Medicine

Characterisation and comparison of semen microbiota and bacterial load in men with infertility, recurrent miscarriage, or proven fertility

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eLife Assessment

This **valuable** study reports a potential connection between the seminal microbiome and sperm quality/male fertility. The data are generally **convincing**. This study will be of interest to clinicians and biomedical researchers who work on microbiome and male fertility.

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Abstract

Several studies have associated seminal microbiota abnormalities with male infertility but have yielded differing results owing to their limited sizes or depths of analyses. The semen microbiota during recurrent pregnancy loss (RPL) has not been investigated. Comprehensively assessing the seminal microbiota in men with reproductive disorders could elucidate its potential role in clinical management. We used semen analysis, terminal-deoxynucleotidyl-transferase-mediated-deoxyuridine-triphosphate-nick-end-labelling, Comet DNA fragmentation, luminol ROS chemiluminescence and metataxonomic profiling of semen microbiota by16S rRNA amplicon sequencing in this prospective, cross-section study to investigate composition and bacterial load of seminal bacterial genera and species, semen parameters, reactive oxidative species (ROS), and sperm DNA fragmentation in men with reproductive disorders and proven fathers. 223 men were enrolled included healthy men with proven paternity (n=63); the male partners in a couple encountering RPL (n=46); n=58,

men with male factor infertility (n=58); the male partners of couples unexplained infertility (n=56). Rates of high sperm DNA fragmentation, elevated ROS and oligospermia were more prevalent in the study group compared with control. In all groups, semen microbiota clustered into three major genera-dominant groups (1, Streptococcus; 2, Prevotella; 3, Lactobacillus and Gardnerella); no species clusters were identified. Group 2 had the highest microbial richness (P<0.001), alpha-diversity (P<0.001), and bacterial load (P<0.0001). Semen analysis, ROS and DNA fragmentation were not associated with overall bacterial composition or load. Whilst, global perturbation of the seminal microbiota is not associated with male reproductive disorders, men with unidentified seminal *Flavobacterium* are more likely to have abnormal seminal analysis. Future studies may elucidate if *Flavobacterium* reduction has therapeutic potential.

Introduction

Mean sperm counts reported within clinical studies have reduced annually by 2.6% since 2000 (1). Male factor accounts for approximately half of all cases of infertility yet there are limited available interventions to improve sperm quality. Understanding the pathogenesis of male infertility may reveal novel therapeutic approaches for treating affected couples.

Symptomatic, genitourinary infection is an established cause of male infertility which may be detected by semen culture and treated with antibiotics (2, 3). The current European Association of Urology guidance states that whilst antibiotics may improve the overall quality of the spermatozoa, there is no evidence of increased pregnancy rates after antibiotic treatment of the male partner (4) (5) . Seminal leukocytes release bactericidal reactive oxygen species (ROS) in response to infection, however paradoxically this may damage sperm DNA and impair semen quality (6). We and others have reported that asymptomatic men affected by recurrent pregnancy loss (RPL), infertility or impaired preimplantation embryo development have increased risks of high seminal ROS and sperm DNA fragmentation (7, 8, 9, 10, 11, 12). It is therefore plausible that asymptomatic seminal infections may be associated with impaired reproductive function in some men. Since semen culture has a limited scope for studying the seminal microbiota due to its inability to identify all present microbiota next generation sequencing (NGS) approaches have been reported recently by a growing number of investigators (13, 14, 15, 16, 17, 18, 19). These studies, with varying methodologies, have produced inconsistent and conflicting results. As such, the composition of semen microbiota and its associations with clinical and molecular markers of male reproduction remains understudied. Elucidation of an association would have wide clinical application with therapeutic potential couple with reproductive disorders (20).

We hypothesised that semen microbiota composition associates with functional semen parameters, including ROS levels and sperm DNA fragmentation. To test this, we explored relationships between metataxonomic profiles of bacteria, bacterial copy number and key parameters of sperm function and quality in semen samples collected from 223 men, including those diagnosed with male factor infertility, unexplained infertility, partners affected by recurrent miscarriage, and paternity-proven controls.

Methods

Ethical approval was granted by the West London and Gene Therapy Advisory Committee (GTAC) Research Ethics Committee (14/LO/1038) and by the Internal Review Board at the Centre for Reproductive and Genetic Health (CRGH) (IRB-0003C07.10.19). Participants were recruited

following informed consent from clinics in Imperial College London NHS Trust and The Centre for Reproductive and Genetic Health (CRGH). Further detailed information on methods used in this study are included in the Supplementary Material.

Semen samples were produced by means of masturbation after 3-7 days abstinence. All semen samples were collected into sterile containers after cleaning of the penis using a sterile wipe. Samples were incubated at 37°C for a minimum of 20 mins prior to analysis. An aliquot was collected in a sterile cryovial and stored at -80°C.

Diagnostic semen analysis was carried out according to WHO 2010 guidelines and UK NEQAS accreditation (21) (22). Seminal analysis was performed in the Andrology Departments of Hammersmith Hospital and CRGH. Microscopic and macroscopic semen qualities were assessed within 60 mins of sample production. Semen volume, sperm concentration, total sperm count, progressive motility and total motility count, morphological assessment, anti-sperm antibodies and leucocyte count were established.

ROS analysis was performed using an in-house developed chemiluminescence assay validated by Vessey et al (23). Results are therefore reported as 'relative light units per second per million sperm'. The upper limit of optimal ROS was internally determined at 3.77 RLU/sec/10^6 sperm (95% CI) (24).

Sperm DNA fragmentation assessment performed by TUNEL (Terminal deoxynucleotidyl transferase biotin- dUTP Nick End Labelling) assay defined elevated sperm DNA fragmentation as >20% (25). Samples for the COMET assay were sent to the Examen Lab (Belfast, UK) for analysis with elevated sperm DNA fragmentation defined as >27% (26).

DNA extraction was performed on 200 µL of semen using enzymatic lysis and mechanical disruption. Bacterial load was estimated by determining the total number of 16S rRNA gene copies per sample using the BactQuant assay (27).

Metataxonomic profiling of semen microbiota was performed using MiSeq sequencing of bacterial V1-V2 hypervariable regions of 16S rRNA gene amplicons 16S rRNA genes using a mixed forward primerset 28F- YM GAGTTTGATYMTGGCTCAG, 28F-Borrellia GAGTTTGATCCTGGCTTAG, 28F-Chloroflex GAATTTGATCTTGGTTCAG and 28F-Bifdo GGGTTCGATTCTGGCTCAG at a ratio of 4:1:1:1 with 388R reverse primers. Sequencing was performed on the Illumina MiSeq platform (Illumina, Inc. San Diego, California). Following primer trimming and assessment of read quality, amplicon sequence variants (ASV) counts per sample were calculated and denoised using the Qiime2 pipeline (28) and the DADA2 algorithm (29). ASVs were taxonomically classified to species level using a naive Bayes classifier trained on all sequences from the V1-V2 region of the bacterial 16S rRNA gene present in the SILVA reference database (release 138.1) (30) (31).

Further methodological detail can be found in supplementary text.

Controls and contamination

3 negative kit/environmental control swabs were included to identify and eliminate potential sources of contamination and false positives in the 16S m*etataxonomic profiles.* These swabs were removed from the manufacturers packaging, waved in air, and then subjected to the same entire DNA extraction protocol. Decontamination of data was done using the decontam package (v1.9.0) in R, at ASV level, using both "frequency" and "prevalence" contaminant identification methods with *threshold* set to 0.1 (31). The "frequency" filter was applied using the total 16S rRNA gene copies measured as the *conc* parameter. For the "prevalence" filter all 3 blank swabs were used as negative controls and compared against all semen samples. ASVs classified as a contaminant by either method (n = 94) were excluded.

Statistical analysis

Hierarchical clustering with Ward-linkage and Jensen-Shannon distance was used to assign samples to putative community state types, with the number of clusters chosen to maximise the mean silhouette score. Linear regression models used to regress microbiota features against semen quality parameters and other clinical and demographic variables were fitted with the base R *lm* function (v4.2.0). The Benjamini-Hochberg false discovery rate (FDR) correction was used to control the FDR of each covariate signature independently (e.g., ROS, DNA Fragmentation, or Semen quality), with a $q < 0.05$, or 5%, cut-off, in both regression and Chi-squared analyses. Detailed information for statistical modelling is presented Supplementary methods.

Results

Study population

Semen samples were collected from a total of 223 men; this included control (n=63) and a study group (n=160) comprised of men diagnosed with male factor infertility (MFI) (n=58), male partners of women with recurrent pregnancy loss (RPL) (n=46) and male partners of couples diagnosed with unexplained infertility (UI) (n=26). The overall mean age of the total cohort was 38.1 ± 6 (mean ± SD). The mean age for controls was 40.1 ± 8, and the mean age for patients undergoing various fertility investigations was 37 ± 4.8. Ethnicity representation amongst recruited cohorts were not significantly different (p=0.38, Chi-square; Supplementary Table 1).

Semen quality assessment

Rates of high sperm DNA fragmentation, elevated ROS and oligospermia were more prevalent in the study group compared with control (**Table 1**). The study group represented 85% of samples with high sperm DNA fragmentation, 85% of samples with elevated ROS and 79% of samples with oligospermia. Rates of abnormal seminal parameters including low sperm concentration, reduced progressive motility and ROS concentrations were found to be highest in the MFI group (Supplementary Figure 1). Baseline characteristics between the RPL, unexplained subfertility and controls groups were similar (Supplementary Figure 1)

The seminal microbiota

Following decontamination, a total of 7,998,565 high quality sequencing reads were identified and analysed. Hierarchical clustering (Ward linkage) of relative abundance data resolved to genera level identified three major clusters, as determined by average silhouette score, amongst all samples (**Figure 1**, Supplementary Figure 2). These were compositionally characterised by highest mean relative abundances of 1. *Streptococcus* (23.8%), 2. *Prevotella* (24.4%), or 3. *Lactobacillus* and *Gardnerella* (35.9% and 35.6%, respectively, **Figure 1C**). Assessment of bacterial load using qPCR showed Clusters 2 and 3 had significantly higher bacterial loads compared to Cluster 1. Similar analyses were performed using sequencing data mapped to species level, however, examination of individual sample Silhouette scores within resulting clusters highlighted poor fitting indicating a lack of robust species-specific clusters (Supplementary Figure 3). To further investigate potential pairwise ecological interactions between taxa, a co-occurrence analysis was performed on the sequencing data, mapped to species level, with the SparCC algorithm (**Figure 2**). 5 major graph communities were detected. Community 1 highlighted a co-occurrence pattern between *Gardnerella vaginalis* and *Lactobacillus iners,* in agreement with the composition of cluster 3 from the hierarchical clustering analysis at genera level. Taxa belonging to communities 3 and 4 had a high number of connections (higher node degree), both within and

Factor	Categories	Controls	Study cases	*p-value*
DNA fragmentation index	Low	45/114 (40%)	69/114 (60%)	0.0002***
	High	12/ 82 (15%)	70/82 (85%)	
ROS	<3.77 RLU/s	53/143 (37%)	90/143 (63%)	0.02*
	>3.77 RLU/s	5/33 (15%)	28/33 (85%)	
Semen volume	Optimal	55/208 (26%)	153/208 (84%)	0.03*
	Sub-optimal	8/15 (53%)	7/15 (47%)	
Age	<34	11/49 (22%)	38/49 (88%)	0.04*
	34-41	31/124 (25%)	93/124 (85%)	
	>41	21/50 (42%)	29/50 (58%)	
Ethnicity	Caucasian	39/156 (25%)	117/156 (75%)	0.10
	Non-Caucasian	24/67 (36%)	43/67 (64%)	
Concentration	>15 M/mL	58/182 (32%)	124/182 (68%)	0.01*
	<15 M/mL	5/41 (21%)	36/41 (79%)	
Progressive motility	>32%	60/207 (29%)	147/207 (71%)	0.56
	<32%	3/16 (19%)	13/16 (81%)	
Sperm morphology	>4%	22/74 (30%)	52/74 (70%)	0.87
	<4%	41/144 (28%)	103/144 (72%)	
Semen quality	Optimal	24/78 (31%)	54/78 (69%)	0.53
	Sub-optimal	39/145 (27%)	106/145 (73%)	

Table I

Patient demographics and notable parameters of seminal quality and function for controls and study subjects.

Fisher's exact tests for all except age. Chi-square test for age. (n=223).

between the two communities, including some anti-co-occurrence patterns (SparCC ρ < 0). These communities included species from Genera *Staphylococcus, Peptoniphilus, Corynebacterium, Prevotella,* among others.

Bacterial richness, diversity and load were similar between all patient groups examined in the study (Supplementary Figure 4). Similarly, no significant associations between bacterial clusters, richness, diversity or load with seminal parameters, sperm DNA fragmentation or semen ROS were observed (Supplementary Tables 2-**3**). No significant differences in relative abundance of bacterial taxa between patient groups were detected, at genus or species-level. Several organisms at the genus level, identified variably in the literature as responsible for genito-urinary infection, were observed in our dataset but their prevalence did not reach our criteria (present in at last 25% of the samples) to be carried forward to regression modelling (32) (33) (24). This included *Chlamydia, Ureaplasma, Neisseria, Mycoplasma* and *Escherichia*. However, several associations (p<0.05) between relative abundance of specific bacterial genera and key sperm parameters were observed (**Table 2**). For example, increased sperm DNA fragmentation was positively associated with increased relative abundance of *Porphyromonas* and *Varibaculum* and inversely correlated with *Cutibacterium* and *Finegoldia.* ROS was positively associated with *Lactobacillus* species relative abundance, with analyses performed at species level taxonomy indicating that this relationship was largely driven by *L. iners* (p=0.04; **Table 3**). In contrast, *Corynebacterium* was inversely associated with ROS and positively associated with semen volume. Of note, the genera *Flavobacterium* was positively associated with both abnormal semen quality and sperm morphology and in both cases, withstood FDR correction for multiple testing (q=0.02 and q=0.01, respectively) (**Table 2**) (**Figure 3**). Consistent with this, a positive association between an unidentified species of *Flavobacterium* and semen quality was also observed (q=0.01, **Table 3**).

To focus analyses toward the most extreme phenotype of poor semen quality, a sub-analysis of controls compared with MFI was performed (**Table 4**). Non-parametric differential abundance analysis again identified a robust relationship between *Flavobacterium* and abnormal sperm morphology (q=0.01, **Table 4**). At species level, this was mapped to an unidentified species of *Flavobacterium* (q=0.01, **Table 5**). Similar to findings observed for all samples, sperm DNA fragmentation was inversely associated with relative abundance of *Cutibacterium* and positively associated with *Porphyromonas* and *Varibaculum* was also observed.

Discussion

We report the largest study to date investigating the seminal microbiota from patients suffering a range of adverse reproductive outcomes including unexplained infertility, male factor infertility and recurrent pregnancy loss. Moreover, we provide a detailed assessment of the relationship between semen microbial diversity, load and compositional structure with both molecular and classical seminal parameters. We identified 3 main clusters present in all study groups. Whilst overall bacterial composition was not association with aberrations in semen analysis, ROS and DNA fragmentation, men with unidentified *Flavobacterium* species were more likely to have abnormal semen quality or sperm morphology.

Several recent studies have indicated the existence of a semen microbiota; however, these studies have been limited to small samples sizes and have failed to reach consensus on the compositional structure of the microbiota, or its biological relevance, particularly in the context of sperm function and quality (13, 14, 16, 17, 18, 19, 34). By analysing the samples of 233 men with various reproductive disorders we offer a robust assessment of association between the microbiota and classical seminal parameters, but also key functional parameters including seminal ROS and sperm DNA damage. We incorporated stringent negative controls to permit removal of sequences likely originating from extraction kits and reagents known to contaminant low biomass samples such as semen (14, 16, 34). Molina et al report that 50%-70% of

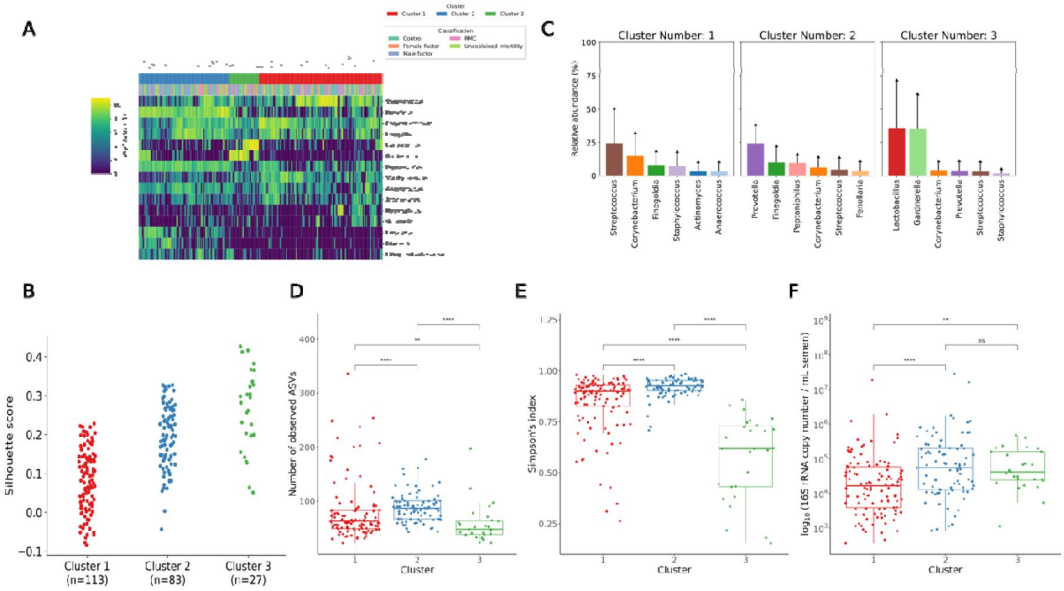


Figure 1.

Characterisation of semen microbiota composition at genera level.

A) Heatmap of Log10 transformed read counts of top 10 most abundant genera identified in semen samples. Samples clustered into three major microbiota groups based mainly on dominance by *Streptococcus* (Cluster 1), *Prevotella* (Cluster 2), or *Lactobacillus* and *Gardnerella* (Cluster 3). (n=223, Ward's linkage). **B)** Silhouette scores of individual samples within each cluster. **C)** Relative abundance of the top 6 most abundant genera within each cluster. **D)** Species richness ($p<0.0001$; Kruskal-Wallis test) and E) alpha diversity ($p<0.0001$; Kruskal-Wallis test) significantly differed across clusters. F) Assessment of bacterial load using qPCR showed Clusters 2 and 3 have significantly higher bacterial loads compared to Cluster 1 Dunn's multiple comparison test was used as a post-hoc test for between group comparisons (*$p<0.05$, ****$p<0.0001$).

Figure 2.

Co-occurrence network estimated with SparCC from 16S sequencing counts at species level.

Network representing co-occurrence patterns (edges), between various taxonomic units, assigned at species level (nodes). Edges are colored by their estimated SparCC correlation coefficient (ρ). Edges with a SparCC bootstrapped *p-value* < 0.05, ρ < 0.25, and singleton nodes are not shown. Node color represents network community membership.

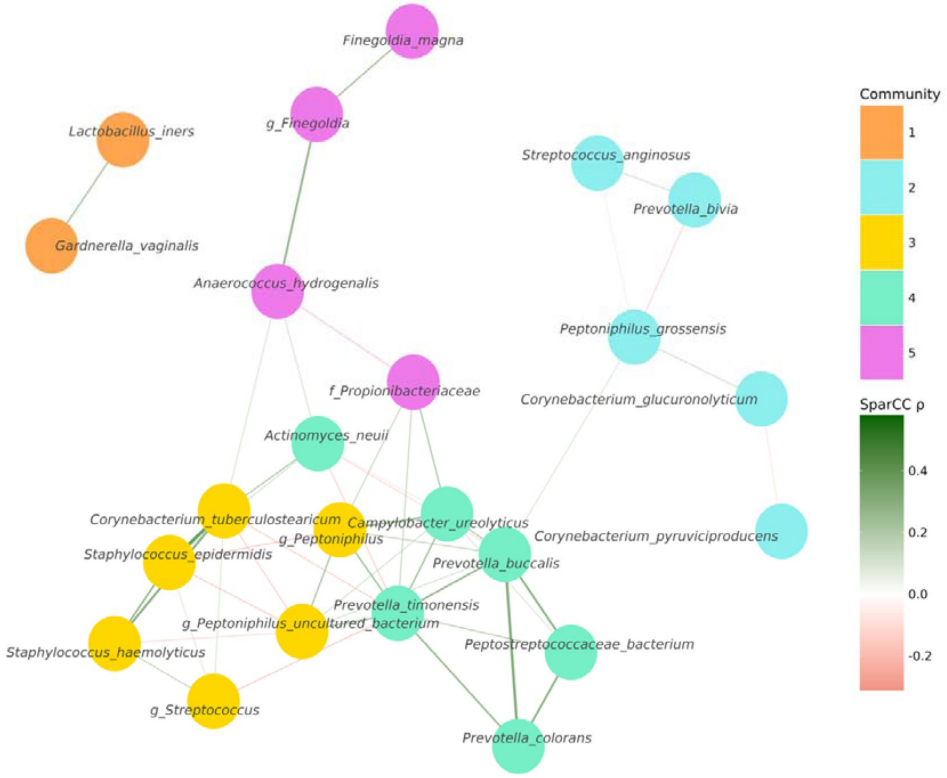
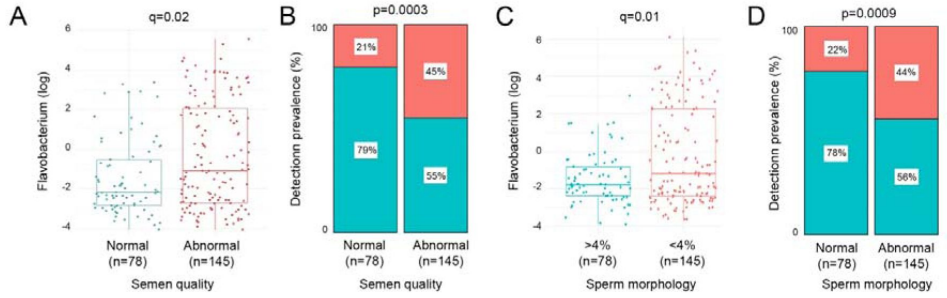


Figure 3.

Relative abundance and prevalence matrices of Flavobacterium in relation to semen quality and morphology.

A) Relative abundance of Flavobacterium was significantly higher in samples with abnormal semen (p=0.0002, q=0.02). **B)** Detection of flavobacterium was significantly more prevalent in abnormal semen quality samples (p=0.0003). **C)** Flavobacterium relative abundance was significantly higher in samples with <4% morphologically normal forms (p=0.0002, q=0.01). **D)** Flavobacterium was also significantly more prevalent in samples with low percentage of morphologically normal sperm (p=0.0009).



Sperm quality and function parameters	Genera	Welch's *t-statistic*	*p-value*	*q-value*
Sperm DNA fragmentation	*Finegoldia*	-2.36	0.01*	0.27
	Cutibacterium	-2.20	0.02*	0.27
	Porphyromonas	2.16	0.03*	0.27
	Varibaculum	2.11	0.03*	0.27
ROS	*Lactobacillus*	2.18	0.02*	0.66
	Corynebacterium	-2.04	0.04*	0.66
Semen quality	*Flavobacterium*	3.39	0.0008***	0.02*
	Prevotella	2.26	0.02*	0.38
Sperm concentration	*Porphyromonas*	-2.08	0.03*	0.61
Sperm morphology	*Flavobacterium*	3.64	0.0003***	0.01*
	Prevotella	2.03	0.04*	0.67
Semen volume	*Corynebacterium*	2.27	0.02*	0.32
	Actinotigum	-2.20	0.02*	0.32
	Varibaculum	-2.16	0.03*	0.32

Table II

Differential abundance analysis for bacterial genera with seminal quality and functional parameters.

Positive t-values indicate a positive relationship, and a negative t-value describes a negative relationship between relative abundance of taxa and seminal quality and function parameters. Significant relationships are indicated using p-values. q-values represent Benjamini-Hochberg false discovery rate corrected p-values for multiple comparisons.

Clinical factor	Species	Welch's *t-statistic*	*p-value*	*q-value*
Sperm DNA fragmentation	*Peptostreptococcaceae bacterium*	2.18	0.03*	0.91
ROS	*Lactobacillus iners*	2.24	0.02*	0.94
	Unidentified Anaerococcus	-2.03	0.04*	0.94
Semen quality	Unidentified Flavobacterium	3.76	0.0002***	0.01*
	Corynebacterium tuberculostearicum	-2.06	0.04*	0.82
Semen volume	*Corynebacterium tuberculostearicum*	2.64	0.008	0.24
	Unidentified Varibaculum	-2.48	0.01	0.24
	Staphylococcus epidermidis	2.35	0.01	0.24
	Unidentified Peptoniphilus	-2.32	0.02	0.24
	Dialister propionicifaciens	-2.24	0.02	0.24
	Prevotella colorans	-2.14	0.03	0.26
Cohorts	*Staphylococcus haemolyticus*	0.04	0.02	0.97

Table III

Differential abundance analysis for bacterial species with seminal quality and functional parameters.

Positive t-values indicate a positive relationship and a negative t-value describes a negative relationship between relative abundance of taxa and seminal quality and function parameters. Significant relationships are indicated using p-values. q-values represent Benjamini-Hochberg false discovery rate corrected p-values for multiple comparisons.

Clinical factor	Genera	Welch's *t-statistic*	*p-value*	*q-value*
Sperm DNA fragmentation	*Cutibacterium*	-2.56	0.01*	0.31
	Porphyromonas	2.34	0.02*	0.31
	Varibaculum	1.96	0.051	0.53
ROS	*Finegoldia*	-1.99	0.04*	0.77
Sperm concentration	*Finegoldia*	2.04	0.04*	0.71
Sperm morphology	*Flavobacterium*	3.64	0.0003***	0.01*
	Prevotella	2.03	0.04*	0.67
Semen volume	*Facklamia*	2.99	0.003**	0.10
	Actinotignum	-2.20	0.02*	0.36
	Dialister	-1.99	0.04*	0.36

Table IV

Differential abundance analysis for specific taxa at genera level for controls and cases with male factor infertility.

Positive t-values indicate a relationship, and a negative t-value describes a negative relationship between relative abundance of taxa and seminal quality and function parameters. Significant relationships are indicated using p-values. q-values represent Benjamini-Hochberg false discovery rate corrected p-values for multiple comparisons.

Clinical factor	Species	Welch's *t-statistic*	*p-value*	*q-value*
Sperm DNA fragmentation	*Staphylococcus hominis*	-2.32	0.02*	0.68
ROS	Unidentified Flavobacterium	2.42	0.01	0.54
	Unidentified Anaerococcus	-2.12	0.03	0.54
	Schaalia radingae	-2.12	0.03*	0.54
	Haemophilus parainfluenza	2.02	0.04*	0.54
Semen quality	Unidentified Flavobacterium	2.36	0.01*	0.91
Semen volume	*Dialister micraerophilus*	-2.66	0.008**	0.41
	Corynebacterium tuberculostearicum	2.27	0.02*	0.44
	Staphylococcus epidermidis	2.22	0.02*	0.44
	Actinotignum schaalii	-2.00	0.04*	0.45
Cohorts	*Staphylococcus haemolyticus*	0.04	0.01*	0.68

Table V

Differential abundance analysis for specific taxa at species for controls and male factor infertility.

Positive t-values indicate a positive relationship and a negative t-value describes a negative relationship between relative abundance of taxa and seminal quality and function parameters. Significant relationships are indicated using p-values. q-values represent Benjamini-Hochberg false discovery rate corrected p-values for multiple comparisons.

detected bacterial reads may be environmental contaminants in a sample from extracted testicular spermatozoa (35); with the addition of passage along the urethra it is likely that contamination of ejaculated semen would be much higher.

Mapping of genera level relative abundance data enabled semen samples to be categorised into 3 major clusters characterised by differing relative abundance of *Streptococcus*, *Prevotella*, *Lactobacillus* and *Gardnerella*. Unlike previous studies, we used an objective statistical approach (i.e. Silhouette methods) to determine the optimal number of microbial clusters supported by the data. These findings are largely consistent with earlier semen metataxonomic profiling studies reporting clusters enriched for *Streptococcus*, *Lactobacillus* and *Prevotella* (13, 14, 16). Moreover, Baud *et al.,* reported increased bacterial richness in the *Prevotella*-enriched cluster, which we also observed (16). This may suggest that certain compositional characteristics of seminal microbiota are conserved across populations. However, similar modelling of species level data, failed to identify statistically robust clusters. This contrasts with other niches such as the vagina where reproducible clusters based on species level metataxonomic profiles have been demonstrated reflecting mutualistic relationships between specific species and the host, which have coevolved over long periods of time (36, 37). It is possible therefore that our findings indicate that microbiota detected in semen are likely the result of transient colonisation events. Consistent with this, several species known to be commensal to the penile skin including *Streptococcus*, *Corynebacterium* and *Staphylococcus*, or the female genital tract including *Gardnerella* and *Lactobacillus*, were observed in semen samples (38). This is in keeping with data suggesting microbiota transference during sexual intercourse (39). It remains possible that a proportion of bacteria detected in semen reflects contamination of the sample acquired during the collection procedure. Studies undertaking assessment of female partner microbiota profiles as well as temporal profiling of semen microbiota would improve understanding of potential dynamic restructuring of semen microbiota compositions. This has been done in part by Baud et al by studying the subfertile couple as a unit to establish if there is a 'couple microbiota'(40) . They took samples from 65 couples with a range of pathologies including idiopathic infertility. From each woman they took vaginal swabs and follicular fluid samples. From each man they took a semen samples and penile swabs. They undertook extensive negative control series and stringent in silico elimination of possible contaminants. The found the male microbiota to be much more diverse than the female, with 90% of female samples being *Lactobacillus*-dominant. Intra-personal male samples i.e. semen and penile swabs from the same man bore more similarity to each other than inter-personal samples of the same sample type i.e. semen *or* penile swab comparisons between men (40). They identified that the male microbiota had very little impact of the microbiota of the female sexual partner(40) . Lack of information regarding the sexual activity of the enrolled couples limits this study somewhat.

Several previous studies have described semen microbiota composition to genera level and some have reported associations between specific genera and parameters of semen quality and function (13, 14, 16, 17, 18, 19, 34). However, in many cases these studies have failed to consider multiple comparisons testing, likely leading the reporting of spurious associations. We did not observe any significant associations between bacterial clusters, richness, diversity or load with traditional seminal parameters, sperm DNA fragmentation or semen ROS. This is in contrast with Veneruso *et al.,* who reported that in infertile patients, semen bacterial diversity and richness was decreased whereas Lundy *et al.,* reported that diversity was increased in infertile patients (17, 34). Further, Lundy et al., reported *Prevotella* abundance to be inversely associated with sperm concentration; this was not replicated in our study (17). There are several possible reasons accounting for the high heterogeneity in results including differences in methodology used to assess the microbial component of semen as well as differences in study design (41). For example, time of sexual abstinence prior to sample production as well as sample processing time often differs between studies, which has been shown to impact microbiological composition of semen (42).

The only association between bacterial taxa and semen parameters to withstand false detection rate testing for multiple comparisons detected in our study was between *Flavobacterium* and abnormal semen quality and sperm morphology (q=0.02). *Flavobacterium* are gram-negative physiologically diverse aerobes, some of which are pathogenic (43). *Flavobacterium* was recently identified as a dominant genus in immature sperm cells retrieved from testicular biopsies of infertile men in a study by Molina et al (35). However, in contrast to these findings, a recent smaller study investigating semen collected from 14 sperm donors and 42 infertile idiopathic patients reported an association between *Flavobacterium* and increased sperm motility but a negative correlation with sperm DNA fragmentation (18).

Though not withstanding multiple correction, we did observe several other associations between specific bacterial taxa and semen parameters. For example, samples enriched with *Lactobacillus* had lower incidence of elevated seminal ROS, a relationship which could largely be accounted for by *Lactobacillus iners*, a common member of the cervicovaginal niche (44). Various studies have also found *Lactobacillus* enrichment in semen to associate with normal seminal parameters, especially morphology (14, 16). were *Lactobacillus*-predominant (14). However, an association between samples enriched with *Lactobacillus* and asthenospermia or oligoasthenospermia has also been described (19). We also observed an association between increased sperm DNA fragmentation and samples enriched with *Varibaculum*, which is consistent with previous reports of increased relative abundance of *Varibaculum* in semen infertile (34).

A limitation of this and other similar studies is that it was a single institutional study with limited ethnic diversity and potential geographical changes induced by environment or dietary habit. Gut microflora is known to display geographical variability; we cannot exclude that similar geographical variability exists for the seminal microbiota (45). The universal primers used during NGS may not be universal and may anneal variably to specific bacteria resulting in over-detection, under-detection, or indeed non-detection of some taxa (46) (47). A further limitation of this study, and others, is the lack of reciprocal genital tract microbiota testing of the female partners, or paired seminal and urinary samples from male participants. Additionally, we did not have other covariables such as smoking status with which to include in further analyses.

In summary, our study confirms that compositionally the semen microbiota can be broadly classified into three major groups based upon relative abundance of key bacterial genera. Despite different methodological approaches a number of studies, including our own, indicate a Prevotella-dominated or Lactobaccilus- dominated seminal microbiota, perhaps suggesting a stable microbiota at genera level. Our species level our data, however, failed to show similar clusters, perhaps instead suggesting transient colonisation. Longitudinal studies are required to ascertain the stability of the seminal microbiota. We provide evidence for an association between *Lactobacillus* abundance and normal seminal parameters. However, our results indicate that no specific semen bacterial composition can robustly differentiate between fertile and infertile men, although a small subset of bacteria may be associated with changes in seminal parameters. Our finding that an unidentified species of *Flavobacterium* impairs seminal parameters warrants further exploration and may offer the potential for targeted therapies. Larger, multi-centred studies as well as mechanistic investigations are required to establish causal links between the semen microbiota and male fertility.

Data availability statement

The 16S rRNA metataxonomic dataset and the data analysis scripts are publicly available at the European Nucleotide Archive (Project accession PRJEB57401) and GitHub (repository link *https://github.com/Gscorreia89/semen-microbiota-infertility*), respectively).

Acknowledgements

We would like to thank the patients and participants for their involvement in the study

Additional information

Authors roles

Mowla, Farahani, Tharakan, Jayasena and MacIntyre made substantial contribution to the study design, acquisition of data, analysis and interpretation of data and critical revision of the article for important intellectual content. Davies and Correia made substantial contribution to the analysis and interpretation of data and drafting the article. Lee, Kundu, Khanjani, Sindi and Khalifa made substantial contribution to the acquisition of data and critical revision of the article for important intellectual content. Rai, Regan, Henkel, Minhas Dhillo, Ben Nagi and Bennett made substantial contribution to the study design and critical revision of the article for important intellectual content. All authors approved the final version to be published and are in agreement to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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Conflict of interest

N/A

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Reviewer #1 (Public review):

Summary:

The authors analyzed the bacterial colonization of human sperm using 16S rRNA profiling. Patterns of microbiota colonization were subsequently correlated with clinical data, such as spermiogram analysis, presence of reactive oxygen species (ROS), and DNA fragmentation. The authors identified three main clusters dominated by Streptococcus, Prevotella, and Lactobacillus & Gardnerella, respectively, which aligns with previous observations. Specific associations were observed for certain bacterial genera, such as Flavobacterium and semen quality. Overall, it is a well-conducted study that further supports the importance of the seminal microbiota.

Strengths:

- The authors performed the analysis on 223 samples, which is the largest dataset in semen microbiota analysis so far
- Inclusion of negative controls to control contaminations.
- Inclusion of a positive control group consisting of men with proven fertility.

Weaknesses:

- The manuscript needs comprehensive proofreading for language and formatting. In many instances spaces are missing or not required.
- Could the authors explore correlation network analyses to get additional insights in the structure of different clusters?
- The github link is not correct.
- It is not possible to access the dataset on ENA.
- Add the graphs obtained with decontam analysis as a supplementary figure.
- There is nothing about the RPL group in the results section, while the authors discuss this issue in the introduction. What about the controls with proven fertility?
- While correctly stated in the title, the term microbiota should be used throughout the manuscript instead of "microbiome"

Comments on revised version:

Discussion: Could the authors discuss more the findings about Flavobacterium? Has it ever been associated with the urogenital tract? What is the relative abundance in the present study: this type of bacterium has been previously associated with contaminations (PMID: 25387460, 30497919).

Figure 1: Increase the size of panel A.

Figure 3: Can the authors indicate the relative abundance of each genus/species by the size of the node?

Supplementary data: I don't see anywhere the decontam plots.

https://doi.org/10.7554/eLife.96090.2.sa1

Author response:

The following is the authors' response to the original reviews.

> ***Reviewer 1:***
>
> *- The manuscript needs comprehensive proofreading for language and formatting. In many instances, spaces are missing or not required.*

Thank you for your comments. The manuscript has been thoroughly proofread for errors in language and formatting.

> *- Could the authors explore correlation network analyses to get additional insights into the structure of different clusters?*

We have added a co-occurrence analysis (at species taxonomic level) based on SparCC to the manuscript (Figure 2).

This is described on Page 9 line 141-148

> *- The GitHub link is not correct.*

The github repository has now been made public.

> *- It is not possible to access the dataset on ENA.*

We have changed the ENA study PRJEB57401 status to open.

> *- Add the graphs obtained with decontam analysis as a supplementary figure.*

We have added the outputs of decontam (.csv files with feature lists of ASVs that were filtered based on the prevalence and frequency tests) to the github repository.

> *- There is nothing about the RPL group in the results section, while the authors discuss this issue in the introduction. What about the controls with proven fertility?*

Thank you. We have amended the manuscript to compare characteristics between the RPL, unexplained subfertility and controls groups.

Line 1279-130 page 8:

"The study group represented 85% of samples with high sperm DNA fragmentation, 85% of samples with elevated ROS and 79% of samples with oligospermia. Rates of abnormal seminal parameters including low sperm concentration, reduced progressive motility and ROS concentrations were found to be highest in the MFI group (Supplementary Figure 1). Baseline characteristics between the RPL, unexplained subfertility and controls groups were similar.

Line 150-154 Page 9:

"Bacterial richness, diversity and load were similar between all patient groups examined in the study (Supplementary Figure 4).

> *- While correctly stated in the title, the term microbiota should be used throughout the manuscript instead of "microbiome"*

Thank you. This misnomer has been amended throughout the manuscript.

> *Minor corrections:*
>
> *Line 25: provoke is not a good term here.*

Thank you. The term ‘provoke’ has been removed

> *Line 26: why does semen culture have a limited scope?*

Thank you. Line 40-41 Page 3 has been amended:

“It is therefore plausible that asymptomatic seminal infections may be associated with impaired reproductive function in some men. Since semen culture has a limited scope for studying the seminal microbiota due to its inability to identify all present microbiota next generation sequencing (NGS) approaches have been reported recently by a growing number of investigators (13, 14, 15, 16, 17, 18, 19)”.

> *Line 68: write µl correctly*

Thank you. This has been corrected

> *Line 131: several organisms at the genus level.*

Thank you. This has been corrected

> *Line 136: what are the relative abundances of these genera? Is this relevant?*

The mean relative abundances for the key taxa mention in each cluster are all above 20%. This information has been added to the manuscript text on page 9, line 153.

> *Line 173: Molina et al.*

Thank you. This has been corrected

> *Line 173: the contaminations are referred to the low biomass nature of testicular samples. If present, bacteria of accessory gland secretions are an integral part of the seminal microbiota itself. Please review these sentences.*

Thank you. This had been reworked to highlight the important of urethral contamination, which you later allude to as a limitation of our study is the failure to provide paired urine and semen samples.

Page 11 line 194-196

“Molina et al report that 50%-70% of detected bacterial reads may be environmental contaminants in a sample from extracted testicular spermatozoa (35); with the addition of passage along the urethra it is likely that contamination of ejaculated semen would be much higher.”

> *Table 1: remove results interpretation from table caption.*

Thank you this has been acted upon.

> *Table 1: why in some cases, like in DNA fragmentation index, the total is not equal to n=223?*

This is due to missing data/ analysis not possible for some men due to the requirement of a minimum number of sperm in the ejaculate to perform DNA fragmentation testing.

> *Table 1: "frag" is not defined.*

Thank you, this has been amended

> *Tables 2, 3 & 4: bacterial genera in italics.*

Thank you, this has been amended

> *Figure 1A: add the fertility status information above the cluster colors.*

Thank you, this has been amended in Figure 1.

> *Figure 1C: the color code is confusing. Use different colors for each cluster.*

Figure 1 legend: bacterial genera in italics.

> *Figures 1 & 2: the authors should use similar chart formatting in the two tables.*

Thank you, this has been amended

> ***Reviewer 2:***
>
> *(1) The patient groups have different diagnoses and should be handled as different groups, and not fused into one 'patient' group in analyses.*
> *Why are the data in tables presented as controls and cases? I would consider men from couples with recurrent pregnancy loss, unexplained infertility, and male factor infertility to have different seminal parameters (not to fuse them into one group). This means, that the statistical analyses should be performed considering each group separately, and not to fuse 3 different infertility diagnoses into one patient group.*

We have conducted detailed analyses, requested by the reviewer, comparing seminal DNA, ROS and microbiota characteristics between each individual patient groups (Supplimental figures 1 and 4). No specific taxa (at either genera or species-level) were found to differ in relative abundance between the diagnostic groups. However, we expect associations between parameters such as reactive oxygen species, or DNA fragmentation, and relative abundance of bacterial species, to be general and not restricted to or specific to each diagnostic group. Therefore, we also conducted further analyses aggregating data from all patient groups to investigate relationships common to these different forms of male reproductive dysfunction.

> *(2) Were any covariables included in the statistical analyses, e.g. age, BMI, smoking, time of sexual abstinence, etc?*

Covariates were not included in the statistical analyses. This has been added in the manuscript to the limitations.

Page 14 line 267-268

"Additionally, we did not have other covariables such as smoking status with which to include in further analyses".

> *(3) Furthermore, it is known that 16S rRNA gene analysis does not provide sensitive enough detection of bacteria on the species level. How much do the authors trust their results on the species level?*

The limitations of taxonomic assignment using 16S rRNA gene metataxonomics are well documented. However, the capacity to assign sequence amplicons at species level depends on the sequence variability of the 16S rRNA gene for each of the taxa reported and the specific gene region chosen. In this study, amplification of the V1-V2 region was performed using a mixed 28f primer set (see methods for details) that enables resolution and assignment of several bacterial species highly relevant to the reproductive tract including *Lactobacillus spp.*, such as *L. crispatus* and *L. iners,* (e.g. https://doi.org/10.3389/fcell.2021.641921, https://doi.org/10.1128/msystems.01039-23, https://doi.org/10.1186/s12915-023-01702-2). In this study, we report the presence of *L. iners*, but not *L. crispatus* in semen samples, and we have also identified a specific association/co-occurrence between *Gardnerella vaginalis* and *Lactobacillus iners*, similar to that observed in vaginal bacterial communities.

> *(4) Were the analyses of bacterial genera and species abundances with seminal quality parameters controlled for diagnosis and other confounders?*

As stated in point 2, no adjustment was made for co-variates. No differences in microbiome composition were observed among the three diagnostic groups, so no adjustments were made to our analysis.

> *(5) The authors stress that their study is the biggest on the microbiome in semen. However, when considering that the study consists of 4 groups (with n=46-63), it does not stand out from previous studies.*

Our study is overall the largest investigating interactions between the seminal microbiome and male reproductive dysfunction. Other studies have included greater numbers of men with infertility.

> *(6) Weaknesses: There is a lack of paired seminal/urinal samples.*

Thank you. This limitation has been added.

Page 14 line 266-267

"A further limitation of this study, and others, is the lack of reciprocal genital tract microbiota testing of the female partners, or paired seminal and urinary samples from male participants".

> ***Recommendation for authors to consider:***
>
> *Including previous classical reviews in the introduction:*
> *DOI:10.1097/MOU.0000000000000742*
> *DOI: 10.1038/s41585-019-0250-y*

Thank you. This has been added.

> *Mentioning in the M&M section that there is a supplementary text with a more detailed M&M part.*

Thank you. This has been added. Further methodological detail can be found in supplementary text.

> *Revising the use of 'microbiota' and 'microbiome', they are not synonyms. When talking of 16S rRNA gene analysis, we consider 'microbiome' analysis.*

Thank you. This misnomer has been amended throughout the manuscript.

> *Revising the text, there are several erratas (e.g. verb missing, etc).*

Thank you for your comments. The manuscript has been thoroughly proofread for errors in language and formatting.

https://doi.org/10.7554/eLife.96090.2.sa0