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Distinct breast milk microbiota, cytokine, and adipokine profiles are associated with infant growth at 12 months: an *in vitro* host–microbe interaction mechanistic approach†

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Breast milk (BM) is important for adequate infant development, and it contains bioactive compounds, such as bacteria, cytokines and some adipokines which play a role in infant microbial, metabolic, and immunological maturation. However, little is known about its impact on growth and development in early life. The objective of this study was to evaluate the impact of milk microbiota, cytokine, and adipokine profiles on the risk of overweight at 12 months of life to find the possible mechanisms of host–microbe interactions. In this study, BM samples from 100 healthy women collected during 15 d after birth were included. BM microbiota was analysed by 16S rRNA gene sequencing, and cytokine and adipokine levels were measured by the Luminex approach. In addition, infant weight and length were recorded during the first 12 months and *z*-scores were obtained according to the WHO databases. Infants were classified as risk of overweight (ROW) and no-risk of overweight (NOROW) based on their body mass index *z*-score (BMIZ) and infant weight-for-length *z*-score (WLZ) at 12 months. In order to study host–microbe interactions, epithelial intestinal and mammary cell lines were exposed to milk microbes to assess the host response by interleukin (IL)-6 production as a potential inflammatory marker. BM was dominated by *Staphylococcus* and *Streptococcus* genera, and the most abundant cytokines were IL-6 and IL-18. Leptin levels were positively correlated with the pregestational body mass index (BMI). Higher relative abundance of the *Streptococcus* genus was associated with higher IL-10 and higher relative abundance of the *Bifidobacterium* genus was associated with lower IL-6 concentrations in milk. Infant WLZ at 12 months could be partially predicted by *Streptococcus* genus proportions and IL-10 and IL-18 levels in BM. BM microbiota significantly induced cytokine responses in mammary epithelial cells. Higher levels of IL-6 production were observed in mammary cells exposed to BM microbiota from mothers with ROW offspring compared to mothers with NOROW offspring. In conclusion, BM microbiota is related to the cytokine profile. IL-10 and IL-18 levels and the abundance of the *Streptococcus* genus could affect early infant development. Further research is needed to clarify the specific impact of BM microbiota and cytokines on infant growth and the risk of overweight.

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Introduction

Early nutrition exerts both short- and long-term effects on human health in programming its immunological, metabolic, and microbiological development. Breastfeeding confers multiple health benefits to offspring, including protection from infections and probable protection against obesity.[1] The child obesity rates are increasing worldwide, and it is estimated that on average nearly one in eight children aged 7–8 years is obese in European countries (World Health Organization (WHO) Europe 2018, Children Obesity Surveillance Initiative, highlights 2015–17, preliminary data), with Spain being one of the

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countries with the highest obesity rates. Thus, European health care systems are supporting strategies to reduce the prevalence of obesity.

Breastfeeding has been associated with a significant reduced risk of obesity, showing a dose–response effect between the breastfeeding duration and the reduction of the risk.[2] In this regard, breast milk (BM) is the best food and the first option for infant nutrition,[3,4] as it has functions with implications for adequate gut microbial assembly and immune system development.[5]

Alterations in microbial colonisation in early life due to C-section,[6–8] antibiotic exposure,[6] and lack of breastfeeding have been associated with a higher prevalence of obesity in children.[1,2]

A distinct microbial pattern has been observed in infants fed with formula compared to breastfed infants,[9] mainly due to the predominance of *Bifidobacteriaceae* in breastfed infants during the first months of life.[10] These observations could be explained by the presence of microbes and oligosaccharides, as well as other components in BM. Thus, it has been hypothesised that shifts in milk microbiota due to some maternal disorders, such as obesity[11,12] or other maternal and infant factors that shape the BM microbiota composition,[13–15] could be transferred to the neonates through an unbalanced microbial colonisation. However, the mechanisms that drive this relationship have been underexplored and these relationships are still poorly understood. To our knowledge, no study has shown the role of BM microbiota in infant growth and development.

Therefore, in this scenario, our main objective was to assess the impact of milk microbiota and immune and adipokine profiles on infant growth and the risk of overweight at 12 months of life, as well as to find potential mechanisms behind the BM microbiota–immune signal–obesity risk relationship using *in vitro* approaches.

Materials and methods

Study design and volunteers

A subgroup of 100 healthy mother–infant dyads ≤15 d post birth following breastfeeding practices from the MAMI cohort (Maternal Microbes, Valencia, Spain)[16] were included in this study based on the availability of clinical and anthropometric data including maternal age, gestational age, delivery mode, maternal body mass index (BMI), and weight gain during pregnancy.

All participants received oral and written information about the study and written consent was obtained from them. This study is registered on the ClinicalTrial.gov platform with registration number NCT03552939 and it is approved by the Ethics Committees of the Hospital Clínico Universitario de Valencia (Spain).

Infant anthropometric measures

Simultaneous weight and length measurements were collected during periodical paediatric visits by clinicians during the first months of life at birth, 1, 6, and 12 months. The age and sex specific BMI *z*-score (BMIZ) and weight-for-length *z*-score (WLZ) were calculated using WHO Anthro software.[17] Children were classified as at risk of overweight (ROW) and no-risk of overweight (NOROW) based on their BMIZ and WLZ at 12 months. BMIZ and WLZ greater than the 85th percentile (*z*-score ≥0.99) were considered as at risk of overweight as has been previously described elsewhere.[18] The changes in the BMIZ and WLZ were calculated by taking the difference in the *z*-score between time points as described elsewhere.[19]

BM samples

Breast milk samples were collected from mothers (n = 100) ≤15 d (average = 11.32 d) after birth following a protocol described previously.[13] The samples were centrifuged at 16 000*g* at 4 °C for 15 min, and the upper fat layer was removed. BM pellets were used for DNA extraction and *in vitro* cell model analysis and the supernatants were used for the determination of cytokine and adipokine profiles.

BM microbiota profile

Total DNA was extracted from the samples using 1.5–2.0 mL of BM using the Master-Pure DNA Extraction kit (Epicentre, Madison, WI, USA) following a previously described protocol.[20] Then, the total DNA was purified using the MagSi-NGS Plus kit (Amsbio, Abingdon, UK) following the manufacturer's instructions. Controls for DNA extraction and PCR amplification were also included.

Milk microbiota composition was assessed by the sequencing of the V3–V4 variable region of the 16S rRNA gene following the Illumina protocols as described by García-Mantrana *et al.*[21] using a MiSeq-Illumina platform (FISABIO sequencing service, Valencia, Spain). Briefly, a Nextera XT Index kit (Illumina, CA, USA) was used for the multiplexing step, and the libraries were sequenced using a 2 × 300 pb paired-end run (MiSeq Reagent kit v3).

Trimmomatic software[22] was used to search and remove the residual adaptors and DADA2 pipeline v.1.16[23] was used for quality filtering, sequence joining, and chimera removal. Taxonomic assignment was achieved using the Silva v132 database, including the species level classification.[24] Additional filtering was performed in which samples with less than 1000 reads, amplicon sequence variants (ASVs) with a relative abundance less than 0.01% and those present less than 3 times in at least 20% of the samples were removed. Also, the decontam package[25] in the Rstudio environment was used to identify the possible contaminants which were removed from the final analysis (n = 48 ASVs).

BM cytokine and adipokine profiles

The quantification of cytokines (GM-CSF, IFN-γ, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-9, IL-10, IL-12p70, IL-13, IL-17A, IL-18, IL-21, IL-22, IL-23, IL-27, and TNF-α) was performed by ProcartaPlex™ Multiplex immunoassay (Thermo Fisher Scientific, Austria) using a Th1/Th2/Th9/Th17 Cytokine 18-Plex Human ProcartaPlex™ panel following the manufacturer's instructions. Assay sensitivity was as follows: 1.2 pg mL^{-1} for GM-CSF; 0.2 pg mL^{-1} for IFN-γ; 0.2 pg mL^{-1} for IL-1β; 0.8 pg mL^{-1} for IL-2; 1.5 pg mL^{-1} for IL-4; 0.3 pg mL^{-1} for IL-5; 0.4 pg

mL^{-1} for IL-6; 0.5 pg mL^{-1} for IL-9; 0.1 pg mL^{-1} for IL-10; 0.04 pg mL^{-1} for IL-12p70; 0.1 pg mL^{-1} for IL-13; 0.1 pg mL^{-1} for IL-17A; 0.4 pg mL^{-1} for IL-18; 0.6 pg mL^{-1} for IL-21; 8.2 pg mL^{-1} for IL-22; 0.9 pg mL^{-1} for IL-23; 5.1 pg mL^{-1} for IL-27; and 0.4 pg mL^{-1} for TNF-α.

The quantification of leptin was performed using a Quantikine® Colorimetric Sandwich ELISA kit (R&D Systems, Minneapolis, MN, USA) following the manufacturer's instructions. Assay sensitivity was 7.8 pg mL^{-1} for leptin. Adiponectin was analysed using the Adiponectin Human ProcartaPlex™ Simplex kit (Thermo Fisher Scientific) with an assay sensitivity of 4.6 pg mL^{-1}.

Host–microbe interactions: *in vitro* approach

Study of the BM microbes exposed to the mammary epithelial cells. A proof-of-concept study was carried out with a subset of milk samples (n = 20). Milk was centrifuged at 16 000*g* at 4 °C for 15 min, and the upper fat layer was removed. The samples were centrifuged again at same conditions to collect the bacterial pellet and this was resuspended in 500 μL of phosphate buffered saline (PBS) to be inactivated for 10 min at 65 °C.

To explore the maternal mammary epithelia and milk microbe interactions, the MCF7 (ATCC HTB-22) mammary epithelial cell line was used. Cells were routinely maintained at 37 °C under a humidified atmosphere of 5% CO_2 in DMEM high glucose with stable glutamine and sodium pyruvate culture medium (Capricorn Scientific, Ebsdorfergrund, Germany), supplemented with 10% v/v inactivated fetal bovine serum (FBS, Biowest, Nuaillé, France), 1% non-essential amino acids (Capricorn Scientific, Ebsdorfergrund, Germany), 10 mM HEPES (Capricorn Scientific, Ebsdorfergrund, Germany), and antibiotics (100 U mL^{-1} penicillin and 100 μg mL^{-1} streptomycin [Sigma-Aldrich, Missouri, USA]), according to a procedure reported elsewhere.[26]

MCF7 cells were seeded in 12-well plates at 30 000 cells per well in complete growth medium without antibiotics. After 24 h, the cells were exposed to bacterial pellet suspension diluted 1 : 10 (v/v) in the specified medium and were co-incubated with human milk bacteria for 20 h at 37 °C and under 5% CO_2 in an incubator. Negative controls consisted of MCF7 cells incubated without bacteria. The experiment was performed in triplicate.

After co-incubation, the cells and supernatants were used for both gene expression determination and cytokine quantification, respectively. IL-6 concentrations were measured by ELISA (Invitrogen, Vienna, Austria) using 100 μL of the supernatant, following the manufacturer's instructions.

RNA was extracted using the NucleoSpin RNA XS kit (Macherey-Nagel, Düren, Germany) following the manufacturer's instructions. cDNA from the total RNA was generated using a high-capacity cDNA reverse transcription kit (Applied Biosystems, CA, USA) after total RNA normalisation. RT-PCR analysis was performed using the SYBR Green PCR Master Mix (Roche), using 1 μL of cDNA and the specific primers (0.15 μM): IL-6 F (5′-GTGTGAAAGCAGCAAAGAGGC-3′), IL-6 R (5′-TGCAGGAACTGGATCAGGACT-3′),[27] actin (ACTB) F (5′-TTGTTACAGGAAGTCCCTTGCC-3′), and ACTB R (5′-ATGCTATCACCTCCCCTGTGTG-3′),[28] which was used as a housekeeping gene. The annealing temperature was 58 °C for both targets. LC480 Conversion version 2014.1 and LinRegPCR v. 11.0 software were used for efficiency calculation,[29,30] and the relative gene expression was quantified according to the efficiency-corrected method using the REST 2009 software tool.[31]

Study of the BM microbes related to the NF-κB pathway activation in intestinal epithelial cells. To test the infant intestinal epithelia and milk microbe interactions, an HT-29-transfected cell line with a plasmid carrying the NF-κB promotor binding site, followed by a reporter gene encoding for a secreted form of the human embryonic alkaline phosphatase (SEAP) (pNiFty2-SEAP; Invitrogen, Carlsbad, CA, USA), was used as described previously.[6,32] The HT-29 cell line was seeded onto 96-well plates with a density of 60 000 viable cells per well and expanded at 37 °C under a humidified atmosphere of 5% CO_2 in DMEM high glucose with stable glutamine and sodium pyruvate culture medium (Capricorn Scientific), supplemented with 10% v/v FBS (Biowest), antibiotics (100 U mL^{-1} penicillin and 100 μg mL^{-1} streptomycin [Sigma-Aldrich]), and zeocin (200 μg mL^{-1}, Invivogen, CA, USA).

The cells were exposed to the same BM pellets as detailed above, and the supernatants were collected after 24 h of stimulation. SEAP activity was measured using *p*-nitrophenyl phosphate as a substrate according to manufacturer's instructions (Thermo Fisher Scientific, Waltham, USA). The signal was quantified using a CLARIOstar microtiter plate reader (BMG Labtech, Ortenberg, Germany) at 405 nm. The cells were lysed in PBS containing 0.1% Triton, 1 mM phenylmethylsulphonyl fluoride (PMSF), and 1 mM ethylenediaminetetraacetic acid (EDTA). The protein content of each well was determined using the Bradford Protein Assay (Bio-Rad, CA, USA). SEAP activity was calculated according to the formula: SEAP activity = ($A_{405\ nm}$ test − $A_{405\ nm}$ initial) × the total assay volume (mL)/mM extinction coefficient of *p*-nitrophenol (18.5) × the cell culture supernatant employed (mL) × time (min) and normalised to the protein content of each well.

Study of the gut microbiota of infants at the risk of obesity related to the NF-κB inflammatory pathways. To test whether the gut microbiota from the infants at ROW induced an inflammatory signal, a pilot test was carried out with faecal samples from a subset of children at 12 months (n = 32, 16 children with NOROW and 16 children with ROW). Faecal supernatants were obtained as follows: 100 mg of faeces were mixed with 1 mL sterile water, vortexed, and incubated for 15 min on ice. Then, the suspension was centrifuged at 16 000*g* for 10 min at 4 °C, and the resulting supernatant was filtered using a 0.20 μm filter and diluted 1 : 10 (v/v) in DMEM. HT-29-transfected cells were exposed to this supernatant as described above, and after 24 h of stimulation, the culture media were collected, and the SEAP activity was measured as detailed before. The experiment was performed in triplicate.

Statistical analysis

A *T*-test and Mann–Whitney analysis were used depending on the data normality assessed by the Kolmogorov–Smirnov and

Shapiro–Wilk tests (GraphPad Prism V5.04). A nonmetric multidimensional scaling (NMDS) analysis based on the Bray–Curtis distance was performed to assess the effect of perinatal factors on the BM microbiota, cytokine, and adipokine compositions.[33]

The Spearman correlations between cytokine and adipokine concentrations and the relative abundances of the bacterial genera, adjusted for mode of birth, intrapartum antibiotic (ATB), and breastfeeding practices at 15 d, were obtained using SPSS V.27 and the heatmap plot was obtained using RStudio.[34–36] Multivariate linear regression (backward regression) analysis was then used to determine the ability of microbiota, cytokine, and adipokine concentrations to predict longitudinal growth outcomes at 12 months post-partum. The following software was used for analysis: SPSS V.27[37] (IBM Corp., released 2020; IBM SPSS Statistics for Windows, version 27.0., Armonk, NY: IBM Corp.), GraphPad Prism v. 5.04 (GraphPad Software, San Diego, CA, USA, https://www.graphpad.com) and RStudio.[34]

Results

Subjects and clinical data

100 families were included in the study (Table 1). 85% of the mother–infant pairs were following exclusive breastfeeding (EBF) at the time of milk collection. Approximately half of the included infants were females (57%), 61% were born vaginally and 39% were born by C-section delivery (Table 1). From the 100 children, 17% were defined as ROW and 82% were defined as NOROW at 12 months according to the BMIZ and WLZ following the WHO growth curves (Table 1). In our cohort, we only observed significant differences in the BMIZ and WLZ between ROW and NOROW infants at 6 and 12 months of life ($p < 0.001$). As expected, a higher weight gain (kg) over pregnancy was observed in mothers with ROW offspring compared to mothers with NOROW offspring ($p = 0.045$). The changes in growth from 6 to 12 months are presented in Fig. S1.† The changes in the WLZ and BMIZ values (Fig. S1A and S1B†) were significantly lower in the NOROW offspring than in the ROW offspring.

Factors shaping the BM microbiota, cytokine, and adipokine compositions at 15 d of lactation

In general, BM microbial communities were characterised by a dominance of Firmicutes [84.49%; interquartile range (IQR): 48.39–94.09] and Proteobacteria [7.88%; IQR: 1.54–38.45] phyla, followed by Actinobacteria [1.82%; IQR: 0.55–5.11] and Bacteroidetes [0.66%; IQR: 0.15–1.73] phyla (Fig. 1A). At the genus level, the most abundant taxa were *Streptococcus*

Table 1 Characteristics of mothers and infants ($n = 100$)

		Total ($n = 100$)	NOROW ($n = 82$)[a]	ROW ($n = 17$)[a]	*p-value*
Maternal characteristics					
Maternal age (years)		34.77 ± 3.74	34.76 ± 3.78	34.76 ± 3.78	0.993
Gestational age (weeks)		40 [39–40]	40 [39–40]	40 [39–41]	0.313
Pre-gestational BMI (kg m^{-2})		22.8 [21.0–25.1]	22.8 [21.0–25.4]	23.0 [20.4–23.6]	0.610
Weight gain (kg) during pregnancy		12 [10–14]	12 [9–14]	14.5 [11–18]	0.045
Antibiotic treatment during pregnancy (%)		31 (31%)	25 (30%)	6 (35%)	0.697
Intrapartum antibiotic exposure (%)		43 (43%)	33 (40%)	10 (59%)	0.160
Infant characteristics					
Gender: female (%)		57 (57%)	46 (56%)	10 (59%)	0.837
Birth mode: vaginal birth (%)		61 (61%)	52 (63%)	8 (47%)	0.209
Antibiotic treatment, 15 days (%)		7 (7%)	6 (7%)	1 (6%)	0.834
Breastfeeding duration (months)		9.5 [6–12]	9.5 [6–12]	12 [6–12]	0.713
Breast feeding at 15 days					
Exclusive breastfeeding (EBF)		85 (85%)	70 (85%)	14 (82%)	0.753
Mixed feeding (MF)		15 (15%)	12 (15%)	3 (18%)	
BMIZ	At birth	−0.11 ± 1.06	−0.17 ± 1.02	0.15 ± 1.20	0.258
	1 month	−0.49 ± 0.99	−0.59 ± 0.96	−0.01 ± 1.03	0.027
	6 months	−0.26 ± 0.87	−0.42 ± 0.77	0.50 ± 0.92	<0.001
	12 months	0.20 ± 0.91	−0.06 ± 0.75	1.48 ± 0.41	<0.001
WLZ	At birth	−0.19 ± 1.15	−0.24 ± 1.12	0.06 ± 1.30	0.330
	1 month	−0.49 ± 1.22	−0.56 ± 1.25	−0.16 ± 1.01	0.220
	6 months	−0.13 ± 0.82	−0.28 ± 0.72	0.58 ± 0.91	<0.001
	12 months	0.20 ± 0.86	−0.06 ± 0.68	1.46 ± 0.35	<0.001

Categorical variables are expressed as positive cases-prevalence and (percentage, %) and a chi-squared test was performed to assess the significance. Normally distributed data are presented as mean ± standard deviation (SD) and non-normal data as median and interquartile range [IQR]. BMI, body mass index; NW, normal weight; OW, overweight; BMIZ, body mass index *z*-score; WLZ, weight-for-length *z*-score; NOROW, no risk of overweight; ROW, risk of overweight. $p < 0.05$ was considered statistically significant. [a] One infant has not available information on weight and length at 12 months and it was not included.

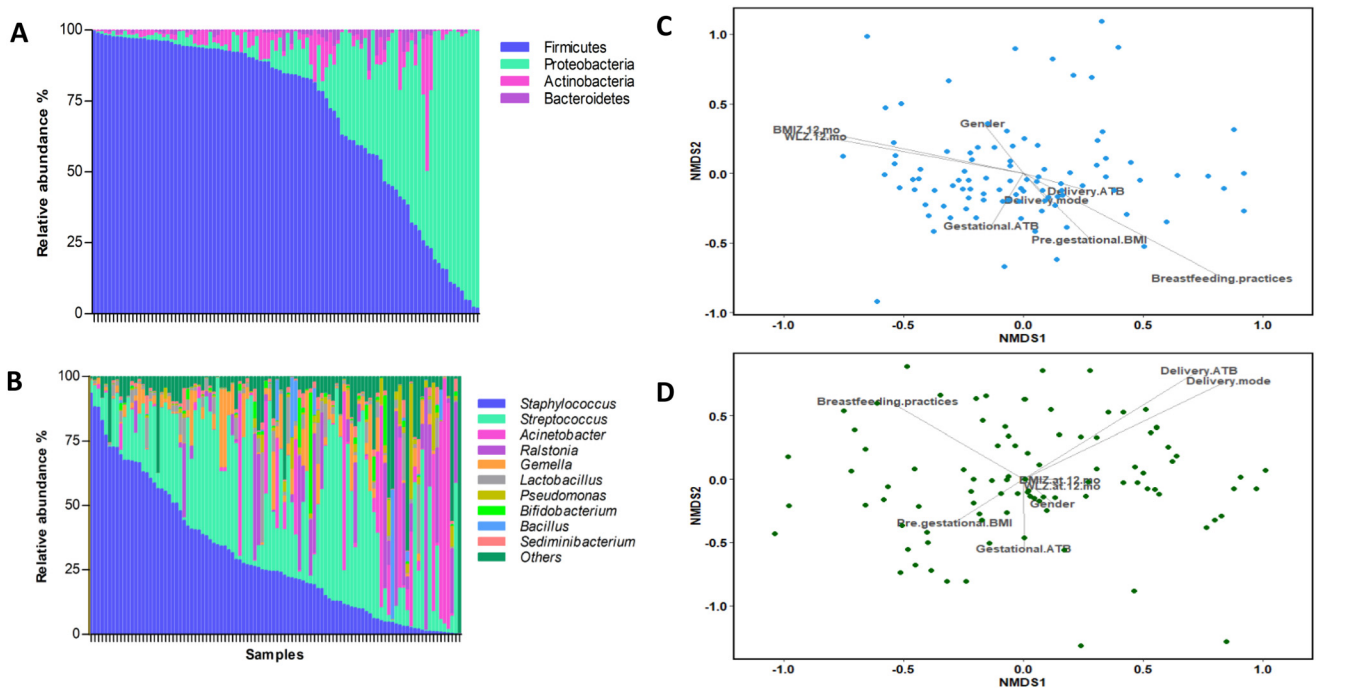

Fig. 1 BM composition in terms of microbiota (n = 100) at the (A) phylum and (B) genus levels. Effect of perinatal factors on the BM microbiota composition assessed by nonmetric multidimensional scaling (NMDS) analysis based on the Bray–Curtis distance (n = 100). (C) The plot of the genus composition and (D) the plot of the composition in terms of cytokines and adipokines.

[28.10%; IQR: 8.48–46.92] and *Staphylococcus* [24.37%; IQR: 8.17–49.96], followed by *Acinetobacter* [0.18%; IQR: 0.04–1.50] and *Ralstonia* [0.08%; IQR: 0–6.24] (Fig. 1B).

We performed a NMDS analysis to identify the main perinatal factors contributing to the BM microbiota. We found that breastfeeding practices significantly affect the NMDS ordination of the samples based on BM microbiota (envfit; breastfeeding practices: R^2 = 0.088, p = 0.011) (Fig. 1C). Additionally, the BMIZ at 12 months showed a significant correlation with the NMDS ordination (envfit; BMIZ at 12 months: R^2 = 0.065, p = 0.044) (Fig. 1C).

The concentration of cytokines, leptin, and adiponectin detected in BM are listed in the ESI (Table S1†). The levels obtained varied among cytokines in terms of concentration and detection, with the most abundant cytokines being IL-6, IL-18, IL-21, and IL-1β (Table S1†), even though the levels of detection of IL-6, IL-18, IL-21, TNF-α and leptin in the BM were above the assay limit of detection in more than 48% of subjects. We explored whether perinatal factors could affect the BM microbiota composition and the cytokine concentration. We found that the delivery mode and ATB significantly impacted the NMDS ordination based on the cytokine and adipokine profiles (envfit; delivery mode: R^2 = 0.0615, p = 0.049; delivery ATB: R^2 = 0.0671, p = 0.042) (Fig. 1D).

To assess the influence of pre-gestational BMI and breastfeeding practices on the cytokine, microbiota, and adipokine concentrations, the correlations between these parameters were also studied.

The pre-gestational maternal BMI was positively correlated with the BM microbiota: *Staphylococcus* genus (ρ = 0.207, p = 0.039) (Table S2†) and the BM cytokines TNF-α (ρ = 0.30, p = 0.03) and IL-22 (ρ = 0.20, p = 0.048) (Table S2†). Additionally, the pre-gestational maternal BMI was positively correlated with leptin (ρ = 0.388, p < 0.001) (Fig. S2A†), while no correlation was observed for BM adiponectin (ρ = −0.109, p = 0.28). Furthermore, mothers with mixed feeding mode showed higher leptin (p = 0.003) (Fig. S2B†) concentration compared with those with exclusive breastfeeding (p = 0.003) (Fig. S2B†), and no significant difference was observed for adiponectin (p = 0.451) (Fig. S2C†) concentration compared with those with exclusive breastfeeding.

Associations between BM microbiota, milk cytokines, and adipokines

Significant associations between cytokines and BM microbial genera were identified (Fig. 2A). Higher *Bifidobacterium* genus was associated with lower IL-6 (ρ = −0.23, p = 0.025), TNF-α (ρ = −0.40, p < 0.001) and IL-21 (ρ = −0.21, p = 0.044). The *Streptococcus* genus was positively related to IL-18 (ρ = 0.28, p = 0.006) and IL-10 (ρ = 0.24, p = 0.018), while a higher abundance of the *Staphylococcus* genus was associated with higher IL-22 (ρ = 0.32, p = 0.002) and IL-23 (ρ = 0.30, p = 0.004).

Negative correlations were also observed between *Acinetobacter* and IL-17 (ρ = −0.26, p = 0.011) and also between *Pseudomonas* and IL-18 (ρ = −0.24, p = 0.021) and IL-22 (ρ = −0.28, p = 0.007).

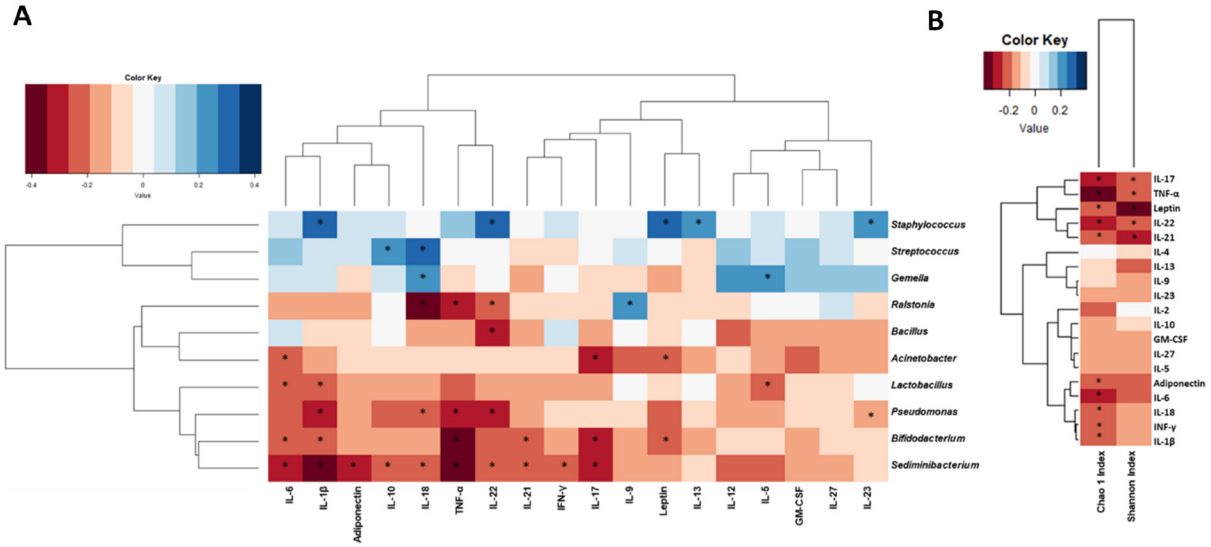

Fig. 2 Heatmaps of Spearman's rank correlations between cytokines and adipokine concentrations and the relative abundances of the bacterial genera from the BM samples (n = 100), adjusted for the mode of birth, intrapartum antibiotic (ATB) and breastfeeding practices at 15 days. (A) Spearman's correlations between the BM microbial genera and the cytokine concentrations. (B) Spearman's correlations between alpha diversity and the cytokine concentrations. Significant correlations ($p < 0.05$) were marked by an asterisk (*). Red squares represent the negative correlations, whereas blue represent the positive correlations.

With regard to the associations between adipokines and BM microbiota (Fig. 2A), negative correlations were observed between leptin and the *Bifidobacterium* ($\rho = -0.20$, $p = 0.048$) or the *Acinetobacter* ($\rho = -0.24$, $p = 0.021$) genus. Similarly, adiponectin also showed a negative relationship with the relative abundance of *Sediminibacterium* ($\rho = -0.27$, $p = 0.010$). Associations between the Chao1 and Shannon indices and cytokines/adipokines were also observed (Fig. 2B). Both leptin ($\rho = -0.21$, $p = 0.037$) and adiponectin ($\rho = -0.22$, $p = 0.031$) showed a negative correlation with the Chao1 index, which was also negatively correlated with IL-1β ($\rho = -0.21$, $p = 0.035$). Similarly, the Shannon index was also negatively correlated with leptin ($\rho = -0.37$, $p < 0.001$) and TNF-α ($\rho = -0.22$, $p = 0.029$).

BM microbiota, cytokine, and adipokine profiles were associated with infant growth trajectories and the risk of overweight at 12 months of life

The concentrations of cytokines, leptin, and adiponectin in BM in mothers with ROW offspring compared to mothers with NOROW offspring are shown in Table 2.

The most predominant cytokine in both groups was IL-6; the concentration of this cytokine was higher in the BM from mothers with ROW offspring compared to those with NOROW offspring ($p = 0.031$) (Table 2).

The relationship between some taxa from the BM microbiota, cytokines and adipokines and infant development is presented in Table 3, including the linear regression β coefficients for the BM microbiota and BM cytokines and adipokines, predicting the BMIZ and WLZ at 12 months, as well as the unadjusted and adjusted models (Tables 3 and S3†).

The unadjusted models showed that lower *Streptococcus* ($p = 0.040$) and IL-10 ($p = 0.010$) and IL-17 ($p = 0.043$) levels in the BM, and higher Chao1 index ($p = 0.012$) and IL-6 ($p = 0.030$) and IL-18 ($p < 0.001$) were associated with a higher WLZ at 12 months.

When these models were adjusted for the mode of birth, ATB and breastfeeding practices at 15 d, lower *Streptococcus* ($p = 0.048$) and IL-10 ($p = 0.009$) levels in the BM and higher Chao1 index ($p = 0.012$) and IL-18 ($p = 0.001$) were associated with a higher WLZ at 12 months (Table 3).

Interestingly, relationship between some taxa from BM microbiota, cytokines and adipokines and BMIZ at 12 months was found in unadjusted analysis. *Streptococcus* ($p = 0.044$) was a predictor of higher BMIZ at 12 months (Table S3†), and in contrast, the IL-18 ($p = 0.008$) concentration and Chao 1 index ($p = 0.007$) were significantly associated with a lower BMIZ at 12 months (Table S3†). Interestingly, in the adjusted model, lower IL-10 ($p = 0.049$) was associated with a higher BMIZ at 12 months.

BM microbes induce IL-6 in the mammary epithelia in a ROW-dependent manner, but do not activate the NF-κB inflammatory pathway in the intestinal epithelia

BM pellets significantly induced cytokine responses in mammary epithelial cells. Higher levels of IL-6 production were observed in mammary cells exposed to pellets from the BM of mothers with ROW offspring compared to those exposed to pellets from the BM of mothers with NOROW offspring ($p = 0.001$) and the control conditions ($p = 0.002$)

Table 2 Cytokines and adipokine concentrations in the BM in mothers with ROW and NOROW offspring (n = 99)

Cytokines and adipokines (pg mL^{-1})	NOROW (n = 82)			ROW (n = 17)		
	IQR	pg mL^{-1}	% det	IQR	pg mL^{-1}	% det
IL-2	0.00–0.00	0.67 ± 2.14	10.98 (9)	0.00–0.00	0.59 ± 1.65	11.76 (2)
IL-4	0.00–0.00	0.19 ± 1.15	3.66 (3)	0.00–0.00	0.04 ± 0.14	5.88 (1)
IL-5	0.00–0.00	0.20 ± 0.72	8.54 (7)	0.00–0.00	0.09 ± 0.34	5.88 (1)
IL-6[a]	**0.00–38.77**	**57.48 ± 162.16**	**54.88 (45)**	**0.00–0.00**	**142.26 ± 418.67**	**17.65 (3)**
IL-9	0.00–0.00	0.24 ± 1.25	4.88 (4)	0.00–0.00	0.04 ± 0.18	5.88 (1)
IL-10	0.00–0.34	1.04 ± 4.98	60.98 (50)	0.00–0.23	0.22 ± 0.55	58.82 (10)
IL-12	0.00–0.10	0.34 ± 2.55	25.61 (21)	0.00–0.10	0.11 ± 0.28	35.29 (6)
IL-13	0.00–0.00	0.01 ± 0.13	1.22 (1)	0.00–0.00	0.07 ± 0.29	5.88 (1)
IL-17	0.00–0.00	0.17 ± 0.76	7.32 (6)	0.00–0.00	0.14 ± 0.59	5.88 (1)
IL-18	2.42–14.88	11.79 ± 18.81	86.59 (71)	3.42–23.59	23.04 ± 39.85	88.24 (15)
IL-21	0.38–4.50	12.60 ± 41.29	79.27 (65)	0.19–10.04	12.11 ± 28.47	76.47 (13)
IL-22	0.00–5.88	4.92 ± 8.68	52.44 (43)	0.00–8.56	5.58 ± 8.26	58.82 (10)
IL-23	0.00–0.00	0.58 ± 1.74	12.20 (10)	0.00–1.73	1.39 ± 2.92	23.53 (4)
IL-1β	0.00–1.88	10.83 ± 55.24	40.24 (33)	0.00–0.67	2.48 ± 8.17	29.41 (5)
IFN_γ	0.00–0.00	1.33 ± 9.73	18.29 (15)	0.00–0.39	0.90 ± 2.89	23.53 (4)
GM_CSF	0.00–0.00	0.13 ± 0.90	2.44 (2)	0.00–0.00	0.00 ± 0.00	0 (0)
TNF_α	1.66–2.44	2.43 ± 2.70	100 (82)	1.66–2.05	2.19 ± 1.39	100 (17)
Leptin	185.90–546.34	446.98 ± 417.23	97.56 (80)	97.64–805.37	521.70 ± 602.82	100 (17)
Adiponectin	7987.05–17 068.39	13 644.08 ± 8626.07	98.78 (81)	7830.93–17 357.13	19 490.01 ± 28 663.63	100 (17)

Data shown are expressed as [IQR]. Detectability frequencies (% det). GM-CSF, granulocyte macrophage colony-stimulating factor; IFN, interferon; IL, interleukin; TNF, tumor necrosis factor; NOROW, no risk of overweight; ROW, risk of overweight. [a] A Mann–Whitney U test was used to determine significant differences between the concentrations and a chi-square test compared detectability. $p < 0.05$ was considered statistically significant. Significances are highlighted in bold.

Table 3 Relationship between the BM microbiota, cytokines and adipokine concentrations and the WLZ at 12 months (n = 99)

Breast milk compounds	Unadjusted analysis			Adjusted analysis[a]		
	β	95% Cl	p	β	95% Cl	p
Chao 1 index	0.20	0.004 to 0.036	**0.012**	0.021	0.005 to 0.037	**0.012**
Streptococcus	−0.008	−0.015 to 0.000	**0.040**	−0.008	−0.016 to 0.037	**0.048**
Gemella	−0.022	−0.044 to 0.000	0.052	−0.021	−0.044 to 0.002	0.070
IL-1β	−0.004	−0.008 to 0.000	0.065	−0.004	−0.008 to 0.001	0.095
IL-6	0.001	0.000 to 0.002	**0.030**	0.001	0.000 to 0.002	0.054
IL-10	−0.054	−0.094 to 0.013	**0.010**	−0.056	−0.098 to 0.014	**0.009**
IL-12	0.061	−0.007 to 0.129	0.079	0.061	−0.008 to 0.131	0.082
IL-13	−0.967	−2.080 to 0.146	0.088	−0.916	−2.058 to 0.226	0.114
IL-17	−0.270	−0.532 to 0.008	**0.043**	−0.264	−0.533 to 0.005	0.055
IL-23	0.077	−0.003 to 0.157	0.060	0.082	−0.001 to 0.164	0.054
IL-18	0.019	0.009 to 0.030	**<0.001**	0.019	0.009 to 0.030	**0.001**

Linear regression β coefficients for the BM microbiota and cytokines predicting the WLZ at 12 months. [a] Adjusted for mode of birth, intrapartum antibiotics (ATB) and breastfeeding practices at 15 days. $p < 0.05$ was considered statistically significant. Cl: confidence interval, IL: interleukin. Significances are highlighted in bold.

(Fig. 3A). In addition, a higher gene expression of IL-6 was observed in those cells exposed to milk bacterial pellets from the mothers with ROW offspring when compared to those exposed to milk bacterial pellets from the mothers with NOROW offspring (p = 0.037) (Table S4†).

However, when the intestinal epithelial cells were tested, the BM bacterial pellet samples did not induce NF-κB activation in the HT-29 reporter cell line (data not shown).

Does the faecal supernatant from ROW infants at 12 months activate the NF-κB inflammatory pathway?

When faecal supernatants from the infants at 12 months were tested, we observed NF-κB activation in the HT-29 reporter cell line. Both groups, ROW ($p < 0.001$) and NOROW (p = 0.006) children, induced NF-κB activation (Fig. 3B); significant differences between NOROW and ROW children were observed (Fig. 3B) (p = 0.029). Also, we observed a positive correlation between the SEAP activity induced by the faecal supernatants

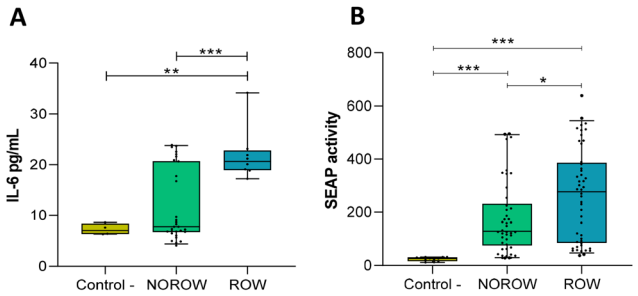

Fig. 3 Host–microbe interactions: *in vitro* approach. (A) IL-6 secretion in MCF-7 mammary cells in response to BM pellets according to the offspring condition (milk from mothers with NOROW offspring and mothers with ROW offspring) (n = 20). (B) NF-κB activation in HT-29 reporter epithelial cells in response to fecal supernatants from children at 12 months of age (children from the ROW and NOROW groups) (n = 32). Whiskers represent the 5–95 percentile interval. Data represent the fold increase related to the control – (only cells). *p < 0.05, **p < 0.01 and ***p < 0.001. ROW (risk of overweight); NOROW (no risk of overweight).

and the concentration of IL-6 in the BM samples (ρ = 0.38, p = 0.037).

Discussion

BM contains bioactive compounds including microbiota and cytokines, which may have an impact on infant growth[38,39] and health outcomes.[40] Our results provide insight on the BM bioactive composition in terms of microbiota, cytokines, and adipokines and their potential link to infant growth and ROW at 12 months of life.

In agreement with previous data,[41] we reported that IL-6, followed by IL-18, was the most abundant cytokine in BM samples.[42,43] The presence and abundance of these cytokines have been described to be relevant in the link between BM and infant development.[41,44,45] Furthermore, *Streptococcus* and *Staphylococcus* were also identified as the most abundant genera.[13] However, the potential interaction between these BM components and the association with the ROW are underexplored. Little is known about the factors influencing the complex association between microbial taxa, cytokines and adipokines in BM. Some studies reported the key relevance of maternal BMI and breastfeeding practices on milk microbiota and infant growth.[11,46] Maternal BMI has been highlighted as one of the factors affecting other components in BM, such as lipids and human milk oligosaccharides.[47,48] While its relationship to other components has been less addressed, significant associations have been described between maternal BMI with milk leptin at 1 month postpartum and milk glucose, insulin, IL-6, and TNF-α with an impact of BMI on the infant body composition.[45] Similarly, our results revealed that higher leptin levels in human milk were associated with higher maternal BMI, which was in accordance with other studies.[46,49,50] These results suggest a potential link between maternal signals in BM and infant growth. Interestingly, although our population was mainly of normal weight and only a few were overweight, the influence of BMI was observed.[11] Indeed, our results also suggested a potential positive association between mixed feeding and leptin concentration. Different studies have shown that both formula feeding[1,51] and high maternal BMI were associated with a higher risk of overweight in children between 2 and 5 years of age.[52] This adipokine is related to the control of food intake and weight regulation;[53] it is commonly observed in higher amounts in obese patients (compared to non-obese population) who also show a phenomenon known as leptin resistance.[54] This indicates that leptin plays an important role in the control of food intake.

Thus, the association described in our study would support the role of BM cytokines as a potential route by which the maternal clinical conditions, such as BMI, could affect infant growth, since higher levels of leptin in BM could exert an effect in children. Apart from leptin, the associations that our analysis has revealed are crucial for infant development, since cytokines could impact infants' immune system and gut epithelium *via* alteration of oral tolerance,[55] among other potential actions.

In the present study, *Streptococcus* genus was positively associated with milk IL-18 and IL-10 concentrations in accordance with a previous study.[12] We also found that *Bifidobacterium* genus abundance was negatively related to pro-inflammatory cytokines, such as IL-6 and IL1-β, and leptin in the BM. In contrast, it has been reported that IL-6 was positively associated with *Staphylococcus.*[12] The *Bifidobacterium* genus has been proposed as one of the most important bacterial taxa affecting immune system development during early life,[56,57] and BM would play a key role in the colonisation of the infant gut;[10,58,59] differences in the gut microbiota composition in children may predict infant growth. Indeed, a study found lower *Bifidobacterium* and higher *Staphylococcus aureus* at 6 and 12 months in obese children.[60] Thus, the negative relationship between this genus in maternal BM and leptin could be one of the potential links between the lower incidence of being overweight observed in breastfed infants.[61]

Our observations suggest that the immune signals present in BM and their relationship with the microbial components could potentially be linked and influence infant development. Higher abundances of *Streptococcus* and IL-10 and lower IL-6 and IL-18 concentrations were found to be predictors of lower WLZ at 12 months, and the Chao1 index and IL-18 were found to be predictors of higher BMIZ at 12 months. These relationships may be explained by the effects of cytokines in the development of the new-born. In this line, IL-10 may have immunomodulatory and anti-inflammatory effects on the alimentary tract of the new-born,[62] and increased levels of IL-6 have been consistently linked to obesity.[63] Contrary to our results, a study reported that BM cytokine levels did not play a substantial role in the growth of children. However, this study performed analysis with data from the first 2–3 months postpartum when the infant development might be influenced by other potential

factors,[41] mainly the maternal nutritional environment during pregnancy that influences growth during the initial months.[64] Cytokines, as well as other BM components, have also been described to influence infant growth. Oligosaccharide composition in human milk 3 months after delivery was significantly associated with child growth throughout the first 5 years of life, since BM oligosaccharide composition may transform into a stronger and better shielded element, linked to a diminished percentage of infections and inflammation, thus allowing infants to fully invest their energy into development.[65] Also, BM lipids could have an important role in the cytokine modulation in the bowels of newborns.[66]

To further explore the relationship between the BM signals and the risk of obesity in infants, we carried out a proof-of-concept study where BM bacteria were exposed to a mammary gland epithelial cell line to ascertain the potential effect on the maternal side and to check the potential impact on the lactating infant. BM microbes induced a response in mammary cells, which were dependent on the infant's risk of overweight at 12 months of life. We observed that milk bacteria from the mothers with ROW offspring induced a higher IL-6 release in MCF7 cells compared to those from the mothers with NOROW offspring, suggesting that the milk microbiome could contribute to the cytokine composition. Previous studies have shown that IL-6 is associated with maturation of the intestinal immune system.[42] This pleiotropic cytokine has a central role in the signalling system of the organism, exerting several, sometimes conflicting, functions,[67] which are also tissue-specific. In fact, the alteration of IL-6 in BM has been associated with maternal obesity;[12,68] however, increased levels of IL-6 have been consistently linked to insulin resistance[69] and the chronic low-grade inflammation that is commonly observed in these diseases. Contrary to our results, a study reported that higher concentrations of IL-6 in BM were also significantly associated with lower relative weight, weight gain, and fat mass in healthy term infants at 1 month of age,[45] yet these previous results do not provide conclusive evidence on how these independent effects of different BM components influence the infant body composition. In this line, despite the IL-6 expression in the mammary epithelial cells due to a pro-inflammatory signal elicited by one or several bacterial species contained in breast milk, we did not find activation of the NF-κB pathway in the epithelial intestinal cells. This observation suggests that intestinal cells are unresponsive to the bacteria contained in BM at 1 month of age to elicit a pro-inflammatory response. However, at 1 year of age, we observed that the faecal supernatants of the ROW offspring showed higher pro-inflammatory activity on intestinal epithelial cells. Most interestingly, NF-κB pathway activation was associated with higher IL-6 BM concentration, suggesting that the pro-inflammatory ability of the initial BM microbiota source and cytokines, although only detected in mammary epithelial cells in our cell culture model, initiated a pro-inflammatory intestinal environment in the infants. Further studies are needed to clarify the potential impact of milk microbiota on the cytokine release by the mammary gland tissue, and how both BM microbiota and cytokines could modulate infant development. Our results suggest that the combination of milk cytokines and microbes is needed to promote a potential gut inflammatory status, leading to a potential higher risk of overweight . The critical combination and interaction of BM compounds and their inter-relations warrant further investigation.

This study has some limitations, including the sample size, which needs to be extended to fully reveal the implications of the results due to the higher intra- and inter-variability among mothers. BM is a complex fluid, and especially the immune signals among others, could be influenced by several factors that contribute to this observed variability across the population. This aspect is especially relevant in *in vitro* experiments, where further analysis with larger cohorts in overweight risk subjects are needed to clarify the influence of BM microbiota on the immunological active content of BM and its impact on child development. Also, the analysis of the potential relationship between the gut microbiota of infants and the BM microbiota is lacking. This would be interesting for understanding the modulation of BM on the infant gut microbiota in those at risk of overweight and those without the risk of overweight.

In conclusion, our study has shown the role of BM microbiota in infant growth and development. Our observations suggest that the association between BM cytokines and microbiota could be related to the growth of children during the first 12 months, although the potential mechanisms behind remain uncovered and this warrants further investigation. Despite that it is required to evaluate other potential environmental and host factors that may influence these associations, our results shed light on the link between BM, cytokines, adipokines, and infant growth and contribute to the knowledge that will be essential for the development of future strategies targeting infant growth modulation through breastfeeding.

Author contributions

EC-M: writing – original draft, methodology, investigation, validation, visualization, and data interpretation. MS-R, KR-A, and CB: investigation, validation, visualization, and data interpretation. MJR-L and FJP-C: supervision, writing – review and editing, visualization, and interpretation. MCC and CM-C: supervision, conceptualization, writing – review and editing, visualization, and interpretation.

Conflicts of interest

There are no conflicts of interest to declare.

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