Biofilm Structure of Foodborne Pathogens

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The ability of many bacteria to adhere to surfaces and to form biofilms has major implications in a variety of industries including the food industry, where biofilms create a persistent source of contamination. It is reported that biofilms are more resistant to antimicrobials compared to planktonic cells. The colonization of bacteria on the food processing surfaces and even the food structure, such as milk molecules, and thus the formation of biofilms has become a major concern for food plants.

Since the biofilm formation has been a major concern to the food processors and medical devices, it is worthwhile to characterize the biofilm formation of foodborne pathogens under food processing-related stress conditions. It is well documented that bacteria, including foodborne pathogens such as *Listeria monocytogenes, Staphylococcus aureus*, *Escherichia coli,* and *Salmonella* can 'stick' to a variety of surfaces found in food industries. In this review, it is focused on biofilm structures and sociobiology of biofilms of food pathogens which are frequently found in food industry.

Keywords: foodborne pathogens; biofilm; signaling molecules; sociobiology

1. Introduction

Biofilms are at least in part the collection of cells surrounded by a matrix of extracellular polymeric material (EPS) produced by cells in the biofilm [1]. Extracellular polymeric substances (EPS) comprises polysaccharides, proteins, phospholipids, teichoic and nucleic acids, and other polymeric substances hydrated to 85 to 95% water [2], [3]. Many environmental determinants, such as carbon and/or nitrogen supply, pH value, cultivation temperature, oxygen limitation and stage of cells growth can affect the EPS synthesis [4]. Within the biofilm by diffusion limitation and/or chemical interaction with the extracellular proteins and polysaccharides the EPS matrix delays or prevents antimicrobials from reaching target microorganisms [3]. Furthermore, EPS protects biofilm inhabitants, metals and toxins by tightly retaining, blocking access to biocides, and preventing desiccation, and also concentrating nutrients [2]. For example, Bacterial hydrophobicity BsIA forms a water-resistant structure on *Bacillus subtilis* biofilm while cellulose produced by *E. coli* biofilms increases resistance to dryness [5].

It is known that EPS is also important for the structural integrity of biofilms, thus sometimes it is emphasised that EPS null mutants tend to be deficient at biofilm formation [6]. Although it is known that the EPS is primarily responsible for the morphology and function of biofilms, on the other hand, some bacteria are able to make biofilms without EPS [7]. Schluter *et. al.* [8] in their study found out that EPS^- producing bacteria (EPS^+) alone produced larger biofilms and also had a significant competitive advantage over a non-biofilm-producing strain (EPS). The transition from planktonic form to biofilm form occurs as a response to environmental and physiological changes [9], [10]. In this process, the bacterial cell is rearranged spatially and temporally with a large number of regulatory networks that turn signals into harmonious gene expression [10].

2. Biofilm Mechanism

Community organization like biofilm is one of the most important pathogenic strategies performed by disease-causing microorganisms that are resistant to the body's natural immune system, antibiotics and biocides. Biofilm formation realises through mechanical, biochemical and genetic factors in bacteria [11].

The formation of biofilms could be described as a five separate-steps including reversible adhesion, irreversible adhesion, micro colony formation, biofilm maturation and dispersion [12].

2.1. Reversible adhesion:

The formation of biofilm begins with the attachment of the cells to the abiotic surfaces [4]. Reversible adhesion is one of the phases of the initial attachment including reversible and irreversible one which are a time dependent process [2]. The cell's adhesion depends on their motility or the gravitational transportation of their planktonic form (free floating), and diffusion or shear force of the surrounding fluid phase [13].

Reversible adhesion occurs in a certain distance among with the bacteria and substratum; this initial weak interaction involves electrostatic, hydrophobic interactions and Van der Waals forces [4], [14]. The adherent cells on a surface forms a small quantity of extracellular polymeric substance (EPS) in this phase [13].

Various researchs has demonstrated that a number of surface proteins have many structural and functional properties which are the important elements in the biofilm formation of various bacterial species [15]. When considered from this point, it is known that these surface proteins includes like Bap in *Staphylococcus aureus,* enterococcal surface protein (Esp) in *Enterococcus faecalis* and BapA protein in *Salmonella enterica* ser. Enteritidis [16]. According to a study by Sinde and Carballo [13], adherences of *Salmonella* and *Listeria* to a surface are reported to be higher in hydrophobic surfaces than in hydrophilic surfaces. Besides, it is reported that many bacteria species have the ability to produce cellulose which is an exracellular polysaccharide. Cellulose have been reported to be an important component of extracellular matrices in *Salmonella* and *Escherichia coli*, pathogenic bacteria. The staphylococcal polysaccharide intercellular adhesin (PIA) that is previously characterized polysaccharides is an another example for the compnnets of extracellular matrices [17].

2.2. Irreversible adhesion:

It is known that physiological properties of bacteria such as flagella, fimbriae and pili, surface polysaccharides, cell hydrophobicity (the tendency of nonpolar molecules to come together in liquid medium) and clustering play a role in the initial adhesion [8], [11], [12], [18], [19]. It has been demonstrated that binding between bacterial appendages, (that is pili, flagella, adhesin protein) and the substratum involves short range forces such as dipole-dipole interaction, hydrogen bonds, hydrophobic, and ionic covalent bonding [2]. After the initial reversible attachment to biotic or abiotic surfaces, microorganisms secrete QS molecules and EPS, which then leads to irreversible attachment, initially controlled by type 1 pili, curli fibers, and Antigen 43 [12]. Flagella motility, for example plays a critical role in the initial adhesion of Escherichia coli, *Listeria monocytogenes* and *Yersinia enterocolitica* bacteria to the cell surface and in biofilm formation. In addition, fimbriae classified as adhesive, antigenic and physical properties or primary amino acid sequences in protein subunits [19]. It is reported that their role in some pathogenic bacteria including *Salmonella enterica* serovar Enteritidis and *E. coli* are investigated with various researches [20]–[22].

2.3. Colonization:

Biofilm, the highest-level organization of bacterial microcolonies, is concerned with 'Quorum sensing', which is defined as the intracellular signaling or the morphogenetic mechanism known as cell-cell communication [11], [19]. When bacteria adhere to biotic and abiotic surfaces, they multiply inside the EPS to form microcolonies by communicating with QS, and secretory EPS pathway. Microcolonies are the cornerstone of biofilms [12]. That means as the biofilm grows, there will be an increase in microorganisms (that are aerobic but also anaerobic microorganisms) encapsulated in the polymer matrix [14].

2.4. Biofilm Maturation:

At this stage, the biofilm turns into an organizational structure that can be single or double layered in a tulip-like structure with flat, mushroom-shaped, liquid and gas channels [12], [13]. This structure varies depending on the type of bacteria, the age of the biofilm and the nutrients available on the surface [12], [23]–[25]. A period of 10 days or more is required for the formation of a mature biofilm [13].

2.5. Dispersion

Dispersion, is the last phase that allows cells that break from the upper part of the biofilm to turn into planktonic cells [13]. It is known that dispersal of monospecies biofilms has been managed by some cues. Some of these cues has been recognised as nutrient fluctuations, changes in oxygen levels, c-di-GMP, cAMP, degradation of the biofilms matrix, induction of motility, nitric acid and increase in toxic products or metabolites and/or surfactants [12]. Many internal factors, such as increased shear stress, or endogenous enzymatic degradation in EPS or release of surface-binding protein, are among the possible causes of this biofilm breakage [13]. Moreover, QS is able to activate death pathways to control colony size. It has been reported that these cells, which are no longer necessary, are disrupted by a process called planned cell death [12], [26].

3. Biofilm Structures of Some Foodborne Pathogens

Food borne pathogens may form biofilms on a variety of food contact surfaces [12], [27]. Biofilms in food premises are important in terms of resistance to cleaning and sanitation, as well as being a chronic source of microbial contamination that causes food spoilage or disease spreading [28]. When pathogens are a major problem for food producers, the formation of biofilms by pathogens causes contamination of foods and post-processing products, not just surfaces and surfaces of food processes [29]. Biofilms may protect bacteria from changes in environmental conditions such as moisture, heat and pH changes, disinfectants, sanitizers, antimicrobials and harmful effects from exposure to ultraviolet light [12], [30]. Removal or inactivation of bacteria from abiotic surfaces by washing with water or treatment with

disinfectant or sanitizers is not always been successful because the cells are enmeshed in biofilms or otherwise protected against exposure to antimicrobials [4].

Biofilms of food pathogens, which are a major risk for food hygiene and public health, are becoming inevitable due to the time-consuming erosion of systems used in food enterprises, the wetting of food enterprises, the presence of food residues microscopically on the surfaces, and microorganism contamination through food microflora raw materials and personnel [31]. Besides, the presence of biofilm surfaces also results in equipment damage, product contamination, energy losses and medical infections, etc. [32].

Many studies have described the ability of foodborne pathogens like *Listeria monocytogenes, Staphylococcus aureus, Escherichia coli O157:H7, Salmonella enterica, Yersinia enterocolitica* and *Campylobacter jejuni* to attach to various surfaces and form biofilms [13], [33]. These pathogens can survive for a long time into the biofilm, protecting themselves from the effects of disinfectants and adverse external factors. Thus, the detection and control of pathogens is difficult and the effectiveness of combat strategies is diminishing. It is known the adhesion of Salmonella to food surfaces was the first published report on foodborne bacterial biofilm [33].

3.1. Biofilm Structure of *Salmonella*

There are four major components in the structure of *Salmonella* biofilms, including curli, cellulose, capsular polysaccharides and lipopolysaccharides [34]. The responsibility of curli are shown some process such as host colonization, cell–cell interactions, persistence, motility and invasion [35]. In a study by Solano *et al.* [34], it was observed that cellulose a glucose polymer of the extracellular matrix, was produced as a result of the regulator AdrA and served as a "sticky component" for attachment to bacterial surfaces.

Latasa *et al.* [36] showed that *S. enterica* genomes contained two open reading frames (ORFs) (stm2689 and sieE) encoding proteins with significant similarities to *Staphylococcus aureus*. It was also provided an evidence that Stm2689, renamed BAPA, either plays a complementary role in the colonization of murine intestine and later in organ invasion and also a complementary role to fimbriae in *Salmonella* biofilms [36], [37].

3.2. Biofilm Structure of *Staphylococcus aureus*

Several genes are involved in the production of staphylococcal biofilm formation [38]. The first member of the BAP family was described during a previous research carried out by a group focused on the development of the *Staphylococcus aureus* biofilm [37]. During the screening of a library of mutants in the bovine mastitis *S. aureus* strain V329, Bap was identified as a protein that is essential for biofilm formation. During the studies on primary attachment, intracellular aggregation and biofilm formation, it was seen that Bap promotes both primary attachment to abiotic surfaces and intercellular adhesion [15]. It is believed and consensus that the main determinants in the mechanism of biofilm formation by *Staphylococcus* are mediated by the production of capsular polysaccharide adhesin (PS/A) and intercellular polysaccharide adhesin (PIA) or poly-Nsuccinyl- β-1,6-glucosamine whose is encoded by the gene products of the locus ica operon of icaABCD [38], [39].

Bap shows typical structural features of cell wall fixing proteins found in gram-positive bacteria. According to in vitro experiments results Bap is not only involved in the primary attachment step, but also, together with the polysaccharide PIA/PNAG, in cell-tocell aggregation and thus biofilm maturation [37].

3.3. Biofilm structure of *Listeria monocytogenes*

Many studies show that *Listeria monocytogenes* can grow and form biofilms on plastic, stainless steel and glass surfaces in food processing environments [40]–[42]. Extracellular DNA is a central component of the biofilm matrix whose playing a vital role in stability in Gram positive cells such as *L. monocytogenes*. eDNA contributes to the early formation of the biofilm [43].

In static incubation conditions, depending on the strain tested, biofilms of *L. monocytogenes* may create of honey comb-like structures, three-dimensional mushroom shaped structures with water filled channels and pores, or more simple structures including monolayers has been observed. On the other hand, in flow cells (dynamic conditions) the presence of grossly spherical microcolonies surrounded by a network of chains was observed in the study [44]. It is emphasized that flagella motility is very important for biofilm formation in *L. monocytogenes* [19], [44]. Once again, the study showed that flagella mediated motility may play an important role in biofilm formation for *L. monocytogenes.* In this study, immobilized flagellum and flagellum minus mutants show biofilm-defective phenotype compared to wild-type bacteria [44]. Besides, it has also been shown PrfA, an important virulence factor and flagellar biosynthesis regulator to be involved in the regulation of biofilm formation [45].

Factors such as the presence of other bacteria, availability of nutrients, the temperature and pH affect the attachment and biofilm-forming capabilities of bacteria. In their work, Monk et. al. (2004) have shown that changes in the peptidoglycan structure exposed on the surface of Gram-positive bacteria can also have an affect on attachment in L. monocytogenes rough colony variants. It is also reported that the cell wall hydrolase A (CwhA), characterized by an impaired cellular localization of several peptidoglycan-degrading enzymes such as showed enhanced attachment to

stainless steel [19]. Besides, the factor affecting the adhesion of *L. monocytogenes* to the polythreitine was associated with which pH of *L. monocytogenes* growth. It was observed that the polystyrene adherence of *L. monocytogenes* at pH 5 was found to be lower after growing at pH 7. This was associated with down regulation of flagellin synthesis [19]. Moreover, how effect of the expressing full-length and truncated InlA (Internalin A) of L. monocytogenes isolates in biofilm formation were evaluated by [16]. It is reported that 8 of *L. monocytogenes* strains (of the total 70 tested in the study) expressed truncated InlA was associated with an increased ability to form biofilms. According to Smoot and Pierson (2009), in the presence of proteolytic enzyme trypsin, of the demonstration that *L. monocytogenes* cells did not adhere to different supports (plastic and non-steel surfaces) suggested that proteins are involved in the initial adhesion of L. *monocytogenes* [16].

3.4. Biofilm structure of *Escherichia coli*

It is reported that Antigen 43 (Ag43), which promotes cell-cell adhesion and aggregation in the early stages of biofilm formation in E. coli, which has been shown to produce a range of autotransporter adhesins, is the most studied. The subfamily of auto-carrier adhesins to which Ag43 belongs also include two other adhesins produced by E. coli that can increase biofilm formation [5]. In the mature biofilm, the main conserved components of the E. coli biofilm matrix have been remarked as the proteinaceous curli fibres and flagella, alongside the polysaccharide cellulose.

In the mature biofilm, they have been defined proteinaceous curli fibres and flagella as alongside the polysaccharide cellulose which are the main conserved components in the matrix of *E. coli* biofilm. Two divergent operons which are csgBAC and csgDEFG encode the curli. It can be seen in some *E. coli* strains in the biofilm matrix some additional components which as β-1,6-N-acetyl-D-glucosamine (PGA) and colanic acid. Two divergent operons as yhjR-bcsQABZC and bcsEFG encode the proteins involved in cellulose synthesis were shown in further analysis of cellulose production of *E. coli* [5]. Besides, the protein synthesized from the surface protein Yeej which could affect the bacteria adhere to abiotic surfaces have shown in many studies in biofilm of *E. coli* [36].

3.5. Biofilm structure of *Campylobacter jejuni*

In the studies, *C. jejuni*, has been shown as a bacterium that is recessive at the onset of biofilm formation but can bind to preformed biofilms and can exist in mixed biofilms [46]–[48]. It is known that *C. jejuni* is microaerophilic, however it was observed that it can survive for a long time in biofilm under normal atmospheric conditions [49]. It is reported that biofilm structure and dynamics of *C. jejuni* can be affected some factors, such as bacterial strain, surface type, temperature, shear stress (quantified by shake rate), and oxygen and nutrient concentrations [46]. On the other hand, It is thought that bacteria may be protected from the toxic effect of high concentration oxygen by the microenvironment formed in the biofilm [49]. Furthermore, in two studies, in a multispecies biofilm which includes *Pseudomonas* spp and *C. jejuni,* was shown that *P. aeruginosa* consumes oxygen and generates a favorable environment for *C. jejuni* growth and survival [46].

Unfortunately, it is known that, there is not enough knowledge about the structure and composition of the *C. jejuni* biofilm EPS. Even so, it is mentioned that in *C. jejuni* biofilm formation and maturation, eDNA is found important, and for reducing the levels of biofilm of a *C. jejuni* 81–176 ΔcprS mutant, DNase can take a role [50]. So far, it was shown that, some genes be responsible for the processes such as cell motility (flaA, flaB, flaC, flaG, fliA, fliS, and flhA), cell surface modifications (peb4, pgp1, and waaF), quorum sensing (luxS), and stress response (ppk1, spoT, cj1556, csrA, cosR, and cprS) [51]. In the studies related to *C.jejuni*, for example Reeser et al. (2007) [49] showed that there is a reduction in biofilm formation in both flaAB and luxS mutants of *C. jejuni* compared to their wild-type strains. Besides, Hanning et al. (2008) [46] reported that can be survival of the bacteria in biofilms depends on the temperature and they showed that *C. jejuni* had longer survival times in biofilms at 32°C than in biofilms at 10°C.

3.6. Mixed Cultured Biofilms

Foods and food contact surfaces cover a special environment and contain various microorganisms. Although studies focus on biofilms containing single / pure species, biofilms contain different types of bacteria, even fungi, algae and protozoans in the natural environment [12]. Numerous studies have been worked about natural flora microbiofilms [52]–[54]. Manios et al. (2013) [12] reported that the natural microflora in fresh products reduced the formation of *Salmonella* Typhimurium biofilms that boosted *L. monocytogenes* biofilms in their mathematical modeling studies using low-level pathogens on fresh vegetables. Recently, it has become important that biofilms containing mixed species are more resistant to disinfectants and sanitizers in food microbiology and food safety [12]. The heterogeneity, different types of bacterial species in a biofilm, increases resistance to antimicrobials, and colonization of the surface by a bacterium results in better adhesion of other bacterial species to the same surface [55]. The heterogeneity in biofilm comes from provides for nutrient exchange, waste products and signaling factors [56]. In a study conducted by Almeida *et. al*. [57], it is investigated that the characterization of biofilms of *Salmonella enterica/Listeria monocytogenes/Escherichia coli* bacteria by using 7 different support materials and combining single, double and three microorganism groups. In the study, *E. coli* had a higher growth rate and EPS productivity, so that led this

microorganism to outcompete the other two microorganisms. When three microorganisms were combined, *E. coli* was a layer on top and the other two formed on bottom as the mixed culture. Furthermore, while *Salmonella* and *E. coli* were present in a mixed biofilm, it is observed that *Listeria* competed against *Salmonella* better in mixed biofilm ad *Salmonella* population decreased. On the other hand, although *Listeria h*as a lower development rate and limited ability than *E. coli*, it is reported that they can be very well adapted in mixed biofilms.

3.7. Alturism in Biofilms

In a biofilm including different types of bacteria, there is a race between the bacteria for using shared and limited resources. For example, it is known that fermentation is an anaerobic metabolism, and when oxidative cells are compared with fermentative cells, it is presumable that fermentative cells could win this race, because of the nutrient content gradually decreases for other cells [6]. In this situation Altruistic behaviour of some bacteria can be mentioned. Altruistic behaviour may be defined more precisely, from the perspective of multi-level selection theory, as behaviour that increases the fitness of the group relative to other groups while it decreases the fitness of the altruist relative to others within the group (The economical use of limiting resources is such a case of altruism because of a trade-off between specific growth rate (rate of biomass increase per time and biomass) and growth yield (biomass formed per amount of resource used). The economical use of common resources means high growth yield, and this situation increases group fitness. However, this can only be achieved at the expense of lowering a growth rate that reduces individual appropriateness [58]. Kreft and Bonhoeffer [59] attempted to explain the competitive situation in biofilms with a simulation study to understand the survival strategies of the bacteria. In the simulation study of survival strategies of microorganisms in biofilm, Kreft was classified bacterial species as RS bacteria (rate strategy) and YS bacteria (yield strategy).It was observed that in this study that RS bacteria have a high productivity growth strategy (Egoist) while low productivity growth strategy (Economic). On the other hand, it has been reported that according to economic uses of resources microorganisms in biofilm, YS bacteria are named as altruistic and RS (*E. coli*, etc.) bacteria are egoist bacteria are named.

4. Results

Biofilms are microbial communities that attached to the surface which form the dominant part of microbial life. The formation of biofilm has been proven in many pathogenic microorganisms. This review summarizes the results of recent studies on biofilm structures of pathogenic microorganisms, one of the most important sources of food contamination on various surfaces in the food industry. Despite recent studies, there are pathogenic microorganisms that have not yet fully elucidated mechanisms that control the structure of biofilms such as quorum sensing. For this purpose, it is necessary to investigate the molecular structures of these pathogenic microorganisms which cause problems in the food industry in the biofilm process. These studies related with biofilms will make it easier to create environmentally friendly and economical solutions for microorganisms which would like to be removed from the food factory.

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