〔Original〕

Metagenomic analyses of oral and vaginal microbiome in ovariectomized rats

Durga PAUDEL[1,2], Osamu UEHARA[3], Tetsuro MORIKAWA[1], Koki YOSHIDA[1], Sarita GIRI[4], Takao KITAGAWA[2], Yuya NAKAO[5], Jin TSUCHIDA[5], Masahiro IIJIMA[5], Hiroko MIURA[3], Yasuhiro KURAMITSU[2], Yoshihiro ABIKO[1]

[1]Division of Oral Medicine and Pathology, School of Dentistry, Health Sciences University of Hokkaido, Hokkaido, Japan
[2]Advanced Research Promotion Center, Health Sciences University of Hokkaido, Hokkaido, Japan
[3]Division of Molecular Epidemiology and Disease Control,
School of Dentistry, Health Sciences University of Hokkaido, Hokkaido, Japan
[4]Division of Periodontology and Endodontology, School of Dentistry, Health Sciences University of Hokkaido, Hokkaido, Japan
[5]Division of Orthodontics and Dentofacial Orthopedics, School of Dentistry, Health Sciences University of Hokkaido

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Abstract

Postmenopausal females complain of dryness and burning sensation in oral cavity and vagina. The common symptoms of oral cavity and vagina in postmenopausal females might have a common etiology which is yet to be clarified. The resident microflora of oral cavity, gut and vagina have role in maintaining the health of those habitats. Alteration of microflora in those habitats have been associated with the pathogenesis of diseases. Although a few studies have demonstrated the alteration of microflora in the vagina of postmenopausal females, it is still unknown how postmenopausal disorder affects the microflora in the oral cavity. Herein, we demonstrate the oral and vaginal microbiome by performing 16S rRNA sequencing in an ovariectomized rats resembling post−menopausal state. The proportion of *Lachnospiraceae* at the family level was significantly increased in the oral cavity of the ovariectomized group rats as compared to the control. The proportion of *Prevotella* at the genus level, *Prevotellaceae* and *Enterobacteriaceae* at the family level, and *Clostridiales* and *Enterobacteriales* at the order level was significantly altered in the vagina between the ovariectomized group and the control. There was significant increase in alpha diversity of vaginal microbiome between the ovariectomized rats and the control. Further studies are required to correlate these findings with clinical symptoms of postmenopausal females.

Introduction

Menopause is the loss of ovarian follicle development occurring in females in late forties. The loss of ovarian follicle makes the ovary unable to respond to the pituitary hormones to produce estrogen and progesterone (Hall et al., 2015). The depletion of these hormones in female body causes several symptoms commonly refereed as postmenopausal symptoms (Saensak et al., 2014). The symptoms are mostly manifested as physical and emotional changes in the female body. The physical symptoms include hot flushes, vaginal atrophy, osteoporosis, headache and sexual problems. The emotional changes include mood swings, irritability, loss of stress coping and depression (Saensak et al., 2014).

Postmenopausal females occasionally complain of dryness and burning sensation called as burning mouth syndrome in oral cavity (Paudel et al., 2020). Similar to the oral symptoms, those postmenopausal females often experience vaginal burning and dryness (Saensak et al., 2014). The common symptoms of oral cavity and vagina in postmenopausal females might have a common etiology which is yet to be clarified.

The resident microflora of oral cavity, gut and vagina have role in maintaining the health of those habitats. Alteration of microflora in those habitats have been associated with the pathogenesis of diseases. Oral bacteria such as *Prevotella*, *Porphyromonas* and *Aggregatibacter* are associated with periodontitis, while *Streptococcus* and *Lactobacil-*

lus are associated with dental caries (He et al., 2014). The reduced alpha diversity in oral habitat correlates with increased mucosal inflammation in diseases such as recurrent aphthous stomatitis and oral lichen planus (Hijazi et al., 2020). The alteration of gut microflora has been associated with various gastrointestinal diseases including Crohn's disease and ulcerative colitis (Zuo & Ng, 2018). The alteration of vaginal microbiome has been linked to diseases such as bacterial vaginosis, cervical cancer and pelvic inflammatory diseases (Chen et al., 2021). Therefore, the alteration of microflora in postmenopausal state may be involved in causing the common symptoms of oral cavity and vagina. Although a few studies have demonstrated the alteration of microflora in the vagina of postmenopausal females, it is still unknown how postmenopausal disorder affects the microflora in the oral cavity (Mitchell et al., 2021 ; Kim et al., 2021). An ovariectomized rats has been widely used for a model of postmenopausal disorder (Høegh−Andersen et al., 2004). Therefore, in the present study, we observed the oral and vaginal microbiome by performing 16S ribosomal RNA (16s rRNA) sequencing in an ovariectomized rats.

Materials and methods

2.1. Animals and sample collection

Six−week−old ovariectomized female Wistar rats (n=5) were purchased from the Sankyo Labo Service Corporation (Sapporo, Japan). Age matched sham operated female rats were used as control (n=5). The rats in both groups were housed for 5 weeks after the arrival. The rats were then anesthetized with an intraperitoneal injection of sodium pentobarbital (50 mg/kg body weight). The oral swab was collected starting from the tongue dorsum and then to palate, buccal mucosa, upper and lower vestibules, and finally the floor of the mouth for 30 seconds. The swab was placed in 200 µl of deionized distilled water in a centrifuge tube and stored at −80°C until further processing. To collect the vaginal lavage, 100 µl of phosphate buffered saline was used twice to lavage the vagina using pipette and then stored at −80°C until further processing. The study was approved by the animal ethics committee of the Health Sciences University of Hokkaido (Approval no : 19−091).

The oral microbial DNA was extracted after adding 200 µl of lysozyme (20 mg/ml, Fujifilm Wako Pure Chemicals, Japan) to the oral swab and incubated at 37°C for 60 min with moderate shaking (Abusleme et al., 2017). Proteinase K (25 µl) and Buffer AL (200 µl) (DNeasy Blood and Tissue kit, Qiagen, Germany) were added to the tube, vortexed and incubated overnight at 56°C. The swab was then pulled out, pressed against the wall of the tube and then discarded. 200 µl of 99% ethanol was added and vortexed. The protocol "Purification of Total DNA of Animal Tissues (Spin−Column Protocol)" from the DNeasy Blood and Tissue kit (Qiagen) was continued from Step 4 for DNA collection. To collect the vaginal microbiome, the vaginal lavage was centrifuged for 10 min at 6000x g. The supernatant was discarded and the pellet collected was further processed for DNA extraction using Protocol for Purification of Total DNA of bacterial cells of DNeasy Blood and Tissue kit. The concentration of DNA extracted from oral swab and vaginal lavage was measured by Nanodrop Spectrophotometer (ThermoFisher Scientific, Waltham, MS, USA) (Table 1).

2.2. 16S rRNA sequencing

16S rRNA sequencing libraries of oral and vaginal DNA were prepared using 16S metagenomic sequencing library preparation (Illumina, USA). The following steps were followed :

Table 1 : DNA concentration of oral swab and vaginal lavage

	Sample	DNA concentration (ng/µl)	260/280 ratio
Control−Oral swab	1	4.26	1.99
	2	4.38	1.90
	3	4.41	1.90
	4	4.40	1.42
	5	3.93	1.99
Ovariectomized−Oral swab	6	5.46	1.63
	7	5.44	1.34
	8	2.94	2.85
	9	4.11	1.48
	10	3.43	1.42
Control−Vaginal lavage	1	77.92	1.93
	2	75.97	1.89
	3	22.32	1.71
	4	22.76	1.86
	5	62.10	1.91
Ovariectomized−Vaginal lavage	6	42.59	1.88
	7	54.20	1.90
	8	31.06	1.91
	9	13.41	1.83
	10	40.34	1.87

i. Amplicon Polymerase Chain Reaction (PCR)

The oral and vaginal DNA was mixed with the V3−V4 region primers and KAPA HiFi HS ReadyMix (Nippon Genetics, Japan) to amplify the V3−V4 regions of the 16S rRNA gene which encodes bacterial 16S rRNA. A mixture of 2.5 µl of microbial DNA, 5 µl of Amplicon PCR forward and reverse primers (Table 2), and 12.5 µl of 2x KAPA HiFi Hotstart ready mix was used for the purpose. PCR was performed in a thermal cycler. The PCR conditions were : 95°C for 3 min, 25 cycles of 95°C for 30 s, 55°C for 30 s, 72°C for 30 s each followed by 72°C for 5 min, and hold at 4°C. AMPure XP bead (Beckman Coulter Life Sciences, Germany) was used to clean up the PCR.

ii. Index PCR

The amplified DNA from the first step was used to attach dual indices and the Illumina sequencing adapter. KAPA HiFi HS ReadyMix and Nextera XT index kit (Illumina) were used for this purpose. 5 µl of PCR clean−up done DNA, Nextra XT index primer 1, and Nextra XT index primer 2 (Table 2) were mixed with 10 µl water and PCR was performed on a thermal cycler. The PCR conditions were : 95°C for 3 min, 8 cycles of 95°C for 30 s, 55°C for 30 s, 72°C for 30 s each followed by 72°C for 5 min, and hold at 4°C. The PCR was cleaned up using AMPure XP beads (Beckman Coulter Life Sciences).

iii. Library quantification, normalization, and pooling

The index PCR done DNA was quantified using a Qubit 3 fluorometer (Thermo Fisher Scientific). The DNA was then normalized and pooled as a 4 nM library which was then denatured with 0.2N NaOH and mixed with PhiX Control v3 (Illumina). Subsequently, heat denaturation at 96°C was performed. The samples were then loaded onto the Illumina MiSeq System for sequencing.

iv. Data Analysis

Quantitative Insights into Microbial Ecology2 (QIIME2 v 2020.4.0) against the 16S rRNA database (Greengenes v 13.8) was used to analyze the data of the metagenomic sequencing. The diversities between the ovariectomized group and the control were analyzed as alpha diversity and beta diversity. For alpha diversity, observed operational taxonomic units (OTUs) and the Shannon group significance were used.

Table 2 : Primer sequences used in this study

Amplicon Primers	
Forward	5' TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG
Reverse	5' GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG
Index Primers 1	
N701	TAAGGCGA
N702	CGTACTAG
N703	AGGCAGAA
N704	TCCTGAGC
N705	GGACTCCT
N706	TAGGCATG
N707	CTCTCTAC
N708	CAGAGAGG
N709	GCTACGCT
N710	CGAGGCTG
N711	AAGAGGCA
N712	GTAGAGGA
Index Primers 2	
S501	TAGATCGC
S502	CTCTCTAT
S503	TATCCTCT
S504	AGAGTAGA
S505	GTAAGGAG
S506	ACTGCATA
S507	AAGGAGTA
S508	CTAAGCCT

Kruskal–Wallis test was used to test the significant differences between two groups in alpha diversity. For beta diversity, unweighted and weighted UniFrac distance metric based on three–dimensional principal coordinate analysis (PCoA) scatterplots were used. Permutational multivariate ANOVA (PERMANOVA) was used to test the significant differences between two groups in beta diversity. The difference in taxonomic abundance between two groups was analyzed using Linear discriminant analysis (LDA) Effect Size (LefSe).

Results

Oral microbiome

The minimum and maximum frequency (sequencing reads) per sample in oral samples were 12,047 and 98,285, respectively with mean frequency of 54,504 (Table 3). The top five OTUs were *Rothia*, *Streptococcus*, *Lactobacillus*, *Facklamia* and *Staphylococcus* at the genus level (Fig. 1a). The rarefaction curve pleatued in both the groups after certain sequencing depth indicating sufficient sequencing (Fig. 1b). The difference in alpha diversity as measured by observed OTUs and Shannon index were not statistically significant. The weighted UniFrac for beta diversity showed some differences in clustering of the ovariectomized rats and the control ; however, did not reach the significance level (Fig. 1c). The LefSe analysis for LDA score showed increased proportion of *Lachnospiraceae* at the family level and an unidentified OTU in the ovariectomized group rats as compared to the control (Fig. 1d).

Vaginal microbiome

The minimum and maximum frequency (sequencing reads) per sample in vaginal samples were 17,925 and 60,006 respectively with mean frequency of 37,425 (Table 3). The alpha diversity as indicated by observed OTUs was significantly higher in the ovariectomized rats as compared to the control rats. (p=0.016 ; Kruskal–Walis test) (Fig. 2a).

Table 3 : Frequency summary of the sequencing

	Oral	*Vagina*
Number of samples	10	10
Number of features	168	2,053
Total frequency	545,047	374,258
Minimum frequency per sample	12,047	17,925
Maximum frequency per sample	98,285	60,006
Median frequency	39,305	35,021

The alpha diversity as indicated by Shannon index was significantly higher in the ovariectomized rats as compared to the control rats (p=0.047 ; Kruskal–Walis test) (Fig. 2b). The rarefaction curve pleatued in both the groups after certain sequencing depth indicating sufficient sequencing (Fig. 2c). The unweighted UniFrac for beta diversity showed some differences in clustering of the ovariectomized rats and the control ; however, did not reach the significance level (p= 0.07 ; PERMANOVA) (Fig. 2d). The top five OTUs were *Enterobacteriaceae* at the family level, unclassified bacteria Bacteria ; __ ; __ ; __ ; __ ; __, *Proteus* and *Enterococcus* at the genus level and OD1 at the phylum level (Fig. 3a). The LefSe analysis for LDA score showed that the altered proportion of bacteria at various taxonomic levels. The proportion of *Bacteroidetes* and *Proteobacteria* at the phylum level was significantly increased in the ovariectomized group as compared to the control. The proportion of *Burkhoideriales* at the order level was significantly increased in the ovariectomized group as compared to the control. The proportion of *Prevotella* at the genus and the family level and *Clostridiales* at the class and the order level was significantly increased in the ovariectomized group as compared to the control. *Enterobacteriaceae* at the family and the order level was significantly downregulated in the ovariectomized group as compared to the control (Fig.3).

Discussion

The present study demonstrated the alteration of microflora in the oral cavity and vagina of the ovariectomized rats. The proportion of *Lachnospiraceae* at the family level was significantly increased in the oral cavity of the ovariectomized group rats as compared to the control. The proportion of *Prevotella* at the genus level, *Prevotellaceae* and *Enterobacteriaceae* at the family level, and *Clostridiales* and *Enterobacteriales* at the order level was significantly altered in the vagina between the ovariectomized group and the control. There was significant increase in the alpha diversity of vaginal microbiome between the ovariectomized rats and the control.

In the present study, we used the ovariectomized rats as a post–menopausal model. The ovariectomized rats and mice have been widely used to observe the effects of post–menopause on many organs including bone, kidney, heart, lungs, and liver (Høegh–Andersen et al., 2004 ; Vorland et al., 2019 ; Sharkey et al., 1999). To the best of our knowledge,

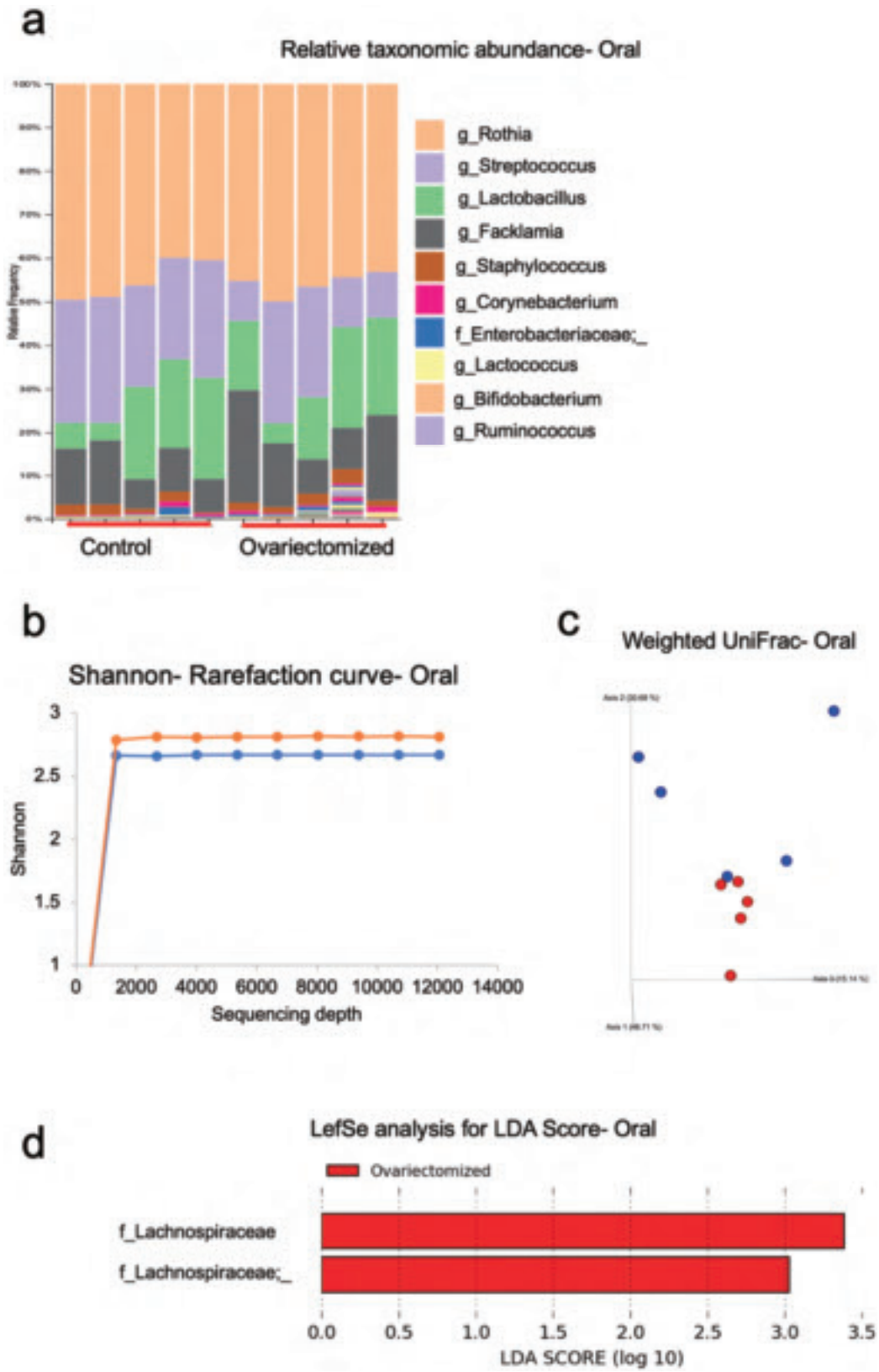

Fig.1. Metagenomic analysis of oral microbiome. (a) The bar graph shows the relative taxonomic abundance in each sample. The oral cavity was dominated with bacteria such as *Rothia*, *Streptococcus*, *Lactobacillus* in both the ovariectomized and the control groups. (b) The Shannon rarefaction curved pleatued in both groups indicating sufficient depth of analysis. (c) The beta diversity as evaluated by weighted UniFrac showed differences in clustering between two groups ; however, did not reach the significance level (ovariectomized–red dots ; control –blue dots). (d) The LefSe analysis for LDA score showed that the Lachnospiraceae at the family level was significantly elevated in the ovariectomized group as compared to the control. f : family ; g : genus

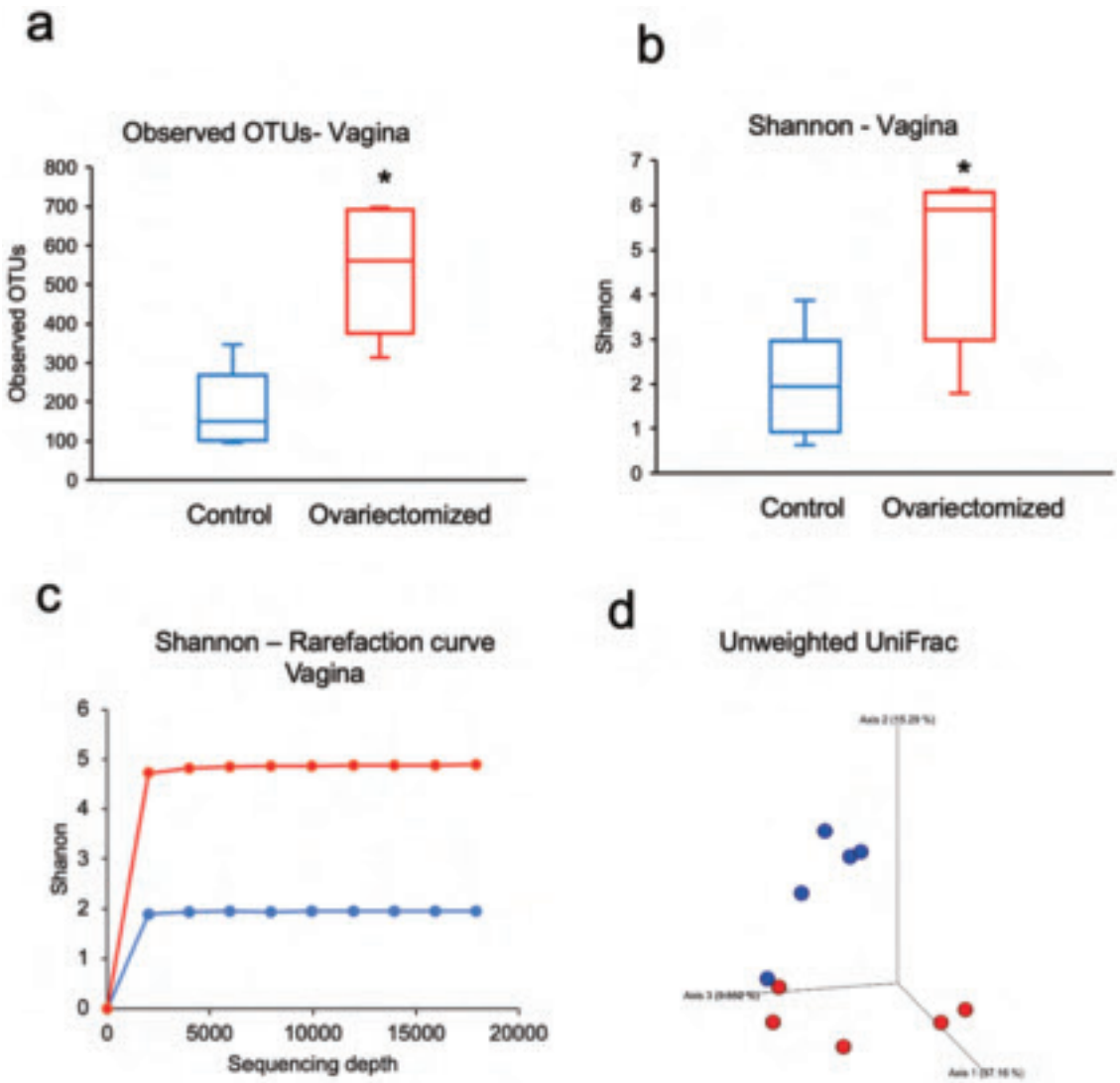

Fig. 2. The alpha and beta diversity of vaginal microbiome. (a, b) the alpha diversity as evaluated by Observed OTUs and Shannon was significantly increased in the ovariectomized group as compared to the control ($p<0.05$; Kruskal–Wallis test). (c) The Shannon rarefaction curved pleatued in both the groups indicating sufficient depth of analysis. (d) The beta diversity as evaluated by unweighted Unifrac showed some differences in clustering of the ovariectomized rats (red dots) and the control (blue dots) ; however, did not reach the significance level ($p=0.07$; PERMANOVA).

it is the first report about the alteration of oral microflora in the post–menopausal model rats. The alterations of oral microflora have been shown in various oral diseases and conditions including dental caries, periodontitis, oral cancer and smoking (Kilan et al., 2016). Moreover, systemic diseases such as diabetes, rheumatoid arthritis, and systemic lupus erythematosus have been shown to affect the oral microbiome (Kilan et al., 2016). The changes in oral microbiome in these diseases were associated with increased inflammation (Graves et al., 2019). Menopause and ovariectomy have been shown to generate low grade systemic inflammation (Abu–Taha et al., 2009). The alteration in microbiomes in post–menopausal state could be attributed to the systemic inflammation. The proportion of *Lachnospiraceae* family in the oral cavity was increased in the ovariectomized rats as compared to the control in the present study. Although no significant role of *Lachnospiraceae* family has been identified in oral cavity, it plays a major role in maintaining gut health (Vacca et al., 2020). *Lachnospiraceae* is an anaerobe producing short chain fatty acids, mainly butyrate. Butyrate is a major energy source for colonic epithelial cells. The human ascending and descending colons are shown to consume more than 70% of oxygen due to butyrate oxidation. Butyrate also maintains the homeostasis of intestine through its anti–inflammatory actions (Parada et al., 2019). On the other hand, increased proportion of *Lachnospiraceae* has been associated with various diseases such as obesity, non–alcoholic steatohepatitis, liver fibrosis, chronic kidney dis-

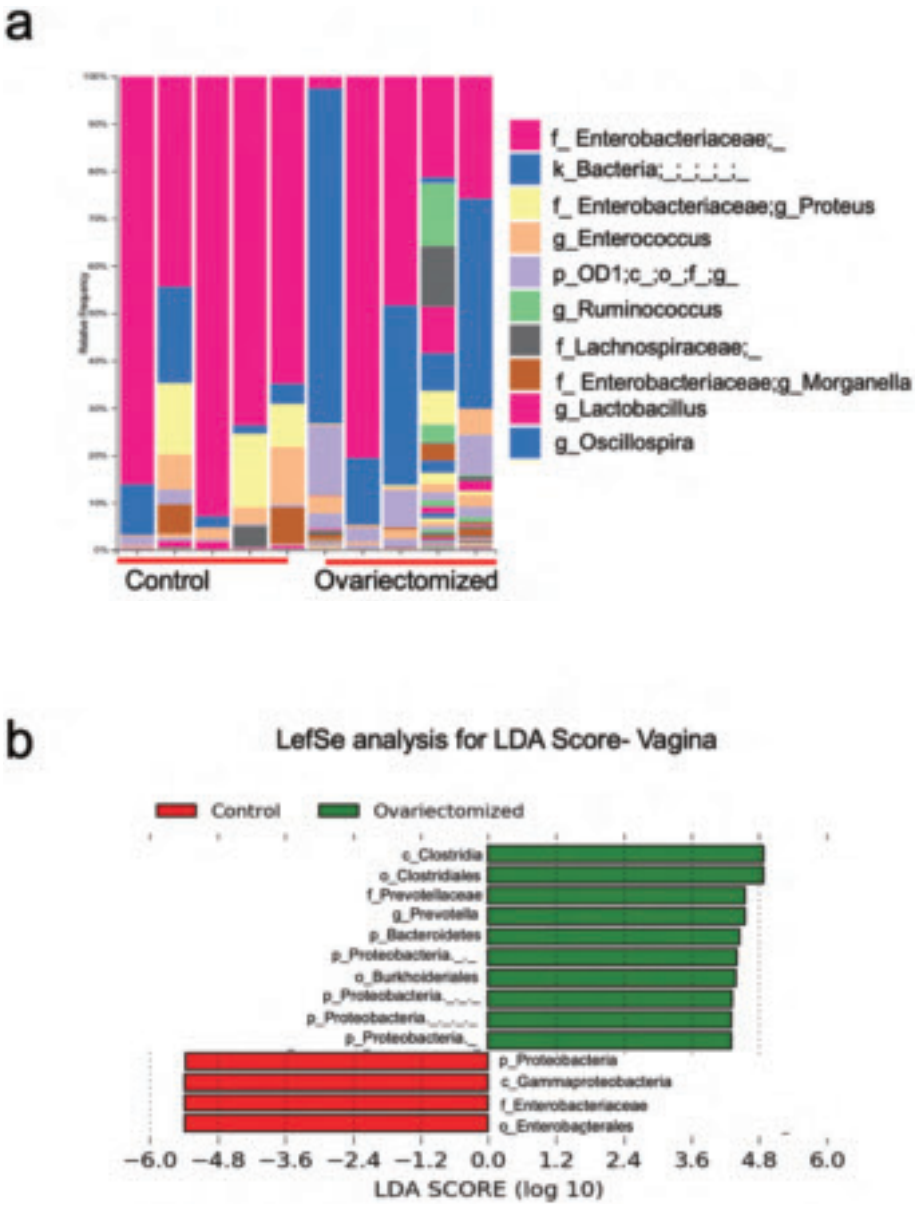

Fig.3. (a) The bar graph shows the relative taxonomic abundance in each sample. The LefSe analysis for LDA score of vaginal microbiomes. (b) The proportion of *Prevotella* at the genus level, *Prevotellaceae* at the family level and *Clostridiales* at the order level, *Clostridia* at the class level and *Bacteroidetes* at the phylum level to be significantly increased in the ovariectomized group as compared to the control. *Enterobacteriaceae* at the family level and *Enterobacteriales* at the order level were significantly decreased in the ovariectomized group as compared to the control. p : phylum ; c : class ; o : order ; f : family ; g : genus.

eases and major depressive disorders (Vacca et al., 2020). It is not known how increased proportion of *Lachnospiraceae* directly affect oral environment and symptoms. Recently, it has been reported that oral microbe may be ingested and naturally translocate to the digestive tract, and may contribute to the development of digestive diseases (Kitamoto et al., 2020). The increased proportion of *Lachnospiraceae* may affect digestive health. Further studies are required to clarify this speculation.

The microbiome in vagina plays an important role in defense system (Smith & Ravel et al., 2017). The dysbiosis of vaginal microbiome has been associated with many vaginal diseases such as bacterial vaginosis, cervical cancer, pelvic inflammatory diseases and pre−term delivery (Chen et al., 2021). Post−menopausal symptoms such as vaginal dryness and inflammation were positively correlated with increased proportion of *Prevotella* and increased bacterial diversity in human vagina (Hummelen et al., 2011 ; Mitchell et al., 2021 ; Kim et al., 2021). Increased abundance of *Prevotella* is associated with increased T helper type 17 (Th17)−medi-

ated mucosal inflammation. *Prevotella* can activate Toll−like receptor 2 leading to production of inflammatory cytokines such as Interleukin (IL)−1, IL−6, IL−8 and IL−23 (Larsen, 2017). In our study, increased proportion of *Prevotella* at the genus levels in the vagina of the ovariectomized rats was observed. Also, increased alpha diversity as Observed OTUs and Shannon index was observed. These findings suggest that the microbiome dysbiosis may be the cause of post−menopausal vaginal symptoms. Overall, our findings of vaginal microbiome in the ovariectomized animal model validate the findings of the human study.

There are few limitations of the present study. A small sample size (n=5, each group) was considered in this study. Also, the role of microbiome in causing common symptoms in oral cavity and vagina of post−menopausal females could not be demonstrated in the present study. Further studies with larger sample size are required to clarify it.

Conclusion

The effect of post−menopausal condition on oral and vaginal microbiome was demonstrated using an ovariectomized rat model. Significant alteration in the bacterial taxa and diversity of oral cavity and vagina were observed. Further studies are required to correlate these findings with clinical symptoms of postmenopausal females.

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Conflict of Interest

There is no conflict of interest associated with the study.

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