Figure 1. Flow chart illustrating the recruitment of GDM and healthy subjects.

Fig 3. Abundances of taxa in GDM and healthy participants. A. Comparison of the relative abundances at the phylum level among the four GDM and non-GDM groups. The Mann–Whitney test was used to evaluate the two groups. * $P < 0.05$. B. PLS-DA score plots based on the relative abundances of microbiota between the GDM and healthy groups. C. Correlation between the relative abundance of the phylum *Acidobacteria* and the 1-h OGTT measurement. Spearman analysis, $R = 0.302$, $P = 0.06$. D. Comparison of the relative abundances of *Acidothermus*, *Granulicella*, *Bryobacter*, and *Candidatus Solibacter* in the phylum *Acidobacteria* in the GDM and healthy groups. Mann-Whitney test, GDM vs. control, ** $P < 0.01$, * $P < 0.01$. E. The relative abundances of *Ruminococcus gauvreaui* and *Eubacterium ventriosum* were highly correlated with the OGTT values at 0 h and 2 h. Mann-Whitney test, GDM vs. healthy, ** $P < 0.01$, * $P < 0.01$.

A

B

C

Fig 4. The microbial pattern after diet management. A. Principal coordinate analysis (PCoA) at the OTU level between the GDM-W2 and healthy-W2 groups. B. Heatmap analysis of the differentially expressed taxa at the family level. C. Ratio of *Firmicutes/Bacteroidetes* among the GDM and non-GDM groups with or without diet intervention.

Fig 5. Heatmap analysis of the correlation between the gut microbiota composition and clinical scores.

The gut microbial signature of gestational diabetes mellitus and the association with diet intervention

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Abstract

Gestational diabetes mellitus (GDM) is a high-risk pregnancy complication that is associated with metabolic disorder phenotypes, such as abnormal blood glucose and obesity. The link between microbiota and diet management contributes to metabolic homeostasis in GDM. Therefore, it is crucial to understand the structure of the gut microbiota in GDM and to explore the effect of dietary management on the microbiota structure. In this study, we analyzed the composition of the gut microbiota between 27 GDM and 30 healthy subjects at two time points using Illumina HiSeq 2500 platform. The taxonomy analyses suggested that the overall bacteria clustered by diabetes status, rather than diet intervention. Of particular interest, the phylum *Acidobacteria* in GDM was significantly increased, and positively correlated with blood glucose levels. Moreover, Partial least-squares discriminant analysis (PLS-DA) revealed that certain genera in the phyla *Firmicutes*, *Bacteroidetes*, *Proteobacteria*, and *Lentisphaerae* characterized the GDM gut microbiota. Correlation analysis indicated that blood glucose levels and BMI index were correlated with the relative abundance of SCFAS-producing genera. Through the comparison between the GDM and healthy samples with or without diet intervention, we discovered that the role of short-term diet management in GDM processes is associated with the change in the *Firmicutes/Bacteroidetes* ratio and some specific taxa, rather than an alternative gut microbial pattern. Our study have important implications for understanding the beneficial effects of diet intervention on the specific gut microbiota and thus possibly their metabolism in pregnant women with GDM.

Importance

Understanding the composition and dynamics of the gut microbiota in GDM women under diet intervention is important because there may be opportunities for preventive strategies. We examined the relationships between GDM gut microbiota at two times before and after the diet intervention during second trimester of pregnancy and clinical characteristics in cohort of GDM women. We found that short-term diet management in GDM processes is associated with changes in the *Firmicutes/Bacteroidetes* ratio and some specific taxa rather than an alternative gut microbial pattern. Our study highlights the importance of considering diet intervention as the rescue of microbial dysfunction of GDM disease and can serve as a strategy for early prevention in future study.

Introduction

The intestinal microbiota is a robust ecosystem inhabited by nearly 100 trillion bacteria (1). In recent years, extensive attention has been given to the gut microbiota during pregnancy. Over the course of a healthy pregnancy, the body undergoes substantial hormonal, immunological, and metabolic changes (2, 3). In predisposed women, these physiological changes may lead to the development of gestational diabetes mellitus (GDM). GDM is defined as abnormal glucose regulation with onset or first recognition during pregnancy and is one of the most common complications during pregnancy, with an incidence of 2–6% of all pregnancies (4, 5). The clinical incidence of GDM in China is currently presenting a dramatic increasing trend (6). In the context of nonpregnant obesity, recent work suggests a role for gut microbiota in driving metabolic diseases, including diabetes, weight gain, and reduced insulin sensitivity (4, 5, 7, 8). Researchers understand that the intestinal flora has an important function in the development of GDM with the notions relating the intestinal flora to metabolic disease (3, 9, 10). GDM is a transient state, and GDM patients are commonly treated by diet management to keep blood glucose within the normal range and reduce the risk of GDM complications (11). However, very few data from observational studies are available about whether diet interventions performed on GDM patients affect the community structure of the gut microbiota. Diet, particularly long-term eating habits, is known to be one of the drivers of microbiota variation (12, 13). Recent clinical studies have shown the importance of routine dietary recommendations for GDM patients, showing a better microbial pattern at the end of the study (14). However, the comparison between healthy pregnant women without dietary recommendations and individuals with GDM under routine dietary management remains uncertain.

In this study, we characterized the different patterns of the gut microbiota between GDM

and healthy pregnancies in the second trimester of pregnancy. Then, comparison of microbial structure between healthy pregnant women without dietary recommendations and individuals with GDM under routine dietary management were assessed, to evaluate the role of short-term diet management on GDM gut microbiota. The aim of the present study was to provide an update on the existing knowledge of the specific structure of the gut microbiota in Chinese GDM women and to elucidate the influence of diet management on the GDM gut microbiota.

Material and methods

Patient recruitment

This study was approved by the Conjoint Health Research Ethics Board of Peking University People's Hospital, and informed consent forms were signed by all of the subjects prior to participation in this study. All experiments were performed in accordance with the approved guidelines and regulations.

Diagnosis of GDM is based on the results of the fasting 75 g OGTT at 24–28 weeks gestation. One or more elevated level(s) is sufficient for a diagnosis of GDM. The threshold values of OGTT (5.1 at 0 hour, 10.0 at 1 hour and 8.5 at 2 hours during OGTT) are based on the diagnostic criteria recommended by the International Association of the Diabetes and Pregnancy

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Thirty healthy subjects were selected based on matched age and pregnancy period, no complicating diseases and no antibiotic use during the 3-month period prior to sample collection. All subjects who met the following criteria were excluded: complicating diseases (such as known diabetes mellitus, hypertension, cardiovascular, pulmonary, autoimmune, joint, liver or kidney diseases; thyroid dysfunction; or any other disease), prebiotics/probiotics use, and antibiotic use

during pregnancy.

The prepregnancy weight was self-reported; weight and height were measured at the time of enrollment. BMI was calculated as weight divided by the square of height. Arterial blood pressure (BP) was measured from the left arm with the participant in a sitting position after at least 10 min of rest with a mercury sphygmomanometer with the appropriate cuff size. The measurements for BP were taken by trained medical personnel at enrollment.

Diet management for the GDM women

The initial treatment of GDM involves diet modification, glucose monitoring, and moderate exercise (15, 16). All the GDM participants in the study received 2 weeks of dietary management and nutritional recommendations at enrollment, which showed the guidelines for the subjects. Participants were considered as adhering to the given dietary recommendations in the presence of all the following criteria: carbohydrates 35–45% of total energy, rapidly absorbed sugars <10% of total energy, proteins 18–20% of total energy, fats 35% of total energy, fiber intake of at least 20–25 g/day, and no alcohol consumption. The nutritionist was in continuous contact with the enrolled GDM subjects, through weekly telephone contact, to remain updated regarding the nutritional condition of the subjects as the study progressed. Patients were instructed to

self-monitor their blood glucose by finger-prick capillary blood glucose tests at least 4 times per day.

To reduce the effect of diet on the composition of the gut microbiota, general 2-week dietary restrictions were imposed on the healthy participants, including no peppery food and no yogurt intake and appropriate fat intake (the intake of calories from fat was no more than 35% of the total calories).

123

124 ***Stool sample collection and DNA extraction***

125 After providing written informed consent, all subjects were contacted for detailed instructions on
126 how to collect and transport the stool sample. Stool samples of 57 subjects were collected at the
127 time of enrollment for the first time. The second stool samples for GDM subjects were collected
128 at the end of the study after the 2-week dietary intervention. For healthy pregnant women, the
129 second stool samples were collected at the end of 2 weeks without dietary management
130 intervention. Stool samples were self-collected by all the participants using the specimen
131 collection kit as instructed. The fecal samples were collected at home, transferred to the hospital
132 and immediately stored at -80°C until DNA extraction. DNA was extracted from stool samples
133 using the QIAamp DNA Stool Mini kit protocol (Qiagen, Germany). During the stool collection,
134 one GDM sample at enrollment from one patient (G28) were limited, and the second sample was
135 collected the other day, which changed the serial number to G28-2 at enrollment and G28-3 at
136 the end of study.

137

138 ***Illumina library generation***

139 The V4 region of the 16S rRNA gene was amplified using 515F

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G-CTGTGCCAGCAGCCGCGGTA-3' and 806R (5'-GGACTACG-5'GGGTWTCTAAT-3').

141 The V4-specific primer regions were associated with the adaptor and the sequences, which were
142 complementary to the Illumina forward and reverse sequencing primers. Each PCR product of
143 the appropriate size was purified and quantified using a Qubit fluorometer and then added to a
144 master pool of DNA for 250-bp nucleotide paired-end read assembly using the HiSeq 2500
145 genome analyzer (Illumina HiSeq 2500, USA).

Bioinformatics

The RDP Classifier was used to assign all of the 16S rRNA gene sequences to a taxonomic hierarchy. The assembled reads were analyzed. The relative abundances of the various phyla, families and genera in each sample were computed and compared between the GDM patients and the healthy subjects. The trimmed reads were clustered into operational taxonomic units (OTUs) at 97 % identity. The comparison of the bacterial diversity of these samples was performed using the Chao1 richness index, ACE index and observed species. The reads displaying greater than 0.1% abundance in both groups were further analyzed via partial least-squares discriminant analysis (PLS-DA) to visualize the differences between two groups using the standard Simca-p1 software (version 12.0; <http://www.umetrics.com/>). The Principal Co-ordinates Analysis (PcoA) analyzed were performed based on Unweighted Unifrac distance metric.

Statistical analysis

The microbial comparisons between the GDM and healthy groups were performed using the Mann-Whitney test. Associations between clinical indices and gut microbiota were evaluated by the Spearman rank correlation coefficient method. The difference in alpha-diversity between groups during GDM and non-GDM was assessed using Student's t test. Statistical analysis of the clinical data was performed using SPSS (Statistical Package for Social Sciences) 22.0 software (SPSS Inc., Chicago, IL, USA). $P < 0.05$ was considered significantly different.

Availability of data

The raw sequences are available from the Genome Sequence Archive (GSA), the

accession is: CRA004782.

Results

Characteristics of the patients

A flow chart illustrating the recruitment strategy of GDM and healthy subjects is shown in Fig 1. Clinical data from 27 GDM patients and 30 healthy controls are shown in Table 1. All 27 GDM patients and 30 healthy pregnant women were from the Peking University People's Hospital. The mean age of the subjects was 32.7 ± 3.3 years for the GDM group and 31.4 ± 2.9 years for the healthy group. There were no differences in age or nulliparity rate between the two groups. The prepregnancy BMI value of the GDM group was 24.2 ± 4.4 , which was significantly higher than the value of 21.4 ± 2.8 of the healthy group ($P=0.0059$), and the same trend was observed for the BMI at enrollment (27.1 ± 4.3 vs. 25.0 ± 2.9 , GDM vs. healthy, $P=0.038$). The GDM group had a markedly higher systolic BP (SBP) value than that of the control group (mean 125.3 ± 11.8 vs. 115.8 ± 14.2 , GDM vs. healthy, $P=0.008$), and an increased diastolic BP (DBP) value was found in GDM women compared to that of healthy women (mean 78.8 ± 9.5 vs. 73.6 ± 8.8 , GDM vs. healthy, $P=0.038$). In the OGTT test, the GDM group had higher values at 0 h, 1 h and 2 h than

the values of the healthy group (all $P < 0.001$).

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Differences in fecal microbial communities between the healthy and GDM groups

To demonstrate the GDM microbiota signature, we explored the microbial composition of pregnant women with GDM. First, we performed PCoA using OTU relative abundance, and we observed discrete clustering of intestinal microbiota in the GDM and healthy groups at

enrollment (Fig 2A). Additionally, shared or unique OTUs in the GDM and control groups were assessed to detect whether GDM has an effect on the gut microbiota. We found that the GDM group had more unique OTUs than the control group, with approximately 60.6% (1458/2404) unique OTUs compared with 14.3% (158/1104) in healthy women, signifying that GDM patients largely harbor unique inhabitant niches (Fig 2B).

The observed species of GDM samples were higher than non-GDM samples (Fig 2C). The ACE and Chao1 indices for alpha-diversity were both significantly increased in the GDM group (Fig 2D&2E), suggesting increased commensal diversity in GDM patients. Similar trends of alpha-diversity were also observed between the Healthy-W2 and GDM-W2 (diet management) groups, suggesting that the microbial pattern of women with GDM is distinct from that of healthy subjects at enrollment and at the end of the study.

Microbiota structure of GDM patients based on taxonomic comparison

To further demonstrate these variations corresponding to the structure of the gut microbiota in GDM, we compared the bacterial abundance between groups at the phylum level (Fig 3A). No significant differences were observed between the healthy subjects and the GDM subjects at enrollment for most of the phyla, with the exception of *Acidobacteria*, which was found to be

0.91% in the GDM group compared with 0.37% in the healthy group ($P=0.001$).

The microbial compositions at the phylum level for each sample at enrollment and at the end of the study are shown in Fig S1. Interestingly, *Acidobacteria* was associated with increased levels of blood glucose in the 0-h OGTT (Fig 3B).

Next, we compared taxa at the genus level. The PLS-DA method was performed (Fig 3C). Forty-nine key genera with variable importance in projection (VIP) scores >1 were identified

215 that differentiated the GDM and healthy groups (Table 2). We then clustered the samples
 216 according to the relative abundance of the 49 genera. Twenty-seven genera were enriched in the
 217 GDM microbiota samples, with 4 genera (*Acidothermus*, *Granulicella*, *Bryobacter*, and
 218 *Candidatus_Solibacter*) belonging to the phylum *Acidobacteria*. Among them, *Acidothermus*
 219 and *Granulicella* were significantly enriched in the GDM group (Fig 3D). Seven genera
 220 belonging to *Proteobacteria*, including *Citrobacter*, *Burkholderia*, *Acidibacter*, and *Bilophila*,
 221 were significantly highly expressed in the GDM intestinal microbiota ($P<0.05$). The genera
 222 *Eubacterium*, *Holdemania*, and *Tyzzereella*, in the phylum *Firmicutes*, were rarely detected in
 223 women with healthy pregnancy microbiota compared with women with GDM. The remaining 22
 224 genera of the 49 key phylotypes were overexpressed in healthy pregnant microbiota, some of
 225 which even disappeared in GDM patients. One genus, *Ruminococcaceae_UCG-010*, belonging
 226 to *Firmicutes*, was highly enriched in the healthy group. Additionally, *Akkermansia* ($P=0.067$)
 227 and *Coprococcus_2* ($P=0.027$) were increased in healthy subjects. *Akkermansia* was recently
 228 proven to be a crucial player in maintaining the integrity of the gastrointestinal tract. In
 229 nonpregnant adults with metabolic syndrome and type 2 diabetes, *Akkermansia* is reported to be
 230 depleted as well (17-19). Our findings suggest that the gut microbiota of women with GDM has
 231 similarities with the microbiota reported in patients with type 2 diabetes and associated
 232 intermediary metabolic traits. At the OTU level, a reduced abundance of *Akkermansia* has
 233 previously been reported in the third trimester of healthy pregnant women (20).

234 To further examine the relationship between these VIP genera in GDM, we evaluated
 235 their abundance based on the results of the OGTT. The threshold values (5.1 at 0 h, 10.0 at 1 h
 236 and 8.5 at 2 h during the OGTT) are based on the diagnostic criteria recommended by the
 237 International Association of the Diabetes and Pregnancy Study Groups in 2011. As shown in Fig

3E, two short chain fatty acids producing and anti-inflammatory bacteria were highly correlated with the OGTT value at 0 h and 2 h. The relative abundance of *Ruminococcus gauvreauii* was observed depleted in GDM women with abnormal OGTT value at 0 h ($P=0.046$), and the relative abundance of *Eubacterium ventriosum* was decreased in GDM women with the abnormal OGTT value at 2 h ($P=0.009$, Mann-Whitney test).

Microbiota signature after dietary intervention

We found that GDM patients developed a microbial pattern with higher alpha-diversity after diet management (Fig 2D & E). Compared with the GDM samples, the GDM-W2 samples showed some distinct taxa with VIP scores >1 , according to the PLS-DA analysis (Fig S2).

At the family level, GDM-W2 samples showed decreased pathogenic taxa (*Acidaminococcaceae*, *Enterobacteriaceae*, and *Bacteroidaceae*) and increased *Bifidobacteriaceae* and butyric acid-producing bacteria (*Prevotellaceae* and *Lachnospiraceae*) compared with the GDM microbial samples at enrollment, suggesting a better pattern driven by the 2 weeks of diet management. One more interesting observation is that because the bacterial lineages were constant within pregnancy over time, communities from the same GDM person were generally more similar to one another than to those from other people from the healthy

group (Fig 4B).

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It is presumed that the influence of maternal gestational diet on the phylogenetic structure of the intestinal microbiota during pregnancy remains underexplored in well-controlled models. To investigate whether the microbiota can be driven by dietary management for GDM in pregnancy, the two dominant groups of beneficial bacteria, *Bacteroidetes* and *Firmicutes*, were analyzed. At the phylum level, a slightly increase in the *Firmicutes/Bacteroidetes* (F/B) ratio in

late pregnancy was exhibited in the GDM group compared with the non-GDM group (Fig 4C). Previous studies indicated that a higher *Firmicutes/Bacteroidetes* ratio was associated with obesity (21) and an aggravation of low-grade inflammation (22). Here, we showed that after 2 weeks of diet therapy, the relative abundance of *Bacteroidetes* in GDM samples increased, and the abundance of *Firmicutes* decreased slightly (Fig 2A). More importantly, the ratio of *Firmicutes/Bacteroidetes* did not increase in GDM-W2 fecal samples compared with GDM samples at enrollment ($P=0.8$) (Fig 4C). However, without diet management, an obviously increased proportion of *Firmicutes/Bacteroidetes* ($P=0.2$) developed in healthy pregnancies (healthy-W2 samples).

Four genera (*Acidothermus*, *Granulicella*, *Bryobacter*, and *Candidatus_Solibacter*) belonging to the phylum *Acidobacteria* were increased in the GDM group, compared with healthy group. Furthermore, we evaluated the levels of the 4 genera in GDM with dietary management (Fig S3). A total of 66.7% (18/27) of GDM subjects showed decreased levels of the genus *Acidothermus* after 2 weeks of diet management. In contrast, 59.3% (16/27) of GDM samples showed decreased levels of the genera *Granulicella*, *Bryobacter*, and *Candidatus_Solibacter* after 2 weeks of diet management.

Association between fecal microbiota and clinical parameters

We examined the correlations between the OGTT values (0 h, 1 h and 2 h), BMI indices (prepregnancy and at enrollment), blood pressure values (SBP and DBP) and the genera of the fecal microbiota (Fig 5).

The distribution of correlation coefficients by heatmap analysis showed that the *Coprococcus_2*, *Christensenellaceae_R.7*, and *Prevotella* groups (*Prevotella_2*, *Prevotella_7*

and *Prevotella_9*) were negatively correlated with the OGTT value, BP values and BMI index ($P<0.05$); among them, *Coprococcus_2* was significantly increased in the healthy group compared with the GDM group.

Parabacteroides showed positive correlations with BMI at enrollment ($P<0.05$). Additionally, *Alloprevotella*, *Megamonas* and *Clostridium_sensu_stricto-1* showed positive correlations with GDM-correlated clinical measures and OGTT values at 0 h ($P<0.05$). Previous studies observed that the genus *Megamonas* was increased in GDM patients in late pregnancy. Elevated genera of *Megamonas* have also been reported to be associated with higher blood glucose at an individual level (9, 23-25).

Discussion

Studies support a causal role for the gut microbiota in the development of type 2 diabetes, insulin resistance and obesity (26). In this study, we compared the composition of the human intestinal microbiota between GDM patients and healthy subjects using a culture-independent Illumina HiSeq 2500 platform. The aim of the present study was to identify gut microbiota dysbiosis in GDM subjects and the associated microbial changes in GDM-W2 samples after diet intervention for 2 weeks and compare them with the basal GDM microbial composition. We observed a

marked shift in the microbiota composition at the phylum and genus levels in GDM samples compared with healthy samples and identified the microbial pattern of GDM-W2 samples after a

2-week dietary intervention.

Gut dysbiosis in women with GDM was mainly characterized by changes in microbiota diversity. It was previously reported that an increase was found in the alpha-diversity in the third trimester of GDM women when compared to the level of the control group (24). Regarding

alpha-diversity, we used the ACE and Chao1 indices and found significant separation in the alpha-diversity between GDM and non-GDM individuals at their enrollment and at the end of the study, indicating dysbiosis of the gut microbiota in GDM women compared with healthy pregnant women. To further identify gut microbial dynamics, the different bacterial taxa were compared within the GDM and non-GDM groups. At the phylum level, the abundance of *Acidobacteria* was significantly greater in the gut microbiota of GDM samples and was associated with increased levels of blood glucose in the 0-h OGTT (Fig 3B). In particular, we observed significant elevation of *Acidothermus* and *Granulicella* belonging to the phylum *Acidobacteria* in the GDM group. The phylum *Acidobacteria* was reported in the gut microbiome of obese individuals (27) and was shown to contain a host of genes involved in diverse metabolic pathways, as evidenced by their pan-genomic profiles in the soil microbiota (28). Further exploration of these genetic attributes and more in-depth insights into GDM mechanics and dynamics would lead to a better understanding of the functions and biological significance of this elevated phylum in the GDM gut environment.

Several bacterial groups at the genus level were detected to be different in the GDM and healthy groups, such as *Megamonas* assigned to the phylum *Firmicutes*. The relationships between gastrointestinal *Megamonas* and metabolic disorders such as obesity and type 2 diabetes

have recently been discovered (29). Differential abundance testing showed that *Megamonas*,

Bacteroides, and *Eubacterium* were statistically associated with food addition (30). A recent study also suggested that the abundance of *Megamonas*, which is closely related to childhood obesity, increased in the gut microbiota of obese children (29). Of particular interest, we revealed the association between gut *Megamonas* and GDM. Our results showed that *Megamonas* was positively correlated with higher blood glucose in the OGTT test at 0 h in the GDM samples at

enrollment (Fig 5). Members of *Megamonas* are known to produce acetic and propionic acid, which is beneficial for the balance of glucose uptake (31). Systemic disorders of glucose metabolism might be modulated by the related gut microbiota. Further study to explore the composition of *Megamonas* and the production of metabolites involved in glucose homeostasis *in vitro* and *in vivo* is very important.

Short-chain fatty acids (SCFAs), especially acetate, propionate and butyrate, are the end products of the intestinal microbial fermentation of dietary fibers and resistant starch. It is well documented that plasma and colonic SCFAs are associated with metabolic syndromes, i.e., obesity and type 2 diabetes (32). SCFAs, namely, acetate, butyrate, and propionate, have been reported to affect metabolic activities at the molecular level. Acetate affects the metabolic pathway through the G protein-coupled receptor (GPCR) and free fatty acid receptor 2 (FFAR2/GPR43). The FFAR2 signaling pathway regulates insulin-stimulated lipid accumulation in adipocytes and inflammation (33, 34). *Coprococcus_2*, an acetate-producing bacteria (25, 35), was found to be negatively correlated with the OGTT value at 1 h, BP values and prepregnancy BMI index ($P<0.05$) by Spearman analysis and was significantly higher in the healthy group than in the GDM group. *Coprococcus* was also proven to be altered in the fecal microbiota of patients with polycystic ovary syndrome, which is a metabolic disorder (36). Guo et al. (37) found that

Coprococcus deletion is implicated in many of the outcomes, including glucose homeostasis.

The importance of an association between the deletion of the *Coprococcus* genus and high levels of blood glucose at 1-h in the OGTT measure is therefore supported by the acetate-producing effect. Furthermore, other SCFA-producing taxa, including *Prevotella_2*, *Prevotella_7*, and *Prevotella_9*, were found to be negatively associated with OGTT measures and the BMI index separately, indicating a beneficial effect on blood glucose in GDM subjects (38). We presumed

that acetate arising from *Coproccoccus_2* members and succinate from *Prevotalla* members are important for energy metabolism and have a mainly protective role in relation to healthy pregnancy. Thus, the observed absence of the *Coproccoccus_2* and *Prevotella* groups in the fecal microbiota of GDM could be a possible microbial driving force for GDM. A better understanding of the microbial ecology of colonic acetate- and succinate-producing bacteria, especially the *Coproccoccus_2* and *Prevotella* groups, may help to explain the influence of diet on the acetate and succinate supply and may contribute to the development of new approaches for optimizing microbial activity for diet management for GDM subjects. *Eubacterium ventriosum*, another SCFAs producer, had been found negative correlated with visceral fat area (VFA) (39). Moraes et al. reported that the abundance of *E. ventriosum* were associated to better cardiometabolic profile (40). Consistent with our study, the data demonstrated a significant decrease of gut *Eubacterium ventriosum* from GDM subjects with abnormal OGTT values at 2 h (Fig 3E). Combined with these findings, we presumed that the expression of the SCFAs producers are critical for energy homeostasis during pregnancy. Further studies investigating the targets and signaling pathways of SCFAs in the GDM microbial, and the modulation of SCFAs-producing bacteria by diet intervention would benefit for GDM management.

Therefore, to further identify the role of diet intervention during GDM pregnancy, we

analyzed the ratio of *Firmicutes/Bacteroidetes* and a higher ratio was proposed as an eventual biomarker of obesity and other metabolic syndromes compared with normal-weight individuals (41). Our data showed different increases in the *Firmicutes/Bacteroidetes* ratio between the GDM and non-GDM groups. Healthy W2 samples without diet management showed a nearly significant increase in the *Firmicutes/Bacteroidetes* ratio, indicating a change in energy homeostasis during pregnancy. Similar to our findings on the *Firmicutes/Bacteroidetes* ratio in

healthy pregnant women, Zheng et al. (42) reported that there were elevations in the *Firmicutes/Bacteroidetes* ratio in the second (T2) trimester compared with the first (T1) trimester. Ley et al. (22) reported that the *Firmicutes/Bacteroidetes* ratio decreases with weight loss on a low-calorie diet. In our observations, the *Firmicutes/Bacteroidetes* ratio did not change in GDM-W2 samples under diet management compared to the ratio in GDM samples, suggesting that the diet intervention could play a positive role during GDM pregnancy by affecting *Firmicutes/Bacteroidetes* ratio. In particular, the gut microbial pattern was not altered in the GDM group with or without 2 weeks of diet intervention (Fig 4A&B). In agreement with our observation, a controlled-feeding study showed that enterotype identity remained stable during the 10-day study, and alternative microbial states were associated with a long-term diet (43). Thus, we presume that the role of short-term diet management in GDM processes is associated with changes in the *Firmicutes/Bacteroidetes* ratio and some specific taxa rather than an alternative gut microbial pattern.

It is well suggested that the diet contributes to the gut microbiota composition in GDM (42). Microbiota-derived metabolites affect glucose homeostasis through intestinal gluconeogenesis (38). A few studies have examined the gut microbiota of GDM and healthy pregnant women before and after diet invention. Uniquely, in the present study, we could

compare gut microbiota in GDM fecal samples, allowing identification of taxa that exhibited

differential abundance at the two time points. We discovered that a short-term diet had a

beneficial effect on GDM by modulating the *Firmicutes/Bacteroidetes* ratio and some taxa. This first observation of the high expression of the phylum *Acidobacteria* in GDM offered an important clue for further study on the subgroup of *Acidobacteria* and the mechanism of GDM.

Several limitations in our study should be considered. One was that we did not have fecal

samples after long-term dietary management. Additionally, our suggestion of the occurrence of specific taxa with divergent metabolites calls for future metagenomic sequencing to reveal the metabolic pathways of the key taxa. In conclusion, our results highlight the relevance of characterizing gut microbial population differences and contribute to understanding the plausible link between diet and specific gut bacterial species that are able to influence metabolic homeostasis and GDM development. Modulating the gut microbiota via short-term diet intervention, especially SCFA-producing bacteria, could be a promising strategy in the search for alternatives for the treatment of metabolic disorders in GDM (44-46). Long-term observation may be more valuable to study the dynamic alteration of the GDM gut microbiota.

Declarations

Funding

This work was supported by the National Natural Science Foundation of China (grant no. 32070116) and the Maternal and Infant Nutrition & Care Research Fund of the Institute of Nutrition and Nursing of Biostime (grant no. 2015-Z-20).

Conflicts of Interest

On behalf of all authors, the corresponding author states that there are no conflicts of interest.

Acknowledgments

We thank all the subjects who made this study possible.

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Figure legends

Fig 1. Flow chart illustrating the recruitment of GDM and healthy subjects.

Fig 2. Comparison of the fecal microbiota composition between the GDM and healthy groups. A. Principal coordinate analysis (PCoA) at the OTU level between the GDM and healthy groups. **B.** Venn diagram illustrating the overlap of the OTUs identified in the fecal microbiota between the GDM and healthy groups. **C.** Observed species of 4 groups, including the GDM and healthy and the GDM-W2 and healthy-W2 groups. **D & E.** Alpha-diversity based on the ACE index and Chao 1 index at the OTU level. Mann-Whitney test, GDM vs. healthy, $**P<0.01$, $*P<0.01$.

Fig 3. Abundances of taxa in GDM and healthy participants. A. Comparison of the relative abundances at the phylum level among the four GDM and non-GDM groups. The Mann-Whitney test was used to evaluate the two groups. $*P<0.05$. **B.** PLS-DA score plots based on the relative abundances of microbiota between the GDM and healthy groups. **C.** Correlation between the relative abundance of the phylum *Acidobacteria* and the 1-h OGTT measurement. Spearman analysis, $R=0.302$, $P=0.06$. **D.** Comparison of the relative abundances of *Acidothermus*, *Granulicella*, *Bryobacter*, and *Candidatus_Solibacter* in the phylum *Acidobacteria* in the GDM and healthy groups. Mann-Whitney test, GDM vs. control, $**P<0.01$, $*P<0.01$. **E.** The relative abundances of *Ruminococcus gnavireus* and *Enterobacter ventriosus* were highly correlated with the OGTT values at 0 h and 2 h. Mann-Whitney test, GDM vs. healthy, $**P<0.01$, $*P<0.01$.

Fig 4. The microbial pattern after diet management. A. Principal coordinate analysis (PCoA) at the OTU level between the GDM-W2 and healthy-W2 groups. **B.** Heatmap analysis of the differentially expressed taxa at the family level. **C.** Ratio of *Firmicutes/Bacteroidetes* among the GAM and non-GDM groups with or without diet intervention.

Fig 5. Heatmap analysis of the correlation between the gut microbiota composition and clinical scores.

Fig S1. Comparison of the relative abundance at the phylum level between the 27 GDM and 30 healthy individuals at the time of enrolment and study end.

Fig S2. PLS-DA analysis indicated 49 distinct taxa with VIP score>1 between GDM samples and GDM-W2 samples. Mann-Whitney test, GDM vs. Healthy, ** $P<0.01$, * $P<0.01$.

Fig S3. The *Acidothermus*, *Granulicella*, *Bryobacter*, *Candidatus_Solibacter* belonging to the phylum *Acidobacteria* were evaluated in GDM and GDM-W2 samples. The 66.7% (18/27) GDM samples was showed decreased level of genus *Acidothermus* after two-week diet management. While 59.3% (16/27) GDM samples was showed decreased level of genus *Granulicella*, *Bryobacter*, *Candidatus_Solibacter* after two-week diet management.

Tables

TABLE 1 The clinical characteristics of all the GDM patients differ from those of the healthy participants

	GDM (Mean $\pm$ SD)	Healthy (Mean $\pm$ SD)	<i>P</i> value
Number	27	30	
Age	32.7 $\pm$ 3.3	31.4 $\pm$ 2.9	0.11
Prepregnancy weight (kg)	63.5 $\pm$ 12.2	57.3 $\pm$ 8.9	0.031
BMI (kg/m ²)	24.2 $\pm$ 4.4	21.4 $\pm$ 2.8	0.0059
Enrollment weight (kg)	71.1 $\pm$ 12.4	66.9 $\pm$ 9.5	0.15
BMI (kg/m ²)	27.1 $\pm$ 4.3	25.0 $\pm$ 2.9	0.038
Nulliparous (number)	22/27	24/30	
Systolic BP (mmHg)	125.3 $\pm$ 11.8	115.8 $\pm$ 14.2	0.008
Diastolic BP (mmHg)	78.8 $\pm$ 9.5	73.6 $\pm$ 8.8	0.038
OGTT (mg/dL)			
0 min	5.2 $\pm$ 1.4	4.3 $\pm$ 0.3	0.001
60 min	10.1 $\pm$ 1.6	7.3 $\pm$ 1.4	<0.0001
120 min	8.8 $\pm$ 1.3	6.4 $\pm$ 1.2	<0.0001

599 **TABLE 2** Forty-nine key genera with VIP >1 that were differentially expressed in the GDM and
600 healthy groups

Genus with VIP ≥ 1	GDM mean	Healthy mean	GDM/Healt hy	P value	Phylum
<i>Citrobacter</i>	0.000316	2.41E-05	up	0.048	<i>Proteobacteria</i>
<i>Bradyrhizobium</i>	0.000422	8.64E-05	up	0.065	<i>Proteobacteria</i>
<i>Eubacterium</i>	0.00012	3.4E-05	up	0.001	<i>Firmicutes</i>
<i>Granulicella</i>	0.000323	0.000102	up	0.001	<i>Acidobacteria</i>
<i>Holdemania</i>	0.000187	7.5E-05	up	0.014	<i>Firmicutes</i>
<i>Succinivibrio</i>	8.81E-05	3.54E-05	up	0.212	<i>Proteobacteria</i>
<i>Oscillibacter</i>	0.000211	9.06E-05	up	0.44	<i>Firmicutes</i>
<i>Tyzzerella</i>	0.000856	0.000368	up	0.007	<i>Firmicutes</i>
<i>Holdemanella</i>	0.009481	0.004176	up	0.162	<i>Firmicutes</i>
<i>Paraprevotella</i>	0.000994	0.000578	up	0.126	<i>Bacteroidetes</i>
<i>Victivallis</i>	0.00056	0.000344	up	0.042	<i>Lentisphaerae</i>
<i>Desulfovibrio</i>	0.000458	0.00029	up	0.479	<i>Proteobacteria</i>
<i>Lachnospiraceae</i>	0.002291	0.001517	up	0.137	<i>Firmicutes</i>
<i>Burkholderia</i>	0.000824	0.000551	up	0.027	<i>Proteobacteria</i>
<i>Acidothermus</i>	0.000499	0.000338	up	0.034	<i>Acidobacteria</i>
<i>Acidibacter</i>	0.000677	0.000508	up	0.405	<i>Proteobacteria</i>
<i>Mucilaginibacter</i>	0.00037	0.00028	up	0.02	<i>Bacteroidetes</i>
<i>Candidatus_Solibacter</i>	0.000474	0.000394	up	0.404	<i>Acidobacteria</i>
<i>Ruminiclostridium_9</i>	0.001163	0.00098	up	0.141	<i>Firmicutes</i>
<i>Ruminococcus_gauvreauii</i>	0.000581	0.000491	up	0.214	<i>Firmicutes</i>
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<i>undemified_Ruminococcus</i>	0.001548	0.001359	up	0.949	<i>Firmicutes</i>
<i>Roseburia</i>	0.028429	0.025656	up	0.482	<i>Firmicutes</i>
<i>Bilophila</i>	0.002439	0.002216	up	0.179	<i>Proteobacteria</i>
<i>Alistipes</i>	0.011983	0.010959	up	0.354	<i>Bacteroidetes</i>
<i>Bryobacter</i>	0.000475	0.00044	up	0.968	<i>Acidobacteria</i>
<i>Odoribacter</i>	0.001495	0.001395	up	0.302	<i>Bacteroidetes</i>
<i>Dorea</i>	0.007676	0.007233	up	0.678	<i>Firmicutes</i>

<i>Lachnospiraceae_NK4A136</i>	0.002712	0.002715	down	0.438	<i>Firmicutes</i>
<i>Eubacterium_ruminantium</i>	0.00633	0.006883	down	0.26	<i>Firmicutes</i>
<i>Bifidobacterium</i>	0.033865	0.038103	down	0.56	<i>Acidobacteria</i>
<i>Ruminococcaceae_UCG-013</i>	0.001251	0.00142	down	0.994	<i>Firmicutes</i>
<i>Tyzzerella_3</i>	0.002118	0.002482	down	0.073	<i>Firmicutes</i>
<i>Ruminococcaceae_UCG-005</i>	0.00281	0.003448	down	0.452	<i>Firmicutes</i>
<i>Ruminococcaceae_UCG-002</i>	0.005792	0.00715	down	0.056	<i>Firmicutes</i>
<i>Ruminococcaceae_NK4A214</i>	0.00144	0.001797	down	0.083	<i>Firmicutes</i>
<i>Eubacterium_ventriosum</i>	0.00239	0.003046	down	0.207	<i>Firmicutes</i>
<i>Enterococcus</i>	0.001193	0.001627	down	0.09	<i>Firmicutes</i>
<i>Megasphaera</i>	0.001971	0.00306	down	0.749	<i>Firmicutes</i>
<i>Lachnospiraceae_UCG-003</i>	0.000269	0.000419	down	0.11	<i>Firmicutes</i>
<i>Coprococcus_2</i>	0.005271	0.008583	down	0.027	<i>Firmicutes</i>
<i>Ruminiclostridium_5</i>	0.001904	0.003111	down	0.009	<i>Firmicutes</i>
<i>Ruminococcaceae_UCG-010</i>	0.000848	0.001403	down	0	<i>Firmicutes</i>
<i>Sarcina</i>	0.000126	0.000252	down	0.001	<i>Firmicutes</i>
<i>Butyrivibrio</i>	0.000455	0.000927	down	0.02	<i>Firmicutes</i>
<i>Intestinimonas</i>	3.93E-05	8.64E-05	down	0.07	<i>Firmicutes</i>
<i>Akkermansia</i>	0.000189	0.000435	down	0.067	<i>Verrucomicrobia</i>
<i>Weissella</i>	7.87E-05	0.000217	down	0.002	<i>Firmicutes</i>
<i>Prevotella_2</i>	0.001153	0.003598	down	0.108	<i>Bacteroidetes</i>

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