequences classified as cyanobacteria and chloroplasts were also removed from the final data set as well as samples with \leq 1000 reads. Predictive inferred functional analysis was performed using the Piphillin pipeline (38).

GraphPad Prism v. 5.04 was used for *t*-test and Mann–Whitney analysis, according to data normality assessed by Kolmogorov–Smirnov and Shapiro–Wilk tests. χ^2 tests (2 \times 2) were performed to assess the significance of the differences in the characteristics according to categorical variables; these were conducted in SPSS v.25 (IBM).

Spearman correlations between relative abundances of bacterial groups and nutrient intakes were also performed in SPSS v.25. Linear discriminant analysis effect size (LEfSe) was used to identify microbial genera enriched in maternal diet clusters. A redundancy discriminant analysis (RDA) was applied to study the statistical effect of maternal diet and delivery mode on breast milk microbiota. A permutational multivariate analysis of variance (PERMANOVA) based on Bray–Curtis distance, and α diversity by Shannon (diversity) and Chao1 indexes (richness) were performed using Calypso online platform (v8.84) software (39). RStudio was also used to represent the heatmaps between bacterial groups at the genus level and dietary components through the ggplot package (40).

Results

Maternal diet clusters and clinical data

A dietary intake–based clustering using Jensen–Shannon distance revealed two distinct clusters validated by the CHI (Figure 1A, B). Cluster I ($n = 44$) was characterized by plant protein, fiber, and carbohydrates, whereas Cluster II ($n = 76$) was characterized by animal protein and lipids (Figure 1C–F). No significant differences were found in the maternal–neonatal clinical characteristics (Table 1). Interestingly, higher pregestational BMI was observed in Cluster II compared with Cluster I ($P = 0.016$); however, both were classified as normal-weight women (BMI \leq 25).

Detailed information about nutrient intakes according to the clustering is shown in Table 2. A significantly higher intake of total protein ($P < 0.0001$), mainly animal protein ($P < 0.0001$), was observed in women from Cluster II compared with those observed in Cluster I. Furthermore, Cluster II also presented a higher total lipid intake, mainly SFAs ($P < 0.0001$) as well as total MUFAs ($P < 0.0001$) and total PUFAs ($P = 0.003$). But there were no significant differences in the intakes of starch and fatty acids, such as ω -6 conjugated linoleic acid, ω -3 α -linolenic acid, ω -3 docosapentaenoic acid (DPA), and

DHA between clusters. However, Cluster I was characterized by significantly higher dietary intakes of plant protein, carbohydrates, and total, insoluble, and soluble dietary fibers (all $P < 0.0001$).

Breast milk microbiota is shaped by maternal diet cluster

In general, the most abundant genera present in all the breast milk samples included in the study were *Staphylococcus* and *Streptococcus* followed by *Ralstonia*, *Gemella*, *Lactobacillus*, and others (Supplemental Figure 3A). We performed a PERMANOVA based on Bray–Curtis distance to identify the main factors contributing to the breast milk microbiota (Figure 2A). The combination of all factors had an impact on the breast milk microbiota (PERMANOVA Bray–Curtis model $P = 0.051$), whereas maternal diet cluster was the main contributor among the others affecting the milk microbiota variation ($P < 0.01$). Breast milk microbiota was significantly different depending on maternal dietary profile (PERMANOVA Bray–Curtis distance $R^2 = 0.021$ and $P = 0.007$). This observation was also confirmed by a multivariate analysis (RDA test, variance = 1.91; $P = 0.002$, Figure 2D).

Distinct bacterial profiles at phylum and genus levels were observed in Clusters I and II (Figure 2B, C). At the phylum level, Cluster I showed higher relative abundance of *Bacteroidetes* ($P < 0.001$) and *Actinobacteria* ($P = 0.014$) whereas at the genus level, a higher relative abundance of *Staphylococcus* ($P = 0.036$) and *Lactobacillus* ($P = 0.022$) compared with Cluster II was observed. In addition, higher relative abundances of *Bifidobacterium* ($P = 0.026$) and *Sediminibacterium* ($P < 0.001$) and lower relative abundances of *Bacteroides* ($P = 0.023$) and *Escherichia/Shigella* ($P = 0.023$) genera were also observed in Cluster I compared with Cluster II. Furthermore, LEfSe [linear discriminant analysis (LDA) >2.5] analysis showed that Cluster II was characterized by *Bacteroides* and *Escherichia/Shigella* genera whereas *Staphylococcus* spp. were characteristic of Cluster I (Figure 2G).

Regarding α diversity, significantly higher bacterial richness (measured by the Chao1 index; $P < 0.0001$) and diversity (measured by Shannon index; $P = 0.0003$) were observed in Cluster I compared with Cluster II (Figure 2E, F). Despite differences in α diversity, the total bacterial counts in milk samples were similar between clusters [median (IQR) \log_{10} bacterial gene copies per milliliter: Cluster I = 6.52 (5.76–7.20) compared with Cluster II = 6.70 (5.77–7.20)]. Additionally, we found that higher bacterial richness but not diversity was associated with lower total bacterial load ($\rho = -0.28$; $P = 0.003$) (Figure 2H, I).

We also found significant differences in breast milk microbiota inferred function, depending on the maternal cluster (Supplemental Table 2).

Association between breast milk microbiota and specific dietary nutrients

Significant associations between dietary nutrients and specific breast milk microbial genera were identified (Figure 3, Supplemental Figure 3). *Staphylococcus* was associated with higher intake of carbohydrates ($\rho = 0.19$; $P = 0.038$) and with lower total protein intake ($\rho = -0.21$; $P = 0.026$) whereas *Streptococcus* genus was directly associated with higher intake of total protein ($\rho = 0.22$; $P = 0.018$) as well as higher EPA ($\rho = 0.19$; $P = 0.040$) and DPA ($\rho = 0.19$; $P = 0.044$), selenium ($\rho = 0.26$; $P = 0.005$), and zinc ($\rho = 0.18$; $P = 0.049$).

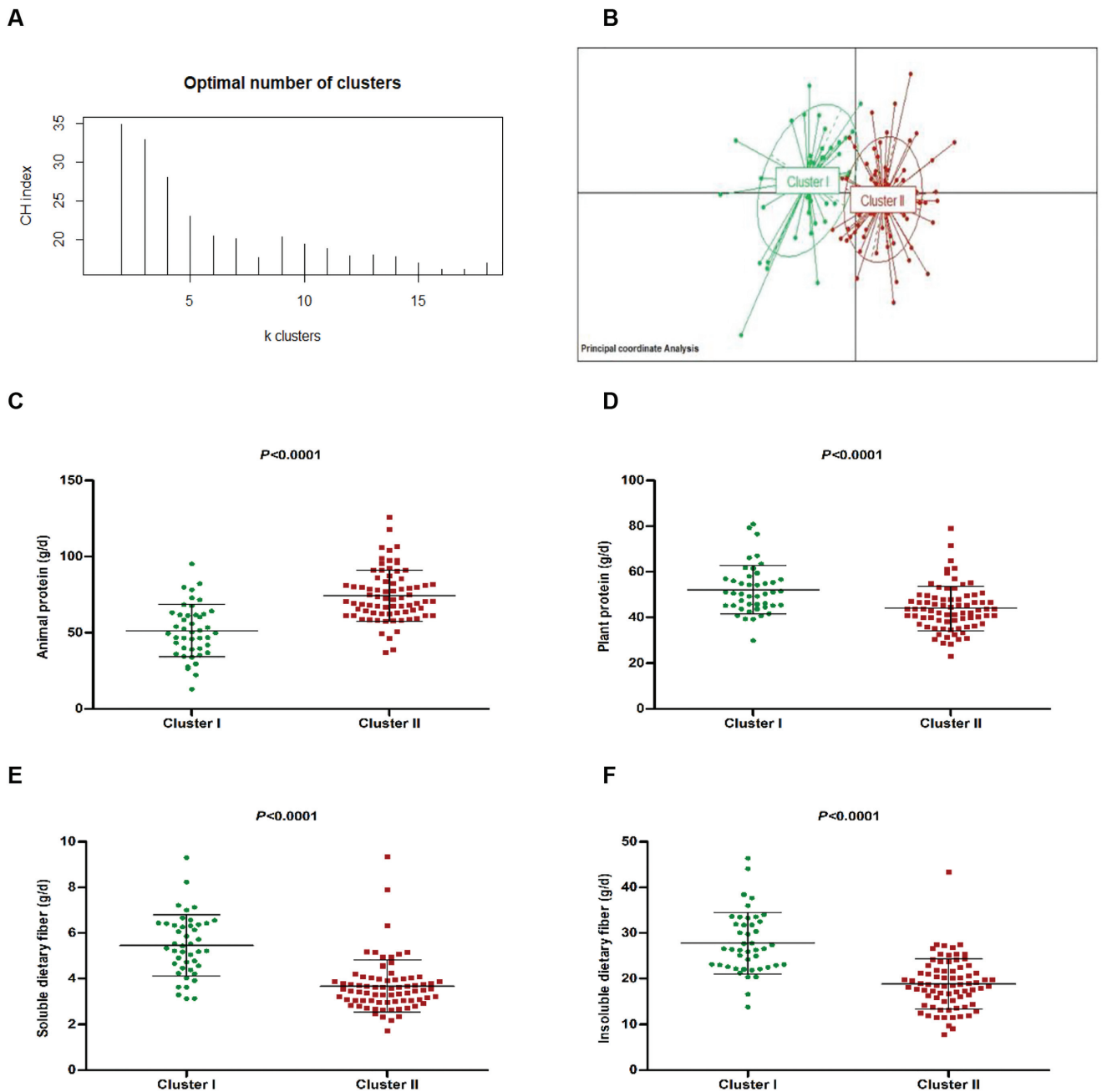


FIGURE 1 Maternal diet clusters and representative dietary components. The partitioning around medoid method showed that women were grouped (A) into 2 clusters and both distinct clusters (Cluster I = green, $n = 44$; and Cluster II = red, $n = 76$) are represented in the Principal Coordinate Analysis (PCoA) (B). The strongest contributors to the clustering were higher intake of animal protein and lower plant protein and dietary fiber. Diet was standardized by total energy intake to 2500 kcal/d (C–F). Middle line represents the media of all values and also, the upper and lower lines represent the standard deviation (SD)..

Furthermore, *Bifidobacterium* abundance was associated with a higher intake of carbohydrates ($\rho = 0.20$; $P = 0.031$) and polyphenols ($\rho = 0.19$; $P = 0.042$), and also with lower total lipid intake ($\rho = -0.23$; $P = 0.012$), mainly with lower total MUFA ($\rho = -0.26$; $P = 0.006$) and total PUFA ($\rho = -0.22$; $P = 0.016$) intakes. Interestingly, whereas *Enterococcus* genus was negatively associated with total animal protein ($\rho = -0.20$; $P = 0.036$) intake and saturated fat ($\rho = -0.30$; $P = 0.001$), the *Gemella* genus was positive correlated with animal protein ($\rho = 0.20$; $P = 0.029$), and also with n-3 PUFAs such as n-3 DPA ($\rho = 0.26$; $P = 0.005$), n-3 DHA ($\rho = 0.26$; $P = 0.004$), and n-3 EPA ($\rho = 0.26$; $P = 0.006$) intake. Additionally,

although total lipid intake presented a positive correlation with *Klebsiella* and *Enterobacter*, both bacterial features showed a negative association with carbohydrates. Calcium intake was associated with lower relative abundance of *Veillonella* genus ($\rho = -0.19$; $P = 0.043$) and higher relative abundance of *Stenotrophomonas* ($\rho = 0.20$; $P = 0.032$). Significantly higher *Veillonella* abundance was associated with higher total fiber ($\rho = 0.20$; $P = 0.034$) and plant protein ($\rho = 0.22$; $P = 0.019$) and insoluble dietary fiber intakes ($\rho = 0.27$; $P = 0.004$). In addition, higher abundance of *Enterococcus* was associated with higher vitamin A ($\rho = 0.20$; $P = 0.037$), and lower vitamin D ($\rho = -0.19$; $P = 0.043$).

TABLE 1 Characteristics of the volunteers included in the analysis ($n = 120$)

	Total sample ($n = 120$)	Cluster I ($n = 44$)	Cluster II ($n = 76$)	P value ¹
Maternal characteristics ²				
Pregestational BMI, kg/m ²	23 ± 3.6	22 ± 2.8	24 ± 3.8	0.016
Weight gain during pregnancy, kg	12 ± 4.6	12 ± 3.9	12 ± 4.9	0.89
Gestational age, wk	40 ± 1.3	40 ± 1.2	39 ± 1.3	0.24
Intrapartum antibiotic exposure, n (%)	44 (37)	17 (39)	27 (36)	0.73
Mode of delivery: vaginal birth, n (%)	78 (65)	29 (66)	49 (64)	0.87
Infant characteristics ²				
Gender: female, n (%)	68 (57)	28 (64)	40 (53)	0.24
Weight, kg				
Birth	3.3 ± 0.46	3.4 ± 0.57	3.3 ± 0.40	0.40
7 d	3.3 ± 0.49	3.4 ± 0.57	3.3 ± 0.43	0.40
15 d	3.6 ± 0.51	3.6 ± 0.59	3.6 ± 0.45	0.42
Length, cm				
Birth	50 ± 2.2	50 ± 2.7	50 ± 1.8	0.57
7 d	51 ± 2.1	51 ± 2.3	51 ± 2.0	0.74
15 d	52 ± 2.1	52 ± 2.3	52 ± 2.0	0.87
Exclusive breastfeeding until 6 mo, n (%)	94 (78)	33 (75)	61 (80)	0.50

¹ Student t test and χ^2 test were performed to assess the significance between maternal clusters' characteristics in numerical variables and categorical variables, respectively. $P < 0.05$ was considered statistically significant.

² Numerical data are expressed as mean ± SD; categorical data as number of cases and percentage.

Higher microbial richness measured by the Chao1 index was associated with lower MUFA intake ($\rho = -0.19$; $P = 0.048$). However, higher microbial diversity measured by the Shannon index was associated with higher maternal total fiber intake ($\rho = 0.18$; $P = 0.055$).

Impact of mode of birth and antibiotics on breast milk microbiota is modulated by maternal diet

Significant differences were observed in the whole microbial community between dietary clusters in mothers under antibiotic exposure and C-section (RDA test variance = 4.9, $P = 0.003$; and PERMANOVA Bray–Curtis $R^2 = 0.046$, $P = 0.037$), whereas no differences were detected in vaginal deliveries (RDA test variance = 1.57, $P = 0.095$; PERMANOVA Bray–Curtis $R^2 = 0.021$, $P = 0.121$) (Figure 4A, B).

Distinct bacterial profiles at the phylum and family level were observed (Figure 4C, D). Lower *Actinobacteria* ($P = 0.043$) and lower *Bacteroidetes* ($P = 0.005$) phylum members were found in C-section/antibiotics Cluster II compared with the other groups. Significantly lower abundances of *Lactobacillus* ($P = 0.042$), *Bacteroides* ($P = 0.026$), and *Sediminibacterium* genera ($P = 0.003$) were observed in C-section/Cluster II compared with the other groups. Interestingly, *Rothia* genus was significantly higher ($P = 0.041$) in vaginal Cluster I compared with the other groups.

Significant differences were observed in microbial communities grouped according to antibiotic exposure/mode of delivery and maternal dietary clusters (RDA test variance = 3.58; $P = 0.002$) as well as in the microbial diversity and richness, depending on antibiotic exposure/mode of birth and diet cluster (Supplemental Figure 3B, D). Higher microbial diversity and richness were observed in women from Cluster I and vaginal/non-antibiotic exposure compared with the other groups. Interestingly, higher microbial richness (Chao1 index) was found in the C-section/antibiotics group in Cluster I than in Cluster II, whereas no differences were found in diversity (measured by Shannon index).

Discussion

Our study showed the influence of maternal diet on the composition and diversity of breast milk microbiota. We observed specific associations between maternal nutrient intake and breast milk microbiota. Maternal diet, mode of delivery, and antibiotic exposure influence the breast milk composition, opening new possibilities to develop targeted dietary interventions during lactation.

In agreement with previous data (16, 41, 42), we reported that *Firmicutes* and *Proteobacteria* followed by *Actinobacteria* were the most abundant phyla in breast milk samples. Furthermore, *Streptococcus* and *Staphylococcus* were also identified as the most abundant genera (11, 43). Other studies have also shown that the genera *Staphylococcus*, *Lactobacillus*, *Enterococcus*, and *Bifidobacterium* in breast milk are shared with mother-to-infant microbiota (5, 44).

Maternal diet, lactation stage, weight status, genetics, and geographical location are known to affect breast milk composition (e.g., protein, fatty acids, immunological profile, oligosaccharides) (12, 45–48). However, the impact of these factors on breast milk microbial communities is still unclear. The vertical transfer of microorganisms through lactation has a relevant role in the correct establishment of the infant gut microbiota (41). Therefore, it is necessary to identify which factors might influence the diversity and composition of breast milk microbiota, with a special emphasis on modifiable factors, such as dietary patterns and lifestyle. For example, specific bacteria from fermented food are present and shared between maternal and neonatal microbiota including breast milk (49); this highlights the key diet–breast milk–microbiota interplay.

In our study, maternal diet intakes were clustered into 2 distinct groups: Cluster I was characterized by a higher intake of dietary fiber, plant protein, carbohydrates, and polyphenols whereas Cluster II was defined by a higher intake of animal protein, total lipids, total PUFAs, total MUFAs, and SFAs. The clusters were each associated with a specific microbial composition and diversity. Cluster I was linked to a higher presence of the *Staphylococcus* and *Bifidobacterium*

TABLE 2 Specific dietary intakes of the healthy mothers involved in the study¹

Dietary intakes ^{2,3}	Total sample (n = 120)	Cluster I (n = 44)	Cluster II (n = 76)	P value ⁴
Total protein, g/d	116 ± 19	106 ± 15	122 ± 19	<0.0001
Animal protein, g/d	66 ± 20	51 ± 17	74 ± 17	<0.0001
Plant protein, g/d	47 ± 11	52 ± 11	44 ± 10	<0.0001
Lipids, g/d	114 ± 16	102 ± 13	120 ± 13	<0.0001
SFAs	24 ± 7.2	21 ± 4.8	26 ± 7.7	<0.0001
<i>Trans</i> fatty acids	0.007 ± 0.01	0.005 ± 0.01	0.008 ± 0.01	0.009
MUFAs	46 ± 11.0	41 ± 10.0	50 ± 10.0	<0.0001
PUFAs	17 ± 4.0	16 ± 2.9	18 ± 4.3	0.003
n-6 CLA	0.003 ± 0.01	0.003 ± 0.01	0.003 ± 0.01	0.57
n-6 EDA	0.007 ± 0.01	0.005 ± 0.01	0.008 ± 0.01	0.14
n-3 ALA	0.14 ± 0.1	0.15 ± 0.1	0.13 ± 0.1	0.34
n-3 EPA	0.17 ± 0.1	0.15 ± 0.1	0.19 ± 0.1	0.025
n-3 DPA	0.09 ± 0.1	0.08 ± 0.1	0.09 ± 0.1	0.10
n-3 DHA	0.41 ± 0.3	0.36 ± 0.2	0.44 ± 0.3	0.10
Carbohydrates, g/d	249 ± 40	285 ± 30	228 ± 28	<0.0001
Total dietary fiber, g/d	36 ± 11	44 ± 11	32 ± 7.3	<0.0001
Insoluble dietary fiber, g/d	22 ± 7.4	28 ± 6.7	19 ± 5.5	<0.0001
Soluble dietary fiber, g/d	4.3 ± 1.5	5.5 ± 1.3	3.7 ± 1.1	<0.0001
Starch, g/d	29 ± 12	30 ± 12	29 ± 12	0.66
Polyphenols, g/d	1.8 ± 759	2.2 ± 941	1.5 ± 497	<0.0001
Folic acid, µg/d	143 ± 142	197 ± 160	112 ± 120	0.001
Vitamin A, mg/d	1.37 ± 533	1.44 ± 528	1.33 ± 535	0.25
Vitamin B-6, mg/d	3.5 ± 1.4	4.1 ± 1.6	3.2 ± 1.2	0.001
Vitamin B-12, µg/d	7.8 ± 3.1	7.3 ± 3.1	8.1 ± 3.0	0.13
Vitamin C, mg/d	156 ± 71	192 ± 73	134 ± 61	<0.0001
Vitamin D, µg/d	103 ± 82	89 ± 67	112 ± 89	0.15
Vitamin E, mg/d	13 ± 5.0	12 ± 3.9	13 ± 4.8	0.34
Iron, mg/d	21 ± 4.9	22 ± 5.6	20 ± 4.3	0.08
Calcium, g/d	1.17 ± 277	1.14 ± 272	1.20 ± 279	0.28
Selenium, µg/d	161 ± 29	154 ± 30	165 ± 28	0.05
Sodium, g/d	2.5 ± 587	2.5 ± 596	2.5 ± 585	0.95
Magnesium, mg/d	566 ± 148	589 ± 118	553 ± 161	0.20
Manganese, mg/d	6.7 ± 1.7	7.6 ± 1.8	6.2 ± 1.5	<0.0001
Zinc, mg/d	14 ± 2.8	15 ± 3.5	14 ± 2.4	0.17

¹n-3 ALA, ω-3 α-linolenic acid; n-6 CLA, ω-6 conjugated linoleic acid; n-3 DPA, ω-3 docosapentaenoic acid; n-6 EDA, ω-6 eicosadienoic acid.

²Numerical data expressed as mean ± SD.

³Diets were standardized by total energy intake to 2500 kcal/d.

⁴Student *t* test was performed to assess the significance of the differences in the dietary intakes between clusters section in numerical variables and categorical variables, respectively. *P* < 0.05 was considered statistically significant.

genera in breast milk. Additionally, we also observed that these bacterial genera were positively related to carbohydrates and polyphenols. Furthermore, these microorganisms, mainly *Bifidobacterium*, are the hallmark of breastfed infant gut microbiota, suggesting a possible link between breast milk and the neonatal gut, as reported previously (44).

A significant impact of specific macronutrient and micronutrient intake on milk microbial communities in healthy lactating women has been reported (50). High maternal protein consumption was associated with high abundance of *Gemella* in breast milk, whereas high consumption of SFAs and MUFAs was associated with low abundance of *Corynebacterium* in breast milk (50). In the present study, *Gemella* was associated with the intake of animal protein and also with PUFAs, such as n-3 DPA, n-3 EPA, and n-3 DHA. Our findings also showed an association between calcium intake and a low relative abundance of *Veillonella* genus. By contrast, it has been reported that calcium was positively associated with *Veillonella* (50). Moreover, a recent study reported an association between

vitamin C intake and *Staphylococcus*, and between total PUFA intake and the *Bifidobacterium* genus (15).

Our findings clearly suggest that the *Bifidobacterium* genus is associated with a high intake of polyphenols and that the *Staphylococcus* genus is related to carbohydrates. Although the relation between polyphenols and breast milk microbiota is poorly understood, several studies have suggested that dietary polyphenols have prebiotic properties and antimicrobial activities as well as modulating the composition and functionality of the gut microbiota (51, 52). Moreover, some phenolic compounds can promote the growth of specific bacterial taxa, such as the *Lactobacillus* and *Bifidobacterium* genera (53, 54).

Microbial diversity measured by the Shannon index, but not microbial richness, was associated with higher total fiber maternal intake. We also observed that Cluster I milk samples, which are associated with higher fiber and plant protein intakes, presented higher microbial diversity and richness than breast milk samples from Cluster II. Thus, we suggest that lower fiber and plant protein intake is linked to lower microbial diversity

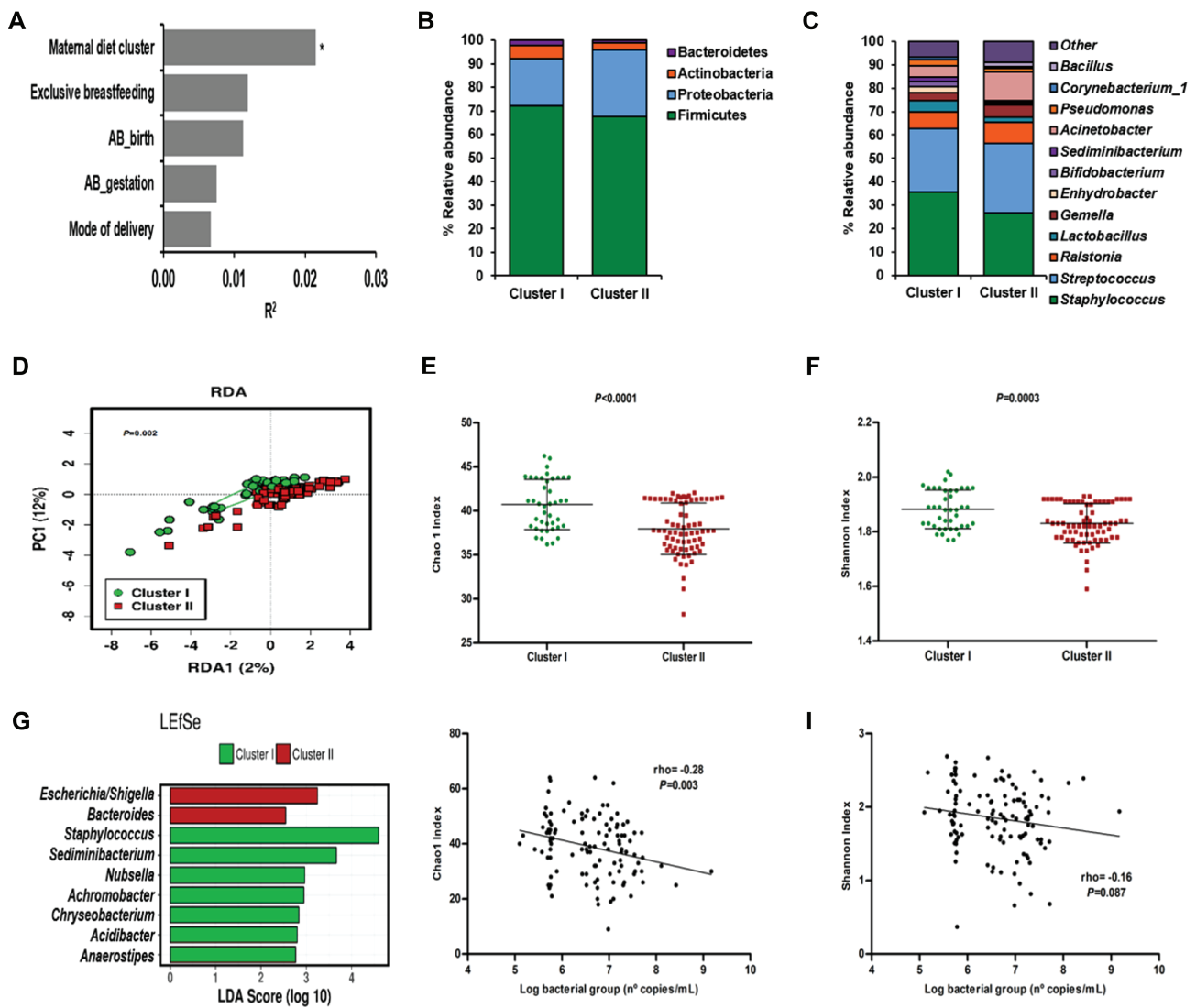


FIGURE 2 Breast milk microbiota is shaped by maternal diet clusters. Maternal factors contributing to milk microbiota variation (PERMANOVA Bray–Curtis model with 5 variables, $P = 0.051$) ($n = 115$) (A). The relative abundance of bacteria present in breast milk at phylum (B) and genus level (C) on maternal diet cluster, Cluster I ($n = 42$) and Cluster II ($n = 73$). Multivariate redundancy discriminant analysis (RDA) showed significant differences in breast milk microbial communities depending on maternal diet clusters, Cluster I ($n = 42$) and Cluster II ($n = 73$) (D). Microbial α diversity indexes according to maternal diet cluster, Cluster I ($n = 42$) and Cluster II ($n = 73$): richness (Chao1 index, E) and diversity (Shannon index, F). Richness and diversity values were adjusted for total bacterial load to normalize the values and avoid the impact of bacterial levels. Linear discriminant analysis (LDA) effect size (LEfSe) plot of taxonomic biomarkers was identified in both clusters: Cluster I ($n = 42$) (green) and Cluster II ($n = 73$) (red) (G). Specific associations between total bacterial load estimated by qPCR and α -diversity indexes, Chao 1 (H) and Shannon index (I) ($n = 115$). AB_birth, intrapartum antibiotic exposure; AB_gestation, antibiotic treatment during gestation; PC, Principal component; PERMANOVA, Permutational multivariate analysis of variance.

and richness. A recent study suggested that the consumption of breast milk with reduced microbial richness during the first month of life is associated with allergy development in children (55). Therefore, maternal diet would shape breast milk microbial diversity and richness with potential impact on infant health outcomes. Furthermore, other studies have reported that breast milk microbiota diversity was higher in women who delivered vaginally than in those who delivered via C-section and had antibiotic exposure (42). Our data showed that antibiotic exposure and/or mode of delivery shaped the breast milk microbiota composition and diversity in a cluster-dependent manner. Higher microbial diversity and richness was observed in breast milk from Cluster I and vaginal/non-antibiotic exposure compared with the other groups. Interestingly, breast milk samples from women who

underwent C-section and were exposed to antibiotics had higher microbial richness (Chao1 index) but not diversity (Shannon index) in Cluster I compared with Cluster II. These results would suggest that fiber and plant protein intakes can modulate the potential impact of antibiotics and C-section on the microbial diversity of breast milk. These data could promote the development of potential new dietary interventions targeted at milk microbiota in women exposed to antibiotics during C-section procedures. Thus, our data together with the available evidence highlight the potential for modulation of breast milk microbiota by perinatal factors, which consequently would influence child microbiota development and health outcomes.

C-sections and antibiotic use have been observed to impact both maternal and neonatal gut microbiota (56). These 2 are

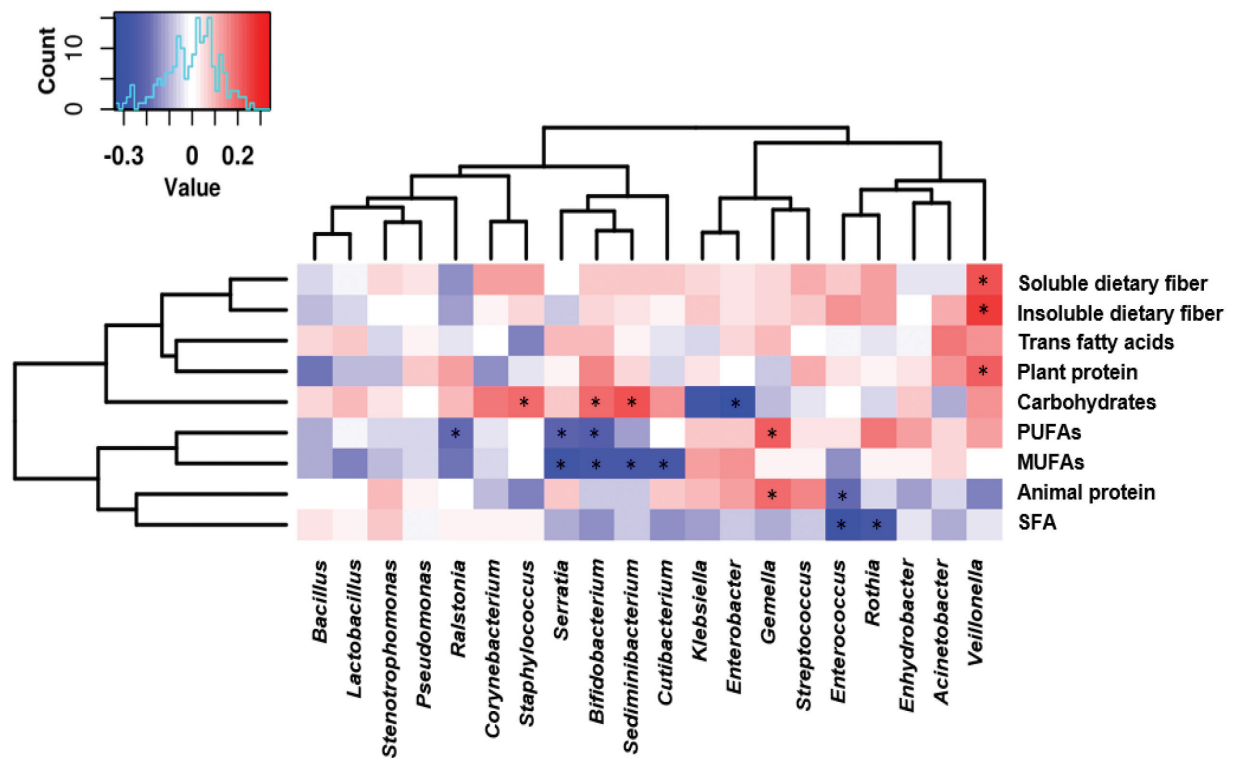


FIGURE 3 Correlations between breast milk microbial top 20 genera and the main dietary nutrients. Heatmaps of Spearman rank correlations between specific dietary nutrient intakes and the top 20 breast milk bacterial genus abundances ($n = 115$). Significant correlations ($P < 0.05$) are marked by an asterisk (*). Blue squares represent negative correlations, whereas red squares show positive correlations.

related factors and cannot be studied separately (42). Although considerable evidence is available regarding the effect of C-section birth and antibiotics on gut microbiota, studies focused on breast milk remain scarce. Contradictory findings regarding the effect of mode of delivery on milk microbiota can be found in the literature. Some studies have shown higher microbial diversity and relative abundance of *Bifidobacterium* and *Lactobacillus* spp. in the breast milk samples of women who delivered vaginally compared with those who delivered via C-section (12, 13, 42), whereas other studies reported no differences (57, 58). Hence, the debate is still open, and additional studies are needed (12). Some studies have shown that breast milk from women undergoing a C-section delivery and/or who received anesthesia during delivery tended to be less likely to contain lactobacilli (5). Mode of delivery and intrapartum antibiotic exposure were both associated with overall microbial composition in breast milk samples at 1 mo postpartum (42). Interestingly, differences between elective and emergency C-section birth seem to influence the microbiota composition (12). Milk samples from women who underwent emergency C-section showed similar microbial profiles to breast milk from those who delivered vaginally, whereas breast milk from those who had elective surgery showed microbial profiles close to those of skin and oral microbial communities (12), although other studies reported no differences (58). These results suggest the contributory role of the physiological labor process, stress, and/or hormonal signals in milk microbiota.

Similarly, antibiotics have been proposed to have an impact on milk microbiota composition (5, 42). Low abundance of *Lactobacillus* and *Bifidobacterium* in breast milk was found in mothers treated with antibiotics during pregnancy or lactation. Chemotherapy during lactation decreased the relative abundance of *Bifidobacterium*, *Staphylococcus*, and

Eubacterium spp. in the milk samples (59). Several authors have related the *Bifidobacterium* and *Lactobacillus* genera to infant health outcomes, proposing the shaping of breast milk microbiota as a gateway to disease prevention (60). Lower *Bifidobacterium* abundances have been observed in the breast milk of allergic mothers and in their offspring's gut microbiota compared with nonallergic healthy ones (61). Thus, the metabolism of dietary compounds by both *Lactobacillus* and *Bifidobacterium* would influence the child's development through several unknown mechanisms. We found a depletion of *Lactobacillus* genus in the breast milk of mothers from the Cluster II/C-section group. However, the breast milk from mothers in Cluster I who had a vaginal birth was enriched in *Actinobacteria* phylum. Interestingly, similar results were found in the analysis of the effect of maternal diet on neonatal gut microbiota (62). These results suggested that the possible interaction between maternal diet and mode of delivery has an effect on breast milk microbiota.

Limitations

This study was a cross-sectional study with a limited sample size and a power analysis that could have hindered—to a certain extent—the possibility of detecting other significant associations. Thus, large-scale prospective longitudinal studies are recommended in the future. Furthermore, an FFQ was chosen for the dietary records as a complete tool to estimate regular intake. However, FFQs can also introduce errors due to a lack of perception of food proportion sizes. Although we validated our data with a random subset of a 3-d diet recall questionnaire, better dietary collection tools and a wider data set would be needed in future studies. Furthermore, other health-related habits, including exercise, stress handling, and home environment have also not been considered in the

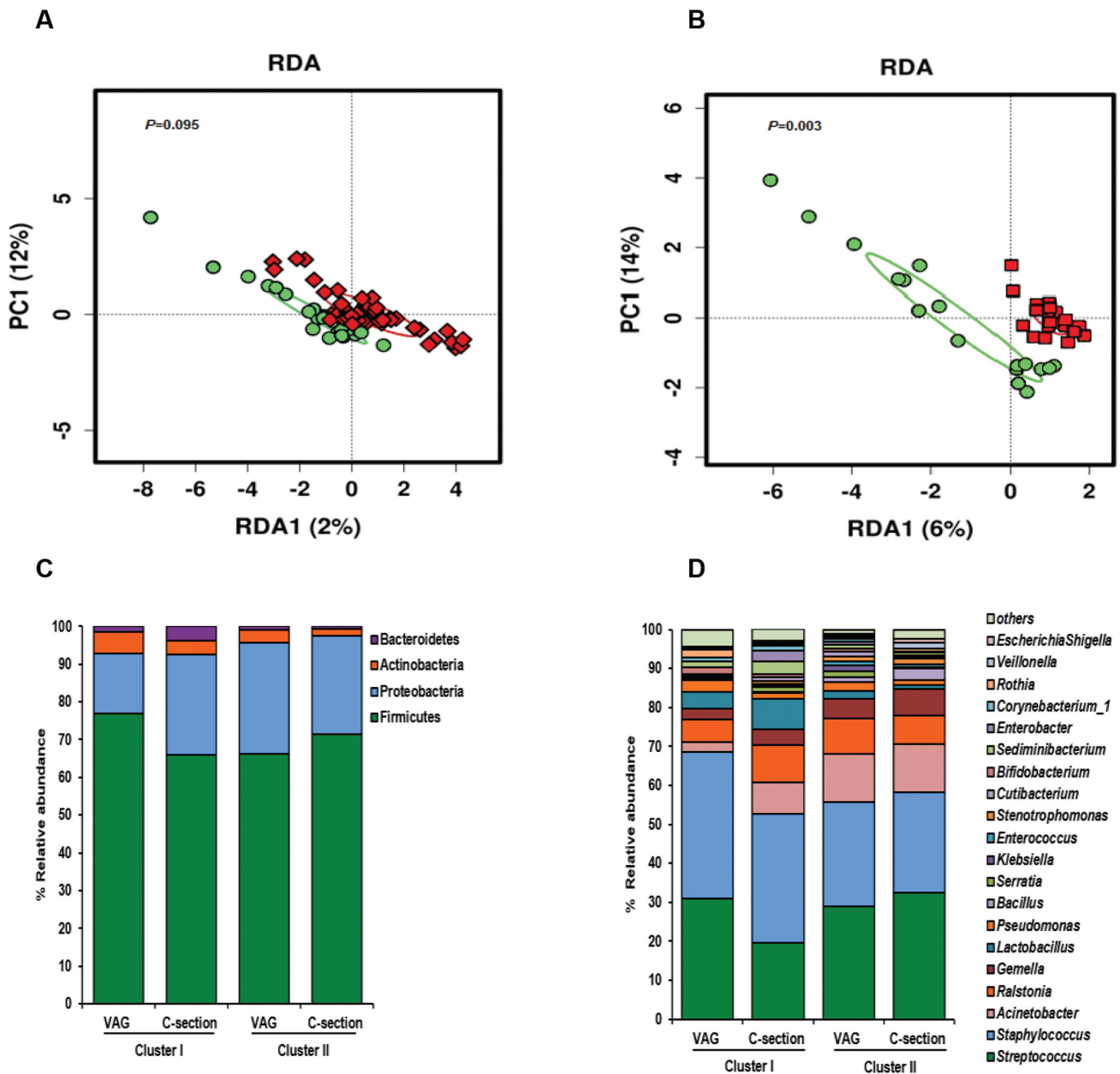


FIGURE 4 Impact of mode of delivery and antibiotic exposure on breast milk microbiota according to maternal diet clusters. Multivariate redundancy discriminant analysis (RDA) showed significant differences in breast milk microbial communities between maternal diet clusters depending on antibiotic exposure and mode of delivery: vaginal/nonantibiotics (A) and C-section/antibiotics (B). Cluster I ($n = 40$) (green) and Cluster II ($n = 72$) (red). Breast milk microbiota profiles at phylum level (C) and at genus level (D) according to maternal diet cluster and mode of delivery and antibiotic exposure. C-section, cesarean section; PC1, principal component 1; VAG, vaginal delivery.

analysis. The potential associations of maternal diet and other breast milk compounds (such as human milk oligosaccharides, cytokine profile, and metabolites, among others) as well as the potential impact of the observed associations on infant microbiota need to be considered and warrant further studies. Moreover, the study of breast milk samples from vaginal deliveries exposed to maternal intrapartum antibiotics would provide novel insights into the impact of maternal diet on milk microbiota.

Despite all these limitations, our study expands knowledge about the impact of maternal diet on breast milk composition and diversity, which can have potential effects in infant microbiota colonization and immune system maturation patterns.

Conclusions

In conclusion, maternal diet shapes the composition and diversity of breast milk microbiota. Macronutrients as well as other specific dietary nutrients, such as soluble and insoluble fiber and polyphenols, influence milk microbial communities. The consumption of food rich in soluble and insoluble fiber and plant protein can be related to microbiota with higher *Staphylococcus*, *Bifidobacterium*, and *Lactobacillus* abundance. The intake of food with high animal protein content and fats, such as n-3 PUFAs and MUFAs, showed a negative correlation with *Enterococcus* and *Bifidobacterium* and a positive correlation with *Gemella*, which might play a role in the composition of the developing infant's gut microbiota. Furthermore, we found an

association among breast milk microbiota composition, dietary pattern, and antibiotic exposure/mode of delivery. This finding suggests a complex interaction between different maternal and perinatal factors that affects breast milk microbiota. Thus, further analysis is warranted to clarify the relations in this network and design interventions that can modify such relations in case one of the factors is affected.

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