Compositional Analysis Of The Bacterial Community In Colostrum Samples From Women With Gestational Diabetes Mellitus And Obesity

July Stephany Gámez-Valdez
Instituto Tecnologico y de Estudios Superiores de Monterrey Escuela de Ingeniería y Ciencias
José Francisco García-Mazcorro
MNA de Mexico, Research and Development
Alan Heriberto Montoya Rincón
Instituto Tecnológico y de Estudios Superiores de Monterrey, Escuela de Medicina y Ciencias de la Salud
Dalia Liliana Rodríguez Reyes
Instituto Tecnológico y de Estudios Superiores de Monterrey, Escuela de Medicina y Ciencias de la Salud
Gelacio Jiménez Blanco
Instituto Tecnológico y de Estudios Superiores de Monterrey, Escuela de Medicina y Ciencias de la Salud
Melissa Tatiana Alanís Rodriguez
Instituto Tecnologico y de Estudios Superiores de Monterrey Escuela de Medicina y Ciencias de la Salud
Rebeca Pérez-Cabeza de Vaca
Centro Medico Nacional 20 de Noviembre
Mario René Alcorta García
Instituto Tecnológico y de Estudios Superiores de Monterrey, Escuela de Medicina y Ciencias de la Salud
Marion Emilie Genevieve Brunck
Instituto Tecnológico y de Estudios Superiores de Monterrey, Escuela de Ingeniería y Ciencias
Víctor Javier Lara Díaz
Instituto Tecnológico y de Estudios Superiores de Monterrey, Escuela de Medicina y Ciencias de la Salud
Cuauhtemoc Licona-Cassani (clicona@tec.mx)
Instituto Tecnologico y de Estudios Superiores de Monterrey - Campus Eugenio Garza Sada
https://orcid.org/0000-0002-0360-3945

Research article

Keywords: Breastmilk Microbiota, Gestational Diabetes Mellitus, Obesity, Colostrum, 16S sequencing

Posted Date: August 1st, 2020

DOI: https://doi.org/10.21203/rs.3.rs-48912/v1

Abstract

Background. Obesity and Gestational Diabetes Mellitus (GDM) are major concerns during pregnancy as they compromise both mother and newborn health. These pathophysiology leads to variations in gut and breastmilk microbiota, threatening the settlement of neonate's gut microbiota and the stimulation of immune cells. While several studies have described compositional changes of breast milk, placenta and maternal and newborn intestinal microbiota, it is unknown how breastmilk microbiota is affected under maternal GDM. The molecular mechanisms in which microbial-unbalanced breastmilk affect development of infants is not well understood for the case of obesity and GDM. As a first step, we used 16S- amplicon sequencing to describe the taxonomical bias of breastmilk of 43 women with GDM and women with obesity. We sampled colostrum within the first 24 h taken after birth in order to identify and quantify the first microbial community potentially colonizing infants gut.

Results. A total of 1,675 amplicon sequence variants (ASVs) were identified, being *Proteobacteria* and *Firmicutes* the dominant phyla. We found *Pseudomonas*, *Gemellales* and *Enterobacter* overrepresented in samples of women with obesity and GDM. We used observed ASV´s, Shannon index and Faith´s phylogenetic diversity scores as alpha diversity indicators and UniFrac and robust Aitchison distances beta diversity indicators. Both metrics show a distinct microbial composition for GDM (female sub-group) and Obesity (male subgroup) compared to controls. Finally, differentially abundance analysis showed that *Rhodococcaceae* was distinct for GDM and 7 families (*Bdellovibrionaceae*, *Burkholderiaceae*, *Halomonadaceae*, *Pseudomonadaceae*, *Shewanallaceae*, TM7-1 and *Vibrionaceae*) with an absolute effect size greater than 1, of which 5 had a q-value ≤ 0.05 were distinct for obesity groups.

Conclusions. To our knowledge, this study represents the first approach to the breastmilk bacteria composition of mothers with GDM. Our work provides the foundation for the design of functional metagnomic studies aiming to understand molecular mechanisms of which breastmilk from individuals with GDM and obesity affect the physiology of the infants.

Background

Breastfeeding during the first semester of life is crucial as the newborn's gastrointestinal tract matures and develops determinants of future health [1, 2]. In addition to nutritional components (proteins, oligosaccharides, short-chain fatty acids) [3], breastmilk contains a diverse microbial population which participates in educating the gastrointestinal immune system, and affect the infant gut-brain axis [4–6]. Compositional microbiota studies suggest that breastmilk contains up to 10^6 bacterial cells / mL and almost 2,000 of Amplicon Sequence Variants (ASVs) under healthy conditions [7, 8]. However, diet [9], body mass index (BMI $\geq$ 25 kg/m^2) [10], mastitis [11], antibiotic use [12] amongst other factors [13, 14] can drastically affect the diversity and composition of the breastmilk microbial community.

Maternal obesity is linked to dysbiosis of the microbiota inhabiting the maternal gastrointestinal tract and breastmilk. This leads to increases in *Bacteroides* and *Staphylococcus* populations in the neonate's

gut [15]. Likewise, a different and less diverse microbiota in the breastmilk of mothers suffering from obesity, compared to normal weight mothers [16, 17]. Increased prevalence of *Staphylococcus* spp. in colostrum, is linked to higher rates of infections, and to decreased proportions of *Bifidobacterium* and *Bacteroides*, which play a protective role against obesity and other metabolism-associated diseases later in life [16, 18].

Pre-conceptional obesity predisposes future mothers to gestational diabetes mellitus (GDM), which is a manifestation of insulin resistance, with a worldwide incidence average of 17% [19]. Together, maternal obesity and GDM are strongly correlated to higher incidence of allergic manifestations and atopic dermatitis (7.5 -fold increase) and allergen sensitization (5.9-fold increase) [20, 21]. The microbiota of the placenta of mothers experiencing GDM show decreased amount of *Pseudomonales* and *Acinetobacter*, together with lower eosinophil leukocytes count and lower placental expression of the immune mediators IL-10 and TIMP3 compared to normoglycemic controls [22]. There is currently a lack of information regarding the microbial composition of breastmilk from mothers with GDM.

Obesity affected more than 650 million adults in 2016 comprising 15% of women worldwide [23]. Globally, Mexico has the second highest prevalence of adult obesity, and the highest rate of childhood obesity in the world [24, 25]. In Mexico, the national prevalence of GDM exceeds the global average with 23.7% of affected pregnancies [26]. Here, we report a comprehensive analysis of the compositional microbiota of colostrum samples of Mexican individuals with GDM and obesity. Using a sample population from Monterrey, the Mexican city with the highest affection of GDM [26], we used a 16S amplicon sequencing approach to identify changes at the taxonomical level in the breastmilk of mothers affected by GDM. The results shown constitute the first characterization of the microbial population in colostrum under the pathophysiological condition of obesity and GDM.

Results

Breastmilk samples (colostrum) from 43 individuals aged 20-32 years from the Monterrey metropolitan area were used in this study. All deliveries were at term with a mean of 39.4 weeks of gestation. A total of 18 samples from mothers with BMI $\leq$ 24.9 kg/m^2 (non-obese), without GDM were considered as controls. Twelve milk samples were collected from mothers with obesity (defined as BMI $\geq$ 30 kg/m^2) and without GDM, as well as 13 samples were obtained from mothers with GDM and a BMI $\leq$ 29.9 kg/m^2. None of the participants in the gestational diabetes study group had obesity; however, eight of them were overweight (BMI $\geq$ 25 kg/m^2). Most of our participants were multiparous, and regarding delivery, 24 neonates were born by caesarean section and 25 of included women were given antibiotics during labor. The sex distribution of the newborn was 48.8% of males and 51.16% of females. Clinical and demographics of the participants are summarized in Table 1.

We classified data according to the pathophysiological condition and the newborn gender resulting in a total of six study subgroups, namely: obesity positive, GDM negative - female (Ob-F; n=8); obesity positive. GDM negative - male (Ob-M; n=4); GDM positive obesity negative - female (GD-F; n=6); GDM

positive obesity negative - male (GD-M; n = 7), healthy normal weight - female (NW-F; n = 8), and healthy normal weight - male (NW-M; n = 10).

After birth, breastmilk was collected within the first 24 hours and colostrum was appropriately stored until analysis. Using NGS, 2,635,830 high quality reads were obtained with a mean of 61,298 ± 16,928 sequencing reads per sample. After the removal of possible contaminants and rare taxa (≤ 25 reads in total), 1,675 amplicon sequence variants (ASVs) were assigned at 29 phyla, 57 classes, 96 orders, 150 families, 224 genera and 126 species using Greengenes database with a confidence identity level set at 99%. We found increased prevalence of *Pseudomonas*, *Gemellales* and *Enterobacter* in samples from participants with GDM and obesity.

Colostrum compositional microbiota was dominated by *Proteobacteria* and *Firmicutes* in all the samples.

We found that breastmilk samples were overrepresented by *Proteobacteria* with a relative abundance mean of 63.3% ± 26.5% (range 17.5% - 97.1%) and *Firmicutes* (21.1% ± 20.7%, range 0.5% - 60.2%). Less represented phyla were *Bacteroidetes* (9.4% ± 12%, range 0.3% - 35.9%), *Actinobacteria* (3.5% ± 4.7%, range 0.2% - 24.1%) and WPS-2 (1.8% ± 5.2%, range 0% - 21.4%) (Figure 1A). The classification of “Other” represents phyla with less than 1% of total relative abundance.

Bacteria belonging to the *Proteobacteria* phylum was more abundant in obesity-male (Ob-M, 88.1% ± 10.1%) but less abundant in obesity-female (Ob-F, 48.9% ± 27.3%). The highest relative abundance of *Firmicutes* was shown for obesity-female (Ob-F, 38.8% ± 17.9). *Actinobacteria* showed higher abundance in all the female groups compared to their male counterpart. *Bacteroidetes* were more abundant in healthy groups with 13.7% ± 14.2% and 11.8% ± 15.7 for female (NW-F) and male (NW-M) respectively. This behavior was also observed for WPS-2, a recently candidate division, which had the highest relative abundance in healthy-female (NW-F, 5% ± 9.2%) (Figure 1A).

Pseudomonas, *Gemellales* and *Enterobacter* are overrepresented in samples of individuals with obesity and GDM

Overall, the most abundant genera were *Pseudomonas* (22.4% ± 24%), *Gemellales* (7.6% ± 10.3), *Ralstonia* (6.7% ± 10.6%), *Herbaspirillum* (5.1% ± 8.6%), *Streptococcus* (4.9% ± 11.8%), *Enterobacteriaceae* (4.5% ± 3.5%), *Chryseobacterium* (4.2% ± 9.7%) and *Sphingomonas* (4% ± 9.7%) (Figure 2). The “Other” category represents the taxa with less than 1% overall. According to the subgroup classification, in the healthy normal weight subgroups, *Chryseobacterium* ($p < 0.05$) and *Sphingomonas* ($p < 0.10$) were more abundant (9% ± 15.1%, 6.1% ± 6.2% for female (NW-F) and 9% ± 13.5%, 5.9% ± 5.7% for male (NW-M) respectively). While not statistically significant, obesity-male (Ob-M) and GD-male (GD-M) have the higher prevalences of *Pseudomonas,* with 55.7% ± 6.6% for obesity and 23.6% ± 23.5 for GDM. A similar pattern was also observed for *Enterobacter* ($p < 0.05$; Ob-M, 12.3% ± 2.2% and GD-M, 3.7% ± 4.8). *Streptococcus* presence was enriched in the obesity-female subgroup (Ob-F, 10.9% ± 13.8%). Breastmilk from subjects with GDM showed higher prevalence of “Other genera” (24.9% ± 12.3%, 18.3% ± 12.3% for female and male respectively), suggesting that the biggest part of this contribution to the

relative abundance of both subgroups is due to genera with less than 1% in total. This is supported by the rarefaction curves which reveal that GDM–female (GD-F), GDM–male (GD-M) and obesity-female (Ob-F) had higher values of estimated number of observed ASVs (Figure 1B). *Gemellales* were more abundant in subgroups with female baby ($p < 0.10$), distinctively in obese (Ob-F, 13.6% ± 14.2%) and with gestational diabetes (GD-F, 12.9% ± 11.5%). A full distribution of taxa is show in Figure 2.

Alpha and beta diversity metrics show a distinct microbial composition for GD-F, Ob-M and newborn gender-related samples

We used a general linear model (glm) using alpha diversity metrics at a sequencing depth of 23,572 (data not shown) in order to quantify the influence of GDM, obesity (BMI ≥ 30 kg/m^2), cervicovaginitis, antibiotic exposure, multiparity and sex of the baby. As a result of the analysis, only maternal physiopathology (GDM, obesity and healthy) and the sex of the baby showed statistically significant association ($p \leq 0.05$) for Shannon index, phylogenetic diversity and observed ASVs (Figure 3A-C). GDM subgroups presented the highest values in all alpha indexes. In addition, our results suggest that, in general, female subgroups had higher diversity compared to male subgroups. Fisher's comparisons indicate that statistical difference was only significant between healthy-female (NW-F) and GDM-female (GD-F) for Shannon index. Breastmilk samples from obesity-male subgroup (Ob-M) had the lowest levels of alpha diversity and were statistically different to all of the subgroups, including their female counterpart (Figure 3A-C).

We estimated microbiome beta diversity using the unweighted UniFrac distance (Figure 3D). Our results show that obesity-male (Ob-M) and healthy-male (NW-M) subgroups cluster separately from the rest of the samples (PERMANOVA; $p = 0.001$; 999 permutations). Using the unweighted distance matrix, we generated a PCoA biplot in order to show that the clustering was significant for obesity-male (Ob-M; $p \leq 0.05$) and GDM-female (GD-F; $p \leq 0.05$) compared to healthy-male (NW-M). Arrows in the plot represent the correlation at family level with PCoA axes, indicating their contribution to the variation (Figure 3D). While samples from GDM-female (GD-F), GDM-male (GD-M), healthy-female (NW-F) and obesity-female (Ob-F) show high similarity regarding microbial composition, the unweighted measurement indicates that there is a phylogenetic difference between obesity-male (Ob-M) and the rest of subgroups ($p \leq 0.05$).

We used the beta-diversity compositional Aitchison's distance in order to assess the compositional nature of data (PERMANOVA; $p = 0.027$; 999 permutations) (Figure 3E). The robust principal component analysis (RPCA) biplot, which allows to examine the variation of samples and taxa, did not show a clear separation of any subgroup. PERMANOVA tests and pairwise comparisons only showed obesity-male (Ob-M) was different to GDM-female (GD-F; $p \leq 0.05$) and that obesity-female subgroup (Ob-F) was statistically different to its male counterpart (Ob-M) and both healthy subgroups ($p \leq 0.05$). The 10 taxa presented as vectors in the plot are the most significant drivers of the location of samples (Figure 3E).

Breastmilk core and differentially abundant taxa

We defined the breastmilk core microbiota as taxonomical families present in all samples with a minimum 1% of total mean relative abundance. Overall, 9 families were identified as the core taxa and comprise 69% ± 23.3% of the total (Table 2). The most abundant were *Pseudomonadaceae* with a general mean of 22.4% ± 24%, followed by *Oxalobacteraceae* (11.8% ± 10.9%) and *Enterobacteriaceae* (11.3% ± 7.2). These results demonstrate the high variability of the core bacteria among subgroups and individuals. The five most abundant families belonging to the core, were found to describe the majority of the variation in the ordination space observed in the unweighted PCoA biplot, and were represented as arrows (Figure 3D). However, no clear participation of families to the formation of subgroups was visualized with the implement of UniFrac metrics.

We used the Aldex2 tool (Fernandes et al., 2013) in order to identify differences in ASV abundance between subgroups. We determined the taxa that were driving the difference between the subgroups and obtained effect plots (based on the effect size), which allowed us to visualize if the variation was higher between or within subgroups. Given the high variability amongst samples, we only observed differentially abundant ASVs with a significant expected Benjamini-Hochberg corrected p value of Welch's t test ($q \leq 0.1$) in three sample pairs (Figure 4). In the GDM-female (GD-F) vs healthy-female (NW-F) the family *Rhodococcaceae* was different (Figure 4A). In obesity-male vs healthy-male (Ob-M vs NW-M) we found a total of 7 families (*Bdellovibrionaceae*, *Burkholderiaceae*, *Halomonadaceae*, *Pseudomonadaceae*, *Shewanallaceae*, TM7-1 and *Vibrionaceae*) with an absolute effect size greater than 1, of which 5 had a q-value ≤ 0.05, and only 1 was part of the breastmilk core (Figure 4B). This phenomenon was also observed in obesity-male vs obesity-female (Ob-M vs Ob-F), in which only 4 of the (*Comamonadaceae*, *Enterobacteriaceae*, *Pseudomonaceae* and *Sphingobacteriaceae*) 12 significant different taxa found corresponded to the core families (Figure 4C). Based on the median difference between subgroups, we observed that in all comparisons, obesity-male (Ob-M) had significant higher abundance for differential taxa found. In addition, GDM-female (GD-F) had higher prevalence of *Rhodococcaceae* compared to healthy-female (NW-F).

Discussion

Here, we present a comprehensive compositional study of colostrum microbiota of 43 individuals with obesity and gestational diabetes mellitus. Breastmilk is considered an essential fluid compiling a plethora of immune regulators, including microbes, that shape the infant's ability to fight pathogens and physiological distress [6]. While many factors including age, ethnicity, weight gain, BMI, diet, antibiotic exposure [13] modify microbial composition, health status has a strong impact and can predispose infant's immune system in subsequent years [27]. Specifically, studying colostrum microbial composition is very important, as this fluid is supposedly the first postnatal maternal fluid inoculating the newborns' gastrointestinal system. Maternal obesity and gestational diabetes mellitus are health problems currently at pandemic scale, and yet little is known about the impact on colostrum microbial composition. In fact, to our knowledge, this is the first study showing a comprehensive analysis of the microbial composition of breastmilk of women with GDM. Recently, changes in placental microbiota and microbiome were reported in women with GDM compared to normoglycemic controls [22]. These authors found decreased

amount of Pseudomonales and Acinetobacter together with lower eosinophil count and lower placental expression of IL-10 and TIMP3. They advance the possibility that GDM may constitute a state of placental microbiota-driven altered immunological tolerance, and signal the possibility that placental microbiota could become a target for comprehensive therapy of GDM.

Overall, we identified *Proteobacteria* and *Firmicutes* as the dominant phyla (Fig. 1A). Our results are consistent with previous breastmilk studies [8, 11, 28] but differ from those published by Meehan et al. [29], and Jost et al. [30] as they reported primarily enrichment of Firmicutes. Differences in relative abundance can be attributed to the sequencing platform used [31, 32] but possibly sample conditions and storage could also interfere. The candidate division WPS-2 was present in 1.8% of total relative abundance in our samples. This phylum has been described in soil, canine and human oral microbiota [33–37]. WPS-2 was incorporated in the Human Oral Microbiome database (HOMD) in 2014 [38]. While it has not yet been described in microbiota of newborns' oral cavity, WPS-2 was present in nasopharynx samples from infants under 6 months of age [39]. This is the first time that WPS-2 is reported in breastmilk samples with high prevalence supporting the retrograde flux theory, in which microbiota from neonate's mouth contributes to the settlement of breastmilk bacteria [40].

Breastmilk samples presented high variability at the genus level (Fig. 2). Overall, *Pseudomonas*, *Gemellales* and *Ralstonia* were the most abundant taxa in our data set, which correlates with the microbial composition of breastmilk from Spanish who delivered by cesarean section and women from Central, East and Northeast China [41, 42]. However, *Staphylococcus* and *Streptococcus* were reported as predominant taxa in breastmilk samples from Taiwanese, Finnish and Chinese (Beijing area) individuals [41, 43]. High variability of microbial composition with geographical location, suggest that deep sequencing approaches are needed in order to identify keystone taxa defining structure and microbiome function.

We identified that breastmilk microbial diversity was specific to the sex of the newborn ($p \leq 0.05$). Our results are in accordance with previous research where BMI and neonate's gender were related to enrichment of *Streptococcus*, *Ralstonia* and *Staphylococcus* [8]. Regardless of the physiopathology (GDM or BMI), female-related colostrum samples showed higher alpha diversity compared to male subgroups, suggesting a more diverse microenvironment (Fig. 3A-C). It has been demonstrated that gut and oral microbiota from children and maternal breastmilk biochemical composition differ between female and male infants, possibly due to variation in hormone recruitment and energetic demand during pregnancy [8, 44–46]. Neonate microbiota sex-bias should be an important consideration for experimental designs trying to explain causality microbial changes due to any pathophysiology.

Despite the sample heterogenicity, we guided our analysis by a general linear model to suggest that colostrum samples of individuals with obesity (Ob-F and Ob-M) are enriched for *Firmicutes* ($p \leq 0.1$; Fig. 1A). It has been observed that higher numbers of *Lactobacillus* and *Staphylococcus* were related to higher maternal BMI [16, 47, 48]. In addition, decreased *Streptococcus* abundance and an increment in breastmilk microbial diversity in Mexican-American subjects with high BMI (> 25 kg/m^2) has been

previously reported [10]. These results also correlate with our study where *Streptococcus* are less abundant and high values of observed ASVs were found in obesity-male (Ob-M, 187 ASVs) and obesity-female (Ob-F, 398 ASVs) subgroups. However, other reports show that colostrum samples from obese mothers presented a less diverse microbiota compared to non-obese samples [16]. This variability can be attributed to differences in study populations (geographical location, diet, socioeconomic status), sample collection at different lactation stage and neonate sex-bias.

We implemented the Aldex2 tool, which performs a log transformation and replacement of the zero values in the obtained results for a matrix creation that allows the determination of significant differences of taxa between subgroups [49]. Interestingly, we observed *Bdellovibrionaceae* and TM7-1, which are ultra-small parasite bacteria, differentially present in Ob-M compared to its corresponding contrast (NW-M; Fig. 4B). While further research is needed, this pattern can be attributed to resilience mechanisms of breastmilk microenvironment to maintain a functional equilibrium through specific predatory interactions with Gram-negative bacteria such as *Pseudomonas* and *Enterobacter*, which also appear to be in higher proportions in obesity-male (Ob-M; Fig. 2). This may be explained by the detection of DNA fragments resulting from the bacterial lysis. *Bdellovibrionaceae* has been found in soil, freshwater and human gut from healthy subjects and patients suffering from inflammatory diseases [50]. This taxon is considered as a potential probiotic, since it could modulate the gut biodiversity by its predation of bacteria correlated in chronic inflammatory diseases, such as obesity and Crohn's disease [51, 52]. On the other hand, TM7 phylum (also known as *Saccharibacteria*), has been reported in human oral cavity, intestines, skin, and female genital tract [53–55]. Lif et al. [56] related the impact of birthing method and a higher prevalence of this novel phylum in oral biofilm samples of infants delivered vaginally compared to infants born by cesarean section. Even though TM7 is a phylum that remains difficult for cultivation, its presence in adult subgingival plaque, vagina and colon has been associated to human inflammatory mucosal diseases [57, 58].

Our results indicate a differential prevalence of *Burkholderiaceae* and *Sphingobacteriaceae* in breastmilk samples from obesity-male (Ob-M) compared to its female contrast (Ob-F; Fig. 4C). Similar results have been described in oral samples from male infants as they reported a higher abundance of *Brachymonas* and *Sphingomonas* [46]. We hypothesize that differences in breastmilk microbiota by infant gender influence the conditioning of the neonate's gut microenvironment for bacterial communities related to the metabolism of nutrients involved in sex-related neurodevelopment.

We observed a higher relative abundance of *Firmicutes* and *Prevotella* in both GDM subgroups compared to their corresponding contrast control (NW). Interestingly, similar profiles have been reported for gut microbiota individuals with GDM [59, 60]. Complementarily, high prevalence of *Prevotella* has been observed in oral cavity, amniotic fluid and gut microbiota of pregnant women with GDM, which confirms vertical transmission mother-to-baby and supports enteromammary theory of breastmilk microbiota origin [59, 61–63]. Functional metagenomic approaches are needed in order to determine what is the exact role of *Prevotella* as keystone taxa, under the specific microenvironment shaped by GDM. *Rhodobacteraceae* was observed in higher proportion in GDM-female (GD-F) compared to healthy-female

(NW-F) (adjusted p-value ≤ 0.1). *Rhodobacteraceae* has been mostly reported in soil [64], but also in breastmilk from healthy Mexican women [65], human skin [66], meconium [67] and fecal samples from patients suffering from diarrhea [68].

Conclusions

The factors determining the establishment of an infant´s gut microbiota are of high scientific interest and could lead to new health policies. In particular, the impact of obesity and subsequent or concurring GDM are relevant, due to the high prevalence worldwide. Amplicon sequencing using NGS technologies are the most reliable methods for large-scale microbial compositional studies [69]; however, in order to obtain relevant functional insights, a robust clinical protocol is required. Our study indicates that *Firmicutes* and *Prevotella* are relevant taxonomic groups within the microbial population of colostrum samples of individuals with GDM. Further studies of the functional role of such taxa are key in order to understand the dynamics of the establishment of infants' gut microbiota under the influence of obesity and GDM. These studies are needed for the potential design of probiotics and in the search of possible therapeutic agents contributing to the infant's gastrointestinal immune system homeostasis.

Material And Methods

Study population

Recruiting was done at Hospital Regional Materno Infantil, the main public perinatal medicine hospital of Servicios de Salud del Estado de Nuevo León, Mexico. We included mother-infant pairs, within 20 and 32 years of maternal age, and with a verified address in the Monterrey Metropolitan Area, who accepted the invitation to participate and signed the informed consent document. Exclusion criteria were: mothers who had a history of antibiotic usage in the 3 months prior to delivery; mothers who had prolonged exposure to antibiotics (more than 3 weeks) at any time during pregnancy; mothers who received immunosuppressive or immunomodulatory corticosteroid therapy; history of a vegan, ovolactovegetarian or exclusion diet (*e.g.*: ketogenic diet); history of bariatric surgery or any complicated surgery; history of feeding disorders; exposure to antineoplastic drugs, histamine-H2 receptor antagonists or proton pump inhibitors and/or monoclonal antibodies; and/or history of diarrhea during the three weeks before delivery. In addition, those with an uncertain last menstrual period date or irregular periods that gave place to an uncertain pregnancy dating were excluded. Elimination criteria were Antibiotics for more than 24 hours post-delivery; need of intensive care (mother or infant) and/or any condition that impeded recollection of breast milk.

Those selected mother infant pairs were further allocated for analysis to one of the three study groups, according to their BMI and health condition: 12 obese women (BMI ≥ 30 kg/m^2), 13 women suffering from gestational diabetes (BMI ≤ 30 kg/m^2) and 18 control healthy women (BMI ≤ 25 kg/m2). The study groups were further sub-divided by sex of the baby. The study protocol was approved by institutional

Review Boards at Escuela de Medicina y Ciencias de la Salud, Tecnológico de Monterrey, with the ID P000185-CarMicrobioLHum2018-CEIC-CR002, on may 6th, 2019.

Samples collection and processing

After gentle cleansing only with sterile water of the breasts, each mother performed a gentle circular massage of each breasts until a few drops of colostrum appeared. These first drops were disregarded, and the mother self-expressed approximately 5 mL of colostrum into a sterile falcon-type 20 mL polypropylene tube, under close medical supervision. The procedure was repeated on the opposite breast. Extreme care was taken to avoid milk contact with breast skin or fingers. Then, the tubes were closed, and kept at -20°C for no more than 48 hours until DNA extraction.

DNA extraction

Genomic DNA was extracted from 1mL of colostrum using the phenol-chloroform protocol with some modifications [70]. Samples were thawed on ice and centrifugated at 16,000 X g for 15 minutes, the fat rim was carefully removed, and PBS washes were performed to eliminate fat residues (0.5 mL PBS), centrifugation at 16,000 X g for 10 minutes). The pellet was resuspended in 0.5 mL of extraction buffer (220 mM Tris-HCl pH 7.5, 110 mM EDTA, 1100 mM NaCl, 20% Triton X-100, 2% SDS) and 0.3 mL of 3 M of sodium acetate. An additional step of mechanical lysis was performed by bead-beating with lysing matrix A using a FastPrep (MP Biomedicals, Santa Ana, CA) disruptor at a speed setting of 5.5 m/s for 25 s. The lysate was submitted to an enzymatic lysis with 10 µL of proteinase K (10mg/mL), 5 µL of lysozyme (10 mg/mL) and 10 µL of RNAse, and incubated at 60°C for 1 hour. After incubation, 100 µL of 1.5 M NaCl were added and carefully mixed, and maintained at room temperature for 5 minutes. Following incubation, the mixture was centrifugated at 16,000 X g for 15 minutes and the supernatant was transferred into a new tube and extracted twice with an equal volume of phenol:chloroform:isoamyl-alcohol (25:24:1). The precipitation of DNA was carried out by the addition of 0.6 volumes of isopropanol and incubation at -80°C for 1 hour. Next, we centrifuged the samples (16,000 X g for 15 minutes), and the isopropanol was retired. The pellet was washed twice with 70% ethanol, air dried and resuspended in pre-heated 50 µL of nuclease free water. The DNA was measured using a NanoDrop ND-1000 UV spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) and the DNA integrity was confirmed through an agarose gel electrophoresis. Unless otherwise specified, all reagents were purchased from Sigma Aldrich.

DNA sequencing and analysis

DNA samples were sequenced at the Advanced Genomics Unit (Langebio, Cinvestav) using an Illumina MiSeq platform (2x300) following the 16S metagenomic sequencing library preparation for the amplification of V3–V4 hypervariable region of the bacterial 16S rRNA gene with the universal primers 341F 5'CCTACGGGNGGCWGCAG3' and 785(R) 5'GGACTACHVGGGTATCTAATCC 3'. We normalized the DNA for sequencing at 25 ng/□l.

Bioinformatic analyzes were carried out using QIIME 2 v.2019.7 [71]. Sequencing readings were quality filtered using the q2-demux plugin with a minimum length at 270 nucleotides followed by denoising with DADA2 [72]. Single-paired filtered readings were used for the taxonomic species profile using amplicon sequence variants (ASVs) with the q2-feature-classifier [73] against the Greengenes database with a limit of sequence identity set at 99% [74]. Removal of potential contaminants include ASVs belonging to Cyanobacteria, Phyllobacterium, Chloroflexi, mitochondria / chloroplast and rare taxa (with less than 25 reads across the entire dataset). Resulting ASVs were aligned with mafft [75] and implemented to create a phylogeny with fasttree2 [76]. Rarefaction of sequences to 23,572 per sample was used to perform alpha and beta diversity analyzes. Observed ASVs, Shannon index and Faith's phylogenetic diversity were used as alpha-diversity metrics; UniFrac (weighted and unweighted) and robust Aitchison distances were used for the creation of PCoA and rPCA respectively. We determined bacterial families present in all samples with a minimum relative abundance of 1% overall as core microbiota. ASVs that were assigned at family level were considered to perform differential abundance analysis with Aldex2 tool [49].

Data processing

QIIME2 α-diversity outputs were imported and processed in Minitab 17. Association of observed ASVs, Shannon index and Faith's phylogenetic distance with maternal health condition, mode of delivery, antibiotic exposure, parity and gender of the neonate was assessed by general linear model (glm) with p-value of ≤ 0.05. β-diversity significance for UniFrac and robust Aitchison distances was calculated using permutational ANOVA (PERMANOVA) with 999 permutations. Differential bacteria were assessed with Welch's t test with a Benjamini-Hochberg's false discovery rate (FDR) p-value correction after a centered log ratio (clr) transformation with a zero-replacement of taxa counts.

List Of Abbreviations

GDM: Gestational diabetes mellitus

ASV: Amplicon sequence variant

BMI: Body mass index

IL-10: Interleukin 10

TIMP3: Tissue inhibitor of metalloproteinase 3

WHO: World Health Organization

OECD: Organisation for Economic Cooperation and Development

NW-F: Healthy normal weight – female neonate

NW-M: Healthy normal weight – male neonate

Ob-F: Obesity positive GDM negative – female neonate

Ob-M: Obesity positive GDM negative – male neonate

GD-F: Gestational diabetes mellitus positive obesity negative – female neonate

GD-M: Gestational diabetes mellitus positive obesity negative – male neonate

GLM: General linear model

PCoA: Principal coordinate analysis

RPCA: Robust principal component analysis

PERMANOVA: Permutational ANOVA

PBS: Phosphate buffered saline

EDTA: Ethylenediaminetetraacetic acid

SDS: Sodium dodecyl sulfate

Declarations

Ethics approval and consent to participate

The clinical protocol was approved by the Ethics Committee of the Escuela de Medicina y Ciencias de la Salud (TecSalud) (Project ID: P000185-CarMicrobioLHum2018-CEIC-CR002).

Consent for publication

All authors have read the manuscript and have provided their consent for publication.

Availability of data and materials

Raw data is available in the NCBI under ID number 638389 and Bioproject accession number PRJNA638389.

Competing interests

All authors have read the manuscript and declare no conflict of interest.

Funding

The project was funded under ITESM-0021C21064 seed grant.

Authors' contributions

BM, LDVJ and LCC designed all experiments and clinical protocol. MRAH, RRDL, JBG, AGMR collected all the samples. GVJS performed all the DNA extractions. GVJS, GMJF, PCVR, BM, LDVJ and LCC analyzed and interpreted all the data. GVJS, BM, LDVJ and LCC wrote the manuscript.

Acknowledgements

First, we wish to express our deepest gratitude to the families that accepted the invitation to participate in this study, and also to the nurses and lactation consultants at the study hospital. We thank critical discussions with Dr. Marcos de Donato, Dra. Alejandra Chavez-Santoscoy and Dra. Karina Gutierrez. In addition, we are thankful to Luz Angela González Salazar for CIRCOS plotting. This work was performed partially through The National Council for Science and Technology (CONACYT) financial support (GVJS postgrad scholarship) and Tecnológico de Monterrey seed grant ITESM-0021C21064.

References

1. Honda K, Littman DR. The microbiota in adaptive immune homeostasis and disease. Nature 2016;535:75–84.
2. van den Elsen LWJ, Garssen J, Burcelin R, Verhasselt V. Shaping the gut microbiota by breastfeeding: the gateway to allergy prevention? Front. Pediatr. 2019;7:47.
3. Bardanzellu F, Fanos V, Reali A. Omics" in Human Colostrum and Mature Milk: Looking to Old Data with New Eyes. Nutrients 2017;9.
4. Anderson G, Vaillancourt C, Maes M, Reiter RJ. Breastfeeding and the gut-brain axis: is there a role for melatonin? Biomol. Concepts 2017;8:185–195.
5. Houghteling PD, Walker WA. Why is initial bacterial colonization of the intestine important to infants' and children's health? J. Pediatr. Gastroenterol. Nutr. 2015;60:294–307.
6. Walker WA, Iyengar RS. Breast milk, microbiota, and intestinal immune homeostasis. Pediatr. Res. 2015;77:220–228.
7. Boix-Amorós A, Collado MC, Mira A. Relationship between Milk Microbiota, Bacterial Load, Macronutrients, and Human Cells during Lactation. Front. Microbiol. 2016;7:492.
8. Moossavi S, Sepehri S, Robertson B, Bode L, Goruk S, Field CJ, et al. Composition and Variation of the Human Milk Microbiota Are Influenced by Maternal and Early-Life Factors. Cell Host Microbe 2019;25:324–335.e4.
9. Williams JE, Carrothers JM, Lackey KA, Beatty NF, York MA, Brooker SL, et al. Human milk microbial community structure is relatively stable and related to variations in macronutrient and micronutrient intakes in healthy lactating women. J. Nutr. 2017;147:1739–1748.
10. Davé V, Street K, Francis S, Bradman A, Riley L, Eskenazi B, et al. Bacterial microbiome of breast milk and child saliva from low-income Mexican-American women and children. Pediatr. Res. 2016;79:846–854.

11. Patel SH, Vaidya YH, Patel RJ, Pandit RJ, Joshi CG, Kunjadiya AP. Culture independent assessment of human milk microbial community in lactational mastitis. Sci. Rep. 2017;7:7804.
12. Hermansson H, Kumar H, Collado MC, Salminen S, Isolauri E, Rautava S. Breast milk microbiota is shaped by mode of delivery and intrapartum antibiotic exposure. Front. Nutr. 2019;6:4.
13. Gomez-Gallego C, Garcia-Mantrana I, Salminen S, Collado MC. The human milk microbiome and factors influencing its composition and activity. Semin Fetal Neonatal Med 2016;21:400–405.
14. Ruiz L, García-Carral C, Rodriguez JM. Unfolding the human milk microbiome landscape in the omics era. Front. Microbiol. 2019;10:1378.
15. Collado MC, Isolauri E, Laitinen K, Salminen S. Distinct composition of gut microbiota during pregnancy in overweight and normal-weight women. Am. J. Clin. Nutr. 2008;88:894–899.
16. Cabrera-Rubio R, Collado MC, Laitinen K, Salminen S, Isolauri E, Mira A. The human milk microbiome changes over lactation and is shaped by maternal weight and mode of delivery. Am. J. Clin. Nutr. 2012;96:544–551.
17. Garcia-Mantrana I, Collado MC. Obesity and overweight: Impact on maternal and milk microbiome and their role for infant health and nutrition. Mol. Nutr. Food Res. 2016;60:1865–1875.
18. Collado MC, Laitinen K, Salminen S, Isolauri E. Maternal weight and excessive weight gain during pregnancy modify the immunomodulatory potential of breast milk. Pediatr. Res. 2012;72:77–85.
19. Coustan DR, Lowe LP, Metzger BE, Dyer AR, International Association of Diabetes and Pregnancy Study Groups. The Hyperglycemia and Adverse Pregnancy Outcome (HAPO) study: paving the way for new diagnostic criteria for gestational diabetes mellitus. Am. J. Obstet. Gynecol. 2010;202:654.e1–6.
20. Kumar R. Prenatal factors and the development of asthma. Curr. Opin. Pediatr. 2008;20:682–687.
21. Peters JL, Suglia SF, Platts-Mills TAE, Hosen J, Gold DR, Wright RJ. Relationships among prenatal aeroallergen exposure and maternal and cord blood IgE: project ACCESS. J. Allergy Clin. Immunol. 2009;123:1041–1046.
22. Bassols J, Serino M, Carreras-Badosa G, Burcelin R, Blasco-Baque V, Lopez-Bermejo A, et al. Gestational diabetes is associated with changes in placental microbiota and microbiome. Pediatr. Res. 2016;80:777–784.
23. Obesity and overweight. https://www.who.int/news-room/fact-sheets/detail/obesity-and-overweight. Accessed 20 May 2020.
24. Obesity Update - OECD. http://www.oecd.org/health/obesity-update.htm. Accessed 20 May 2020.
25. Turnbull B, Gordon SF, Martínez-Andrade GO, González-Unzaga M. Childhood obesity in Mexico: A critical analysis of the environmental factors, behaviours and discourses contributing to the epidemic. Health Psychol. Open 2019;6:2055102919849406.
26. Dainelli L, Prieto-Patron A, Silva-Zolezzi I, Sosa-Rubi SG, Espino Y Sosa S, Reyes-Muñoz E, et al. Screening and management of gestational diabetes in Mexico: results from a survey of

multilocation, multi-health care institution practitioners. Diabetes. Metab. Syndr. Obes. 2018;11:105–116.

27. García-Mantrana I, Bertua B, Martínez-Costa C, Collado MC. Perinatal nutrition: How to take care of the gut microbiota? Clinical Nutrition Experimental 2016;6:3–16.
28. Murphy K, Curley D, O'Callaghan TF, O'Shea C-A, Dempsey EM, O'Toole PW, et al. The composition of human milk and infant faecal microbiota over the first three months of life: A pilot study. Sci. Rep. 2017;7:40597.
29. Meehan CL, Lackey KA, Hagen EH, Williams JE, Roulette J, Helfrecht C, et al. Social networks, cooperative breeding, and the human milk microbiome. Am. J. Hum. Biol. 2018;30:e23131.
30. Jost T, Lacroix C, Braegger C, Chassard C. Assessment of bacterial diversity in breast milk using culture-dependent and culture-independent approaches. Br. J. Nutr. 2013;110:1253–1262.
31. LeMay-Nedjelski L, Copeland J, Wang PW, Butcher J, Unger S, Stintzi A, et al. Methods and strategies to examine the human breastmilk microbiome. Methods Mol. Biol. 2018;1849:63–86.
32. Douglas CA, Ivey KL, Papanicolas LE, Best KP, Muhlhausler BS, Rogers GB. DNA extraction approaches substantially influence the assessment of the human breast milk microbiome. Sci. Rep. 2020;10:123.
33. Nogales B, Moore ER, Llobet-Brossa E, Rossello-Mora R, Amann R, Timmis KN. Combined use of 16S ribosomal DNA and 16S rRNA to study the bacterial community of polychlorinated biphenyl-polluted soil. Appl. Environ. Microbiol. 2001;67:1874–1884.
34. Dewhirst FE, Klein EA, Thompson EC, Blanton JM, Chen T, Milella L, et al. The canine oral microbiome. PLoS One 2012;7:e36067.
35. Camanocha A, Dewhirst FE. Host-associated bacterial taxa from Chlorobi, Chloroflexi, GN02, Synergistetes, SR1, TM7, and WPS-2 Phyla/candidate divisions. J. Oral Microbiol. 2014;6.
36. Lazarevic V, Gaïa N, Emonet S, Girard M, Renzi G, Despres L, et al. Challenges in the culture-independent analysis of oral and respiratory samples from intubated patients. Front. Cell Infect. Microbiol. 2014;4:65.
37. Marsh P, Lewis M, Rogers H, Williams D, Wilson M. The resident oral microbiota. In: Oral microbiology. 2016. p. 26–50.
38. Chen T, Yu W-H, Izard J, Baranova OV, Lakshmanan A, Dewhirst FE. The Human Oral Microbiome Database: a web accessible resource for investigating oral microbe taxonomic and genomic information. Database (Oxford) 2010;2010:baq013.
39. Prevaes SMPJ, de Winter-de Groot KM, Janssens HM, de Steenhuijsen Piters WAA, Tramper-Stranders GA, Wyllie AL, et al. Development of the Nasopharyngeal Microbiota in Infants with Cystic Fibrosis. Am. J. Respir. Crit. Care Med. 2016;193:504–515.
40. Fernández L, Langa S, Martín V, Maldonado A, Jiménez E, Martín R, et al. The human milk microbiota: origin and potential roles in health and disease. Pharmacol. Res. 2013;69:1–10.

41. Kumar H, du Toit E, Kulkarni A, Aakko J, Linderborg KM, Zhang Y, et al. Distinct patterns in human milk microbiota and fatty acid profiles across specific geographic locations. Front. Microbiol. 2016;7:1619.
42. Lackey KA, Williams JE, Meehan CL, Zachek JA, Benda ED, Price WJ, et al. What's normal? microbiomes in human milk and infant feces are related to each other but vary geographically: the INSPIRE study. Front. Nutr. 2019;6:45.
43. Chen P-W, Lin Y-L, Huang M-S. Profiles of commensal and opportunistic bacteria in human milk from healthy donors in Taiwan. J. Food Drug Anal. 2018;26:1235–1244.
44. Markle JGM, Frank DN, Mortin-Toth S, Robertson CE, Feazel LM, Rolle-Kampczyk U, et al. Sex differences in the gut microbiome drive hormone-dependent regulation of autoimmunity. Science 2013;339:1084–1088.
45. Galante L, Milan AM, Reynolds CM, Cameron-Smith D, Vickers MH, Pundir S. Sex-Specific Human Milk Composition: The Role of Infant Sex in Determining Early Life Nutrition. Nutrients 2018;10.
46. Raju SC, Lagström S, Ellonen P, de Vos WM, Eriksson JG, Weiderpass E, et al. Gender-Specific Associations Between Saliva Microbiota and Body Size. Front. Microbiol. 2019;10:767.
47. Koren O, Goodrich JK, Cullender TC, Spor A, Laitinen K, Bäckhed HK, et al. Host remodeling of the gut microbiome and metabolic changes during pregnancy. Cell 2012;150:470–480.
48. Jost T, Lacroix C, Braegger C, Chassard C. Stability of the maternal gut microbiota during late pregnancy and early lactation. Curr Microbiol 2014;68:419–427.
49. Fernandes AD, Macklaim JM, Linn TG, Reid G, Gloor GB. ANOVA-like differential expression (ALDEx) analysis for mixed population RNA-Seq. PLoS One 2013;8:e67019.
50. Iebba V, Santangelo F, Totino V, Nicoletti M, Gagliardi A, De Biase RV, et al. Higher prevalence and abundance of *Bdellovibrio bacteriovorus* in the human gut of healthy subjects. PLoS One 2013;8:e61608.
51. Mosca A, Leclerc M, Hugot JP. Gut microbiota diversity and human diseases: should we reintroduce key predators in our ecosystem? Front. Microbiol. 2016;7:455.
52. Gagliardi A, Totino V, Cacciotti F, Iebba V, Neroni B, Bonfiglio G, et al. Rebuilding the gut microbiota ecosystem. Int. J. Environ. Res. Public Health 2018;15.
53. Eckburg PB, Bik EM, Bernstein CN, Purdom E, Dethlefsen L, Sargent M, et al. Diversity of the human intestinal microbial flora. Science 2005;308:1635–1638.
54. Fredricks DN, Fiedler TL, Marrazzo JM. Molecular identification of bacteria associated with bacterial vaginosis. N. Engl. J. Med. 2005;353:1899–1911.
55. Bor B, Bedree JK, Shi W, McLean JS, He X. Saccharibacteria (TM7) in the human oral microbiome. J. Dent. Res. 2019;98:500–509.
56. Lif Holgerson P, Harnevik L, Hernell O, Tanner ACR, Johansson I. Mode of birth delivery affects oral microbiota in infants. J. Dent. Res. 2011;90:1183–1188.

57. Brinig MM, Lepp PW, Ouverney CC, Armitage GC, Relman DA. Prevalence of bacteria of division TM7 in human subgingival plaque and their association with disease. Appl. Environ. Microbiol. 2003;69:1687–1694.
58. Kuehbacher T, Rehman A, Lepage P, Hellmig S, Fölsch UR, Schreiber S, et al. Intestinal TM7 bacterial phylogenies in active inflammatory bowel disease. J. Med. Microbiol. 2008;57:1569–1576.
59. Ferrocino I, Ponzo V, Gambino R, Zarovska A, Leone F, Monzeglio C, et al. Changes in the gut microbiota composition during pregnancy in patients with gestational diabetes mellitus (GDM). Sci. Rep. 2018;8:12216.
60. Cortez RV, Taddei CR, Sparvoli LG, Ângelo AGS, Padilha M, Mattar R, et al. Microbiome and its relation to gestational diabetes. Endocrine 2019;64:254–264.
61. Asnicar F, Manara S, Zolfo M, Truong DT, Scholz M, Armanini F, et al. Studying Vertical Microbiome Transmission from Mothers to Infants by Strain-Level Metagenomic Profiling. mSystems 2017;2.
62. Wang J, Zheng J, Shi W, Du N, Xu X, Zhang Y, et al. Dysbiosis of maternal and neonatal microbiota associated with gestational diabetes mellitus. Gut 2018;67:1614–1625.
63. Crusell MKW, Hansen TH, Nielsen T, Allin KH, Rühlemann MC, Damm P, et al. Gestational diabetes is associated with change in the gut microbiota composition in third trimester of pregnancy and postpartum. Microbiome 2018;6:89.
64. Pujalte MJ, Lucena T, Ruvira MA, Arahal DR, Macián MC. The Family Rhodobacteraceae. In: Rosenberg E, DeLong EF, Lory S, Stackebrandt E, Thompson F, editors. The Prokaryotes. Berlin, Heidelberg: Springer Berlin Heidelberg; 2014. page 439–512.
65. Corona-Cervantes K, García-González I, Villalobos-Flores LE, Hernández-Quiroz F, Piña-Escobedo A, Hoyo-Vadillo C, et al. Human milk microbiota associated with early colonization of the neonatal gut in Mexican newborns. PeerJ 2020;8:e9205.
66. Hospodsky D, Pickering AJ, Julian TR, Miller D, Gorthala S, Boehm AB, et al. Hand bacterial communities vary across two different human populations. Microbiology (Reading, Engl.) 2014;160:1144–1152.
67. Wang Y, Liu Y, Bai J, Chen X. The effect of maternal postpartum practices on infant gut microbiota: A chinese cohort study. Microorganisms 2019;7.
68. Sangster W, Hegarty JP, Schieffer KM, Wright JR, Hackman J, Toole DR, et al. Bacterial and Fungal Microbiota Changes Distinguish C. difficile Infection from Other Forms of Diarrhea: Results of a Prospective Inpatient Study. Front. Microbiol. 2016;7:789.
69. Tessler M, Neumann JS, Afshinnekoo E, Pineda M, Hersch R, Velho LFM, et al. Large-scale differences in microbial biodiversity discovery between 16S amplicon and shotgun sequencing. Sci. Rep. 2017;7:6589.
70. Jiménez E, de Andrés J, Manrique M, Pareja-Tobes P, Tobes R, Martínez-Blanch JF, et al. Metagenomic Analysis of Milk of Healthy and Mastitis-Suffering Women. J Hum Lact 2015;31:406–415.

71. Bolyen E, Rideout JR, Dillon MR, Bokulich NA, Abnet CC, Al-Ghalith GA, et al. Reproducible, interactive, scalable and extensible microbiome data science using QIIME 2. Nat. Biotechnol. 2019;37:852–857.
72. Callahan BJ, McMurdie PJ, Rosen MJ, Han AW, Johnson AJA, Holmes SP. DADA2: High-resolution sample inference from Illumina amplicon data. Nat. Methods 2016;13:581–583.
73. Bokulich NA, Kaehler BD, Rideout JR, Dillon M, Bolyen E, Knight R, et al. Optimizing taxonomic classification of marker-gene amplicon sequences with QIIME 2's q2-feature-classifier plugin. Microbiome 2018;6:90.
74. McDonald D, Price MN, Goodrich J, Nawrocki EP, DeSantis TZ, Probst A, et al. An improved Greengenes taxonomy with explicit ranks for ecological and evolutionary analyses of bacteria and archaea. ISME J. 2012;6:610–618.
75. Katoh K, Misawa K, Kuma K, Miyata T. MAFFT: a novel method for rapid multiple sequence alignment based on fast Fourier transform. Nucleic Acids Res. 2002;30:3059–3066.
76. Price MN, Dehal PS, Arkin AP. FastTree 2 — approximately maximum-likelihood trees for large alignments. PLoS One 2010;5:e9490.

Tables

Table 1. Clinical characteristics of subjects included in the study (n = 43).

	n	Values
Maternal age (years)	43	24.6 ± 3.4
Maternal BMI		
Normal	23	22.6 ± 1.8
Overweight	8	27.3 ± 1.0
Obese	12	33.2 ± 3.0
Gestational age (weeks)		
Term	43	39.4 ± 1.3
Preterm	0	0.0 ± 0.0
Problems during pregnancy		
None	22	51.2%
Gestational diabetes	13	30.2%
Urinary tract infection	9	20.9%
Cervicovaginitis	6	14.0%
Mode of delivery		
Vaginal	19	44.2%
Cesarea	24	55.8%
Parity		
1	10	23.3%
≥ 2	33	76.7%
Maternal exposure to antibiotics		
During delivery	25	58.1%
None	18	41.9%
Sex of the baby		
Female	22	51.2%
Male	21	48.8%

Table 2. Breastmilk core microbiota at the taxonomical family level (% relative abundance ± standard deviation).

Core family	Overall (%)	NW-F	NW-M	Ob-F	Ob-M	GD-F	GD-M
Pseudomonadaceae	22.42 ± 24.02	21.37 ± 27.30	22.50 ± 22.32	10.89 ± 15.02	55.79 ± 6.62	15.35 ± 24.78	23.69 ± 25.51
Oxalobacteraceae	11.84 ± 10.94	12.54 ± 10.57	11.60 ± 10.68	15.83 ± 14.32	6.30 ± 3.55	7.19 ± 6.99	13.99 ± 13.42
Enterobacteriaceae	11.33 ± 7.20	8.48 ± 7.61	11.74 ± 8.35	8.42 ± 5.60	16.86 ± 3.09	14.22 ± 8.27	11.69 ± 6.41
Gemellales	7.61 ± 10.32	5.05 ± 10.39	1.95 ± 2.07	13.60 ± 14.18	1.95 ± 2.61	12.88 ± 11.52	10.47 ± 9.79
Streptococcaceae	4.87 ± 11.79	5.83 ± 15.87	6.68 ± 15.16	10.94 ± 13.82	0.24 ± 0.36	1.05 ± 1.01	0.15 ± 0.15
Sphingomonadaceae	4.22 ± 4.93	6.23 ± 6.25	6.12 ± 5.73	3.46 ± 6.03	1.17 ± 0.68	3.44 ± 2.59	2.47 ± 2.18
Bradyrhizobiaceae	3.74 ± 6.51	4.47 ± 7.61	2.71 ± 6.40	4.15 ± 8.80	0.10 ± 0.05	7.86 ± 6.34	2.48 ± 3.65
Xanthomonadaceae	1.71 ± 2.36	2.43 ± 3.49	3.20 ± 2.96	0.55 ± 0.46	1.34 ± 1.03	0.56 ± 0.42	1.30 ± 1.69
Comamonadaceae	1.22 ± 1.57	1.65 ± 2.27	2.31 ± 1.97	0.32 ± 0.38	0.91 ± 0.32	0.74 ± 0.58	0.81 ± 1.03
Total relative abundance (%)	**68.97 ± 23.27**	**68.05 ± 26.19**	**68.80 ± 24.01**	**68.16 ± 27.53**	**84.66 ± 7.67**	**63.30 ± 19.99**	**67.08 ± 26.05**

Figures

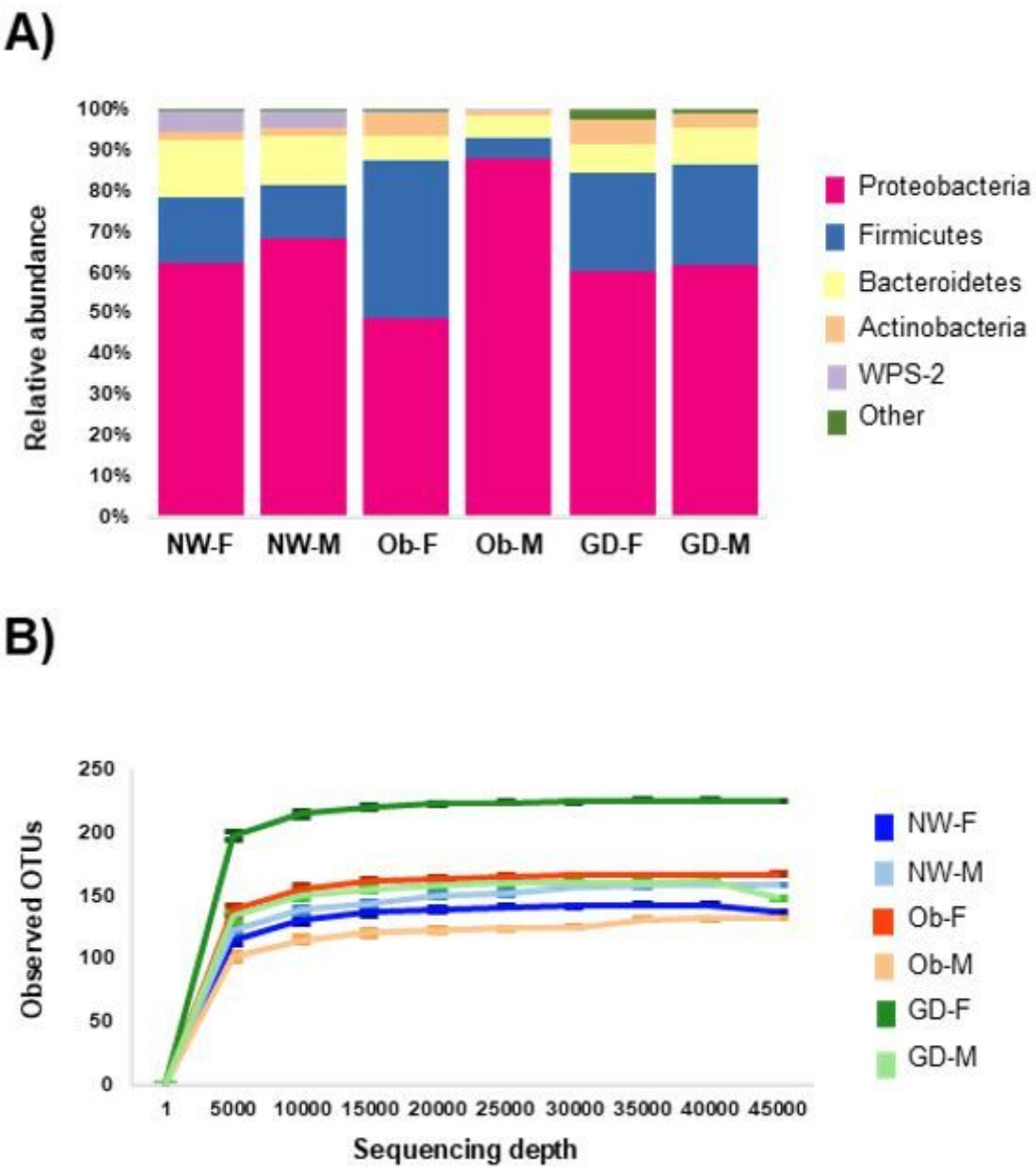
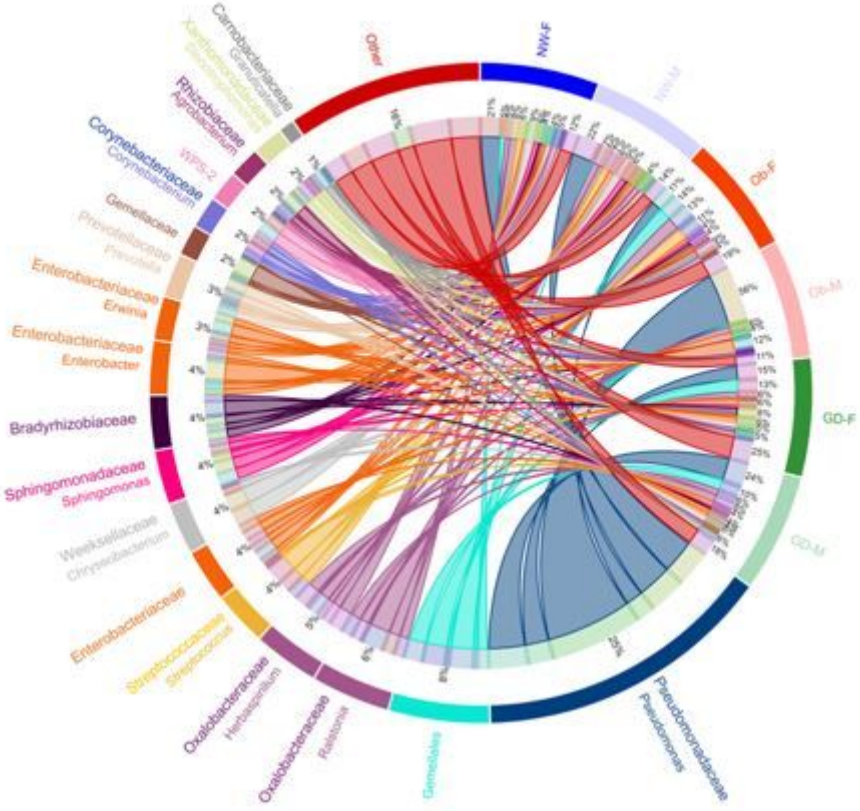


Figure 1

Bacterial diversity of breastmilk samples. A) Taxonomic profile at phylum level divided by study subgroups (maternal health condition and gender of the newborn). B) Rarefaction curves from subgroups of breastmilk samples relating the sequencing depth and the estimated number of bacteria. ASVs, amplicon sequence variants; NW-F, healthy normal weight – female (n=8); NW-M, healthy normal weight – male (n=10); Ob-F, obesity positive GDM negative– female (n=8); Ob-M, obesity positive GDM negative – male (n=4); GD-F, GDM positive obesity negative – female baby (n=6); GD-M, GDM positive obesity negative – male (n=7).



Family / Order	Total % (n=43)	NW-F (n=8)	NW-M (n=10)	Ob-F (n = 8)	Ob-M (n = 4)	GD-F (n = 6)	GD-M (n = 7)
Pseudomonas	22.4	21.4	22.5	10.9	55.8	15.4	23.7
O_Gemellales	7.6	5.1	2	13.6	2	12.9	10.5
Ralstonia	6.8	4.9	6.8	13	0.3	5.7	6.3
Herbaspirillum	5.1	7.6	4.8	2.9	6	1.4	7.7
Streptococcus	4.9	5.8	6.7	10.9	0.2	1	0.2
Enterobacteriaceae	4.5	2.9	6.1	3.8	4.3	5.6	4.2
Chryseobacterium	4.2	9	9	0.2	2.8	0.2	0.7
Sphingomonas	4	6.1	5.9	3.3	1.1	3.1	2.4
Bradyrhizobiaceae	3.7	4.5	2.7	4.1	0.1	7.9	2.5
Enterobacter	3.7	3.3	3.4	1.1	12.3	2.1	3.7
Erwinia	3	2.1	2.1	3.3	0.2	6.3	3.7
Prevotella	3	4	1.8	0.1	2	4.6	5.9
Gemellaceae	2.1	0.4	1.2	9.3	0	0.2	0.1
Corynebacterium	2.1	1.5	1.2	3.8	0.5	2.8	2.5
WPS-2	1.9	5	3.9	0	0.2	0	0
Agrobacterium	1.9	1.4	2.7	0.3	0.5	5.4	0.6
Stenotrophomonas	1.7	2.4	3.1	0.5	1.2	0.5	1.3
Granulicatella	1.1	0.5	0	0.1	0	0.1	6.1
Other	18.3	12.2	14.1	18.7	10.5	24.9	18.3

Figure 2

Circos representation of the top most abundant genera amongst healthy, obesity and Gestational Diabetes Mellitus groups. The table shows the relative abundance per subgroup of the 18 most abundant bacteria. “Other” represents all the taxa with less than 1% of total abundance. NW-F, healthy normal weight – female; NW-M, healthy normal weight – male; Ob-F, obesity positive GDM negative– female; Ob-

M, obesity positive GDM negative – male; GD-F, GDM positive obesity negative – female baby; GD-M, GDM positive obesity negative – male.

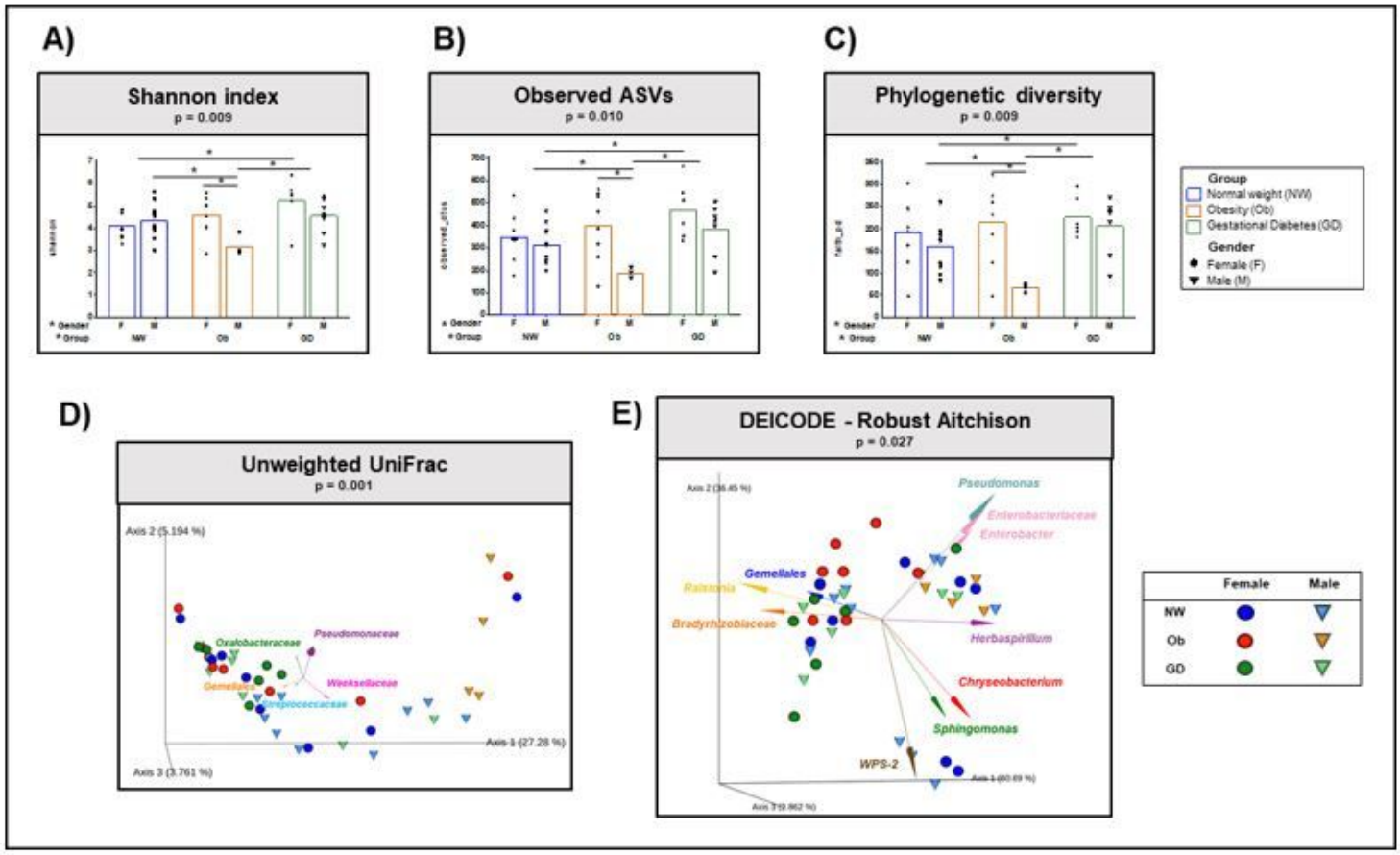


Figure 3

Alpha and Beta diversity indexes of breastmilk. A-C, Alpha diversity. D-E, Beta diversity. A) Shannon index. B) Number of observed ASVs. C) Phylogenetic diversity. All the Alpha indexes showed significant differences after a general linear model (glm) with a confidence level of 95% ($p \leq 0.05$). Fisher test was implemented for comparisons. D) Unweighted principal coordinate analysis (PCoA) biplot of UniFrac distances with vectors at family level. E) Robust principal component analysis (RPCA) biplot using DEICODE (robust Aitchison). Beta indexes showed significant differences after being assessed by permutational ANOVA (999 permutations). *$p \leq 0.05$.

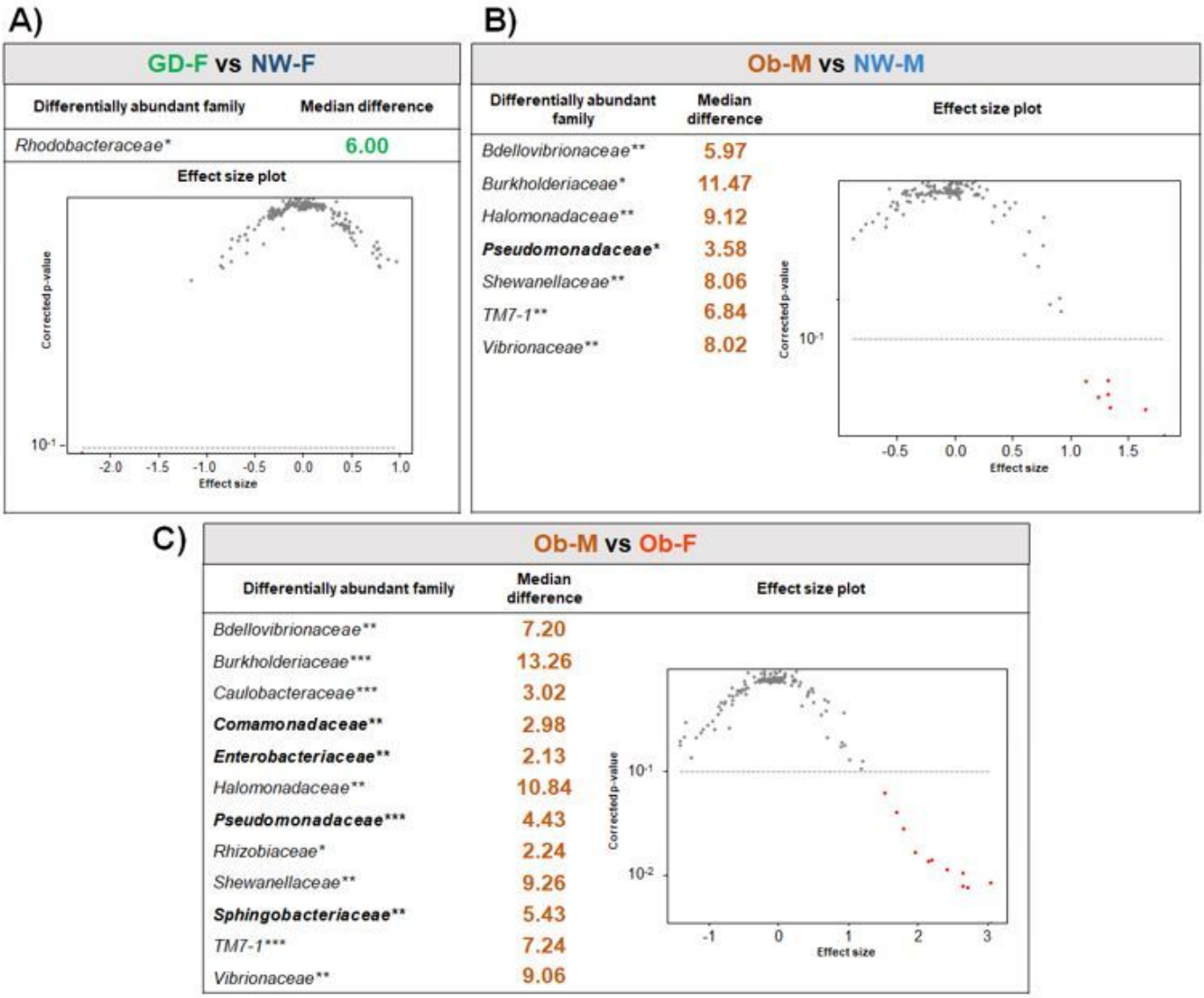


Figure 4

Differential bacteria at family level between subgroups. Each panel shows an effect size plot (effect versus Benjamini-Hochberg corrected p-value of Welch's t test) and a table illustrating differentially abundant taxon for each comparison and their median difference of centered log-ratio (clr) transformation, which indicates the dimension of the difference in abundance. Taxa with bold letters represent members of the core microbiota. Red dots in the plot represent significant features after a Welch's t test (adjusted p-value ≤ 0.1 and effect size ≥ 1). Values in tables are colored according to the subgroup that contained a greater fraction. A) Comparison of GDM-female versus healthy-female. B) Comparison of obese-male versus healthy-male. C) Comparison of obesity-male versus obese-female. *q ≤ 0.1; **q ≤ 0.05; ***q ≤ 0.001.