Acta Biologica Turcica 32 (4) 220-235 2019 Received 02 July. 2019; Accepted 27 Aug. 2019

ACTA BIOLOGICA TURCICA

E-ISSN: 2458-7893, http://www.actabiologicaturcica.com

Review

Insight on microbial biofilms and recent antibiofilm approaches

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Abstract: Biofilms are organized sessile microbial communities embedded in a self-secreted extracellular matrix. These cells exhibit modified morphological, physiological and metabolic characteristics. Biofilms represent a survival strategy that confers protection against wide range of adverse environmental conditions (UV, antimicrobial, host immune system). The persistence of these microbial communities on different biotic and abiotic surfaces represents a real problem for both food and medical sectors. Their formation on food processing surfaces and biomedical devices is responsible for almost 80% of microbial infections. In addition to the health problems, the economic burden caused biofilms is enormous which necessitates the development of biofilm prevention or elimination strategies. Conventional approaches based on physical, mechanical and chemical interventions are not efficient anymore due to increased resistance of biofilm organisms. Biofilm cells exhibit higher resistance to antimicrobial agents by 100- 1000 folds when compared to their planktonic counterparts. Such findings prove the seriousness of the threat imposed by these communities and emphasize the urgent need to come up with new antibiofilm approaches.

The increased interest in biofilm research has provided deeper knowledge concerning these communities, which led to impressive progress in developing novel antibiofilm tools. The bulk of these emerging strategies focuses on green technology or development new molecules that can be used as adjuvants in combination with antimicrobial agents. On the other hand, some approaches aim to inhibit adhesion via targeting microbial surface proteins such as adhesins, or through engineering antiadhesive surfaces through chemical or mechanical modifications.

Keywords: biofilms, antibiofilm, antiadhesion, biofilm formation

Citing: Shatila, F., Yalçın, H.T., & Yaşa, İ., 2019. Insight on Microbial Biofilms and Recent Antibiofilm Approaches. *Acta Biologica Turcica, 32*(4): 220-235.

Introduction

The ability of microorganisms to form biofilms on biotic surfaces is responsible for tissue related infections. Moreover, the formation of these sessile communities on food processing surfaces and in hospital settings is responsible for born outbreaks and hospital acquired infections. The appearance of antimicrobial resistant strains has made treatment of biofilm related infections even more challenging (Lebeaux et al., 2014; Hughes and Webber, 2017). The current review article provides some general information concerning microbial biofilms and highlights some of the most recent antibiofilm strategies.

History of Biofilms

In the seventeenth century, Antonie van Leeuwenhoek described the existence of microbial aggregates on teeth surfaces. Zobell (1943) who was concerned in studying the interactions between solid or adsorbing surfaces and bacterial activity, reported that sessile bacteria secrete mucilaginous exudates to support their growth on solid

surfaces. Costerton et al. (1978) reported that a bacterial cell can adhere to a plant, animal or another bacterial cell by juxtaposing its own glycocalyx to the surface of the cell it adheres to.

After 25 years, Donlan and Costerston (2002) provided the most prominent definition for a biofilm. The new definition took into consideration the readily observable modifications as well as the non-observable characteristics. Since then a biofilm is defined as "a microbially derived sessile community characterized by cells that are irreversibly attached to a substratum or interface or to each other, embedded in their own extracellular polymeric substances. The cells of a sessile community exhibit an altered phenotype with respect to growth rate and gene transcription" (Donlan and Costerton, 2002).

Biofilm cells demonstrate differences from their planktonic counterparts with respect to morphological appearance, metabolic state and physiology (Yssel, 2017). Previous studies also mentioned the presence of differences in gene expression levels between biofilms and planktonic cells. It is estimated that as much as 40% of bacterial genome is either up regulated or down regulated in response to the transition from planktonic to biofilm state (Prakash et al., 2003). Biofilm cells are also characterized by increased gene transfer rate and communication through quorum sensing (Chandki et al., 2011).

Importance of Biofilm Formation for Bacterial Cells

Free living planktonic mode is common in laboratory conditions. However, in natural environments bacterial cells are in continuous search for advantageous niches suitable to start the biofilm formation process (Moldoveanu, 2012). Around 99% of the world's bacterial populations exist as biofilms at various stages of growth (Toyofuku et al., 2016). They ensure bacterial colonization in niches that ensure continuous nutrients supply, relatively stable water content and oxygen availability (Jefferson, 2004). On the other hand, biofilm formation also promotes survival and persistence of bacterial cells in stressful conditions such as starvation, temperature shocks, pH alterations, hypoxia or anoxia conditions, UV exposure, predation, exposure to antimicrobial agents (antibiotics, biocides and disinfectants) and immune host defences (Flemming and Wingender, 2010; Sousa et al., 2011).

Biofilm cells have 100-1000 fold increased resistance for antibiotics when compared to their planktonic counterparts. Such resistance is attributed to the following mechanisms:

1. Limited access of antimicrobial agents into the biofilms which function as physical barriers. For example, the binding of positively charged aminoglycosides to the negatively charged polymers within the biofilm matrix slows their penetration (Lewis, 2001; Hall and Mah, 2017).
2. Adaptive response is triggered by biofilm cells to overcome environmental fluctuations (oxidative stress, temperature fluctuations, starvation etc…) and confer protection against the deleterious effects of antibiotics (Butt and Khan, 2015). The retarded diffusion of antibiotics along with the presence of degrading enzymes decrease the concentration of antibiotics within the biofilm. For example the release of ß-lactamases can effectively degrade ampicillin before reaching its target cells. Moreover, elevated expression of multidrug efflux pumps genes in biofilms when challenged with antibiotics can directly contribute to biofilm resistance (Stewart, 2002; Poole, 2012).
3. The stratification of biofilm into microenvironments: the gradual decrease in oxygen concentration and nutrient level towards the centre of biofilm causes altered metabolic activity for biofilm cells. The low oxygen concentration and restricted nutrient diffusion in the centre can even inhibit bacterial growth. Taking into consideration that antimicrobials are more effective in killing metabolically active growing cells, the stated mechanism can explain the reduced susceptibility of biofilms to antibiotics (Poole, 2012; Singh et al 2017).
4. The presence of Persisters. Persister cells are defined as highly resistant phenotypic variants that arise due to state of dormancy. They represent a highly protected subpopulation that is characterized by being metabolically inactive and tolerant to antibiotics without undergoing any genetic change (Stewart, 2002; Paraje, 2011; Wood et al., 2013).

Biofilm Formation Steps

The process of biofilm formation is orchestrated by the expression of distinct set of genes at each stage. It starts by the attachment of planktonic bacterial cells to conditioned surfaces. The conditioning layer results from

the adsorption of organic and inorganic molecules in the bulk fluid on biotic or abiotic surfaces. The deposition of molecules is facilitated by Brownian motion, sedimentation and movement with liquid flow. The changes in surface properties (surface charge, potential and tension) conferred by the conditioning layer renders it more favourable for bacterial attachment (Garrett et al., 2008). Initial attachment is ensured by the expression of genes which are required to make contact with surfaces. As a result, the bacterial cells use their extracellular organelles (flagella, fimbria, pili) and outer membrane proteins to sense and provide initial attachment to the underlying substratum (Renner and Weibel, 2011). The initial interaction between the cell surface and substratum, mediated by Van der Wall forces, Lewis acid-base, and electrostatic forces, is reversible (Kaplan, 2010). Therefore, the bacterial cells at this stage can leave the surface and return to planktonic life style or persist as sessile community. The cells become irreversibly attached as a result of down regulation of motility genes and up regulation of genes that encode extracellular matrix components. The extracellular matrix, which assists the adhesion between cells and surfaces, is a dense, chemically inert mixture of DNA, proteins, lipids and lipopolysaccharide surface proteins (Zhao et al., 2017). The time required for transition from reversible to irreversible attachment can be as short as several minutes (Palmer et al., 2007). Simultaneous EPS production and cellular division of the irreversibly attached cells result in micro colonies that are encapsulated by EPS (Donlan, 2002). The exopolysaccharides function as a physical barrier that can protect the biofilm cells from extracellular environment (Kaplan, 2010). Moreover, the presence of EPS supports the complex structure of the multi-layered macro colonies in mature biofilms. The macrocolonies comprise channels that allow the circulation of signalling molecules, nutrients and disposal of waste products inside mature biofilms (Toyofuku et al., 2016). Eventually, changes in the environmental and growth conditions (starvation, oxygen deficiency and metabolites accumulation) serve as signal to trigger dispersal of mature biofilm cells. The active dispersal process can be mediated by matrix degrading enzymes produced by the biofilm cells (dispersin) or through up regulation of motility genes and down regulation of genes involved in attachment such as fimbria synthesis (Toyofuku et al., 2016; Fleming and Rumbaugh, 2017). On the other hand, passive dispersal is mediated by external forces such as mechanical intervention or fluid and solid shear (Banerjee et al., 2015; Toyofuku et al., 2016; Fleming and Rumbaugh, 2017). Detachment of cells from mature biofilms represent another survival strategy and an opportunity of self-renewal since dispersed cells tend to colonize new niches and start a new biofilm cycle (Fleming and Rumbaugh, 2017).

Biofilm formation is a complex dynamic process that is genetically regulated and controlled by the interaction between cell surface properties, surface properties as well as the environmental conditions (media, pH, temperature) (Van Houdt and Michiels, 2010).

Factors Affecting Biofilm Production

Biofilm formation process affected by the interactions between the (1) bacterial cells, (2) attachment surface and (3) environmental conditions.

Properties related to bacterial cells include cell hydrophobicity, surface charge, and outer membrane proteins. Bacterial hydrophobicity, which varies among different organisms and strains, enhances attachment of bacterial cells. It is influenced by the growth medium, bacterial age, and bacterial surface structures such as pili, flagella and fimbria. Pili function as adhesins which enhance nonspecific interaction between bacterial cells and the cell-surface. On the other hand, flagella are considered critical for initial cell to surface contact and biofilm formation. Under stagnant conditions, flagellar motility favours adherence by facilitating movement along the surface and reducing the repulsive forces between the cells and the surface (Van Houdt and Michiels, 2010). Similarly, fimbria is suggested to aid attachment by overcoming the electrostatic repulsion barrier that exists between the cell and the surface (Krasowska and Sigler, 2014). Moreover, the presence of high hydrophobic amino acid residues in flagella favours attachment due to increased cell surface hydrophobicity (Choi et al., 2015). In addition to nucleic acids, proteins, glycoproteins and lipoproteins, the EPSs constitute an important portion of the extracellular matrix. Negatively charged bacterial cells can attach readily to positively charged surfaces. On the other hand, cell contact is destabilized due to electrostatic repulsion upon interaction with anionic surfaces (Renner and Weibel, 2011). However, the presence of extracellular organelles (flagella, fimbriae, pili and curli fibers) and deposition of

molecules carrying different functional groups (carboxylates, hydroxyl, phosphate and amine moieties) over the attachment surface promote adsorption of bacteria to surfaces and biofilm formation (Renner and Weibel, 2011).

Attachment surface: Biofilms can form on wide variety of surfaces. The physicochemical properties (surface topography, hydrophobicity and surface charge) of the substratum has an important role in the adhesion process.

Surface roughness and topography: There is no general rule controlling the exact effect of surface topography on bacterial adhesion and biofilm formation. Yet, its effect is controlled by the size and shape of bacterial cells as well as environmental conditions (Song et al., 2015). In general, smooth surfaces can reduce biofilm formation. Hence, any increase in surface roughness due to mechanical intervention favours biofilm formation. Rough surfaces favour entrapment of nutrient residues, increase the contact area between the surface and the bacterial cells and protect them from shear forces (Graham and Cady, 2014; Garcia-Gonzalo and Pagan, 2015). Bacterial cells with hydrophobic surfaces tend to colonize hydrophobic substrata (such as plastics) more readily when compared to the ability of their hydrophilic counterparts to colonize hydrophilic surface (glass or metals) (Garcia-Gonzalo and Pagan, 2015).

Biofilms are observed on many food processing surfaces, including stainless steel, glass, rubber, polycarbonate, polyurethane, teflon, nitrile rubber, titanium, aluminium and ceramic (Garcia-Gonzalo and Pagan, 2015). Moreover, medical implants (such as catheters and pace makers) are synthesized from hydrophobic materials (such as silicon, stainless steel, teflon) which favour adherence of hydrophobic microorganisms (Krasowska and Sigler, 2014).

Environmental conditions (pH, nutrient composition of the food matrix, temperature, presence of mixed microbial community) (Borges et al., 2018; Van Houdt and Michiels, 2010). Environmental conditions can modify gene regulation and the physicochemical properties of the cell surface, moreover, they can change the physicochemical properties of the substratum (Garcia-Gonzalo and Pagan, 2015). For example, the availability of nutrients, constituents of the food matrix and temperature can alter the physicochemical properties of the bacterial surface such as surface charge and its hydrophobic/ hydrophilic nature. Additionally, temperature and pH fluctuations can affect the genetic expression of biofilm regulating genes. For example, the expression of genes responsible for curli production, a main constituent of biofilms, is higher at lower temperatures (28, 20 °C) when compared to that at higher temperatures such as 37 °C (Van Houdt and Michiels, 2010). Acquiring better understanding of the factors controlling biofilm formation can be exploited to control biofilm formation especially in food industrial settings (Garcia-Gonzalo and Pagan, 2015).

Biofilm Analysis Techniques

The methodologies applied in biofilm studies demonstrate variations according to the aim of the study. Biofilms can be cultivated in two different approaches dynamic or static (Azeredo et al., 2016). Different types of biofilm reactors are developed. Such systems allow biofilms analysis under dynamic conditions and provides continuous nutrient supply. Such reactors allow observation of biofilms grown under shear forces and evaluate the effect of different antibiofilm agents (Franklin et al., 2015; Azeredo et al., 2016). In these systems, biofilms can be grown on coupons made from different substrates or materials that are specifically selected or designed to fit the aim of the study (Azeredo et al., 2016, Ergin, 2017). After which, the coupons can undergo microscopic inspection, cell viability assessment or used in different omics studies (Franklin et al., 2015). Moreover, flow reactors that are designed for imaging can provide 3D images that allows monitoring of biofilm formation under continuous growth conditions (Franklin et al., 2015).

On the other hand, biofilms cultivated under static conditions don't mimic real environmental conditions in nature or industry due to absence of continuous nutrient supply. Yet, static biofilms grown in microtiter plates remains the most common approach due to its feasibility and high output (Corcoran 2013). After incubation period under conditions that favour biofilm formation, the resulting biofilm can be quantified using variable colorimetric assays. The choice of colorimetric assay depends on the aim of experiment. Crystal violet assay estimates the entire biomass of a biofilm. It estimates both living and non-living cells. On the other hand, tetrazolium dyes (such MTT, XTT and TTC) quantify only the metabolic activity of the biofilm cells (Franklin et al., 2015, Azeredo et al., 2016). Similarly, safranin has also been used to stain the biomass of a biofilm, while

Resazurin, a stable redox indicator, is reduced to a pink highly fluorescent resorufin by metabolically active cells (Azeredo et al., 2016). Such indirect quantification assays are based on the assumption that a certain marker can infer biofilm quantity (Wilson et al., 2017). Indirect quantification methodologies are numerous, they also include dry mass estimation, total organic carbon and ATP bioluminescence (Ergin, 2017; Wilson et al., 2017).

Biofilms can also be statically grown on coupons placed in the wells of the microtiter plates. After incubation, the coupons can be visualized under microscope or used to evaluate biofilm growth (Corcoran, 2013).

On the other hand, colony biofilm represents another type of static biofilm models. In this approach, biofilms are grown on filter papers already placed on the surface of agar plates. The regular transfer of filter papers to fresh medium provides semi continuous supply of new nutrients and results in thick biofilms that can be used various analysis (Franklin et al., 2015).

Quantification of biofilm cells can also be evaluated through direct colony forming unit (CFU) counts. Cell counts can also be assessed using light microscopy, fluorescence microscopy or flow cytometer (Wilson et al., 2017). Flow cytometer provides additional information concerning cell properties such as cell dimensions and metabolic activity (Wilson et al., 2017).

Tube adherence test and Congo Red agar test are among the commonly used qualitative tests to detect biofilm formation. Tube adherence test involves the overnight incubation of an activated microorganism in test a tube. After incubation period is over, the growth is aspirated, tube is washed, dried and stained with crystal violet or safranin. Tubes that contain visible coloration lining the walls and the bottoms of test tubes are considered positive for biofilm formation (Ergin, 2017).

On the other hand, the addition of Congo red to Luria Bertani without salt allows the detection of curli and cellulose when incubated at 28°C. Biofilms formed on Congo red agar reveal different morphotypes, these include: RDAR (red, dry and rough indicating the presence of curli and cellulose) PDAR (pink, dry and rough indicating the presence of curli and absence of cellulose) BDAR (brown, dry and rough indicating the absence of curli and presence of cellulose) and SAW (smooth and white indicating the absence of both curli and cellulose).

On the other hand, qualitative determination of a biofilm also evaluates the effect of different environmental factors on the surface roughness, morphology and spatial organization of a biofilm (Wilson et al., 2017). Hence, Biofilms grown on coupons or cover slips, whether in static or dynamic conditions, can be inspected using microscopy techniques.

While light and fluorescent microscopes can be used to estimate the biofilm volume or total surface coverage (Wilson et al., 2017) other microscopy techniques can provide more valuable information. Scanning electron microscopy remains the most commonly applied high resolution imaging method that provides details about biofilm structure and topography. It allows assessment of bacterial interactions, EPS organization and biofilm morphology (Pan et al., 2016; Dhayakaran and Neethirajan, 2017).

Confocal laser scanning microscopy provides high resolution, three dimensional visualization of hydrated, living undisturbed microbial biofilms. Moreover, its coupling with fluorescent markers allows reliable detection and quantification of the EPS and macromolecules of a biofilm (Franklin et al., 2015; Dhayakaran and Neethirajan, 2017; Ergin, 2017; Wilson et al., 2017).

On the other hand, atomic force microscopy, which is a surface scanning microscope, provides better understanding of different biofilm characteristics such as roughness, topography and stiffness (Wilson et at., 2017). The three dimensional images at nanoscale resolution can clearly show the secretion of EPS along with the entrapment of bacterial cells (Pan et al., 2016). Moreover, AFM force microscope can provide information about the forces of attachment, as well as the influence of other factors affecting biofilm growth (Azeredo et al., 2016; Dhayakaran and Neethirajan, 2017).

Some Recent Antibiofilm Approaches

Antibiofilm approaches include the natural and induced process that leads to reduction of bacterial biomass through the alteration of biofilm formation integrity and or quality". Antibiofilm approaches can target either the adhesion stage of biofilms or mature biofilms (Miquel et al., 2016).

Anti-adhesion Approaches

Anti-adhesion strategies can cause either general or specific inhibition of adhesion depending on its target. Nonspecific inhibition of adhesion is conferred through the modification of surfaces chemistry or topography (Beloin et al., 2014; Neoh et al., 2017). Engineering surface topography or its manipulation at micro and nanoscale seems to be an advantageous approach. It is non-toxic and independent of material type. Moreover, it can also be chemically modified (Graham and Cady, 2014). Yet, this approach has not been fully explored. Existing studies infer that there is no rule concerning the effect of nanoscale topographical modifications on bacterial attachment (Hsu et al., 2013). Lagree et al., (2018) studied the effect of surface topography on *Candida albicans* biofilm formation. Biofilm formation surfaces were coated with particles of different sizes of polydimethyl siloxane (PDMS) solids. The study reported that higher biofilm formation was observed on surfaces coated with particles of size range 4-8 μm when compared to surfaces coated with particles of size range 0.5-5 μm. Perera-Costa et al., (2014) reported that biofilms grown on spatially organized microtopographic surface patterns generated on polydimethylsiloxane reduced the adhesion of three bacterial strains (*S. epidermidis*, *E. coli* and *Bacillus subtilis*) by 30 to 45% more when compared to smooth control surfaces.

The inhibitory effect of surface topography has been attributed to the presence of fewer binding sites when compared to flat surfaces. The presence of similar curvature between the solid surface and the microorganism can also make the adhesion process more challenging. Additionally, the topography of a solid surface can trap air which in return reduces the access of microorganisms to the solid (Lagree et al., 2018).

Chemical modification of surfaces involves coating with anti-adhesive materials, chosen on the basis of their anti-adhesive properties. Anti-adhesive materials include synthetic polymers such polyethylene glycol (Bangerjee et al., 2011; Zhou, 2014; Zhang and Chiao, 2015) and phosphorylcholine containing polymers (Lewis, 2000; Beloin et al., 2014). On the other hand, natural or modified polysaccharides such as ulvan, hyaluronic acid and agarose have also demonstrated promising anti adhesive activity (Junter et al., 2015, Neoh et al., 2017). However, anti-adhesive coatings don't kill the bacterial cells. As a result, the continuous release of bacterial proteins and surfactants will eventually mask the underlying chemical modifications. Therefore, coating surfaces with anti-adhesive and bactericidal materials seem to be a more effective approach. Such approach suggests coating with antibiotics, or surface immobilized antimicrobial moieties such as quaternary ammonium compounds, microbial peptides or nanoparticles (Ramasamy and Lee, 2016; Neoh et al., 2017). Peng et al., (2018) tested the antibiotic and antibiofilm effect of a commercial thermoplastic polyurethane surface modified with peptide-like cationic functional groups. The cationic coating demonstrated significant ability to kill and slow the growth of *E. coli* biofilms.

On the other hand, targeting specific adhesins demonstrated even stronger anti-adhesive potential. Pilicides which targeted type I fimbriae and P pili reduced *E. coli* biofilm by approximately 90% while curlicides which targeted curli fimbriae inhibited both curli dependent and type 1 dependent biofilms (Cegelski et al, 2009). Totsika et al., (2013) demonstrated the potency of FimH inhibitors to treat the acute UTI caused by the multidrug resistant *E. coli*.

Antibiofilm Strategies

On the other hand, different approaches that target microbial biofilms' adhesion or maturation are being developed. Antibiofilm agents can be used as adjuvants in combination with antimicrobial agents (Roy et al., 2018).

Nanoparticles

Nanoparticles are defined as materials that have one dimension (1-100 nm) in the nanometer scale range or whose basic unit in the three dimensional space is in this range. Their broad spectrum antibacterial activity against gram positive and gram negative bacteria is attributed to the large surface area to volume ratio as well as to their unique chemical and physical properties. In addition to their antibacterial activity, nanoparticles have recently become a promising approach to control or prevent biofilms (Wang et al., 2016). The silver nanoparticles developed by Namasivayam et al. (2013) reduced the carbohydrate and protein content of biofilm matrix, which weakened the biofilm and allowed the penetration of drugs. The Au nanoparticles loaded with gentamicin (GPA NPs) produced by Mu et al. (2015) effectively damaged the established biofilms of gram positive (*L. monocytogenes* and *S. aureus*) and gram negative bacteria

(*E. coli*, *P. aeruginosa* and *S.* Typhimurium). Moreover, the nanoparticles didn't demonstrate toxicity to RAW 264.7 cell line. Costa et al. (2017) produced nanoparticles from the non-toxic poly chitosan. The particles demonstrated bactericidal activity and anti-adhesive activity. Moreover, they reduced biofilm formation by the Methicillin-susceptible and Methicillin-resistant *S. aureus* strains. The silver nanoparticles produced by Kyaw et al. (2017) were able to inhibit biofilm formation by *S.* Typhimurium, *B. subtilis*, *S. aureus*, *P. aeruginosa* and *E. coli* DH-5α at concentration equivalent to 6.25 ppm. Moreover, they destroyed *Salmonella*, *Pseudomonas*, and *B. subtilis* biofilms at concentrations ranging from 25-50 ppm. Ramachandran and Sangeetha (2017) assessed the antibiofilm activity of silver nanoparticles against *E. coli*, *K. pneumonia*, *P. aeruginosa*, *P. mirabilis* and *A. baumanni*. The AgNPs effectively restricted biofilm formation of the tested bacteria within range of 12.5 – 100 µg/ml. Ravindran et al. (2017) also tested AgNPs synthesized using *V. zizanioides* aqueous root extract which turned out to be an ideal anti-QS and antibiofilm agent against *S. marcenscens*. Oliver et al. (2018) prepared AgNPs using catechin, cat-borax or polycat. Silver nanoparticles prepared using polycat demonstrated superior antibacterial and enhanced antibiofilm activity against *P. aeruginosa* biofilms. Li et al. (2018) reported a novel polymeric NPs (block copolymer Nanoparticles) that can diffuse into the biofilms, and cause dispersal of preformed biofilms upon binding to the bacterial cells of various clinically multidrug resistant gram positive bacteria including *S. aureus*, *Enterococci* and *E. faecalis.* Slomberg et al. (2013) tested the effect nitric oxide (NO) -releasing silica nanoparticles shape on *P. aeruginosa* and *S. aureus* biofilms. The study reported that rod shaped NPs were more effective in delivering NO to the biofilms and induced greater antibacterial action when compared to spherical shaped ones. The antibiofilm effect of nanoparticles is attributed to their antibacterial characteristics as well as to other properties (extra small sizes, shapes and surface charges) which result in increased penetration ability and makes them potent drug delivery agents (Hu, 2017).

Photo-Dynamic Therapy

Photodynamic therapy (PDT) is based on the application of a nontoxic photosensitizer (PS) that can be activated upon exposure to a specific wavelength. Such activation results in cytotoxic reactive oxygen species that can directly damage sub-cellular components. PDT has a broad spectrum activity against biofilm microorganisms including resistant pathogens. Photosensitizers have been suggested to exert their effect by destroying either the components of the biofilm matrix, cell surface or intracellular damage after penetrating the cytoplasmic membranes (Hu et al., 2018).

Misba et al. (2016) conjugated a photosensitizer toluidine blue O (TBO) with silver nanoparticles (AgNP). The conjugate inhibited *S. mutans* biofilm upon exposure to laser light (630 nm). Upon comparison to TBO when applied alone, the conjugate increased the leakage of cellular constituents and resulted in more evident down regulation of biofilm related genes. Pourhajibagher et al. (2016) characterized the effect of sublethal doses of PDT using Toluidine Blue O (TBO) Methylene Blue (MB) and Indocyanine Green (ICG) on *E. faecalis* biofilms. The sub lethal dose reduced biofilm formation up to 22.6%, 19.5% and 42.8% respectively. The obtained results indicate that ICG-PDT demonstrated higher antibiofilm activity when compared to other photosensitizers. Chiniforush et al. (2016) tested the effect of Indocyanin Green (ICG) on *E. faecalis* biofilms. Photodynamic therapy mediated through ICG significantly reduced bacterial counts and inhibited biofilm formation. Beytollahi et al. (2017) assessed the effect of PDT with Toluidine Blue O on *S. mutans* biofilms. The study demonstrated that 0.1 mg/ml TBO-PDT resulted in biofilm inhibition equivalent to 63.87%. Akbari et al. (2017) reported that the efficacy of PDT depends on the type, concentration and incubation time of photosensitizer and laser parameters including wavelength power density and time of eradication. Haris and Khan (2017) reported that selenium nanoparticles-toluidine blue O conjugate demonstrated 2 fold higher antibiofilm activity (60% Inhibition) against *S. mutans* when compared to TBO alone upon activation by diode laser. Similarly, the Indocyanine Green-Nano-Grahene Oxide conjugate (NGO-ICG) synthesized by Akbari et al. (2017) inhibited *E. faecalis* biofilms by 99.4%, which was higher than that observed when ICG was used alone (21%). Diogo et al. (2017) assessed the efficacy of PDT with the Zn (II) chlorine 6 methyl ester (Zn (II) e6Me) against mono species and mixed biofilms of *C. albicans* and *E. feacalis*. The treatment was able to remove around 60% of the biofilm's mass once activated by the red light. Pourhajibagher et al. (2018) also targeted the biofilms of

root canal-infecting microorganism *E. faecalis*, using curcumin (Cur) and Indocyanin Green as photosensitizers. The CUR and ICG mediated PDT reduced the bacterial biofilm by 83.6% and 75.2% respectively. Nemezio et al. (2017) tested PDT against *S. mutans* biofilms using diode laser combined with Methylene Blue. PDT weakened the biofilm as it lowered concentration of its intra and extra cellular polysaccharides. Misba et al. (2018) attributed the antibiofilm activity of the photosensitizers to their ability to produce singlet oxygen. TBO, which recorded higher singlet oxygen production, demonstrated better antibiofilm activity against *S. mutans* when compared to that of MB.

Anti-Quorum Sensing

Many studies reported the vital role of quorum sensing in regulating biofilm formation. Therefore, targeting the bacterial communication system remains a convenient approach to block biofilms formation. Rahman et al. (2017) evaluated the anti-quorum activity of the *Amomum tsaoko* fruit extract against different bacterial biofilms. The extract at concentration of 4 mg/ml inhibited the biofilms of *S.* Typhimurium, *S. aureus* and *P. aeruginosa* by 51.96%, 47.06% and 45.28% respectively. The extract constituents are thought to accelerate the activity of proteins with or HD-GYP domains which regulates c-di-GMP. The study also reported that tsaoko extract contains tsaokoaryline. The active ingredient is suspected to affect gene expression, hence, inhibit biofilm formation and swarming motility. Zhou et al. (2018) reported the effect of Hordenine, a dietary phenolic phytochemical from sprouting barley, as an anti-quorum sensing agent against *P. aeruginosa*. Hordenine resulted in a concentration dependent reduction in AHLs (acyl homoserine lactones) production as well as in biofilm formation. The bacterial biofilm was reduced by 30% while preformed biofilms were reduced by 23%. The correlation between AHL and QS related genes expression wasn't strong since biofilm formation is a complex process and is regulated only partially by quorum sensing. Singh et al. (2017) extracted a quorum sensing inhibitor from *Delftia tsuruhatensis* SJ01, a gram negative bacterium isolated from the rhizoshpere of *Cyperus laevitagus*. Even though the extract didn't demonstrate any toxicity against the bacterial cells, yet it exhibited antibiofilm activity against *P. aeruginosa* PAH and *P. aeruginosa* PAO. The antiadhesive effect was attributed to changes in the topography of the biofilm. Li et al. (2017) reported that the disruptive effect of Anthranilate on *P. aeruginosa*, *Vibrio vulnificus*, *B. subtilis*, and *S.* Typhimurium. The tryptophan degradation product, anthranilate, reduced c-di-GMP level and increased the dispersal of bacterial cells due to enhanced swimming and swarming motility. On the other hand, the inhibition of non-flagellated *S. aureus* biofilms, which has no c-di GMP signalling, was attributed to decreased slime production. Skogman et al. (2016) screened 465 natural and synthetic compounds in search for quorum sensing inhibitors. Flavones, which were the most potent quorum sensing inhibitors, inhibited the transition of *E. coli* and *P. aeruginosa* strains from microcolonies to mature biofilms. Zhang et al. (2017) investigated the effect of MomL, an enzyme that has been reported to degrade different N-acyl homoserine lactones (AHLs) of various gram negative pathogens. The study recorded a reduced biofilm formation and increased susceptibility to different antibiotics when tested on two nosocomial pathogens *P. aeruginosa* and *A. baumannii*.

Phages

The interest in phages as antibiofilm agents is supported by their appealing features. Kumaran et al. (2018) investigated the synergistic effect of the lytic phage SATA-8505 with different antibiotics (cefazolin, vancomycin, dicloxacillin, tetracycline and linezolid) against *S. aureus* biofilms. A significant reduction in biofilms was recorded when phage treatment preceded antibiotics. Such results illustrate the synergistic effect of combined treatment. Similarly, Chaudhry et al. (2017) also observed the importance of treatment sequence of phage-drug combinations. The study reported that treatment with phages before drugs achieved maximum killing in *Pseudomonas* biofilms. Melo et al. (2018) characterized a new broad host range bacteriophage vB_sauM-LM12 (LM12) which significantly reduced the number of biofilm viable cells. Moreover, the purified encoded endolysins recorded one order of magnitude reduction in the biofilm's biomass during prolonged periods of treatment.

Phages can simply diffuse into the biofilms or secrete enzymes (such as polysaccharide depolymerase) which affect the biofilm architecture (Harper et al., 2014; Abedon, 2015). For example, T4 phage bind to their receptors on *E. coli* surface, replicate within the biofilm cells and cause their lysis, which compromise the integrity

of biofilm matrix. On the other hand, genetically engineered phages that can secrete biofilm degrading enzymes have been developed (Sadekuzzaman et al., 2015; Gutierrez et al., 2016). Lu and Collins (2007) cloned dispersin (dspB) gene into an *E. coli* specific phage T7. The engineered phage demonstrated higher antibiofilm activity when compared to its non-genetically engineered counterpart. The engineered phage resulted in 4.5 order decrease in biofilm cell counts which was about two orders of magnitude higher than that recorded by control T7 phage.

Antimicrobial Peptides

Costa et al. (2018) tested the antibacterial and antibiofilm effect of antimicrobial peptide produced by *Bacillus* sp. P34 against both *E. faecalis* and *S. aureus*. The tested antimicrobial peptide demonstrated an antibacterial effect against the planktonic *E. faecalis*. Moreover, the ability of the bacteriocin like antimicrobial peptide BLS P34 to reduce the percentage of adhered *S. aureus* cells by 46.9% suggested the presence of antibiofilm effect. Similarly, Guo et al. (2017) reported an antimicrobial activity of human cationic antibacterial peptide hCAP18/LL-37 as well as its strong inhibitory and dispersal effect against the drug resistant *A. baumanni* biofilms. Cao et al. (2018) designed two types of synthetic peptides that were strongly coupled by dopamine. The modified peptides demonstrated inhibited *S. aureus* biofilms formed on stainless steel surfaces. Such findings provide valuable information for antifouling material research and can find applications as antibiofouling agents in ships and medical implants. Dawgul et al. (2014) synthesized different antimicrobial peptides and tested their antimicrobial and antibiofilm effect against several gram positive and gram negative bacterial strains. The antibiofilm activity of synthetic antimicrobial peptides was recorded against gram positive strains (*S. aureus S. epidermidis S. pneumoniae* and *S. pyogenes*) at concentrations equivalent to (32-64 mg/l). Kim et al. (2018) synthesized several antimicrobial peptides, based on a parent peptide Hp1404. The synthetic antimicrobial peptide had improved activity and reduced toxicity. One of the synthesized peptides Hp1404T1e exhibited a strong antibacterial and antibiofilm activity against the multidrug resistant *P. aeruginosa*. The antimicrobial peptides might have exerted their effect due to their ability to bind strongly to the lipopolysaccharides and kill bacteria through membrane disruption. Another explanation resides in their ability to enter the bacterial cells and interact with their DNA. Almaaytah et al. (2018) demonstrated an antibiofilm effect of the ultra-short antimicrobial peptide UP-5 against several multidrug resistant gram positive and negative bacteria. The MBEC value recorded was equivalent to (20 µM) which is higher than MIC value by two folds. Zaptoczna et al. (2017) tested different synthetic AMPs against methicillin susceptible and methicillin resistant *S. aureus* biofilms. Among the AMPs tested, D-Bac8c variant maintained the highest antibiofilm activity against mature biofilms with MBC equivalent to 256 µg/ml. Gordya et al. (2017) studied the antibiofilm activity of the antimicrobial peptide complex produced by blowfly *Calliphora vicinia* maggots. The complex, named FLIP7, was able to destroy the biofilm matrix of several human antibiotic resistant pathogens such as *E. coli*, *S. aureus* and *A. baumanni*. Mohamed et al. (2017) designed a more potent and less toxic antimicrobial peptides termed D-RR4. It demonstrated high antimicrobial activity as well as antibiofilm activity against drug resistant *P. aeruginosa* and *A. baumannii*. The antibiofilm activity caused by the antimicrobial peptides (AMP) can be attributed to their bactericidal effect. Yet, other mechanisms have been suggested, these include: 1-interfere with adhesion through binding to biomaterial surfaces or bacterial surfaces. Such event prevents interactions between bacterial cells and the surface. On the other hand, AMP can bind to mature biofilm bacterial cells and detach them from mature biofilms. The ability of antimicrobial peptides to bind to the Exopolysaccharide matrix also affect the integrity of biofilm architecture. 2: antimicrobial peptides can also interfere with gene expression such as the genes that control motility, stringent response, matrix synthesis and other genes that are involved in the sessile life style (Batoni et al., 2016).

Enzymes

Enzymes compromise the physical integrity of EPS through degrading the proteins, carbohydrates and lipids components (Kaplan, 2010; Sadekuzzaman et al., 2015). PelAh and PslGh, two glycoside hydrolases, were utilized to degrade the key component of *P. aeruginosa* biofilms, Pel and Psl polysaccharides. The enzymes were able to inhibit biofilm formation and reduce pre-existing biofilms by 58% to 94%. Moreover, the non-cytotoxic enzymes

demonstrated synergistic effect when combined with colistin antibiotic (Baker et al., 2016).

Vaikundamoorthy et al. (2018) used the thermostable amylase enzyme, extracted from *Bacillus cereus*, against *P. aeruginosa* and *S. aureus* biofilms. The highest biofilm inhibitory effect was equivalent to 40 -60 % at 20-25 µl enzyme concentration.

Kalpana et al. (2012) and Hogan et al. (2017) used several enzymatic agents either alone or in combination with antibiotics (vancomycin and rifampicin). Synergistic effect was recorded especially when lysostaphin and serrapeptase were tested against methicillin resistant and methicillin susceptible *S. aureus* strains.

Torelli et al. (2017) tested the synergistic effect of vancomycin with two biofilm matrix degrading enzymes (alginate lyase and deoxyribonuclease I) against *E. faecalis* and *E. faecium* biofilms. The combination treatment affected the biofilm structure, cell viability and reduced MBEC (minimum biofilm eradication concentration) of both *E. faecalis* and *E. faecium*. The extra cellular matrix constituents function as a diffusion barrier. Hence, combination approaches with antibiotics allow their penetration upon degradation of the biofilm matrix.

The activity of recombinant nucleolytic enzymes, derived from the genome of the marine bacterium *C. amphilecti* KMM296, was tested against *P. aureginosa* and *S. aureus* biofilms. The study reported that the highly active alkaline phosphatase CmAP can be exploited as an antibiofilm agent. The enzyme had significant effect at a low concentration equivalent to 2.5 units per ml (Balabanova et al., 2017).

Nasayif et al. (2016) isolated a *P. stutzeri* strain which produced significant amounts of pectinase. The treatment of *P. aeruginosa* biofilms with the purified enzyme resulted in anti-adhesive and anti-biofilm effect equivalent to 72% and 37% respectively. Lesser antiadhesive activity (equivalent to 67% and 53%) and antibiofilm activity (equivalent to 30% and 28%) were recorded against *E. faecalis and S. aureus* biofilms respectively.

Mohamed et al. (2018) tested the efficiency of papain enzyme against the biofilms of different *K. pneumonia* strains. The proteolytic enzyme recorded a maximum inhibitory activity equivalent to 56% and 59% and eradication ability equivalent to 54.9 % and 55.6% when applied at two different concentrations (50 and 100 mg/ ml) respectively.

Snarr et al. (2017) produced recombinant hydrolase domains $sph3_h$ from *A. fumigatos* and $PelA_h$ from *P. aeruginosa*. The purified enzymes demonstrated antibiofilm activity only against *A. fumigatos* biofilms which suggested that glycoside hydrolases can exhibit cross-kingdom activity. In a new approach, proteases immobilized on a polypropylene surface inhibited *C. albicans* biofilms. The N hydroxysuccinimiede proteases were covalently linked with glutaraldegyde or N-di isoroycarbodimide and plasma treated polypropylene surfaces. The coated surfaces recorded dispersal activity up to 55% higher than controls (Glutaraldehyde-linked) (Andreani et al., 2017).

Watters et al. (2016) tested several enzymes (lysostaphin, α-amylase, bromelain and papain) against *S. aureus* biofilms. The enzymes reduced the biomass of biofilms by 76, 97, 98 and 98% respectively. They caused detachment of the biofilm exopolysaccharide as well as bacterial cells from the growth surface. Even though the dispersal effect of lysostaphin was less visible, yet its ability to damage the bacterial cells was evident.

Trypsin, α-mannosidase and β-mannosidase enzymes were also tested for their antibiofilm activity against *P. aeruginosa* burn wound infections. All the tested enzymes were able to destroy the biofilms and reduce ceftazidime MBECs. Moreover, trypsin demonstrated no cytotoxic effect on A-431 human epidermoind carcinoma cell lines. Such data makes trypsin a better candidate combat *P. aeruginosa* burn wound infections (Banar et al., 2016).

Baidamshina et al. (2017) treated *S. aureus* and *S. epidermidis* biofilms with ficin, a non-specific plant protease. The study recorded a 2 fold and 6 fold decrease in biofilm thickness when ficin was used at concentrations 10 µg/ ml and 1000 µg/ ml respectively. The protease also potentiated the effect of ciprofloxacin and bezalkonium chloride which are used to suppress viable *Staphylococci* b.

Aptamers

Aptamers are single stranded DNA or RNA sequences that can specifically bind and often inhibit their targets. Only a few studies have investigated aptamers as antibiofilm agents. In an approach to block the motility of flagella as a potential strategy to inhibit biofilm formation, Ning et al. (2015) developed a single stranded DNA aptamer that specifically targeted *S.* Choleraesuis flagellin protein. The characterized aptamer inhibited the early attachment by

restricting cellular aggregation and production of mature biofilms. Moreover, flagellin aptamer demonstrated synergistic effect with ampicillin antibiotic. Cheng et al. (2017) further upgraded the flagella targeting aptamer by linking it with ampicillin. The conjugate had a distinctive antibacterial activity and higher antibiofilm activity when compared to those when either components were applied separately. The aptamer is thought to ensure facilitated entry of ampicillin into the biofilm which decreased its tolerance to the antibiotic. Moreover, loss of bacterial motility due to fli aptamer can also result in decreased adherence to the matrix surface. Moreover, the developed aptamer might have also served as an antibiotic carrier that can help ampicillin to penetrate the biofilm, eradicate its cells and overcome biofilm tolerance to drugs. Wang et al. (2017) developed an aptamer that targeted *P. aeruginosa* biofilms. The aptamer which acted as a targeted delivery agent was used to develop two complexes, aptamer-SWNT (Single-walled carbon nanotubes) and aptamer-ciprofloxacin-SWNT. The former complex caused a higher biofilm inhibition by 36% when compared to SWNT alone. The three-component complex demonstrated higher antibiofilm activity than that when the complex components applied separately or as a two - component complex. Mao et al. (2018) targeted *S.* Typhimurium biofilms with Graphene oxide and Graphene oxide aptamer conjugates. The ST-3-GO conjugate inhibited and dispersed biofilms within 93.5% and 84.6% respectively. ST-3 aptamer might have facilitated the entry of GO and caused a decrease the cellular membrane potential.

Conclusion

Prevention of microbial biofilms in food and health care setting is inevitable to limit food borne out breaks, device acquired infections and recurrent infections. Taking into consideration that traditional methods have lost their efficiency novel approaches are required.

Biofilm formation is a complex multifactorial process that has not been full elucidated yet. Better understanding of the interactions leading to biofilm formation would be a step forward to develop more efficient and practical antibiofilm approaches. While designing antibiofilm surfaces seem to be an appealing approach, targeting the key components of microbial biofilms represent another strategy. The development of antibiofilm agents against different microbial targets and their subsequent application as adjuvents with antimicrobial agents seems to be more efficient.

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