**Unravelling the origin of the seminal microbiome: comparative analysis of semen and urine samples before and after vasectomy**

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## Abstract

The semen harbours a polymicrobial community; however, the origin of the seminal microbiome has not yet been clearly established. One-third of the seminal microbes originate from the urethra, whereas a considerable part could come from the upper genital tract. Similarly, male reproductive organs, such as the prostate, seminal vesicles, and testicles contain their own microbiome. Recent pioneering studies with a limited sample size indicate that the vasectomy procedure alters the seminal microbiome, suggesting a testicular or epididymal microbial origin. This cohort study included 82 men who were planning to undergo a vasectomy and provided paired semen and urine samples before and after this surgical procedure. The seminal microbiome was analysed by sequencing the V4 hypervariable region of the 16S rRNA gene. We found that vasectomy influences the seminal microbial composition and that the semen shares 50% of bacterial communities with urine, altogether indicating paracrine effects of the genitourinary system on the seminal microenvironment. Our study provides new insights into the origin of seminal microbes, suggesting that part of the seminal microbiome could originate from the testicular and urinary environment. Furthermore, we confirm the effect of the vasectomy procedure on seminal microenvironment, which could have a short- and long-term effect on male urogenital health.

## Introduction

The human microbiota, consisting of trillions of microorganisms inhabiting various anatomical sites, has emerged as a crucial player in human health and disease (Rowe et al., 2020). Broad research has shed light on the diverse microbial communities residing in the gut, oral cavity, and urogenital tract, influencing numerous physiological processes, and contributing to overall wellness (Altmäe et al., 2019; Gilbert et al., 2018). However, despite its significance, the exploration of the microbiome (i.e., microorganisms and their genomes) in certain human niches remains uncovered. Especially, the seminal microbiome has received relatively limited attention compared to other body sites (Altmäe et al., 2019).

Understanding the seminal microbiome and its origin is essential because it may play a pivotal role in male reproductive health (Altmäe & Kullisaar, 2022; Lundy et al., 2021; Suarez Arbelaez et al., 2023). Semen, traditionally considered as a sterile fluid, has been recognised as an emerging niche for microbial colonisation (Contreras et al., 2023; Venneri et al., 2022). Accordingly, investigating the seminal microbiome has gained

attention due to its potential implications in male fertility, reproductive disorders, and overall reproductive health. Even with the role of the semen microbiome not completely elucidated, studies indicate its association with seminal quality and its influence on inflammation and immune responses (Altmäe et al., 2019).

Despite growing interest in the seminal microbiome, a few studies have investigated the sources and acquisition pathways of microorganisms present in semen, by comparing the microbial composition of semen samples before and after vasectomy or assessing the disparities between seminal and urinary microbiomes (Cao et al., 2023; Kermes et al., 2003; Kiessling et al., 2008; Lundy et al., 2021; Suarez Arbelaez et al., 2023). These pioneering studies have highlighted alterations in the seminal microbial diversity and composition following male sterilisation through vasectomy, suggesting paracrine contribution of upstream anatomic locations such as testis and epididymis as contributors to the seminal microbiome (Kiessling et al., 2008; Lundy et al., 2021; Suarez Arbelaez et al., 2023). Likewise, comparative studies between semen and urine samples have revealed distinct semen microbiome with modest similarity (~30%) to the urinary microbiome (Cao et al., 2023; Kermes et al., 2003; Lundy et al., 2021), suggesting that the microbial composition in these fluids exhibit distinct characteristics and origin. Indeed, seminal microbiome could partly originate from the upper genital tract as existence of microorganisms in the testis (Alfano et al., 2018; Molina, Plaza-Díaz, et al., 2021) and prostate (Cavarretta et al., 2017; Feng et al., 2019; Jain et al., 2020; Wu et al., 2020; Yow et al., 2017) has been identified.

On the other hand, vasectomy is a common procedure for sterilisation, whose prevalence in Europe and North America is approximately 10%, with certain countries reaching 20% among reproductive-aged men (Degraeve et al., 2022; Jacobstein, 2015). This procedure causes changes in semen viscosity, pH, and prostaglandin levels that affect inflammation in addition to other functions (Brummer, 1973; Nikkanen, 1979). These oscillations in seminal characteristics could in part be the result of microbial alterations, as microbiome is an important regulator of inflammation and autoimmunity (Ding et al., 2020). Therefore, changes in the microbial composition following vasectomy could lead to dysbiosis in the seminal microbiome which might have long-term effects on men's reproductive health (Suarez Arbelaez et al., 2023).

In the current study, we set out to explore the seminal microbiome changes induced by vasectomy by analysing paired seminal and urine samples collected from the same individuals before and after vasectomy. We aimed to investigate the potential contribution

of the upper reproductive tract together with the urinary microbiome to the microbial composition in semen to uncover potential sources and routes of microbial colonisation in the seminal microenvironment.

## Materials and methods

*Study population*

The study was carried out in accordance with the ethical guidelines of the Declaration of Helsinki and the legally enforced Spanish regulation, which standardises the clinical investigation of human beings (RD 223/04). All procedures were approved by the Ethics Committee of the Investigación Biomédica de Andalucía (ref. CEIM/CEI 0463-M1-18r). Written informed consent was obtained from all subjects prior to inclusion.

Eighty-two men who were planning to undergo vasectomy were recruited at the University Hospital Virgen de las Nieves, Granada between February 2021 and October 2022. All participants donated urine and semen samples before the vasectomy and 3 months after the procedure with confirmed azoospermia in the semen analysis. In the case of presence of spermatozoa after surgery, an additional sample was collected 3 months later with confirmed azoospermia. No preoperative or postoperative antibiotics were prescribed.

Participants were requested to maintain a minimal sexual abstinence of 3-5 days before sample collection. All semen samples were self-collected at the Hospital by masturbation into a sterile polypropylene 120ml-container (DELTALAB, Barcelona, Spain). Patients performed hand sterilisation and collected semen sample after washing the glans penis with soap and water, and after urinating. Samples were immediately provided to andrology lab technicians for processing. Before liquefaction and routine semen analysis, 200µl-aliquot from each semen sample was placed in a cryovial (VWR®, part of Avantor, Barcelona, Spain), snap-frozen in the gas phase of liquid nitrogen and stored at -80°C for further analysis.

Urine samples were collected from the midstream into a sterile polypropylene 120ml-container (DELTALAB) prior to the semen sampling. Next, 3 ml were pipetted into 1 ml of nucleic acids' stabiliser medium (eNAT® 608CS01R, COPAN Italia, Brescia, Italy), kept at room temperature max 6 hours, and stored at -80°C for further analysis.

Additionally, participants completed a questionnaire that included demographic characteristics, lifestyle factors, and sexual activities. Body mass index (BMI) was calculated from the self-reported weight and height data.

*Semen analysis*

The rest of the sample was taken for the assessment of the sperm parameters (i.e., sperm volume, concentration, and total progressive motility) according to the World Health Organisation guidelines (World Health Organization, 2021) and the semen analysis methodology checklist (Björndahl et al., 2022).

*DNA extraction*

For microbiome analysis, genomic DNA was extracted from semen samples using the QIAamp DNA Microbiome Kit (QIAGEN, Venlo, The Netherlands) and the QIAamp UCP Pathogen Mini Kit (QIAGEN) for urine samples, following the manufacturer's instructions. The purity, quality, and yield of the extractions were determined by measuring the A260/A280 and A260/A230 ratios with the NanoDrop ND1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). DNA concentration was quantified by fluorimetry with Qubit 4 (Thermo Fisher Scientific) and normalised.

Negative and positive controls were included and processed along with the biological samples to monitor the potential microbial contamination. Negative controls included sample collection controls for each tissue source, DNA extraction (e.g., reagent) controls, library preparation controls, and sequencing controls (**Supplementary Table 1**). Positive controls included the ZymoBIOMICS (Zymo Research, Irvine, CA, USA) mock community standard.

*Analysis of 16S rRNA gene sequencing*

Seminal and urinary microbiomes were profiled by amplifying the V4 hypervariable region of the 16S rRNA gene and sequencing. The primers used were 515F (5'-GTGYCAGCMGCCGCGGTAA) and 806R (5'- GGACTACNVGGGTWTCTAAT). All PCRs were performed in 50µl-reaction volume containing 20 µl 2X Platinum Hot Start PCR Master Mix (Invitrogen, Waltham, MA, USA), 2 µl of forward primer (5 µM), 0.1 µl of reverse primer (5 µM), MilliQ lab water, and extracted DNA (20 ng) under the following cycling conditions using Applied Biosystems Veriti 96-Well Thermal Cycler (Thermo Fisher Scientific): initial denaturation at 94°C for 3 min, followed by 35 cycles of denaturation at 94°C for 45 s, annealing at 50°C for 1 min and elongation at 72°C for 90 s, with a final extension at 72°C for 10 min. A quality control was performed using 2% agarose gel electrophoresis to verify that each sample had been amplified. The

expected amplicon size was around 380 bp. Each sample was quantitated separately by fluorimetry with Qubit 4 (Thermo Fisher Scientific) and pooled equimolarly with an optimal amount of 50 ng per sample. PCR products were first purified by column using MicroElute Cycle Pure Kit (Omega Bio-tek, Norcross, GA, USA) and next with AMPure XP magnetic beads (Beckman Coulter, Brea, CA, USA). To check the absence of primer residues and that the library size was as expected, a quality control was performed with an HS bioanalyser (Agilent technologies, Santa Clara, CA, USA). Illumina Nextera library preparation was performed according to the manufacturer's specifications, combining PhiX phage (20%) with the amplicon library to give diversity to the run. The final library was paired-end sequenced (2 × 300 bp) using a MiSeq Reagent Kit v.3 on the Illumina MiSeq sequencing system (Illumina, San Diego, CA, USA).

*Bioinformatic and statistical analyses*

Raw data were demultiplexed with Illumina bcl2fastq2 Conversion Software (v2.20) and imported to QIIME2 software (v.2022.11) with a PairedEndFastqManifestPhred33 input format. Divisive Amplicon Denoising Algorithm 2 (DADA2) was used for the denoising step. Low-quality regions were trimmed considering a quality score below 25 to create high quality forward and reverse reads, using the "q2-dada2" function. Taxonomy assignment of amplicon sequence variants (ASVs) was performed using the "classify-sklearn" function against the SILVA 16S v132_99 database, along with a similarity threshold of 99%. Microbial taxa were aggregated to phylum and genus level in further analysis.

The resulting ASV tables were decontaminated based on proportions of contaminant sequences in negative controls, identifying and removing contaminating reads from biological samples. The decontamination approach was performed in R (v.4.2.2) under RStudio (v.2022.12.0+353). In particular, the "decon()" function from microDecon package was run on its default values. Additionally, the decontaminated tables were filtered to consider only those taxa that were present in ≥30% samples included in each comparison group in order to capture microorganisms consistently present in the niches.

Two sets of analyses were performed. The first compared paired pre- and post-vasectomy microbial profiles in semen samples. Paired sample analysis aims to mitigate the impact of population and lifestyle factors on the microbial composition outcomes, while providing a more comprehensive understanding of the specific microbiome changes associated exclusively with vasectomy (Suarez Arbelaez et al., 2023). The second

analysis compared paired seminal and urinary microbiomes to assess the possible microbial contribution of the urinary tract to the seminal environment.

Microbiome diversity analyses were also conducted under RStudio using phyloseq, vegan, microviz, and ggplot2 R packages. Within-sample microbiome diversity (i.e., α-diversity) was estimated by Shannon diversity index and richness (i.e., number of microbial taxa), using the "diversity" and "specnumber" functions from the vegan package. Between-sample microbiome dissimilarity (i.e., β-diversity) was visualised using nonmetric multidimensional scaling (NMDS) ordination, based on the Bray Curtis distance. For α-diversity comparisons in paired samples, Wilcoxon signed-rank test was used for significance testing with the function "wilcox.test()". For β-diversity testing, PERMANOVA was permuted using the "adonis2" function from vegan package. Differential abundance analysis was performed on those bacterial genera present using an Analysis of Compositions of Microbiomes with Bias Correction (ANCOM-BC) (Lin & Peddada, 2020) from the ancombc2 R package. All *p*-values were corrected for the multiple comparison testing applying the Benjamini-Hochberg false discovery rate (FDR) method (Benjamini et al., 2006). Statistical significance was set $p$-value<0.05 after FDR correction.

**Results**

From the total of 82 men recruited into the prospective study, the final study population comprised 55 participants, as certain individuals either lacked paired urine sample (n=3), failed to provide post-vasectomy samples (n=16), or had samples excluded from the analysis due to technical issues such as low DNA yield and/or poor sequencing quality (n=8). All vasectomies were uncomplicated.

Forty-six men were considered for the first comparison analysis between paired pre- and post-vasectomy semen samples; 43 post-vasectomy samples were collected 3 months after the surgery, while 3 post-vasectomy samples were taken 6 months after the intervention due to the presence of spermatozoa after 3 months of the vasectomy (n=2) or insufficient sample to evaluate the seminal parameters (n=1). For the second analysis 55 men provided paired semen and urine samples before the vasectomy. Baseline demographics, seminal parameters, and lifestyle habits are presented in **Table 1**. Regarding negative controls, 3 of them were included in the first set of analyses whereas 5 were considered for the second analyses (**Supplementary Table 1**).

First, we characterised the semen and midstream voided urine microbiomes separately. After applying contamination correction with microDecon and filtering out genera present in less than 30% of the samples (**Supplementary Table 2**), the dominant phylum in semen was *Firmicutes* (45%), distantly followed by *Proteobacteria* (19%), *Actinobacteria* (16%), and *Epsilonbacteraeota* (16%). Similarly, we identified *Proteobacteria* (31%) as the most abundant phylum in urine samples, closely followed by *Firmicutes* and *Bacteroides* (25% and 17%, respectively). At genus level, semen showed high abundance of *Campylobacter* (15%), *Finegoldia* (9%), and *Ezakiella* (9%) while urine presented *Prevotella* (14%), *Acinetobacter* (7%), and *Lactobacillus* (6%) as dominant genera.

*Pre- vs. post-vasectomy seminal microbial analysis*

Seminal microbiome composition, α-, β-diversity, and relative abundances were compared between paired pre- and post-vasectomy samples. In total, 39 genera were identified in the semen samples. Of these, 4 genera (*Acinetobacter*, *Brevundimonas*, *Altererythrobacter*, and *Escherichia-Shigella*, representing 10% of seminal genera) were exclusively found in pre-vasectomy semen samples and 5 genera (*Arcanobacterium*, *Actinobaculum*, *Murdochiella*, *Howardella*, and *Fastidiosipila*, 13%) were unique to post-vasectomy samples. A total of 30 genera (77%) remained after the surgery (**Figure 1A**, **Supplementary Table 3**).

Post-vasectomy semen samples had significantly higher α-diversity (observed richness Wilcoxon signed-rank *p*-value=0.011; **Figure 1B**) compared to pre-vasectomy samples. β-diversity analysis based on Bray Curtis distances indicated a significant microbial dissimilarity between seminal samples collected before and after vasectomy (PERMANOVA, $R^2$=0.031, *p*-value=0.004; **Figure 1C**).

Further, we performed a differential abundance analysis using ANCOM-BC to detect specific genera that could be differentially abundant in the semen microbiome of pre- and post-vasectomy samples. Ten genera showed significantly different relative abundances between pre- and post-vasectomy semen samples: *Acinetobacter*, *Brevundimonas*, *Altererythrobacter*, and *Escherichia-Shigella* showed markedly increased abundance in pre-vasectomy samples while *Arcanobacterium*, *Porphyromonas*, *Actinobaculum*, *Murdochiella*, *Howardella*, *Fastidiosipila* genera were more abundant in the post-vasectomy samples (FDR *p*-value<0.05; **Figure 2**; **Supplementary Table 4**).

*Semen vs. urine microbial analysis*

Seminal and urinary microbiome composition, α-, β-diversity, and relative abundances were compared between paired urine and semen samples. A total of 38 genera were identified. Of these, 20 genera were exclusively identified in urine samples (53% of urinal genera, **Supplementary Table 5**), while no genera were exclusively related to semen. The remaining 18 genera (47%) were shared by both niches (**Figure 3A**, **Supplementary Table 5**).

Urine samples revealed significantly higher α-diversity (Shannon index and observed richness Wilcoxon signed-rank $p$-value<0.001; **Figure 3B**) compared to semen samples. β-diversity analysis based on Bray Curtis distances revealed a discernible clustering between semen and urine samples (PERMANOVA, $R^2$=0.117, $p$-value=0.001; **Figure 3C**).

ANCOM-BC analysis revealed that 31 identified genera exhibited differential abundance between semen and urine samples. Among them, 21 were more abundant in urine, standing out *Prevotella* and *Escherichia-Shigella*, which showed noticeably increased abundance compared to semen samples (FDR $p$-value<0.05, log fold change≥2.5; **Figure 4**; **Supplementary Table 6**). On the contrary, ten genera significantly prevailed in semen samples, with particular emphasis on *Anaerococcus*, *Finegoldia*, and *Corynebacterium* (FDR $p$-value<0.05, fold change≥2.5; **Figure 4**; **Supplementary Table 6**).

## Discussion

In the current study, we described and compared the semen and urine microbiomes in paired samples from the same individuals before and after a vasectomy to better understand the origins and dynamics of the seminal microenvironment. To the best of our knowledge, this is the biggest study performed so far in the paired pre- and post-vasectomy samples. Our results indicate that vasectomy procedure influences the seminal microbial composition and that semen shares 50% of bacterial communities with urine, altogether suggesting paracrine effects of the upper reproductive tract (testis and epididymis) on seminal microenvironment.

Semen harbours its microbial communities, where we detect abundantly *Lactobacillus*, *Corynebacterium*, *Staphylococcus*, *Prevotella*, and *Finegoldia*, which is in line with previous studies (Altmäe et al., 2019). When comparing the effect of vasectomy on seminal microbial composition, ten genera were differentially abundant between pre- and post-vasectomy semen samples: *Acinetobacter*, *Brevundimonas*, *Altererythrobacter*, and

*Escherichia-Shigella* were decreased in post-vasectomy samples while *Arcanobacterium*, *Porphyromonas*, *Actinobaculum*, *Murdochiella*, *Howardella*, and *Fastidiosipila* were more abundant after the vasectomy. Particularly, *Brevundimonas* has exhibited a reduction in vasectomised samples also in a previous study (Suarez Arbelaez et al., 2023). This genus has been observed to be the most abundant in individuals exhibiting lower levels of oxidative stress, increased progressive sperm motility, and reduced levels of overall DNA fragmentation (Garcia-Segura et al., 2022).

When evaluating the genera richness, the vasectomy procedure had an effect of increasing the α-diversity among the seminal samples. In line with our finding, a pioneering study found that only two of the pre-vasectomy samples, but all five of the post-vasectomy samples, tested positive for bacteria (Kiessling et al., 2008). However, other studies with limited sample size detected the contrary and linked vasectomy to a reduced α-diversity in paired and unpaired semen samples (Suarez Arbelaez et al., 2023). There seems to be consistency in vasectomy affecting the composition and abundance of the seminal microbiome. This suggests that the upstream anatomic locations such as testis and epididymis have their unique microbiome and that the paracrine contribution of these sites can influence the seminal microbial composition. Indeed, a testicular microbiome has been described, albeit as low-biomass site (Alfano et al., 2018; Molina, Plaza-Díaz, et al., 2021). Further, supporting our results earlier studies on the semen microbiome have demonstrated a positive correlation between dysbiosis and a higher bacterial richness (Altmäe et al., 2019; Contreras et al., 2023; Kiessling et al., 2008; Zuber et al., 2023). In our study, an increase in the bacterial richness in the post-vasectomy samples could be explained by the absence of testicular and epididymal influence on semen microbiota after the vasectomy. The removal of these contributions may allow for other bacterial sources, possibly from the urinary tract or external genitalia, to become more prominent in the semen, thereby increasing the diversity and richness of the microbiome. Further, this rise in bacterial richness may be also linked to the epididymis-unique defensins (Yamaguchi et al., 2002; Yenugu et al., 2004). Defensins are a group of antimicrobial proteins recognised as vital in response to pathogens. Humans are known to produce a reasonably large quantity of these defensins in their epididymis, including certain types that are exclusive to this organ (Kiessling et al., 2008). In light of our study results, one could conjecture that these epididymal defensins might act as a protective shield against bacterial infections in downstream tissues. Thus, further studies are needed to identify the

specific bacteria that are lost post-vasectomy and to understand the exact biological mechanisms they may have in a short and long term on male health.

Our study findings exposed a statistically significant difference in α-diversity between semen and urine samples, with urine exhibiting higher diversity. Previous studies have obtained contrary results, detecting higher α-diversity in semen (Lundy et al., 2021) or no differences in microbial α-diversity between semen and urine (Cao et al., 2023). The difference could arise from small sample size analysed in previous studies, and additionally Cao *et al.* collected semen samples first, followed by urine samples, while our study followed the reverse order, collecting urine before the semen.

In addition to α-diversity, β-diversity significantly changed after vasectomy, supporting that semen microbial communities fluctuate after male sterilisation. In line, the only study where diversity between non- and vasectomised samples has been analysed so far, the bacterial composition of the samples did show a tendency for distinct clustering between the two groups, nevertheless it could be due to the small sample size (i.e., 16 individuals) the result was not statistically significant (Suarez Arbelaez et al., 2023).

Our analysis of the microbiome profiles between the paired semen and urine samples revealed that the β-diversity analysis resulted in discernible clustering patterns meaning that the two types of samples have unique and distinct sets of bacterial genera, which is also observed in previous studies (Cao et al., 2023; Lundy et al., 2021).

When we compared the microbial composition between the semen and urine to disentangle further the seminal microbial origin, semen displayed higher *Anaerococcus, Finegoldia*, and *Corynebacterium* abundances and reduced *Prevotella and Escherichia-Shigella*, among others. All these genera have been previously described in both niches (Cao et al., 2023; Lundy et al., 2021). Interestingly, *Prevotella* has been broadly linked to reduced parameters of semen quality (Baud et al., 2019; Cao et al., 2023; Farahani et al., 2020; Nguyen et al., 2014; Weng et al., 2014) and its abundance has shown significant differences between urine and semen. In our study and others, *Prevotella* has been found to be more abundant in urine (Lundy et al., 2021), while contrasting findings from other authors have reported lower abundances in urine (Cao et al., 2023). Likewise, we observed a similar pattern for other bacterial genera, which were more abundant in semen, occasionally coinciding with the literature, as is the case for *Finegoldia* (Cao et al., 2023), *Lactobacillus* (Lundy et al., 2021), and *Enterococcus* (Lundy et al., 2021). However, disparities arise when considering *Anaerococcus, Veillonella*, *Corynebacterium*, and *Streptococcus*, as our findings indicate greater abundance in semen, in contrast to other

studies (Cao et al., 2023; Lundy et al., 2021). Also for bacteria abundant in the urine in our study, such as *Prevotella, Lactobacillus, Escherichia-Shigella*, and *Porphyromonas* contradicting results in other studies have been obtained (Cao et al., 2023; Lundy et al., 2021). However, we did observe a slightly higher abundance of *Bifidobacterium* in urine, consistent with another study (Cao et al., 2023). These contradicting results between studies could arise from different sample size and study design, protocol used and analysis methods (Molina, Sola-Leyva, et al., 2021). Indeed, the biggest discordancy between our study findings and others was found with the study by Cao *et al*., where the semen samples were collected first, followed by urine samples (while in our study the order was reverse). Further, one plausible explanation for the shared presence of these genera in both urine and semen could be the anatomical proximity of the urethra (through which urine passes) and the *vas deferens* (which transports sperm). Cross-contamination could occur during urination or ejaculation due to their common exit pathway from the body. Bacterial colonisation from the urethra could subsequently influence the seminal microbiome. Another possible explanation could be related to biofilm formation. Many of these genera are known for their biofilm-forming capabilities (Brook, 2007; Davey & O'toole, 2000; Hall-Stoodley et al., 2004; Kolenbrander et al., 2006; Souza et al., 2015; Turroni et al., 2014), which could allow them to persist in the genitourinary tract, colonise both the urinary and reproductive systems, and possibly influence the microbiome composition of both niches. Nonetheless, the mechanisms behind the microbial differences in semen and urine, as well as its potential effects on sperm quality, require further investigation.

The strength of our study is the increased sample size and that the same individuals were assessed before and after the vasectomy which allowed to perform paired comparisons for semen and urine microbiomes. This eases the impact of population and lifestyle factors on the microbial composition outcome while providing a more comprehensive understanding of the specific microbiome changes. In fact, a previous study where paired and unpaired seminal samples before and after the vasectomy were analysed, the paired samples presented significantly less bacterial species between study groups than the unpaired samples (Suarez Arbelaez et al., 2023). Also, contamination in microbiome analysis was stringently controlled including negative and positive controls together with *in silico* decontamination methods. Nevertheless, our study has limitations that should be mentioned. Initially, it is worth noting that mid-stream urination and masturbation involve the urethra, which harbours the urethral microbiome. Although catheterisation and

seminal vesicle aspiration are more suitable collection methods to elucidate seminal microbial origin, it is improbable that volunteers would accept. Another limitation was obtaining sufficient DNA yield from semen samples, which presents a challenge during sequencing. This difficulty in obtaining an adequate amount of bacterial DNA complicates the sequencing process and requires a larger initial sample size to account for potential sample dropouts.

## Conclusion

The seminal microbiome origin was explored through comparative analysis between paired seminal and urine pre- and post-vasectomy samples, in the largest cohort reported to date. Our findings reveal considerable differences in both α- and β-diversity when comparing pre- and post-vasectomy semen samples as well as urine and semen samples. Intriguingly, we have also pinpointed several bacterial genera that show significant variations in abundance across the different niches examined. Altogether, our study underscores the intricate relationships between anatomically close but functionally distinct sites within the male reproductive and urinary systems. The differential microbial community structures and compositions might be associated with different physiological states and could potentially influence different health outcomes. Our study findings provide new insight into the origin of seminal microbes, indicating that some accompanying bacteria could already originate from the testicular and urinary environment.

By elucidating the origins of the seminal microbiome, this work provided crucial insights into the factors influencing male reproductive health and demonstrate that the vasectomy procedure might have long lasting effects on male health via modulation of seminal microenvironment. A comprehensive understanding of the seminal microbiome's origin and its impact on male fertility will pave the way for novel diagnostic approaches, therapeutic interventions, and strategies for promoting reproductive health.

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**Figure legends**

**Figure 1.** Comparison of bacterial genera, α-, and β-diversity between paired pre- and post-vasectomy samples. **A.** Venn diagram showing the distribution of identified genera according to sample source: pre- (pink), post-vasectomy (orange). **B.** Shannon diversity index (left) and observed richness (right). **C.** Nonmetric multidimensional scaling (NMDS) ordination based on the Bray Curtis distance.

**Figure 2.** Relative abundance of bacterial genera in semen samples before and after the vasectomy. Genera with abundance less than 1% were grouped as 'others'.

**Figure 3.** Comparison of bacterial genera, α-, and β-diversity between paired urine and semen samples. **A.** Venn diagram showing the distribution of identified genera according to sample source: urine (pink), semen (orange). **B.** Shannon diversity index (left) and observed richness (right). **C.** Nonmetric multidimensional scaling (NMDS) ordination based on the Bray Curtis distance.

**Figure 4.** Relative abundance of bacterial genera in urine and semen samples. Genera with abundance less than 1% were grouped as 'others'.

**Table legend**

**Table 1.** Baseline demographics, lifestyle habits, and seminal parameters of the study participants.

**Supplementary material**

**Supplementary Table 1.** Negative controls included in the study.

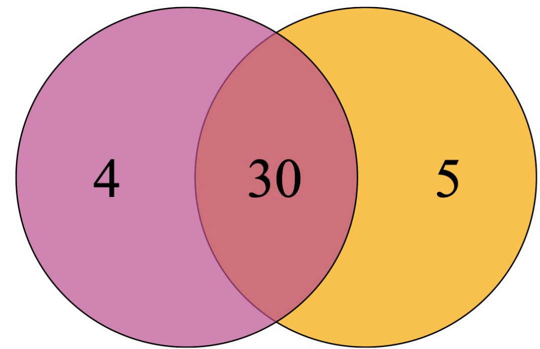
**Supplementary Table 2.** Number of reads in semen and urine samples after the microDecon decontamination approach and additional low-prevalence filtering.

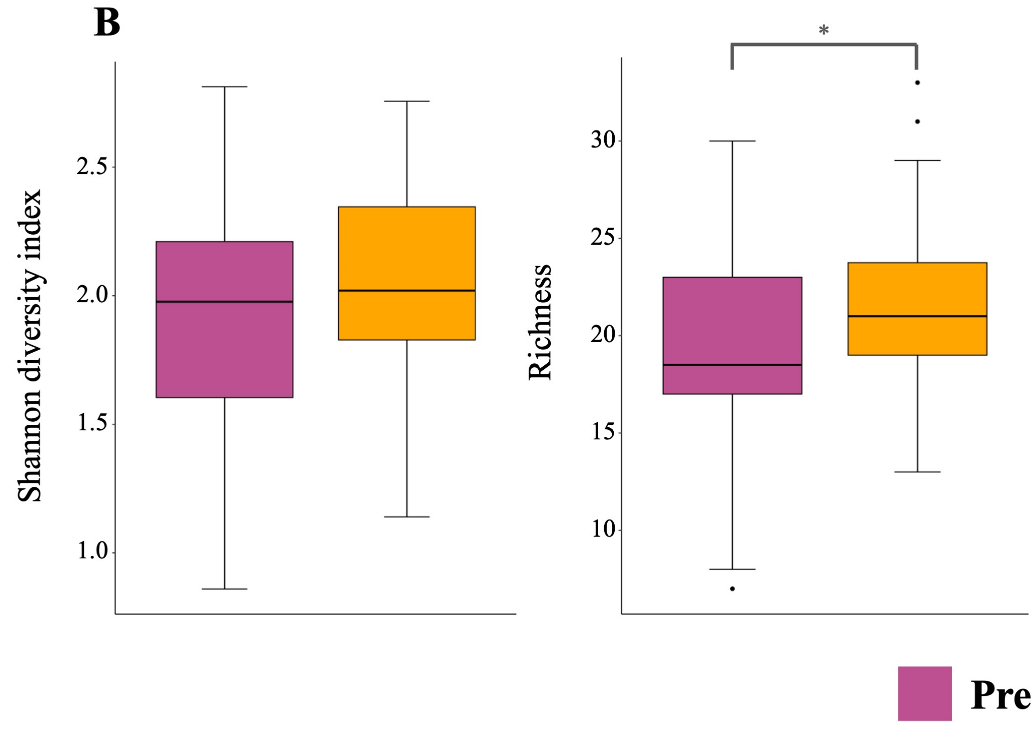
**Supplementary Table 3.** Mean number of reads of specific or common bacterial genera in pre- *vs.* post-vasectomy samples.

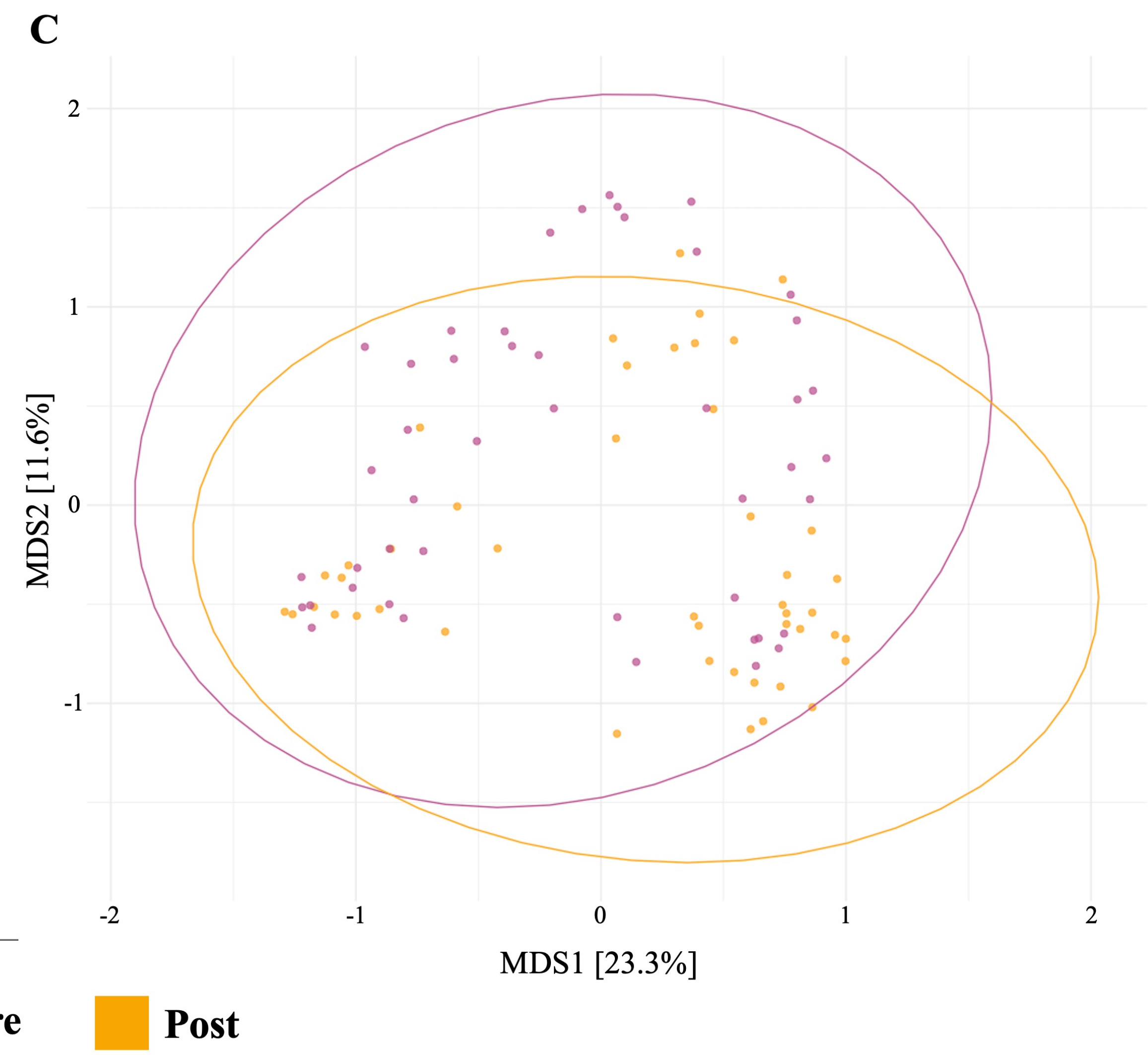
**Supplementary Table 4.** Statistical results after applying Analysis of Compositions of Microbiomes with Bias Correction (ANCOM-BC) in the analysis of pre- *vs.* post-vasectomy samples.

**Supplementary Table 5.** Mean number of reads of specific or common bacterial genera in urine *vs.* semen samples.

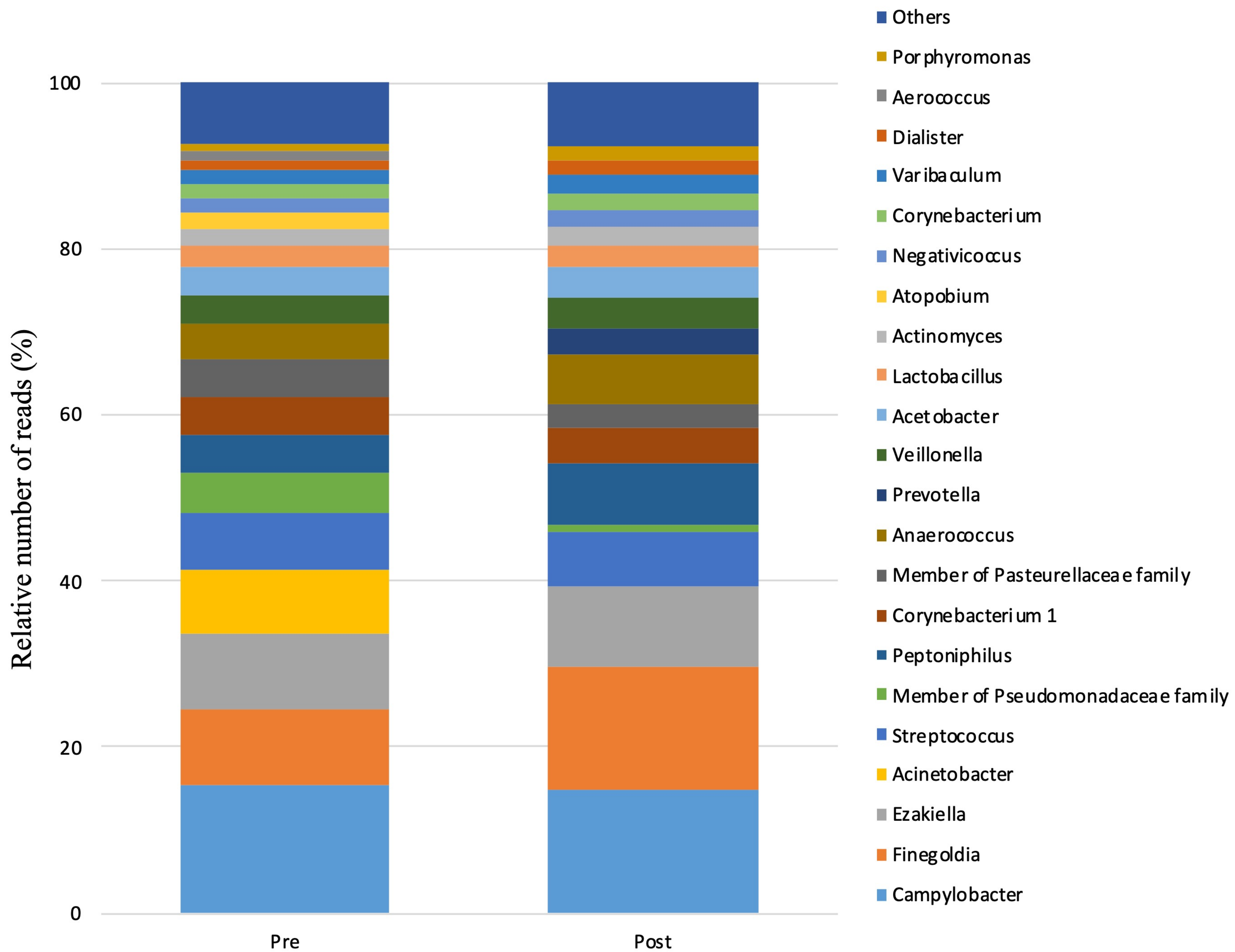
**Supplementary Table 6.** Statistical results after applying Analysis of Compositions of Microbiomes with Bias Correction (ANCOM-BC) in the analysis of urine *vs.* semen samples.

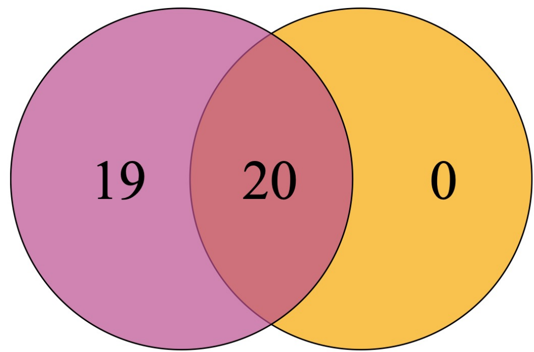


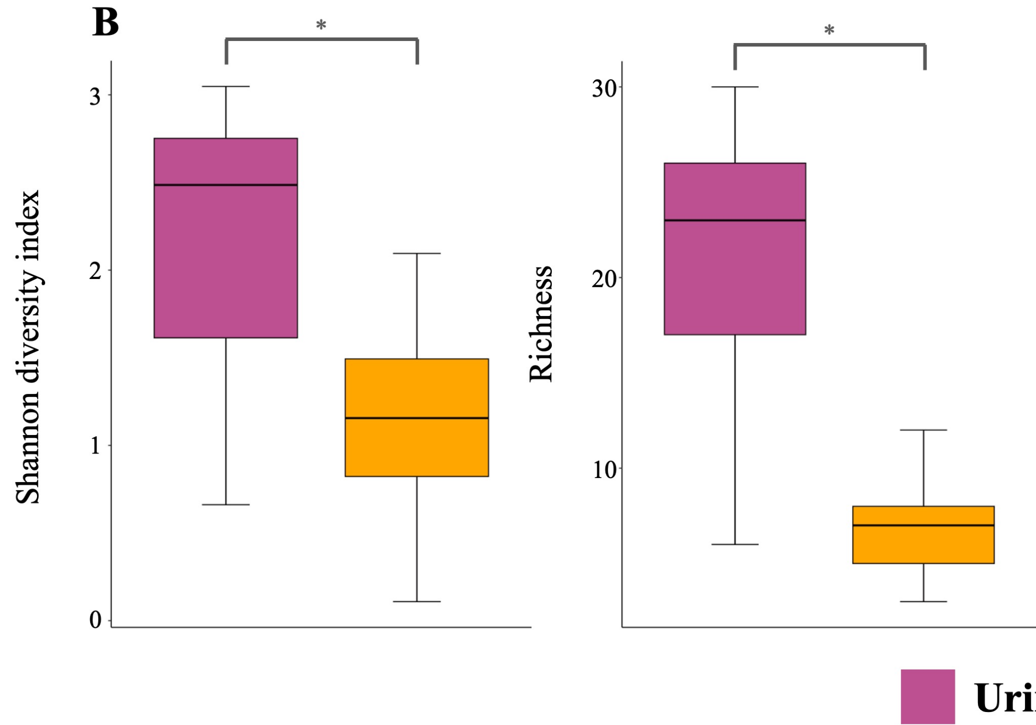



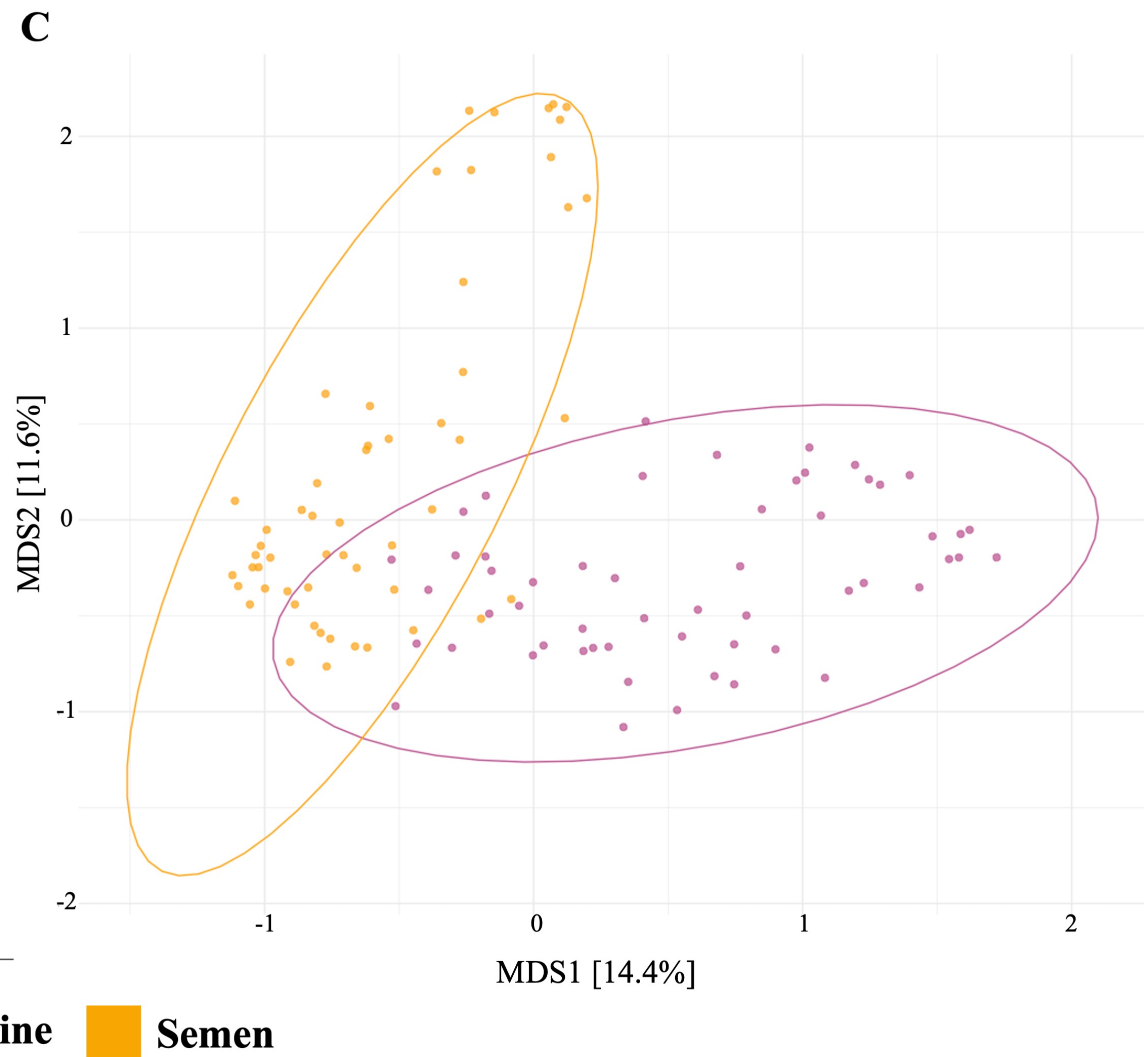



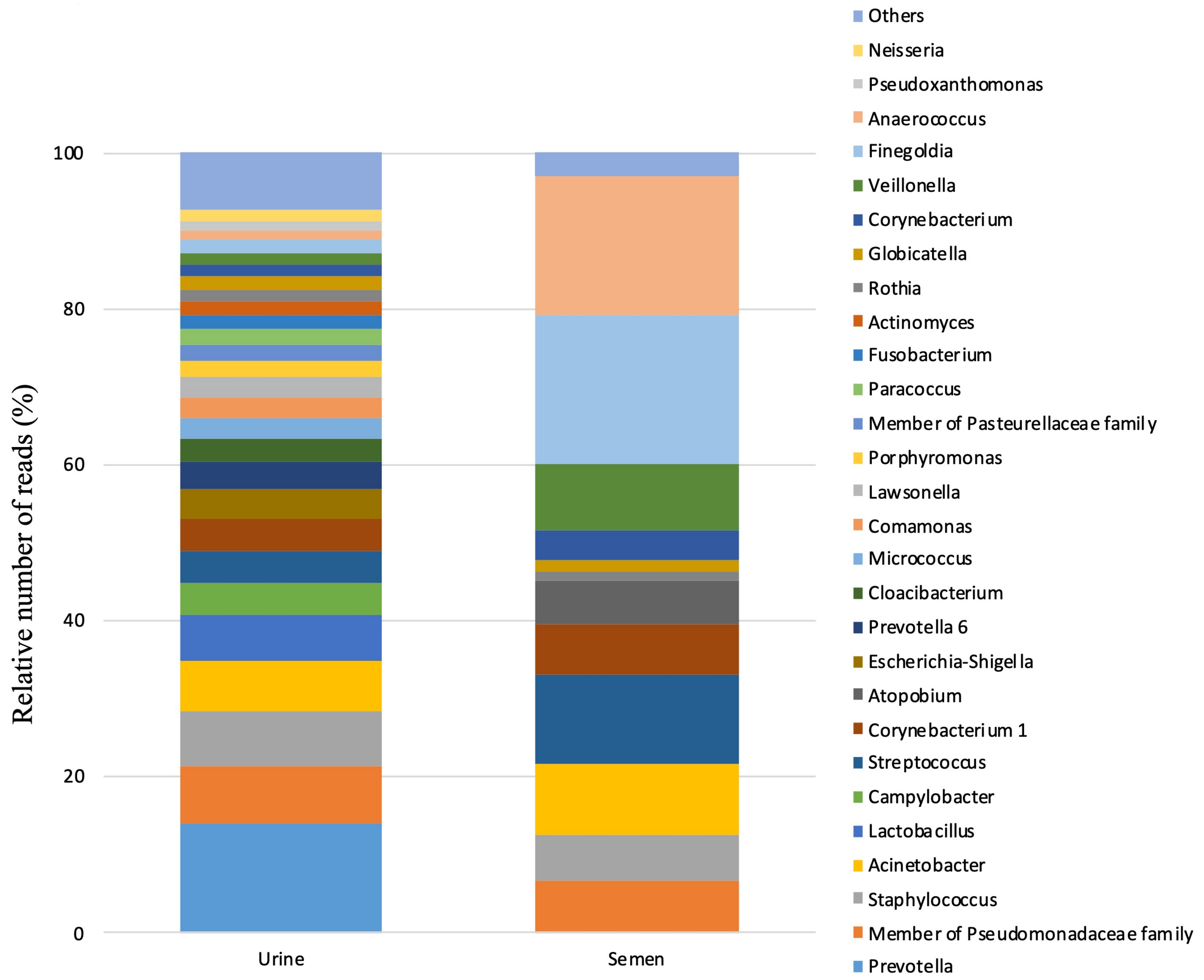

Pre Post


Relative number of reads (%)
100
80
60
40
20
0
Pre
Post
Others
Porphyromonas
Aerococcus
Dialister
Varibaculum
Corynebacterium
Negativicoccus
Atopobium
Actinomyces
Lactobacillus
Acetobacter
Veillonella
Prevotella
Anaerococcus
Member of Pasteurellaceae family
Corynebacterium 1
Peptoniphilus
Member of Pseudomonadaceae family
Streptococcus
Acinetobacter
Ezakiella
Finegoldia
Campylobacter


A
19
20
0


B
Shannon diversity index
3
2
1
0
*
Richness
30
20
10
*


C
MDS2 [11.6%]
2
1
0
-1
-2
MDS1 [14.4%]
-1
0
1
2
Urine
Semen


Relative number of reads (%)
100
80
60
40
20
0
Urine
Semen
Others
Neisseria
Pseudoxanthomonas
Anaerococcus
Finegoldia
Veillonella
Corynebacterium
Globicatella
Rothia
Actinomyces
Fusobacterium
Paracoccus
Member of Pasteurellaceae family
Porphyromonas
Lawsonella
Comamonas
Micrococcus
Cloacibacterium
Prevotella 6
Escherichia-Shigella
Atopobium
Corynebacterium 1
Streptococcus
Campylobacter
Lactobacillus
Acinetobacter
Staphylococcus
Member of Pseudomonadaceae family
Prevotella