

Assessment of the Microbiome Collected from the Reproductive Tracts of Women from Saudi Arabia and Its Potential Influence on Infertility

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Abstract

Background and Objective: Female infertility may be attributed to several causes that are fundamentally related to the health status of women. The main objective of this project was to correlate the abundance of the microbiota in cervicovaginal fluid to infertility. **Materials & Methods:** A total of 36 married women who voluntarily came to the hospital in Riyadh for a routine visit participated in the study. To collect the cervicovaginal liquid, a Softcup™ menstrual device was used by the participant; the cup was then transported in a liquid nitrogen box to the laboratory for analysis. **Results:** The mean vaginal pH in normal women and infertile women was 3.96 and 5.06, respectively, and the difference between the two cohorts was significant ($p < 0.0001$). The results showed a clear correlation between the pH value and the floral species detected in the cervicovaginal liquid. In fact, normal flora is primarily composed of a heterogeneous group of *Lactobacillus* species that have been detected at pH range 3.0 - 4.5, whereas a second group of bacteria mainly composed of *Gardnerella vaginalis*, *Peptostreptococcus anaerobius* and *L. iners* has been detected in a pH range between 4.5 and 5.5. The third group, with a pH range >5.5 , is primarily composed of *G. vaginalis*, *P. anaerobius*, *Mycoplasma hominis*, *Mobiluncus species* and *Atopobium vaginae*. The protein content and viscosity of the cervicovaginal liquid were significantly lower in infertile women compared to normal women ($p < 0.05$).

Keywords

Female Infertility, Microbiota, Cervicovaginal Fluid, pH, Viscosity

1. Introduction

The World Health Organization estimates that approximately 8% of couples, or 50 - 80 million people worldwide, have some forms of infertility [1]. In industrialized countries, 10% - 15% of married couples have had either primary or secondary infertility experiences [2]. This finding may be due to several causes that are fundamentally related to the healthy state of women. Some environmental factors such as smoking and alcohol consumption were linked to infertility. In addition, the exposure to some reproductive toxicants including lead and zinc was also correlated to infertility [2]. The microbiota composition of the reproductive organs is one of the multiple factors that modulate animal reproduction and may also affect embryonic development.

The human microbiota is found various organs including the skin, mouth, gut and vagina. This microbiota plays a crucial role in the health/disease status in both man and women. While the gut is populated with more than 800 microbial species, the vaginal tract hosts approximately only 50 [3]. Vaginal microbiota (VMB) are mainly dominated by Lactobacilli (particularly *Lactobacillus iners*, *L. crispatus*, *L. jensenii* and *L. gasseri*), which are associated with a healthy state in childbearing women [4]. Previous studies have reported the prevalence of at least six types of VMB that have been denoted as community state types (CSTs) [5] [6]. The VMB play key protective roles by maintaining low vaginal pH levels (<4.5) through the production of lactic acid [7] [8] and antimicrobial compounds, or through competitive exclusion of non-indigenous pathogens [9] [10].

It is important to study the microbial communities in the reproductive organs of women within fertility risk and compare them to those in normal women. Such study will be able to determine whether or not the presence of some genera is significantly different between the two cohorts. While most of the bacteria found in the female reproductive organs are considered beneficial, some are considered as pathogens. Thus, the variation in bacterial communities between fertile and infertile women could be associated with level of infertility.

2. Material and Methods

2.1. Data Collection

The study was approved by the Research Ethics Committee in King Saud University. The study has been conducted on married women who voluntarily came to the hospital for a routine visit on a random day of their cycle. The subjects were assigned into two groups according to the level of their fertility. The first group includes married non-pregnant women who have been pregnant at least one time since mirage but without clinical intervention (control, $n = 22$). The second group (Infertile group, $n = 14$) includes women who were married to normal men but were unable to get pregnant after at least one year of marriage. These women are defined as infertile according to WHO. The sampling criteria for the infertile group were: at least one year of marriage without successful fertilization, more than 10 times intercourses per month, have no antibiotics and

have not used any vaginal medication in the last two months. Twenty five women with risk of fertility were recorded, however only 14 have matched the inclusion/exclusion criteria.

At admission, clinical measurements and health history were obtained. These include age, weight, height, date of marriage and number intercourse. Subjects were asked to provide information about any medications they used in the last two months. Women from both groups were asked to obtain a self-sample of the cervicovaginal liquid at admission using Softcup™ menstrual device (Evofem Inc., San Diego, CA, USA). A leaflet of clear instructions was provided to all subjects. Briefly, the Softcup was inserted by the participant into the vagina up to the cervix and removed after 20 minutes. This was repeated in the second admission. The Softcup was quickly placed inside an open 50 ml centrifuge tube and immediately placed into a box containing nitrogen. All cervicovaginal liquid samples were then transported to the laboratory for analysis.

2.2. pH, Viscosity and Total Protein Measurements

The pH was measured using a digital pH meter (Φ11 pH meter, Beckman Instruments Inc., Fullerton, CA, USA) and a glass microelectrode designed to measure pH in small volumes. The pH level was calibrated each time using standard buffer solutions (Sigma-Aldrich pH 4.0, 7.0 and 10.0). The microelectrode was washed with deionized water and disinfected with a solution of benzalkonium chloride each time.

The viscosity of each sample was measured using the Cambridge MicroSample Viscometer (Cambridge Viscosity, Medford, MA, USA). The cervicovaginal liquid of all samples was centrifuged at 2000g for 10 minutes at room temperature (25°C), and the protein phase was removed for quantification. Finally, the samples were stored at -70°C for future study.

2.3. Isolation and Determination of Vaginal Microbiota

Self-collected vaginal swabs from all subjects were treated according to the method described by Verstraelen *et al.* [11]. The swabs were incubated for 4 days at 37°C on a Schaedler agar enriched with 5% sheep blood, vitamin K, haemin and sodium pyruvate. The isolates were then selected for identification. DNA was extracted through simple alkaline lysis before at DNA-PCR and capillary electrophoresis were performed as. The results were analyzed, and the microbiome composition in each sample was determined by comparing the tDNA-PCR fingerprint obtained from each isolate with a library of tDNA-PCR fingerprints. The presence, absence and prevalence of bacterial species/genera were assessed. Correlations between the microbiome composition and infertility were analyzed.

2.4. Statistical Analysis

We used GraphPad prism version 5 for the statistical analysis. Statistical comparisons were made using a two-tailed independent sample t-test. All values are presented as the mean ± SD. Significance was assumed when $p < 0.05$.

3. Results

3.1. Demographic Characteristics of the Studied Women

The ages of the infertile women group (mean 25.27 years) were not significantly different ($p = 0.42$) compared to control women (mean 27.95 years). The mean weight of the normal women was higher (mean 67.86 kg) than that of the infertile women (mean 66.93 kg), but the difference was not significant ($p = 0.73$), **Figure 1**. The mean height of women from normal group (162.8 cm) is higher than that of infertile women (160.8 cm) whereas their BMI is lower (25.65 kg/m) than that of infertile women (26.05 kg/m). However, the differences for both parameters are not significant ($p = 0.747$ and 0.741, respectively).

3.2. The pH level of the Cervicovaginal Liquid

The pH levels of 36 Cervicovaginal samples were measured (22 normal women and 14 infertile women). The present study demonstrated that the mean vaginal pH levels in normal women and infertile women were 3.96 and 5.06, respectively, and the difference between the two cohorts was statistically significant ($p < 0.0001$). For normal women, the lowest pH value was 3.2, and the highest was 5.1 whereas for the infertile women, the lowest pH value was 3.9, and the highest was 5.8 (**Figure 2**).

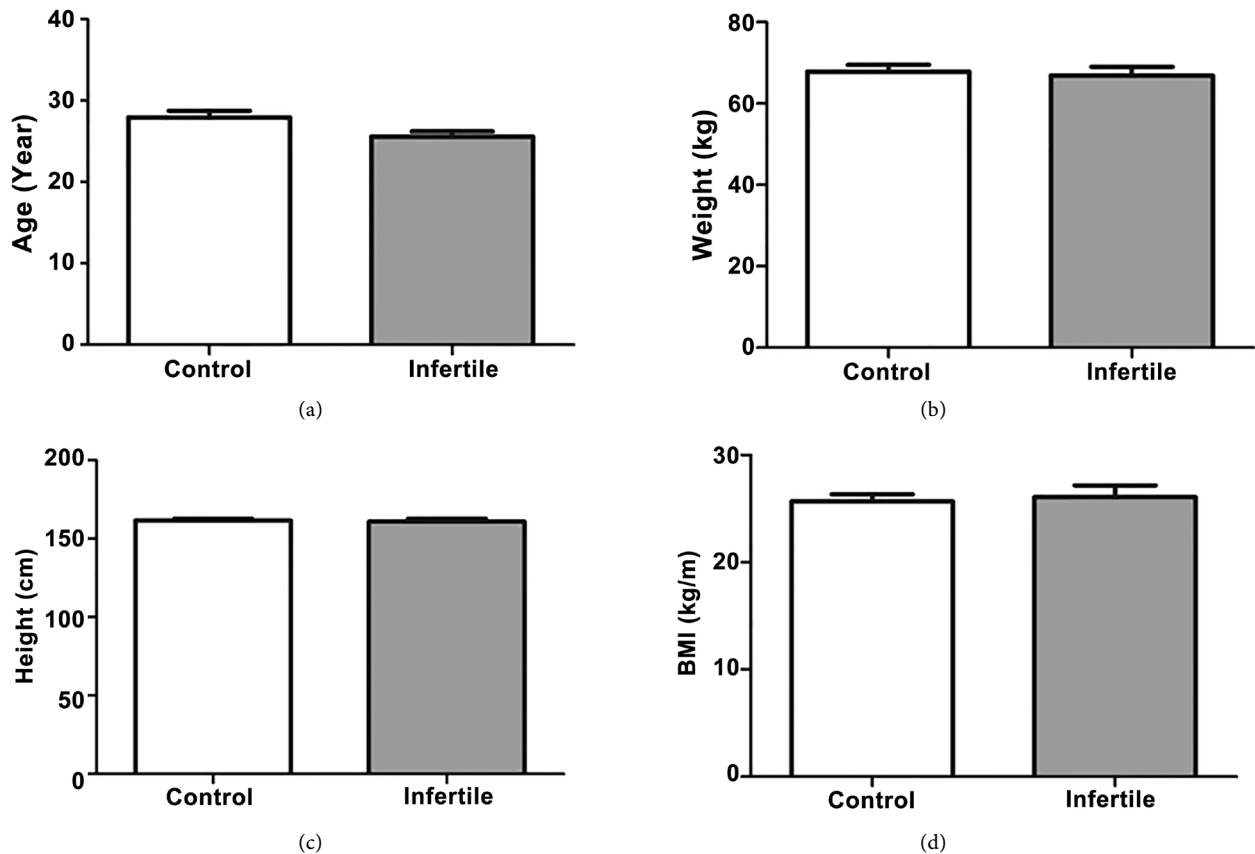


Figure 1. The mean values of age (a), body weight (b), height (c) and body mass Index (d) in the two studied groups of normal women compared to the infertile women.

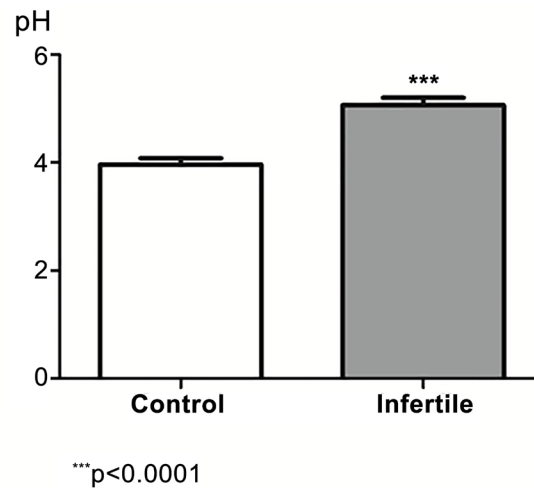


Figure 2. The mean pH values in the two studied groups of normal women compared to the infertile women.

The pH results show a clear correlation between the pH value and the floral species detected in the cervicovaginal liquid (**Figure 3**). In fact, normal flora, primarily composed of *Lactobacillus* species (i.e., *L. gasseri* L., *crispatus*, *L. iners*, *L. vaginalis*, *L. jensenii*, *L. buchneri*, *L. cellobiosus*, and *L. fermentum*) were detected at pH ranges from 3 to 4.5 (with the exception of one patient). Another subgroup of bacteria, primarily composed of *Gardnerella vaginalis*, *Peptostreptococcus anaerobius* and *L. iners*, was detected in a pH range between 4.5 and 5.5. The last subgroup, pH range >5.5, was mainly composed of *Gardnerella vaginalis*, *Peptostreptococcus anaerobius*, *Mycoplasma hominis*, *Mobiluncus species* and *Atopobiumvaginae*.

3.3. Total Protein in the Cervicovaginal Liquid (mg)

The protein content of the cervicovaginal liquid was significantly higher in the normal women compared to the infertile women ($p < 0.0001$) (**Figure 4**). The mean value was 26.36 for the normal women and 20.27 for the infertile women.

3.4. Viscosity of Cervicovaginal (Centipoise)

Normal women were found to have higher cervicovaginal viscosities (mean value 1.56) compared to women who were determined to be infertile (mean value 1.10). This difference was significant ($p < 0.0001$) (**Figure 5**).

4. Discussion

Female infertility may be caused by many factors, including polycystic ovary syndrome, endometriosis, and fallopian tube problems. Many other factors could cause infertility in women. The physical properties of the cervicovaginal liquid must have an adequate consistency and a pH value for suitable proliferation of *lactobacillus* bacteria.

What attracted our attention in this study is the fact that in the second subgroup, *L. iners* (normal flora beneficial for vaginal health) coexisted with a

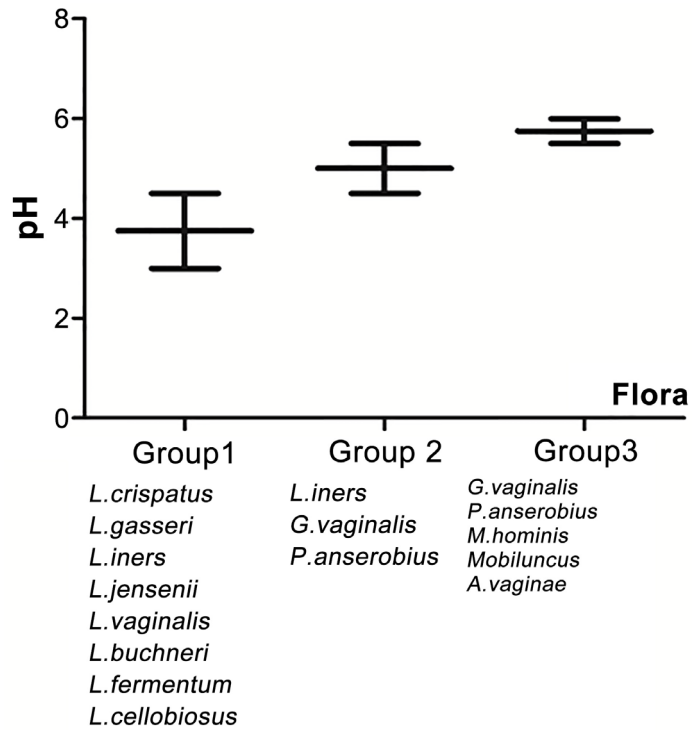


Figure 3. Relationship between the cervicovaginal pH values and the species of cervicovaginal bacterial flora. We determined 3 bacteria groups based on pH values: group 1 includes bacteria living at cervicovaginal acidic pH levels (from 3 to 4.5), group 2 includes bacteria living at pH levels 4.5 - 5.5, and group 3 includes bacteria living at pH levels >5.5.

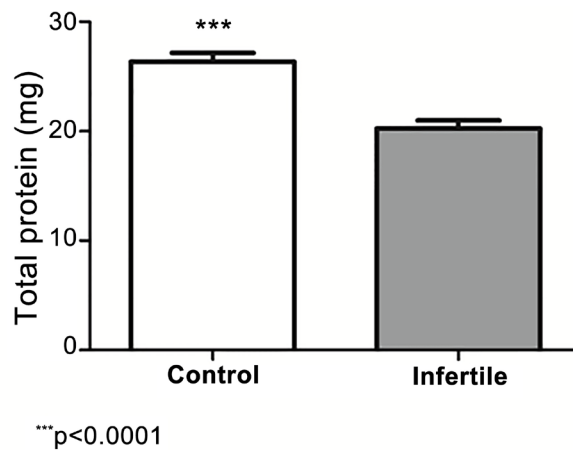


Figure 4. Total protein in the cervicovaginal mucus of the two studied groups of normal women compared to infertile women.

second type of harmful bacteria represented by *Gardnerella vaginalis* and *Peptostreptococcus anaerobius*. Our findings align with those of Jakobsson and Forsum [12] who have suggested that *L. iners* is a dominant part of the cervicovaginal flora when the flora is in a transitional stage between abnormal and normal. Moreover, *Gardnerella vaginalis* and *Peptostreptococcus anaerobius* have been reported to be the most acid resistant of the harmful species [7], which may

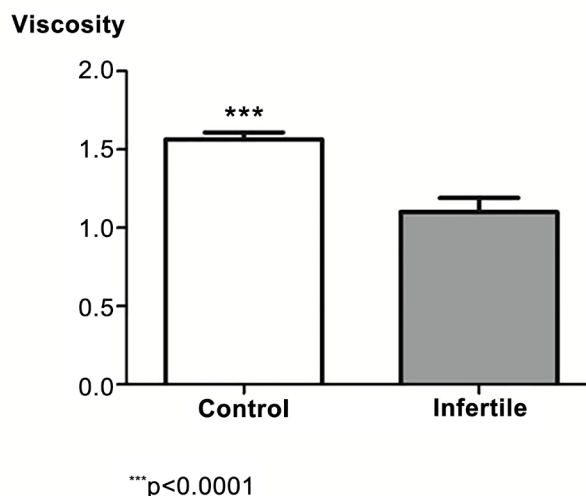


Figure 5. Viscosities of the cervicovaginal mucus in the two studied groups of normal women compared to infertile women.

explain their presence in the pH range of 4.5 to 5. Even *G. vaginalis* is not currently considered to be harmful because this species has been reported to be present in women without any symptoms [13]. In fact, a healthy vaginal pH is somewhere between 3.5 and 4.5 (slightly acidic) for most of the menstrual cycle, and it is generally self-regulating [14] [15]. This particular physical property of the cervicovaginal liquid represents the ideal environment for *acidophilus bacilli*, whereas the pathogenic saprophytes do not find the conditions favorable to proliferate [16]. A normal properly estrogenized vagina is characterized by a predominance of *Lactobacillus* species, such as *L. crispatus*, *L. gasseri*, *L. iners*, *L. jensenii*, *L. vaginalis*, and *L. buchneri* [13] [17]. Indeed, the glycogen is deposited in the epithelial cells of the vagina, which allows the proliferation of lactobacilli and the breakdown of this cellular glycogen. As a result, large amounts of lactic acid (generated by glucose anaerobic fermentation) and hydrogen peroxide are produced to decrease the vaginal pH level to the range of 3.5 - 4.5 [14] [15]. The latter provides a natural barrier to infections and prevents the uncontrolled growth of other bacteria present in the vagina. Moreover, this characteristic is thought to protect against sexually transmitted infection [14].

However, during ovulation and under the effect of ovarian hormones (primarily LH), an increase in cervical mucus causes pH to move more alkaline (7 - 14), which allows sperm to travel through the vagina and cervix into the fallopian tubes for fertilization. Many other factors may also cause the vaginal alkalinization, such as long menstrual periods, inadequate douches and the use of broad-spectrum antibiotics [16]. These factors allow the decrease of the glucose metabolism by *acidophilus bacilli*, and, therefore, the decrease of lactic acid production, which represents an adequate environment to the proliferation of harmless and pathogenic vaginal microorganisms, such as *Escherichia coli*, *Bacteroides fragilis*, *Mobiluncus spp.*, *Neisseria gonorrhoeae*, *Gardnerella vaginalis*, *Peptostreptococcus anaerobius* and *Staphylococcus aureus* [16]. We suggest that

this environment may be one cause of infertility in the second cohort of women studied. In fact, many studies have demonstrated that an elevated vaginal pH level (>5) correlates with recovering potentially pathogenic bacteria from the vagina [13]. Moreover, some studies have reported that an elevated pH greater than 4.5 increased the risk of preterm delivery of low-birth-weight infants [13] [18].

The differences in the protein quantity in the cervicovaginal liquid between the two groups suggest that the vaginal environments of the patients in the two groups are different and may be the cause of infertility. Chappell *et al.* [14] have reported that the protein content of cervicovaginal liquid was significantly lower in postmenopausal women compared to that of premenopausal women, which aligns with our results. Moreover, decreased protein levels in the cervicovaginal liquid could indicate a decreased amount of mucus and a decreased quantity of antimicrobial proteins, such as albumin and globulins, in the vaginal mucus [19].

Cervical mucus is considered to be a hydrogel that is composed of a high-molecular-weight component (gel phase) and a low-molecular-weight component (cervical plasma) [15]. Cervical mucus abnormalities can result in infertility (approximately 3% of couples are facing infertility due to cervical mucus problems in females). In fact, alterations in the cervicovaginal mucus can affect its composition, which is generally associated with cervicitis [15] and acute inflammatory conditions. Therefore, the secreted mucus will be of low viscosity but with high leukocyte content. This physical feature of the mucus (viscosity) forms an environment that is not adequate for sperm to swim easily in, and it is even unable to maintain sperm vitality, which may be a cause of infertility.

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