Biofilm formation in food processing environments is still poorly understood and controlled

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Abstract

The presence of undesirable biofilms on food processing contact surfaces may lead to: 1) transmission of diseases; 2) food spoilage; 3) shortened time between cleaning events; 4) contamination of product by nonstarter bacteria; 5) metal corrosion in pipelines and tanks; 6) reduced heat transfer efficacy or even obstruction of the heat equipment.

Despite the significant problems caused by biofilms in the food industry, biofilm formation in these environments is still poorly understood and effective control of biofilms remains challenging. Although it is understood that cell attachment and biofilm formation are influenced by several factors, including type of strain, chemical-physical properties of the surface, temperature, growth media, and the presence of other microorganisms, some conflicting statements can be retrieved from the literature and there are no general trends yet that allow us to easily predict biofilm development. It is likely that still unexplored interaction of factors may be more critical than the effect of a single parameter.

New alternative biofilm control strategies, such as biocontrol, use of enzymes and phages, and cell-to-cell communication interference, are now available that can reduce the use of chemical agents. In addition, as preventing biofilm formation is a more efficient strategy than controlling mature biofilm, the use of surface-modified materials have been suggested. These strategies may better reveal their beneficial potential when the ecological complexity of biofilms in food environments is addressed.

Introduction

Some microbes, including *Listeria monocytogenes*, *Campylobacter jejuni*, *Salmonella* spp., *Staphylococcus* spp., *Pseudomonas* spp., *Escherichia coli* O157:H7 and *Bacillus cereus*, are a concern in the food processing industry. Indeed, the presence of detrimental bacteria on food processing surfaces may lead to: transmission of diseases; food spoilage; shortened time between cleaning; reduced heat transfer efficacy or even equipment obstruction; metal corrosion in pipelines and tanks resulting at least in metal loss; and contamination of product by nonstarter bacteria (e.g. cheese by nonstarter lactic acid bacteria) (Wong 1998; Gram et al. 2002; Chmielewski and Frank 2003; Beech and Sunner 2004; Wijman et al. 2007; Shi and Zhu 2009; Malek et al. 2012).

Biofilms are matrix-enclosed microbial accretions that adhere to biological or non-biological surfaces (Hall-Stoodley et al. 2004). The extracellular polymeric substances (EPS), which are mainly polysaccharides, proteins, nucleic acids and lipids, are responsible for the morphology, structure and physico-chemical features of these aggregates (Flemming and Wingender 2010). Since biofilm is a universal phenomenon, i.e. microbes prefer to live on surfaces rather than in the liquid phase, it is very likely that most of the microbial contamination of food products is biofilm-related (Brooks and Flint 2008). The biofilm formation mechanisms in a number of environments have been the subject of debate and were reviewed in many comprehensive and authoritative books and reviews (Hall-Stoodley et al. 2004; Costerton 2007). Biofilm-associated cells can be differentiated from their freely suspended counterparts, called planktonic microorganisms, by generation of EPS, reduced growth rates, the up- and down- regulation of specific genes and cell-to-cell communication (Donlan 2002). Interestingly, among the genes differentially regulated in biofilms are those involved in metabolism or starvation responses (Stewart 2003).

Biofilm formation is a dynamic process that in a sequential manner involves attachment, maturation and dispersal (Fig 1). In the first step of biofilm formation, planktonic microorganisms move into close proximity with the surface, which may have several physico-chemical characteristics that are important to determine the rate and extent of the attachment process. Additionally, the substratum exposed in an aqueous medium can become coated by molecules, the so-called conditioning layer, and the resulting physico-chemical modification may inhibit or promote microbial attachment (Percival et al. 2011). The transport of microbial cells to a surface is generally achieved by a number of well-established fluid dynamic processes, including Brownian motion and gravitational effects (Characklis 1981).

At the beginning of the process adhesion is reversible, and cell surface characteristics such as hydrophobicity, presence of appendages (fimbriae and flagella), and surface-associated polysaccharides or proteins, may have a role in overcoming the initial electrostatic repulsion between the cell and substratum. When the loosely bound microorganisms consolidate the adhesion process by producing exopolymers and adhesins that complex with surface materials, adhesion to the surface becomes irreversible, i.e. microorganisms are not dislodged by gentle rinsing (Dunne 2002). The attachment of microbial cells to a substratum is followed by microbial growth, development of microcolonies and recruitment of additional microorganisms from the local environment. As attachment of microorganisms occurs, microorganisms grow with the production and accumulation of extracellular polymers and are eventually become embedded and immobilised in this hydrated polymeric matrix. Biofilms at the solid/liquid interface are very heterogeneous, they are formed by microcolonies encased in an EPS matrix are separated from other microcolonies by interstitial voids (water channels), and behave like viscoelastic materials (Klapper et al. 2002). Diffusion governs most transport of solutes within the biofilm and between the biofilm and its environment. As diffusion is slow compared with cellular metabolism, the chemical environment often differs greatly at different biofilm depths (Stewart 2003; Nadell et al. 2009). These chemical gradients create environmental microniches that allow for the coexistence of diverse species.

Biofilm provides an optimal environment for the exchange of genetic material and communication among cells as microorganisms are immobilised in relatively close proximity to one another. Genetic transfer rates in biofilms are orders of magnitudes higher than between free-living cells (Donlan 2002). Within the biofilm community, microorganisms communicate with each other by using chemical signal molecules, termed autoinducers, in response to population density in a process that is called quorum sensing (QS) (Waters and Bassler 2005). Several physiological activities are regulated via QS, including biofilm formation, expression of virulence factors and dispersal.

Multi-species biofilms are generally encountered as the activity of specific functional types of microorganisms may create conditions that favour other complementary functional groups of microorganisms. This leads to the establishment of spatially separated, but interactive, functional groups of microorganisms, supporting physiological cooperation (Percival et al. 2011). Coordinated by signals, biofilm communities also have the option of moving through their environment in swarming masses, while retaining their spatial associations and metabolic integration (Costerton 2007).

Cells detached from the biofilm colony can translocate to a new location and attach again. Mechanisms of biofilm dispersal are active and passive. Active dispersal refers to mechanisms that are initiated by the bacteria themselves. Detachment can be dictated by low nutrient conditions as a survival mechanism, which may be genetically determined. Therefore, detachment is important for escaping unfavourable habitats aiding in the development of new niches (Percival et al. 2011). Passive dispersal refers to biofilm cell detachment that is mediated by external forces such as human intervention. To date three distinct modes of biofilm dispersal have been identified: i) erosion, the continuous release of single cells or small clusters of cells from a biofilm at low levels over the course of biofilm formation, ii) sloughing, the sudden detachment of large portions of the biofilm, usually during the later stages of biofilm formation, and iii) seeding, the rapid release of a large number of single cells or small clusters of cells from hollow cavities that form inside the biofilm colony (Kaplan 2010). All these dispersal phenomena can be active and passive, the only exception being seeding dispersal that is always an active process.

A remarkable feature of biofilm cells is that they can become 10–1000 times more resistant to the effects of antimicrobial agents than the same cells grown in the planktonic mode, depending on the species-drug combination (Mah and O'Toole 2001). A number of biofilm characteristics contribute of biofilm cells to the antimicrobial agents resistance, such as the presence of a diffusion barrier provided by the EPS, the interaction with the exopolymers, the slow growth mode of sessile cells, and the possible genetic expression of certain resistance genes.

Here biofilm formation refers to food processing plants, the reader is redirected to other reviews for biofilms on produce (Jahid and Ha 2012; Srey et al. 2013).

Equipment contamination has been accounted for 40% of food-borne disease in France (Lequette et al. 2010). Product contact surfaces may contaminate the product directly as the product touches or passes over the surface and indirectly by environmental surfaces, such as floors and walls. In the latter case, microorganisms may be transferred to the product by vectors such as the air, personnel and cleaning systems (Gibson et al. 1999). The ways in which microorganisms may be introduced into the processing plants are probably numerous, being some of them, like *L. monocytogenes*, ubiquitous bacteria. The raw materials processed are not necessary the main cause. According to Rørvik (2000) raw salmon does not seem to be an important source of *L. monocytogenes*, although slaughtered fish from colonized slaughterhouses may introduce the bacteria into a plant. The thermophilic bacteria are normally present in low levels in raw milk, but may reach high amounts in the final product after 15–20 h of milk powder plant operation (Hinton et al. 2002). Biofilm formation can occur on milk contact surfaces also after pasteurization (Salustiano et al. 2009).

Valderrama and Cutter (2013) proposed that biofilm formation may be an alternative surrogate for fitness, a set of properties that an organism possesses to enhance its survival, spread, and/or transmission within a specific ecological niche (Preston et al. 1998). An ecological niche refers to the combination of biotic and abiotic factors needed to persist and maintain stable populations (Valderrama and Cutter 2013). The factors governing the adhesion of microorganisms to surfaces have not been fully elucidated yet. Nevertheless, scientists agree that there is hardly a material that does not allow biofilm formation (da Silva Meira et al. 2012), including stainless steel and buna-n rubber (acrylonitrile butadiene), materials commonly used in food processing equipment (Beresford et al. 2001). However, differences have been reported in both the extent and rate of attachment depending on the surface type (Boistier-Marquisa et al. 2000; Silva et al. 2008).

Substratum and cell features

Relationships between surface hydrophobicity and the amount of biofilm have been investigated, and the correlation between them are only sometimes evident (Srey et al. 2013). Data presented by Wang et al. (2013b) showed that cell hydrophobicity of different serotypes of *Salmonella* correlates with biofilm formation on polystyrene. Some authors claimed that spores are very hydrophobic and this characteristic causes them to have strong adhesive properties and easily attach to food processing equipment (Verran et al. 2002; Lindsay et al. 2006; Wijman et al. 2007). Hydrophobicity of 11 yeast strains from a fouled ultrafiltration membrane of an apple juice processing plant correlated positively with the rate of adhesion (Tarifa et al. 2013). On the contrary, studying the attachment of *Geobacillus* spp. spores to casein-modified glass surfaces and the less hydrophobic glass surfaces, Han and colleagues (2011) demonstrated that spore attachment rates were higher on non-modified glass surfaces. Nevertheless, hydrophobicity plays a role in multicellular behavior as it was demonstrated that *C. jejuni* grown in biofilm mode is more hydrophobic than the same microorganism in planktonic mode (Trachoo and Brooks 2005).

Some researchers support the thesis that attachment occurs most readily on surfaces coated by surface conditioning films (Barish and Goddard 2013). On the contrary, Wong (1998) claimed that milk and milk proteins significantly reduced *L. monocytogenes* adhesion and that the presence of lactose did not affect adhesion. In another research, for the majority of the strains tested, stainless steel coupons pretreated with milk showed a lower adherence of *B. cereus* spores and therefore a smaller amount of biofilm formed (Wijman et al. 2007).

Some authors could not find a direct correlation between surface roughness and initial bacterial attachment (Schlisselberg and Yaron 2013), in contrast to the previously implied connection between the two (Tang et al. 2011). On the contrary, it has been observed that small irregularities may greatly favour colonization by pioneer species (Palmer et al. 2007; Silva et al. 2008; Schlisselberg and Yaron 2013).

Conflicting opinions also exist on the importance of flagella in initial attachment. Simões et al. (2010) suggested that flagella-mediated motility overcomes repulsive forces of the substratum. Similarly, Lemon et al. (2007) demonstrated that *L. monocytogenes* nonmotile mutants were defective in attachment and subsequent biofilm formation. In contrast, Di Bonaventura et al. (2008) found a negative correlation of flagellum production in *L. monocytogenes* strains with biofilm-forming ability. Understanding the relation between motility and biofilm formation is not an easy task because both processes might involve similar components at certain stages and specific conditions, e.g. motility on a surface can be crucial for biofilm architecture and is also involved in the dispersal of cells (Verstraeten et al. 2008).

Research performed so far on biofilm formation by a species has generally focused on a single strain and did not take diversity among strains into account. The few studies carried out with many strains indicate differences in biofilm-forming capacity among the strains tested. Considering 17 *Salmonella* strains, Wang et al. (2013b) indicated that the strain type played a pivotal role in the development of microbial biofilms rather than the incubation conditions. Similarly, significant variation in biofilm level produced by different *L. monocytogenes* serotypes was detected at 37°C (Di Bonaventura et al. 2008). Therefore, some researchers suggested to carry out experiments choosing only strains previously isolated from food processing environments. Adhesion of many isolates of *Salmonella* Sofia, *S.* Typhimurium, *S.* Infantis and *S.* Virchow to Teflon, stainless steel, glass, rubber and polyurethane were studied by Chia et al. (2009). The findings showed that *S.* Sofia isolates adhered in higher numbers to all materials compared to other serovars. Biofilm formation by *B. cereus* was assessed using 56 strains (Wijman et al. 2007). Interestingly, in the microtiter plate assays, the biofilm formation ability was different among the strains used and took place preferentially at the liquid-air interface, while biofilm formation was much lower in submerged systems. Sporulation occurred mainly in the biofilm phase rather than in suspension under almost all conditions tested.

Although many researches have focused upon biofilms of pure strains, in the food-processing environment microorganisms most likely exist as complex community consisting of several species (Djordjevic et al. 2002). Some microorganisms can form biofilms that harbour other microorganisms less prone to biofilm formation, increasing the probability of the many strains survival (Whitchurch et al. 2002; Djordjevic et al. 2002). Kalmokoff et al. (2001) reported that several strains of *L. monocytogenes* did not form biofilm under the tested conditions but adhere to the surface as isolated cells. *Staphylococcus xylosus* and *Ps. fragi* acted as primary colonizing bacteria for *L. monocytogenes* allowing the formation of multispecies

biofilm found on stainless steel (Norwood and Gilmour 2000). A *Flavobacterium* sp. strain and *Enterococcus faecium* have also been shown to favour *L. monocytogenes* and *C. jejuni* attachment respectively (Bremer et al. 2001; Trachoo and Brooks 2005). Tang and co-workers (2011) reported that *Acinetobacter* is a secondary colonizer as cells were often found attached to a monolayer of *Pseudomonas* cells. Habimana et al. (2010) proved that biofilm formed by an *Acinetobacter calcoaceticus* isolate from meat-processing environments enhanced colonization by *E. coli* O157:H7. Some strains of *Staphylococcus* species decreased *L. monocytogenes* populations while others increased them (Carpentier and Chassaing 2004).

Coculture of *Burkholderia cepacia* and *P. aeruginosa* resulted in a higher chlorine tolerance of both species in biofilm or clusters detached from biofilm in comparison to single species (Behnke et al. 2011). Similarly, mixed species biofilm of *L. monocytogenes* and *Lactobacillus plantarum* showed less than 2 log10 cfu/well inactivation after exposure for 15 min to 100 μg/ml benzalkonium chloride, while single species biofilms of *L. monocytogenes* showed 4.5 log10 cfu/well inactivation and single species biofilms of *L. plantarum* showed 3.3 log10 cfu/well inactivation (van der Veen and Abee 2011). In contrast, dual biofilm of *L. monocytogenes* and *S. enterica* did not show more resistance to benzalkonium chloride (50 ppm), sodium hypochlorite (10 ppm), and peracetic acid (10 ppm) in comparison to mono-biofilm of each species (Kostaki et al. 2012).

On a food processing surface *P. aeruginosa* and *Salmonella* can co-exist. The presence of cell-free culture supernatants containing N-acyl-L-homoserine lactones from *P. aeruginosa* significantly inhibited biofilm development of *Salmonella* (Wang et al. 2013b). These researches corroborated the hypothesis that the indigenous microflora has a strong effect on the settlement of *L. monocytogenes* as well as other microorganisms on surfaces in food processing environments.

These studies suggest that the strain type may be even more important than the substrate features in determining the pattern of biofilm formation and that considering in the experiments more than one species would be necessary to mimic microbial life on food processing surfaces where different bacterial species clearly live intermingled.

Effects of nutrients and environmental conditions on foodborne microorganisms

Nutrient availability and many environmental factors are known to modulate attachment and biofilm behaviour and therefore have been considered essential for the prevention of the biofilm formation (Wang et al. 2013b; Pilchová et al. 2014). Bacteria growing in a food-processing environment are exposed to fluctuating levels of nutrients, depending upon location in the plant, whereas environmental bacteria survive under reduced nutrient conditions (Djordjevic et al. 2002).

At lower temperatures (4, 12 and 22°C), the amount of *L. monocytogenes* biofilm was significantly higher on glass in comparison with polystyrene and stainless steel (Di Bonaventura et al. 2008). At 37°C, both stainless steel and glass allowed formation of similar biofilm levels, significantly higher than polystyrene did. Swimming by *L. monocytogenes* strains was observed only at 22°C, confirming the temperature-dependent flagellum production in this bacterium (Di Bonaventura et al. 2008). Studying the influence of the incubation temperature on the production of biofilm by 30 *Salmonella* strains, Stepanović et al. (2003) showed that it was statistically higher after 24 h at 30°C than at 37°C or ~22°C. However, after 48 h of incubation the statistically highest amounts of biofilm were observed at ~22°C. According to Cappello and Guglielmino (2006), higher temperatures (47 °C in comparison with 15 and 30 °C) seemed to increase cell surface hydrophobicity of *P. aeruginosa* ATCC 27853 and, in turn, its attachment. In the experiments by Hamanaka et al. (2012), *Pseudomonas* biofilm was considerably affected by incubation temperature (5 and 30°C) and nutrient condition (trypticase soy broth; TSB, and 1:20 diluted TSB), and physically weak biofilms were developed under high nutrient conditions, especially at low temperature. In Luria-Bertani broth, *B. cereus* ATCC 10987 produced thick biofilms at 30°C after 48 h, while *B. cereus* ATCC 14579 did not form biofilms under these conditions (Wijman et al. 2007). In the defined Y1 medium, after 24 h at 20 and 30°C both strains formed biofilms, but after 48 h the biofilm formed by *B. cereus* ATCC 14579 had dispersed, whereas that of *B. cereus* ATCC 10987 did not. Finally, spores were present in biofilms after 24 h, indicating that biofilm dispersion resulted also in the spread of highly resistant spores (Wijman et al. 2007). Few biofilm studies have focused on fluctuating temperature despite the fact that food processing plant frequently experience fluctuating environmental conditions. Morimatsu et al. (2013) studied the effect of temperature fluctuation on biofilm formation of a

co-culture of *S. enterica* and *P. putida*. At constant temperature of 5°C, *P. putida* became the dominant biofilm species and no bacterial interaction was reported, whereas, at constant temperature of 30°C, under poor nutrient condition, biofilm formation was enhanced. Inhibition of biofilm formation by temperature fluctuation was observed but this fluctuation helped *S. enterica* survival at low temperature, indicating that unsuitable temperature fluctuations in food processing pose a food safety risk.

The effect of different NaCl concentrations (0%, 2%, 4%, 6%, 8%, and 10%) on adhesion and detachment kinetics of *L. monocytogenes*, *S. aureus*, *S. boydii*, and *S.* Typhimurium was evaluated during 10 d of incubation at 37°C (Xu et al. 2010). All strains showed detachment at low NaCl concentrations, while no detachment was observed at high NaCl concentrations. Hingston and colleagues (2013) highlighted the importance of ensuring complete removal of salt and fat soils from food contact surfaces in order to limit the dessication survival of *L. monocytogenes*.

The obligate microaerophile *C. jejuni* is a very successful pathogen that survives during transmission under stressful aerobic conditions. Reuter and co-workers (2010) resolved this apparent paradox claiming that life in a biofilm would be an explanation, as they demonstrated that *C. jejuni* biofilm formation is clearly increased under aerobic conditions. The key target for the regulation of multicellular behaviour expression of *Salmonella* Typhimurium strains are the agfD promoters. Expression maxima of AgfD promoter activities were observed in rich medium under microaerophilic conditions and in minimal medium under aerobic conditions and in the environment characterized by low levels of nitrogen and phosphate (Gerstel and Römling 2001). Kives and co-workers (2005) reported on the sessile cocultivation of anaerobic *Lactococcus lactis* ssp. *cremoris* and aerobic *P. fluorescens* in skim milk at 7°C. The most significant advantages for the poor biofilm former *L. lactis* was the enhanced attachment provided by the *P. fluorescens* matrix and the low available oxygen in the mixed biofilm due to *P. fluorescens* methabolism. In return, *P. fluorescens* benefited from the lactic acid produced by the lactococci as a nutrient source.

Xu and colleagues (2011) evaluated the potential of biofilm formation of foodborne pathogens under different acidity conditions. The adhered cells of *L. monocytogenes* KACC 12671 and *Serratia liquefaciens* in TSB at pH 7 were relatively denser than those at pH 6 after 12 h of incubation. Also, the biofilm architecture was influenced by the pH. *L. monocytogenes* KACC12671 cells formed netlike structures at neutral pH and a monolayer biofilm at pH 6. *S. liquefaciens* cells cultivated in TSB at pH 6 produced cluster biofilms compared to the cells cultivated in TSB at pH 7. In all pH-unadjusted samples of *B. licheniformis* and *Lactobacillus paracasei* biofilm formation increased rapidly while pH decreased in the media to 5.7 and 5.5 respectively (Dat et al. 2012). As a consequence, the control of environmental pH at neutral range mitigated long-term biofilm formation in milk.

In general, dynamic flow conditions have also been observed to enhance bacterial attachment by bringing bacteria closer to a surface when compared to static conditions (Rijnaarts et al. 1993). Thus, also the transport of cells from the bulk liquid to the surface must be taken into account for a proper assessment of bacterial attachment.

The lack of consistent results under similar processing environmental and nutrient stress conditions can be explained by the application of different methods, conditions and bacterial strains but also by the fact that it is likely that no one factor can be responsible for the overall attachment (Palmer et al. 2007).

Paradigms of biofilms development monitoring

The occurrence of biofilms in plant environments, associated with human health problems and food spoilage, has increased the need for rapid, reliable and sensitive methods to sample sessile cells. The intensity in frequency and number of samples must be determined for each processing plant. Most of the surfaces presenting biofilms are difficult to access and, whether the analyses of water phases are possible, these do not reveal the site and extent of colonization. Due to the current lack of early warning systems the presence of biofilms is often still presumed when poor process performance and product quality is observed (Strathmann et al. 2013).

If physical access to the surface is possible, total viable count analysis is an easy option. Standard plate counts, often preceded by swabbing or scraping, first detach the microorganisms from the surface and then count them. Several researchers claimed standard plate count methods result in some inaccuracy on the number of viable microorganisms in biofilm since only its top might be removed (Oulahal-Lagsir et al. 2000; Verran et al. 2002) and swabs are not suited for sampling large surface areas (Schirmer et al. 2012). Additionally, two commercially available all-in-one swab rapid detection systems for *Listeria* spp. tested in cheese production environments and salmon processing facilities returned significant amounts of false positives (Schirmer et al. 2012). Other authors reported surface scraping to remove up to 97% of the cells adhered to stainless steel, and vortexing 99% of cells attached to silicone tubing (Djordjevic et al. 2002). Surfaces could be overlay with agar containing a tetrazolium salt, which stained the growing colonies red, with direct moulding (Husmark et al. 1999). However, cells may be difficult to remove and methods relying on cultivation fail to detect viable but non-culturable microorganisms that may threaten the quality of the food product. Additionally, chromogenic and blood agars are routinely used to distinguish between pathogenic and nonpathogenic *Listeria* species (Willis et al. 2006). Nevertheless, a nonhemolytic *L. monocytogenes* strain repeatedly isolated from a smoked salmon processing plant injected intraperitoneally into mice led to 60% lethality (Lindbäck et al. 2011).

Test surfaces can be used in laboratory, such as microtiter plates, or located at representative sites of the processing plants and removed at intervals. Number of attached microorganisms to these surfaces can be evaluated by staining. To better mimic food industry conditions, media in microtiter plates and other biofilm forming apparata are frequently used diluted (e.g. 1/20) (Castelijn et al. 2012). Comparing the biofilm architecture in standard and diluted media, Castelijn and colleagues (2012) reported a dense layer of *S.* Typhimurium cells in standard TSB while observed cells clusters embedded in cellulose in 1/20 TSB. Wang et al. (2013a) claimed that in vitro biofilm formation has limited significance to the understanding of biofilm formation under actual conditions in the food industry. Importantly, most of the in vitro biofilm were grown at the solid—liquid interface while biofilm at the solid—air or air-liquid interface has received considerably less attention. Biofilms formed by different strains of *B. cereus* in microtiter were seen to develop preferentially at the air-liquid interface. In real settings, they might develop in industrial storage and piping systems that are only partly filled during a production cycle or where some residual fluid has remained after operation (Wijman et al. 2007).

Microscopy techniques (light, laser-scanning confocal, transmission electron, and scanning electron microscopy) have the advantage of allowing direct observation of microbial colonization on the surface (Djordjevic et al. 2002). Attached microorganisms and matrix on these surfaces can be monitored by staining and specific stains can be used to label specific microorganisms or extracellular polymeric substances (Fig. 2). These techniques have the potential for quantifying microorganisms and provide qualitative data via image analysis (Jun et al. 2010). Confocal microscopy and quantitative image analysis of biofilms formed by fluorescent protein-tagged bacteria were used to investigate the development, structure and resilience of multispecies biofilm in comparison with single-species biofilms (Lee et al. 2014). Up to now, the architecture of few biofilms has been described in any detail in the food processing environment (Verran et al. 2002). Many biofilm growth simulating devices allow observation with microscopy techniques (McLandsborough et al. 2006). Finally, early biofilm detection can be achieved by installing sensors that provide in situ, online, real time information (Strathmann et al. 2013). Strathmann and co-workers (2013) reported a pioneer optical sensor that can distinguish biotic and abiotic deposits and provide information on total biomass and microbial activity.

Identification of sessile cells is important to evaluate if some strains are found repeatedly over long periods of time (even years) at the same location, while other strains (transient ones) appear just occasionally (Orgaz et al. 2013). A key difficulty in studying persistent strains is that strains may be persistent but missed because of the locations surveyed (Pan et al. 2006).

The information retrieved from genome sequences of microbial communities in the food processing industry, together with the development of DNA microarrays and improved proteomics techniques, might provide invaluable tools for the rapid detection and identification of pathogens, for assessing biological diversity and for understanding microbial ability to respond to stress (Abee et al. 2004). The direct detection and quantification of microorganisms from complex communities on food without cultivation using a DNA array-based method that targets 16S ribosomal DNA was proposed by Rudi et al. (2002). Recently, Xu and colleagues (2013) have written a review about the application of proteomics in safety assessment

and monitoring of food microorganisms. The identification of proteins associated with biofilm formation may lead to new strategies for controlling pathogens in food processing facilities.

Current methods remove the biofilm materials from the surface and generally investigate them in the laboratory. Despite all the efforts to mimic food industry conditions, it is worth noting that biofilms under laboratory conditions may have little resemblance with biofilms grown on food processing surfaces. Further, most of these methods are more or less time consuming. The implementation and use of sensors and probes will open new field of analysis in the food industry.

Traditional biofilms control

A pre-requirement is that the plant equipment has to be designed with high standards of hygiene in mind, so that the number of harborage sites, e.g. crevices, dead spaces, corners, gaskets, valves and joints, are reduced to a minimum. However, good design is not sufficient and an effective cleaning and disinfection (sanitation) programme is the traditional method of control of surface contamination (Møretrø et al. 2012).

Cleaning is a key procedure as, generally, disinfectants do not penetrate the intact biofilm matrix and thus do not destroy all the biofilm living cells (Simões et al. 2010). The cleaning process can remove up to 90% or more of sessile microorganisms, but cannot kill them. Importantly, cleaning methods can create aerosols that are known to transport microbial cells (Carpentier and Cerf 1993) and relocate viable cells from some areas to others receiving less disinfection (Taormina and Beuchat 2002).

The disinfectants currently used have been the focus of several reviews (Simões et al. 2010; Srey et al. 2013) and will not be treated here. In this review we want nevertheless hightlight that not only xenobiotics but also products of natural origin can effectively been used as disinfectants. Disinfectant solutions containing peppermint and lemongrass essential oils against *S. enterica* serovar Enteritidis S64 biofilm formation on stainless steel exhibited powerful antibacterial properties (Valeriano et al. 2012). According to the researchers, the essential oils solutions fulfilled the characteristics of having broad spectrum activity, environmental resistance and were easy to use.

In the food processing industry the time frame for biofilm development will depend on the frequency of sanitation regimes. A biofilm can build up in a few hours. The investigated sites of biofilm formation in an ice cream plant showed that most of the biofilm formations were seen on the conveyor belt of a packaging machine 8 h after the beginning of the production (Gunduz and Tuncel 2006). Some food processing surfaces, such as the milking machines, may be cleaned several times per day, while environmental substrata such as walls are cleaned less frequently (Gibson et al. 1999; Simões et al. 2010). Therefore, there is more time for biofilm formation on environmental surfaces rather than on food contact surfaces. The disinfectant concentration, type and exposure appeared to have a more important role in successful sanitation of *Salmonella* biofilm rather the age of the biofilm (Wong et al. 2010).

It has frequently been observed that biofilm cells can tolerate much higher concentrations of biocides than their planktonic counterpart, and consequently, cleaning and sanitation procedures used in the food industry are sometimes ineffectual in their removal (Romanova et al. 2007; Di Bonaventura et al. 2008; Lewis 2010). Of nine disinfectants used against *Salmonella* spp., all were efficient against planktonic cells while the bactericidal effect varied considerably and was less on sessile cells (Møretrø et al. 2009). Moreover, it has been shown that the chemical agent most effective on planktonic cells is not necessarily the most active against sessile cells and that the most active compound against monospecies biofilm is not necessarily the most effective against multispecies biofilm (Van Houdt et al. 2010). Trachoo and Brooks (2005) demonstrated that heat resistance is enhanced when *C. jejuni* forms a multispecies biofilms with *E. faecium*. Similarly, Norwood and Gilmour (2000) demonstrated the increased protective properties of multispecies biofilms containing *L. monocytogenes* compared with those of monoculture biofilms of the same microorganism.

Behaviour of sessile microorganisms to disinfectants is strongly influenced by the type of substratum. The resistance of sessile *L. monocytogenes* cells to a sanitizer was greater on the Teflon substrate than on the stainless steel substrate (Pan et

al. 2006). Disinfection of biofilms with chlorine proved to be up to 130 times more effective on the electropolished stainless steel than on the stainless steel surface polished by bright-alum, mechanically sanded or untreated, suggesting that surface finish is a key characteristic in a food processing plant (Schlisselberg and Yaron 2013). Chlorine, iodine, quaternary ammonium compounds, and anionic acid were tested against *L. monocytogenes* biofilm (Wong 1998). Bacterial biofilm cells were reduced 3 to 5 log colony forming units (cfu)/cm^2 and <1 to 2 log cfu/cm^2 on stainless steel and buna-n rubber respectively. Slow growth on buna-n rubber could partially explain the reason why sessile cells that formed on BN were more resistant to these disinfectants.

Apart from the type of substratum, other factors such as temperature of contact, environmental pH and the presence of organic matter (food particles, dirt and extracellular polymeric substances) can exert a considerable effect on the activity of an antimicrobial agent (Di Bonaventura et al. 2008), i.e. disinfectants are generally more effective in the absence of organic material. To enhance the penetration of the biocidal molecule through the biofilm, thereby abating the concentrations needed to eradicate sessile bacteria to levels very close to those effective against planktonic bacteria, Costerton et al. (1994) suggested the use of an electric current (the so-called 'bioelectric effect').

It may well be necessary to use much higher concentrations of disinfectant than the manufacturer suggests for specific areas in the food-processing environment to ensure lethal concentrations (Norwood and Gilmour 2000). Resistant species are expected to prevail over the rest of microbial community upon exposure to sublethal biocide concentrations, i.e. at concentrations below that which is recommended by the manufacturer or if it becomes diluted accidentally, increasing the chances for food contamination (Ortega et al. 2013). The fact that some bacteria have developed resistance to conventional antimicrobials once effective in killing them, clearly shows that new biofilm control strategies are required (Torlak and Sert 2013). On food processing surfaces, in addition to apply biocidal solutions at recommended concentrations, Ortega and colleagues (2013) suggested the use of biocidal solutions containing more than one bioactive compound.

Even with diligent cleaning and sanitizing, low numbers of microorganisms may remain on equipment surfaces and this residual viable population, even if lower than 1% of the total population can reseed the biofilm (Simões et al. 2010). Persistence, the ability of a microorganism to survive for months or years on a surface despite intensified sanitation efforts and periods of inactivity, causes more risk of cross contamination (Hingston et al. 2013). The nonstarter lactic acid bacterium *L. curvatus*, which can potentially cause quality defects in the final cheese product at very low level, persisted on the surface of the vat (ca. 10 cfu/cm^2) after cleaning and disinfection (Wong 1998). Orgaz and co-workers (2013) did not find a correlation between biofilm-forming ability of *L. monocytogenes* strains and persistence. However, after 72 h two non persistent *L. monocytogenes* strains showed more detachment than the other strains tested. In addition, when immersed into fresh medium with biocidal chitosan and then re-incubated, persistent strains took longer to start resuscitation, but then they grew at a faster rate suggesting a peculiar damage and/or recovery mechanism. Also Møretrø and colleagues (2003) did not relate persistence of certain *Salmonella* isolates in fish feed factories with enhanced resistance to disinfectants.

The use of approved antimicrobial agents presents some issues related to disposal and worker's safety (Yang et al. 2013) and scientific evidence indicates that biocides may contribute to the increased occurrence of antibiotic resistant bacteria (SCENIHR 2009). Therefore, the possible consequences to human health of biocide tolerance in the food industry are very relevant. This fact is particularly important considering that *Salmonella* isolates from a chicken slaughter plant and from humans in the same area of the plant suffering from *Salmonella* infections showed similar antimicrobial resistance patterns, namely resistance to ampicillin, trimethoprim/sulfamethoxazole, gentamicin, chloramphenicol and tretracycline (Wang et al. 2013a). *Salmonella* Enteritidis mutants obtained following exposure to the chlorine or to various preservatives showed less susceptibility to multiple antibiotics (tetracycline, chloramphenicol, nalidixic acid, and ciprofloxacin) (Potenski et al. 2003).

As conventional control methods sometimes fail to adequately remove adhered bacteria from the process equipment (Srey et al. 2013), new control strategies or supplemental control methods have to be developed.

Alternative biofilm control strategies

The antibacterial photosensitization-based treatment has been recently proposed as a novel method for decontamination of food-processing and food-handling environment from biofilm (Luksiene and Brovko 2013). The method is based on combined action of a nontoxic dye or photosensitizer, visible light, and oxygen. The photosensitization diminished population of *L. monocytogenes* $ATC_{L3}C$ 7644 biofilms by 3.1 log when supplying 5-aminolevulinic acid, the precursor of the endogenous photosensitizer porphyrins (Buchovec et al. 2010). Moreover, thermosensitive *L. monocytogenes* $ATC_{L3}C$ 7644 and thermoresistant 56 Ly strain biofilms were removed by 4.5 log from the surface by photosensitization with Na-Chlorophyllin at a concentration of 1.5 $x10^{-4}$ M (Luksiene et al. 2010).

Greer (2005) claimed that exogenously introduced phages have a lot of potential for food preservation being self-perpetuating, highly discriminatory, natural, and cost-effective, whereas the main drawback is the limited host range vs. the diversity of bacteria found in different settings. Little information is available on the action of bacteriophage on biofilm (Simões et al. 2010). Some of the work using phages against in vitro biofilms formed by spoilage and pathogenic bacteria showed that under ideal conditions significant viable cell reductions were achieved and thus, their use for biosanitation is promising (Sillankorva et al. 2012). Recently, coliphage ECP4 applied to *E. coli* O157:H7 sessile cells has been shown to efficiently reduce biofilm (Lee et al. 2013). Importantly, some researchers claim that biofilm cell lysis provoked by phages might promote persistence and survival of the remaining live cells, as survived cells gain nutrients from the dead microorganisms (Brooks and Flint 2008).

Oulahal-Lagsir and colleagues (2000) reported the in situ use of an ultrasonic technique for the removal of biofilms on a meat processing equipment. They claimed that ultrasound removed a significant amount of biofilm up to four times greater compared to the swabbing method. In a later study by the same authors (Oulahal et al. 2004), two ultrasonic devices were developed to remove biofilms from opened and closed stainless steel surfaces. A total removal of *E. coli* and *S. aureus* biofilms was obtained with the device used on opened surfaces (10 s at 40 kHz). However, the curved ultrasonic transducer developed for closed surfaces used under the same conditions failed to completely remove *E. coli* and *S. aureus* biofilms. This device used in combination with a chelating agent (EDTA or EGTA) completely dislodged *E. coli* biofilm but not significantly improve *S. aureus* biofilm removal.

Enzymes can be used to degrade the biofilm matrix. A mixture of enzymes is generally necessary due to the variety of polymers composing the matrix. A commercial product containing a *Bacillus* protease, altered the biofilm formed by *E. coli* K-12 MG 1655, leading to a fragile and easily peeled off biofilm (Furukawa et al. 2010). Lequette and co-workers (2010) reported that on stainless steel coupons serine proteases were more efficient in removing cells of *Bacillus* biofilms than polysaccharidases. On the contrary, polysaccharidases were more efficient in removing *P. fluorescens* biofilms than serine proteases. Surfactants, and dispersing and chelating agents added to the buffer improved the efficiency of both the enzymes. The researchers suggested that a mixture of enzymes, surfactants, dispersing and chelating agents could reduce the use of chemical cleaning agents. Nevertheless, these mixtures are generally more expensive than conventional products and must be stable under the plant working conditions (Brooks and Flint 2008). Interestingly, a synergetic effect of ultrasound and enzymatic activity has been reported. Using a combined treatment consisting in ultrasonic waves and proteolytic or glycolytic enzyme preparations, Oulahal-Lagsir et al. (2003) demonstrated a two to three times greater removal compared to sonication alone.

The research on new control methods for biofilms showed the potential of the atmospheric-pressure/cold plasmas (Abramzon et al. 2006). The technique has been applied several times on fresh produce and on different surfaces (Critzer 2007, Misra et al. 2011, Niemira 2012).

Another alternative approach involves interrupting communication in bacteria instead of killing them (Bai and Rai 2011). A deeper appreciation of signaling at the cellular level may lead to novel control targets for combating single- and mixed-microbial communities and conversely may also allow the enhancement of beneficial microorganisms (Skandamis and Nychas 2012). Importantly, several proteolytic, lipolytic, chitinolytic, and pectinolytic activities associated with the deterioration of foods as well as virulence are regulated by quorum sensing (Bai and Rai 2011). Davies and Marques (2009) found that *P. aeruginosa* produces the signalling molecule cis-2-decenoic acid, which is capable of inducing established biofilm dispersion and biofilm development inhibition. This molecule was successfully applied exogenously to induce

dispersion of sessile *B. subtilis*, *E. coli*, *S. aureus*, *Klebsiella pneumoniae*, *Proteus mirabilis*, *Streptococcus pyogenes* and *Candida albicans*.

Combining more than one approach, called 'hurdle technology', is expected to have greater effectiveness at controlling microorganisms than the use of any single factor (Lee 2004). This strategy can be better pursued in the future to control biofilm formation on food processing surfaces. As current control strategies are not entirely satisfying, much attention should be paid at preventing biofilm formation. Management programmes should include both prevention and control strategies, undertaking a more comprehensive approach.

Biofilm prevention

Biofilm formation by the bacteriocin-producing strains, in particular *Lactobacillus sakei* CRL1862, with anti-*Listeria* activity was tested on inert materials regularly used in meat processing facilities (Pérez et al. 2014). Studies on the inhibition of *L. monocytogenes* by bacteriocinogenic lactic acid bacteria sessile cells are in progress and early results show potential for developing new strategies for controlling this pathogen.

Pre-conditioning the surface with a surfactant has been reported to prevent microbial attachment (Nitschkea and Costab 2007). Biosurfactants that have anti-adhesive properties have been reported to be active against bacteria important to the dairy manufacturers (Flint et al. 1997). Surfactin from *B. subtilis* disperses biofilms without affecting cell growth and prevents biofilm formation by microorganisms such as *S. enterica*, *E. coli*, and *P. mirabilis* (Mireles et al. 2001). Splendiani and co-workers (2006) screened twenty-two surfactants for their potential to increase the cell wall negative charge or the electrostatic repulsion between *Burkholderia* sp. JS150 cells and reduce the ability to attach to a surface. The authors demonstrated that teepol had the best characteristics in relation to the reduction of biofilm accumulation.

A future goal of the applied research in this field is to functionalize food industry materials or blend compounds into a polymer coating in order to make the food contact materials resistant to microbial colonization (Villa et al. 2009). Materials with (3-N,N,N--triethanolammoniopropyl)trimethoxysilane chloride, and (3-N,N-dimethyl-3-N-n-octylammoniopropyl)trimethoxysilane chloride seemed promising as suitable coatings for materials that come into contact with drinking water, due to their significant antibacterial properties and their ability to repel *Aeromonas hydrophila*, an emerging player causing infectious disease, implicated in the contamination of water (Kregiel 2013). Kregiel and Niedzielska (2014) showed that also polyethylene modified with dimethoxydimethylsilane inhibited cell attachment and biofilm formation of *A. hydrophila* LOCK0968. Beside xenobiotics, microorganisms and plants offer a virtually inexhaustible and sustainable resource of very interesting classes of biologically active compounds that, used at sub-lethal doses, reveal mechanisms subtler than the killing activity, offering an elegant way to develop novel biocide-free antibiofilm strategies (Villa and Cappitelli 2013). Not exerting their action by killing cells, these compounds do not impose a selective pressure causing the development of resistance.

Along with experimental methods, mathematical models for describing the processes in living systems at various organization level, starting with macromolecules and then at level of organisms, and finally, at the level of biofilm as a whole, are considered very useful for predicting biofilm formation in many environments. In 1986 Wanner and Gujer (1986) proposed, for the first time, an analytical mathematical model of microbial interaction in biofilms predicting changes in biofilm thickness and describing the dynamics and spatial distribution of microbial species and substrates in the biofilm. Later, based on the assumptions used in the one dimensional model by Wanner and Gujer, a multidimensional continuum model for heterogeneous growth of biofilm systems with multiple species and multiple substrates was developed by Alpkvista and Klapper (2007).

Although this area arguably remains in its infancy, promising present research suggests that some prevention applications in the form of modified surfaces are now feasible and other may be available in the next future. Predicting biofilm development on a surface would decrease the risk of cross contamination and increase time between cleaning events and, in turn, reduce losses and abatement costs to the food industry.

Conclusion

In the future, predictive models should be the dominant research theme when dealing with the ecological complexity of biofilm in food environments. In addition, recent ecological developments recognize that the microbial community cannot be defined without reference to its abiotic environment and multiple factors are associated with attachment, maturation and detachment processes (Konopka 2009). To date, significant progresses on understanding biofilm formation in food processing environments have likely not be achieved as microbial environmental sensing and factor interactions have been not fully elucidated and most of the biofilm studies adopted a reductionist approach, trying to oversimplify complex ecological systems, e.g. using the conventional 'one-variable-at-a-time' approach in laboratory-based model systems with monospecies biofilms. More robust use of the design of experiment (Olsson et al. 2006) and high-throughput methodologies (Vinten et al. 2011; Hook et al. 2012), and correlations between sequenced genomes and biofilm phenotype features (Toussaint et al. 2003) in the food environment field will be needed to more effectively address many unanswered questions.

Fig 1. Biofilm formation. A) microorganisms move into close proximity with the surface. The first step is the adhesion, first reversible and then irreversible, to the surface. B) The maturation of biofilms involves production of an extracellular polysaccharide matrix, cellular division, recruitment of additional microorganisms from the local environment and cell-to-cell communication. C) The final stage is the detachment of microbial cells and related biofilm material.

Fig 2. Image of biofilm formed by *Listeria monocytogenes* on stainless steel surface. The biofilm was grown in a lab-scale system, called the CDC biofilm reactor (CBR) system (Biosurface Technologies, Bozeman, MT, USA), operating under continuous flow configuration. Cells were stained in green with Syto9, whereas the polysaccharide component of the EPS matrix was stained in red with the Texas red-labelled Concanavalin A. Biofilm was visualized using a Leica TCSNT confocal laser scanning microscope with excitation at 488 nm, and emission ≥ 530 nm (green and red channels). The image was captured with a 63X 0.9NA water immersion objective and analyzed with the software Imaris (Bitplane Scientific Software, Zurich, Switzerland). Bar represents 20 um.

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