Effects of Probiotics Supplementation on Placental Microbiome in Healthy Women Undergoing Spontaneous Delivery

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Research Article

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ABSTRACT

Objective: To investigate the effect of orally supplemented probiotic on term placental microbiota and provide possible evidences for clinical management of pregnant women.

Methods: A population-based cohort of specimens were collected from 37 healthy nulliparous pregnant women who underwent systemic examination. The pregnant women were divided randomly into probiotics group and control group. We collected placental specimens during spontaneous delivery at term. The placental samples were taken for analyzing microbiome with the 16S rRNA amplicon sequencing of V4 region.

Results: There were no significant differences between two groups in clinical characteristics. The placental microbiota from normal vaginal delivery were composed of *Bacteroides*, *Lactobacillus* and *Ralstonia*. The relative abundant of *Prevotella*_9, *Faecalibacterium*, and *Escherichia*_*Shigella* etc., in probiotic group was significantly higher than that control group ($p<0.05$). Probiotics supplementation could affect the network structure of placental microbiota.

Conclusion: The characteristics of the placental microbiome changed after probiotics supplementation and the network structure of interaction tended to be loose. The probiotic supplementation may be useful in regulating the interaction network of placental microbiota.

Keywords: Probiotic; Full-term pregnancy; 16S rRNA sequencing; Interaction network; Placental microbiota; Supplements; Specimen

INTRODUCTION

Probiotic supplements are widely promoted as "over-the-counter medicines" and can be taken as an auxiliary medicine for human health by modifying the existing microbiota community. Furthermore, it is gradually applied to pregnant women. Therefore, many pregnant women can voluntarily supplement some probiotics during pregnancy, such as golden bifid, acidophilus milk and so on. The use of probiotics in the United States and Canada ranges from 1.3% to 3.6%, whereas the probability of using probiotics in pregnant women in Netherlands has risen to 13.7% [1]. Meanwhile, the International Scientific Association for Probiotics and Prebiotics (ISAPP) defined the probiotics as an active microorganism, the supplementation of appropriate amounts of which may have a positive effect on human metabolic function and health, where *Lactobacillus* and *Bifidobacterium* are widely used [2]. Furthermore, it is used in obstetrics to prevent premature birth and reduce the occurrence of gestational diabetes mellitus. Studies have shown that pregnant women who take probiotics during the first trimester can reduce the diagnostic rate of gestational diabetes mellitus by 60% [3]. What's more, the risk of spontaneous preterm birth is lower in pregnant women who often take probiotics [4]. An improved maternal microbiota is likely to provide the beneficial microbes through either direct colonization of the neonatal gastro-intestinal tract or indirect effect on the succession of indigenous intestinal bacteria [5]. Despite the popularity of golden bifid in the application in the obstetrics, questions remained: How would the intake of golden bifid affect the placental microbiota, and subsequently influence the perinatal outcomes in women with normal pregnancy?

Therefore, this study aimed to investigate the effect of orally supplemented probiotic on term placental microbiota and provide possible evidences for clinical management of pregnant women.

MATERIALS AND METHODS

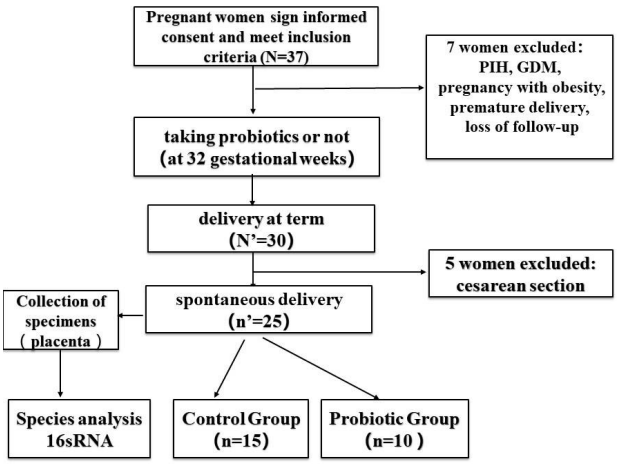
Participants

According to the principle of informed consent, 37 patients with normal physiological pregnancy who planned to give birth in the first affiliated hospital of Jinan University (Guangzhou, Guangdong Province, China) and met the inclusion criteria were recruited. All the participants were Chinese, conceive naturally, singleton pregnancy, nulliparous and under 35 years old. Pregnant women with complications and caesarean section were excluded in the follow-up period, and finally 25 pregnant women with spontaneous delivery were enrolled (Figure 1). The probiotic group (10 cases were supplemented with golden bifid through the oral cavity, and they took 2 tablets twice a day (2 grams/day) from 32 weeks of gestation until delivery. Each tablet contains 0.5×10^7 CFU of live *Bifidobacterium*, 0.5×10^6 CFU of *Lactobacillus* and *Streptococcus*. Yet the control group took no pills. All the participants were kept in touch with us and their dietary habits were relatively consistent. The antibiotics or other foods contained probiotics during the study were not allowed.

In this study, after consent was obtained, collected and analyzed clinical characteristics from participants included age, height, weight before delivery, BMI before delivery, apgar score, birth weight, head circumference and length of new-born. Placental specimens were collected under clean and sterile conditions within 30 minutes of spontaneous delivery at term. The placental surfaces were excised and discarded, and the area of necrosis and calcification of the maternal surface of the term placenta were avoided. In the fetal face of the placenta, four 1 × 1 × 1 cm cuboidal sections are circumferentially excised from separate areas of the placenta, each located 3 cm from the

cord insertion site. Finally, placental specimens were immediately placed in dedicated specimen box, transported to the laboratory within half an hour, and then stored at -80°C until DNA extraction.

Figure 1. Technical flow chart for screening research subjects.



High-throughput sequencing

After extraction of the DNA of microorganisms in specimens, we used the IonS5TM XL sequencing platform to sequence the 16s rRNA V4 regions. Cutadapt (V1.9.1,http://cutadapt.readthedocs.io/en/stable/) was used to perform low-quality partial shearing on the reads, then the sample data were split from the obtained reads according to barcode; we then cut off the initial data of barcode and primer sequences to obtain the original data. The Reads obtained were further processed to remove the chimera sequence, and the reads sequence is passed [6]. We then compared the chimeric sequences with the database (gold database, http://drive5.com/uchime/uchime_download.html) and finally removed the chimeric sequences [7], and got the final valid data (clean reads). All procedures strictly followed the product manual.

OTU clustering and annotation

The uparse software (uparse v7.0.1001, http://drive5.com/uparse/) [8] was used to cluster all clean reads from all samples. By default, the sequences are clustered into Operational Taxonomic Units (OTUs) with 97% identity. At the same time the most frequently occurring sequence of OTUs was selected as representative sequence according to its algorithm principle. Specimen annotation of OTUs representative sequences and species annotation analysis (set threshold of 0.8-1) were performed using the mothur method and the SSU rRNA database of SILVA (http://www.arb-silva.de/) [9,10], taxonomic information was obtained and the community composition of each sample was counted at each taxonomic level (such as kingdom, phylum, class, order, family, genus and species). Fast multi-sequence alignment was performed using MUSCLE [11] (Version 3.8.31, http://www.drive5.com/muscle/), where a systematic relationship of all OTUs representative sequences was obtained.

Statistical analysis

Clinical characteristics processing was performed using SPSS19.0, t-test was used to determine the significance of continuous metadata for study subjects. The basic data were summarized by means of descriptive statistics, including proportion, and mean ± standard deviation (SD). The LDA effect size (LEfSe: Linear discriminant analysis

effect size) was used to detect significant features, which differentiate groups and rank these features by effect size (http://huttenhower.sph.harvard.edu/galaxy/). The threshold used on the logarithmic linear discriminate analysis score for discriminative feature was 3.0. The Spearman analysis was used to analyze the correlation of interaction network structure. $P<0.05$ was considered statistically significant. The R language and Origin 8.0 were applied to statistical plotting.

RESULTS

Clinical characteristics of the study subjects

For this study, the clinical and demographic characteristics of pregnant women who had a term delivery are displayed in Table 1. There are no significant differences on dietary habits, age, height, weight before delivery, BMI before delivery and birth weight of new-born between two groups.

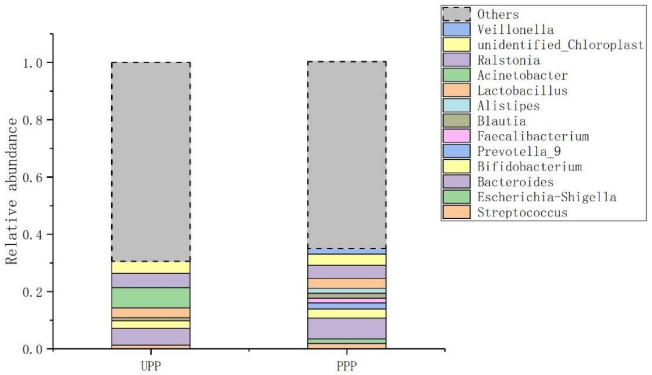
Table 1. The clinical and demographic characteristics of pregnant women.

Clinical characteristics	Control group	Probiotics group	P value
Age (years)	27.7 ± 1.10	27.2 ± 1.16	0.763
Height (cm)	162.5 ± 2.12	158.6 ± 1.15	0.171
Weight before delivery (kg)	65.5 ± 2.27	65.3 ± 2.86	0.958
BMI before delivery (kg/m^2)	24.8 ± 0.57	25.9 ± 0.98	0.286
Birth weight of newborn (kg)	3.5 ± 0.18	3.3 ± 0.17	0.671
Birth length of newborn (cm)	49.7 ± 0.41	50.1 ± 0.51	0.595
Head circumference of newborn (cm)	35.6 ± 1.85	33.9 ± 0.30	0.48
1-minute Apgar score	9	9	-
5-minute Apgar score	10	10	-

The feature of full-term placental microbiota in the two groups at the genus level

At the genus level, the distribution of microbial communities with Operational Taxonomic Units (OTUs) contents above 0.01 in the placental microbiota based on 16S rRNA gene deep sequencing analysis was illustrated in the Figure 2.

Figure 2. Relative abundances of Operational Taxonomic Units (OTUs) at genus level. The relative abundances of OTUs accounting for above 0.01 in the placental microorganisms are shown for each group using the 16S Ribosomal RNA gene deep sequencing analysis. OTUs representing less than 0.01 are grouped as others. **Note**: UPP: Control group; PPP: Probiotic group; Others; *Veillonella*; *Unidentified_Chloroplast*; *Ralstonia*; *Acinetobacter*; *Lactobacillus*; *Alistipes*; *Blautia*; *Faecalibacterium*; *Prevotella_9*; *Bifidobacterium*; *Bacteroides*; *Escherichia-Shigella*; *Streptococcus*.



By screening the OTUs-table, there are 12 genera whose relative abundance of 0.01 or higher (i.e. high-abundant microbial communities) in term placental microbiome (Figure 2). Among them, 8 genera were detected in control group (UPP), namely *Streptococcus*, *Bifidobacterium*, *Lactobacillus*, *Bacteroides*, *Blautia*, *Acinetobacter*, *Ralstonia* and *unidentified_Chloroplast*. Yet, the relative abundance of the OTUs more than 0.01 only accounted for 31% (Table 2). The remainder represents low-abundant bacteria or unidentified genus. However, in addition to the above-mentioned genus, *Escherichia_Shigella*, *Prevotella_9*, *Faecalibacterium*, and *Alistipes* were detected in probiotic group (PPP), and the relative abundance of the OTUs more than 0.01 only accounted for 35% (Table 2). As shown is Table 3, the OTUs contents of the high-abundant microbial communities of placental microbiome were showed.

Table 2. Relative abundance and proportion of placental microbiome in two group.

Group	Control group (UPP group)	Probiotic group (PPP group)
OTU＞0.01 (count)	8	12
Classification genus (count)	953	1032
Proportion with OTU＞0.01 (%)	0.80%	0.10%
OTU abundance ratio (%)	31%	35%
Others (uncategorized)	69%	65%

Table 3. OTUs content of high-abundant microbial communities of placental microbiome in two groups.

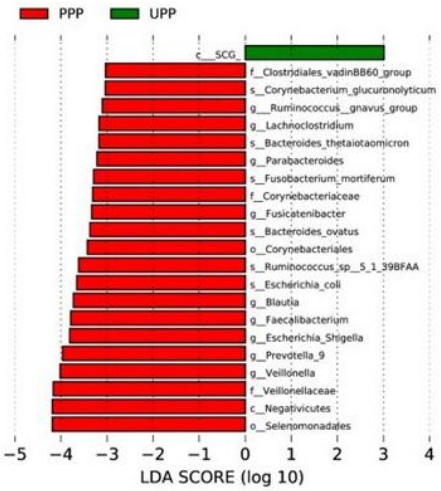
Taxonomy (genus)	Control group (UPP group)	Probiotic group (PPP group)
Bacteroides	0.058442	0.072816
Ralstonia	0.049748	0.044852
Lactobacillus	0.034534	0.03516
Bifidobacterium	0.026674	0.03164
unidentified_Chloroplast	0.041402	0.039873
Streptococcus	0.012821	0.017911
Blautia	0.010709	0.017453
Acinetobacter	0.071167	-
Prevotella_9	-	0.020718
Veillonella	-	0.019272
Escherichia-Shigella	-	0.016882
Faecalibacterium	-	0.016934
Alistipes	-	0.017011

The difference of placental microbiome between the two groups

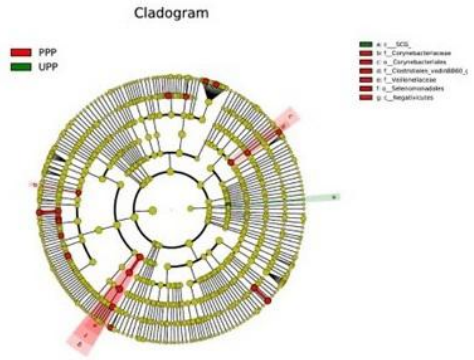
Applying LEfse to identify significant features of the full-term placental microbiome of two groups. In Figure 3a, there were significant differences about full-term placental microbiome between the control group (UPP) and the probiotic group (PPP) ($p<0.05$). Namely, the relative abundance of placental microbiome in probiotic group were significantly higher than those in control group, and including *Prevotella_9*, *Faecalibacterium*, *Escherichia_Shigella*, *Blautia*, *Parabacteroides*, *Veillonella*, *Fusicatenibacter*, *Lachnoclostridium* and *Ruminococcus gnavus* group (Table 4). Moreover, those genera were all labelled in Figure 3b. In all panels, red regions and dots are used to designate cases with probiotic group, and green to designate control group.

Figure 3. LEfSe analysis of full-term placental microbiome in two groups. **Note**: PPP; UPP; 3a: UPP: control group; PPP: probiotic group; The abscissa represents LDA score greater than 3, the ordinate represents the species of the flora, and the length of the histogram represents the influence of the differential flora. The LDA threshold is set to 3 (Log LDA > 3.00). If the Group, LDA, and P values are all empty, it means that the species has no difference between the groups. In the above picture. c: class, o:order, f: family, g: genus, s:species;

3b: UPP: control group; PPP: probiotic group; Red regions represent the PPP group, and green regions represent the UPP group. The red node indicates the microbial group that plays an important role in the red group, the green node indicates the microbial group that plays an important role in the green group, and the yellow indicates the species that have no significant difference between the groups.



3a.Differences in key OTUs identified as differentiating between probiotic and control group visualized using a LDA columnar distribution



3b.Bacterial taxa that were differentially abundant in probiotic and control group visualized using a cladogram generated from LEfSe analysis

Table 4. The difference of placental microbiome between the two groups.

Taxonomy	Control group	Probiotic group	P value
Prevotella_9	0.0095	0.0296	0.015
Faecalibacterium	0.0091	0.0197	0.048
Escherichia_Shigella	0.0071	0.0193	0.017
Blautia	0.0065	0.0221	0.045
Parabacteroides	0.0036	0.0066	0.013
Veillonella	0.0023	0.0289	0.016
Fusicatenibacter	0.0016	0.0095	0.018
Lachnoclostridium	0.0016	0.005	0.046
Ruminococcus gnavus group	0.0003	0.0032	0.007

The interaction network structure of placental microbiome in the two group

In this study, Figure 4 presented the interaction network structure between the placental microbiome in the control group, with significant statistical support ($r \geq \pm 0.6$, $p<0.05$). In probiotic group, we also displayed with statistical significance the interaction network structure between placental microbiome (Figure 5). As shown, we observed that each genus is related to one or more genera (i.e. high-density correlation). In the control group, the interaction network of the placental microbiome was close, with the clustering coefficient of 0.606 (Table 5).

However, the interaction network structure of the probiotics group became loose, with the clustering coefficient of 0.469 (Table 5).

Figure 4. The interaction network structure of placental microbiome in control group (UPP group).

Note: *Cyanobacteria*; *Actinobacteria*; *Proteobacteria*; *Bacteroidetes*; *Fusobacteria*; *Verrucomicrobia*; *Firmicutes*; The *Lactobacillus*, *Bifidobacterium*, and *Streptococcus* were pointed by blue, green and black arrow respectively. The nodes in the above figure are identified by different colours, representing each dominant genus. The line between the nodes indicates a correlation between the two genera, the red line indicates a positive correlation, the blue line indicates a negative correlation, and the thickness of the line indicates the intensity of correlation. The node size represents the content of the OTUs, and the dots of the same colour represent the genus classified into the same phylum. The more connections through a node, the more associations the genus has with other members of the genus.

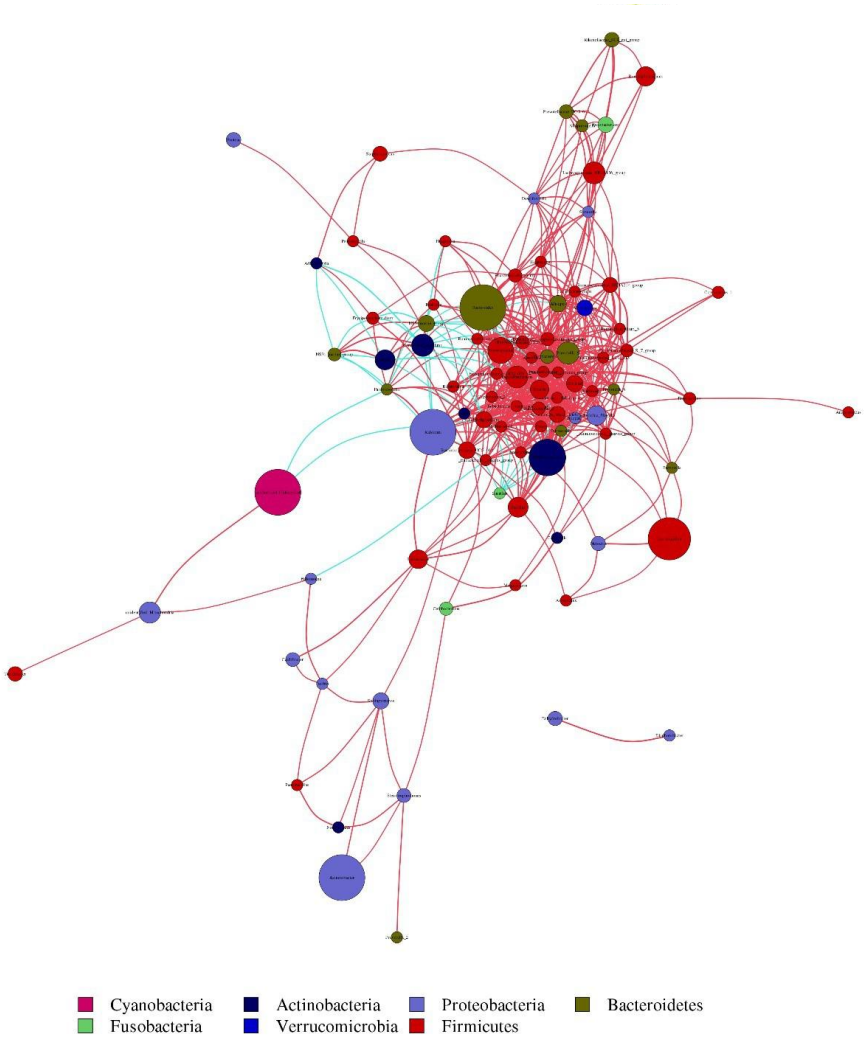
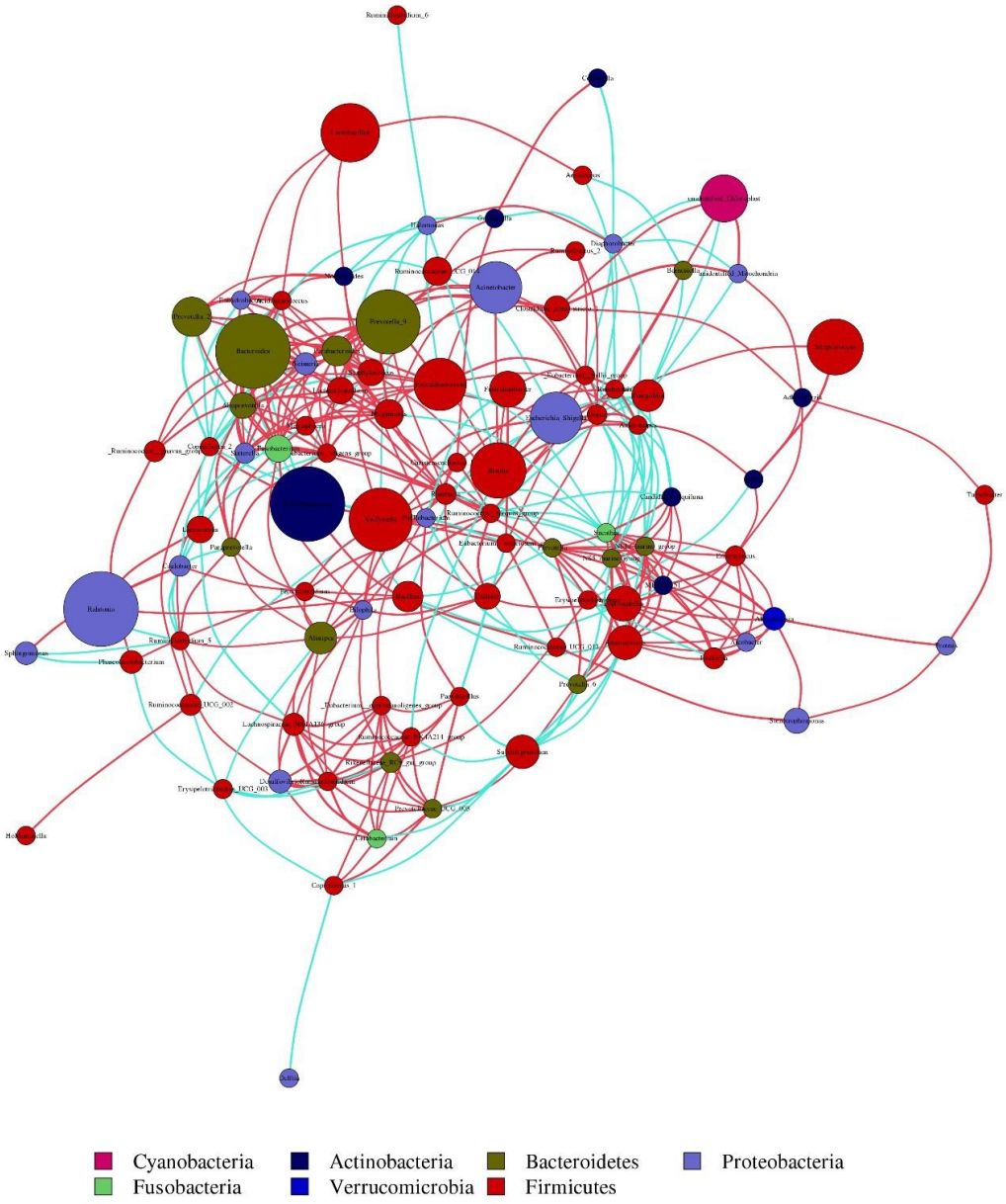


Figure 5. The interaction network structure of placental microbiome in probiotic group (PPP group).

Note: *Cyanobacteria*; *Actinobacteria*; *Proteobacteria*; *Bacteroidetes*; *Fusobacteria*; *Verrucomicrobia*; *Firmicutes*; The *Lactobacillus*, *Bifidobacterium*, and *Streptococcus* were pointed by blue, green and black arrow respectively. The nodes represent each dominant genus. The line between the nodes indicates a correlation between the two genera, where the red line indicates a positive correlation, and the blue line indicates a negative correlation, and the thickness of the line indicates the intensity of correlation. The node size represents the content of the OTUs, and the dots of the same colour represent that the genus is classified into the same phylum. The more connections through a node, the more associations the genus has with other members of the genus.



What is more, in control group, the number of significant correlations between three genera (i.e. *Lactobacillus*, *Streptococcus* and *Bifidobacterium*) and other genera were 5, 38 and 24, respectively (Table 6).

In probiotic group, the number of significant correlations between three genera and other genera were 4, 2 and 8, respectively (Table 6).

Table 5. Topological parameter of interaction network structure of placental microbiome in the two groups.

Topological parameter	Control group	Probiotic group
Network diameter	8	10
modularity	0.192	0.482
Clustering coefficient	0.606	0.469
graph density	0.102	0.045
Average degree	20.54	9.06
Average path length	2.629	3.475

Table 6. Number of significant positive and negative correlations with three genera in two groups.

Group	Relationship	*Lactobacillus*	*Streptococcus*	*Bifidobacterium*
Control Group	+	5	37	24
	-	0	1	0
Probiotic Group	+	4	2	3
	-	0	0	5
Note: += Positive relationship; -=Negative relationship.				

DISCUSSION

In this study, the probiotic supplementation showed that there was no adverse effect on pregnant women and fetus. The pregnant women in the control group and probiotics group were full-term vaginal delivery, and there was no significant difference in fetal growth and development indicators between the two groups.

Microbiota are the earliest life form on the earth. Without microbiota, human beings will not be able to survive healthy. The human body is a “super organism” composed of its own cells and microbial cells. Similarly, the fetus has lived in a micro-ecological environment composed of commensal bacteria. In particular, the placenta, which provides nutrition for the fetus, contains a unique microbiota. Our results show that the placental tissue of normal full-term healthy pregnant women contains symbiotic bacteria. These symbiotic bacteria have the characteristics of various species and low relative abundance, the top three genera were *Bacteroides*, *Lactobacillus* and *Ralstonia* (Table 3). At present, with the development of high-throughput sequencing and the increasing research of placenta by related scholars, it has been confirmed that there was microbiota in umbilical cord blood, amniotic fluid, and placental tissue from healthy maternal and fetal [12-15]. In addition, many researchers reported that the placental microbiome is crucial for the maintenance of normal pregnancy and offsprings growth and development [16-19]. Full-term vaginal delivery is the basic law of pregnant women’s reproduction, and also in line with the normal process of pregnancy and delivery. Thus, the placental tissue in this study is all vaginal delivery, and the normal microbiota in the vagina may affect the placental microbiome, which is the highlight of this project, that is to explore the characteristics of placental microbiome under normal physiological conditions. Meanwhile, the pregnant women included in this study had no symptoms of vaginitis, such as pruritus, abnormal leucorrhea, etc. There are many hypotheses about the origin of placental microbiota, such as oral cavity, vagina and gut of pregnant women, but the specific path is still in the exploration stage. Thought the specific source pathway of the placental microbiota is still unknown, the connection and circulation among different bacterial banks has been reported and proven [20,21]. The similarities between the human oral, placental epithelia and the microbial communities have been suggested to explain this phenomenon [15,22]. What’s more, scholars have found that the placental microbiome can also affect

the establishment of gut microbiome, and thus influence the growth and development, as well as susceptibility to immune diseases and neurological diseases [19,23].

Through LEfse analysis in this study, we found that probiotics supplementation can increase the relative abundance of *Prevotella*_9, *Faecalibacterium*, *Escherichia*_*Shigella*, *Blautia*, *Parabacteroides*, *Veillonella*, *Fusicatenibacter*, *Lachnoclostridium* and *Ruminococcus gnavus* group. In placenta microbiome (Figure 3 and Table 4). However, there was no difference in the relative abundance of *Lactobacillus*, *Streptococcus* and *Bifidobacterium* between the control group and probiotics group. The reason for the above results may be that the dosage of probiotics supplement is too small or the lack of sample size fails to make significant difference between the two groups. What's more, the above-mentioned increased bacteria may play a potential role in placental function and the growth and development of fetus and infant.

As shown in Figures 4 and 5 the interaction network structure of the control group was closely connected, that is, there was a significant correlation between bacteria. Among them, *Streptococcus* may be dominant in placental microbiome. However, in the probiotic group, the interaction network structure had tended to be loose. Furthermore, *Bifidobacterium* maybe dominant in the placental microbiome. *Streptococcus* is gram-positive, aerobe or facultative anaerobe. Most of them are commensal bacteria. In the placental microbiome of control group, it plays a synergistic role with many bacteria through positive correlation, so as to maintain the physiological function of placenta and the growth and development of fetus (Table 6). However, *Bifidobacterium* is gram-positive and anaerobic. It, as a kind of physiological beneficial bacteria, provides nutrition and immune regulation for the body. In the probiotics group, *Bifidobacterium* has a negative correlation with other bacteria and mainly plays an antagonistic role (Table 6). Meanwhile, it can inhibit the growth of harmful bacteria, resist the infection of pathogens, synthesize vitamins needed by human body, and its metabolites can regulate pH, activate the immune system and play an important role [24]. Thus, probiotics supplementation made the structure of the interaction network structure of the placental microbiome tend to be loose, and enhance the effect of *Bifidobacterium*, which may provide a theoretical basis for drug intervention of placental diseases.

Golden bifid, live combined *Bifidobacterium* and *Lactobacillus* tablets, is a way to supplement probiotics during pregnancy. Probiotics have long been renowned for its benefits to human health [24,25], especially *Lactobacillus* and *Bifidobacterium*, which are closely related to reduce body weight gain, fat mass development, inflammation, and hepatic steatosis or to improve glucose homeostasis [26]. Research suggested that probiotic supplementation could change the structure of gut microbiome, by promoting the growth of beneficial commensal bacteria and inhibiting the growth of harmful commensal bacteria [27]. In this study by Zhou, et al. [28] the intervention of probiotics can increase the beneficial anaerobic bacteria and maintain the stability of gut microbiome in the mice model. Meanwhile, the application of probiotics in obstetrics is also very extensive, which may play a positive role in the prevention of premature delivery and gestational diabetes [3,4,29]. In addition, Jeanjacques, et al. [30] related scholars reported that probiotics supplementation during pregnancy maybe not have adverse effects on cesarean section, neonatal birth weight and gestational age. This is also consistent with our results.

Overall, the placental tissue of normal full-term healthy pregnant women contains symbiotic bacteria. These symbiotic bacteria have the characteristics of various species and low relative abundance. Probiotics supplementation can increase the relative abundance of *Prevotella*_9, *Faecalibacterium*, *Escherichia*_*Shigella*, *Blautia*, *Parabacteroides*, *Veillonella*, *Fusicatenibacter*, *Lachnoclostridium* and *Ruminococcus gnavus* group. What's more, probiotics supplementation maybe significantly modify the interaction network structure of full-term placental microbiome. Whether this change can lead to better perinatal outcomes are not aware. However, the

effect of probiotics supplementation may provide a theoretical basis for drug intervention of placental diseases. Meanwhile, other researchers of our research group also collected oral cavity, gut and vaginal secretion of the same subjects. Then this study is mainly aimed at the analysis of placental microbiome. In the future, we may analyze the flora structure of different parts.

CONCLUSION

Though the above limitations, our study demonstrated that the placental tissue of normal full-term healthy pregnant women have the characteristics of various species and low relative abundance. Probiotics supplementation made the structure of the interaction network structure of the placental microbiome tend to be loose, and enhance the effect of *Bifidobacterium*. Thus, the probiotic supplementation may be useful in regulating the interaction network of placental microbiome. The effect of probiotics supplementation may provide a theoretical basis for drug intervention of placental diseases.

LIMITATIONS

There are some limitations to our study. First, the choosing of the women undergoing spontaneous delivery guaranteed the normal physiological integrity, but we cannot exclude the impact of bacterial transfer during vaginal delivery. Second, the limited amount of subject may eventually affect the reliability of our results. Third, we failed to collect indicators of growth and development of infants after delivery.

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AUTHORS CONTRIBUTIONS

XM. X. and Z.L. conceived and designed the study, P.Y., J.Z., and T.K.D performed placenta tissue sampling, P.Y., Z. L., J.Z. and T.K.D traced the subjects, P.Y. and Z.L. performed experiments, P.Y. and YY.C. analyzed the data, P.Y., T.L., and ZL. H. wrote the article. All authors made comments on the manuscript. All authors read and approved the final manuscript.

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ETHICS APPROVAL AND CONSENT TO PARTICIPATE

The contents and methods of the study conform to the medical ethics and requirements, and finally approved. We carry out clinical studies in strict accordance with the scheme approved by the ethics committee and informed consent to protect the health and rights of the subjects. The study project was authorized by the Institutional Review Board (IRB) for human subjects research at the first affiliated hospital of Jinan University and the approval number was: 2019–011.

CONFLICTS OF INTEREST

No author has any potential conflict of interest statement.

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