Peer Review File

Article Information: http://dx.doi.org/10.21037/atm-20-2813

Reviewer A

#1 The level of English language is not satisfactory. The manuscript should be rewritten (including figure legends) and edited for the language from a native English speaker; There are a plethora of typing errors…

Response: Thank you for your correction. We are really very sorry for our incorrect writing to confuse the reviewer. And accordingly our revised manuscript have been edited by a native English speaker.

#2 The introduction lacks almost completely the description of previous studies associated with endometrial microbiota.

Response: We appreciate your suggestion, which reminds us that we should describe the previous studies associated with endometrial microbiota. And this topic has been revised in the **2nd paragraph** of **Introduction** as followings:

"As we known, the balance of micro-ecology on the female reproductive tract plays a key role in health. An increasing body of evidence suggests that the change of composition and distribution of endometrial microbiota is closely relevant to endometrial diseases such as endometrial polyps, endometrial cancer, infertility and so on [4-6]. Theoretically, the endometrial infection may be related with micro-ecological imbalance. With recent researches, patients with IUA have a micro-ecological imbalance in lower genital tract, and the V4 region or the V3 and V4 region of the 16S rDNA genes in each sample was amplified by PCR method [7-8]. However, there were few reports on the endometrial microbiota in patients with IUA and their association."

#3 Use the term microbiota instead of microbiome.

Response: Thank you for your suggestion, we should be strict with our words. And accordingly the term microbiome has been revised as microbiota in the entire

manuscript.

#4 A major issue is the absence of the inclusion of negative controls processed as regular samples. With such low abundance microbiota this type of control is essential. The authors should include them if possible or largely discuss this point and the limitations to their study.

Response: Thank you for this valuable suggestion. Theoretically we should include negative controls processed as regular samples due to such low abundance microbiota, but now it is difficult for us to include negative controls during this study period. However, this limitation has been stated in the **7th paragraph** of **Disccusion** as followings:

"Moreover, this study lacked the inclusion of negative controls processed as regular samples because of ethical restriction. With such low abundance microbiota, this type of control is essential. We would take into serious consideration to this matter in further research."

#5 Line 90: "After vaginal and cervical canal disinfection". This description is not satisfactory: contamination by vaginal microbiota is a main problem in obtaining endometrial samples. Also in this case, controls would be greatly strengthen the results. Discuss this limitation.

Response: Thanks for your thoughtful suggestions, which reminds us that we should make the statement clearly. Actually, during the collection of endometrial samples from intrauterine to outside, the samples were protected by hysteroscope sheath so as not to be contaminated by vaginal microbiota. And accordingly this topic has been revised in the **2nd paragraph** of **Methods** as followings:

"After vaginal and cervical canal disinfection, endometrial tissues were taken gently from the intrauterine cavity using a hysteroscopic cutting ring without electricity. During the collection of endometrial samples from intrauterine to outside, the samples were protected by hysteroscope sheath so as not to be contaminated by vaginal microbiota."

#6 Why did the authors choose variable region 4 of the 16S rRNA gene? Motivate this decision.

Response: We appreciate your suggestion. We referred to the previous studies on microbiota in patients with IUA. And this topic has been added in the **2nd paragraph** of **Introduction** as followings:

"As we known, the balance of micro-ecology on the female reproductive tract plays a key role in health. An increasing body of evidence suggests that the change of composition and distribution of endometrial microbiota is closely relevant to endometrial diseases such as endometrial polyps, endometrial cancer, infertility and so on [4-6]. Theoretically, the endometrial infection may be related with micro-ecological imbalance. With recent researches, patients with IUA have a micro-ecological imbalance in lower genital tract, and the V4 region or the V3 and V4 region of the 16S rDNA genes in each sample was amplified by PCR method [7-8]. However, there were few reports on the endometrial microbiota in patients with IUA and their association."

#7 Do not use the symbol "&" in the text.

Response: Thanks for your suggestion, we should be strict with our words. And accordingly the symbol "&" has been corrected as the term "and" in the revised manuscript.

#8 Figure 1: Remove the Phyla without hits in panel A. Panel C and D could go to a supplementary figure.

Response: Thanks for your suggestion. And accordingly the Phyla without hits in panel A were removed in Figure 1, while Panel C and D were put as Supplementary Figure 1.

#9 Figure 2: improve the quality of the figure.

Response: Thanks for your suggestion, we should be strict with our figure quality.

And accordingly the quality of the figure has been improved.

#10 Figure 3: put as supplementary data.

Response: Thanks for your suggestion, and the Figure 3 has been put as Supplementary Figure 2.

#11 Figure 4: if possible, use colours, since the different greys do not allow a clear distinction between groups. Again, remove the Phyla without hits in panel A.

Response: We appreciate your suggestion. All figures have been drawn using colous, and the Phyla without hits in panel A has been removed.

#12 Table 2: not necessary or may be included as supplementary data.

Response: We appreciate your suggestion. Table 2 has been put as Supplementary Table 1.

#13 Line 200: these are genera, not species…

Response: Thanks for your correction. And this correction has been made in the revised manuscript.

#14 Line 205: do not compare directly vaginal microbiota with endometrial microbiota, as these two ecological niches are different.

Response: Thanks for your thoughtful suggestion, which reminds us that we should make the statement clearly.

Actually we want to compare different studies on the microbiota in the patients with intrauterine adhesions. Although the results of two studies are inconsistent, we have also explained the factors including different severity of IUA, different sites of samples and different ecological niches in the reproductive tract. Moreover, another research on association between IUA and microbiota has been recently reported. And we have referred this publication.

To be more clearly and accurately, this issue has been revised in the **2nd paragraph** of

Disccusion as followings:

"But our results are not completely consistent with the IUA study reported previously[7, 8]. Liu Z et al[7] showed that patients with IUA had a significantly lower percentage of Firmicutes and a higher percentage of Actinobacteria in the vagina. And half of these patients were found to have overgrowth of Gardnerella and Prevotella accompanied with reduction of Lactobacillus in the vagina. Xingping Zhao et al [8] demonstrated that the proportion of Firmicutes was higher in vagina and cervical canal from most cases with IUA, but some species including Acidobacteria, Euryarchaeota, Chlamydiae, Chlorobi, Planctomycetes and TM6 (Dependentiae), almost disappeared. We found that the proportion of Actinobacteria was lower than Firmicutes, while Lactobacillus increased among the endometrial microbiota in patients with IUA. This discrepancy might be related to different severity of IUA, different sites of samples and different ecological niches in the reproductive tract."

#15 Lines 223-230: overinterpretation. It is not possible to define which Klebsiella species is actually present in the samples and whether is capable of inducing a pathogenic state. Reference 18 points to a study on hepatic stellate cells, thus not relevant to the endometrial milieu. Modify this part of the text.

Response: We are very grateful for your critical comments and thoughtful suggestions, which remind us that we should make the interpretation properly. And accordingly this part has been revised in the **5th paragraph** of **Disccusion** as followings:

"Klebsiella is a kind of encapsulated Gram-negative bacilli in Enterobacteriaceae, and it is also a typical conditional pathogen[18]. Some certain strains of Klebsiella can produce virulence factors Lipopolysaccharide (LPS), which acts on its receptor Toll-like receptors 4 (TLR4) and ultimately induces fibrosis and inflammatory effect[19]. As we known, the expression of TLR4 is constant in female endometrium[20]. Liu F et al has also proved that LPS-induced endometrial infection plays an important part in the occurrence of IUA[2]. Hence, the higher number of endometrial Klebsiella may involve the occurance of IUA. Further research is needed to define which Klebsiella species

are actually present in the endometrial samples and whether is capable of inducing a pathogenic state.”

#16 Lines 232-245: Please refer to more recent publications to make conclusions.
The most recent and important studies in the field are note cited and discussed.
Response: We appreciate your thoughtful suggestions, which reminds us that we should make the references to cite more recently and properly. And this topic has been revised in the **5th and 6th paragraph** of **Disccusion** and in the **references** part.

“Klebsiella is a kind of encapsulated Gram-negative bacilli in Enterobacteriaceae, and it is also a typical conditional pathogen[18]. Some certain strains of Klebsiella can produce virulence factors Lipopolysaccharide (LPS), which acts on its receptor Toll-like receptors 4 (TLR4) and ultimately induces fibrosis and inflammatory effect[19]. As we known, the expression of TLR4 is constant in female endometrium[20]. Liu F et al has also proved that LPS-induced endometrial infection plays an important part in the occurrence of IUA[2]. Hence, the higher number of endometrial Klebsiella may involve the occurance of IUA. Further research is needed to define which Klebsiella species are actually present in the endometrial samples and whether is capable of inducing a pathogenic state.”

“It has been proved that most strains of Lactobacillus such as Lactobacillus jensenii, Lactobacillus crispatus, and Lactobacillus gasseri are probiotics[21], while Lactobacillus iners produces less hydrogen peroxide and has weaker ability to resist pathogens[22]. Previous report by Fang et al[5] is consistent with our result. She found that the relative abundance of Lactobacillus in uterine cavity of patients with endometrial polyps was significantly higher than that of "healthy people". Therefore, endometrial Lactobacillus could exist in the micro-ecological imbalance or balance state. In different state, more than 10% of the gene expression of the strain is different, and the expression of related metabolic enzymes increases[23].”

“”

“[18]José A Bengoechea, Joana Sa Pessoa. Klebsiella pneumoniae infection biology: living to counteract host defences. FEMS Microbiol Rev 2019; 43: 123-144.

[19] Clegg S, Murphy CN. Epidemiology and Virulence of Klebsiella pneumoniae. Microbiol Spectr Actions 2016; 4(1). doi: 10.1128/microbiolspec.

[20] Yun BH, Chon SJ, Choi YS, et al. Pathophysiology of Endometriosis: Role of High Mobility Group Box-1 and Toll-Like Receptor 4 Developing Inflammation in Endometrium. PLoS One 2016; 11:e0148165.

[21] Zhongwang Zhang, Jianliang Lv, Li Pan, et al. Roles and applications of probiotic Lactobacillus strains. Appl Microbiol Biotechnol 2018; 102:8135-8143.

[22] Mariya I Petrova, Gregor Reid, Mario Vaneechoutte, et al. Lactobacillus iners: Friend or Foe?. Trends Microbiol 2017; 25:182-119.

[23] Jean M Macklaim, Andrew D Fernandes, Julia M Di Bella, et al. Comparative meta-RNA-seq of the vaginal microbiota and differential expression by Lactobacillus iners in health and dysbiosis. Microbiome 2013; 1: 12."

Reviewer B

#1 Overall, throughout the manuscript, there are some grammar errors and the misuse of the English language that needs to be addressed before the MS is considered for publishing.

Response: Thank you for your correction. We are really very sorry for our incorrect writing to confuse the reviewer. And accordingly our revised manuscript have been edited by a native English speaker.

#2 The classification used for IUA (low, middle, and high) are misleading and are not in accordance with the literature (American Fertility Society classifications... Fertil Steril. 1988;49:944–955). The American Fertility Society classifies as Stage I (Mild) 1-4 / Stage II (Moderate) 5-8 / Stage III (Severe) 9-12.

Response: We are very grateful for your critical comments and thoughtful suggestions. And we have made the classification used for IUA in accordance with the literature in the revised manuscript, the revised figures and the revised table.

#3 The Methods section is not thoroughly detailed, and some information is missing. On page 4, the DNA input and cycling conditions for the PCR, for example, are not described.

Response: Thanks for your thoughtful suggestion, which reminds us that we should make the methods section thoroughly detailed. And accordingly this topic has been added in the **3rd paragraph** of **Methods** as followings:

"The cycling conditions for PCR were as follows: 94˚C for 5 min, followed by 30 cycles of 94˚C for 30 sec, 52˚C for 30 sec,72˚C for 30 sec and another 10 minutes at 72°C before the end."

#4 The authors present only the total of reads obtained per sample but did not seem concerned in assessing if these reads were enough to reach sequencing saturation. Rarefaction curves might have given more confidence in the data presented, since it is a measure of sequencing quality/depth per sample and give an idea of the general number of OTUs per sample. A rarefaction curve is shown in Figure 1C, but not with this purpose. Nonetheless, they used the Simpson index (not as informative as the Observed number of OTUs in this matter) and the image has poor quality (I cannot distinguish the legend color in the figure).

Response: We are very grateful for your critical comments and thoughtful suggestions. Firstly, we used the rarefaction curves of the Observed_species or the Simpson index to assess if these reads were enough to reach sequencing saturation. When the rarefaction curves tend to be flat, it indicates that the amount of sequencing data is large enough to reflect most of the microbial diversity information in the sample. Secondly, in this study we have made the Observed number of OTUs instead of the Simpson index in the Rarefaction curves. And the revised figures have been improved and put as Supplementary data. Moreover, this revision has been made in the **2nd paragraph** and **3rd paragraph** of **Results** as followings:

"The rarefaction curves of Simpson index showed the α-diversity in the Group IUA was statistically lower than that in the Group C (P = 0.01) (Supplementary Figure 1A). But the results for the observed_species indicated that the Group IUA has relatively

more OTUs than the Group C (P =0.257) (Supplementary Figure 1B). ”

“There was no difference between the groups in the observed species (P = 0.491, Supplementary Figure 2B). As shown in the Supplementary Figure 2A, rarefaction curves of Simpson index indicated that there was a statistical difference in the α-diversity of endometrial bacteria between the different stage of IUA and Group C.”

#5 The data only shows the top 4 Genera found on both IUA and control, but no additional statistics test was performed to find significantly different bacteria between the two groups (such as Lefse or any other test, such as logistic regression or any other statistical appropriate test for the data). Another concern of mine is that the results the authors consider statistically significant and which the entire manuscript is dependent on for the conclusions (considered by the authors they're major findings) are hardly significant. Numbers are shown with three digits (0,048 or 0,047 …etc) in order to "force" their significance ($P < 0,05$). For me, 0.048 is the same as 0,05, and therefore, not significant at all.

Response: Thank you for your concern, which reminds us that we should give a more detailed explanation and description.

Firstly, as the Methods part shown, data of top 4 Genera were compared by the Kruskal-Wallis analysis of variance on ranks between Group IUA and control. And as the **2nd paragraph** of **Results** shown:

“The mean proportion of Acinetobacter was statistically lower in Group IUA than that in the Group C（P =0.005）, while the mean proportion of Klebsiella was statistically higher in Group IUA than that in the Group C (P = 0.006).”

Secondly, statistical analyses were performed with Statistical Product and Service Solutions, thus the statistical results were objective. Moreover, for the proper interpretation of clinical research, we should consider its statistical significance and clinical significance simultaneously. Of course, the statistical results are affected by the sample size and other factors. Indeed, we have stated this limitation in the **7th paragraph** of **Disccusion** as following:

“First, the sample size may be small, which might cause that some statistical difference can not be detected. However, previous studies on endometrial flora

showed that the total sample size ranged from 10 to 110 cases, and the sample size fluctuated between 4 and 79 cases per group[25]. Thus, our sample size is consistent with previous reports, but a statistics-based sample size is more rigorous and persuasive. ”

#6 On page 7, lines 180-181, I do not understand how the results presented in this paragraph leads the authors to the conclusion presented here. There is a lack of evidence and stretching of the results. The same thing on page 8, lines 198-199.
Response: We appreciate your suggestion, which reminds us that we should make the interpretation properly in our study. And this topic has been revised in the **3rd paragraph** of **Results** and the **1st paragraph** of **Disccusion** as followings:
“To some extent, these above results indicated that there may be a relationship between IUA and the variation of endometrial microbiota.”
“and all the potential variation of endometrial microbiota might be related with the occurrence of IUA.”

#7 Figure 2 is in bad quality in the pdf, and it is difficult to see the figure legend (to distinguish colors).
Response: Thanksfor your suggestion, we should be strict with our figure quality. And accordingly the quality of the figure has been improved.