Effects of deferoxamine on intrinsic colistin resistance of *Proteus mirabilis*

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Abstract. *Proteus mirabilis* is a common pathogen, which is responsible for urinary tract infections. Iron is a critical element necessary for both humans and pathogens to maintain their biological functions, and iron limitation via chelator agents may be useful in the treatment of infections. The present study aimed to investigate the synergistic interactions between the iron chelator agent deferoxamine (DFO) and the antibacterial drug colistin. The minimum inhibitory concentration (MIC) values of DFO and colistin for *P. mirabilis* isolates were determined by broth microdilution. The checkerboard technique was used to examine the potential synergy between DFO and colistin. Furthermore, time-kill assays were used for the confirmation of synergy detected by the checkerboard assay, as well as for determining bacteriostatic and bactericidal interactions throughout a 24-h period. As expected, all *P. mirabilis* isolates were resistant to colistin. DFO did not inhibit *P. mirabilis* growth when used alone, even at very high doses (10 μg ml^{-1}). Notably, when in combination with DFO, the MIC values of colistin were markedly reduced, and the checkerboard assay results showed synergy between colistin and DFO for all isolates. In addition, in time-kill assays, colistin + DFO exhibited synergistic activity against all strains at most time intervals and concentrations tested. Colistin + DFO showed bactericidal activity at colistin concentrations of 1xMIC and 2xMIC, although a degree of re-growth was observed in one of the strains at 12-24 h. These findings indicated that DFO has the potential for use as an adjunct to colistin through iron sequestration, thus providing synergistic activity to an antibiotic that would not normally be considered a treatment option against *P. mirabilis*. *In vivo* experiments in the future may provide useful information on the efficacy of DFO/colistin since these models effectively reflect physiological parameters.

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Introduction

Proteus is a gram-negative bacterium, which is widely distributed in a range of settings, including water sources, soil and sewage, but it is primarily a flora member of the gastrointestinal systems of humans and animals (1). Swarming motility, and the production of urease, hemolysin and numerous fimbrial adhesions are the phenotypic characteristics of this bacterium (2). *Proteus mirabilis* is the most prevalent cause of human infections among all *Proteus* species (3). *P. mirabilis* is the most frequently detected bacteria in long-term urinary catheterization and is a significant cause of complicated urinary tract infections (UTIs), wound infections, gastroenteritis, and, in rare cases, bacteremia (2,3). A distinctive feature of *P. mirabilis* is that it produces crystalline biofilms, leading to encrusted and clogged catheters in long-term urinary catheterization, which aggravates catheter-associated urinary tract infections (CAUTIs) (4). Therefore, urine retention and reflux, as well as severe bladder distension and pyelonephritis, may develop. Furthermore, crystalline biofilms have been reported to be associated with the persistence of *P. mirabilis* in the urinary system via shielding it from antimicrobial agents and the host immune mechanisms (5).

Colistin exhibits a broad spectrum of antibacterial activity, mostly against gram-negative bacteria since its antibacterial activity occurs on the outer membrane (6). However, some gram-negative bacteria are naturally resistant to colistin, such as *Neisseria meningitidis*, *Burkholderia* species and *P. mirabilis* (7). Antimicrobial resistance is a major public health issue and antimicrobial resistance in numerous bacterial species has an impact on a number of facets of medical practice, from treatment of infections in primary healthcare to the clinical management of patients with severe diseases in tertiary care (8). The worldwide spread of antibiotic-resistant bacterial strains poses a considerable obstacle to appropriate treatment, as there are few clinically available antibiotics that maintain adequate action against these strains (9).

Iron is a vital element for growth, and is necessary for the activity of numerous proteins and enzymes participating in various physiological pathways, such as oxygen transportation, gene regulation and nitrogen fixation (10). In the mammalian host, the majority of intracellular iron is stored in ferritin or bound to haem or haem-containing substances, whereas extracellular iron is bound to transferrin, lactoferrin, haemopexin and haptoglobin, making it unavailable for bacterial uptake (11). Bacterial pathogens

that have the ability to colonize humans use several strategies to scavenge essential elements, such as iron and zinc; therefore, there is a perpetual competition between bacteria and the host for micronutrients (12). Pathogenic bacteria have evolved multiple iron transportation pathways, intracellular iron stores, redox stress resistance systems and iron-sensitive regulatory sequences to control the expression of genes involved in several cellular activities to counterbalance iron-deficient situations and sustain iron homeostasis (10). A number of pathogenic bacteria use siderophores to overcome the iron-limiting environment in the host. Siderophores are low molecular weight iron-binding substances that are secreted and imported by microorganisms for iron acquisition (13). The iron is released from the siderophore after cellular uptake to aid microbial metabolism and multiplication. During infection with bacterial and fungal pathogens, siderophores are thought to be important virulence components (14). The existence of these regulatory and competing mechanisms underlines the significance of iron in the survival of bacteria.

Given the critical function of iron in the growth and survival of numerous pathogenic bacteria, minimizing the amount of iron available at the infection site may help to improve the treatment outcome. Using chelator compounds that sequester various metals and impede bacterial iron uptake is one of the approaches for attaining iron limitation (8). Iron chelating agents can form complexes with iron in both its ferrous (Fe^{2+}) and ferric (Fe^{3+}) states, although chelators generally exhibit different levels of affinity for the different states (15,16). Deferoxamine (DFO) is a semi-synthetic drug derived from the bacterial siderophore desferrioxamine B, which has been licensed for medical use in the treatment of iron excess in patients (17). DFO also has an impact on the amount of iron available to microorganisms, this feature constitutes the founding of new microbial control strategies, such as novel treatment regimens or even preservation systems (18,19). Considering the ongoing occurrence and spread of antibiotic resistance, and the limited prospect that the current development process will be able to meet the demand for new antimicrobial agents with novel mechanisms of action, it is necessary to investigate the potential of alternatives (8). Iron chelators that have already withstood toxicity and preclinical testing in animals may provide an alternative therapeutic technique in the case of multidrug-resistant bacteria, for which entire classes of antibiotics are no longer considered treatment options (20). Siderophores may also serve as a facilitator for antibiotics across the cell membrane because of increased cell membrane permeability induced by iron deprivation (21). We hypothesize that iron deprivation or interactions with cell membranes caused by DFO or increased siderophore synthesis may cause inhibition or inactivation of proteins and enzymes necessary for critical processes of bacteria, as well as synergy with membrane-active antibiotics, such as colistin.

Materials and methods

Bacterial strains. Clinical *P. mirabilis* isolates (n=11) recovered from blood culture samples between July and December 2021 in the Research and Application Hospital of Gaziantep University (Gaziantep, Turkey), a tertiary care center, were used in the present study. The isolates were collected from 11 individual patients. *Escherichia coli* ATCC25922 (American Type Culture Collection) and *E. coli* NCTC 13846 (National Collection of Type Cultures) strains were provided by the Microbiology Laboratory of Gaziantep University's Medical Faculty Hospital (Gaziantep, Turkey) and employed as quality control in the broth microdilution tests. Prior to testing, the isolates were cultured from frozen stocks onto Columbia Agar with 5% sheep blood (BD Biosciences) and incubated overnight at 35˚C.

Determination of minimum inhibitory concentration (MIC). MIC values of DFO and colistin against *P. mirabilis* were determined using the reference broth microdilution method according to the International Organization for Standardization (ISO) standards (ISO 20776-1:2019) (22). Colistin (Biosynth Ltd.) stock solutions were prepared in sterile distilled water (dH_2O) and stored in aliquots at -20˚C. DFO (Desferal®; Novartis Corporation) was freshly prepared as a 50 mg/ml stock solution in sterile dH_2O prior to the study. Test solutions of DFO and colistin were prepared immediately before use. Briefly, serial two-fold dilutions of DFO and colistin in cation-adjusted Mueller-Hinton Broth (CAMHB; Oxoid; Thermo Fisher Scientific, Inc.) were prepared in a 96-well plate. The inoculum to be tested was prepared from overnight cultures by dilution in MHB to provide a final bacterial density of $5x10^5$ colony-forming unit (CFU)/ml. Colistin was tested over a range from 0.062 to 128 μg/ml, and DFO was tested over a range from 0.062 to 10 mg/ml. Each well was loaded with bacterial suspensions, and the plates were incubated at 35˚C for 24 h. Controls for positive growth and negative sterility were also performed. The lowest concentrations of DFO and colistin with no visible signs of turbidity were defined as MIC values.

Checkerboard assay. Fractional inhibitory concentration index (FICI) values of combinations of DFO and colistin against a total of 11 *P. mirabilis* isolates were determined using the checkerboard technique (23,24). In brief, serial two-fold dilutions of the first compound (colistin) were prepared across the columns and the second compound (DFO) dilutions were prepared across the rows of a 96-well plate. Individual wells were inoculated with suspensions of overnight cultures in MHB to provide a final inoculum density of $5x10^5$ CFU/ml. The plates were incubated for 24 h at 35˚C. FIC values were defined by broth microdilution on separate checkerboard panels containing increasing concentrations of DFO (rows G through A, 8 to 512 μg/ml) and colistin (columns 1 through 11, 0.06 to 64 μg/ml). The FIC of both drugs was calculated using the formula: FIC=MIC of Drug in combination/MIC of Drug alone. The FICI was then calculated from the sum of the FIC values using the following formula: FICI=(MIC of colistin in combination/MIC of colistin alone) + (MIC of DFO in combination/MIC of DFO alone). The interpretation of the findings was as follows: Synergy if FICI $\leq$0.5; no interaction if FICI 0.5-4; and antagonism if FICI >4 (23). On an occasion where a MIC for one of the test compounds was off-scale (greater than the highest concentration tested), the MIC was set to the next highest two-fold concentration for calculation of the FIC (e.g. if the highest MIC was tested 32 μg/ml, the FIC was calculated based on a MIC of 64 μg/ml) (25).

Time-kill assay. In accordance with the results from the checkerboard assays, two isolates were randomly selected for further evaluation of the DFO-colistin combination using a time-kill assay. Colistin was tested alone and in combination

Table I. Antimicrobial activity of colistin with DFO against PM strains.

	MIC value, μg/ml				
Isolate	Colistin	DFO	Colistin MIC in combination with DFO	FICI	Interpretation
PM1	16	>512	0.5	0.06	Synergy
PM2	16	>512	4	0.5	Synergy
PM3	16	>512	0.5	0.06	Synergy
PM4	16	>512	1	0.12	Synergy
PM5	16	>512	0.5	0.06	Synergy
PM6	16	>512	0.5	0.06	Synergy
PM7	16	>512	4	0.5	Synergy
PM8	16	>512	4	0.5	Synergy
PM9	16	>512	0.5	0.06	Synergy
PM10	8	>512	0.5	0.09	Synergy
PM11	16	>512	0.5	0.06	Synergy

PM, *Proteus mirabilis*; MIC, minimum inhibitory concentration; DFO, deferoxamine; FICI, fractional inhibitory concentration index.

with DFO for the selected isolates at 0.5X, 1X and 2X the MIC concentrations of colistin. For isolates where the DFO MIC was >512 μg/ml, a DFO concentration of 128 μg/ml (0.25X the highest concentration tested) was used during testing. In preparation for the study, bacterial suspensions were prepared in MHB from freshly-grown blood agar plates, diluted and grown to the log-phase. The turbidity of bacterial cultures was adjusted to form a final inoculum density of $5x10^5$ CFU/ml, as verified by viable count, and added to flasks containing 20 ml MHB. Then, treatments were added to broth cultures to yield desired concentrations. Growth controls without any treatment were also included. Test and control flasks were incubated at 35˚C and viable counts were performed at 0, 1, 3, 6, 9, 12 and 24 h by serial dilution plating. Samples were spread onto Mueller-Hinton Agar (MHA; Difco; BD Biosciences) plates, because swarming ability of *Proteus* makes colony counting difficult on media other than MHA. All plates were incubated for 20-24 h at 35˚C. Colonies were counted manually, and the CFU/ml was determined from the average count of the duplicate plates, followed by the calculation of the log10 CFU/ml. Antimicrobial activity was calculated for each isolate as the change in the bacterial count of ($\Delta\log_{10}$ CFU/ml) obtained in 24 h compared with the count at the start and defined as $\Delta\log_{10}$ CFU_{24}. Bactericidal activity was evaluated as a ≥3 $\log_{10}$ decrease in CFU/ml over the time period examined, whereas synergy was considered a ≥2 $\log_{10}$ decrease in CFU/ml for the antibiotic combination in comparison with the most effective monotherapy (26,27). Time-kill studies were performed as three independent replicates and graphs displaying the results were generated using Excel (version 16; Microsoft Corp.).

Results

Broth microdilution and checkerboard method. MICs, detected by broth microdilution method, were 8-16 μg/ml for colistin. However, DFO did not affect bacterial growth even at a concentration of 10 mg/ml (Table I). Because the DFO may easily transfer iron to bacteria with a homologous siderophore receptor and because CAMHB is a rich broth with abundant iron, the outcome was not surprising. Using EUCAST criteria, all *P. mirabilis* strains were classified as resistant to colistin (28). After combination with DFO, the MIC values of colistin were reduced. The results of a checkerboard assay showed synergy (i.e. FICI ≤0.5) between colistin and DFO for all of the isolates (Table I). No antagonism was observed for the combination.

Time-kill method. The two randomly selected *P. mirabilis* isolates (PM2 and PM7) were used in the time-kill assays. The time-kill profiles of isolates and changes in $\log_{10}$ CFU/ml from the initial inoculum to 24 h are shown in Fig. 1. DFO monotherapy (512 μg/ml) and colistin monotherapy at a concentration of 0.5xMIC produced little or no bacterial killing at any time-point, with bacterial growth close to control values across the 24 h. Colistin monotherapy at concentrations of 1xMIC and 2xMIC showed bacteriostatic activity maintained for 9 h, with subsequent re-growth in varying amounts. Colistin + DFO showed synergistic activity against all strains at most time intervals and concentrations tested. Colistin + DFO showed bactericidal activity at colistin concentrations of 1xMIC and 2xMIC although a degree of re-growth was observed in isolate PM7 at 12-24 h (Fig. 1).

Discussion

A limitation of the present study is that there were no animal experiments conducted, in which the physiological environment could be better evaluated, since they were not included in the initial research design and ethics committee approvals. Excess iron has been shown to aggravate the situation in various infections, including tuberculosis, malaria, invasive bacterial infections, cystitis, keratitis and wound infections (29-31). *P. mirabilis* is a significant causative agent

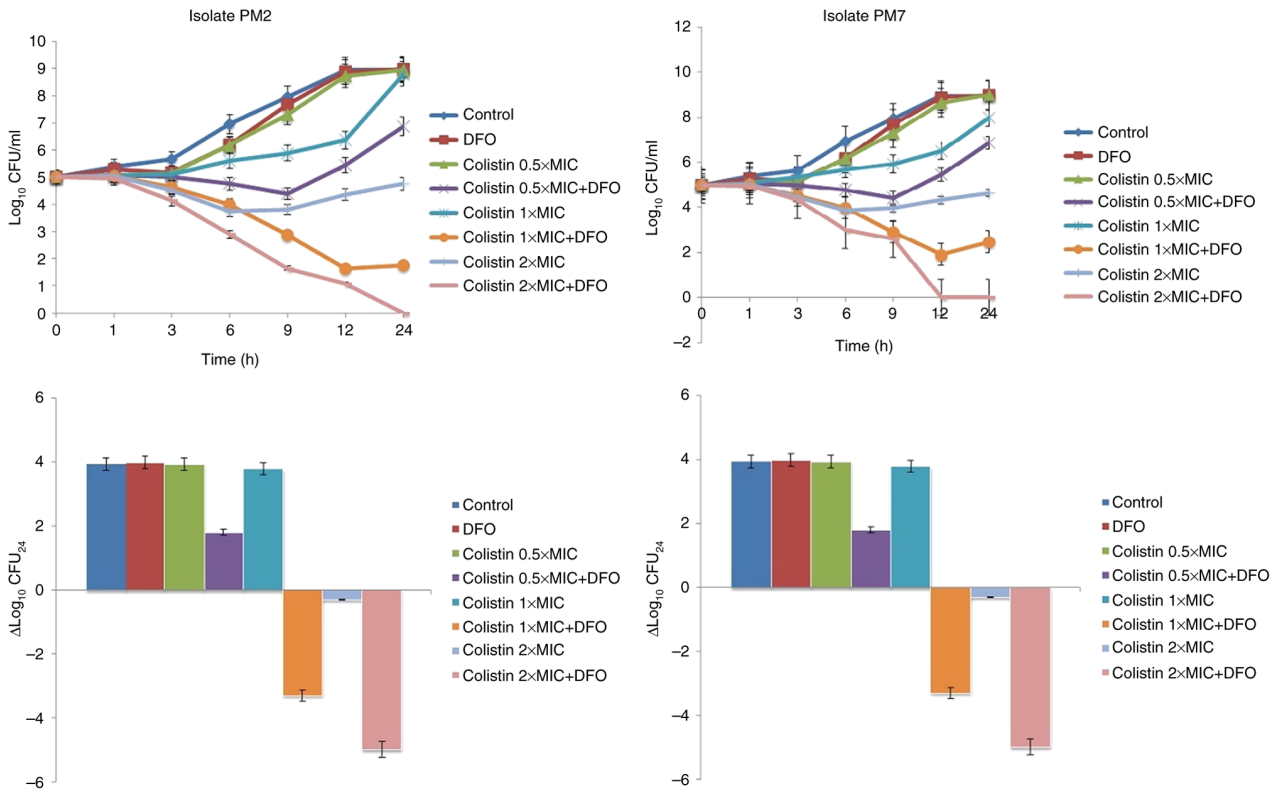

Figure 1. Time-kill profiles of colistin and DFO monotherapy and their combination against two *Proteus mirabilis* isolates. $\log_{10}$ CFU/ml indicates logarithmic calculation of CFU over time. $\Delta\log_{10}$ CFU_{24} indicates changes in $\log_{10}$ CFU/ml at 24 h. CFU, colony-forming units; DFO, deferoxamine; MIC, minimum inhibitory concentration.

of UTIs, especially CAUTIs. Bacterial capability to import Fe^{3+}, Zn^{2+} and Ni^{2+}, and export Cu^{+} is essential for efficacious colonization of the urinary system (32). In the course of a UTI, it has been shown that iron overload in the bladder or in the urine can exacerbate inflammation and escalate urothelial cell death (33). Urine samples from patients with acute UTIs have markedly higher iron levels and sloughed epithelial cells than urine samples from the healthy population (32). In addition, it has been reported that, for infants, having higher basal iron serum concentrations or increased iron supplementation is associated with an increased risk of UTIs (34-36). Similarly, iron concentrations in the urine of postmenopausal women who are prone to recurrent and chronic UTIs have been shown to be higher than those in the general postmenopausal female population (37-39). Increased ambient iron has also been reported to lead to enhanced growth and a significant increase in the intracellular bacterial load of *E. coli*, another frequent causative agent of UTIs, in bladder epithelial cells (33). Given that high iron levels enhance bacterial colonization, infection development and infection chronicity in specific systems, limiting ambient iron appears to be an appropriate strategy to battle pathogenic microorganisms.

Sequestration of iron by chelation may be a beneficial adjunct for the treatment of infections, given the relationship between iron excess or dietary iron supplementation and infection (40). For a number of years, iron chelators have been used to treat iron excess conditions, and their pharmacological and safety profiles have been widely investigated (41). Depletion of iron through synthetic iron chelators can be effective in inhibiting bacterial growth (29). DFO, as an iron chelator, aids the host's intrinsic iron-withholding systems and appears as a promising treatment option for local infections (21). Iron chelation with DFO has been reported to improve host cell survival, reduce bacterial proliferation in urothelial cells and reduce autophagy (33). Furthermore, DFO has shown antibacterial activity as a single agent, and can inhibit the growth of *Acinetobacter baumannii*, *Pseudomonas aeruginosa* and *Staphylococcus aureus* at varying concentrations (21). Furthermore, impaired iron acquisition can reduce the urinary tract colonization of *P. mirabilis* (42). To the best of our knowledge, the present study is the first investigation to determine DFO MIC values against *P. mirabilis* isolates using the standard broth microdilution method. However, the results revealed that DFO does not have the potential to be an antibacterial agent on its own.

An overabundance of iron is hazardous to the host, not just because it enhances bacterial growth, but also because it induces increased inflammatory activity and epithelial cell stress (33,43). Furthermore, local iron deprivation may have an effect on the host immune system response by reducing local reactive oxygen species (ROS) formation (29). It has been demonstrated that the cumulation of iron causes an elevated inflammatory response in local bladder tissue, as well as increased bacterial colonization, whereas these findings can be reversed by a low-iron diet (44). During the course of infection, unbound labile iron accumulates in the plasma and induces a variety of responses, including elevated cell proliferation rates, persistent suppression of cell proliferation, and apoptotic or necrotic cell death via ROS. Therefore, pharmacological regulation of iron by chelation therapy is crucial for achieving

a balance between inhibiting cell damage and supplying the cellular demands (29). Although the most concrete indicator of the success of medical treatment against infections is the discontinuation of bacterial growth, since limiting ambient iron reduces inflammation, iron chelators combined with antibiotics can reduce patient complaints and symptoms.

Medical device-associated infections are frequently related to indwelling objects, such as urinary catheters and prosthetic joints, and are responsible for ~50% of all nosocomial infections (45). These objects provide an abiotic surface and facilitate the formation of biofilms (46). Biofilm production is an important virulence factor in the emergence of CAUTIs (42). Biofilm-related infections are notoriously difficult to treat due to the adjustments enabled by biofilm development, which result in increased antibiotic resistance as well as increased resistance to host immune mechanisms (46). Higher levels of iron are required for the production of biofilms than for bacterial proliferation (47,48). A recent study showed that iron can trigger biofilm formation in *P. mirabilis* (42). Similarly, biofilm production is decreased when lactoferrin, a physiological iron chelator, is added to *P. aeruginosa* cultures (49). A previous study also observed that DFO can improve the ability of tobramycin to dissolve formed biofilms grown on human airway epithelial cells (50). Iron chelation may be useful in infection treatment due to the crucial function of iron in biofilm development and bacterial pathogenicity (29). Inhibition of bacterial iron acquisition via catheters made of iron-scavenging materials or coated with chelators may decrease biofilm development and further infection, but this approach has yet to be tested in a clinical setting (29,42). However, generally, it has been assumed that the local administration of iron-chelating agents represents a safe pharmacological therapy with a low risk of side effects (29). Iron chelation may be employed as a prophylactic strategy to minimize medical device-associated infections, especially for certain systems, such as the urinary system.

Bacteria sense an iron-deficient environment and react correspondingly by upregulating iron-acquisition pathways as well as virulence genes (51). A β-barrel receptor on the outer membrane of gram-negative bacteria recognizes iron-bound siderophores. The iron-bound siderophore is translocated into the periplasm after ligand engagement causes a conformational alteration (29). An ATP-binding cassette transporter in the inner membrane mediates conformational alterations in transportation into the cytoplasm and iron reduction. This complex will then attach to certain receptor proteins on the bacterial cell surface, allowing it to be taken through active transport (29). It is hypothesized that iron limitation conditions may result in increased production of siderophores, which are specific molecules for transporting iron. Siderophore secretion has the physical outcome of allowing molecules to diffuse away from producers while possibly preventing diffused molecules from returning producer cells. Diffusion can still result in significant siderophore loss, putting bacterial fitness at risk (52). In the present study, in addition to discovering a synergistic interaction between DFO and colistin in all *P. mirabilis* isolates using the checkerboard method, the MIC values of the DFO and colistin combination were in the susceptible ranges of colistin against Enterobacterales for the vast majority of the isolates, according to EUCAST guidelines (28). However, *in vivo* experiments would be necessary to confirm the *in vitro* findings. According to data not shown, no significant synergy was detected in *E. coli*, *K. pneumoniae*, *P. aeruginosa* and *A. baumannii* isolates in the subsequent investigation to assess whether DFO can exhibit synergistic effects when used with colistin against other gram-negative bacteria. From this point of view, we hypothesize that the ability of all bacteria to produce siderophores and their responses to iron restriction is not the same, and the synergy of DFO and colistin detected in the present study is based on a specific mechanism. Effective siderophore production was previously thought to be a feature of aerobic gram-negative bacteria (53). However, it has been discovered that some gram-positive bacteria may generate siderophores (54). The identification of bacterial species that lack effective siderophore production will become more relevant if iron chelators are combined with antimicrobial treatment.

Colistin is a polymyxin antibiotic with rapid bactericidal activity against gram-negative bacteria (55). Similarly, the bactericidal action of the DFO-colistin combination (colistin 1xMIC + DFO, colistin 2xMIC+ DFO) was evident from the onset of the present study, according to our time-kill assay results. As a result, it was hypothesized that several intrinsic mechanisms related to colistin resistance of *P. mirabilis* were rapidly eliminated via the addition of DFO. Furthermore, the fact that the bactericidal effect of the DFO-colistin combination was maintained for ≥12 h in the present experiments indicated that it could be beneficial without the requirement for frequent dose repetitions since colistin has a long half-life (14.4 h) (56). This insight can be beneficial for optimizing doses and improving treatment approaches when combined with the pharmacokinetic profile of the targeted patient population. Conformational changes in the outer membrane of the bacteria during both increased secretion and uptake of siderophores may be responsible for vulnerabilities against colistin activity. Similarly, vancomycin, which is not preferred in *P. aeruginosa* infections due to its low gram-negative activity, has been reported to be effective when used with the DFO-gallium complex due to disturbance of the outer membrane via a combination of electrostatic and hydrophobic interactions coupled with selective binding and resulting in enhanced permeability of vancomycin as a result of the membrane damage (57). Under low iron concentrations, several physiological changes may occur in the bacterial pathogens, including a shift to a planktonic state (58,59). Bacteria in a planktonic state are known to be more susceptible to certain antimicrobials, suggesting a potential mechanism of iron chelation-induced sensitization to antimicrobials (8). Because of the increased permeability induced by iron deprivation, siderophores may potentially serve as a facilitator for colistin across the cell membrane. The use of antivirulence compounds combined with antibiotics may be a promising approach for virulence attenuation and pathogen elimination (60). In addition to binding iron, DFO may also have an affinity for zinc. The ability to sequestrate zinc is thought to be responsible for making metallo-β lactamase producers susceptible to β-lactams (61). Similarly, deprivation of iron reduces the activity of key proteins and enzymes, such as cytochromes, which are examples of iron-dependent proteins that are crucial for energy metabolism and ribonucleotide reductase, which is involved in DNA synthesis. If any of these are disrupted, the multiplication of the microorganism may

be halted (21). Briefly, inhibition of bacterial growth, reducing biofilm formation, membrane disruption, inactivation of specific enzymes or proteins that are essential for cell replication, and reducing oxidant stress on host cells may constitute potential pathways for the enhanced bactericidal effect of colistin via iron chelation. We are currently investigating whether the synergistic interaction discovered between DFO and colistin extends to other antibiotics. Identifying patient groups and explaining the mechanisms underlying the synergistic interaction will be aided by determining the interactions between different drug groups and iron chelators.

In conclusion, the present study suggested that DFO has the potential for use as an adjunct to colistin through iron sequestration, thus providing synergistic activity to an antibiotic that is not normally considered a treatment option against *P. mirabilis*. *In vivo* experiments will provide useful information on DFO-colistin efficacy, since these models are better in terms of reflecting physiological conditions such as metal ion levels. Also, *in vivo* models account for parameters, such as compound biodistribution, pH and the presence of host factors. Because CAMHB is a rich broth with substantially higher iron, carbon sources and other cofactors than the levels in the human body, the synergy that was detected *in vitro* may be greater *in vivo*.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

ME and YZ contributed to the study conception and design, and performed material preparation, data collection and analysis. The first draft of the manuscript was written by ME and both authors commented on previous versions of the manuscript. ME and YZ confirm the authenticity of all the raw data. Both authors have read and approved the final manuscript.

Ethics approval and consent to participate

The present study was performed in line with the principles of The Declaration of Helsinki. Approval was granted by the Gaziantep University Clinical Research Ethics Committee (date, January 27, 2021; approval no. 2021/11; Gaziantep, Turkey).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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