BIOFILM AND HOSPITAL ACQUIRED INFECTION: MECHANISM, TOLERANCE AND TREATMENT.

Thesis submitted in partial fulfillment of the requirements for the award of the degree of

MASTER OF SCIENCE

IN

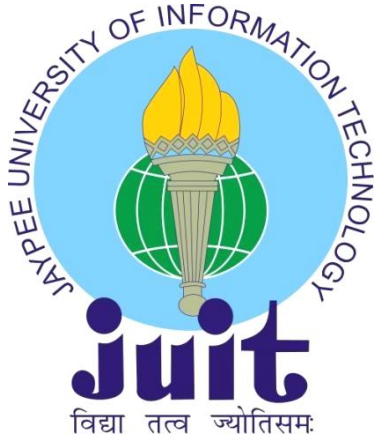
BIOTECHNOLOGY

By

ANAMIKA VERMA (197819)

UNDER THE GUIDANCE OF

DR. RAHUL SHRIVASTVA



DEPARTMENT OF BIOTECHNOLOGY AND BIOINFORMATICS

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MAY- 2021.

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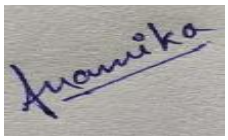
DECLARATION BY THE SCHOLAR

I hereby declare that the review of literature reported in the M.Sc. thesis entitled "**Biofilm and hospital acquired infection: mechanism, tolerance and treatment**" submitted at Jaypee University of Information Technology, Waknaghat, India, is an authentic record of work done by me (**Anamika Verma- 197819**) carried out under the supervision of Dr. Rahul Shrivastava (Associate Professor) Department of Biotechnology and Bioinformatics. I have not submitted this work elsewhere for any other degree or diploma.



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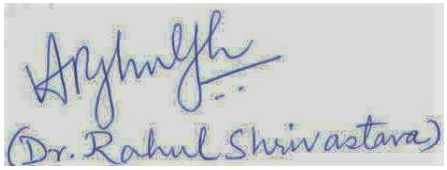
SUPERVISOR'S CERTIFICATE

This is to certify that the work reported in the M.Sc. thesis entitled "**Biofilm and hospital acquired infection: mechanism, tolerance and treatment**", submitted by **Anamika Verma (197819)** at Jaypee University of Information Technology, Waknaghat, India, is a bonafide record of her original work carried out under my supervision. This work has not been submitted elsewhere for any other degree or diploma.



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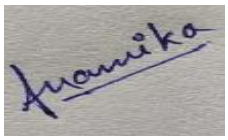

Anamika Verma (197819)

LIST OF ABBREVIATIONS

Am	Ampicillin
AMP	Antimicrobial peptide
AI	Autoinducing
Bf	Belofloxacin
Cip	Ciprofloxacin
Cf	Ceftazidime
EPS	Extracellur Polymeric substance
Gm	Gentamycin
HAI	Hospital Acquired Infections
HBOT	Hyperbaric Oxygen Therapy
Km	Kanamycin
MRSA	Methicillin Resistant *Staphylococcus aureus*
Of	Ofloxacin
QS	Quorum Sensing
Tb	Tobramycin

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ABSTRACT

Sessile cells secrete extracellular polymeric substance (EPS) comprising of polysaccharides, proteins, lipids and eDNA which forms the matrix in which cells are embedded and held together, forming a biofilm. The EPS acts as a barrier and is necessary for biofilm survival. In nature, more than 90% bacteria live and grow in biofilms. Surface attachment, microcolony formation, maturation and dispersal are general steps of biofilm formation. Phenotypic shift from planktonic (free living) cells to sessile (surface attached) cells is mediated via changes in genetic expression through quorum sensing and cGMP mediated pathway.

Pathogenic biofilms in human health cause tenacious clinical problems from non-healing chronic wounds to lung cystic fibrosis. Hospital acquired infections (HAI) are a major set back in health care industry and biofilms are the root cause. They from on the surfaces of medical devices and implants infecting various patients worldwide. Dynamic and ever evolving nature of biofilm makes it difficult to diagnose and treat infections. Therefore, better and advanced diagnostic tools like transcriptomic and wound bed analysis is required to effectively target biofilms.

Persistor cells cause recurring infections that delays the process of wound healing and generate continuous albeit low levels of inflammatory response from the host which cause damage to the surrounding tissue in the long run. Currently, antibiotics is the major treatment for bacterial biofilm infections but, traditional methods have failed time and again in complete eradication of biofilms owing to antibiotic resistance (which is increased up to 1000 folds in comparison to their planktonic counterparts) and protection from the EPS matrix (which acts a barrier against antimicrobial agents, host immune response etc.). Hence, a combination of traditional methods with new advanced therapy like hyperbaric oxygen therapy, modulating microbial metabolism, probiotics etc. is a necessity to control biofilms and the spread of drug resistance.

Keywords: Biofilms, Hospital acquired infections, Chronic infections, Biofilm treatment, Biofilm tolerance.

1. INTRODUCTION

Aggregates of single or multiple microbial species that can form on biotic or abiotic surfaces is known as biofilms. They produce extracellular polymeric substance (EPS) which approximates to 90% of the total biofilm mass along with proteins and DNA. Microbes in biofilm are embedded in this self-produced matrix which plays major role in mediating surface adhesion and providing stability to the cells [1,2]. EPS also acts as barrier against the antimicrobial agents, host immune response, enzymatic degradation, along with trapping nutrients and retaining water to be utilized by cells during starvation, thereby protecting and providing for the cells in biofilms.

Biofilms are generally complex in nature and has an overall dynamic structure as different parts of the biofilms can be in various stages of development. Cells in biofilms undergo transition from planktonic (free living) to sessile (surface attached) stage in response to various environmental signals and changes in molecular pathways. Sessile cells have general metabolism and are actively involved in reproduction with reduced expression of surface structures that help in motility whereas planktonic cells have motility required for colonization of new surfaces. These changes are due to change in genetic expression of the planktonic and sessile cells.

Varied conditions within the biofilms like nutrient availability, oxygen concentration, waste accumulation, gives rise to non-homogenous conditions. Spatial heterogeneity, due to non-homogeneous environmental conditions within biofilms, results in different microbial physiology and metabolism in different sections of the biofilms [3]. Spatial heterogeneity also gives advantages to cells in terms of survival, enhanced antimicrobial tolerance and resistance, for example aerobic population can reside in the periphery of the biofilm where there is continuous supply of oxygen, such populations also utilize the proton motive force to drive the efflux pumps to toss antibiotics out of the cells.

In multispecies biofilms, the interspecies interactions along with the spatial heterogenicity, may give the sessile cells enhanced resistance to antimicrobial agents [4,5] and increased virulence [6], as compared to their counter planktonic cells. Multispecies biofilms are prevalent in most environments; however, single species biofilms exist in various infections and on surfaces of medical devices [7-10].

Biofilms have been associated with 65-80% of infections in humans. They cause chronic and recurring infections in humans. Approximately 80% of biofilm forming pathogens are involved in persistent infections [11,12]. Persistent cells are those that hide within the host cells and hence are saved from antimicrobials and host immune response. Such cells are responsible for reestablishment of biofilms after an infection as they are more resistant to antibiotics [13-15]. Typical examples include *Pseudomonas aeruginosa* and *Staphylococcus aureus* responsible for cystic fibrosis and most wound infections respectively [16,17].

In hospital settings biofilms play a major role in causing hospital acquired infections or nosocomial infection. They grow on medical devices and implants which can cause tenacious problem in patients. Biofilms are frequent in pulmonary infections like chronic obstructive pulmonary disease (COPD), cystic fibrosis (CF), bronchiectasis and ventilator-associated pneumonia (VAP). Catheter associated urinary tract infection is the most common nosocomial infection. Finding efficient ways to prevent biofilm formation in medical settings is a necessity to provide effective treatment to patients and to control spread of antibiotic resistance. Use of antimicrobial peptides, probiotics and superhydrophobic coating on implants and surfaces have been reported to reduce or prevent biofilm formation.

The challenge in treating biofilm infections in patients lies in late diagnosis of disease and the failure to pin point the causative organism in the biofilm responsible for resistance or virulence. Use of antibiotics is still the major treatment in biofilm infections. As the metabolic activity of the cells in the biofilm is lowered and also, they are protected by EPS matrix, antibiotics like β-lactams which targets the actively dividing cells are inefficient in eradicating biofilms. Spread of resistance and adaptation of the cells to increase tolerance against particular antibiotics have rendered antibiotic treatment ineffective. Many techniques are available like adjuvant HBOT treatment, bacteriophages and lysin, enzymes to degrade biofilm matrix, quorum signal inhibitors, modulating microbial metabolism, probiotics, antimicrobial peptides, superhydrophobic coating of surfaces which can be used in combination with antibiotics treatment that makes the cells vulnerable to the antibiotics for complete eradication of biofilms.

2. MECHANISM OF BIOFILM FORMATION

Biofilm formation is initiated when single bacterial cell adheres to the surface using extracellular polymeric substance. The cell then divides within the extracellular matrix producing sister cells which gradually forms adherent microcolonies [18]. Growth of biofilm is a result of continuous division of cells within the microcolonies and addition of planktonic bacteria. As a result, biofilm constitutes of single cells and colonies of sister cells which are embedded in highly hydrated self-produced matrix of exopolymers [19].

2.1 ROLE OF EXTRACELLULAR POLYMERIC SUBSTANCE

Biofilm matrix comprises of polysaccharide, proteins and teichoic acid actively secreted by bacteria along with trapped macromolecules like DNA giving it a diverse chemical nature. DNA released form dying cells gets trapped in the matrix and is known as extracellular DNA (eDNA) [20]. In well-developed biofilms, 90% of the biofilm volume is EPS along with protein and DNA, and only 10% of biofilm volume is bacteria [21].

Extracellular polymeric substance (EPS) is responsible to hold multiple groups of bacteria in biofilms together. Polysaccharides facilitates adhesion and aggregation of cells. It forms a protection barrier against host immune system, antimicrobial agents, oxidizing agents. It traps nutrients and retains water which is to be utilized by the sessile cells growing within the matrix [22].

2.2 STAGES OF BIOFILM FORMATION [Fig 1]:

(i) Bacterial attachment: Reversible and Irreversible

(ii) Maturation

(iii) Dispersion or detachment.

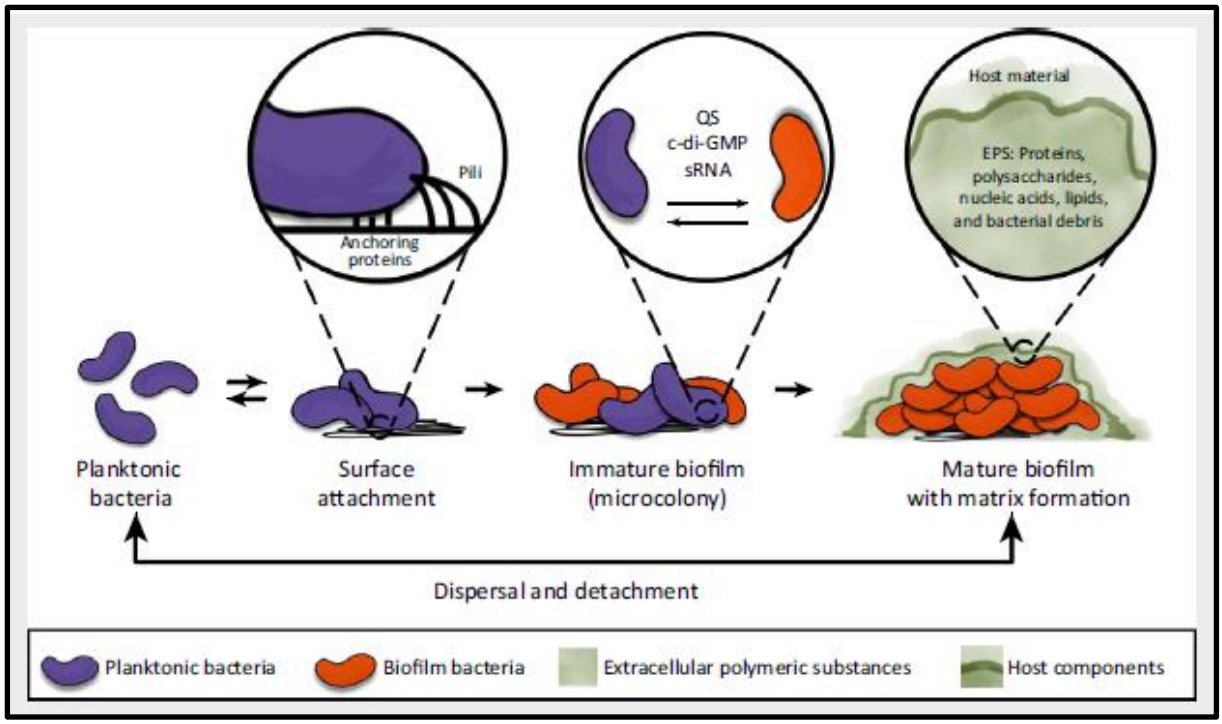


Fig 1: Various steps involved in biofilm formation. Planktonic bacteria attach to the surface via various reversible(electrostatic) and irreversible attachment, changing its phenotype to sessile stage by change in genetic expression. Division within the microcolony gives rise to mature biofilms which is characterized by increased EPS secretion. *[Reference: Y.-K. Wu, N.-C. Cheng, and C.-M. Cheng, "Biofilms in chronic wounds: Pathogenesis and diagnosis," Trends Biotechnol., vol. 37, no. 5, pp. 505–517, 2019..]*

A. BACTERIAL ATTACHMENT

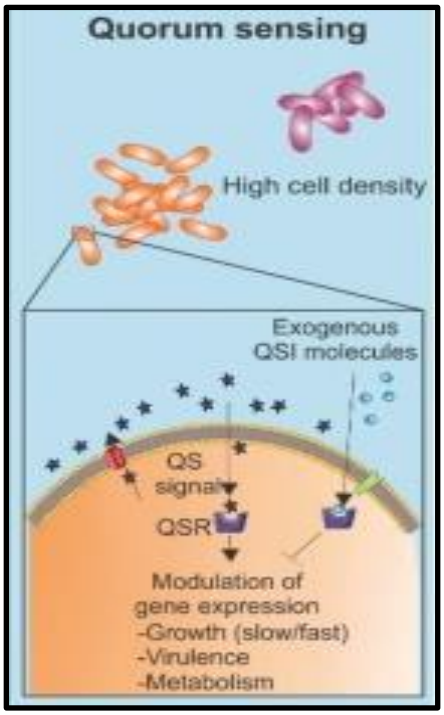
First step in biofilm formation is the surface attachment of planktonic cells. Chemotaxis is the phenomenon by which bacterial cells are attracted towards a source of nutrients or chemo attractants in a concentration gradient dependent manner. Surface attachment of planktonic cells depends on the net repulsive or attractive force between the two surfaces. Net attractive force more than repulsive force results in initial, reversible and unstable attachment via electrostatic, physicochemical and hydrophobic interactions [22-24]. Higher hydrophobicity has been reported to promote stronger attachment. Surface charge also plays an important role in initial attachment to substratum as most bacterial surfaces are considered to be negatively charged owing to the presence of phosphate, carboxy and amino groups, hence a positively charged surface will likely promote attachment while a negatively charged surface will resist it.

More stable attachment is mediated by receptor-ligand interactions via the flagellum, type 1 and type 4 pili, antigen and curli fibres. In non-motile bacteria stable attachment is mediated through adhesins and pili. Once stable and irreversible attachment is established, complex signalling events in the cell changes the phenotype from planktonic to sessile state. This is facilitated by bacteria that uses mechano-sensing, mechano-transduction and chemo-sensing properties to sense various physical and chemical signals in the microenvironment. Successful attachment also initiates matrix formation that strengthens the attachment along with providing biochemical and structural support [25-27].

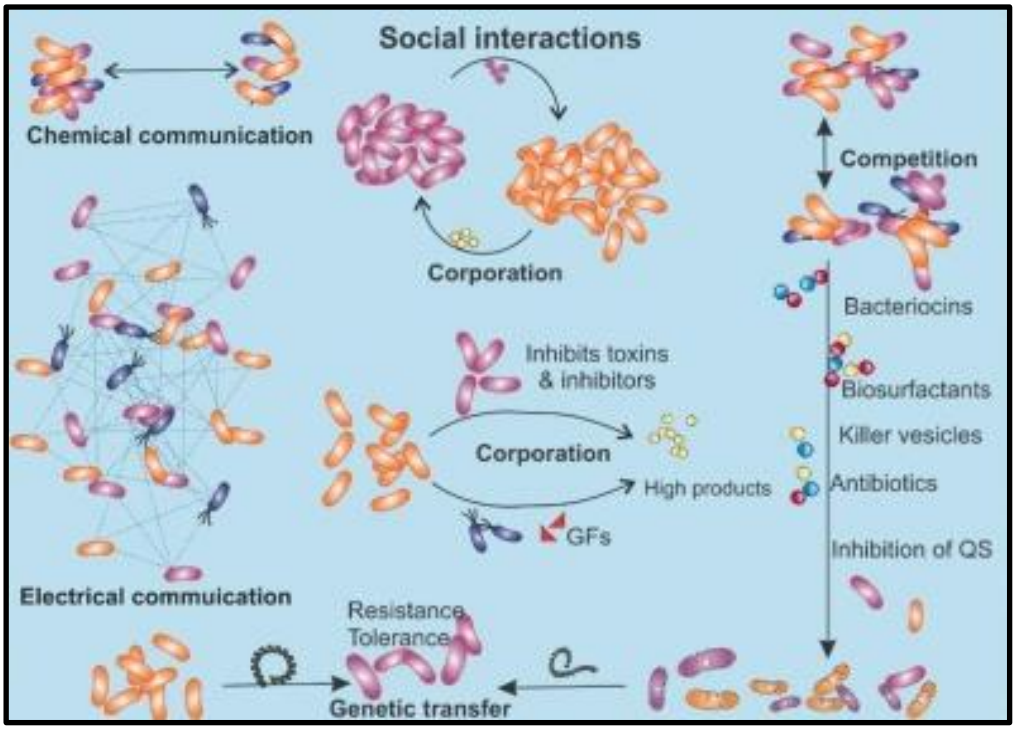
B. BIOFILM MATURATION

Bacterial attachment is followed by secretion of EPS and divisions within the matrix to form dense clonal structures. Some reported structures include pillar-like, capped mushroom, overall flat or unstructured biofilms. In *Pseudomonas aeruginosa* biofilm, a layer of biofilm develops into stalk and then gradually into a capped mushroom like structure [28]. Transport of nutrients and removal of waste products in biofilms are done via channels filled with water that acts as a circulatory system. Also, while maturating mobility of bacteria in sessile stage is restricted, as change in the genetic expression limits the formation of surface structures of bacteria.

Two properties, increased EPS synthesis and the growth of antibiotic resistance, are often observed in mature biofilm. Such characteristics tend to create a defensive atmosphere and make biofilms a tenacious clinical problem. Other properties can also be developed by biofilm bacteria, which includes, increased UV light resistance, increased genetic exchange rates, changes in biodegrading capabilities and increased secondary metabolites production [29-38].



A



B

Fig 2: Various mechanisms and social interactions undergoing in a mature biofilm. (A) Process showing the effect of a quorum signal in a cell and the effect of a quorum signal inhibitor molecule within the same cell. (B) Social interaction in biofilms consists of

cooperation or positive and competition or negative interactions which is responsible for the complex and dynamic nature of the biofilm. Positive interaction is mediated by electrical and chemical signals while negative interaction is mediated by antibiotics, enzymes etc. *[Reference: A. Barzegari, K. Kheyrolahzadeh, S. M. Hosseiniyan Khatibi, S. Sharifi, M. Y. Memar, and S. Zununi Vahed, "The battle of probiotics and their derivatives against biofilms," Infect. Drug Resist., vol. 13, pp. 659–672, 2020.]*

C. DISPERSION

The last stage in development of biofilm is marked by the change in bacterial phenotype from sessile to planktonic stage. Planktonic cells travel by twitching motility through the EPS matrix using pili (type IV), from the centre of the biofilm to the periphery of the structure [30]. These planktonic cells may initiate a new biofilm at a different site or integrate into an existing one. Nutritional and environmental signals control the development of this tertiary structure; for instance, limited iron supply induces twitching motility in P. aeruginosa biofilms [31]. Also, c-di-GMP (secondary messenger), quorum-sensing compounds, and small non-coding RNAs (sRNAs) regulated molecular pathways coordinate the transition. [39-41].

C-di-GMP is a secondary messenger that controls the motility of the bacteria by binding to its receptors. Amounts of cyclic-di-GMP in the cells influence bacterial motility. High level of cyclic-di-GMP is often associated with sessile stage whereas low levels promote planktonic lifestyle. Two enzymes regulate c-di-GMP levels i.e., diguanylate cyclases and phosphodiesterases that produce and degrade cyclic-di-GMP respectively. c-di-GMP level is non-uniform throughout the biofilms, high levels of it are observed in the periphery as compared to the centre of a mature biofilm where as a more uniform distribution was observed in less developed biofilms.

Communication between bacteria, through chemical signals to carry out physiological processes is known as quorum sensing shown in fig 3. Along with mediating various cellular properties like virulence quorum sensing also helps in biofilm development in most bacteria. The three main signaling systems for quorum-sensing are:

- N-acyl homoserine lactone-based signaling in Gram-negative bacteria;
- Autoinducing peptide-based signaling in Gram-positive bacteria;
- Autoinducer-2-based signaling in both types of bacterium [41].

QS in Gram-Positive Bacteria

Communication between the cells and density dependent regulation of response in gram positive bacteria by quorum signals is mediated by secretory peptides known as autoinducers. The precursor peptide chain is first cleaved to produce a signal which is then exported through ABC transporters. Prior to extracellular transfer peptides undergo post translational modification or cyclization. The signal is then detected by an extracellular histidine kinase receptor that autophosphorylates, it then phosphorylates response regulator molecules that has DNA binding capacity which is activated upon phosphorylation. This response regulator molecules then activate gene expression and regulate response.

QS in Gram-Negative Bacteria

First identified in *Vibrio fischeri*, N-acyl homoserine lactone is the quorum signaling molecule in Gram-negative bacteria. In *Vibrio fischeri*, *N*-(3-oxohexanoyl)-L-homoserine lactone (OHHL) is the AHL signal, which is biosynthesized by AI synthase LuxI. The signalling molecule OHHL is then passively diffused out of the cell. With increase in density of the cell, the concentration of signal increases and when it crosses a critical threshold OHHL binds to its receptor LuxR which is also a DNA binding transcriptional regulator hence it activates expression of genes associated with biofilm formation. This is a typical model for quorum sensing in gram negative bacteria.

For interspecies communication, autoinducer AI-2 signalling molecule common in both gram-positive and gram-negative bacteria could serve as a "universal signal".

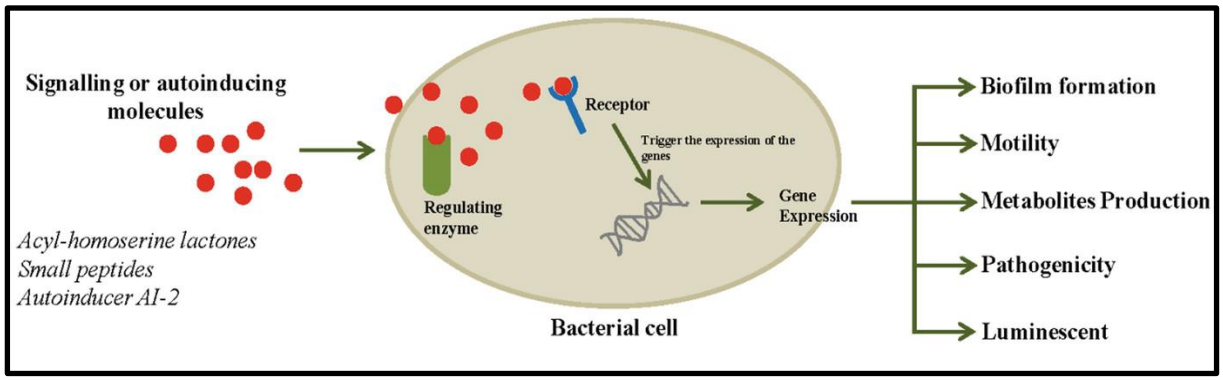


Fig 3: Quorum sensing mechanism of bacteria responsible for biofilm formation. *[Reference: R. Subramani and M. Jayaprakashvel, "Bacterial quorum sensing: Biofilm formation, survival behaviour and antibiotic resistance," in Implication of Quorum Sensing*

and Biofilm Formation in Medicine, Agriculture and Food Industry, Singapore: Springer Singapore, 2019, pp. 21–37.]

sRNAs regulate attachment and biofilm formation by modulating the expression or activity of transcriptional regulators and components of regulatory network by:

1. Base-pairing with other target RNAs turning them into stable double stranded structures which are unavailable for translation.
2. Binding with target regulatory proteins. They mimic the targeted mRNAs binding sequences and antagonize the regulatory proteins [42].

Detachment of cells in the biofilm developmental pathway is a significant area of research. One potential signal for detachment can be starvation. As reported by Boyd & Chakrabarty, the process of detachment in P. aeruginosa, is regulated by an enzyme, alginate lyase. They reported that alginate lyase overexpression could increase detachment and dispersion of the cells from biofilms [43].

3. BIOFILMS IN HOSPITAL ACQUIRED INFECTIONS

3.1 Hospital acquired infections

Hospital acquired infections (HAIs) are infections which patients acquire while being treated for medical conditions. Infections that are present or incubating during or before admission does not fall under HAIs. It also includes occupational infections among staff and infections acquired by patients in the hospital or facility but appearing after discharge. It can occur in all settings of health care including surgical centres, nursing homes, rehabilitation centres and hospitals.

3.2 MICROBIAL BIOFILMS

Device related HAIs accounts for approximately half of all HAIs [44]. Microbial biofilms can form on surfaces of bio materials like plastic, rubber or metals used to make various medical instruments and implants. Patient's skin microflora, contaminated intravenous fluids or exogenous microflora may give rise to biofilm formation. Once formed biofilms are difficult to remove from reusable devices as they are more resistant to antimicrobials, up to 1000 times, as compared to vegetative cells. Extracellular polymeric substance provides a barrier against

cleaning and antimicrobial agents. Both single species and multispecies biofilm may form on medical devices as shown in Fig 4.

Infections caused by devices include:

1. Blood Stream Infections via Central Line

2. Urinary Tract Infections via Catheter

3. Pneumonia Infections via Ventilator

4. Infections of Surgical Sites.

Urinary Tract Associated Infections via Catheter are the most frequently observed device-associated HAI.

Table 1: Various medical devices and their corresponding biofilm forming bacteria contributing in various hospital acquired infections. *[Reference: M. H. Muhammad et al., "Beyond risk: Bacterial biofilms and their regulating approaches," Front. Microbiol., vol. 11, p. 928, 2020.]*

S.No.	Medical Devices	Biofilm-forming bacteria
1.	Contact lenses	*P. aeruginosa, S. aureus, S. epidermidis, S. saprophyticus, Klebsiella spp.*
2.	Central venous catheters	*Coagulase-negative Staphylococci, S. aureus, Enteric Gram-negative Bacilli*
3.	Urinary catheters	*S. aureus, Enterococcus faecalis, P. aeruginosa*
4.	Peritoneal dialysis catheters	*S. epidermidis, P. acnes, S. warneri, S. lugdunensis, R. mucilaginosa*
5.	Mechanical heart valves	*Streptococcus spp., S. aureus, S. epidermidis, Gram-negative Bacillus, Enterococcus*
6.	Cerebrospinal fluid shunts	*S. aureus, S. epidermidis, Enterococcus faecalis, Enterococcus faecium*
7.	Breast implants	*S. epidermidis, Coagulase-negative Staphylococci, Propionibacterium acnes*

8.	Orthopaedic implants	*S. aureus, S. epidermidis, P. aeruginosa, E. coli, S. haemolyticus*
9.	Voice prostheses	*S. aureus, P. aeruginosa, Klebsiella spp., Enterobacterspp., R. dentocariosa, and Proteus spp.*
10.	Intrauterine devices	*E. coli, Streptococcus agalactie, S. aureus, Enterococcus faecalis, Lactobacillus spp., Prevotella spp., Porphyromonas spp., Bacteroides, Fusobacterium spp.*
11.	Biliary stents	*Pseudomonas, Citrobacter, Klebsiella, Staphylococcus, Enterococcus, Aeromonas, Proteus, Enterobacter*
12.	Cardiac pacemakers	*S. aureus, S. epidermidis*
13.	Dental implants	*Gram-positive cocci, Actinomyces spp., Gram-negative anaerobic oral bacteria*

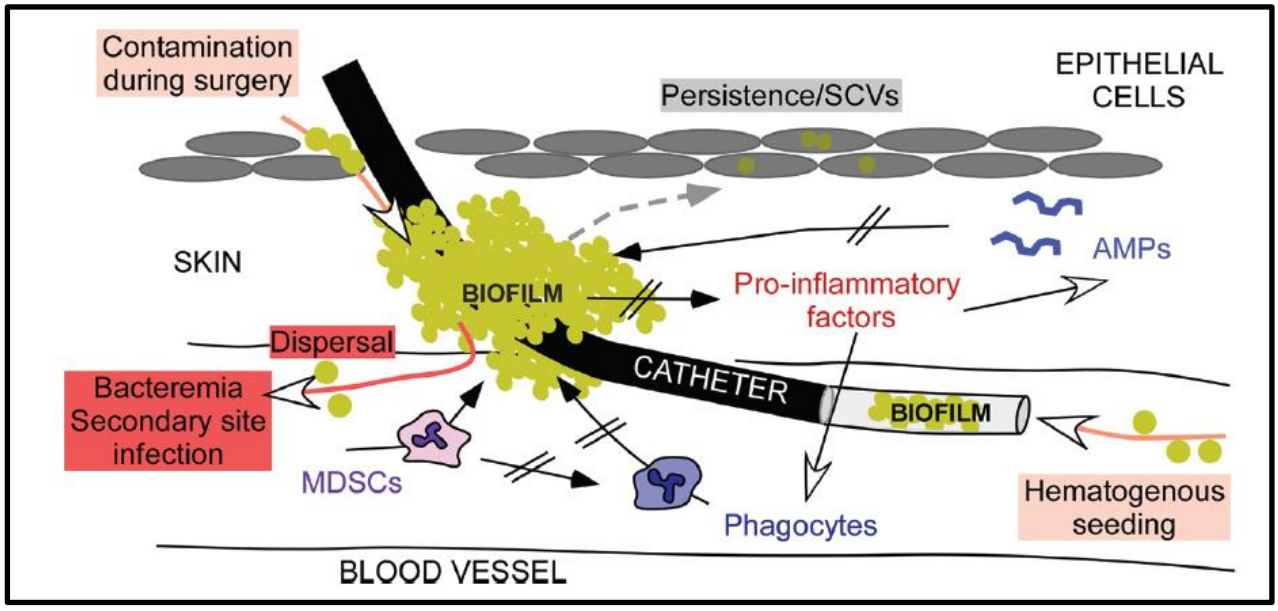


Fig 4: Biofilm-associated Staphylococcal infection on medical devices. *[Reference: M. Otto, "Staphylococcal biofilms," Curr. Top. Microbiol. Immunol., vol. 322, pp. 207–228, 2008.]*

4. RISK ASSESSMENT

Hospital acquired infection, affects hundreds of millions of patients each year, resulting in high mortality rate and, great financial loses in health sectors worldwide. Every hospitalized patient is at the risk of acquiring HAI but elderly, children and, immunocompromised patients are comparatively at higher risk of being infected. Risk factors for acquiring HAI are prolonged

hospital stays, contaminated medical implants and intravenous devices and, unhygienic medical staff. Improper use of antibiotics, spread of resistance and no new antibiotics have combinedly increased morbidity and mortality in health sectors.

Table 2: Prevalence of health care-associated infection in developed and low- and middle-income countries. *[Reference: K. Lillis, Device-Associated Infections: Evidence-based Practice Remains the Best Way to Decrease HAIs. Infection Control Today, 2015.]*

Country	Prevalence of HAI	No. of HAI in every 100 hospitalized patients
Developed	3.5% - 12%	7
Low- and middle-income	5.7% - 19.1%	10

A survey of Centre for Disease Control (CDC) reports, 1.7 million estimated infections and associated deaths of approximately 99,000 each year due to HAI in America. Table below shows prevalence percentage of various HAIs in America [46-48]:

Table 3: Prevalence percentage of various HAIs in America. ***[Reference:*** *K. Facts, "Health care-associated infections FACT SHEET," Who.int. [Online]. Available: https://www.who.int/gpsc/country_work/gpsc_ccisc_fact_sheet_en.pdf?ua=1. [Accessed: 20-Jun-2021]*

HAI	Percentage
Urinary tract	32 %
Surgical site	22 %
Pneumonia	15 %
Bloodstream	14 %

Although infection in urinary tract is the most common nosocomial infection in high-income countries, in settings with limited resources, infection of surgical site is the foremost cause of nosocomial infection, impacting one-third of surgical patients which is nine times more than in developed countries.

In high-income nations, at least one HAI affects nearly 30 percent of patients in intensive care units (ICUs). The prevalence of infection is 2-3 times less as compared to under developed and developing countries.

Cases of infections associated with device is up to 13 times more in low income and middle-income nation than in the USA. In developing countries, nosocomial infection rate of new-born is 3-20 times higher than in developed countries. [45]

Table 4: Device associated infection rate of adult and neonatal ICU patients in India. *[References: "Hospital-acquired Infection a high risk for patients in India," Expresshealthcare.in, 04-Jun-2019. [Online]. Available: https://www.expresshealthcare.in/interviews/hospital-acquired-infection-a-high-risk-for-patients-in-india/411904/.]*

Device associated infection	Infection rates for adult and paediatric ICUs	Infection rates for neonatal ICUs
Blood Stream Infections via Central Line	5.1 /1000 central line-days	36.2 /1000 central line-days
Pneumonia via Ventilator	9.4 /1000 mechanical ventilator-days	1.9/1000 mechanical ventilator-days
Urinary Tract Infections via Catheter	2.1 /1000 urinary catheter-days	-

Data collected from 20 cities of India during 10 years including 236,700 ICU patients for 970,713 bed-days. Less than 10 percent of all hospital acquired pneumonia is non-ventilator-associated pneumonia and is typically triggered after aspiration [49].

4.1 CASE STUDIES [50]

4.1.1

In late 2018, CDC reported an epidemic of infections in individuals who were operated at Grand View Hospital in Tijuana, Mexico. Several states in the United States have reported infections among travellers with highly resistant *Pseudomonas aeruginosa* who were operated at different hospitals since August 1, 2018, in Tijuana, Mexico. While an epidemic that mostly occurred in a single facility seems to be over, reports of infections following surgery in Tijuana continue to be received by the CDC.

As of April 30, 2019, this outbreak appears to be over. Although as precautionary measure, CDC continues to advise operated patients (between August 1, 2018 and January 30, 2019) to

test for hepatitis B, hepatitis C and HIV. And to those experiencing symptoms like fever, pus or swelling at the incision site to seek urgent medical attention.

4.1.2 Heart surgery

In the United States more than 250,000 heart bypass procedures are performed annually using heater-cooler systems, and infection associated with the system was seen in approximately 60% of these procedures. Heater-cooler systems are an integral part of these life-saving operations because they help maintain the circulating blood and organs of a patient at a particular temperature throughout the procedure.

Slow-growing bacteria such as Nontuberculous Mycobacteria (NTM) infections can take months to develop. Cases of infections within months to several years after open-heart surgery involving heater-cooler system, were diagnosed. CDC estimates that in hospitals where at least one infection has been identified, the risk of bacterial infection in patients ranged from about 1 in 100 to 1 in 1,000.

5. ANTIMICROBIAL TOLERANCE IN BIOFILMS

Exposure of microorganism to high concentration of antimicrobials for temporary period and its ability to withstand it without affecting the minimal inhibitory concentration (MIC) is known as tolerance. Biofilm-associated sessile cells differ from planktonic cells both phenotypically and physiologically. The target sites for the antimicrobial agents are involved in these phenotypic changes, along with the extracellular polymeric substance that creates a barrier and prevents entry of various antimicrobial agents. Reduced antimicrobial agent penetration into biofilms, persistent cell occurrence, decreased growth, and protective stress response gives tolerance to the cell.

Metabolic activity of the cell along with the microenvironment dominant at the site of infection plays an important role in antimicrobial activity. Persister cell and small colony variant phenotypes have low metabolic activity and are commonly found in biofilms [51, 52]. Overexpression of efflux pumps in biofilms have been observed that help cells to expel antibiotics. The PA1874-1877 efflux pump was expressed more in number in biofilm-associated *P. aeruginosa* cells than in planktonic cells [52]. In addition, inactive efflux pumps in cells could have decreased biofilm-forming ability. Therefore, to prevent biofilm formation,

antimicrobial agents like (thioridazine and PAN (Phe-Arg-naphthylamide)) that target and inactivate efflux pumps may be effective [53].

5.1 Biofilm Heterogenicity and Microenvironment Plays Role in Tolerance.

The heterogeneity of biofilms and the in vivo tolerance of antibiotics appear to be controlled by accessibility to nutrients. Oxygen alone plays a crucial role in tolerance. This implies that microenvironment plays a major part in development of tolerance [54-56]. These tolerant subpopulations within the biofilm makes it difficult to eradicate using different antimicrobial agents. As biofilm mature, its thickness and biomass increase with time. This generates gradient of metabolite and oxygen which gives heterogenicity to the biofilm. Due to this heterogenicity, zones with different metabolic activity, oxygen requirement, growth rate, and tolerance is observed within the biofilms [57].

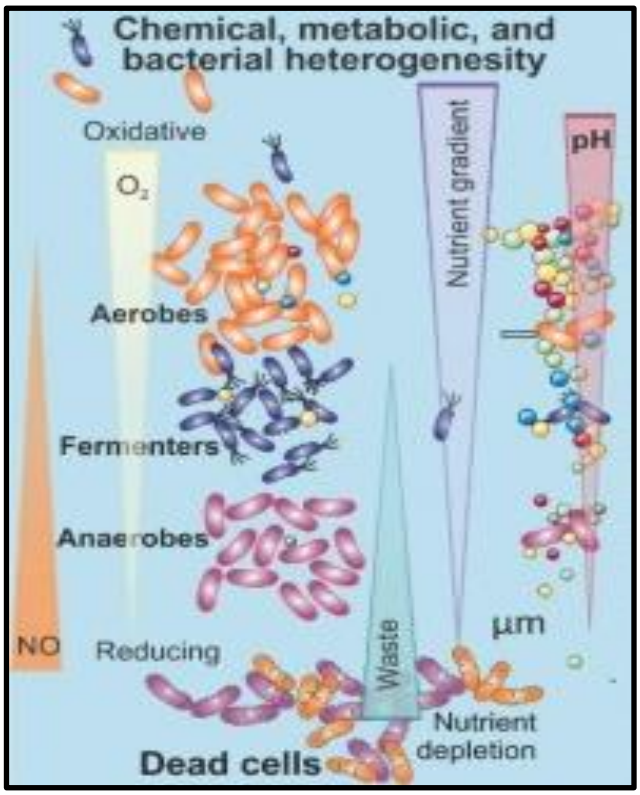


Fig 5: Chemical, metabolic and bacterial heterogenicity in a biofilm. Spatial heterogenicity within the biofilm due to non-homogenous environmental conditions like gradients of oxygen, nutrients, waste and pH. *[Reference: A. Barzegari, K. Kheyrolahzadeh, S. M. Hosseiniyan Khatibi, S. Sharifi, M. Y. Memar, and S. Zununi Vahed, "The battle of probiotics and their derivatives against biofilms," Infect. Drug Resist., vol. 13, pp. 659–672, 2020.]*

For example, in *Pseudomonas aeruginosa* biofilms, ciprofloxacin and tobramycin tolerant subpopulations are internally located, in areas of low oxygen, having slow growth rates and less metabolic activity [54-56]. Whereas, colistin-tolerant subpopulations of *P. aeruginosa* require proton motive force to produce sufficient ATP required to drive efflux pump to flush out antibiotics and for lipopolysaccharide modifications of the antimicrobial target sites. And as continuous supply of oxygen is likely to promote generation of strong PMF, these subpopulations are usually located in the periphery of the biofilm, where they receive continuous supply of oxygen and nutrients [55].

5.2 ROLE OF METABOLIC ADAPTATIONS IN TOLERANCE

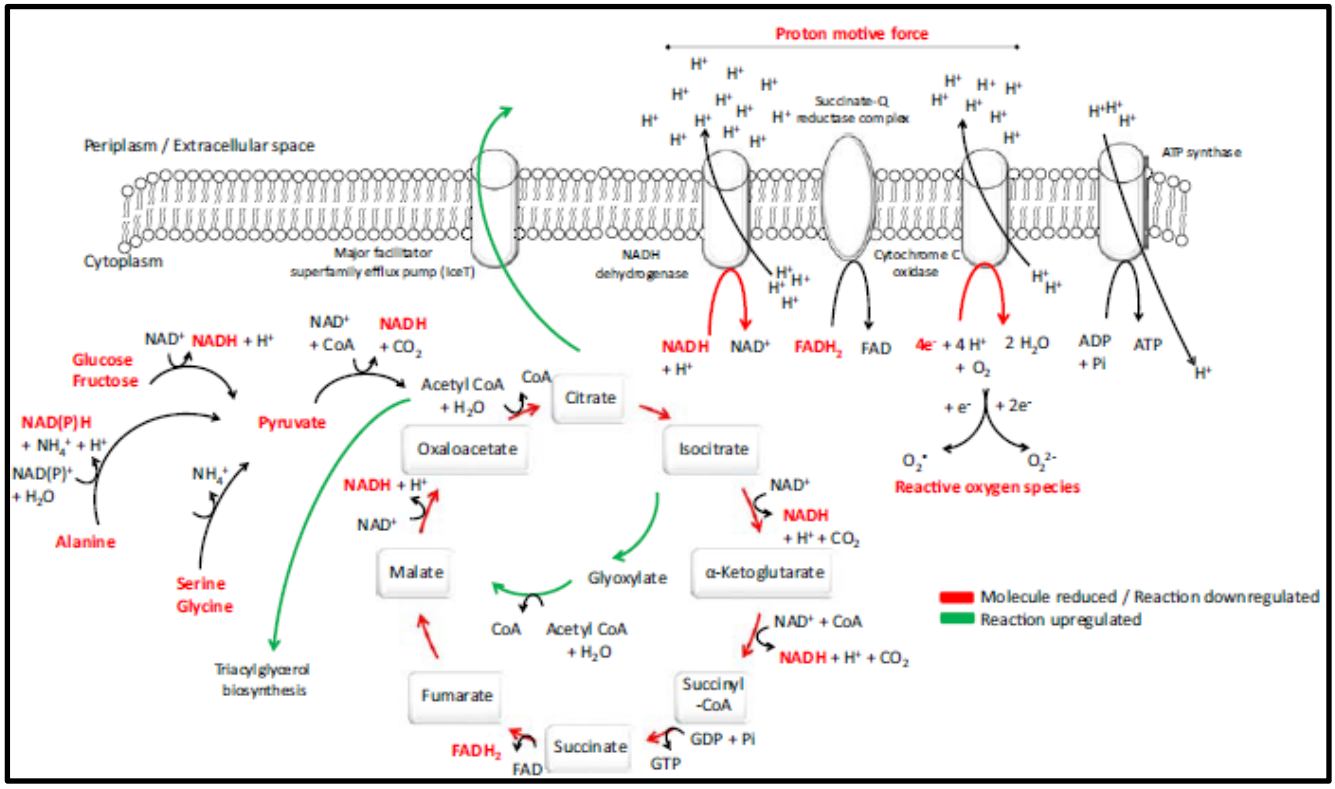


Fig 6: Metabolic adaptation in bacteria leading to antibiotic tolerance via reduced production of NADH or FADH electron donors. Mechanism of metabolic adaptations in various bacterial species observed in patients or in vitro conditions after exposure to antibiotics is compiled in the given image. Most reported mechanism of increased tolerance was found to be reduction in the levels of electron donors (NADH, FADH) present in the cells. This may be possible via (1) Reducing intracellular concentration of upper glycolysis intermediates like glucose, fructose or lower glycolysis product like pyruvate. (2) Reduced or non-expression of enzymes used in TCA cycle like citrate synthase, isocitrate dehydrogenase through altered gene expression, mutation etc. (3) Decreased intracellular TCA metabolite concentrations by using

efflux pumps to export metabolites out of the cell or by activating biosynthetic pathways, such as triacylglycerol biosynthesis, that utilize these metabolites. Reduced production of electron donors decreases the proton motive force which is essential for many antibiotic uptake or it can reduce the levels of ROS within the cell which can interfere with the activity of antibiotics. Figure shows simplified steps involved in glycolysis, TCA and ETC (electron transport chain) reactions. Metabolic reactions indicated in red shows adaptive mechanism involved in downregulating levels of specific metabolites. Metabolic reactions labelled in green shows adaptive mechanism involved in inducing levels of specific metabolites. *[Reference: A. Crabbé, P. Ø. Jensen, T. Bjarnsholt, and T. Coenye, "Antimicrobial tolerance and metabolic adaptations in microbial biofilms," Trends Microbiol., vol. 27, no. 10, pp. 850–863, 2019.]*

The majority of metabolic adaptations, in increased tolerance, are reported to be mediated by decreased production of NADH and FADH2 electron donors [Fig 4]. For instance, 14.2 percent of S. epidermidis isolates had extreme TCA dysfunctions, and rest 57.9 percent had metabolic adaptations that decreased metabolic flux through the citric acid cycle [58].
This may be achieved by:

i. Reduced intracellular concentration of glycolysis intermediates like glucose, fructose or pyruvate.
ii. Reduced or non-expression of enzymes used in TCA cycle through altered gene expression mutation etc.
iii. Decreased intracellular TCA metabolite concentrations.
iv. By activating biosynthetic pathways, such as triacylglycerol biosynthesis, that utilize these metabolites.

Decreased levels of electron donor:

i. Decreases the PMF (proton motive force) which is important for antibiotic uptake.
ii. Decreases intracellular levels of ROS (reactive oxygen species) that complements certain antibiotics for effective killing.

Antibiotic treatment of microbial biofilms may be unsuccessful due to the microenvironment and metabolic adaptations. Presence of other competitive microbes combined with host response may affect nutrient levels in the microenvironment which may further contribute to metabolic adaptations in cells. Metabolic adaptations are usually associated with

downregulation of electron transport chain and proton motive force in the cells which leads to tolerance. Replenishing missing metabolites and/or supplying electron acceptors (NADH and FADH2) can be of considerable clinical potential as it may reverse tolerance.

Table 5: Different metabolites effect on bacterial tolerance to antibiotics. *[Reference: A. Crabbé, P. Ø. Jensen, T. Bjarnsholt, and T. Coenye, "Antimicrobial tolerance and metabolic adaptations in microbial biofilms," Trends Microbiol., vol. 27, no. 10, pp. 850–863, 2019.]*

Metabolite	Antibiotic	Effect on tolerance	Species
Upper glycolysis			
Glucose	Km, Gm	Decrease	*Escherichia coli*
	Km, Gm	No effect	*Staphylococcus aureus*
	Km, Gm	Decrease	*Edwardsiella tarda*
	Am, Cf, Bf	No effect	*E. tarda*
	Km	Decrease	*Edwardsiella piscicida*
	Tb	Decrease	*Pseudomonas aeruginosa*
Glucose 6-P	Cip	Increase	*E. coli*
Fructose	Km, Gm	Decrease	*E. coli, E. tarda, S. aureus, Klebsiella pneumoniae, Vibrio parahemolyticus*
	Am, Of	No effect	*S. aureus*
	Tb	Decrease	*P. aeruginosa*
Mannitol	Km, Gm	Decrease	*E. coli*
	Am, Of	No effect	*E. coli*

	Gm	No effect	*S. aureus*
	Tb	Decrease	*P. aeruginosa*
Maltose	Km	Decrease	*E. tarda*
Lower glycolysis			
Pyruvate	Km, Gm	Decrease	*E. coli*
	Tb	Decrease	*P. aeruginosa*
	Gm	No effect	*S. aureus*
Phosphoenolpyruvate	Cip	Increase	*E. coli*
Glycerol	Gm	No effect	*E. coli*
Glycolate	Gm	No effect	*E. coli*
Galactarate	Gm	No effect	*E. coli*
Acetate	Tb	Decrease	*P. aeruginosa*
(Acetyl-/Malonyl-) coenzyme A	Cip	Increase	*E. coli*
1,3-Bisphosphoglyceric acid	Cip	No effect	*E. coli*
2-Phosphoglycerate	Cip	No effect	*E. coli*
Dihydroxyacetone phosphate	Cip	No effect	*E. coli*
TCA cycle			
Oxaloacetic acid	Km	Decrease	*E. coli*
Citrate	Km	Decrease	*E. coli*
		No effect	*P. aeruginosa*
	Cip	No effect	*E. coli*
Fumarate	Tb	Decrease	*P. aeruginosa*
Succinate	Tb	Decrease	*P. aeruginosa*
α-ketoglutarate	Km	Decrease	*E. tarda*
	Tb	Decrease	*P. aeruginosa*
Glyoxylate shunt			
Glyoxylate	Tb	Increase	P. aeruginosa
	Cip	Increase	E. coli

Entner–Doudoroff pathway			
Gluconate	Gm	No effect	*E. coli*
	Tb	Decrease	*P. aeruginosa*
Pentose-phosphate pathway			
Arabinose	Gm	No effect	*E. coli*
Ribose	Gm	No effect	*E. coli*
	Tb	Decrease	*P. aeruginosa*
Ribulose 5-P	Cip	No effect	*E. coli*
Sedoheptulose 7-phosphate	Cip	No effect	*E. coli*
Fatty acid metabolism			
Propionate	Tb	Decrease	*P. aeruginosa*

Amino acids			
Alanine	Km, Gm	Decrease	*E. tarda*
	Am, Cf,Bf	No effect	*E. tarda*
	Cip	No effect	*E. coli*
Glutamate	Km	Decrease	*E. coli*
	Cip	Increase	*E. coli*
Isoleucine	Km	Decrease	*E. tarda*
Threonine	Km	Decrease	*E. tarda, E. piscicida*
Glycine	Km	Decrease	*E. piscicida*
Serine	Km	Decrease	*E. piscicida*
Phenylalanine	Km	Decrease	*E. piscicida*

6. BIOFILMS IN CHRONIC INFECTIONS

Biofilms can cause chronic infections at various places in a human body. They can cause persistent recurring infection resulting in delayed healing. To establish a chronic infection, interaction between host microbiota and pathogen forming biofilm plays a major role. The biofilm is dynamic and continuously evolving in nature, they adapt according to the prevalent microenvironment. Non-healing wounds, cystic fibrosis lung infections, periodontitis, diabetic foot ulcer are some examples where biofilms have been known to cause tenacious clinical problems.

Dead cells, low oxygen, and prolonged albeit reduced host immune response forms the microenvironment in chronic wounds that facilitates bacterial growth in biofilms thereby delaying the process of wound healing. In chronic wounds, biofilm formation is the primary reason that resist the treatment and healing of infection. Microorganisms can cause both chronic and acute infections. For instance, if untreated, bloodstream infections by *Pseudomonas aeruginosa* can lead to death within hours. But, at the same time, in case of cystic fibrosis, it can survive for decades in the respiratory tracts of infected patients at high densities (108 to 1010 cfu/ml) without causing any invasive infection or spreading outside of the lungs [59].

Neutrophil infiltration is one of the most immediate cellular responses in biofilms; for instance, the presence of a biofilm initiated by *Pseudomonas aeruginosa*, is associated with striking increase in neutrophils [64]. Study of biofilm in chronic wounds and in the lungs of cystic fibrosis patients showed that large number of neutrophils surround biofilms, but do not penetrate. This may be because biofilms actively recruit neutrophils, however they paralyze and/or lyse the cells via their quorum sensing mediated signalling [60-64].

Pseudomonas aeruginosa takes advantage of the host immune system by using polymers and actin from dead neutrophils as scaffolds for biofilm formation [65]. Similarly, Biofilms of *Staphylococcus aureus* can also draw macrophages, but upon contact the macrophages are modified to M2 macrophage which is an alternate activated state. Such cells show reduced migration and microbicidal activity [66].

7. DIAGNOSTICS

Colonization of wrong type of bacteria at the wrong place has been associated with many diseases. For example, in case of head and neck cancer, change in the oral biofilms influenced by unhealthy lifestyle like smoking and drinking negatively affect health associated biofilms and is considered to be one of the causative reasons for tumor. In case of chronic wounds, persistent cells can cause recurring infections, which delays wound healing process. *Streptococcus pyogenes* forms biofilms and necrotize soft tissue which is an acute infection progressing rapidly associated with higher bacterial load along with an increased immune response.

Challenges in early diagnosis of persistent cells and undetectable infections caused by biofilms include type of sample and method of sampling, microorganism's identification, heterogenous distribution of cells in the biofilms and biofilm composition. Various traditional methods like morphology assay and microbiology assay are currently in use for diagnostics but emerging technologies like wound bed analysis and transcriptomic analysis of the sample gives a clear idea about the distribution of the cells in biofilms, stages of wound recovery and an accurate analysis of types of species and even strain present in the biofilm via transcriptomics for effective treatment strategies.

7.1 Currently used methodology [67]

a. Morphology assay

Tissue sampling: To locate the biofilm, determining the morphology of the chronic wound is important. In histological analysis, bacteria and biofilms are frequently located on ulcerated wounds and several samples are obtained from different superficial and deep wounds to improve the sensitivity of the test. Confocal laser scanning microscopy and scanning electron microscopy are also more objective and accurate alternatives for biofilm diagnosis.

b. Microbiology assay

Standard clinical microbiology culturing methods are difficult to perform in biofilm associated infection as in a typical infection four to five species of bacteria forms biofilms and only 1% of the bacteria are identified using traditional method as most of them are in slow growth phase.

However, chemical methods like dithiothreitol treatment of prosthesis and sonication have been reported to acquire clinical samples. Both higher sensitivity and specificity of 71.4% and

94.1% respectively was observed with sonication of sample, whereas, dithiothreitol treatment gave specificity similar to sonication but higher sensitivity of 85.7%.

c. Molecular assay:

A highly conserved sequence in bacterial genome known as 16S ribosomal RNA or 16S rRNA is commonly used for microorganism recognition. Due to its highly conserved nature, it gives species specific data for pathogen identification. It also acts as a primer binding site.

7.2 Emerging diagnostics methodology [67]

a. Wound blotting:

Application of a nitrocellulose membrane on wound beds can absorb and immobilize biomolecules such as proteins and polysaccharide within the membrane. This technique provides a qualitative assessment of the desired biomolecule along with providing a spatial information about the homogenous or heterogenous distribution of desired biomolecule across the entire wound bed.

Immunostaining a wound blotting membrane give three types of TNF-alpha distribution pattern namely edge, bed or mostly negative pattern. Increased intensity of TNF-alpha signal along the wound bed or edge is called as bed pattern or edge pattern respectively. And a distribution pattern is called mostly negative when there is no obvious TNF-alpha signalling observed. These TNF-alpha patterns are linked to wound healing prognosis where in edge pattern delayed healing was observed and in bed pattern and mostly negative pattern enhanced healing was observed.

b. Transcriptomics approach

In addition to recognizing bacteria, transcriptomics can be used to distinguish between bacteria of the same genus by their ability to form a biofilm and their antibiotic resistance pattern. It can also help distinguish between the biofilm and the planktonic state as in *Gardnerella vaginalis* by detecting a lower level of vaginolysin expression, a virulence factor that induces cytotoxicity of epithelial cells.

Methicillin-susceptible *S. aureus* (MSSA) containing biofilms are more resistant to antibiotic therapy than methicillin-resistant *S. aureus* (MRSA) containing biofilms. Transcriptomics can distinguish between the two S. Aureus species, due to its antibiotic resistance. One of the

master regulators of virulence and the quorum sensing system of bacteria is accessory gene regulator (agr) system. RNA-seq evaluation revealed its absence in MSSA which allows it to form biofilms thicker and at higher rate as compared to MRSA.

8. TREATMENT

8.1 Hyperbaric oxygen therapy (HBOT)

Oxygen is one of the bacterial growths limiting factors. In biofilms containing anoxic population responsible for tolerance, like in P. aeruginosa biofilms, the increased susceptibility to antibiotics was demonstrated with high supply of oxygen. The oxygenated subpopulation significantly expanded when HBOT was combined with ciprofloxacin therapy, leading to decrease in the anoxic zone and increased subpopulation with increased ciprofloxacin susceptibility. Similarly, in case of *S. aureus* HBOT therapy combined with tobramycin increased the susceptibility of microbes to the antibiotic. Experimental studies in rats with endocarditis due to MRSA (methicillin-resistant *S. aureus*) and *S. aureus* showed improved results and increased bacterial clearance when treated with tobramycin combined with HBOT. The clinical importance of adjuvant HBOT during antibiotic treatment of biofilm-related infection has recently emerged, with evidence of improved outcomes of brain abscesses, refractory osteomyelitis and device-related infections.

Even though HBOT treatment reduces the time of treatment along with less use of antibiotics to inhibit resistance, in some instance it has been reported that lack of oxygen strongly decreases antibiotic resistance induction by sublethal concentration of antibiotic treatment so it has been suspected that reoxygenation during HBOT treatment promotes antimicrobial resistance if lethal amount of antibiotics is not used. However, such instance has only been seen in overnight or longer culturing of microbes in sublethal antibiotic treatment at atmospheric O2 which is far long than the 90 min typical sessions with HBOT. Therefore, further research is necessary to completely determine the efficacy of HBOT treatment in biofilm removal and resistance development [68].

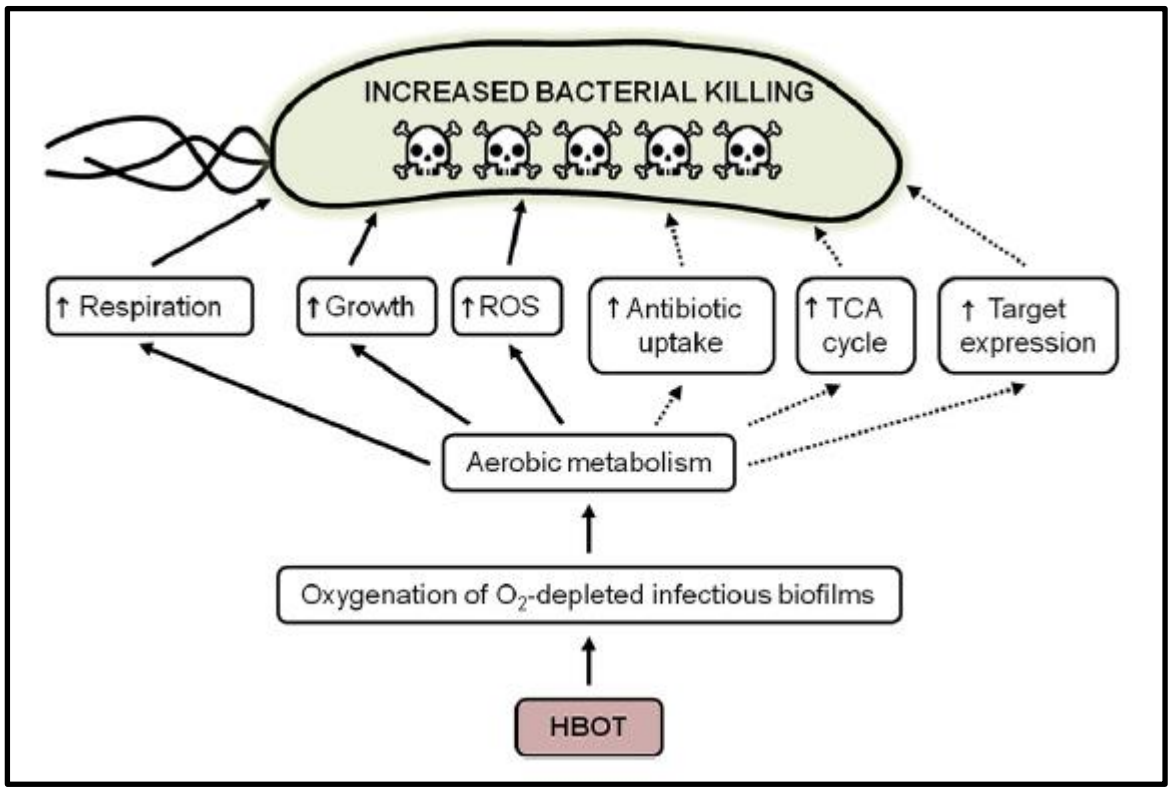


Fig 7: HBOT mediated killing of bacteria. Solid lines and dotted lines indicate confirmed and putative mechanisms respectively that results in effective and enhanced bacterial death in biofilms when HBOT combined with antibiotic treatment is used. *[Reference: P. Ø. Jensen et al., "Improving antibiotic treatment of bacterial biofilm by hyperbaric oxygen therapy: Not just hot air," Biofilm, vol. 1, no. 100008, p. 100008, 2019.]*

8.2 Modulation of microbial metabolism

Microbial metabolism plays an important role in tolerance, therefore by regulating metabolic pathways by either (a) activation of metabolic pathways that improve antibiotic killing or (b) inhibition of alternative metabolic pathways, biofilms and related infections can be tackled.

In 2011, eradication of persister cells from biofilms of *Escherichia coli* and *Staphylococcus aureus* was shown by giving a combined treatment of discrete carbon sources like mannitol with aminoglycoside antibiotics that resulted in the proton motive force (PMF) induction, that promotes the intake of aminoglycosides [69].

In killing of bacteria using antibiotics, reactive oxygen species (ROS) plays an important role [70,71] and, in aerobic bacteria with high metabolic rate and ample supply of oxygen, ROS levels are expected to increase, as it is an obligate by-product. Interfering with defence

mechanisms of the cell against such reactive species, by deactivating genes coding for catalase and superoxide dismutases (SOD), by supressing the function of these enzymes, or by lowering intracellular antioxidant compounds, improves susceptibility of the cells to antibiotics [72,73].

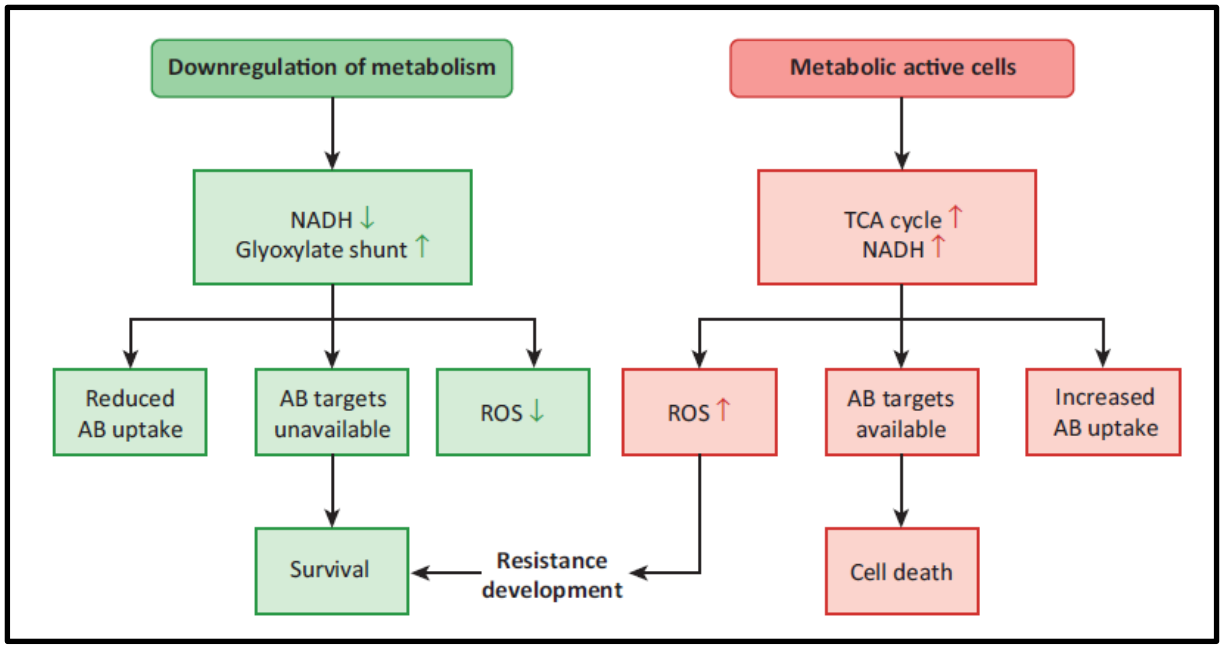


Fig 8: Role of metabolism in tolerance. Slow growing cells express a smaller number of pumps which reduced antibiotic (AB) uptake by the cells resulting in survival. However, supplying cells with discrete carbon sources like mannitol along with antibiotic treatment resulted in the proton motive force (PMF) induction and increased AB uptake resulting in death of the cells. *[Reference: H. Van Acker and T. Coenye, "The role of reactive oxygen species in antibiotic-mediated killing of bacteria," Trends Microbiol., vol. 25, no. 6, pp. 456–466, 2017.]*

8.3 Antimicrobial peptides (AMPs)

Antimicrobial peptides are part of the innate immunity in eukaryotes and prokaryotes, produced in defence against pathogens. These are generally small in size, up to 15 to 30 amino acids, and possess net positive charge with specific target sites on cell membranes.

Bacterial cell membrane and biofilm surfaces are negatively charged due to which they attract often positively charged AMPs. In biofilms, AMPs target bacteria with both high or low metabolic activity, by either formation of pore or disruption of the cell membrane [74,75]. By manipulating the amino acid composition of AMP its antimicrobial activity can be increased.

AMPs like synthetic cathelicidin and LL-37 have been reported to prevent formation of biofilm in *S. aureus* and *P. aeruginosa* respectively, when used at levels below the minimal inhibitory concentration [76,77].

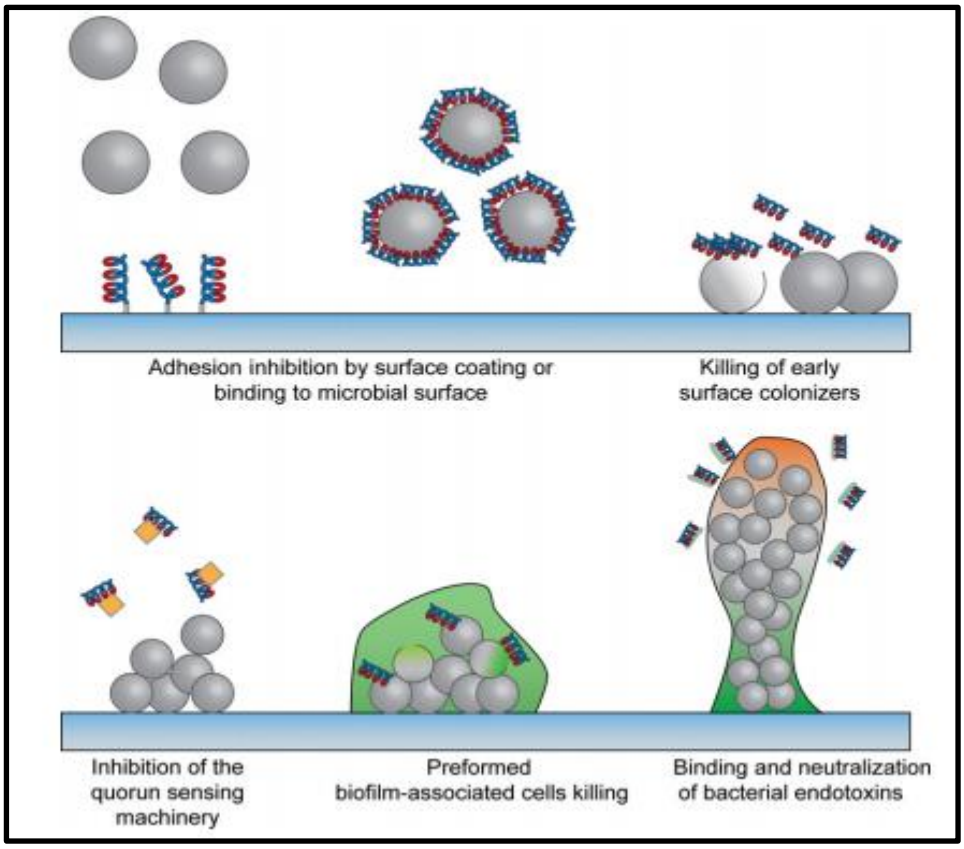


Fig 9: AMP-mediated mechanism of preventing biofilm formation. Top panel shows inhibition mechanism of biofilm formation. By coating of medical device surface or by interaction with microbes AMP prevent microbial adhesion. By killing of early surface colonizers biofilm maturation can be prevented. Bottom panel shows AMP mediated eradication strategies of biofilms via binding quorum-sensing signals to inhibit communication, penetrating mature biofilm matrix and killing associated cells, by binding and neutralizing endotoxins released by biofilm-associated bacterial cells. *[Reference: M. Di Luca, G. Maccari, and R. Nifosì, "Treatment of microbial biofilms in the post-antibiotic era: prophylactic and therapeutic use of antimicrobial peptides and their design by bioinformatics tools," Pathog. Dis., vol. 70, no. 3, pp. 257–270, 2014.]*

8.4 Biofilm degrading enzymes

The EPS matrix consisting of polysaccharides, proteins, and nucleic acids could be blocked and disrupted by enzymes. The matrix works to provide both structure and defence to the cells by holding the cells in biofilms together and by blocking the entry of antimicrobials respectively. Use of enzymes that degrade biofilms have been reported to decrease mass of the matrix along with the cell numbers in the biofilms.

DNase work by altering the morphological and textural properties of the biofilms, and may affect the number of cells in biofilm. This modification, increases the action of antibiotics as observed in *P. aeruginosa* and *S. aureus* biofilms. However, DNase I can be inactivated as mature biofilms secrete more EPS and proteolytic enzymes. Amylase and dispersin B (DspB) have also been reported to degrade biofilms [78-80].

Alginate lyase along with gentamicin showed excellent results in elimination of *P. aeruginosa* associated mucoid biofilm [81-82]. Combination treatment of Methicillin resistant staphylococcus aureus (MRSA) biofilms with Nafcillin (50 mg/kg) and Lysostaphin (15 mg/kg) on a medical device showed effective killing of biofilm [83,84].

Table 6: Various strategy and mechanism of biofilm matrix disruption. *[Reference: M. H. Muhammad et al., "Beyond risk: Bacterial biofilms and their regulating approaches," Front. Microbiol., vol. 11, p. 928, 2020.]*

S.No.	Strategy	Examples	Mechanism of action
1.	Matrix targeting enzymes	DNase I, restriction endonucleases, glycoside hydrolases, proteases, and dispersin B	EPS degradation
2.	Bacteriophages	SAP-26	EPS degradation
3.	Small molecules	Cis-2 decenoic acid (C2DA)	Biofilm dispersal
4.	Natural agents	Furanone, ajoene, naringin, musaceae, andcurcumin	Prevention of bacterial biofilm
		Honey	Restrict biofilm development

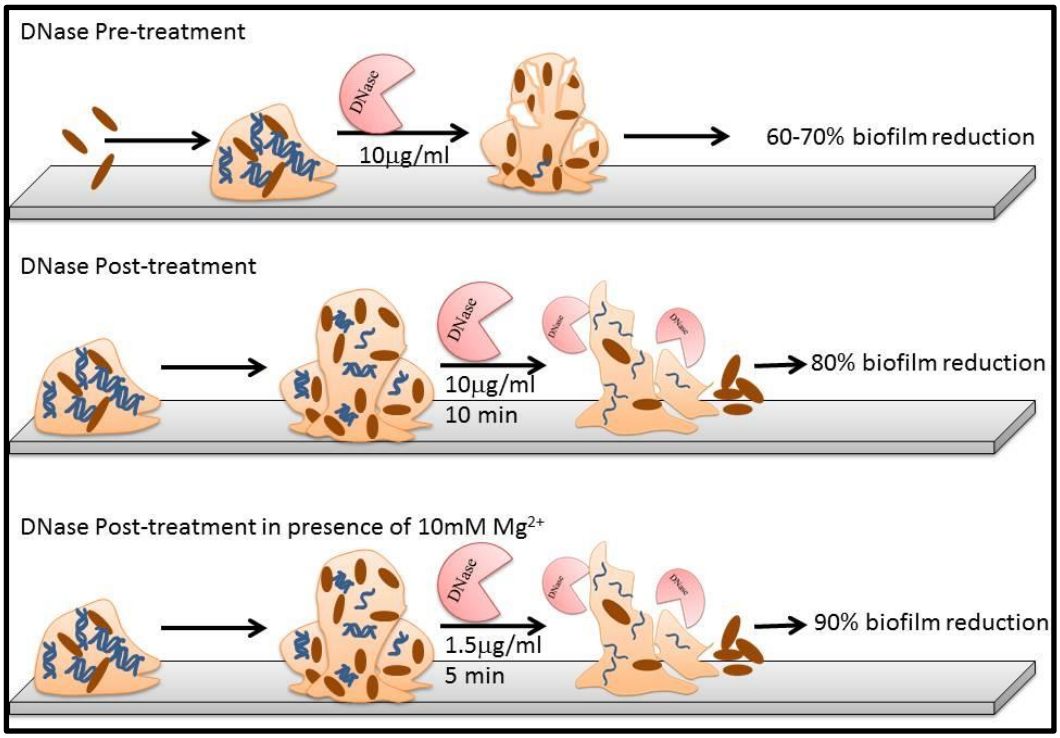


Fig 10: Effect of DNase treatment on biofilms. Biofilm grown with DNase in Pre-treatment setup showed 60-70% reduction in biofilm formation. DNase treatment given in Post-treatment setup after biofilm formation showed 80% reduction in Biofilm formation. DNase treatment along with Mg2+ in Post-treatment setup showed 90% biofilm reduction. *[Reference: K. Sharma and A. Pagedar Singh, "Antibiofilm effect of DNase against single and mixed species biofilm," Foods, vol. 7, no. 3, p. 42, 2018.]*

8.5 Quorum sensing inhibitors

Quorum sensing compounds like N-acyl-homoserine lactones are produced by gram negative bacteria and autoinducing peptides are produced by gram positive bacteria. Quorum sensing (QS) controls activities of virulence along with biofilm formation in bacteria, hence use of quorum sensing quenchers may help inhibit biofilm formation [85].

Regulating the expression of QS genes, using quorum sensing quenchers that attenuate quorum signals and use of enzymes like oxidoreductase, lactonase, and acylase that degrade QS signals, may help to control biofilm formation [86]. Lichen secondary metabolite, usnic acid, prevents biofilm formation in S. aureus by interacting with QS signals and in P. aeruginosa biofilms, it alters its morphology [87].

By targeting the signal or receptor via natural analogs that blocks the receptor, using brominated furanones that acts as a quorum sensing inhibitor, other compounds like fatty acids and peptide-based inhibitors can also be used to either quench the signal or inhibit biofilm

formation. However, use of synthetic compound analogs has been best researched for inhibiting quorum sensing signals. These work by blocking or interrupting receptor and ligand interaction or by destructing downstream signalling.

Table 7: Various synthetic synthase inhibitors and receptor inhibitors and their targets. *[Reference: P. L. Bhukya, R. Nawadkar, P. V. Bramhachari, and G. M. Sheela, "Significance of quorum sensing and biofilm formation in medicine and veterinary sciences," in Implication of Quorum Sensing and Biofilm Formation in Medicine, Agriculture and Food Industry, Singapore: Springer Singapore, 2019, pp. 87–99.]*

Category	Synthetic Inhibitor	Targets
Synthase inhibitors	Compound 10	LuxS
	pClPhT–DADMe–ImmA	MTAN (Methylthioadenosine/s-adenosylhomocysteine nucleosidase enzyme)
	JA-C8	TofI (LuxI family, B. glumae)
Receptor inhibitors	trAIP-II	AgrC
	CTL, CL	CviR
	Compounds 19 and 20	PqsR
	Itc-11, -12	LasR
	TP-5	LasR
	4606-423	LuxN, CviR

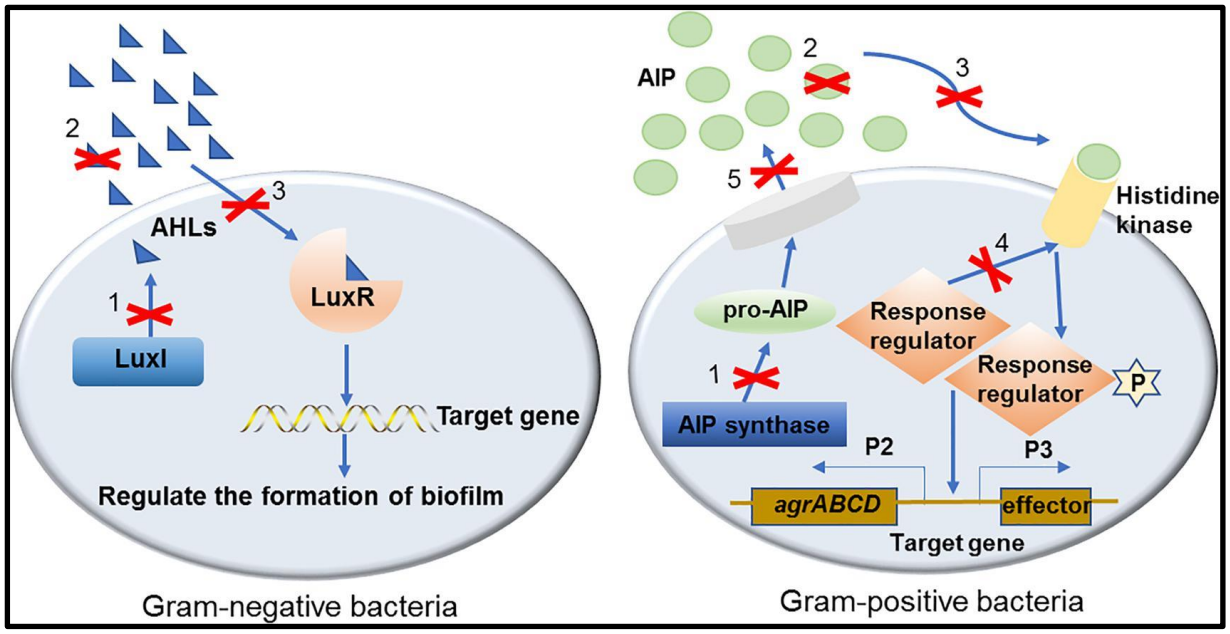


Fig 11: Various mechanism of quorum sensing inhibiting agents in controlling biofilm formation in bacteria. (1) By inhibiting AIs synthesis; (2) By degrading or inactivating AIs by AHL-lactonases, oxidoreductases, antibodies etc.; (3) By interfering with the signal receptors using AI antagonists or synthetic analogs; (4) By interfering with the response regulators thereby disturbing signaling cascade; (5). By reducing the accumulation of extracellular AIs by inhibiting AIs efflux thus inhibiting cell-to-cell signaling. *[Reference: L. Zhou, Y. Zhang, Y. Ge, X. Zhu, and J. Pan, "Regulatory mechanisms and promising applications of quorum sensing-inhibiting agents in control of bacterial biofilm formation," Front. Microbiol., vol. 11, p. 589640, 2020.]*

Table 8: Various quorum quenching molecules along with their mechanism of action and target. *[Reference: S. Challa, T. Dutta, P. V. Bramhachari, and N. N. Rao Reddy, "Quorum Sensing and Multidrug Resistance Mechanism in Helicobacter pylori," in* Implication of *Quorum Sensing and Biofilm Formation in Medicine, Agriculture and Food Industry, Singapore: Springer Singapore, 2019, pp. 101–119.]*

S.No.	Inhibitors	Class of quorum quenching molecule	Mechanism	Target
1.	t 5Z-4-bromo-5-bromomethylene-3- butyl-2(5H)-furanone	Halogenated furanones	Blocks reception of quorum signal	Acyl homoserine lactone (AHL) signals
2.	Synthetic AI peptides (AIPs)	Synthetic AI peptides (AIPs)	Blocks reception of quorum signal	Autoinducer Peptides (AIP) signals
3.	Triclosan	Enzyme inhibitor	Enzyme degrades or inhibits signalling molecule	Reduces production of AHL by inhibiting enoylacyl carrier protein (ACP) reductase involved in acyl-ACP synthesis and intermediate AHL biosynthesis
4.	Closantel	Enzyme inhibitor	Enzyme degrades or inhibits signalling molecule	Inhibits two component system histidine kinase sensors
5.	AHL lactonases	Enzyme inhibitor	Enzyme degrades or inhibits signalling molecule	AHL lactonases hydrolyse the lactone ring in the homoserine moiety of AHLs
6.	AHL acylase	Enzyme inhibitor	Enzyme degrades or inhibits signalling molecule	Degradation of AHL signals by hydrolysing the amide bond of AHLs

8.6 Material Alterations

Alterations like lowering the adhesiveness of the surface or antimicrobial coating on the surface, using metals like copper or silver, in combination with antimicrobial peptides or antibiotics can target bacterial adhesion to device surfaces thereby preventing biofilm formation. Such optimized Catheters are in clinical use, with some success, as it is impossible to inhibit adhesion completely. Since, device surfaces are prone to biofilm formation, as they are covered in host matrix proteins, which facilitates biofilm formation despite the alterations in device surfaces. Altered surfaces do not impact growth of biofilm once adhesion is successful [88].

8.7 Superhydrophobic Coating on Titanium surface.

Superhydrophobic surfaces have contact angles more than 150°, such coatings on medical implants have been investigated for its anti-biofilm properties. A new technique, single step glow discharge plasma, used to create superhydrophobic coating on titanium based medical implants was tested against polymicrobials where these implants showed increased resistance to corrosion, host biocompatibility and enhanced antimicrobial activity [89].

The investigation of the implant found a significant reduction, approximately 7 times, in the concentration of few pathogenic bacteria along with an overall reduction in surface biofilm formation by polymicrobials. In dental implants, the biofilm composition showed a positive effect with decreased numbers of oral pathogens. The implant showed non cytotoxicity along with enhanced antibiofilm activity, wherein they significantly reduced fungal and bacterial attachment and in situ formation of biofilms. These results indicated that such surfaces can be used to avoid polymicrobial biofilm infection in dental implants and can also be exploited for other medical devices and implants [89].

8.8 Bacteriophages and Lysins

Bacteriophage is bactericidal in nature thus it has the advantage of not being affected by the efficacy lowering morphological and physiological changes in the bacterial biofilms or persister cells. However, owing to biofilm matrix, the cell surface receptor for bacteriophages may not be accessible. Even so, bacteriophages have shown to be effective against in vitro staphylococcal biofilms in many cases. In in-vitro setup, the bacteriophages jIPLA-RODI and jIPLA-C1C reduced S. aureus and S. epidermidis biofilms. [90]

Bacteriophage K is often regarded as a staphylococcal biofilm disruptor. Bacteriophage lysins, such as CF-301 [87], can also be used to disrupt biofilms enzymatically.
However, in vivo studies of bacteriophage and bacteriophage lysin in humans still needs to done against staphylococcus biofilm-associated infection to determine its efficacy.

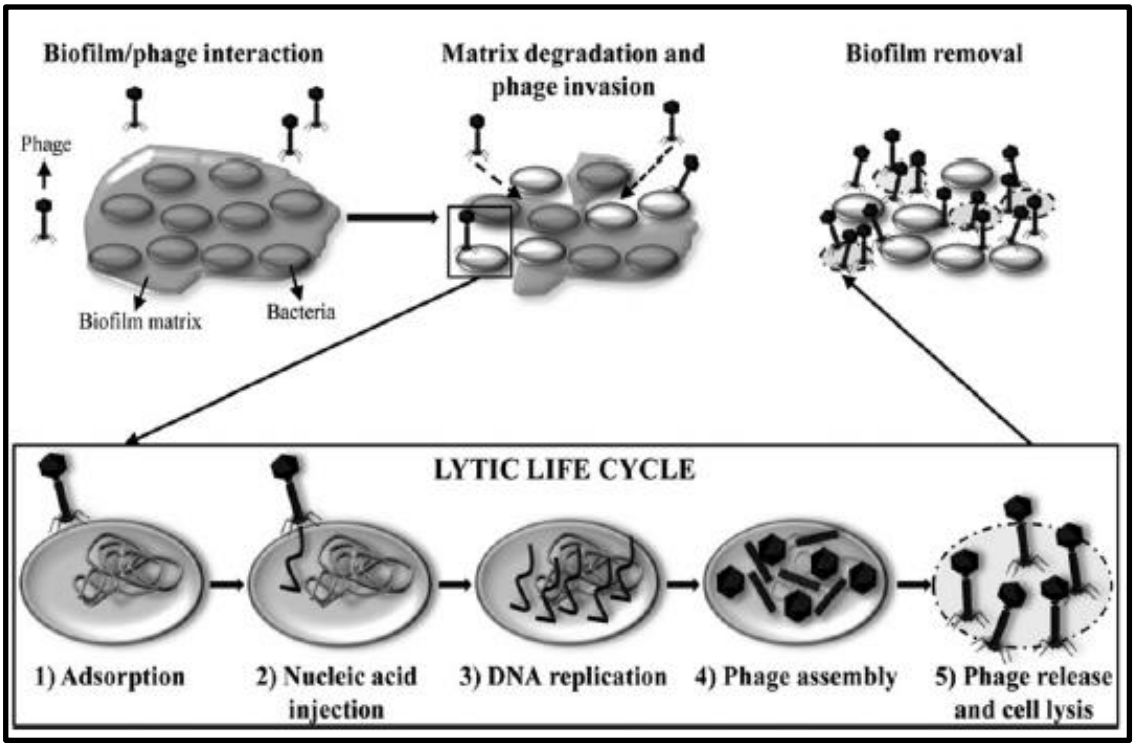


Fig 12: Mechanism by which phages disrupt biofilms. *[Reference: L. Geredew Kifelew, J. G. Mitchell, and P. Speck, "Mini-review: efficacy of lytic bacteriophages on multispecies biofilms," Biofouling, vol. 35, no. 4, pp. 472–481, 2019.]*

8.9 Use of Probiotics

Human microbiome has trillions of different species, that help humans in day-to-day functioning, ranging from metabolism to immune response of an individual. Probiotics are concoction of beneficial microrganisms and can be used for treatment and prevention of pathogenic microbial biofilm formation. They produce antagonistic substances such as enzymes (lipase, amylase), EPS, organic acids, surfactants, lactic acid, bacteriocins, fatty acids, and hydrogen peroxide that can inhibit pathogenic bacterial activity and their adhesion the surfaces of medical devices and implants. Along with preventing biofilm formation by interfering with quorum sensing signals they also interfere with formed biofilms integrity and quality leading to their eradication. They can alter the environmental conditions like pH

alterations thereby generating unfavourable conditions for pathogen survival. Competition for surface and nutrients between the probiotics and pathogenic bacteria can also be a cause for pathogen elimination. Probiotics preferably adhere to human tissues or medical devices (prostheses, catheters etc.) and acts as a barrier for colonization of pathogenic bacteria. Additionally, probiotics can modulate host immune response targeting pathogenic bacteria and form non-pathogenic biofilms which prevents pathogenic biofilm formation [91].

Table 9: List of various probiotics and their mechanism of action against pathogenic bacteria to inhibit biofilm formation. *[Reference: A. Barzegari, K. Kheyrolahzadeh, S. M. Hosseiniyan Khatibi, S. Sharifi, M. Y. Memar, and S. Zununi Vahed, "The battle of probiotics and their derivatives against biofilms," Infect. Drug Resist., vol. 13, pp. 659–672, 2020.*

Biofilm Former	Probiotic	Mechanism of action
C. albicans, #	*L. rhamnosus* supernatant	Secretes biosurfactants that disrupt the physical membrane structure or protein conformations; results in cell lysis, destroys the hyphae formation and interferes with the interaction between the cells and material.
Vibrio cholera and *V. parahaemolyticus*	*L.* spp. L13 (KY780504), ##	Inhibited the adherence of *Vibrio* spp. to the epithelial cells and dispersed the preformed-*V. cholerae* biofilms
P. aeruginosa	*Pediococcus acidilactici* M7 strain isolated from new born faeces	Lactic acid produced by the strain: - Inhibited the Rh1 system signaling molecule (C4-HSL) ↓Virulence factors regulated by the Rhl including protease, pyocyanin, elastase, and biofilm production - Did not reduce/inhibit the Las system signaling molecule (3-oxo-C12-HSL)

B. subtilis BM19	*L. acidophilus* ATCC 4356	Bacteriocin from this probiotic inhibits the growth of *B. subtilis* BM19 planktonic cells and biofilm formation
Propionibacterium acnes, P.aeruginosa, S. aureus, E. coli	*L. delbrueckii subsp. Bulgaricus,*###	Due to organic acid production, all probiotics except *L. delbrueckii*, had antimicrobial activity. Probiotics inhibit the AHL production and prevent biofilm formation, *P. innocua* was able to destroy pre-formed biofilms of *E. coli, P. aeruginosa* and *S. aureus*
P. aeruginosa PAO1, MRSA and their hospital-derived strains	*L. plantarum F-10* supernatant	↓QS signals, ↓Oxidative stress in wound healing stages, Co-aggregated with all pathogens, inhibited the virulence factors (motility, activity of protease and elastase, production of pyocyanin and rhamnolipid)

E. coli ATCC35218	EPS-Lp from *L. plantarum* and EPS-B from *Bacillus spp.,*	EPSs: ↓cell surface hydrophobicity level, ↓indole production, prevent biofilm formation, ↓efflux pumps involved in bacterial adhesion and antimicrobial resistance.
*Staphylococcus aureus,**	*Streptococcus salivarius* 24SMB and *oralis* 89a	↓ pH and ↓biofilm biomass prevents biofilm formation of selected pathogens, disperse the pre-formed biofilms, secret diffusible molecules that are implied in their anti-biofilm activity
EHEC, *P. aeruginosa, Staphylococcus aureus, S. epidermidis*	*E. coli Nissle* 1917	*S*ecretes DegP, a bifunctional protein with protease and chaperone activity outside the cells and controls other biofilms.

S. aureus	*L. fermentum TCUESC01* and *L. plantarum TCUESC02*	Inhibition of biofilm by alteration of the ica operon (icaA and icaR) involved in the biofilm matrix synthesis.
C. albicans, C. tropicalis, C. krusei.	*L. pentosus*/strain LAP1	Probiotic indicated an anti-Candida activity and antibiofilm property
C. albicans	*Pediococcus acidilactici* HW01	It has antifungal agent against *C. albicans* by reducing the growth and biofilm formation.
Clinical *Salmonel la* species and uropathogenic *E. coli*	*L. rhamnosus GG*	Lectins are involved in the adhesion capacity of *L. rhamnosus* to vaginal and gastrointestinal epithelial cells.
Cronobacter sakazakii	*L. casei, L. sporogenes,* **	With antimicrobial activity, production of bioactive molecules to limit the emerging infections.
P. aeruginosa PAO1	*L. fermentum* (KT998657) isolated from neonatal fecal samples	↓Biofilm forming due to postbiotics (bacteriocin and EPS), bacteriocins make pores in the cell membrane, change membrane integrity of cells, and cause cell death, EPS alter the matrix and restrict cell assembly, cell-cell interaction and *Pseudomonas* attachment to form biofilms.
C. glabrata	*L. rhamnosus GR-1, L. reuteri RC-14*	↓EPA6 and YAK1 expression (biofilm-related genes)

Notes: #*Candida tropicalis, Streptococcus salivarius, R. dentocariosa, Staphylococcus epidermidis,* ##*L. plantarum* L14(KY582835), *L.* spp. L18 (KY770976), *L. fermentum* L32 (KY770983), *L.* spp. S30 (KY780503), *L. pentosus* S45 (KY780505), *L.* spp. S49 (KY770966) isolated from the fecal samples of healthy children, ###*Bifidobacterium animalis subsp. Lactis, L. acidophilus, L. brevis, Bifidobacterium lactis, L. salivarius Bifidobacterium longum subsp. Infantis, L. plantarum, L.acidophilus, L. casei, Propioniferax innocua, L. casei subsp. Rhamnosus,* MRSA: methicillin-resistant *Staphylococcus aureus, *Streptococcus pyogenes,*

*Propionibacterium acnes, Streptococcus pneumoniae, Moraxella catarrhalis, Staphylococcus epidermidis, **L. sporogenes, B. mesentericus,C. butyricum L. sporogenes, S. faecalis, L. sporogenes, S. faecalis, Clostridium butyricum, Bacillus mesentericus.*

Abbreviations: *L, Lactobacillus; S, Streptococcus; P, Pseudomonas; C, Candida*; EPS, exopolysaccharides; NEC, necrotizing enterocolitis; *E, Escherichia*; EHEC, *enterohemorrhagic E. coli*; QS, quorum sensing; A, *Aggregatibacter.*

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Table 10: Summary of various methods available for targeting microbial biofilms. *[Reference: T. Bjarnsholt et al., "Biofilm formation - what we can learn from recent developments," J. Intern. Med., vol. 284, no. 4, pp. 332–345, 2018.]*

S.No.	Methods	Action	Applications
1.	Antimicrobial peptides	Dispersing formed biofilms and preventing new biofilm formation on various surfaces	Medical devices such as urinary catheters
2.	Atmospheric cold plasma	Generate reactive species of oxygen and nitrogen that target macromolecules like lipids, proteins etc. within the cell.	Wound infections
3.	Acetic acid	Traditionally used antimicrobial	Used to treat infections like Swimmer's ear, chronic wounds and prosthetic joint infection
4.	Hypochlorous acid, HOCl	Peroxidase-generated anti-bacterial innate immune molecule	Wound infections
5.	Nitric oxide, NO	Innate immune and signalling molecule that is sensed by sensory protein domains to subsequently induce biofilm dispersion in a broad range of bacteria	Inhalation to treat cystic fibrosis lung infection caused by *P. aeruginosa*

6.	Antimicrobial surface material	Prevention of cell adhesion and formation of pathogenic biofilm on surfaces	Medical devices and implants
7.	Enzymes - DNases, glycosidase proteases,	Dispersion of the biofilm by destruction of the extracellular matrix in combination with antimicrobials	Skin and wound biofilm, cystic fibrosis lung infection
8.	Photodynamic therapy	Stimulating photoreactive components to create reactive nitrogen and oxygen species.	Wound infection, Skin biofilm
9.	Low frequency ultrasonic therapy	Dispersal of biofilms with the help of mechanical energy used in combination with chemical therapy to reduce biofilm load.	Wound infection, periprosthetic joint infection
10.	Probiotics	Prevention of cell adhesion and pathogenic biofilm formation on surfaces	Hospital acquired infections via medical devices and implants.
11.	Bacteriophage and Lysin	Lysin degrade EPS; phage invade EPS to infect cells causing lysis and cell death, thereby disrupting biofilms.	Potential application in Chronic wounds
12.	Hyperbaric oxygen therapy	Generation of ROS species in anaerobic population and promotes growth of antibiotic susceptible aerobic species.	Chronic wounds
13.	Quorum Sensing Inhibitors	Interfere with signalling molecule or receptor to inhibit biofilm formation.	Medical devices and implants to prevent biofilm formation.
14.	Modulating microbial metabolism	Reversing metabolism adaptation leading to tolerance for effective killing by microbes	Chronic wounds

9. CONCLUSION AND FUTURE RECOMMENDATION

As mentioned earlier, almost 80% of biofilm forming bacteria causes persistent infections. Device associated and non-device associated hospital acquired infections are the most adverse event in healthcare facilities causing high number of deaths per year. Especially in chronic wounds, it is critical to eradicate biofilms to prevent recurring infections and for effective healing. Research in biofilms is often conducted in in vitro models and such models vary substantially from in vivo conditions, in terms of, microenvironment resulting from interaction among immune system, host proteins and biofilm. Due to this, observations from such models are not applicable at in vivo level. Research in Ex vivo models should be able to generate more reliable results. Also, multispecies biofilms are more common in infections, which also imparts distinguishing qualities to the biofilm for numerous applications like resistance. But our current knowledge of the interspecies interactions is still very limited. Further research should be conducted using multiple species, Ex vivo model simulating maximum natural conditions.

The microenvironment and metabolic adaptations play important role in the persistence of biofilms by rendering antibiotic treatment ineffective. High supply of oxygen or addition of metabolites that initiate the pathways like TCA and Glycolysis which is downregulated in biofilms and blocking the genes for the production of SOD and catalases may lead to killing of the cells by reactive oxygen species, an essential by product of aerobic respiration. Many such metabolic pathways can be targeted for biofilm destruction. Excess secretion of EPS is a key feature in surface attached bacteria, this matrix protects bacteria from invasion of phagocytic cells and other antibiotics. Research regarding targeted biofilm degrading enzymes in combination with other conventional treatment may prove to be effective. Conventionally used antibiotics have not only failed to treat infections but have also resulted in spread of resistance therefore alternatives like use of either new antibiotics or combining new strategies with traditional treatments to target a biofilm is critical.

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