Introduction
Joseph Bigger first observed the phenomenon that cultures of *Staphylococcus aureus* in the exponential phase showed resistance to penicillin. This phenotype was first described by Hobby in 1942, observing that 1 in 100 cells was not affected by penicillin and developed into persisters. A genetically-identical bacterial population is heterogeneous in terms of phenotypic characteristics, with phenotypic variants arising due to fluctuations in gene expression. The subpopulations of phenotypic variants that tolerate high doses of antibiotics are termed as persisters. In contrast to antibiotic resistance, persistence against antibiotics is temporary and cannot be inherited.

Bacterial persistence has been attributed to a change in the physiology of a cell. Microfluidic analysis revealed that persisters are either not growing or slow-growing, suggesting that they are dormant. Additionally, persistence was found to increase when attempts were made to interfere with essential cellular processes, such as replication, transcription, translation, and energy production. Upon removal of antibiotics, persisters may resume growth and metabolic activity that again makes them susceptible to antibiotics.

While studies suggest that stochastic gene expression results in the spontaneous formation of phenotypic variants including persisters, a large body of evidence suggests that persister formation is induced by environmental factors/stress such as antibiotic treatment, signalling molecule indole, quorum sensing molecules, nutrient starvation, oxidative stress, heat stress, hyperosmotic stress, and acid stress. Since persisters are clinically relevant, it is important to study the mechanisms responsible for persister formation so that therapies can be designed to curtail these infections.

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have been found to be responsible for the recalcitrance of chronic infections. They are abundant in biofilms as well as immune cells, indicating their role in chronic and relapsing infections such as cystic fibrosis and tuberculosis, respectively. Persistence has also been suggested to contribute to evolution to give rise to antibiotic resistance. Additionally, the phenomenon of persistence is also implicated to play a role in tumour relapse after chemotherapy. Thus, it is important to dissect the mechanisms that are responsible for persister formation, which would help to design therapies to curtail the persistence of infections. Several methods have been found to kill persister cells but in vivo application has not been studied. Combination of antibiotics with metabolites (such as aminoglycosides combined with specific metabolites) has shown efficacy in killing persisters. Other efficient strategies include HipA inhibitors, membrane-penetrating peptides, peptidoglycan-degrading endolysins, and acyldepsipeptides. FDA-approved anti-cancer drugs mitomycin C and cisplatin have been shown to be efficient in killing persister cells but have an adverse effect in also targeting host cells.

This review provides a summary of recent literature that sheds light on the regulatory mechanisms involved in persister formation, persister physiology, experimental methods to study persister cells, survival mechanisms of persisters in the host as well as new strategies to combat persisters.

Bacterial Persistence
What are Persisters?
There are naturally occurring fluctuations in the expression of genes, termed as noise. Regulatory mechanisms such as positive or double-negative feedback could add even more noise in the gene expressions. This results in the development of distinct phenotypic variants within a genetically-identical population. Persisters are such phenotypic variants in a clonal bacterial population that are tolerant to lethal antibiotic treatment.

The antibiotic-killing curve provides the evidence for the existence of persisters in a bacterial population, where the rapid-killing represents the death of sensitive cells while the slow-killing represents the tolerant persisters. They are found in bacteria, fungi and tumour cells. This tolerance to antibiotics, also called as persistence, is not limited to bacteria and even observed in eukaryotic microorganisms such as Candida albicans, Saccharomyces cerevisiae, and even in tumour cell populations. It is also thought to contribute to the evolution of antibiotic resistance. Persisters are abundant in biofilms, and it is suggested that they have a significant contribution to relapse of infections. Persistence is also thought to play a role in tolerance to chemotherapy and the relapse of tumours.

How Is Persistence Different From Resistance?
The reasons due to which efficacy of antibiotics gets impeded are of two categories: permanent and temporary. The permanent cause is the development of antibiotic resistance via alterations in the genetic structure, which allows antibiotic targets to evade the treatment. The temporary cause of antibiotic failure is entrance into a dormant state, called persistence, which allows bacteria to survive via a transitory/temporary tolerant phase. Persisters of Candida albicans differ from resistant cells, in which they show biphasic killing pattern against microbicidal agents to create highly tolerant cells instead of resistant cells. However, persisters switch to an actively growing state at a later time when the antibiotic stress has been removed. Unlike resistance, persistence is not inherited since there is no genetic change involved. Although persisters are genetically identical to the rest of the bacterial population, they differ in their physiological state: there is a lack of translation, transcription, proton motive force, and ATP.

Persisters are rare in exponentially growing cultures but increase in numbers when the culture reaches the stationary phase (1%) as well as in biofilms. These persisters regrow in the fresh culture and produce a population similar to the original culture, consisting of both antibiotic-sensitive as well as antibiotic-tolerant cells.

Persistence Comes With Age
Persisters are a phenomenon where few bacterial cells develop mechanisms to bypass antibiotic stress so it may be possible that it occurs as bacterial cells age. Stewart et al reported that Escherichia coli divides asymmetrically, owing to stochastic fluctuations, and gives rise to an actively growing daughter cell and another daughter cell that inherits the old spindle pole has diminished growth rate. They suggest that the daughter cell with old spindle pole should be considered an aging parent which further reproduces to give rise to an actively growing rejuvenated daughter cell. Such asymmetric division may generate phenotypic variation for adapting to variations in environments. Persistence may result from asymmetrical division and the daughter cells that inherit the old spindle pole may actually form the persisters. This population of ‘old’ daughter cells is heterogeneous, consisting of phenotypically variable cells. Some of these die over time while few of them accumulate mechanisms that allow them to adapt to environmental stresses such as antibiotic stress. This explains the extremely few numbers of persisters under normal growth conditions. Upon cessation of stress, persisters resume growth to divide and give rise to daughter cells, one of which carries the legacy of persistence while the other daughter cell does not, giving rise to a population of antibiotic-sensitive and antibiotic-tolerant cells.
Formation of Persisters

Stochastic Persister Formation

The persister formation depends on the expression of persister protein; stochastic/noisy expression results in the formation of spontaneous persisters while deterministic/environmentally-triggered expression results in induced persisters. Microfluidic observations of exponentially growing cells of E. coli confirmed that stochastic expression of toxin-antitoxin (TA) operon genes causes cessation of growth and that this correlates with high tolerance against lactam antibiotics. Persister formation in E. coli has been shown to be related to stochastic or unregulated expression of HipA kinase, a type II toxin that inhibits protein synthesis by phosphorylating Glu-tRNA synthetase.\(^\text{27}\)

For the persister formation in E. coli, mRNAs are cleaved by released toxins, or a signal transduction that is ppGpp-dependent induces growth arrest. Changes in metabolic flux result in a decreased TCA cycle activity, which increases persistence.

Induced Persister Formation

Certain environmental factors and stresses have been implicated in the development of persisters (Figure 1). The change of growth phase - from exponential to stationary-results in a significant increase in the number of persisters. Certain signalling molecules such as indole and quorum sensing molecules have also been associated with increase in persister formation. Various forms of stress such as carbon source shifts, oxidative stress, heat stress, hyperosmotic stress, acid stress, antibiotic stress, and phage infection have been shown to increase persister formation. Recent studies have suggested that reactive oxygen species (ROS) play a role in increasing bacterial persistence and protecting against antibiotic treatment.\(^\text{29}\)

However, the detailed mechanisms are not known. In a recent study, salicylate was found to increase bacterial persistence in E. coli by the generation of ROS.\(^\text{30}\)

Active Mechanism of Persister Formation

Recently, persisters were shown to express efflux pumps (ToIC protein) in response to antibiotic treatment, suggesting an active response in persisters in contrast to passive dormant state as hypothesized before.\(^\text{31}\) In summary, ToIC is a trimer that forms a channel across the membrane to serve as a duct for substances. The protein forms a pore of dimensions 140 A; sealed at the end of the periplasm but open at the end of the medium. The protein is opened transiently through a mechanism similar to that of an iris to throw out certain substrates from the cytoplasm to the outside.\(^\text{32}\)

Regulatory Mechanisms of Persister Formation

The molecular mechanisms involved in the formation of persisters have started to be elucidated, but complete pathways are still to be determined. Genetic studies failed to identify any specialized persister genes that function exclusively in persister formation,\(^\text{33}\) suggesting a lack of a specialized persister pathway. However, identified genes (relA, spot, dksA, ssrA, lon) have multiple roles in many different cellular pathways. Other genes have been found to be involved in metabolism (sucB, glpD, ubiF) or in stress responses (recA).

Stress and Toxin-Antitoxin Pathways

Stress responses play a prominent role in persister formation pathways. The SOS response upon DNA damage stress by fluoroquinolone antibiotics results in not only the expression of repair enzymes but also the production of type I TisB toxin, which forms an ion channel in the cytoplasmic membrane that reduces the proton motive force and ATP levels leading to drug tolerance.\(^\text{34}\) Recently, a new pathway of persister formation in the stationary phase was elucidated.\(^\text{35}\)

Tkachenko et al initially showed that inhibition of polyamine synthesis enzymes leads to decrease in bacterial tolerance to antibiotics.\(^\text{36}\) Later it was shown in E. coli that polyamine putrescine can trigger persister cell formation via stimulation of rpoS gene expression, which regulates general stress response.\(^\text{37}\)

In their recent work, they show that putrescine, spermidine, and cadaverine upregulate persistence via stimulation of rpoS, rmf and yqiD genes, which are involved in translational arrest as the cells enter the stationary phase.\(^\text{38}\)

Lewis and colleagues have elucidated the complete persistence pathway for tisB/istR-1 type 1 TA module (Figure 2). Ciprofloxacin treatment causes DNA damage and induces the SOS response, activating RecA and relieving the LexA-mediated transcriptional inhibition of the tisB/istR-1 operon. TisB protein toxin disrupts the proton motive force (pmf) and ATP production, resulting in multidrug tolerance.

Initially, TA modules were identified due to their role in post-segregation killing to select for plasmid-containing cells, now they have been attributed stress adapting roles including persistence.\(^\text{9}\) Due to stress activation, the antitoxins that inhibit the toxin are degraded. The active toxins suppress bacterial growth by inhibiting essential cellular functions such as replication, transcription, and translation. Thus, TA systems play an important role in the formation of dormant drug-tolerant persisters.\(^\text{38}\)

Based on the mechanism of detoxification, TA modules are divided into six types - type I to VI. The different modes of antitoxin detoxification include inhibiting toxin synthesis (type I and type V), blocking toxin activity (type II and III), promoting degradation (type VI), and protecting the toxin’s target (type IV).\(^\text{39}\)

Although TA systems remain quiescent under normal growth conditions via antitoxin detoxification, they can be activated temporarily by environmental stress such as...
nutrient starvation, antibiotic treatment, oxidative stress, and phage infection.\textsuperscript{40} In \textit{E. coli}, multiple TA systems seem to cross-interact and activated in a synchronous manner in response to stress.\textsuperscript{41} This synchronized response of TA systems is controlled by (p)ppGpp-mediated stringent response that activates antitoxin degradation not only by Lon protease\textsuperscript{42} but also by cross-activation between different TA systems.\textsuperscript{43}

However, TA toxin-mediated persistence can also be achieved in the absence of (p) ppGpp.\textsuperscript{44} A recent study on \textit{S. aureus} identified a mechanism of persister formation that was independent on the TA system. It senses the intracellular ATP to trigger the formation of persister.\textsuperscript{45} In \textit{Salmonella typhimurium}, the toxicity of Hha toxin of the Hha-TomB toxin-antitoxin system was shown to be conditional; i.e., Hha triggered cell death when subjected to acid stress, however, under antibiotic stress, the toxin-mediated persistence by inhibiting apoptosis-like death.\textsuperscript{46}

Recently a new TAS was characterized called DarT-DarG TAS, which is found in many bacteria including \textit{Mycobacterium tuberculosis}. It consists of DarT toxin that acts as an enzyme that specifically catalyzes ADP-ribosylation of thymidines on single-stranded DNA in a sequence-dependent manner while its antitoxin DarG removes this modification.\textsuperscript{47}

The GTPase Obg has been found to be a central player in mediating bacterial persistence, downstream to HipA toxin, by activating the transcription of type I toxin hokB and resulting in membrane depolarization. Recently, the crystal structure of \textit{E. coli} ObgE protein in a GDP-bound state was determined. Their results showed that GTP binds to the C-terminus and that ObgE was stimulated upon ribosome binding, confirming the ribosome-dependent GTPase activity of ObgE.\textsuperscript{48} These results may further help in unravelling the pathway by which HipA activates ObgE in activating persister cell formation (Figure 3).

**Regulation of TAS at the Transcriptional Level**

Recently, the crystal structures of type II TAS VapBC1 complex as well as VapBC1 bound to DNA were determined in \textit{Caulobacter crescentus}. The antitoxin VapB1 was found to have pseudo-palindromic protein sequences at the C-terminus that bind to the complementary pseudo-palindromic sequences in the operator region of TA operon. These palindromic protein sequences were found to be a common feature of VapB antitoxins indicating a common regulatory mechanism of VapBC TA systems. The crystal structure of VapBC pointed to structural changes as the basis for activation of VapC toxin.\textsuperscript{49}

**Regulation of TAS at the Post-transcriptional Level**

Investigation of the type I TisB/IstR-1 TAS revealed that
the tisB toxin expression was under double regulation. The first layer of regulation is already known, where the antitoxin RNA IstR-1 binds and inhibits the translation of toxin mRNA. The second layer of regulation is provided by an inhibitory 5’-UTR, inherently present in the tisB-mRNA, which delays TisB translation to avoid membrane depolarization in non-persistent cells.39

Crosstalk Between TA Modules
TA modules cross-talk with each other for persistence.40 VapC toxin overexpression induces yefM-yoeB TAS.40 HipA activates multiple type II toxins.41 RelE expression induces transcription of mqsRA, mazEF, dinJ-fayQ, hicAB, yefM-yoeB and prlF-yyaV. In return, relBE operon is upregulated upon the expression of MazF, MqsR, HicA, and HipA.41 Doc toxin overexpression transactivates relBE expression.42 MqsR activates expression of ghoT.42 The number of TA systems varies throughout the bacterial kingdom, and still remains poorly understood. The TA modules display tight coordination in toxic events, which could occur mainly due to rapid proteolytic coupling that happens post-translation, and may be the key to understanding the persister cell survival. Then, it would be possible use the drugs interfering with proteolytic crosstalk as a major tool for eliminating persistence and provide novel therapies for infectious bacteria.

Physiology of Persisters
The persistence of cells against lethal antibiotic treatment has been shown to be derived from reduction in metabolic activity, replication, transcription, translation, and membrane polarization. Inactivity of the antibiotic’s primary target lends protection against antibiotic stress.43 The original paper by Bigger described antibiotic-tolerant persisters as dormant cells that did not proliferate.4 He also showed that growth inhibitory conditions such as low temperature, lack of nutrients and boric acid treatment increased the number of persisters. Since antibiotic targets are mainly involved in cell growth processes, dormancy or metabolic inactivity best explains the antibiotic-tolerance exhibited by persisters. Balaban et al5 performed single-cell analysis and showed that persisters do have reduced growth rates.5 Later, transcriptome analysis of persisters revealed down regulation of metabolic and biosynthetic pathways.53 On the same lines, interference with essential physiological functions was found to increase persistence. For example, over expression of translation-inhibitory toxins increased persistence, while antitoxin-mediated toxin inhibition reversed the effect.54 Further the inhibition of translation, transcription or ATP synthesis was found to increase the number of persisters significantly.5

Modifications in genes encoding metabolic enzymes or regulatory molecules have been shown to affect persistence. For example, overexpression of an enzyme involved in glycerol metabolism, called glycerol-3-phosphate dehydrogenase (GlpD), was found to increase tolerance to ampicillin and ofloxacin.55 Another study found that deletion of GlpD results in increased persistence via accumulation of growth-inhibitory metabolite methylglyoxal.56 The contradictory results from the two studies may be due to different assay conditions but glycerol metabolism seems to play an important role in persistence.

Gene mutations affecting amino acid metabolism also significantly affect persistence. Screening of transposon mutants in E. coli for persistence against ticarcillin or ofloxacin revealed 18 mutants with increased persistence,
of which 16 had mutations in the genes involved in amino acid synthesis. In *Pseudomonas aeruginosa*, mutations in a gene coding for lysine decarboxylase that converts lysine to cadaverine have been found to increase persistence against carbencillin. Also, increased persistence against ofloxacin was observed for a mutation in the gene *pheA*, which is involved in phenylalanine biosynthesis and metabolism. This strong relation between amino acid metabolism and persistence also suggests a link between persistence and stringent response, which involves ppGpp and DksA.

Persistence is also shown to result from downregulation of energy metabolism. Screening of an *E. coli* transposon library found that deactivation of *phoU*, a gene whose product is involved in negative regulation of phosphate metabolism, which in turn led to an increased metabolic activity and reduced persistence. An *E. coli* deletion mutant screen revealed that mutations in *sucB* and *ubiF* genes, which are involved in energy production, decreased persister survival.

Few studies attempted to show that persisters employ active mechanisms to tolerate antibiotics. In *Mycobacterium marinum* and *M. tuberculosis*, persistence was shown to be a result of macrophage-induced expression of efflux pumps. In *Pseudomonas aeruginosa*, persistence was found to result only when starvation-induced growth arrest was accompanied by an active stringent response.

Recent studies point towards persisters being both active as well as passive, with active mechanisms resulting in energy depletion, growth retardation, and dormancy. In *Salmonella*, the expression of virulence factor is heterogeneous and confers metabolic cost leading to growth retardation and antibiotic-tolerance. In *E. coli*, activation of amino acid synthesis and motility were found to contribute to persister formation tolerant to gentamicin. Research also points out to the role of increased activities of efflux pumps in persister cells. For instance, bacterial persister cells reported increased levels of efflux mechanisms that led to lower concentrations of a fluorescent β-lactam antibiotic BOCILLIN™ FL Penicillin (BOCILLIN) inside the cell.

The inhibition of synthesis of macromolecules (i.e., transcription or translation) was associated with a decrease in bacterial cellular respiration. Research has indicated that bacteriostatic antibiotics target the central metabolism that in turn affect respiration and the production of ATP. The efficacy of targets can be increased by increasing the rates of cell respiration that results in a futile cycle. Certain drugs have been reported that target members of the electron transport chain such as bedaquiline that targets the F1F0 ATPase of *M. tuberculosis*. Overall, this is suggestive that the suppression of respiration and metabolism can be used for targeting such cells.

In bacteria, antitoxin proteins are degraded by activated proteases. The mRNA are cleaved by released toxins, or a signal transduction that is ppGpp-dependent induces growth arrest. The synthesis of the second messenger cAMP by adenylate cyclase is favoured by nutrient limitation. A decreased TCA cycle activity results because of metabolic flux alterations and increases persistence (Figure 4).

Different branches of metabolism can produce reactive oxygen species (ROS) as hazardous side products, impairing persister formation. Biofilm made of aminosugar or proteins to enhance survival and favors formation of persisters. The various aspects associated with persisters are explained briefly in the following (Table 1).

**Combating Persister Cells**

The phenomenon of formation of persister cells in a bacterial population causes the formation of diseases that are refractory such as tuberculosis, cystic fibrosis etc. Hence, it is essential that techniques or molecules are developed to eradicate these cells. The following section summarizes instances of the use of peptides, drugs or other molecules in quelling such cells. Antimicrobial peptides (AMPs) are present in several organisms as the host defence system against pathogens. There are reports of the use of certain AMPs in the treatment of bacterial diseases such as gramicidin. Persisters are implicated in several clinical abnormalities: especially recurrent infections as discussed in the examples that follow as they are formed in several pathological species such as *M. tuberculosis*, *S. aureus*, and *P. aeruginosa*. Additionally, the stress response pathway associated with horizontal gene transfer or mutations linked with resistance to antibiotics. In 2011, a study reported the use of cationic peptides in killing persister cells of a very active persister bacterium: *Escherichia coli* HM22. The team tested the activity of linear peptides of Trp/Arg repeats designated as [(RW)n-NH2, where n is 2, 3, or 4] and a dendrimer molecule represented as (RW)4D. They found that the peptides (RW)4D and (RW)4-NH2 showed high potency in killing persister cells with (RW)3-NH2 following closely behind. Additionally, one of the peptides called (RW)4-NH2 could disperse biofilms that were rendering the bacteria resistant to antibiotics and thus increased the susceptibility of the cells to antibiotics. Thus, this study showed the potential of AMPs in the combat against persister cells and even their refractory biofilms.

A research team in 2015 examined the use of FDA-approved anti-cancer drug mitomycin C to target these persisters. It was observed that the drug was up taken by the cells and induced the crosslinking of DNA in dormant cells too. They reported the efficacy of mitomycin C in killing biofilm and planktonic cells of several genera such as pathogenic species of *E. coli*, *S. aureus* and *P.
aeruginosa. In another recent study in 2016, scientists tested the efficacy of 36 diverse (halogenated) indole derivatives in order to target persister cells, especially in biofilms. They reported that the derivatives 4-fluoroindole, 7-chloroindole, and 7-bromoindole caused the death of persister cells of *E. coli* and *S. aureus*. The most potent result was observed with that of 5-iodoindole that also removed biofilm formation and prevented the formation of persister cells along with an inhibition of virulence factor such as carotenoid staphyloxanthin in *S. aureus*. Biofilms that cause increased resistance to antimicrobial agents this has spawned the testing of electrochemically produced H2O2 to target persister cells. This entails the use of an e-scaffold that generates Hydrogen peroxide that exerts several effects including the formation of toxic hydroxyl free radicals that degrade several biomolecules. H2O2 is also reported to target persister cells and disrupt biofilms. A research team examined the potential of such an e scaffold that generated H2O2 that induced the formation of hydroxyl free radicals in the cells and caused an increase in membrane permeability. This, in turn, increased the susceptibility of the test organism *P.*

![Diagram](image)

Figure 4. Physiological Aspects Associated With the Persister State

| Table 1. Summary of Several Physiological Aspects Associated With Persisters |
|------------------|------------------|
| **Mechanisms** | **Examples Of Bacterial Species** |
| Asymmetric cell division | Mycobacteria<sup>46</sup> |
| Upregulation of stress response genes such as SOS response, phage-shock response, heat-shock response, cold-shock response and oxidative stress | *Escherichia coli*,<sup>43,44</sup> *Staphylococcus aureus*<sup>44</sup> |
| Stochastic expression of HipA kinase: a toxin system | *Escherichia coli*,<sup>41</sup> *Staphylococcus aureus*<sup>41</sup> |
| Certain persistence-inducing molecules such as polyamine putrescine, spermidine, and cadaverine | *Escherichia coli*,<sup>42,43</sup> |
| Toxin-antitoxin systems such as Hha-TomB, Dsr-DarG, and HipA-ObgE | *Salmonella typhimurium*,<sup>46</sup> *Mycobacterium tuberculosis*<sup>47</sup> |
| Inhibition of translation, transcription, or ATP synthesis | *Escherichia coli*,<sup>41</sup> |
| Certain metabolites such as glycerol-3-phosphate dehydrogenase, glycerol-3-phosphate acyltransferase as enzymes involved in persister formation | *Escherichia coli*<sup>45</sup> |
| Downregulation of the genes governing metabolism, such as *phoU, sucB*, etc | *Escherichia coli*,<sup>46,47</sup> *Streptococcus pyogenes*<sup>11</sup> |
| Increased activities of active efflux pumps | *Escherichia coli*,<sup>41</sup> |

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aeruginosa PA01 biofilms to the antibiotic tobramycin. The researchers reported complete elimination of all viable persister cells and biofilms in the experiment, highlighting the importance and efficacy of this electrical approach to enhance the activity of antibiotics to in turn destroy persister cells.\cite{2004} Reports indicate that studies to target persisters have revolved around antibiotic resistance; with heavy metal resistance, another feature of persisters. Similar to cancer cells, persisters display heterogeneity with a sub-population emerging following antibiotic administration that presents a potential strategy similar to such cancer cells to escape treatment.\cite{2016, 2017, 2018}

**Conclusion**

The review has discussed several mechanisms and the physiology of cells that display persistence. The mechanisms range from metabolic changes to the upregulation of certain active efflux pumps to remove antibiotics. Toxin-Antitoxin systems have been found to play a key role in the development of persisters. It can also be seen that certain changes in metabolites such as amino acids or enzymes also seem to increase the numbers of persisters. Apart from the mechanisms of persistence, several techniques to combat persisters such as drugs, AMPs and even the use of an electrochemical technique were discussed. Understanding the details of persister cell survival may enable us to test drugs that interfere with different modules of persister formation pathway, and provide treatments for infectious bacteria that affect millions each year. Overall, the mechanisms underlying persisters and biofilm formation can aid in the combat against several dreadful diseases.

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**Competing Interest**

There is no conflict of interest.

**Ethical Approval**

Not applicable.

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