

1 Introduction to Biofilms: Definition and Basic Concepts

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1.1 Definition of biofilms

In 2012, the term 'biofilm' was defined by the International Union of Pure and Applied Chemistry (IUPAC), Polymer Division as an 'Aggregate of micro-organisms in which cells that are frequently embedded within a self-produced matrix of extracellular polymeric substances (EPS) adhere to each other and/or to a surface'. IUPAC included the following notes after the definition:

Note 1: A biofilm is a fixed system that can be adapted internally to environmental conditions by its inhabitants.

Note 2: The self-produced matrix of EPS, which is also referred to as slime, is a polymeric conglomeration generally composed of extracellular biopolymers in various structural forms.

The idea behind the development of this definition was to provide a terminology usable, without any confusion, in the various domains dealing with biorelated polymers, namely, medicine, surgery, pharmacology, agriculture, packaging, biotechnology and polymer waste management (Vert *et al.*, 2012).

Bearing this definition in mind, in this book we use the term 'biofilm' to refer to 'microorganisms attached to and growing, or capable of growing, on a surface'. This definition is broader than the IUPAC definition, as it includes cells or spores that are attached to a surface but have yet to produce a biofilm matrix. We have included attached cells not within a matrix in order to acknowledge that in many instances the act of attaching induces phenotypic changes to a cell. We have included the phrase 'growing or capable of growing' to reinforce the point that many of the unique features associated with biofilms arise as a result of the

growth and replication of microorganisms on a surface, such as the production of EPS and the development of a complex three-dimensional structure.

In this chapter, we briefly discuss the importance of biofilms to the dairy industry, before introducing their general features, including their development, composition and structure, the advantages they confer to microorganisms living in them and how they may be controlled. This chapter serves as an introduction to the other chapters in the book, and includes cross-references to more detailed information on dairy-specific features in other chapters.

1.2 Importance of biofilms in the dairy industry

On a global basis, the dairy industry produces a wide range of perishable (milk and cream) and semiperishable foods (cheese, butter and yoghurt) and food ingredients (milk powders, whey protein concentrates and caseinates). Microbial contamination of dairy products is of great concern to the dairy industry. Strict adherence to microbiological guidelines is essential to maintain product quality, functionality and safety (see Chapter 4) and to allow companies to remain competitive in the international market.

Those microorganisms associated with bovine raw milk and dairy manufacturing plants that are of particular interest to the dairy industry can be divided into three major categories, namely, spoilage, pathogenic and beneficial microorganisms. Spoilage microorganisms can have an impact on the quality and sensory properties of milk and other dairy products, through the production of metabolic byproducts and/or extracellular enzymes. Pathogenic microorganisms (see Chapter 9) have the potential to cause human illness and to have significant economic repercussions. Beneficial microorganisms generally belong to a diverse group loosely termed 'lactic acid-producing bacteria' (LAB) and are used as starter cultures for the manufacture of cheese, yoghurt and other fermented dairy products. A subgroup of LAB that is becoming more commonly used in fermented dairy products, such as yoghurt, is the probiotic bacteria, which include strains of *Lactobacillus* and *Bifidobacterium* (Jamaly *et al.*, 2011; Quigley *et al.*, 2013).

Biofilms have become a major issue within the dairy industry and are now recognised as sources, or potential sources, of contamination by spoilage or pathogenic microorganisms, which can decrease product safety, stability, quality and value. Many manufacturing processes provide unique niches, within processing equipment, where bacteria are able to grow and survive. Examples are thermoresistant streptococci in pasteurisation equipment (see Chapter 6) and thermophilic spore-forming bacteria in milk powder production equipment (see Chapter 7). Within the last 2–3 decades the importance of biofilms in the processing environment has also been recognised, particularly around drains and other locations that are difficult to reach and where cleaning and sanitation applications may be inadequate to eliminate bacteria present within biofilms.

In dairy manufacturing plants, biofilms can be divided into two categories: process biofilms, which are unique to processing plants and form on surfaces in direct contact with flowing product; and environmental biofilms, which form in the processing environment, such as in niches where cleaning and sanitation is poor and around drains. Process biofilms differ from environmental biofilms in two key ways. First, in a process biofilm, one or a few species may dominate, as the unit operation employed (e.g. pasteurisation equipment) may

select for particular groups of bacteria (e.g. thermophilic). Second, process biofilms are frequently characterised by rapid growth rates. An example of this is the increase in numbers from ‘not detectable’ to 10^6 bacteria per cm^2 within 12 hours of operation that occurs in the regeneration section of a pasteurisation plant (Bouman *et al.*, 1892). In contrast, environmental biofilms can take several days or weeks to develop (Zottola & Sasahara, 1994).

1.3 Biofilm formation

The development of a biofilm on a surface follows a logical series of steps, in which the first step is the initial contact of the free-living microorganism with the surface. The initial interaction of cells with a surface is influenced by a wide range of chemical, physical and biological cues, as outlined in detail in Chapter 2. In general, the initial interactions are influenced by: (i) the surface topography, chemistry (functional groups, surface charge, presence of antibacterial compounds) and free energy (hydrophobicity); (ii) environmental conditions, including temperature, pH, nutrients and the presence of other microorganisms, which can either inhibit or enhance contact; (iii) processing factors such as fluid velocity and shear force; and (iv) the various mechanisms employed by the cell (quorum sensing, nutrient sensing, production of EPS) and the cell surface structures (such as pili, flagella, fimbriae, adhesins) to interact with the surface (Figure 1.1).

Once on or near a surface, a bacterium has to commit to adopting either an attached or a planktonic lifestyle based on a series of signals or cues it receives (Karatani & Watnick, 2009). An obvious cue for settlement is nutrient concentration, with high or low concentrations of nutrients

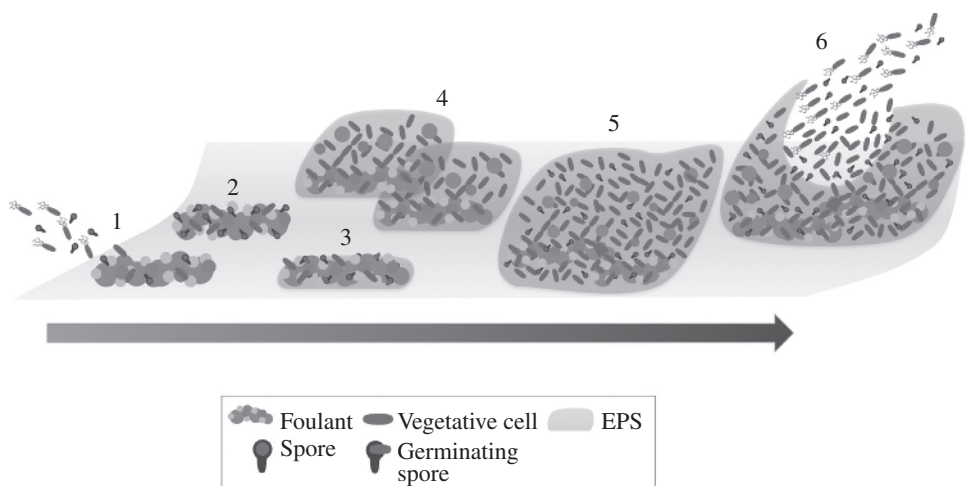


Figure 1.1 Steps involved in biofilm formation over time (arrow) in a dairy processing plant under conditions of flow. (1) Cells and/or spores come into contact with a surface that may be fouled with protein, fat and salts. (2) Cells and spores attach to the fouled surface. (3) Spores germinate and cells grow, beginning to produce EPS. (4) Cells replicate, forming microcolonies enclosed in EPS. (5) Microcolonies increase in size and coalesce, forming complex three-dimensional aggregates of cells and EPS that may contain a variety of niches. (6) Dispersal of cells and spores from the biofilm occurs.

promoting biofilm formation for different bacterial species. Bacteria, such as *Salmonella* spp., are more likely to join a multilayer biofilm in response to nutrient limitation (Gerstel & Romling, 2001), while for *Vibrio cholera*, the presence of glucose and other sugars induces production of a biofilm matrix and multilayer biofilm formation (Kierek & Watnick, 2003).

The second step in biofilm formation requires the cell to form at least a semipermanent association with the surface. This step is frequently referred to as the 'attachment phase'. Many authors have broken this down into a reversible and an irreversible phase, but with increasing knowledge on cell dispersal, the term 'irreversible attachment' is proving to be overstated. In dairy processing plants, there is a wide range of different materials to which bacteria can attach, including 304 and 316 stainless steel, plastic, elastomer (rubber) materials, polyester/polyurethane (conveyor belt materials), epoxy surface coatings and tiles. Bacteria will attach at different rates and strengths to these materials. The ability of bacteria to attach to a surface and the rate at which they attach will, however, change as material (proteins, carbohydrates) from the processing environment comes into contact with the surface and modifies its characteristics. Such so-called 'conditioning films' (see Chapter 3) occur almost as soon as a clean surface comes into contact with a liquid. In addition, the rate of attachment and the ease with which bacteria can be removed from the surface will change as the surface material ages, becomes damaged through mechanical operation or is exposed to cleaning agents and sanitisers.

The effect of surface roughness on the propensity of cells to attach is unclear. Some research reports greater cell attachment on surfaces with high surface roughness, while other research reports that there is no correlation between surface roughness and cell attachment to inert surfaces (Vanhaecke *et al.*, 1990; Flint *et al.*, 2000; Mitik-Dineva *et al.*, 2008, 2009; Truong *et al.*, 2010). While there may be some debate about the influence of surface roughness on attachment, there appears to be general agreement about the importance of using surfaces with minimal cracks and crevices in order to reduce bacterial adherence and biofilm growth and to enhance cleaning effectiveness.

In the next step of biofilm formation, the cells on the surface begin to replicate and produce EPS, which can include polysaccharides, proteins, eDNA and lipids. The production of EPS and the incorporation of extraneous material from the environment, such as food residues (soil) and other microorganisms, into the biofilm, results in an increase in the biofilm's bulk and complexity.

In the final stages of biofilm development, the growth and replication of the primary colonisers (the first cells to attach to the surface) lead to the formation of microcolonies on the surface. These microcolonies independently increase in size over time until they form a series of macrocolonies, which can eventually coalesce to varying degrees, forming complex three-dimensional aggregates of cells and EPS on the surface, variously described as being 'mushroom' - or 'pillar' -like. As the biofilm develops, the presence and metabolic activity of the bacteria within it, coupled with the production of EPS and its associated impact on the diffusion of compounds and gases into, out of and through the biofilm, can lead to the development of a wide variety of microenvironments or niches within the biofilm.

The ultimate structure of the biofilm is dependent on the bacterial species involved in its creation and the chemical and physical characteristics of its environment. Individual macrocolonies may merge together or may remain separated by narrow channels, through which nutrients and other molecules can readily diffuse. The developed biofilm is in a state

of flux, where cells within it react to changes in the physical (flow rate, shear) and chemical (nutrient gradients, oxygen concentration) nature of the environment. The variety of conditions occurring within a biofilm can result in the development of phenotypically or genotypically distinct cell populations within it and can ultimately lead to the dispersion or release of cells from the biofilm.

Dispersal from biofilms may be either initiated by the bacteria themselves or mediated by external forces such as fluid shear, abrasion and cleaning. At least three distinct modes of biofilm dispersal have been identified: erosion, sloughing and seeding. Erosion is the continuous release of single cells or small clusters of cells from a biofilm at low levels, owing to either cell replication or an external disturbance to the biofilm. Sloughing is the sudden detachment of large portions of the biofilm, usually during the later stages of its growth, perhaps as conditions with it change or it becomes unstable due to its size. Seeding dispersal is the rapid release of a large number of single cells or small clusters of cells and is always initiated by the bacteria (Kaplan, 2010).

In the 1980s and 90s, interest in biofilms rapidly increased and there were many reports of biofilm formation and development following the generalised steps just described, leading to the proposal of a developmental model of microbial biofilms (O'Toole *et al.*, 2000). This model received wide interest, but, 10 years after it was first proposed, Monds and O'Toole (2009) published a paper expressing concern that evidence in its support had not been forthcoming and that it should not be considered as dogma.

It is known that many, if not all, bacteria are capable of forming or at least living within a biofilm and that living within a biofilm is frequently their normal mode of existence in natural environments (Costerton *et al.*, 1995; Stoodley *et al.*, 2002). As living within a biofilm requires extensive changes in both cell form and function, this strategy entails a significant commitment (Monds & O'Toole, 2009). Once a cell is committed to a biofilm, the spatial stratification within the biofilm can drive an additional physiological differentiation of the population. However, rather than being seen as an indication of the presence of specialised developmental stages, this is increasingly being considered as simply a reflection of the microorganism's response to the development of niches or a microenvironment within the biofilm. In short, it is the ability of bacteria to sense and to respond to their localised environment by regulating gene expression that leads to the development of a sustainable and complex biofilm, rather than an overarching bacterial community-focused goal.

1.4 Biofilm structure

While the structure of a biofilm is ultimately dependent on the species growing within it and the specific physical and chemical conditions in the environment surrounding it, a mature biofilm generally comprises clusters or layers of cells, which form a structure that can vary in thickness from a few micrometres to several millimetres. The cells are surrounded by EPS, which can contain up to 97% water (Zhang *et al.*, 1998). In general, the bacterial cells within a biofilm make up only about 15–20% of its volume, with the remainder being taken up by EPS.

Based on modelling studies, classical porous biofilms containing channels and voids between the mushroom-like outgrowths are predicted to occur under a substrate-transport-limited regime, while compact and dense biofilms are predicted in systems limited by

biofilm growth rate and not by the substrate transfer rate. Surface complexity measures, such as roughness and fractal dimension, will increase with increasing transport limitations, while compactness will decrease as the biofilm changes from being dense to being highly porous and open (Picioareanu *et al.*, 1998).

Physical conditions, such as temperature, impact on the species composition (see Chapter 4) and growth rate of bacteria within a biofilm, while in pipelines, fluid flow dynamics can influence biofilm structure. Biofilms grown under laminar flow are reported to be patchy and to consist of aggregates of cells (mushrooms) separated by interstitial voids. Biofilms grown under turbulent flow may also be patchy but are characterised by the occurrence of chains of cells (streamers) that run from the biofilm surface into the bulk fluid phase (Stoodley *et al.*, 1998a). The biofilm as a whole, and the streamers in particular, exhibits viscoelastic properties, which means that it elongates and deforms as flow velocity increases and retracts as velocity decreases (Stoodley *et al.*, 1998b). Recently, it has been shown that the flow of liquid through porous materials, such as industrial filters, can stimulate the formation of streamers, which, over time, can bridge the spaces between surfaces and cause rapid clogging (Drescher *et al.*, 2013).

For many years, it has been known that some bacterial species, growing either as free living cells or within a biofilm, produce or release diffusible signal molecules that increase in concentration as a function of cell numbers. In a process termed 'quorum sensing', bacteria communicate with each other via these signal molecules or autoinducers to regulate their gene expression in response to population density (Miller & Bassler, 2001). The role of quorum sensing in biofilm formation was first reported for biofilms of *Pseudomonas aeruginosa* growing in a flow-through reactor, where it was found that the quorum sensing signal molecule 3OC₁₂-homoserine lactone (C12) was required for normal biofilm differentiation (Davies *et al.*, 1998). The role of quorum sensing molecules in biofilm formation and differentiation has subsequently received considerable interest. While quorum sensing may not be significant in the structural development of all biofilms, there is evidence that for some species it can be important in events such as the attachment of bacteria to a surface, structural development and maturation and even the control of events leading to the dispersion or release of cells (Davies *et al.*, 1998; Boles & Horswill, 2008; Periasamy *et al.*, 2012; Lv *et al.*, 2014).

1.5 Composition of the EPS

As previously discussed, as cells attach, replicate and grow on a surface they produce EPS. EPS is recognised as playing an important role in the formation and function of biofilms of many species in many different environments. In addition, EPS, which is usually the major component of biofilm matrix, can act as an impermeable or at least semipermeable barrier, limiting the penetration of compounds into and out of the biofilm, and thereby facilitating the establishment of ecological niches within the biofilm and protecting the cells against the actions of antimicrobial compounds.

The composition and structure of components within EPS is varied and complex, being dependent on the bacterial species involved and the environment (Sutherland, 2001; Flemming & Wingender, 2010). EPS compounds that originate from microorganisms

include polysaccharides, proteins, lipids and extracellular DNA (eDNA) (Flemming & Wingender, 2010). Polysaccharides have been identified as one of the major components of EPS. However, in many cases, the biochemical properties and functions of polysaccharides remain elusive, due to their complex structures, unique monomer linkages and the fact that their composition and concentration can change over time. Most of the polysaccharides that have been described are long linear or branched molecules, with molecular masses of $0.5\text{--}5.0 \times 10^5$ Daltons, and they may be homo- or heteropolysaccharides and either poly-anionic (e.g. polysaccharides, such as algininate or xanthan) or polycationic compounds (Flemming & Wingender, 2010).

The biofilm matrix can also contain a considerable number of proteins. A wide range of enzymes has been detected within biofilms. Many of these are reported to have bipolymer degrading ability, enabling them to break down complex compounds, such as polysaccharides, proteins, nucleic acids, cellulose and lipids, into nutrients that are more readily available to bacteria. Biopolymer degrading enzymes also play a role in the dispersal of cells from the biofilm. Nonenzymatic proteins in the EPS or biofilm matrix are often involved in the formation and stabilisation of the EPS matrix and are often therefore termed 'structural proteins'. These include the cell surface-associated and extracellular carbohydrate-binding proteins, known as lectins, which form links between the bacterial surface and the EPS (Flemming & Wingender, 2010).

In addition to the obvious role of transferring genetic material between bacteria, via conjugation and DNA transformation, eDNA also appears to play a structural role in maintaining biofilms. The expression of conjugative pili has been shown to stimulate biofilm formation and can stabilise and influence the biofilm structure by forming connections between cells (Ghigo, 2001). The presence of eDNA has been shown to stabilise the young biofilms (Whitchurch *et al.*, 2002). eDNA also has antimicrobial activity and causes cells to lyse by chelating cations that stabilise lipopolysaccharides in the outer membranes of bacterial cells (Flemming & Wingender, 2010).

Lipids, lipopolysaccharides and surfactants can also be found to varying degrees within some EPS, where they are believed to play a role in the initial attachment of the cell to the surface, the development of the biofilm structure and the dispersal of cells from the biofilm (Flemming & Wingender, 2010).

1.6 Composition of the biofilm population

Most biofilms found in nature comprise a range of bacterial species. However, in specialised niches within processing plants, especially in those areas subjected to extremes of temperature, or where the product has been treated to inactivate most microorganisms, it is possible for biofilms dominated by one or a few species to develop. An example of this is in the production of milk powder, where it is possible to find biofilms developing within the evaporators that are dominated by one or two species of thermophilic spore-forming bacteria (Burgess *et al.*, 2010, 2013).

In general, biofilms are very heterogeneous environments characterised by a large degree of chemical, physical and biotic diversity. Variation in diffusion rates into and out of biofilms, as well as in the rates at which compounds are produced or metabolised, can lead

to the development of concentration gradients for nutrients, oxygen, ions and signalling molecules. This can result in the creation of microenvironments and biotic diversity, even in monospecies biofilms, as cells adapt to changes in their local environment.

Like any other ecological niche, conditions within biofilms select for cells that are best suited to survive. This means that the resulting population is a reflection of the cells that come into contact with the niche, their ability to grow within the niche and the impact that cell growth and metabolism have on the niche. Based on the diversity of the planktonic population and the selective pressure at the surface and within the developing biofilm, biofilms can comprise one or a small number of species. In most instances, however, it is expected that a biofilm will contain a number of microbial species, with interactions occurring between them. In some cases, such interactions can facilitate the growth and survival of species that may be less suited to survival in a monospecies biofilm under the same environmental conditions (Bremer *et al.*, 2001).

Biotic diversity therefore occurs through a number of mechanisms. In the simplest instance, phenotypic changes take place due to variations in the cell's physiological status, dictated by nutrient or oxygen gradients (Stewart & Franklin, 2008). For example, cells located in the outermost layers of a biofilm that have a ready supply of nutrients and oxygen available can easily grow aerobically. The facultatively anaerobic cells in underlying layers may be oxygen-deprived and so will need to shift to an anaerobic metabolism in order to grow. This can encourage the growth of obligate anaerobic microflora. Cells at deeper layers within the biofilm may be nutrient-limited and have limited growth rates or be metabolically inactive. The response of individual bacterial cells to the local conditions drives phenotypic heterogeneity.

Phenotypic diversity may also arise due to variations in gene expression resulting from differences in transcription initiation or mRNA degradation. So-called 'stochastic gene expression' has been hypothesised to be a cell population's insurance against potential dramatic changes in environmental conditions (Veening *et al.*, 2008).

A third source of phenotypic heterogeneity is genetic mutations. Genetic variation occurring through point mutation, insertion or deletion can potentially increase the phenotypic variability within the biofilm. If such spontaneous mutants confer a significant selective advantage, especially in the presence of a stressor, they will confer a fitness advantage to the mutated cell and its offshoots and promote the survival of the cell population (Plakunov *et al.*, 2010).

Gene transfer within biofilms is enhanced by the close proximity of cells and the ability of the biofilm matrix to trap gene products within the biofilm. Gene transfer occurs within biofilms by two main mechanisms: plasmid conjugation and DNA transformation. In conjugation, direct cell-to-cell contact is required for plasmid transfer. Therefore, while DNA transfer can occur at high rates within a biofilm (Hausner & Wuertz, 1999), the structure of the biofilm and the degree to which cells can move within the biofilm to establish direct contacts will ultimately limit the extent to which conjugation occurs (Molin & Tolker-Nielsen, 2003). DNA transformation occurs when DNA (chromosomal or plasmid) released by one cell is picked up by another. It has been reported that most, if not all, bacteria have the ability to release DNA (Lorenz & Wackernagel, 1994). Cells that have the ability to efficiently take up macromolecular DNA are defined as having developed natural competence. Transformation rates for *Streptococcus mutans* growing within a biofilm have been reported to be 10–600-fold higher compared to the rate in planktonic cultures (Li *et al.*, 2001). Given

that the presence of conjugative pili and eDNA, as discussed above, can stabilise biofilms (Whitchurch *et al.*, 2002), it appears that efficient gene transfer is both a consequence of and a contributor to biofilm development (Molin & Tolker-Nielsen, 2003).

1.7 Enhanced resistance of cells within biofilms

A large number of authors have compared the resistance of bacteria within biofilms to their free-living counterparts and declared that the former are far more resistant to a wide range of stressors, including antibiotics, ultraviolet (UV) damage and sanitisers (Costerton *et al.*, 1995; Elasmri & Miller, 1999; Langsrud *et al.*, 2003; Bridier *et al.*, 2011). This protection has been postulated to result from a number of factors associated with living within a biofilm, including the binding of EPS to antimicrobial compounds, physical inhibition of the diffusion of antimicrobial compounds by the EPS or chemical reaction of antimicrobial compounds with components of the EPS matrix, all of which decrease the concentration of antimicrobial compounds reaching microorganisms within the biofilm (Thurnheer *et al.*, 2003). For example, chlorine (in a 25 ppm solution), which chemically reacts with organic material, has been shown to only be able to penetrate to a depth of 100 µm into a complex 150–200 µm-thick dairy biofilm (Jang *et al.*, 2006). In addition, chlorine concentrations within a mixed *Pseudomonas aeruginosa* and *Klebsiella pneumoniae* biofilm reached only 20% of the concentration measured in the bulk liquid (De Beer *et al.*, 1994). In contrast, it has been shown that EPS generally does not pose much of a barrier to relatively uncharged molecules, such as the antibiotic rifampin (Zheng & Stewart, 2002).

A direct result of the development of microenvironments within a biofilm is that the physiological state of cells in different parts of the biofilm can be varied. An example of this is the occurrence of so-called ‘persister cells’: the subpopulation of cells that are not growing (Lewis, 2010). It is postulated that these dormant cells are well suited to survival in stressful environmental conditions, and especially to exposure to antimicrobials, such as antibiotics, which target sites within actively growing cells. Most research on persister cells has focused on their high tolerance to antibiotics, with it being postulated that these cells are not antibiotic-resistant mutants, but rather phenotypic variants that occur stochastically within a clonal population of genetically identical cells (Levin & Rozen, 2006). It is thought that persister cells maintain dormancy due to the overexpression of a broad variety of genes that produce products which induce dormancy if present at high enough levels. Persister cells have been shown to occur at low numbers within stationary phase planktonic cultures and biofilms and it is postulated that such cells may be able to withstand the initial antimicrobial challenge and subsequently grow, reestablishing the population (Lewis, 2008, 2010).

Persister cells aside, the reduced metabolic activity of cells in nutrient-deficient areas within a biofilm may in part account for their increased resistance to antimicrobial agents (Stewart & Olson, 1992; Lisle *et al.*, 1998; Sabev *et al.*, 2006; Soto, 2013). Further, the stress of living in the biofilm (nutrient limitations, cell density triggers, pH changes, oxygen limitations, accumulation of waste products) can induce cells to express stress-responsive genes and to switch to more tolerant phenotypes. For example, in *E. coli*, environmental stress induces a transcriptional regulator that controls the rate at which the alternative sigma

factor *RpoS* is produced. This sigma factor can help to prevent DNA damage and its production has been shown to be linked to biofilm formation (Foley *et al.*, 1999).

Biofilm formation may also result in the induction or inhibition of genes, which may specifically or inadvertently, either directly or indirectly, make the cells resistant to the stressor (Sauer *et al.*, 2002; Tremoulet *et al.*, 2002; Schembri *et al.*, 2003; Beaudoin *et al.*, 2012; Zhang *et al.*, 2013).

1.8 Controlling biofilms

Within a processing environment, the renowned difficulty in removing biofilms is caused by a wide variety of factors associated with plant design and operation, as well as the inherent properties of biofilms and the cells within them. Five factors are involved in the development of biofilms in dairy processing plants, namely: the species of microorganisms involved; the type of product being manufactured; the operational conditions (runtime and temperature); the surface material and its condition; and the cleaning and sanitation regimes (chemicals, use and frequency) employed. Given these variables, the factors that can be most easily controlled are the runtime, the cleaning and sanitation regime and, in some cases, the surface materials.

Cleaning and sanitation regimes are required to remove food residues, microorganisms and the cleaning and sanitation agents from food contact surfaces. The effectiveness of a cleaning and sanitation regime is dictated by chemical, thermal and mechanical processes, with combinations of cleaning and sanitation agents, chemical additives (surfactants, wetting agents, chelating agents), the correct temperature and the use of mechanical force (brushing, turbulent flow) being required. It is also essential to have a good understanding of the microorganisms involved – especially whether they are spore-forming or non-spore-forming microorganisms – and of the nature of any fouling material (protein, fat, carbohydrates, mineral salts) associated with the process, which may be incorporated into or cover the biofilm. As the effectiveness of cleaning and sanitation is dependent on a number of factors, it is vital that indicators of cleaning efficacy (microbial numbers, food residue) are monitored on a routine basis.

In dairy processing plants, equipment is normally cleaned-in-place (CIP) by circulating warm or hot cleaning solutions at high velocity (Stewart & Seiberling, 1996), thus satisfying the requirements for chemical, physical and thermal energy input (see Chapters 4 and 12 for more details). A feature of CIP regimes, evident in both industrial- and laboratory-scale systems, is their variable efficiency in eliminating surface-adherent bacteria (Austin & Bergeron, 1995; Faille *et al.*, 2001; Dufour *et al.*, 2004; Bremer *et al.*, 2006). The most important factors influencing the effectiveness of a CIP are: cleaning agent concentration and chemistry; cleaning agent temperature; cleaning time; degree of turbulence of the cleaning solution; and the characteristics of the surface being cleaned. The standard chemicals used in CIP regimes can be formulated to contain compounds, such as surfactants, that improve surface wetting, soil penetration and cleaning properties (Bremer *et al.*, 2006).

As concerns associated with the growth of bacteria within biofilms and their inherent increased resistance to cleaning agents and sanitisers have increased, increasing care has been taken in the design of systems and the specification of materials that will limit biofilm

formation and enhance cleaning effectiveness. Dead ends, corners, cracks, crevices, gaskets, valves and joints have long been recognised as being difficult to clean and vulnerable to biofilm formation (Chmielewski & Frank, 2006). It is important to appreciate that any flaws in the design or physical location of equipment that decrease cleaning efficacy will enhance biofilm formation.

1.9 Emerging strategies for biofilm control

It is now well recognised that the removal of microbial cells from surfaces, once they have become attached (biofilms), can be very challenging. For this reason, recent interest has focused on the development of surfaces that either prevent or reduce attachment or contain compounds that are antibacterial and can therefore act against attached cells. It has recently been suggested that antibacterial surfaces should be categorised as being either antibiofouling or bactericidal, depending on the effect that they have on biological systems (Hasan *et al.*, 2013). In a recent review, Hasan *et al.* (2013) defined antibiofouling surfaces as surfaces that resist or prevent cellular attachment due to the presence of an unfavourable surface topography or surface chemistry. They defined bactericidal surfaces as surfaces that disrupt the cell on contact and cause cell death. They also stated that, in some instances, antibacterial surfaces may exhibit both antibiofouling and bactericidal characteristics, giving the example of a surface coated with N,N-dimethyl-2-morpholinone (CB ring), which is capable of inactivating bacteria in a dry environment, and with a zwitterionic carboxybetaine (CB-OH ring), which will resist bacterial attachment in a wet environment (Cao *et al.*, 2012).

Many approaches to the development of antibacterial surfaces involve the immobilisation of an antibacterial agent on the surface to be protected. The classic example of this approach is the historical widespread use of a number of antifouling paints containing either tributyl tin- or copper-based antimicrobial agents in the marine environment. In the food industry, it is important to develop antibacterial surfaces that in themselves will not impact on the safety or quality of the food with which they come into contact. While a number of coatings containing either silver, titanium, hydroxyapatite, antibiotics, quaternary ammonium compounds or fluoride ions (Price *et al.*, 1996; Ding, 2003; Hume *et al.*, 2004; Murata *et al.*, 2007; Zhao *et al.*, 2009) have been explored for their suitability as food contact surfaces, there are safety concerns over the possibility of the compounds being leached from them. In addition, there are a number of other limitations to this approach, including the potential for bacteria to develop resistance, the time it takes for the antibacterial agent to be released from the surface, the low concentration that may result, the potentially short lifetime of the antibacterial functionality and the ability of food components (proteins, lipids) to coat the surfaces, reducing their efficacy (Hasan *et al.*, 2013). Lee *et al.* (2004) proposed an approach to produce permanent, nonleaching antibacterial surfaces by utilising atom-transfer radical polymerisation to modify surfaces with quaternised ammonium groups. This approach is controllable and is reported to present a permanent antibacterial effect, as the surface can be reused without loss of activity (Lee *et al.*, 2004; Yang *et al.*, 2011). While such an approach is believed to potentially have application in the food industry, its commercial applications are still in development (Hasan *et al.*, 2013).

The observation that a number of naturally occurring surfaces in nature, such as insect wings, shark skin and lotus leaves, have the ability to resist fouling by preventing particles, algal spores and bacteria from sticking to them has led researchers to attempt to mimic their activity via microfabrication or nanotechnology (Chung *et al.*, 2007; Anselme *et al.*, 2010; Bazaka *et al.*, 2012). While this field of research is considered to be increasingly promising, with methods to modify the nanotopography of surfaces developing, it seems likely that the degree to which bacterial attachment is inhibited will be species-dependent (Ivanova *et al.*, 2011; Hasan *et al.*, 2013). The impact of surface topography and especially surface roughness on bacterial attachment will be discussed further in Chapter 2 (Section 2.4.3). Further, to be applicable for use in the dairy industry, the antifouling surface will need to be able to work in the presence of not only bacteria but also proteins, fat, sugar and inorganic salts – all compounds which have the potential to attach to and change the chemical and physical nature of a surface.

1.10 Conclusion

It is important to appreciate that microorganisms have been evolving and refining survival strategies for many millions of years. The ability to attach to surfaces and form biofilms is not new, and evidence from the fossil record indicates that microorganisms were living within biofilms at least 500 million years ago (Westall *et al.*, 2001). Over the last 30 years, as our knowledge of the features of biofilms and their way of life has developed, it has become increasingly obvious that the interactions associated with biofilms at the genetic, cellular, population and community level are extremely complex and that the challenge of preventing, controlling or eliminating biofilms is a daunting one.

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