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REVIEW ARTICLE

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The semen microbiome and its impact on sperm function and male fertility: A systematic review and meta-analysis

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Abstract

Background: Male factor is attributable in up to 50% of cases of infertility. In vitro studies demonstrate that bacteria can negatively impact sperm function. The use of next-generation sequencing techniques has provided a better understanding of the human microbiome, and dysbiosis has been reported to impact health. Evidence regarding the impact of the semen microbiome on sperm function and fertility remains conflicting.

Materials and methods: A systematic search was conducted in accordance with the Preferred Reporting Items for Reviews and Meta-analysis (PRISMA) statement. The databases MEDLINE, OVID and PubMed were searched to identify English language studies related to the identification of bacteria in the semen of infertile and fertile men, between 1992 and 2019. Fifty-five observational studies were included, with 51 299 subjects. We included studies identifying bacteria using next-generation sequencing, culture or polymerase chain reaction.

Results: The semen microbiome was rich and diverse in both fertile and infertile men. Three NGS studies reported clustering of the seminal microbiome with a predominant species. Lactobacillus and Prevotella were dominant in respective clusters. Lactobacillus was associated with improvements in semen parameters. Prevotella appeared to exert a negative effect on sperm quality. Bacteriospermia negatively impacted sperm concentration and progressive motility, and DNA fragmentation index (DFI; MD: 3.518, 95% CI: 0.907 to 6.129, $P = .008$).

There was an increased prevalence of ureaplasma urealyticum in infertile men (OR: 2.25, 95% CI: 1.47-3.46). Ureaplasma urealyticum negatively impacted concentration and morphology. There was no difference in the prevalence of chlamydia trachomatis between fertile and infertile men and no significant impact on semen parameters. Enterococcus faecalis negatively impacted total motility, and Mycoplasma hominis negatively impacted concentration, PM and morphology.

Discussion and conclusions: Ureaplasma urealyticum, Enterococcus faecalis, Mycoplasma hominis and Prevotella negatively impact semen parameters, whereas

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Lactobacillus appears to protect sperm quality. These findings may facilitate the development of novel therapies (eg probiotics), although the evidence regarding the impact of the seminal microbiome on fertility is inconclusive and further studies are needed to investigate this association.

KEYWORDS
semen microbiome, male fertility, bacteriospermia

1 | INTRODUCTION

Infertility affects 8%-12% of couples worldwide[1] and is defined as the failure to conceive after 12 months of regular unprotected intercourse.[2.] Male factor infertility is attributable in up to 50% of cases,[3] and potential causes include urogenital tract infections (eg prostatitis, epididymitis).[4] In vitro studies have highlighted the mechanisms through which bacteria affect sperm function, including agglutination of motile sperm, induction of apoptosis, production of immobilization factors and impairment of the acrosome reaction.[5-9] However, the evidence for using empiric antibiotics in the clinical setting is controversial,[10] as there are conflicting data as to whether such pathogens cause abnormalities in semen parameters in vivo and whether treatment leads to an improvement in semen parameters and reproductive potential. Leucocytospermia has been posited as the pathogenesis for male factor infertility, and has been associated with elevated reactive oxygen species (ROS),[11] which are associated with DNA damage of the spermatozoa.[12] Sperm DNA damage is associated with adverse reproductive outcomes.[13-15] However, there are also conflicting data on the significance of leucocytospermia, with some studies reporting an association between bacteriospermia and leucocytospermia,[16,17] whilst others have found no such association.[18-20]

The human microbiome is composed of the genetic material of the microbial community (eg bacteria, fungi and viruses) and is more complex than the human genome. The advent of next-generation sequencing (NGS), which uses the 16s ribosomal RNA region of the bacterial genome to identify bacteria,[21,22] has enabled more accurate characterization of the human microbiome, and large-scale microbial genome sequences can now be analysed. Previously undetectable pathogens have been discovered using this novel technique.[23] The human microbiome project[24] has characterized the microbiome of the airway, skin, oral cavity, gut and vagina. Metagenomic research has increased our understanding of the microbiome and how dysbiosis plays a role in conditions, such as mental health disorders and cancers.[25-27] Research on the vaginal microbiome has identified over 100 bacterial species, and its impact on pregnancy, premature birth, infertility and gynaecological cancer has been studied.[28-32] Further evidence suggests that male and female interactions may influence the composition of the microbiome,[33] and further research is needed to understand how this may impact on reproductive health and pregnancy, and whether novel therapies targeting the seminal microbiome improve outcomes.[34]

The objectives of this systematic review and meta-analysis were as follows:

1. To describe the species and communities present in semen
2. Assess the prevalence of bacteriospermia and the association with male infertility
3. Assess the association between bacterial species and semen quality
4. Provide a contemporary understanding of the seminal microbiome and its potential effects on reproductive health.

2 | EVIDENCE ACQUISITION

A systematic search was conducted in accordance with the Preferred Reporting Items for Systematic Reviews and Meta-Analysis (PRISMA) statement[35] (Table 1). The systematic review was registered with PROSPERO (ID Number CRD42019124483). The databases MEDLINE, OVID and PubMed were searched to identify studies related to the identification of bacteria in the semen of infertile men, between 1 January 1992 and 1 September 2019. This time frame was chosen to facilitate the identification of studies using the 3rd to 5th editions of the World Health Organization (WHO) laboratory manual for the examination and processing of human semen.[36] The following terms were used in the search: "sperm OR semen OR seminal," AND "microbiome OR microbiota OR bacteria OR microorganisms," AND "fertility OR fertile OR infertility OR infertile" AND "man OR men OR male OR males". Outcomes of interest were as follows: (a) prevalence of bacteriospermia, (b) genera/species of bacteria present in semen, (c) clustering of the microbiome, and (d) impact of bacteriospermia on semen parameters and fertility.

This systematic review and meta-analysis aimed to evaluate the bacterial composition of semen and compare this between men who are infertile with those who are fertile, and to assess the association of bacteriospermia with semen parameters in both healthy and infertile men. Therefore, the inclusion criteria for this review were as follows:

1. The specimen analysed was semen obtained by masturbation.

TABLE 1 PRISMA checklist

Section/topic	#	Checklist item	Reported on page #
TITLE			
Title	1	Identify the report as a systematic review, meta-analysis, or both.	1
ABSTRACT			
Structured summary	2	Provide a structured summary including, as applicable: background; objectives; data sources; study eligibility criteria, participants, and interventions; study appraisal and synthesis methods; results; limitations; conclusions and implications of key findings; systematic review registration number.	2-3
INTRODUCTION			
Rationale	3	Describe the rationale for the review in the context of what is already known.	4
Objectives	4	Provide an explicit statement of questions being addressed with reference to participants, interventions, comparisons, outcomes, and study design (PICOS).	5
METHODS			
Protocol and registration	5	Indicate if a review protocol exists, if and where it can be accessed (eg, Web address), and, if available, provide registration information including registration number.	5
Eligibility criteria	6	Specify study characteristics (eg, PICOS, length of follow-up) and report characteristics (eg, years considered, language, publication status) used as criteria for eligibility, giving rationale.	5
Information sources	7	Describe all information sources (eg, databases with dates of coverage, contact with study authors to identify additional studies) in the search and date last searched.	5
Search	8	Present full electronic search strategy for at least one database, including any limits used, such that it could be repeated.	5
Study selection	9	State the process for selecting studies (ie, screening, eligibility, included in systematic review, and, if applicable, included in the meta-analysis).	5-6
Data collection process	10	Describe method of data extraction from reports (eg, piloted forms, independently, in duplicate) and any processes for obtaining and confirming data from investigators.	5-6
Data items	11	List and define all variables for which data were sought (eg, PICOS, funding sources) and any assumptions and simplifications made.	5-6
Risk of bias in individual studies	12	Describe methods used for assessing risk of bias of individual studies (including specification of whether this was done at the study or outcome level), and how this information is to be used in any data synthesis.	6
Summary measures	13	State the principal summary measures (eg, risk ratio, difference in means).	6
Synthesis of results	14	Describe the methods of handling data and combining results of studies, if done, including measures of consistency (eg, I^2) for each meta-analysis.	6
Risk of bias across studies	15	Specify any assessment of risk of bias that may affect the cumulative evidence (eg, publication bias, selective reporting within studies).	Supplementary
Additional analyses	16	Describe methods of additional analyses (eg, sensitivity or subgroup analyses, meta-regression), if done, indicating which were pre-specified.	na
RESULTS			
Study selection	17	Give numbers of studies screened, assessed for eligibility, and included in the review, with reasons for exclusions at each stage, ideally with a flow diagram.	Figure 1
Study characteristics	18	For each study, present characteristics for which data were extracted (eg, study size, PICOS, follow-up period) and provide the citations.	Tables 2-4
Risk of bias within studies	19	Present data on risk of bias of each study and, if available, any outcome level assessment (see item 12).	Table 5-6
Results of individual studies	20	For all outcomes considered (benefits or harms), present, for each study: (a) simple summary data for each intervention group (b) effect estimates and confidence intervals, ideally with a forest plot.	Tables 2-4

(Continues)

TABLE 1 (Continued)

Section/topic	#	Checklist item	Reported on page #
Synthesis of results	21	Present results of each meta-analysis done, including confidence intervals and measures of consistency.	Figures 4-24
Risk of bias across studies	22	Present results of any assessment of risk of bias across studies (see Item 15).	Table 5-6
Additional analysis	23	Give results of additional analyses, if done (eg, sensitivity or subgroup analyses, meta-regression [see Item 16]).	na
DISCUSSION			
Summary of evidence	24	Summarize the main findings including the strength of evidence for each main outcome; consider their relevance to key groups (eg, healthcare providers, users, and policy makers).	13
Limitations	25	Discuss limitations at study and outcome level (eg, risk of bias), and at review-level (eg, incomplete retrieval of identified research, reporting bias).	12
Conclusions	26	Provide a general interpretation of the results in the context of other evidence, and implications for future research.	13-14
FUNDING			
Funding	27	Describe sources of funding for the systematic review and other support (eg, supply of data); role of funders for the systematic review.	16

Notes: From: Moher D, Liberati A, Tetzlaff J, Altman DG, The PRISMA Group (2009). Preferred Reporting Items for Systematic Reviews and Meta-Analyses: The PRISMA Statement. PLoS Med 6(7): e1000097. https://doi.org/10.1371/journal.pmed1000097 For more information, visit: www.prisma-statement.org.

2. The bacterial composition was assessed by either culture, PCR or NGS.
3. The semen analysis was undertaken as per the WHO criteria.
4. In studies investigating the association of bacteriospermia with fertility, the male population were diagnosed with infertility.

The exclusion criteria were as follows:

1. Evidence of male urogenital infection such as male accessory gland infections (MAGI) or bacterial prostatitis.
2. Evidence of other causes of male infertility, for example drug use, hypogonadism.
3. Specimens other than semen used for assessment of microbiome composition (eg urine, urethral culture).
4. Studies reporting only on bacteriospermia without analysing the impact on semen parameters or the association with male fertility.

Observational studies (cross-sectional and case-control) were included as higher levels of evidence were not available in the literature. Studies reporting duplicates, non-English language, animal or in vitro studies, reviews, letters and non–full-text articles were excluded. Studies that reported on bacteriospermia in semen without referencing the association with fertility or the impact on semen parameters were excluded.

A tool developed by the National Institute of Health (NIH) was used to assess the risk of bias and the methodological quality of the studies, in order to ascertain whether they should be included in the systematic review. The NIH quality assessment tool was used as it can be specifically applied to observational studies.[37] Study titles and abstracts were screened before full-text review was completed in duplicate by two study investigators independently (LF and TT). Discrepancies were resolved after resolution with a third author (CJ).

Data were extracted using a pre-designed form: date of publication; country of investigation; number of participants; study design; patient group; detection methods used (eg culture, polymerase chain reaction (PCR), NGS); prevalence of bacteriospermia; types of bacterial genera or species identified; and semen parameters affected. Measures of associations between bacteriospermia and infertility/abnormal semen parameters were reported as one of the following: (a) differences in means, odds ratios (OR), relative risk (RR), chi-square as quoted in the original study; (b) OR and RR derived by LF from the raw data published in the original studies; and (c) not reported as data not available.

3 | EVIDENCE SYNTHESIS

A total of 55 studies fulfilled the criteria for inclusion to this systematic review, with a total of 51 299 subjects. 24 studies were included in the meta-analysis, with a total of 29 358 subjects. The PRISMA flow chart describes the cases excluded from this review (Figure 1). All the studies were observational, assessing the prevalence and impact of bacteriospermia in infertile men. Thirty-nine cross-sectional studies and 16 case-control studies were included (Tables 2-4). Risk of bias for each study was ascertained (Tables 5 and 6).

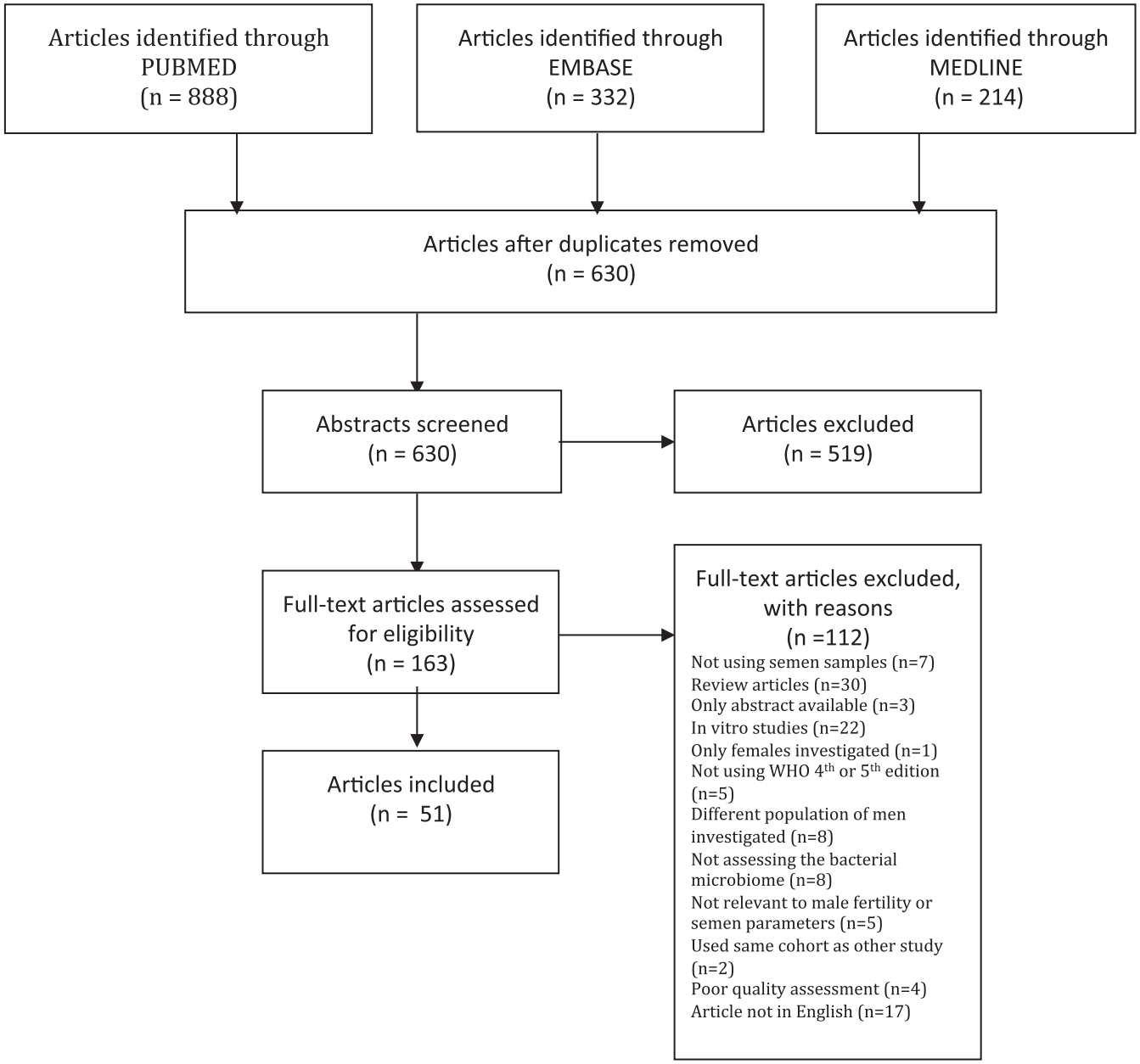


FIGURE 1 PRISMA flow chart

Extracted data were collated in Excel 2007 (Microsoft Corporation, Redmond, CA, USA), and analysis was performed using Stata v.12.0SE (College Station, TX, USA). Meta-analyses were performed to analyse the pooled data for the impact of bacteriospermia and specific bacterial species on the various semen parameters, as well as the difference in prevalence between fertile and infertile groups. The semen parameters that were studied are semen volume (mL), mean sperm concentration (million/mL), total motility (%), progressive motility (%), normal morphology (%) and DNA fragmentation index (%).

Meta-analysis of proportions was performed using the metaprop command in Stata.[38] A random-effects model was applied using the method of DerSimonian and Laird. Proportions were transformed with the Freeman-Tukey double inverse sine transformation, and confidence intervals (CIs) were calculated with the Score method. This form of transformation is preferred for meta-analysis as it stabilizes the variance of the estimates, and studies with zero or one effect size can be included.[38,39] Heterogeneity within and between subgroups was assessed with the I^2 statistic.[40] Significance was set at the .05 level.

Twenty-four studies included in this systematic review used methods to identify any and all bacterial species present in the semen, using differing techniques that included culture (n = 20) and NGS (n = 4). The majority of bacteria identified were members of four phyla: Firmicutes, Actinobacteria, Proteobacteria and Bacteroidetes. Firmicutes were represented most abundantly. There were also representations from other phyla, such as Tenericutes, Chlamydiae and Parcubacteria. The remaining

TABLE 2 Summary of studies that used culture methods to detect pathogens

Author	Nos	Study design	Findings
Vilvanathan[45] (India) 2016	85	Cross-sectional study IF Culture Specimens considered positive if organisms isolated in a concentration of > 10^3 colony-forming units (cfu)/mL	Prevalence of BS 35.3% *E faecalis* 11%, *Coagulase-negative staphylococcus* 8%, *S aureus* 7%, *E coli* 4%, *Klebsiella pneumonia* 2%, *Proteus* spp 2%, *Citrobacter* 1% BS and OS = RR 0.658, 95% CI 0.3708 to 1.1563, $P = .14$ BS and AS = RR 0.9167, 95% CI 0.4450 to 1.8885, $P = .8135$ BS and TS = RR 0.8582, 95% CI 0.6739 to 1.0929, $P = .2149$ No significant association between abnormal semen parameters and BS ($P > .05$) No significant association between individual bacterial species and semen parameters ($P > .05$)
Fraczek[18] (Poland) 2016	101	Cross-sectional study Normozoospermic men Culture Specimens considered positive if organisms isolated in a concentration of > 10^4 colony-forming units (cfu)/mL	Prevalence of BS 51% 27 bacterial species identified *Coagulase-negative staphylococcus* 22.9% *Streptococcus* spp 18.3% *Enterococcus* spp 13.8% *Mycoplasma* spp 4.6% BS significantly associated with reduced sperm count ($P < .01$), reduced normal forms ($P < .05$), higher numbers of dead cells ($P < .05$), increased DFI in both live and dead spermatozoa ($P < .05$, $P < .01$), and with lower mitochondrial membrane potential ($P < .01$)
Zeyad[16] (Germany) 2018	120	Cross-sectional study IF Culture Specimens considered positive if organisms isolated in a concentration of > 10^3 colony-forming units (cfu)/mL	Prevalence of BS 30% *Staphylococcus* spp 15%, *Escherichia* spp 5%, *Streptococcus* spp 4%, *Enterococcus* spp 4%, *Klebsiella* spp 1.66% The most prevalent bacterial species identified was *S aureus* 6.66% Sperm concentration, motility and PM significantly negatively impacted by BS ($P = .000$) Sperm protamine deficiency was significantly higher in infected patients ($P = .000$) Morphology and DFI did not differ significantly in BS patients
Zeyad[46] (Germany) 2018	84	Cross-sectional study IF Culture Specimens considered positive if organisms isolated in a concentration of > 10^3 colony-forming units (cfu)/mL	Prevalence of BS 34.5% (n = 29) *S aureus* 9% *S epidermis* 6% *S haemolyticus* 5% *E coli* 7% *E faecalis* 5% *S agalactiae* 2% Sperm concentration and progressive motility were significantly lower ($P < .001$), and sperm protamine deficiency was significantly higher ($P < .1$) in patients with BS ($P < .001$) There was no significant difference in DFI ($P = .72$)
Mashaly[44] (Egypt) 2016	60	Cross-sectional study IF Culture	Prevalence of BS 33.3% *Corynebacteria* 18%, *S*. aureus 12%, *Alpha-haemolytic streptococcus*, *E coli* BS significantly negatively impacted sperm concentration ($P = .0006$), motility ($P = .0004$) and morphology ($P = .0003$) Corynebacteria associated with a significantly lower sperm motility ($P < .05$) Seminal pus cells were significantly negatively correlated with motility (r=−0.340, $P = .008$)
Ruggeri[48] (Italy) 2016	246	Cross-sectional study IF Culture	Prevalence 6% (a further 8% showed mixed flora) *E faecalis* 2.8%, *UU* 3.2%, *E Coli* 0.8% In 3.2% (n = 8) of cases, both of the partners had an infection. 2% (n = 5) had the same infective agent, whilst the remaining 1.2% (n = 3) had differing infective agents

(Continues)

TABLE 2 (Continued)

Author	Nos	Study design	Findings
Bussen[49] (Germany) 1997	8888	Cross-sectional IF Culture	Prevalence of BS 68% *S aureus* 9% *S epidermis* 33% *E coli* 8% *Enterobacter* spp. 7% Group B streptococcus 8% Corynebacteria 8%
Nasrallah[20] (Egypt) 2018	200	Cross-sectional study IF Culture The bacterial concentration of greater than 10^3 CFU/mL for certain pathogens and greater than 10^4 for occasional pathogens was considered as significant	Prevalence of BS 54% Isolated microorganisms • Certain pathogens (24.1%). *E coli*, *Klebsiella* spp, *Pseudomonas* • Occasional pathogens (59.3%). *S aureus*, *Enterococcus* spp, *Streptococcus* spp • Sexually transmitted diseases (9.3%). *Neisseria* • Skin commensals possible pathogens (7.4%). *S epidermis*, *Alpha-haemolytic streptococcus* No significant difference in semen parameters in the presence of BS No statistically significant differences in semen parameters in association with type of bacterial species No significant difference in leucocyte concentration between the BS and non-bacteriospermic group ($P = .147$)
Aghazarian[59] (Iran) 2013	171	Cross-sectional study IF Culture	Prevalence 36.2% *UU and Gardenerella* 25.8%, *UU* 19.4% *G vaginalis* 16.1%, *E coli* 12.9%, *E faecalis* 9.7% No significant differences in any semen parameters in BS patients ($P > .05$) No significant correlation between BS and leucocytospermia
Eggert-Kruse[50] (Germany) 1995	126	Cross-sectional study IF Culture	Prevalence of bacterial species *Staphylococcus epidermis* 63.5% *Enterococcus* spp. 19.8% Non-haemolytic streptococcus 45% Alpha-haemolytic streptococcus 36% Anaerobes 11.9% *Gardnerella vaginalis* 3% *Actinomyces* spp. 1.6% *Veillonella* spp. 28% Gram-negative anaerobes 5.6% *Lactobacillus* spp. 21% *Peptococcus* spp. 38% *Peptostreptococcus* spp. 33% *Bacteroides* spp. 8% *Propionibacteria* spp 7% *Fusobacteria* spp 3%
Virecoulon[54] (France) 2005	600	Cross-sectional study IF Culture Specimens considered positive if organisms isolated in a concentration of $> 10^3$ colony-forming units (cfu)/mL	Prevalence of BS 30.5% (n = 183) Coagulase-negative staphylococcus 3.5% (n = 21) *Enterococcus* spp 1.5% (n = 9) UU 15.5% (n = 90) *Mycoplasma hominis* 2.5% (n = 15) *Escherichia coli* 1.3% (n = 8) *Proteus mirabilis* 1.5% (n = 9) *Enterobacter* spp 2.8% (n = 17) Non-haemolytic streptococcus 1.2% (n = 7) *Streptococcus* spp 8.3% (n = 50) *Streptococcus aginosus* 3.2% (n = 19) *Gardnerella vaginalis* 5.8% (n = 35) *Corynebacteria* spp 1.3% (n = 8) *Lactobacillus* spp 0.2% (n = 1)

(Continues)

TABLE 2 (Continued)

Author	Nos	Study design	Findings
Gdoura[55] (Tunisia) 2008	116	Cross-sectional study IF Culture and PCR	Prevalence of BS 56.9% (n = 66) Culture *E faecalis* 0.9% (n = 1) *E coli* 1.7% (n = 2) *S agalactiae* 0.9% (n = 1) *G vaginalis* 0.9% (n = 1) PCR CT 41.4% (n = 48) UU 15.5% (n = 18) UP 4.3% (n = 5) MH 10.3% (n = 12) MG 5.2% (n = 6)
Moretti[56] (Italy) 2009	1085	Cross-sectional study of men attending fertility clinic Case-control study comparing those with infection (n = 246) with controls (fertile men without infections n = 20) Culture	Prevalence of BS 22.6% (n = 246) Staph epidermis 2.2% (n = 24) *E faecalis* 7.3% (n = 79) UU 2.7% (n = 29) *E coli* 4.6% (n = 50) *S agalactiae* 3% (n = 33) *S aginosus* 2.1% (n = 23) *Morganella morganii* 0.7% (n = 8) UU—significant decrease in motility ($P = .0001$) *E faecalis*—significant decrease in concentration ($P = .0001$) and motility ($P = .0002$) *E coli*—significant decrease in concentration ($P = .0001$) and motility ($P = .0005$)
Isaiah[57] (Nigeria) 2011	140	Cross-sectional study IF Culture	Prevalence of BS 65.7% (n = 92) *Staph aureus* 18.6% (n = 26) *S saprophyticus* 8.6% (n = 12) *E coli* 12.9% (n = 18) *Proteus mirabilis* 7.1% (n = 10) *Proteus vulgaris* 7.1% (n = 10) *Klebsiella* spp. 7.1% (n = 10) *Pseudomonas* spp 4.3% (n = 6)
Domes[58] (Canada) 2012	4593	Cross-sectional study IF Culture	Prevalence of BS 15% (n = 1200) *S aureus* 0.8% (n = 60) *E faecalis* 8.6% (n = 672) *E coli* 2.5% (n = 192) *Proteus mirabilis* 0.3% (n = 20) Group B streptococcus 2% (n = 156) *Klebsiella* spp 0.3% (n = 24) *Citrobacter* spp 0.2% (n = 18) *Morganella morganii* 0.2% (n = 16) No significant differences in sperm concentration, motility or morphology in the presence of BS or with any specific bacterial species DFI significantly higher in bateriospermic samples ($P = .04$)
Ricci[47] (Italy) 2018	285	Cross-sectional study IF Culture	Prevalence of BS 29.1% (n = 83) *E faecalis* 11.5% (n = 33) *S agalactiae* 4.6% (n = 13) *E coli* 6.7% (n = 19) MH 1% (n = 3) UU 2% (n = 6) *S aureus* 0.7% (n = 2) *S haemolyticus* 2% (n = 6) *P aeruginosa* 0.3% (n = 1) BS associated with a significant decrease in total motility ($P = .012$) and progressive motility ($P = .0098$) Only *E faecalis* associated with a difference in semen parameters. Associated with a significant decrease in total motility ($P < .01$), progressive motility ($P < .01$) and morphology ($P < .05$)

(Continues)

TABLE 2 (Continued)

Author	Nos	Study design	Findings
Sellami[60] (Tunisia) 2014	85	Cross-sectional study IF Culture and PCR	Prevalence of BS 7% (n = 6) Culture *S aureus* 1.1% (n = 1) *Enterococcus* spp. 1.1% (n = 1) Group B streptococcus 3.5% (n = 3) Corynebacteria 1.1% (n = 1) PCR CT 15.2% (n = 15) UU 5.8% (n = 5) UP 1.1% (n = 1) MH 1.1% (n = 1) MG 3.5% (n = 3) CT associated with significant reduction in rapid progressive motility (P = .04), but not other semen parameters
Kjaergaaed[51] (Denmark) 1997	**201**	Cross-sectional study IF Culture and PCR	Prevalence of bacteriospermia 57% Culture *S aureus* 0.5% Coagulase-negative staphylococcus 5*% *Enterococcus* spp. 10% *E coli* 3.5% *Enterobacter* spp. 3% Non-haemolytic streptococcus 50% Group B streptococcus 3% Corynebacteria 58% *Lactobacillus* spp. 3% PCR CT 4.5% UU 32% MG 2.5%
Levy[52] (France) 1999	**92**	Cross-sectional study IF Culture and PCR	Culture UU 13% PCR CT 11%
Shalika[53] (USA) 1996	**342**	Cross-sectional study IF Culture	Prevalence of bacteriospermia 32% *S aureus* 3% *Enterococcus* spp. 23% *Ureaplasma* spp. 11% *E coli* 3% *Proteus mirabilis* 0.5% *Streptococcus* spp. 2%

Abbreviations: AS, asthenospermia; BS, bacteriospermia; CIs, confidence intervals; DFI, DNA fragmentation index; F, fertile men;IF, infertile men; IL-17, interleukin 17; IL-18, interleukin 18; NO, nitric oxide; NOS, non-oligospermia; OR, odds ratio; OS, oligospermia; ROS, reactive oxygen species. CT, Chlamydia trachomatis. UU, Ureaplasma urealyticum. UP, Ureaplasma parvum. MH, Mycoplasma hominis. MG, Mycoplasma genitalium; RR, relative risk; TS, teratospermia.

studies (n = 35) used PCR or culture to identify pre-specified pathogens.

3.1 | Types of bacteria

Four studies used NGS to identify and quantify the bacterial species present in the semen. Two of the studies used the V1-V2 region of the gene,[41,42] one used V4,[43] and the fourth used the V3-V6 region.[17] The genera identified consisted of aerobic, facultative anaerobic and strictly anaerobic bacteria, and many types of species that are considered to be opportunistic pathogens. Three studies[41-43] were in agreement that there was clustering of the semen microbiome, with a predominant species. All three studies reported that *Lactobacillus* was the dominant species in one cluster, and *Prevotella* was the dominant species in another. These three studies reported that the most abundant genera were *Ralstonia*, *Lactobacillus*, *Prevotella*, *Corynebacterium*, *Pseudomonas*, *Streptococcus*, *Staphylococcus*, *Ureaplasma*, *Clostridiales*, *Atopobium*, *Anaerococcus*, *Gardnerella*, *Rhodanobacter*, *Finegoldia*, *Haemophilus*, *Planococcaceae and Burkholderia*. Conversely, Monteiro et al[17] did not identify clustering of species, and this study reported that the most abundant genera were *Enterococcus* (>21.8%), *Staphylococcus* (>5.8%) and

TABLE 3 Summary of studies that employed next-generation sequencing techniques

Author	Nos	Study design	Findings
Hou[42] (China) 2013	77	Case-control study 2 arms • IF men (58) • Healthy donors (19)NGS V1-V2 regions of the 16S rRNA genes using 'universal primers'	Clusters of six SM communities No significant differences in SM between IF men and healthy donors ($P > .477$) *Anaerococcus* associated negatively with sperm quality ($P = .0012$)
Baud[41] (Switzerland) 2019	94	Cross-sectional IF men NGS V1-V2 region of the 16S rRNA gene using primers	Clusters of three SM communities Prevotella, Lactobacillus and Polymicrobial No significant differences in SM between normal and abnormal semen parameter groups Bacterial load highest in *Prevotella* group compared with the other two groups ($P < .005$ and $P < .001$) *Prevotella* associated with abnormal sperm parameters *Staphylococcus* and *Lactobacillus* associated with normal sperm parameters
Weng[43] (Taiwan) 2014	96	Cross-sectional IF men NGS V4 region of the 16S rRNA gene using primers	Clusters of three SM communities *Pseudomonas*, *Lactobacillus* and *Prevotella* Significantly higher proportion of abnormal samples in the Pseudomonas and Prevotella groups compared with Lactobacillus group ($P = .009$ and 0.008) The *Lactobacillus* group is most frequent The majority of normal samples (80.6%) were found in the Lactobacillus group In the *Pseudomonas* group, there was a higher proportion of normal samples in the participants who also had an abundance of *Lactobacillus* ($P = .041$) The proportion of *Lactobacillus* ($P < .05$) and *Gardnerella* ($P < .05$) was significantly abundant in the normal samples The proportion of *Prevotella* was significantly abundant in the abnormal samples
Monteiro[17] (Portugal) 2018	118	Case-control study 2 arms IF men Healthy controls NGS V3-V6 region of the 16S rRNA gene using primers The samples were merged into four pools and analysed C, OAT, AT, H	Most common bacteria *Enterococcus* (>21.8%) *Staphylococcus* (>5.8%) *Anaerococcus* (1.2%-8.7%) *Peptoniphilus* (2.4%-10.6%) Higher prevalence of bacterial pathogens in H and OAT *Aerococcus* and *Pseudomonas* increased incidence in H and OAT The prevalence of STD agents was below the threshold of < 0.1, except for H which had Neisseria 0.2% *Lactobacillus* found in low abundance in all pools Highest in C, 0.6% Lowest in H, >0.1%

Abbreviations: AT, asthenospermia; C, controls; H, hyperviscosity without a teratozoospermia phenotype; IF, infertility/infertile; NGS, next-generation sequencing; OAT, oligoasthenospermia; rRNA, ribosomal RNA; SM, semen microbiome.

Anaerococcus (1.2%-8.7%), with a low abundance of *Lactobacillus*, *Chlamydia* and *Ureaplasma*.

Twenty studies used culture-based methods to isolate all the bacteria present in the specimens.[16,18,20,44-60] The prevalence of bacteriospermia in infertile men was reported as ranging from 6% to 68%. The most commonly isolated bacterial genera using culture methods were *Escherichia* spp (16/20, 0.8%-20%), *Staphylococcus* spp (16/20 studies, 0.8%-30%), *Streptococcus* spp (15/20, 0.9%-20%), and *Enterococcus* (16/20, 0.9%-13.8%) and Ureaplasma spp. (8/20, 1.7%-19.4%; Figure 2). The most commonly isolated species were Escherichia coli (14/20, 0.8%-20%), followed by Staphylococcus aureus (12/20, 0.7%-30%), Enterococcus faecalis (8/20, 0.9%-11.5%) and Ureaplasma urealyticum (5/20, 1.7%-5.5%; Figure 3).

Thirty-five studies used PCR or culture to isolate specific bacteria, namely *Chlamydia trachomatis*, *Ureaplasma* spp. and *Mycoplasma* spp.[9,19,51,52,55,60-89] The PCR technique identified *C trachomatis* in 15 studies with reported prevalence of between 0.3% and 43.3% in infertile men, which is in contrast to the culture technique where this species was not identified in any of the studies (0/20). The following species were also more commonly identified using PCR: Ureaplasma urealyticum (5%-63%),

TABLE 4 Summary of studies that employed techniques to identify pre-specified pathogens (culture and PCR)

Author	Nos	Study design	Findings
Lopez-Hurtado[70] (Mexico) 2018	116	Cross-sectional study IF PCR	Prevalence of CT 31.9% (n = 37) No significant difference in semen parameters between CT-positive and CT-negative individuals ($P > .05$) Significantly higher numbers of leucocytes and erythrocytes in CT-negative group ($P = .024$, $P = .041$, respectively) RR calculated for semen parameters and bacteriospermia exposure Only significant finding is that RR of lower semen volume is greater in bacteriospermic patients (RR: 2.847, 95% CI: 1.065-7.61)
Qian[72] (China) 2016	81	Case-control 3 arms • IF with UU (C) • IF without UU (B) • Controls (A)Culture	UU associated with a significant decrease in concentration ($P = .0009$), total motility ($P = .0005$), progressive motility ($P = .0001$) and morphology ($P = .004$) NO concentrations higher in group C than group B ($P < .05$). NO concentration correlated positively with IL-17 and IL-18 concentrations (r = 0.7303, r = 0.7076, $P < .01$)
Rybar[19] (Czech Republic) 2012	293	Cross-sectional study IF Culture and immunofluorescence	Prevalence *Mycoplasma* (8.9%) *Ureaplasma* (14%) *Chlamydia* (13%) *Mycoplasma*: Significantly associated with reduced sperm count ($P < .01$), reduced motility ($P < .05$), reduced normal forms ($P < .05$), increased defective chromatin condensation ($P < .05$) *Chlamydia:* Significantly associated with reduced sperm count ($P < .01$) *Ureaplasma* No significant differences noted No significant difference in sperm DFI in the presence of bacteria
Zhang[87] (China) 2014	369	Case-control 2 arms • IF (223) • F (146)Culture and real-time PCR	No difference in age between groups ($P = .552$) Prevalence of UU not statistically significantly different between IF and F (33.6% versus 24.7%, $P = .066$) Biovar II infection more prevalent in IF ($P = .036$) Infection with biovar II significantly associated with reduced sperm count ($P = .041$) and lower total motility ($P = .015$) ROS levels significantly higher in biovar II than in controls ($P = .001$) DFI significantly higher in biovar II groups than controls ($P = .014$)
Yang[73] (China) 2018	480	Cross-sectional study IF Culture and PCR Expanded multilocus sequencing (eMLST) to determine different strains	Median age did not differ significantly between the infected and non-infected groups Prevalence *Ureaplasma* spp 22% UP *16%* UU *5%* *Ureaplasma* was divided into seven subgroups RR of OS is 2.03 times greater in men with *Ureaplasma* than in non-infected men (RR 2.03, 95% CI 1.3316 to 3.1228, $P = .0010$) Three of the subgroups of *Ureaplasma* spp were significantly associated with OS($P < .05$) No significant differences between the seven subgroups (all positive for *Ureaplasma*) and the negative group in sperm concentration ($P > .05$)
Wang[75] (China) 2005	160	Cross-sectional study IF Culture	Prevalence of UU 49% (n = 79)
Wang[74] (China) 2006	**346**	Cross-sectional study Culture	Prevalence of UU 39.3% (n = 136) Sperm concentration significantly lower in UU-positive individuals ($P < .01$) No significant differences in progressive motility or morphology

(Continues)

TABLE 4 (Continued)

Author	Nos	Study design	Findings
Zhang[76] (China) 2011	1168	Case-control study IF 967 F 201 Culture	Prevalence of UU in IF 16.1% (n = 156) Percentage of normal forms significantly lower in UU-positive men ($P < .0001$)
Lee[77] (Korea) 2013	98	Case-control IF 50 F 48 Culture	Significantly higher prevalence of UU in IF ($P = .02$) No difference in prevalence of MH ($P = .32$) UU associated with a significant reduction in total ($P = .015$) and progressive motility ($P = .03$) MH associated with a significant reduction in concentration ($P = .01$), total motility (0.001) and progressive motility (0.001)
Liu[86] (China) 2014	1236	Case-control IF 621 F 615 Culture	No difference in prevalence of BS between IF and F No difference in prevalence of CT, UU or MH UU associated with a significant reduction in concentration ($P = .03$) MH not associated with a significant difference in routine semen parameters CT not associated with a significant difference in routine semen parameters
Huang[78] (China) 2016	22 466	Case-control study IF 19 098 F 3368 Culture	Significant difference in prevalence of UU and MH between IF and F No significant differences in routine semen parameters in the presence of MH UU associated with a significant reduction in progressive motility ($P < .05$) and normal forms ($P < .001$)
Zhou[81] (China) 2018	800	Case-control study IF 540 F 260 Culture and PCR	There was a significant difference in the prevalence of Ureaplasma spp. between IF and F ($P < .001$)
Zhou[88] (China) 2018	5428	Case-control study IF 5016 F 412 Culture	The prevalence of Ureaplasma spp. was significantly higher in IF than F ($P < .05$)
Esmailkhani[79] (Iran) 2018	100	Cross-sectional study IF Culture and PCR	Prevalence of *S aureus* 16% (n = 16)
Gdoura[89] (Tunisia) 2001	92	Cross-sectional study of infertile men PCR	Prevalence of CT 16.3% (n = 15) CT associated with a decrease in motility
Hosseinzadeh[61] (UK) 2004	642	Cross-sectional study IF PCR	Prevalence of CT 4.9% (n = 31) CT associated with a significantly higher semen volume ($P = .042$) CT associated with a significantly higher concentration of leucocytes ($P = .0285$) No differences in any other routine semen parameter
Bezold[62] (USA) 2007	241	Cross-sectional study IF PCR	Prevalence of CT 2.5% (n = 6) Routine semen parameters not impacted by the presence of CT
Zeighami[63] (Iran) 2007	200	Case-control study IF 100 F 100 PCR	Higher prevalence of UU in IF men ($P = .03$) UU associated with significant decrease in sperm concentration ($P = .02$)
Gdoura[64] (Tunisia) 2007	120	Cross-sectional study IF PCR	Prevalence Ureaplasma spp. 19% (n = 23) UU 15% (n = 18) UP 4.2% (n = 5) MH 10.8% (n = 13) MG 5% (n = 6) MH associated with a significant decline in semen concentration ($P = .007$) and morphology ($P = .03$) UU, UP and MG did not impact semen parameters significantly

(Continues)

TABLE 4 (Continued)

Author	Nos	Study design	Findings
Peerayeh[65] (Iran) 2008	146	Case-control study IF + varicocoele 81 IF + no varicocoele 65 Controls 100 PCR	Prevalence of UU IF (no V) 9% (n = 6) Prevalence of UU controls 3% (n = 3) UU associated with a significant decline in concentration ($P = .0001$), total motility ($P = .0001$) and morphology ($P = .0001$) semen
Zeighami[66] (Iran) 2009	200	Case-control study IF 100 F 100 PCR	Prevalence: UU 9/100, 9% (INF) 1/100 1% (F) UP 3/100 3%, 2/100 2% Ureaplasma spp. associated with a significant decline in concentration
Kokab[67] (Iran) 2010	255	Cross-sectional study IF PCR	Prevalence of CT 6% (n = 16)
Al-Sweih[68] (Kuwait) 2012	315	Case-control IF 127 F 188 PCR	No significant difference in BS between IF and F men (38.6% versus 48.9%, $P = .0832$) No significant difference in prevalence of CT, UU or MH No significant differences in routine semen parameters in the presence of CT, UU, MH or MG
Ruan[69] (China) 2017	15	Cross-sectional study IF PCR	Prevalence of Ureaplasma spp. 66.6% (n = 10) Prevalence of UU 33.3% (n = 5) Prevalence of UP 40% (n = 6)
Domes[80] (Canada) 2012	5588	Cross-sectional study IF PCR	Prevalence of CT 0.3% (n = 17) Prevalence of NG 0.05% (n = 3)
Mohseni Moghadam[71] (Iran) 2014	100	Cross-sectional study IF PCR	Prevalence of Mycoplasma (genus) 45% (n = 45) Prevalence of MG (species) 28.8% (n = 13) Presence of MG associated with low sperm concentration ($P = .001$), and motility ($P = .002$), and with greater abnormal sperm morphology ($P = .000$)
Ma[9] (China) 2017	49	Case-control IF 37 F 12 Culture	Significant difference in prevalence of UU between IF and F men (32.4% (n = 12 and 0% n = 0, respectively, $P = .02$) Presence of UU associated with lower sperm concentration ($P < .05$), progressive motility ($P < .05$) and with greater abnormal sperm morphology ($P < .05$)
Wang[82] (China) 2005	185	Case-control IF F Culture	Significantly higher prevalence of UU in infertile group (63% versus 39%, $P = .004$)
Gdoura[83] (Tunisia) 2008	104	Cross-sectional IF PCR	Prevalence of CT 43.3% (n = 45), UU 15% (n = 16), UP 3% (n = 3), MH 9.6% (n = 10) and MG 4.8% (n = 5) Routine semen parameters not impacted by the presence of CT, UU, MH or MG
Sellami[60] (Tunisia) 2014	85	Cross-sectional study of infertile men Culture and PCR	Prevalence of BS 7% (n = 6) Culture S. aureus 1.1% (n = 1) Enterococcus spp. 1.1% (n = 1) Group B streptococcus 3.5% (n = 3) Corynebacteria 1.1% (n = 1) PCR CT 15.2% (n = 15) UU 5.8% (n = 5) UP 1.1% (n = 1) MH 1.1% (n = 1) MG 3.5% (n = 3) CT associated with significant reduction in rapid progressive motility ($P = .04$), but not other semen parameters

(Continues)

TABLE 4 (Continued)

Author	Nos	Study design	Findings
Gdoura[55] (Tunisia) 2008	116	Cross-sectional study IF Culture and PCR	Prevalence of BS 56.9% (n = 66) Culture E. faecalis 0.9% (n = 1) E. coli 1.7% (n = 2) S. agalactiae 0.9% (n = 1) G. vaginalis 0.9% (n = 1) PCR CT 41.4% (n = 48) UU 15.5% (n = 18) UP 4.3% (n = 5) MH 10.3% (n = 12) MG 5.2% (n = 6)
Kjaergaaed[51] (Denmark) 1997	201	Cross-sectional study IF Culture and PCR	Prevalence of bacteriospermia 57% Culture S. aureus 0.5% Coagulase-negative staphylococcus 5*% Enterococcus spp. 10% E. coli 3.5% Enterobacter spp. 3% Non-haemolytic streptococcus 50% Group B streptococcus 3% Corynebacteria 58% Lactobacillus spp. 3% PCR CT 4.5% UU 32% MG 2.5%
Levy[52] (France) 1999	92	Cross-sectional study IF Culture and PCR	Culture UU 13% PCR CT 11%
Debata[84] (India) 1999	197	Cross-sectional study IF Culture	Culture UU 43% MH 17%
Ochsendorf[85] (Germany) 1999	125	Cross-sectional study IF PCR	CT 1.6%

Abbreviations: AS, asthenospermia; CI, confidence intervals; CT, *Chlamydia trachomatis;* DFI, DNA fragmentation index; F, fertile men; IF, infertile men; IL-17, interleukin 17; IL-18, interleukin 18; MG, *Mycoplasma genitalium;* MH, *Mycoplasma hominis;* NG, *Neisseria gonorrhoea;* NO, nitric oxide; NOS, non-oligospermia; OR, odds ratio; OS, oligospermia; PCR, polymerase chain reaction; ROS, reactive oxygen species. BS, bacteriospermia; RR, relative risk; UP, *Ureaplasma parvum;* UU, *Ureaplasma urealyticum*.

Ureaplasma parvum (1.1%-40%), *Mycoplasma hominis* (1.1%-17%) and *Mycoplasma genitalium* (2.5%-28.8%).

3.2 | Fertility

All four NGS studies reported a seminal microbiome that was rich and diverse in both infertile and fertile men. One case-control study[42] identified no significant difference in the semen microbiome between infertile men and healthy, fertile controls. Baud et al[41] found that there were no shifts in the composition of the microbiome community when comparing controls and infertile men with different sperm abnormalities.

Eleven studies compared the presence of *Ureaplasma urealyticum (UU)* between fertile and infertile men, and nine of these studies reported that the presence of this species was significantly higher in infertile men ($P < .05$).[9,63,65,66,68,75,77,78,86-88] These studies used a variety of diagnostic techniques, with five studies employing culture of semen, four studies using PCR, and the remaining two studies using both culture and PCR.

A meta-analysis was conducted on these studies, and the results show that there is an increased prevalence of UU in infertile men (OR: 2.25, 95% CI: 1.47 to 3.46: I^2: 82.96%, $P = .0$).

A sub-analysis found that the higher prevalence of UU in infertile men was reported whether the isolation was with PCR (OR: 2.122, 95% CI: 1.207 to 3.731, I^2: 62%) or culture (OR: 2.388, 95% CI: 1.266 to 4.504, I^2: 90.5%; Figures 4 and 5).

Four case-control studies identified *Mycoplasma hominis* (MH)[68,77,78,86] and one found the prevalence of MH was significantly

higher in infertile men,[78] whereas another found it was significantly higher in fertile men ($P = .02$).[68] A meta-analysis conducted on these four studies found that there was no significant difference in the prevalence of this species between fertile and infertile men (OR: 1.34, 95% CI: 0.51-3.5, I^2: 91.73%; Figure 6).

Two case-control studies identified *Chlamydia trachomatis* (CT)[68,86] and found no significant difference in the prevalence between infertile and fertile men (OR: 1.11, 95% CI: 0.601-2.064, I^2: 0%; Figure 7).

3.3 | Impact on semen parameters

All four NGS studies found an association between certain bacterial species and semen parameters. *Lactobacillus* was associated with improvements in sperm quality in two studies, and one study reported an abundance of *Lactobacillus* in samples with normal morphology.[42,43] Baud et al also reported an improvement in samples enriched with *Staphylococcus*.[41]

Two studies reported that *Lactobacillus* may have a protective effect on semen quality.[17,43] Monteiro et al found that there was a lower prevalence of *Lactobacillus* in the groups with oligoasthenoteratozoospermia and hyperviscosity phenotypes, and Weng et al found that in clusters where the predominant species was *Pseudomonas*, a negative effect was observed when the abundance of *Lactobacillus* was low.

Two studies reported that *Prevotella* appeared to exert a negative effect on sperm quality, as there was an increased abundance of this genus in specimens that had abnormal parameters. One study reported that *Anaerococcus* was negatively associated with semen quality ($P = .0012$).[42] Monteiro et al reported that oligoasthenospermia and hyperviscosity phenotypes were associated with a higher prevalence of *Pseudomonas* and *Klebsiella*.

Ten culture-based studies reported on the impact of bacteriospermia on semen parameters.[16,18,20,44-47,56,58,59] Six studies reported on the impact of bacteriospermia on mean sperm concentration, and five of these reported a significant negative impact on concentration. A meta-analysis was conducted on these six studies that provided continuous data (mean ± SD) which showed that there is a negative impact on mean sperm concentration in the presence of bacteriospermia (difference in means: −15.654, 95% CI: −22.653 to −8.656, I^2 95%; Figure 8).

Four studies reported on the impact of bacteriospermia on progressive motility,[16,20,46,47] and a meta-analysis of the pooled data found that there is a significant deterioration in percentage progressive motility in the presence of bacteriospermia (difference in means: −8.105, 95% CI: −13.568 to −2.642, I^2: 98%; Figure 9).

The majority of studies reported no significant impact on morphology (78%), and the meta-analysis of the pooled data concurred with this (difference in means: −1.722, 95% CI: −5.7 to 2.256, Q value: 0.97, $P = .614$, I^2 0.0%; Figure 10).

Four studies reported on the impact of bacteriospermia on DNA fragmentation. Two studies reported a significant increase in the DFI,[18,58] whilst two others reported an increase in DFI that was not statistically significant.[16,46] Two of these studies assessed DNA fragmentation using the same technique (TUNEL assay) and reported results enabling a pooled analysis, which noted a significantly higher DNA fragmentation index in the presence of bacteriospermia (difference in means: 3.518, 95% CI: 0.907 to 6.129, $P = .008$, I^2: 0.0%; Figure 11). Two studies found that there was a significant impact on protamine deficiency, reporting increased chromomycin levels (CMA3) in the presence of bacteria.[16,46] A pooled analysis found that there was a significant difference (difference in means: 21.268, 95% CI: 16.163 to 26.372, $P = .000$, I^2: 70.42%; Figure 12).

A number of different species of bacteria were assessed to investigate their impact on semen parameters. Thirteen studies investigated the impact of *Ureaplasma urealyticum*[9,56,63-65,68,72,75-78,83,86] and meta-analyses found that there was a significant deterioration in mean sperm concentration (difference in means: −13.851, 95% CI: −20.938 to −6.763, $P = .00$, I^2: 93%; Figure 13), and morphology (difference in means: −2.823, 95% CI: −3.546 to −2.10, $P = .00$, I^2: 97%; Figure 14), but no differences in motility (difference in means: 1.471, 95% CI: −5.911 to 8.853, $P = .696$; Figure 15).

Two studies performed further sub-analyses, with one reporting that UU biovar II was associated with reduced sperm count, reduced motility, increased ROS and increased DFI, whilst another reported that clonal variants of *Ureaplasma* were associated with oligozoospermia.

Seven studies investigated the impact of *Mycoplasma hominis*,[19,64,68,77,78,83,86] and meta-analyses found that there was a significant decrease in mean sperm concentration (difference in means: −24.497, 95% CI: −44.334 to −4.661, $P = .015$, I^2: 96%), percentage progressive motility (difference in means: −3.976, 95% CI: −7.208 to −0.745, $P = .016$, I^2: 78%) and morphology (difference in means: −3.745, 95% CI: −5.90 to −1.586, $P = .001$, I^2: 94%; Figures 16-18). Three studies investigated *Mycoplasma genitalium* and meta-analyses found a significant decrease in mean concentration (difference in means: −27.918, 95% CI: −33.280 to −22.555, $P = .00$, I^2: 95%), but no difference in progressive motility (difference in means: 5.110, 95% CI: −5.099 to 15.320, $P = .327$, I^2: 89%)[64,68,83] (Figures 19 and 20).

Nine studies investigated the impact of *Chlamydia trachomatis*,[19,60-62,68,70,83,86,89] and pooled analysis of these studies found that CT had no significant impact on mean sperm concentration, progressive motility or morphology (Figures 21-23). One study found no significant difference in DFI in the presence of CT.[60]

The impact of other bacterial species was also investigated in a number of other studies. Two studies found that there was a significant decrease in total motility in the presence of *Enterococcus faecalis*[47,56] with a pooled analysis concurring with these findings (difference in means: −11.034, 95% CI: −17.845 to −4.223, $P = .001$, I^2: 99%; Figure 24). One study found a significant decrease in motility with *Corynebacteria* spp.[44] The majority of studies that reported on it found that there were no differences in semen parameters with *S aureus*, or *E coli*.[45,47,58]

TABLE 5 Quality assessment of study methodology of cross-sectional studies[a]

	Rybar 2012	Yang	Lopez-Hurtado 2018	Vilvanathan 2016	Fraczek 2016	Zeyad 2018	Mashaly 2016	Ruggeri 2016	Nasrallah 2018
Was the research question or objective in this paper clearly stated?	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
Was the study population clearly specified and defined?	No	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
Was the participation rate of eligible persons at least 50%?	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
Were all the subjects selected from the same or similar populations?	No	Yes	Yes	No	Yes	Yes	No	No	Yes
Was a sample size justification or power description provided?	No	No	No	No	No	No	No	No	No
Were the exposures of interest measured prior to the outcome being measured?	No	No	No	No	No	No	No	No	No
Was the timeframe sufficient to see an association between exposure and outcome?	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
Did the study examine different levels of the exposure as related to the outcome?	na	na	na	na	na	na	na	na	na
Were the exposure measures clearly defined?	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
Was exposure assessed more than once over time?	No	No	No	No	No	No	No	No	No
Were the outcome measures clearly defined?	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
Were the outcome assessors blinded to the exposure status?	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
Was loss to follow-up after baseline < 20%?	na	na	na	na	na	na	na	na	na
Were key potential confounding variables measured and adjusted for?	No	Yes	No	No	Yes	No	No	No	No
Quality assessment	Fair	Good	Fair	Fair	Good	Fair	Fair	Fair	Fair

	Isaiah 2011	Domes 2012	Sellami 2014	Ricci 2018	Gdoura 2001	Hosseinzadeh 2013	Gdoura 2007	Gdoura 2008	Kokab 2010
Was the research question or objective in this paper clearly stated?	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
Was the study population clearly specified and defined?	No	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
Was the participation rate of eligible persons at least 50%?	Cd	Yes	Cd	Cd	Cd	Cd	Cd	cd	Yes
Were all the subjects selected from the same or similar populations?	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
Was a sample size justification or power description provided?	No	Yes	No	No	No	No	No	No	No
Were the exposures of interest measured prior to the outcome being measured?	No	No	No	No	No	No	No	No	No
Was the timeframe sufficient to see an association between exposure and outcome?	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
Did the study examine different levels of the exposure as related to the outcome?	na	No	No	No	No	No	No	No	Na
Were the exposure measures clearly defined?	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes

Aghazarian 2013	Esmailkhani 2018	Baud 2019	Weng 2014	Gdoura 2008	Moretti 2008	Eggert-Kruse 1995	Ochsendorf 1999	Shalika 1996	Zeyad 2018
Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
Yes	Yes	Yes	Yes	Yes	No	Yes	Yes	Yes	Yes
Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	cd	Yes
Yes	Yes	Yes	Yes	Yes	No	Yes	Yes	Yes	Yes
No	No	No	No	No	No	No	No	No	No
No	No	No	No	No	No	No	No	No	No
Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
na	na	na	na	Na	Na	Na	Na	Na	Na
Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
No	No	No	No	No	No	No	No	No	No
Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
na	na	na	na	na	Na	Na	Na	No	Na
Yes	No	No	Yes	No	No	No	No	No	No
Good	Fair	Fair	Good	Fair	Fair	Good	Fair	Fair	Fair

Ruan 2017	Domes 2011	Mohseni Moghadam 2014	Wang 2005	Wang 2006	Virecoulon 2005	Bussen 1997	Kjaergard 1997	Levy 2001	Debata 1999	Bezold 2007
Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
Yes	Yes	Yes	No	Yes	Yes	Yes	Yes	Yes	Yes	Yes
Cd	Yes	Cd	Cd	Cd	Yes	Cd	Cd	Cd	Cd	Cd
Yes	Yes	Yes	Cd	Yes	Yes	Yes	Yes	Yes	Yes	Yes
No	No	No	No	No	No	No	No	No	No	No
No	No	No	No	No	No	No	No	No	No	No
Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
na	Na	No	No	No	No	No	No	Na	Na	Na
Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes

(Continues)

TABLE 5 Continued

	Isaiah 2011	Domes 2012	Sellami 2014	Ricci 2018	Gdoura 2001	Hosseinzadeh 2013	Gdoura 2007	Gdoura 2008	Kokab 2010
Was exposure assessed more than once over time?	No	No	No	No	No	No	No	No	No
Were the outcome measures clearly defined?	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
Were the outcome assessors blinded to the exposure status?	Yes	No	No	No	No	No	Yes	Yes	Yes
Was loss to follow-up after baseline < 20%?	Na	Na	Na	Na	na	na	Na	Na	na
Were key potential confounding variables measured and adjusted for?	No	Yes	Cd	No	No	No	No	No	No
Quality assessment	Fair	Fair	Fair	Fair	Fair	Fair	Fair	Fair	Fair

Abbreviation: na, not applicable.

[a]Quality Assessment Tool for Observational Cohort and Cross-Sectional Studies. National Institutes of Health (NIH; National Institute of Health, 2018).

4 | STRENGTHS AND LIMITATIONS

We conducted a thorough systematic review and meta-analysis of the literature, using standardized tools for the assessment of study methodology.

When assessing the risk of bias, the majority of studies included were of fair quality; however, some were of poor quality. In view of the low quality of the studies, it was impossible to control for confounding variables, particularly between the fertile and infertile populations. The majority of the included studies clearly stated the research objectives, and whilst patient selection was generally acceptable, a few studies did not clearly report the inclusion criteria. Additionally, it could not be determined for the majority of studies whether the outcome assessors were blinded to the exposure status. There was a great deal of methodological variability between studies (eg different hypervariable regions (NGS), different culture media), which may explain the differences in the reported prevalence of organisms. As the aim of this systematic review was to investigate the association of bacterial species in semen with fertility and semen parameters, studies that used other specimens for investigation (eg urethral swabs) were not included, and therefore, a possible limitation is that not all species in the male genital tract were identified.

A meta-analysis was conducted on various outcome data, and it should be noted that within these groups there was significant heterogeneity, and therefore, the results should be interpreted with caution.

There are currently no published core outcome sets for male fertility, which limits meaningful comparison of the data.[90]

There are limited published data using NGS to characterize the seminal microbiome and its impact on fertility. Culture and PCR have their place in the clinical setting, but their inherent weakness is the inability to identify all bacterial species, leading to inaccuracies in the reported outcomes.

5 | DISCUSSION

This systematic review identified 55 studies that investigated the bacterial composition in the semen of infertile men, and the various isolation techniques included culture, PCR and NGS. There was a wide range in the reported prevalence of bacteriospermia (6%-68%), and significant differences in the composition of the microbiome between the studies. Semen is not sterile, and the microbiome is noted to be a rich and diverse community in both fertile and infertile men. Culture-based studies frequently observed *Staphylococcus*, *Enterococcus*, *Escherichia* and *Ureaplasma*, whereas NGS studies reported a high abundance of *Lactobacillus*, *Prevotella* and Pseudomonas, as well as other opportunistic and strictly anaerobic pathogens. PCR techniques identified *Chlamydia* and *Mycoplasma* species in greater abundance than the culture or NGS studies. These conflicting results highlight the difficulty in interpreting outcomes as species could be underrepresented due to the limitations of these detection methods. For example, culture-based studies detected fewer anaerobic bacteria than NGS. NGS has the ability to detect bacterial species that were not previously isolated (due to cultivation difficulties), and at very low levels of abundance, making this a superior detection method. But it should be noted that there is variation within the NGS studies. Of the four NGS studies, one reported significantly different outcomes, namely a high abundance of *Enterococcus* and a low abundance of *Lactobacillus*. These differences could be explained by the use of different hypervariable regions of the genome or primers in the methodology, or the different populations studied.[17]

Only a few case-control studies directly compared the microbiome of infertile and fertile men and did not find any major differences in the bacterial composition of semen between these two groups. However, a pooled analysis was conducted to determine the prevalence of individual species. A meta-analysis of UU studies found that *Ureaplasma* is more prevalent in infertile men and that it has a negative impact on concentration and morphology

Ruan 2017	Domes 2011	Mohseni Moghadam 2014	Wang 2005	Wang 2006	Virecoulon 2005	Bussen 1997	Kjaergard 1997	Levy 2001	Debata 1999	Bezold 2007
No	Yes	No	No	No	Yes	Yes	No	No	No	No
Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
Cd	Cd	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
Na	Na	na	Na	Na	Na	Na	Na	No	Na	Na
No	Yes	No	No	Yes	No	No	No	No	No	No
Fair	Good	Fair	Poor	Good	Good	Fair	Fair	Good	Fair	Fair

TABLE 6 Quality assessment of study methodology of case-control studies[a]

	Qian 2016	Zhang 2014	Hou 2013	Monteiro 2018	Zhang 2011	Lee 2013	Liu 2014	Huang 2015	Zhou 2018	Zhou 2018	Zeighami 2007	Peerayeh 2008	Zeighami 2009	Al-Sweih 2012	Ma 2016	Wang 2005
Was the research question or objective in this paper clearly stated?	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
Was the study population clearly specified and defined?	Yes	Yes	Yes	Yes	No	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	No
Was a sample size justification or power description provided?	No	No	No	No	No	No	No	No	No	No	No	No	No	No	No	No
Were the controls selected or recruited from a similar population?	Yes	Yes	Yes	Yes	No	Yes	Yes	Yes	Yes	Yes	Cd	Yes	Yes	Yes	Yes	cd
Were the definitions, inclusion and exclusion criteria, valid and reliable?	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Cd	Yes	Yes	Yes	Yes	cd
Were the cases clearly defined and differentiated from controls?	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	cd
Were the cases and/or controls randomly selected from those eligible?	cd	cd	cd	cd	Cd	Cd	Cd	Cd	Nr	Cd	Cd	Cd	Cd	Cd	Cd	cd
Was there use of concurrent controls?	nr	nr	nr	nr	Cd	Cd	Cd	Cd	Nr	Nr	Cd	Cd	Cd	Cd	Nr	cd
Were the investigators able to confirm that the exposure occurred prior to the development of the condition?	No	No	No	No	No	No	No	No	No	No	No	Yes	No	No	No	No
Were the exposure measures clearly defined?	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Cd	Yes	Yes	Yes	Yes	No

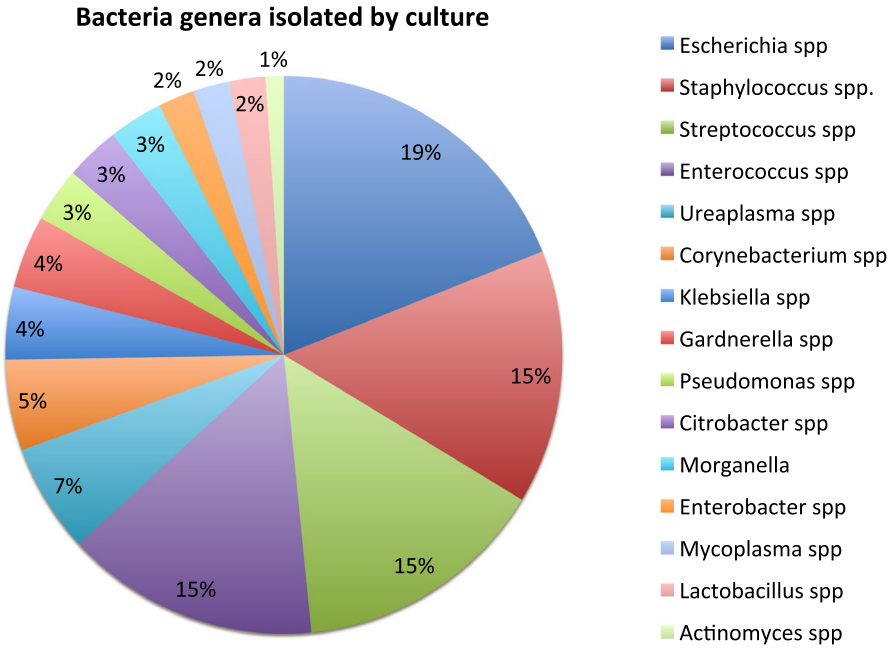


FIGURE 2 Bacteria genera identified by culture

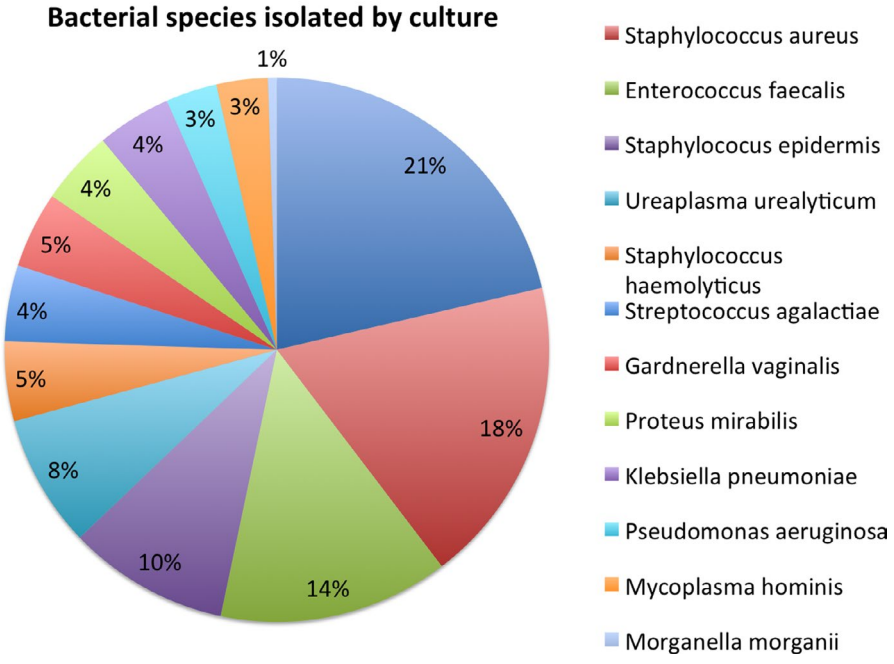


FIGURE 3 Bacterial species identified by culture

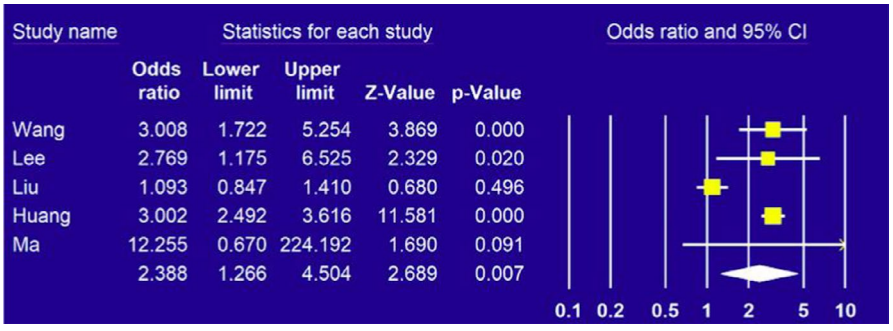


FIGURE 4 Forest plot. Ureaplasma urealyticum (culture) and fertility. Wang (n = 185), Lee (n = 98), Liu (n = 1236), Huang (n = 22 466), Ma (n = 49)

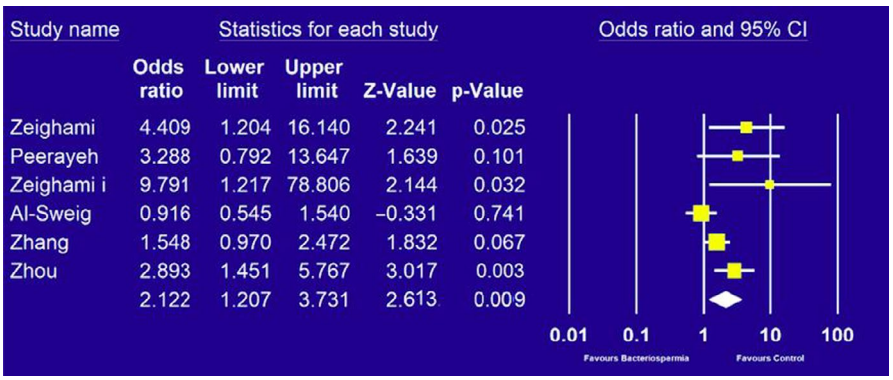


FIGURE 5 Forest plot. Ureaplasma urealyticum (PCR) and fertility. Zeighami (n = 200), Peerayeh (n = 165), Zeighami (i) (n = 200), Al-Sweih (n = 315), Zhang (n = 369), Zhou (n = 800)

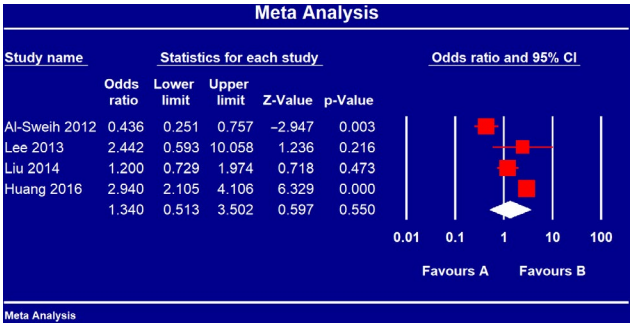


FIGURE 6 Forest plot. Mycoplasma hominis and fertility. Al-Sweih (n = 315), Lee (n = 98), Liu (n = 1236), Huang (n = 22 466)

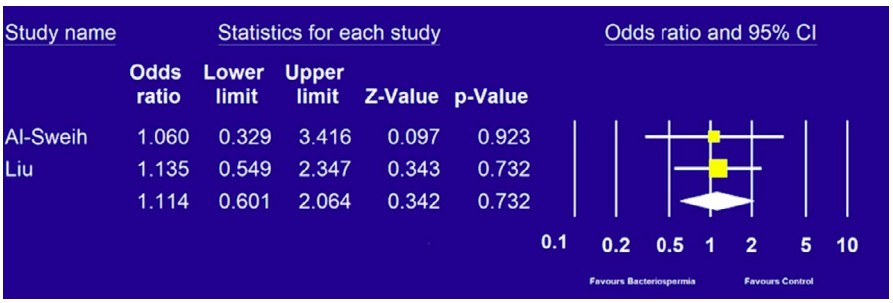


FIGURE 7 Forest plot. Chlamydia trachomatis and fertility. Al-Sweih (n = 315), Liu (n = 1236)

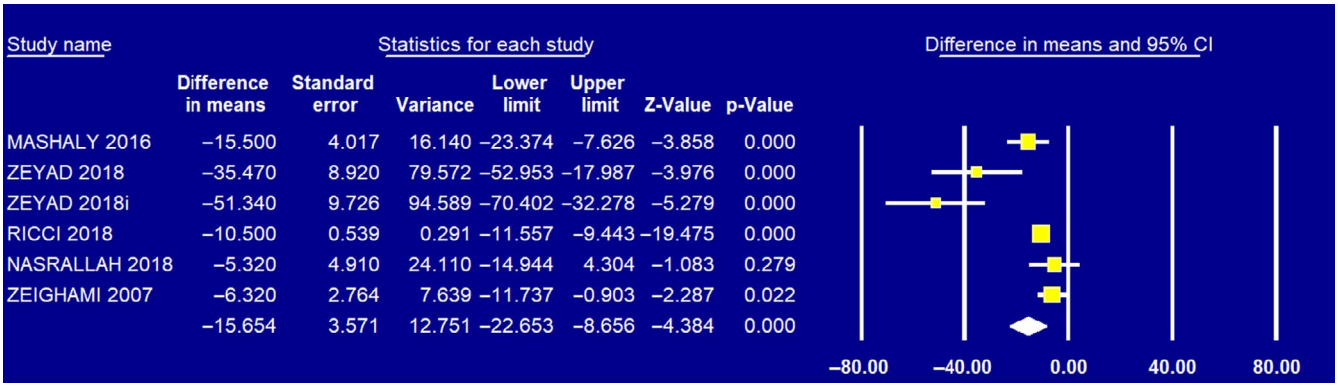


FIGURE 8 Forest plot. Bacteriospermia and mean sperm concentration. Mashaly (n = 60), Zeyad (n = 120), Zeyad (n = 84), Ricci (n = 285), Nasrallah (n = 200), Zeighami (n = 200)

parameters. Two studies investigated different variants of UU and found that only certain variants of this species had a significant impact on concentration, in addition to other parameters, which may explain why some men with UU are not reported to have negative outcomes. The impact of CT on male fertility is controversial with different studies reporting contradictory effects; however, this meta-analysis found no difference in the prevalence of CT between infertile and fertile men, and CT does not have any significant negative impact on semen parameters. The results of this meta-analysis report that there is a reduction in the mean concentration of

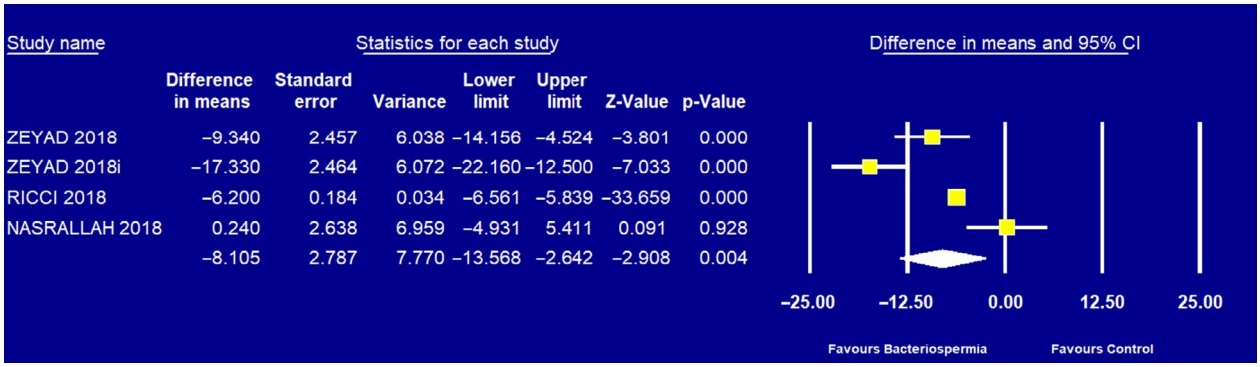


FIGURE 9 Forest plot. Bacteriospermia and progressive motility. Zeyad (n = 120), Zeyad (n = 84), Ricci (n = 285), Nasrallah (n = 200)

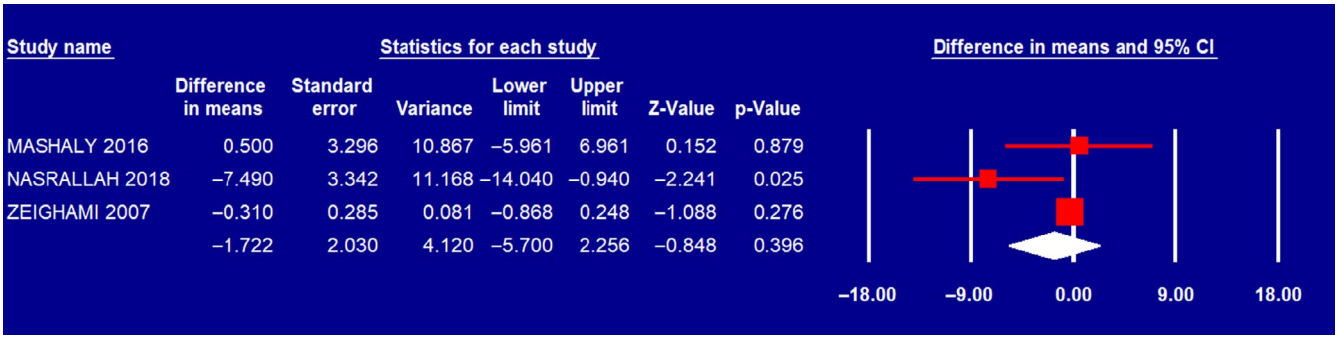


FIGURE 10 Forest plot. Bacteriospermia and morphology. Mashaly (n = 60), Nasrallah (n = 200), Zeighami (n = 200)

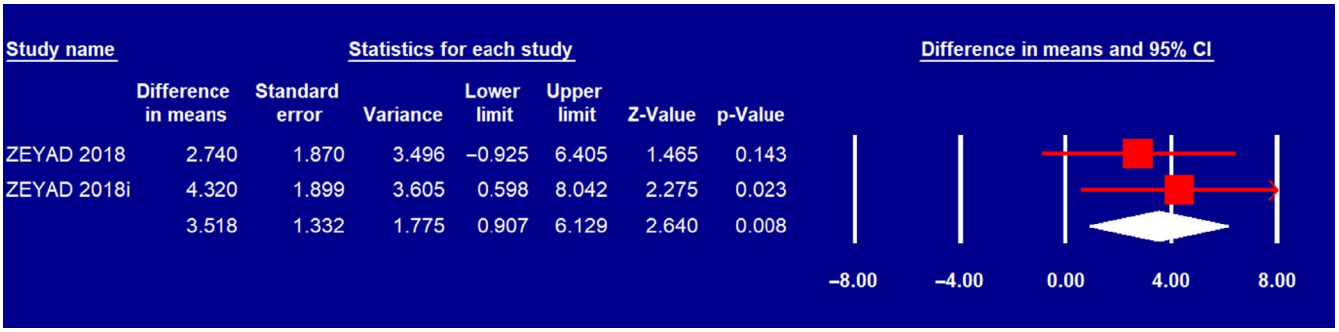


FIGURE 11 Forest plot. Bacteriospermia and DNA fragmentation index (DFI). Zeyad (n = 120), Zeyad (n = 84)

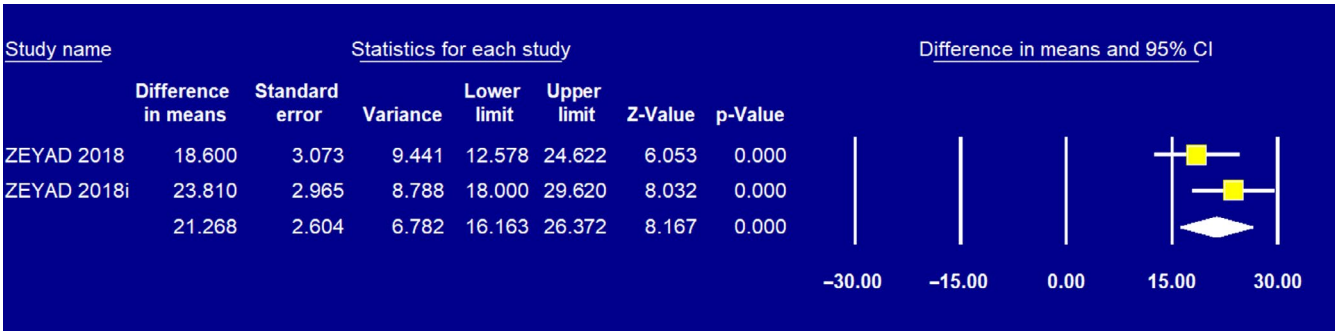


FIGURE 12 Forest plot. Bacteriospermia and protamine deficiency. Zeyad (n = 120), Zeyad (n = 84)

spermatozoa of 15 million/mL in the presence of bacteria, which was statistically significant. There was an association between certain bacterial species and reduced mean concentration (13 million/mL (UU), 25 million/mL (MH) and 27 million/mL (MG), which was statistically significant, and although may be clinically significant, this study is not able to make that determination.

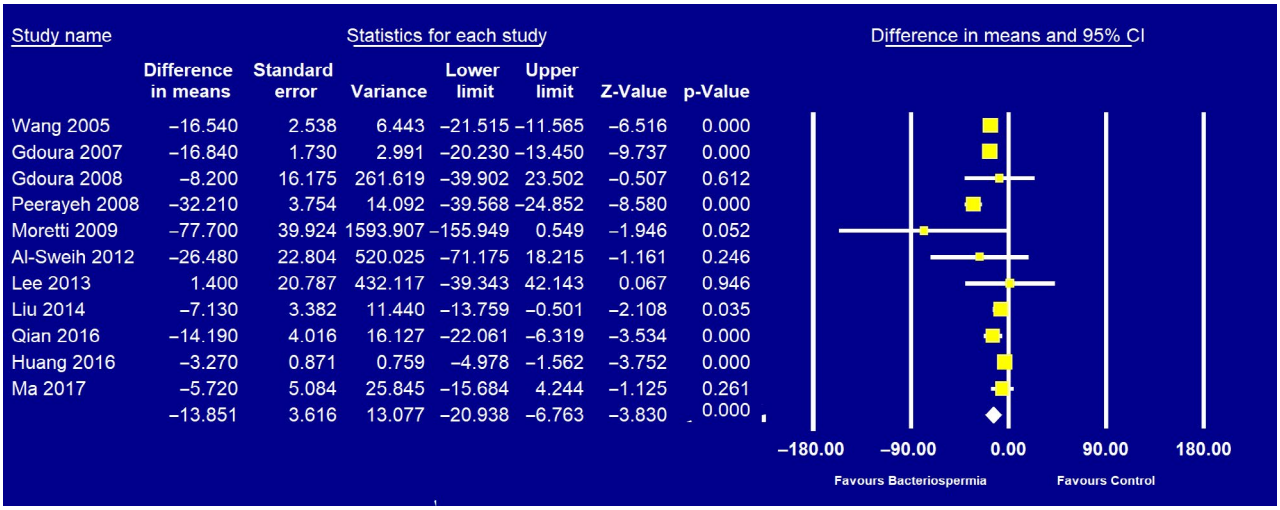


FIGURE 13 Forest plot. Ureaplasma urealyticum and mean sperm concentration. Wang (n = 185), Gdoura 2007 (n = 120), Gdoura 2008 (n = 104), Peerayeh (n = 165), Moretti (n = 1085), Al-Sweih (315), Lee (n = 98), Liu (n = 1236), Qian (n = 81), Huang (n = 22 466), Ma (n = 49)

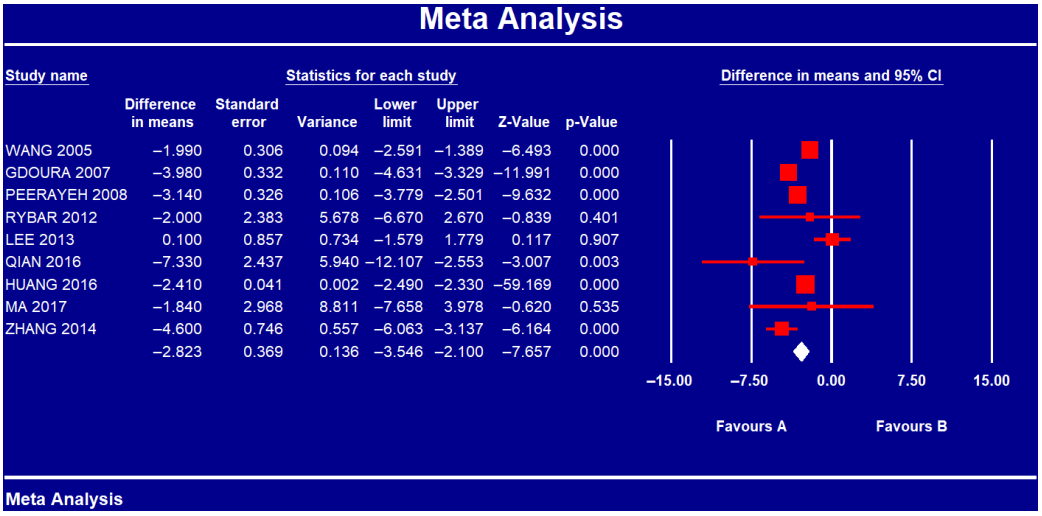


FIGURE 14 Forest plot. Ureaplasma urealyticum and morphology. Wang (n = 185), Gdoura 2007 (n = 120), Peerayeh (n = 165), Rybar (n = 293), Lee (n = 98), Qian (n = 81), Huang (n = 22 466), Ma (n = 49), Zhang (n = 369)

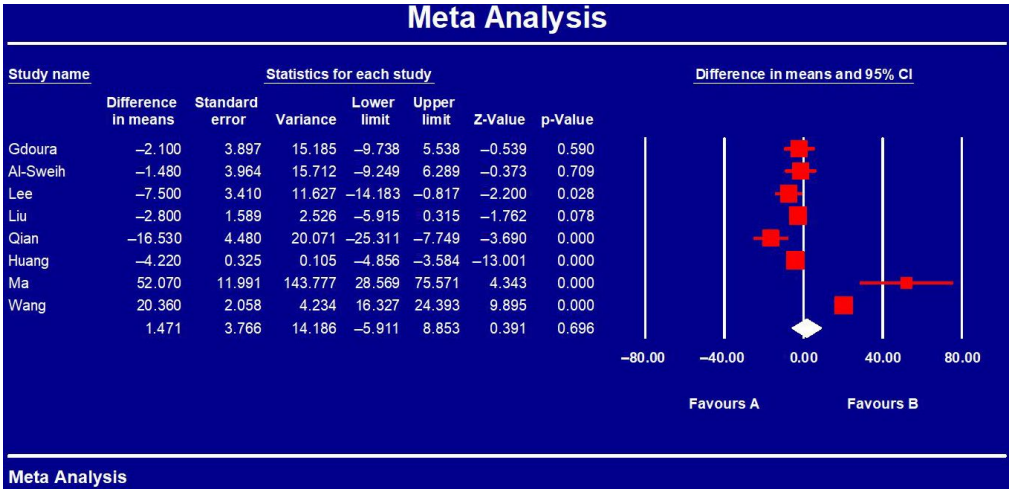


FIGURE 15 Forest plot. Ureaplasma urealyticum and motility. Gdoura (n = 120), Al-Sweih (n = 315), Lee (n = 98), Liu (n = 1236), Qian (n = 81), Huang (n = 22 466), Ma (n = 49), Wang (n = 185)

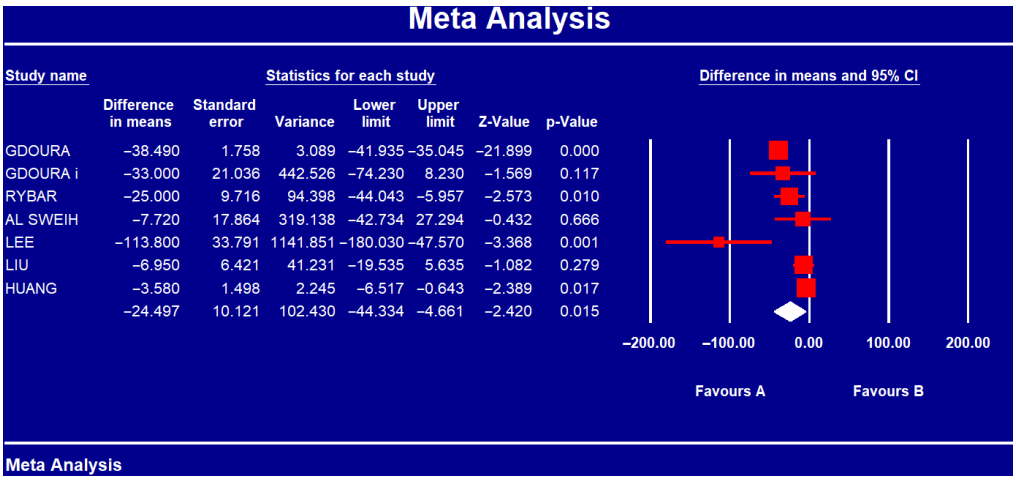


FIGURE 16 Forest plot. Mycoplasma hominis and mean sperm concentration. Gdoura (n = 120), Gdoura (n = 104), Rybar (n = 293), Al-Sweih (n = 315), Lee (n = 98), Liu (n = 1236), Huang (n = 22 466)

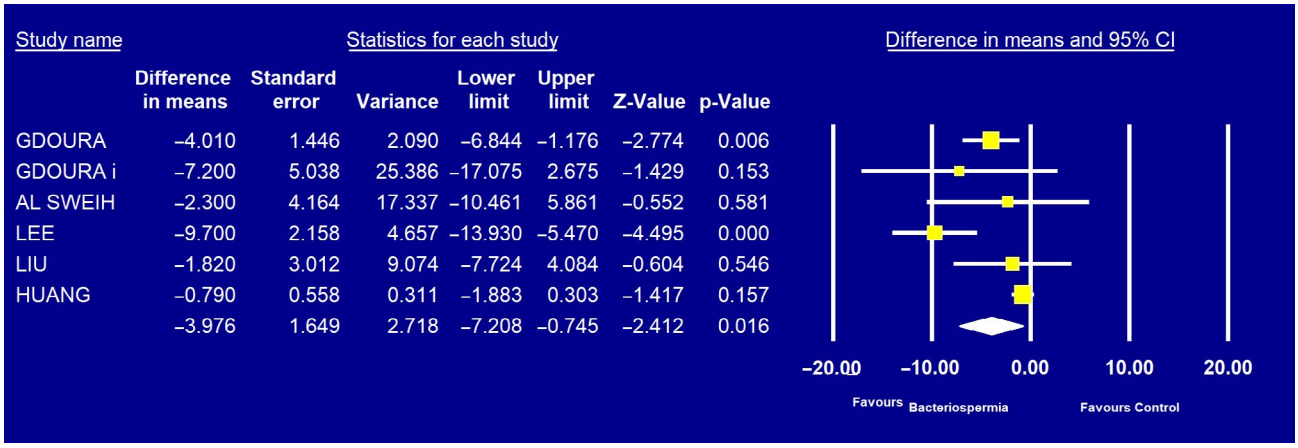


FIGURE 17 Forest plot. Mycoplasma hominis and progressive motility. Gdoura (n = 120), Gdoura (n = 104), Al-Sweih (n = 315), Lee (n = 98), Liu (n = 1236), Huang (n = 22 466)

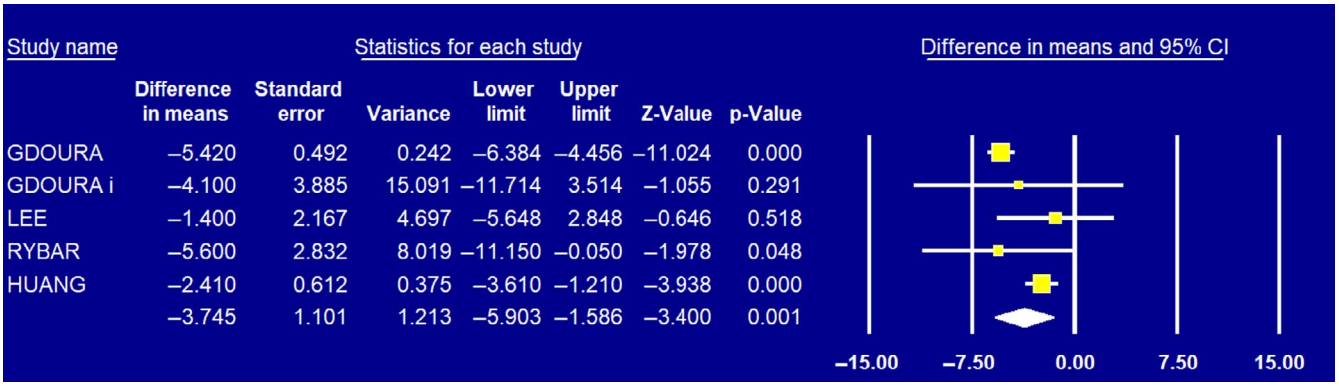


FIGURE 18 Forest plot. Mycoplasma hominis and morphology. Gdoura (n = 120), Gdoura (n = 104), Lee (n = 98), Rybar (n = 293), Huang (n = 22 466)

These studies have also reported an association between *E faecalis* and *Corynebacteria* and decreased motility, *Pseudomonas* and oligoasthenospermia, and *Prevotella* and *Anaerococcus* with decreased semen quality. Conversely, *Lactobacillus* has been associated with an improvement in semen quality.

In vitro studies have investigated the negative impact of bacteria on spermatozoa,[91,92] implicating apoptosis, necrosis and lipid membrane

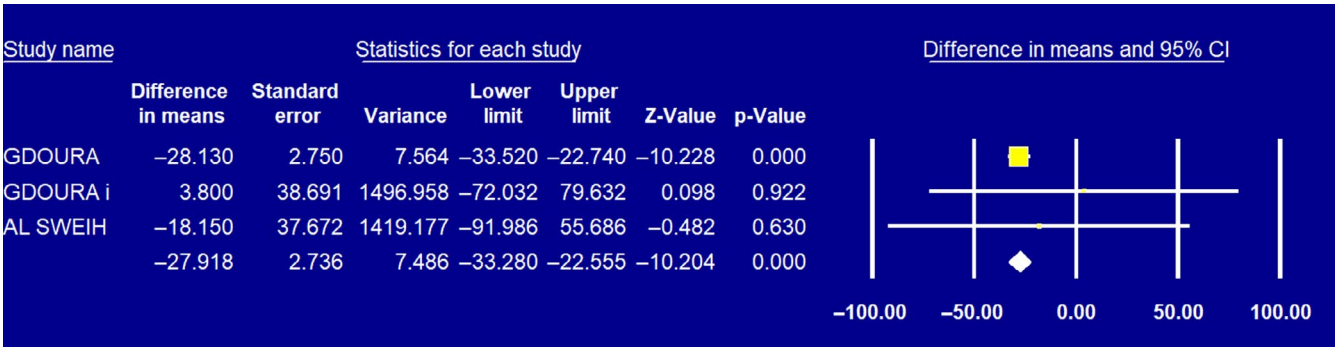


FIGURE 19 Forest plot. Mycoplasma genitalium and mean sperm concentration. Gdoura (n = 120), Gdoura (n = 104), Al-Sweih (n = 315)

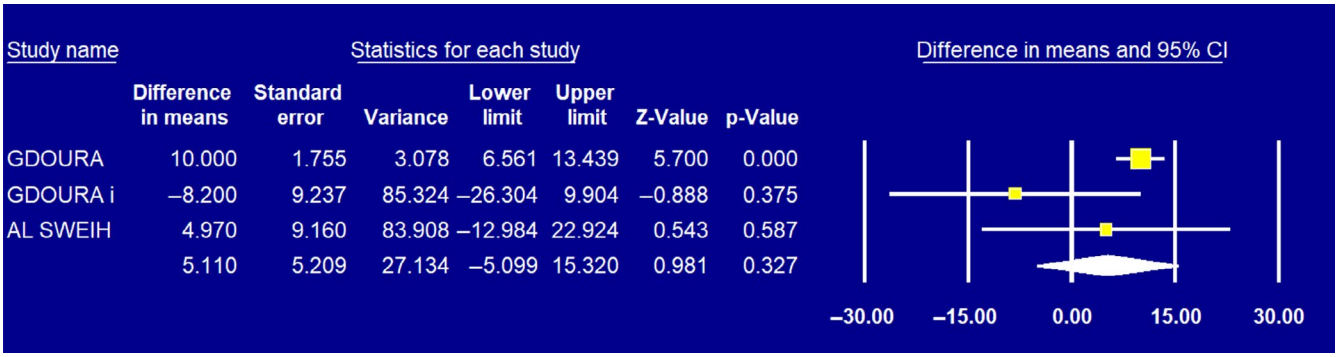


FIGURE 20 Forest plot. Mycoplasma genitalium and progressive motility. Gdoura (n = 120), Gdoura (n = 104), Al-Sweih (n = 315)

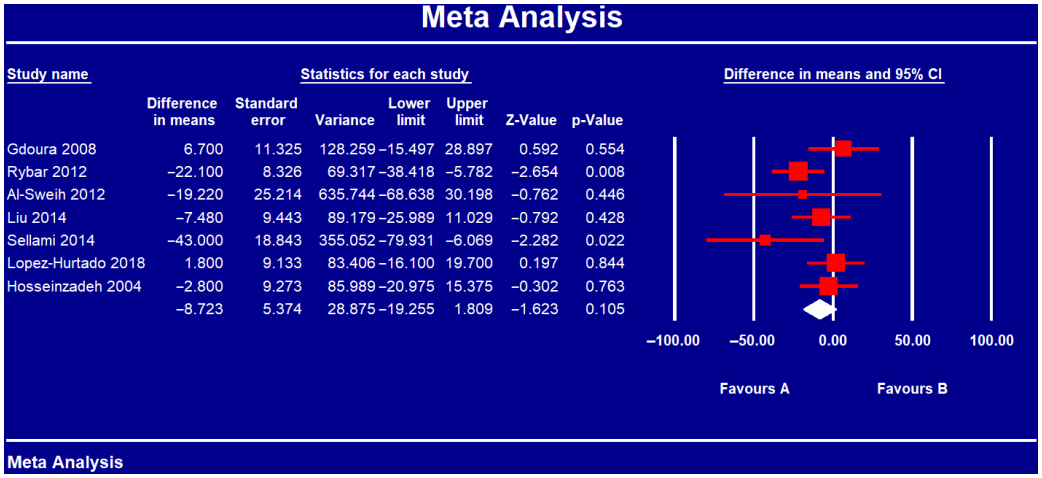


FIGURE 21 Forest plot. Chlamydia trachomatis and mean sperm concentration. Gdoura (n = 104), Rybar (n = 293), Al-Sweih (n = 315), Liu (n = 1236), Sellami (n = 85), Lopez-Hurtado (n = 116), Hosseinzadeh (n = 642)

injury. There is little in vivo evidence to explain the mechanisms by which bacteria cause male infertility, with some studies implicating leucocytospermia, and others suggesting that bacteria act independently of leucocytes. The proposed mechanisms include lowering the mitochondrial membrane potential causing apoptosis, and increased protamine deficiency. A previously published meta-analysis concluded that protamine deficiency was significantly associated with sperm DNA damage,[93] and this study found that protamine deficiency and DNA fragmentation were increased in the presence of bacteriospermia.

Studies analysing the microbiome of other body sites suggest that there is a fine balance of the community, with dysbiosis leading to domination by opportunistic or occasional pathogens, causing infections and inflammatory responses, for example *E coli* and inflammatory bowel disease.[94] This would suggest that the microbiome is necessary for the normal functioning of the semen and sperm, rather than having a strictly deleterious effect, similar to the vaginal microbiome, which plays a role in host defence.[95] Two studies in this review found that only certain strains or clonal variants

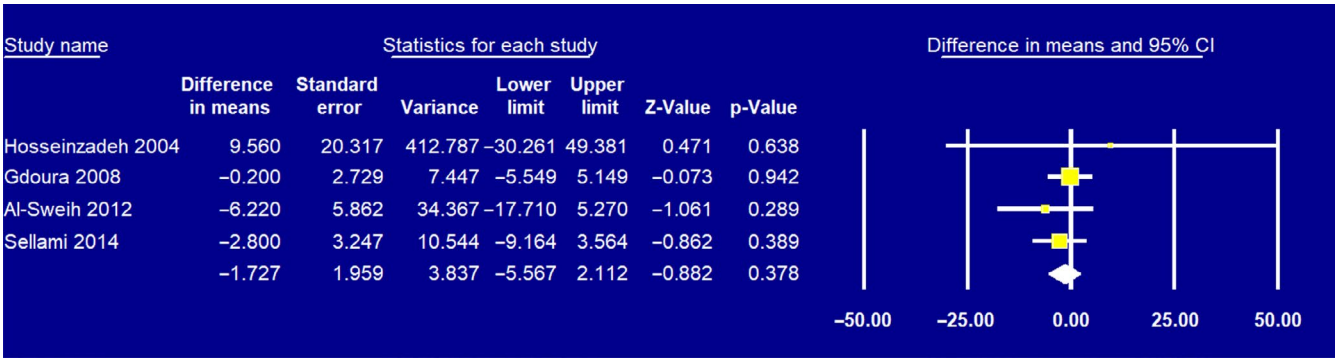


FIGURE 22 Forest plot. Chlamydia trachomatis and progressive motility. Hosseinzadeh (n = 642), Gdoura (n = 104), Al-Sweih (n = 315), Sellami (n = 85)

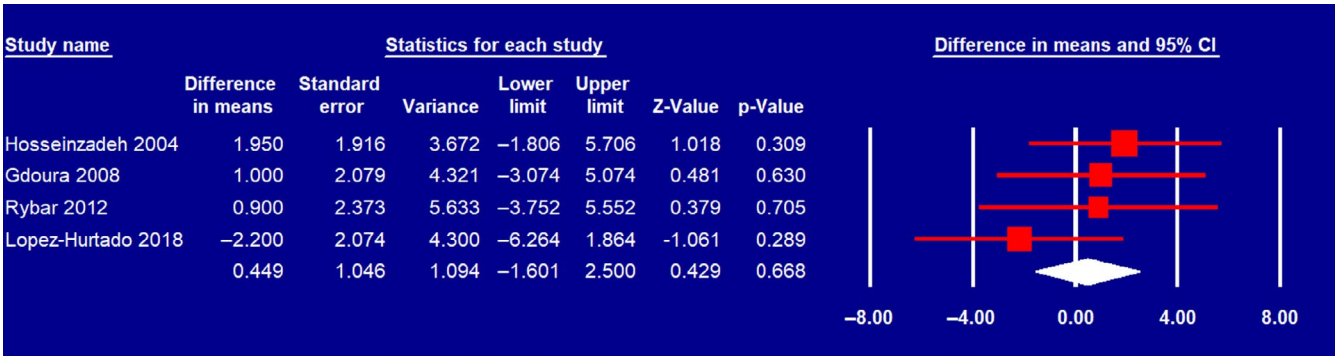


FIGURE 23 Forest plot. Chlamydia trachomatis and morphology. Hosseinzadeh (n = 642), Gdoura (n = 104), Rybar (n = 293), Lopez-Hurtado (n = 116)

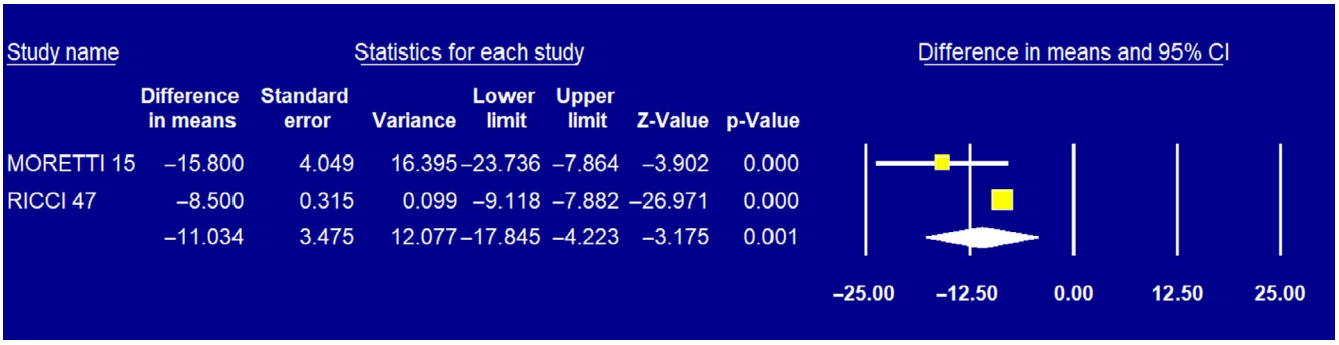


FIGURE 24 Forest plot. Enterococcus faecalis and total motility. Moretti (n = 1085), Ricci (n = 285)

of *Ureaplasma* had negative effects on semen, and a review of the species *Lactobacillus Iners* similarly proposed that clonal variants could either be health-promoting or implicated in disease.[96] These factors may explain why it has been difficult to reach a consensus as to whether bacteriospermia has a negative impact on semen and fertility.

The studies sampled the semen at a single time point; therefore, we cannot conclude from the available evidence whether the microbiome is transient or permanent. An acute infection is likely to alter the microbiome composition, but could it play a part in male infertility, or is this more likely to be caused by chronic, asymptomatic infections or other host factors? As many studies in this review reported a similar prevalence of bacteriospermia in fertile and infertile men, a greater understanding of host factors and their impact on the microbiome is needed. Host factors (eg environmental, immune response, genome) certainly impact the microbiome in other sites,[97-99] and may explain how dysbiosis occurs, and how it may affect developing spermatozoa.

The majority of the NGS studies identified microbiome clusters. There is evidence that the gut microbiome can be classified in this way, with studies also reporting *Prevotella*-enriched clusters,[100,101] as seen in the semen microbiome. The clustering in semen appeared to be grouped by bacteria that had similar requirements (eg oxygen), suggesting that

the semen of individuals creates specific environments enabling certain bacteria to grow and thrive, implying that the semen microbiome is unique and personalized. However, as three NGS studies reported similar abundant bacterial species within the clusters, there is evidence that the microbiome of the semen is conserved across individuals and perhaps across ethnic groups and geographical populations, which is in agreement with a gut microbiome study.[100] The NGS studies focused on small cohorts in China, Taiwan and Europe, and therefore, further larger-scale research is needed to answer these questions.

Next-generation sequencing and culture studies detected a number of species associated with the vaginal flora which supports findings from other studies that report sharing of bacteria between men and women, and that sexual intercourse can induce changes in the microbiome.[33,102] Further research investigating the sharing of taxa and the implications for infertility, pregnancy and disease processes is warranted.

Although we identified 55 studies investigating the semen microbiome and fertility, only a few performed case-control studies, and therefore, the data comparing infertile men with healthy controls are limited. With the advent of NGS, our knowledge of the semen microbiome is increasing, but it is clear that further work must be carried out using larger sample sizes with consistent and multiple sampling of individuals across different populations, as has been undertaken for other body sites, to gain a firm understanding of the semen microbiome and how it may vary through puberty, sexual debut, adult life and its effect on reproductive functions and disease processes. Future research must focus on the importance of host factors, and how they may affect the microbiome (eg age, ethnicity, BMI, behaviours), and strive to closely match these, in addition to using robust reproducible methodologies with standardized outcome measures.

This study has found that despite no major differences in the semen microbiome between those who are infertile or fertile, or between those with normal and abnormal semen quality, there are certain bacterial species or variants of species that do have a negative or positive effect on semen quality. UU, MH, MG, *Prevotella*, *Pseudomonas*, *E faecalis* and *Anaerococcus*, *and Corynebacteria* have been shown to exert a negative effect. Whilst *Lactobacillus* spp has a positive effect on morphology and may protect spermatozoa from the negative effect of opportunistic pathogens such as *Pseudomonas*. This study has not found any differences in the presence of CT, *S aureus* or *E coli*.

Future research should therefore also focus on non-pathogenic organisms that may have a protective role and how these can be developed as therapeutic options (eg probiotics), and well-designed randomized-controlled studies should be conducted to assess the impact of these interventions.

Currently, for clinicians who are managing couples with male infertility, it would be prudent to investigate leucocytospermia and bacteriospermia and treat seminal infections according to speciality guidelines. Additionally, couples should be informed that given the limited evidence available, the impact of the semen microbiome on fertility is inconclusive, and further studies are required.

CONFLICT OF INTEREST

None declared.

AUTHORS' CONTRIBUTIONS

L. Farahani wrote the article under the supervision of C. N. Jayasena and S. Minhas, who conceived of the article. L. Farahani, T. Tharakan and C. N. Jayasena were involved in the acquisition of the data. L. Farahani and T. Yap were involved in the analysis and interpretation of the data. J. W. Ramsay, C. N. Jayasena, T. Tharakan and S. Minhas critically reviewed the article and were involved in revising the article. All authors were involved in final approval of the article.

ETHICAL APPROVAL

Not applicable.

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