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Review Article

Bacterial Biofilms and Application of Plasma Activated Liquid for Inactivation of Biofilm-Associated Diseases

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Abstract

Microbial challenges in the food industry and clinical sectors have become a matter of concern and a subject for expansive research. The noxious and increasing resilience towards existing biocidal approaches has made it more strenuous to eliminate biofilms and disinfect surfaces. The prevalence of biofilms on food contact surfaces and food products can be very dangerous and continues to be a health threat. One promising strategy to decontaminate food contact surfaces and food products is the application of non-thermal or even cold plasma. It is an expeditiously advancing field to increase the efficiency of bacterial biofilm treatment and control. Cold plasma is essentially an ionized gas comprising of charged particles, reactive species, UV photons, an electric field along other elements. A novel method to implement this technology is by utilizing plasma-activated liquids (PAL) to inactivate a wide variety of microorganisms. PAL is produced by discharging the plasma in the liquid leading to the transference of the reactive species into the liquid. The ease of administration, low-cost treatment, and environmental safety are a few of the main reasons to adopt this strategy.

A lot about the optimum parameters of the technique is unresolved regarding biofilm inactivation by PAL, and the search for an ideal approach to disinfect (delicate) food (contact) surfaces is still on.

The primary goal of this review article is to understand food safety and list the effects of PAL on biofilms and their applications in the food industry. Secondly, the process of inactivation by PAL and its advantages over conventional methods is also explained in this article.

Keywords: Plasma-Activated Liquids (PAL); Biofilms; *E. coli, Listeria monocytogenes and Salmonella Typhimurium*

Abbreviations

EU: European Union; UV: Ultraviolet; PAL: Plasma Activated Liquid; PAW: Plasma Activated Water; GI: Gastro-intestinal; EPEC: Enteric Pathogenic *E. coli*; ExPEC: Extraintestinal pathogenic *E. coli;* STEC: Shiga Toxin-Producing *E. coli;* UPEC: Uropathogenic *E. coli;* UTI: Urinary Tract Infection; HIV: Human Immunodeficiency Virus; SCV: *Salmonella* Containing Vacuole; LPS: Lipopolysaccharides; SP: *Salmonella* Pathogenicity; EPS: Extracellular Polymeric Substances; TSB: Tryptic Soy Broth; CaMHB: Cation-Adjusted Mueller Hinton Broth; DNA: Deoxyribonucleic Acid; c-di-GMP: Bis-(3'-5')-cyclic Dimeric Guanosine Monophosphate; FDA: Food and Drug Administration; DBD: Dielectric Barrier Discharge; OAUGDP: One Atmosphere Uniform Glow Discharges Plasma; ROS: Reactive Oxygen Species; RNS: Reactive Nitrogen Species; RNA: Ribonucleic Acid

Food Safety

Some major problems for the food industry are the bacterial pathogens *Listeria monocytogenes, Salmonella Typhimurium, Escherichia coli*, and *Campylobacter jejuni* due to their prevalent existence and the ability to form highly resistant biofilms. Inefficient disinfection of food contact surfaces and products is potentially harmful and can lead to rare and critical food-borne infections called listeriosis, salmonellosis, colitis, and campylobacteriosis [1]. *Listeria*

was responsible for 2,621 confirmed invasive human infections in the year 2019 according to a study conducted in 28 member states of the EU. The fatality rate was 17.6% which makes listeriosis a serious concern for food safety. *Salmonella* on the other hand accounted for 87,923 cases in humans in the EU in the same year. Salmonellosis was the second most commonly reported gastrointestinal infection followed by campylobacteriosis according to the 2019 data affecting more than 220,000 people. Additionally, a total number of 8313 cases of *E. coli* infections were reported in 2019 in the EU, majorly affecting children between 0-4 years. *Mycobacterium bovis, Brucella, Trichinella,* and *Echinococcus* are some of the other commonly reported zoonotic agents responsible for food-borne outbreaks in the EU [2].

Listeria: Background and characteristics

L. monocytogenes has been a major challenge for food technologists for a long time due to its high resistance to environmental stress as well as high fatality rate. Biofilms of the microorganism are centrally responsible for its pathogenic nature and research is underway to inactivate or prevent the presence of this bacterial pathogen [3].

It was introduced by Murray., *et al*. [4] and was named *Bacterium monocytogenes* after monocytosis was observed in infected rabbits and guinea pigs. It was relabelled *Listerella hepatolytica* in 1927 by Pirie and received its current name by him in 1940 [3].

It is a Gram-positive rod, non-spore-forming, facultative anaerobe approximately 0.5-4 micrometers in size existing intracellularly. It uses peritrichous flagella (Figure 1) which provides it with a distinct motility in cold temperatures. It can withstand environmental stress like low temperature, high concentration of NaCl, and low pH to a certain extent. It has been shown to exhibit methods of survival and adaptation like biofilm formation and quorum sensing [5]. It is widely present in food, water, wastewater, soil, decaying vegetation, silage, and animal and human feces. It is frequently found in food products like fish, meat, soft cheese, and raw vegetables [2]. It has been isolated from goats, sheep, and poultry but it does not show a dominant presence in wild animals [3].

Listeriosis

It is a rare but potentially fatal disease caused by the bacteria *L. monocytogenes* which mostly targets pregnant women, newborns, and immunocompromised individuals. The symptoms are generally muscle aches, diarrhea, fever, and fatigue. In severe cases, it can lead to abortion, meningitis, or encephalitis. Although it is a critical illness and necessitates medical attention, it can go undiagnosed in some of the infections specifically in cases of stillbirth or miscarriages as it cannot be detected in routine cultures [2].

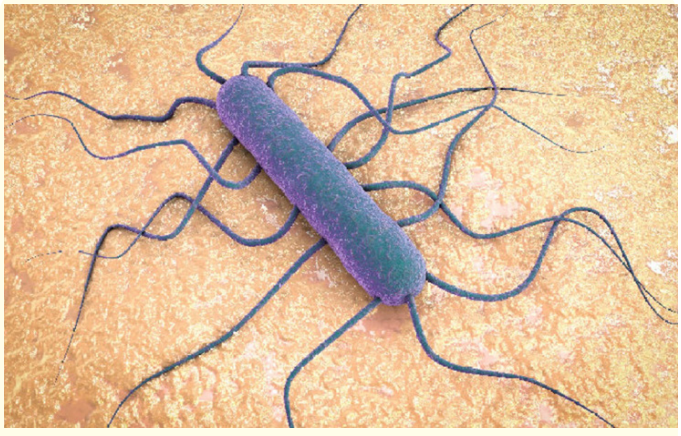

Figure 1: *Listeria monocytogenes.*
Source: Millipore M. (2019). Compliant Detection of *Listeria monocytogenes* in Food & Environmental Samples. https://www.rapidmicrobiology.com/news/compliant-detection-of-listeria-monocytogenes-in-food-environmental-samples

The prime route of infection is through the consumption of contaminated food products by the host. In the case of healthy individuals, the bacterial pathogen mostly ends up in the liver or spleen of the host as a huge number of microphages attack the bacterial pathogen as soon as it reaches the bloodstream as represented in Figure 2 [6]. In individuals with a weak immune system, the bacterial pathogen escapes the innate immune response and continues to divide and replicate. Its ability to divide in the cytosol of the infected host cells and spread to other cells protects the bacteria from humoral immune response as well. This enables the microorganism to reappear in the bloodstream and to cause severe and fatal systemic and central nervous system ailments [7].

L. monocytogenes uses several different elements to facilitate invasion of the host cells. It uses D-galactose from its teichoic acids to initiate macrophage phagocytosis by binding to polysaccharides present on the macrophages. It also uses internalin proteins A and B to bind to the cellular receptors to access the host cell via the indirect zipper mechanism. In this mechanism, two surface proteins of the bacteria (internalins) function as ligands to interact with the host cell receptors called E-cadherin and Met. This interaction leads

to ubiquitination of the receptors followed by recruitment of Cathrin, the protein centrally responsible for endocytosis of the bacterial cells [8]. The bacteria are encapsulated by an acidic organelle called phagolysosome following phagocytosis. The bacteria use the exotoxin listeriolysin O to disintegrate the vacuole membrane which enables it to replicate in the cytosol of the host cell. The next step in the bacterial invasion is migration to surrounding cells by using the cytoskeleton of the host cell and infecting other host cells intracellularly [9].

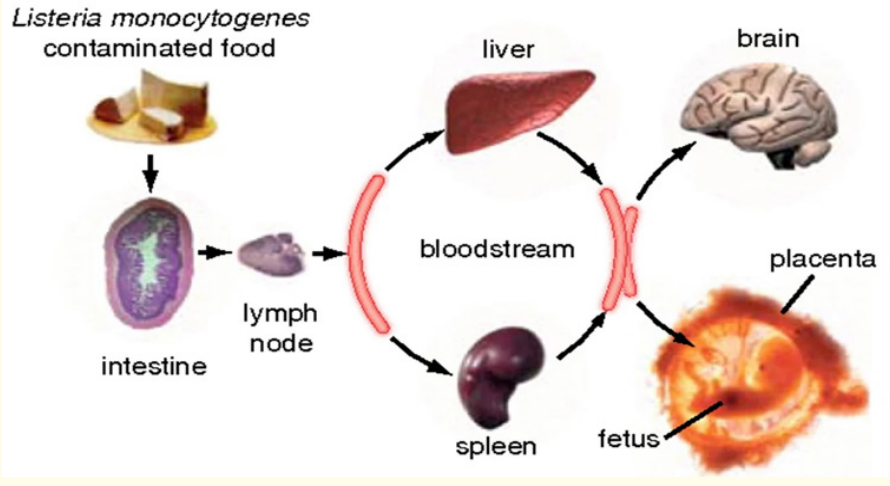

Figure 2: Schematic representation of Listeria infection.

Source: Cossart P. (2011). Illuminating the landscape of host-pathogen interactions with the bacterium *Listeria monocytogenes.* https://doi.org/10.1073/pnas.1112371108

Salmonella: Background and Characteristics

Salmonella is a serious concern for food safety all around the globe and has the highest cost burden annually in many developed and developing nations. The main source of salmonellosis has been attributed to the consumption of contaminated poultry products like chicken, turkey or eggs majorly by human and animal feces [10]. It is a Gram-negative, rod-shaped, facultatively anaerobic bacteria of the family Enterobacteriaceae. It is a gastrointestinal pathogen equipped with peritrichous flagella responsible for causing gastroenteritis, a food-borne diarrheal disease (Figure 3). It is approximately 2-5 microns in length and 0.5-1.5 microns in width and is the causative agent of the highest number of food-borne outbreaks in the EU [11]. This bacterial pathogen is known to reside in the intestinal tract of humans and farm animals. As these bacterial pathogens colonize the gastrointestinal tract, they are excreted in form of faeces and can potentially contaminate a number of places leading to infection of other host organisms [12].

Figure 3: *Salmonella Typhimurium.*

Source: Davis, C.P. (2021). Salmonella symptoms: food poisoning, other causes, and treatment. https://www.onhealth.com/content/1/Salmonella_outbreak

Salmonella has two major serotypes, typhoidal and non-typhoidal. The non-typhoidal strains cause gastrointestinal infections called salmonellosis and can be transferred from animal to human and human to human. On the other hand, typhoidal strains cause typhoid fever and can be transferred only from human to human [11].

Salmonellosis

Infection from *Salmonella Typhimurium* leads to the inflammation of the gastrointestinal tract, also called gastroenteritis. The symptoms are typically diarrhea, vomiting, fever, and abdominal cramps and usually last about 2-7 days [11]. Infections by *S.* Typhimurium are rarely severe in normal individuals but can be dangerous for people with a weak immune system like older people, infants, and immunocompromised persons. The infection normally takes around 6-72 hours to develop observable symptoms in infected individuals and can go undiagnosed in several cases because of its sporadic nature [13].

The mechanism of infection by *Salmonella* consists of four stages involving adhesion, invasion, SCV (*Salmonella*-containing vacuole) maturation, and bacterial replication. After ingestion of contaminated food, the bacterial cells reach the mucosal cells and Peyer's patches where they adhere to the host cells. To facilitate adhesion, the pathogen expresses factors like fimbriae, flagella, capsule, and lipopolysaccharides (LPS) which are encoded in six *Salmonella* pathogenicity islands [14]. The next step in bacterial pathogenesis is invasion of the host cell which is majorly regulated by SPI-1 genes also called the trigger process. In this step, the bacterial effectors initiate rearrangement of the mammalian cytoskeleton leading to ruffling at the host-pathogen interaction site which eventually accommodates invasion [15]. In *Salmonella Typhimurium*, the bacteria are enclosed in a phagosomal vacuole called SCV (*Salmonella*-containing vacuole) after entering the host epithelial cells [16]. In the next step of the infection process, these vacuoles undergo maturation enabling the movement of bacterial cells to a perinuclear region in proximity of the Golgi apparatus. This allows the pathogenic cells to capture nutrients from endocytic and exocytic transport vesicles involved in several transport pathways. In the final step, the bacteria start the replication process after being supplied with enough nutrients which leads to host cell apoptosis followed by systemic spread of the pathogen [17].

Escherichia coli

The bacterium *Escherichia coli* was first reported by Theodor Escherich in the late 19th century and belongs to the Enterobacteriaceae family and is facultatively anaerobic [18]. It is a gram-negative, rod-shaped, and non-spore-forming bacterium. It can be either non-motile or motile due to the presence of peritrichous flagella [19]. It is the most prevalent commensal inhabitant of warm-blooded animals, especially the gastrointestinal (GI) tract of humans, and a very important pathogenic microbe [18]. In its morbific form, *E-coli* is prominently responsible for enteritis, urinary tract infections, septicemia, meningitis, and diarrhea [20]. *E. coli* strains are divided based on virulence factors and host clinical symptoms. There are majorly two subcategories i.e., enteric pathogenic *E. coli* (EPEC) which has seven pathotypes, and extraintestinal pathogenic *E. coli* strains (ExPEC) which have three pathotypes. The main route of spread of the intestinal pathogen is via the fecal-oral route by ingestion of infected food or water [18]. The pathogenic form of the bacterium is responsible for a high number of nosocomial and community-associated disease spread. Due to strains with antibiotic resistance, *E. coli* has become a major global healthcare threat in its pathogenic form. *E. coli* is naturally resistant to several anti-microbial agents like penicillin G, precursory beta-lactams, and quinolones [21].

E. coli infections

Infections caused by *E. coli* vary in severity due to the presence of different types of strains. They can be acquired by consumption of raw or undercooked meat, untreated milk, vegetables, and fruits washed with infected water, contaminated water, or other beverages [22].

Pathogenic strains of *E. coli* are responsible for different types of diseases such as pneumonia, urinary tract infections, food poisoning, etc. In the majority of cases, abdominal cramps, diarrhea with or without blood, nausea and constant fatigue are the most common symptoms as shown in Figure 4. Some variants of *E. coli* produce a toxin called Shiga-like toxins resembling the ones synthesized by Shigella dysenteriae which in turn damages the lining of the human intestine. These strains are called STEC [Shiga toxin-producing *E. coli*] and are the most virulent diarrheagenic *E. coli* strains [22]. On the other hand, ExPEC is predominantly responsible for nosocomial and community-associated infections, whereas

uropathogenic *E. coli* (UPEC) is linked with urinary tract infections (UTIs) in humans responsible for 80% of the total number of infections caused [23]. Some variants like O127:H7 can cause very serious symptoms like cramps, vomiting, and bloody diarrhea and can be life-threatening if not attended to properly [22].

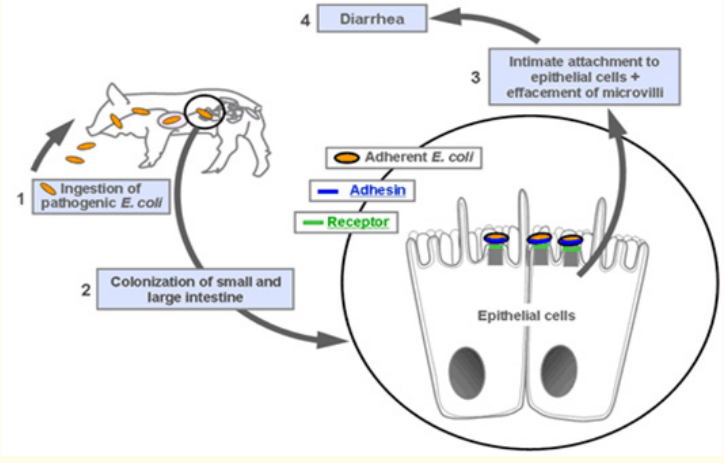

Figure 4: Pathogenesis of EPEC.

Source: Taming bacteria to promote animal and public health. University of Montreal. http://www.ecl-lab.ca/en/ecoli/pathogenesis.asp

Campylobacter jejuni

Campylobacters were discovered in 1886 by Theodor Escherich and were isolated from human feces and blood in 1972 by Dekyser and Butzler [24]. Members of the *Campylobacter* family are typically motile in nature via a single polar unsheathed flagellum present at either one or both ends of their cells. *C. jejuni* are S-shaped gram-negative rod-like bacteria. They are nonspore-forming non-saccharolytic bacteria with the activity of catalase, oxidase, and hydrolysis [25]. Thermophilic *Campylobacters* like *C. jejuni*, *C. coli,* and *C. lardis* are majorly linked to human gastrointestinal disease. According to a study conducted by Thomas., *et al*. (1999), *C. jejuni* is the most common species related to human illness and together with *C. coli*, accounts for 95% of all clinical isolates in the UK [26]. *C. jejuni* does not multiply in foods like other bacteria due to its high optimal growth temperature and hence does not cause large outbreaks of campylobacteriosis. Irrespective of the ability to multiply in food, *Campylobacters* are responsible for most of the intestinal infections caused globally [27].

Campylobacteriosis

The main route of invasion for this bacterium is ingestion of contaminated water and food. In addition to that, it enters the host intestine via the stomach acid barrier colonizing the mucous blanket and the epithelium of the distal ileum and colon as a result of that [28]. Due to the high number of infections in humans by antibiotic-resistant strains, the clinical management of campylobacteriosis has become very strenuous. The clinical symptoms can vary from being discreet to life-threatening depending on the individual's state of health. In cases like pregnancy, HIV, or susceptible immune system, campylobacteriosis can even lead to sepsis and death if not treated timely [29].

Biofilms and their role in the disease

Biofilms are defined as complex microbial consortia arranged in a three-dimensional structure containing multicellular communities formed of eukaryotic (e.g., fungi) and/or prokaryotic (e.g., bacterial) cells embedded in a matrix of extracellular polymeric substances [30]. Biofilms help the bacteria to tolerate harsh environmental conditions like low pH and high temperatures making, them more tolerant to environmental stress and more resistant to disinfecting agents and antibiotics, causing dangerous bacterial diseases like listeriosis and salmonellosis [31].

Biofilm composition

Biofilms are majorly composed of extracellular polymeric substrates and microbial cells. The matrix mainly consists of proteins, lipids, polysaccharides, and extracellular DNAs. Some non-cellular elements like minerals, corrosion particles, and clay or slit particles are also present depending on the environment during the biofilm development [32]. The extracellular polymeric substances are discussed in detail below. A number of media like tryptic soy broth (TSB) and cation-adjusted Mueller Hinton broth (CaMHB) are used to form biofilms in a lab environment and the composition differs according to the parameters like temperature, water content, pH, etc. [33]. Biofilms can grow on different types of surfaces like rocks, freshwater, or salty water and can use any kind of naturally occurring moisture as nutrients. The contents of the bacterial biofilm along with environmental conditions like temperature and pH determine the strength of its attachment and adhesion with the substratum. The amount of EPS and lipopolysaccharides (LPS) produced, and the presence of flagella and fimbriae are two examples of factors that determine the adhesion strength of the biofilm. The nature of the attachment is also dependent upon the charge of the substratum available. The presence of EPS and LPS promote biofilm adhesion on hydrophilic surfaces while the existence of fimbriae, flagella, and other cell surface polymers with non-polar sites promote attachment with hydrophobic surfaces [34].

EPS accounts for around 50-90% of the carbon content of the biofilm and the main constituent of it is polysaccharides. In the case of Gram-negative bacteria, the polysaccharides possess an overall ionic charge in most cases, and for Gram-positive cells on the other hand, the net charge is cationic. These charges play an important role in the binding of biofilm constituents within the matrix [35]. EPS in different types of biofilms also vary in their solubility and conformation, for instance, in some bacterial EPS, the structure has 1,3 or 1,4-β-linked hexose which makes them rigid and insoluble whereas other types of EPS molecules are readily soluble [36].

The formation of biofilm is dependent on several dynamics like shear stress, gene expression, motility, quorum sensing, etc. Another major parameter for the production of EPS is the nutrient content; the amount of nitrogen, potassium, phosphate, and carbon are important parameters for the development of EPS and biofilm [36]. Additionally, the EPS content also increases with the age of the biofilm in the suitable environment and the growth rate of bacteria can also determine the amount of EPS synthesized by the bacterial cells in the biofilm as it has been shown to have more EPS when the bacteria proliferate at a slow rate [37]. As the EPS is highly hydrated due to hydrogen bonding with hydrophilic polysaccharides, it prevents desiccation in natural biofilms. Apart from that, it also enables resistance against antibiotics possibly by binding to the antimicrobial agents directly [38].

Biofilm formation

The development of a biofilm is a highly dynamic process leading to the formation of a three-dimensional structure consisting of bacterial cells, EPS, flagella, pili, and other adhesive fibers [39]. The content of nutrients available not only determines the growth rate but also alters the composition of the biofilm modifying it to the given habitat. The components of a biofilm synthesize a vigorous network of bacterial cells with a matrix capable of cell-to-cell interactions, DNA exchange, and protection of the bacteria against desiccation, oxidation, predation, radiation and other harmful agents [40].

Biofilm attachment and maturation have several reversible and irreversible stages and a lot of parameters can vary depending on the species of bacteria. The first step is the encounter of bacteria with a surface majorly led by gravitational forces and hydrodynamic forces present in the surroundings [41]. Factors like pH, temperature, and nutrient medium determine the strength of adhesion and further development. It has been observed that it is relatively amiable for motile bacteria to overcome repulsive forces due to the presence of flagella [42]. In the initial phase of adherence, the attachment of bacteria with the surface is reversible, enabling it to detach and re-join the planktonic population depending thus on the availability of nutrients, and repulsive and hydrodynamic forces present [43]. Bacteria use adhesins, flagella, fimbriae, and pili to irreversibly bind to the surface in the later phase of the first stage [42].

As soon as the bacteria attach to a surface, changes in the gene expression direct a rise in up-regulating factors boosting sessility and formation of EPS. The second step essentially comprises of

maturation of the biofilm by increasing the amount of matrix and complexity of the structure. As mentioned before, the constituents of the EPS are varied depending on the species of the bacteria. To cite a few components, EPS of *Listeria* comprises mainly teichoic acids, fatty acids polysaccharides, and proteins potentially internalins [44].

Dispersion of the biofilm comprises the last step of biofilm development. In a mature biofilm, all components function together by exchanging and sharing products in order to stabilize and proliferate the community of cells in addition to providing the bacterial cells with a favorable environment to grow. However, after a certain period of maturation, the dispersal of biofilms can be advantageous for the bacterial cells to protect them from stress like fluid shear, collision, human intervention, and nutrient deficiency. The bacterial cells in the biofilm are capable of determining the suitable conditions to change into planktonic forms by sensing cues like the amount of oxygen, nutrients, toxic substances, and other stress factors [40]. A number of sensory systems keep track of small molecules and modify the gene expressions accordingly directing the biofilm to dispersion [45]. Factors like c-di-GMP and EPS degrading enzymes are shown to be the intermediates between sessility and motility [46]. In addition to that, surfactant molecules are formed to loosen the bonds between bacterial cells also leading to dispersal of the biofilm [47].

Biofilm removal

Bacterial biofilms are responsible for several health and hygiene problems around the globe which makes it essential to devise effective strategies for their removal from a number of products. Existing technologies such as rinsing, disinfecting, scrubbing, high-pressure cleaning, anti-microbial agents, UV light, acid exposure, and biocides are not suited for all kinds of food products as their efficiency and ability to maintain quality are not sufficient [48]. A huge number of researchers are working on introducing novel and efficacious techniques and technologies to combat biofilm formation. Several of these novel methods including ultrasound power, electric field in combination with biocides, UV radiation, anti-biofilm agents such as imidazole and indoles, magnetic fields with antimicrobial agents, bioactive compounds, enzymes and plasma have been assessed to eliminate biofilms from food, food contact surfaces and medical equipment. Different methods follow different mechanisms of action to eradicate the biofilm-like interruption in the quorum sensing pathways, hampering the EPS and adhesion mechanism [49].

The extra polymeric substance protects the cells inside the biofilm by inhibiting processes like neutrophil-mediated phagocytosis. The EPS not only plays a role in the pathogenesis of microbial infection but also imparts resistance against UV, metal toxicity, acid exposure, desiccation, pH gradients, antimicrobial agents, etc. [50]. Approaches that inhibit EPS growth and destabilize its production are shown to be potent in the eradication of biofilms also because they reduce the physical, physiological and passive tolerance of the bacterial biofilm as displayed in Figure 5. The presence of EPS prevents the anti-microbial agents from penetrating and coming into action giving more time for bacterial cells to become tolerant and provide physical tolerance as a result. The physiological tolerance is imparted by the metabolically inactive cells in the deeper layers of the biofilm matrix which have the ability to adapt to conditions like starvation, and ecological factors and exhibit stress-adaptive responses [51]. Enzymes present in the EPS play a major role in the neutralization of antimicrobial molecules providing it with passive tolerance. In addition to that, slow-dividing bacteria called persister cells are present in the biofilm that upregulate toxin-antitoxin genes leading to a lower cellular metabolism rate by blocking translation and enabling survival of the bacteria in the presence of antibiotics [52]. It has been observed that cell diversity and metabolism are important factors contributing to antibiotic resistance and as a result, biofilms with more than one species are less susceptible to antibiotics than single-species biofilms and are more resistant to removal treatment [53].

The initial two stages of the biofilm development process i.e., attachment of bacterial cells to a surface and development of the biofilm structure are targeted for inactivating it in most of the methods as these are critical for biofilm development. The internal cell-to-cell interaction in a bacterial biofilm is called quorum sensing and is acute for biofilm production and maturation. A high number of biofilm removal strategies involve disruption and inhibition of quorum sensing by blocking the inducers of quorum sensing to disable biofilm proliferation. Other mechanisms for biofilm removal involve substrate deprivation and membrane disruption [54].

An important aspect of cleaning biofilms is to take the type of surface used for attachment by the bacteria into consideration as a number of methods can cause corrosion on the surface leading to damage and degradation, specially by repetitive use [55]. Physical methods like high magnetic fields and ultrasound in association with organic acids and antimicrobial agents have shown biocidal effects and strategies involving low currents via electrodes also represented eradication by killing planktonic cells of Gram-positive and Gram-negative bacteria. It has been shown that applications of disinfectants after mechanical removal of microorganisms adhered to the surface increase the effectiveness of biofilm control. In addition to that, methods enabling mechanical or chemical breakage of EPS matrix are equally essential for proper biofilm inactivation [56].

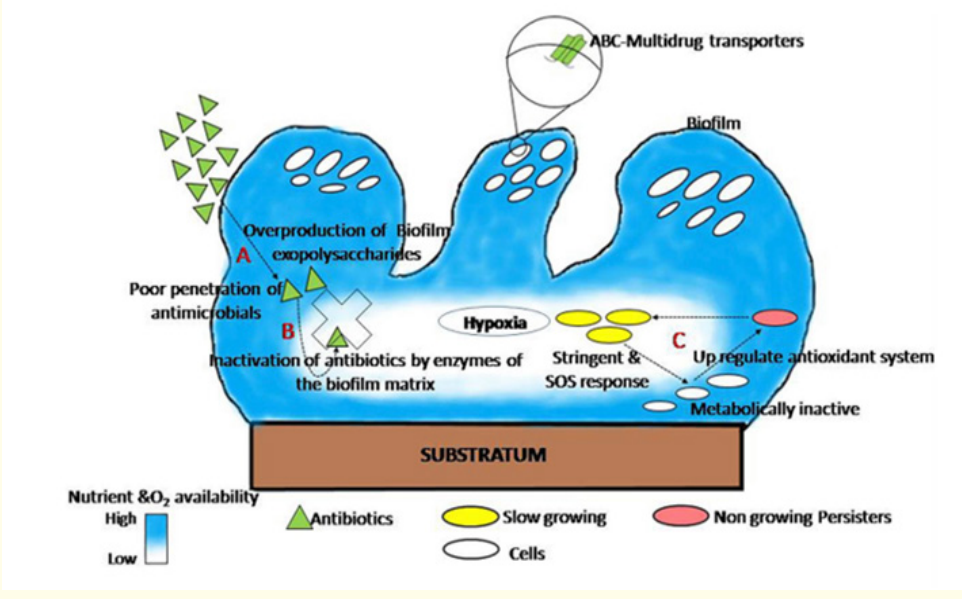

Figure 5: The general mechanism of biofilm tolerance to various antimicrobials.
(A) Physical tolerance: biofilm matrix limits the diffusion of antimicrobials (B) Passive tolerance: matrix enzymes inactivate the penetrated antibiotics molecules (C) Physiological tolerance: persister cells in the deeper layer of biofilm induce adaptive SOS response and thus become more tolerant [73].

Bioactive compounds like bacteriocins prevent the adhesion of bacteria to the food-contact surfaces. Bacteriocins are proteinaceous compounds exhibiting antimicrobial properties. A commonly used bacteriocin is the compound nisin, it has been approved by the FDA as a safe additive in food products to combat infections against *Listeria* and *Clostridium*. Additionally, enzymes were speculated to play a major role in the control of biofilms, specific enzymes are used depending on the microflora of the biofilm and their function in the degradation of EPS contributes to essential biofilm removal [57].

A relatively novel technique called Plasma Activated Liquid [PAL] has been researched vigorously to develop ways for biofilm control in the food and medical sectors. It uses the same principle as Cold Atmospheric Plasma [CAP] and is adapted in order to enable use on sensitive food surfaces and enable transportation and storage of liquids pre-treated with CAP making it easier and cost-efficient to use. A more detailed account of PAL and CAP is discussed in the following sections.

Cold atmospheric plasma

The term 'plasma' stands for a fractionally or completely ionized gas composed of electrons, photons and other atoms in their excited or ground state consisting of a net neutral charge. Due to its unique characteristics, plasma is also known as the fourth state of matter having higher energy level than that of gas. Plasma is mainly of two types; thermal and non-thermal depending on the temperatures used during its formation. Thermal plasma is generated at higher temperatures ranging between 2000-20000 K and pressure and exhibits a thermal equilibrium between the electrons and the heavy species. On the other hand, non-thermal plasma is developed in relatively low temperatures and pressure. It is of significance for the food industry due to its colder and moderate development conditions [58].

Cold Atmospheric Plasma (CAP) is essentially electrically energized matter composed of highly reactive species including gas molecules, positive and negative ions, free radicals, electrons and photons, and UV radiation at near-room temperatures (Figure 6). CAP has shown influential and coherent results in surface disinfection of raw produce like dried nuts and packaging material. It is a highly potent technology for sterilization of e.g., food as it uses safe temperatures during decontamination which prevents nutritional and quality damage to the intended products [58].

CAP: Characteristics and formation

There are various methods to synthesize cold atmospheric plasma, the most common way is to expose a neutral gas to a high-intensity electromagnetic field which results in the ionization of the gas. A wide range of electrical discharges are used to obtain cold plasma such as corona discharge, micro-hollow cathode discharge, gliding arc discharge, dielectric barrier discharge (DBD), etc. For applications concerning the food and biomedical industry, DBD and plasma jets are the most commonly used platforms [59]. These devices share the basic working principle, components, and materials. In both devices, a violet plasma is formed between the cathode and the anode, and one of the electrodes is covered with a dielectric material like quartz. Plasma jet devices require a carrying gas to sustain the synthesis of CAP while the DBD device forms plasma directly in the air. DBD has a wider plasma, and the sample is also a part of the discharge in this method [60].

The device used to generate CAP determines the inactivation efficiency and mechanism of action used to inactivate microorganisms. In direct treatment, the plasma discharge directly interacts with the sample, and all electrical charges along with short-lived species like hydroxyl radical (OH^-), nitric oxide (NO), and superoxide (O_2^{*-}) interact with the sample whereas, in indirect plasma treatment, the sample is placed at a distance which prevents electrical charges and short-lived species to come in contact with the sample. The long-lived species namely hydrogen peroxide (H_2O_2), ozone (O_3), nitrites (NO_2^-), and nitrates (NO_3^-) are responsible for a major part of the inactivation of bacterial biofilms in both direct and indirect treatment [61].

Other than the parameters of the plasma setup like power voltage, amount of oxygen and humidity, frequency of excitation, gas flow, type of gas, and treatment media, characteristics of the sample like bacterial serovar, attachment surface, and microbial load also determine the rate and efficacy of treatment by cold atmospheric plasma. Additionally, conditions of contamination like the age of biofilm and storage conditions also impact the inactivation process [62].

Potential applications of CAP are in the food industry due to its ability to treat biofilms in a dry disinfection process. It can be used for the decontamination of products like meat, poultry, dried milk, herbs, sprouted seeds, etc. as well as the packaging material used. A prominent reason for its potential as a disinfectant is its ability to prevent arching, therefore preventing damage to fragile surfaces such as fresh and pre-packaged produce [63].

Inactivation mechanism

Cold plasma has various sterilizing properties against bacterial infections like the removal of biofilms. The low-pressure plasma deteriorates lipids, proteins and DNA of the bacterial cells [61]. Another means that the plasma utilizes to degrade the bacterial cells is oxidation by reactive species like NO_2^-, O_3, OH^-, NO_3^-, etc. present

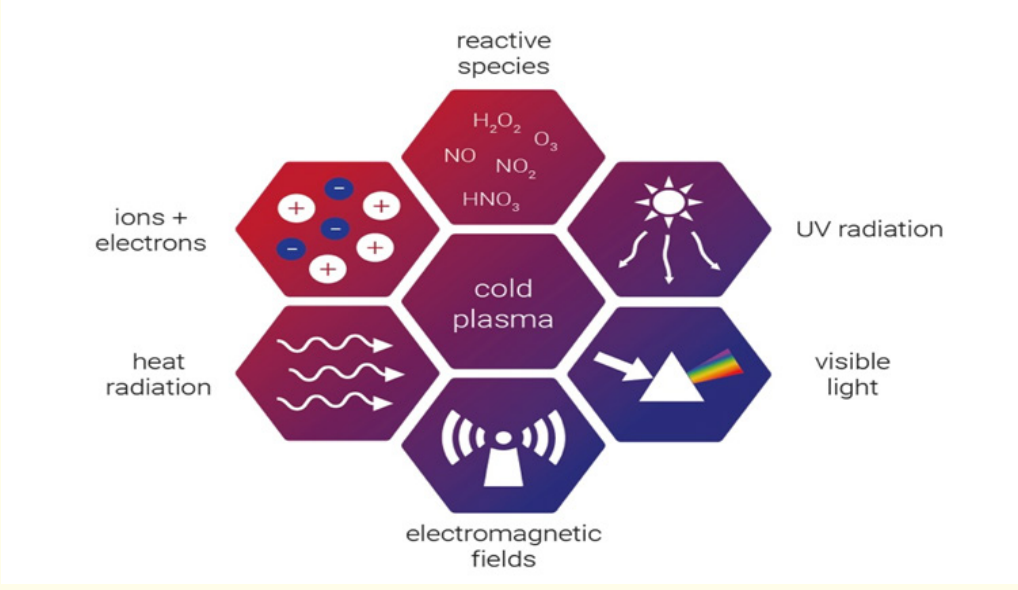

Figure 6: Composition of Cold Atmospheric Plasma.
Source: terraplasma. https://www.terraplasma.com/en/cold-plasma/

in its composition. These reactive species act on unsaturated fatty acids of the lipid bilayer and obstruct the biomolecule transport resulting in hampering of biofilm synthesis. The membrane lipids are attacked by reactive oxygen species and are intensely bombarded by the oxidizing agents due to their position alongside the surface of bacterial cells [64]. Additionally, the proteins of the cells are also ambushed by these species leading to cell leakage and denaturation [61]. The radicals present in the plasma strike the microorganisms enormously causing lesions on the surface leading to rapid destruction, this phenomenon is called "etching". Another course of action utilized by plasma is the accumulation of charges at the outer surface of cell membranes leading to electrostatic forces rupturing the cell wall [65]. One key route to disperse biofilms taken by plasma is the acidification of the biofilm matrix leading to degradation of biofilm [66].

Ultraviolet photons have been shown to act on dimerizing the thymine bases of DNA contributing to bacterial deformation. In a study conducted by Roth., *et al*. [67], it was concluded that UV-C radiation is a major element is the inactivation of bacterial biofilm. Another study by [68]. revealed that heat and UV do not affect the inactivation process highly and identified oxygen atoms as the key elements in biofilm eradication. The role of UV photons is in a contradictory state and more studies are required to get a better understanding [68].

The contact of the substratum with the plasma affects the efficacy of the treatment as mentioned above. It has been speculated that remote exposure of substratum with plasma reduces the efficiency of the treatment as the amount of heat reaching the target is decreased and all elements of CAP do not reach the sample [69].

Plasma activated liquids

Recent studies have shown the high potential and efficiency of cold atmospheric plasma to treat biofilms, one alternative approach to using CAP is by using plasma-activated liquids (PAL). PAL is an improved technique due to its transportability, storage, and flexible course of action which are key in industries like food and medicine [70]. PAL essentially uses CAP to activate solutions in an indirect manner and the samples are then treated with those liquids leading to log reduction of bacterial cells in biofilm and planktonic forms. This technique is more feasible for fresh produce and sensitive surfaces as its application is safe on the samples and prevents damage and injury [71].

PAL technology executes inactivation by employing reactive oxygen and nitrogen species that are synthesized during the CAP operation. The reactive species mainly nitrites, nitrates, and hydrogen peroxide are the resulting long-lived reactive species present in the solution. Application of the activated liquids on the target samples leads to the removal of bacterial cells. In addition to that, the low pH of the activated solutions also contributes to the inactivation process by creating a pH imbalance [71].

Formation of PAL

There are two ways to generate plasma-activated liquids; plasma discharge in the liquid or plasma discharge above the liquid surface (Figure 7). It has been seen that plasma discharge directly in the liquid has lower energy efficiency when compared with the gas plasma system. In order to have better energy efficiency, the latter is used, and the reactive species generated in the gas phase can be transferred to the liquid or the liquid-gas interface [72]. A number of electrode types like DBD, plasma jets, glow discharges, etc. are available to facilitate the formation of PAL through the gaseous phase which contacts the liquid surface. It has been reported that the distance between the electrode and plasma surface can be changed to selectively produce different reactive oxygen and nitrogen species according to Giichiro Uchida., *et al*. [73]. Solutions like phosphate buffer saline [PBS], phosphate buffer [PB], and water are used in order to generate PAL. The PAL setup comprises a number of parameters like the plasma power supply, discharge frequency, type of gas, distance between the electrode and the liquid, generation time, electrode configuration, rotations per minute etc. All these parameters determine the composition of the activated liquid like the aggregate of long-lived species, electrons, UV radiation, and pH [74]. Factors like the type of gas and power supply can be altered to customize PAL to suit to different application as shown by Girard., *et al*. [75]. PAL has also shown considerable activity when used after a few days of production indicating potential storage and transport capability. Factors like temperature and humidity also play a role in the efficiency of the PAL formed [70]. In an experiment performed by Ma., *et al*. (2015), strawberries inoculated with *S. aureus* were treated with PAL and it was discovered that the presence of short-lived reactive oxygen species was the most important agent in inactivation. It was also observed that application of PAL had no considerable effect on the color, firmness, or pH of the strawberries establishing that PAL is safe for application on sensitive and fragile food surfaces [76].

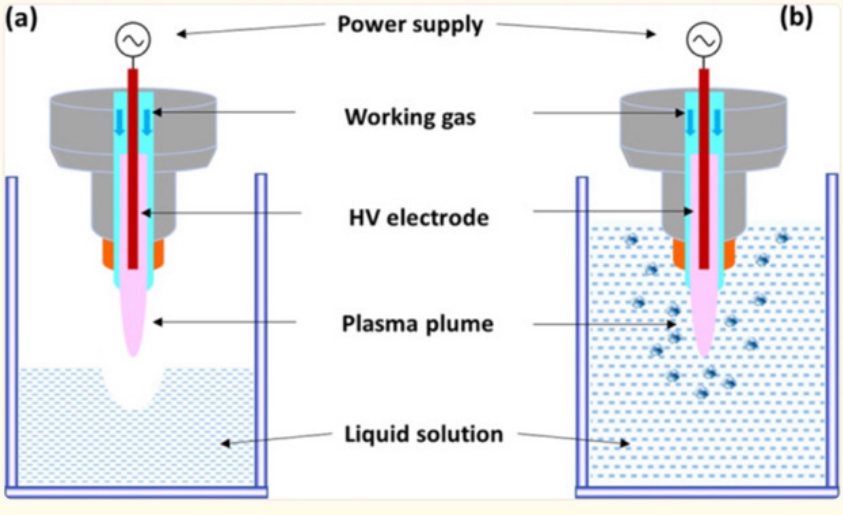

Figure 7: a) Discharge over the water surface with indirect contact of the plasma plume with the water surface b) and direct contact of the plasma plume with the water surface (107).

Inactivation mechanism

The key elements in the inactivation of bacterial biofilms are the long-lived reactive species namely hydrogen peroxide (H_2O_2), ozone (O_3), nitrites (NO_2^-), and nitrates (NO_3^-), and short-lived reactive species like hydroxyl radical (OH^-), nitric oxide (NO) and superoxide (O_2^{*-}). The short-lived species have an essential contribution in bacterial treatment in the food industry and fresh produce disinfection [77].

Amongst the long-lived species, hydrogen peroxide (H_2O_2) is one of the major constituents of PAL as it functions in cell oxidative stress, pathogen inactivation, and cell redox signaling pathways. It is formed in two ways; the direct transfer from the gaseous phase and the reaction of OH radicals dissolved in the gas phase [78]. Another important long-lived species is ozone (O_3), it is known to possess very high oxidizing potential when dissolved in an aqueous solution. Ozone carries out microbial inactivation and water purification in the eradication process and supposedly contributes 14% in bacterial removal. It is essentially produced by solubilizing gaseous ozone in liquid or by direct generation in the aqueous phase [79]. Additionally, nitrites and nitrates [RNS] are generated in PAL by the reactions and dissolution of N_2 and O_2 in gaseous plasma discharges. They have exhibited high eradicating activity in acidic conditions [76].

In the short-lived reactive species, hydroxyl radicals (OH^-) are generated by impact dissociation of electrons and by secondary reactions. They play a major part in microbial inactivation as they facilitate the production of other short-lived and long-lived species, therefore contributing to the anti-microbial activity of PAL. Another important short-lived species is nitric oxide (NO). It is a signaling molecule capable of penetrating cell membranes and organelles which leads to organelle damage and cell death [80]. The formation procedure for nitric oxide is not properly defined and more research is needed to get a better understanding. It has been shown in previous studies that it is synthesized in the gas phase, gas-liquid interface, and liquid phase. It has also been speculated that the formation of NO by the reaction of nitrogen species with O/O_3 in the liquid is crucial for the NO amounts present in PAL [81]. Moreover, another short-lived reactive species in plasma is superoxide (O_2^{*-}). Its production is mostly carried out by reactions between OH and H_2O and it reacts with other nitrogen species like N and N_2 leading to the formation of nitrites. They serve as intermediates in the production of long-lived species and are potentially vital for the antimicrobial activity of PAL [77]. The amount of oxygen present in the gas, the generation time, and the treatment time are important factors in the inactivation of bacterial cells by PAL [70].

The bactericidal activity of PAL is an accumulation of several RNS, ROS, low pH, UV radiation, and gas flow that leads to a collaboration of physical and chemical factors acting upon the bacterial cells. The gas flow along with the UV radiation acts on the cell membrane and leads to an etching and drying effect. The RONS cause damage to the DNA, proteins, RNA, and lipids of the bacterial cell [82]. In a study conducted by Naïtali., *et al*. it was observed that a synergistic activity of nitrites, nitrates, hydrogen peroxide, and low pH to treat bacterial biofilm shows similar results as when treated with PAL [83]. Studies are performed by mimicking the mechanism of action of PAL to observe the role of different components and reactive species in the PAL and their contribution to inactivation. A large number of factors can influence the removal activity of PAL like the generation conditions, type of media, type of set up, etc. The type of microorganism and the surrounding conditions like temperature and humidity also impact the inactivation efficiency. Another critical feature determining the PAL activity is the type of cell wall and its components. In an experiment conducted by Smet., *et al*. it was demonstrated that *Listeria monocytogenes* being a Gram-positive bacteria showed less susceptibility to PAL treatment than *Salmonella Typhimurium* being a Gram-negative species. It was postulated that the presence of a thicker peptidoglycan layer in Gram-positive bacteria functions as a physical shield towards the PAL treatment making it more resistant to disinfection [70].

Advantages of PAL

PAL technology has several applications in the food and medical industry. It offers a promising solution to the problem of biofilms and related diseases as it has shown significant results in the inactivation of bacterial cells. The amount of different long-lived and short-lived reactive species can be adjusted in PAL production and therefore, it can be customized by changing parameters in the production process enabling its use in different areas [84].

Additionally, the possibility of storing and transferring PAL majorly contributes to its potential of being a suitable agent for biofilm control. Apart from that, it has been observed that applications of PAL on sensitive food surfaces caused no harmful effect on the texture, color, firmness, and nutrient quality according to a study conducted by Ma., *et al*. [76]. The formation of PAL is a cost-effective strategy with a relatively short generation time along with a straightforward procedure. Research is underway to find out more about PAL in order to simplify the biofilm eradication process and prevent a huge number of infections leading to severe diseases around the globe.

Conclusion

Plasma-activated liquids have shown considerable inactivation effects on bacterial biofilms in vitro and have vast potential for use in the food and medicine industry according to recent research. Ongoing studies have represented that the PAL setup, biofilm age, and type of microorganism can significantly affect the efficiency of inactivation by PAL. Researchers have been working with both single and dual-species biofilm to see the inactivation efficiency of PAL in different settings closely resembling the environmental conditions.

Published data shows that the low pH and reactive species are majorly responsible for the inactivation of biofilms. Characterization of PAL showed that acidification of bacterial cell membranes along with inactivation by nitrates and nitrites are the most important parameters to facilitate inactivation.

Moreover, the data shows that PAL exhibits less inactivation when stored and more research is required to find a solution to it as it can be a limiting factor in the application of PAL on an industrial scale. More work in regards to optimum treatment time, mechanism of inactivation as well as PAL formation parameters is also needed to understand the precise process of inactivation employed by PAL and if it can be achieved in big-scale industry setup. Advantages like low operating cost and low operating temperatures along with maintaining the quality and texture of target products are the major merits in the implementation of PAL as a biocidal agent specifically for fragile contact surfaces like fruits and meat products where other methods are not as effective for disinfection.

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