Gestational diabetes is associated with changes in placental microbiota and microbiome

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BACKGROUND: The human microbiota is a modulator of the immune system. Variations in the placental microbiota could be related with pregnancy disorders. We profiled the placental microbiota and microbiome in women with gestational diabetes (GDM) and studied its relation to maternal metabolism and placental expression of anti-inflammatory cytokines.

METHODS: Placental microbiota and microbiome and expression of anti-inflammatory cytokines (IL10, TIMP3, ITGAX, and MRC1MR) were analyzed in placentas from women with GDM and from control women. Fasting insulin, glucose, O'Sullivan glucose, lipids, and blood cell counts were assessed at second and third trimester of pregnancy.

RESULTS: Bacteria belonging to the Pseudomonadales order and Acinetobacter genus showed lower relative abundance in women with GDM compared to control ($P < 0.05$). In GDM, lower abundance of placental Acinetobacter associated with a more adverse metabolic (higher O'Sullivan glucose) and inflammatory phenotype (lower blood eosinophil count and lower placental expression of *IL10* and *TIMP3*) ($P < 0.05$ to $P = 0.001$). Calcium signaling pathway was increased in GDM placental microbiome.

CONCLUSION: A distinct microbiota profile and microbiome is present in GDM. Acinetobacter has been recently shown to induce IL-10 in mice. GDM could constitute a state of placental microbiota-driven altered immunologic tolerance, making placental microbiota a new target for therapy in GDM.

Elevated prepregnancy BMI, excessive gestational weight gain, and gestational diabetes (GDM) are prevalent risk factors in inner-city populations and determinants of fetal growth (1). Placental size is an important mediator between prepregnancy BMI, gestational weight gain, GDM, and increased fetal growth and has also been linked with adult diseases including hypertension and cardiovascular disease (2,3).

Establishment and maintenance of placental integrity and function are critical to fetal growth, development, and survival. A unique placental microbiota niche, composed of nonpathogenic commensal microbiota from the Firmicutes, Tenericutes, Proteobacteria, Bacteroidetes, and Fusobacteria phyla has been recently described (4). In contrast to the gut microbiota, in which Firmicutes and Bacteroidetes are the most abundant Phyla (80%), placental microbiota is mainly formed by Proteobacteria.

It is increasingly recognized that GDM and allergy are interrelated and that both prenatal and early life factors are associated with maternal obesity or allergic manifestations in the child (5–10). For instance, term infants of pregnancies with GDM had a 7.57-fold increased risk of developing atopic dermatitis and a 5.91-fold increased risk of allergen sensitization. But only a small part of this increased risk was accounted by the inclusion of fetal growth as a covariate (11). These data suggest that, at least in term infants, prenatal metabolic factors increase the risk of allergic disease and sensitization in early life.

As environmental biodiversity, human microbiota and allergy are interrelated; we postulated that placental microbiota could be related to GDM. This hypothesis is supported by the fact that bacterial DNA has been recently identified in the blood of patients and predicts the incidence of type 2 diabetes (12). The bacterial translocation toward tissues is causal of inflammation in metabolic tissues (13,14) and hence could also be responsible for placenta immune cell infiltration and inflammation. We aimed to profile the human placental microbiota and investigate the placental microbiome in women with gestational diabetes and study whether they relate to maternal metabolism and placental expression of anti-inflammatory cytokines.

RESULTS

Placental Bacterial 16SrRNA DNA Profile

Table 1 shows the clinical and anthropometric characteristics of the studied subjects. To analyze the placental bacterial profile, the overall microbiota from control nondiabetic pregnant women and women with GDM was sequenced. The operational taxonomic unit (OTU) count in placenta from GDM

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Table 1. Clinical characteristics of pregnant women enrolled in the study

	Control	GDM	
	n = 11	*n* = 11	*P* value
Mother			
Age (years)	32 ± 1	31 ± 1	Ns
Pregestational weight (kg)	61 ± 3	60 ± 3	Ns
Pregestational height (m^2)	162 ± 2	164 ± 2	Ns
Pregestational BMI (kg/m^2)	23 ± 1	22 ± 1	Ns
Second trimester			
Weight (kg)	69.5 ± 4.2	69.3 ± 3.4	Ns
BMI (kg/m^2)	26.3 ± 1.2	26.0 ± 1.2	Ns
Fasting glucose (mg/dl)	74.1 ± 1.6	80.0 ± 1.2	0.011
O'Sullivan glucose (mg/dl)	98.4 ± 5.1	154.7 ± 17.0	0.005
$HbA1_C$(%, mmol/mol)	4.8 ± 0.1 (29 ± 1)	4.4 ± 0.5 (25± l)	Ns
HOMA-IR	1.2 ± 0.2	1.5 ± 0.3	Ns
TG (mg/dl)	125.4 ± 14.0	131.6 ± 17.9	Ns
HDL-c (mg/dl)	94.7 ± 5.2	70.82 ± 8.2	0.024
Blood eosinophil count (%)	2.0 ± 0.5	1.3 ± 0.2	Ns
Blood neutrophil count (%)	69.2 ± 1.8	71.6 ± 2.1	Ns
Blood lymphocyte count (%)	21.5 ± 1.6	20.0 ± 1.3	Ns
Third trimester			
Weight (kg)	74.2 ± 4.9	72.1 ± 4.4	Ns
BMI (kg/m^2)	28.2 ± 1.4	27.0 ± 1.6	Ns
Fasting glucose (mg/dl)	54.9 ± 10.9	55.4 ± 11.0	Ns
$HbA1_C$ (%)	3.4 ± 0.8 (14 ± 1)	3.2 ± 0.9 (11 ± 1)	Ns
HOMA-IR	1.4 ± 0.4	0.8 ± 0.2	Ns
TG (mg/dl)	142.1 ± 32.2	128.8 ± 34.7	Ns
HDL-c (mg/dl)	62.5 ± 12.9	47.9 ± 11.2	Ns
Blood eosinophil count (%)	1.8 ± 0.6	1.3 ± 0.3	Ns
Blood neutrophil count (%)	69.9 ± 1.2	72.0 ± 3.1	Ns
Blood lymphocyte count (%)	20.6 ± 1.0	19.7 ± 2.4	Ns
Newborn and placenta			
Gender (% female)	42.3%	45.5%	Ns
Gestational age (weeks)	39 ± 0.3	39 ± 0.4	Ns
Birth weight (g)	3,165.4 ± 87.3	3,209.1 ± 105.7	Ns
Birth weight SDS	−0.1 ± 0.2	0.2 ± 0.2	Ns
Birth height (cm)	48.8 ± 0.5	50.0 ± 1.1	Ns
Birth height SDS	−0.4 ± 0.2	0.2 ± 0.5	Ns
Placental weight (g)	555.5 ± 45.1	544.0 ± 25.8	Ns
Placental IL10	1.5 ± 0.3	1.0 ± 0.2	Ns
Placental TIMP3	0.4 ± 0.1	0.7 ± 0.2	Ns
Placental ITGAX	0.4 ± 0.1	0.5 ± 0.2	Ns
Placental MRC1MR	3.7 ± 0.9	1.9 ± 0.4	Ns

Data are shown as mean ± SEM. Placental gene values are shown as relative expression (2-ACCT).
GDM, gestational diabetes; NS, not significant.

was similar to that in control placentas (45,076 ± 3,288 vs. 42,501 ± 4,742).

Our results showed that the diversity of placental microbiota was mainly related to 4 phyla: Proteobacteria, Bacteroidetes, Firmicutes, and Actinobacteria (3%) (**Figure 1a** and **Supplementary Figure S1** online). In GDM, relative abundance of these 4 phyla was different. The proportion of Firmicutes was increased by 7% and the proportion of Proteobacteria and Bacteroides was reduced (by 5 and 2%, respectively) in GDM compared to control women. Within the Proteobacteria, the classes including Betaproteobacteria, Alphaproteobacteria, and Gammaproteobacteria were reduced in GDM by 2.5, 1.1, and 2.1%, respectively. Within the Bacteroidetes, the class of Flavobacteria was reduced in GDM by 4%. Within the Firmicutes, the class of Bacilli was increased in GDM by 4% (**Figure 1** and **Supplementary Figure S1** online). Pseudomonadales order ($P = 0.03$) and Acinetobacter genus ($P = 0.03$) showed significantly lower relative abundance in women with GDM compared to controls (**Figure 1** and **Supplementary Figure S1** online).

A Specific Pattern Distinguishes Placental Microbiota in GDM

Next, we performed a microbial biomarker analysis to identify bacterial groups specifically related to either GDM or control patients. As shown in **Figure 2A**, the cladogram identified and confirmed the significant enrichment of Acinetobacter in control group. By contrast, GDM group was characterized by a significant increase in: Coriobacteriales (Order) and Coriobacteriaceae (Family), both from phylum Actinobacteria; Parabacteroides (Genus) from Bacteroidetes phylum; Lachnospiraceae (Family) from phylum Firmicutes; and Bradyrhizobiaceae (Family) and Escherichia both from phylum Proteobacteria. **Figure 2B** shows the full list of bacterial groups significantly increased in either GDM or control group, based on the Linear Discriminant Analysis (LDA) score, used to build the cladogram.

Maternal Metabolism and Placental Expression of Anti-inflammatory Cytokines

In women with GDM, lower relative abundance of Acinetobacter was associated with increased O'Sullivan glucose, lower blood eosinophil count in the second and third trimester of pregnancy and lower placental expression of *IL10* and *TIMP3* (all $P < 0.05$ to $P = 0.001$; **Table 2**; **Figure 3**; and **Supplementary Figure S2a** online).

In addition, lower relative abundance of Pseudomonadales associated with lower blood neutrophil and lymphocyte counts in the second and third trimester of pregnancy and with lower placental expression of *IL10*, *ITGAX*, and *MRC1MR* (all $P < 0.05$ to $P = 0.001$, **Table 2**; **Figure 3**; and **Supplementary Figure S2b** online). Interestingly, even in placenta from control women, increased relative abundance of bacteria belonging to Pseudomonadales order was negatively associated to placental weight ($P = 0.001$, **Table 2** and **Supplementary Figure S2b** online).

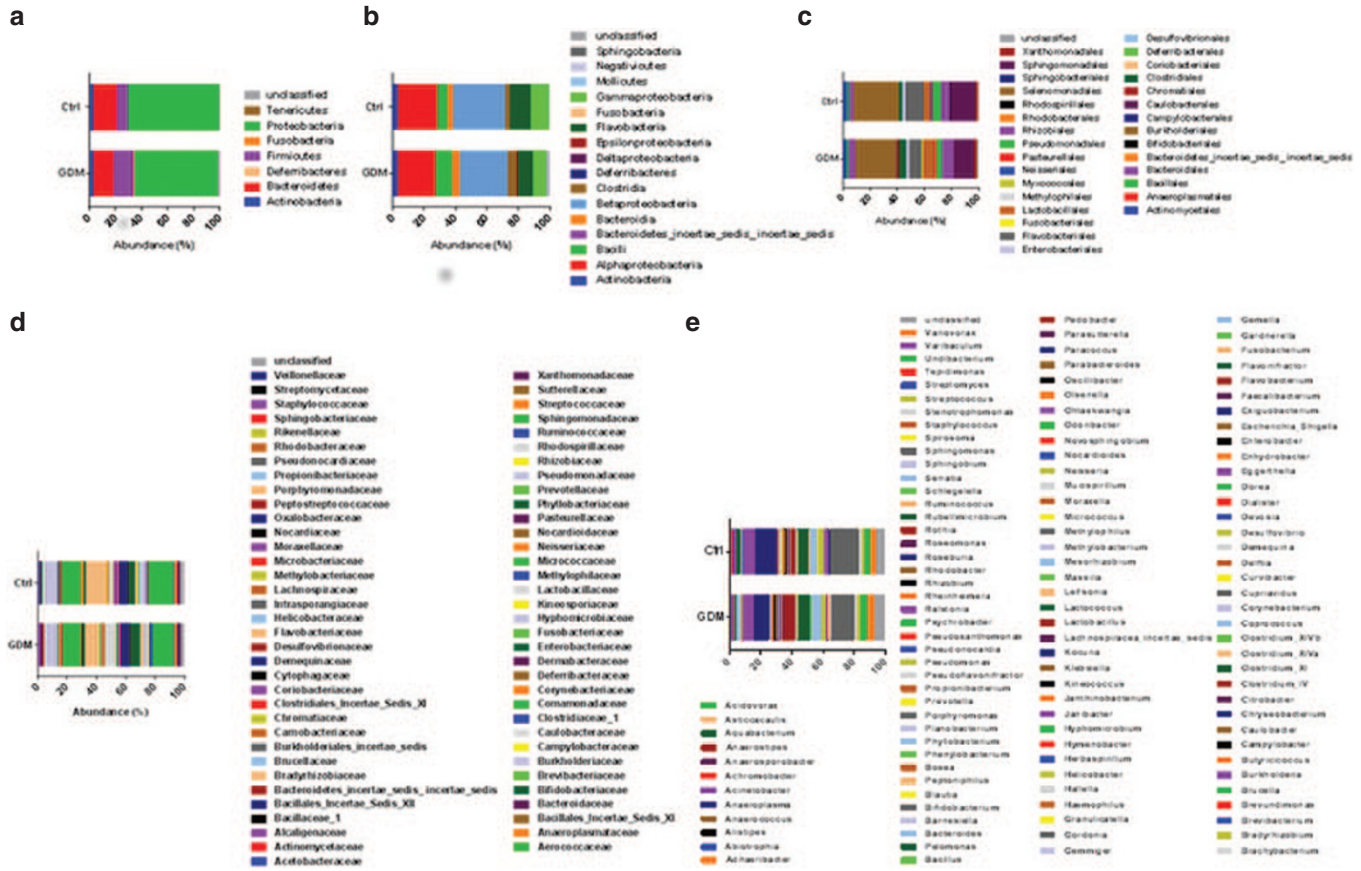

Figure 1. Bacterial taxonomic characterization of placental microbiota in gestational diabetes vs. control group. Relative abundance (%) of taxa at the level of (**a**) "Phylum", (**b**) "Class", (**c**) "Order", (**d**) "Family", and (**e**) "Genus".

The Placental Microbiome Is Functionally Different in GDM

Finally, we analyzed the function of the placental microbiota by studying the placental microbiome. Based on the overall analysis of placental microbiome in GDM and control group, patients appeared not to be neatly separated (**Figure 4a**). However, we identified a significant increase in the calcium signaling pathway in GDM placental microbiome vs. control group (**Figure 4b**).

DISCUSSION

Our results showed that the placental microbiota and microbiome from women with GDM differ from control women with lower levels of Pseudomonadales order and Acinetobacter genus. Lower abundance of placental Acinetobacter was associated to a more adverse metabolic and inflammatory phenotype.

Similarly to Aagaard K (4), our results showed that the placental microbiota is composed of nonpathogenic commensal microbiota from Proteobacteria, Bacteroidetes, Firmicutes, and Actinobacteria, being the Proteobacteria the most abundant. However, we showed for the first time, that the placental microbiota from women with GDM had a different taxonomic profile than control women, with significantly lower relative abundance of Pseudomonadales order and Acinetobacter genus. Moreover, in women with GDM, lower relative abundance of Acinetobacter was associated with lower blood eosinophil count and lower placental expression of several anti-inflammatory genes including *IL10*. The metagenomic sequencing methodology differed from that of Aagaard *et al.* (4), however, the mirrored taxonomic findings in terms of taxa suggests the true presence of a placental microbiome that indeed varies by GDM and other comorbidities. In these sense, Anthony *et al.* (15) showed that the preterm placental microbiome varies according to excess gestational weight gain and were accompanied by variations in some bacterial-encoded metabolic pathways. Even though high rates of gestational weight gain associated with woman's risk of GDM, differences in Acinetobacter genus were not found, probably because they studied placentas with preterm delivery, whose placental microbiome profile significantly varied from term pregnancies (4).

Eosinophils are innate immune leukocytes implicated in the initiation and maintenance of type 2 immune responses, including asthma and allergy. Eosinophils are distinguished from most lymphocytes in their capacity for rapid cytokine secretion as they can produce, store, and secrete over 30 cytokines, including IL10 (16).

IL-10 is one of the key anti-inflammatory cytokines in immunologic tolerance and is produced by many different immune cells. Of the different leucocytes, monocytes are the most efficient producers of IL-10 (17). Thus the expression of IL-10,

which correlated with the relative abundance of Acinetobacter species on the placenta in GDM subjects, is likely derived from monocytes within the placenta.

GDM has been associated with asthma and allergy in early childhood (11,18). The biological mechanism explaining the interrelation between GDM and allergy remains unclear. Kumar *et al.* (11) showed that GDM was still associated with atopic dermatitis and allergen sensitization despite the inclusion of LGA. This implies that the effects were likely due to exposure to GDM *in utero* but unlikely to occur via the same pathway which leads to fetal macrosomia. The effects of GDM were also independent of maternal prepregnancy BMI. The association of GDM with early childhood atopy mirrors some findings in a large Dutch epidemiological study of adults which found that insulin resistance may have mediated the association of obesity on allergen sensitization (19).

A relationship between commensal microbiota, anti-inflammatory parameters and allergy has been previously reported (20,21). Changes in gut microbiota have been reported in patients with allergic diseases (22,23). Endotoxin derived from Gram-negative bacteria, such as the Gammaproteobacteria genus Acinetobacter, is known to have immunomodulatory and allergy-protective potential (24). A positive association between the abundance of Acinetobacter and IL-10 expression in peripheral blood mononuclear cells (PBMCs) was found in healthy individuals, but not in atopic individuals (25). This is consistent with IL-10's central role in maintaining immunologic tolerance to harmless substances (25). Hessle *et al.* (26) reported that seven Gram-negative bacteria (including four Gammaproteo bacteria) significantly stimulated IL-10 secretion in PBMCs obtained from healthy blood donors, and Zhang *et al.* (27) found significant IL-10 expression in Acinetobacter-pulsed dendritic cells. Finally, a series of experimental studies using the mouse model have demonstrated strong allergy-protective properties for Acinetobacter species (20,28–30).

Thus, current results imply that placental microbiota, may play a special role in the development and maintenance of the fetal homeostasis and healthy barrier function, similar to that of certain gut bacteria (31,32). Similarly to what happens with the skin, placental microbiota, including Acinetobacter species, should have a strong modulatory effect of the immune system, producing a local anti-inflammatory environment, which is translated into systemic protection from the GDM status.

Finally, a significant increase in the calcium signaling pathway in GDM placental microbiome was remarkable. Interestingly, altered calcium signaling proteins have been described in skeletal muscle from women with GDM (33) and pancreatic B-cells of diabetic mice (34). Different Ca^{2+} signaling components are disturbed in a wide range of organelles in diabetic animals and patients, suggesting a pivotal role for the dysregulation of Ca^{2+} signaling in the development of diabetes (35). Moreover, calcium homeostasis and calcium-dependent signaling play important roles in many processes

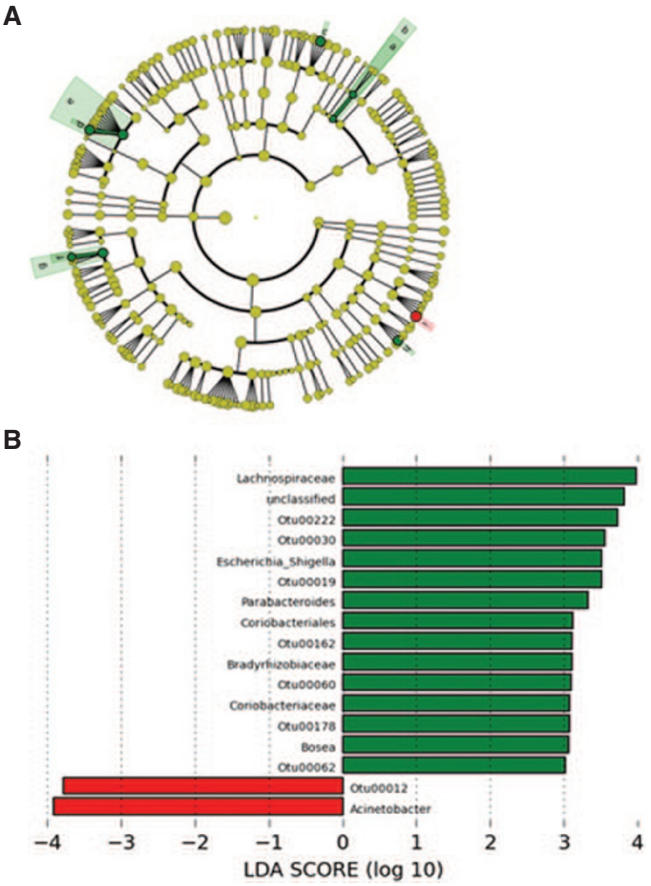

Figure 2. Bacterial biomarker identification in placental microbiota in gestational diabetes (GDM) vs. control groups. (A) Cladogram analysis of placental microbiota. (a) Coriobacteriaceae, (b) Coriobacteriales, (c) Parabacteroides, (d) unclassified, (e) Lachnospiraceae, (f) Bosea, (g) Bradyrhizobiaceae, (h) Escherichia Shigella, (i) Acinetobacter. (B) Linear discriminant analysis score, used to build the cladogram in GDM (green) vs. control groups (red).

such as insulin-mediated glucose uptake, AMPK signaling, and mitochondrial biogenesis (36). In GDM, placental microbiota may be linked to disrupted insulin signaling and reduced mitochondrial activity. Such disturbances in calcium homeostasis proteins could contribute to the inflammation, insulin resistance, and altered substrate metabolism observed in GDM patients, consequently shunting maternal nutrients to the growing fetus.

We acknowledge the limitations of our study. The cross-sectional design does not allow inferring a causal relationship between Acinetobacter abundance and anti-inflammatory response in GDM. As the microbiota of the placenta is similar to that of the skin (4), microbiota of the skin should be investigated to confirm a decreased abundance of Acinetobacter genus. If this is the case, skin vaccination or oral probiotics before pregnancy could be an alternative therapy for this prevalent metabolic disorder in women at risk.

In conclusion, our results indicate that GDM could constitute a state of placental microbiota-driven altered immunologic tolerance, making placental microbiota a new target for therapy in GDM.

Table 2. Spearman correlations of placental *Acinetobacter* and *Pseudomonadales* relative abundance with maternal and placental parameters in women with and without GDM

	All $n = 22$		Control $n = 11$		GDM $n = 11$	
GENUS: Acinetobacter						
Second trimester	r	*P*	r	*P*	r	*P*
O'Sullivan glucose (mg/dl)	−0.581	0.006	0.073	Ns	−0.790	0.007
Fasting glucose (mg/dl)	−0.002	Ns	0.237	Ns	0.534	Ns
$HbA1_C$ (%)	−0.105	Ns	0.323	Ns	−0.146	Ns
Blood eosinophil count (%)	0.550	0.008	0.082	Ns	0.831	0.001
Blood neutrophil count (%)	−0.223	Ns	−0.118	Ns	−0.255	Ns
Blood lymphocyte count (%)	0.121	Ns	0.132	Ns	−0.027	Ns
Third trimester						
Fasting glucose (mg/dl)	0.430	Ns	0.667	Ns	0.167	Ns
$HbA1_C$ (%)	−0.094	Ns	0.771	0.025	−0.709	Ns
Blood eosinophil count (%)	0.366	Ns	−0.396	Ns	0.750	0.020
Blood neutrophil count (%)	−0.176	Ns	0.195	Ns	−0.367	Ns
Blood lymphocyte count (%)	0.199	Ns	0.073	Ns	0.267	Ns
Placenta						
Placental weight (g)	−0.022	Ns	−0.233	Ns	0.079	Ns
Placental IL10	0.314	Ns	−0.164	Ns	0.707	0.015
Placental TIMP3	0.251	Ns	−0.109	Ns	0.679	0.022
Placental ITGAX	0.122	Ns	0.169	Ns	0.009	Ns
Placental MRC1MR	0.167	Ns	0.236	Ns	−0.105	Ns
ORDER: Pseudomonadales						
Second trimester						
O'Sullivan glucose (mg/dl)	−0.282	Ns	0.236	Ns	−0.018	Ns
Fasting glucose (mg/dl)	0.217	Ns	0.712	0.014	−0.046	Ns
$HbA1_C$ (%)	0.188	Ns	0.180	Ns	0.488	Ns
Blood eosinophil count (%)	−0.006	Ns	−0.206	Ns	0.000	Ns
Blood neutrophil count (%)	0.229	Ns	−0.091	Ns	0.709	0.015
Blood lymphocyte count (%)	−0.264	Ns	0.041	Ns	−0.764	0.006
Third trimester						
Fasting glucose (mg/dl)	0.275	Ns	0.190	Ns	0.310	Ns
$HbA1_C$ (%)	0.595	0.019	0.482	Ns	0.600	Ns
Blood eosinophil count (%)	0.099	Ns	−0.293	Ns	0.067	Ns
Blood neutrophil count (%)	0.211	Ns	−0.243	Ns	0.667	0.050
Blood lymphocyte count (%)	−0.223	Ns	0.195	Ns	−0.717	0.030
Placenta						
Placental weight (g)	−0.508	0.026	−0.900	0.001	−0.067	Ns
Placental IL10	0.255	Ns	−0.100	Ns	0.591	0.050
Placental TIMP3	0.080	Ns	0.409	Ns	−0.191	Ns
Placental ITGAX	0.204	Ns	0.369	Ns	0.636	0.035
Placental MRC1MR	0.516	0.014	0.327	Ns	0.802	0.003

GDM, gestational diabetes.

METHODS

Subjects and Ethics

The study population consists of 22 Caucasian women (11 with gestational diabetes (GDM) and 11 control non-GDM women with normal pregnancies) who were consecutively recruited among those seen within a setting of prenatal primary care in Girona (Spain). The protocol was approved by the Institutional Review Board of Dr. Josep Trueta Hospital. Informed written consent was obtained from the women.

Clinical Assessments

A close prenatal follow-up, consisting of predefined clinical exams, ultrasonograms, and laboratory tests (urine and blood), was performed in all subjects. Information on maternal pregnancy characteristics was abstracted from standardized medical records. Pregestational BMI was calculated as weight divided by height squared, kg/m^2. Control non-GDM women were matched in terms of pregestational BMI to avoid any bias according to gestational obesity.

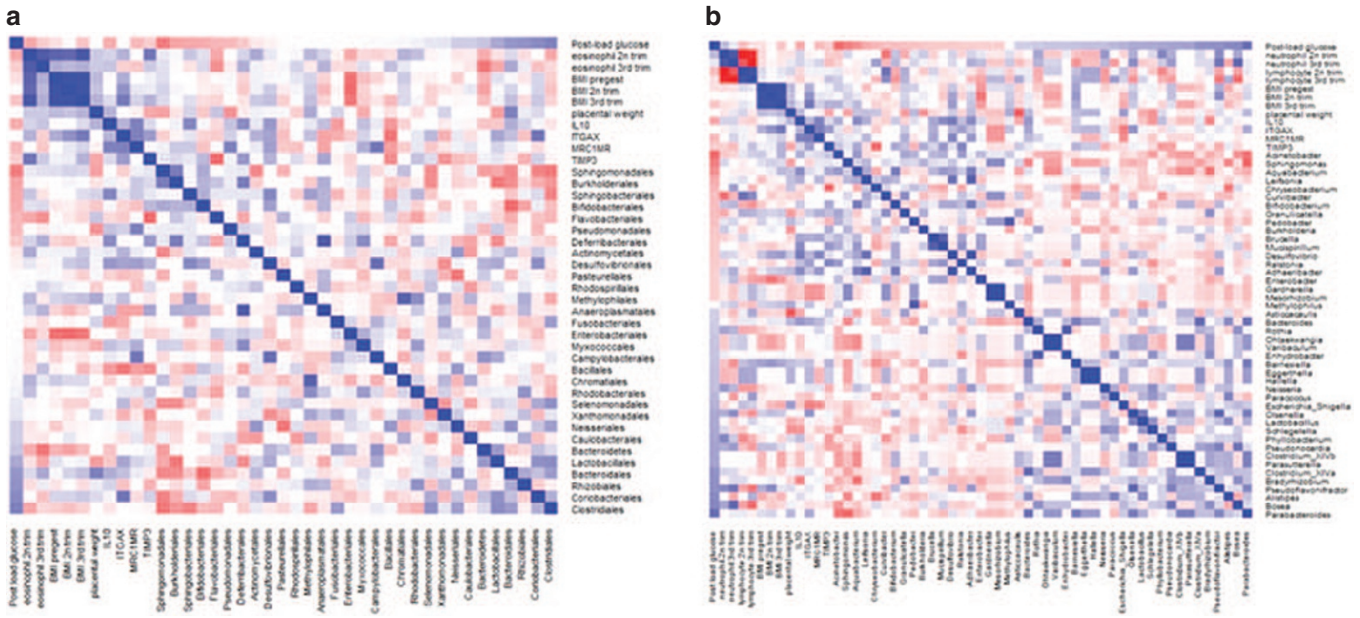

Figure 3. Identification of correlation link between placental microbiota and both metabolic and inflammatory parameters in gestational diabetes vs. control groups. Heat-maps of placental microbiota at the "Order" (**a**) and "Genus" (**b**) level and selected metabolic and anti-inflammatory parameters.

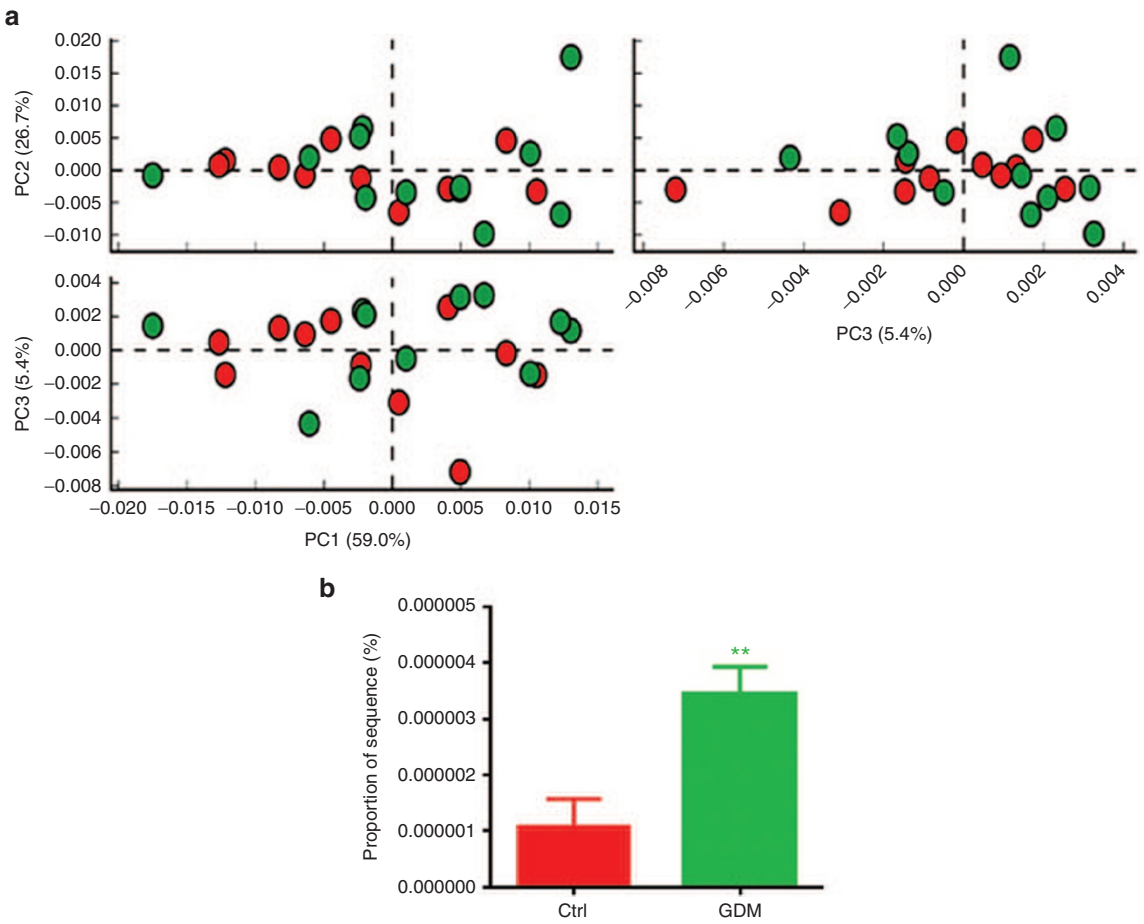

Figure 4. Bacterial functional characterization of placental microbiome in gestational diabetes (GDM) vs. control groups. (**a**) Principal component analysis of the overall placental microbiome study; (**b**) proportion of sequences (%) in GDM (green) vs. control group (red) for the calcium signaling pathway in placental microbiome.

A 50-g, 1-h oral glucose-challenge test was also performed between 24 and 28 wk of gestation. Women with serum glucose levels ≥ 140 mg/dl were subjected to a 100-g, 3-h glucose tolerance test to diagnose GDM.

All infants were born at term (37–42 wk). Infants were weighed and measured after delivery using a calibrated scale for weight and a measuring board for length.

The placentas were collected after childbirth in delivery room or operating room to ensure sterility. Briefly, following standard obstetrical practice, the placenta was delivered and immediately passed off to trained personnel. Placentas were weighed and three 1 cm^3 cuboidal sections were collected from separate areas of the placenta. To avoid the placenta being contaminated by the vagina, samples were collected from the inner surface of the placenta. The personnel were wearing facial masks and sterile gloves and using a sterile scalpel and instruments. The samples were stored within 1 h at −80 °C until DNA and RNA extraction.

Analytical Methods

Blood tests were performed under fasting conditions in all women at second and third trimester of pregnancy. Fasting glucose was analyzed by the hexokinase method. Serum immunoreactive insulin was measured by immune-chemi-luminescence (Immulite 2000; Diagnostic Products, Los Angeles, CA). The lower detection limit was 0.4 mIU/l, and intra- and inter-assay coefficients of variation (CV) were less than 10%. Fasting insulin sensitivity was estimated from fasting insulin and glucose levels using the following formula: HOMA-IR = (fasting insulin in mU/l) × (fasting glucose in mmol/l)/22.5. HbA1c was measured by high-performance liquid chromatography (Bio-Rad, Muenchen, Germany) and a Jokoh HS-10 auto-analyzer. Total serum triacylglycerol (TG) were measured by monitoring the reaction of glycerol-phosphate-oxidase and peroxidase. High-density lipoprotein (HDL) cholesterol was quantified by the homogeneous method of selective detergent with accelerator.

RNA Extraction and Real-Time PCR

Total RNA was isolated from placenta using RNeasy Fibrous Mini Kit (Qiagen, Hilden, Germany). RNA was reverse transcribed using High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA).

Real time PCR was performed using the following Taqman Gene Expression Assays: *IL10*, *TIMP3*, *ITGAX*, *MRC1-MR*, and the housekeeping controls *TBP* and *SDHA* (Applied Biosystems). Reactions were run on a LightCycler 480 Real-Time PCR System (Roche Diagnostics, Indianapolis, IN), using the default cycling conditions. Relative expression was calculated according to the 2-ÄÄCT method.

DNA Extraction and Bacterial Microbiota

Genomic DNA was extracted from placental tissue using DNeasy Mini Kits (Qiagen), following the standard protocol. Total DNA was eluted from the columns in 50 µl of sterile water and aliquoted for storage at −80 °C. Total DNA concentration was calculated by determining the absorbance at 260 nm (Spectramax Plus 384 spectrophotometer, Molecular Devices, Sunnyvale, CA).

Bacterial populations contained in DNA placental samples were determined using next generation high throughpout sequencing of variable regions of the 16S rRNA bacterial gene (Vaiomer SAS, Labège, France). PCR amplification was performed using 16S rRNA universal primers targeting the V3-V4 region of the bacterial 16S rRNA gene (Vaiomer universal 16S primers). The length of the assembled paired sequence reads 476 base pairs. Amplicons were sequenced on a Illumnia MiSeq instrument using the 300 paired-end sequencing kit V3. For each sample, a sequencing library was generated by addition of sequencing adapters. The targeted metagenomic sequences from placental microbiota was analyzed using the bioinformatics pipeline established by Vaiomer. Data was quality-filtered, aligned against a 16S rRNA gene sequence reference database and clustered into OTU (Operational Taxonomic Unit) with a 97% identity threshold. The cladogram was drawn using the Huttenhower Galaxy web application, (The Huttenhower Lab, Boston, MA) website via the LEfSe algorithm (37).

Functional Study of Placental Microbiota via Placental Microbiome Analysis

Functional analysis of placental microbiota was performed via Picrust (38). Principal component analysis in **Figure 4a** was drawn via Statistical Analysis of Metagenomic Profiles (STAMP) software (39).

Statistical Analyses

Statistical analyses were performed using SPSS 12.0 (SPSS, Chicago, IL). Results are expressed as mean ± SEM. Nonparametric variables were mathematically transformed to improve symmetry. Unpaired *t*-test or one-way ANOVA was used to study differences in continuous variables among groups. The relation between variables was analyzed by Spearman correlation. Significance level was set at $P < 0.05$.

Role of the Funding Source

The funding sources had no role in the design or conduct of the study; the collection, management, analysis, or interpretation of the data; the preparation, review, or approval of the manuscript; or the decision to submit the manuscript for publication.

SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at http://www.nature.com/pr

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AUTHOR CONTRIBUTIONS

J.B. contributed to the design and acquisition of data and drafted the manuscript. G.C.B. contributed to data acquisition and helped writing the first version of the manuscript. V.B.B. helped with microbiome figures generation. R.B. reviewed the manuscript. A.L.B. contributed to conception and interpretation of data and reviewed the manuscript. M.S. performed cladogram and placental microbiome analysis, generated figures, wrote, and reviewed the manuscript. J-M.F-R. contributed to conception and interpretation of data and wrote and reviewed the manuscript.

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Dislosure: The authors report no conflict of interest.

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