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Identification of Bacterial Vaginal Microbiota via Metagenomic Approach

Bakteriyel Vajinal Mikrobiyotanın Metagenomik Yaklaşımla Tanımlanması

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ABSTRACT

Aim: The aim of the current study was to identify vaginal bacterial microbiota of 38 Turkish women using the high-throughput next-generation sequencing and metagenomic approach at different taxonomic levels from the kingdom to the species level.

Materials and Methods: Vaginal swab samples (n=38) were collected in the DNA/RNA shield collection tubes at Yeditepe University Hospital, Department of Obstetrics and Gynecology in June 2021 and DNA extraction was performed by ZymoBIOMICS DNA miniprep kit. The information related to age, marital status, preliminary diagnosis and anamnesis status of patients were collected. To determine the vaginal microbiota, a metagenomic approach was applied using 16S rRNA amplicon sequencing.

Results: The dominant phylum Firmicutes was followed by Proteobacteria, Actinobacteria, Tenericutes, Fusobacteria, and Synergistetes in the vaginal samples. *Lactobacillus* was the most abundant genus followed by *Prevotella*, *Enterobacter*, *Gardnerella*, and *Dialister*. *Lactobacillus iners* was dominant at the species level in vaginal swab samples, followed by *Gardnerella vaginalis*, *Enterobacter tabaci*, *Prevotella timonensis*, *Prevotella bivia*, and *Lactobacillus jensenii*. Canonical correspondence analysis (CCA) showed that Proteobacteria and Fusobacteria were mainly related to married/single variable with the highest percentages, whereas Actinobacteria and Tenericutes were related to age variable at the phylum level. *Campylobacter*, *Atopobium*, *Enterobacter*, and *Lactococcus* were mainly found in married/single variable with the highest percentages, whereas *Anaerococcus*, *Streptococcus*, *Sutterella*, and *Veillonella* were related to age. Moreover, CCA showed that *Campylobacter ureolyticus*, *Lb. jensenii*, and *Atopobium vaginae* were associated with married/single variable, whereas *Lactobacillus johnsonii* and *G. vaginalis* were found in age variable with the highest percentages at the species level.
Conclusion: Vaginal diseases are still a major public health concern. The vaginal microbiota, which has been studied in more depth in recent years, has been discovered to be more complicated than previously imagined thanks to technological developments. More patient investigations are needed to confirm and develop these findings.

Keywords: Metagenomics, microbiota, next-generation sequencing, vaginal tract

ÖZ

Amaç: Bu çalışmanın amacı, alem düzeyinden tür düzeyine kadar farklı taksonomik seviyelerde yüksek verimli yeni nesil dizileme ve metagenomik yaklaşım kullanarak 38 Türk kadınının vajinal bakteriyel mikrobiyotasını belirlemektir.

Gereç ve Yöntem: Yeditepe Üniversitesi Hastanesi Kadın Hastalıkları ve Doğum Kliniği'nde Haziran 2021'de DNA/RNA koruma toplama tüplerine vajinal sürüntü örnekleri (n=38) alındı ve ZymoBIOMICS DNA miniprep kiti ile DNA ekstraksiyonu yapıldı. Hastaların yaşı, medeni durumu, ön tanı ve anamnez durumu ile ilgili bilgiler toplandı. Vajinal mikrobiyotayı belirlemek için 16S rRNA amplikon DNA dizilimi kullanılarak metagenomik bir yaklaşım uygulandı.

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Bulgular: Vajinal örneklerde baskın filum Firmicutes'i Proteobacteria, Actinobacteria, Tenericutes, Fusobacteria ve Synergistetes izledi. *Lactobacillus* en fazla bulunan cins düzeyinde bakteri olup onu *Prevotella, Enterobacter, Gardnerella* ve *Dialister* izledi. Vajinal sürüntü örneklerinde tür düzeyinde *Lactobacillus iners* baskın bulundu, bunu *Gardnerella vaginalis, Enterobacter tabaci*, *Prevotella timonensis*, *Prevotella bivia* ve *Lactobacillus jensenii* izledi. Kanonik uyum analizi (CCA), filum düzeyinde Proteobacteria ve Fusobacteria'nın en yüksek yüzdelerle evli/bekar değişkeni ile ilişkili olduğunu, ancak Actinobacteria ve Tenericutes'in yaş değişkeni ile ilişkili olduğunu gösterdi. *Campylobacter, Atopobium, Enterobacter* ve *Lactococcus* en yüksek yüzdelerle evli/bekar değişkeni ile ilişkili bulunurken, *Anaerococcus, Streptococcus, Sutterella* ve *Veillonella* en yüksek yüzdelerle yaşla ilişkili bulundu. Ayrıca, CCA, *Campylobacter ureolyticus, Lb. jensenii* ve *Atopobium vajinae* türlerinin evli/bekar değişkeni ile en yüksek yüzdelerle ilişkilendirirken, *Lactobacillus johnsonii* ve *G. vaginalis* en yüksek yüzdelerle yaş değişkeninde ilişkili bulundu.
Sonuç: Vajinal hastalıklar hala önemli bir halk sağlığı sorunudur. Son yıllarda teknolojik gelişmeler sayesinde daha derinlemesine çalışılan vajinal mikrobiyotanın sanıldığından daha karmaşık olduğu keşfedilmiştir. Bu bulguları doğrulamak ve geliştirmek için daha fazla hasta araştırmasına ihtiyaç vardır.

Anahtar Kelimeler: Metagenomik, mikrobiyota, yeni nesil dizileme, vajinal sistem

INTRODUCTION

Human lifestyle shifts do not only impact the health of the biosphere but likely affect our health as a result of changes in our microbial ecology. In the microscopic world, it is well known that humans are ecological harbors. The female genital tract is increasingly becoming one of the important habitats for human microbiota because the vaginal tract contains remarkably complex microbial communities[1].

Vaginal illnesses are among the most frequent gynecological issues. The vaginal microbiota's stability is influenced by a variety of variables. Age, menses, menarche, diseases, pregnancy, birth control, and sexual practices all affect the composition of the vaginal microbiota[2]. Infections are a common occurrence in vaginal disorders. The human vaginal microbiota tends to play a crucial role in avoiding a variety of urogenital diseases, including bacterial vaginosis, yeast infections, sexually transmitted diseases, urinary tract, and HIV-related infections[3]. A significant number of cases of vulvitis, cervicitis, and pelvic infections are associated with urogenital diseases[4]. The healthy vaginal microbiota is dominated by the hydrogen peroxide-forming *Lactobacillus* spp.[5-7], such as *Lb. crispatus*, *Lb. acidophilus*, *Lacticaseibacillus rhamnosus,* and others (except *Lb. iners*). This feature suppresses the growth of other organisms and excludes other bacteria unable to synthesize catalase[7]. Both healthy habitat and vaginal dysbiosis are often identified in the vaginal habitat of *Lb. iners*. It seems representative of a symbiotic or parasitic lifestyle, as opposed to other niche-flexible lactobacilli[8,9]. Except for 9% of the strains[10], *Lb. iners* are unable to produce hydrogen peroxide[11,12]. However, facultative or obligate anaerobic bacteria, which are 100 to 1000 times more abundant than hydrogen peroxide-producing *Lactobacillus* spp., dominate a typical unhealthy vaginal flora[13,14]. Furthermore, some non-infectious diseases, such as intrauterine adhesions, preterm birth, induced abortions, polycystic ovarian syndrome, miscarriage, uterine fibroid, infertility, and menstrual disorders, have been linked to microbial dysbiosis, represent a major threat to women's reproductive health[15].

The function of many bacteria that are assumed "normal" within the vagina has been redefined thanks to the introduction of NGS technology. Scientists are concerned not only about potentially "harmful" bacteria but also about changes in the organization of the vaginal microbiota as a whole[16]. Although there are some disadvantages using this method, such as a lot of technical difficulties which need to be thoroughly studied and solved, and high costs, time-consuming protocols, NGS has managed to solve the drawbacks of conventional DNA sequencing approaches and find the use in a wide variety of applications[17]. NGS could also be applied effectively to classify a large variety of taxonomic species that would not be possible with other methods.

The present study aimed to identify bacterial vaginal microbiota using the NGS method and metagenomic approach at different taxonomic levels from the kingdom to the species level. Furthermore, the relationships of identified bacterial communities present in vaginal microbiota with age and marital status were determined by canonical correspondence analysis (CCA).

MATERIALS AND METHODS

Samples

In this study, a total of 38 vaginal swab samples were collected from patients admitted to Yeditepe University Hospital, Department of Obstetrics and Gynecology in June 2021, using the DNA/RNA shield collection tubes with a swab (R1107, Zymo Research, USA). The swabs were stored at -20 °C till DNA extraction. The information related to patients' age, marital status, preliminary diagnosis, and anamnesis status is given at Table 1.

The collection of all human materials was approved by Yeditepe University Clinical Research Ethics Committee (approval number: 1274, date: 20.08.2020).

DNA Extraction

Total DNA extraction was carried out using a ZymoBIOMICS DNA miniprep kit (D4300, Zymo Research, USA). The DNA

samples were quantitated spectrophotometrically by the Take3 plate of the microplate reader (Epoch-2, BioTek, USA). Then, DNA samples were stored at -20 °C up to amplicon PCR experiments for NGS.

NGS and Metagenomic Analysis

DNA library was prepared according to the 16S metagenomic sequencing library preparation guide instructions (Illumina, Inc., California, USA). In 16S rRNA amplicon PCR, the primer pairs F-primer: 5'-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG-3' and R-primer5'-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC-3' were used. In total extracted DNA of vaginal swab samples, bacterial 16S rRNA V3–V4 regions were amplified by PCR using KAPA HiFi HS Mix (Roche, Germany). Then, the amplicons of each swab sample were indexed with dual indexes by the Nextera XT index Kit v2 Set-A (Illumina). All PCR products and indexed samples were cleaned using AMPure XP beads (Beckman Coulter, USA) in a magnetic rack (DynaMag™-96 Side, Invitrogen, Norway). The equimolar (10 nM) proportions of the samples were pooled in a tube, then it was diluted to 1 nM, and it was finally diluted to a 35 pM DNA library. The diluted library (20 µl) containing 5% (v/v) PhiX control DNA (Illumina) was loaded into an iSeq100 v1 cartridge (Illumina). The sequencing was performed in the iSeq100 system (Illumina) with a pair-end read type and two reads of 151 bp read length of the sequence.

The NGS data were analyzed using the 16S Metagenomics, Version: 1.1.0 software (Illumina). The sequence identity of clustered sequences by NGS was determined by an operational taxonomic unit (OTU) approach. Alpha diversity values (Shannon species diversity index), the number of species, evenness, and taxonomic distributions of bacterial communities were determined by the 16S Metagenomics software Version 1.1.0 (Illumina) using RefSeq RDP 16S v3 May 2018 DADA2 32 bp taxonomical interference and the Ribosomal Database Project (RDP) Classifier[18]. CCA was applied using the PAleontological STatistics Software version 4.06b package (2021) to consider the variables of the patients' age and marriage status[19].

RESULTS

In the present study, the swab samples for vaginal bacterial microbiota taken from 38 Turkish patients were evaluated. The patients' ages ranged between 22 and 46 years. Out of 38 patients, 21 of them were married, and 17 of the patients were single (Table 1). In addition, five of the patients applied for general control and no symptoms related to any diseases was observed (v2, v11, v22, v29, and v30) and they were considered healthy controls. A preliminary diagnosis of acute vaginitis was made only in one patient with pregnancy status (v35). Moreover, HPV with mild cervical dysplasia was prediagnosed in two patients (v7 and v13). Mastodynia with acute vaginitis was prediagnosed in three patients (v4, v5, and v10). Abnormal uterine and vaginal bleeding were prediagnosed in five patients (v3, v15, v16, v32, and v39). The remaining 22 patients were prediagnosed with acute vaginitis. Patient information with age, marital status, preliminary diagnosis, and anamnesis are given in Table 1.

The vaginal microbiota analysis was generated from a total of 2,047,376 high-quality NGS reads obtained from the total

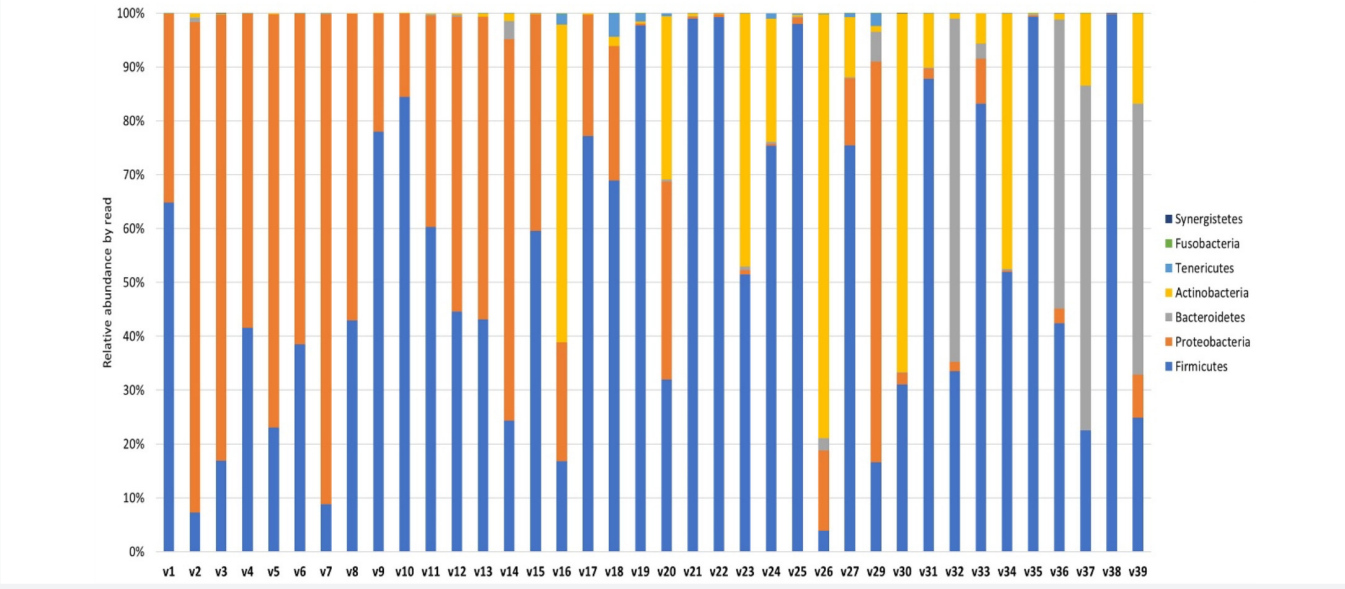

Figure 1. Relative abundance of bacterial communities at the phylum level determined by 16S metagenomic analysis

DNA of 38 vaginal swab samples and all belonged to the bacteria kingdom. The percentages of OTUs allocated to each bacterial phylum are given in Figure 1. Vaginal swab samples revealed the percent abundance of the top six phyla at the phylum level. The dominant phylum Firmicutes was followed by Proteobacteria, Actinobacteria, Tenericutes, Fusobacteria, and Synergistetes in the vaginal samples (Figure 1).

The percentages of bacterial OTUs assigned to the genus level were given in Figure 2. At the genus level, vaginal swab samples revealed the percent abundance of the top twenty genera. In addition, *Lactobacillus* was the most abundant genus in the vaginal swab samples, followed by *Prevotella, Enterobacter, Gardnerella*, and *Dialister*. The percent abundance of the top twenty species was shown in Figure 3. *Lb. iners* was the most abundant species in vaginal swab samples, followed by *Gardnerella vaginalis, Enterobacter tabaci, Prevotella timonensis, P. bivia,* and *Lb. jensenii.*

The alpha diversity of the samples determined by Shannon species diversity index values and evenness is presented in Table 2. The lower evenness indicates the diverse vaginal microbiota. In this context, the highest diversity was found in v38 and v19 samples. The preliminary diagnosis of the v19 and v38 samples was acute vaginitis with vaginal discharge for 2 days. The lowest diversity was found in v39 and v36 samples. The preliminary diagnosis of the v36 sample was acute vaginitis with smelly vaginal discharge for 2 days and menstrual

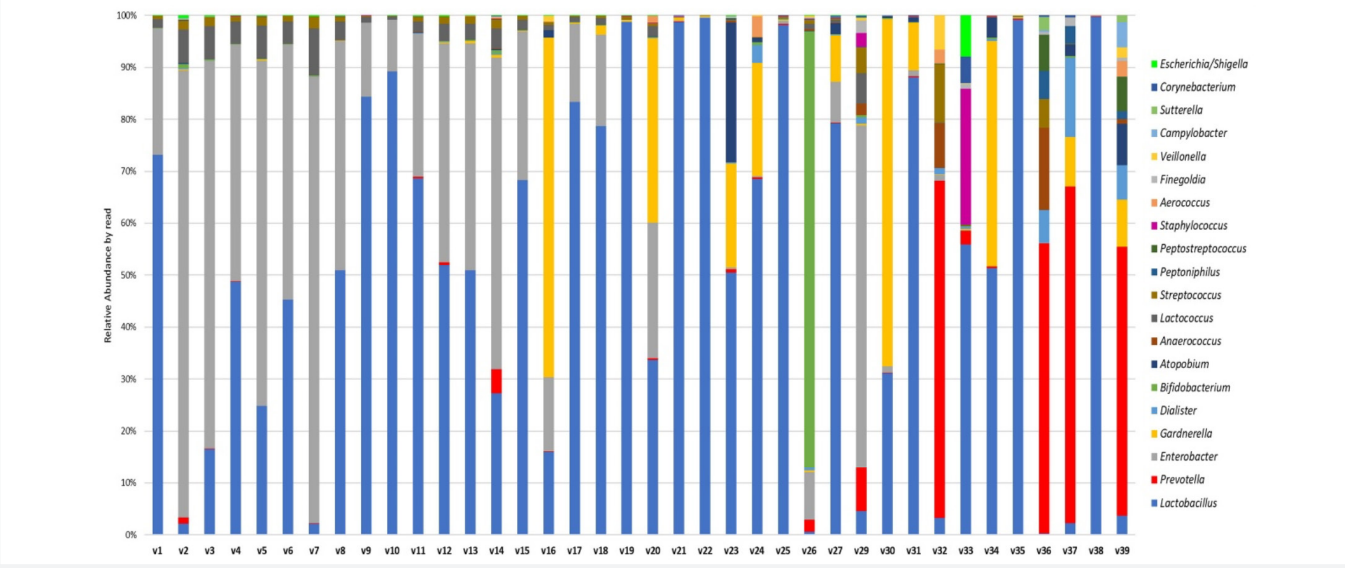

Figure 2. Relative abundance of bacterial communities present in the vaginal microbiota at the genus level

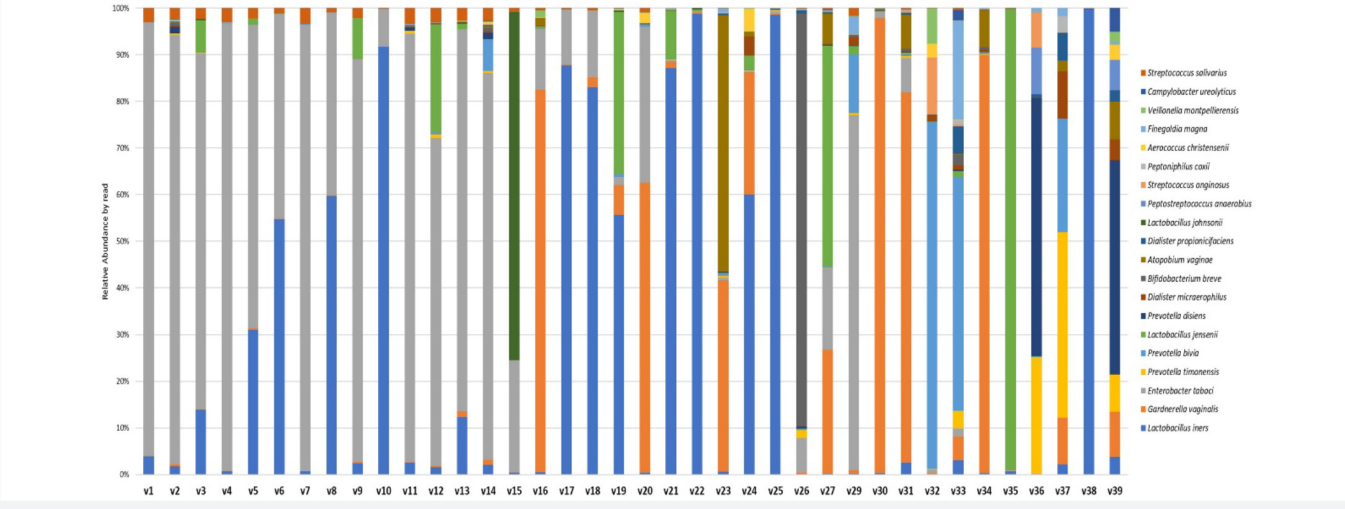

Figure 3. Relative abundance of bacterial communities at the species level

Table 1. Patient information on age, marital status, preliminary diagnosis, and anamnesis

Sample ID	Age	Marital status	Preliminary diagnosis	Anamnesis
V1	29	Married	Acute vaginitis	Menstrual pain for 3 months.
V2	28	Married	Healthy	General control
V3	29	Married	Abnormal uterine, vaginal bleeding	Vaginal discharge for 2 days, Menstrual irregularity for 4 months.
V4	34	Single	Mastodynia, acute vaginitis	Vaginal discharge for 2 days. Pain in the breast.
V5	40	Married	Mastodynia, acute vaginitis	Vaginal discharge for 2 days. Pain in the breast for 1 week.
V6	36	Married	Acute vaginitis	Vaginal discharge for 2 days.
V7	27	Single	HPV with mild cervical dysplasia	Vaginal discharge for 2 days.
V8	46	Married	Acute vaginitis	Spotting in the vagina 4 days ago, Vaginal discharge for 1 week.
V9	31	Single	Acute vaginitis	Menstrual irregularity for 2 months
V10	41	Married	Mastodynia, acute vaginitis	Vaginal discharge for 2 days. 2 months delay in menstruation, anemia.
V11	33	Single	Healthy	General control
V12	30	Married	Acute vaginitis	Vaginal discharge for 2 days, Abnormal uterine bleeding for 2 months.
V13	36	Single	HPV with mild cervical dysplasia	Vaginal discharge for 2 days.
V14	31	Married	Acute vaginitis	Vaginal discharge for 3 months, Abdominal pain.
V15	41	Single	Abnormal uterine, vaginal bleeding	Vaginal discharge for 2 days, Menstrual irregularity for 2 months.
V16	35	Single	Abnormal uterine, vaginal bleeding	Vaginal discharge and itching for 2 days, Menstrual irregularity for 3 months.
V17	27	Single	Acute vaginitis	Vaginal discharge for 2 days.
V18	36	Single	Acute vaginitis	Vaginal discharge and bleeding for 2 weeks.
V19	41	Married	Acute vaginitis	Vaginal discharge for 2 days.
V20	28	Married	Acute vaginitis	Vaginal discharge for 2 days.
V21	28	Single	Acute vaginitis	Vaginal discharge for 2 days, uterine bleeding, Menstrual irregularity for 2 months
V22	32	Single	Healthy	General control
V23	36	Married	Acute vaginitis	Pain, burning, itching in the vagina for 2 days.
V24	36	Single	Acute vaginitis	Vaginal discharge for 2 days.
V25	41	Single	Acute vaginitis	Vaginal discharge for 2 days.
V26	37	Married	Acute vaginitis	Vaginal discharge for 2 days.
V27	22	Married	Acute vaginitis	Vaginal discharge for 2 days, 27-week premature birth and baby died.
V29	28	Married	Healthy	General control
V30	38	Single	Healthy	General control
V31	35	Married	Acute vaginitis	Vaginal discharge for 3 days.
V32	43	Married	Abnormal uterine, vaginal bleeding	Vaginal discharge for 2 days, Menstrual irregularity for 4 months.
V33	26	Single	Acute vaginitis	Vaginal discharge 1 week, Epigastric pain.
V34	32	Single	Acute vaginitis	Frequent urination and burning for 2 weeks, vaginal discharge for 1 week.
V35	32	Married	Acute vaginitis with pregnancy status	Vaginal discharge for 2 days.
V36	43	Married	Acute vaginitis	Smelly vaginal discharge for 2 days, Menstrual irregularity for 2 months.
V37	27	Single	Acute vaginitis	Vaginal discharge for 2 days.
V38	34	Married	Acute vaginitis	Vaginal discharge for 2 days.
V39	30	Married	Abnormal uterine, vaginal bleeding.	Menstrual irregularity for 4 months.

HPV: Human papillomavirus

Table 2. The number of NGS reads per sample, Shannon species diversity index, the number of identified species, and evenness values of the vaginal swab samples

Sample number	Sample ID	Number of reads	Shannon species diversity index	Number of identified species	Evenness
1	v1	59915	0.533	60	0.130
2	v2	15919	0.974	61	0.237
3	v3	25743	1.129	86	0.253
4	v4	32897	0.695	53	0.175
5	v5	47252	1.069	60	0.261
6	v6	49192	1.088	56	0.270
7	v7	41878	0.856	75	0.198
8	v8	24303	1.077	39	0.294
9	v9	69532	0.465	60	0.114
10	v10	142675	0.770	64	0.185
11	v11	22403	0.556	63	0.134
12	v12	73060	0.778	91	0.172
13	v13	12341	0.803	47	0.209
14	v14	8023	1.033	58	0.254
15	v15	38851	1.025	64	0.246
16	v16	17526	0.872	45	0.229
17	v17	46192	0.881	55	0.220
18	v18	37863	1.143	51	0.291
19	v19	86663	0.375	76	0.087
20	v20	30058	1.097	67	0.261
21	v21	42604	0.847	60	0.207
22	v22	26622	0.789	32	0.228
23	v23	21180	1.177	66	0.281
24	v24	50556	1.507	68	0.357
25	v25	27031	0.730	59	0.179
26	v26	36660	0.983	105	0.211
27	v27	68742	1.151	135	0.235
28	v29	19078	0.942	76	0.218
29	v30	83561	0.769	101	0.167
30	v31	51999	0.445	73	0.104
31	v32	54969	1.324	91	0.294
32	v33	31191	0.745	171	0.145
33	v34	67697	0.925	93	0.204
34	v35	115832	0.762	82	0.173
35	v36	93089	2.098	166	0.410
36	v37	236825	2.035	175	0.394
37	v38	46293	0.308	41	0.083
38	v39	91161	2.208	114	0.466

irregularity for 2 months, and the v39 sample was abnormal uterine and vaginal bleeding with menstrual irregularity for 4 months (Tables 1 and 2). In the vaginal samples, the number of identified bacterial species ranged from 32 to 175 (Table 2).

CCA is a correspondence analysis extension that uses direct gradient analysis[20]. CCA was used to determine the associations between vaginal swab samples and their bacterial populations at the phylum, the genus, and the species levels, as well as their variables like marital status and age of examined patients.

The phylum points were presented in Figure 4A. In combination with the arrows for variables, it was accounted for 99.75% of the weighted averages of the 6 phyla of bacteria regarding the two variables. The sum of all eigenvalues was 0.03. Proteobacteria and Fusobacteria were mainly found in variable married/single with the highest percentages, whereas Actinobacteria and Tenericutes were found in variable age with the highest percentages. At the phylum level, a negative correlation was found between variables married/single status and age (Figure 4A).

The genus points were shown in Figure 4B. In combination with the arrows for variables, it was accounted for 99.69% of the weighted averages of the 20 genera of bacteria about the two variables, the sum of all eigenvalues being 0.10. *Campylobacter*, *Atopobium*, *Enterobacter,* and *Lactococcus* were mainly found in variable married/single with the highest percentages, whereas *Gardnerella* was found in the lowest percentages. *Anaerococcus*, *Streptococcus*, *Sutterella,* and *Veillonella* were mainly found in variable age with the highest percentages, whereas *Staphylococcus* and *Escherichia/Shigella* were found in the lowest percentages. In addition, a positive correlation was found between variables married/single and age at the genus level (Figure 4B).

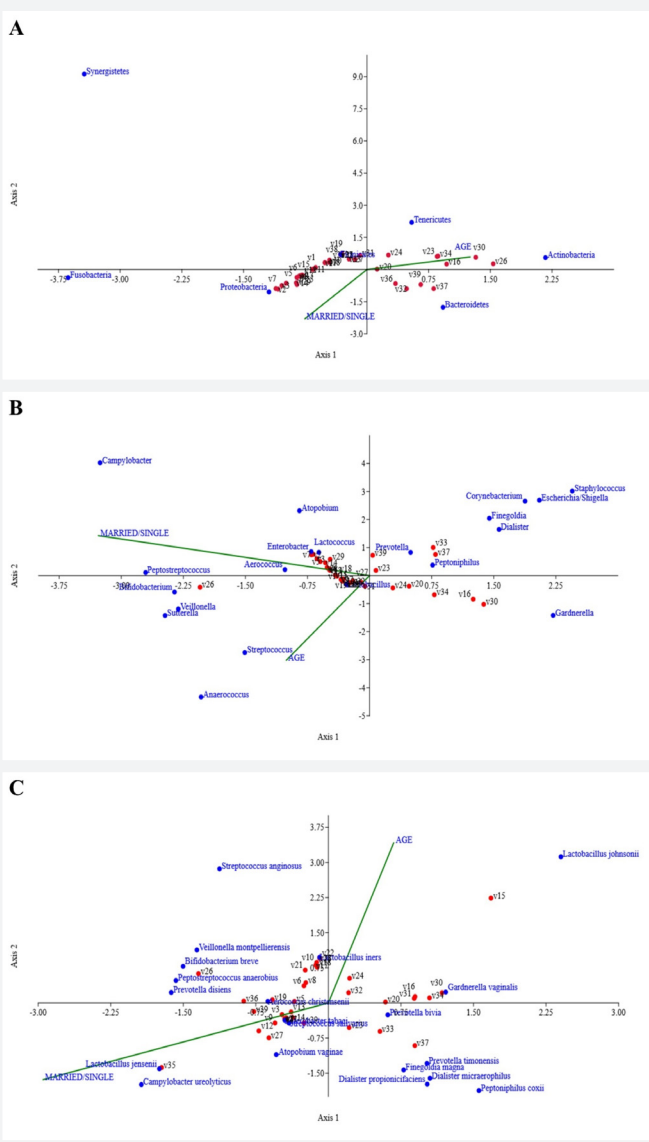

Figure 4. CCA results of the identified bacterial communities in the vaginal swab samples with respect to variables age and married/single status (A) at the phylum level, (B) at the genus level, and (C) at the species level

CCA: Canonical correspondence analysis

It was accounted for 99.42% of the weighted averages of the 20 species of bacteria regarding the two variables, the sum of all eigenvalues being 0.33. *Campylobacter ureolyticus, Lb. jensenii*, and *Atopobium vaginae* were mainly found in variable married/single with the highest percentages, whereas *Lb. johnsonii* and *Gardnerella vaginalis* were mainly found in variable age with the highest percentages. In addition, at the species level, a negative correlation was found between variables married/single and age (Figure 4C).

DISCUSSION

The human vaginal microbiome is complex, and researchers are only now beginning to understand its role in health and disease. The human microbiome is a collection of microorganisms like viruses, bacteria, and fungi that live in a symbiotic relationship with the human body[21]. The microbiome profile of each anatomical component of the body is unique. In the female vaginal tract, virus and bacterial profiles are complex, with a substantial inter-individual variation[22].

Hočevar et al.[23] studied preterm delivery with 155 Caucasian women and they found the dominant phyla in the vaginal microbiota were Firmicutes and Actinobacteria, with lower contributions from Fusobacteria, Proteobacteria, and Tenericutes. At the genus level, the dominant members were Lactobacillus, Gardnerella, Atopobium, Streptococcus, and *Sneathia*. At the species level, *Lb. iners* and *Lb. crispatus* were most abundant bacteria. Their results were similar to our findings in the current study in which Firmicutes were the dominant phyla. However, we found Proteobacteria as the second dominant phyla, and Fusobacteria and Synergistetes were found to be the lower contributors for bacterial microbiota profile (Figure 1). Similarly, we found *Lactobacillus* was the most abundant genus. Indeed, in our study, *Atopobium* and *Streptococcus* were detected, but *Sneathia* was not found in our metagenomics study (Figure 2). At the species level, similarly, we found *Lb. iners* was the most abundant species. Indeed, we did not identify *Lb. crispatus* in our study (Figure 3). Many studies have detected that having *Lb. crispatus* in the vaginal area is connected with fine health; however, having *Lb. iners* in the vaginal area does not provide adequate protection against vaginal dysbiosis[24-26]. Exogenous bacteria are inhibited more effectively by D-lactic acid than by L-lactic acid. As a result, it appears that L-lactic acid makes *Lb. iners* less effective at preventing pathogen invasion[27]. A recent study has reported that bacterial vaginosis patients exhibit vaginal colonization with a wide range of bacteria, including numerous previously uncultivated species that appear to be highly specific for bacterial vaginosis, as well as the absence of *Lb. crispatus*[28].

In a recent study, the microbiota of asymptomatic bacterial vaginosis patients was compared with that of healthy women

using NGS in India. Their results showed that *Lactobacillus* was dominant especially in normal healthy vaginal microbiota compared to dysbiotic microbiota with the abundance of *Gardnerella, Sneathia, Prevotella, Atopobium, Ureaplasma*, and *Dialister* genera[29]. Similar to our results, *Prevotella, Gardnerella,* and *Dialister* were also identified with high read numbers. Furthermore, *Atopobium vaginae, Sneathia amnii, Mycoplasma hominis*, and *Prevotella disiens* were significantly demonstrated as biomarkers for dysbiosis, and *Lb. jensenii* as a biomarker for a healthy microbiota[29]. The decrease in the number of protective *Lactobacillus* population in bacterial vaginosis patients leads to an increase *Gardnerella vaginalis* and *Prevotella species*[30]. Indeed, *G. vaginalis* is the most isolated bacteria in bacterial vaginosis patients[13]. *Prevotella* spp. are associated with menopause, bacterial vaginosis, and body mass index (obesity), as well as they are the most heritable vaginal microbiota members among twins. Moreover, *Prevotella* spp. are negatively correlated with *Lactobacilli* and obese individuals have an abundance of *Prevotella* populations in their vaginal microbiota[31].

The link between ethnicity and health is gaining attention in global health studies. Varied ethnic origins as Algerian women[32], South African women[33], large North American cohorts consisting of four ethnic groups (Caucasian, African, Hispanic, and Asian)[34], Dutch, African Surinamese, South-Asian, Surinamese, Ghanaian, Turkish, and Moroccan women[35] have been documented in earlier research to have different geographical settlements and vaginal flora. The causes of these differences within ethnic groups are unknown, but they could be linked to genetic variations in innate and adaptive immune systems[31].

In a prior study, Komesu et al.[36] discovered that *Lactobacillus* had the greatest connection with age. *Lactobacillus* content in vaginal microbiota was shown to be decreased as people became older, according to their research. The quantity of *Lactobacillus* has therefore been demonstrated to be age-dependent. Similarly, according to our CCA result related to the genus level (Figure 4B), we did not find a positive relationship with the genus *Lactobacillus.* In addition, *Anaerococcus, Streptococcus, Sutterella,* and *Veillonella* were mainly found to have a positive relationship with age variable. Dysbiosis, or changes in the microbiota, has been linked to reproductive failure, and changes in the microbiota may affect susceptibility to gynecological problems. For example, *Anaerococcus, Streptococcus,* and *Veillonella* were found in abnormal vaginal bacterial microbiota[37]. *Sutterella* is a major component of the intestinal microbiota and plays an important role in the dysfunction of human microbiota[38]. Previous results showed that marital status was significantly associated with vaginal bacterial microbiota and the incidence of vaginitis was shown to be more common in married women than in unmarried women[39]. Likewise, we also found the relationships between vaginal bacterial microbiota and marital status by using CCA in the present study (Figures 4A-C).

Study Limitations

The small number of patients in our study is a constraint. More research with a larger number of patients is required.

CONCLUSION

In conclusion, vaginal diseases continue to be a significant public health issue. Thanks to technological developments, it has been seen that the vaginal microbiota, which has been examined in more detail in recent years, is more complex than previously thought. It is critical to swiftly and precisely identify the bacteria involved in the etiology of vaginal diseases for proper therapy. Further studies are needed in more patients to confirm and develop current findings.

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Ethics

Ethics Committee Approval: All procedures used in studies involving human volunteers complied with the institutional and/or national research committee's ethical requirements, as well as the 1964 Helsinki Statement and its subsequent revisions or comparable ethical standards. The collection of all human materials was approved by Yeditepe University Clinical Research Ethics Committee (approval number: 1274, date: 20.08.2020).

Informed Consent: Consent form was filled out by all participants.

Peer-review: Externally peer-reviewed.

Authorship Contributions

Concept: S.U., B.G.T., S.D., V.C.Ö., Design: S.U., M.S., B.G.T., S.D., V.C.Ö., Data Collection or Processing: S.U., M.N.Z.Y., B.B.T., E.E.A., B.G.T., S.D., Analysis or Interpretation: S.U., M.S., M.N.Z.Y., B.B.T., E.E.A., Literature Search: S.U., Writing: S.U., M.S., V.C.Ö.

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REFERENCES

1. Turnbaugh PJ, Ley RE, Hamady M, Fraser-Liggett CM, Knight R, Gordon JI. The human microbiome project. Nature. 2007;449:804-10.

2. Huang B, Fettweis JM, Brooks JP, Jefferson KK, Buck GA. The changing landscape of the vaginal microbiome. Clin Lab Med. 2014;34:747-61.
3. Gupta S, Kakkar V, Bhushan I. Crosstalk between Vaginal Microbiome and Female Health: A review. Microb Pathog. 2019;136:103696.
4. White BA, Creedon DJ, Nelson KE, Wilson BA. The vaginal microbiome in health and disease. Trends Endocrinol Metab. 2011;22:389-93.
5. Stout MJ, Wylie TN, Gula H, Miller A, Wylie KM. The microbiome of the human female reproductive tract. CurrOpin Physiol. 2020;13:87-93.
6. Mancabelli L, Tarracchini C, Milani C, Lugli GA, Fontana F, Turroni F, et al. Vaginotypes of the human vaginal microbiome. Environ Microbiol. 2021;23:1780-92.
7. Shazadi K, Arshad N. Evaluation of inhibitory and probiotic properties of lactic acid bacteria isolated from vaginal microflora. Folia Microbiol (Praha). 2022;67:427-45.
8. Petrova MI, Reid G, Vaneechoutte M, Lebeer S. Lactobacillus iners: Friend or Foe? Trends Microbiol. 2017;25:182-91.
9. Kusters JG, Reuland EA, Bouter S, Koenig P, Dorigo-Zetsma JW. A multiplex real-time PCR assay for routine diagnosis of bacterial vaginosis. Eur J Clin Microbiol Infect Dis. 2015;34:1779-85.
10. Tamrakar R, Yamada T, Furuta I, Cho K, Morikawa M, Yamada H, et al. Association between Lactobacillus species and bacterial vaginosis-related bacteria, and bacterial vaginosis scores in pregnant Japanese women. BMC Infect Dis. 2007;7:128.
11. Mitchell C, Fredricks D, Agnew K, Hitti J. Hydrogen Peroxide-Producing Lactobacilli Are Associated With Lower Levels of Vaginal Interleukin-1β, Independent of Bacterial Vaginosis. Sex Transm Dis. 2015;42:358-63.
12. Balkus JE, Mitchell C, Agnew K, Liu C, Fiedler T, Cohn SE, et al. Detection of hydrogen peroxide-producing Lactobacillus species in the vagina: a comparison of culture and quantitative PCR among HIV-1 seropositive women. BMC Infect Dis. 2012;12:188.
13. Chen X, Lu Y, Chen T, Li R. The Female Vaginal Microbiome in Health and Bacterial Vaginosis. Front Cell Infect Microbiol. 2021;11:631972.
14. Mohseni R, Sadeghi F, Mirinargesi M, Eghbali M, Dezhkame S, Ghane M. A study on the frequency of vaginal species of Mycoplasma genitalium, Gardnerella vaginalis and Neisseria gonorrhoeae among pregnant women by PCR technique. IJMCM. 2013;3:231-6.
15. Han Y, Liu Z, Chen T. Role of Vaginal Microbiota Dysbiosis in Gynecological Diseases and the Potential Interventions. Front Microbiol. 2021;12:643422.
16. Hong X, Ma J, Yin J, Fang S, Geng J, Zhao H, et al. The association between vaginal microbiota and female infertility: a systematic review and meta-analysis. Arch Gynecol Obstet. 2020;302:569-78.
17. Ari Ş, Arikan M: Next-generation sequencing: advantages, disadvantages, and future. In: Hakeem K, Tombuloğlu H, Tombuloğlu G (eds), Plant Omics: Trends and Applications.Switzerland: Springer, Cham; 2016;109-135.
18. Wang Q, Garrity GM, Tiedje JM, Cole JR. Naive Bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy. Appl Environ Microbiol. 2007;73:5261-7.
19. Hammer Ø, Harper DAT, Ryan PD. Past: Paleontological statistics software package for education and data analysis. Palaeontol Electron. 2001:4;1-9.
20. Ter Braak CJF. Canonical correspondence analysis: a new eigenvector technique for multivariate direct gradient analysis. Ecology. 1986;67:1167-1179.
21. Siqueira JD, Curty G, Xutao D, Hofer CB, Machado ES, Seuánez HN, et al. Composite Analysis of the Virome and Bacteriome of HIV/HPV Co-Infected Women Reveals Proxies for Immunodeficiency. Viruses. 2019;11:422.
22. Ling Z, Liu X, Luo Y, Yuan L, Nelson KE, Wang Y, et al. Pyrosequencing analysis of the human microbiota of healthy Chinese undergraduates. BMC Genomics. 2013;14:390.
23. Hočevar K, Maver A, Vidmar Šimic M, Hodžić A, Haslberger A, Premru Seršen T, et al. Vaginal Microbiome Signature Is Associated With Spontaneous Preterm Delivery. Front Med (Lausanne). 2019;6:201.
24. Verstraelen H, Verhelst R, Claeys G, De Backer E, Temmerman M, Vaneechoutte M. Longitudinal analysis of the vaginal microflora in pregnancy suggests that L. crispatus promotes the stability of the normal vaginal microflora and that L. gasseri and/or L. iners are more conducive to the occurrence of abnormal vaginal microflora. BMC Microbiol. 2009;9:116.
25. France MT, Mendes-Soares H, Forney LJ. Genomic Comparisons of Lactobacillus crispatus and Lactobacillus iners Reveal Potential Ecological Drivers of Community Composition in the Vagina. Appl Environ Microbiol. 2016;82:7063-73.
26. Tortelli BA, Lewis WG, Allsworth JE, Member-Meneh N, Foster LR, Reno HE, et al. Associations between the vaginal microbiome and Candida colonization in women of reproductive age. Am J Obstet Gynecol. 2020;222:471.e1-471.e9.
27. Basavaprabhu HN, Sonu KS, Prabha R. Mechanistic insights into the action of probiotics against bacterial vaginosis and its mediated preterm birth: An overview. Microb Pathog. 2020;141:104029.
28. Fredricks DN, Fiedler TL, Thomas KK, Oakley BB, Marrazzo JM. Targeted PCR for detection of vaginal bacteria associated with bacterial vaginosis. J Clin Microbiol. 2007;45:3270-6.
29. Pramanick R, Nathani N, Warke H, Mayadeo N, Aranha C. Vaginal Dysbiotic Microbiome in Women With No Symptoms of Genital Infections. Front Cell Infect Microbiol. 2022;11:760459.
30. Randis TM, Ratner AJ. Gardnerella and Prevotella: Co-conspirators in the Pathogenesis of Bacterial Vaginosis. J Infect Dis. 2019;220:1085-8.
31. Si J, You HJ, Yu J, Sung J, Ko G. Prevotella as a Hub for Vaginal Microbiota under the Influence of Host Genetics and Their Association with Obesity. Cell Host Microbe. 2017;21:97-105.
32. Ouarabi L, Drider D, Taminiau B, Daube G, Bendali F, Lucau-Danila A. Vaginal Microbiota: Age Dynamic and Ethnic Particularities of Algerian Women. Microb Ecol. 2021;82:1020-9.
33. Anahtar MN, Byrne EH, Doherty KE, Bowman BA, Yamamoto HS, Soumillon M, et al. Cervicovaginal bacteria are a major modulator of host inflammatory responses in the female genital tract. Immunity. 2015;42:965-76.
34. Ravel J, Gajer P, Abdo Z, Schneider GM, Koenig SS, McCulle SL, et al. Vaginal microbiome of reproductive-age women. Proc Natl Acad Sci U S A. 2011;108 Suppl 1:4680-7.
35. Borgdorff H, van der Veer C, van Houdt R, Alberts CJ, de Vries HJ, Bruisten SM, et al. The association between ethnicity and vaginal microbiota composition in Amsterdam, the Netherlands. PLoS One. 2017;12:e0181135.
36. Komesu YM, Dinwiddie DL, Richter HE, Lukacz ES, Sung VW, Siddiqui NY, et al. Defining the relationship between vaginal and urinary microbiomes. Am J Obstet Gynecol. 2020;222:154.e1-154.e10.
37. Kiefer ZE, Koester LR, Studer JM, Chipman AL, Mainquist-Whigham C, Keating AF, et al. Vaginal microbiota differences associated with pelvic organ prolapse risk during late gestation in commercial sows†. Biol Reprod. 2021;105:1545-61.
38. Williams BL, Hornig M, Parekh T, Lipkin WI. Application of novel PCR-based methods for detection, quantitation, and phylogenetic characterization of Sutterella species in intestinal biopsy samples from children with autism and gastrointestinal disturbances. mBio. 2012;3:e00261-11.
39. Razzak MS, Al-Charrakh AH, Al-Greitty BH. Relationship between lactobacilli and opportunistic bacterial pathogens associated with vaginitis. N Am J Med Sci. 2011;3:185-92.