Distinct composition of gut microbiota during pregnancy in overweight and normal-weight women

Maria Carmen Collado, Erika Isolauri, Kirsi Laitinen, and Seppo Salminen

ABSTRACT
Background: Results of experimental studies suggest that deviations in gut microbiota composition predispose to excessive energy storage and obesity. The mother influences the original inoculum and the development of infant microbiota, which in turn is associated with later weight gain.

Objective: We characterized the gut microbiota in women according to their body mass index (BMI) and the effect of weight gain over pregnancy on the composition of microbiota before delivery.

Design: Overweight women (n = 18) were selected according to their prepregnancy BMI from a prospective follow-up study. Normal-weight women (n = 36) were selected as controls in consecutive order of recruitment. Excessive weight gain during pregnancy was defined as >16.0 kg for normal-weight and >11.5 kg for overweight states according to Institute of Medicine recommendations. The composition of gut microbiota was analyzed by fluorescent in situ hybridization coupled with flow cytometry (FCM-FISH) and by quantitative real-time polymerase chain reaction (qPCR).

Results: Bacteroides and Staphylococcus were significantly higher in the overweight state than in normal-weight women as assessed by FCM-FISH and qPCR. Mother’s weight and BMI before pregnancy correlated with higher concentrations of Bacteroides, Clostridium, and Staphylococcus. Microbial counts increased from the first to third trimester of pregnancy. High Bacteroides concentrations were associated with excessive weight gain over pregnancy (P = 0.014).

Conclusions: Gut microbiota composition and weight are linked, and mother’s weight gain is affected by microbiota. Microbiota modification before and during pregnancy may offer new directions for preventive and therapeutic applications in reducing the risk of overweight and obesity.

INTRODUCTION
The gut microbiota influences human health through its effect on the gut defense barrier, immune development, and nutrient utilization (1). Microbiota deviations were associated with an enhanced risk of atopic and diarrheal diseases (2, 3) and recently also obesity (4–6). One mechanism here could lie in the ability of specific microbes to induce excessive energy harvest and storage (5). The importance of this process may culminate in pregnancy; overweight pregnant women supply excessive energy to the fetus, resulting in higher birth weight and risk of complications to both mother and newborn (7–10). Further, a vicious circle may ensue because overweight infants frequently become overweight adolescents and obese adults with a heightened risk of Western lifestyle diseases. Finally, excessive weight gain in pregnancy results in deteriorated glucose tolerance and a risk of gestational diabetes (11, 12). Here, also the role of the gut microbiota was recently uncovered in an animal model with reduced numbers of bifidobacteria and enhanced endogenous lipopolysaccharide production, leading to endotoxemia associated with obesity and insulin resistance (13).

We now hypothesized that the gut microbiota composition differs in obese pregnant women compared with normal-weight women and is further associated with weight gain during pregnancy. For this purpose we studied by fluorescent in situ hybridization (FISH) and quantitative real-time polymerase chain reaction (qPCR) the gut microbiota composition in the first and third trimesters of pregnancy.

SUBJECTS AND METHODS

Design
Subjects for the study were selected according to their prepregnancy body mass index (BMI; in kg/m²) from a prospective follow-up study of 256 women (14). Altogether 18 women fulfilled the criterion of the present study, a BMI > 30 (Table 1). For each overweight woman, 2 normal-weight women with a BMI < 25 were selected as controls in consecutive order of recruitment. Height was measured at the first visit and weight at each trimester. The prepregnancy weight was taken from well-women clinic documents in which the self-reported prepregnancy weight is recorded during the first visit.

Stool samples were collected for analysis of gut microbiota composition at the first (10–15 wk of gestation) and third (30–35 wk of gestation) trimesters of pregnancy. Birth data on the infants were obtained from hospital records. Differences in microbiota...
Microbiota during pregnancy depends on weight

To analyze the effect of weight gain on the microbiota over pregnancy, the women were classified into 2 groups, normal weight gain and excessive weight gain over pregnancy, according to the Institute of Medicine recommendations for total weight gain in pregnant women (15). The total normal weight gain ranges according to BMI over pregnancy were 11.5–16.0 kg for normal-weight women (BMI: 19.8–26) and 7.0–11.5 kg for overweight women (BMI > 26). Total weight gains above these values, >16 kg for normal-weight women and >11.5 kg for overweight women, were considered excessive weight.

Analytic methods

Fecal samples were weighed, suspended in phosphate-buffered saline (PBS), and homogenized. An aliquot of the first 10-fold dilution of each sample was mixed thoroughly with glass beads and centrifuged (2000 × g, 2 min, 4 °C) to remove debris. Different aliquots were taken for DNA extraction and to fix cells for in situ hybridization (FISH) analysis.

One volume of the supernatant fluid was transferred into 3 volumes of fresh 4% paraformaldehyde and fixed at 4 °C overnight. After fixation, the bacteria were centrifuged at 22 000 × g for 3 min and washed with PBS. The centrifuging and washing were repeated 3 times. The bacteria were stored in 50% ethanol-PBS at −20 °C until analysis. One volume was used for DNA extraction with the use of the QIAamp DNA stool Mini kit (Qiagen, Hilden, Germany) following the manufacturer’s instructions.

Microbiota analysis by FISH combined with flow cytometry

The group- or genus-specific probes used in this study targeted the eubacterial 16S rRNA, including the Bacteroides-Prevotella group (Bac303, 5′-CCAATGGGGGACCTT), the Bifidobacterium genus (Bif164, 5′-CATCCGGCATACCACC), the Clostridium histolyticum group (CHis150, 5′-CATCCGGCATACCACC), and the Lactobacillus-Enterococcus group (Lab158, 5′-GTTAGGACGCATTTTCCTA). Specific cell enumeration was performed by combining each of the group-specific Cy3 probes with the EUB 338-FICT probe. Samples were analyzed as previously described (6). Briefly, fixed cell suspensions were incubated in the presence of each fluorescent probe (50 ng/μL) in hybridization buffer (10 mmol/L Tris-HCl, 0.9 mol/L NaCl, and 10% sodium dodecyl sulfate). The hybridized cells were collected after washing by centrifugation (22 000 × g for 5 min) and were resuspended in PBS. Flow cytometric analyses were performed with the use of a BD LSR II flow cytometer (Becton Dickinson and Co, Franklin Lakes, NJ) equipped with a 488-nm laser at 15 mW. Data were stored as list-mode files and analyzed off-line with the use of the BD FACSDIVA software, version 4.1.1 (Becton Dickinson and Co). Absolute bacterial cell counts were determined by addition of Flow-Count fluorospheres (Beckman Coulter, Fullerton, CA), to avoid loss of the signal intensity of hybridized cells, they were kept in the dark on ice at 4 °C until the flow cytometry (FCM) assay. Results were expressed as the numbers of cells hybridizing with the specific group-Cy3 probe and total bacteria EUB 338-FICT probe.

Microbiota analysis by qPCR

DNA extraction from pure cultures of the different microorganisms and fecal samples was conducted with the use of the QIAamp DNA stool Mini kit (Qiagen) following the manufacturer’s instructions. qPCRs were conducted as previously described (18). For characterization of fecal microbiota PCR primers were used to target the Bifidobacterium genus group (19), the Bacteroides fragilis group (18), Staphylococcus aureus (20), and Akkermansia muciniphila (21). These oligonucleotides were purchased from the Thermo Electron Corporation (Thermo Biosciences, Ulm, Germany).

PCR amplification and detection were performed with an ABI PRISM 7300-PCR sequence detection system (Applied Biosystems, Foster City, CA). Each reaction mixture of 25 μL was composed of SYBR Green PCR Master Mix (Applied Biosystems), 1 μL of each of the specific primers at a concentration of 0.25 μmol/L, and 1 μL of template DNA. The fluorescent products were detected in the last step of each cycle. A melting curve analysis was made after amplification to distinguish the targeted from the nontargeted PCR product.
TABLE 2

Bacterial counts in fecal samples analyzed by fluorescent in situ hybridization coupled with flow cytometry at the first and third trimesters of pregnancy in normal-weight and overweight women

|                        | Normal-weight women (n = 36) | Overweight women (n = 18) | Ratio of overweight to normal-weight women | P for significance between groups
<table>
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<tr>
<td></td>
<td>log fecal cells/g</td>
<td>log fecal cells/g</td>
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<tr>
<td><strong>Bifidobacterium genus</strong></td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>First trimester</td>
<td>9.85 (9.72, 9.98)1</td>
<td>9.78 (9.51, 10.05)1</td>
<td>0.91 (0.61, 1.35)1</td>
<td>0.627</td>
</tr>
<tr>
<td>Third trimester</td>
<td>10.47 (10.38, 10.56)2</td>
<td>10.45 (10.30, 10.60)2</td>
<td></td>
<td></td>
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<tr>
<td><strong>Clostridium histolyticum group</strong></td>
<td></td>
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<tr>
<td>First trimester</td>
<td>9.79 (9.63, 9.96)3</td>
<td>9.56 (9.25, 9.87)3</td>
<td>0.89 (0.53, 1.49)3</td>
<td>0.649</td>
</tr>
<tr>
<td>Third trimester</td>
<td>10.08 (9.95, 10.20)4</td>
<td>10.19 (9.99, 10.38)4</td>
<td></td>
<td></td>
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<tr>
<td><strong>Bacteroides-Prevotella group</strong></td>
<td></td>
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<tr>
<td>First trimester</td>
<td>9.74 (9.62, 9.87)5</td>
<td>9.88 (9.67, 10.09)5</td>
<td>1.45 (1.03, 2.06)5</td>
<td>0.035</td>
</tr>
<tr>
<td>Third trimester</td>
<td>10.36 (10.27, 10.45)6</td>
<td>10.55 (10.44, 10.65)6</td>
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| First trimester        | 11.33 (11.24, 11.42)1        | 11.22 (11.03, 11.41)1      | 0.99 (0.71, 1.38)1                     | 0.943                         |
| Third trimester        | 11.10 (11.00, 11.20)1        | 11.21 (11.09, 11.32)1      |                                      |                               |

1 ANOVA was used for repeated measures with time and weight group as factors and interaction between weight group and time.
2 P for time effect < 0.001; P for interaction = 0.760.
3 r; 95% CI in parentheses (all such values).
4 P for time effect < 0.001; P for interaction = 0.054.
5 P for time effect < 0.001; P for interaction = 0.546.
6 P for time effect = 0.034; P for interaction = 0.051

Statistical analyses

Because of nonnormal distribution, clinical data (Table 1) are expressed as medians with interquartile ranges. The Mann-Whitney U test was applied in comparisons between normal-weight and overweight women. A P value < 0.05 was considered statistically significant.

Bacterial counts as log10 units are given as means with 95% CIs. Microbial data at first and third trimesters were analyzed with the use of analysis of variance for repeated measures, in which the factors were weight group (overweight compared with normal-weight states) and time. The interaction term was introduced to test whether there was a difference between weight groups in changes in bacterial counts from the first trimester to the third trimester. Microbial data at third trimester were compared between weight gain groups (excessive weight gain compared with normal weight gain) with the use of analysis of covariance, in which the baseline bacterial count was included as a continuous covariate. The results of all group comparisons are given as ratios of overweight to normal-weight states or of excessive weight gain to normal weight gain with 95% CIs. The association between bacterial counts and weight gain was analyzed with the use of the Pearson correlation coefficient. A P value < 0.05 was considered statistically significant. The SPSS package version 11.0.2 (SPSS Inc, Chicago, IL) was used to analyze the data.

RESULTS

Subjects

Clinical characteristics of the women at recruitment were not significantly different except for BMI and weight before pregnancy (Table 1). All subjects were white and in good health; most were expecting their first child (58% and 61% in normal-weight and overweight women, respectively; P = 0.98). No differences in weight gain were detected between women with BMI < 25 and BMI > 30 (Table 1). The infants were born at term; infants born to overweight women tended to be heavier than infants of normal-weight women (Table 1).

Microbiota composition in normal-weight and overweight women

Significant differences were observed in microbiota composition according to weight and normal-weight or overweight states before pregnancy. The distinction was mainly due to significantly higher numbers of Bacteroides group and S. aureus assessed by FCM-FISH and qPCR in the overweight state compared with normal-weight women (Table 2 and Table 3). Concentrations of Staphylococcus and Clostridium group during the first trimester of pregnancy also showed correlations with BMI (r = 0.26, P = 0.059; r = −0.251, P = 0.067, respectively).

Microbiota composition changes during pregnancy in normal-weight and overweight women

Increases in bacteria as assessed by FCM-FISH and qPCR were manifest in both overweight and normal-weight women over pregnancy (Tables 2 and 3). These increases throughout pregnancy, ie, time effect, for each weight group, normal-weight and overweight, were significant. In addition, the increase or decrease from first trimester to third trimester between groups (overweight and normal-weight) were close to significant in total counts (P = 0.051) and in the Clostridium group (P = 0.054) count assessed by FCM-FISH. The total cell counts decreased more in normal-weight subjects, whereas concentrations of the Clostridium group increased more in overweight subjects. These interactions were not found in bacterial groups analyzed by qPCR. To analyze the effect of early microbiota on late microbiota, paired correlations were compared. Clostridium, Bacteroides, and Staphylococcus groups and Akkermansia muciniphila showed significant effects (r = 0.29, P = 0.036; r = 0.32, P = 0.021; r = 0.37, P = 0.008; r = 0.33, P = 0.021, respectively) on late microbiota (third trimester microbiota).
pregnancy. No correlations were found between third and first trimesters of pregnancy showed a correlation (r = 0.38, P = 0.010; P for interaction = 0.965).

In addition, the difference in Bifidobacterium genus numbers between the third and first trimesters of pregnancy showed a correlation (r = −0.38, P = 0.03, Pearson correlation) with normal weight gain over pregnancy, suggesting that Bifidobacterium counts were higher in women with lower weight gain over pregnancy. No correlations were found between third and first trimester bacterial groups and weight gain over pregnancy.

**DISCUSSION**

Our results show that there are indeed differences in the gut microbiota between overweight and normal-weight women, as reported in animal studies (22, 23). The gut microbiota also appear to be different in subjects with excessive weight gain compared with normal weight gain during the relatively short period of time over pregnancy. Indeed, pregnancy may be an ideal model to assess obesity and weight gain separately.

Recent studies have reported microbiota aberrations in the proportion of Firmicutes bacteria in obese compared with lean mice (4, 5, 23). The preliminary report in human subjects on a weight-reducing diet also suggests differences in gut microbiota between lean and obese persons (22). However, the outcomes cannot be directly compared with our results. In the earlier studies, the Firmicutes phyla composition, including *Clostridium* groups (C. coccoides group and C. leptum group) and *Eubacterium rectale* was compared with the composition of Bacteroidetes phyla (Bacteroides group) by sequencing 16S ribosomal RNA genes from stool samples (4, 5).

In our study we selected FCM-FISH and qPCR as methods that were successfully used to analyze microbial composition in complex communities (6, 24). The *Clostridium histolyticum* group was analyzed as representatives of the Firmicutes phyla, and this group was present in higher counts in obese women in agreement with previous reports (4, 5). Clearly, the Firmicutes phyla composition should be characterized in detail with respect to *Clostridium* groups (C. coccoides group and C. leptum group) and *Eubacterium rectale* to be compared with those obtained for the Bacteroidetes phyla (Bacteroides group). In general, Bacteroides and S. aureus numbers are significantly higher in overweight women than in normal-weight women, suggesting a link between obesity, overweight, and microbiota.

Thus, these results and also genetic studies are based on a specific animal model of genetically obese and gnotobiotic mice, which does not allow direct comparison with our results. In addition, pregnant mothers in our study had a diet higher in fiber content as a result of dietary counseling in the well-women clinics (14), and this may have provided a greater source of nutrition for the lower gut microbiota, thus promoting a specific microbiota composition depending on the source of the fiber (13, 25–27).

As a theoretical basis for the microbiota-obesity link, 3 possible mechanisms were suggested. First, the gut microbiota may enable hydrolysis of indigestible polysaccharides to easily absorbable monosaccharides activating lipoprotein lipase. Consequently, glucose will be rapidly absorbed and fatty acids excessively stored with de novo synthesis of liver-derived triglycerides; both phenomena boosting weight gain (28). Second,
specific microbiota compositions were showed to modulate fasting-induced adipocyte factor. The third possible explanation for the association is AMP-activated protein kinase activation, leading to suppression of anabolic and induction of catabolic pathways (28).

We suggest that, in addition to energy harvest and storage, the proinflammatory and antiinflammatory properties attributed to specific strains of the gut microbiota could also be associated with the mechanism of action. Obesity is not only caused by overnutrition but also associated with low-grade systemic inflammation (29–33), and the gut microbiota was shown to regulate inflammation. Our results further emphasize the possible role of the Bacteroides group in energy storage and weight gain because of its high presence in samples from obese women and also a high presence in women showing excessive weight gain over pregnancy. It was also suggested that S. aureus overgrowth is associated with inflammatory bowel disease, because S. aureus superantigens were shown to elicit inflammation in a mouse model (34, 35). Our results suggest that high concentrations of S. aureus in overweight mothers may be associated with inflammatory processes and also with fat storage. It is of importance to assess further the Staphylococcus species composition and activity and their toxin production potential.

We also showed a tendency in the presence of high numbers of clostridia (Firmicutes phylum; Table 2) in obese women, as previously reported (4, 5). In addition, the Bifidobacterium group (Actino bacteria phylum) was present in higher numbers in normal-weight than in overweight women in agreement with our previous findings (6) and also in women with lower weight gain over pregnancy. A recent study reports high-fat feeding to be associated with lower concentrations of bifidobacteria (13). High numbers of bifidobacteria may correlate positively with the normalization of inflammatory status and improved glucose tolerance and glucose-induced insulin secretion (13). These findings, together with our results, would suggest that higher numbers of bifidobacteria are associated with the normalization of inflammatory processes, thus supporting the hypothesis envisaging a modulating role of bifidobacteria within the intestinal microbiota. Our results may be interpreted to indicate that low numbers of bifidobacteria in overweight mothers may be associated with inflammatory processes. It is important to analyze further bifidobacterial species composition, because these bacteria have an important effect on microbiota transfer from mothers to infants (36).

Aberrant compositional development of the gut microbiota was associated with specific diseases (2–6). Our data suggest that it may be important to further monitor and modify the microbiota of pregnant women to influence the first inoculum and the transfer of microbiota to the infant. This may have a significant effect on the later health of the infant.
In conclusion, our findings show that there are aberrancies in gut microbiota associated with overweight and weight gain and that the gut microbiota can thus be seen as one factor contributing to obesity over and above nutrition. Particularly, the *Bacteroides* group and *S. aureus* were altered, and a high concentration of these microorganisms may predispose to enhanced energy storage and obesity and the decreased control of systemic low-grade inflammation typical of obesity. Because overweight pregnant women have heavier newborns with an increased risk of subsequent overweight, our results offer new directions for preventive and therapeutic applications of obesity prevention during pregnancy.

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The author’s responsibilities were as follows—SS and EI: study concept and design; MCC and KL: acquired data and analyzed statistics; all authors participated in preparation of the manuscript. None of the authors had a personal or financial conflict of interest.

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