Bacterial vaginosis (BV) is the most common vaginal infection found in women in the world. Due to increasing drug-resistance of virulent pathogen such as Gardnerella vaginalis (G. vaginalis), more than half of BV patients suffer recurrence after antibiotics treatment. Here, metastable iron sulfides (mFeS) act in a Gram-dependent manner to kill bacteria, with the ability to counteract resistant G. vaginalis for BV treatment. With screening of iron sulfide minerals, metastable Fe₃S₄ shows suppressive effect on bacterial growth with an order: Gram-variable G. vaginalis > Gram-negative bacteria >> Gram-positive bacteria. Further studies on mechanism of action (MoA) discover that the polysulfide species released from Fe₃S₄ selectively permeate bacteria with thin wall and subsequently interrupt energy metabolism by inhibiting glucokinase in glycolysis, and is further synergized by simultaneously released ferrous iron that induces bactericidal damage. Such multiple MoAs enable Fe₃S₄ to counteract G. vaginalis strains with metronidazole-resistance and persister in biofilm or intracellular vacuole, without developing new drug resistance and killing probiotic bacteria. The Fe₃S₄ regimens successfully ameliorate BV with resistant G. vaginalis in mouse models and eliminate pathogens from patients suffering BV. Collectively, mFeS represent an antibacterial alternative with distinct MoA able to treat challenged BV and improve women health.

1. Introduction

Bacterial vaginosis (BV) is the most common vaginal infection among women with the population prevalence up to 29% in the world.¹ The estimated economic burden for BV treatment may cost the world around $5 billion annually. Of clinical consequence, BV is associated with preterm birth and increased risk for acquisition of human immunodeficiency virus (HIV) and other sexually transmitted infections (STIs).² Normally BV is treated with oral metronidazole, vaginal metronidazole gel, or vaginal clindamycin.³ Despite the availability of these treatments, more than half of women suffer recurrence of BV within one year of treatment. As a primary pathogen in BV, G. vaginalis reside in vaginal flora which is normally predominated by the Lactobacilli species. But when Gardnerella bacteria become the dominant species, this leads to BV progress with the common symptom of vaginal discharge. G. vaginalis has been known to easily evolve metronidazole resistance, form persistent strains in biofilm or...
in host cells that protect them from antibiotics treatment. These resistance and persistence of pathogens result in the recurrence and relapse of BV. To tackle these issues, new concepts and approaches that differ from traditional antibiotics mechanism are required for the discovery of antibacterial alternatives that can overcome bacterial resistance and persistence without developing new drug-resistance and impacting probiotic species.

The development of nanotechnology provides lots of cutting-edged nanomaterials and strategies with nonantibiotic mechanisms against antimicrobial resistance. For instance, silver nanoparticles demonstrate a strong antibacterial activity to resistant bacteria and biofilm by penetrating or release silver ion into bacterial microenvironment. Although highly effective, silver nanomaterials perform high toxicity to host cells and also cause bacterial resistance. Recently, a class of nanomaterials with enzyme-like properties, which is termed as nanozymes, have shown promising capability to eliminate biofilm or intracellular bacteria. For instance, iron oxide nanozymes with peroxidase-like activity can disrupt biofilm by degrading matrix and suppress intracellular bacteria. However, these nanozymes often rely on the presence of hydrogen peroxide (H$_2$O$_2$) to achieve bacterial killing, which reduces the biocompatibility and thus limits their application for in vivo antibacterial therapy. Our recent studies discovered that iron sulfide nanomaterials (nFeS) exhibited high antibacterial activity by sustained releasing iron and polysulfides. Impressively, the nFeS not only exhibited a broad spectrum to both Gram-positive and Gram-negative bacteria, but also eliminated biofilm and intracellular bacteria. Further studies revealed that the antibacterial activity was dependent on a ferroptosis-like death in bacteria dominated by the released ferrous iron from nFeS under the condition without molecules that can chelate iron or resist oxidative damage. Unfortunately, the antibacterial performance of nFeS dramatically decreased for bacteria like *Escherichia coli* or *Staphylococcus aureus* in nutrient-rich media or physiological environment, making them equivocal for in vivo antibacterial therapy.

Here, we conducted a screen of iron sulfides including Fe$_3$S$_4$ (greigite), Fe$_S$S$_6$ (pyrrhotite), FeS and FeS$_2$ (pyrite) (Figure 1A) and investigated their MoA against Gram-positive *S. aureus*, Gram-negative *E. coli* and Gram-variable *Gardnerella vaginalis* under culture media conditions. Our experimental design allows to identify the exact crystal phase and components of iron sulfides in antibacterial process, as there are many crystal phases for iron sulfides and mutually transformation may occur between them. We found that metastable iron sulfides (mFeS), in particular for Fe$_3$S$_4$, exhibited highly antibacterial activity preferably against *G. vaginalis* through the MoAs of glycolysis inhibition and ferroptotic damage. This synergistic manner of action ensured that Fe$_3$S$_4$ counteracted the drug-resistance of *G. vaginalis*, and demonstrated superior effects than silver nanoparticles and the first line medication of metronidazole for treating BV in mouse models and eliminating pathogens from clinical samples.

2. Results

2.1. Metastable Iron Sulfides Exhibit Antibacterial Activity with Gram-Dependent Activity

To assess the activity of iron sulfides against bacteria under culture conditions, we first chose four types of iron sulfide phases both via nanosynthesis and from natural mineral sources, including DADS-derived Fe$_3$S$_4$ (D-Fe$_3$S$_4$), Zirantong (z-Fe$_3$S$_4$), which was processed by a hyperthermia-vinegar quenching method from traditional Chinese medicine), commercialized FeS (Sigma, s-FeS), commercialized natural FeS$_2$ (Sigma, s-FeS$_2$) respectively. The D-Fe$_3$S$_4$ was synthesized using previous hydrothermal reaction with amended parameters to produce pure-phase greigite. We then examined these minerals using SEM imaging. As shown in Figure S1A (Supporting Information), D-Fe$_3$S$_4$ exhibited nanoscale morphologies of nanosheets and z-Fe$_3$S$_4$ exhibited bulk block with nanorod-like surface. In contrast, both s-FeS and s-FeS$_2$ displayed as bulk blocks with flatten surface. Our subsequent X-ray diffraction (XRD) analysis confirmed that the prepared D-Fe$_3$S$_4$, z-Fe$_3$S$_4$, s-FeS$_2$ and s-FeS were in pure phases of Fe$_3$S$_4$, Fe$_S$S$_6$, FeS$_2$ and Fe$_S$, respectively (Figure S1B, Supporting Information). A schematic of atom structures representing the phases of Fe$_3$S$_4$, Fe$_S$S$_6$, FeS$_2$ and Fe$_S$ was shown according to the reported crystal information of iron sulfides (Figure 1A,B).

To test the antibacterial activity of D-Fe$_3$S$_4$, z-Fe$_3$S$_4$, s-FeS$_2$, and s-FeS, we assessed the bacterial growth by measuring the optical density of the culture at 600 nm (OD$_{600}$) in liquid media post 24 h. We first tested the inhibition efficiency of iron sulfides against Gram-positive strain *S. aureus* as well as Gram-negative strain *E. coli*. Surprisingly, we failed to detect any inhibitory effects for *S. aureus* using the above iron sulfides (at 500 × 10$^{-6}$ m) (Figure 1C), while we found that D-Fe$_3$S$_4$ and z-Fe$_3$S$_4$ exhibited partial inhibition at the early stages of growth for *E. coli* (Figure 1D).

We next tested *G. vaginalis* (GV14018) as a representative of Gram-variable bacteria as it has a thin wall consisted of peptidoglycan without outer membrane, but appears to be Gram-negative species under Gram staining. As shown in Figure 1E, *G. vaginalis* was completely suppressed in the presence of synthesized D-Fe$_3$S$_4$ at 128 × 10$^{-6}$ m, and partially inhibited in the presence of z-Fe$_3$S$_4$ at 500 × 10$^{-6}$ m. In contrast, neither s-FeS nor s-FeS$_2$ inhibited bacterial growth. In addition, we determined the minimal inhibitory concentration (MIC) of D-Fe$_3$S$_4$ against *G. vaginalis* and found that D-Fe$_3$S$_4$ exhibited MIC at 89.6 × 10$^{-6}$ m, while metronidazole at 256 × 10$^{-6}$ m inhibited bacterial growth (Figure S1C,D, Supporting Information). When measuring bacterial availability using agar plate counting, we found that 99.99% of *G. vaginalis* bacteria were dead after 6 h incubation (Figure S1E, Supporting Information). Furthermore, while D-Fe$_3$S$_4$ showed long-term (up to 96 h) suppression of *G. vaginalis* growth at 128 × 10$^{-6}$ m, metronidazole failed to completely inhibit bacterial growth after 24 h (at 1024 × 10$^{-6}$ m) as well 72 h (at 2048 × 10$^{-6}$ m) (Figure 1F). We also tested the antibacterial activity of DADS, an antibacterial ingredient in garlic oil that is used as a S donor for D-Fe$_3$S$_4$ preparation. As shown in Figure 1C–E, DADS failed to inhibit the growth of *S. aureus*, *E. coli* or *G. vaginalis*. Together, these results showed that antibacterial activity of iron sulfides follows the order as: D-Fe$_3$S$_4$ > z-Fe$_3$S$_4$ > s-FeS and s-FeS$_2$. Among them, D-Fe$_3$S$_4$ possesses the highest antibacterial activity and preferentially affects bacteria in the following order: Gram-variable *G. vaginalis* > Gram-negative *E. coli* > Gram-positive *S. aureus*, indicating strict Gram-selectivity. For all subsequent mechanisms and characterizations of antibacterial activity, we chose D-Fe$_3$S$_4$.

Further characterizations with high-resolution SEM and TEM demonstrated that the lateral size and the thickness of D-Fe$_3$S$_4$ nanosheets were at 491.10 ± 264.50 nm and 47.92 ± 10.72 nm (mean ± SD), respectively. The HR-TEM pat-
Figure 1. Metastable iron sulfides exhibit antibacterial activity in a Gram-dependent manner. A, B) A schematic atom structure represents crystal phases of four iron sulfides with different bond length B) of Fe–S. Left to right: Fe₃S₄, Fe₇S₈, FeS₂, FeS. C) Antibacterial activity of iron sulfides against Gram-positive S. aureus in culture media. D) Antibacterial activity of iron sulfides against Gram-negative E. coli in culture media. Both D-Fe₃S₄ and z-Fe₇S₈ exhibited partial inhibitory effects on E. coli. E) Antibacterial activity of iron sulfides against Gram-variable G. vaginalis in culture media. D-Fe₃S₄ exhibited the robust antibacterial activity followed by z-Fe₇S₈. In contrast, neither s-FeS nor s-FeS₂ exhibited any antibacterial effects. Concentration for z-Fe₇S₈, s-FeS and s-FeS₂ was adjusted to 500 × 10⁻⁶ M. F) D-Fe₃S₄ completely suppressed G. vaginalis without regrowth. In contrast, metronidazole (at 1024 × 10⁻⁶ M or 2048 × 10⁻⁶ M) cannot achieve complete suppression, as bacteria regrow after 24 h or 72 h. G) Fe²⁺ released from different iron sulfides. H) Polysulfides released from different iron sulfides. n = 3, ****p < 0.0001. Representative images are shown. Mean±SD are shown.

terns illustrated that the lattice fringe spacing of 0.298 nm was consistent with the interplanar distance of the (311) plane of the Fe₃S₄ phase and the electron diffraction pattern (the inset) indicated that the nanostructure was single crystal (Figure S2A–C, Supporting Information).

To better understand the differences in antibacterial activity of our four selected iron sulfides, we analyzed their ability to release iron and polysulfide into aqueous media. In our previous studies, we previously found that ferrous iron and polysulfides released from nanoiron sulfide are critical for antibacterial reactivity.\textsuperscript{[11,13]} We thus speculated that the difference in antibacterial activity of D-Fe₃S₄, z-Fe₇S₈, s-FeS and s-FeS₂ may derive from the efficiency of releasing ferrous iron and polysulfides. To confirm this hypothesis, we first analyzed the bond length of Fe–S in iron sulfide structure. In the spinal structure of Fe₃S₄,\textsuperscript{[17]} the length of 3 Fe–S bond is 2.39 Å (Figure 1B). Fe₇S₈ have three Fe–S bonds which are at 2.29–2.34 Å and two Fe–S bonds which are 2.30 and 2.40 Å,\textsuperscript{[18]} respectively (Figure 1B). In contrast, the Fe–S bonds in FeS and FeS₂ are 2.17 Å\textsuperscript{[19]} and 2.25 Å,\textsuperscript{[20]} respectively (Figure 1B). Together, these data indicated that the Fe–S in Fe₃S₄ and FeS₈ is easily broken upon iron and sulfur release. To test this hypothesis, we next measured that the release of ferrous iron and polysulfides with Iron Assay Kit (SigmaAldrich) and sulfane sulfur probe 4 (SP4), respectively. We found that among the four iron sulfides, D-Fe₃S₄ released ferrous iron most efficiently and z-Fe₇S₈ showed half part of releasing, while FeS
and s-FeS2 showed negligible release of ferrous iron (Figure 1G). The release of polysulfides showed a trend similar to that of ferrous iron (Figure 1H). To identify the species of polysulfides, we used electrospray ionization mass spectrometry (ESI-MS) and identified that persulfide (S2−) and trisulfide (S3−) were released (Figure S1F, Supporting Information). In addition, SEM images demonstrated that a structure transformation occurred as small curl structure formed on the surface of nanosheet of D-Fe3S4 when dissolved in water for 72 h (Figure S2D,E, Supporting Information). Furthermore, energy dispersive X-ray spectrometry (EDS) analysis of the precipitate of D-Fe3S4 (incubated in water for 72 h) showed a decreased atom ratio (atom%) of S (from 37.36% (0 h) to 9.20% (72 h), while an increase ratio for oxygen (O) (Figure S2F–H, Supporting Information). Consistently, XRD analysis of D-Fe3S4 precipitate (incubated in water for 72 h) showed low and hetero peaks, which indicated that the precipitate was not the single Fe3S4 with good crystallinity, but a mixture with poor crystallinity probably containing oxyiron compounds (FeO or FeO2) (Figure S2I, Supporting Information). These data demonstrated a transformation from iron sulfide to iron oxide during polysulfide species release in aqueous condition, further indicating the metastable property of D-Fe3S4 under aqueous condition. Therefore, we hypothesized that the difference of releasing iron and polysulfide species is correlated with the antibacterial performance of the metastable iron sulfides.

### 2.2. Polysulfide Species Induce Gram-Dependent Antibacterial Action

To confirm this hypothesis, we tested the antibacterial activity of both ferrous iron and polysulfide species in vitro culture. In our previous study, we showed that under aqueous condition (e.g., water), ferrous iron induces ferroptotic damage in *S. aureus* and *E. coli*, which is synergized by glutathione (GSH) depletion mediated by polysulfide species. However, we found here that such antibacterial action is suppressed in culture media. In agreement with our earlier results, the D-Fe3S4 showed high antibacterial activity to both *S. aureus* and *E. coli* in water, while polysulfide species including S2− and S3− only showed negligible bactericidal effect (Figure S3A–C, Supporting Information), indicating that polysulfides lack the ability to kill bacteria in water. In contrast, when the test was conducted in culture media (e.g., LB), the polysulfide species showed no inhibitory effects on Gram-positive *S. aureus* (Figure 2A) and *Streptococcus mutans* (Figure 2D, Supporting Information), however, they partially suppressed the growth of Gram-negative *E. coli*, *Pseudomonas aeruginosa* (*P. aeruginosa*) and *Acinetobacter baumannii* (*A. baumannii*) (Figure 2B, and Figure S3E,F, Supporting Information) and completely suppressed the growth of Gram-variable *G. vaginalis* (Figure 2C). In the test for *E. coli*, S2−, S3− and S4− showed significant inhibition (at 500 × 10−6 M) on bacterial growth in the early stages of growth. In contrast, in the test for *G. vaginalis*, bacterial growth was completely suppressed at much lower concentration (at 128 × 10−6 M) of S2− and S3−. Even S2− exhibited achieved similar antibacterial effect at 250 × 10−6 M. However, neither SH− nor S2− showed inhibition on *S. aureus*, *E. coli*, or *G. vaginalis*. Furthermore, ferrous iron failed to affect bacterial growth when cultured in media (Figure 2E,F). Together, these data clearly demonstrated that polysulfide species are able to suppress bacterial growth in the presence of culture media. The antibacterial potency follows below orders: 

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S_2^{2−} > S_3^{2−} > S_4^{2−} > S^{2−} > HS^{−}.
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The bacteria tested in our experiments can be divided into three types: I) *S. aureus* and *S. mutans* are Gram-positive strains possessing thick bacterial wall. II) *E. coli*, *P. aeruginosa* and *A. baumannii* are Gram-negative strains whose cell wall is thin wall yet protected by additional outer membrane; III) *G. vaginalis* consists of cell wall without LPS but appears Gram-negative under the microscope[21] (Figure S3G,H, Supporting Information). We further characterized the wall thickness of the three bacteria using TEM and found that the wall thickness for *S. aureus*, *E. coli* and *G. vaginalis* were approx. 40.5 nm, 17.3 nm and 14.6 nm, respectively (Figure S3J, Supporting Information). Therefore, we speculated that antibacterial effects of polysulfide species occur in a Gram-dependent manner, and thus is likely to be related to the structure of the bacterial wall. To confirm this, we characterized the entry of polysulfide species and ferrous iron into bacteria using either a SSP4 probe (Green) or a FeRhoNoxTM-1 (Red) probe for our confocal microscopy analysis. As shown in Figure 2G, bright green signal was observed in *G. vaginalis* treated with D-Fe3S4 or Na2S3, which indicated that either polysulfide species or the released moieties enter bacteria. Similarly, strong red signal was present in *G. vaginalis* treated with D-Fe3S4 or FeCl2, indicating that ferrous iron readily accessed the bacterial cell wall (Figure 2H). However, we detected negligible signals for SSP4 and FeRhoNoxTM-1 probes in *E. coli* and *S. aureus* in the presence of D-Fe3S4, Na2S3, or FeCl2, indicating that neither polysulfide species nor ferrous iron access the barrier of bacterial wall (Figure S4A–D, Supporting Information). To verify that it is necessary for polysulfide species to cross the bacterial cell wall to exhibit antibacterial action, we next digested bacterial walls of *S. aureus* and *E. coli* using lysozyme. As shown in Figure S4E,F (Supporting Information), both D-Fe3S4 and Na2S3 exhibited significant inhibitory effects on *S. aureus* predigested with lysozyme. In addition, both D-Fe3S4 as well as Na2S3 completely blocked the growth of *E. coli* pre-digested with lysozyme (Figure S4G, Supporting Information). CFU counting assay showed that Na2S3 killed all pre-digested *E. coli* within 3 h in culture media (Figure S4H, Supporting Information). These results strongly suggest that the Gram-specific effects of polysulfide species on bacterial growth are directly related to the differences in bacterial wall structures (Figure 2I).

In order to assess the antibacterial efficiency of polysulfide species, we next measured their minimal inhibitory concentration (MIC) using *G. vaginalis* as model bacteria in culture media. As shown in Figure 2I, S2−, S3−, S4− exhibited the highest antibacterial activity against *G. vaginalis*, with MIC values of 115.2 × 10−6 M for S2− and 102.4 × 10−6 M for S3−, followed by S4− with MIC value at 460.8 × 10−6 M. Surprisingly, we found that the presence of iron dramatically reduced the MIC of polysulfide species, especially for S2−, for which we obtained values of 115.2 × 10−6 M for *G. vaginalis*. This phenomenon suggested that iron acts synergistically with polysulfides. To identify the mechanism behind this observation, we prepared a mixture of Na2S3 and either ferrous or ferric chloride. The resulting colloid solution primarily consists of nanoparticles of iron sulfide (Figure S4J, Supporting Information). When we measured the amount of polysulfide species
Figure 2. Polysulfide species exhibit Gram-dependent antibacterial activity. A–C) Polysulfide species exhibited different antibacterial activity against bacteria. Polysulfide species (Na$_2$S$_2$, Na$_2$S$_3$, and Na$_2$S$_4$) failed to suppress the growth of A) Gram-positive *S. aureus*, but partially suppress B) Gram-negative *E. coli*, and heavily blocked growth of C) *G. vaginalis*. The concentration of polysulfide species was $500 \times 10^{-6}$ M if not specified. The significant suppression on *G. vaginalis* was achieved with Na$_2$S$_2$ ($125 \times 10^{-6}$ M), Na$_2$S$_3$ ($125 \times 10^{-6}$ M), and Na$_2$S$_4$ ($250 \times 10^{-6}$ M), respectively. D–F) Iron (at $500 \times 10^{-6}$ M) failed to affect the growth of either D) *S. aureus*, E) *E. coli*, or F) *G. vaginalis*. G) Confocal imaging with SSP4 probe in *G. vaginalis* treated by D-Fe$_3$S$_4$ or Na$_2$S$_3$. Scale bars: 1 μm. H) Confocal imaging with FeRhoNoxTM-1 probe in *G. vaginalis* treated by D-Fe$_3$S$_4$ or FeCl$_2$. Scale bars: 10 μm. I) A schematic for antibacterial selectivity of polysulfide as a function of the bacterial wall. G+: Gram-positive. G-: Gram-negative. J) Minimal inhibitory concentration (MIC) of polysulfide species for *G. vaginalis*. Both S$_2^{2-}$ and S$_3^{2-}$ showed high antibacterial activity, while S$_4^{2-}$ exhibited limited antibacterial effects by itself, though its effects were enhanced in the presence of iron. n = 3, **p < 0.0001. Representative images are shown. Mean±SD are shown.
using SSP4 probe that strongly reacts with $S_2^{2–}$, we found that the added ferrous iron increased the $S_2^{2–}$ signal of Na$_2$S$_3$ (Figure S4K,L, Supporting Information). In particular, ferrous chloride generated more $S_2^{2–}$ species compared to ferric chloride. Therefore, iron may synergize the antibacterial activity of $S_2^{2–}$ by converting $S_2^{2–}$ into $S_2^{–}$ as the latter has superior antibacterial activity. Since our D-Fe$_3$S$_4$ released both ferrous iron and polysulfides and performed the lowest MIC value to $G. vaginalis$, this feature may ensure that large amount of $S_2^{2–}$ is generated in the antibacterial process. Therefore, we chose D-Fe$_3$S$_4$ for all subsequent experiments with $G. vaginalis$.

### 2.3. Polysulfide Induces Glycolysis Inhibition and Is Synergized by Ferrous Iron in $G. vaginalis$

To assess how D-Fe$_3$S$_4$ achieves selective antibacterial activity against $G. vaginalis$, we analyzed its effects at the biochemical level, energy metabolism level and transcription level. Since bacterial growth was suppressed in culture media, we therefore speculated that D-Fe$_3$S$_4$ interferes with the metabolism of $G. vaginalis$. To confirm this hypothesis, we assessed the metabolome and transcriptome of $G. vaginalis$ treated with D-Fe$_3$S$_4$ under non-lethal conditions ($50 \times 10^{-6}$ m D-Fe$_3$S$_4$). Compared to untreated $G. vaginalis$, the levels of phosphoenolpyruvate (PEP) were increased 5.7-fold in D-Fe$_3$S$_4$-treated bacteria compared to untreated $G. vaginalis$. While the levels of glucose 6-phosphate and fructose 6-phosphate decreased by 0.4-fold and 0.68-fold, respectively (Figure 3B). Next, we performed an in vitro enzyme assay to assess the effect of D-Fe$_3$S$_4$ on glycolytic enzymes. Due to the decrease of Glucose 6-phosphate, we thought the first step of glycolysis may be blocked. To this end, we cloned the gene of glucokinase in $G. vaginalis$ and transformed the gene into E. coli for protein expression (Figure S5A, Supporting Information). For the purified glucokinase, we found that the activities of glucokinase (GLK), a key enzyme involved in glycolysis,[22] was inhibited by D-Fe$_3$S$_4$ (Figure 3C). The inhibition was also observed in the lysate of D-Fe$_3$S$_4$-treated bacteria (Figure S5B, Supporting Information). We next assessed the transcriptional levels of energy metabolism using a transcriptome assay. We found that starch, sucrose, glycolysis/glucoseogenesis pathways were downregulated and the transcription for PTS sugar transporter subunit (EIIC) was increased in the presence of D-Fe$_3$S$_4$ (Figure S5C and Table S1, Supporting Information), which further confirms the lethal effects of D-Fe$_3$S$_4$ on the energy metabolism of $G. vaginalis$. Accumulation of PEP and the lack of glucose 6-phosphate are reliable indicators showing that the bacteria attempt to regulate bacterial phosphotransferase system (PTS) to acquire energy for bacteria growth.[23] The levels of ABC transporters related to sugar import processes[24] were also significantly suppressed (Figure S5C, Supporting Information). Together, these features clearly demonstrated that D-Fe$_3$S$_4$ causes cell death of $G. vaginalis$ by disrupting energy metabolism.

When we treated $G. vaginalis$ with $50 \times 10^{-6}$ m Na$_2$S$_3$, we found that Na$_2$S$_3$ exerted similar effects on metabolic and transcriptome levels to those caused by D-Fe$_3$S$_4$ (Figure S5D and Table S2, Supporting Information). Importantly, we found that GLK activity was also inhibited by polysulfides, thus showing that GLK activity is not affected by the presence of iron (Figure 3C). To understand the inhibitory mechanism of polysulfides, we predicted the 3D structure of GLK ($Gardnerella vaginalis$ ATCC 14018 = JCM 11026, GenBank: BAQ33377.1) in $G. vaginalis$ using I-TASSER server. As shown in Figure 3D, the $G. vaginalis$ GLK structure comprises a typical pocket-like active center. We then predicted glucose binding to the pocket using molecular dynamic (MD) calculation, which showed that the glucose (green molecule) bound to the inner side of the pocket and interacted with residues including Pro78, Asn117, Asp118, Val163 (Figure 3E,F). In addition, it clearly showed that a proximal loop connecting two β-sheets extended into the pocket. There are three cysteine residues located on the loop, which are Cys179, Cys181, and Cys186 (Figure 3G). We speculated that the thiols in these residues are the target of polysulfides to inhibit GLK activity. To confirm it, we first evaluated the feasibility of polysulfides ($S_2^{2–}$) reacting with thiol group in cysteine by calculating the energy barrier under aqueous condition (physiological pH, 37 °C). It showed that the energy barrier for the reaction of $S_2^{2–}$ and thiol group was only 15.6 kcal mol$^{-1}$, indicating the reaction readily occurs under physiological condition. This result suggested that $S_2^{2–}$ can react with the three cysteine residues on proximal loop of active pocket in GLK (Figure 3G). We then analyzed the binding conformation and calculated the values of interaction energy ($E_{int}$) for glucose with GLK before and after polysulfidation. Before polysulfidation, the GLK combines with glucose in its active pocket (complex I, Figure 3E,F) with an $E_{int}$ of $–220.3$ kJ mol$^{-1}$ (Figure 3J). Notably, the best binding site of glucose and GLK changed after the polysulfidation of cysteine residues as denoted in Figure 3H where the glucose was shown in blue. The corresponding $E_{int}$ was $–192.5$ kJ mol$^{-1}$ (complex 2, Figure 3J). The relative position of glucose before the polysulfidation of GLK was illustrated in red (Figure 3H) as well for clarity. If the glucose was docked into polysulfidated GLK at the location similar to that of the complex 1, as illustrated in Figure 3I, their $E_{int}$ increased to $–166.4$ kJ mol$^{-1}$ (complex 3, Figure 3J), which indicates that the polysulfidation was adverse for glucose binding to the active center of GLK. Collectively, based on MD analyses, the inhibition of polysulfides on GLK activity is possibly through the polysulfidation of cysteine residues proximally located on the active center of GLK.

In addition to the changes on metabolome and transcriptome, we also tested the ability of D-Fe$_3$S$_4$ to damage the bacterial morphology using TEM imaging. As shown in Figure 4A, when treated with $100 \times 10^{-6}$ m D-Fe$_3$S$_4$, the bacterial cells exhibited a deformed surface structure. In addition, the cytosol displayed a large hollow area, indicating that the membrane was disrupted and the cytoplasm content leaked into the extracellular space. To assess lipid peroxidation in $G. vaginalis$, we measured the fluorescent intensity using a C11 BODIPY probe, which stains lipid ROS. As shown in Figure 4B, lipid peroxidation increased when $G. vaginalis$ was treated with either D-Fe$_3$S$_4$ or FeCl$_3$. In contrast, we detected no signal for $G. vaginalis$ treated with Na$_2$S$_3$. When we used an iodonitrotetrazolium chloride assay to assess respiration of $G. vaginalis$, we found that the respiratory chain of complex I (NADH dehydrogenase) was inhibited (Figure 4C). Furthermore, when we measured the level of glutathione in $G. vaginalis$ treated with D-Fe$_3$S$_4$, we found that the ratio of GSH/GSSG was also inhibited by polysulfides.
Figure 3. Polysulfide kills *G. vaginalis* by disrupting energy metabolism. A) The heatmap of metabolome analysis showing that abnormal change of intermediates occurred in the glycolytic pathway in *G. vaginalis*. A is control group and B is Fe3S4 treated group (*n* = 8). B) Incubation of *G. vaginalis* in D-Fe3S4 and Na2S3 exhibited similar effects on glycolytic metabolism. C) Addition of D-Fe3S4 inhibited glucokinase (GLK) activity in *G. vaginalis*. The polysulfide species, such as S22– and S32– also showed inhibitory effects on GLK. D) Predicted protein structure of glucokinase (*Gardnerella vaginalis* ATCC 14018 = JCM 11026, GenBank: BAQ33377.1) shows an active pocket. E) The final conformation of glucose and glucokinase complex after 20 ns MD simulation. F) The key residues around the glucose in the active center of glucokinase. G) Polysulfidation of cysteine residues (Cys-179, Cys-181, and Cys-186) by polysulfides (S32–). H) The binding conformations of glucose with glucokinase with the most negative interaction energy (*E*int) before (red, complex 1) and after (blue, complex 2) polysulfidation of cysteine residues. I) The binding conformations of glucose and glucokinase with similar ligand positions before (red) and after (blue, complex 3) polysulfidation of cysteine residues. J) The *E*int between glucose and glucokinase in different binding conformations. *n* = 3, ***p* < 0.001, ****p < 0.0001. Representative images are shown. Mean±SD are shown.
Figure 4. Iron synergizes antibacterial activity by inducing ferroptotic damage. A) TEM image of G. vaginalis treated with D-Fe$_3$S$_4$ showing leakage of intracellular cytoplasm. Concentration for D-Fe$_3$S$_4$ was 100 $\times$ 10$^{-6}$ m. Scale bar = 500 nm. B) Lipid peroxidation in G. vaginalis induced by D-Fe$_3$S$_4$. FeCl$_2$ alone resulted in high levels of lipid peroxidation, while Na$_2$S$_4$ failed to induce lipid peroxidation, indicating that the increase in lipid peroxidation was caused by iron. C) The respiratory chain of G. vaginalis was interrupted by D-Fe$_3$S$_4$. The activity of NADH dehydrogenase in complex I of respiratory chain was dramatically reduced. D) The ratio of GSH/GSSG decreased upon D-Fe$_3$S$_4$ treatment, indicating that D-Fe$_3$S$_4$ is causing GSH depletion in G. vaginalis. E) ATP production was reduced in G. vaginalis upon D-Fe$_3$S$_4$ treatment, indicating that energy metabolism was affected by D-Fe$_3$S$_4$. F–H) the inhibition of D-Fe$_3$S$_4$ on G. vaginalis is countered by F) Ferrostatin-1 (inhibitor of ferroptosis), TCEP (an iron chelator and reducing agent of G) S–S bond), and H) GSH. I) Drug resistance of G. vaginalis treated under sub-lethal concentration (32 $\times$ 10$^{-6}$ m) of D-Fe$_3$S$_4$. The MIC remained constant after 20 passages of G. vaginalis. J) A schematic summary of the mechanisms causing bacterial death of G. vaginalis upon ferrosulfide effect induced by iron polysulfide. 1, glucokinase inhibition, 2, glutathione (GSH) depletion, 3, respiratory chain depression, 4, lipid peroxidation. n = 3, ***p < 0.001, ****p < 0.0001. Representative images are shown. Mean±SD are shown.

was considerably lower compared to levels in untreated bacteria (Figure 4D), indicating that GSH was depleted in G. vaginalis upon D-Fe$_3$S$_4$ treatment. When we assessed the energy level of bacteria cells using an ATP kit, we found that ATP levels decreased upon D-Fe$_3$S$_4$ treatment in G. vaginalis (Figure 4E), which confirmed that the energy metabolism was interfered by D-Fe$_3$S$_4$.

Our observed changes in morphology, lipid peroxidation, GSH depletion and respiratory chain function indicated that treatment of G. vaginalis with D-Fe$_3$S$_4$ caused ferroptotic damages, ulti-
mately resulting in bacterial death. To confirm these findings, we performed gain of function experiments using inhibitors of ferroptosis. As shown in Figure 4F, addition of ferrostatin-1, a ferroptosis inhibitor that scavenges lipid peroxide, reduced the antibacterial activity of D-FeS$_4$. When we added TCEP, an iron chelator that also reduces polysulfide, we found that TCEP strongly inhibited antibacterial activity of D-FeS$_4$ (Figure 4G). Importantly, addition of GSH also reduced the antibacterial activity of D-FeS$_4$ (Figure 4H). These data strongly suggested that D-FeS$_4$ primarily induces ferroptotic damages in G. vaginalis affected. Presumably, this ferroptotic damage is mainly the result of the iron present. When incubating bacteria with D-FeS$_4$ in water, both iron ions (in particular for Fe$^{2+}$) and D-FeS$_4$ caused cell death in G. vaginalis as indicated by lipid peroxidation (Figure S5E,F, Supporting Information). Another chelating agent, EDTA, achieved the same effects as TCEP by chelating iron (Figure S5G, Supporting Information). However, when we incubated G. vaginalis with D-FeS$_4$ in culture media, D-FeS$_4$ induced cell growth of G. vaginalis, not ferrous or ferric iron (Figure S5H, Supporting Information), indicating that iron induced ferroptotic damages in the antibacterial action of D-FeS$_4$ represents an auxiliary effect.

The above dual antibacterial mechanisms target metabolism inhibition and oxidative damage in bacteria, which may prevent drug resistance. To prove this, we incubated G. vaginalis under sublethal concentration ($32 \times 10^{-6}$ m) of D-FeS$_4$ and monitored changes of the MIC values. As shown in Figure 4I, MIC values remained constant after 20 passages of G. vaginalis under continuous pressure of D-FeS$_4$, indicating that bacteria did not generate drug resistance to D-FeS$_4$. Together, these data clearly demonstrated that the polysulfides rather than the iron are responsible for inhibiting the glycolytic energy metabolism in G. vaginalis, which is the primary factor causing bacterial death in culture media. Moreover, both D-Fe$_4$S$_4$ and Na$_2$S$_4$ reduced the transcription of vaginolysin (VLY) in G. vaginalis which is a key virulent factor causing damage to vaginal epithelial cells$^{[25]}$ (Figure S5I, Supporting Information). Taken together, these results reveal that Fe$_4$S$_4$ kills G. vaginalis by integrating polysulfide-induced suppression on energy metabolism and iron-induced ferroptotic damage, which is termed as ferrosulfucidal effect (Figure 4J).

### 2.4. Metastable Iron Sulfide Counteracts Antibiotic Resistance of G. Vaginalis

G. vaginalis is characterized by resistance to antibiotic treatments, resulting in common failure of antibiotic treatment and ultimate recurrence of vaginosis. It remains unclear whether D-Fe$_4$S$_4$ represents a suitable candidate for clinical treatment of G. vaginalis. To evaluate the clinical use of D-Fe$_4$S$_4$, we first established four resistance models, namely antibiotics-resistant bacteria, antibiotics-induced persistent bacteria, biofilm-embedded bacteria, as well as intracellularly growing bacteria. In the model of antibiotic-resistant G. vaginalis, we exposed a clinical strain to D-Fe$_4$S$_4$, which is able to evade antibiotic killing.$^{[26]}$ To assess the cellular antibacterial activity of D-Fe$_4$S$_4$, we then constructed an intracellular model of G. vaginalis. Human immortalized VK2 cells (ATCC 2616) were infected with G. vaginalis, and internalization of bacteria was characterized using both TEM as well as CFU counting assay. When we evaluated the morphology of intracellular G. vaginalis using TEM, we found that G. vaginalis was readily internalized into VK2 cells (6–8 bacteria per cell), but the bacteria were found to be deformed in the presence of D-Fe$_4$S$_4$, which appeared to enter cytoplasm of VK2 (Figure 5G). When we evaluated the viability of intracellular G. vaginalis using CFU counting assay, we found that D-Fe$_4$S$_4$ ($1024 \times 10^{-6}$ m) reduced bacteria numbers by 2-log10 in VK2 cells within 6 h. In comparison, metronidazole showed negligible antibacterial effects under identical con-
Figure 5. D-Fe₃S₄ counteracts antibiotic resistance of *G. vaginalis*. A, B) D-Fe₃S₄ suppressed metronidazole-resistant strain of *G. vaginalis* (MRGV) by A) measuring bacterial growth (OD₆₀₀) and B) viability (CFU mL⁻¹). While metronidazole at 2048 × 10⁻⁶ m showed no any inhibition on MRGB, D-Fe₃S₄ at 125 × 10⁻⁶ m, 250 × 10⁻⁶ m or 500 × 10⁻⁶ m killed GRSV eventually within 6 h (B). C) D-Fe₃S₄ killed tolerant *G. vaginalis* induced by metronidazole. The tolerant bacteria were challenged with metronidazole stress and regrown in fresh culture media. D-Fe₃S₄ achieved identical MIC for both tolerant and normal strain of *G. vaginalis*, while metronidazole failed to inhibit persistent strain. D–F) D-Fe₃S₄ suppressed *G. vaginalis* biofilm characterized by D) dry weight assay, E) SEM imaging, and F) 3D imaging with confocal microscopy. Scale bars E): left side 50 μm; right side 300 nm. Scale bars F): 30 μm. G, H) D-Fe₃S₄ suppressed intracellular *G. vaginalis* in VK2 cells characterized by G) TEM imaging and H) CFU counting. Scale bars: left side 2 μm, right side 500 nm. n = 3, ***p < 0.01, ****p < 0.001, *****p < 0.0001. Representative images are shown. Mean±SD are shown.
Figure 6. D-Fe₃S₄ is biocompatible with probiotic lactobacillus. A,B) D-Fe₃S₄ showed minimal antibacterial activity to vaginal lactobacillus in culture media with A) different growth time and B) concentration. D-Fe₃S₄ or Na₂S₃ at 500 × 10⁻⁶ m only partially suppressed the growth of A) lactobacillus, B) even D-Fe₃S₄ at 1600 × 10⁻⁶ m only suppressed 50% growth, indicating a high value of MIC (>1600 × 10⁻⁶ m) for D-Fe₃S₄ toward lactobacillus. C–E) D-Fe₃S₄ suppressed mixed species biofilm composed of G. vaginalis and vaginal lactobacillus as assessed by C) CFU counting, D) dry weight assay, and E) SEM imaging. CFU counting showed that vaginal lactobacillus became prevalent in mixed-species biofilm treated by D-Fe₃S₄. SEM imaging showed that G. vaginalis was deformed while vaginal lactobacillus remained intact in the mixed-species biofilm treated by D-Fe₃S₄. Scare bars: left side 25 μm, middle 5 μm, right side 1 μm. GV: G. vaginalis, LV: vaginal lactobacillus. n = 3, **p < 0.01, ****p < 0.0001. Representative images are shown. Mean±SD are shown.

2.5. D-Fe₃S₄ Is Biocompatible with Probiotic Lactobacillus and Nontoxic to Epithelial Cells

Vaginal lactobacillus (LV) is a Gram-positive probiotic bacterium that exists in a symbiotic relationship with G. vaginalis under biofilm condition. LV forms an acid microenvironment and produces H₂O₂, thus controlling the balance between species present in the vaginal microbiota.[28] To assess the biocompatibility of D-Fe₃S₄, we evaluated the viability of vaginal lactobacillus following exposure to various concentrations of D-Fe₃S₄. As shown in Figure 2J, G. vaginalis growth in the presence of D-Fe₃S₄ is characterized by a MIC at 89.6 × 10⁻⁶ m. In contrast, LV growth was largely unaffected, as indicated by D-Fe₃S₄ up to 1600 × 10⁻⁶ m (Figure 6A,B). In agreement with these results, D-Fe₃S₄ failed to affect cell viability of vaginal lactobacillus under in vitro aqueous condition (water), even after exposure for 3 h incubation (Figure S6J, Supporting Information). To compare the biosafety of D-Fe₃S₄, silver nanoparticles (AgNPs), which are characterized by high antibacterial activity, were tested in parallel. Silver nanoparticles exhibited antibacterial activity against G. vaginalis with a MIC value of 76.8 × 10⁻⁶ m. However, the silver particles also showed stronger suppression on vaginal lactobacillus...
lus with MIC value at 256 × 10^{-6} m. We found that under in vitro aqueous conditions, 100 × 10^{-6} m silver nanoparticles caused a four-log reduction in vaginal lactobacillus cell numbers after only 1 h incubation (Figure S6K, Supporting Information). In addition, our cytotoxicity assay showed that silver nanoparticles at 64 × 10^{-6} m caused cell death of more than 90% VK2 cells (Epithelial cell line from human normal vaginal mucosal tissue) (Figure S6L, Supporting Information). In contrast, D-Fe_{3}S_{4} only caused cell death of 11% VK2 cells, at concentration as high as 512 × 10^{-6} m (Figure S6M, Supporting Information) and the released polysulfides and ferrous iron showed no any cytotoxicity to VK2, RAW264.7 (macrophages from mouse) and HaCaT (The immortalized human keratinocytes) (Figure S6N–P, Supporting Information).

To test if D-Fe_{3}S_{4} prefer to kill virulent pathogen, we prepared a dual species biofilm with G. vaginalis and vaginal lactobacillus. As shown in Figure 6C, exposure to D-Fe_{3}S_{4} reduced G. vaginalis growth by 9 logs as a result, LV established itself as the dominant strain following D-Fe_{3}S_{4} treatment for 12 h. Next, we assessed dry weight of dual species biofilm exposed to D-Fe_{3}S_{4} treatment. As shown in Figure 6D, the influence on dry weight of biofilm mass was also reduced dramatically. SEM images showed that vaginal lactobacillus retained intact shape and all G. vaginalis were disrupted in the dual species biofilm (Figure 6E). Together, these results clearly demonstrated that D-Fe_{3}S_{4} is superior to silver nanoparticles in its biocompatibility with both probiotic bacteria and vaginal cells.

### 2.6. D-Fe_{3}S_{4} Is a Suitable Candidate for Treating Bacterial Vaginosis

Given that D-Fe_{3}S_{4} exhibited robust antibacterial activity against G. vaginalis and high biocompatibility to probiotics and vaginal cells, we sought to determine if D-Fe_{3}S_{4} can be used to treat bacterial vaginosis. We then evaluated the therapeutic effects of D-Fe_{3}S_{4} in a bacterial vaginosis model. To this end, we infected female Balb/C mice with G. vaginalis. Mice infected for 3 d (1 × 10^{8} CFU/mouse/day) were treated with D-Fe_{3}S_{4} by direct injection of 10 μL D-Fe_{3}S_{4} (500 × 10^{-6} μm) or 10 μL of metronidazole (500 × 10^{-6} μm) into the vagina once per day for 7 d. As shown in Figure S7A (Supporting Information), the infected mice discharged large amount of vaginal secretion. The group treated with D-Fe_{3}S_{4} showed negligible vaginal secretion 4 d postinfection, while those treated with metronidazole reached the similar effect 8 d postinfection. Ten days postinfection, the mice were sacrificed and the vaginal secretion was assessed for viability of G. vaginalis using histochemistry, CFU counting and enzyme assay. Our histochemistry analysis showed that the vaginal epithelial tissue of mice treated with D-Fe_{3}S_{4} recovered on 8 days post infection (Figure S7B, Supporting Information). We determined bacterial viability of in vaginal tissues using CFU counting. As shown in Figure 7A, the group treated with D-Fe_{3}S_{4} exhibited the lowest number of colonies (3.83 lg (CFU mL^{-1})), while those without treatment or treated with metronidazole exhibited colonies number at 7.72 and 7.47 lg (CFU mL^{-1}), respectively. Next, we used neuraminidase assay to determine the virulence of G. vaginalis. Our analysis showed that the activity of neuraminidase was reduced in the group treated with D-Fe_{3}S_{4} (Figure 7B), further indicating that the growth of vaginal bacteria was suppressed.

To confirm the therapeutic effect of D-Fe_{3}S_{4}, we used the metronidazole-resistant strain MRGV to construct another in vivo infection model. To this end, we introduced a combination treatment consisting of D-Fe_{3}S_{4} and probiotic LV. LV has previously been recognized as a promising treatment for vaginosis.

As shown in Figure S7C (Supporting Information), the mice infected with MRGV failed to recover from the infection by treatment with metronidazole alone. However, upon treatment with D-Fe_{3}S_{4} + LV, the mice recovered within 7 d. When we evaluated bacterial viability of G. vaginalis in vaginal tissues using CFU counting assay, we found that D-Fe_{3}S_{4} alone and LV alone reduced the number of G. vaginalis from 5.3 lg (CFU mL^{-1}) to 2.1 and 1.1 lg (CFU mL^{-1}). In contrast, a combination of D-Fe_{3}S_{4} and LV showed the best therapeutic effect, characterized by bacterial death of nearly 100% (Figure 7C). All treatments with D-Fe_{3}S_{4} as well as a combination of D-Fe_{3}S_{4} and LV showed little effect on the body weight of treated mice (Figure S7D, Supporting Information). Histochemistry analysis showed that the organs including heart, liver, spleen, lung, kidney and uterus are not affected after 7 d continuously vaginal administration, indicating good biocompatibility of D-Fe_{3}S_{4} with in vivo system (Figure S7E, Supporting Information).

To render D-Fe_{3}S_{4} suitable for in vivo application, we prepared a suppository by mixing D-Fe_{3}S_{4} with gelatin and glycerol (Figure 7D–F), with the characteristic of dissolution to release D-Fe_{3}S_{4} under physiological conditions (e.g., water, 37 °C). We found that the suppository with 1 mg mL^{-1} D-Fe_{3}S_{4} was solid with black color (Figure 7E) and the inside D-Fe_{3}S_{4} remained sheet-like structure as characterized by SEM (Figure 7F), indicating that D-Fe_{3}S_{4} is stable in this suppository. Using our CFU counting assay, we found that D-Fe_{3}S_{4} suppository (After one year storage at room temperature) exhibited MIC at 117 × 10^{-6} μm to both GV14018 and MRGV, indicating that its antibacterial activity is comparable with free D-Fe_{3}S_{4} and stable for long-term storage. In addition, D-Fe_{3}S_{4} suppository showed comparable therapeutic efficacy with D-Fe_{3}S_{4} in the same infected mouse model, which enhanced the recovery of vaginal tissue by suppressing G. vaginalis (Figure 7G, H). Together, these results demonstrated that D-Fe_{3}S_{4} possesses therapeutic characteristics superior than metronidazole for in vivo treatment of bacterial vaginosis.

We further evaluated antibacterial performance of D-Fe_{3}S_{4} using clinical samples from patients affected by bacterial vaginitis. The vaginal secretion from patients was first identified and scored based on the bacterial infection with G. vaginalis, vaginal lactobacillus or campylobacter mobilis using the Nugent score system. The samples with score ≥7 were chosen for antibacterial testing (Table S3, Supporting Information). As shown in Figure 7I, 99.9% G. vaginalis were eliminated in the samples of 82 patients. In addition, our treatment also eliminated Streptococcus, staphylococcus, or E. coli infection in the samples of other three patients. However, in the samples of patients carrying fungus (11 cases) and vaginal lactobacillus (4 cases), our treatment with D-Fe_{3}S_{4} only showed limited antibacterial effects. Together, these results clearly demonstrated that D-Fe_{3}S_{4} is able to effectively eliminate G. vaginalis in clinical samples from patients.
Figure 7. D-Fe₃S₄ is able to treat bacterial vaginosis in mice and clinical samples. A) CFU counting showed D-Fe₃S₄ reduced the number of G. vaginalis in mice, while metronidazole failed. B) Neuraminidase activity of G. vaginalis was reduced following treatment with D-Fe₃S₄ and metronidazole, indicating that the virulence of G. vaginalis was reduced. C) Combination therapy of D-Fe₃S₄ and probiotic lactobacillus in the model infected with metronidazole-resistant G. vaginalis. Both D-Fe₃S₄ alone and lactobacillus alone showed considerable suppression on bacterial growth compared to metronidazole, however, combination of D-Fe₃S₄ and lactobacillus showed maximum therapeutic effects. D) A schematic of a suppository prepared from D-Fe₃S₄, gelatin and glycerol. E) The prepared suppository containing 1 mg mL⁻¹ D-Fe₃S₄. Scale bar 1 cm. F) SEM image of sectioned suppository composed of D-Fe₃S₄. Scale bar 2 μm. G) D-Fe₃S₄ suppository showed comparable antibacterial effects in mouse model with D-Fe₃S₄ treatment. H) Histochemistry staining demonstrated that the vaginal tissue recovered quickly in the group treated with D-Fe₃S₄ and D-Fe₃S₄ suppository compared to control group (the red arrow indicated a microcystis). The normal group represents the mouse without infection. Scale bar = 100 μm. I) Statistical analysis of 100 clinical samples from patients with bacterial vaginosis following the treatment with D-Fe₃S₄. 82 samples were confirmed with G. vaginalis infection and could be eliminated completely. Three samples infected with Streptococcus, Staphylococcus or E. coli also can be eliminated, while 11 samples infected with Fungi and four samples with lactobacillus showed very limited or no antibacterial effect. n = 5, **p < 0.05, ***p < 0.01, ****p < 0.001, *****p < 0.0001. Representative images are shown. Mean±SD are shown.
3. Discussion

In this work, we demonstrated that mFeS materials (e.g., Fe$_7$S$_8$) are potent antibacterial candidates that kill bacteria in a Gram-dependent manner, namely by releasing polysulfide species and ferrous iron that exhibit biocidal effects in a highly selective fashion. In particular, the mFeS showed a great potential for bacterial vaginosis treatment for those challenged by resistant G. vaginalis. Although BV is treated with a number of effective available antibiotics, such as metronidazole, it has been reported that large number of BV patients experience recurrence within 1 year of treatment for incident disease.[31] The major reasons for recurrence are ascribed to the antibiotics resistance and persistence of residual infection. In particular, the persistence may be ascribed to the formation of a biofilm or intracellular strain that protects G. vaginalis from antibiotics therapy. Such resistance and persistence are also confirmed in our experimental models. We indeed observed that G. vaginalis quickly became tolerant to metronidazole after several passages. Moreover, metronidazole failed to kill the bacteria embedded in biofilm matrix or intracellular vacuole, because it cannot penetrate the barrier to reach the inside bacteria. In contrast, our D-Fe$_3$S$_4$ showed high potency against both resistant and persistent G. vaginalis, but without causing drug resistance. First, the high antibacterial activity is ascribed to the dual mechanisms of action (MoAs) of D-Fe$_3$S$_4$ including polysulfide disrupting glycolysis-related energy metabolism and iron inducing ferroptotic damage, rendering G. vaginalis unable to generate resistance. Second, the antibacterial action mode of D-Fe$_3$S$_4$ is releasing polysulfide species and ferrous iron, which can cross the barriers of biofilm matrix and cell membrane as both polysulfides and iron have strong diffusibility. In particular, we discovered that the permeability of polysulfide species is strongly correlated with the thickness of bacterial wall, enabling an antibacterial selectivity. Thus, D-Fe$_3$S$_4$ not only effectively overcomes metronidazole resistance, but also eliminates persistence strains of G. vaginalis, showing superior advantage than the first-line medication of metronidazole in BV treatment.

In addition, our study provided a readily available candidate of antibiotic alternative as metastable iron sulfides are plenty in nature or can be easily synthesized. There are many iron sulfides present in nature with various interchangeable physical phases including greigite, pyrrhotite, pyrite, marcasite, etc.[15] Our study showed that metastable Fe$_3$S$_4$ exhibited high antibacterial activity, followed by Fe$_7$S$_8$ with limited antibacterial activity, while stable FeS and Fe$_9$S$_8$ (including synthesized Fe$_7$S$_8$ (pyrite) with nanoscale size or microscale size, data not shown) lacked antibacterial activity in our culture assays. The ability of releasing ferrous iron and polysulfide is strongly correlated with the antibacterial activity of iron sulfide minerals. We verified that converting sulfur into iron polysulfides is an effective approach to improve antibacterial performance of sulfur-containing compounds. We found that hydrothermal method may be an approach to prepare Fe$_7$S$_8$ with pure phase by supplying organic sulfur (e.g., DADS) (Figure S1A,B, Supporting Information). Such system also can convert other organosulfurs, sodium sulfides (Na$_2$S, Na$_2$S$_2$) or elemental sulfur into metastable iron sulfides (Fe$_7$S$_8$, Fe$_9$S$_8$ or Fe$_{12}$S$_{17}$) (Data not shown). In addition, we found that directly mixing Na$_2$S and FeCl$_2$ also forms a colloid (Figure S4J, Supporting Information). Although the phase of colloidal product has not yet been clarified, the addition of FeCl$_2$ into Na$_2$S increased the production of polysulfides (Figure S4L, Supporting Information) and thus enhanced the antibacterial activity of Na$_2$S (Figure 2J). In addition, phase change is achieved in processed Ziriontang, a mineral traditional Chinese medicine, by combining high temperature calcination and vinegar quenching, which can gradually change the phase of pyrite (Fe$_3$S$_4$) to Fe$_7$S$_8$. In addition, many microorganisms, such as magnetotactic bacteria, can synthesize iron sulfide magnetosome containing Fe$_3$S$_4$ or mackinawite (tetragonal FeS) and cubic FeS which are thought to be precursors of Fe$_7$S$_8$, providing high quality of iron sulfide with specific crystal and narrow size ranges.[31] In view of the abundant resource in geological ore in nature, biomineralization in biological system as well as chemical synthetic technologies, it is possible to prepare metastable iron sulfides at large scale with low cost, which is advantageous for drug development and clinical translation. In particular, these materials can be formulated into powder and suppository for long-term storage, showing high practicability as medications.

Furthermore, we found that polysulfide species play a bacterial role by penetrating bacterial wall and inhibiting glycolysis-related energy metabolism. Our previous studies discovered that polysulfides act as an oxidizer to deplete GSH and thus aggravate redox imbalance in ferrous iron induced ferroptosis-like death of bacteria under in vitro aqueous conditions.[13] Suchaction of polysulfides was thought to be auxiliary in antibacterial process and the antibacterial efficacy of nFeS toward S. aureus decreased dramatically in culture media or in vivo system. In contrast, significant differences of antibacterial activity of Fe$_3$S$_4$ are evident for these bacteria under culture media with the degree of antibacterial activity changing in the order: G. vaginalis > Gram-negative E. coli > Gram-positive S. aureus, showing a strong dependence on the type of bacterial wall, as classified by Gram-staining (Figure 2A–C). The Gram-dependent antibacterial mode of polysulfides may explain why nFeS failed to kill S. aureus in culture media or physiological conditions. In addition, owing to the selective antibacterial mode, D-Fe$_3$S$_4$ showed minimal toxicity to vaginal lactobacillus (Gram-positive species) at the MIC for G. vaginalis, making it feasible for combination therapy with D-Fe$_3$S$_4$ and probiotic lactobacillus for BV. These features render D-Fe$_3$S$_4$ superior to other antimicrobial nanomaterials, such as silver nanoparticles which exhibit undifferentiated toxicity to both pathogenic and probiotic bacteria and host cells.[24]

There are several limitations to our study. First, although the antibacterial mechanism of D-Fe$_3$S$_4$ was revealed as targeting to glucokinase in glycolysis, other related pathways may be affected by polysulfide species which induce polysulfidation in molecules containing free thiol group. We will further conduct more detailed analyses of metabolome, transcriptome, and proteome and screen potential targets of polysulfide species in different levels in our future work. Second, there are diverse pathogens in BV progress, and although we determined that D-Fe$_3$S$_4$ killed G. vaginalis and was safe to vaginal lactobacillus, it could be that more pathogens such as Atopobium vaginae, Neisseria gonorrhoeae, Mobiluncus spp., Bacteroides spp., and Prevotella spp.,[35] and fungi (Candida albicans).[36] might be affected in BV treatment with D-Fe$_3$S$_4$. Hence, the broad antimicrobial efficacy will be investigated in our future study to evaluate the potential advantages.
and limitations of D-Fe₃S₄ in treating BV and restoring microbiota balance.

In conclusion, our data demonstrate that mFeS exhibit unique mechanism different from traditional antibiotics, and the antibacterial action of iron–polysulfide coordination may provide insight for the design of iron-sulfur antibacterial. Importantly, mFeS represent a promising candidate specifically killing G. vaginalis and countering drug resistance in BV treatment with high biocompatibility. With the challenge of fast-evolved resistance in bacteria, mFeS might provide a valuable non-antibiotic alternative option to help treating BV with broad prevalence and frequent recurrence and improving women’s health and life quality.

4. Experimental Section

Materials: All chemicals were of analytical grade. Ethylene glycol, FeCl₃, NaAc·3H₂O, FeCl₂, FeCl₄, Fe₂S₃, diethyl disulfide (DADS), monobromobimane, and lysozyme were purchased from Sigma-Aldrich. Processed Zirantong was purchased from Kangmei Pharmaceutical Co., Ltd (Sichuan, China). SulfoBiotics-SSPA, NaHS, Na₂S, Na₂S₂, and Na₂S₃ were purchased from DOJINDO (Japan). Na₂S₄ was purchased from Shanghai Key Industrial Co. LTD (Shanghai China). Tryptone, brain heart infusion, porcine skin (LP0008), yeast extract (LP0021), glucose, starch and drug sensitive piece were purchased from Oxoide (UK). Glutathione (GSH), dimethylsulfoxide (DMSO), and agar were purchased from San- gon Biotech (China). Columbia blood AGAR plate were purchased from Cm micro (Jiangmen, China). Cell Counting Kit-8, Crystal violet, metronidazole and hydrogen peroxide (H₂O₂) were bought from Aladdin Chemistry (Shanghai, China). GSH and GSSG Assay Kit, Enhanced ATP Assay Kit, propidium iodide (PI), 2,7-dichlorofluorescein diacetate (DCFH-DA) were purchased from Beyotime Biotechnology (Shanghai, China). SYTO9 green fluorescent nucleic acid stain was purchased from Thermo Fisher Scientific (Waltham, MA). G. vaginalis ATCC 14018 were purchased from the American Type Culture Collection (ATCC). Streptococcus mutans UA159 (ATCC 700610), Escherichia coli (E. coli, CMCC(B)44102), Staphylococcus aureus (S. aureus, ATCC 29213) were purchased from the Institute of Microbiology of the Chinese Academy of Science.

Ethics Statement: All animal studies were performed following the protocols approved by the Institutional Animal Care and Use Committee of Yangzhou University and the Institutional Animal Care and Use Committee of the Institute of Biophysics, Chinese Academy of Sciences, respectively. The experimental research for antibacterial test using human vaginal secretion from patients was approved by the Ethics Committee of Xishan People’s Hospital of Wuxi City and conducted after obtaining informed consent agreement by all participating patients.

Synthesis of D-Fe₃S₄: D-Fe₃S₄ synthesis was based on a previously described hydrothermal synthesis method established in the laboratory. Briefly, 0.82 g FeCl₃ was dissolved in 40 mL ethylene glycol, stirred at room temperature for 30 min, and exposed to ultrasound for 10 min to ensure complete dissolution of FeCl₃. Once the solution was clear, 3.6 g NaAc·3H₂O was added, stirred at room temperature for 30 min. One mL of diethyl disulfide (DADS) was added with continuous and vigorous stirring for 30 min. The mixture was transferred to a 50 mL Teflon-lined stainless steel autoclave and reacted at 200 °C for 12 h. After the reaction was completed, the autoclave was cooled to room temperature. The product of black precipitate was washed alternately with H₂O until the reaction was completed, the autoclave was cooled to room temperature. The effluent was then applied to tandem mass spectrometry triple TOF 5600 plus. Briefly, 30 μL supernatant of D-Fe₃S₄ (1 mg mL⁻¹) was added into a tube containing 70 μL 10⁻³ M Tris–HCl buffer (pH 9.5, with 0.1 × 10⁻⁵ M diethyl- etiamine pentaacetic acid (DTPA)) and then 50 μL monobromobimane (mBBr) (10 × 10⁻³ M, in acetoni trile solution) was added and incubated at 25 °C for 30 min, followed by adding 50 μL of 5-sulfosalicylic acid (200 × 10⁻³ M). The pretreated samples were injected into a reverse phase spectromer C18 column (ACQUITY UPLC CSH C18: 2.1 × 100 mm, 1.7 μm particle, Waters). The mobile phases were A (0.1% FA in water) and B (acetoni trile in 0.1% FA), and the flow rate was 0.25 mL min⁻¹. The samples were separated with a gradient as follows: 0–1 min, 15%B; 1–10 min, 15%–45%B; 10–11 min, 45%–95%B; 11–12 min, 95%–15%B; 13–15 min, 15%B and hold for 5 min. The mass range parameters were as follows: CE 10.000; DP 100.000; IDx 0.000; IDUx 5.000; IRDX 15000.000; IRWx 10000.000; IWx 0.000; IWUX 5.000; XA1 136.617; Start Mass:50.0; End Mass: 1000.0. The effluent was then applied to tandem mass spectrometer using an electrospray ionization (ESI) interface, and operated in the positive-ion mode. The results were monitored in multiple reaction monitoring mode. The ion released from D-Fe₃S₄ was measured using a Serum Ion Concentration Assay kit (Solarbio, Beijing, China).

Preparation of Vaginal D-Fe₃S₄ Suppository: Suppository formulations were prepared with the following percentage by mass: 20% gelatin, 70% glycerol, 9.9% distilled water, 0.1% D-Fe₃S₄. Gelatin was soaked in a few mL of water until it became swollen and softened. Gluten was then added and mixed well. Next, D-Fe₃S₄ was added and mixed well. The mixture was heated to 80 °C with continuously stirring for 4 h. After standing at 75 °C for 1 h, the mixture was injected into the lubricant-coated and pre-cooled mold. After cooling down, the black, translucent and elastic suppository was formed and collected for experimental use.

In Vitro Antibacterial Assays: Five to seven colonies of G. vaginalis on Columbia blood agar plates were randomly selected and inoculated into 5 mL of BHIs culture and was cultured at 37 °C with 5% CO₂ for 12–18 h. One day post-inoculation, required amounts of G. vaginalis bacterial solution was taken and transferred into fresh medium at a ratio of 1:100 and cultured at 37 °C with 5% CO₂ for 6–8 h. When the OD₆₀₀ reached a value of 0.5, 100 μL of bacterial inoculation was mixed with BHIs (900 μL) as the control. An additional 100 μL of bacterial inoculation was mixed with D-Fe₃S₄ and BHIs (800 μL) as the experimental group. After incubation at 37 °C for certain time, bacterial viability was checked by plating bacteria with proper dilution and calculating the bacterial number in CFU mL⁻¹. The inhibition assays using either FeCl₃, Na₂S₄, EDTA, ferrostatin-1 or other inhibitors were conducted in the same system and using the same procedure. Glutathione levels were measured using GSH and GSSG assay kits (Beyotime, China). The fluorescent probe Bodipy581/591 (C11) was used to measure the lipid peroxidation level of bacteria. ATP Content Assay Kit and Micro Glucokinase (GLK) Assay Kit (Solarbio, China) were used to measure ATP and glucokinase activity levels.

Bacterial Strains and Growth Conditions: G. vaginalis ATCC 14018 were obtained from the American Type Culture Collection (ATCC), vaginal clinical isolates were obtained from vaginal swabs from the Department of Microbiology, Xishan People’s Hospital, Wuxi city (based on Nugent score, BV ≥ 7 points). The swabs were immediately used for inoculation on a Columbia blood agar plate, and the bacteria were cultured under anaerobic atmosphere at 37 °C with 5% CO₂ for 48 h. Finally, candida Gardnerella species were identified by stroma-assissted laser desorbed/ionization time-of-flight (MALDI-TOF, Bruker Daltonics, Mabilika, USA). G. vaginalis was cultured in BHI media composed of brain/heart infusion broth containing 1% gelatin, 1% yeast extract, 0.1% soluble starch, 0.1% glucose and 1% fetal bovine serum. Lactobacillus vaginalis was isolated from vaginal secretions of healthy women and screened for antibacterial activity against G. vaginalis, and production of hydrogen peroxide, lactic acid and form biofilm, vaginal lactobacillus was cultured in MRS broth. Agar plates and liquid cultures were incubated at 37 °C with 5% CO₂, frozen stocks of strains were stored at −80 °C in BHIs containing 30% (v/v) glycerol.

Identification of Polysulfide and Iron Released from D-Fe₃S₄: The polysulfide species released by D-Fe₃S₄ were identified by liquid chromatography-mass spectrometry triple TOF 5600 plus. Briefly, 30 μL supernatant of D-Fe₃S₄ (1 mg mL⁻¹) was added into a tube containing 70 μL 10⁻³ M Tris–HCl buffer (pH 9.5, with 0.1 × 10⁻⁵ M diethyl- etiamine pentaacetic acid (DTPA)), and then 50 μL monobromobimane (mBBr) (10 × 10⁻³ M, in acetoni trile solution) was added and incubated at 25 °C for 30 min, followed by adding 50 μL of S-sulfosalicylic acid (200 × 10⁻³ M). The pretreated samples were injected into a reverse phase spectromer C18 column (ACQUITY UPLC CSH C18: 2.1 × 100 mm, 1.7 μm particle, Waters). The mobile phases were A (0.1% FA in water) and B (acetoni trile in 0.1% FA), and the flow rate was 0.25 mL min⁻¹. The sample was separated with a gradient as follows: 0–1 min, 15%B; 1–10 min, 15%–45%B; 10–11 min, 45%–95%B; 11–12 min, 95%–15%B; 13–15 min, 15%B and hold for 5 min. The mass range parameters were as follows: CE 10.000; DP 100.000; IDx 0.000; IDUx 5.000; IRDX 15000.000; IRWx 10000.000; IWx 0.000; IWUX 5.000; XA1 136.617; Start Mass:50.0; End Mass: 1000.0. The effluent was then applied to tandem mass spectrometer using an electrospray ionization (ESI) interface, and operated in the positive-ion mode. The results were monitored in multiple reaction monitoring mode. The ion released from D-Fe₃S₄ was measured using a Serum Ion Concentration Assay kit (Solarbio, Beijing, China).
Intracellular Antibacterial Assays: VK2 cells were cultured to exponen-
tial phase and divided into six well plate with complete DMEM medium
(90% DMEM and 10% FBS without antibiotics) with 80–90% coverage
of plate surface at 37 °C with 5% CO₂. In parallel, G. vaginalis was cultured,
collected and suspended into DMEM medium at 5 × 10⁶ CFU mL⁻¹. G. va-
ginalis infected VK2 cells for 3 h at 37 °C with 5% CO₂. After discard-
ing the supernatant and washing twice with PBS, the extracellular bac-
teria were killed with clindamycin (0.1 μg mL⁻¹) for 3 h. 1024 × 10⁶ m
metronidazole and D-Fe₃S₄ was then introduced to kill intracellular bac-
teria by 3 h incubation at 37 °C with 5% CO₂. A sample of the cells was
lysed using buffer containing 0.1% Triton-X. The number of viable bacteria
was counted by plating serial dilutions of bacteria on Columbia blood agar
plate.

Drug Sensitivity Test: The G. vaginalis isolates were evaluated for in
vitro antimicrobial susceptibilities to metronidazole, D-Fe₃S₄, Ag NPs,
FeCl₃, NaHS, Na₂S, Na₂S₂, Na₂S₃, Na₂S₄, MNPs, Na₂S₅S₆, EDTA using the
Brodth dilution method previously described by the Clinical and Laboratory
Standards Institute. When the G. vaginalis culture was determined to be
at its logarithmic growth stage, (OD₆₀₀ = 0.5), containing approximately
1–2 × 10⁶ CFU mL⁻¹, G. vaginalis was diluted 100 times in BHI solution,
to approximately 1–2 × 10⁵ CFU mL⁻¹. Next, different concentrations of
drugs were added to the prepared bacterial suspension. After cultured
for 24 h, the end reading of the OD₆₀₀ point was measured with a microplate
reader to evaluate the growth of bacteria. The minimum inhibitory concen-
tration (MIC) was defined as the lowest antibiotic concentration at which
growth is significantly reduced or no growth at all.

RNA-Seq for Transcriptome Analysis and Metabolome Analysis: Five to
seven colonies of G. vaginalis on Columbia blood agar plates were ran-
domly selected and inoculated into 5 mL of BHIs culture, and G. vaginalis
was cultured at 37 °C with 5% CO₂ for 12–18 h. One day post inoculation,
0.5 mL of G. vaginalis bacterial solution was transferred into the fresh
medium at a ratio of 1:100 and cultured at 37 °C with 5% CO₂ for 6–8 h.
When the OD₆₀₀ reached a value of 0.8, 900 μL of the bacterial solution
was mixed with either 100 μL BHIs (Control) or 100 μL Na₂S₁, Na₂S₂, D-
Fe₃S₄ (100 × 10⁻⁶ m) and incubated at 37 °C with 5% CO₂ for 60 min.
Following drug treatment and washing the G. vaginalis bacteria cells twice
with PBS, the supernatants were completely removed following centrifuga-
tion at 4 °C at 10 000 rpm for 2 min and the bacteria pellets were collected.
After washing twice with PBS, the G. vaginalis pellets were immediately
placed into liquid nitrogen and frozen for 15 min. After freezing, the G.
vaginalis was stored at -80 °C. Bacteria were processed for transcriptome
analysis and metabolome analysis. Total RNA extraction, RNA sequencing
and bioinformatic data collection were performed by Shanghai Personal-
bio (Shanghai, China).

Molecular Dynamic Simulation for Glucose Interaction with Glucokinase:
The energy profile for the insertion reaction of polysulfide (the H₂S₄⁻⁻ was
used for calculation based on the estimated pKa value of oligosulfanes
[41]) and Cys in water was calculated using the B3LYP functional [39] in
conjunction with the 6-31G* basis sets[40] integrated in the Gaussian09
package.[4] For the molecular dynamic (MD) simulations, the initial con-
formations for the complexes of glucokinase and glucose were obtained by
docking using the AutoDock Vina package.[28] For each complex, five con-
formations with top 5 docking scores were dissolved in water for 20 ns MD
simulations. To capture the strength of the interaction between glucoki-
nase and glucose, the interaction energy (E_{interaction} in kJ mol⁻¹) including
the short-range Coulombic interaction (E_{Coul}) and the short-range Lennard-
Jones energy (E_{LJ}) were calculated. The conformations with the most neg-
ative E_{interaction} were selected for further investigations. All the MD simulations
and analysis were performed by GROMACS software.[43]

Vaginalis Biofilm Formation: Cell slides were placed at the bottom of
the 24-well sterile plate and incubated with 2.5 mL BHIs for 30 min. Then
the culture solution was exchanged with 2.5 mL of prepared bacterial sus-
pension (approximately 10⁶ CFU mL⁻¹) in the BHIs with 0.1% (w/v) gluo-
ses per well and incubated at 35 °C for 24 h. The planktonic cells were
carefully removed, and 2.5 mL of fresh medium was added to each
well before the plates were further incubated for 24 h. After 48 h of cul-
turing, the biofilms were treated with D-Fe₃S₄ for 3 h. The biofilms were
then washed once with PBS. Either 24 or 48 h monospecies biofilm of G.
vaginalis was used as a control. BHI solution was used as a negative con-
trol in all experiments to exclude any possible contamination of bacteria.
The biofilms were collected for dry weight measurement, viability assay of
G. vaginalis.

The dual-species biofilm of G. vaginalis and lactobacillus was established
using similar procedure. Briefly, following 24 h of G. vaginalis biofilm for-
mation, the planktonic cells remaining in the solution were carefully re-
moved, before 2 mL of fresh BHI solution was added to each well. Simulta-
neously, the suspension of lactobacillus was added (10⁸ CFU mL⁻¹) to each
well and the plates were further incubated for 24 h. Next, the dual-species
biofilms were washed once with phosphate buffer saline (PBS) solution.
All assays were repeated three times with five technical replicates.

SEM and Confocal Microscopy for G. Vaginalis Biofilms: The structure of
G. vaginalis biofilm treated by D-Fe₃S₄ was examined by scanning electron
microscope (SEM). First, G. vaginalis biofilms were resuspended in glu-
teraldehyde (2.5%, Sigma-Aldrich) for 24 h at 4 °C under dark conditions.
Bacterial cells were then washed and treated with ethanol gradient dehy-
dration (30%, 50%, 70%, 90%, and 100% twice), before being dried using
a critical point dryer and coated with platinum sputter. Finally, scanning
electron microscope (SEM) images were obtained on a Hitachi S-4800
FE-SEM at a working voltage of 15.0 kV and a working current of 10 μA
under magnification of 40 K. The 3D structure of biofilm was also char-
acterized using confocal microscopy. Once the biofilm were grown, 1 μg
mL⁻¹ PI and 5 × 10⁻⁶ m Syto9 staining solution were added to the surface
of the biofilm and incubated for 20 min avoiding light. Next, the samples
were washed twice with PBS before being analyzed using laser confocal
microscope FV1000 (OLYMPUS FV 1000).

Mouse Vaginal Infection Model: Seven week old female Balb/C mice
were obtained from the Institute of Biophysics, Chinese Academy of
Sciences, Beijing. Mice were inoculated vaginally with 1 × 10⁶ CFU G. va-
ginalis (ATCC 14018) in 10 μL sterile PBS, and inoculated for three consec-
tive days. Three days of post infection, the mice were divided into three
groups (five mice per group). The infected mice in the different groups
were treated with 10 μL PBS, 500 μM D-Fe₃S₄, 500 μM metronidazole
for 72 h, respectively. Vaginal washes were collected by flushing vaginas
with 10 μL sterile PBS using a P10 pipet, followed by rinsing into an addi-
tional 90 μL PBS in a sterile 1.5 mL Eppendorf tube. G. vaginalis titers
were determined from washes by preparing 10-fold serial dilutions in PBS
(in the anaerobic chamber), before spotting 100 μL of each dilution onto
Columbia blood agar plates. Colonies were then enumerated and reported
as recovered colony forming units (CFU) per mL of vaginal fluid. The
vaginal orifices of mice were photographed. The mice were sacrificed 7 d
postinfection, and the vagina, uterus, heart, liver, spleen, lung, kidney, and
tissue were collected and fixed with formalin. Tissues were then dissected
and analyzed using high-resolution (HR) histological staining. The tissue
sections were examined using a Nikon Eclipse C1 microscope in bright field
mode.

Another infection model was established using clinical metronidazole-
resistant strain MRGV with similar infection procedure as above. Mice suc-
cessfully infected with MRGV were then randomly divided into 5 groups
(five mice per group), 1: Control, 2: D-Fe₃S₄, 3: Metronidazole, 4: LV, 5:
D-Fe₃S₄+LV. Mice in groups 1–3 were inoculated with PBS, and groups 4
and 5 were inoculated with 1 × 10⁶ CFU mL⁻¹ Lactobacillus vaginalis with
10 μL sterile PBS at 8:00 am. Groups 1 and 4 were treated with PBS, groups
2, 3 and 5 were treated with D-Fe₃S₄ (3.38 × 10⁻³ μM) and metronidazole
(3.38 × 10⁻³ μM) of 8:00 pm, respectively. The treat-
m ent was performed for 3 d and all mice were sacrificed for bacterial via-
bi lity assay.

D-Fe₃S₄ to Eliminate Bacteria in Clinical Samples: The clinical samples
were collected from 100 women from the Department of Obstetrics and
Gynecology of Wuxi Xishan People’s Hospital. This study was approved by
the Ethics Committee of Wuxi Xishan People’s Hospital (approval number:
LLS2020KY036 27/07/2020). All samples were examined by Gram stain-
ing and microscopy to assess their Nugent score, patients with Nugent
score ≥ 7 were selected. Vaginal secretion was collected with a sterile swab
and then resuspended with 1 mL water. After mixed thoroughly,
0.5 mL of suspension was added D-Fe₃S₄, the left was taken as the control.
After 3 h incubation at room temperature, colonies counting of microbes
were conducted and presented in CFU mL⁻¹.
Biosafety Assessment of D-Fe₃S₄: The cytotoxicity of D-Fe₃S₄ was evaluated in VK2 cells (ATCC 2616) using CCK8 kit. VK2 cells were inoculated into a sterile 96-well plate (Corning, NY) and incubated in DMEM with 10% FBS under 5% CO₂ at 37 °C for 12 h. The initial concentration of the VK2 cells was 5000 × 10⁴ cells per well. VK2 cells were treated with a series of concentrations of D-Fe₃S₄ (64 × 10⁻⁶, 128 × 10⁻⁶, 256 × 10⁻⁶, 512 × 10⁻⁶ and 1024 × 10⁻⁶ M) for 24 h. Finally, CCK8 was added to a 96-well plate and incubated with VK2 cells for 2–3 h. The supernatant were extracted, and measured at 532 nm using a miniature tablet reader. Relative cell viability was calculated by comparing with untreated control cells. The cytotoxicity of the supernatant containing polysulfide species and ferrous iron released from D-Fe₃S₄ was evaluated with the VK2, macrophages (RAW264.7) and HaCaT (human keratinocytes) using the above same procedure. The supernatant was prepared by incubating D-Fe₃S₄ in water for 24 h at room temperature and collected via a centrifugation at 10,000 g for 10 min.

For animal test, ten healthy Balb/C mice (7 week old female mice) were randomly divided into two groups with five mice in each group. 10 μL (1024 × 10⁻⁶ M) D-Fe₃S₄ was injected into the vagina in one group and PBS was injected into the mice of the control group. Vaginal inoculation was performed once a day for seven days for each mouse. The mice’s behavior, including feeding, drinking, activity, and weight, was then continuously observed and recorded. Finally, three mice in each group were sacrificed for vaginal histopathological examination and major organ histochemical examination.

Statistical Analysis: Each experiment was repeated at least three times and the data were interpreted as means ± SD. GraphPad Prism8.3 was used for statistical analysis. The t-test was used to make a comparison (p < 0.05, **p < 0.01, ***p < 0.001, and ****p < 0.0001).

Supporting Information
Supporting Information is available from the Wiley Online Library or from the author.

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Conflict of Interest
The authors declare no conflict of interest.

Author Contributions
L.F. and R.M. contributed equally to this work. L.G. conceived and organized the project and wrote the manuscript. L.F. and Q.W. conducted in vitro and in vivo antibacterial tests of iron polysulfide. Y.L. and X.W. helped synthesis and characterization of iron sulfide minerals. L.C. conducted theoretical analysis of Fe–S bonds and X.J.G. analyzed the binding of glucokinase with glucose. Y.H. and T.W. performed structure prediction of glucokinase and purified glucokinase. H.W., Q.S., and C.C. provided clinical guideline and samples. J.J. guided the cell and animal tests. L.G. contributed to the data discussion and drafted the manuscript. All authors contributed to the editing of the manuscript.

Data Availability Statement
The data that support the findings of this study are available from the corresponding author upon reasonable request.

Keywords
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