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Original article

Complementary seminovaginal microbiome in couples

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Abstract

The genital tract microbiome is tightly associated with reproductive health. Although many research studies have been performed on the vaginal microbiome, current knowledge of the male microbiome is scarce, and parallel studies examining couples are extremely rare. In this work, we aimed to compare seminal and vaginal microbiomes in couples and to assess the influence of sexual intercourse on vaginal microbiome.

The study included 23 couples. Microbiomes of semen and vaginal fluid (pre- and post−intercourse) were profiled using Illumina HiSeq2000 sequencing of the V6 region of 16S rRNA gene.

Seminal communities were significantly more diverse, but with lower total bacterial concentrations than those of the vagina. *Gardnerella vaginalis* was predominant in half of the women whose partners had significant leukocytospermia, but only in one of 17 women who had a partner without leukocytospermia. There was significant decrease in the relative abundance of *Lactobacillus crispatus* after intercourse, and high concordance between semen and vaginal samples. Our data support the hypothesis that semen and vaginal microbiomes are in association, inasmuch as the predominance of *G. vaginalis* in female partners was significantly related to inflammation in male genital tracts.

Keywords: Couple; Leukocytospermia; Seminal microbiome; Seminovaginal microbiome; Sexual intercourse; Vaginal microbiome

1. Introduction

The vaginal microbiota is comprised of a moderately diverse community of microbes that play a mutualistic role in maintaining vaginal health. Disrupting of the vaginal microbiota can lead to increased susceptibility to infectious diseases, increased likelihood of adverse pregnancy outcomes and infertility. Recently, the use of next-generation sequencing (NGS) techniques has provided a high-throughput method for determining detailed taxonomic and abundance information regarding the microbes present in diverse microbial communities. This technique has significantly improved the efficiency of studying the vaginal microbiota [1,2]. The vaginal microbiota is an open ecosystem and, in the case of unprotected sex, the female genital microbiota is highly likely to be impacted by the male genital tract microbiota.

Semen contains microorganisms along with other constituents, such as male reproductive proteins and markers of inflammation. Thus, semen serves as a medium for the transmission of microorganisms between men and women and

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contributes to the development of sexually transmitted diseases (STDs). Microbial communities in semen are associated with inflammation in the upper genital tract, and it has been estimated that infections of the genitor-urinary tract may account for 15% of male infertility [3]. Previous studies demonstrated an increase in bacterial vaginosis (BV)-type microbiota after intercourse [4,5], whereas others did not confirm this association [6,7]. Fluctuations in non-BV bacteria have been noted as well [7,8].

Despite its importance for men and their partners, the male genital tract microbiota has been much less frequently studied than the female genital microbiota; indeed, the first NGS studies were only recently published [3,9–11]. In men, the genital tract microbiota exists primarily in the urethra and in the coronal sulcus, whereas the upper genital tract is normally germ-free. Current knowledge about the male microbiota is scarce, and parallel studies examining the microbiota of couples are extremely rare [12,13]. In this work, we aimed to apply NGS to compare the seminal and vaginal microbiome in couples and to assess the influence of sexual intercourse on the composition of the vaginal microbiome.

2. Materials and methods

2.1. Formation of the study group

The study was carried out at the Andrology Center of Tartu University Hospital from 2009 to 2012. It included 23 couples who had consulted a physician due to infertility (trying to conceive >1 year) of diverse etiologies. The male partners (mean age 32.2, range 24–43 years) consulted an andrologist, while the female partners (mean age 29.9, range 21–39 years) were investigated for causes of infertility at the same time. The inclusion criterion for female partners was contraception-free partnership with the current partner for at least 12 months. Exclusion criteria for male partners were defined according to suggestions of an NIH workshop on chronic prostatitis in Bethesda, MD [14]. Exclusion criteria for both partners were antimicrobial therapy within 3 months and anti-inflammatory medication for at least 2 weeks before evaluations.

2.2. Ethical considerations

Participation in the study was voluntary. All subjects were at least 18 years old. Written informed consent was obtained from all study subjects. The study was approved by the Ethics Review Committee on Human Research of Tartu University, Estonia (permission No 174/T-16, 22.09.2008).

2.3. Specimens

Semen samples were collected during menstruation of the female partner, after the 4- to 7-day abstinence. Samples were collected after washing the glans penis with soap and water and after urinating. Semen was obtained by masturbation, ejaculated into a sterile collection tube and incubated at 37 °C for 25–45 min for liquefaction. Detection of basic semen parameters and identification of inflammatory prostatitis using a white blood cell (WBC) count in semen (leukocytospermia) is described elsewhere [15]. These data are presented in Table S1 [41].

Each female participant collected two vaginal samples 3–5 days later (on days 6, 7 or 8 of her menstrual cycle). Samples were collected in the evening before intercourse and the next morning 8–12 h after intercourse. The 3- to 5-day interval between the samples of the male and female counterpart was necessary to ensure optimal semen quality for intercourse related to female sample collection. Female partners were instructed to wash their hands and external genitals with water (without soap) and to collect specimens by inserting a swab 6–8 cm into the vagina. Subsequently, the swab was inserted into an empty tube. The evening specimens were stored overnight in a 4 °C refrigerator. After the morning specimens had been collected, all of the specimens were transported to the laboratory within 2 h. The absence of STDs in both male and female samples was confirmed by PCR (polymerase chain reaction), as described previously [16].

2.4. DNA extraction for NGS

Material from vaginal swab specimens was suspended in 1 ml of phosphate-buffered saline (PBS) and collected by centrifugation at 16,000 × g for 20 min. The supernatant was discarded and the remaining pellet was resolved in PBS. DNA was extracted with the High Pure PCR Template Preparation Kit (Roche Molecular Biochemicals) according to the manufacturers' instructions. DNA extraction from semen samples was performed with the QIAamp Blood Kit (Qiagen, Hilden, Germany) according to the manufacturers' instructions.

Quantitative PCR reactions for counting the 16S rRNA gene copies in semen and vaginal samples were performed as described earlier [17].

2.5. Illumina sequencing

Seminal and vaginal samples were characterized by profiling the microbial community on the basis of the 16S rRNA gene by using the Illumina HiSeq2000 sequencing combinatorial sequence-tagged PCR products. Forward (5′-CAACGCGARG AACCTTACC-3′) and reverse (5′-ACAA-CACGAG CTGACGAC-3′) primers were used to amplify the bacterial-specific V6 hypervariable region of the 16S rRNA gene [18]. The PCR mixture for each sample contained a unique primer pair combination that differed from the rest of the reactions by a 6-base pair (bp) long barcode sequence at the 5′ end [19].

The Phusion Hot Start High Fidelity Polymerase (Thermo Fisher Scientific, Waltham, MA, USA) reaction mixture was used to perform PCRs according to the manufacturer's instructions. The following PCR program was used: 3 min of denaturation at 98 °C, 6 thermal cycles of denaturation at 98 °C for 5 s, annealing at 62 °C for 30 s with a reduction of 1 °C each cycle and extension at 72 °C for 10 s, followed by 19 cycles of denaturation at 98 °C for 5 s, annealing at 57 °C

for 30 s and extension at 72 °C for 10 s. The final extension step was performed at 72 °C for 5 min. For each sample, three replicates of PCR products (20 µl each) were prepared and pooled. The PCR product concentrations of each composite sample were determined on a 2% agarose gel using a MassRuler Express DNA Ladder (Fermentas) and Quantity One software (Bio-Rad Laboratories, Hercules, CA, USA).

Finally, the PCR products of all samples were pooled in equal proportions and the mixture was cleaned and concentrated (4.5 times) with the NucleoSpin Extract II kit (Macherey–Nagel GmbH & Co., Düren, Germany) according to the manufacturers' instructions. The final concentration of the PCR product mixture was measured with a Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA, USA). Preparation of the paired-end DNA library was performed with the NEXTflex PCR-Free DNA Sequencing Kit (BIOO Scientific Corp., Austin, TX, USA) according to the manufacturers' instructions. Sequencing was performed with the Illumina HiSeq2000 system. A mock community consisting of 11 human related stains was included, together with seminal and vaginal samples in the NGS pipeline in order to reveal possible contamination and sequencing errors.

2.6. Sequence data preparation and taxonomic assessment

Paired-end reads were assembled into composite reads with SHERA [20]. Sequences with an SHERA quality score of less than 0.5 were discarded. Custom Perl scripts were used to sort reads to samples according to barcodes, while also removing barcodes and primers. Assembled reads were processed with the program Mothur v. 1.27 [21], following the standard operating procedure guidelines.

Sequences were discarded if they met any of the following criteria: the average sequencing quality score dropped below 35 over a 25-bp sliding window, the sequence was shorter than 70 bp, had any ambiguous bases, or had homopolymers longer than 6. Remaining sequences were aligned to the SILVA-compatible reference alignment [22]. Only sequences that overlapped in the same alignment space (in total, 176,358 effective reads) were analyzed. Within the samples, the number of effective reads was between 268 and 7177.

The Greengenes reference database [23] was trimmed to the 16S rRNA V6 region by using V-Xtractor v. 2.0 [24]. This trimmed database was used with the 6-nearest neighbor algorithm for taxonomic assignment. De-noised sequences were clustered into operational taxonomic units (OTUs) using CROP v 1.33 with 95% similarity level [25]. OTUs with less than 10 sequences were discarded to reduce false diversity because of sequencing errors [26]. The analysis of mock community sequencing data did not reveal any typical contaminating taxa listed in Salter et al. [27]. Analysis of mock community data also confirmed that applied primers did not amplify the genus Mycoplasma. Sequencing error estimated based on mock community analysis results was 0.01%. Sequence data files are deposited in the European Nucleotide Archive under accession number PRJEB8658.

2.7. Data analysis

The LDA Effect Size (LEfSe) algorithm [28] was applied to assess the differences between seminal and vaginal microbiota. For 16S rDNA amplicon sequencing data, principal coordinate analysis (PCoA) was used to explore and visualize similarities between seminal and vaginal microbiota samples based on the obtained Yue and Clayton measure of dissimilarity. The Wilcoxon signed-rank test was used to determine changes in the relative abundance of phylotypes and community diversity indices before and after intercourse in vaginal samples. Procrustes analysis [29], together with the PROTEST permutation test (9999 permutations), were used to assess the overall degree of association between ordinations of seminal and vaginal microbiota samples based on 16S rDNA amplicon sequencing data. This test was also used to assess the association between ordination of seminal microbiota samples and ordination of changes in relative OTU abundances in vaginal microbiota samples (ordination obtained from principal component analysis based on differences in OTU relative abundances before and after intercourse). The m^2 statistics, which are analogous to the r^2 of a correlation and significance value, are reported.

3. Results

Bacterial diversity in vaginal and semen samples was characterized by sequencing the V6 region of 16S rRNA genes. In total, 176,358 sequences were obtained, with an average of 2854 reads from each of the 46 vaginal fluid samples and an average of 1712 reads from the 23 semen samples (Table 1).

Seminal and vaginal bacterial communities had a high number of shared phylotypes (85% of all detected phylotypes). The most abundant genera among shared phylotypes were *Lactobacillus, Veillonella, Streptococcus, Porphyromonas and Atopobium*. Seminal communities were significantly more diverse, having a higher number (n = 90) of unique phylotypes compared to vaginal communities (n = 12), while at the same time, the semen samples had lower total bacterial

Table 1
Average number (±SD) of sequences, phylotype abundance (Sobs), diversity and total 16S rRNA gene copies in the studied samples.

Sample group	Number of sequences	Sobs (number of observed species)	Inverted Simpson index (species richness)	Total 16S rRNA gene copies per ml of sample as revealed by qPCR
Semen	1712 ± 1673	84.1 ± 27.1	15.0 ± 6.0	$2 \times 10^7 \pm 2 \times 10^7$ (range 2×10^6 … 8×10^7)
Vaginal A (before intercourse)	3190 ± 1884	24.9 ± 12.8	1.81 ± 0.63	$8 \times 10^9 \pm 5 \times 10^9$ (range 9×10^8 … 2×10^{10})
Vaginal B (after intercourse)	2357 ± 1037	27.1 ± 12.4	1.70 ± 0.54	$1 \times 10^{10} \pm 9 \times 10^9$ (range1 $\times 10^8$ … 3×10^{10})

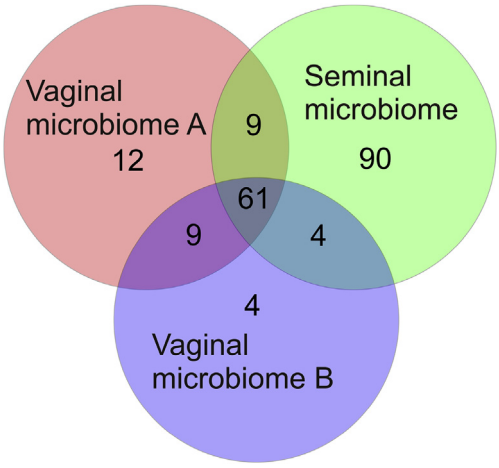

Fig. 1. Venn diagram showing overall overlap of OTUs between the three studied sample groups: vaginal microbiome before intercourse (A), vaginal microbiome after intercourse (B) and seminal microbiome. OTUs are defined at 95% sequence similarity level.

concentrations than vaginal samples (Fig. 1, Table 1). In LEfSe analysis, the vaginal microbiota harbored more species from the genera *Lactobacillus, Streptococcus and Gardnerella* compared to semen samples. Semen samples contained more species from the phyla Firmicutes *(g. Varibaculum),* Bacteroidetes *(g. Flavobacterium, Prevotella, Porphyromonas, Dysgonomonas),* Actinobacteria *(Atopobium, Corynebacterium, Varibaculum)* and β-Proteobacteria (Fig. 2).

Fig. 3a illustrates the microbiome abundance profiles of each semen sample. In a PCoA plot of semen samples, seminal microbiomes did not group according to the level of inflammation based on WBC counts (Fig. 3b). However, the mean proportion of proteobacteria was higher in leukocytospermic men (>1 M WBC/ml) compared to men without leukocytospermia ($p = 0.045$). A higher proportion of *Corynebacterium*, *Flavobacterium* and *Lactobacillus* genera characterized most of the semen samples scattering to the right. The small group on the left (Fig. 3b) was characterized by a high proportion of *Porphyromonas* (samples 17 and 23, Fig. 3a), whereas another small group at the bottom (Fig. 3b) was identified by a high proportion of *Prevotella* sp. that was accompanied by an increased proportion of *Porphyromonas* (samples 26 and 30, Fig. 3a).

In most of the vaginal samples, lactobacilli predominated (mainly *Lactobacillus iners* and *Lactobacillus crispatus,* but also *Lactobacillus jensenii* and *Lactobacillus gasseri*). *Gardnerella vaginalis* was the dominant species in four women, whereas other bacteria (*Streptococcus, Enterobacteriaceae, Veillonella, Pseudomonas, Atopobium and* others) predominated in three women (Fig. 4). *G. vaginalis* was the predominant microorganism in half of the women (three out of six) whose partners had significant leukocytospermia, but in only one of the 17 women who had a partner without significant leukocytospermia (chi-squared test, $p = 0.04$) (Fig. 4). In PCoA (Fig. S1), the vaginal samples were spread along the primary axis depending on the relative abundance of *L. iners* (proportion higher to the right of the origin) and *G. vaginalis*

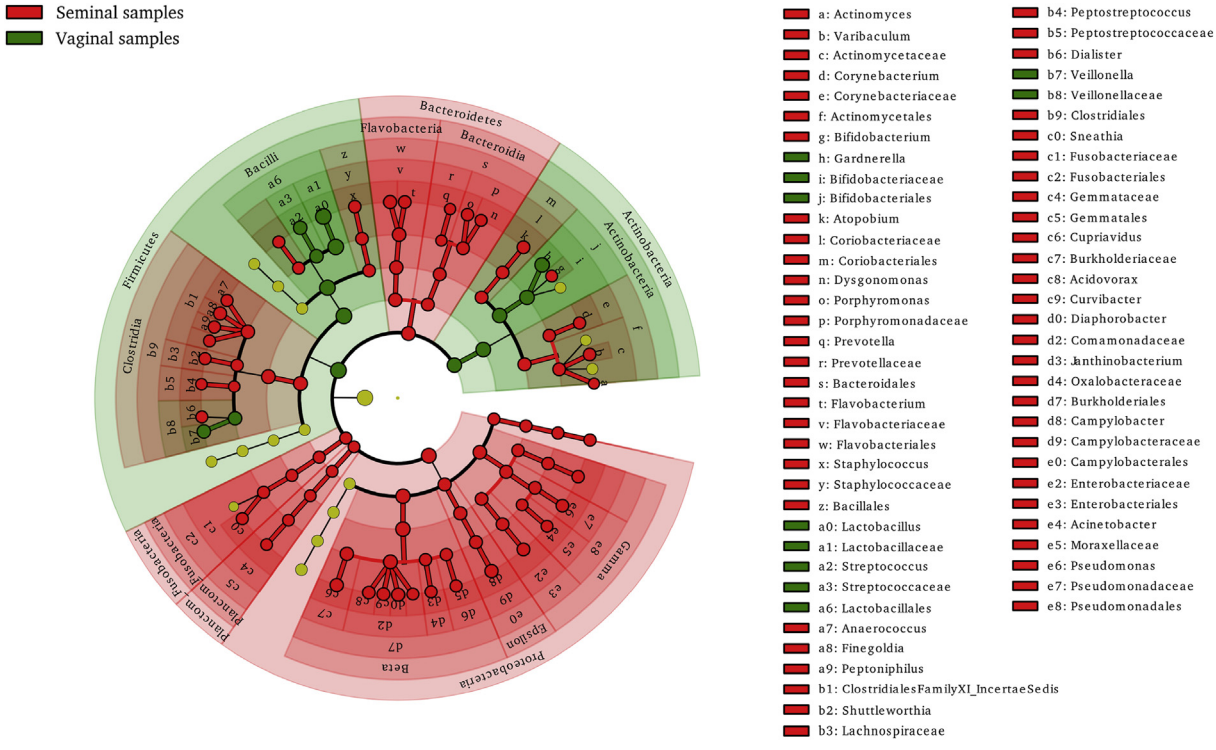

Fig. 2. LEfSe analysis results. Taxonomic representation of statistically significant differences between semen and vaginal microbiomes. Differences are represented in the color of the most abundant classes (red indicating semen, green vaginal and yellow non-significant). Each circle's diameter is proportional to the taxon's abundance.

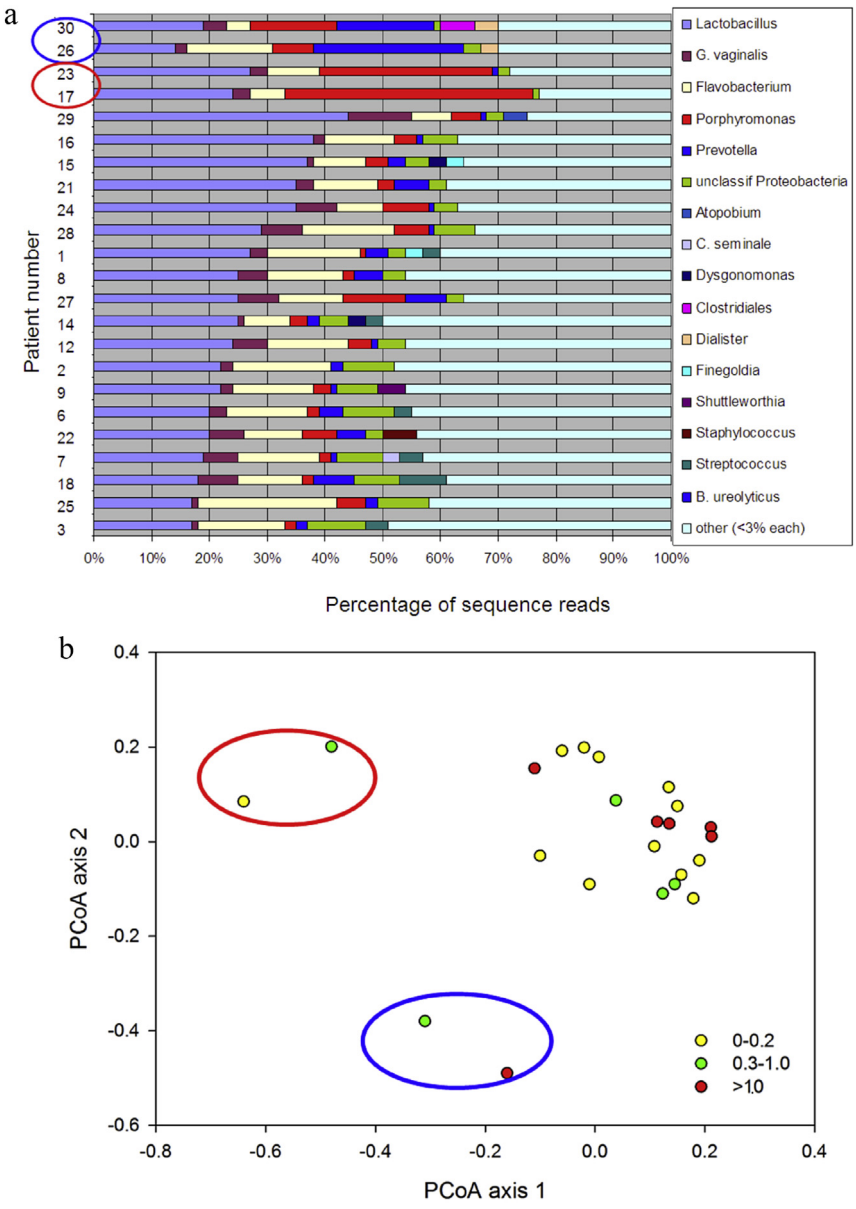

Fig. 3. a. The microbiome abundance profiles of semen samples (% of total microbiome). Only the OTU's above 3% are displayed. The small group at left on Fig. 3b was characterized by a high proportion of *Porphyromonas* (samples 17 and 23 on Fig. 3a, red circle), while another small group at the bottom (Fig. 3b) was distinct in its high proportion of *Prevotella* sp. and *Porphyromonas* (samples 26 and 30 in Fig. 3a, blue circle). b. Ordination plot showing grouping of semen samples according to PCoA based on the Yue and Clayton measure of dissimilarity obtained from 16S rDNA amplicon sequencing data. The first two principal coordinate axes account for 29.8% and 17.4% of overall data variance, respectively. Different levels of inflammation in semen are indicated by different colors (yellow: 0–0.2 M WBC/ml – normal, green: 0.3–1.0 M WBC/ml – weak inflammation; red: >1.0 M WBC/ml – severe inflammation).

(higher to the left of the origin). The distribution of samples along the secondary axis for PCoA was related to the relative abundance of *L. crispatus* in the community. Notable shifts after intercourse in vaginal bacterial community structure appeared in four women and were related to *L. iners* and/or *G. vaginalis*. There was a significant decrease in the relative abundance of *L. crispatus* after intercourse (Wilcoxon signed-rank test, $p < 0.01$).

On the basis of the first four principal components, Procrustes analysis revealed a statistically significant concordance between data sets of semen and vaginal samples before intercourse ($m_{12} = 0.870$, $p < 0.001$). The same strong concordance remained between two PCoA ordinations of semen and vaginal samples after intercourse ($m_{12} = 0.867$, $p < 0.001$). When ordination of samples based on change in OTU relative abundances in vaginal microbiome before and after intercourse was compared to ordination of seminal samples, a strong correlation was found as well ($m_{12} = 0.843$, $p < 0.001$).

4. Discussion

To the best of our knowledge, this is the first study to compare seminal and vaginal microbiomes using NGS, and

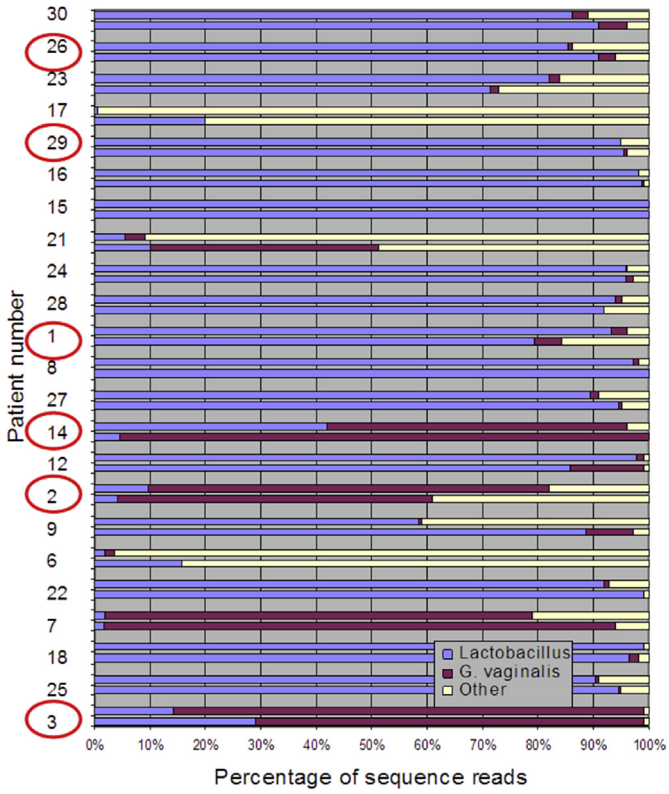

Fig. 4. Microbiome abundance profiles according to Illumina sequencing (% of total microbiome) in 23 women before (lower bar) and after intercourse (upper bar). Red circles indicate women whose male partners had severe inflammation in their semen (>1 M WBC/ml).

to examine the dynamics of vaginal communities related to intercourse. We discovered that semen microbiomes are of low abundance, but of high diversity, whereas vaginal microbiomes are more homogenous than seminal microbiomes. After intercourse, the seminal microbiome caused unexpected but significant shifts in the vaginal microbiome. Major shifts in vaginal communities were seen after intercourse in four out of 23 women. The relative abundance of *L. crispatus* decreased after intercourse. Based on Prokrustes analysis results, both long- and short-term interactions between seminal and vaginal microbiomes of couples was delineated. A predominance of *G. vaginalis* in women was significantly associated with leukocytospermia in their partners.

There has been substantially more investigation regarding the microbiota of the female genital tract, especially that of the vagina, compared to the male genital tract. The healthy vagina contains mostly *Lactobacilli,* which participate in mutualism with the host by resisting colonization by other microorganisms and preventing ascending or systemic infection [30]. The mucosal surface of the female genital tract is a complex biosystem that acts as a barrier against the outside environment and provides innate and acquired immune defense systems. Thus, this compartment has adapted to a dynamic, non-sterile environment that is challenged by various inflammatory stimuli associated with sexual intercourse and endogenous vaginal microbiota [31].

To date, only a few studies have used molecular methods to detect a wide spectrum of microorganisms in semen [3,9–11,32,33]. In the male genital tract, the microbiota exists primarily in the lower genital tract, mostly in the urethra and the coronal sulcus. Semen samples of healthy men tend to have low concentrations of individually different microbiota originating from their urethra. The male upper genital tract is generally germ-free, except in the case of infection. For example, prostatitis patients frequently have abundant polymicrobial communities in their semen and in other prostate-specific materials (expressed prostatic secretion and post-massage urine) [34]. Unlike the vaginal communities, the possible changes in male genital tract communities over time are under-researched.

In contrast, the vaginal microbiota is an open ecosystem and thus can be significantly affected by sexual intercourse. Frequent sexual intercourse, multiple sex partners, frequent episodes of receptive oral sex, receptive anal sex before vaginal intercourse and sex with an uncircumcised male partner may cause fluctuations in vaginal microbial communities and contribute to BV episodes [4,5,35]. Verstraelen et al. [30] suggested that bacterialvaginosis (BV) should be considered a sexually enhanced disease (SED) because of two distinct pathogenetic mechanisms: (1) during unprotected intercourse, alkalinization of the vaginal niche enhances a shift from *Lactobacilli*-dominated microbiota to a BV-like type of microbiota; and (2) intercourse enhances mechanical transfer of perineal enteric bacteria to the vagina. Similar observations supporting the SED pathogenetic model were made for vaginal candidiasis and urinary tract infections [36]. In this study we did not aim to follow our study subjects over time, but if the same study could be done repeatedly during a certain time period, the communities would probably differ to some extent.

Although a man's genital tract microbiota directly influences his partner's vaginal microbiota, there are very few studies examining the relationship between couples' genital tract microbiota. Wittemer et al. [13] cultured endocervical, vaginal and seminal microbiota before in vitro fertilization in 951 couples and found that positive bacterial cultures from both the vagina and semen decreased the clinical pregnancy rate and increased the spontaneous miscarriage rate significantly more than vaginal infection alone. Kjaergaard et al. [12] investigated 11 couples with preterm prelabor rupture of membranes (PPROM) and 18 couples with normal pregnancies. They found pyospermia in three men from the PPROM group and no cases of pyospermia in the control group.

In the present study, we examined the seminal and vaginal microbiota of 23 couples. Although the study group was comprised of infertile couples, we did not aim to reveal any specific infertility-related change in microbiota, since we did not have a control group. This kind of study is quite complicated and healthy couples are not motivated enough to participate. Previous studies that investigated men and women separately have shown that a balance of genital tract microbiota is closely associated with reproductive function, whereas its imbalance may explain reproductive failure in some cases. A common disturbance of vaginal microbiota, BV, may induce shifts in cytokine profiles or disturb the

immuno-endocrinological milieu during implantation and early embryo development. Also, BV may predispose individuals to STDs and thus promote tubal infertility [37,38]. From the male side, genital tract dysbiosis-associated prostatitis may cause obstruction of the genital tract and alter the metabolism, motility and DNA integrity of spermatozoa [39,40]. Hou et al. [3] found a negative association between sperm quality and the presence of *Anaerococcus*. In our study, this genus made up more than 1% of the semen microbes in five men, and two of those men were leukocytospermic. Inflammation of the male genital tract was associated with a somewhat higher proportion of proteobacteria. This observation coincides with current knowledge that coliforms belong to acknowledged urinary tract pathogens. In addition, in four men, Gram-negative anaerobes *Prevotella* and *Porphyromonas* a comprised high proportion, that may indicate inflammation in the upper genital tract.

The present study revealed some intriguing associations between partners' genital tract microbiota. Seminal microbiota communities were significantly more diverse than vaginal microbial communities and there were no predominant microorganisms in most semen samples; at the same time, in most vaginal samples, lactobacilli or *G. vaginalis* predominated. Our data support the hypothesis that semen and vaginal microbiomes are in association, inasmuch as the predominance of *G. vaginalis* in female partner was significantly related to inflammation in the male genital tract. Further studies investigating the complementarity of the partner's microbiome should reveal the functional meaning of these observations.

Conflict of interest

None.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.resmic.2015.03.009.

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