RBMO

ARTICLE

Assessing the testicular sperm microbiome: a low-biomass site with abundant contamination

BIOGRAPHY

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KEY MESSAGE

The human testicle harbours bacterial signature, albeit in a low biomass, in which *Bacteroides*, *Akkermansia*, *Faecalibacterium*, and *Alistipes* genera prevail. These microbes could play a role in functional sperm development. Rigorous control and elimination of contaminants is crucial for analysing low microbial biomass site to obtain reliable data.

ABSTRACT

Research question: The semen harbours a diverse range of microorganisms. The origin of the seminal microbes, however, has not yet been established. Do testicular spermatozoa harbour microbes and could they potentially contribute to the seminal microbiome composition?

Design: The study included 24 samples, comprising a total of 307 testicular maturing spermatozoa. A high-throughput sequencing method targeting V3 and V4 regions of *16S rRNA* gene was applied. A series of negative controls together with stringent in-silico decontamination methods were analysed.

Results: Between 50 and 70% of all the detected bacterial reads accounted for contamination in the testicular sperm samples. After stringent decontamination, *Blautia* ($P = 0.04$), *Cellulosibacter* ($P = 0.02$), *Clostridium* XIVa ($P = 0.01$), *Clostridium* XIVb ($P = 0.04$), *Clostridium* XVIII ($P = 0.02$), *Collinsella* ($P = 0.005$), *Prevotella* ($P = 0.04$), *Prolixibacter* ($P = 0.02$), *Robinsoniella* ($P = 0.04$), and *Wandonia* ($P = 0.04$) genera demonstrated statistically significant abundance among immature spermatozoa.

Conclusions: Our results indicate that the human testicle harbours potential bacterial signature, though in a low-biomass, and could contribute to the seminal microbiome composition. Further, applying stringent decontamination methods is crucial for analysing microbiome in low-biomass site.

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KEYWORDS
Male infertility
Microbiome
Microbiota
Semen
Sperm

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Declaration: The authors report no financial or commercial conflicts of interest.

INTRODUCTION

Few, if any, tissues in the human body are entirely sterile, and it is becoming clear that the microorganisms on and in the human body have important functions in health and disease (*Power et al., 2017*). The Human Microbiome Project has assessed that the urogenital tract accounts for about 9% of the whole human microbiota (*NIH HMP Working Group et al., 2009*). Nevertheless, little is known about the microbial communities found in the male reproductive tract, and this microbial niche is currently understudied compared with other areas of microbiome research (*Altmäe et al., 2019*).

With the advancement in technologies for detecting microorganisms, it is now acknowledged that semen harbours a diverse range of bacteria, plays a role in male reproductive health and acts as a medium for the transmission of microbes, with the ability to affect both the couple's and the newborn's health (*Altmäe, 2018*; *2019*; *Farahani et al., 2020*; *Osadchiy et al., 2020*). Direct sperm–bacteria cellular interactions have been demonstrated, and the possible function of some bacteria in semen could result from these cellular attachments; bacteria seem to firmly attach to the spermatozoon to evade immune responses and to successfully reach the female reproductive tract (*Fraczek et al., 2012*; *Rowe et al., 2020*).

The origin and function of the seminal microbes, however, has not yet been established. One-third of the seminal microbes originate from the urethra (*Kermes et al., 2003*), whereas a substantial part could originate from the upper genital tract. Indeed, the existence of the testicular microbiome was recently presented (*Alfano et al., 2018*). *Alfano et al. (2018)* identified bacterial DNA fingerprints within testicular samples from men with idiopathic non-obstructive azoospermia and found that bacterial dysbiosis was associated with idiopathic non-obstructive azoospermia and complete germ cell aplasia (*Alfano et al., 2018*). This study provides the first insight into the possible existence of testicular microbiome and its potential role in functional sperm development (*Alfano et al., 2018*); nevertheless, no rigorous controlling for contamination was applied. Testicles, like other tissues in the human body (*Zheng et al., 2020*), harbour limited amount of commensal bacteria, and adequate microbiome identification over the host material is technically challenging and requires well-controlled experiments with rigorous bioinformatic analyses (*O'Callaghan et al., 2020*).

The aim of the present study was to investigate the existence of microbes in human testicular samples by analysing maturing spermatozoa using 16S ribosomal RNA (*16S rRNA)* gene sequencing and following stringent decontamination protocols together with internal contamination controls at every step throughout the study.

MATERIALS AND METHODS

Study design and participants

Testicular biopsies from infertile men were collected at MAR&Gen Assisted Reproduction Clinic, Granada, Spain, when attending for assisted reproductive technology (ART) treatment between September 2014 and April 2016. The study participants presented with azoospermia, severe oligoasthenoteratozoospermia, or DNA fragmentation (TABLE 1). Men with DNA fragmentation underwent testicular biopsy as five or more previous ART cycles had failed. In total, 307 testicular spermatozoa at different maturation stages from 11 men distributed into 24 samples (TABLE 1). All men were screened for sexually transmitted infections (hepatitis B and C, human immunodeficiency virus, cytomegalovirus, syphilis and *Chlamydia*) and no infections were detected. The study was carried out in accordance with the Declaration of Helsinki, and the procedures was approved by the Ethics Committee of the University of Granada (number 927/2014). All participants gave written consent for the donation of testicular cells for research.

Collection of testicular spermatozoa

Testicular samples were obtained in the air-purificated operating room by open testicular biopsy and were subjected to in-vitro culture for 5–48 h as previously described (*Tesarik et al., 1998*). Briefly, an antiseptic was used to clean the scrotum and allowed to dry before the incision for the testicular biopsy. The pieces of testicular tissue were placed in G-GAMETE™ medium (Vitrolife, Gothenburg, Sweden) and disintegrated mechanically by stretching between two microscope slides, followed by repeated aspirations into a 1-ml tuberculin syringe. Large tissue pieces were removed, and the remaining small fragments of the seminiferous tubules were cultured *in vitro*. All cell cultures were carried out in G-GAMETE™ in a water bath set to 30°C. Recombinant human FSH (Puregon, Organon, Oss, the Netherlands) was added at 50 IU/l final activity concentration, and water-soluble testosterone (T-5035) (Sigma-Aldrich, St Louis, MO, USA) was added at a concentration of 1 µmol/l. The cultures were maintained at 30°C.

Testicular cells in in-vitro cultures could be found both isolated and forming small groups of cells. To achieve the disintegration of the cell clusters, aliquots of all cultures were prepared and incubated with 1000U/ml of collagenase IV (C-5138) (Sigma-Aldrich Indicated before) at 37°C for 1 h and shaken every 10–15 min during the incubation period followed by recovery in G-MOPS™ medium (Vitrolife). Cells that were not used for clinical procedures were donated for research. In total, 307 testicular spermatozoa at different developmental stages were picked one by one into cell-type specific pools for the present study (TABLE 1). The collection of the 24 cell pool samples from the culture was carried out under the Olympus IX71 microscope (Olympus Corporation, Shinjuku, Tokyo, Japan) using the Tokai-Hit thermal plate (Olympus Corporation, Shinjuku, Tokyo, Japan), the IM-9B microinjector (Narishige Group, Setagaya-ku, Tokyo, Japan) and hatching pipettes (Humagen, Charlottesville, VA, USA). The droplets of cell pools were placed into a 0.2 ml-sterile polymerase chain reaction (PCR) tube containing cell lysis buffer (with added 3.6 µl Tween-20 [10%], 60 µl dithiothreitol [100 mM] and 6.4 µl RiboLOCK RNase inhibitor [40 U/µl in 30 µl Milli-Q water]), and stored at −80°C for further analysis.

Pre-treatment and DNA extraction

Pre-treatment by bead-beating protocol was carried out to achieve a more efficient bacterial cell lysis. QIAamp *cador* Pathogen Mini Kit was used (Qiagen, Venlo, the Netherlands) following the protocol for difficult-to-lyse bacteria in whole blood or pre-treated tissue by using lysis tubes. As the volume of our starting material was limited (<10 µl), 200 µl of the ATL solution was used.

TABLE 1 PATIENT DATA

Sample[a]	Cell type	Number of cells	Patient	Age (years)	Infertility diagnosis	Sperm concentration (mill/ml)	Progressive motility (%)	Normal morphology (%)	DNA fragmentation (%)
S1	Spermatozoa	5	1	65	Sperm DNA fragmentation	65	73	5	35
S2	Round spermatid	5							
S3	Spermatocyte	5							
S4	Round spermatid	15	2	44	Sperm DNA fragmentation	165	33	3	52
S5	Round spermatid	15							
S6	Spermatocyte	15							
S7	Spermatocyte	15							
S8	Spermatozoa	20	3	32	Sperm DNA fragmentation	20	50	4	45
S9	Spermatocyte	10							
S10	Spermatozoa	5	4	28	Azoospermia	0	–	–	–
S11	Round spermatid	5							
S12	Spermatocyte	5							
S13	Round spermatid	15	5	38	Azoospermia	0	–	–	–
S14	Spermatozoa	10	6	33	Azoospermia	0	–	–	–
S15	Round spermatid	10							
S16	Round spermatid	16	7	52	Azoospermia	0	–	–	–
S17	Spermatocyte	16							
S18	Round spermatid	15	8	41	Azoospermia	0	–	–	–
S19	Spermatocyte	15							
S20	Round spermatid	20	9	45	Azoospermia	0	–	–	–
S21	Non-classified spermatid	15	10	40	Azoospermia	0	–	–	–
S22	Non-classified spermatid	15							
S23	Elongated spermatid	20	11	42	Severe oligoasthenoteratozoospermia	0.1	1	0	–
S24	Elongated spermatid	20							

[a] Indicates the name of the sample for downstream analyses.

'-' not assessed

Next, DNA was extracted from the testicular spermatozoa using QIAamp *cador* Pathogen Kit as directed by the manufacturer and the extracted DNA was eluted in 20 µl of AVE solution. Negative controls from the culture media and laboratory reagents were processed in parallel with the 24 testicular sperm samples to control for the possible microbial contamination (TABLE 2).

Sequencing V3 and V4 hypervariable regions of 16S rRNA gene

To characterise the composition of bacterial communities, hypervariable regions V3 and V4 of *16S rRNA* gene were amplified by PCR from each sample and sequenced. The primers used were: 5′CCTACGGGNGGCWGCAG3′ (forward primer) and 5′GACTACHVGG GTATCTAATCC3′ (reverse primer). All PCRs were carried out in 25 µl reaction volume containing 12.5 µl 2x KAPA HiFi Hotstart ready mix (KAPA Biosystems, Woburn, MA, USA), 5 µl of each primer (1 µM), and 2.5 µl of extracted DNA (10 ng) under the following cycling conditions using Applied Biosystems 2720 Thermal Cycler (Thermo Fisher Scientific): initial denaturation at 95°C for 3 min, followed by 35 cycles of denaturation at 95°C for 30 s, annealing at 55°C for 30 s and elongation at 72°C for 30 s, with a final extension at 72°C for 5 min. PCR products were analysed on 1% (weight/volume) agarose gel electrophoresis in which 1 kb Plus DNA Ladder (catalogue number 10787018) (Thermo Fisher Scientific, Waltham, MA, USA), DNA Gel Loading Dye (6X) (catalogue number R0611) (Themo Fisher Scientific) were used and run under 80V for 35 min to confirm the amplification of a single product. Amplicons were purified with AMPure XP beads (Beckman Coulter, Indianapolis, IN, USA). Next, a PCR to index the amplicons was carried out using the Nextera XT Index Kit (Illumina, San Diego, CA, USA). Index PCR conditions using Applied Biosystems 2720 Thermal Cycler were as follows: 95°C for 3 min; eight cycles of 95°C for 30 s, 55°C for 30 s, 72°C for 30 s, with a final extension step of 5 min at 72°C and kept at 4°C. The pooled PCR products were purified using AMPure XP beads (Beckman Coulter, Brea, CA, USA) before quantification. Then, the samples were quantified in a Qubit 4 Fluorometer (Thermo Fisher Scientific). Briefly, the two standards were added in the Qubit 4 equipment (Thermo Fisher Scientific), and the relative fluorescence unit values were checked from 0 to 100 ng/µl. All the samples were measured, including

TABLE 2 NEGATIVE CONTROLS INCLUDED IN THE STUDY

Negative controls[a]		DNA amplification	Total number of reads (Decontam)	Total number of reads (microDecon)
Culture media				
NC1	G-GAMETE™	+	48,349	39,753
NC2	G-MOPS™	+	47,379	36,386
NC3	Cell lysis buffer	+	20,580	14,849
Laboratory reagents				
NC4	VXL solution[b]	+	66	0
NC5	AW1 solution[b]	+	7	0
NC6	ACB solution[b]	–	–	–
NC7	AVE solution[b]	–	–	–
NC8	2x KAPA HiFi HotStart ReadyMix + primers[c]	+	0	0
NC9	AMPure XP beads + 80% Ethanol + 10 mM Tris pH 8.5[c]	+	0	0
NC10	2x KAPA HiFi Hotstart ready mix + index primers + PCR Grade water[c]	–	–	–
NC11	4 pM PhiX library[c]	–	–	–

[a] The number and the type of negative control used in downstream analyses. After applying the Decontam and microDecon decontamination procedures, the final number of contaminant reads are indicated for each negative control that was taken into account when identifying sperm-specific bacteria and contaminant bacteria in each sample.
[b] DNA extraction kit.
[c] Sequencing library preparation kit.
'-' DNA not amplified

the controls, and after quantification, the samples were normalised at 4 nM using 10 mM Tris pH 8.5 (Sigma-Aldrich). Finally, the samples were denaturalised with 0.2 N NaOH (Sigma-Aldrich), and diluted to a final concentration of 4 pM. The final library was paired-end sequenced at 300-bp using a MiSeq Reagent Kit v.3 on the Illumina MiSeq sequencing system (Illumina.).

Bioinformatic and statistical analyses

Bioinformatic analysis of the sequencing data was carried out as previously described (Sydor et al., 2020). All fastQ files, generated after sequencing and demultiplexing, were analysed using DADA2 package version 1.10.1 (Callahan et al., 2016) and, as result, a unique table containing all samples with the sequence reads and abundances was generated. Phylotypes were assigned to a taxonomic affiliation based on the naïve Bayesian classification with a pseudo-bootstrap threshold of 80%. Further annotation of phylotypes was performed with the RDP database using the Seqmatch function to define the discriminatory power of each sequence read; annotation was carried out according to the criteria published previously (Schulz et al., 2018). The resulting phylotype table was filtered to consider only those phylotypes that were present in 50% or more of samples to capture microbes consistently present in the dataset. Microbial communities were analysed at genera phylogenetic rank.

To discern between the true bacterial sequences and potential contaminant DNA, two different decontamination approaches were applied: Decontam v.1.6.0 (Davis et al., 2018) and microDecon v.1.0.2 (McKnight et al., 2019). Given that the characterisation of the low microbial biomass requires in-silico contaminant removal to ensure that DNA from biological samples can be effectively distinguished from amplified exogenous DNA, the R packages Decontam and microDecon are the most used approaches in the low-biomass microbiome studies (Karstens et al., 2019; O'Callaghan et al., 2020).

Decontam

Decontam identifies background DNA contaminants based on their pattern of occurrence in biological versus control samples (Davis et al., 2018). A sequence is classified as contaminant by comparing its associated score statistic P-value to a user-defined score threshold P^*, where P can be the frequency, prevalence or composite score (Davis et al., 2018) . Specifically, the Decontam score threshold was set to 0.5 to define contaminating phylotypes using the prevalence-based method, as it is recommended for the low microbial biomass environments, e.g. tissue samples (Davis et al., 2018). The prevalence-based method calculates a score for each phylotype (ranging from 0 to 1) that is used by Decontam to distinguish between contaminant and non-contaminants, presenting contaminant phylotypes small scores P ($P < 0.5$). With the score threshold of 0.5, the Decontam package is able to identify 70–90% of contaminant phylotypes (Karstens et al., 2019). Further, the remaining contaminant phylotypes present in low abundance were removed by an additional filtering step, by transforming the testicular microbial community data set to relative abundances and then setting any phylotype values below 0.1% to zero as described previously (Karstens et al., 2019).

MicroDecon

MicroDecon, a newer decontamination method, is based on the principle that all the samples will receive the same proportions of contamination from a common source and thereby uses the proportions of contaminant sequences in negative controls to identify and remove contaminating reads (McKnight et al., 2019). More specifically, this package identifies a phylotype that is complete contamination, i.e., the 'constant', and uses it to calculate the number of reads in each sample that arise from

the contamination, and those reads are then subtracted (McKnight et al., 2019). MicroDecon method is suggested to have two advantages over Decontam: first, microDecon treats each sample completely independently and, second, it is not affected by the sample size. MicroDecon can correct phylotypes that occur in both negative controls and real samples, as it is able to remove contaminant reads rather than entire phylotype (McKnight et al., 2019). In the present study, the decon() function was run on its default values, which first decontaminates the data and then applies filtering thresholds to remove residual contamination that should have been removed from all samples but is retained in low numbers in a few samples.

One-way analysis of variance (ANOVA) was used to assess differences in microbial signatures between infertility diagnoses and between cell types. Differences in read counts between testicular cells and internal negative controls were evaluated by Welch's *t*-test. Benjamini and Hochberg correction (false discovery rate) for multiple testing was applied. $P < 0.05$ was considered statistically significant.

RESULTS

In total, 307 testicular spermatozoa at different maturing phases that grouped into 24 samples were analysed, together with 11 negative controls for the microbial profiles. After quality filtering, the total number of paired-end reads and phylotypes in the sperm samples was 3,486,343 and 13,885, respectively. Of the 11 negative controls, six were excluded from further analyses as they did not show any DNA amplification or obtained zero reads after sequencing, i.e. clean controls (TABLE 2). The most contaminated negative controls were the initial in-vitro culture media, in which the fresh testicular biopsies were placed and cells were cultured (G-GAMETE^{TM} and G-MOPS^{TM}). Indeed, it has been recently demonstrated that in-vitro culture media contains a wide range of microbes (Štšepetova et al., 2020).

Decontamination with Decontam

After applying the contamination correction with Decontam, a total of 1,958,794 paired-end reads were obtained and grouped into 205 phylotypes (Supplementary Table 1), with a mean of 81,616 reads and 119 phylotypes per sample.

Contaminant bacteria were detected in all testicular sperm samples, with an average of 45% of contaminant bacterial sequences per sample (ranging from 32–64%) (FIGURE 1A). Decontam analysis identified *Pseudarcicella* (Phy175), *Phascolarctobacterium* (Phy101), *Vampirovibrio* (Phy98), *Barnesiella* (Phy122), *Alistipes* (Phy170), *Bacteroides* (Phy178 and Phy208), and *Prevotella* (Phy279) as contaminant phylotypes (Decontam score <0.5) (FIGURE 1B), and these taxa were removed from downstream analyses. Further, after abundance filtering, two additional phylotypes with zero reads corresponding to *Bacteroides* genus (Phy932 and Phy973) were identified and removed.

Although negative controls presented similar bacterial profile to testicular samples, the number of the reads differed significantly (FIGURE 1C, FIGURE 1D and Supplementary Table 2). The testicular samples contained 66 genera and the negative controls 63 genera (FIGURE 1C). Genera not identified in negative controls included *Delftia*, *Prolixibacter* and *Robinsoniella*. Sequencing of testicular maturing spermatozoa revealed that the dominant genera included *Bacteroides*, *Akkermansia*, *Faecalibacterium*, *Alistipes*, and *Prevotella* (FIGURE 1E).

Decontamination with microDecon

Decontaminated output of microDecon analysis contained 976,323 paired-end reads grouped into 171 phylotypes (Supplementary Table 1), with a mean of 40,680 reads and 96 phylotypes per sample. This method detected and removed contaminant reads in all testicular sperm samples, rather than assigning an entire phylotype as contaminant, which resulted in an average of 72% of contaminant bacterial sequences per sample (ranging from 65–78%) (FIGURE 2A).

Negative controls and testicular samples presented similar bacterial profiles; however, the number of the reads differed statistically, being higher in sperm samples (FIGURE 2B, FIGURE 2C and Supplementary Table 2). With microDecon approach, 60 genera in testicular sperm and 59 in negative controls were detected (FIGURE 2B). *Robinsoniella* was the only genus not identified among negative controls. The dominant genera detected in the immature spermatozoa included *Bacteroides*, *Akkermansia*, *Faecalibacterium*, *Alistipes*, and *Flavobacterium* (FIGURE 2D).

To compile the contamination results, the number of detected DNA sequences in the negative controls (contaminant reads) was lower than in the biological samples. After subtracting these contaminant reads from the testicular samples (applying Decontam and microDecon methods), a microbial signature in the testicular cells was identified. Significantly more abundant genera were found in the testicular samples compared with controls after applying both decontamination approaches (FIGURE 2E).

No statistically significant differences in microbiome profiles were detected between individuals and between testicular spermatozoa in different developmental stages (Supplementary Figure 1 and Supplementary Figure 2).

DISCUSSION

The present study findings help to unravel the microbial composition in the testicle; however, it seems to be a low microbial biomass site. Microbiome analysis of a low microbial biomass site requires specific focus on combating host and laboratory reagent microbial contamination to identify true bacterial sequences (Karstens et al., 2018; 2019; Eisenhofer et al., 2019; Stinson et al., 2019; Weyrich et al., 2019; O'Callaghan et al., 2020; Molina et al., 2021). In the present study, internal negative controls were used throughout all the experimental steps and additionally applied rigid in-silico decontamination methods for unravelling the non-contaminant microbiome in the testicular sperm samples. Altogether, 10 bacterial genera were identified as testicle sperm specific. These included *Blautia* (phylum *Firmicutes*), *Cellulosibacter* (*Firmicutes*), *Clostridium* XIVa (*Firmicutes*), *Clostridium* XIVb (*Firmicutes*), *Clostridium* XVIII (*Firmicutes*), *Collinsella* (*Actinobacteria*), *Prevotella* (*Bacteroidetes*), *Prolixibacter* (*Bacteroidetes*), *Robinsoniella* (*Firmicutes*), and *Wandonia* (*Bacteroidetes*). The detected bacteria *Blautia*, *Clostridium*, and *Prevotella* have also been identified in previous studies among the seminal samples (Weng et al.,

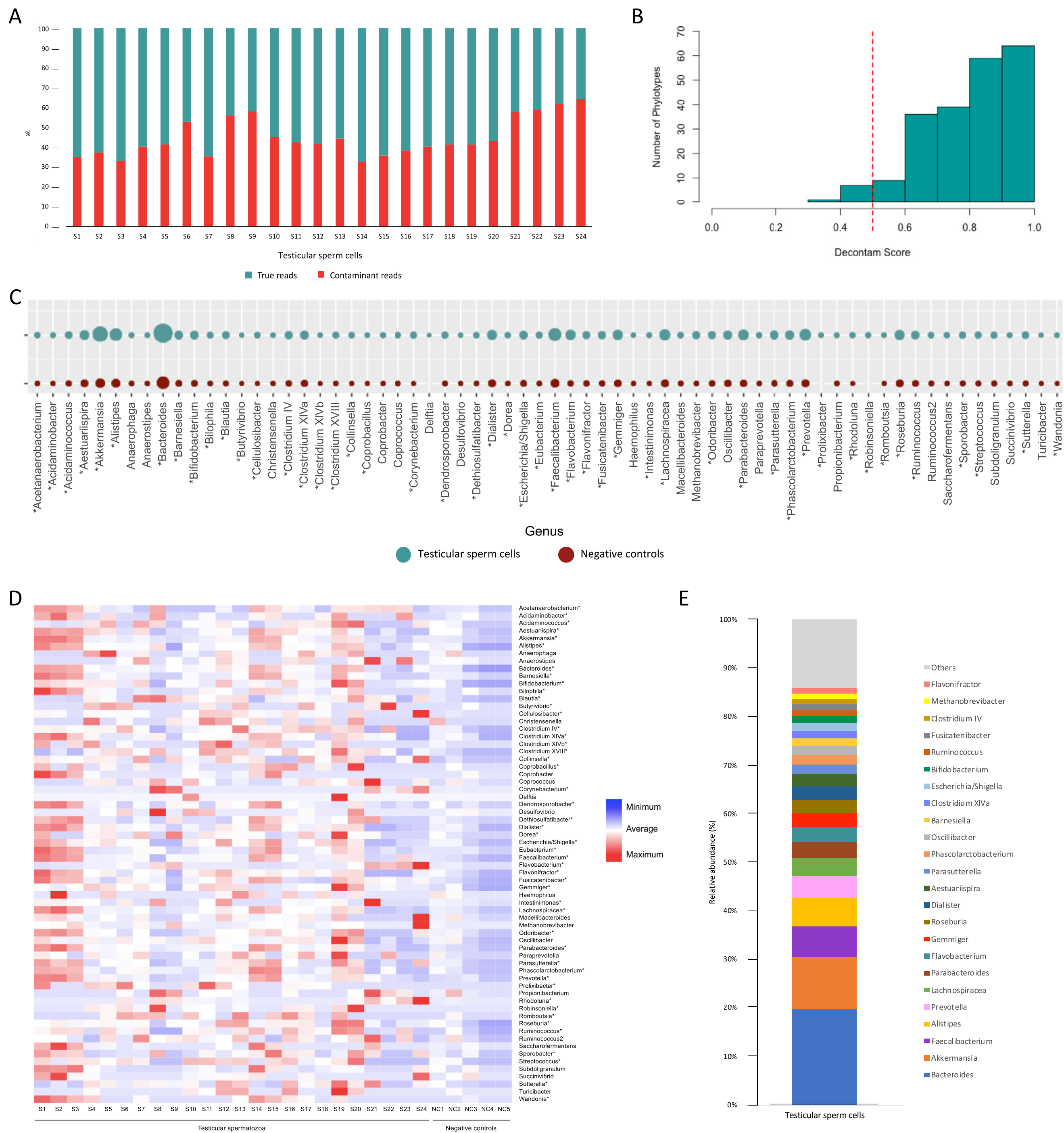

FIGURE 1 Analysis of microbial sequences in testicular immature spermatozoa using Decontam approach. (A) Percentage of true (blue) and contaminant (red) reads obtained in testicular samples; (B) histogram of prevalence-based scores assigned by Decontam to each phylotype. The x-axis represents the prevalence-based score assigned by Decontam, and y-axis shows the number of phylotypes assigned to a given score. The represented Decontam scores were computed with *IsContaminant* function. The distribution of Decontam scores shows that most of the phylotypes in our samples were assigned high scores (>0.5), suggesting non-contaminant origin; (C) average reads of each bacterial genus in testicular samples (blue) versus negative controls (red). The circle size denotes the average reads of each genus; (D) heatmap illustrating the number of reads at genus level in each testicular sample and negative control. 'Average' indicates average score that has the same raw value as the row mean, 'Maximum' indicates maximum score that has SD above the row mean, and 'Minimum' denotes minimum score that has SD below the row mean; (E) the 'clean' bacterial composition in testicular samples at genus level. Genera with abundance less than 1% were grouped as 'others'. *, indicates genera that differed statistically ($P < 0.05$) (Supplementary Table 2) in the number of reads between the testicular sperm samples versus negative controls.

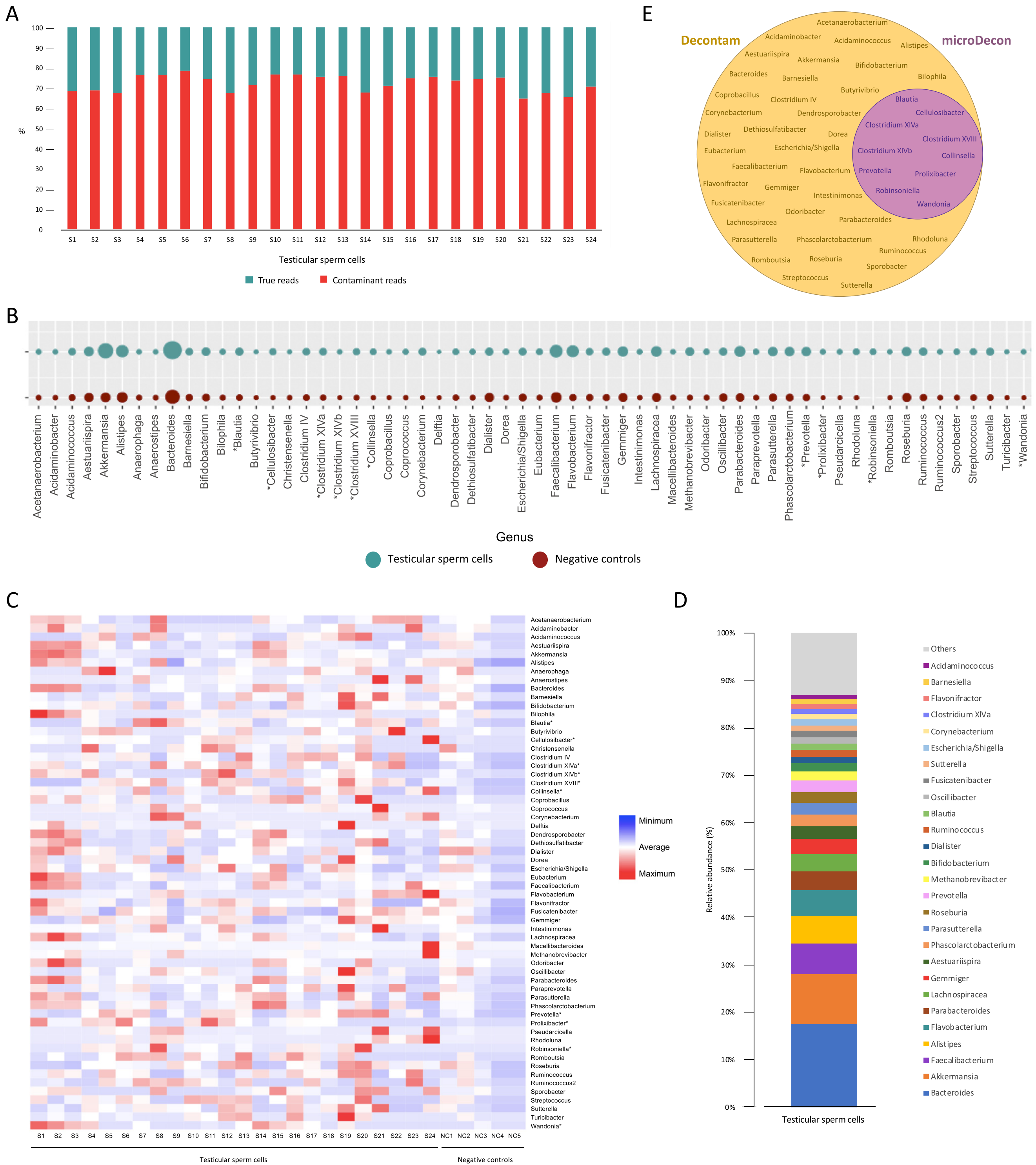

FIGURE 2 Analysis of microbial sequences in testicular immature spermatozoa using microDecon approach. (A) Percentage of true (blue) and contaminant (red) reads obtained in testicular samples; (B) average reads of each bacterial genus in testicular samples (blue) versus negative controls (red). The circle size denotes the average reads of each genus; (C) heatmap illustrating the number of reads at genus level in each testicular sample and negative control. 'Average' indicates average score that has the same raw value as the row mean, 'Maximum' indicates maximum score that has SD above the row mean, and 'Minimum' denotes minimum score that has SD below the row mean; (D) the 'clean' bacterial composition in testicular samples at genus level. Genera with abundance less than 1% were grouped as 'others'; (E) significantly more abundant genera in testicular samples versus negative controls in both decontamination approaches ($P < 0.05$). *Blautia, Cellulosibacter, Clostridium* XIVa, *Clostridium* XIVb, *Clostridium* XVIII, *Collinsella*, *Prevotella*, *Prolixibacter*, *Robinsoniella*, and *Wandonia* are considered to be testicle sperm-specific bacteria.*, indicates genera that differed statistically ($P < 0.05$) (Supplementary Table 2) in the number of reads between testicular sperm samples versus negative controls.

2014; Altmäe et al., 2019; Campisciano et al., 2020; Štšepetova et al., 2020; Yao et al., 2020), demonstrating that the most abundant bacteria in the testicular sperm samples are also present in the semen and supporting the possible contribution of the upper genital tract microbes to the downstream seminal microbiome composition. Interestingly, *Prevotella* was identified in over 90% of our testicular samples. *Prevotella* genus has been associated with low-quality semen when analysing semen samples from humans (Jarvi et al., 1996; Nguyen et al., 2014; Weng et al., 2014; Baud et al., 2019; Campisciano et al., 2020; Farahani et al., 2020; Yang et al., 2020), suggesting that species within *Prevotella* could contribute to the spermatogenesis defects and male infertility (Ding et al., 2020; Yang et al., 2020). The pioneering study of the testicular microbiome (Alfano et al., 2018) did not present their results on bacterial genus level; therefore, our study results on specific testicular bacteria are not comparable, whereas, on phylum level, our identified phyla were also reported in the previous study.

Another important result of our study is that contamination comprised 50–70% of all the detected bacterial reads in our testicular cell samples, supporting the hypothesis that assisted reproductive technology is not carried out in sterile conditions (Štšepetova et al., 2020), and highlighting the importance of controlling for the possible contaminants when dealing with low microbial biomass tissue. Indeed, it has been demonstrated that contaminant microorganisms, specifically the contaminants arisen before amplification, can dominate the composition of low-microbial-biomass samples, which could lead to inaccurate data interpretation (Salter et al., 2014; Glassing et al., 2016).

In the present study, all contaminating steps in analysing microbiome were controlled for; however, the study has limitations that should be highlighted. One limitation is the analysis of cultured spermatozoa instead of untreated cells, which might have favoured the growth of some bacteria. The culturing media, however, were treated as negative controls, and the results were rigorously controlled for a possible contamination arising from this step. Also, inclusion of positive control (mock microbial community) would have helped to assess the amplification efficiency and the possible cross-contamination during sample processing. Furthermore, although we analysed microbial composition of testicular sperm samples from infertile men, whose testis microbiome could be altered, knowledge of the healthy commensal microbiome in the human testicles was lacking.

In conclusion, our study results indicate that the testicle harbours its unique low-biomass microbial signature, with a possible role in functional sperm development, and could be one source of the seminal microbial composition. Nevertheless, further research is required for assessing the potential effect of short microbial DNA fragments as determinants of spermatogenesis and male reproductive health outcomes. We also conclude that when analysing low microbial biomass tissue, such as the testicle, systematic control and elimination of possible contamination is crucial to obtain reliable microbiome data over the host information and to minimise misinterpretation of the results.

ACKNOWLEDGEMENTS

We thank all men who generously donated testicular material for the purpose of this study. We also acknowledge the research support by Copan Italia S.p.A Inc., and Clearblue, SPD Swiss Precision Diagnostics GmbH. This study is part of a PhD Thesis conducted at the Official Doctoral Program in Biomedicine of the University of Granada, Spain. This work was supported by the Spanish Ministry of Economy, Industry and Competitiveness (MINECO) and European Regional Development Fund (FEDER): grant numbers RYC-2016-21199 and ENDORE (SAF2017-87526-R); by FEDER/Junta de Andalucía-Consejería de Economía y Conocimiento: MENDO (B-CTS-500-UGR18); by Junta de Andalucía: (PAIDI P20_00158) by the University of Granada, Plan Propio de Investigación 2016, Excellence actions: Units of Excellence; Unit of Excellence on Exercise and Health (UCEES), and the Junta de Andalucía, Consejería de Conocimiento, Investigación y Universidades and European Regional Development Fund: (SOMM17/6107/UGR); by Spanish Ministry of Science, Innovation, and Universities: (PRE2018-085440 and FPU19/01638); and by Spanish Ministry of Education, Culture, and Sport: (FPU15/01193). Funding for open access charge: Universidad de Granada/CBUA Sequence data of all testicular spermatozoa and negative control samples have been deposited in the National Centre for Biotechnology Information (NCBI) Sequence Read Archive (SRA) database (http://www.ncbi.nlm.nih.gov/sra) under the BioProject ID PRJNA643898. The preliminary results of this study were presented as a poster communication at the 35th Annual ESHRE Meeting (Vienna, 2019).

SUPPLEMENTARY MATERIALS

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.rbmo.2021.06.021.

REFERENCES

Alfano, M., Ferrarese, R., Locatelli, I., Ventimiglia, E., Ippolito, S., Gallina, P., Cesana, D., Canducci, F., Pagliardini, L., Viganò, P., Clementi, M., Nebuloni, M., Montorsi, F., Salonia, A. **Testicular microbiome in azoospermic men-first evidence of the impact of an altered microenvironment.** Hum. Reprod. 2018; 33: 1212–1217. doi:10.1093/humrep/dey116

Altmäe, S. **Commentary: Uterine Microbiota: Residents, Tourists, or Invaders?** Front. Immunol. 2018; 9: 1874. doi:10.3389/fimmu.2018.01874

Altmäe, S., Franasiak, J.M., Mändar, R. **The seminal microbiome in health and disease.** Nat. Rev. Urol. 2019; 16: 703–721. doi:10.1038/s41585-019-0250-y

Baud, D., Pattaroni, C., Vulliemoz, N., Castella, V., Marsland, B.J., Stojanov, M. **Sperm Microbiota and Its Impact on Semen Parameters.** Front Microbiol. 2019; 10: 234. doi:10.3389/fmicb.2019.00234

Callahan, B.J., McMurdie, P.J., Rosen, M.J., Han, A.W., Johnson, A.J.A., Holmes, S.P. **DADA2: High-resolution sample inference from Illumina amplicon data.** Nat. Methods 2016; 13: 581–583. doi:10.1038/nmeth.3869

Campisciano, G., Iebba, V., Zito, G., Luppi, S., Martinelli, M., Fischer, L., De Seta, F., Basile, G., Ricci, G., Comar, M. **Lactobacillus iners and gasseri, Prevotella bivia and HPV Belong to the Microbiological Signature Negatively Affecting Human Reproduction.** Microorganisms 2020; 9: 39. doi:10.3390/microorganisms9010039

Davis, N.M., Proctor, D.M., Holmes, S.P., Relman, D.A., Callahan, B.J. **Simple statistical identification and removal of contaminant sequences in marker-gene and metagenomics data.** Microbiome 2018; 6: 226. doi:10.1186/s40168-018-0605-2

Ding, N., Zhang, Xin, Zhang, Xue Di, Jing, J., Liu, S.S., Mu, Y.P., Peng, L.L., Yan, Y.J., Xiao, G.M., Bi, X.Y., Chen, H., Li, F.H., Yao, B., Zhao, A.Z. **Impairment of spermatogenesis and sperm motility by the high-fat diet-induced dysbiosis of gut microbes.** Gut. 2020; 69: 1608–1619. doi:10.1136/gutjnl-2019-319127

Eisenhofer, R., Minich, J.J., Marotz, C., Cooper, A., Knight, R., Weyrich, L.S. **Contamination in Low Microbial Biomass Microbiome Studies: Issues and Recommendations.** Trends Microbiol 2019; 27: 105–117. doi:10.1016/j.tim.2018.11.003

Farahani, L., Tharakan, T., Yap, T., Ramsay, J.W., Jayasena, C.N., Minhas, S. **The semen microbiome and its impact on sperm function and male fertility: A systematic review and meta-analysis.** Andrology andr. 2020: 12886. doi:10.1111/andr.12886

Fraczek, M., Piasecka, M., Gaczarzewicz, D., Szumala-Kakol, A., Kazienko, A., Lenart, S., Laszczynska, M., Kurpisz, M. **Membrane stability and mitochondrial activity of human-ejaculated spermatozoa during in vitro experimental infection with Escherichia coli, Staphylococcus haemolyticus and Bacteroides ureolyticus.** Andrologia 2012; 44: 315–329. doi:10.1111/j.1439-0272.2012.01283.x

Glassing, A., Dowd, S.E., Galandiuk, S., Davis, B., Chiodini, R.J. **Inherent bacterial DNA contamination of extraction and sequencing reagents may affect interpretation of microbiota in low bacterial biomass samples.** Gut. Pathog. 2016; 8: 24. doi:10.1186/s13099-016-0103-7

Jarvi, K., Lacroix, J.M., Jain, A., Dumitru, I., Heritz, D., Mittelman, M.W. **Polymerase chain reaction-based detection of bacteria in semen.** Fertil. Steril. 1996; 66: 463–467. doi:10.1016/0167-7012(96)00844-5

Karstens, L., Asquith, M., Caruso, V., Rosenbaum, J.T., Fair, D.A., Braun, J., Gregory, W.T., Nardos, R., McWeeney, S.K. **Community profiling of the urinary microbiota: considerations for low-biomass samples.** Nat. Rev. Urol. 2018; 15: 735–749. doi:10.1038/s41585-018-0104-z

Karstens, L., Asquith, M., Davin, S., Fair, D., Gregory, W.T., Wolfe, A.J., Braun, J., McWeeney, S. **Controlling for Contaminants in Low-Biomass 16S rRNA.** Gene Sequencing Experiments mSystems 2019; 4: 1–14. doi:10.1128/mSystems.00290-19

Kermes, K., Punab, M., Lõivukene, K., Mändar, R. **Anaerobic seminal fluid micro-flora in chronic prostatitis/chronic pelvic pain syndrome patients.** Anaerobe 2003; 9: 117–123. doi:10.1016/S1075-9964(03)00085-4

McKnight, D.T., Huerlimann, R., Bower, D.S., Schwarzkopf, L., Alford, R.A., Zenger, K.R. **microDecon: A highly accurate read-subtraction tool for the post-sequencing removal of contamination in metabarcoding studies.** Environ. DNA 2019; 1: 14–25. doi:10.1002/edn3.11

Molina, N.M., Sola-Leyva, A., Haahr, T., Aghajanova, L., Laudanski, P., Castilla, J.A., Altmäe, S. **Analysing endometrial microbiome: methodological considerations and recommendations for good practice.** Hum. Reprod. 2021; 36: 859–879. doi:10.1093/humrep/deab009

Nguyen, P.V., Kafka, J.K., Ferreira, V.H., Roth, K., Kaushic, C. **Innate and adaptive immune responses in male and female reproductive tracts in homeostasis and following HIV infection.** Cell. Mol. Immunol. 2014; 11: 410–427. doi:10.1038/cmi.2014.41

NIH HMP Working Group. Peterson, J., Garges, S., Giovanni, M., McInnes, P., Wang, L., Schloss, J.A., Bonazzi, V., McEwen, J.E., Wetterstrand, K.A., Deal, C., Baker, C.C., Di Francesco, V., Howcroft, T.K., Karp, R.W., Lunsford, R.D., Wellington, C.R., Belachew, T., Wright, M., Giblin, C., David, H., Mills, M., Salomon, R., Mullins, C., Akolkar, B., Begg, L., Davis, C., Grandison, L., Humble, M., Khalsa, J., Little, A.R., Peavy, H., Pontzer, C., Portnoy, M., Sayre, M.H., Starke-Reed, P., Zakhari, S., Read, J., Watson, B., Guyer, M. **The NIH Human Microbiome Project.** Genome Res. 2009; 19: 2317–2323. doi:10.1101/gr.096651.109

O'Callaghan, J., Turner, R., Dekker Nitert, M., Barrett, H., Clifton, V., Pelzer, E. **Re-assessing microbiomes in the low-biomass reproductive niche.** BJOG An Int. J. Obstet. Gynaecol. 2020; 127: 147–158. doi:10.1111/1471-0528.15974

Osadchiy, V., Mills, J.N., Mayer, E.A., Eleswarapu, S.V. **The Seminal Microbiome and Male Factor Infertility.** Curr. Sex Heal. Reports 2020; 12: 202–207. doi:10.1007/s11930-020-00273-5

Power, M.L., Quaglieri, C., Schulkin, J. **Reproductive Microbiomes: A New Thread in the Microbial Network.** Reprod. Sci. 2017; 24: 1482–1492. doi:10.1177/1933719117698577

Rowe, M., Veerus, L., Trosvik, P., Buckling, A., Pizzari, T. **The Reproductive Microbiome: An Emerging Driver of Sexual Selection, Sexual Conflict, Mating Systems, and Reproductive Isolation.** Trends Ecol. Evol. 2020; 35: 220–234. doi:10.1016/j.tree.2019.11.004

Salter, S.J., Cox, M.J., Turek, E.M., Calus, S.T., Cookson, W.O., Moffatt, M.F., Turner, P., Parkhill, J., Loman, N.J., Walker, A.W. **Reagent and laboratory contamination can critically impact sequence-based microbiome analyses.** BMC Biol. 2014; 12: 87. doi:10.1186/s12915-014-0087-z

Schulz, C., Schütte, K., Koch, N., Vilchez-Vargas, R., Wos-Oxley, M.L., Oxley, A.P.A., Vital, M., Malfertheiner, P., Pieper, D.H. **The active bacterial assemblages of the upper GI tract in individuals with and without Helicobacter infection.** Gut. 2018; 67: 216–225. doi:10.1136/gutjnl-2016-312904

Stinson, L.F., Keelan, J.A., Payne, M.S. **Identification and removal of contaminating microbial DNA from PCR reagents: impact on low-biomass microbiome analyses.** Lett. Appl. Microbiol. 2019; 68: 2–8. doi:10.1111/lam.13091

Štšepetova, J., Baranova, J., Simm, J., Parm, Ü., Rööp, T., Sokmann, S., Korrovits, P., Jaagura, M., Rosenstein, K., Salumets, A., Mändar, R. **The complex microbiome from native semen to embryo culture environment in human in vitro fertilization procedure.** Reprod. Biol. Endocrinol. 2020; 18: 3. doi:10.1186/s12958-019-0562-z

Sydor, S., Best, J., Messerschmidt, I., Manka, P., Vilchez-Vargas, R., Brodesser, S., Lucas, C., Wegehaupt, A., Wenning, C., Aßmuth, S., Hohenester, S., Link, A., Faber, K.N., Moshage, H., Cubero, F.J., Friedman, S.L., Gerken, G., Trauner, M., Canbay, A., Bechmann, L.P. **Altered Microbiota Diversity and Bile Acid Signaling in Cirrhotic and Noncirrhotic NASH-HCC.** Clin. Transl. Gastroenterol. 2020; 11: e00131. doi:10.14309/ctg.0000000000000131

Tesarik, J., Guido, M., Mendoza, C., Greco, E. **Human spermatogenesis in vitro: respective effects of follicle-stimulating hormone and testosterone on meiosis, spermiogenesis, and Sertoli cell apoptosis.** J. Clin. Endocrinol. Metab. 1998; 83: 4467–4473. doi:10.1210/jcem.83.12.5304

Weng, S.-L., Chiu, C.-M., Lin, F.-M., Huang, W.-C., Liang, C., Yang, T., Yang, T.-L., Liu, C.-Y., Wu, W.-Y., Chang, Y.-A., Chang, T.-H., Huang, H.-D. **Bacterial communities in semen from men of infertile couples: metagenomic sequencing reveals relationships of seminal microbiota to semen quality.** PLoS One 2014; 9e110152. doi:10.1371/journal.pone.0110152

Weyrich, L.S., Farrer, A.G., Eisenhofer, R., Arriola, L.A., Young, J., Selway, C.A., Handsley-Davis, M., Adler, C.J., Breen, J., Cooper, A. **Laboratory contamination over time during low-biomass sample analysis.** Mol. Ecol. Resour. 2019; 19: 982–996. doi:10.1111/1755-0998.13011

Yang, H., Zhang, J., Xue, Z., Zhao, C., Lei, L., Wen, Y., Dong, Y., Yang, J., Zhang, L. **Potential Pathogenic Bacteria in Seminal Microbiota of Patients with Different Types of Dysspermatism.** Sci. Rep. 2020; 10: 6876. doi:10.1038/s41598-020-63787-x

Yao, T., Han, X., Guan, T., Wang, Z., Zhang, S., Liu, Changhui, Liu, Chao, Chen, L. **Effect of indoor environmental exposure on seminal microbiota and its application in body fluid identification.** Forensic Sci. Int. 2020; 314110417. doi:10.1016/j.forsciint.2020.110417

Zheng, D., Liwinski, T., Elinav, E. **Interaction between microbiota and immunity in health and disease.** Cell Res. 2020; 30: 492–506. doi:10.1038/s41422-020-0332-7

Received 10 February 2021; received in revised form 19 May 2021; accepted 22 June 2021.