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Stability of the Maternal Gut Microbiota During Late Pregnancy and Early Lactation

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Abstract Scarce research has been performed to assess whether the human maternal gut microbiota undergoes changes during the perinatal period. Therefore, in the present study, gut microbiota composition of seven healthy mothers(to-be) was assessed at different time points during the perinatal period (i.e. weeks 3–7 prepartum and days 3–6, 9–14, and 25–30 postpartum) using quantitative polymerase chain reaction (qPCR) and pyrosequencing, and was complemented by short-chain fatty acids (SCFA) and calprotectin quantification using high-performance liquid chromatography and enzyme-linked immunosorbent assay, respectively. qPCR revealed the predominance of members of the Firmicutes, *Bacteroides*, and *Bifidobacterium* without detectable changes over the perinatal period. Pyrosequencing supported these data in terms of microbiota stability for any population at any taxonomic level, although ratios of members of the Actinobacteria and Bacteroidetes differed between the two methods. However, the number of operational taxonomic units observed by pyrosequencing was subjected to fluctuations and the relative abundance of *Streptococcus* decreased numerically postpartum ($P = 0.11$), which may indicate that aberrancies in subdominant populations occur perinatally. Furthermore, total fecal SCFA concentrations, particularly the branched-chain fatty acids isobutyrate and isovalerate, were higher than for non-pregnant subjects throughout the perinatal period. This suggests metabolic changes and increased energy extraction via proteolytic, in addition to saccharolytic fermentation, accompanied by low-grade inflammation based on fecal calprotectin levels. Our data show that the maternal gut microbiota remained stable over the perinatal period despite altered metabolic activity and low-grade inflammation; however, it remains to be confirmed whether changes preceded earlier during pregnancy and succeeded later postpartum.

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Introduction

The human gut microbiota profoundly influences health and disease throughout the life by complex microbe–microbe and mutualistic host–microbe interactions [9]. A healthy microbiota plays important functions in energy harvest and storage, trophic and metabolic functions (e.g. production of short-chain fatty acid (SCFA)), and protection against pathogens [15]. Moreover, the microbiota interacts with mucosal epithelial cells, leading to maturation and maintenance of the host immune system, which in turn impacts the composition of the microbiota [28]. Consequently, microbial dysbiosis has been associated with a range of immune-related and metabolic diseases, including inflammatory bowel diseases, gastric cancer, obesity, and type-2 diabetes [9].

Besides factors, such as diet, host genome, lifestyle, disease, and medication, the gut microbiota has been shown to undergo dramatic changes during early and late life, but remains relatively stable in healthy adults [28]. However, recent studies have shown that the microbiota

can become aberrant over the course of a healthy pregnancy, accompanied by metabolic changes that resemble obesity-associated metabolic syndrome (e.g. reduced insulin sensitivity, elevated blood glucose, and fecal cytokine levels) [12, 23]. Collado et al. [12] reported significantly increasing population levels from the first to the third trimester of pregnancy using fluorescence in situ hybridization coupled to flow cytometric detection (FISH-FC) (i.e. total bacteria, *Bifidobacterium* spp., *Clostridium hystolyticum* group, *Bacteroides–Prevotella* group), as well as using quantitative polymerase chain reaction (qPCR) (i.e. *Bifidobacterium* spp., *Bacteroides fragilis*, *Staphylococcus aureus*, and *Akkermansia muciniphila*). Koren et al. [23], when analyzing a subset of the same study cohort by pyrosequencing, observed dramatic changes in microbiota composition, involving loss of diversity and increasing Proteobacteria and Actinobacteria populations from the first to the third trimester of pregnancy, which persisted in 1 month postpartum samples. However, in contrast to metabolic syndrome, the authors suggested these changes to be beneficial during pregnancy and to support growth of the fetus, as well as to prepare for energetic demands of lactation.

In the present study, we used a narrower sampling schedule compared with Koren et al. [23], focusing on the gut microbiota composition during the perinatal period. Therefore, the bacterial composition of maternal feces collected during the third trimester of pregnancy and at three time points within the first month postpartum was analyzed using qPCR and pyrosequencing, and complemented by quantification of glucose and metabolites (i.e. SCFA and lactate) by high-performance liquid chromatography (HPLC) and calprotectin, a marker of neutrophil intestinal inflammation, and by enzyme-linked immunosorbent assay (ELISA).

Methods

Study Design and Sample Collection

Healthy mothers-to-be carrying a healthy baby, and planning to deliver vaginally and to exclusively breast feed during the neonatal period, were recruited for this observational clinical study by the University Children's Hospital Zurich and the Hospital Zollikerberg (Zurich, Switzerland). Exclusion criteria were preterm and/or cesarean delivery, any formula feeding, as well as any variables known to affect the balance of the maternal microbiota, including any dietary supplementation (e.g. probiotic supplements) and drug administration (e.g. antibiotics) during lactation and at least four months prepartum. This study was conducted according to the guidelines laid down in the Declaration of Helsinki, and all procedures involving human subjects were approved by the Ethics Committee of the Canton of Zurich (Zurich, Switzerland) (project identification code: StV47/08; date of approval: 12.10.2009) and written informed consent was obtained from all subjects.

A total of 21 healthy mothers-to-be were recruited for this study, however, due to the stringent inclusion criteria, 12 mothers-to-be were excluded before the first postpartum sampling point and 2 mothers thereafter, mainly due to cesarean delivery and/or antibiotic treatment. Thus, maternal feces were successfully collected from seven mothers(-to-be) (aged 34.1 $\pm$ 3.9 years) at four sampling points, i.e. at 3–7 weeks prepartum and at days 3–6, 9–14, and 25–30 postpartum.

Fresh feces were collected into fecal collection containers provided with an anaerobic gas generation system that creates a CO_2 atmosphere, while chemically binding O_2 (Anaerocult A, Merck KGaA, Darmstadt, Germany). Samples were transported at 4 °C, aliquoted in an anaerobic chamber (Coy Laboratory Products Inc., Grass Lake, MI, USA) with the atmosphere of 85 % N_2, 10 % CO_2, and 5 % H_2, and stored at −80 °C within 4 h upon sampling.

DNA Extraction

Total DNA was extracted from 0.2 g of fecal aliquots using a FastDNA SPIN Kit for Soil and the FastPrep-24 instrument for mechanical lysis (MP Biomedicals, Illkirch, France) according to the manufacturer's instructions. DNA concentration and quality were assessed spectrophotometrically and aliquots were stored at −20 °C prior to the molecular analyses.

Quantitative PCR

Different qPCR assays were performed, using a 7500 Fast Real-Time PCR System with SYBR Green chemistry (Applied Biosystems Europe BV, Zug, Switzerland), for the absolute quantification of the major gut-associated bacterial populations, *Bacteroides*, *Bifidobacterium*, Firmicutes, *Roseburia* and *Eubacterium rectale*, *Faecalibacterium prausnitzii*, *Lactobacillus*, *Streptococcus*, *Staphylococcus*, *Enterobacteriaceae,* and total bacteria. The corresponding primer sets targeted the 16S rRNA gene, except for the *Bifidobacterium* assay, in which the xylulose-5-phosphate/fructose-6-phosphate phosphoketolase gene (*xfp*) was targeted, as well as for the *Staphylococcus* and *Streptococcus* assays, in both of which the gene encoding the elongation factor Tu (*tuf*) was targeted. The advantage of the *xfp* and *tuf* assays is that they overcome the 16S rRNA gene multiple copy number bias [25], as

only one copy of the *xfp* gene has been detected in *Bifidobacterium* spp. to date, and low-GC gram-positive bacteria, such as *Staphylococcus* and *Streptococcus* spp., generally contain only one copy of the *tuf* gene [11, 22]. Primer sequences, reaction mixtures, cycling conditions, and melt curve analysis have been described in detail previously [19]. Type strain DNA for the generation of standard curves consisted of purified 16S rRNA gene amplicons of *Bacteroides thetaiotaomicron* DSM2079T; *Lactobacillus delbrueckii* DSM20081T; *Escherichia coli* DSM5698; and *Faecalibacterium prausnitzii* DSM17677 for the *Bacteroides*, *Lactobacillus*, *Enterobacteriaceae*, and *Faecalibacterium* assays, respectively, and *Roseburia intestinalis*, DSM14610T for both the *Firmicutes* and *Roseburia* assays; while, plasmid pLME21 containing the 16S rRNA gene from *Bifidobacterium lactis,* DSM10140T was used in the total bacteria assay, the *xfp* amplicon of *Bifidobacterium longum* DSM20219T for the *Bifidobacterium* assay, and the *tuf* amplicon of *Staphylococcus epidermidis*, DSM20044T and *Streptococcus mitis*, DSM12643T in the *Staphylococcus* and *Streptococcus* assays, respectively. Sample gene copy numbers per gram of wet feces were extrapolated from standard curves generated in triplicate in each run by linear regression of C_t values from serial tenfold dilutions of appropriate type strain DNA.

Pyrosequencing

Total fecal DNA was used for unidirectional barcoded pyrosequencing of the V5–V6 hypervariable 16S rRNA region, which was chosen since the V6 region exhibits the highest sequence variability and therefore, a high discriminatory power [1]. Reactions were carried out at DNAVision SA (Charleroi, Belgium), using a 454 Life Sciences GS FLX system in combination with Titanium chemistry (Roche AG, Basel, Switzerland) as described previously [19, 20].

Sequence quality was verified according to the criteria: maximum of one mismatch in the barcode and primer, length of at least 240 bp, and a maximum of two undetermined bases per sequence (excluding barcode and primers). The dataset has been deposited to the National Center for Biotechnology Information (NCBI) Sequence Read Archive (SRA) under accession number SRA065797. Phylum-, family- and genus-level taxonomic assignments of quality-controlled sequences were made using the Ribosomal Database Project (RDP) Classifier [36] with a stringent confidence threshold of 80 %. The Mothur software package was used for nearest neighbor clustering of the sequences into operational taxonomic units (OTU), based on which richness (Chao index and rarefaction curves) and diversity (Shannon index) were calculated [7, 32].

HPLC and ELISA

Fecal concentrations of SCFA, acetate, butyrate, formate, propionate and valerate, branched-chain fatty acids (BCFA), isobutyrate and isovalerate, as well as glucose and lactate were measured by HPLC as described previously [10], and reported as mM fecal water. Fecal calprotectin concentrations were measured by ELISA (Eurospital SpA, Trieste, Italy) according to the manufacturer's instructions and reported as $\mu g\ g^{-1}$ feces. Calprotectin is a highly stable antimicrobial protein accounting for $\sim$60 % of the soluble cytosol proteins in neutrophils. Thus, its concentration correlates with the intensity of intestinal neutrophil infiltration and can be considered as reliable, noninvasive method for assessing intestinal inflammation [6].

Statistical Analysis

Quantitative data obtained from qPCR (triplicates) were averaged and $\log_{10}$-transformed, while ratios of individual populations over total bacteria were calculated from absolute values. For statistical analysis, mean $\log_{10}$-transformed qPCR data, mean relative abundance and OTU data from pyrosequencing, mean metabolites and calprotectin concentrations were calculated at each time point (mean $\pm$ SD), and differences over time were assessed by one-way repeated measures ANOVA and Tukey post-test using Prism 5 (GraphPad Software Inc, La Jolla, CA, USA) with significance levels of $P < 0.05$.

Results

Quantitative PCR

Quantification of the major gut-associated bacterial populations using qPCR showed that the maternal fecal microbiota was dominated by members of the Firmicutes phylum at all four sampling points during the perinatal period (overall mean of 10.6 $\pm$ 0.5 log gene copies/g feces, representing about 32 % of total bacteria) including the genera *Faecalibacterium* and *Roseburia* (overall means of 9.7 $\pm$ 0.6 and 9.6 $\pm$ 0.7 log gene copies/g feces, i.e. each $\sim$5 % of total bacteria) followed by *Bacteroides* and lower counts of *Bifidobacterium* (overall means of 10.3 $\pm$ 0.7 and 8.9 $\pm$ 0.6 log gene copies/g feces, i.e. $\sim$14 and $\sim$0.6 %) of total bacteria, respectively) (Table 1). *Enterobacteriaceae*, *Lactobacillus*, and *Streptococcus* were observed at subdominant levels only (7.4 $\pm$ 0.9, 7.7 $\pm$ 1.7 and 5.6 $\pm$ 3.0 log gene copies/g feces i.e. each $\leq$0.02 % of total bacteria), and *Staphylococcus* population levels were below the detection limit in all but one sample. Population densities detected for total bacteria, *Bifidobacterium*,

Table 1 Quantification of the major gut-associated bacterial populations by qPCR

Target population	MF0	MF1	MF2	MF3	*P* value[a]
Total bacteria	11.1 ± 0.4	10.9 ± 0.5	11.2 ± 0.8	11.2 ± 0.4	0.74
Bifidobacterium	9.0 ± 0.5	8.7 ± 0.6	8.8 ± 0.6	9.0 ± 0.5	0.36
Bacteroides	10.1 ± 0.5	10.2 ± 0.6	10.3 ± 0.9	10.5 ± 0.5	0.51
Firmicutes	10.6 ± 0.4	10.5 ± 0.4	10.6 ± 0.7	10.7 ± 0.3	0.89
Faecalibacterium	9.6 ± 0.4	9.7 ± 0.4	9.8 ± 0.9	9.6 ± 0.5	0.84
Roseburia	9.3 ± 0.6	9.6 ± 0.4	10.0 ± 0.8	9.7 ± 0.6	0.19
Lactobacillus	8.3 ± 1.1	7.6 ± 1.0	7.1 ± 3.0	8.0 ± 0.4	0.56
Staphylococcus	n.d.[b]	n.d.	1.7 ± 3.0[c]	n.d.	0.44
Streptococcus	6.4 ± 2.7	5.0 ± 3.2	6.2 ± 2.6	4.9 ± 3.1	0.36
Enterobacteriaceae	7.6 ± 0.9	7.2 ± 0.8	7.3 ± 0.7	7.6 ± 0.6	0.44

Gene copy numbers detected in maternal feces during pregnancy (MF0, i.e. 3–7 weeks prepartum) and lactation (MF1, MF2, and MF3, i.e. days 4–6, 9–14, and 25–28 postpartum, respectively; $N = 7$ at each time point). Values are expressed as mean ± SD log gene copy numbers/g feces

[a] differences over time were assessed by one-way repeated measures ANOVA with significance levels of $P < 0.05$

[b] not detected

[c] one positive sample only at 6.8 log gene copies/g feces

Bacteroides, Firmicutes, *Faecalibacterium*, *Roseburia,* and *Enterobacteriaceae* did not vary significantly from the third trimester of pregnancy (3–7 weeks prepartum) throughout the first month postpartum (Table 1).

High inter-individual variations in population densities (and thus high standard deviations, i.e. >1 log unit) were observed for *Lactobacillus* and *Streptococcus*, however, no significant differences were detected over time for both of these populations, nor for any other population (Table 1).

Pyrosequencing

High-throughput sequencing performed on maternal fecal DNA generated a total of 278,084 quality-filtered, taxonomically classifiable 16S rRNA gene sequence reads with a mean of 9,932 ± 2,474 reads per sample, and mean read lengths of 258 ± 1 bp. The slopes of the rarefaction curves generated at 3, 5, and 10 % OTU dissimilarity cut-off, which are generally accepted cut-offs for species, genus, and family/class level differentiation, respectively, level-off toward the right, indicating that the majority of the diversity has been covered and that a considerable additional sequencing effort would be required to detect additional OTU (Fig. 1). The overall mean OTU number at 3 % similarity cut-off was 842 ± 331, based on which richness and diversity were calculated (Chao1 index: 1,924 ± 829 and Shannon index: 4.41 ± 0.49, respectively). However, the mean number of observed OTU at 3 % similarity cut-off was 875 ± 363 prepartum, and 956 ± 479, 734 ± 265, and 803 ± 221 over the three sampling points during the first month postpartum. Thus, OTU numbers varied during the perinatal period, however, these variations were not significant over time ($P = 0.12$). The same variations were observed for richness and diversity indexes over time.

Phylum-level taxonomic classification using RDP classifier revealed that the Firmicutes and Actinobacteria dominated the maternal microbiota (overall mean relative abundance of 84.9 ± 10.5 and 11.6 ± 9.7 %, respectively), followed by the Bacteroidetes and Proteobacteria phyla (1.5 ± 2.2 and 0.8 ± 3.4 %, respectively) (Fig. 2a). Classification at the family-level (Fig. 2b) and genus-level (Fig. 3) showed the highest diversity within the Firmicutes phylum, comprising six families and thirteen genera that were detected at overall mean relative abundances of ≥1 and ≥0.5 %, respectively. However, members of the *Lachnospiraceae* family (*Blautia*, *Coprococcus*, *Dorea* and *Roseburia*) and the *Ruminococcaceae* family (*Faecalibacterium*, *Ruminococcus* and *Subdoligranulum*) dominated the maternal microbiota. In contrast, less diversity was observed for the other three phyla: the Actinobacteria phylum comprised members of the *Bifidobacteriaceae* (*Bifidobacterium*) and *Coriobacteriaceae* families (*Collinsella*). The Bacteroidetes and Proteobacteria phyla comprised only members of the *Bacteroidaceae* (*Bacteroides*) and *Enterobacteriaceae* families (*Escherichia*/*Shigella*), respectively, which was reflected in their mean relative abundance pattern at all taxonomic levels (Figs. 2a, b, 3).

Despite that no statistically significant variations in mean relative abundances over time were observed at any taxonomic level, both *Streptococcaceae* and *Streptococcus* decreased numerically over the perinatal period ($P = 0.13$ and 0.11, respectively), which is in agreement with data obtained from qPCR; while on the other hand, *Dialister* increased numerically over the perinatal period ($P = 0.43$).

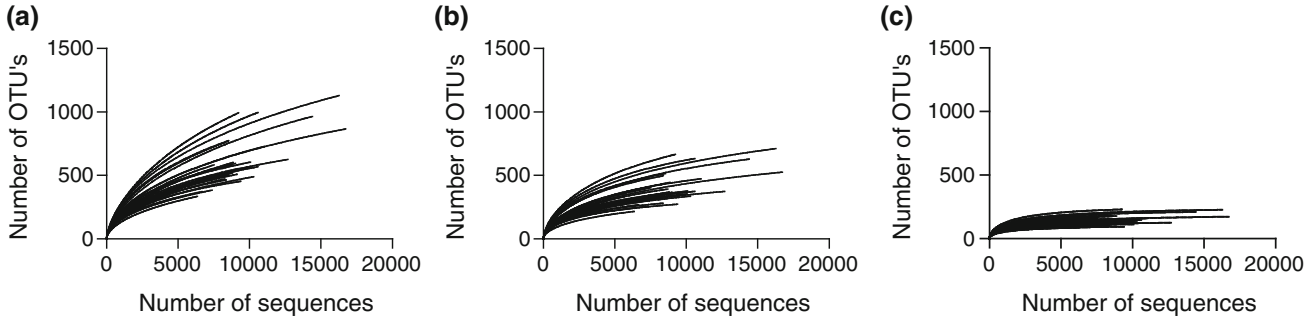

Fig. 1 Rarefaction curves showing the number of observed OTU at **a** 3 %, **b** 5 %, and **c** 10 % dissimilarity cut-off as a function of the number of pyrosequences, which are generally accepted cut-offs for species, genus, and family/class level differentiation, respectively

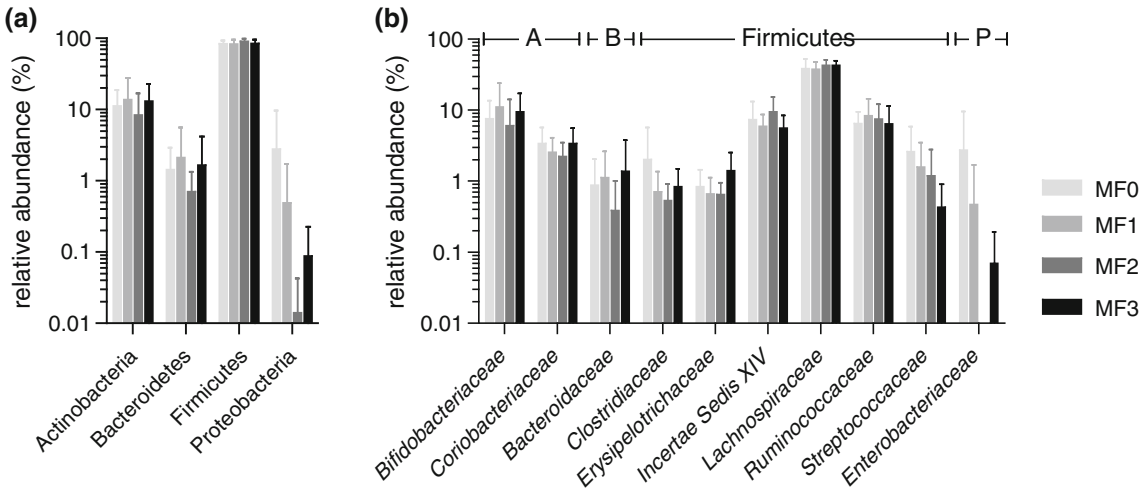

Fig. 2 Phylum- and family-level gut microbiota composition assessed by pyrosequencing. Mean relative 16S rRNA gene abundances detected in maternal feces during pregnancy (MF0, i.e. 3–7 weeks prepartum) and lactation (MF1, MF2, and MF3, i.e. days 4–6, 9–14, and 25–28 postpartum, respectively; $N = 7$ at each time point). **a** Taxonomic assignments at the phylum-level and **b** at the family-level considering phyla and families detected at an overall mean relative abundance ≥ 0.5 and ≥ 1 %, respectively ($N = 28$). Values are expressed as mean $\pm$ SD. The abbreviations *A*, *B*, and *P* stand for the phyla Actinobacteria, Bacteroidetes, and Proteobacteria, respectively

HPLC and ELISA

Assessment of fecal metabolites using HPLC revealed that overall mean total SCFA concentration was 156.6 $\pm$ 27.1 mM. The most abundant SCFA was acetate (overall mean of 79.8 $\pm$ 19.2 mM), followed by isobutyrate, butyrate, and propionate (24.6 $\pm$ 12.3, 23.0 $\pm$ 6.6, and 18.4 $\pm$ 5.7 mM, respectively), while all other metabolites, as well as glucose, were detected at lower concentrations (overall means < 6 mM) (Fig. 4). Fecal calprotectin was detected at an overall mean concentration of 18.8 $\pm$ 6.1 μg g^{-1} feces using ELISA (Fig. 4). No significant differences in any fecal SCFA, glucose, lactate, or calprotectin were detected over time.

Discussion

Scarce research has been performed to assess whether the maternal gut microbiota undergoes changes during the perinatal period. In the present study, we used state-of-the-art molecular methods, qPCR and pyrosequencing, and successfully characterized the bacterial composition of feces collected from seven mothers at four time points during the perinatal period.

Quantitative PCR revealed that the major gut-associated bacterial populations did not undergo detectable changes throughout the perinatal period. Members of the Firmicutes and *Bacteroides* predominated the maternal microbiota, followed by *Bifidobacterium*, while facultative anaerobic populations, *Enterobacteriaceae*, *Streptococcus*, and *Lactobacillus* were detected at subdominant levels. These population levels largely reflect the composition of a healthy adult gut microbiota [31], which indicates that delivery and lactation do not induce significant alteration of the gut microbiota.

Compared with absolute quantification by qPCR, assessment of the mean relative population abundances by pyrosequencing at phylum-, family-, and genus-level,

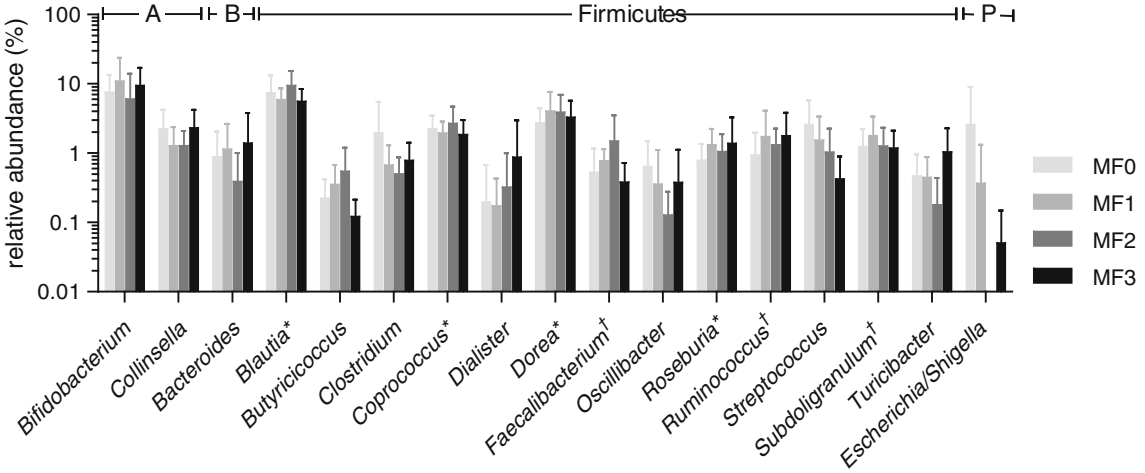

Fig. 3 Genus-level gut microbiota composition assessed by pyrosequencing. Mean relative 16S rRNA gene abundances detected at the genus level in maternal feces during pregnancy (MF0, i.e. 3–7 weeks prepartum) and lactation (MF1, MF2, and MF3, i.e. days 4–6, 9–14, and 25–28 postpartum, respectively; $N = 7$ at each time point). Only genera detected at an overall mean relative abundance $\geq$0.5 % ($N = 28$) are shown. Values are expressed as mean $\pm$ SD. The abbreviations *A*, *B*, and *P* stand for the phyla Actinobacteria, Bacteroidetes, and Proteobacteria, respectively. Members of the *Lachnospiraceae* and *Ruminococcaceae* families are indicated with *asterisks* and *dagger*, respectively

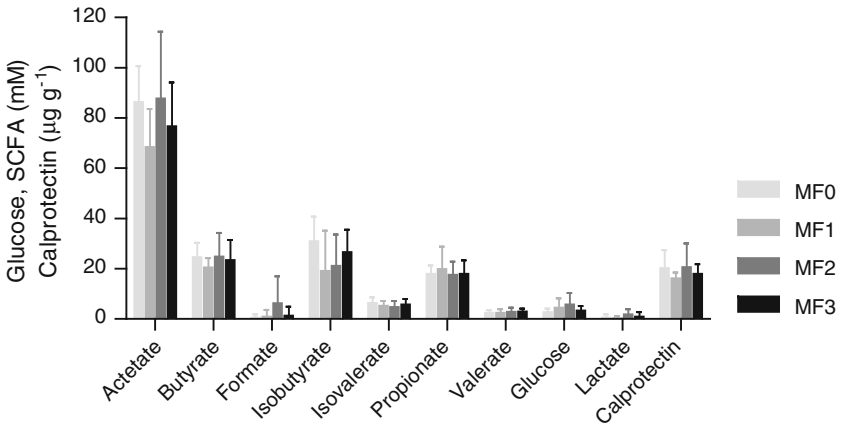

Fig. 4 Quantification of fecal metabolites and calprotectin by HPLC and ELISA, respectively. Mean SCFA, glucose, lactate, and calprotectin concentrations detected in maternal feces during pregnancy (*MF0*, i.e. 3–7 weeks prepartum) and lactation (*MF1*, *MF2*, and *MF3*, i.e. days 4–6, 9–14, and 25–28 postpartum, respectively; $N = 7$ at each time point). Values are expressed as mean $\pm$ SD

allowed gaining a more comprehensive insight into the predominant bacterial diversity. Pyrosequencing supported qPCR data in terms of microbiota stability throughout the perinatal period for any population at any taxonomic level, which is consistent with the fact that Koren et al. [23] did not observe significant changes in microbiota composition between the third trimester of pregnancy and one month postpartum. However, differences in some population levels were observed between those methods: both methods confirmed the predominance of members of the Firmicutes, but ratios of Bacteroidetes and Actinobacteria phyla, comprising mainly *Bacteroides* and *Bifidobacterium*, respectively, were inversed. The high quantitative accuracy of qPCR and the fact that the Firmicutes and *Bifidobacterium* levels detected in the present study are consistent with previous research using qPCR on healthy, non-pregnant subjects [3, 16, 26, 33], suggest an underestimation of Bacteroidetes and/or overestimation of Actinobacteria by pyrosequencing due to primer bias rather than due to DNA extraction, since the same extracts have been used for both methods. The mean relative phylum-level abundances detected in the present study are consistent with the previous pyrosequencing analyses of fecal samples from healthy subjects [1, 35]. Andersson et al. [1], using the same primers targeting the V5-V6 16S rRNA gene region, suggested an underestimation of Bacteroidetes and a higher primer snsitivity for Actinobacteria. On the other hand, Collado et al. [12], when analyzing for the first time the gut microbiota during pregnancy using qPCR and FISH, detected slightly higher *Bifidobacterium* compared with *Bacteroides–Prevotella* group levels and an increase in both populations from the first to the third trimester of pregnancy. In line with these findings, Koren et al. [23] reported an increase in Actinobacteria pyrosequences

during pregnancy when analyzing a subset of the same study cohort. It remains, however, to be elucidated whether differences between these and our study are attributable to methodological factors (e.g. DNA extraction procedures), nutritional (e.g. probiotic consumption) and/or geographic factors as pointed out in previous research [14, 37], or if an inversed ratio of Actinobacteria to Bacteroidetes may be attributable to changes in host-microbiota interactions during pregnancy. It should be noted that the abundance of Bacteroidetes detected by qPCR and pyrosequencing (i.e. ~14 and ~1.5 %) was relatively low compared with several previous studies where abundances of up to ~50 % have been detected [2, 4, 34]. This discrepancy may be explained by the DNA extraction procedure, which involved a relatively harsh beat beating step that may have partly destroyed shear sensitive gram-negative bacteria, such as members of the Bacteroidetes. On the other hand, the relatively low abundance of the Firmicutes detected by qPCR compared with pyrosequencing (i.e. 32 and 84.9 %, respectively) may be explained by the difficulty in designing primers that are specific to all members of a group as diverse as the Firmicutes, whereas specificity is less of an issue for the design of universal bacterial primers.

Variations in mean OTU number and relative abundance of facultative anaerobes, such as *Streptococcus* and members of the Proteobacteria observed over time using pyrosequencing, although not statistically significant, may indicate that aberrancies in subdominant populations occur perinatally. Koren et al. [23] reported a reduction in OTU (loss of diversity) from the first to the third trimester, accompanied by an increase in Proteobacteria. In this context, the slight decrease in *Escherichia/Shigella*, as well as *Streptococcus* during the perinatal period in our study may thus reflect resilience to normal physiologic population and diversity levels. It should be noted that no *Shigella* are expected in the group reported as *Escherichia/Shigella*, however, the length of the pyrosequences is not sufficient for differentiating between those closely related genera.

Assessment of SCFA concentrations by HPLC revealed as expected acetate, butyrate, and propionate as major metabolic products resulting from saccharolytic bacterial fermentation. Although, ratios of individual to total SCFA concentrations corresponded largely to those reported previously for non-pregnant subjects, except BCFA, total SCFA concentrations were higher than those detected in both normal-weight and obese subjects (i.e. 156.6 ± 27.1 vs 124.9 ± 31.5 and 142.8 ± 28.6 mM, respectively) [29]. Furthermore, concentrations of the BCFA, isobutyrate and isovalerate, which result from proteolytic fermentation and usually represent only a minor fraction of total SCFA, surprisingly exceeded those reported previously [29]. This suggests metabolic changes and highly efficient dietary energy extraction, which has been hypothesized as beneficial in the context of pregnancy [23]. This observation is moreover supported by the fact that fecal glucose (i.e. 4.1 ± 3.1 mM) was similarly depleted as in obese (i.e. 4.3 ± 5.2 mM), while considerably higher concentrations have been observed in normal-weight subjects (i.e. 26.6 ± 61.7 mM) [29]. One explanation for increased energy extraction may be an increase in gastrointestinal transit time due to hormonal (i.e. increased progesterone and estradiol levels) and metabolic changes during late pregnancy, as reported previously [8, 24]. In contrast to butyrate, whose beneficial functions on colonocytes have been studied extensively [17], it remains to be elucidated whether isobutyrate is beneficial or harmful to colonocytes. Despite that butyrate is preferentially metabolized by colonocytes, research has shown that colonocytes are able to metabolize isobutyrate under conditions of low butyrate concentrations [18], which may be a reason for fecal isobutyrate accumulation observed in our study.

In the present study, fecal calprotectin levels were assessed as a marker of intestinal inflammation by ELISA, and no significant differences over the perinatal period could be observed. Costa et al. [13] reported median fecal calprotectin levels of 11 μg g^{-1} (95 % CI 3–18 μg g^{-1}) in healthy subjects versus ~22 μg g^{-1} (95 % CI 9–35 μg g^{-1}) in patients with inflammatory bowel disease. Thus, mean levels observed in the present study (18.8 ± 6.1 μg g^{-1}) may represent low-grade signs of intestinal inflammation, which has also been observed by Koren et al. [23] on the basis of elevated pro-inflammatory cytokine levels during the third trimester of pregnancy. Low-grade inflammation may reduce epithelial barrier integrity [5], which would support the hypothesis of increased bacterial translocation during pregnancy, and the presence of a bacterial entero-mammary pathway as a novel way of mother-neonate communication via breast-feeding, as proposed previously [20, 21, 27, 30].

The use of both qPCR and pyrosequencing, as benchmark for absolute bacterial quantification and diversity assessment, respectively, at four different time points during the perinatal period is a major strength of the present study. Compared with the study by Koren et al. [23], a higher mean number of pyrosequences was generated per sample (~2,873 vs ~9,932), allowing to gain deeper insight into subdominant populations, which may also explain the higher OTU number observed in the present study. On the other hand, sample size was smaller in the present study, which limits statistical power. Furthermore, although the observation of stability of the maternal microbiota from the third trimester of pregnancy to one month postpartum is consistent with the previous research [23], the first trimester samples would be required in future research to confirm the changes observed by Koren et al. [23] and Collado et al. [12].

In conclusion, in the present study, we observed quantitative or qualitative stability of the maternal gut microbiota throughout the perinatal period, despite that OTU number was subjected to fluctuations. Furthermore, elevated fecal SCFA and calprotectin concentrations suggest metabolic changes and low-grade inflammation, which in contrast to obesity has been hypothesized as beneficial in the context of a healthy pregnancy [23]. However, in future research these observations should be verified based on a larger number of subjects, extended sampling schedule (including non-pregnant controls), and methodology (e.g. functional genomics), to confirm whether changes in microbiota composition and metabolism preceded earlier during pregnancy and succeeded later postpartum.

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Conflict of interest The authors declare that they have no conflict of interest.

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