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Bidirectional Mendelian randomization to explore the causal relationships between the gut microbiota and male reproductive diseases

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Gut bacteria might play an important role in male reproductive disorders, such as male infertility and sperm abnormalities; however, their causal role is unclear. Herein, Mendelian randomization (MR)-Egger, weighted median, inverse variance weighting, Simple mode, and Weighted mode were used to test the causal relationship between gut microbes and male reproductive diseases. The MR results were validated using various metrics. The MR results were also consolidated using reverse causality speculation, conducted using two-way MR analysis and Steiger filtering. Biological function was analysed using enrichment analyses. The results suggested that eight intestinal microflorae were causally associated with male infertility. The *Eubacterium oxidoreducens* group was associated with an increased risk of male infertility, while the family *Bacteroidaceae* was negatively associated with male reproductive diseases. Eight intestinal microflorae were causally associated with abnormal spermatozoa. The family *Streptococcaceae* was associated with a high risk of abnormal spermatozoa, whereas the family *Porphyromonadaceae* was associated with a low risk of abnormal spermatozoa. No pleiotropy was observed, this study identified a high correlation between the gut flora and the likelihood of male reproductive diseases. Future research will attempt to advance microbial-focused treatments for such diseases.

Keywords Male reproductive disease, Mendelian randomization, Gut microbiota, Sperm, Genome wide association study, GWAS

Clinical infertility is commonly defined as the persistent inability of a couple to achieve pregnancy after 12 months of active attempts to conceive. Approximately 30–50% of these cases are associated with male infertility due to a variety of causes[1]. Primary male infertility is usually caused by congenital developmental anomalies, such as mutations in the *CFTR* gene (encoding cystic fibrosis transmembrane conductance regulator) leading to a congenital absence of the vas deferens and microdeletions of the Y chromosome at the azoospermia locus[2]. Secondary male infertility is often caused by acquired factors, including medical injury, reproductive tract infections, and poor lifestyle[3]. Most infertile men have problems with the quality of their sperm, the quantity of sperm production, or both. Common clinical conditions such as azoospermia, oligospermia, hypocupremia, and sperm incompetence are summarized under the term sperm abnormalities. Frequently, the issue can be rectified by changing their lifestyle, drug treatment, or using assisted reproductive technology[4].

Intestinal microorganisms can be functionally divided into three main groups: commensal, probiotic, and pathogenic microorganisms, which mainly comprise bacteria, but also include fungi, viruses, and phages, which maintain a dynamic balance in the human intestines[5]. This large microbial community in the gut has become an important acquired "organ" that is inextricably linked to the human body through long-term co-evolution with the host[6]. The gut microbiota performs a variety of functions, including metabolism, biological barriers, immune regulation, and host defence[7]. The intestinal microorganisms not only help the body absorb nutrients from food, but also synthesize amino acids, organic acids, vitamins, and antibiotics[8]. They can also metabolize the

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toxins produced by pathogenic bacteria, thus reducing their toxicity to the human body. Therefore, gut microbes are considered the second genome of the human body[9]. Together with the human genome, the genome of the gut microbiome influences our health in various ways through interactions with environmental factors[10]. Commensal *Lactobacilli* were observed to enhance sperm qualitative parameters in dogs[11]. Moreover, the intestinal microbiota plays an important role in the fertility of hens storing sperm[12]. In addition, the gut microbiome can influence spermatogenesis by controlling and metabolizing androgens and influencing the blood–testis barrier. It also raises serum levels of trimethylamine N-oxide (TMAO), which can cause vascular inflammation and erectile dysfunction[13]. Furthermore, some researchers have used tretinoin-induced testicular injury of mice to investigate the connection between intestinal microbes and testicular dysfunction. They discovered that the gut flora primarily aided the restoration of testicular injuries by controlling the metabolism of polyamines. However, in many previous studies, most of which were observational, the relationship between the gut flora and male reproductive disorders was also influenced by confounding factors, such as age, environment, dietary habits, and lifestyle. These circumstances have limited the investigation of causality between the gut microbiota and male infertility.

Mendelian randomization (MR)[14] is an epidemiological method used to analyse causal relationships between traits. The core of MR is based on the law of independent gametes, which states that the assignment of genes is random when parents with multiple pairs of traits cross to produce gametes. Genotype determines phenotype; therefore, MR studies use genetic variation as an instrumental variable of exposure to explore its causal relationship with outcomes[15]. Randomized classification and constant germline genotypes in MR reduce the impact of confounding by reverse causation, as well as confounding factors, such as the environment, lifestyle, and dietary habits. These factors tend to detract from traditional observational studies and significantly affect the inference of causal relationships between risk factors and outcomes[16]. The data used for the MR approach are based on genome-wide association studies (GWAS). In MR, single nucleotide polymorphisms (SNPs) are used as instrumental variables (IVs) to infer causal relationships between exposure factors and endpoints[17]. No published studies have fully elucidated the causal relationship between the gut flora and male reproductive diseases.

In this study, we used a two-sample MR study to investigate whether there is a causal relationship between the gut microbiota and male reproductive diseases. The findings of this investigation might yield new ideas for disease therapy.

Methods

Study design

We used a two-sample MR design based on summary GWAS statistics to provide compelling evidence for a causal relationship between the gut microbiota and male infertility. The role of the gut microbiota in male reproductive diseases was investigated at five levels (phylum, class, order, family, and genus). In addition, Fig. 1 shows that the study design is consistent with three hypotheses: (1) IVs are strongly associated with the exposure of interest; (2)

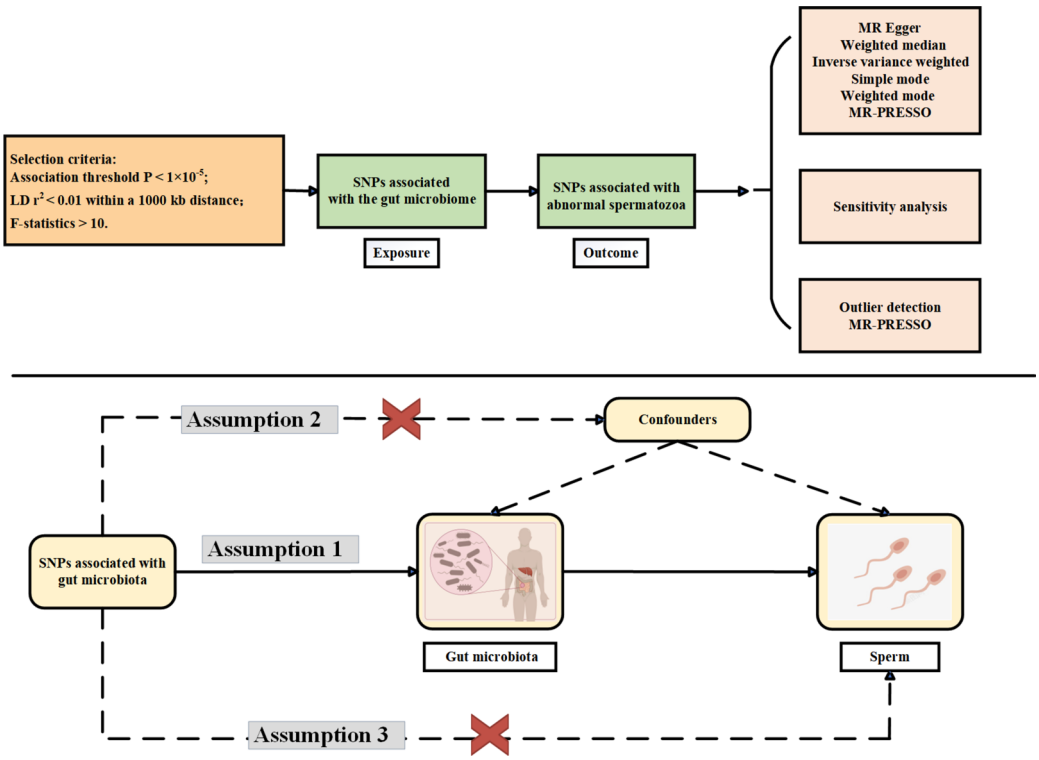

Figure 1. An overview of the study design. *LD* linkage disequilibrium, *SNPs* single nucleotide polymorphisms, *MR-PRESSO* Mendelian Randomization Pleiotropy RESidual Sum and Outlier.

genetic instrumentation is independent of potential confounding factors; and (3) IVs influence outcomes only through the pathway of exposure factors[18].

Data sources

Gut microbiome

Summary statistics from a GWAS of the human gut microbiome were extracted from The MiBioGen project (http://www.mibiogen.org)[19], which is a large-scale, multiethnic GWAS investigation that recruited 18, 340 people (24 cohorts) from various nations with 122,110 loci of variation. A total of 211 taxa were classified into five biological groups: 9 phyla, 16 classes, 20 orders, 35 families, and 131 genera[20]. Most of the participants in this study were the United States, Europe (including the United Kingdom, Germany, the Netherlands, and Denmark), and Korea. The microbial composition of the V4, V3–V4, and V1–V2 variable regions of the 16S rRNA gene was investigated and identified using direct taxonomic grading. The microbiota quantitative trait locus (mbQTL) positioning analysis revealed host genetic variation connected with genetic loci related to the abundance of bacterial taxa in the gut microbiota. MiBioGen has standardised all the methods and protocols required to analyse the cohort, such as microbiome data processing, genotype data processing, genome-wide association analysis, and meta-analyses. This standardization has contributed to the trustworthiness and authenticity of the records, allowing robust prospective analysis.

GWAS summary data for diseases

The GWAS summary datasets for male infertility were retrieved from the IEU OpenGWAS database. The diagnostic criteria of male infertility was based on the 10th code of the International Classification of Diseases and GWAS ID finn-b-N14_MALEINFERT, which included 680 cases and 72,799 controls[21]. Abnormal spermatozoa cases were diagnosed according to the WHO Laboratory Manual for the Examination and Preparation of Human Semen. The GWAS data for abnormal sperm was also acquired from the IEU Open GWAS project, which included 915 cases and 209,006 controls of European ancestry[22]. The GWAS ID was finn-b-R18_ABNORMAL_SPERMATOZ.

There was no significant overlap between gut microbiota and male reproductive diseases samples. The original GWAS were all approved by their respective agencies. All data used in our study is publicly available. No additional ethical approval is required.

Selection of IVs

The criteria were as follows: (1) SNPs with a genome-wide motif significance threshold ($p < 1.0 \times 10^{-5}$) were selected as potential IVs[23]. (2) Human genome GRCh38 data were used as reference data to exclude SNPs with linkage disequilibrium (LD) effects ($r^2 < 0.001$, clump kb = 10,000 kb). Proxy SNPs were not searched by default if a specific SNP was not available in the resulting GWAS. Echo SNPs were also excluded[24]. (3) The intensity of included IVs was assessed using F-statistics and R2[25]. R2 reflects the degree of exposure explained by the IVs and is calculated as $R2 = 2 \times EAF \times (1 - EAF) \times b^2 / [2 \times EAF \times (1 - EAF) \times b2 + 2 \times EAF \times (1 - EAF) \times N \times SE(b^2)]$ (EAF: effect allele frequency, SE: standard error of effect size, b: effect size, N: sample size). The F-statistic was calculated according to the formula $F = R2 \times (N - 2)/(1 - R^2)$ (N: sample size), where the weak instrumental bias is relatively small and the F-statistic is greater than 10 (MR assumption I)[26]. SNPs that were significantly associated with the outcomes ($p < 5e^{-8}$) were excluded (MR hypothesis III). We screened all eligible SNPs using PhenoScanner (http://www.phenoscanner.medschl.cam.ac.uk/) with filtering according to $r^2 > 0.8$ and $p < 1 \times 1\ 0^{-5}$ to exclude SNPs associated with confounding factors (MR hypothesis II)[27].

MR analysis

In this study, we used inverse variance weighting (IVW), MR-Egger regression, weighted median, weighted model, and simple mode to test whether there is a causal relationship between the gut flora and the risk of male infertility. The IVW method was the earliest and most commonly used method, which requires the SNPs to be fully consistent with the three principles of the MR study to obtain a correct causal estimate. It is characterized by regressions that do not take into account the presence of an intercept term and are adjusted using the inverse of the variance of the outcome (the squared value of SE) as weights to obtain an overall estimate of the impact of the gut microbiota on the disease[28]. The major difference between the MR-Egger method and IVW is that the regression takes into account the presence of an intercept term and uses the inverse of the variance of the outcome (the squared value of SE) as a weighting factor for the adjustment. If the intercept term is zero, there is no horizontal pleiotropy and the results of the MR-Egger regression are compatible to those of IVW[29]. The weighted median method combines the results of several MR estimates. This method weights the causal effects of different genetic variants on a trait and then uses the weighted median as the final estimate of the causal effect. The weighted median method is robust and can reduce bias caused by variation in the estimation results of specific genetic variants[30]. The weighted mode and the simple mode are also commonly used for MR of causality. The ability to detect causal effects is lower than the IVW and weighted median methods, but greater than that of the MR-Egger method.

Sensitivity analysis

This study tested heterogeneity using sensitivity analysis employing Cochran's Q-test[31]. The MR-Egger regression test was used to detect pleiotropy Horizontal multinomiality is present if the intercept is not zero. Mendelian Randomization Pleiotropy RESidual Sum and Outlier (MR-PRESSO) reduces horizontal pleiotropy by detecting and removing final outliers[32]. A leave-one-out sensitivity analysis was performed to check the robustness of the

results by removing one SNP at a time. All statistical analyses were performed using the packages "Two Sample MR" and "MR-PRESSO" in R version 4.3.0[33].

Reverse causality analysis

To evaluate the causal relationship between the gut microbiota and disease, we also performed reverse MR analysis for the bacteria that were found to be causally associated with disease in the forward MR analysis. The methods and settings used were the same as those for forward MR.

Enrichment analysis

To thoroughly examine the physiological impact of the gut microbiota on two male reproductive diseases, we performed an enrichment analysis based on lead SNPs for the chosen gut microbiota[34]. We mapped causative microbial lead SNPs to neighbouring genes using the GWAS4D website (http://www.mulinlab.org/varnote/index.html)[35]. Next, we conducted an enrichment analysis using the Metascape website (https://metascape.org). Clusters were created by grouping significantly enriched phrases with $p < 0.01$, the least number > 3, and an enrichment factor > 1.5[36].

Ethics statement

This human research did not require ethical approval as we used publicly available data approved by competent ethical and institutional review boards. This research was carried out in compliance with local legal and institutional guidelines. According to national legislation and institutional standards, written informed consent from the participants or their legal guardians/next of kin was not required for this study.

Results

Instrumental variables selection

Using the entire locus statistical significance criterion ($p < 1 \times 10^{-5}$), 2256 SNP were retrieved. No genus had a single SNP in each of the resulting datasets. There was no indication of mild instrumental bias in the current investigation because all the IVs' F statistic values were higher than 10. Supplementary Tables 1A and 2A include comprehensive details on the effect alleles, other alleles, beta, SE, and p-values for the IVs. We evaluated each genus's causal influence on the resultant data.

Mendelian randomization and sensitivity analysis of the association between the gut microbiota and male infertility

The IVW method identified eight bacterial genera that might be causally associated with male infertility (Supplementary Table 1B–F). Among them, five bacterial genera were negatively associated with male infertility, suggesting a potential protective effect against male infertility: the family *Bacteroidaceae* (id.918) (odds ratio (OR) 0.34, 95% confidence interval (CI) 0.15–0.73, p = 0.006); the family *Pasteurellaceae* (id.368)9 (OR 0.61, 95% CI 0.42–0.89, p = 0.01); the genus *Bacteroides* (id.918) (OR 0.34, 95% CI 0.15–0.73, p = 0.006); the genus *Ruminococcaceae NK4A214 group* (id.11358) (OR 0.54, 95% CI 0.29–0.99, p = 0.045); and the order *Pasteurellales* (id.3688) (OR 0.61, 95% CI 0.42–0.89, p = 0.010). In addition, three bacterial genera were positively associated with male infertility: the genus *Lactococcus* (id.1851) (OR 1.45, 95% CI 1.01–2.06, p = 0.042); the genus *Eubacterium oxidoreducens group* (id.11339) (OR 2.05, 95% CI 1.20–3.49, p = 0.008); and the genus *Eubacterium ventriosum group* (id.11341) (OR 1.66, 95% CI 0.98–2.83, p = 0.061) (Figs. 2, 4). Similar risk estimates were obtained in this study using the MR-Egger, weighted median, weighted model, and simple mode methods, although sometimes these associations were not statistically significant. The p-values for the Cochran Q-test and MR-Egger intercept test were larger than 0.05, showing that there was no heterogeneity or multiplicity in this investigation (Fig. 2, Supplementary Fig. 1A–C).

Mendelian randomization and sensitivity analysis between the gut microbiota and abnormal spermatozoa

For abnormal spermatozoa, IVW initially identified eight bacterial genera with potential causal effects on abnormal spermatozoa (Supplementary Table 2B–F). Two bacterial genera were negatively associated with the likelihood of abnormal spermatozoa, including the family *Porphyromonadaceae* (id.943) (OR 0.38, 95% CI 0.20–0.71, p = 0.003) and the genus *Prevotella9* (id.11183) (OR 0.66, 95% CI 0.46–0.94, p = 0.02); six bacterial genera were negatively correlated with abnormal spermatozoa, suggesting that they have a protective effect against abnormal spermatozoa, including the class *Erysipelotrichia* (id.2147) (OR 1.77, 95% CI 1.03–3.03, p = 0.038); *ebi-a-GCST90016952* (family *Streptococcaceae*; id.1850) (OR 2.54, 95% CI 1.50–4.31, p = 0.001); the genus *Lachnospiraceae UCG001* (id.11321) (OR 1.59, 95% CI 1.07–2.3, p = 0.02); the genus *Ruminococcaceae UCG009* (id.11366) (OR 1.44, 95% CI 1.02–2.04, p = 0.04); the genus *Streptococcus* (id.1853) (OR 2.31,95% CI 1.38–3.88, p = 0.002); and the order *Erysipelotrichales* (id.2148) (OR 1.77, 95% CI 1.03–3.03, p = 0.04) (Figs. 3, 4, 5). In summary, the IVW estimates were significant ($p < 0.05$), and the dependability of the results was confirmed by the consistent direction of IVW, MR-Egger, weighted median, weighted model, and simple mode. The results were strong because the p-values of the MR-Egger intercepts for each of the eight bacterial genera were higher than 0.05 (Supplementary Fig. 2A–C).

Reverse MR analysis

To determine whether reverse causality existed, we performed MR analyses to determine reverse causality between each of the two Male reproductive diseases and the identified gut microbiota. There were no significant

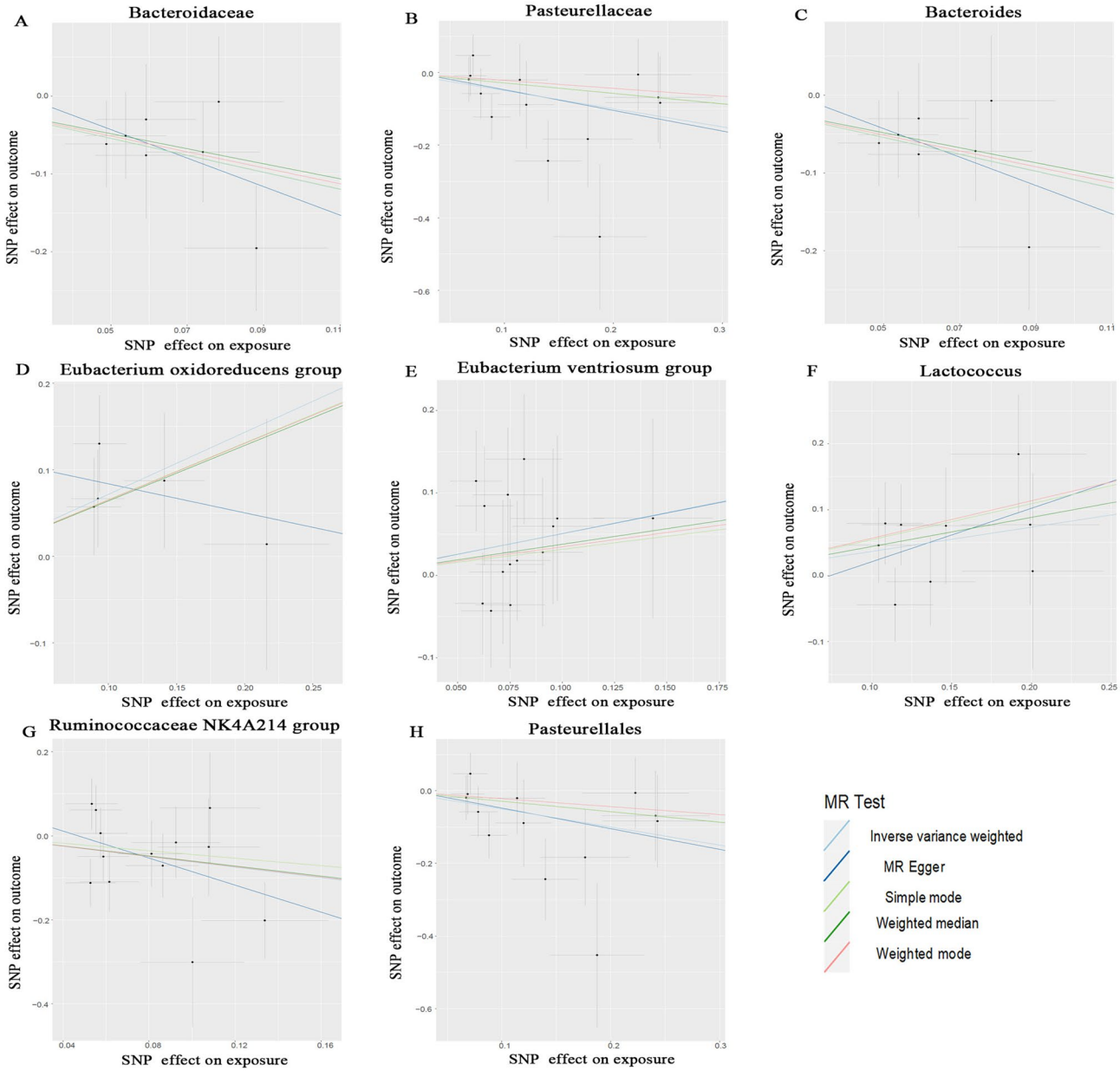

Figure 2. Scatter plots for the casual association between gut microbiota and male infertility. *SNP* single nucleotide polymorphism, *MR* Mendelian randomization.

associations between the two Male reproductive diseases and the previously identified gut bacteria (Supplementary Tables 1G–I, 2G–I). Cochran's Q-test showed no heterogeneity.

Enrichment analysis

The gut microbiome enrichment analysis identified 158 regulatory pathways associated with male infertility (Supplementary Table 1J, Fig. 6). The gut microbiome enrichment analysis also identified 198 regulatory pathways associated with abnormal spermatozoa (Supplementary Table 2J, Fig. 7). Thirty significantly enriched pathways were selected.

Discussion

The incidence of male infertility is steadily rising due to the widespread adoption of contemporary lifestyles[37]. There is insufficient data to determine the precise function played by the gut microbiota in male infertility, despite research indicating that it might be linked to infertility. Therefore, to thoroughly analyse the data and investigate the causal relationship between male reproductive diseases and gut microbiota, we used a two-sample MR technique based on gut microbiota GWAS data. Our study provided a thorough and complete MR examination of our current understanding, looking at the genetic link between the gut microbiota and male reproductive diseases. We identified a genetic susceptibility to gut bacteria that is causally associated with male reproductive

Outcome	Exposure	HR（95%CI）	P value
Male infertility	family Bacteroidaceae id.918	0.336（0.155-0.730）	0.006
	family Pasteurellaceae id.3689	0.607（0.416-0.886）	0.01
	genus Bacteroides id.918	0.336（0.155-0.730）	0.006
	genus Eubacterium oxidoreducens group id.11339	2.048（1.203-3.486）	0.008
	genus Eubacterium ventriosum group id.11341	1.663(0.977-2.828)	0.061
	genus Lactococcus id.1851	1.445(1.013-2.061)	0.042
	genus Ruminococcaceae NK4A214 group id.11358	0.537(0.292-0.987)	0.045
	order Pasteurellales id.3688	0.607(0.416-0.886)	0.01

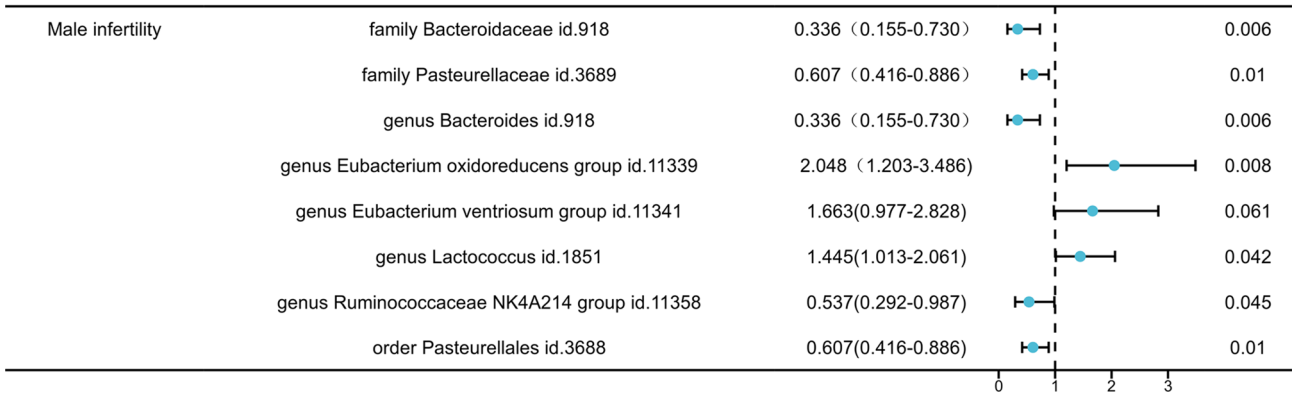

Figure 3. Forest plot illustrating the causal effect of eight gut microbiota members on male infertility using inverse variance weighted (IVW) methods. *HR* hazard ratio, *CI* confidence interval.

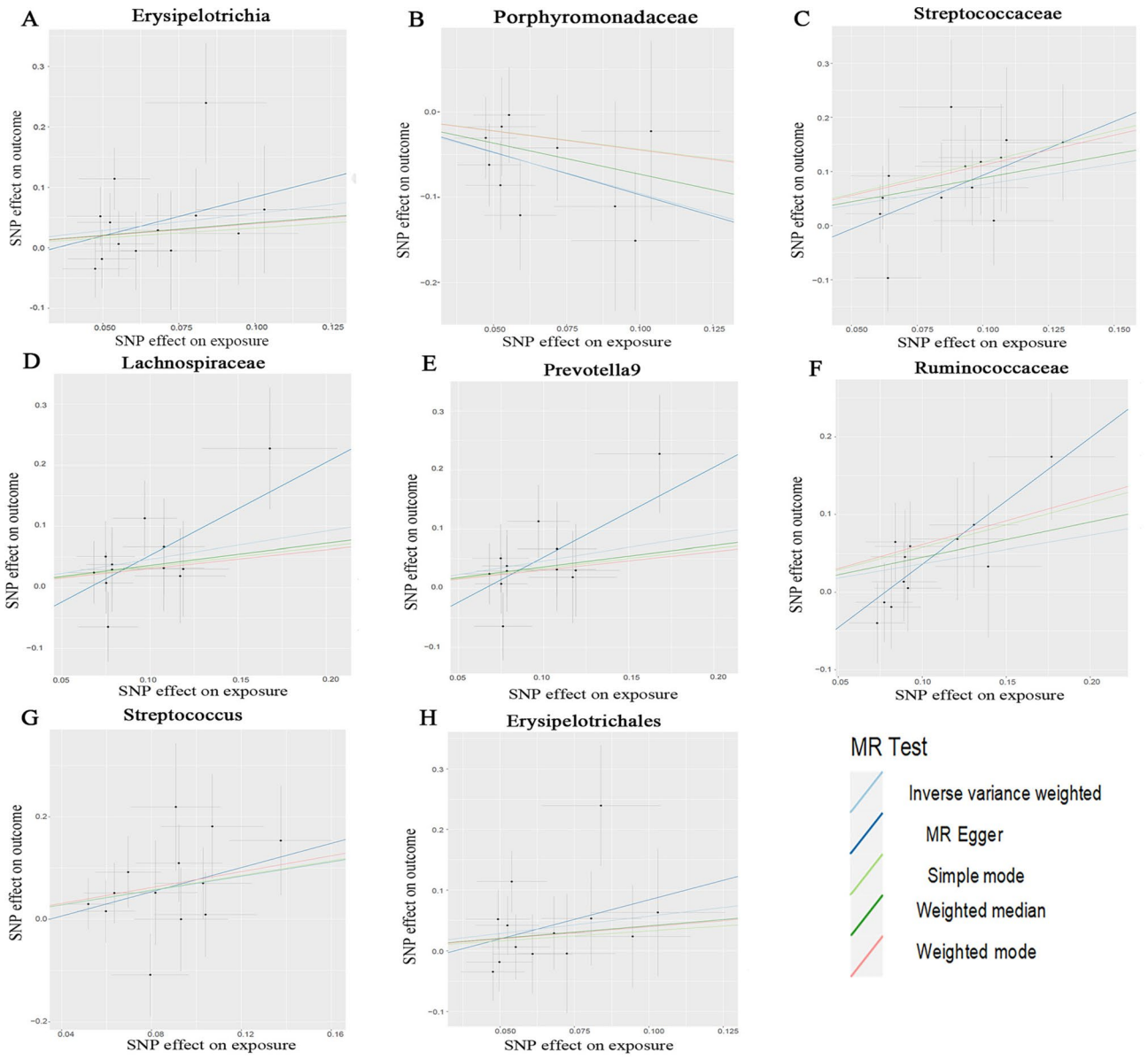

Figure 4. Scatter plots for the casual association between the gut microbiota and abnormal spermatozoa. *SNP* single nucleotide polymorphism, *MR* Mendelian randomization.

Outcome	Exposure	HR(95%CI)	P value
abnormal spermatoz	ebi-a-GCST90016916 (class Erysipelotrichia id.2147)	1.769(1.031-3.034)	0.03839143
	ebi-a-GCST90016947(family Porphyromonadaceae id.943)	0.385(0.207-0.715)	0.0025
	ebi-a-GCST90016952 (family Streptococcaceae id.1850)	2.543(1.502-4.306	0.000512
	ebi-a-GCST90017025(genus Lachnospiraceae UCG001 id.11321)	1.586(1.074-2.342)	0.02
	ebi-a-GCST90017045(genus Prevotella9 id.11183)	0.657(0.459-0.942)	0.02
	ebi-a-GCST90017057(genus Ruminococcaceae UCG009 id.11366)	1.443(1.020-2.040)	0.04
	ebi-a-GCST90017070(genus Streptococcus id.1853)	2.313(1.377-3.885)	0.00153
	ebi-a-GCST90017099(order Erysipelotrichales id.2148)	1.769(1.031-3.034)	0.04

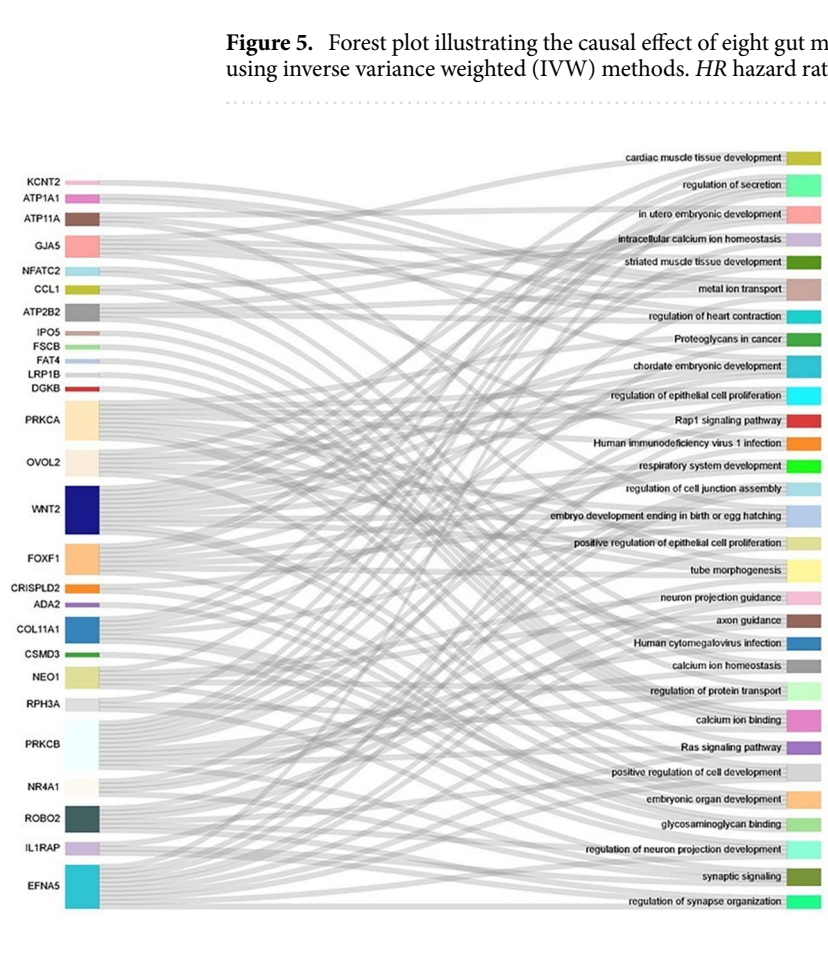

Figure 5. Forest plot illustrating the causal effect of eight gut microbiota members on abnormal spermatozoa using inverse variance weighted (IVW) methods. *HR* hazard ratio, *CI* confidence interval.

Figure 6. Gene ontology (GO) enrichment analysis of the gut microbiota of male infertility (30 pathways were significantly enriched).

disorders, such as male infertility and sperm abnormalities. Overall, we identified 16 different gut bacteria as potential risk factors for these diseases. Our research will contribute to a better understanding of the causal relationship between male reproductive diseases and the gut microbiota, laying a solid scientific foundation for the clinical treatment of these diseases.

Our IVW analysis yielded conclusive findings regarding the causal relationship between the gut microbiota and male infertility at various taxonomic levels, including phylum, class, order, family, and genus. Among them, we identified two families, two genera, and one order that exhibited a negative correlation with male infertility. These are the family *Bacteroidaceae* (id.918), the family *Pasteurellaceae* (id.3689), the genus *Bacteroides* (id.918), the genus *Ruminococcaceae NK4A214 group* (id.11358), and the order *Pasteurellales* (id.3688). This is consistent with previous studies. Hao et al. showed that faecal microbiota transplantation could improve the high-fat diet (HFD)-disrupted gut microbiota by increasing *Bacteroidales*, which in turn improved the damaged testicular

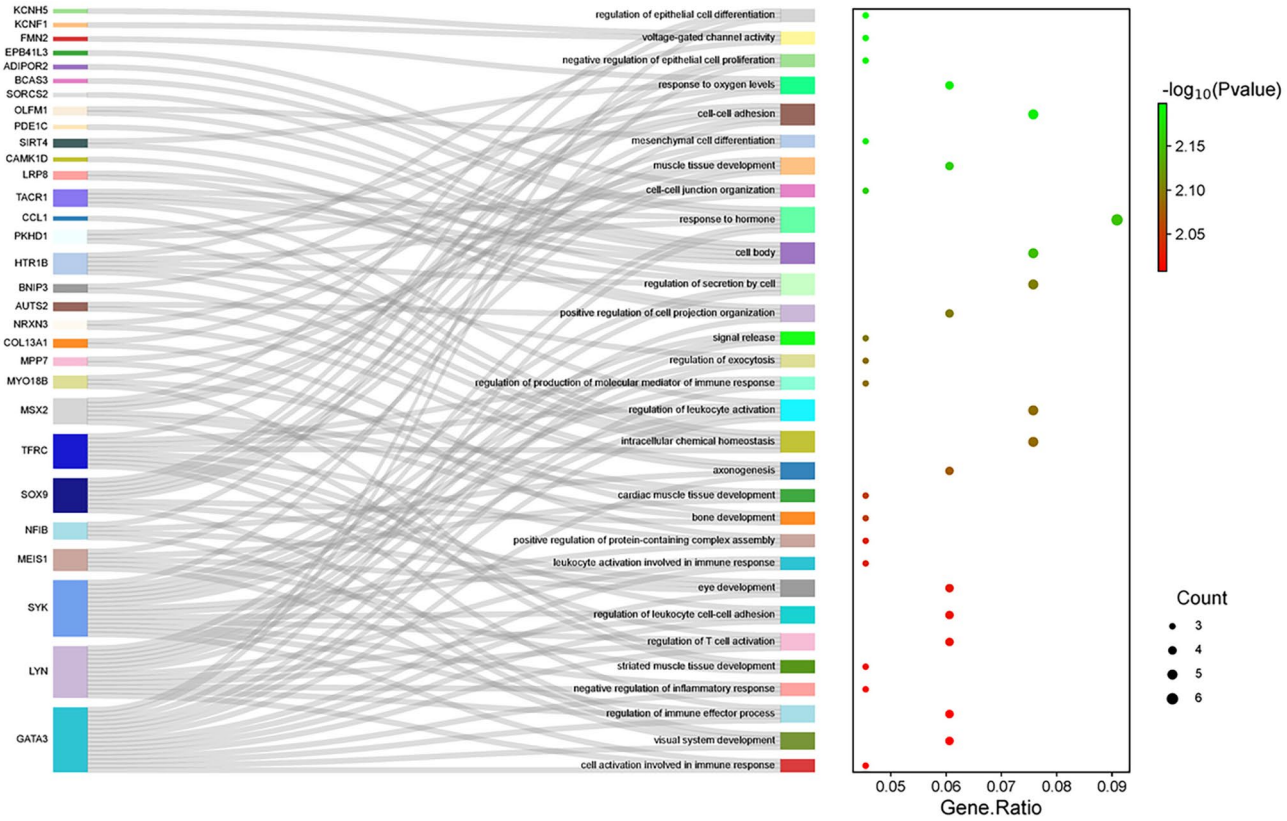

Figure 7. Gene ontology (GO) enrichment analysis of the gut microbiota of abnormal spermatozoa (30 pathways were significantly enriched).

microenvironment, rescued spermatogenesis, and improved semen quality and fertility in HFD-treated patients[38]. *Mycobacterium* species have a positive impact on male fertility through their influence on spermatogenesis signalling and the gut microbiota-testis axis. Reduced abundance of the *Ruminococcaceae NK4A214* cluster might lead to abnormal spermatogenesis by reducing bile acid levels and vitamin A absorption[39]. Combining the results of our study and a literature search, the family *Pasteurellaceae* and the order *Pasteurellales* were not found to be associated with male infertility, which provides a potential new avenue for microbiological studies of male infertility. Furthermore, we found three taxa that showed a positive association with male infertility: the genus *Lactococcus* (id.1851), the genus *Eubacterium oxidoreducens grou*p (id.11339), and the genus *Eubacterium ventriosum group* (id.11341). Consistent with previous studies, *Lactococcus* is a risk factor for male infertility[21]. *Eubacterium*, which belong to the phylum *Firmicutes*, are important intestinal bacteria found in the colon of healthy people. Decreased numbers of *Eubacterium* correlates with many diseases, such as depression and/or fatigue, obesity, inflammatory bowel disease, type II diabetes mellitus, cardiovascular and cerebrovascular diseases, colorectal cancer, autism, senile sarcosis, intestinal health, good tumour prognosis, and intestinal homeostasis[40]. However, the relationship between *Eubacterium* and male infertility has not been reported in previous studies.

In this study, 8 gut microbiota members were found to be associated with abnormal sperm. The family *Porphyromonadaceae* (id.943) and the genus Prevotella9 (id.11183), were found to be negatively correlated with abnormal sperm. *Porphyromonadaceae* and *Prevotella* are associated with metabolic syndrome indexes, including atherosclerosis and diabetes, in mice and humans[41]. They might protect sperm by controlling the body's metabolic index, thereby reducing chronic inflammation. Six other bacteria correlated positively with abnormal spermatozoa, including the class *Erysipelotrichia* (id.2147); *ebi-a-GCST90016952* (family *Streptococcaceae*, id.1850); the genus *Lachnospiraceae UCG001* (id.11321); the genus *Ruminococcaceae UCG009* (id.11366); the genus *Streptococcus* (id.1853); and the order *Erysipelotrichales* (id.2148). Certain pathogenic bacteria in the gut, such as *Erysipelotrichia*, *Streptococcus*, *Lachnospiraceae*, *Ruminococcaceae*, and the order *Erysipelotrichales*, can produce an endotoxin-induced inflammatory response. The endotoxin binds to human spermatozoa and supports cells' toll-like receptor-4 (TLR-4), activating the TLR-4-myeloid differentiation primary response protein 88 (MyD88)-dependent pathway, which releases several molecules, such as mitogen-activated protein kinases, interferon-regulating factors, and inducible nitric oxide synthase (iNOS)[42]. Together, these transcription factors activate and regulate the expression of numerous pro-inflammatory factors that damage the blood-testis barrier's endothelium, impair spermatogenesis, and ultimately result in male infertility. Furthermore, dysbiosis causes aberrant expression of related genes, such as *SYCP* genes (encoding synaptonemal complex proteins), which are essential for chromosome binding or segregation, as well as DNA double-strand breaks. Their decreased

expression also results in a decrease in the expression of the *GGNBP2* gene (encoding gametogenetin binding protein 2), which is necessary for spermatocytes to repair meiotic DNA double-strand breaks[43].

The relationship between the gut microbiota and male infertility was substantially influenced by several signalling pathways. Enrichment analyses showed that calcium (Ca^{2+}) signalling controls several crucial stages in the fertilization process, such as sperm overactivation, acrosome response, and sperm-egg fusion. Antonouli et al. demonstrated that several Ca^{2+}-dependent physiological responses during fertilization are mediated by the sperm-specific cation channel CatSper, which is primarily located in the main part of the flagellum of mature spermatozoa. Meanwhile, Ca2+ signalling mediated by CatSper initiates a tyrosine phosphorylation cascade that controls sperm motility[44]. Therefore, it is reasonable to speculate that gut microbes might cause male infertility by affecting calcium ion binding and calcium ion homeostasis. Furthermore, enrichment analysis revealed that multiple signalling pathways have a significant impact on the link between abnormal spermatozoa and the gut microbiome, including cell activation involved in the immune response; regulation of the immune effector process; negative regulation of the inflammatory response; regulation of T cell activation; regulation of leukocyte cell–cell adhesion; leukocyte activation involved in the immune response; regulation of leukocyte activation; and regulation of the production of molecular mediator of immune response. Duan et al. found that Th17 causes antigen-presenting cells to become chronically inflamed in patients with azoospermia and that T lymphocytes, as regulators, are crucial to the pathophysiology of male infertility[45]. Losdat et al. conducted related studies on great tits and found that an enhanced immune response affects sperm quality through the damaging effects of oxidative stress[46]. Therefore, we speculated that the relevant gut microbes might affect sperm by affecting immunity.

To date, there have been few reports on the relationship between the gut microbiota and male fertility. Through MR Analysis, we revealed that the family *Bacteroidaceae*, the family *Pasteurellaceae*, the genus *Bacteroides*, the genus *Ruminococcaceae NK4A214 group*, and the order *Pasteurellales* are protective against male infertility. The family *Porphyromonadaceae* and the genus *Prevotella9* were found to correlate negatively with the likelihood of abnormal sperm. These gut bacteria were primarily linked to immunological reactions and calcium ion channels, according to further pathway enrichment analysis. However, the exact mechanism between gut microorganisms and male fertility is unclear and further research is needed. Our analyses demonstrated the existence of a gut-fertility axis, highlighting the interconnectedness and feedback between the two. Disruption of this balance can lead to the development of disease.

Mendelian Randomization is a statistical method based on whole genome sequencing data that is effective in reducing bias, similar to RCT studies, and is used to uncover causal relationships[47]. The benefits are as follows: first, individual genetic diversity predates the course of disease, eliminating confounding bias resulting from problems with reverse causation; second, the study met all three MR analysis hypotheses and ensured the IVs' power for MR analysis by obtaining genetic variations of the gut microbiota via the broadest feasible GWAS meta-analysis. Third, the exclusion of pleiotropy was tested using the MR-PRESSO and MR-Egger regression intercept terms. This study has certain limitations. First, the gut microbiome GWAS data came from a varied sample of 18,340 adults from various ethnic backgrounds. Extrapolating the results to other populations with various lifestyles, cultural backgrounds, and genetic backgrounds should be carried out with caution, because the specific traits of different races and ethnic groups might alter due to differences in living circumstances and genetic backgrounds. Second, we employed a public GWAS database that cannot be accessed individually for men and women. We were unable to prevent bias by using subgroup analyses because the analyses employed summary statistics rather than raw data. As a result, this might affect the generalisation of the finding to the male population. Finally, further experimental and clinical validation is required to establish whether a specific microbial species has any appreciable impact on humans, given that the MR analysis is predicated on untestable assumptions.

Conclusion

Using MR analysis, we were able to identify microorganisms linked to two male reproductive diseases, while avoiding the frequent biases seen in observational studies. Our research creates new opportunities for therapeutic intervention with this group of patients. To investigate the precise physiological processes and the role played by bacteria in the pathophysiology of male reproductive diseases, more research is necessary.

Data availability

The article and Supplementary material contain the original contributions made during the study. For additional information, contact the corresponding author.

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Author contributions

X.H. wrote the main manuscript text. L.Y., H.T. and Y.J. prepared Figs. 1, 2, 3, 4, 5, 6 and 7. All authors reviewed the manuscript.

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