

Effect of Electro-Acupuncture on Gut Microbiome in Cisplatin-Induced Premature Ovarian Failure Mice

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Research

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Abstract

Background

Growing evidences showed that gut microbiota is associated with premature ovarian failure (POF). Many case reports had shown that electro-acupuncture was effective in the treatment of POF. However, there was little research on regulating gut microbiome of POF mice by electro-acupuncture. Therefore, this study aimed to verify whether electro-acupuncture could improve ovarian function and regulate gut microbiome in mice with POF. In addition, the therapeutic effects of electro-acupuncture at acupoints and non-acupoints were compared.

Methods

POF mice were established by injected intraperitoneally with cisplatin (2mg/kg) for 2 weeks. Guanyuan (CV4) and Sanyinjiao (SP6) were selected in the electro-acupuncture at acupoints group (EA group). Non-acupoints which around CV4 and SP6 were selected in the electro-acupuncture at non-acupoints group (EN group). EA group and EN group were treated for 3 weeks. The ovarian function was evaluated by assays of histopathological and molecular. Meanwhile, the gut microbiome of POF mice was detected by 16S rDNA sequencing.

Results

The results showed that EA could restore the estrous cycle and reduce the number of atresia follicles in POF mice. The levels of serum follicle stimulating hormone and luteinizing hormone were decreased by EA. As well as, the levels of serum estradiol, anti mullerian hormone and β -glucuronidase were increased by EA. The relative expressions of PI3K, AKT and mTOR were increased to promote the proliferation of ovarian cells in EA group. According to the results of 16S rDNA sequencing, we found that the diversity of gut microbiome in mice with POF was changed. However, the abundance and diversity of gut microbiome could be regulated by EA in POF mice. The relative abundance of beneficial bacteria was increased by EA. The KEGG pathway analysis showed that the gut microbiome which associated with estrogen signaling pathway, oocyte maturation and PI3K-AKT signaling pathway were regulated by EA.

Conclusions

EA could provide protection against cisplatin-induced ovarian damage. The abundance and diversity of probiotics could be increased by EA. Moreover, EA might treat POF via regulating the gut microbiome.

Background

Nowadays, more and more patients were diagnosed as premature ovarian failure (POF) in clinics. The epidemiology showed that the incidence rate of women with POF in childbearing age was over 4% [1]. POF was the premature decline or failure of ovarian function before 40 years old. The level of serum follicle stimulating hormone (FSH) was increased and estradiol (E₂) was decreased in patients with POF [2]. POF could be caused by metabolic disorders, gonadotropin dysfunction, immune damage, chemical damage, genetic factors and others. However, the etiology remains unclear in nearly half of the patients with POF [3]. Hormone therapy (HT) could supplement exogenous hormones and relieve the symptoms caused by hormone deficiency in time [4]. However, the risk of breast cancer would be increased by long-term using of HT [5]. Therefore, an alternative or complementary therapy was needed for the treatment of POF.

Acupuncture is one of the most popular therapies in Chinese traditional medicine. Electro-acupuncture is an improved version of acupuncture, which is an electrical stimulation on the needle handle after acupuncture. Several clinical randomized controlled trials had shown that electro-acupuncture could improve ovarian function and increase the pregnancy rate in patients with POF [6].

Recently, the gut microbiome had been proved to participate in the enterohepatic circulation of estrogen to regulate the level of estrogen in serum [7]. As reported, the level of estrogen could induce a positive and negative feedback mechanism through the hypothalamus pituitary ovary (HPO) axis [8]. Although mainly estrogen was secreted from ovary, the level of estrogen in serum might also affect the functions of ovary [9]. Therefore, the gut microbiome might be closely related to ovarian function. However, to the best of our knowledge, there were few studies on the correlation between gut microbiome and electro-acupuncture for POF.

In this study, 16S rDNA sequencing assay was applied to investigate the effect of electro-acupuncture on gut microbiome in mice with POF. In addition, the assays of histopathological and molecular biological were used to evaluate the effect of electro-acupuncture for ovarian function.

Materials And Methods

Animals

All animals were raised in the experimental animal center of Xiamen University. The study was approved by the Animal Care and Use Committee of Xiamen University (Permit Number: SCXK: 2018-0003). The experimental procedures were in accordance with the ethical guidelines of International Council for Laboratory Animal Science (ICLAS).

40 ICR female mice (30g ± 5g) were randomly divided into 4 groups (n = 10 each group), which were: control group, POF group, electro-acupuncture at acupoints group (EA group), and electro-acupuncture at non-acupoints group (EN group). The POF mice were established by intraperitoneal injection of cisplatin (2mg/kg) for 2 weeks in groups except control group [10].

Electro-acupuncture treatment

According to the “Experimental acupuncturelog”, Guanyuan (CV4), Sanyinjiao (SP6) and non-acupoints (5 mm horizontally beside CV4 and 3 mm vertically above SP6) were selected. The non-acupoints did not belong to any meridians. Subsequently, all acupoints and non-acupoints were disinfected by 75% alcohol before electro-acupuncture. The 0.25mm×13mm acupuncture needles (Suzhou Medical Appliance Factory, JiangSu, China) were be selected. The depth of acupuncture was about 3-5mm. The current output mode of the electro-acupuncture instrument (Model SDZ-II; Suzhou Medical Appliance Factory, Jiangsu, China) was alternate of sparse wave and dense wave (intermittent wave: 4 Hz; irregular wave: 50 Hz). The POF mice in EA group and EN group were treated by electro-acupuncture 30 min daily for 3 weeks (Fig. 1A and B).

Afterwards, all mice were sacrificed by enucleated eyes at the end of electro-acupuncture treatment.

Observation of estrous cycle

20μL of 0.9%NaCl solution was infused into the vagina of mice by the pipette gun and pumped gently several times. Then, the solution was sucked out onto the glass slide and stained. Vaginal exfoliated cells were observed under the light microscope. The above steps were repeated daily during the period of experiment.

Histopathology examination

Ovaries were collected and dehydrated in 10% paraformaldehyde for 48 hours after mice were sacrificed, and embedded with paraffin after ethanol dehydration. Subsequently, ovaries were sliced to a thickness of 5mm, and the morphology of ovary was observed under light microscope after stained.

Enzyme Linked Immunosorbent Assay (ELISA)

Blood samples were collected via enucleated eyes. Blood samples were centrifugated (10000rpm 10 min 4°C) to obtain the serum, and the FSH, E₂, luteinizing hormone (LH), anti mullerian hormone (AMH) and β-glucuronidase were detected by ELISA kit. All experimental processes were according to the protocol provided by the manufacturer.

Quantitative Real-time PCR (qPCR)

The relative expressions of PI3K, AKT and mTOR in ovary were detected by qPCR. Then, the ovary was put into phosphate buffer solution (PBS) and homogenized on ice. The homogenate was centrifuged (3000r/min 15min 4°C) and the upper liquid was collected. Afterwards, total RNA was extracted by the method of TRIpure and reverse transcribed into cDNA. The CT value was derived and the relative expression of PI3K, AKT and mTOR were calculated according to the $2^{-\Delta\Delta CT}$ value after amplification.

Detection and analysis of gut microbiome

Fecal samples were collected in the sterile laboratory before sacrificed. The samples of fecal were placed into sterile centrifuge tubes with PBS and mixed. All samples were centrifuged (4000r/min 4°C 30min) and precipitates were retained. After, total DNA was extracted according to the protocol of kit (Thermo Fisher, DNAzol, USA). The DNA of bacillus coli was applied as a template for amplification. Clone databases of 16S rDNA gene were constructed according to DNA Sample Prep Kit (Illumina, TruSeq™, USA), and DNA sequences were detected (Illumina, MiSeq, USA). Finally, original sequences were spliced and filtered.

Principal Component Analysis (PCA) was applied to compare the bacterial diversity of gut microbiome. The analysis of similarities (ANOSIM) was applied to analyze whether there was comparability between each group. In addition, the linear discriminant analysis (LDA) was applied to screen out the different species through LEfSe. Finally, the function of gut microbiome was annotated according to Kyoto Encyclopedia of genes and genomes database (KEGG database).

Results

Weight

All mice were weighted after treated for 3 weeks. The weight of POF group was significantly lower than control group after modeling. Although the weight of EA group showed a recovery trend and besides, a significant increase could also be observed than the POF group, the weight of EA group was still slight lower than control group. Compared with the POF group, the weight of EN group had no significant difference (Fig. 1C).

Observation of estrous cycle

Vaginal exfoliated cells examination was applied to confirm the estrous cycle in mice. The vaginal exfoliated cells of all mice were evaluated, and the representative pictures which could clearly show the estrous cycle were selected. In the control group and EA group, nucleated epithelial, cornified epithelial, and leukocytes were observed alternately. There were long-term existence of nucleated epithelial and leukocytes, but cornified epithelial was not observed in the POF group and EN group. These results indicated that POF mice were established successfully. (Fig.S1)

Histopathology examination

As shown in Fig. 3, the antral follicles and corpus luteum could be observed in the control group. On the contrary, the number of atresia follicles increased in the POF group, which indicated the POF model was established successfully. There were more intact follicles in the EA group than POF group. Moreover, atresia follicles were decreased in the EA group. Although there were a few antral follicles in the EN group, atresia follicles and the structure of vacuoles could still be observed (Fig. 2).

ELISA examination

In this study, it was found that the high levels of FSH and LH in POF mice. On the contrary, the levels of E₂, AMH, and β -glucuronidase were decreased significantly in POF mice. Compared with the POF group, the levels of FSH and LH were significantly decreased, while the levels of E₂, AMH, and β -glucuronidase were increased after EA. There was no significant difference in the levels of FSH, LH, AMH and β -glucuronidase between EN group and POF group. However, the level of E₂ in EN group was significantly higher than that in POF group (Fig. 3).

QPCR examination

The results showed that the relative expressions of PI3K, AKT and mTOR in POF group were lower than those in control group. Obviously, there were no significant difference in the relative expression of PI3K, AKT and mTOR between EA group and control group. In addition, the expressions of PI3K, AKT and mTOR in EA group were significantly increased from those in EN group (Fig. 4).

Relative content of gut microbiome

The dual terminal sequence data were obtained by MiSeq sequencing platform. Non-repetitive sequences of OTUs were clustered according to more than 97% similarity. Moreover, chimeras were removed in the clustering process to obtain OTU sequences. The rarefaction curves were constructed according to the sobs index of each sample at different sequencing depths. The sequencing depth of all samples was more than 25000. With the increase of sequencing depth, the dilution curve tended to be flat. All in all, the sequencing depths of all samples were reasonable (Fig. 5A).

In this study, there were no significant difference in the sobs index of OUT level between control group, POF group and EN group. However, the sobs index of OUT level in EA group was significantly higher than POF group (Fig. 5B).

Diversity of gut microbiome

As shown in **Fig.S2-S5**, ANOSIM analysis and PCA analysis indicated that the diversity of gut microbiome in each group was significant different. PCA analysis showed that the gut microbiome of POF group and EA group had obvious dispersion, and there was significant difference between POF group and EA group (Fig. 6). Similarly, although the PCA showed that diversity of gut microbiome in POF group and EN group had poor dispersion, there was significant difference between POF group and EN group (Fig. 7).

Species Changes of gut microbiome

To screen the different species of microbiome in each group, LDA was applied. And the top three most diverse and highest expressions dominant microbiomes in each group were selected. According to the results, the dominant microbiomes of the control group were *Lactobacillus*, *Muribaculaceae*, *Monoglobus*. Meanwhile, the dominant microbiomes in POF group were *Lachnospiraceae*, *Eubacterium*

coprostanoligenes and *Blautia*. Then, *Anaeroplasma*, *Ruminococcaceae* and *Eubacterium ventriosum* were the dominant microbiomes of EA group. In addition, the dominant microbiomes of EN group were *Tannerellaceae*, *Parabacteroides* and *Streptococcaceae* (Fig. 8).

To compare the relative abundance of gut microbiome, three highest expressions dominant microbiomes in the control group were compared with other groups. The relative abundance of *Lactobacillus* in EA group was significantly higher than that in POF group and EN group. Then, the relative abundance of *Muribaculaceae* in EA group was significantly lower than that in control group. However, there was no significant difference in the relative abundance of *Muribaculaceae* between EA group and POF group. Furthermore, the relative abundance of *Monoglobus* in the control group was significantly higher than that in other groups, but there was no significant difference between POF group and EA group (Fig. 9).

The *Firmicutes/Bacteroidetes* (F/B) ratio was considered to be an important indicator of gut microbiome homeostasis [11]. Although the F/B ratio in EA group was significantly higher than that in control group, EA group was significantly lower than that in POF group. Meanwhile, there was significant difference between EA group and EN group. (Fig. 10)

Functionality of the gut microbiome

According to the KEGG database, estrogen signaling pathway, oocyte maturation and PI3K-AKT signaling pathway were selected which were the most related to POF and the number of sequences was calculated.

The results showed that the numbers of sequence which associated with estrogens signaling, oocyte nutrition and PI3K-AKT signaling pathway were increased in POF group. Meanwhile, the numbers of sequence in EA group was lower than that of POF group. There was no significant difference between EA group and control group. At the same time, there was no significant difference in the numbers of sequence between EN group and POF group (Fig. 11).

Discussion

Patients with POF usually have symptoms such as amenorrhea for more than four months, hot flashes, night sweats, low libido and infertility [2]. Cisplatin as one of the most common-seen drugs for chemotherapy and considered to activate PTEN through the PI3K-AKT signaling pathway, which might potentially lead to follicular atresia [12]. Herein, the POF mice were established by cisplatin in this study. The pathological morphology of ovary and vaginal exfoliated cells could reflect the ovarian status of mice. According to the above results, we found that EA could repair the injured of ovary and restore estrous cycle in cisplatin-induced POF mice. On the contrary, EN had little effect on the regulation of ovarian pathological morphology and estrus cycle.

FSH, LH, E2 and AMH are the important sex hormones that can reflect ovarian function [2], while both FSH and LH are thought to be secreted by hypophysis, while E2 is mainly secreted by granulosa cells in ovary. Serum AMH is applied to detect ovarian reserve function in clinic as reported [13]. The decrease of

AMH level indicated premature depletion of primordial follicles. E2 can affect the levels of FSH and LH through the mechanism of negative feedback in HPO axis, which makes the sex hormones maintain a dynamic balance [8]. Follicular atresia causes the decrease of granulosa cells in follicles when POF occurs [14]. Therefore, POF can cause E2 and AMH to maintain at a low level. The long-term maintenance of low serum E2 level will break the negative feedback mechanism between ovary and pituitary, and lead to the levels increased of FSH and LH [15]. Therefore, both the high levels of FSH and LH in patients with POF, while low levels of E2 and AMH. According to the results, it was found that EA could effectively restore the levels of serum FSH, LH, E2 and AMH in POF mice. However, EN did not restore the levels of FSH, LH, E2 and AMH. These results might be related to EA increasing the number of antral follicles in ovary. It showed that acupoints could improve ovarian function more effectively than non-acupoints.

PI3K-AKT signaling pathway is involved in the process of oocyte growth, primordial follicle development and granulosa cell proliferation [16]. FSH is a glycoprotein hormone synthesized by hypophysis. FSH can combine with the receptor on the membrane of ovarian granulosa cells to activate PI3K-AKT pathway to promote the maturation of ovarian granulosa cells [17]. On the other hand, E2 can combine with the estrogen receptor α (ER- α) and estrogen receptor β (ER- β) in ovary [18]. PI3K has subunits of p85 and P110. ER- α combines with the subunit p85 in PI3K to activate AKT [19, 20]. Meanwhile, mTOR is activated by AKT, which leads to proliferation and development of follicle [21]. In this study, we found that the PI3K-AKT signaling pathway in ovary of cisplatin-induced POF mice was inhibited. The relative expression of PI3K, AKT, and mTOR were reversed to normal in POF mice by EA. The result indicated that PI3K-AKT signaling pathway could be activated by electro-acupuncture at acupoints to promote the proliferation of ovarian cells. The results were consistent with the previous research results of other subjects.

There are a large number of microbiomes in human intestine, including probiotics, pathogenic bacteria and conditioned pathogen [22]. Gut microbiome is considered to be closely related to human health [23]. Growing evidence shows that the disorder of gut microbiome can lead to ovarian diseases, endocrine diseases and cardiovascular diseases [24, 25]. It had been reported that the disorder of gut microbiome would break the barrier of intestinal mucosa [26]. Pathogenic bacteria and their metabolites reached the ovary through the circulation of blood to reduce ovarian function [27]. On the other hand, the enterohepatic circulation of estrogen was an important way to maintain the stability level of human serum estrogen [28]. Serum free estrogen is converted into conjugated estrogen by the liver. Then, the conjugated estrogen is excreted into the intestine with the bile [29]. β -glucuronidase was considered to be one of the metabolites of gut microbiome. Conjugated estrogen was converted into free estrogen by β -glucuronidase [30]. Free estrogen enters the circulatory system of blood through intestinal resorption to promote growth of follicle and relieve the symptoms of POF.

In the control group, the three highest relative expression dominant microbiomes were *Lactobacillus*, *Muribaculaceae* and *Monoglobus*. *Lactobacillus* plays an important role in maintaining human health and is considered as one of the most important probiotics in human intestinal [31]. It had been reported that *Lactobacillus* could inhibit the growth and reproduction of pathogenic bacteria [32]. *Muribaculaceae* is widely distributed in the intestines of mice. *Muribaculaceae* can reduce the colonization of *Clostridium*

difficile in the intestine [33]. Meanwhile, *Muribaculaceae* can degrade carbohydrates. In addition, *Monoglobus* can degrade pectin and maintain intestinal health [34]. According to the above results, we found that the three highest relative expression dominant microbiomes were probiotics in control group. They are important to maintain the health physiological function of intestine.

Lachnospiraceae, *Eubacterium coprostanoligenes* and *Blautia* were the dominant microbiomes in the POF group. *Lachnospiraceae* was considered to protect the intestinal mucosa [35]. On the contrary, *Lachnospiraceae* also could damage the pathway of glucose metabolism and promote the process of inflammation [36]. Therefore, *Lachnospiraceae* was considered as a conditional pathogen. *Eubacterium coprostanogenes* was considered to be pathogenic bacteria. It had been reported that *Eubacterium coprostanogenes* could reduce the intestinal absorption of cholesterol [37, 38]. *Blautia* could prevent inflammation and promote the production of short chain fatty acids (SCFAs) to maintain intestinal homeostasis, so it was considered to be a potential probiotic [39, 40]. The existence of beneficial bacteria in the first three dominant bacteria of POF mice whether be related to the self-healing function of mice, and it still needed to be further researched.

In the EA group, *Anaeroplasma*, *Ruminococcaceae*, and *Eubacterium ventriosum* were the three microbiomes with the highest relative expression. *Anaeroplasma* was an agent of anti-inflammatory. In addition, *Anaeroplasma* could maintain the homeostasis of immune in intestinal mucosal [41]. Similarly, *Ruminococcaceae* and *Eubacterium ventriosum* had the function of anti-inflammatory, and both were the common beneficial bacteria in the intestine [42, 43]. As a member of SCFAs producer, *Ruminocaceae* was considered to maintain intestinal immune homeostasis. All the first three dominant microbiomes in POF mice were probiotics after treating by EA. The result indicated that EA could increase the abundance and diversity of probiotics.

The dominant microbiomes of three highest relative expressions in the EN group were *Tannerellaceae*, *Parabacteroides*, and *Streptococcaceae*. Several studies had found that *Tannerellaceae* promoted the occurrence of intestinal inflammation [44]. *Parabacteroides* were considered to be beneficial bacteria [45]. Moreover, it had been reported that *Parabacteroides* could alleviate liver injury and reduce the expression of inflammatory genes in liver [46]. *Streptococceae* is a conditioned pathogen, which was a common microbiome in human oral cavity, skin, intestinal, and upper respiratory tract [47]. Although the content of probiotics could be increased by EN, the dominant bacteria were pathogenic bacteria.

According to the results, EA could significantly reduce the F/B ratio, which indicated that EA had a good effect on improving the structure of gut microbiome. The abundance and diversity of gut microbiome in POF mice could be regulated by EA. According to the results of KEGG pathway analysis, the gut microbiome which related to estrogen signaling pathway, oocyte nutrition and PI3K-AKT signaling pathway were regulated by EA. These findings indicated that EA might play a role in the treatment of POF by regulating the abundance and diversity of gut microbiome.

In the future, antibiotic cocktail mice would be selected to further research the therapeutic mechanism of EA on POF by fecal microbial transplantation and macrogene sequencing.

Conclusion

EA could restore ovarian pathological morphology and estrous cycle of mice with POF. At the mean time, EA was also found to regulate sex hormones and PI3K-AKT signaling pathway in mice with POF. Further, we speculated that estrogen level might be restored by EA through regulating the abundance and the diversity of gut microbiome in POF mice. Estrogen combined with the estrogen receptor on follicular granulosa cells to activate PI3K-AKT signaling pathway, and that could promote the growth and development of follicles.

The disease from POF would become an increasing public health burden. Regulating the gut microbiome by EA to impact ovarian function provides an exciting future therapeutic. This study provided a preliminary verification for revealing the mechanism of EA in the treatment of POF.

Declarations

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Authors' Contributions

Q-DH, Z-BY and MC designed the project; Q-DH, J-JG, QZ, Z-ZH and YY conducted the experiments; YY and MC analyzed all data; Q-DH and Z-YT wrote original draft preparation; Z-BY and MC revised the manuscript. All authors had read and agreed to the published version of the manuscript.

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Availability of data and materials

All data of this study were supplied from the corresponding author (Min Chen, mchen@must.edu.mo) upon reasonable request.

Ethics approval and consent to participate

The study was approved by the Animal Care and Use Committee of Xiamen University (Permit Number: SCXK: 2018-0003).

Consent for publication

The manuscript is approved by all authors for publication.

Competing interests

All authors have no competing interests with respect to the manuscript.

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Figures

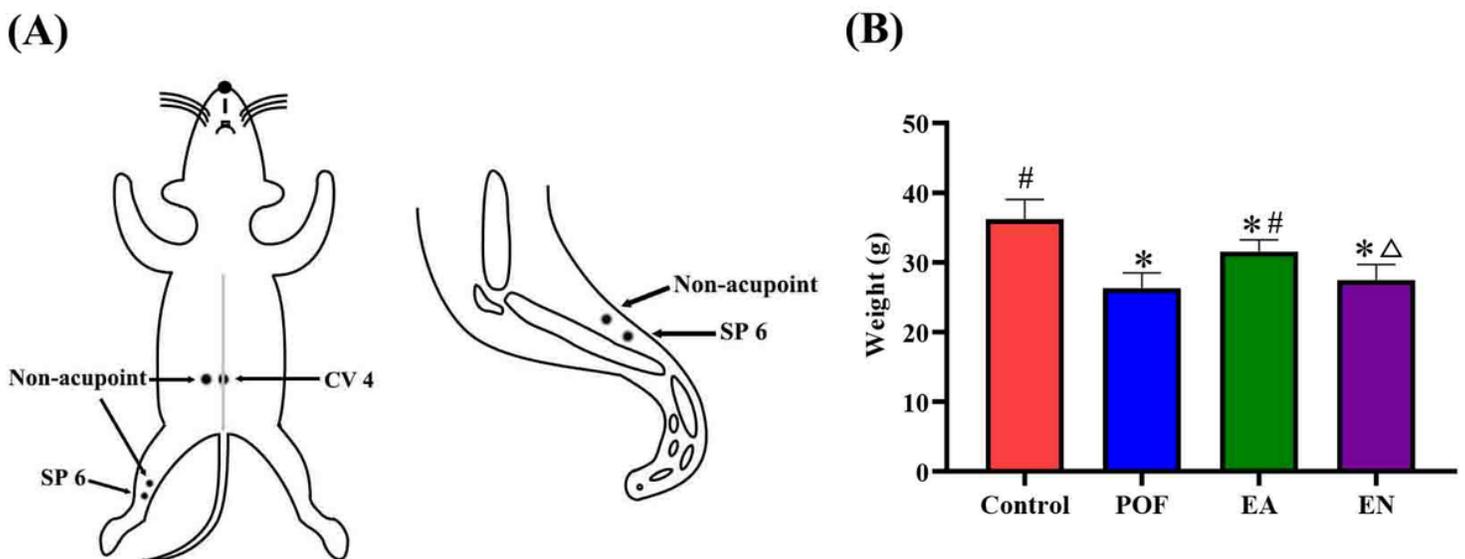


Figure 1

The selected points and weight of mice. A and B: acupoints and non-acupoints. C, the weight of mice in each group (*mean significant difference from the control group at $P < 0.05$; # mean significant difference from the POF group at $P < 0.05$; Δ mean significant difference from the EA group at $P < 0.05$).

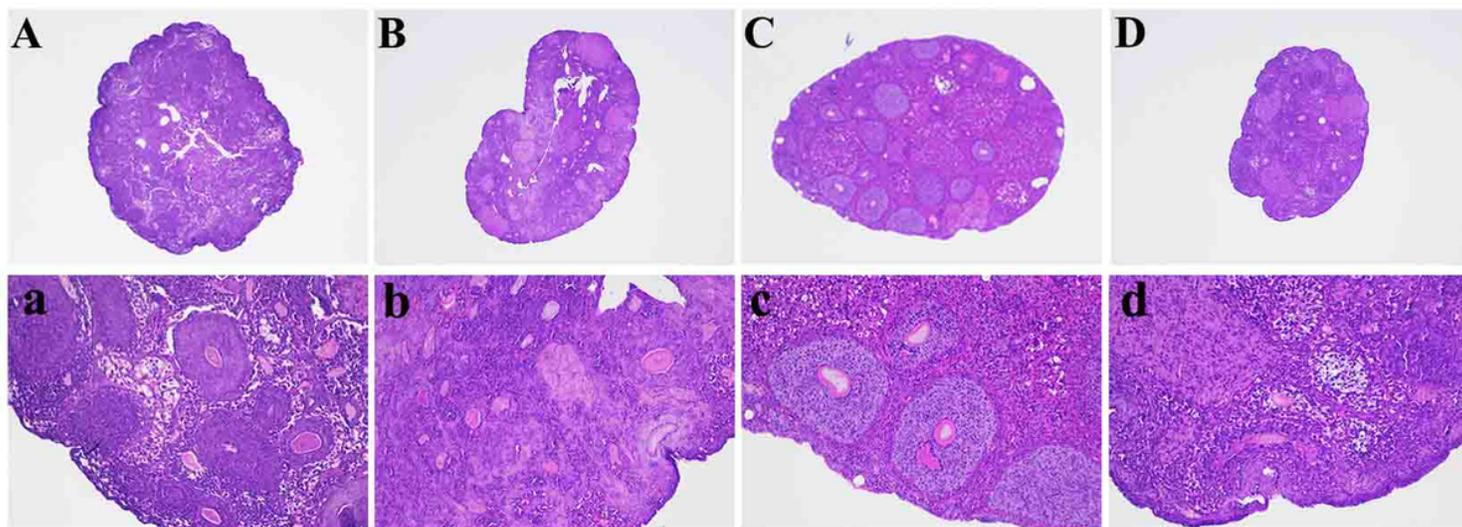


Figure 2

The pathological examination of ovaries in mice of each group. (A and a, mean control group; B and b, mean POF group; C and c, mean EA group; D and d, mean EN group)

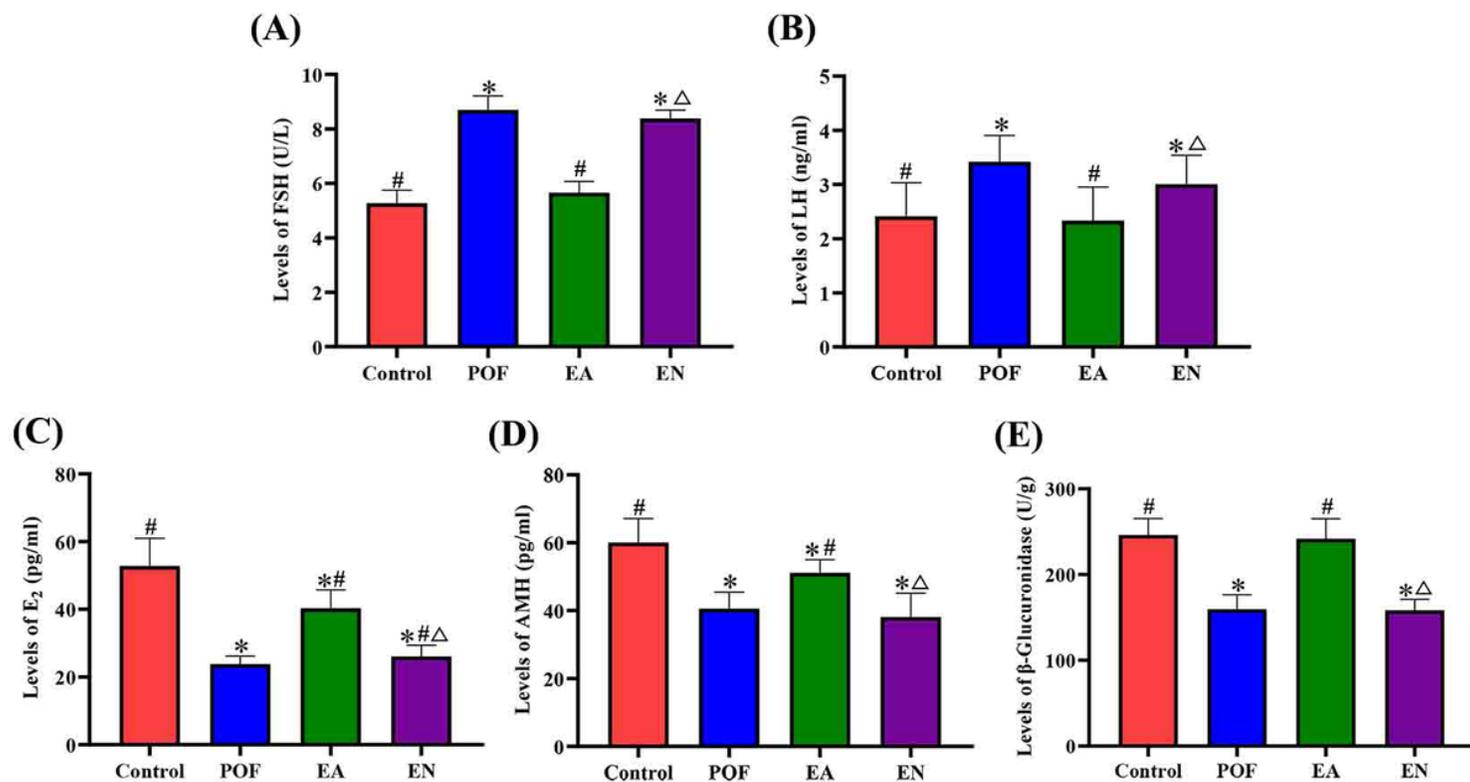


Figure 3

The levels of serum (A) FSH, (B) LH, (C) E2, (D) AMH and (E) β -glucuronidase in different groups. (*means significant difference from the control group; # means significant different from the POF group; Δ means significant different from the EA group.)

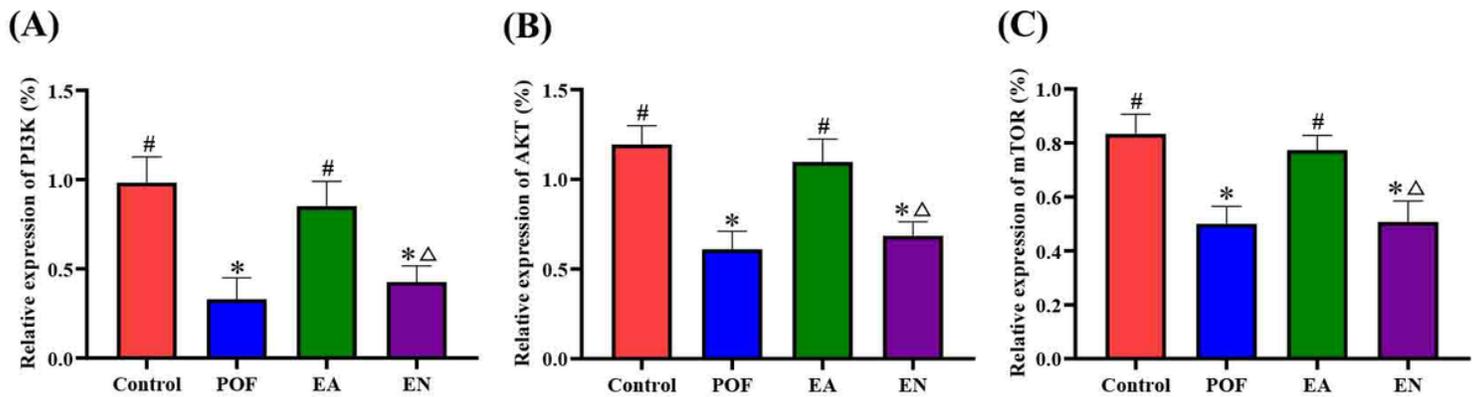


Figure 4

The relative expressions of PI3K, AKT and mTOR in ovary. (*means significant difference from the control group; # means significant different from the POF group; Δ means significant different from the EA group.)

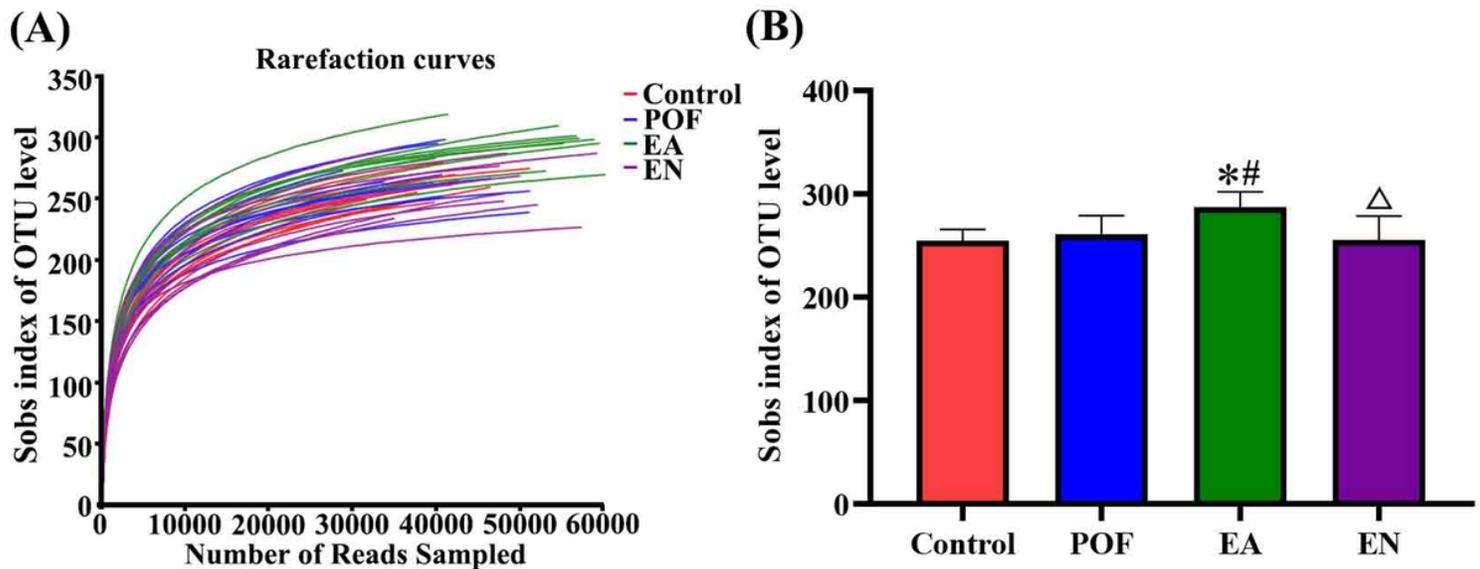


Figure 5

Sobs index and rarefaction curves of gut microbiome at OUT level. (*means significant difference from the control group; # means significant different from the POF group; Δ means significant different from the EA group.)

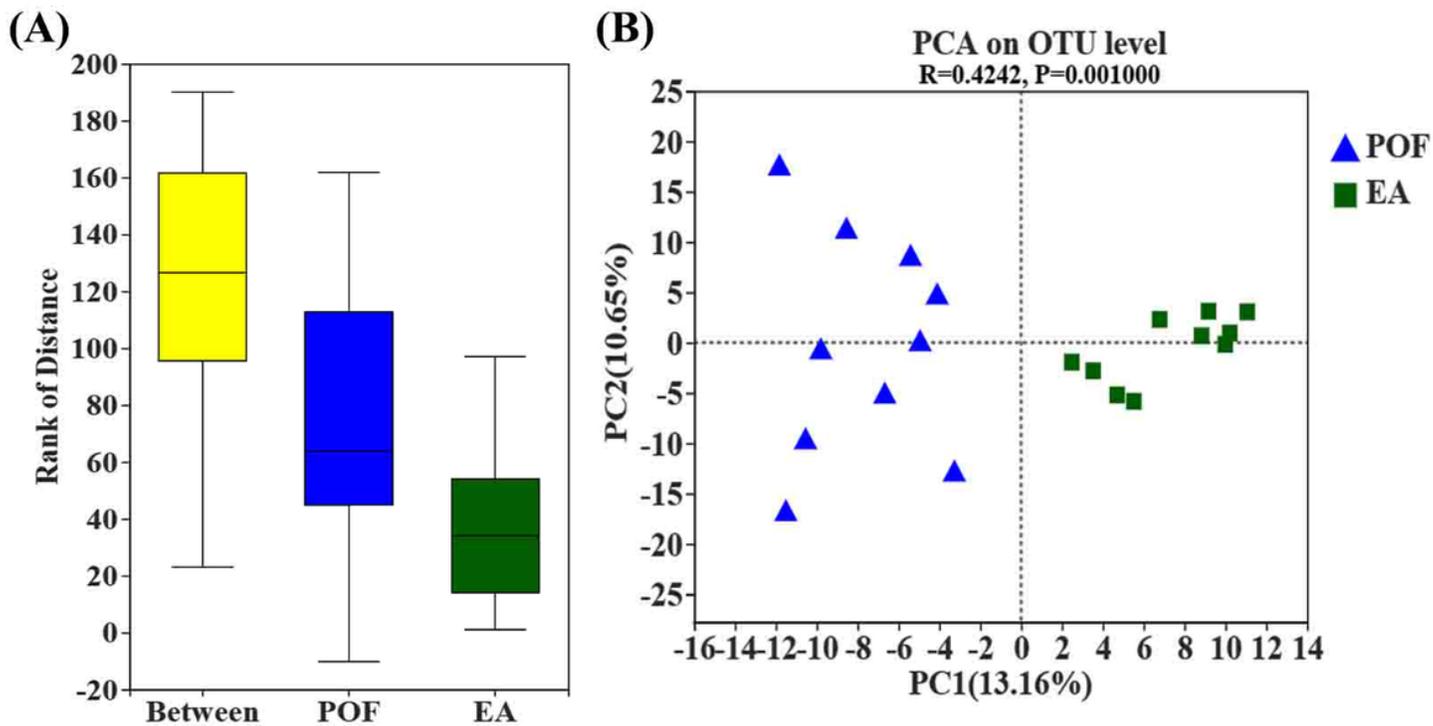


Figure 6

ANOSIM and PCA in (A and B) the POF group and EA group.

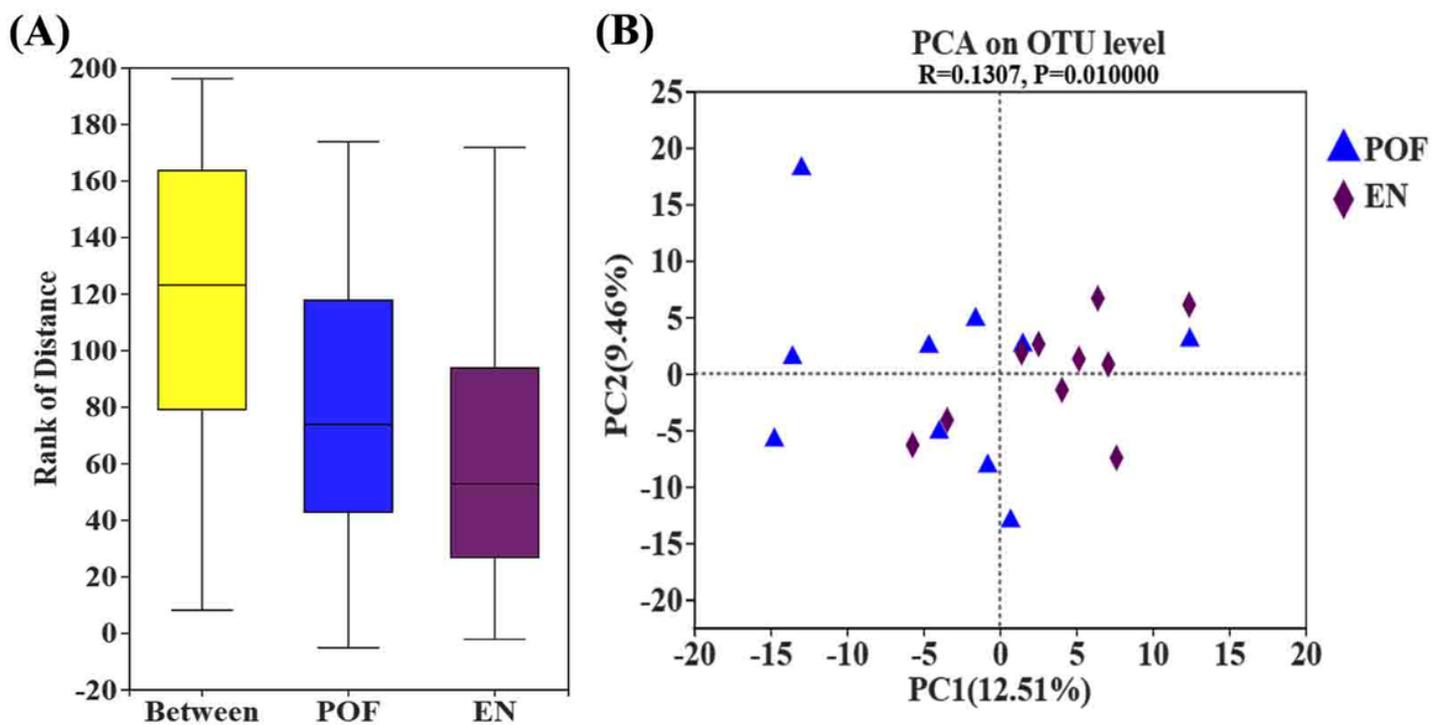


Figure 7

ANOSIM and PCA in (A and B) the POF group and EN group.

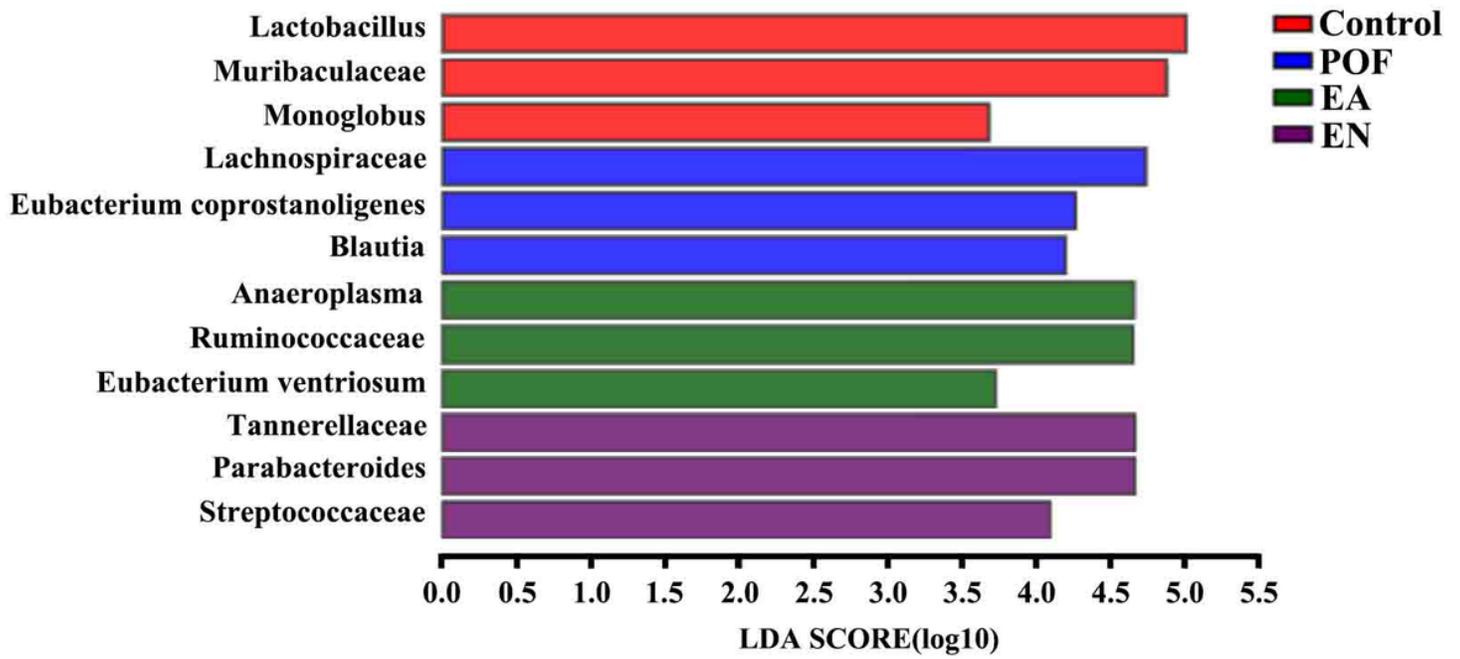


Figure 8

The histogram of LDA value distribution in each group. Three microbes with the most difference significantly were selected in each group.

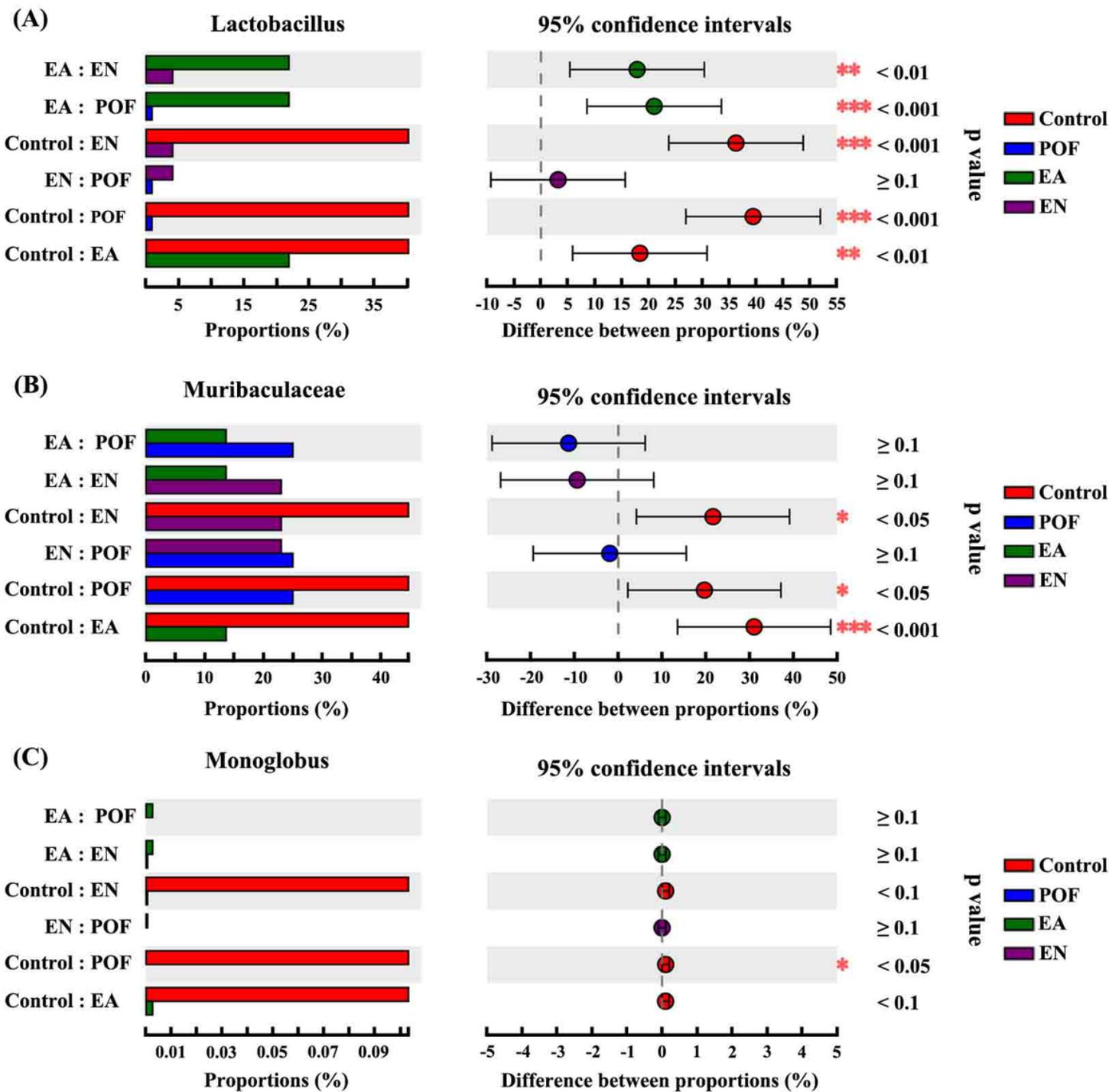


Figure 9

The relative expression levels of the first three dominant bacteria in control group were compared with those in the other groups.

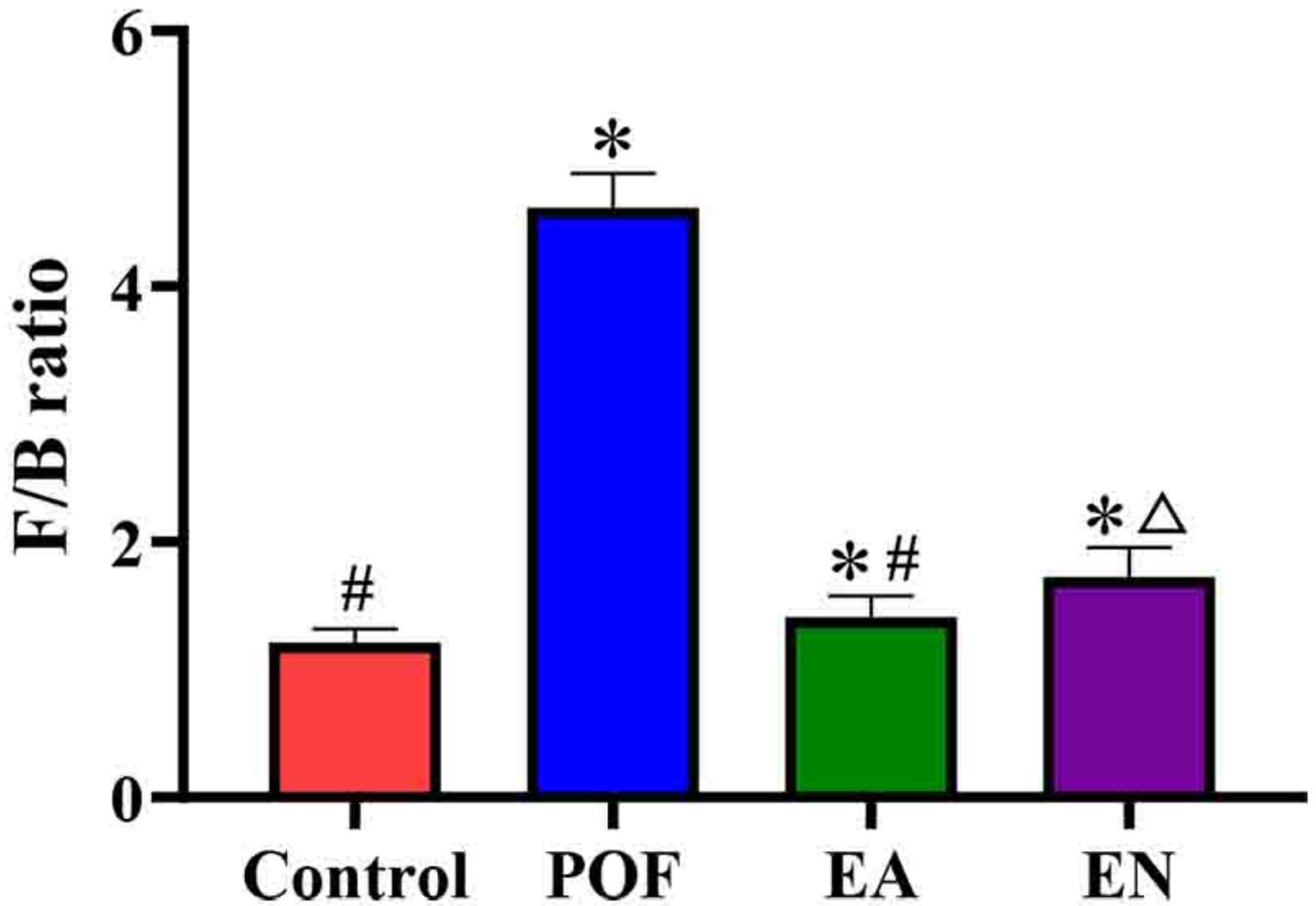


Figure 10

The Firmicutes/Bacteroidetes (F/B) ratios in groups. (*means significant difference from the control group; # means significant different from the POF group; Δ means significant different from the EA group.)

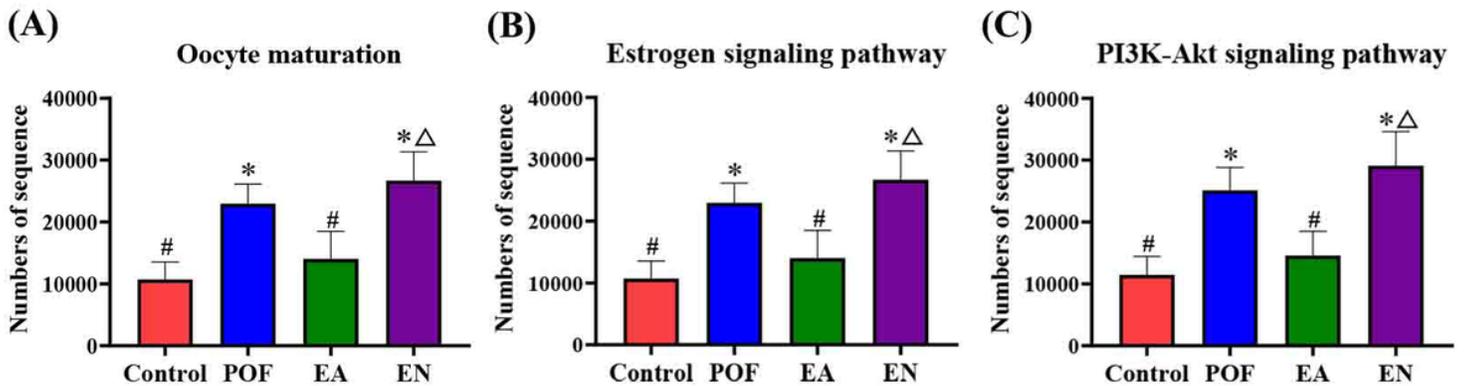


Figure 11

The KEGG database was applied to compare the levels of gut microbiome which related to estrogen signaling pathway, oocyte nutrition and PI3K-Akt signaling pathway in each group. (*means significant difference from the control group; # means significant different from the POF group; Δ means significant different from the EA group.)

Supplementary Files

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