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REVIEW ARTICLE

Effect of supplementation on biofilms and antibiotic efficacy against *Pseudomonas aeruginosa*

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ABSTRACT

Pseudomonas aeruginosa, a Gram-negative opportunistic pathogen, is one of the leading causes of nosocomial infections, particularly in vulnerable patients. However, treating these infections is challenging due to its various antibiotic resistance mechanisms and biofilm formation ability. As traditional antibiotic development struggles to keep pace with evolving resistance, this review explores a promising alternative strategy which is enhancing existing antibiotic efficacy by combining them with nutritional supplements that modulate *P. aeruginosa* physiology. Specifically, it focuses on studies investigating the effects of diverse carbon, nitrogen, and iron sources on bacterial response to antibiotics, and the mechanisms underlying observed synergy. To achieve this, published literature on *P. aeruginosa* metabolism, antibiotic resistance, and nutritional influences was comprehensively analyzed and summarized. The findings highlight specific carbon, nitrogen, and iron sources that can enhance various antibiotic classes against *P. aeruginosa*. These include supplements capable of disrupting biofilm formation, reducing efflux pump activity, or interfering with other resistance mechanisms, thereby increasing antibiotic susceptibility. The specific mechanisms by which these supplements interact with bacterial physiology and antibiotic action are thoroughly discussed. Ultimately, modulating *P. aeruginosa* physiology through strategic supplementation alongside existing antibiotics offers a promising approach to overcome drug resistance in this pathogen.

Keywords: *Pseudomonas aeruginosa*; Anti-bacterial agents; Biofilms; Persister cells; Xylitol; Arginine; Iron

INTRODUCTION

Pseudomonas aeruginosa is a Gram-negative, heterotrophic, and motile bacterium measuring 0.5-1.0 µm in width and 1-5 µm in length. It typically affects immunocompromised or vulnerable patients, and it is rarely pathogenic in healthy individuals. This facultative aerobe generates metabolic energy through both aerobic and anaerobic respiration mechanisms, utilizing nitrate or arginine as the final electron acceptor when oxygen is not present. Additionally, *P. aeruginosa* exhibits limited fermentative capabilities, often resulting in extremely slow or no growth. As a prototroph, the organism can thrive on minimal salt growth media with a single carbon and energy source, while it can utilize over 100 diverse organic molecules as sources of carbon and/or energy. *P. aeruginosa* often exhibits resistance to various antibiotics and other treatments, making its infections notably challenging to treat. Consequently, the World Health Organization has prioritized *P. aeruginosa* as a Gram-negative bacterium urgently requiring new treatment strategies (1, 2).

The available therapeutic options for these resis-

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tant infections are severely restricted, contributing to high mortality rates. Given the time-consuming and costly nature of discovering new antibiotics, recent efforts have shifted towards innovative therapeutic strategies that aim to alter the metabolic state of *P. aeruginosa* cells, thereby rendering them susceptible to existing antibiotics. Crucially, these strategies are designed not to activate novel resistance mechanisms, offering a unique advantage over traditional approaches. This review therefore explores the impact of various supplementation sources on *P. aeruginosa* metabolism and biofilm formation, both of which influence antibiotic resistance. It also describes key antibiotic resistance mechanisms and discusses novel therapeutic strategies involving these nutritional approaches.

ANTIBIOTIC RESISTANCE

As previously mentioned, eradication of *P. aeruginosa* infections is particularly challenging because of its resistance to several antibiotic groups, including *β*-lactams, quinolones, and aminoglycosides (3). The bacterium's primary resistance mechanisms generally fall into the following categories: inherent, acquired, and adaptive. Intrinsic resistance involves the synthesis of enzymes that degrade or modify antibiotics, the development of efflux pumps that expel drugs from the cell, and poor outer membrane permeability. Acquired resistance occurs through horizontal gene transfer and mutational modifications. Furthermore, *P. aeruginosa* employs its adaptive resistance mechanism to form biofilms in infected patients. These biofilms serve as diffusion barriers, significantly limiting antibiotic penetration to bacterial cells (4, 5).

Biofilms are aggregates of bacteria embedded in a protective matrix made up of extracellular polymeric substances (EPS). EPS increase bacterial persistence on both living and non-living surfaces while also protecting them from harsh conditions (6). A unique feature of biofilms is the presence of persister cells within them; these are dormant bacteria showing tolerance to antibiotics (7). The initial stage of biofilm formation involves the reversible attachment of planktonic or free-living bacteria to a surface. In the subsequent step, known as growth or microcolony formation, the attached bacteria multiply and aggregate within the EPS, eventually forming microcolonies. EPS are central to the formation of biofilms by facilitating bacterial attachment to surfaces, maintaining the biofilm's three-dimensional structure, promoting cell aggregation, and protecting them from external stresses. Finally, matured biofilms disperse either passively (e.g., due to physical forces like liquid flow-dependent dispersion) or actively (e.g., due to EPS degradation), allowing bacteria to spread and form new biofilms (8, 9).

POSSIBLE THERAPEUTIC STRATEGIES

Numerous therapeutic strategies have been investigated to manage *P. aeruginosa* infections. These strategies can be categorized as follows.

Combination therapy. One way to treat antibiotic-resistant infections is by combining two or more antibiotics with different mechanism of action. This type of therapy is often more effective than single-agent therapy. However, combining antibiotics can increase the risk of adverse effects and toxicity. A number of recently approved combination drugs demonstrate promising activity against multi-drug-resistant *P. aeruginosa*. For instance, ceftolozane/tazobactam, a combined *β*-lactamase inhibitor-cephalosporin, has demonstrated efficacy against *P. aeruginosa* infections (10).

Bacteriophage therapy. Another approach to treat *P. aeruginosa* infections is using bacteriophages, which are viruses that specifically target bacteria. Bacteriophages attach to receptors on bacteria, inject their genetic material into bacterial cells, and replicate, ultimately causing the bacterial cell to burst. Researchers have explored bacteriophage therapy as a potential complementary or alternative therapy to antibiotics, particularly in cystic fibrosis (CF) patients. Bacteriophage therapy is already being implemented in certain regions (11).

Immunotherapy. Strengthening immune system responses against *P. aeruginosa* through either immunomodulatory drugs or vaccinations is another promising strategy for treating these infections. Researchers are developing various vaccine candidates, ranging from live-attenuated or killed *P. aeruginosa* to those which target specific bacterial components. The latter candidates include DNA sequences, flagella, pili, LPS O-antigen, surface polysaccharides, outer-membrane

proteins (like OprF and OprI), and Type III Secretion System (T3SS) component PcrV (12, 13).

Antimicrobial peptides. Antimicrobial peptides are also being investigated as potential therapeutic agents to treat infections. These peptides are derived from natural sources, including bacteria, fungi, protists, plants, and animals. Antimicrobial peptides primarily target the bacterial cytoplasmic membrane, disrupting it to kill cells (14). Moreover, promising results have been shown by peptides that target other bacterial components, such as antibiotic-hydrolyzing or -modifying enzymes (15).

Anti-biofilm strategies. Anti-biofilm strategies are also promising when it comes to eradicate *P. aeruginosa* infections. This bacterium's biofilm-forming capability represents a major factor in its resistance to antimicrobial treatments, as biofilms create a physical shield that restricts the penetration of antibiotics and other antibacterial agents (16). This protective effect, coupled with changes in bacterial growth and physiology (such as persister cell formation) within the biofilm, significantly reduces antibiotic effectiveness, leading to difficult-to-treat and recurring infections (17). Recognizing this challenge, researchers are actively investigating various strategies to improve biofilm treatment efficacy. These approaches include using enzymes to break down the biofilm matrix, applying sound waves or magnetic fields to disrupt the biofilm structure, and chemically modifying existing antibiotics to enhance their penetration (18-21). Given the limitations of current methods to treat biofilm-related infections, research directions are focusing on more innovative strategies. Ideally, these strategies target mechanisms that reverse antibiotic tolerance in persister cells by activating their low metabolic phenotype, thereby rendering them susceptible to conventional antibiotics. This approach holds promise for a more effective and long-lasting solution against *P. aeruginosa* infections. The usage of nutritional supplements is among the promising therapeutic approaches and will be discussed in detail below.

SUPPLEMENTS

Regarding antimicrobial resistance, biofilms frequently exhibit hundreds of times greater resistance than planktonic cells, owing to the reasons previously mentioned (22). Recent studies have revealed that adding carbon sources such as glucose, mannitol, fructose, and pyruvate to *Escherichia coli* persister cells can increase their metabolic activity and restore antibiotic sensitivity (23). Comparable findings were also observed in *P. aeruginosa* biofilms (24). Numerous researchers have shown that microbial lipids can be modified by the type of carbon sources supplied to the microbes. These variations in cellular structure undoubtedly reflect variations in antibiotic susceptibility among various species and strains. The exceptional nutritional capacities of *Pseudomonas* spp. allow them to catabolize a wide range of chemical molecules and produce diverse catabolites, which may affect their antibiotic resistance. For instance, Conrad et al. demonstrated that the acyl derivatives of branched-chain amino acids and their metabolism have a significant impact on *P. aeruginosa*'s susceptibility to colistin and polymyxin B (25).

According to the above statements, supplements can alter bacterial metabolism to increases their susceptibility to antibiotics. This is a significant outcome, as it allows for the re-sensitization of bacteria to existing antibiotics, circumventing the time-consuming and expensive process of discovering new antimicrobial agents. This review will therefore discuss various supplements and their effects on *P. aeruginosa* antibiotic resistance. The supplements are categorized into carbon sources, nitrogen sources, and metals.

CARBON SOURCES

Mannitol. Mannitol is a six-carbon polyol found in various natural sources including some microorganisms (26). This polyol serves as a carbon source for certain bacteria, including *P. aeruginosa* (27) and has demonstrated potential efficacy against *P. aeruginosa* biofilms. Studies suggest it may enhance the effectiveness of antibiotics against persistent infections by affecting persister cells. Notably, Barraud et al. investigated mannitol's potential to alter the metabolism of biofilm-associated persister cells. Their research showed that growing biofilms of *P. aeruginosa* strain PAO1 in the presence of 40 mM mannitol, followed by treatment with 80 mg/L tobramycin, significantly decreased biofilm viability and enhanced antibiotic activity by 99.96%, compared to controls lacking mannitol. They concluded that man-

nitol's presence during biofilm formation and antibiotic treatment prevented persister cell formation. Beyond preventing persister cell formation, Barraud et al. also observed that 40 mM mannitol was able to reverse the persister phenotype and significantly increase 80 mg/L tobramycin's effectiveness when a 20-hours, pre-grown biofilm was treated with this combination for 3 h, compared to a control treated with tobramycin alone. The reason for this persister cell reversion appears to be a metabolic pathway induction by mannitol, as a *P. aeruginosa* mutant strain with impaired mannitol metabolism in this study did not show a similar effect. The increased inflow of tobramycin to the cells in the presence of mannitol may also contribute to its enhanced activity. These findings collectively imply that mannitol modulates biofilm antibiotic susceptibility via an active physiological response (24). This aligns with the concept proposed by Allison et al., who suggested that activating metabolites could transform persister cells into normally developing cells, rendering them susceptible to antibiotics (23). Further supporting the potential for mannitol's synergistic effects, Žiemytė et al. demonstrated that mannitol at various doses (0.0625 to 3200 mg/L) did not exhibit any antimicrobial impact on planktonic growth of *P. aeruginosa* strains PAO1 or MF120. While their results indicated that mannitol alone is not an agent for biofilm dispersal, they found that the combined treatment of a 48-hour biofilm with 3200 mg/L of mannitol and 0.25 mg/L of ciprofloxacin could 100% destroy bacterial cells within the biofilm (28).

However, contradictory findings have emerged regarding mannitol's impact on antibiotic efficacy. A study by Price et al. on an in vivo-like model system, which involved bacterial biofilms cultivated on CF-derived airway cells directly, contradicted Barraud et al.'s results. In this in vivo-like context, mannitol failed to show synergism with tobramycin in eradicating *P. aeruginosa* biofilms, whether formed by isolates recovered from sputum of individuals with CF or by laboratory strains (PAO1 and PA14). Specifically, co-treating *P. aeruginosa* with 8 µg/mL of tobramycin and 60 mM mannitol did not make *P. aeruginosa* more sensitive to tobramycin. This discrepancy was attributed to inherent differences between in vivo-like biofilms formed on living surfaces (like the cell cultures), which benefit from better access to nutrients, compared to in vitro biofilms formed on non-living surface, as used in Barraud's research (29).

Beyond its metabolic roles, mannitol also offers promise in improving drug delivery in challenging environments like CF lungs. Yang et al. highlighted that while respiratory drugs can be effective locally in CF patients, the impenetrable lung mucus presents a significant challenge for antibiotic therapy. They hypothesized that an osmotic agent like mannitol, when applied to the lung epithelial mucosal layer, would draw water from epithelial cells, diluting the mucus and enhancing drug penetration. In their research, they evaluated the efficacy of spray-dried microparticles containing ciprofloxacin and either mannitol or NaCl. They demonstrated that 1.6 mg of mannitol-containing microparticles (each with 80 µg of ciprofloxacin) significantly enhanced the antibacterial activity against *P. aeruginosa* grown in 1 mL of artificial mucus, substantially reducing cell numbers compared to NaCl-containing particles. This marked antibacterial activity of ciprofloxacin-mannitol was likely due to mannitol's capacity to improve antibiotic penetration into the mucus, a result of mannitol-induced increases in local water content. This mechanism is supported by the observation that the MIC values of ciprofloxacin alone and ciprofloxacin-mannitol were not different, suggesting enhanced delivery rather than altered intrinsic activity (30). It is also worth mentioning that dry powder mannitol is utilized for mucus clearance in CF patients (31), implying potential dual benefits if it also influences biofilm antibiotic sensitivity through a dynamic, physiological mechanism.

Collectively, these studies highlight mannitol's multifaceted potential in combating *P. aeruginosa* infections, particularly through mechanisms such as metabolic activation of persister cells, enhanced antibiotic penetration in mucus, and modulation of biofilm structure, both through direct effects on biofilms and by enhancing drug delivery, although further research is needed to reconcile conflicting findings.

Xylitol. Xylitol is a naturally occurring sugar-alcohol (polyol) characterized by its five-carbon structure which is found in some fruits and vegetables. While structurally a polyol and grouped here for organizational purposes, it is notably poorly metabolized by bacteria including *P. aeruginosa*. Instead, its unique properties, coupled with low transepithelial permeability in human cells, contribute to its antibacterial effects through other pathways. A number of investigations have revealed that xylitol possesses antibac-

terial properties through several pathways, offering a significant advantage over most sugars. Unlike readily utilized sugars, xylitol's unique five-carbon polyol structure makes it unsuitable for many microorganisms. This structure prevents its use in the pentose phosphate pathway in most bacteria, rendering it an ineffective energy source (32-34). One possible antibacterial mechanism is metabolic inhibition, which has been proposed in *Streptococcus* species. Xylitol accumulates in the bacterial cell as a toxic molecule, xylitol phosphate. This phosphorylated form cannot be further metabolized and hinders bacterial growth. Due to its cytotoxicity, xylitol 5-phosphate must be dephosphorylated and removed from the cell as xylitol, which is an energy-demanding process for bacteria (34, 35).

Researchers have extensively investigated the antibacterial and anti-biofilm effects of xylitol on numerous microorganisms, including *P. aeruginosa* (36). Several in vitro studies highlight xylitol's efficacy in preventing biofilm formation and, to a lesser extent, eradicate established biofilms. Siroosi and Jabalameli investigated xylitol's impact on *P. aeruginosa* strain PAO1 biofilm in an alginate bead model, finding that xylitol significantly inhibited biofilm formation by 53 and 90% in a concentration-dependent manner at 2 and 4 M, respectively. For pre-established biofilms, xylitol concentrations of 1, 2, and 4 M eliminated statistically significant 17, 19, and 26% of the biofilm, respectively, showing promising results (37). Similarly, Zhou et al. explored the effects of 50, 100, and 200 mM xylitol on *P. aeruginosa* ATCC 9027. Their findings indicated dose-dependent inhibition of both free-living bacterial growth and initial biofilm development, with 200 mM xylitol achieving approximately 15.1% reduction in planktonic growth and 43.1% reduction in initial biofilm formation, compared to controls. This concentration also reduced bacterial motility. No effect was observed at lower concentrations (38). Extending these findings to a mixed-species context, Dowd et al. utilized an in vitro Lubbock Chronic Wound Biofilm model, reporting that 2, 10, and 20% xylitol prevented biofilm formation of a mixture of *Staphylococcus aureus, P. aeruginosa*, and *Enterococcus faecalis* in a concentration-dependent manner, with 20% xylitol achieving 100% inhibition of the mixed biofilm (39). While some studies show direct anti-adherent properties, the antibacterial effect on planktonic growth can vary. For instance, Sousa et al. demonstrated that 0.5, 2.5, and 5% (w/v) xylitol did not exhibit any antibacterial effect on planktonic *P. aeruginosa* strain ATCC 9027, but significantly prevented bacterial cell attachment to glass slides when added to the culture medium, indicating anti-adherent properties (40).

Beyond its direct effects, xylitol has shown potential in enhancing the activity of other antimicrobial agents. Ng et al. assessed the inhibitory effect of alginate films containing 1.5% (w/v) xylitol and/or 0.1% (w/v) gentamicin on *P. aeruginosa* growth. While xylitol alone did not inhibit growth, its combination with gentamicin showed a greater, albeit not statistically significant, reduction in bacterial growth compared to gentamicin alone. The authors proposed that xylitol destabilized the bacterial membrane, altering its permeability and allowing gentamicin better access to the bacteria. Consequently, films containing both xylitol and gentamicin demonstrated superior anti-biofilm activity compared to gentamicin alone (41). In a related approach, Ammons et al. studied the efficacy of a hydrogel containing 2% lactoferrin and 5% xylitol in reducing viable *P. aeruginosa* cells in an in vitro wound model. Their research showed that treating a 72-h biofilm with this hydrogel significantly reduced cell numbers compared to a gauze dressing (42).

The potential for clinical application of xylitol, particularly in conditions like CF, is also being explored. Durairaj et al. investigated the safety of aerosolized xylitol in CF patients. In their study, six CF patients received 10 mL of aerosolized normal saline followed by 10 mL of 5% (w/v) xylitol. The authors concluded that nebulizing xylitol in CF patients is likely safe and well-tolerated. Their research suggests that iso-osmotic xylitol administered to airways may decrease airway salt concentration and reduce bacterial colonization rates, positioning xylitol as a potential therapeutic adjunct to prevent or treat bacterial airway colonization (43).

Glucose. Glucose is a monosaccharide with the chemical formula $C_6H_{12}O_6$. Its primary role as the essential energy source for cellular activity makes its metabolism critically controlled in the cells (44).

Recent research has explored the potential antibacterial and anti-quorum sensing properties of hypertonic glucose against *P. aeruginosa*. Chen et al. investigated the effects of hypertonic glucose on *P. aeruginosa* PAO1 and four clinical multidrug-resistant strains. Their findings demonstrated that 50

mg/mL of hypertonic glucose significantly inhibited 50% of biofilm formation across all tested *P. aeruginosa* isolates. Moreover, all *P. aeruginosa* strains exhibited concentration-dependent inhibition of their swimming motility when exposed to hypertonic glucose (50-200 mg/mL); with complete inhibition observed at 300 mg/mL. The study also revealed a significant reduction (at least 80%) in pyocyanin and elastase production at a glucose concentration of 100 mg/mL in PAO1 and three clinical strains. Critically, hypertonic glucose notably down-regulated the expression of key *P. aeruginosa* quorum sensing genes (*lasI*, *lasR*, *rhlI*, and *rhlR*) at 200 mg/mL (45).

Galactose-fucose. Galactose is a monosaccharide found in most cells, including microorganisms and common dietary sources such as fruits, vegetables, and dairy products (46). While *P. aeruginosa* typically prefers organic acids like succinate, pyruvate, and acetate as carbon source over sugars, research shows it responds to different monosaccharides, such as galactose, in a time-dependent manner. This response can increase the production of virulence factors when the bacteria are cultivated under aerobic conditions (47).

Some studies have shown the efficacy of galactose and fucose in eradicating *P. aeruginosa* infections, likely by targeting *P. aeruginosa* lectins, specifically LecA and LecB. These proteins play important roles in *P. aeruginosa* biofilm formation, adhesion to human cells, and damage to epithelial tissue. LecA and LecB bind to oligo- and polysaccharides, as well as individual galactose (LecA) and fucose (LecB) residues (48). A compelling case study illustrated the potential of this approach, showing that inhaling galactose and fucose alongside tobramycin was effective when traditional antibiotic therapy failed to eradicate *P. aeruginosa* from a patient's airways. Bismarck and colleagues successfully eliminated antibiotic-resistant *P. aeruginosa* in a 1.6-year-old patient within two weeks using this combined therapy. The treatment involved twice-daily inhalation via nebulizer of tobramycin and a 2 mL solution containing fucose (0.01 M) and galactose (0.01 M) in 0.9% (w/v) NaCl. Notably, this treatment produced no side effects such as mucosal irritations or bronchial spasms. The authors hypothesized that while antibiotic treatment might stimulate the release of the lectins, which aids bacterial attachment to epithelial surfaces, the sugar solution could neutralize these lectins. This neutralization would then prevent bacterial attachment, leading to infection clearance (49). In a subsequent clinical trial, Hauber et al. discovered that fucose/galactose inhalation, administered either alone or combined with intravenous antibiotics, significantly decreased the number of *P. aeruginosa* in the sputum of 11 adult CF patients with chronic *P. aeruginosa* infection. Subjects received 10 mL of a 0.1 M fucose/0.1 M galactose solution in 0.9% (w/v) NaCl for 21 days twice a day, either with (4 patients) or without (7 patients) concurrent intravenous antibiotic therapy (cephalosporin + aminoglycoside). The authors reported that patients tolerated these sugars well, with no negative side effects and no signs of altered lung function (50).

Citric acid. Citric acid (citrate), a key metabolite produced by the tricarboxylic acid cycle (44), is known to alter the general structure of biofilms and functions as a chelating agent (51). In a research study conducted by Sauvage et al., citrate supplementation inhibited three types of *P. aeruginosa* motility (twitching, swarming, and swimming). Citrate also lowered its biofilm formation, possibly because lectin A, an adhesion factor crucial for biofilm development, was less abundant in citrate-grown strains (52). Similarly, Sauer et al. found that 20 mM citrate could induce biofilm dispersal (51). Ross et al., further explored the synergistic effect of various antibiotics on *P. aeruginosa* biofilms when combined with 0.01 M citrate. The combination of citrate and amikacin (1 mg/mL) resulted in a significant 91.7% reduction of viable bacteria, a marked improvement compared to amikacin alone (49.6% reduction). Since amikacin targets metabolically active bacteria, the addition of citrate likely enhanced its efficiency by activating bacterial cells. Erythromycin (2 mg/mL) in combination with citrate also exhibited a significant synergistic effect in destroying biofilms achieving a 93.5% reduction in live bacteria compared to the antibiotic control (33% reduction). Given that high concentrations of erythromycin inhibit protein production (thus stopping bacterial growth) and require metabolically active bacterial cells for efficacy, the addition of citrate likely enhanced its action by promoting dispersion and increasing bacterial metabolic activity. In this study, the most effective combination therapy was colistin methanesulphonate (2.2 mg/mL) with citrate, which resulted in a significant 97.2% decrease of living bacteria. Cyclic polypeptides such

as colistin methanesulphonate, work well against planktonic bacteria at all developmental stages. As a prodrug requiring hydrolysis into its active form, the delayed effect of colistin methanesulfonate may have allowed dispersion to occur before the drug was fully activated, likely contributing to the observed synergistic killing. Conversely, co-treatments with citrate and polymyxin B or tobramycin showed a tendency toward antagonistic effects, as the combinations did not result in decreased viable cells compared to antibiotics alone. While cyclic polypeptides like polymyxin B efficiently eradicate internal bacteria in biofilms characterized by minimal metabolic activity, reducing membrane permeability allows cells on the outer layer of the biofilm to resist against therapy. Therefore, concurrent administration of nutrients like citrate with polymyxin B sulfate may inadvertently promote cell adaptability, facilitate growth, and hinder antibiotic penetration (53).

Succinate. Succinate, a dicarboxylic acid dianion, forms when both carboxy groups of succinic acid are deprotonated. This water-soluble, colorless crystal is found in various bacteria, including *P. aeruginosa*, where it serves as an intermediary in the tricarboxylic acid cycle (44, 54). Studies have demonstrated the dispersion effect of succinate. For instance, a study by Sauer et al. showed a significant dispersal effect when the succinate concentration was suddenly increased in *P. aeruginosa* strain PAO1 biofilms. In their experiment, treating a 4-day biofilm grown in a minimal medium in a biofilm tube reactor with 20 mM succinate led to an 80% reduction in biomass (51). Moreover, Ross et al. further highlighted succinate's potential by showing promising results in reducing the number of live bacteria when *P. aeruginosa* biofilms were simultaneously treated with succinic acid as a dispersion agent and colistin methanesulphonate. Specifically, the combination of colistin methanesulphonate (2.2 mg/mL) and succinic acid (0.01 M) resulted in a significant reduction in viable bacteria compared to either the dispersion agent or the antibiotic alone (53).

NITROGEN SOURCES

Nitrate. Nitrate (NO_3^-) is a monovalent inorganic anion and a reactive nitrogen species formed when a proton is lost from nitric acid. It is naturally present in the environment as well as some foods, and it is also produced in the human body (55). *P. aeruginosa* is well-known for its ability to grow in anaerobic environments, a process that can occur slowly through substrate-level phosphorylation of arginine or, more rapidly, through anaerobic respiration utilizing nitrate or nitrite as the final electron acceptor instead of oxygen (56). Studies have confirmed that the mucus of CF patients contains enough nitrate to sustain *P. aeruginosa* growth (57). Under anaerobic conditions and when nitrate is available, *P. aeruginosa* generates ATP using a membrane-bound nitrate reductase (58).

Given *P. aeruginosa*'s metabolic reliance on nitrate in anaerobic conditions, researchers have investigated how nitrate supplementation might influence antibiotic efficacy, particularly within biofilms where anoxic environments can reduce bacterial metabolic activity and, consequently, antibiotic effectiveness. Borriello et al. hypothesized that providing nitrate during antibiotic exposure would improve antibiotic effectiveness in biofilms by restoring bacterial metabolic activity. They treated 48-hour colony biofilms of *P. aeruginosa* strain PAO1 for 12 hours with 10 µg/mL tobramycin or 1 µg/mL ciprofloxacin, both alone and in combination with 1% (w/v) potassium nitrate. Their findings demonstrated that the activity of both antibiotics was significantly enhanced in the presence of nitrate. Specifically, tobramycin alone resulted in a 0.52 log reduction, which increased to 1.90 log reduction when combined with nitrate. Similarly, ciprofloxacin alone yielded a 1.1 log reduction, which rose to 3.2 log reduction with nitrate. This observed enhancement was not due to nitrate's direct antimicrobial effect, as 12 hours of nitrate exposure alone had no effect on viable cell counts. Instead, the mechanism is attributed to nitrate's ability to restore metabolic activity in the anoxic regions of mature biofilms, thereby increasing antibiotic efficacy. Borriello et al. also indicated that nitrate's effects on antibiotic susceptibility are influenced by biofilm age, observing no effect on 4-hour colony biofilms (59). This concept of biofilm age influencing nitrate's impact was further supported by another study from Borriello et al., which similarly reported no, or even adverse efficacy of nitrate supplementation on antibiotics in 4-hour colony biofilms under anaerobic conditions (60). Further exploring the intricate interplay between nitrate and antibiotic susceptibility, Jabalameli et al. investigated the effect of varying nitrate concentrations (50, 100, and 200 mM) com-

bined with sub-MBEC concentrations of amikacin, tobramycin, and ciprofloxacin on biofilms of clinical and reference (*P. aeruginosa* PAO1) strains in an alginate bead biofilm model. Their study revealed that the impact of nitrate on antibiotic sensitivity was complex and dependent on both the specific antibiotic and the nitrate concentration. They observed that, when bacterial aggregates were treated with a mixture of nitrate and an antibiotic, differing amounts of nitrate could either increase or reduce the antibiotic sensitivity of *P. aeruginosa* strains. Notably, nitrate was less successful in enhancing susceptibility when combined with tobramycin (61). Nitrate enhances antibiotic efficacy in *P. aeruginosa* biofilms by restoring bacterial metabolic activity in anoxic conditions. While its effect varies with biofilm age and specific antibiotic combinations, nitrate holds promise as a valuable adjunctive therapy.

L-Glutamate. L-Glutamic acid, or its ionic form, L-glutamate, is one of the most prevalent amino acids in nature, playing vital roles in both cellular and systemic processes (62). For *P. aeruginosa*, L-glutamate is a preferred or even exclusive source of carbon and nitrogen for its development (63). Interestingly, adding this amino acid to the culture medium has been shown to alter *P. aeruginosa*'s sensitivity to antibiotics.

Warraich et al. demonstrated an increase in antibacterial activity of ciprofloxacin when combined with glutamic acid. Specifically, combining 7.5 and 15 mM of the amino acid with 0.13 and 0.27 μM ciprofloxacin generally reduced *P. aeruginosa* strain PAO1 growth. This enhanced antibacterial activity could be attributed to glutamate's modulation of efflux mechanisms, leading to increased drug accumulation and improved drug permeation into the cells (64). However, antibiotic enhancement isn't glutamate's only effect. Sauer et al. reported that a sudden increase in glutamate concentration in the minimal culture medium induced biofilm dispersion formed by *P. aeruginosa* strain PAO1. They showed that treating 4-day biofilms of this bacterium with 18 mM glutamate reduced the biofilm by 72% compared to a control and dispersed the cells. These results align with the observation that high concentrations of nutrients can repress biofilm formation (51).

L-Aspartate. L-Aspartate is a necessary amino acid for *P. aeruginosa* growth and metabolism. It can serve as a nitrogen source, and *P. aeruginosa* can even thrive on L-aspartate as its sole nitrogen source (63). Warraich et al. discovered that using the combination of aspartic acid and ciprofloxacin could boost its antibacterial effectiveness against *P. aeruginosa* strain PAO1 in a concentration-dependent manner. Their findings revealed that while combining 7.5, 15, and 30 mM of aspartic acid with 0.53 and 1.06 μM ciprofloxacin appeared to aid the pathogen's growth, the same concentrations of aspartic acid, when paired with lower ciprofloxacin concentrations (0.13 and 0.27 μM), actually increased the antibiotic's antibacterial activity. Moreover, pyocyanin synthesis was significantly decreased when 0.13 and 0.27 μM ciprofloxacin combined with 30 mM aspartic acid, compared to ciprofloxacin alone. This effect appears to stem from aspartic acid’s ability to alter the efflux mechanism, evidenced by decreased efflux and increased drug accumulation inside the cell (64). In a similar study, Silva et al. found that 20 mM aspartic acid combined with 2 mg/L of ciprofloxacin significantly reduced the entire population of *P. aeruginosa* strain PAO1 in a 24-hour biofilm formed in an artificial sputum medium, with 4 mg/L of ciprofloxacin achieving complete eradication of the cells (65).

L-Glutamine. Glutamine is an amino acid utilized by *P. aeruginosa* as a nitrogen source (63). A systematic review reported that glutamine administration in surgical patients reduces infection rates and shorten hospital stays (66), suggesting a potential role in modulating host-pathogen interactions.

Building on this, Zhao et al., investigated the effect of glutamine on the susceptibility of multi-drug-resistant *P. aeruginosa* both in vitro and in vivo. In the in vitro experiments, bacterial cells were exposed to 20 mM glutamine in combination with varying concentrations of ampicillin (0.256, 0.4, and 0.8 mg/mL) for 6 hours. Compared to treatment with ampicillin alone, the combination resulted in a significant reduction in viable bacterial cells at all concentrations. In the in vivo model, a catheter-associated urinary tract infection was established in mice by inserting a catheter pre-colonized with *P. aeruginosa* into the bladder. After 48 hours, treatment started with a combination of 320 mg/kg ampicillin and 100 mg/kg glutamine. This combination significantly reduced bacterial load on the catheter and also decreased secondary kidney infections compared to control groups. The enhanced antibacterial effect was at-

tributed to glutamine-induced increases in bacterial membrane permeability, which facilitated intracellular accumulation of ampicillin. Although the exact mechanism remains unknown, these findings suggest that glutamine may potentiate antibiotic efficacy. Notably, glutamine is a naturally occurring nutrient in humans, and doses up to 300 mg/kg in rats have shown no adverse effects, supporting the safety of this adjunctive therapy (67).

L-Arginine. L-Arginine is an amino acid that provides bacteria with both carbon and nitrogen, supporting their growth. It can also be used for ATP synthesis in *P. aeruginosa* in low-oxygen environments, allowing this bacterium to thrive in anaerobic conditions. L-Arginine concentration has been linked to antibiotic resistance, pathogenicity, and biofilm formation (68). In hypoxic microenvironments, electron acceptors such as arginine and nitrate influence biofilm growth and metabolism. This can be beneficial in making cells more susceptible to antibiotics and eradicating infections (69). Studies on L-arginine's effect on *P. aeruginosa* have shown a concentration-dependent impact on biofilm formation: high concentrations favor a sessile, biofilm-forming lifestyle over a more active, motile alternative, while low concentrations increase swimming motility (70, 71).

In a study by Eisha et al., the combination effect of tobramycin and L-arginine on *P. aeruginosa* strain PAO1 biofilm reduction was evaluated. They observed that L-arginine at physiological ranges (50 and 100 mM) when combined with 1000 µg/mL tobramycin, significantly promoted biofilm growth compared to the control (0 mM L-arginine and 1000 µg/mL tobramycin). Conversely, higher concentrations of L-arginine (300 and 600 mM) combined with 1000 µg/mL tobramycin significantly prevented biofilm growth in this strain compared to the control (72). Another study also revealed that susceptibility of biofilms formed by clinical strains of *P. aeruginosa* to amikacin, tobramycin, and ciprofloxacin was elevated by 0.8% (v/w) arginine. Specifically, sub-MBEC concentrations of amikacin completely eradicated the biofilms at this arginine concentration (61). In an animal model study, Everett et al. observed an increase in animal survival and a decrease in sepsis when 0.125 g/mL of L-arginine was administered to a mice burn wound model infected with *P. aeruginosa*. Given the low L-arginine levels typically found in burn wounds, and the fact that these low levels enhance *P. aeruginosa* motility, the authors hypothesized and subsequently proved that local L-arginine supplementation would slow *P. aeruginosa* growth in burned mice by increasing the amino acid's concentration at the wound site (71).

METALS

Iron. Iron is essential for bacterial cell viability, serving as a crucial component in numerous biological processes, including DNA replication and repair (73). In order to obtain iron, *P. aeruginosa* uses siderophores, small organic molecules with iron-chelating activity, which transfer iron from host's transferrin, lactoferrin, and ferritin to bacterial cell (74). Consequently, iron concentration significantly impacts *P. aeruginosa* physiology and pathogenicity. It has been proposed that *P. aeruginosa* forms biofilms optimally within a specific range of iron concentrations; outside this range, the pathogen is restricted to a planktonic lifestyle. Studies on iron's effect on *P. aeruginosa* have led to two distinct approaches for eradicating infections: high concentrations of iron, which alter *P. aeruginosa*'s physiology and iron chelators, which limit bacterial access to iron.

Expression-profiling studies in *P. aeruginosa* have shown that elevated iron levels suppress the expression of genes necessary for iron scavenging, critical genes for virulence. Musk et al. demonstrated that high concentrations of iron salts, such as ferric ammonium citrate (up to 500 µM), prevented biofilm formation of strain PA14 in a dose-dependent manner in vitro. Specifically, 250 µM of this salt completely or partially inhibited biofilm production by clinical *P. aeruginosa* strains isolated from sputum of CF patients. Furthermore, 200 µM ferric ammonium citrate could completely disrupt a preformed biofilm of strain PA14 in a flow-cell chamber after 10 days (75). In another study, Jabalameli et al. explored the combined effect of ferrous supplementation and amikacin. They found that 2 mM ferrous sulfate and sub-MBEC concentrations of amikacin completely destroyed biofilms of PAO1 and two clinical strains of *P. aeruginosa* in an alginate biofilm model after 24 h (61). Interestingly, iron concentration can regulate immune responses during *P. aeruginosa* infections. According to research by Vuong et al., iron overload could be an innovative and successful strategy for managing and preventing bacterial-induced

lung inflammation. They demonstrated that during a *P. aeruginosa* lung infection, iron regulated immune cell recruitment, alveolar capillary permeability, and the production of some cytokines (76).

Using iron chelators, which absorb the body's free iron and reduce its availability to the pathogen, represents another cutting-edge treatment strategy for *P. aeruginosa* infections (77). Oglesby et al. explained that FDA-approved iron chelators can be used to treat multidrug-resistant microbial infections, such as *P. aeruginosa* infections in CF patients' lungs. They showed that *P. aeruginosa* growing in iron-replete conditions is more susceptible to several antibiotics, including tigecycline and tobramycin (at varying concentrations), when iron chelation (330 µM deferasirox, an FDA-approved iron chelator) was present, while, the MBEC for PAO1 biofilms was elevated by iron, from 2 µg/mL in low iron concentration to 8 µg/mL in high iron concentration (78). In another study conducted by Moreau-Marquis et al., 1 µM deferasirox (which chelates the approximately 0.1 mM Fe in the medium), reduced *P. aeruginosa* biofilm formation on CF airway epithelial cell cultures by 99% (79).

Summary of supplement efficacy in *P. aeruginosa*. A summary of the key findings regarding the efficacy of these various carbon, nitrogen, and metal-based supplements in inhibiting *P. aeruginosa* biofilms, their mechanisms, and relevant experimental details is presented in Table 1.

SAFETY CONSIDERATION

While the reviewed supplements show considerable promise as adjunctive therapies against *P. aeruginosa* infections, particularly in biofilm contexts, their clinical translation necessitates a thorough evaluation of safety, potential adverse interactions, and patient-specific contraindications. The complex interplay between supplements, antibiotics, host physiology, and the pathogen demands a nuanced understanding.

Not all combinations of supplements and antibiotics will yield beneficial outcomes; some may be inert, or in certain contexts, even antagonistic. For instance, while mannitol demonstrated impressive synergy with tobramycin in in vitro biofilm models, these effects did not translate to in vivo-like models (29). This highlights a critical limitation in directly extrapolating in vitro findings. Similarly, citric acid, despite its broad synergistic effects, exhibited antagonistic tendencies when combined with antibiotics such as polymyxin B and tobramycin in certain studies (53). The efficacy of some supplements can also be highly context-dependent. For example, nitrate supplementation, while generally beneficial in promoting ATP synthesis in anoxic biofilm regions, showed no effect or even adverse effects on younger *P. aeruginosa* biofilms (60). Furthermore, L-arginine presents a compelling case of dose-dependent interaction: while high concentrations proved beneficial, physiological concentrations were reported to paradoxically promote biofilm growth when co-administered with tobramycin (72). The observation that aspartate could, at certain ciprofloxacin concentrations, even aid pathogen growth further complicates its clinical application.

The inherent safety profile of each supplement is paramount for clinical use. Xylitol, a common sugar alcohol, is generally recognized as safe. However, excessive oral intake can lead to gastrointestinal discomfort, including bloating and diarrhea (80). Despite these potential oral side effects, its established safety and good tolerability when administered via nebulization in CF patients suggests a promising route for respiratory infections (43). Similarly, in a clinical case using nebulized fucose/galactose, no side effects were reported following administration of these sugars (50). Moreover, glutamine has been reported to be safe as an adjuvant in treating infections in mice (67), and human studies have shown no side effects from consuming this amino acid at doses up to 50-60 g/day (81).

The nuanced findings regarding supplement-antibiotic interactions and varying efficacy based on context highlight the critical need for robust future research. Rigorous preclinical in vivo studies in relevant animal models are essential to assess the true efficacy, safety, and interactions within a complex biological system. Ultimately, well-designed human clinical trials are indispensable to systematically evaluate the safety, optimal dosing, pharmacokinetics, and pharmacodynamics of these adjunctive therapies.

CONCLUSION

This review systematically investigated a range of carbon, nitrogen, and metal-based supplements

Table 1. Summary of in vitro and in vivo findings on the impact of various supplements, alone or combined with antibiotics, on bacterial biofilm formation, viability, and eradication.

Supplement & Conc.*	Antibiotic & Conc. (if applicable)	Observed Impact on Biofilm	Reference
Mannitol, 40 mM	Tobramycin (80 mg/L)	Significant reduction in biofilm viability	(24)
Mannitol, 3200 mg/L	Ciprofloxacin (0.25 mg/L)	Complete destroy of bacterial cells within the biofilm	(28)
Xylitol, 4 M	-	Significant inhibition of biofilm formation	(37)
Glucose, 50 mg/mL	-	Significant inhibition of biofilm formation	(45)
Citric acid, 0.01 M	Colistin methanesulphonate, 2.2 mg/mL	Significant reduction in biofilm viability	(53)
Succinate, 20 mM	-	Significant biomass reduction in the biofilm	(51)
Nitrate, 1% (w/v)	Ciprofloxacin (1 µg/mL)	Significant reduction of bacterial cells in the biofilm	(59)
Glutamate, 18 mM	-	Significant dispersion of the biofilm	(51)
Aspartate, 20 mM	Ciprofloxacin, 4 mg/L	Complete destroy of bacterial cells within the biofilm	(65)
Glutamine, 100 mg/kg	Ampicillin, 320 mg/kg	Significant reduction of bacterial cells in an in vivo biofilm model	(67)
L-Arginine, 300 and 600 mM	Tobramycin, 1000 µg/mL	Significant prevention of biofilm growth	(72)
Iron, 200 µM	-	Complete disruption of the biofilm	(75)

* Concentration

that show promise in augmenting antibiotic efficacy against *P. aeruginosa* infections, particularly within challenging biofilm settings. These diverse compounds exert their beneficial effects through various mechanisms, including direct interference with bacterial metabolism, modulation of motility, disruption of efflux pumps, inhibition of key virulence factors, and alterations in gene expression. Critically, many of these supplements demonstrate the ability to impact persistent bacterial populations by reversing the persister cell phenotype, preventing biofilm formation, or enhancing the eradication of established biofilms, thereby increasing bacterial susceptibility to conventional antibiotics.

Among the extensively studied supplements, xylitol stands out due to its multifaceted antimicrobial actions. It exhibits direct anti-growth and anti-adhesive properties, and significantly enhances the anti-biofilm activity of antibiotics, even against preformed structures. Furthermore, xylitol's established use in improving mucus clearance in CF patients suggests a dual benefit, offering both direct antimicrobial effects and enhanced drug delivery in respiratory infections.

However, the reviewed literature also highlights key complexities that necessitate further investigation. For instance, mannitol shows promising synergistic effects with tobramycin in in vitro biofilm models by altering persister cell metabolism, but these findings were directly contradicted in more in vivo-like models, emphasizing the critical role of environmental context in supplement efficacy. Similarly, the impact of nitrate and L-arginine on antibiotic susceptibility can be highly dependent on biofilm age and concentration, respectively, sometimes even showing adverse effects. Conversely, iron presents a unique duality, with both high concentrations and iron chelators demonstrating anti-biofilm potential, underscoring the delicate balance of this essential metal in *P. aeruginosa* physiology.

These discrepancies and context-dependent effects underscore the need for more sophisticated in vivo and clinical studies that mimic physiological conditions more closely. Future research should prioritize elucidating the precise molecular mechanisms underlying these synergistic and, at times, antagonistic interactions. Ultimately, the integration of these novel supplements, particularly those like xylitol with an established safety profile, represents a compelling strategy to enhance existing antibiotic therapies and overcome the pervasive challenge of *P. aeruginosa* biofilm-associated infections.

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