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REVIEW

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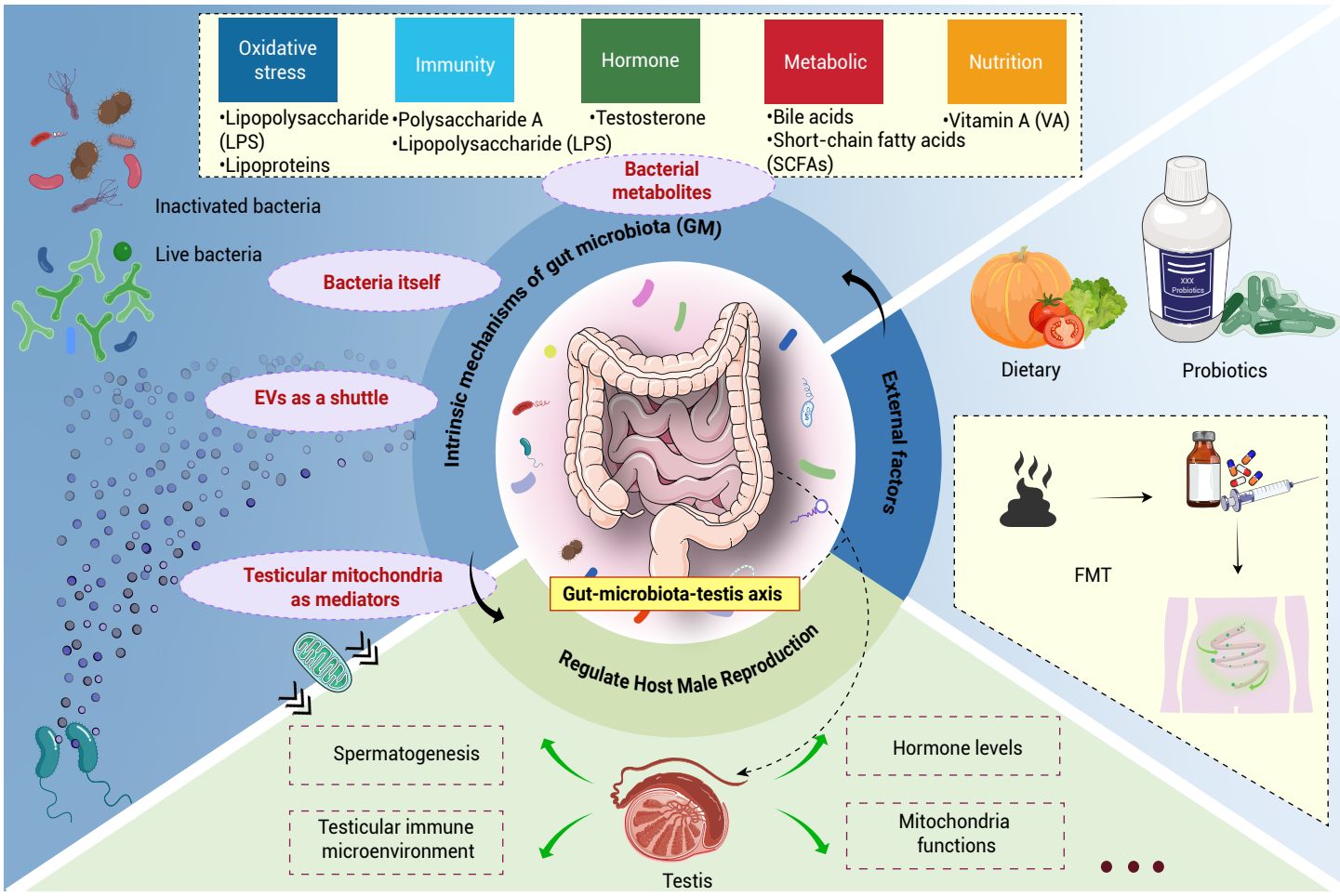
GRAPHICAL ABSTRACT



PUBLIC SUMMARY

- The gut-microbiota (GM)-testis axis lets gut bacteria regulate male reproduction via multiple key pathways.
- GM-host interaction includes response to bacterial metabolites and direct interaction with bacteria themselves.
- Extracellular vesicles (EVs) shuttle biomolecules to testes; testicular mitochondria mediate microbial effects.
- Diet and lifestyle also play a role in this regulation, linking gut health to male reproductive function.

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Gut microbiota (GM), a dynamically changing community of bacterial species and other microorganisms, has become a crucial factor in host physiology. Mounting evidence indicates its communication with distal organs like the testis via intricate mechanisms, establishing the gut-microbiota-testis axis. This axis has emerged as a key focus in investigations into the regulation of male reproduction by GM. However, several critical aspects remain unaddressed comprehensively and systematically. There is a dearth of knowledge regarding the detailed characterization of GM components relevant to male reproduction. The mechanisms underlying the translocation of GM components to the testes remain poorly understood. Additionally, how GM impacts testicular function to modulate male reproductive processes has not been thoroughly investigated. Here, we reviewed the gut microbiota and gut-microbiota-testis axis and discussed the main mechanisms of GM regulating host male reproduction. It is noted that GM regulates host male reproduction through bacterial metabolites and/or direct interactions with the bacteria themselves. Moreover, extracellular vesicles (EVs) may serve as shuttle vehicles for transporting bioactive substances such as microbial metabolites, and testicular mitochondria can act as mediators for gut microbiota to regulate host male reproduction. Finally, gut microbiota is a highly dynamic and complex community that is influenced by factors such as diet, living environment, and lifestyle, which is of great significance for clinical interventions. This review will assist with clinical targeted intervention of GM and explore new therapies for male reproductive disorders.

INTRODUCTION

The gut microbiota (GM) is the most prominent microbial community within the human body.[1] With its wide-ranging gene set and diversity, it is capable of performing many beneficial functions for the host, such as providing nutrition, regulating physiology, and modulating the reproduction system, all of which collectively contribute to human well-being.[2,3] It has been discovered that the GM is sufficiently versatile, interacting not only locally with the intestinal tract but also engaging in an interconnected network with a range of distant organs and tissues. To underscore the GM's pivotal role within this network, a number of hypotheses have been formulated, such as the gut-brain axis,[4] the gut-liver axis,[5] the gut-kidney axis,[6] and the gut-muscle axis.[7] The latest research showed that changes in GM could affect the function of testes and the hypothesis of the gut-microbiota-testis axis was proposed.[8,9] Thus, this field has gradually become the focus of male reproduction. Although the exact mechanism of the gut-microbiota-testis axis has not yet been fully understood and clarified, the evidence from animals and human studies has shown that the GM may regulate host male reproduction through pathways such as microbiota itself, *de novo* synthesis or microbial metabolism to produce key molecules, extracellular vesicles (EVs) derived from bacteria and so on.[10-12]

In this review, we first introduce the gut microbiota and the gut-microbiota-testis axis, laying a foundational understanding of their basic concepts and significance. Then, in the core mechanism section, we elaborate on how gut microbiota regulates male reproduction from four aspects: interactions with bacterial metabolites (including effects related to nutrition, immunity, hormones, inflammation, oxidative stress, and metabolic pathways), direct interactions with bacteria themselves, microbiota-derived extracellular vesicles (EVs) as cargo shuttles, and testicular mitochondria as mediators. Next, we discuss the impact of dietary and lifestyle interventions and their clinical implications, covering the effects of high-fat diets and the Mediterranean diet, as well as the potential and limitations of probiotics and fecal microbiota transplantation (FMT). Finally, we summarize the overall content and propose future research perspectives, aiming to provide support for the diagnosis and treatment of male reproductive diseases.

GUT MICROBIOTA

The GM forms a dynamic ecosystem distributed throughout the digestive tract, reaching maximal density in the colon.[13,14] Essential bacterial, fungal, and viral symbionts colonize humans at trillion-scale concentrations, maintaining core physiological functions.[15,16] 80% of them exist in the gut, about 10 times as many as cells in the human body. The gut microbial DNA content is 150 times that of the host and more than 99% of this DNA belongs to bacteria.[13,17] The human gut is believed to house between 200 and over 1,000 distinct bacterial species.[18-20] Metagenomics has been employed to investigate the human gut microbial community at the species level. For instance, Qin and colleagues found that a total of around 1,150 bacterial species were present in 124 European subjects, with the majority of individuals hosting roughly 160 bacterial species.[21] Metagenomic studies have revealed that the main microbial communities are those belonging to the eubacterial phyla *Firmicutes*, *Bacteroidetes*, *Proteobacteria*, *Fusobacteria*, *Cyanobacteria*, *Verrucomicrobia* and the archaebacterial phylum *Euryarchaeota*.[22,23]

GM composition is a dynamic entity that changes throughout human life and is affected by a range of factors. Infants acquire the initial collection of microbes from their mothers.[24,25] Once the infants reach the age of one, their gut microbiome has developed into a complex community, comparable to that of adults.[26,27] The dynamic changes also show significant differences among different individuals, but the common result is a macrocosmic balance.[28] A change in beneficial bacteria can have significant effects on an individual's health, especially by inducing certain aspects of disease development. Factors like diet, illness, medication, and infection can also modify the microbiota.[29,30]

The gut microbiota performs a multitude of functions, including preserving intestinal integrity, generating mucus, prompting the regeneration of the intestinal epithelium, and regulating the production of short-chain fatty acids (SCFAs).[31] The GM regulates the maturation of innate immunity during the early phases of life and senses and modulates a large number of signals from the environment, influencing processes throughout the body.[32] It serves as a mediator between the host and the environment and could potentially impact human health.[33] Of note, the GM also participates in the sexual maturation of males. In reality, various intestinal metabolites—including secondary bile acids, indole, and soy-derived compounds—are capable of regulating male sexual organs.[34-36] Dysfunction of GM can lead to abnormal spermatogenesis.[12,37] Gut microbiota translocation can lead to an overactive immune response and a persistent inflammatory state, or changes in hormone levels, which can cause endothelial damage, disruption of the blood-testis barrier (BTB), and impaired spermatogenesis and viability.[10,38,39] As male reproductive issues are becoming an increasingly serious global concern, people are showing a growing interest in the relationship between gut microbiota (GM) and male reproductive health.

GUT-MICROBIOTA TESTIS

The "gut-microbiota-testis axis" refers to a bidirectional crosstalk between intestinal microbes and testicular function, mediated through circulatory, neural, immune, and metabolic pathways. Its core lies in the interaction axis

formed between the gut microbiota (and its metabolites) and testicular tissues (including spermatogenic cells, Sertoli cells, Leydig cells, etc.) via specific anatomical structures and physiological mechanisms. This axis not only participates in maintaining normal testicular functions (such as spermatogenesis and hormone secretion) but also can affect testicular homeostasis through the axis when the microbiota is disturbed. Conversely, the functional state of the testis can also react on the composition of the gut microbiota through systemic regulation. The balance or health status of GM affects the development and well-being of the male reproductive system.[40] The development of the testes depends on germ cells and somatic cells. When a testicle reaches full maturity, it produces sperm, which accumulate in the epididymis and are subsequently released from the penis via the deferent ducts to complete ejaculation. Nutrients like water, amino acids, lipids, carbohydrates, vitamins, and minerals are essential for this process. Through the exchange of nutrients and metabolic waste with Sertoli cells, germ cells accomplish differentiation and maturation.[41] Furthermore, penile erection relies on stimulation from various gas signaling molecules, which are largely generated by cyclic metabolism in the GM.[42] In summary, GM can regulate host testicular function and affect the development and health of the host male reproduction, through various complex mechanisms, forming the gut-microbiota-testis axis.

Despite the robust support provided by multi-omics technologies (metabolomics, transcriptomics, and metagenomics) for investigating the gut microbiota-testis axis, these methodologies have limitations. For instance, metabolomics analysis struggles to fully elucidate the functions of all metabolites, and metagenomics studies have difficulty differentiating between the contributions of live and dead bacteria.[10] Current research is largely correlational, revealing associations between the gut microbiota and testicular function, but causal relationships remain incompletely defined.[43] The composition and function of the gut microbiota vary among individuals, leading to differential responses to dietary interventions or probiotic supplementation, which may impact the generalizability and reproducibility of research findings. Furthermore, the composition of the gut microbiota is influenced by a combination of genetic, environmental, and lifestyle factors.[44]

Many studies on the gut microbiota-testis axis have been conducted in animal models, and the complete translatability of their findings to humans requires further validation. Gut microbiota modulation strategies also face challenges in clinical application; for example, the effects of probiotic supplementation and dietary interventions vary among individuals, and standardized treatment protocols are lacking.[45] Moreover, the safety and effectiveness of fecal microbiota transplantation (FMT) in large-scale clinical applications require further verification.[46] Current research often focuses on single factors or localized mechanisms, lacking systematic integrative studies. For example, the interactions between the gut microbiota, metabolites, the immune system, and testicular function necessitate more comprehensive systems biology research.

Therefore, this article further explores gut microbial metabolites and other mechanisms, aiming to provide new perspectives and assistance for the diagnosis and treatment of male infertility and other reproductive issues.

THE MECHANISM OF GUT MICROBIOTA REGULATING HOST MALE REPRODUCTION

Interaction with the bacterial metabolites

The most common way GM influences male reproduction is through its metabolites. Key molecules made or modified by GM have diverse functions: they support nutrient supply to the testis, adjust immune responses, regulate hormone levels, and mediate processes like inflammation and oxidative stress (Figure 1).

GM metabolites affect testicular function through nutrition. The testes have little to no capacity for de novo nutrient synthesis. Instead, blood vessels within the testes transport nutrients metabolized by the gut microbiota (GM) from the digestive system to the testicular interstitium. This process occurs via the convoluted seminiferous tubules, aided by Sertoli cells and their intercellular junctions. Those nutrients such as vitamins and minerals are essential for the testes. For example, vitamin A (VA) is essential for male reproduction, and its main metabolic product—retinoic acid (RA)—drives two critical steps in spermatogenesis: the differentiation of spermatogonia and the start of meiosis. Without sufficient RA, spermatogonial stem cells cannot mature properly, disrupting sperm production.[47] *STRA8* (Stimulated By Retinoic Acid Gene 8) is a gene induced by retinoic acid (RA) that regulates spermatogenesis in a PCNA-dependent manner through Cdl4-Clu4A-Ddb1 ubiquitinated degradation axis;[48] RA can decrease the expression of miR-34c, which also plays an important role in spermatogenesis;[49] All-trans retinoic acid (ATRA) is another metabolic intermediate of vitamin A (VA). It can induce the activities of superoxide dismutase (SOD) and glutathione transferase (GST) in both varicocele and healthy sperm, and reduce the production of malondialdehyde and reactive oxygen species (ROS), thereby protecting spermatogenesis.[50] A deficiency of vitamin A leads to the failure of type A spermatogonial stem cells to differentiate into type A1.[51] In a sheep model with diet-induced metabolic disorders, gut microbiota (GM) dysbiosis results in reduced bile acid levels. This impairs the transport of fat-soluble vitamin A within the gut-microbiota-testis axis, consequently affecting spermatogenesis.[10]

GM metabolites affect testicular function through immunity. GM preserves the tolerogenic state of immune-privileged compartments in the testis by regulating the balance between pro-inflammatory and anti-inflammatory cells. For example, *Bacteroides fragilis* produces polysaccharide A, which triggers TLR2 signaling in the testis. This signaling prompts the formation of Foxp3+regulatory T (Treg) cells, which then boost secretion of the anti-inflammatory cytokine IL-10 while blocking the pro-inflammatory effects of Th17 cells—ultimately protecting the testis from excessive inflammation;[52] GM also produces lipopolysaccharide (LPS), an endotoxin from gram-negative bacteria in the gut, which promotes the secretion of inflammatory factors (TNF-α, IL-1β, and IL-6) in testicular cells through the TLR4/MyD88 pathway.[53] When the gut microbiota is disrupted, the integrity of the intestinal barrier may be compromised. This allows LPS to travel through the epithelial cells of the intestinal barrier and enter the bloodstream. Once in the bloodstream, in cases of inflammation or when the BTB is damaged, LPS can potentially reach the testis. Studies have also shown that inflammatory factors promoted by LPS can increase the permeability of the BTB. For example, extracellular signal-regulated protein kinase (ERK) can be activated by tumor necrosis factor-α (TNF-α) and p38 signaling pathways in Sertoli cells. This activation reduces the steady-state levels of occludin and zonula occludens-1 (ZO-1), proteins that are important for maintaining the integrity of the tight junctions in the BTB. As a result, the BTB becomes more permeable, and LPS may have an opportunity to cross.[54,55] Exposure to glyphosate can alter the composition of GM, causing Th17 induced by GM to produce interleukin (IL)-17A, leading to oxidative damage to the testes, resulting in structural damage to the testes, reduced sperm motility, and a higher rate of sperm deformity.[56] In addition, GM dysbiosis disrupts testicular immune homeostasis via aberrant macrophage polarization and disrupted cytokine gradients, impairing spermatogenesis: under normal conditions, testicular macrophages are primarily M2-type, secreting anti-inflammatory factors (e.g., IL-10, TGF-β) to maintain testicular microenvironment stability, promote spermatogonial stem cell proliferation/differentiation, and phagocytose apoptotic germ cells, but GM dysregulation increases harmful metabolites like LPS, inducing polarization to pro-inflammatory M1-type macrophages that secrete TNF-α, IL-1β, and IL-6, triggering local testicular inflammation, damaging spermatogenic cells, and impairing Sertoli cell function.[57] Meanwhile, the testicle normally maintains precise cytokine gradients (higher anti-inflammatory factors in the seminiferous tubule basal compartment for spermatogonial stem cell self-renewal, appropriate pro-inflammatory factors in the luminal compartment for germ cell maturation/apoptosis regulation), which are disrupted by excessive pro-inflammatory factors (e.g., IL-17 from Th17 cells) from dysregulated GM, disturbing spermatogenic cell-Sertoli cell interactions, hindering spermatogonial stem cell differentiation, increasing germ cell apoptosis, and ultimately impairing spermatogenesis.[58,59] Rongkang et al. also reported the role of extracellular and intracellular microorganisms in cancer immunotherapy.[60]

GM metabolites affect testicular function through hormones. Studies show that the GM also possesses a robust capacity to elevate testosterone levels. In adult mice, the fecal concentration of dihydrotestosterone (DHT) is over 20 times higher than that in serum. In normal mice, the level of free DHT in the intestine is greater than that in germ-free mice. Testosterone levels, as

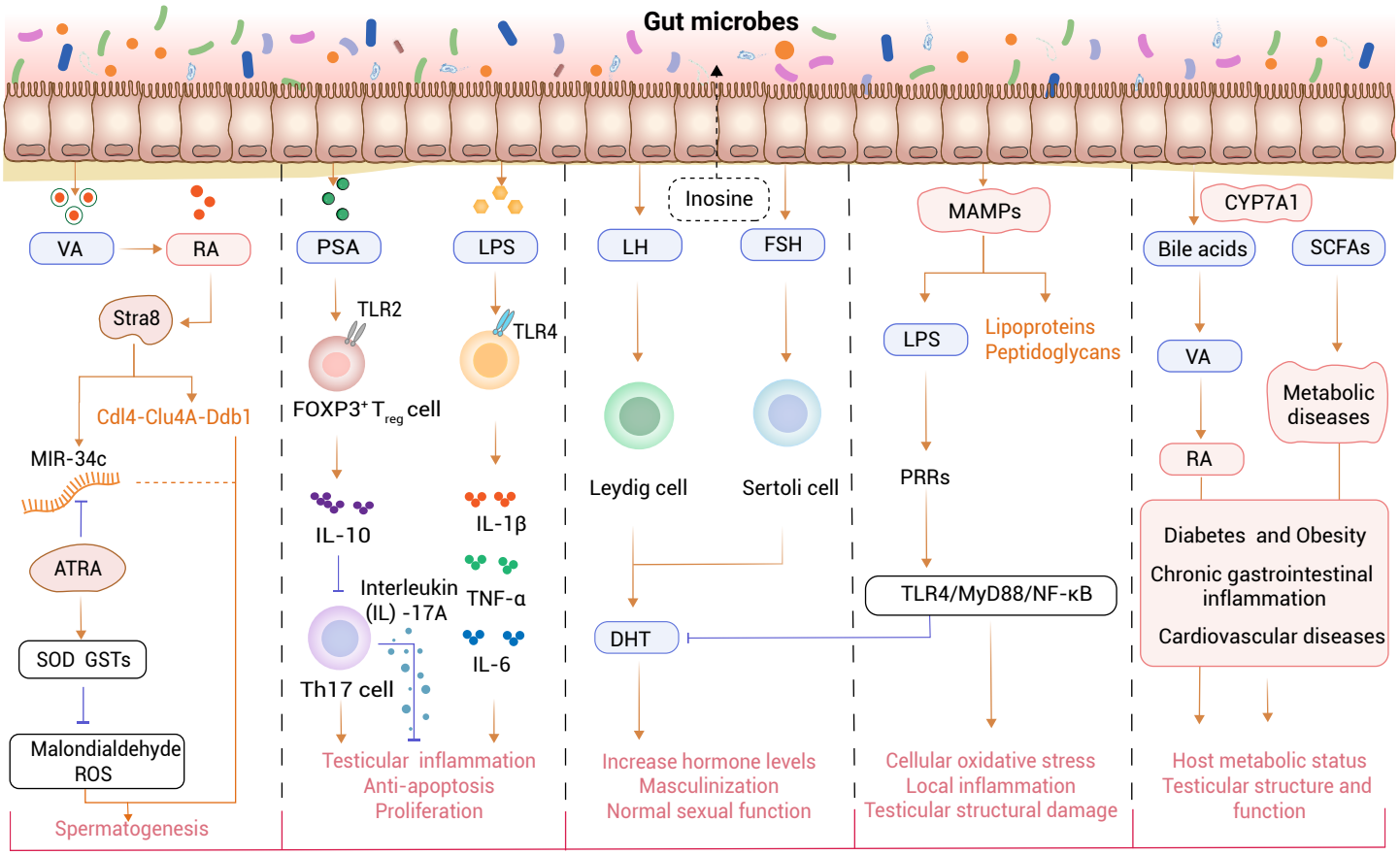

Figure 1. Modulation of host male reproduction by gut microbiota metabolites through five primary pathways (1) Nutritional effects: Vitamin A metabolites, such as retinoic acid and all-trans retinoic acid, promote spermatogenesis; (2) Immunomodulation: Polysaccharide A activates TLR2 signaling, promoting anti-inflammatory responses and enhancing testicular resilience to inflammation. Lipopolysaccharide (LPS) activates TLR4 signaling, triggering pro-inflammatory responses that may disrupt testicular function; (3) Hormonal effects: Luteinizing hormone (LH), follicle-stimulating hormone (FSH) and dihydrotestosterone (DHT) play a crucial role in the development and maintenance of masculinization, and normal sexual function; (4) Oxidative stress: Microbe-associated molecular patterns (MAMPs), such as lipopolysaccharide (LPS), lipoproteins, and peptidoglycans, bind to PRRs on the testicular cell membrane and trigger cellular oxidative stress, local inflammation, and testicular structural damage; (5) Metabolic pathways: Bile acid levels can affect vitamin A absorption, causing testicular dysfunction and abnormal spermatogenesis. Short-chain fatty acids (SCFAs) play a crucial role in the development of various metabolic disorders and host metabolic status, affecting testicular structure and function.

well as serum levels of gonadotropins, luteinizing hormone (LH), and follicle-stimulating hormone (FSH), are higher in the testes of normal mice or probiotic-colonized mice compared to germ-free mice.[61] These hormones play a crucial role in the development and maintenance of masculinization and normal sexual function.[62,63] In addition, inhibition of inosine metabolism of the gut microbiota can decrease testosterone secretion in the testis. Disrupting inosine metabolism through inhibition of *Akkermansia* leads to the downregulation of serum testosterone levels in male mice. Inosine supplementation may restore testosterone secretion, likely by promoting the recovery of the intestinal mucus barrier and regulating serum lipopolysaccharide levels.[64] However, whether gut microbiota or its metabolites directly affect the sex hormone biosynthesis in the gonad remains largely unknown.

GM metabolites affect testicular function through inflammation and oxidative stress. Inflammation, a rapid immune response to injury or infection, is initiated by the innate immune system to combat damage or pathogen invasion by activating innate immunity and initiating the recruitment of adaptive immune responses.[65] The initiation of inflammation and innate immunity relies on pattern recognition receptors (PRRs), such as Toll-like receptors (TLRs) and nucleotide-binding oligomerization domain-like receptors (NLRs), with TLRs being the most typical PRRs. PRRs recognize microbe-associated molecular patterns (MAMPs), such as lipopolysaccharide (LPS), lipoproteins, and peptidoglycans, triggering downstream signaling pathways that converge on the regulation of key transcription factors like NF-κB.[66] The entry of LPS into the bloodstream is a common consequence of systemic inflammation and oxidative stress, pathological processes closely associated with idiopathic male infertility.[67] LPS can inhibit the formation of testicular interstitial fluid and reduce the levels of testosterone within the testes and circulation across a wide range of doses. When intestinal barrier permeability increases, MAMPs can cross the intestinal barrier and enter the circulation, reaching the testes via the testicular artery, binding to PRRs on the testicular cell membrane, triggering cellular oxidative stress, local inflammation, and testicular structural damage.[68] Studies by Reddy et al. using a high-dose LPS-induced acute inflammation model in rats have shown a significant association between early and more severe damage events and testicular oxidative stress responses, specifically manifested by significantly increased lipid peroxidation levels, decreased antioxidant enzyme activity, mitochondrial dysfunction, and increased germ cell apoptosis.[69]

GM metabolites affect testicular function through metabolic pathways. Bile acids, crucial microbial metabolites synthesized from hepatic cholesterol, undergo further metabolism by the gut microbiota.[70] These molecules modulate various host physiological processes, including metabolic functions, by activating receptors in the intestine, liver, and peripheral tissues. The synthesis of bile acids is a complex process involving multiple enzymatic reactions, with at least 17 different bile acids identified to date.[71] The gut microbiota can regulate the expression of several key enzymes, including CYP7A1, CYP7B1, and CYP27A1, with CYP7A1 acting as the rate-limiting enzyme that determines the amount of bile acid produced.[72,73] A significant reduction in bile acid levels in the intestine can affect vitamin A absorption, leading to vitamin A deficiency in the testes. Retinoic acid, a metabolite of vitamin A, is an essential factor for the initiation of meiosis in germ cells; its deficiency can lead to

the blockage of spermatogonia differentiation, ultimately causing testicular dysfunction and abnormal spermatogenesis. Furthermore, the gut microbiota generates short-chain fatty acids (SCFAs) through the degradation of indigestible polysaccharides, such as dietary fiber and resistant starch. SCFA concentrations exhibit dynamic fluctuations throughout an individual's lifespan, closely correlated with the composition of the gut microbial community, which itself undergoes significant alterations across the life cycle.[74] SCFAs are implicated in the regulation of metabolic pathways and play a crucial role in the development of various metabolic disorders, including chronic gastrointestinal inflammation, cardiovascular diseases, diabetes, and obesity. Obesity has been demonstrated to impair male fertility and reproductive potential, primarily through the disruption of the hypothalamic-pituitary-gonadal axis, impairment of testicular steroidogenesis, and the induction of metabolic dysregulation.[75] Although direct evidence linking SCFAs to testicular structural damage is currently lacking, it is plausible, based on the aforementioned indirect evidence, that SCFAs may influence testicular structure and function, necessitating further experimental validation. Moreover, the study by Zhang et al. (2022) indicates that fecal microbiota transplantation can significantly increase "Alistipes" in the small intestine, consequently altering testicular lipid metabolism and the microenvironment. This leads to an elevation in testicular metabolites positively correlated with sperm quality, thereby contributing to the improvement of spermatogenesis and the restoration of sperm production.[76]

However, the gut microbiota generates a diverse array of metabolites, the functionality of which can be modulated by host metabolic status and environmental factors. For instance, short-chain fatty acids (SCFAs) may elicit varying physiological effects under different conditions. Consequently, advancements in quantitative metabolomics techniques, such as mass spectrometry, are essential to comprehensively capture the dynamic alterations in microbial metabolites.

Direct interaction with the bacteria

The interaction between GM and host not only involves sensitivity to bacterial metabolites but also direct interaction with the bacteria themselves. It is mainly reflected in the fact that the effects of live bacteria are usually different from those of inactivated bacteria. The functions of inactivated bacteria are actually the functions of metabolites, as inactivating bacteria can still retain pre-formed metabolites in their cellular structure. Live bacteria can continuously produce metabolites and exert dynamic regulatory effects. Compared with inactivated bacteria, live bacteria can improve the intestinal environment. Supplementation of beneficial bacteria in the intestine can increase the number of beneficial bacteria and inhibit the growth of harmful bacteria. Oral administration of live *Lactobacillus plantarum* TW1-1 reversed the increase in *Bacteroidetes* and decrease in *Firmicutes* induced by Diethylhexylphthalate (DEHP), and restored the presence of *Deinococcus* in mice exposed to DEHP, thereby alleviating DEHP-induced testicular damage in mice.[77] When gut microbiota from a high-fat diet are transplanted into mice on a normal diet, it causes disruption of spermatogenesis and a decrease in sperm motility.[78,79] The beneficial taxa such as *Bacteroidetes* and *Lactobacillus* in the mice intestine increase, while harmful bacteria such as *Desulfovibrio* decrease following fecal microbiota transplantation from alginate oligosaccharide-dosed mice, thus rescuing the spermatogenesis destroyed by busulfan.[10]

Microbiota-derived EVs as a shuttle for transferring bioactive cargoes

Although GM metabolites can affect host spermatogenesis and other traits, how these molecules cross the blood-testis barrier to reach the testes remains unclear. Shen et al. first demonstrated in 2012 that commensal bacteria can produce EVs, which seems to be able to reply.[80] Their report indicated that administering EVs isolated from *Bacteroides fragilis* yielded similar benefits as administering the bacteria themselves. This discovery, soon followed by others, provided a new perspective for understanding how gut bacteria affect host homeostasis and, more importantly, for understanding the systemic and distant effects of gut bacteria on the host. EVs are a crucial means of communication between cells, and both bacteria and mammals can produce EVs.[81,82] This article focuses on bacteria-derived EVs (BEVs) as a shuttle for transporting bioactive substances. BEVs are tiny membrane-bound vesicles vesicles that contain a wide range of biologically active compounds (such as mRNA, miRNA, DNA, proteins, lipids and carbohydrates),[83] thus enabling the horizontal transfer of their cargo to occur across intracellular and intercellular space (Figure 2).

According to their outer membrane properties, bacteria can be divided into two categories: Gram-negative (G−) bacteria and Gram-positive (G+) bacteria. The vesicles produced by the outer membrane of G-bacteria are called outer membrane vesicles (OMV).[84,85] In addition, some pathogenic G-bacteria can produce another type of vesicle, called inner and outer membrane vesicles (IOMV).[86] *C. Volgers* and his team reviewed three models showcasing OMV production in 2018.[86] Correspondingly, OMVs are generated under three scenarios: when outer membrane asymmetry is established (Model A), when misfolded proteins accumulate in the outer membrane (Model B), and when lipopolysaccharides undergo modification (Model C). Compared with G-bacteria, G+bacteria utilize different mechanisms to release BEVs. Vesicles are pushed through thick membranes by them through swelling pressure, protease-induced lysis, or protein channels.[87] BEVs are rich in molecular components such as nucleic acids, lipids, and various proteins, including microbial metabolites.[88,89] Moreover, due to their small size and good stability, they are easy to cross physiological barriers. Therefore, BEVs can serve as a shuttle for transporting bioactive goods such as microbial metabolites to influence the host. *O'Donoghue* et al. proposed three main mechanisms in 2016 to describe the interaction between BEVs and their hosts: binding to cellular receptors and activating cellular responses; completely integrating into the cytoplasm of the cell; delivering their contents to the host cells.[90] However, a comprehensive view of how the BEVs interact with human cells is not yet available.

At present, the impact of extracellular vesicles of GM on the host mainly focuses on the gut-microbiota-brain axis and immune regulatory function. For example, EVs derived from *Bifidobacterium longum* and *Bifidobacterium bifidum* can inhibit allergic diarrhea by inducing mast cell apoptosis and facilitating the differentiation of naive T cells into Tregs, respectively.[91,92] Although there have been numerous reports that GM affects host male reproduction (discussed in previous sections); there are also reports that EVs derived from testicular cells play an important role in maintaining normal spermatogenesis in the testes, such as cell-secreted vesicles containing microRNAs as regulators of gamete maturation.[93] However, the role of EVs derived from GM in host male reproduction is still unclear. Therefore, future research is required to clarify whether and how EVs derived from GM affect host male reproduction.

Furthermore, the methodologies for the isolation and characterization of bacterial extracellular vesicles (BEVs) are still in their nascent stages, with technical limitations affecting purity and source identification, thereby restricting the precision of research. Concurrently, the physiological roles of BEVs, encompassing mechanisms such as trans-organ transport, cellular uptake, and signal transduction, remain incompletely elucidated. To comprehensively investigate the association between BEVs and male reproductive function, research necessitates the collection of fecal, blood, and seminal fluid samples from males with reproductive disorders. Employing multi-omics approaches, including 16S rRNA sequencing, metagenomic sequencing, and metabolomics analyses, is crucial for a comprehensive assessment of the correlations between gut microbiota, BEV characteristics, and reproductive function indicators. This approach aims to offer novel perspectives for the diagnosis and treatment of male reproductive disorders.

Testicular mitochondria as mediators for gut microbiota to regulate host male reproduction

Mitochondria are intracellular organelles that play a crucial role in energy production.[94,95] Sperm is a highly specialized cell that requires a sustained and effective energy supply at different stages of the male reproductive process to perform its various functions.[96] Mitochondria can provide energy to sperm through the energy produced by cellular respiration.[97] Therefore, the complete physiological function of mitochondria is crucial for maintaining the vitality and vigor of sperm.[98] In sperm, mitochondria are not only responsible for cell energy supply but also for the regulation of apoptosis,[99] Ca^{2+} homeostasis required for flagellum motility,[100] for capacitation, and acrosomal reaction.[98,101] In addition, in the male reproductive system, mitochondria are

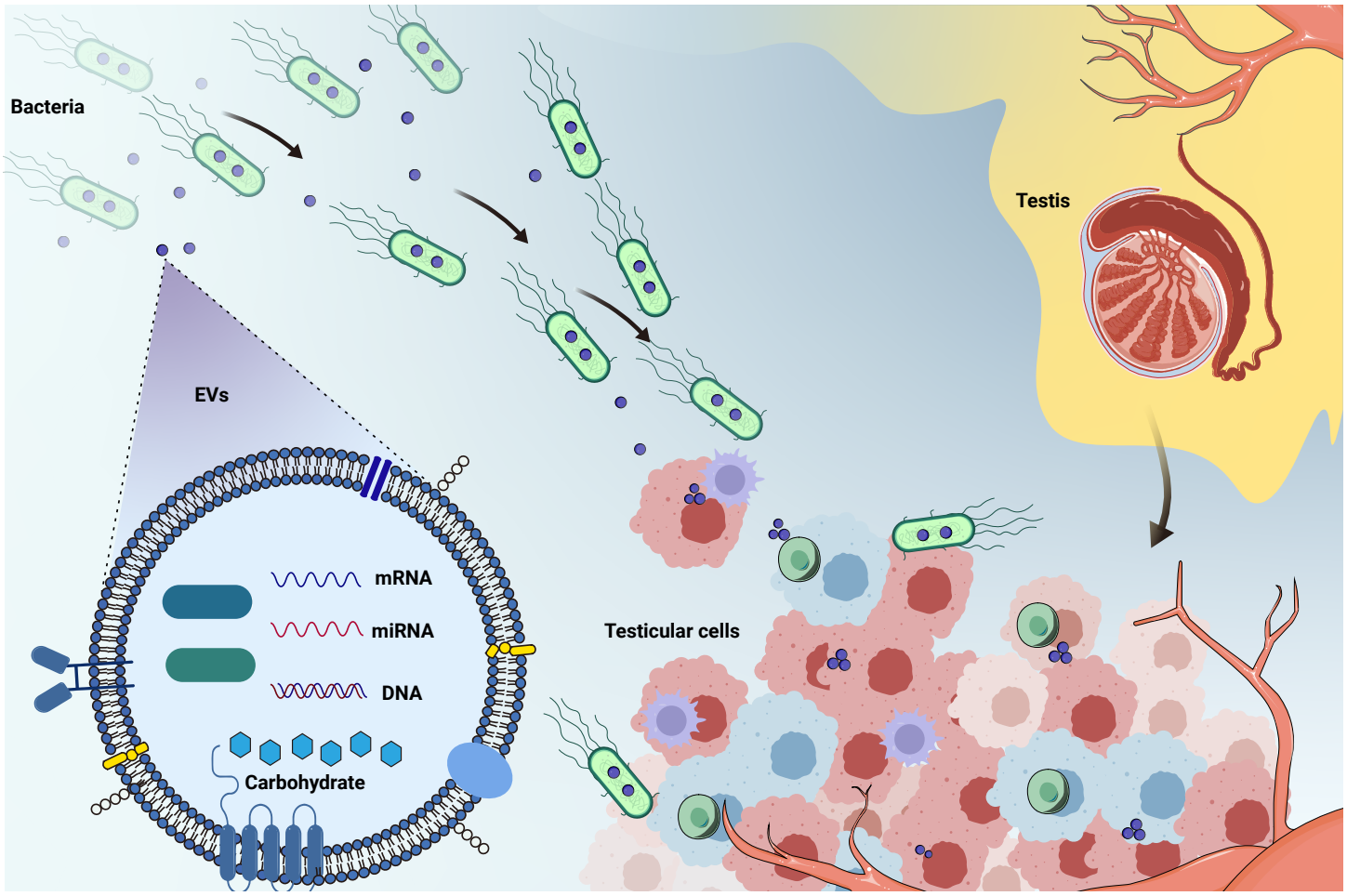

Figure 2. Mechanism by which bacterial-derived extracellular vesicles (EVs) act as mediators to transfer bioactive molecules (e.g., proteins, RNA, lipids) to host cells such as testicular cells EVs, produced by both Gram-negative and Gram-positive bacteria, interact with host cells via three primary mechanisms: (1) receptor binding, (2) cytoplasmic integration, and (3) cargo delivery. These interactions may modulate male reproductive function by influencing spermatogenesis, immune responses, and hormonal homeostasis.

the starting point for steroid hormone biosynthesis, as the conversion of cholesterol to pregnenolone (a common precursor of all steroid hormones) is achieved through the activity of cytochrome P450 side-chain cleavage enzyme (P450scc) on the inner membrane of mitochondria.[102] Mitochondria are also crucial for Leydig cells, which are essential somatic cells in the testes as they produce high levels of testosterone.[103] Testosterone is essential for male physiological processes, including spermatogenesis. In summary, testicular mitochondria are vital in the male reproductive system.

The mitochondrial endosymbiosis hypothesis suggests that mitochondria evolved from bacteria, meaning that after being engulfed by eukaryotes, bacteria evolved to form mitochondria through long-term symbiosis.[104] Mitochondrial components have significant similarities with bacterial components, making them more likely to become "immune recognition receptors". Therefore, when the GM reaches the testes in some way, it is highly likely to act on the mitochondria. Recent studies have demonstrated that the gut microbiota exerts its influence on host male reproduction through testicular mitochondria.[11,105-111] (Table 1) Key findings encompass: (1) Bisphenol A-induced gut dysbiosis exacerbates oxidative stress and mitochondrial apoptosis, culminating in reduced sperm counts; (2) High-fat diets compromise sperm motility through the downregulation of mitochondrial gene expression; This bidirectional regulation indicates that a balanced gut microbiota can maintain sperm quality through mitochondrial mechanisms; and disrupted gut microbiota can disrupt this balance, leading to mitochondrial dysfunction and affecting sperm quality or normal male function. Overall, testicular mitochondria can serve as a mediator for GM to regulate host male reproduction.

The impact of dietary and lifestyle interventions and their clinical implications

High-fat diet-induced gut microbiota dysbiosis can initiate metabolic syndrome (MetS), subsequently impacting male reproductive function.[44] Both obesity and high-fat diets lead to reducing gut microbiota diversity and richness. This dysbiosis may indirectly impair testicular function by disrupting the immune system.[112] Furthermore, an imbalance in the gut microbiota elevates epididymal inflammation, diminishes sperm motility, and significantly downregulates the expression of mitochondrial function-related and meiosis-related genes in testicular cells.[11]

The Mediterranean Diet (MD), rich in fruits, vegetables, whole grains, nuts, fish, and olive oil, has been shown to benefit cardiovascular health and significantly enhance male fertility through multiple mechanisms. Population studies indicate that high adopting the mediterranean diet is strongly linked to an increase in the *Bacteroidetes phylum* within the gut microbiota and elevated fecal short-chain fatty acid levels.[113] Long-term adherence to the Mediterranean diet may exert a more positive influence on the composition and diversity of the gut microbiota compared to short-term dietary adjustments. This dietary pattern, by providing abundant dietary fiber and antioxidants, improves the stability and diversity of the gut microbiota, thereby reducing systemic inflammation and optimizing the testicular microenvironment.[114]

Probiotics, including strains like Lactobacillus and Bifidobacterium, show potential in indirectly supporting male reproductive health by regulating the composition of gut microbiota, improving intestinal barrier function, and mitigating inflammatory responses. While promising, current evidence is primarily derived from preclinical models: supplementation with probiotics can restore testosterone levels in aging mice, improve seminiferous tubule structure, and facilitate the generation of beneficial metabolites like short-chain fatty acids (SCFAs), thereby optimizing the testicular microenvironment and reducing systemic inflammation.[115] In relevant studies, the probiotic strains Lactobacillus fermentum NCDC 400 and Lactobacillus rhamnosus NCDC 610 have been found to ameliorate reproductive function in high-fat diet-induced

Table 1. Studies elucidating the regulatory role of the gut microbiota on host male reproduction via testicular mitochondria.

Cause	Gut microbiota	Effect	Mechanism	Reference
Bisphenol A	Gut microbiota disorder	Sperm count↓, and sperm abnormalities↑	Oxidative stress↑and mitochondrial apoptosis↑	105
Vitamin B12	*Escherichia coli*	Late fertility↑	mtGTP level↑	106
Bacterial-derived mitochondrial co-enzymes	Gut microbiota	Gamete generation and fecundity↑	Mitochondrial activity↑ and ATP levels↑	107
High-fat diet	Gut microbiota disorder	Sperm motility↓	Mitochondrial gene expression associated with sperm motility↓	11
Incubation of sperm suspension with Escherichia coli	*Escherichia coli* and its soluble factors	Sperm motility↓	Mitochondrial membrane potential↓	108
Sperm agglutinating factor	*Escherichia coli*	Sperm motility and viability↓	Mitochondrial ATPase activity↓	109
Menadione	-	Flagellar ATP utilization or contractile↓	Mitochondrial ROS↑	110
Doxycycline	Gut microbiota disorder	Sperm motility and count↓	Mitochondrial function in Leydig cells↓	111

obese mouse models, restore testosterone levels, and significantly enhance sperm motility.[116] Furthermore, the gut microbiome can adjust the permeation capacity of the blood-testis barrier (BTB), consequently impacting testicular function.[117] These findings support the significant efficacy of probiotics in ameliorating male infertility issues, highlighting their substantial therapeutic potential and promising application prospects, which warrant further investigation. However, clinical translation faces critical limitations: individual responses vary significantly due to baseline gut microbiota composition and host genetics; standardized protocols for strain selection, dosage, and duration remain lacking; and long-term safety data (e.g., potential overgrowth of supplemented strains disrupting native microbial balance) are insufficient. These factors necessitate cautious interpretation of preclinical efficacy when extrapolating to human applications.

Fecal microbiota transplantation (FMT) facilitates functional crosstalk between the gut microbiota and the testes.[118] By transplanting the gut microbiota from a healthy donor into a recipient animal, FMT can re-establish the equilibrium of the recipient's gut microbial community. For instance, FMT of the gut microbiota from high-fat diet-induced obese mice into normal diet mice can lead to an increase in Bacteroides and Prevotella in the recipient mice, subsequently triggering elevated levels of endotoxins within the gut. This, in turn, activates systemic inflammatory responses, ultimately resulting in testicular inflammation and impaired sperm motility.[11]

Furthermore, FMT is capable of restoring the diversity and stability of the gut microbiota, increasing the proportion of beneficial bacteria, such as Parabacteroides distasonis. These beneficial bacteria can synthesize polyamines, such as spermine, which play a crucial role in testicular function, including antioxidant activity, regulation of ion channels, maintenance of reproductive physiology, and reversal of testicular damage.[119] As an emerging therapeutic approach, FMT, by modulating the composition and function of the gut microbiota, holds promise as an innovative and effective treatment for male reproductive dysfunction, offering new possibilities for improving semen quality and enhancing male fertility. For example, research by Zhao et al. demonstrated that the combination of alginate oligosaccharide (AOS) and FMT could increase the proportion of beneficial microorganisms in the gut microbiota, improve the systemic metabolic environment and testicular microenvironment, thereby promoting spermatogenesis.[120] Nevertheless, clinical application is hindered by significant challenges: variable individual responses, lack of standardized protocols, and potential risks (e.g., FMT-related infections). Most current evidence remains preliminary, with large-scale, long-term clinical trials needed to validate therapeutic potential.

The gut microbiota exhibits significant dynamism, with its composition and functionality being profoundly influenced by various factors, including diet, antibiotic exposure, and stress.[121,122] Clinical investigations have demonstrated that interventions such as dietary modifications, probiotic supplementation, lifestyle adjustments, and fecal microbiota transplantation (FMT) can effectively modulate the structure and function of the gut microbiota,[123-125] though their efficacy in improving male reproductive health ranges from well-supported (e.g., dietary fiber's role in SCFA production) to preliminary (e.g., FMT's direct impact on spermatogenesis). These interventions offer potential novel strategies for male infertility, but their clinical utility requires further validation through rigorous, standardized research.

CONCLUSION AND FUTURE

Accumulating evidence suggests that GM affects the development and function of the male reproductive system. The gut-microbiota-testis axis will be a reliable hypothesis to explain the correlation between GM and testis. In order to clarify the mechanism by which GM regulates male reproduction, this article summarizes the mechanisms by which GM regulates male host reproduction from the perspectives of what components of GM, how they reach the testis, and how they affect testicular function. From the perspective of what components of GM, we reviewed how the metabolites of GM regulate host male reproduction through nutritional, immune, and hormonal functions. In addition, GM itself, as live bacteria, can also have an impact on the male reproductive system of the host. From the perspective of how they reach the testis, we reviewed EVs as a shuttle for GM to transport active ingredients and proposed that GM may transport its metabolite components to the testes through EVs to regulate the male reproductive system. Furthermore, from the perspective of how they affect testicular function, considering the hypothesis of mitochondrial endosymbiosis and the importance of testicular mitochondria to the male reproductive system, we pointed out that testicular mitochondria can serve as a key mediator for GM regulation of host male reproduction. Finally, dietary and lifestyle factors may affect the dynamic changes of gut microbiota, and interventions such as probiotics and prebiotics can be used as a starting point to improve male reproductive health issues.

To further systematize these findings, we propose integrating the gut-microbiota-testis axis regulatory effects into a four-level multidimensional framework: (1) System: Gut-testis bidirectional communication via circulatory (e.g., metabolite transport), neural (e.g., gas signaling), and immune (e.g., cytokine diffusion) pathways; (2) Organ: Testicular homeostasis (spermatogenesis, steroid synthesis, immune balance); (3) Cell: Regulation of spermatogenic (differentiation), Sertoli (nutrient supply), and Leydig (testosterone secretion) cells; (4) Genetic: Modulation of spermatogenesis-related (e.g., STRA8) and mitochondrial function genes (e.g., ATP synthesis, redox). This framework clarifies the hierarchical regulatory logic underlying the mechanisms summarized above, thereby offering a more systematic basis for translating these insights into targeted clinical interventions

Hope it will contribute to the development of promising diagnostic and treatment strategies for both male infertility treatment and contraception. For infertility, targeted GM modulation (e.g., probiotics or inosine supplementation) may improve spermatogenesis and sperm quality by regulating metabolites, EV signaling, or mitochondrial function. For contraception, disrupting key crosstalk (e.g., inhibiting testosterone-related bacterial metabolites) could offer reversible, non-invasive options. This links basic research to clinical needs in male reproductive health.

However, there are still many issues to be solved. The investigation of bacterial extracellular vesicles (BEVs) in male reproductive disorders is poised to advance through innovative methodologies and multi-omics integration, addressing current limitations in isolation, characterization, and mechanistic understanding. Future research could consider leveraging multi-omics

frameworks—including 16S rRNA sequencing for gut microbiota profiling, organ-on-chip models to study gut-testis crosstalk, metagenomics for functional pathway analysis, and metabolomics to comprehensively assess the correlations between gut microbiota, BEV characteristics, and reproductive function indicators. These efforts will not only refine diagnostic biomarkers but also inspire BEV-based therapeutics, such as engineered vesicles for targeted drug delivery across the blood-testis barrier, ultimately bridging mechanistic insights with clinical translation. In addition, in the following research, scholars need to pay attention to the impact of partial and/or complete functions of GM on male reproductive ability, CRISPR-based modulation of specific bacterial pathways to validate mechanisms, and develop a range of microbial compound preparations aimed at enhancing or suppressing fertility without compromising normal health.

ABBREVIATIONS

GM	Gut microbiota
EVs	Extracellular vesicles
SCFAs	Short-chain fatty acids
BTB	Blood-testis barrier
FMT	Fecal microbiota transplantation
VA	Vitamin A
RA	Retinoic acid
STRA8	Retinoic Acid Gene 8
ATRA	All-trans retinoic acid
SOD	Superoxide dismutase
GST	Glutathione transferase
LPS	Lipopolysaccharide
DHT	Dihydrotestosterone
LH	Luteinizing hormone
FSH	Follicle-stimulating hormone
PRRs	Pattern recognition receptors
TLRs	Toll-like receptors
NLRs	Nucleotide-binding oligomerization domain-like receptors
MAMPs	Microbe-associated molecular patterns
MetS	Metabolic syndrome
MD	Mediterranean Diet
AOS	Alginate oligosaccharide
BEVs	Bacterial extracellular vesicles

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DECLARATION OF INTERESTS

The authors declare no competing interests.

ETHICAL STATEMENT AND PATIENT CONSENT

Not applicable.

DATA AND CODE AVAILABILITY

Not applicable.