Gut Microbiota Composition and Functional Prediction in Recurrent Spontaneous Abortion

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Abstract

Objective

The changes of microbial community in pregnant women, let alone those of patients with recurrent spontaneous abortion (RSA), remain unclear. We analyzed the differences of gut microbiota (GM) between RSA patients and pregnant women to find the possible mechanism of RSA.

Methods

We enrolled 30 RSA patients (RSA group) and 30 pregnant women who terminated their pregnancy and did not have a history of spontaneous abortion (NR group) in our hospital from June 2020 to August 2020, and fecal samples were obtained to analyze the GM using 16S rDNA V3–V4 sequencing.

Results

At the phylum level, we found that there is no significant difference in composition of GM between RSA and NR. But at the genus level, compared with NR, *Roseburia* significantly decreased ($P<0.01$), and *Ruminococcus* significantly increased in RSA patients ($P<0.05$). Further analysis indicated that *Klebsiella* ($P<0.05$) was significantly increased, *Prevotella.9* ($P<0.05$) and *Roseburia* ($P<0.05$) were significantly decreased in RSA2 group (BMI>23.9 in RSA). Moreover, *Agathobacter* ($P<0.01$) was significantly increased in NR2 group (no delivery in NR). Functional prediction indicated that GM may interfere with RSA through membrane transport, carbohydrate metabolism, amino acid metabolism and other pathways.

Conclusion

Decreased *Roseburia* in GM of pregnant women maybe related to RSA. Our results provide the basis for in-depth studies of the composition of gut microbial communities in RSA.

Background

The microbial communities of humans are characteristic and complex mixtures of microorganisms that have co-evolved with their human hosts [1]. Microbes colonize the neonatal gut immediately following birth [2], maternal factors are important determinants of early infant colonization, many studies indicated that maternal microbiota have an important impact on infant gut microbiota, as prevention of allergic diseases, affect the infant's immunity, and so on [3, 4, 5]. The composition and development of infant gut microbiota can be influenced by many prenatal factors, such as maternal diet, obesity, smoking status, and use of antibiotic agents during pregnancy [6]. In addition, the mode of delivery, type of infant feeding, gestational age, infant hospitalization were considered to be important determinants of the gut microbiotic composition in infants [7].

In view of the important influence of microbiota on the mother and fetus, some scholars have studied the microbiota in natural cavities of pregnant women, such as the oral cavity, vagina, uterus and gut, to observe their influence on the pregnancy outcome. Recently, based on 16SrDNA sequencing, several studies have shown that the structure and stability of maternal microbiota changed during pregnancy, for example, *Lactobacillus* in the maternal vagina and intestinal tract have a potentially important impact on fetal development and may affect the outcome of pregnancy. But the results of these studies varied widely, which possibly due to the differences in race, diet, antibiotic use and education level [8-12]. The research on the gut microbiota (GM) of pregnant women focuses on the aspects of pregnant women with diabetes mellitus, pre-eclampsia and [13-14].

Recurrent spontaneous abortion (RSA) is a common gynecological disease in China, defined by two or more failed pregnancies, its etiology remains unclear, genetics, autoimmune abnormalities, endocrine, anatomy, or pre-thrombotic state are thought to be related to its pathogenesis [15, 16]. Previous proteomic study had shown that there is a significant changes occurred in the pathway of Fc gamma R-mediated phagocytosis in RSA, this result indicated that autoimmunity is involved in its pathogenesis [17]. A study suggested that RSA may be caused by an imbalance of vaginal flora (especially that of *Pseudomonas*) in RSA patients [18]. In this study, we focused on the relationship of maternal GM and RSA. So far, no such study has been reported.

Through this study, we want to explain the following questions: 1) Whether there is a difference in GM between RSA patients and normal abortion patients, whether there is statistical significance; 2) Is the maternal gut microbiota related to RSA, and if so, which bacteria play an important role? 3) How these bacteria involved in mechanisms of RSA and whether it is related to body mass index (BMI).

Methods

Study design

From June 2020 to August 2020, in the case group (RSA), we recruited 30 RSA patients who met the diagnostic criteria of Royal College of Obstetricians and Gynecologists (RCOG) consensus [16]. Meanwhile, in the control group (NR), we recruited 30 women who terminated their pregnancy and did not have a history of spontaneous abortion. Exclusion criteria for all subjects included: 1) Complicate with chromosomal abnormalities, anatomical abnormalities, surgery and trauma, etc; 2) taking antibiotics, probiotics, or other treatments, within 4 weeks; 3) Complicate with endocrine diseases, infectious diseases, immune diseases; 4) any psychiatric comorbidity; 5) excessive physical exercise. Characteristics of participants were summarized in Table 1.

Sample collection and DNA Extraction

A single fecal sample was collected by each participant at home, and immediately stored at -20°C, then transferred to -80°C for longer-term storage. Fecal bacteria genomic DNA was extracted with

Cetyltrimethyl Ammonium Bromide (CTAB)/ Sodium dodecylsulfate (SDS).

16S rDNA gene sequencing

The following steps and methods refer to the implementation method of *Lundberg DS* [19].

Amplicon Generation:

Primer: 16S V3-V4: 341F-806R. 16S rDNA genes were amplified used the specific primer with the barcode. All PCR reactions were carried out in 30µL reactions with 15µL of Phusion® High-Fidelity PCR Master Mix (New England Biolabs).

PCR Products treatment:

PCR products were mixed with same volume of 1X loading buffer (contained SYB green), electrophoresis were operated on 2% agarose gel for detection, mixture PCR products were purified with AxyPrepDNA Gel Extraction Kit (AXYGEN).

Library preparation and sequencing:

Following manufacturer's recommendations, sequencing libraries were generated using NEB Next® Ultra™ DNA Library Prep Kit for Illumina (NEB, USA) and index codes were added. The quality of library was assessed on the Qubit® 2.0 Fluorometer (Thermo Scientific) and Bioanalyzer 2100 system (Agilent). Then, paired-End (PE) amplicon library was constructed using a TruSeq® DNA PCR-Free Sample Preparation Kit (Illumina, US) and quantified by Qubit, then sequencing was performed using the Illumina HiSeq platform (APT BIO Technology, Shanghai, China).

Data and bioinformatics analysis

The following steps and methods refer to the implementation methods of *Lundberg DS* [19] and *Avershina E* [20].

Paired-end reads assemblies:

Paired-end reads from the original DNA fragments were merged using FLASH (<http://ccb.jhu.edu/software/FLASH/>), Quality filtering on the raw tags was performed under specific filtering conditions to obtain high-quality. clean tags according to the Fast QC (V 0.10.1).

OTU cluster and Species annotation:

Chimeric sequences were filtered using Usearch software (10 version). Sequences with 97% similarity were assigned to the same operational taxonomic units (OTUs) by Usearch (10 version). Representative sequences were chosen for each OTU, and taxonomic data were then assigned to each representative sequence using the RDP (Ribosomal Database Project) classifier. Sequences were processed with the software package of the QIIME (V1.9.0) toolkit. Taxonomy-based analyses were conducted by classifying each sequence using the SILVA database (<https://www.arb-silva.de/>). In order to compute Alpha Diversity, we rarify the OTU table and calculate seven indexes: Ace, Chao1, Goods coverage, Observed Species, PD whole tree, Shannon, Simpson.

Phylogenics distance and community distribution:

Graphical representation of the relative abundance of bacterial diversity from phylum to species can be visualized using Krona chart. Cluster analysis was preceded by principal component analysis (PCA), which was applied to reduce the dimension of the original variables using the QIIME (Version 1.9.1) software package. QIIME calculates both weighted and unweighted unifracs distance, which are phylogenetic measures of beta diversity. We used unweighted unifracs distance for Principal Coordinate Analysis (PCoA) and Unweighted Pair Group Method with Arithmetic mean (UPGMA) Clustering.

Statistical analysis:

STAMP software was utilized to confirm differences in the abundances of individual taxonomy between the two groups. LDA Effect Size (LEfSe) was used for the quantitative analysis of biomarkers within two groups. To identify differences of microbial communities between the two groups, ANOSIM and ADONIS were performed based on the Bray-Curtis dissimilarity distance matrices.

Results

The number of OTUs

The number of common OTUs between the patients of RSA and NR was 23672. Meanwhile, the number of proper OTUs in the patients of RSA was 17847, and that in NR was 43885, as shown in Table 1.

Table 1

Characteristics and OTUs of two groups

Group	RSA	NR	P-value
Number	30	30	/
Age	30.00±1.41	28.03±3.54	0.11
Body mass index (BMI)	21.74±0.35	20.58±2.09	0.07
The number of spontaneous abortions			
2 times	18	/	/
more than 3 times	12	/	/
OTUs	41529	77577	/

Characterization of fecal microbiota

The phylum and genus level were chosen to show a histogram of relative abundance of species. As shown in Figure 1, at the phylum level, we found that there is no significant difference in composition of GM between RSA and NR. But at the genus level, the results were shown in Figure 2 that compared with NR, *Roseburia* significantly decreased ($P<0.01$), and *Ruminococcus* significantly increased in RSA patients ($P<0.05$).

The normal group was divided into two groups according to whether to give birth or not, those who did not give birth were included in NR2. According to BMI, uterine and ovarian lesions, patients with intrauterine adhesion, polycystic ovary syndrome (PCOS), and BMI > 23.9 among RSA patients were classified as RSA1 group, and the remaining patients were classified as RSA2 group. We analyzed the relative abundance of different groups again. As shown in Figure 3, the results indicated that at the genus level, *Klebsiella* ($P<0.05$) were significantly increased, *Prevotella.9* ($P<0.05$) and *Roseburia* ($P<0.05$) were significantly decreased in RSA1 group. Moreover, *Agathobacter* was significantly increased in NR2 group ($P<0.01$).

NR1 was pregnant women who had given birth, NR2 was pregnant women who had not given birth, RSA1 was RSA patients with intrauterine adhesion, PCOS, and BMI > 23.9, RSA2 was other RSA patients.

Alpha diversity

Alpha diversity is used to analyze the microbial community diversity within samples or groups. Observed species is the number of OUT, Shannon and Simpson were the bacterial diversity index, Chao1 and ACE are the bacterial abundance index. Coverage was the depth index of sequencing. PD whole tree is the phylogenetic diversity index. Comparison of alpha diversity difference indexes between RSA and NR was

shown in Table 2. The results of difference analysis of alpha diversity between two groups were represented by a boxplot (Figure 4).

Table 2

Comparison of alpha diversity difference index between groups

index	Ace	Chao1	Goods coverage	Observed species	PD whole tree	Shannon	Simpson
<i>P</i> value	0.00033	0.00035	0.000044	0.00098	0.00499	0.00021	0.00577

Beta diversity difference index

The t-test was used to analyze whether the difference of species diversity among groups was significant. Based on Weighted Unifrac and Unweighted Unifrac distances, the results of difference analysis of beta diversity between two groups were represented by a boxplot (The upper two figures in Figure 5). In order to study the similarity of community composition, we performed Principal Co-ordinates Analysis (PCoA) based on weighted unifrac distance and unweighted unifrac distance (The lower two figures in Figure 5). The closer the sample distance is, the more similar the species composition.

Functional prediction

Based KEGG and COG datashops, we performed functional prediction of GM in each sample or group. The community structure component map shows the functional composition and relative abundance of each sample or group. TOP10 KEGG function items were selected to generate the relative abundance column accumulation diagram, as shown in figure 6. Membrane transport, carbohydrate metabolism, amino acid metabolism were first three pathway in function of GM. The COG functional prediction results were analyzed by Heatmap, as shown in figure 7. Moreover, STAMP results of COG was shown in figure 8.

Different colors represent different functional items, corresponding to the legend on the right; The horizontal axis represents different samples or groups, and the vertical axis represents the relative abundance of each functional item.

The horizontal axis represents different samples, and the vertical axis represents different functional items. The shade of color is related to the abundance of functional items. The darker the color, the higher the abundance.

The figure on the left shows the abundance ratio of different functions in the two samples or groups; the figure in the middle shows the difference ratio within the 95% confidence interval; the value on the far

right is the P value, P value < 0.05, indicating significant difference.

Discussion

In the last decades, using 16S rDNA sequences, there have been an increasing amount of research into GM, and it seems that most systemic diseases in the body are related to them [21]. In our previous study, we found that there were differences in GM between irritable bowel syndrome patients and normal population in Nanchang, China [22]. In this study, we focused on the RSA patients and wanted to find the difference in GM between RSA patients and normal pregnant women in Nanchang, China. It is the first to analyze the differences in GM between RSA patients and normal pregnant women.

The structure of gut microbiota changes during pregnancy

The changes of GM in different stages of pregnancy have attracted much attention [23]. Studies from different regions have shown clear differences in the characteristics of GM between pregnant women and normal women. A US study reported that GM changed dramatically from first to third trimesters, and showed vast expansion of diversity between mothers, an overall increase in *Proteobacteria* and *Actinobacteria* [24]. A Saudi Arabia study indicated that bacterial diversity decreased in pregnant woman, whereas phylum Bacteroidetes declined significantly ($p < 0.05$) in the first trimester, and a relatively high abundance of butyrate-producing bacteria (eg, *Faecalibacterium spp.* and *Eubacterium spp.*) in the gut of pregnant women [25]. A Japan research focused the differences in GM between early and late pregnancy, and they suggested that there were no obvious differences among four major phyla (*Firmicutes*, *Bacteroidetes*, *Actinobacteria* and *Proteobacteria*) between early and late pregnancy, although the proportion of the TM7 phylum decreased in late pregnancy compared with that in early pregnancy [26]. A study in China reported that at genus level, *Akkermansia*, *Bacteroides*, *Subdoligranulum*, *Oscillospira*, *Ruminococcaceae UCG-004*, and *Alistipes* showed higher abundance during pregnancy [27].

RSA patients have abnormal gut microbiota

There has been no report on the correlation between RSA and GM, but there are many reports focused on the correlation between abortion and vaginal or oral bacteria. A China study was aimed at analyzing the changes in gut microorganism of patients with positive immune antibody-associated recurrent abortion using the 16s rRNA gene sequencing microbiome assay, and found that *Bacteroides* had the highest relative abundance in the positive group, *Bacteroides*, *Erysipelotrichaceae UCG-003*, *Faecalibacterium*, and *Prevotella_9* had high relative abundance in the negative group [28]. First trimester miscarriage associated with reduced prevalence of *Lactobacillus spp.*-dominated vaginal microbiota classified using hierarchical clustering analysis [29]. A study of vaginal microbiota showed that at the genus level, the relative abundance of *Fam_Finegoldia* and *Lac_Roseburia* significantly differed in the embryonic miscarriage group [30]. In the present study, although there was no significant difference in the abundance of dominant bacteria (*Firmicutes*, *Bacteroidetes*, *Actinobacteria* and *Proteobacteria*) between

the two groups, but *Actinobacteria* and *Proteobacteria* tended to be higher in RSA patients at the phylum level. GM dysbiosis can cause insulin resistance (IR), which is closely linked to the occurrence of polycystic ovary syndrome (PCOS), and is associated with chronic inflammation, hormonal changes, follicular dysplasia, endometrial receptivity changes, and abortion or infertility [31].

***Roseburia* and *Agathobacter* may have a protective effect against RSA**

In our study, one of the most striking results was a significant decrease in the abundance of genera *Roseburia* in the RSA group ($P < 0.05$). In addition, *Agathobacter* was significantly increased in NR2 group, while *Klebsiella* ($P < 0.05$) were significantly increased, *Roseburia* ($P < 0.05$) and *Prevotella.9* ($P < 0.05$) were significantly decreased in RSA1 group. These results suggest that these bacteria play an important role in pregnancy, and the following studies can provide certain support for our research.

A Japan study in GM of infertile women showed that the abundance of the genera *Roseburia* and *Phascolarctobacterium* were decreased in patients with infertility [32]. A mice study indicated that the microbiota of conventional mice were significantly different at the end of pregnancy (day 18) as compared with pre-pregnancy ($p < 0.05$), and the abundance of *Roseburia faecis* was significantly different at day 18 compared with pre-pregnancy [33].

Moreover, autism spectrum disorder (ASD) children with sleep disorder exhibited declines in the abundance of *Agathobacter*, decreased levels of 3-hydroxybutyric acid and melatonin [34]. Jin M et al found that *Prevotella.9* were significantly decreased in patients with positive immune antibody-associated recurrent abortion [28]. The enrichment of *Faecalibacterium*, *Agathobacter* and *Roseburia* were related to geriatric depression [35].

Gut microbiota mechanism associated with RSA

Functional prediction analysis indicated that GM may play their role through membrane transport, carbohydrate metabolism, amino acid metabolism and other mechanisms.

Dietary composition:

The composition of the GM can be influenced by dietary composition. In our study, *Roseburia* ($P < 0.05$) were significantly decreased in RSA group; furthermore, compared with RSA2 group, *Roseburia* ($P < 0.05$) and *Prevotella.9* ($P < 0.05$) were significantly decreased in RSA1 group (patients with BMI > 23.9 in RSA). The human gut Firmicute *Roseburia intestinalis* is a primary degrader of dietary β -mannans, gut *Roseburia spp.* metabolize dietary components that stimulate their proliferation and metabolic activities. They are part of commensal bacteria producing short-chain fatty acids, especially butyrate, affecting

colonic motility, immunity maintenance and anti-inflammatory properties [36, 37]. In early pregnancy, the relative abundances of *Roseburia* and *Lachnospiraceae* increased in GM of vegetarian compared with omnivorous diet, and sub-analysis of GM showed an alterations in fermentation end products from a mixed acid fermentation towards more acetate/butyrate [38].

Immune maintenance and anti-inflammatory:

Roseburia is one of the most important microorganisms in human intestinal tract, and its main metabolite is butyric acid. Of note, the GMs that we found to differ significantly at the genus level, such as *Roseburia*, *Prevotella.9*, *Agathobacter*, were butyrate producing bacteriums. A ulcerative colitis (UC) study suggested that butyrate-producing species like *Roseburia hominis*, involved in the development of UC [39]. Several animal studies reported that abundance of *Roseburia intestinalis* (R.I), decreased significantly in patients with inflammatory bowel disease (IBD) and exerted an anti-inflammatory function in dextran sulfate sodium (DSS)-induced colitis [40, 41, 42]. Another animal study on UC indicated that R.I flagellin plays an important role in the treatment of UC by inhibiting activation of the NLRP3 inflammasome and pyroptosis, [43]. In a murine model study suggested that *Roseburia*, a prominent gut-associated butyrate-producing bacterial genus, may provide a importion protection against atherosclerosis [44]. These studies suggest that R.I may be involved in the mechanism of immune maintenance and anti-inflammatory, which is related to butyrate.

Butyrate-producing bacteria may prevent RSA by regulating BMI

Previous evidence from animal studies suggests that butyrate-producing bacteria prevents high fat diet-induced obesity [45]. In the present study, the patients with BMI>23.9 in RSA have decreased *Roseburia* ($P<0.05$) and *Prevotella.9* ($P<0.05$), both well-known butyrate-producing bacteria. Study suggested that chronic butyrate supplementation can prevent diet-induced obesity, hyperinsulinaemia, hypertriglyceridaemia and hepatic steatosis [46]. Abundances of butyrate-producing bacteria, such as R.I and *Faecalibacterium prausnitzii*, were lower in patients with type 2 diabetes (T2D). This result support that butyrate and other short-chain fatty acids are able to exert profound immunometabolic effects [47]. The abundance and butyrate-producing bacteria and butyrate production of overweight and obese women at 16 weeks gestation were significantly negatively correlated with blood pressure and plasminogen activator inhibitor-1 levels. These results suggest that increased butyrate production may help obese pregnant women maintain normal blood pressure. [48].

Conclusion

RSA patients have abnormal gut microbiota compared with normal pregnant women. Butyrate-producing bacteria, like *Roseburia* *Prevotella.9*, *Agathobacter*, maybe play an important role in pregnant women, and

are associated with RSA. Our results provide the basis for in-depth studies of the composition of gut microbial communities in RSA.

Abbreviations

ASD: autism spectrum disorder; BMI: body mass index; COG: clusters of orthologous groups; GM: gut microbiota; IBD: inflammatory bowel disease; IR: insulin resistance; KEGG: Kyoto encyclopedia of genes and genomes; NLRP3: NOD-like receptor protein 3; OTU: operational taxonomic units; PCA: principal component analysis; PCoA: principal co-ordinates analysis; PCOS: polycystic ovary syndrome; PCR: polymerase chain reaction; RSA: recurrent spontaneous abortion; T2D: type 2 diabetes; UC: ulcerative colitis; UPGMA: unweighted pair group method with arithmetic mean

Declarations

Ethics approval and consent to participate

All procedures complied with the principles of the Declaration of Helsinki, approved by our institutional review board and Ethics Committee of Jiangxi Maternal and Child Health Hospital, and documented in writing (No. 2020L005). Healthy controls were recruited among students and volunteers. All participants had signed informed consent.

Consent for publication

Not applicable.

Availability of data and material

All available data and material are in the manuscript and Supplementary Material.

Competing interests

All authors declare no conflict of interest of this work.

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Authors' contributions

YC and LH conceived the original idea of the study. LH analyzed data and wrote this paper. YC, QY, LZ, DL and HW collected cases and samples. All authors discussed the results and contributed to the final manuscript. All authors read and approved the final manuscript.

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Figures

Figure 1

Top10 histogram of relative abundance of species (phylum level)

Figure 2

Top10 histogram of relative abundance of species (Genus level)

Figure 3

Top10 histogram of relative abundance of species in four groups (Genus level)

Figure 4

Boxplot of Alpha diversity index between RSA and NR

Figure 5

Characteristics of Beta diversity index between RSA and NR

Figure 6

Barchart of relative abundance of KEGG functional items

Figure 7

Functional annotation clustering heatmap of COG

Figure 8

STAMP results of COG

Supplementary Files

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