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MINIREVIEWS

Unraveling Microbial Biofilms of Importance for Food Microbiology

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Abstract The presence of biofilms is a relevant risk factors in the food industry due to the potential contamination of food products with pathogenic and spoilage microorganisms. The majority of bacteria are able to adhere and to form biofilms, where they can persist and survive for days to weeks or even longer, depending on the microorganism and the environmental conditions. The biological cycle of biofilms includes several developmental phases such as: initial attachment, maturation, maintenance, and dispersal. Bacteria in biofilms are generally well protected against environmental stress, consequently, extremely difficult to eradicate and detect in food industry. In the present manuscript, some techniques and compounds used to control and to prevent the biofilm formation are presented and discussed. Moreover, a number of novel techniques have been recently employed to detect and evaluate bacteria attached to surfaces, including real-time polymerase chain reaction (PCR), DNA microarray and confocal laser scanning microscopy. Better knowledge on the architecture, physiology and molecular signaling in biofilms can contribute for preventing and controlling food-related spoilage and pathogenic bacteria. The present study highlights basic and applied concepts important for understanding the role of biofilms in bacterial survival, persistence and dissemination in food processing environments.

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Introduction

It is known that bacteria are remarkably capable of adjusting their needs for survival in environments. Among the microbial characteristics enabling these adaptations one of the most important is the ability of the microorganism to form biofilms, since it facilitates adaptation to harsh environmental conditions.

In the last decades a substantial effort has been put forth to improve our understanding on microbial biofilms, defined as complex and well-organized biological communities embedded in a self-produced extracellular polymeric matrix that can develop into moist surfaces, either biotic or abiotic [102, 180].

The presence of biofilms is common in food industry and represents a concern because bacteria can adhere to almost any type of surface, such as plastic, metal, glass, soil particles, wood and food products [7, 54]. The attachment of bacteria to food products or to product contact surfaces leads to economic losses and also to a higher risk of occurrence of bacterial foodborne diseases. For example, contamination of equipment with biofilms was a contributing factor to 59 % of food-borne disease outbreaks investigated in France [120]. Bacterial in biofilms confer survival advantages to its members since they are protected from environmental stress such as ultraviolet light, dehydration or treatment with antimicrobial and sanitizing agents, which makes their elimination a huge challenge [7, 37]. On the other hand, microbes in biofilms can also be beneficial to food industry and may be used for biotechnological applications [41, 172]. For instance, it helps the production of fermented food and in the waste treatment.

Biofilm in the Food Industry

The attachment of bacteria with subsequent development of biofilms on food industry surfaces has important consequences

[169]. The occurrence of such structured microbial communities in food processing plants represents a reservoir of microorganisms and serves as a potential source of contamination of raw materials and processed products as they pass through various stages of food production operations. Moreover, the presence of biofilms may lead to food spoilage, economical losses, reduced shelf life of products or transmission of pathogens [27, 82, 152, 169].

The first report published on foodborne bacterial biofilm described the adhesive properties of *Salmonella* sp. [46], and since then, many bacteria have been identified to form biofilm in the food industry premises, such as *Listeria monocytogenes*, *Yersinia enterocolitica*, *Campylobacter jejuni*, *Staphylococcus* spp. and *Escherichia coli* O157:H7 [47, 161].

L. monocytogenes is commonly found in food processing environment, and it has been isolated from both meat and dairy processing plants. This microorganism can adhere rapidly and firmly to inert surfaces and may survive in the sessile form for a long period of time [32, 58, 71, 97, 129, 161, 176]. For example, Unnerstad et al. [177] found the same clone of *L. monocytogenes* persisting in a dairy plant for 7 years and Miettinen et al. [121] demonstrated that isolates of *L. monocytogenes* PFGE type II had also survived in an ice cream plant for at least 7 years.

Staphylococcus aureus is one of the most frequent foodborne pathogens in the food industry [116, 152]. Some researchers have observed the ability to adhere and form biofilm by *Staphylococcus* genus [116]. Rode et al. [152] studied biofilm formation by both food-related and clinical *S. aureus* strains grown under different stress conditions (temperature, sodium chloride, glucose and ethanol), and those authors observed that some ingredients, food such as sodium chloride and glucose, could promote biofilm formation by *S. aureus.*

E. coli O157:H7 is known to produce exopolysaccharides (EPS) and to form biofilm on food-contact surfaces and equipment used in beef processing plant [20, 44]. Recently, Mendonça et al. [117] also demonstrated that *E. coli* O157:H7 had the potential to form biofilm on different surfaces commonly used by food industry while Dourou et al. [44] observed that *E. coli* O157:H7 attachment occurred not only at temperatures of beef slaughter houses areas (15 °C), but also during cold storage (4 °C) temperatures, indicating more effective sanitation programs are necessary.

Lactic acid bacteria (LAB) may cause biofilms and in this sense, it represents a concern for the food industry undesirable changes in foods [87, 95]. One example is *Lactobacillus curvatus*, a nonstarter lactic acid bacterium that is capable of forming biofilm and produce an D-(−)-lactic acid, an isomer responsible for the formation of calcium lactate crystals that could lead to sensorial defects in cheeses [95]. Also, in situ biofilm formation by *Bacillus* has been previously reported, e.g., in milk powder and whey processing plants, indicating a risk factor for foodborne diseases [47, 101, 158].

Beneficial aspects of biofilms to the food industry should also be mentioned and are related to biotechnological applications. Biofilms are essential elements in the production of fermented foods; for example, during the production of vinegar, the acetic acid bacteria are grown on wood chips and the attachment of bacteria promote a more efficient conversion of substrate to acid [172]. Moreover, results from studies by Demirci et al. [41] demonstrated that the production of ethanol by *Saccharomyces cerevisiae* in biofilms presented advantage in comparison to conventional fermentation since it had a higher productivity. According to Gamarra et al. [53], *Aspergillus niger* biofilms may be used for the commercial production of cellulases since biofilms formed on polyester cloth yielded 70 % more cellulase activity than freely suspended mycelial culture. These enzymes are of importance due to their potential industrial applications for the processing of food, textile, laundry, pulp and paper.

Morikawa [125] highlighted good aspects related to biofilm formation by *B. subtilis* and other bacilli due to the control of infection caused by plant pathogens, the reduction of steel corrosion, and the exploitation of novel compounds. Biofilm reactors were also applied to promote effective microbial growth for the treatment of sewage, industrial waste streams or contaminated groundwater [48]. It is very important to control harmful biofilm formation, but beneficial biofilms formed by industrial bacteria may contribute for the development of new biotechnological processes.

Biofilm Formation

Biofilms can be defined as structured communities of bacteria, which are adhered to each other and/or to surfaces or interfaces and embedded in a self-produced exopolysaccharide matrix [38]. Their formation involves (1) initial attachment of planktonic cells to the surface, (2) production of extracellular polimeric matrix (EPS), (3) microcolony formation and secretion of chemical signals, (4) maturation of biofilm architecture and (5) dispersion of cells [62, 68, 123, 154]. However, in this process of biofilm formation and detachment, some factors should be considered in more detail, as will be described in sequence.

Biofilm Architecture

Biofilms formed by pathogenic bacteria, including *Salmonella* sp., *L. monocytogenes*, *S. aureus*, *E. coli* O157:H7, *Bacillus cereus* and *Vibrio cholerae* have been extensively studied on biotic and abiotic surfaces [10, 17, 25, 164, 168, 179]. However, unattached aggregates (flocs) and pellicle at the air–liquid interface may be formed by some pathogens, such as *C. jejuni*, *B. cereus* and *Chronobacter sakasakii* [79, 96, 187].

Generally, bacteria form a non-uniform structured biofilm characterized by variable thickness, cell distribution and polymer densities as per bacterial strain, genetic features, environmental conditions, and also according to the experimental model used in laboratory (Table 1).

Microscopic methods, such as scanning electron microscopy (SEM), confocal laser scanning microscopy (CSLM), and cry embedding followed by sectioning and microscopic examination provide a consistent picture of the structural heterogeneity of microbial biofilms. However, CSLM and cry embedding approaches have more ability to image the biofilm interior and more potential to provide quantitative information than SEM [17, 65, 66, 171].

In the past years, the microscopy approach allowed viewing of mature biofilms as an interesting construction, that can be made by layers, clumps and ridges, or even more complex microcolonies that are arranged into stalks or mushroom-like formations. Furthermore, cell-free pores and channels in the biofilm interior can also be visualized, suggesting a local cell dispersion and/or a place for nutrient and waste exchange. Some cell-free spots can be found filled by EPS, which is important for biofilm organization and maintenance [17, 68, 69, 91, 171].

Factors Involved in Biofilm Formation: Surface Properties, Environmental Factors and EPS

The physicochemical characteristics of bacterial surface and solid surfaces in the food processing industry are very important for biofilm formation because they influence the initial cell attachment [1]. Some surface properties such as hydrophobicity, electrical charge, cation bridging, roughness and topography are good examples [133, 150, 181].

There is no consensus regarding the bacterial ability to attach to hydrophilic or hydrophobic surfaces, but the variety of surface materials (metal, plastic, glass, granite and marble) commonly used in domestic kitchens or in the food processing industry is admittedly involved in the retention of foodborne pathogens [160, 174].

The most common food contact material used in the food industry is stainless steel type 304, because it is chemically inert at a variety of processing temperatures, easy to clean, and highly resistant to corrosion [194]. However, in general, due to continuous use, the topography of this material presents cracks and crevices, which protect the bacteria from mechanical cleaning methods and sanitizing treatments [161]. Also, several food processing equipment's and utensils may have inaccessible areas difficult to clean, as for example slicers, dicers, floor drains, rollers, and conveyor belts [32, 165].

Moreover, any food residues or molecules like proteins from milk, pork or beef left on food processing or handling equipment's can be adsorbed to the surface, forming a conditioning film. This first stage usually starts within the first 5–10 s of contact and may provide a niche in which microorganisms can rapidly grow [82, 88, 165, 194]. Thus, food matrix can highly interfere with the cleaning and disinfecting of food processing surfaces.

Furthermore, bacteria can use a variety of cellular membrane components (e.g., protein and lipopolysaccharide), appendages (e.g., flagella, pili, fimbriae and curli fibers) and sporulation for sensing and/or attaching to surfaces [15, 19, 36, 78, 138, 157, 166, 179, 187]. In addition, some environmental factors including pH, temperature and nutrient composition of the medium and population characteristics of bacteria play an important role in cell wall change and on physicochemical properties of solid surfaces [34, 64, 81, 83, 152].

The EPS amount, composition, chemical and physical properties may vary significantly among different species and between different strains of the same species [40, 49, 111, 114, 150].

Among the Gram-positive bacteria, staphylococci are excellent producers of EPS, forming a *slime* composed of a mixture of polysaccharide intercellular adhesin (PIA) — a polycationic exopolysaccharide termed poly-*N*-acetylglucosamine (PNAG) — teichoic acid and small quantities of proteins [135]. Similarly, the Gram-negative bacteria species (e.g., *E. coli*) appear to produce PIA-like polymers that also function as adhesins [185]. However, the eDNA has been the most studied component of EPS over the last years, since it was discovered as a major structural component of *P. aeruginosa* biofilm by Whitchurch et al. [186]. Their function and possible origin was recently shown for other

Table 1 Biofilm architecture of some foodborne pathogens according to experimental laboratory model used

Bacteria	Experimental model	Biofilm architecture	Reference
C. sakasakii	Static condition	Pellicles and flocks	[96]
	Flow condition	Basal layer of cells and microcolonies	[70]
L. monocytogenes	Static condition	Homogeneous layer and microcolonies of rod cells	[151]
	Flow condition	Ball-shaped microcolonies surrounded by network of knitted chains composed of elongeted cells	
C. perfringens	Static condition	Flat and thickness biofilm encased in a dense EPS	[179]
P. aeruginosa	Static and flow condition	Flat biofilm during early colonization and mushroom-like microcolonies surrounded by water-filled voids latter	[106]
S. aureus	Static and flow condition	Dense layer of cells	[144]

pathogens, including *B. cereus*, *S. aureus*, *L. monocytogenes* and *V. cholerae* [21, 39, 67, 106, 144, 156, 182].

Thus, the identification of these novel matrix components, their relationship with other cell components, and also their functions in biofilm formation and dispersal may provide clues for the development of new strategies to prevent and control biofilms.

Bacterial Biofilm Dispersal

Bacterial dispersal may occur naturally during biofilm development in the environment, likely due to nutrient depletion or accumulation of toxic waste products [81, 155].

According to Kaplan [80], biofilm dispersal generally occurs due to active or passive mechanisms. The former is associated with the presence of bacterial structures (e.g., flagella) and/or with the production of extracellular substances (e.g., enzymes and signaling molecules). The latter is mediated by external forces through different process: (1) shearing or erosion, which refers to the continuous removal of single cells or small clusters of cells from a biofilm at low levels over the course of biofilm formation; (2) sloughing, which refers to rapid and massive removal of large portions of the biofilm, usually during the later stages of biofilm formation; and (3) abrasion, which refers to the detachment due to collision of solid particles with the biofilm.

Bacterial detachment can also be viewed from the perspective of control when induced by human intervention in food processing facilities or medical devices by chemical, physical and biological removal [29, 63]. Furthermore, both in monospecies and multispecies biofilms close relationships between species based on competition, mutualism, predation, or parasitism may result in cell dispersal by producing interspecific antimicrobial compounds, *quorum sensing* (QS) signals or matrix-degrading enzymes [30, 112, 115, 124, 173, 178]. Thus, it points out that numerous factors are 'essential' or 'required' for biofilm dispersal and some of them may be an important key to remove pre-formed biofilms on solid surfaces.

Methods to Prevent and to Control Cells in Biofilms

Good Manufacturing Practice (GMP) and Hazard Analysis and Critical Control Points (HACCP) have been established for controlling food quality and safety [158] and one of the first steps to prevent and control biofilms is to identify Critical Points where it can grow and develop [158, 194].

Microorganisms within biofilms are able up to 1,000-fold more resistant to disinfectants and biocides than planktonic counterparts and this characteristic may be associated with aspects related to the architecture and physiology of biofilms, such as reduced diffusion, anaerobic growth, physiological changes due to reduced growth rates and the production of enzymes that degrade antimicrobial substances [73, 88, 163, 165]. Because of those factors, the most common disinfectants used by food industries including: acidic compounds, aldehyde-based biocides, caustic products; chlorine, hydrogen peroxide, iodine, isothiazolinones, ozone, peracetic acid, phenolics, biguanidines, surfactants halogens, and quaternary ammonium compounds is not enough to remove the biofilms [129, 161, 189, 194].

Thus, sometimes a combination of sanitizers with other methods is required [118, 161, 163]. Steam vapor technology and aerosolized sanitizer present potential use for disinfection of biofilms on environmental surfaces because of their ability to penetrate and to reach difficult places [141]. Park et al. [141] found that the treatment with 100 ppm of aerosolized peracetic acid was more effective to inactivate *E. coli* O157:H7, *Salmonella* Typhimurium, and *L. monocytogenes* in biofilms than the same concentration of sodium hypochlorite. In dairy manufacturing plants (DMP) biofilm control generally involves a process called Clean-In-Place (CIP), which consists in cleaning and sanitizing by means of a combination of mechanical, thermal, and chemical processes [24, 88, 95, 128].

Another possibility for controlling biofilms is to prevent cell adhesion by modifying the chemical properties of surfaces [27, 163]. Surfactants have general structural characteristic with the presence of a hydrophilic and hydrophobic structure and they may help to improve physical cleaning actions through emulsification, penetration, spreading, foaming, and wetting [27, 163]. Biosurfactants produced by a broad variety of bacteria, actinobacteria and fungi had been studied for that purpose and may be useful to prevent biofilm formation [57, 161, 167]. Gómez et al. [58] proved that the pre-conditioning of polystyrene surfaces with surfactin (0.25 %) reduced by 42.0 % the adhesion of *L. monocytogenes* and *S. enteritidis*, whereas the treatment using rhamnolipids (1.0 %) reduced by 57.8 % adhesion of *L. monocytogenes* and by 67.8 % adhesion of *S. aureus*.

Studies have been currently focusing on the molecular and genetic basis of pathogenic biofilm formation, since it could enable the development of new strategies to block relevant pathways for biofilm development [70, 90, 94, 97, 176]. The molecular mechanisms that govern biofilm formation by the vast majority of foodborne pathogens are not completely understood but it is known that thin aggregative fimbriae (Tafi) and cellulose are the two main matrix components in *Salmonella* biofilms [168, 169]. Biofilm formation and maturation in *S. aureus* is related to various regulators encoded mainly by *sarA*, *agr*, *ica*, and *sigB* [2].

As shown on the literature, QS systems may affect biofilm formation as well as developmental regulatory networks, such as sporulation, competence and virulence factors [2, 84, 161, 169]. Thus, QS inhibitors (QSI) secreted by other microbial,

vegetal and animal organisms and Quorum Quenching (QQ) enzymes, which produce the enzymatic degradation of signal molecules, are also a very attractive alternative to control and to regulate biofilms but is still a challenge, due to the uncertainty associated with their safety [2, 81, 93, 161].

The potential application of enzymatic cleaning can also be used in the food industry as an adjuvant technology to remove biofilms. Augustin et al. [11] studied the use of proteinase disinfectants (alkalase, chymotrypsin, cryotin and krilltrypsin) against biofilms of *Lactobacillus bulgaricus*, *Lactobacillus lactis* and *Streptococcus thermophilus*. Despite good results reported by the authors, the use of enzymes is still limited because it is difficult to identify which enzymes are effective against different types of biofilms and due to high cost [118, 161].

Viruses that target bacteria (bacteriophages) may be also a good option to kill undesirable bacteria in biofilms. Phage therapy is based on the use of lytic phages that also can potentially destroy the integrity of biofilm EPS matrix by enzymatic mechanism [43, 147, 161]. Pires et al. [147] described the isolation and characterization of lytic phages capable of infecting antibiotic-resistant *P. aeruginosa* strains and a reduction of 3 log of cell counts in biofilms.

Antimicrobial molecules known as bacteriocins have been well documented due to their biofilm control potential [55, 161]. The exact mechanism of action in biofilms has not been completely elucidated but bacteriocins are known to form pores in bacterial cell membrane [3]. Winkelströter et al. [188] demonstrated that culture medium supernatant containing bacteriocin produced by *Lactobacillus sakei* 1 was able to control *L. monocytogenes* biofilm formation. Spray-dried crude bacteriocin fermentate (CBF) of *Lactococcus lactis* UQ2, or *Lactococcus lactis* UQ2 reduced more than 5 log of planktonic and sessile cells of *L. monocytogenes* Scott A attached to stainless steel chips [55].

Other natural antimicrobial compounds had been studied to control microorganisms in biofilms [8, 20]. Laird et al. [89] demonstrated that essential oil vapors (orange/bergamot, 1:1, v/v) reduced the populations of *Enterococcus* sp. and *S. aureus* in biofilms by 1.5 and 3 log after 24 h exposure. Furukawa et al. [51] showed that sugar fatty acid esters reduced the attachment of *S. aureus*, *Streptococcus mutans* and *L. monocytogenes* to abiotic surface. Bodur and Cagri-Mehmetoglu [20] showed that scallop shell powder (SSP) reduced biofilm formation by *L. monocytogenes*, *S. aureus* and *E. coli* O157:H7 in biofilms on stainless steel surfaces whereas Arevalos-Sánchez et al. [8] demonstrated that acidic and neutral electrolyzed waters had strong antimicrobial activity against listerial biofilms at 65 ppm or higher concentrations, after 10 min contact time. Despite important progress have been achieved in understanding mechanisms to control undesirable biofilms, no method is considered completely effective and more knowledge is needed in this area.

Methods for Detection and Quantification of Microbes in Biofilms

Sessile cells can be dislodged from surfaces by sonication, vortexing, sponge and swabbing methods, followed by agar plating or enrichment for detection of pathogens. Some authors demonstrated that the swabbing method may not be efficient to detach all microorganisms [26, 50, 56, 119, 137], and the ultrasonic apparatus may render results more reproducible [9, 136, 137]. Luppens et al. [104] found that the combined swab-vortex method was more efficient to detached microorganisms from stainless steel compared with shaking, vortexing with glass beads, vortexing and sonication. Other authors did not find significant differences among these methods [100, 122].

Bremer et al. [24] verified by direct microscopic observation, that bacterial cells in mixed biofilms remained on the surface studied after the vortexing method but not after the swabbing method. Lindsay and von Holy [100] observed by SEM, that shaking with beads removed cells as well as residues of EPS more efficiently from the surfaces than vortexing and sonication.

A broad range of microscopy techniques such as fluorescence microscopy, CLSM and SEM have been used for qualitative and/or quantitative analyses of biofilm architecture in different surfaces.

Fluorescence microscopy and CLSM are used for studying specimens that fluoresce. While multilayered biofilms can only be analyzed two-dimensionally by fluorescence microscopy, CLSM allows non-invasive images of biofilm structures with high resolution, the elimination of defocused haze, and quantitative visualization of two-dimensional (2D) (both *xy* and *xz*), three-dimensional (3D) and four-dimensional (4D) reconstructions of biofilm [45, 93]. The volume of the biofilm, volume to surface ratio, roughness coefficient, mean and maximum thickness can also be calculated, but these features depend on availability and expertise on dedicated software to perform calculations [18, 92].

Numerous fluorescent probes are used for the detection of total numbers of bacteria in biofilms and the most common is to use acridine orange (AO) to label DNA and RNA or other DNA-specific dyes [25, 30, 77, 113, 145, 148, 159]. There are several stains that target either viable or nonviable bacteria, and the most commonly used viability-staining systems for biofilm samples are CTC-DAPI (5-cyano-2,3-ditolyl tetrazolium chloride [CTC] and 4′,6-diamidino-2-phenyindole [DAPI]) and the LIVE/DEAD bacterial viability kit *Bac*-Light™ which can be used to detect metabolic activity and cell viability, respectively, differing total cells from living cells [23, 75, 85, 134].

Multiple fluorophores are also used to characterize biofilms by binding to total cells (e.g., SYTO 9, SYTO63), dead cells (e.g., SYTO X blue), proteins (e.g., SYPRO Ruby and

fluorescein isothiocyanate [FITC]), lipids (e.g., DiD, Nile red), extracellular polysaccharides as part of the EPS (e.g., Calcofluor white, concanavalin A–tetramethylrhodamine conjugate, FITC, tetramethyl rhodamine isothiocyanate [TRITC] and cyanine [CY5]) [12, 35, 127].

Another alternative to study microorganisms in biofilms is genetically modify them to express fluorescent protein (FP), which does not require substrate or cofactors for its activity [162]; the use of dual technique involving two FP variants or one FP variant combined with a fluorescent dye is used to study multi-species biofilms [38, 61, 86, 105, 108, 126, 151].

Detailed ultra structure of biofilms is difficult to capture by light microscope, and electron microscopy (such as SEM) has higher resolution and may show the location of a single bacterium cell and 3D structure of biofilm. However for SEM studies, it is necessary to dehydrate the biofilm samples during preparation and biofilm structure may be altered [5].

Alhede et al. [5] compared four different SEM techniques: conventional SEM, Focused Ion Beam (FIB)-SEM and CLSM with SEM techniques [cryo-SEM and environmental-SEM (ESEM)], and concluded that there is not a single method that allows to visualize the true structure of biofilm matrix. Lawrence [91] also demonstrated the need to use multimicroscopic analysis to determine in detail biofilm structure and composition.

AFM is an additional technique for high-resolution images, requires simple sample preparation [190], and it is useful for measuring critical dimensions such as cell size, appendage length and surface roughness [46]. However AFM has some limitations such as inability to obtain a large area survey scan, to observe the side walls of bacterial cells, and the soft and gelatinous nature of the biofilm [191].

Díaz et al. [42] observed by AFM the orientation of flagella in the biofilm formed by of *P. fluorescens* on solid opaque surfaces avoiding sample pre-treatment. Teixeira et al. [174] used AFM to perform quantitative measurements of surfaces topography and roughness, but those authors had difficulty to establish a relationship between surface properties and the extent of adhesion. Touhami et al. [175] showed that AFM can give high-resolution images of *P. aeruginosa* type IV pili and it is sufficient to probe their elastic properties.

Fluorescent in situ hybridization (FISH) has emerged as a molecular tool to identify and quantify bacterial species or genera in microbial communities by hybridization to ribosomal RNA. FISH or peptide nucleic acid fluorescence in situ hybridization (PNA-FISH) consist in rRNA-targeted oligonucleotide probes based on phylogenetic markers at 16S and 23S rDNA sequences labeled with a fluorescent dye (Cy3, FAM, FITC, rhodamine) or with an enzyme, which deposits fluorescent molecules [59, 107, 139, 153]. PNA-FISH method uses shorter peptides, and present higher specificity and sensitivity than conventional DNA probes [6, 110].

Methods for rapid detection of foodborne pathogens on surfaces are important in order to avoid contamination of food, and molecular techniques can be an alternative to detect and quantify bacterial pathogens on contaminated surfaces such as real-time polymerase chain reaction (RT-PCR). This technique is rapid, sensitive, specific, and minimizes post-PCR contamination because reactions are performed in a one-tube system, and can detect low number of microorganisms on surfaces. Although, the amplification of DNA from dead cells causes false-positive PCR results, an alternative to avoid it is to use an enrichment procedure that increases the concentration of target cells and allows only living bacteria to be detected [60, 109].

In addition, PCR has been used to measure live cells in biofilms as described by Nogva et al. [131] based on membrane integrity. Those authors used ethidium monoazide (EMA) and propidium monoazide (PMA), which are DNA-intercalating dyes that enter only membrane-compromised cells (dead cells). Extraction of genomic DNA is performed and analyzed by quantitative PCR (qPCR), excluding the cells with damaged membranes from analysis. PMA assay may present a major advantage over the EMA assay, because EMA can also penetrate in live cells of some bacterial species and lead to substantial loss of DNA [130, 140].

Many authors have quantified the number of RNA transcripts of specific genes by qRT-PCR using fluorescent dyes, to evaluate the bacterial populations in biofilms, to estimate the number of copies of a target gene. The use of dual-labeled probes allows the analysis of multiple genes simultaneously, and studies on gene expression in biofilms using different fluorescently labeled probes may target different genes [60, 98, 143].

qRT-PCR has a large dynamic range and may be used to verify gene expression data obtained from microarrays. DNA microarray is another molecular tool that facilitates the study of large numbers of genes simultaneously. Microarrays consist of thousands of unique DNA sequences each attached at a known location to a small solid surface, and complementary, labeled mRNA or DNA can bind to the fixed sequences to produce a pattern indicative of nucleic acid sequences, generating a gene expression profile for a particular microorganism and can provide information on the molecular basis of microbial diversity, evolution, and epidemiology [13, 103, 104, 191].

Microarrays have been mostly used to study gene expression changes due to environmental stressors or treatments that mimic food industry conditions comparing planktonic and biofilm cells, or comparisons of mutant *vs.* wild-type [149, 184, 192, 193].

More recently, many authors have used molecular techniques and proteomic assays to elucidate different structural components of the biofilm, regulatory pathways and signaling molecules involved in biofilm formation [72, 76, 183, 192].

There are several high-throughput methods that use 96-well microtiter plates to grow and to quantify biofilms by

measuring fluorescence or absorbance: (a) the biofilm biomass assay which measures live and dead cells and matrix such stained with crystal violet (CV) [99, 170], (b) SYTO 9 [22], and (c) the BioFilm Ring Test®. The quantification of viable cells can be done using XTT assay [4, 52, 146]; by resazurin or Alamar Blue [132] and by non-fluorescent fluorescein diacetate (FDA) [74, 142]. The biofilm matrix measurement is possible to be measured by the 1,9-dimethyl methylene blue (DMMB) dye released by adding a decomplexation solution that reflects the amount of sulphated polysaccharides present in the biofilm matrix [14].

Peeters et al. [142] verified broad applicability and high repetition in microtiter plate assays such as CV, SYTO 9, FDA, resazurin, XTT and DMMB. The BioFilm Ring Test® was faster than the CV method due to less handling steps, such as no washing and staining [33]. The Alamar Blue method presents several benefits over other methods such as relative cost, compatibility with high throughput, lack of toxicity of chemicals used and measurement of viability [146].

Burton et al. [28] developed a spectrofluorometric assay to quantify bacterial biofilms grown on a microtiter plate, staining biofilms with wheat germ agglutinin–Alexa Fluor 488 conjugate, which selectively binds to *N*-acetylglucosamine residues in biofilms, and observed that spectrofluorometric method was more sensitive and specific than CV staining.

Some authors used specific microplates coupled with microscopy to visualize and quantify biofilms such as the Calgary Biofilm Device (CBD) coupled with SEM [31] or with CLSM and SEM [68]. Bridier et al. [25] used a high-throughput method combined with CLSM; Benoit et al. [16] developed a Bioflux device with controlled flow rates and determination of viability in real time and enables microscopic examination.

Conclusions and Perspectives

Many microorganisms are able to adhere and to form biofilms in food industry premises, which may be an important reservoir of microbial contamination of food products. Biofilms present a high level of complexity and many mechanisms involved are still not fully understood. In recent years, the knowledge regarding mainly genomic and proteomic analyses have been extensively used to screen and to identify genes related to biofilm formation or dispersal. These new approach may enhance the understanding of the molecular basis of biofilms regulatory pathways and will advance the development of new strategies and technologies in an attempt to control microorganisms in biofilms.

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