Association between Premature Ovarian Insufficiency and Gut Microbiota

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Abstract

Premature ovarian insufficiency (POI) is characterized by impairment of ovarian function on a continuum before the age of 40 years. POI is affected by multiple factors. Considering new insights from recent gut microbiome studies, this study aimed to investigate the relationship between gut microbial community structure and POI. Subjects were recruited at the Shenzhen Maternity&Child Healthcare Hospital. Fecal microbial community profiles of healthy women (n = 18), women with POI (n = 35) were analyzed using 16S rRNA gene sequencing based on Illumina NovaSeq platform. Compared to the controls, the serum levels of FSH, LH, T and FSH/LH ratio significantly increased in women with POI, whereas E2 and AMH decreased significantly. Higher weighted UniFrac value was observed in POI women compared with healthy women. Phylum *Firmicutes*, genera *Bulleidia* and *Faecalibacterium* were more abundant in healthy women, while phylum *Bacteroidetes*, genera *Butyricimonas, Dorea, Lachnobacterium* and *Sutterella* enriched significantly in women with POI. Moreover, these alterations of the gut microbiome in women with POI was closely related to FSH, LH, E2, AMH level and FSH/LH ratio. Women with POI had altered microbial profiles in their gut microbiome, which were associated with serum hormones levels. These results will shed a new light on the pathogenesis and treatment for POI.

Introduction

Premature ovarian insufficiency (POI) is an ovarian insufficiency syndrome before the age of 40 years affecting approximately 1–2% women (Luisi et al., 2015)(Fenton, 2015). It is characterized by a continuous decline in ovarian function, and resulting in an earlier cessation of menstruation than normal (Fenton, 2015). Women with POI are faced with increased risk of low chance of natural conception (Bidet et al., 2011)(Sassarini et al., 2015), urogenital atrophy (Portman and Gass, 2014), decrease in bone mineral density (Bakhsh et al., 2015), autoimmune and thyroid disease risk (Goswami et al., 2006), cognitive dysfunction (Bove et al., 2014), shortened life expectancy (Mondul et al., 2005), and cardiovascular disease (Yorgun et al., 2013)(Goldmeier et al., 2013). POI is a multifactorial disease (Podfigurna-Stopa et al., 2016). Spontaneous POI is associated with genetic defects, autoimmunue diseases, enzyme deficiency and environmental factors, and iatrogenic POI occurs mainly due to surgical intervention, chemotherapy and radiotherapy (Gupta and Tiwari, 2019)(Podfigurna-Stopa et al., 2016).

In recent years, increasing evidences have strongly suggested that gut microbiome play an important role in autoimmune dysfunction (Siljander et al., 2019)(De Luca and Shoenfeld, 2019)(De Luca and Shoenfeld, 2019), bone health (Ibáñez et al., 2019)(Medina-Gomez, 2018)(Yan and Charles, 2017), cognitive and neurological health (Martin et al., 2018)(Galland, 2014). Gut microbiota and its metabolites also have the ability to regulate inflammation pathway activation, brain-gut peptide secretion and the destruction of islet β-cell (Zheng et al., 2018)(Lach et al., 2018). All these syndromes are closely related to POI, that a relationship may exist between the gut microbiome and POI.

In order to study the community profile of gut microbiome in women with POI, and how the changes of gut microbiota correlated with the sex hormones, 35 women with POI and 18 healthy women were

recruited in this study. Sequencing of the V3-V4 region of 16S rRNA gene in fecal samples was performed to reveal the substantial differences of gut microbiota between the POI subjects and controls.

Materials And Methods

Study cohort

A total of 35 women with spontaneous POI and 18 healthy women, aged 24 to 40 years, were recruited at the Shenzhen Maternity&Child Healthcare Hospital from August 2019 to September 2019. Spontaneous POI was diagnosed and assessed according to the previously reported (Guo et al., 2020), primary or secondary amenorrhea for at least 4 months before 40 years, and with at least two instances of serum follicle-stimulating hormone (FSH) levels exceeded 40 IU/L with an interval of 4–6 weeks. All the control women had normal ovarian function, without history of menstrual dysfunction and infertility, with regular menstruation and normal levels of FSH (< 10 IU/L). Participants were excluded if with following situation: non-46-XX karyotype, POI with family history, pregnancy, tumor, chronic diarrhea, autoimmune diseases, use of antibiotics/medications with the preceding three months, pelvic surgery, gastrointestinal disease, active infections, body mass index < 18.5 or > 23.9, smoking and chemo/radiotherapy treatment. The study protocol was approved by the ethics committee of Shenzhen Maternity&Child Healthcare Hospital. Written informed consents were obtained from all participants prior to enrollment. And clinical characteristics were extracted from the health records.

Sampling

All participants were examined in the morning after > 8 h fasting. Fecal samples were collected using empty stool collection tubes with an inbuilt sterile swab. Samples were stored at -80°C until further analysis.

DNA extraction and sequencing

DNA was extracted from fecal samples using QIAamp DNA stool mini kit (Qiagen, Germany) according to the manufacturer's instructions. PCR amplification was conducted using 338F forward primer 5'-ACTCCTACGGGAGGCAGCAG-3' and 806R reverse primer 5'-GGACTACHVGGGTWTCTAAT-3' targeting the variable V3-V4 regions of 16S rRNA gene. All samples were pooled equally and sequenced on an Illumina NovaSeq 6000 machine with 2 × 250 flow cell. Raw sequencing data in this study were deposited into the NCBI's Sequence Read Archive database (SRA BioProject ID PRJNA615330).

Sequencing data analysis

Sequencing reads were assigned to each sample based on dual-index barcodes using custom Perl script. Reads were processed using the bioinformatics software package QIIME2 (version 2019.10). Firstly, the reads were imported to a QIIME2 artifact with command "qiime tool import". Then the reads were denoised with command "qiime data2 denoise-paired" to exclude chimeric sequences and phiX sequences. Next, taxonomy was assigned with command "qiime2 feature-classifier classify-sklearn"

against Greengenes (13_8 revision) database. Meanwhile, Shannon index and weighted UniFrac distance were generated with command “qiime phylogeny align-to-tree-mafft-fasttree” and “qiime diversity core-metrics-phylogenetic” at a sample depth of 1000. Principal coordinate analysis (PCoA) based on weighted UniFrac distances were also calculated.

Statistical analysis

Permutational multivariate analysis of variance (PERMANOVA) was performed on weighted UniFrac distance to investigate the differences of microbial community structure between POI group and healthy control group using package vegan (999 permutations) in R software.

Statistical calculations were performed using R software, and P value < 0.05 was considered significantly different. Normality test was conducted using Shapiro-Wilk test. Unpaired t-tests were used for comparisons of clinical characteristics, indices of diversity between the two groups. All these continuous data were expressed as mean value ± standard deviation (SD). While Wilcoxon Rank Sum tests were used for taxa comparisons at the phylum and genus level. Partial correlation analysis was used to investigate the relationships between microbes and clinical characteristics in R packages “ggm” and “psych” according to previous study (Liu et al., 2017).

Results

Study subject characteristics

Study subject characteristics were summarized in Table 1. All participants aged from 24 to 40 years old (average: 34.6 years old), accompanied with body mass index (BMI) ranging from 18.6 to 23.9 (average: 21.2). Statistical analysis demonstrated that there were no differences at age, progesterone (P), prolactin (PRL) and glucose (GLU) between women with and without POI. In addition, women with POI had significantly higher levels of BMI, FSH, luteinizing hormone (LH), testosterone (T) and FSH/LH ratio, but significantly lower levels of estradiol (E2) and anti-Mullerian hormone (AMH), compared to healthy control women.

Table 1
Demographic and clinical characteristics of the two groups

Characteristic	POI = 35	NG = 18	*P* value
Age (years)	35.23 ± 4.62	33.5 ± 4.05	0.19
BMI (kg/m^2)	21.5 ± 1.35	20.54 ± 1.61	0.02*
FSH (mIU/mL)	45.60 ± 28.77	5.39 ± 1.74	< 0.01**
LH (mIU/mL)	15.79 ± 9.02	4.04 ± 1.07	< 0.01**
E2 (pg/L)	30.71 ± 11.7	54.56 ± 9.0	< 0.01**
P (nmol/L)	0.48 ± 0.33	0.35 ± 0.14	0.12
T (nmol/L)	0.43 ± 0.22	0.31 ± 0.11	0.03*
PRL (nmol/L)	14.37 ± 7.12	11.49 ± 4.57	0.13
AMH (ng/mL)	0.49 ± 0.35	3.94 ± 2.04	< 0.01**
FSH/LH (ratio)	2.88 ± 0.68	1.32 ± 0.18	< 0.01**
GLU (nmol/L)	5.07 ± 0.41	4.99 ± 0.32	0.48

Overall community structure of POI gut microbiome

Sequencing was performed on the V3-V4 regions of 16S rRNA to evaluate the community structure of gut microbiota in women with and without POI. In total, 3,163,487 usable reads (59,688 ± 11,095 reads per sample) were obtained from all 53 samples, and the mean and median sequence lengths were 411 and 410 bp separately. The number of reads analyzed did not differ between POI and control samples (60,943 ± 11,393 versus 57,247 ± 10,326, $P = 0.25$), indicating comparable and adequate sequencing coverage.

To explore the dissimilarity of gut microbiota between the two groups, PCoA analysis was performed based on the weighted UniFrac distance. The results showed the subjects of the two groups did not separate (Fig. 1A) ($P = 0.16$, PERMANOVA analysis with 999 permutations). Further, POI subjects exhibited a higher Shannon index without significant difference between the two groups (4.97 ± 0.74 versus 4.71 ± 0.39, $P = 0.09$) (Fig. 1B). The average weighted UniFrac value within subjects of POI group was significantly higher than control group (0.41 ± 0.13 versus 0.37 ± 0.11, $P < 0.01$) (Fig. 1C).

Characterizing the gut microbiome in POI group

Gut microbiome communities were dominated by phyla *Firmicutes*, *Bacteroidetes, Actinobacteria* and *Proteobacteria* in both POI and control groups (Fig. 2A). And *Firmicutes* was the predominant microbe, accounting for 65.35% ± 18.71% and 76.83% ± 14.90% in POI and control group, respectively. The top 10 abundant genera in both two groups were *Bacteroides*, *Bifidobacterium*, *Blautia*, *Clostridium*,

Coprococcus, *Faecalibacterium*, *Megamonas*, *Prevotella*, *Roseburia* and *Ruminococcus* (Fig. 2B). Compared to the control group, *Bacteroides* (13.42% ± 11.06% versus 8.45% ± 10.14%), *Bifidobacterium* (6.30% ± 11.76% versus 5.0% ± 8.84%), *Megamonas* (2.97% ± 12.90% versus 1.37% ± 4.61%), *Prevotella* (4.09% ± 9.90% versus 2.37% ± 4.62%) increased, whereas *Blautia* (9.60% ± 7.37% versus 11.72% ± 7.90%), *Clostridium* (1.61% ± 2.13% versus 4.31% ± 7.99%), *Coprococcus* (3.0% ± 2.93% versus 3.91% ± 4.12%), *Faecalibacterium* (13.35% ± 10.39% versus 21.65% ± 14.80%), *Roseburia* (3.63% ± 4.72% versus 3.84% ± 3.98%) and *Ruminococcus* (3.73% ± 3.46% versus 4.75% ± 5.90%) decreased in POI group.

Through Wilcoxon Rank Sum tests, phylum *Bacteroidetes* (21.10% ± 14.01% versus 12.72% ± 11.96%, $P = 0.04$), genera *Butyricimonas* (0.12% ± 0.16% versus 0.04% ± 0.08%, $P = 0.03$), *Dorea* (1.91% ± 1.72% versus 1.02% ± 0.83%, $P = 0.049$), *Lachnobacterium* (0.26% ± 0.72% versus 0.0011% ± 0.0047%, $P = 0.007$) and *Sutterella* (0.34% ± 0.46% versus 0.08% ± 0.11%, $P = 0.02$) significantly increased in POI women, compared to control group. Whereas phylum *Firmicutes* ($P = 0.03$), genera *Bulleidia* (0.0006% ± 0.0033% versus 0.0094% ± 0.021%, $P = 0.007$) and *Faecalibacterium* ($P = 0.04$) significantly decreased. Moreover, *Bacteroidetes/Firmicutes* ratio (0.41 ± 0.44 versus 0.20 ± 0.24, $P = 0.03$) in POI group was significantly higher than control group.

Association between differential gut microbiota and clinical characteristics

Pearson correlation analysis was performed after adjusting for BMI to evaluate the association between the differential microbes and serum hormones (Fig. 3). The results showed that E2 level was significantly negatively correlated with relative proportion of *Bacteroidetes* (R = -0.41, $P = 0.002$) and *Bacteroidetes/Firmicutes* ratio (R = -0.45, $P < 0.01$), while positively correlated with *Firmicutes* (R = 0.30, $P = 0.03$) and *Faecalibacterium* (R = 0.28, $P = 0.047$). FSH was significantly positively correlated with *Bacteroidetes* (R = 0.31, $P = 0.02$), *Bacteroidetes/Firmicutes* ratio (R = 0.35, $P = 0.01$), and negatively correlated with *Firmicutes* (R = -0.28, $P = 0.04$). LH was significantly positively correlated with *Bacteroidetes/Firmicutes* ratio (R = 0.31, $P = 0.02$). FSH/LH ratio was significantly positively correlated with the relative proportion of *Bacteroidetes* (R = 0.35, $P = 0.01$), *Bacteroidetes/Firmicutes* ratio (R = 0.34, $P = 0.01$), *Dorea* (R = 0.40, $P < 0.01$), and negatively correlated with the relative proportion of *Firmicutes* (R = -0.30, $P = 0.03$) and *Faecalibacterium* (R = -0.34, $P = 0.01$). AMH level was significantly correlated with the relative proportion of *Bacteroidetes* (R = -0.28, $P = 0.04$), *Bacteroidetes/Firmicutes* ratio (R = -0.30, $P = 0.03$), *Butyricimonas* (R = -0.31, $P = 0.02$) and *Faecalibacterium* (R = 0.33, $P = 0.02$).

Discussion

This study aimed to reveal the overall composition of gut microbiota in women with POI. As 15–30% of POI occurrences are considered to be familial (Fenton, 2015). To rule out genetic influences, all subjects recruited in this study were without blood relationship. The results showed all recruited subjects composed primarily of phyla *Actinobacteria*, *Bacteriodetes*, *Firmicutes* and *Proteobacteria*. The sum relative abundance of *Bacteriodetes* and *Firmicutes* accounted for more than 90%, which was consistent

with previous studies (Turnbaugh et al., 2006)(Baker et al., 2017). A balance between *Bacteriodetes* and *Firmicutes* is important to maintain intestinal homeostasis (Baker et al., 2017), while a significant higher *Bacteriodetes/Firmicutes* ratio was observed in POI subjects compared to control subjects in this study. Furthermore, the abundant genera were *Bacteroides*, *Bifidobacterium*, *Blautia*, *Clostridium*, *Coprococcus*, *Faecalibacterium*, *Megamonas*, *Prevotella*, *Roseburia* and *Ruminococcus* in this study. Most of them play important roles in the maintenance of host gastrointestinal homeostasis and health (Sarkar and Pitchumoni, 2017)(Lopetuso et al., 2013). Notably, genera *Bifidobacterium*, *Blautia, Clostridium, Faecalibacterium, Roseburia* and *Ruminococcus* can produce short chain fatty acids (SCFAs) in human gut, such as acetate, butyrate and succinate and so on (Parada Venegas et al., 2019)(Ríos-Covián et al., 2016). SCFAs not only have anti-inflammatory and immunomodulatory properties, but also can influence psychological function and cognitive processes (Ríos-Covián et al., 2016)(Dalile et al., 2019). While the relative abundances of *Blautia, Clostridium*, *Faecalibacterium, Roseburia* and *Ruminococcus* presented decrease trends, especially *Faecalibacterium* decreased significantly in POI women in this study. Their reduction may impact the cognitive function and inflammation in POI subjects.

There were significant decrease of *Firmicutes*, and significant increase of *Bacteroidetes* and *Bacteroidetes/Firmicutes* ratio in POI women. It is consistent with the microbial community structure of type 1 diabetes and systemic lupus erythematosus patients, with a low proportion of *Firmicutes*, high proportion of *Bacteroidetes* and *Bacteroidetes/Firmicutes* ratio (Demirci et al., 2020)(He et al., 2016). These changes of gut microbiome also play key roles in the pathogenesis of autoimmune disease (De Luca and Shoenfeld, 2019). Moreover, genera *Dorea* and *Sutterella* were more abundant in POI group. Increase proportion of *Dorea* is related to multiple multiple sclerosis, an autoimmune condition (Chen et al., 2016). *Sutterella* is a mildly pro-inflammatory genus, and its elevated level is associated with cognitive function (Wang et al., 2013)(Ticinesi et al., 2018). As SCFAs producing members with anti-inflammatory properties, *Faecalibacterium* decreased significantly in POI group in this study, its low proportion is also related to multiple sclerosis (Cantarel et al., 2015). Butyrate producer *Butyricimonas* (de Oliveira et al., 2017) increased significantly in POI group in this study. On the contrary, a low abundance of *Butyricimonas* was observed in multiple sclerosis fecal samples (Forbes et al., 2016), that maybe due to different species under *Butyricimonas*. Type 1 diabetes, systemic lupus erythematousus and multiple sclerosis, and cognitive dysfunction are all closely related to POI. Thus, the alterations of gut microbiome observed in this study may contribute to the development of POI.

Considering hormones, significant lower level of E2 was observed in POI subjects, and it was significantly correlated with the proportion of *Bacteroidetes*, *Firmicutes* and *Faecalibacterium* by adjusting for BMI. Moreover, the FSH, LH and AMH levels were also affected by gut microbiome observed in this study. Accumulating researches indicates that estrogens regulate glucose and lipid metabolism, bone formation and inflammatory response, its reduction can impair estrogen-dependent processes, triggering cardiovascular disease, osteoporosis and so on (Baker et al., 2003)(Li et al., 2016). The gut microbiome has been shown to play a important part in impacting estrogen level through the secretion of β-glucuronidase, which could deconjugate estrogen and affect related physiological process (Plottel and Blaser, 2011)(Baker et al., 2017). It indicates that the altered gut microbiota of POI is associated with the

sex hormones. Yet the mechanism under the relations between gut microbiome and sex hormones is not clear due to limited data in this study.

POI leads to several complications, including decrease in bone mineral density, autoimmune, thyroid disease risk and cognitive dysfunction. This study revealed the association between gut microbiota and these complications. These indicate that the dysbiosis of gut microbiota may contribute to the development of POI discussed above. However, limited sample size, participants from the same hospital and the observation of association but not causality, large sample size and multi-center are needed in the further studies. Moreover, metagenome sequencing, measurements of metabolites produced by gut microbiota, animal experiments should also be considered to explore the potential causal mechanism.

In summary, this study demonstrated an altered gut microbial pattern in women with POI against healthy controls. These changes of microbes were closely related to serum hormones. It lays a foundation for revealing the interaction between gut microbiota and POI certainly.

Declarations

Ethics approval and consent to participate

This study was approved by the Ethics Committee of Shenzhen Maternity&Child Healthcare Hospital (No. SFYLS[2020]012), Shenzhen, China.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Availability of data and material

The dataset supporting the conclusion of this article is available in the NCBI's Sequence Read Archive database (SRA BioProject ID PRJNA615330).

Authors' contributions

1. Yao and Y. Ning conceived of the presented idea and planned the experiments. Y. Zhuo and Y. Chen carried out the experiments. J. Yu, and Y. Liu designed the computational framework and analyzed

the data. J. Wu, J. Yao, Y. Ning and Y. Zhuo wrote the manuscript. All authors discussed the results and contributed to the final manuscript.

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Figures

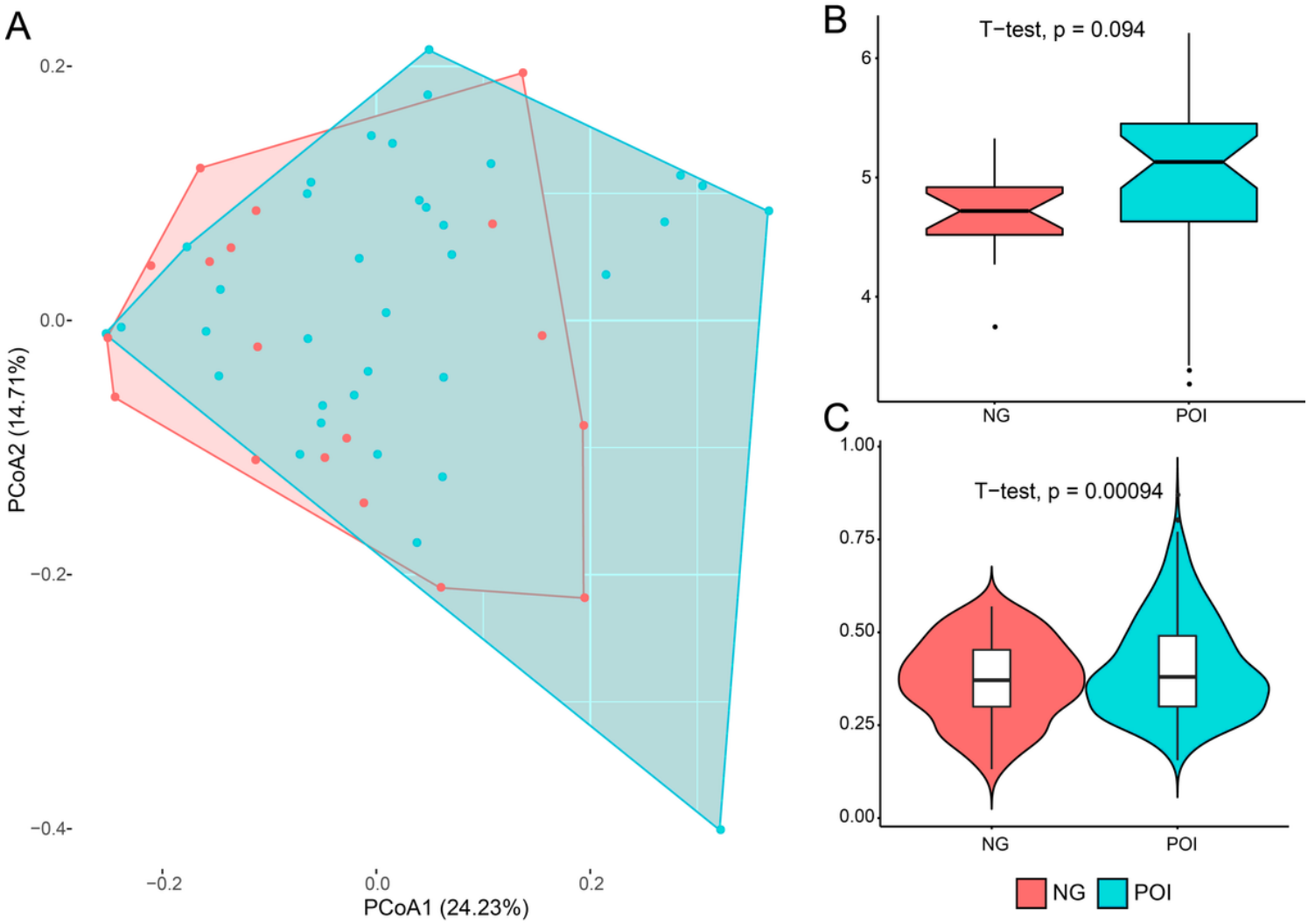

Figure 1

Overall structural differentiation of gut microbiota between the two groups. POI represented women with premature ovarian insufficiency, NG represented healthy control women. (A) PCoA analysis plot based on weight UniFrac value. (B) Shannon index between the two groups. (C) Weighted UniFrac value between the two groups.

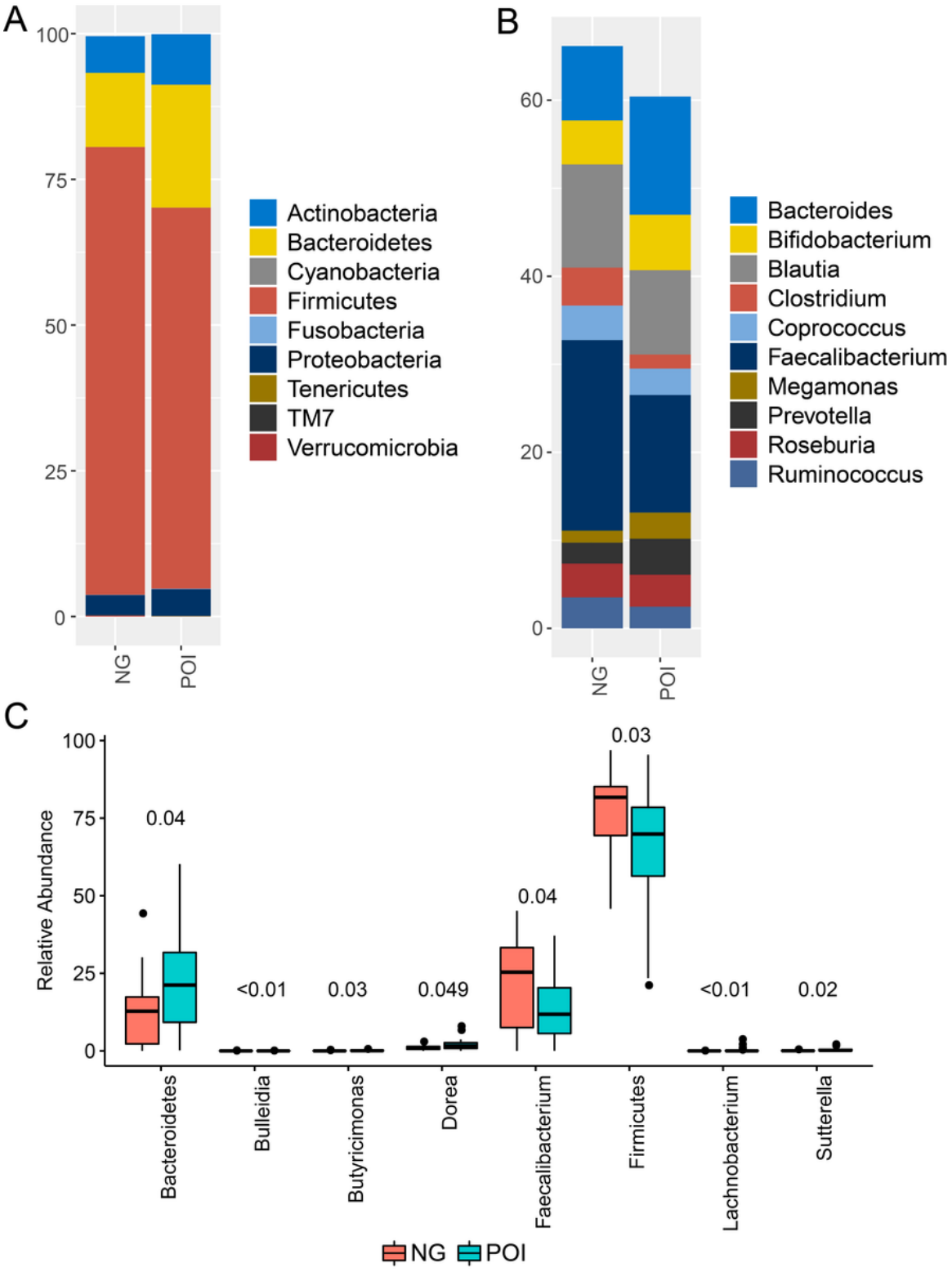

Figure 2

Microbial community profiles of gut microbiota between POI and control group. POI represented women with premature ovarian insufficiency, NG represented healthy control women. (A) Relative abundances of the dominant phylum. (B) Relative abundances of the top 10 genera according to the relative abundance. (C) Significant different and important microbes between POI and control group.

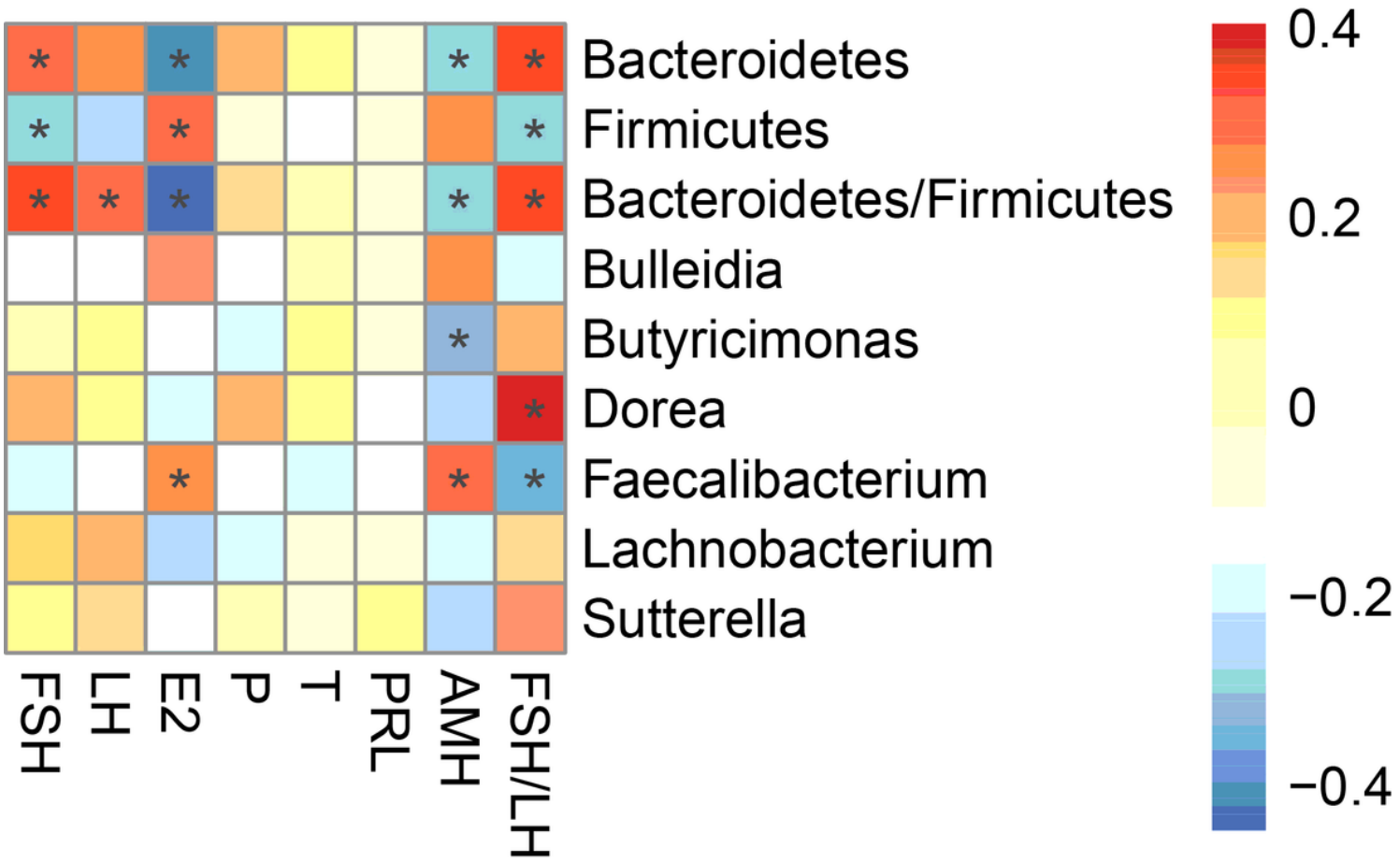

Figure 3

Associations between gut microbiota and serum hormones. The star indicate the significant correlation at 5% level.