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ORIGINAL ARTICLE

Semen Analysis

Semen microbiota in normal and leukocytospermic males

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Large numbers of microbes can be present in seminal fluid, and there are differences in the semen microbiota between normal and abnormal semen samples. To evaluate the semen microbiota in patients with leukocytospermia, 87 seminal fluid samples, including 33 samples with a normal seminal leukocyte count and 54 samples with leukocytospermia, were obtained for a cross-sectional analysis. Twenty samples with a normal seminal leukocyte count had normal sperm parameters (Control group), and 13 samples with a normal seminal leukocyte count were from asthenozoospermia patients (Ast group). However, 32 samples with leukocytospermia were from asthenozoospermia patients (LA group), and only 22 samples with leukocytospermia had normal sperm parameters (Leu group). The 16S ribosomal RNA (rRNA) gene sequencing method was used to sequence the microbiota in the seminal fluid, and multiple bioinformatics methods were utilized to analyze the data. Finally, the results showed that the worse sperm parameters were observed in the leukocytospermia-related groups. Semen microbiota analysis found that there was increased alpha diversity in the leukocytospermia-related groups. *Firmicutes*, *Proteobacteria*, *Actinobacteria*, and *Bacteroidetes* were the primary phyla in the seminal fluid. Two microbiota profiles, namely, *Lactobacillus*-enriched and *Streptococcus*-enriched groups, were identified in this study. The majority of the samples in the groups with a normal seminal leukocyte count could be categorized as *Lactobacillus*-enriched, whereas the majority of the leukocytospermia samples could be categorized as *Streptococcus*-enriched. Our study indicated that males with leukocytospermia have worse sperm parameters and a different semen microbiota composition compared to males with a normal seminal leukocyte count.

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INTRODUCTION

Leukocytospermia, which is defined by the World Health Organization (WHO) as a seminal leukocyte count of more than 1×10^6 ml^{-1}, may indicate an obstruction or infection in the genitourinary tract.[1] Several studies have found that leukocytospermia is closely related to sterility.[1–4] Most of the evidence suggests that an increase in leukocyte count results in worse semen parameters such as lowered sperm count, concentration, and motility, and abnormal morphology, as more leukocytes, can increase reactive oxygen species (ROS), acrosomal damage, and DNA fragmentation. Consequently, a reduction in the seminal leukocyte count may have a favorable effect on sperm function.[5,6] However, this idea remains controversial, as some studies have failed to associate leukocytospermia with decreased motility or fertilization ability.[7–9] Therefore, further investigation is required to understand the relationship between leukocytospermia and sperm parameters.

Microbes, particularly bacteria, have been implicated in the pathogenesis of many human diseases. For a long time, microbes were considered exclusively harmful to human health. However, with the development of next-generation sequencing (NGS), researchers have realized that microbiomes play an important role not only in disease but also in maintaining human health. The microbiota in the gut, oral cavity, skin, and vagina are necessary for the immune response, energy metabolism, and pathological processes.[10–14] Compared to studies pertaining to other locations in the body, reports on the microbiota in seminal fluid are relatively low, and most of them have focused on the relationship between sperm quality and microbiota. Hou *et al.*[15] identified six groups of bacteria in seminal fluid, with *Anaerococcus* being significantly negatively correlated with sperm quality. Weng *et al.*[16] found that seminal fluid samples could be categorized into three groups based on the predominance of *Lactobacillus*, *Pseudomonas*, or *Prevotella*, and most normal samples could be categorized in the *Lactobacillus* group. Baud *et al.*[17] reported similar results, which indicated that semen samples broadly had three microbiota profiles, namely, *Prevotella* enriched, *Lactobacillus* enriched, and polymicrobial. Furthermore, vaginal microbiota were also found in the seminal fluid samples in that study. Pagliuca *et al.*[18] study found that a relationship between microbiological evaluation and sperm DNA fragmentation in semen samples of patients undergoing fertility investigation.

The mechanism of leukocytospermia is still unclear, and bacterial infection may contribute to this process.[19,20] Although the correlation

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between leukocyte count and bacterial infection of the semen remains controversial, treatment with antibiotics improves sperm parameters in patients with leukocytospermia, suggesting that the modulation of seminal microorganisms could affect sperm quality and fertilization ability.[6,21,22]

This study aimed to assess the relationship between leukocytospermia and sperm parameters in normal and asthenozoospermic males. Furthermore, we performed 16S ribosomal RNA (rRNA) gene sequencing on the MiSeq platform (Illumina Co., Ltd., San Diego, CA, USA) to analyze the composition of the semen microbiota in 87 samples, and the results related to the effects of leukocytospermia on the semen microbiota are described here.

PARTICIPANTS AND METHODS

Participants

For this retrospective study, patient medical records dated between December 2018 and December 2020 were obtained from Xiangya Hospital, Central South University (Changsha, China). The subjects were men seen for premarital checkups or patients with premature ejaculation and sterility. Because the influence of leukocytospermia on azoospermia and oligospermia (sperm concentration $<15 \times 10^6$ ml^{-1}) is minimal, the primary etiologies are endocrine abnormalities, intrinsic disorders of spermatogenesis, and obstruction of the ductal system.[23–25] Patients with documented azoospermia and oligospermia were not included in this study. Patients with any other chronic diseases were excluded from this study. Data on age, medical history, semen volume, sperm concentration, progressive sperm motility, total sperm motility, sperm acrosin activity, and leukocytospermia were extracted from these records. The use of the hospital's database was approved by the Ethical Review Board at Xiangya Hospital, Central South University (No. 201906122).

Based on the WHO guidelines, asthenozoospermia was defined as a sperm concentration higher than 15×10^6 ml^{-1}, with progressive sperm motility <32% or total sperm motility <40%. Leukocytospermia was defined as a seminal leukocyte count $>1 \times 10^6$ ml^{-1}.[26] Sperm parameters including sperm concentration, progressive sperm motility, and total sperm motility were determined in our laboratory by a skilled technician. The seminal leukocyte count was stained using the peroxidase method, and acrosin was quantified using an enzyme-linked immunosorbent assay (ELISA) kit. Finally, the selected samples were categorized into four groups. The Control group included subjects with normal sperm parameters and a normal seminal leukocyte count, the asthenozoospermia (Ast) group included subjects with a normal seminal leukocyte count and asthenozoospermia, the leukocytospermia (Leu) group included subjects with normal sperm parameters, but with a seminal leukocyte count $>1 \times 10^6$ ml^{-1}, and the final group (LA group) included subjects with asthenozoospermia and a seminal leukocyte count $>1 \times 10^6$ ml^{-1}.

Sample collection

In addition to the retrospective study, a cross-sectional study with 87 subjects with normal sperm parameters or leukocytospermia was conducted at Xiangya Hospital, Central South University. As described above, subjects were divided into four groups: the Control group ($n = 20$), the Ast group ($n = 13$), the Leu group ($n = 22$), and the LA group ($n = 32$). Subjects were excluded if they had any systemic diseases (*e.g.*, cardiac, pulmonary, digestive, neural, and renal diseases, hypertension, and diabetes) or any type of tumor, zoospermia, or oligospermia; a fever or any infection in the previous two months; a personal or family history of genetic or immune diseases; or a history of use of antibiotics, immunosuppressive drugs, or corticosteroids in the previous two months. All participants provided written informed consent. The study protocol was reviewed and approved by the Ethical Review Board at the Xiangya Hospital, Central South University (No. 201906122).

Seminal fluid was collected as per the WHO guidelines.[26] The seminal fluid was collected by masturbation after 3 to 7 days of abstinence. Before sample collection, the hands and penises of the subjects were washed twice with soap. The seminal fluid was ejaculated into a sterile receptacle; 1 ml of seminal fluid sample was transferred to sterile microcentrifuge tubes and immediately stored at −80°C, and the rest of the sample was used for clinical tests. Venous blood samples from all subjects were collected, and white blood cell (WBC), hemoglobin (Hb), and platelet (PLT) counts were analyzed in our biochemistry laboratory.

Semen microbiota analysis

The QIAamp DNA mini kit (Qiagen Biotechology Co., Ltd., Dusseldorf, Germany) was used to extract genomic DNA, and the manufacturer's instructions were strictly followed. Bacterial DNA amplification was performed as previously described.[27] Bacterial DNA was amplified via PCR (ABI GeneAmp® 9700, Boston, MA, USA) using custom-barcoded primers targeting the V3–V4 regions (338F: ACTCCTACGGGAGGCAGCAG, −806R: GGACTACHVGGGTWTCTAAT) of the 16S rRNA. Next, we purified the amplified sequences with the AxyPrep DNA Gel Extraction Kit and quantified the purified products with QuantiFluor™-ST (Promega Co., Madison, WI, USA). Finally, the TruSeqTM DNA Sample Prep Kit (Illumina Co., Ltd.) was used to construct the sequencing library, and the MiSeq platform was used to sequence the eligible library.

The raw sequences were analyzed by FLASH (https://ccb.jhu.edu/software/FLASH/index.shtm) for quality control and nonredundant gene building high-quality sequences reached a 97% nucleotide similarity were clustered into operational taxonomic units (OTUs). Nonredundant genes were stored in the Silva 138 release database (http://www.arb-silva.de) for species annotation and assessment, and the relative abundance was calculated at different levels (Domain, kingdom, Phylum, Class, Order, Family, Genus, and Species). Alpha diversity was evaluated using the Shannon index, ace index, and chao index with mothur software (https://www.mothur.org/wiki/Download_mothur). Beta diversity was analyzed using principal coordinate analysis (PCoA), with the Bray Curtis dissimilarity distance matrix at the OTU level by R software (https://www.r-project.org/). The relative abundance of genera was normalized by the log2 method. The microbiota clusters at the genus level were determined using the partitioning around medoids (PAM) algorithm with the Bray Curtis distance matrix. A heatmap of the top 15 most abundant genera was generated using R software. The semen microbiota analysis in our study was completed at Shanghai Majorbio Bio-pharm Technology Co., Ltd. (Shanghai, China). The experimental design is shown in **Figure 1**.

Statistical analyses

Differences in sperm parameters and detailed subject information between the Control and Leu groups or the Ast and LA groups in the retrospective study were evaluated using the nonparametric Kruskal–Wallis test followed by *post hoc* Wilcoxon rank sum test with continuity correction. Sequence number, alpha diversity indices, and the relative abundance of phyla were determined using Student's *t*-tests and expressed as the mean ± standard deviation (s.d.). All statistical analyses were conducted using SPSS 23.0 (SPSS Inc., Chicago, IL, USA). $P < 0.05$ was considered statistically significant.

RESULTS

Relationship between leukocytospermia and sperm parameters

Based on the inclusion criteria, a total of 1161 samples were selected for further analysis in the retrospective study. In total, 460 samples with a normal seminal leukocyte count and 701 samples with leukocytospermia were included in this study (**Table 1** and **Supplementary Table 1**). In the non-leukocytospermia groups, 66.1% of samples had normal sperm parameters, and only 33.9% of samples were from asthenozoospermia patients. However, 63.6% of samples with leukocytospermia fulfilled the diagnostic criteria for asthenozoospermia. Moreover, compared to the Leu group, the seminal leukocyte count was significantly increased by 20.0% in the LA group ($P < 0.001$).

No significant differences in age or ejaculated volume were observed among the four groups (**Table 1**). The other four sperm parameters, including sperm concentration, progressive sperm motility, total sperm motility, and sperm acrosin activity, were significantly decreased in the groups with leukocytospermia (all $P < 0.001$). Compared to those of the Control group, sperm concentration, progressive sperm motility, total sperm motility, and sperm acrosin activity were significantly decreased in the Leu group by 10.2%, 11.2%, 9.5%, and 11.8%, respectively.

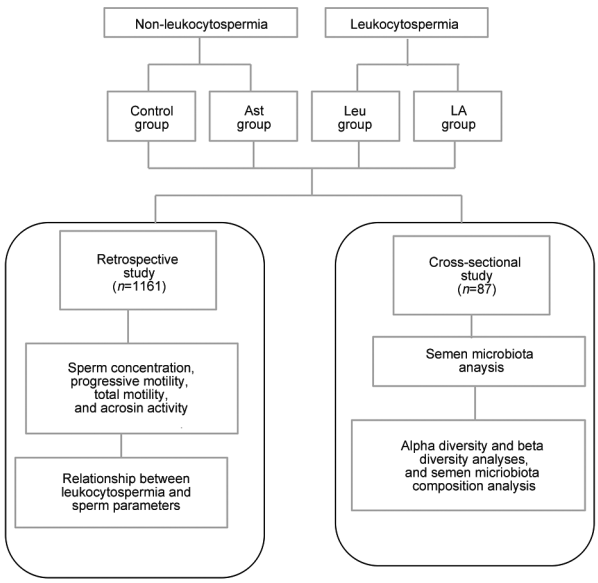


Figure 1: Experimental design. Ast group: asthenozoospermia patients with a normal seminal leukocyte count; Leu group: patients with leukocytospermia and normal sperm parameters; LA group: asthenozoospermia patients with leukocytospermia.

Similarly, compared to those of the Ast group, sperm concentration, progressive sperm motility, total sperm motility, and sperm acrosin activity decreased in the LA group by 10.7%, 8.4%, 6.4%, and 17.7%, respectively. These results indicated that samples from patients with leukocytospermia had worse sperm parameters than samples with a normal seminal leukocyte count.

Patient information

Detailed information on the samples from the 87 subjects who were included in the cross-sectional study is given in (**Supplementary Table 2**). We did not observe any differences in age, WBC, Hemoglobin, PLT, or smoking ratio among the groups. Similar to the retrospective study, sperm parameters, including sperm concentration, progressive sperm motility, total sperm motility, and sperm acrosin activity, were found to be significantly decreased in the leukocytospermia-related groups (all $P < 0.05$; **Figure 2** and **Supplementary Table 3**).

Semen microbiota analysis at the OTU level

A total of 3 945 550 high-quality sequences were detected, and 15 937 OTUs were clustered from the 87 semen samples (**Supplementary Table 4**). We found that the number of OTUs in the LA group was as high as 10 679, and only 5399 OTUs were identified in the Ast group (**Figure 3a**). The number of OTUs in the Control group was 8107 and that in the Leu group was 8855. The Leu group had 1780 unique OTUs, and 2896 unique OTUs existed in the LA groups. However, only 1513 and 666 unique OTUs were identified in the Control and Ast groups, respectively. The Shannon index, ace index, and chao index were used to assess the alpha diversity of the semen microbiota. Compared to the Control group, the Shannon index was found to be significantly increased ($P < 0.05$), and the ace index and chao index were slightly increased in the Leu group (**Figure 3b**). Similar results were observed in the Ast and LA groups, indicating that semen samples from subjects with leukocytospermia had increased alpha diversity (**Figure 3b**). To examine the bacterial community structure in all semen samples, a beta diversity analysis was performed with PCoA at the OTU level. However, the semen samples could not be separated into their four groups by PCoA (**Figure 3c**).

Semen microbiota analysis at the phylum level

To further investigate the sperm microbial composition, we analyzed the results at the phylum level. *Firmicutes*, *Proteobacteria*, *Actinobacteria,* and *Bacteroidetes* were the primary phyla in every group (**Figure 4a**). We did not observe significant differences in the proportion of these three phyla (including *Firmicutes*, *Proteobacteria*, and *Actinobacteria*) among the four groups (**Figure 4a** and **4b**). In the Control group, the composition of *Firmicutes*, *Proteobacteria*, and

Table 1: Sperm parameters in the nonleukocytospermia and leukocytospermia groups

Variable	*Non-leukocytospermia*		*Leukocytospermia*	
	Control	*Ast*	*Leu*	*LA*
Patient, *n* (%)	304 (66.1)	156 (33.9)	255 (36.4)	446 (63.6)
Leukocytospermia ($\times10^6$ ml^{-1})	0.70 (0.50–0.90)	0.73 (0.60–0.90)	1.40 (1.10–1.50)[ab]	1.68 (1.20–1.70)[ab]
Age (year)	30.6 (28.0–33.8)	30.2 (28.0–33.0)	30.0 (27.0–33.0)	30.5 (28.0–33.0)
Semen volume (ml)	3.0 (1.5–4.5)	3.1 (2.0–4.0)	3.0 (2.0–4.0)	3.0 (1.5–4.0)
Sperm concentration ($\times10^6$ ml^{-1})	195.6 (140.8–237.9)	106.7 (61.8–140.8)[a]	177.4 (132.8–207.2)[ab]	96.4 (64.0–122.2)[ab]
Progressive sperm motility (%)	44.0 (37.4–49.3)	24.6 (21.6–29.6)[a]	39.6 (34.6–43.2)[ab]	22.7 (18.2–28.5)[ab]
Total sperm motility (%)	67.5 (60.3–74.7)	43.1 (38.6–50.2)[a]	61.9 (55.1–68.5)[ab]	40.5 (35.0–48.0)[ab]
Sperm acrosin activity ($\times10^6$ μIU^{-1})	108.0 (95.3–118.3)	75.0 (56.9–91.2)[a]	96.6 (83.8–106.9)[ab]	63.7 (50.8–72.0)[ab]

[a]The Ast, Leu or LA group compared to the Control group, $P<0.001$; [b]The Leu or LA group compared to the Ast group, $P<0.001$. Data are shown as median (IQR). Ast group: asthenozoospermia patients with a normal seminal leukocyte count; Leu group: patients with leukocytospermia and normal sperm parameters; LA group: asthenozoospermia patients with leukocytospermia; IQR: interquartile range

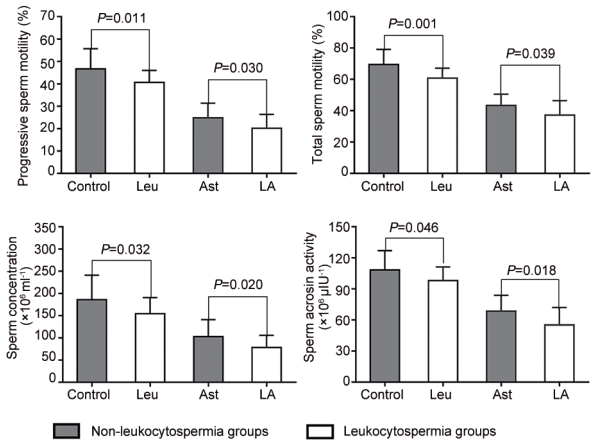


Figure 2: Sperm parameters of the 87 subjects. The leukocytospermia groups had worse sperm parameters. Ast group: asthenozoospermia patients with a normal seminal leukocyte count; Leu group: patients with leukocytospermia and normal sperm parameters; LA group: asthenozoospermia patients with leukocytospermia.

Actinobacteria accounted for 38.7%, 26.3% and 13.6%, respectively. The composition of *Firmicutes*, *Proteobacteria*, and *Actinobacteria* in the Leu group was 37.6%, 25.0%, and 13.4%, respectively. Similarly, the proportions of these three phyla were 35.3%, 28.9%, and 18.1% in the Ast group and 41.2%, 25.2%, and 12.6% in the LA group (**Figure 4a**). However, the relative abundance of *Bacteroidetes* was different in each group. The proportion of *Bacteroidetes* in the Control group was 3.9% but that in the Leu group was 9.4% (**Figure 4a**). Compared to the Control group, *Bacteroidetes* was significantly increased in the Leu group ($P < 0.05$; **Figure 4b**). As a result, the ratio of *Firmicutes*/*Bacteroidetes* was significantly decreased in the Leu group. Compared to that in the Ast group, the relative abundance of *Bacteroidetes* moderately increased in the LA group, accompanied by a slight decrease in the ratio of *Firmicutes*/*Bacteroidetes* (**Figure 4b**).

Semen microbiota analysis at the genus level

At the genus level, the composition of the semen microbiota was analyzed. Overall, the top 15 most abundant genera in the semen were *Streptococcus*, *Lactobacillus*, *Burkholderia-Caballeronia-Paraburkholderia*, *Staphylococcus*, *Gardnerella*, *Ralstonia*, *Corynebacterium*, *Veillonella*, *Acinetobacter*, *Rhodococcus*, *Finegoldia*, *Peptoniphilus*, *Enterococcus*, *Prevotella*, and *Haemophilus* (**Figure 5a**), and most of these genera are also found in the vagina.[12,28–33] Microbiota profile analysis revealed that the seminal genera were clustered into two main groups: *Lactobacillus*-enriched and *Streptococcus*-enriched groups (**Figure 5b**); 60.0% of Control subjects and 53.9% of Ast subjects belonged to the *Lactobacillus*-enriched group, whereas only 45.5% of Leu subjects and 50.0% of LA subjects belonged to the *Lactobacillus*-enriched group. In other words, 57.6% of non-leukocytospermia subjects but only 48.1% of leukocytospermia subjects belonged to *Lactobacillus*-enriched group. In contrast, 51.9% of leukocytospermia subjects and only 42.4% of subjects with normal seminal leukocyte counts exhibited to *Streptococcus*-enriched group. This indicated that there was a decrease in *Lactobacillus* and an increase in *Streptococcus* in samples from subjects with leukocytospermia.

DISCUSSION

In this retrospective study, based on the diagnostic criteria of asthenozoospermia and leukocytospermia, 1161 samples were obtained

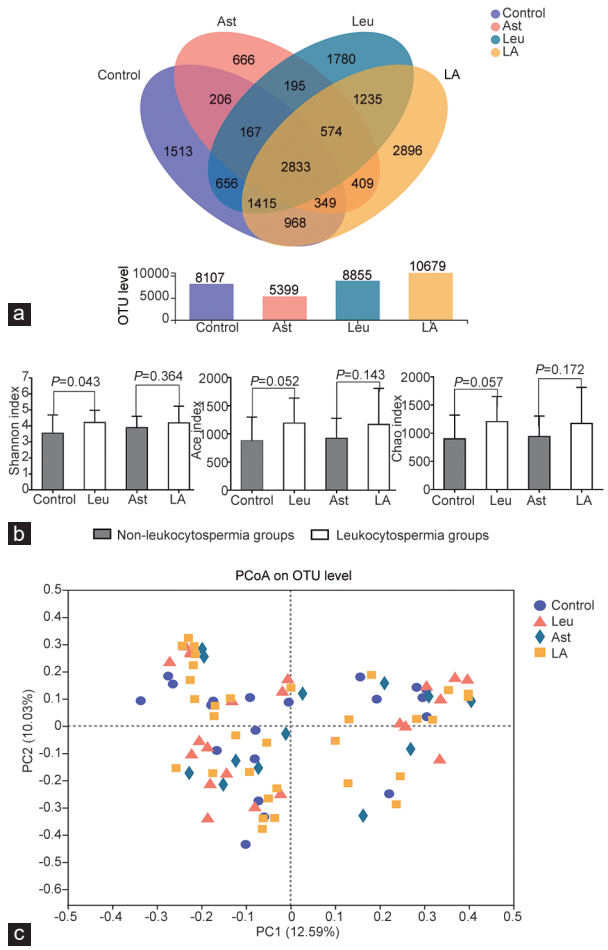


Figure 3: Semen microbiota was analyzed at the OTU level. (**a**) Venn diagram of Control, Leu, Ast, and LA groups at the OTU level. The leukocytospermia-related groups had more OTUs. (**b**) Alpha diversity analysis. The leukocytospermia-related groups had an increase in alpha diversity. (**c**) PCoA of the microbial composition. No significant separation between the different groups at the OTU level. Ast group: asthenozoospermia patients with a normal seminal leukocyte count; Leu group: patients with leukocytospermia and normal sperm parameters; LA group: asthenozoospermia patients with leukocytospermia; OTU: operational taxonomic unit; PCoA: principal coordinate analysis.

and categorized into four groups. The results revealed that samples from subjects with leukocytospermia in the Leu and LA groups had lower sperm quality, indicating that the seminal leukocyte count was negatively correlated with sperm parameters not only in normal males but also in asthenozoospermic males. These results are consistent with those of previous studies[1–3,34] and reconfirmed the relationship between leukocytospermia and sperm parameters.

Owing to the results indicating an important effect of sperm microbes on seminal leukocyte count and sperm parameters, we used 16S rRNA gene sequencing to identify the microbiota in additional 87 seminal samples. Several studies have observed that higher alpha diversity in the gut, skin, and oral cavity is beneficial for human health.[35–37] However, this rule does not seemingly apply to the microbiota in genital tract, and some studies have found that higher diversity of microbiota is a negative factor for sperm health.[38,39] Semen from men with chronic prostatitis/chronic pelvic pain syndrome has been shown to have a higher species diversity than that of healthy men, and women with bacterial vaginosis have communities that are more polymicrobial.[40,41] In

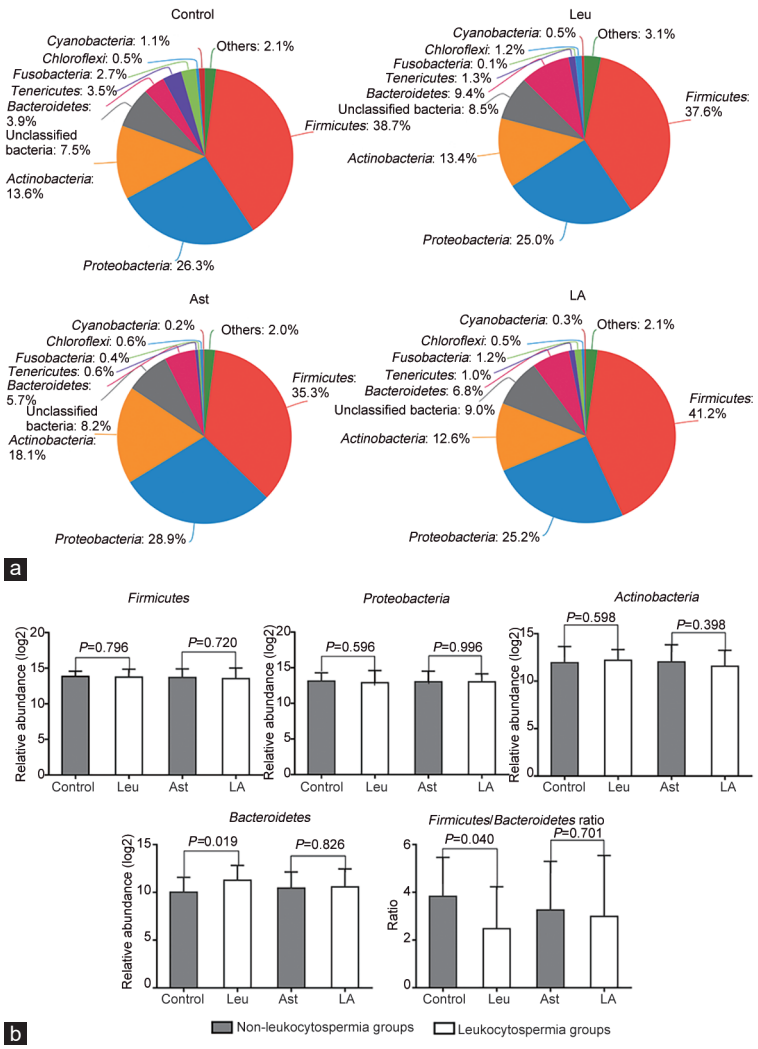
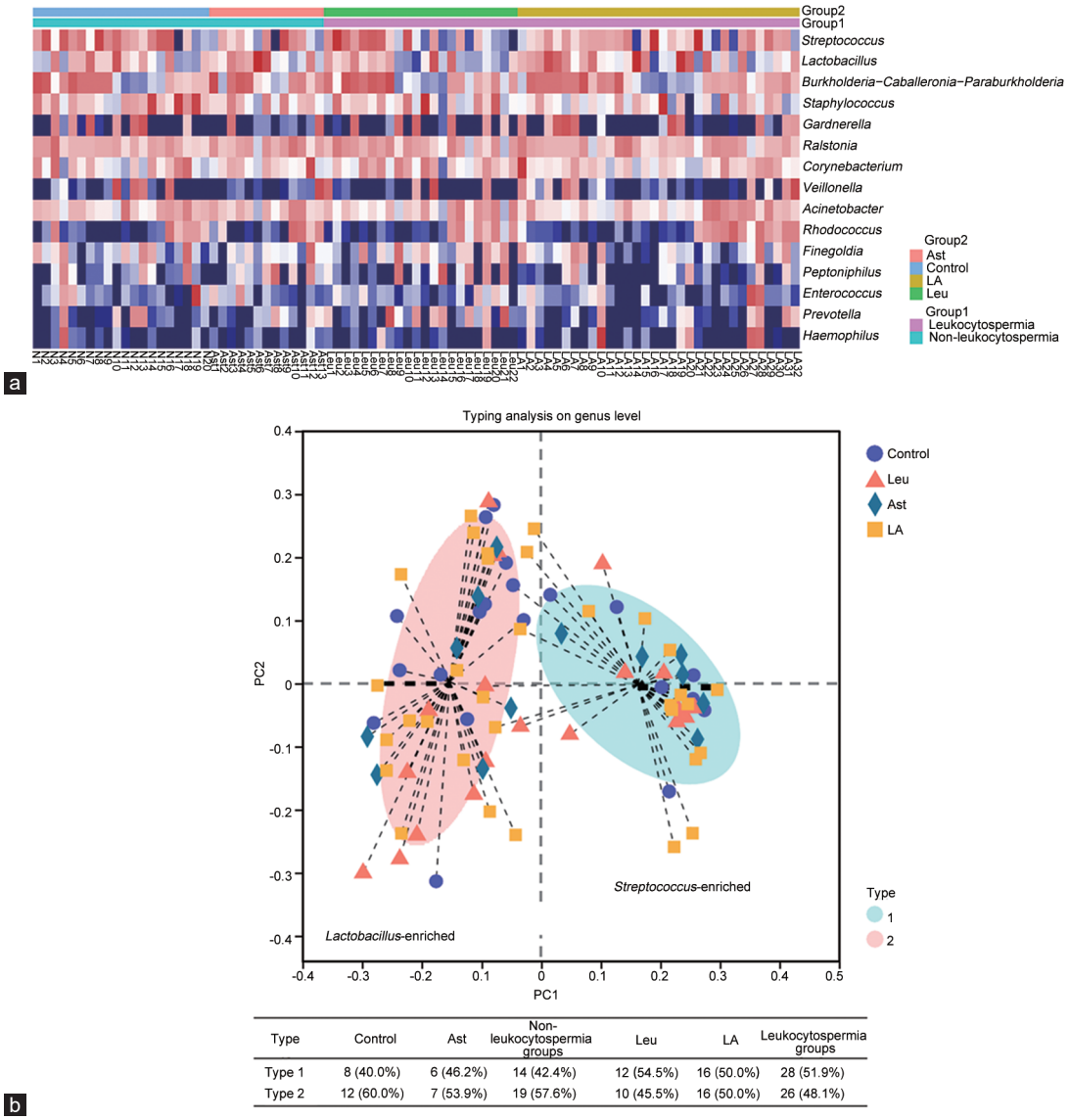


Figure 4: Semen microbiota at the phylum level. (**a**) Pie plot of the main semen microbiota at the phylum level. *Firmicutes*, *Proteobacteria*, *Actinobacteria*, and *Bacteroidetes* were the primary phyla. (**b**) Relative abundances of the different phyla in each group. Leukocytospermia-related groups had a different semen microbiota composition, with an increase in *Bacteroidetes* and a decrease in the *Firmicutes/Bacteroidetes* ratio. Ast group: asthenozoospermia patients with a normal seminal leukocyte count; Leu group: patients with leukocytospermia and normal sperm parameters; LA group: asthenozoospermia patients with leukocytospermia.

this study, the results showed that alpha diversity increased in the leukocytospermia-related groups. A possible reason for this could be the increased microbes in samples from subjects with leukocytospermia.

Although there are multiple microbiota communities in different parts of the urogenital tract in males, *Firmicutes*, *Proteobacteria*, *Actinobacteria,* and *Bacteroidetes* are the most abundant in seminal fluid.[37,40] We found that *Firmicutes*, *Proteobacteria*, *Actinobacteria,* and *Bacteroidetes* were the major phyla in sperm samples, and this result supported the above conclusion. It is well known that these four phyla are also abundant at other body sites.[42,43] A study of 1135 Dutch participants showed that *Firmicutes*, *Proteobacteria*, *Bacteroidetes,* and *Actinobacteria* are the dominant bacterial communities in the gut, and other studies have found that *Firmicutes*, *Proteobacteria*, *Actinobacteria*, and *Bacteroidetes* are the most abundant phyla in the oral tract, skin, and vagina.[43–45] Disorder in the dominant phyla has a severe effect on host health, and several studies have indicated that obesity, diabetes, and cardiovascular diseases are associated with an increase in the *Firmicute*s phylum and a relatively lower abundance of the *Bacteroidetes* phylum in the gut.[46] Another study found that azoospermic men associated with a decreased of *Bacteroidetes.*[47] However, different results are found in another study that compared with healthy men, patients with prostatitis have a higher abundance of *Bacteroidetes* in seminal fluid.[40] In this study, the results showed that a significant increase of *Bacteroidetes* was observed in the Leu group, with a slight increase in the LA group. Correspondingly, the ratio of *Firmicutes*/*Bacteroidetes* was decreased in the subjects with leukocytospermia. Hence, this study found that leukocytospermia-related groups have a different microbiota composition at the phylum level.

Subsequently, we analyzed the composition of the semen microbiota at the genus level. *Streptococcus*, *Burkholderia-Caballeronia-Paraburkholderia*, *Lactobacillus*, *Staphylococcus*, *Gardnerella*, *Ralstonia*, *Corynebacterium*, *Veillonella*, *Acinetobacter*, *Rhodococcus*, *Finegoldia*, *Peptoniphilus*, *Enterococcus*, *Prevotella*, and *Haemophilus* were the most abundant genera in the sperm samples. Some 16S rRNA-based NGS studies have produced similar results.[15–17,48–51] Interestingly, the majority



Type	Control	Ast	Non-leukocytospermia groups	Leu	LA	Leukocytospermia groups
Type 1	8 (40.0%)	6 (46.2%)	14 (42.4%)	12 (54.5%)	16 (50.0%)	28 (51.9%)
Type 2	12 (60.0%)	7 (53.9%)	19 (57.6%)	10 (45.5%)	16 (50.0%)	26 (48.1%)

Figure 5: Semen microbiota on the genus level. (**a**) Heatmap of top 15 most abundant genera. Most of the semen microbiota was also been found in the vagina. (**b**) Community cluster of semen microbiota in each sample at the genus level. The semen samples clustered into two main groups *Lactobacillus*-enriched and *Streptococcus*-enriched, 57.6% of the samples in the groups with a normal seminal leukocyte count could be categorized as *Lactobacillus*-enriched, with 48.1% of leukocytospermia-related samples being *Lactobacillus*-enriched. In contrast, 51.9% of the leukocytospermia samples could be categorized as *Streptococcus*-enriched, whereas only 42.4% of the non-leukocytospermia samples were *Streptococcus*-enriched. Ast group: asthenozoospermia patients with a normal seminal leukocyte count; Leu group: patients with leukocytospermia and normal sperm parameters; LA group: asthenozoospermia patients with leukocytospermia.

of these genera can also be found in the vaginal microbiota.[12,29–33,38,49] Studies have demonstrated that bacteria are shared among partners, and young men with no sexual experience have been shown to have a lower bacterial concentration and lower bacterial diversity in their semen than sexually experienced men of the same age.[52] Correspondingly, an abnormal semen microbiota could generate disorders of vaginal bacteria and finally influence the fertility.[38,49] In our study, the presence of these bacteria in males may be attributed to the exchange of bacteria in the reproductive tracts between sexual partners. However, a distinction in the seminal microbiota at the genus level was not observed between the non-leukocytospermia and leukocytospermia groups.

The samples in this study could be clustered into two groups, namely, *Lactobacillus*-enriched and *Streptococcus*-enriched groups. Furthermore, we found that most of the subjects with normal seminal leukocyte counts exhibited to the *Lactobacillus*-enriched group. Our results corroborate those of previous studies that have used NGS to analyze semen microbiota and found that normal samples had a higher abundance of *Lactobacillus*.[15–17] *Lactobacillus* is usually considered to be beneficial for human health, and it has been isolated from the gut, skin, oral cavity, and vagina.[53,54] *Lactobacillus* species are the most commonly found microbes in the vagina of healthy women. These microbes can produce large amounts of lactic acid, maintaining the normal microenvironment of the vagina.[53,55,56] Some studies have found that transplantation of *Lactobacillus* in subjects with vaginosis was effective.[55,56] The genus *Streptococcus* comprises more than one hundred species and is a prevalent human pathogen causing pneumonia, otitis media, bacteremia, and meningitis.[57] Some studies

revealed that patients with prostatitis had a higher relative abundance of *Streptococcus* in their seminal fluid.[58,59] Our study found that more subjects with leukocytospermia belonged to the *Streptococcus*-enriched group, while more subjects with non-leukocytospermia belonged to the *Lactobacillus*-enriched group. However, the cluster relationship in this study was weak, and more samples should be collected for further study to verify these results. Changes of seminal microbiota in leukocytospermia-related groups were observed, so we could infer that if the abnormal semen microbiota are corrected in patients with leukocytospermia, there might be an improvement in their sperm parameters and benefit for the treatment of sterility.

CONCLUSION

This study provided evidence indicating that males with leukocytospermia have worse sperm parameters. The results of semen microbiota analysis showed that the leukocytospermia-related groups had a higher alpha diversity. *Firmicutes, Proteobacteria, Actinobacteria, and Bacteroidetes* were the primary phyla in the seminal fluid. The *Bacteroidetes* phylum was more prevalent in leukocytospermia-related subjects. Several bacterial genera, including *Streptococcus*, *Lactobacillus*, *Burkholderia-Caballeronia-Paraburkholderia*, *Staphylococcus*, *Gardnerella*, *Ralstonia*, *Corynebacterium*, *Veillonella*, *Acinetobacter*, *Rhodococcus*, Finegoldia, *Peptoniphilus*, *Enterococcus*, *Prevotella*, and *Haemophilus,* were abundantly found in sperm samples, and most of these genera have been previously found in the vaginal microbiota. Moreover, subjects with leukocytospermia have a characteristic semen microbiota composition, with a decrease in *Lactobacillus* and an increase in *Streptococcus*.

AUTHOR CONTRIBUTIONS

This study was designed by YY, XJQ, DSW, and LLZ. YY, JKL, TT, YHL, and CHZ collected the sperm samples. YY, XJQ, and LLZ collected the clinical data. The data were analyzed by YY, DSW, HL, and LLZ. The draft was written by YY and critically reviewed by all the authors. All authors read and approved the final manuscript.

COMPETING INTERESTS

All authors declare no competing interests.

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Supplementary Table 3: Detailed patient information for sperm microbiota analysis

	Control group	*Asthenozoospermia group*	*Leukocytospermia group*	*LA group*
Number	20	13	22	32
Age (year), median (IQR)*	29.0 (27.3–30.0)	31.2 (29.0–33.5)	29.3 (26.8–30.3)	32.4 (28.3–37.8)
Semen volume (ml), median (IQR)	3.4 (2.1–4.9)	3.8 (3.0–4.5)	3.8 (3.0–5.0)	3.8 (3.0–4.5)
WBC (10^9 l^{-1}), median (IQR)*	7.0 (6.0–7.9)	6.8 (5.8–7.5)	7.0 (5.4–8.2)	6.8 (5.6–7.0)
Hemoglobin (g l^{-1}), median (IQR)*	147.4 (134.8–160.2)	149.4 (137.3–157.5)	147.5 (130.0–159.0)	151.4 (145.0–161.0)
PLT (10^{12} l^{-1}), median (IQR)*	222.6 (192.5–253.3)	223.5 (201.5–248.8)	234.7 (201.5–248.8)	219.3 (200.0–234.0)
Smoking ratio (%)*	25.0	30.7	27.3	25.8

*No significant difference between each group. LA group: asthenozoospermia patients with leukocytospermia; IQR: interquartile range; WBC: white blood cell; PLT: pallet