Control of Biofilm Formation: Antibiotics and Beyond

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ABSTRACT

Biofilm-associated bacteria are less sensitive to antibiotics than free-living (planktonic) cells. Furthermore, with variation in concentration of antibiotics throughout a biofilm, microbial cells are often exposed to levels below inhibitory concentrations and may develop resistance. This, and the irresponsible use of antibiotics, lead to the selection of pathogens that are difficult to eradicate. The Centers for Disease Control and Prevention use the terms “antibiotic” and “antimicrobial agents” interchangeably. A clear distinction between these two terms is required for the purpose of this assessment. Therefore, we designate “antibiotics” as pharmaceutically formulated and medically administered substances, and identify “antimicrobials” as a broad category of substances which are not regulated as drugs. This comprehensive mini-review evaluates the effect of natural antimicrobials on pathogens in biofilms when used instead of, or in combination with, commonly prescribed antibiotics.
INTRODUCTION

As many as eighty percent of pathogens that form biofilms are associated with persistent infections (1, 2). Approximately ninety percent of the biofilm mass is composed of extracellular polysaccharides (EPS), proteins and DNA (3). EPS provides stability to the cells, mediates surface adhesion, and serves as a scaffold for cells, enzymes and antibiotics to attach to (4-8).

*Pseudomonas aeruginosa*, associated with cystic fibrosis (9) and *Staphylococcus aureus*, responsible for most wound infections (10), are typical examples of persistent pathogens that form biofilms.

Cells in biofilms experience stringent growth conditions. Survival depends on their ability to mutate and exchange genetic information, e.g. through horizontal gene transfer (4, 11). Resistance to antibiotics may thus be seen as a phenotypic shift in behavior when cells adapt to a sessile life style (12). This hypothesis is supported by cells developing tolerance to antimicrobial peptides and phagocytosis (13). Some staphylococci produce poly-γ-DL-glutamic acid (PGA) that binds to antimicrobial peptides and protects bacterial cells from neutrophil phagocytosis (14). Other physiological changes occur due to oxygen deprivation or nutrient deprivation, especially in deeper layers of the biofilm. Oxygen deprivation and low metabolic activity in biofilms render *P. aeruginosa* more tolerant to antibiotics (15). Rapid changes in pH between layers in a biofilm may lead to the accumulation of organic acids and the deactivation of penetrating compounds (15). Complex (polymicrobial) biofilms composed of multiple species are generally more resistant to antibiotics than biofilms composed of a single species (16, 17).

The diversity and metabolic state of cells in a biofilm play a key role in antibiotic resistance. Persistent cells are generally more resistant to antibiotics and they play an important role in supporting the re-establishment of the biofilm community (8, 18, 19). Cells in dormant sections...
of a biofilm are usually not affected by antibiotics, as recorded with studies on β-lactams, ciprofloxacin, tetracycline and tobramycin (9, 20).

*P. aeruginosa* showed an increase in antibiotic tolerance when the cells were immobilized in a biofilm. The efflux pump PA1874-1877 in *P. aeruginosa* was more actively expressed in biofilm-associated cells compared to planktonic cells (21). Efflux pumps in *Pseudomonas* spp. are also used for secretion of biocides such as glutaraldehyde (22). Moreover, cells with inactive efflux pumps may have diminished ability to form biofilms (23). Therefore, antimicrobial agents inactivating efflux pumps such as thioridazine and Phe-Arg -naphthylamide (PAN) (23), might be helpful in the prevention of biofilm formation.

Failure to develop new antibiotics, combined with the spread of resistance may result in increased morbidity and mortality, especially in health care facilities. Challenges to the discovery of alternative treatments have been mentioned in other reviews (7, 24, 25). Estrela et al. (26) discussed the potential of combining antimicrobial compounds with antibiotics to inhibit quorum sensing in a biofilm.

In this review, the combination of different classes of antimicrobial compounds with antibiotics to control biofilm formation is discussed and summarized (Figure 1). This is in line with the recent approach taken by the National Center for Complementary and Integrative Health (NCCIH) of NIH, i.e. combining conventional treatments with complementary methods to uncover “potential usefulness and safety issues of natural products” (27).

**ANTIMICROBIAL PEPTIDES: SYNERGY WITH ANTIBIOTICS WHEN AIMED AT DIFFERENT TARGETS**
Antimicrobial peptides (AMPs) are naturally produced by eukaryotes and prokaryotes as a part of their innate immune/defense system (28). The unique features of many AMPs are their small size (15-30 amino acids), charge (often, overall positive), and that they target cell membranes (28, 29). The positively charged peptides are attracted to the negatively charged cell membranes of bacteria and biofilm surfaces. Active and slow-growing bacteria in biofilms are killed by AMPs (30) and manipulation of the AMPs’ amino acid composition may result in increased antimicrobial activity (31-33). One example of genetic manipulation is construction of the broad-spectrum bactericidal peptide R-FV-I16 by removing the functional ‘defective’ sequence RR7 and by inserting the anti-biofilm sequence FV7 embedded in peptide RI16 (32). The specificity of AMPs can also be manipulated by designing specifically targeted AMPs (STAMPs) highly selective against pathogens but harmless to non-pathogenic bacteria (34,35).

Many AMPs either form pores in the cell membrane or act as membrane perturbers (36). However, at low concentrations AMPs may act bacteriostatically (7). De la Fuente-Nunez and co-workers have shown that AMP 1037 stimulates the swarming of *P. aeruginosa* PA2204 cells, but inactivates twitching motility and biofilm formation (37). The peptide antimicrobial NA-CATH:ATRA1-ATRA1, a synthetic cathelicidin, inhibited *S. aureus* biofilm formation, and the peptide LL-37 controlled *P. aeruginosa* biofilm formation when used at levels below MIC (38, 39). These AMPs prevented expression of the genes encoding proteins involved in biofilm formation. In *P. aeruginosa* the down-regulated genes are coding for type IV pili, rhamnolipid synthesis, quorum sensing, and the assembly of flagella (38). Some AMPs have specific antimicrobial features, for example milk lactoferrin chelates iron and inhibits biofilm formation by *P. aeruginosa* (40). Moreover, binding of AMPs to extracellular DNA may enhance the detachment of biofilms (41).
To survive in the presence of AMPs, bacteria utilize various approaches, e.g. mutations that change the structure and charge of the cytoplasmic membrane, modification of lipopolysaccharides in the cell wall, secretion of AMPs by specific efflux pumps, etc. (42). In addition, *S. aureus* biofilm formation regulatory system (GraRS) plays an important role in the microorganism’s resistance to AMPs (43). This resistance was reversed when AMPs were added in combination with other antimicrobial compounds. AMPs from various sources, when combined with commonly prescribed antibiotics, effectively prevented biofilm formation by *P. aeruginosa* (44 - 48). Moreover, STAMP G10KHc synergized with tobramycin against planktonic and biofilm-associated cells of *P. aeruginosa* (44). This peptide destabilized the cell membrane, which enhanced the penetration of tobramycin into bacterial cells. Tobramycin and AMP GL13K synergized in 67.5% eradication of *P. aeruginosa* (47). Similarly, broad-spectrum AMP tachyplesin III synergized with antibiotic piperacillin tazobactam (TZP) against biofilm-associated *P. aeruginosa* (49). Inhalation of the amphipathic polypeptide colistin, combined with ciprofloxacin, killed biofilm-associated cells of *P. aeruginosa* and improved the lung functions of cystic fibrosis (CF) patients (46) over a 4-weeks treatment. Noticeably, colistin inhibited persister cells.

*In vivo* studies showed a synergistic effect on biofilms of methicillin-resistant *S. aureus* (MRSA) when nisin was combined with daptomycin/ciprofloxacin, indolicidin with teicoplanin, and CAMA with ciprofloxacin (50, 51). Pre-treatment of central venous catheters (CVC) with cathelicidin peptide BMAP-28, in combination with traditional antibiotics quinupristin/dalfopristin (Q/D), linezolid (LZD) and vancomycin, reduced *S. aureus* on CVC and prevented bacteremia (52). To effectively eradicate biofilms on CVC, the "antibiotic-lock" technique (also called "intraluminal therapy") was suggested, which involves the filling of CVCs...
with a predetermined concentration of AMPs. A combination of the cationic peptide IB-367 and LZD in the antibiotic-lock technique eradicated *S. aureus* biofilms on CVC (53). A noticeable reduction in biofilm-associated *S. aureus* on vascular grafts was observed when sub-MIC levels of vancomycin were combined with the lipopeptides Pal–Lys–Lys–NH2 and Pal–Lys–Lys (54).

Some AMPs with broad anti-biofilm activity, such as peptide 1018, blocked or degraded (p)ppGpp, which is essential for biofilm formation. At low concentrations, peptide 1018 inhibited biofilm formation, but eradicated pre-formed biofilms when applied at higher concentrations (55). In a separate study, the same authors reported on the *in vivo* and *in vitro* anti-biofilm activity of newly synthesized broad-spectrum D-enantiomeric AMPs (56). These peptides synergized with antibiotics in the inhibition and eradication of pathogenic biofilms of *P. aeruginosa*.

AMPs combined with conventional antibiotics may be a better alternative than antibiotics alone. The synergy of AMPs and antibiotics against biofilm-associated pathogens should attract the attention of scientists to explore the mechanistic actions of these combinations.

**BIOFILM-DEGRADING ENZYMES: EFFECTIVE HELPERS WHEN IT COMES TO THE MATRIX DESTRUCTION**

Adhesion to surfaces stimulates bacterial cells to produce EPS (57), which is mainly composed of polysaccharides, proteins, and nucleic acids (58). These components play a key role in cell-cell or cell-surface attachment, supporting the integrity of biofilm architecture and protecting biofilm cells from the shearing stress factors (59, 60). Enzymes could inhibit and disrupt the EPS matrix formation and then facilitate the detachment of biofilm. However, a second antimicrobial substance is required to target the detached cells (61, 62).
The biofilm-degrading enzymes DNase I, α-amylase and dispersin B (DspB) reduced the EPS mass and biofilm cell numbers (62-65). However, the older the *P. aeruginosa* biofilm, the more difficult it was dissolved by DNase I. Production of high quantities of EPS and proteolytic exoenzymes by the mature biofilms inactivated DNase I (64). Nevertheless, purified recombinant DNase I derivative (DNase1L2), extracted from human stratum corneum, effectively controlled biofilm-associated *P. aeruginosa* and *S. aureus* (63). *Bacillus subtilis* S8-18 α-amylase was evaluated against biofilms of a clinical MRSA strain and *P. aeruginosa* ATCC10145 (65). An efficient biofilm inhibition and degradation of mature biofilms were reported due to disruption of EPS. Interestingly, *B. subtilis*-derived α-amylase was more effective in degrading the EPS in biofilms of *S. aureus* and *P. aeruginosa* compared to amylases from human saliva and sweet potato (66). In a different study (67), strong degradation activity of α-amylase (82% biofilm reduction) against biofilm-associated *P. aeruginosa* was also reported. Biofilm-degrading enzymes such as lysostaphin (68) and alginate lyase (69) showed anti-biofilm activity against various pathogenic bacteria. Although these enzymes destroy and detach biofilms, the biofilm re-establishment is not guaranteed.

Treatment of *S. aureus* biofilm with combinations of recombinant human DNase I (rhDNase I) and topical antiseptics (chlorhexidine gluconate and povidone iodine) reduced the number of viable cells by an additional 4 to 5 logs compared to treatment with only antibiotics (70). DNase is likely acting against biofilms by changing their texture and morphology, and influencing biofilm-associated cell numbers (71). In turn, alteration of biofilm structure enhances the activity of antibiotics against biofilms of *P. aeruginosa* and *S. aureus*.

Donelli and co-workers (72) found that dispersin B (DspB), produced by *Actinobacillus actinomycetemcomitans*, alone or in synergy with cefamandole nafate, hydrolyzed the EPS of a
staphylococcal biofilm, promoted antibiotic penetration, and augmented the killing of microbial
cells. Furthermore, DspB synergized with triclosan against *S. aureus* biofilms formed on
vascular catheters (61).

Using a continuous flow culture, alginate lyase showed a remarkable sensitization and
elimination of mucoid biofilm-associated *P. aeruginosa* when administrated with gentamycin
(69). The enzyme lysostaphin, extracted from *Staphylococcus simulans*, killed *S. aureus* by
cleaving the pentaglycine cross-bridges in the cellular membrane’s peptidoglycan, and destroyed
extracellular polymeric substance matrix of lysostaphin sensitive staphylococci (73).
Furthermore, lysostaphin (15 mg/kg) combined with nafcillin (50 mg/kg), effectively killed
MRSA biofilms on a medical device (68). Lysostaphin has shown synergy with five of nine
antibiotics, with highest eradication of MRSA in combination with clarithromycin, which
suppresses hexose polymerisation (74). Early adhesion and dispersion of *S. aureus* in mature
biofilms was inhibited by proteinase K (75).

Despite the high cost of production, biofilm eradicating enzymes could possibly be used as an
alternative or as a synergistic helper to antibiotics in treatments of persistent infections.

**QUORUM SENSING INHIBITORS: STOP TALKING, HELP KILLING**

Quorum sensing (QS) regulates virulence behaviors, including biofilm formation (76). QS
compounds include N-acyl homoserine lactones (AHLs), produced by Gram-negative bacteria,
and auto-inducing peptides (auto-inducer-2, AI-2) produced by Gram-positive bacteria.
Inhibition of biofilm formation by QS quenchers or inhibitors (QSQ or QSI) may play a
significant role in preventing biofilm formation by many pathogens. Therefore, enzymatic
degradation of QS signals such as lactonase, acylase, oxidoreductase and paraoxonase could be
considered a promising approach in controlling biofilm formation (77). QSQ can also attenuate QS by blocking or shutting down the expression of QS-genes in pathogens, which leads to biofilm inhibition without killing planktonic cells or influence normal growth.

Recently, inhibition of QS and biofilm formation had been reported in several studies (78-80). The injection of RNAIII-inhibiting peptide (RIP) in rats with MRSA graft infection repressed staphylococcal RNAIII-activating protein (RAP) and agr QS systems, which are required for staphylococcal biofilm formation (79). In addition, usnic acid, a natural secondary metabolite of lichen, interfered with QS which resulted in prevention of *S. aureus* biofilm formation and changed the morphology of *P. aeruginosa* biofilms (78). A pungent oil of fresh ginger (6 gingerol) adhered to QS receptors in *P. aeruginosa* and paused biofilm maturation. Transcriptomic analysis confirmed that 6-gingerol inhibited QS-induced gene expression and the production of virulence factors (81).

Several compounds were reported to have QS inhibitory effects, including penicillic acid, solenopsin A, catechin, ellagic acid derivatives and curcumin (82). Rasmussen et al. (83) referred to the activity of patulin and penicillic acid, which were isolated from *Penicillium* species, as active QSI compounds that controlled QS-gene expression in *P. aeruginosa*. Using QSI alone, or in combination with antibacterials, creates an opportunity for their implementation in biofilm controlling formulations.

Interestingly, a clearance of *P. aeruginosa* biofilm and reduction in pyocyanine production were reported when phenyl-DPD, an AI-2 analog, was combined with gentamicin, indicating the possible role of QS systems in biofilm maturation and/or dispersion (84). Furthermore, while lactonase from *Bacillus* spp. did not affect the growth of *P. aeruginosa*, it reduced biofilm
formation (85). The perturbation of biofilm formation by lactonase increased the susceptibility of biofilms to antibiotics and significantly reduced the production of virulence factors when lactonase was used in combination with ciprofloxacin and gentamicin. It is still to be explored if QSI s from various sources possess similar anti-biofilm activity.

Plant-derived QSI s often exhibit remarkable biofilm reduction ability, especially when combined with antibiotics. An *in vivo* study by Brackman and co-workers (86) assessed the activity of tobramycin against *P. aeruginosa* biofilm, and activities of clindamycin and vancomycin against a *S. aureus* biofilm, alone and in combination with QS inhibitors (baicalin hydrate, cinnamaldehyde and hamamelitannin). The combined treatments strengthened the antibiotics’ potential (86).

Traditional Chinese medicine baicalein proteolytically digested the signal receptor (TraR protein) in *P. aeruginosa*, which is likely contributing to its anti-biofilm activity as a QSQ (87). In addition, 14-alpha-lipoyl andrographolide (AL-1), an antimicrobial diterpenoid lactone from green chiretta (*Andrographis paniculata*), inhibited the Las and Rhl QS systems in *P. aeruginosa* by suppressing the transcriptional level of QS-regulated genes (88). Both baicalein and AL-1 synergized with the tested antibiotics against *P. aeruginosa* biofilm. Furthermore, fruit extract of *Lagerstroemia speciosa* (LSFE) caused the down-regulation of QS genes (*las* and *rhl*) and N-acylhomoserine lactones in *P. aeruginosa* PAO1 (89). Also, LSFE increased the antibiotic potential of tobramycin in *P. aeruginosa* PAO1 biofilms. A garlic extract, ajoene, inhibited or controlled QS-associated virulence factors, such as rhamnolipids, in *P. aeruginosa* (90). In the same study, ajoene synergized with tobramycin, killed *P. aeruginosa* in biofilms and prevented lytic necrosis of PMN cells (91). Recently, a comprehensive *in vivo* study was performed by Christensen et al. (92), evaluating differences between single treatments (ajoene or horseradish...
juice extract) and combination treatment (QS inhibitors with tobramycin) of BALB/c mice in which wild-type *P. aeruginosa* were injected into the peritoneal cavity. Mice treated with a combination of the antimicrobials showed a significant decrease in the number of biofilm-associated *P. aeruginosa* compared to mice treated with a single formulation.

Overall, QSIs combined with antibiotics could have a great impact on future applications to prevent biofilm formation of clinically-important pathogens, especially *P. aeruginosa*.

**ESSENTIAL OILS: BROAD-SPECTRUM COMPOSITIONS, MULTIPLE MECHANISMS OF ACTION IN ASSISTING ANTIBIOTICS**

Essential oils (EOs) are natural antimicrobial formulations with broad-spectrum activity against bacteria, fungi, and viruses (93). EOs may inhibit ATP production and ATPase activity. Moreover, EOs disrupt membrane permeability, modify proton motive forces and membrane fatty acids, leading to the leakage of metabolites and ions. Some EOs are acting as QSI by interfering with and regulating of QS genes, leading to reduction of biofilm formation and virulence factor production (see table 1 in the review of Nazzaro et al. [94]). The easiness of EOs extraction, non-toxicity to the tissue culture, quick degradation in water and positive health impacts (95-97) may increase the value of EOs as alternative antimicrobial agents.

Kavanaugh and Ribbeck (98) referred to the high biofilm-eradication effect of three EOs: cassia, Peru balsam, and red thyme when compared to ofloxacin and gentamicin against biofilms of *Pseudomonas* and *S. aureus*. Biofilm formation was also inhibited when oregano essential oils, carvacrol and thymol were used against *S. aureus* (99). Five of nine biofilms formed by coagulase-negative staphylococci (CoNS) strains were completely eradicated when 5% tea-tree oil (TTO) was used while the same concentration of TTO achieved complete eradication of...
MSSA and MRSA biofilm growth as microcolonies in glycocalyx during 1h treatment (100). The antimicrobial function of TTO was attributed to the disruption of the hydrophobic phospholipid bilayers in the cell membrane.

Few studies focused on the antimicrobial combinations of EOs and antibiotics. EOs modify the tolerance of bacterial cells to antibiotics (reviewed by Yap et al. [101]). In this regard, synergistic activity was reported when Pelargonium graveolens essential oil was used in combination with norfloxacin against two strains of S. aureus (102). In the same study, EOs increased the norfloxacin’s uptake by bacterial cells. This may reduce the side effect(s) of antibiotics. Moreover, the anti-biofilm potential of several Eos, including eugenol, cinnamaldehyde, citral and geraniol, had been elucidated (103). Three essential Eos, cinnamon (Cinnamomum zeylanicum), TTO (Melaleuca alternifolia) and Palmarosa (Cymbopogon martini) synergized with ciprofloxacin against pre-formed biofilm of P. aeruginosa (104).

The antimicrobial tolerance of P. aeruginosa was effectively controlled when two antimicrobials that targeted more than one component of the bacterial cell (105) were combined in a single formulation. The targets included DNA synthesis (106) and the cytoplasmic membrane (107). EOs from Origanum vulgare L., carvacrol and thymol were identified as putative efflux pump inhibitors facilitating the uptake of antibiotics, norfloxacin, erythromycin and tetracycline (108).

More in vitro and in vivo studies are required to verify the safety and efficacy of EOs as ‘drug resistance modulators,’ alone, or in combination, with conventional antibiotics.

NANOPARTICLES: NEW GENERATION OF ANTIBIOTIC HELPERS
Various nanoparticles (NPs) are often reported having inhibitory effect against planktonic and biofilm cells. This activity is related to ATP-associated metabolism, permeability of the outer membrane and the generation of hydroxyl radicals that are induced by bactericidal compounds (109). Silver nanoparticles (Ag NPs) at concentrations of 100 mg/ml showed anti-biofilm activity by causing a 4-log reduction of *P. aeruginosa* cell growth (110). Moreover, a 95% inhibition in biofilm formation by *P. aeruginosa* was noted when Ag NPs were used as an anti-biofilm agent for 24 h (111). A synergism was noticed when NPs were combined with antibiotics against *S. aureus*, leading to disruption of the biofilm architecture and modulation of the antibiotic resistance of pathogens. Ag NPs were reported inhibiting QS and preventing biofilm formation by *S. aureus* (112). In the same study, a synergistic anti-biofilm effect was noticed when Ag NPs were combined with chloramphenicol and gentamicin. The antimicrobial activity of Ag NPs is influenced by their net charge and their ability to diffuse through a biofilm (113).

Recently, Gurunathan et al. (114) generated new, cost effective Ag NPs prepared by combining silver ions with leaf extract of *Allophylus cobbe*. These NPs showed a higher antibacterial and anti-biofilm activity against *P. aeruginosa* and *S. aureus* when combined with ampicillin and vancomycin, rather than using NPs or antibiotics alone. The interaction of Ag⁺ with the bacterial cell membrane disrupted membrane permeability, inhibiting their respiratory enzymes and thus production of reactive oxygen species (ROS) (115). It had been suggested that at higher production of ROS, cellular membranes become more damaged, leading to increase ampicillin and vancomycin uptake (115).

In an attempt to improve the bactericidal activity of NPs, Habash and co-workers (116) evaluated different sizes of citrate-capped Ag NPs, alone and in combination with aztreonam,
against *P. aeruginosa*. Ten nm capped Ag NPs synergized with aztreonam, efficiently disrupting the biofilm structure of *P. aeruginosa*.

Combination of antibiotics (ampicillin, oxacillin and penicillin) with selenate NPs (SeNPs), was more effective (94%) in disrupting and inhibiting MRSA biofilms than the antibiotics alone (117).

Overall, the antimicrobial potential of NPs compounds may depend on their sizes, charges and stability in order to enhance antibiotics and control biofilm. However, the safe consumption of NPs must be established before using them in pharmaceutical formulation as antibacterial agents.

**CONCLUSION: COMPLEMENTARY APPROACH MAY BE A SOLUTION THAT WORKS WITHOUT ABANDONING ‘OLD-TIMER’ ANTIBIOTICS**

In this mini-review, the attempt was made to bring the reader’s attention to the mentioned challenges, illustrating them with two ‘representative’ organisms from the groups of Gram-positive and Gram-negastive pathogens. With the goal of provoking and inspiring the reader’s interest to the topic of this review, the authors considers it important to mention another target, the so-called ‘Gram-variable’ pathogens such as *Gardnerella vaginalis*, one of the major contributors to a multi-microbial infection known as bacterial vaginosis (118). Similar to discussed representatives of two groups of infectious bacteria, *G. vaginalis* biofilms are effectively controlled by combinations of DNase (enzyme) and metronidazole (antibiotic) (119). Subtilosin (AMP) inhibited biofilms of *G. vaginalis* when combined in synergistic formulations with antibiotics (metronidazole or clindamycin) or naturally-derived substances such as lauramide arginine ethyl ester (120, 121). Also, thymol had been found to interfere with adhesion of *G. vaginalis* to human vaginal cells (122), and combination of thymol and eugenol showed...
synergistic activity against newly established and matured *G. vaginalis* biofilm, reducing the microbial adhesion to the human vaginal epithelial cells (122). Moreover, in the *in vivo* study, a synergistic activity between thymol and eugenol vaginal douche was reported reducing the recurrence rate of BV infection (123). All of these are sound example of the complementary approach’s validity and significance when applied to ‘traditional’ and ‘unorthodox’ pathogens.

Finding an effective strategy to control biofilm formation remains a challenge (Table 1). Antibiotic resistance and the recurrence of infections reflect the failure of conventionally used antibiotics for the treatment of biofilm-associated persistent infections. Alternative methods for biofilm prevention and/or eradication are urgently required to modify the traditional treatments. The ability of several novel, natural antimicrobial compounds to efficiently control biofilm formation on biotic and abiotic surfaces has been identified. Compared to the activity of each one individually, a stronger anti-biofilm activity (synergistic or enhancement) was reported when traditional antibiotics were used in combination with alternative antimicrobials reviewed here, or when used in the presence of other recently reported compounds such as chitosan (124-129), nitric oxide (130) and Cis-2 (131). The potency of antimicrobial combinations is ultimately determined by the synergy of interacting antimicrobials where each one of them is acting on different targets (Table 2 and Figure 1).

Overall, the beneficial properties of complementary approach in controlling biofilms of health-threatening bacteria is a promising strategy and could be used in personal care and pharmaceutical applications.
Figure legend

Figure 1. Persistence of microbial pathogens in biofilms requires a sophisticated arsenal of killing machines to break their party.
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TABLE 1 Mode of action, advantages and limitations of the reviewed anti-biofilm agents.

<table>
<thead>
<tr>
<th>Antimicrobial compounds</th>
<th>Proposed Mode(s) of Action on Biofilm</th>
<th>Advantage(s)</th>
<th>Limitation(s)</th>
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<tbody>
<tr>
<td><strong>Antimicrobial Peptides</strong></td>
<td>- Cationic AMPs interact with anionic biofilm surface (37). - AMPs inactivate the twitching motility of <em>P. aeruginosa</em> and inhibit biofilm formation (37). - Disrupt expression of biofilm formation essential genes, down-regulate expression of type IV pili, rhamnolipid, quorum sensing, and flagella assembly genes (38). - Bind to eDNA and accelerate detachment of the biofilm (41).</td>
<td>- Relative selectivity, broad-spectrum activity, cationic and amphiphatic properties, disruption of bacterial cell membrane with low frequency and slow emerge of bacterial tolerance (28, 29). - Bactericidal activity against slow growing bacteria within biofilms (30). - Antimicrobial activity improved and cytotoxicity reduced by modifying and hybridization the sequences of primary amino acids (31, 32).</td>
<td>- Development of resistance to AMPs via modification of the bacterial lipopolysaccharides or using efflux pumps (43). - Susceptibility of some AMPs to proteolytic enzymes as well as their high cost of purification and sequences (140). - Insufficient selectivity of STAMPs (140). - Possible hemolytic and potential cytotoxic effect (140).</td>
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<tr>
<td><strong>Nanoparticles</strong></td>
<td>- Interference with ATP-associated metabolism, change of the outer membrane’s permeability and generation of hydroxyl radicals (109). - Inhibition of quorum sensing and prevention of biofilm formation (112). - Changing of bacterial protein profile and modifying their pathogenesis by interaction with bacterial DNA (137).</td>
<td>- New technique, simple method, cost effective compounds, and delivers strong antimicrobial activity (114). - As antimicrobial carriers (139): • High stability in the biological environment and high carrier capacity. • The possibility to incorporate both hydrophilic and hydrophobic molecules. • Its viability using different courses (oral, parenteral and inhaled) of administration. - Design nanoparticles (NPs) to ensure release of an efficient drug concentration from the matrix (139).</td>
<td>- The antimicrobial potential of NPs depends on size, charge, stability and biocompatibility (116). - Cytotoxicity (142).</td>
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<tr>
<td><strong>Essential Oils</strong></td>
<td>- Degradation of extracellular matrix components (polysaccharides, protein and eDNA) (63).</td>
<td>- Efficient inhibition of biofilm formation, disruption of the EPS production, dispersing the pre-formed biofilm (64, 66). - Reduces the number of biofilm viable cells (70).</td>
<td>- High quantities of EPS and proteolytic exoenzymes by mature biofilm counteract the enzymatic activity (64).</td>
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<tr>
<td><strong>Enzymes</strong></td>
<td>- Inhibition of cell-to-cell or cell-to-surface attachment. - Inhibition of binding of QS signals to receptor proteins, antagonizing quorum signal biosynthesis, or degrading QS signals (136). - QSIs possess “high species specificity” and effectively act against certain pathogens (138).</td>
<td>- Development of resistance to AMPs via modification of the bacterial lipopolysaccharides or using efflux pumps (43). - Development of resistance to AMPs via modification of the bacterial lipopolysaccharides or using efflux pumps (43). - Development of resistance to AMPs via modification of the bacterial lipopolysaccharides or using efflux pumps (43).</td>
<td>- Lack of bacterial activity (85). - Reported toxicity of and resistance to QSI (136).</td>
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<tr>
<td><strong>Quorum Sensing Inhibitors</strong></td>
<td>- Attack cellular ATP and ATPase, acting on cytoplasmic enzyme and membrane proteins/fatty acids leading to the leakage of metabolites and ions (94). - Anti-quorum sensing activity by down regulation of QS genes leading to reduce virulence factors production and biofilm formation (94). - Putative efflux pump inhibitors facilitating the uptake of antibiotics (108).</td>
<td>- Have a broad-spectrum activity against a wide range of pathogenic microbes (93). - Have been used as ethnomedicine against bacterial infection and cancer for a long time (95, 96). - Simple extraction, non-toxic to the tissue environment and high carrier capacity. - Biofilm inhibition is noticed at the sub-MICs of many-tested EOs, such as <em>T. vulgaris</em> EO (103).</td>
<td>- Some EOs produce oxidative stress and possess toxic properties inducing killing activity against eukaryotes (141). - Increased albumin level and skin irritation (see review of Patel [141]).</td>
</tr>
<tr>
<td><strong>Biofilm Degradating Enzymes</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Natural anti-biofilm compounds</td>
<td>Antibiotics in combination</td>
<td>Interaction activity</td>
<td>Study type(s)</td>
</tr>
<tr>
<td>-------------------------------</td>
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</tr>
<tr>
<td>Dispersin B</td>
<td>Tocilizumab</td>
<td>Synergistic effect</td>
<td>In vivo</td>
</tr>
<tr>
<td>Lysophospholipid (LPL)</td>
<td>Nafcilin</td>
<td>Synergistic effect</td>
<td>In vivo</td>
</tr>
<tr>
<td>Alkaline lyase</td>
<td>Gentamicin, Ceftazidime</td>
<td>Alkaline lyase enhances antibiotic activity</td>
<td>In vivo</td>
</tr>
<tr>
<td>Recombinant human DNase I (rDNase)</td>
<td>Porfione, enol, chlorella plaque, benzalkonium chloride</td>
<td>DNase I enhances antibiotics activity</td>
<td>In vivo</td>
</tr>
<tr>
<td>DNase I, RNase A, porin K</td>
<td>Ampicillin, cefazolin, rifampin, levofloxacin, azithromycin</td>
<td>DNase I enhances antibiotics activity</td>
<td>In vivo</td>
</tr>
<tr>
<td>Dispersin B</td>
<td>Cefazolin, levofloxacin, imipenem</td>
<td>Synergistic effect</td>
<td>In vivo</td>
</tr>
<tr>
<td>Lysophospholipid (LPL)</td>
<td>Streptomycin, garamycin, erythromycin, ampicillin</td>
<td>Pseudomonas K enhances antibiotics activity</td>
<td>In vivo</td>
</tr>
<tr>
<td>DNase I</td>
<td>Metronidazole</td>
<td>DNase I enhances antibiotics activity</td>
<td>In vivo</td>
</tr>
<tr>
<td>Phenolic, phenolic acid</td>
<td>Tetracycline</td>
<td>Synergistic effect</td>
<td>In vivo</td>
</tr>
<tr>
<td>Phenol DPD</td>
<td>Gentamicin</td>
<td>QSI enhances antibiotic activity</td>
<td>In vivo</td>
</tr>
<tr>
<td>Lactose</td>
<td>Cefazolin, garamycin</td>
<td>QSI enhances antibiotic activity</td>
<td>In vivo</td>
</tr>
<tr>
<td>Bacillus hydroxy, cinemaldehyde, humulonitrile</td>
<td>Tetracycline, Chlortetracycline, vancomycin</td>
<td>QSI enhances antibiotic activity</td>
<td>In vivo and in vitro</td>
</tr>
<tr>
<td>Bacillus</td>
<td>Ampicillin, cefazolin, levofloxacin, azithromycin, erythromycin, garamycin</td>
<td>QSI enhances antibiotic activity</td>
<td>In vivo and in vitro</td>
</tr>
<tr>
<td>Aragonite flavus spicatus extract (LEF)</td>
<td>Tetracycline</td>
<td>LEF enhances antibiotic activity</td>
<td>In vivo</td>
</tr>
<tr>
<td>Dispersin B</td>
<td>Tetracycline</td>
<td>Synergistic effect</td>
<td>In vivo</td>
</tr>
<tr>
<td>P. aeruginosa essential oil</td>
<td>Norflloxacin</td>
<td>Synergistic effect</td>
<td>In vivo</td>
</tr>
<tr>
<td>Thyme oil, eglandin</td>
<td>Pefloxacin</td>
<td>Synergistic effect</td>
<td>In vivo</td>
</tr>
<tr>
<td>TTO (Teucrium fruticosum)</td>
<td>Ciprofloxacin</td>
<td>Synergistic effect</td>
<td>In vivo</td>
</tr>
<tr>
<td>Silver nanoparticles (AgNPs)</td>
<td>Chlorophenol, garamycin</td>
<td>Synergistic effect</td>
<td>In vivo</td>
</tr>
<tr>
<td>Gold nanoparticles (AuNPs)</td>
<td>Ampicillin, cefazolin, levofloxacin, azitromycin, erythromycin, garamycin</td>
<td>NPs enhances antibiotic activity</td>
<td>In vivo</td>
</tr>
<tr>
<td>Chloroquine-phospholipid (Clq)</td>
<td>Tetracycline</td>
<td>Chloroquine-phospholipid enhances antibiotic activity</td>
<td>In vivo</td>
</tr>
<tr>
<td>Nitric oxide</td>
<td>Tetracycline</td>
<td>Nitric oxide enhances antibiotic activity</td>
<td>In vivo</td>
</tr>
<tr>
<td>Chlorophyll a</td>
<td>Tetracycline</td>
<td>Chlorophyll a enhances antibiotic activity</td>
<td>In vivo</td>
</tr>
<tr>
<td>Citrus leaf extract</td>
<td>Tetracycline</td>
<td>Citrus leaf extract enhances antibiotic activity</td>
<td>In vivo</td>
</tr>
<tr>
<td>Ultrasonic exposure</td>
<td>Tetracycline</td>
<td>Ultrasonic increases transport of gentamicin</td>
<td>In vivo</td>
</tr>
</tbody>
</table>

**TABLE 2** In vivo and in vitro studies of natural anti-biofilm agents in combination with antibiotics for combating biofilm-associated pathogens.
Erratum for Algburi et al., Control of Biofilm Formation: Antibiotics and Beyond

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