3 Resistance of *Listeria Monocytogenes* Biofilms to Sanitizing Agents

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3.1 INTRODUCTION

Listeria monocytogenes is notorious for its capacity to colonize the environment and equipment of food processing facilities and to persist in the processing plant ecosystem, sometimes for decades (Carpentier and Cerf, 2011; Ferreira *et al.* 2014; Gandhi and Chikindas, 2007; Kathariou 2002). Such persistence is mediated by multiple attributes of *L. monocytogenes*, including the pathogen's capacity to form biofilms, replicate in the cold, tolerate disinfectants, and resist attack by phages. Biofilm forming potential is of pivotal importance: not only are bacteria in the sessile biofilm community more difficult to physically remove (e.g., by scrubbing or rinsing) than loosely adherent cells, but also existence in a biofilm impacts adaptive attributes, potentially resulting in enhanced tolerance towards various stresses. Indeed, extensive evidence indicates that bacteria in biofilms from diverse ecosystems exhibit enhanced tolerance to disinfectant and other antimicrobial agents, including antibiotics (Bridier *et al.* 2011; Costerton *et al.* 1995).

There is a substantial body of literature on the role of specific *L. monocytogenes* determinants and environmental conditions (e.g., type of solid substrate, nutrients, presence of other microbial partners, and temperature) on biofilm formation. In addition, several investigations have addressed the relative ability of various strains of *L. monocytogenes* to form biofilms, in order to ascertain potential impact of attributes such as serotype, genotype, and origin (e.g., environmental, food, clinical) on biofilm forming potential. Since several relevant reviews are already available (Carpentier and Cerf, 2011; da Silva and de Martinis, 2013; Ferreira *et al.* 2014; Gandhi and Chikindas, 2007; Renier *et al.* 2011; Sofos and Geornaras, 2010; Valderrama and Cutter, 2013; Wong, 1998; Zottola and Sasahara, 1994), here we will direct our attention on certain key trends and new findings and on their potential

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implications for the focus of this chapter (i.e., resistance of biofilm-associated *L. monocytogenes* to disinfectants and other agents or treatments employed for cleaning and sanitation in food processing ecosystems).

In food processing environments, the presence of water and organic residues such as complex mixtures of carbohydrates, proteins, fats and minerals, the constant influx of microbes, and the availability of numerous potential harborage sites are major contributors to the formation and persistence of biofilms that may include *L. monocytogenes* and other pathogens (Tompkin, 2002). In these biofilms, *L. monocytogenes* and other microbes form aggregates that are cemented together and stabilized via an extracellular polymeric substance (EPS) (Flemming and Wingender, 2010; Ghannoun, 2004). EPS not only mediates adherence of microbes to each other and to the solid substrate, but also protects the cells from dehydration and other stresses. The biochemical profile of EPS in biofilms varies based on the composition of the biofilm community as well as environmental factors such as temperature, nutrients, and type of contact surface (Flemming and Wingender, 2010; Ghannoun, 2004).

As mentioned previously, from the point of view of food safety the interest in *L. monocytogenes* biofilms largely stems from their ability to serve as stable assemblages that facilitate survival and persistence of the pathogen in harborage sites within the food processing plant ecosystem, compromising not only physical removal efforts but also the efficacy of disinfectants. The impacts of biofilm associations on resistance to sanitizers are complex and variable, depending on a multitude of parameters. To date, such impacts have been characterized in model systems that vary markedly in their experimental design and the extent to which they are able to simulate the food processing plant ecosystem, the microbial components of which largely remain poorly characterized themselves. The result is a relatively large but often conflicting and confusing literature, which still fails to address major knowledge gaps and thus is hampered in its ability to identify and assess science-based mitigation strategies. Therefore, this chapter will discuss the major sources of variation in studies and outcomes of sanitizer resistance of biofilm-associated *L. monocytogenes*. However, attention must first be paid to the methodology for assessment of resistance, since this is a critical source of variation in the experimental design and interpretation of findings from relevant investigations.

3.2 ASSESSMENT OF SANITIZER EFFICACY AND RESISTANCE IN BIOFILM-ASSOCIATED *LISTERIA MONOCYTOGENES*

The extent to which biofilm-associated *L. monocytogenes* is resistant to a sanitizer is typically determined by treating the biofilms of interest with specific concentrations of the sanitizer for specific lengths of time and determining the effect on *L. monocytogenes*. Even though crystal violet staining has been frequently

employed in assessing amounts of biofilm, the extent of staining reflects overall biofilm biomass and frequently does not correlate with viable counts of *L. monocytogenes* in the biofilm (Combrouse *et al.* 2013; Kadam *et al.* 2013; Romanova *et al.* 2007). Therefore, enumerations of viable *L. monocytogenes* in sanitizer-treated biofilms have utilized neutralization of the sanitizer and detachment of the cells from treated biofilms by methods such as by swabbing, scraping, sonication, or vortexing with glass beads (Giaouris *et al.* 2014; Kastbjerg and Gram 2009; Manios and Skandamis, 2014; Minei *et al.* 2008) followed by direct culture-based determinations of viable counts for the detached cells or indirect metabolic/physiological assays such as ATP bioluminescence (Nakamura *et al.* 2013; Romanova *et al.* 2007), conductance (Chorianopoulos *et al.* 2008, 2011a, b, Giaouris *et al.* 2014), cellular viability XTT assays (Torlak and Sert, 2013), real time quantitative polymerase chain reaction (qPCR) in the presence of propidium monoazide (Pan and Breidt, 2007) and LIVE-DEAD stains (e.g., the BacLight RedoxSensor CTC Vitality kit) (Kadam *et al.* 2013; Weiss *et al.* 2011; Skovager *et al.* 2013). Besides providing estimates of numbers of viable cells, conductance assays were able to determine differential anti-listerial impacts of lactic versus hydrochloric acid on biofilm-associated *L. monocytogenes* (Giaouris *et al.* 2014).

In order to determine overall efficacy of sanitizers on biofilms, direct or indirect viability assays such as the ones listed previously should be ideally complemented with assays that measure overall biofilm biomass, such as crystal violet staining. Such combined approaches will assess not only the extent of pathogen inactivation in the biofilm but also the extent to which the biofilm (regardless of whether cells are alive or not) has been removed (Romanova *et al.* 2007). Residual biofilms, even without viable microbes, can serve as foci for settlement of in-coming live cells of *L. monocytogenes* and other microbes followed by their growth as sessile populations.

In certain investigations such as van der Veen and Abee (2010a, b), sanitizer resistance of biofilm-associated *L. monocytogenes* was measured utilizing cells that were detached from the biofilm by methods as described previously. The detached and dispersed cells were then treated with the sanitizer and reductions in populations determined by direct or indirect viability assessments. Detached cells are clearly of great importance, because they not only can contaminate food products, but can also serve to seed a new biofilm at another harborage site. Thus, assessing their susceptibility to sanitizers in their detached state (prior to adherence and growth onto food or on new surfaces) is informative in measurements of sanitizer efficacy. However, even though detached cells from static biofilms of *L. monocytogenes* EGD-e were more resistant to peracetic acid and benzalkonium chloride than their planktonic counterparts (van der Veen and Abee 2010a, b), such assessments may over-estimate sanitizer efficacy for *L. monocytogenes* in biofilms, as detached cells may lose the protective impact of EPS and other biofilm components (Behnke *et al.* 2011; Pan *et al.* 2006; Stopforth *et al.* 2002).

In efforts to standardize and streamline efficacy determinations for multiple sanitizers against biofilm-associated *L. monocytogenes*, certain investigations have

employed the Calgary Biofilm Device (CBD), originally developed to facilitate testing of multiple antibiotics against biofilm-associated pathogens on indwelling medical devices (Ceri *et al.* 2001). Pegs attached to the lid of a 96-well plate serve as sites for formation of biofilms, allowing replicate testing of multiple compounds in the same plate. *Listeria innocua*, alone and in mixed cultures with *Escherichia coli*, was used to optimize conditions for establishment of the biofilms, treatment with disinfectant and release of the cells from the pegs for determinations of viable counts (Ali *et al.* 2006). The CBD has been used to compare efficacy of various disinfectants and *L. monocytogenes* cells released from the pegs were suitable for RNA extraction to assess expression of specific genes subsequent to treatment (Rodrigues *et al.* 2011). The device was also used to determine minimum biofilm eradication concentrations (MBECs) for four disinfectants in biofilms of *L. monocytogenes* alone and in coculture with *Pseudomonas aeruginosa*, revealing that MBECs were generally greater for mixed-species biofilms than *L. monocytogenes* alone and for biofilms produced at 12°C than those produced at 37°C (Lourenco *et al.* 2011).

3.3 FACTORS AFFECTING *L. MONOCYTOGENES* BIOFILM RESISTANCE

3.3.1 Contact surfaces for biofilm establishment

Most investigations of sanitizer resistance of *L. monocytogenes* in biofilms have employed static multi-well formats (typically polystyrene). However, surfaces more relevant to materials encountered in food processing have been increasingly incorporated in model systems to assess resistance, including stainless steel, plastic, glass, and so on.

One study focusing at simulating materials and conditions likely to be encountered in the ready-to-eat meat processing environment investigated resistance of biofilms following treatment by combinations of detergents and sanitizers (Somers and Wong, 2004). Biofilms were formed at 10°C with and without meat and fat residues on different types of conveyor materials, stainless steel, rubber, wall surface, and brick. Sanitizer efficacy was decreased for biofilms formed on surfaces with meat and fat residues and depended on type of material, with lowest efficacy noted for biofilms on brick and conveyor materials (Somers and Wong, 2004).

Floor drains and drain pipes are important harborage sites for *L. monocytogenes* and other microbes in food processing plants (McBain *et al.* 2003; Tompkin, 2002). In one study, biofilms on polyvinyl chloride model drain pipes were treated with quaternary ammonium, peroxide, or chlorine sanitizers applied with or without a 30 s ultrasonication (Berrang *et al.* 2008). The peroxide sanitizer, either alone or in combination with ultrasonication, resulted in 4 log reductions of *L. monocytogenes* from the inner wall surface of the pipes. Efficacy of the other two sanitizers was

lower (~2 log reductions), but could be further enhanced by at least 10-fold by ultrasonication (Berrang *et al.* 2008).

Inactivation of *L. monocytogenes* from floor drains of a ready-to-eat poultry processing plant was examined in a study employing biocontrol as sanitation tool (Zhao *et al.* 2013). Following four administrations of two combined competitive exclusion (CE) cultures (*Lactococcus lactis* C-1–92 and *Enterococcus durans* 152) to the floor drains in the first week, *L. monocytogenes* could not be recovered from five of the six drains; the five drains remained *L. monocytogenes*-free for the following 13 weeks. The two CE cultures also exhibited efficacy against *L. monocytogenes* (~2 log reductions or higher) on model biofilms formed on stainless steel, plastic, rubber, glass, and silicone at 4 and 8°C (Zhao *et al.* 2013).

Conveyors have attracted special interest as potential food contact surfaces (Somers and Wong, 2004). *L. monocytogenes* in biofilms on conveyor belts was found to have remarkable resistance to chlorine (whether adjusted to pH 6.5 or not) with less than 2 log reductions following treatment (200, 400, and 600 ppm chlorine) (Bremer *et al.* 2002). In the same study, efficacy of chlorine at the same concentrations was noticeably higher when biofilms were formed on stainless steel coupons (Bremer *et al.* 2002).

Three different conveyor belt materials (polypropylene, acetal, and cold-rolled stainless steel) exposed to milk residues were investigated for the ability of ultrasonic cleaning (30 and 45°C) with two different alkaline detergents to reduce *L. monocytogenes* viable counts on 72-h biofilms (Tolvanen *et al.* 2007). Such ultrasonic cleaning, especially with KOH detergent at 45°C, was found highly effective even after only 30 s treatment, and significantly more for stainless steel (>4 log reductions) than for plastic (Tolvanen *et al.* 2007). In a subsequent pilot study, further increasing the temperature of ultrasonic washing with a KOH detergent to 50°C resulted in >5 log reductions of *L. monocytogenes* from 72-h biofilms formed on stainless steel conveyor belt material in the presence of meat residues, even after only 10 s of treatment (Tolvanen *et al.* 2009). In another pilot study with a different potential food contact surface (polystyrene containers), ultrasonic cleaning for 1–5 min at room temperature also enhanced the efficacy of the quaternary ammonium disinfectant benzalkonium chloride to reduce viable counts of *L. monocytogenes* in biofilms formed in tryptic soy broth over 6 days at 20°C (Torlak and Sert, 2013).

Cutting boards represent a major site for biofilm establishment and cross-contamination both in industrial and home settings. To assess impact of surface roughness of polyethylene cutting boards, biofilms were produced on polyethylene coupons of different surface roughness and employing conditions likely to be employed in the home (Yang *et al.* 2009). Assessing efficacy of a panel of sanitizers (quaternary ammonium compounds, lactic acid-based and sodium hypochlorite-based sanitizers) allowed ranking of efficacy against mature (7 days or older) biofilms, with the lactic acid sanitizer (pH 3.0) being most effective. The study also revealed that efficacy was reduced with increasing biofilm age (21 vs 7 days) and with increasing surface roughness (Yang *et al.* 2009).

3.3.2 Biofilm types and models

Static biofilms where biofilms are formed in the presence of batch media on the surface of the wells of polystyrene microwell plates and on other, more industry-relevant surfaces have been employed in the majority of studies of sanitizer resistance of biofilm-associated *L. monocytogenes*. Several alternative models have been developed and employed in investigations of sanitizer resistance of *L. monocytogenes*. These utilize four major approaches; specifically (1) repeated cycles of growth, rinsing and treatment with a sanitizer, (2) establishment of steady-state biofilms using constant-depth film fermenters, (3) continuous-flow systems, and (4) the Calgary Biofilm Device.

3.3.2.1 Repeated-cycle models

Pan *et al.* (2006) first described a simulated food processing (SFP) system involving repeated 24-h cycle treatments of coupons harboring biofilms of a five-strain mixture of *L. monocytogenes* for 3 weeks. After a brief exposure to disinfectant (mixture of peroxide-based compounds) the biofilm-laden coupons were rinsed with saline or water followed by extended drying (15–16 h) at room temperature. This was followed by 8-h exposure to diluted culture media simulating limited availability of nutrients (Pan *et al.* 2006). The biofilms developed resistance not only to the peroxide-based sanitizer but also to quaternary ammonium compounds and chlorine. However, resistance was abolished when cells were removed from the biofilms and then treated with the peroxide sanitizer (Pan *et al.* 2006). This SFP model system was also employed in studies of the relative fitness of different strains in the biofilms (Pan *et al.* 2009).

A model system employing repeated 24-h cycles was also employed to assess survival and resistance of *L. monocytogenes* (five-strain mixture) in biofilms formed on high-density polyethylene, material used in cutting boards (Yang *et al.* 2009). To simulate procedures in the home, each cycle consisted of growth in diluted media, rinsing and treatment with multiple types of sanitizers. As discussed earlier, using this model system it was possible to rank the sanitizers based on their efficacy, while also identifying impacts of biofilm maturity and surface roughness on resistance (Yang *et al.* 2009).

3.3.2.2 Constant-depth film fermenters

Constant-depth film fermenters have been employed to investigate steady-state multi-species biofilms of *L. monocytogenes* in the presence of *Pseudomonas fragi* and *Staphylococcus xylosus* (Norwood and Gilmour 2000). Steady-state was achieved after 17 days of growth, with *L. monocytogenes* constituting a minority (1.5%) of the biofilm population. This system was employed to assess tolerance of *L. monocytogenes* to increasing concentrations of sanitizer, with the biofilm

cells exhibiting >100-fold tolerance to chlorine than the planktonic counterparts (Norwood and Gilmour 2000).

3.3.2.3 Continuous-flow biofilms

Continuous-flow systems may better simulate conditions in food processing ecosystems than biofilms formed under static conditions and are accompanied not only by greater volume and thickness but also by distinctly different microstructure than static biofilms (Rieu *et al.* 2008). Initial adherence of *L. monocytogenes* depends on multiple factors, including strain type and flow rate/shear stress (Skovager *et al.* 2012). Under continuous flow conditions *L. monocytogenes* was found to be unable to adhere to wetted fine polished stainless steel but adhered onto stainless steel with nanocomposite TiN and TiN/Ag (silver) coatings; the latter surface exhibited pronounced anti-listerial potential, the extent of which differed between the two tested strains (Skovager *et al.* 2013).

In spite of the intrinsic interest and relevance of continuous-flow biofilms, we currently lack reports on systematic comparisons of sanitizer resistance of *L. monocytogenes* in such biofilms versus static biofilms with batch nutrients. Investigations of *L. monocytogenes* EGD grown in brain heart infusion broth for 48 h in static 12-well polystyrene plates and in continuous-flow biofilms indicated that dispersed cells from static biofilms were more resistant than planktonic cells to both benzalkonium chloride and peracetic acid; dispersed cells from continuous-flow biofilms, however, exhibited similar resistance to benzalkonium chloride as their planktonic counterparts (albeit more resistant to peracetic acid, as also observed for the static biofilm cells) (van der Veen and Abee 2010a, b). Such differences suggest sanitizer-specific impacts of continuous-flow versus static biofilms on resistance. However, as also discussed earlier, full assessments of possible impacts are compromised by the fact that comparisons involved cells detached from the biofilms, which may or may not reflect resistance of the cells in the actual biofilm matrix (Manios and Skandamis, 2014; Pan *et al.* 2006; Romanova *et al.* 2007; Stopforth *et al.* 2002).

Formation of *L. monocytogenes* biofilms in continuous-flow systems is accompanied by SOS responses that would be expected to enhance mutation frequency (van der Veen and Abee 2010c). Indeed, the frequency of rifampicin-resistant mutants was higher in *L. monocytogenes* from continuous-flow than in static biofilms. These findings correspond to enhanced formation of superoxide and hydroxyl radicals in the former, resulting in oxygen radical-induced DNA damage and mutagenic repair by RecA (van der Veen and Abee 2011b). It has been hypothesized that such DNA damage and repair mechanisms would promote genetic diversity within biofilms, thereby allowing adaptation to the changing biofilm environment including exposure to different types and concentrations of disinfectants. However, it remains unknown whether mutants with enhanced tolerance to sanitizers are also more likely to be generated in continuous flow than static biofilms, especially in the presence of sanitizers, which further enhance production of oxygen radicals.

3.3.2.4 The Calgary biofilm device

The CBD (Ceri *et al.* 2001) discussed earlier represents an ingenious system especially useful for high-throughput comparisons of the effectiveness of different sanitizers. Modifications of this design (e.g., employing different materials and surface texture) can be valuable in future studies.

3.3.3 Environmental conditions (temperature, nutrients, humidity)

Environmental conditions such as pH, humidity, nutrients, and temperature can impact efficacy of sanitizers against biofilm-associated *L. monocytogenes*. In certain cases, this is based on requirements of specific sanitizers for optimal efficacy. For instance, testing of a panel of quaternary ammonium sanitizers revealed that their efficacy against *L. monocytogenes* in biofilms formed on high-density polyethylene was higher at pH 10.4–11.5 than 6.2–8.7 (Yang *et al.* 2009). Generally, however, and as will be discussed further later, environmental conditions impact sanitizer resistance of biofilm-associated *L. monocytogenes* via effects on the physiology of the cells, especially by means of changes in EPS amounts, composition, and topology.

The acid tolerance response brought about following exposure of *L. monocytogenes* Scott A to sublethal acid stress during planktonic growth in brain heart infusion medium (15 days at 5, 16 or 30°C) conferred significantly greater ability of the cells in the biofilm to tolerate lethal acid conditions (pH 2.0, adjusted with either hydrochloric or lactic acid) (Belessi *et al.* 2011; Chorianopoulos *et al.* 2011a). Similar trends were noted with certain *L. monocytogenes* cheese isolates (Adrião *et al.* 2008). Planktonic cultures adapted over moderately acidic conditions (lactic acid; pH 5.5) were more tolerant to low pH and high salt concentration in their sessile forms. Furthermore, some of the acid-adapted strains exhibited greater propensity to bind to polystyrene (Adrião *et al.* 2008).

Temperature has been shown to play a crucial role in biofilm formation, even though the findings have often been conflicting, possibly reflecting differences in strains and biofilm conditions (Combrouse *et al.* 2013; Kadam *et al.* 2013; Kalmokoff *et al.* 2001; Nilsson *et al.* 2011; Norwood and Gilmour 2001). To assess impact of temperature and salt content on sanitizer resistance of *L. monocytogenes* in biofilms on stainless steel coupons, biofilms were formed at 5 and 20°C in tryptic soy broth with 0.5, 7.5, and 9.5% NaCl and then treated with warm water (20 min at 60°C) and 2% peroxyacetic acid. Viability of *L. monocytogenes* by the warm water treatment was not impacted in any of the biofilms, but *L. monocytogenes* in biofilms formed at 20°C exhibited greater tolerance to peroxyacetic acid than in 5°C biofilms; no impact of salt content on resistance was noted (Belessi *et al.* 2011). An opposite trend for impact of temperature was noted in a determination of the efficacy of four disinfectants (alkyl amine acetate, chlorine, and phosphoric acid-based) for a panel of strains using the CBD, with biofilms formed at 12°C being

generally more resistant to the sanitizers than those at 37°C (Lourenco *et al.* 2011). Differences in type of sanitizer, biofilm model type, temperature, and strains are among factors potentially responsible for different outcomes in response to temperature.

Humidity was found to influence resistance of biofilms of *L. monocytogenes* (and other foodborne bacterial pathogens) on stainless steel (Bae *et al.* 2012). *L. monocytogenes* in biofilms stored at 25°C, 100% relative humidity (RH) for 5 days displayed the highest levels of resistance to inactivation following treatment with chlorine and alcohol-based commercial sanitizers, in comparison to biofilms stored at 23, 43, 68, and 85% RH, even though viable counts in the absence of disinfectant decreased in biofilms exposed to low relative humidity (Bae *et al.* 2012).

3.3.4 Strain differences and relative fitness in resistance of biofilm-associated *L. MONOCYTOGENES*

L. monocytogenes is known for its clonal structure with most strains from human listeriosis belonging to two lineages: lineage I includes strains of serotypes 1/2b, 3b, and most strains of serotype 4b, while lineage II is comprised of strains of serotype 1/2a, 1/2c, 3a, and 3c. Serotype 4b, 4d, and 4e strains ("serotype 4b complex") are typically difficult to differentiate except by classical antibody-based serotyping. Serotypes 1/2a, 1/2b, and 4b are most frequently implicated in human listeriosis (Kathariou, 2002; Orsi *et al.* 2011; Painter and Slutsker, 2007; Swaminathan and Gerner-Smidt, 2007). A number of clonal groups have been identified within each of the serotypes, with certain of these groups ("epidemic clones"), especially of serotype 4b, being implicated in multiple outbreaks of human foodborne listeriosis (Cheng *et al.* 2008; Haase *et al.* 2014; Orsi *et al.* 2011; Ragon *et al.* 2008).

The fact that *L. monocytogenes* strains from food processing plants and foods tend to be of serotype 1/2a and 1/2b, and generally less commonly of serotype 4b (Kathariou, 2002), has prompted numerous investigations on differences among different strains and serotypes regarding biofilm forming potential (e.g., Borucki *et al.* 2003; Djordjevic *et al.* 2002; Kadam *et al.* 2013). However, few studies have reported the relative fitness of different *L. monocytogenes* strains in biofilms in the presence or absence of sanitizer treatment. Furthermore, these investigations have employed different experimental systems (i.e., microbial strain panels, materials for biofilm formation, sanitizer types and concentrations, and environmental conditions such as pH, nutrients, and temperature), compromising comparisons of findings from different studies.

Sanitizer tolerance has been investigated to assess its possible contribution to persistence of strains in the processing plant environment. Persistence is typically empirically evidenced by repeated isolations of strains with the same or closely related genotype over prolonged periods of time and is itself a complex attribute that can lead to confusion and controversy in the literature (Carpentier and Cerf, 2011; Ferreira *et al.* 2014; Holah *et al.* 2004; Kathariou, 2002; Keto-Timonen *et al.* 2007; Lundén *et al.* 2008; Wulff *et al.* 2006). However, findings related to relationships

between persistence and sanitizer tolerance of biofilm-associated *L. monocytogenes* must be viewed with caution. With a few exceptions (Kastbjerg and Gram, 2009; Nakamura *et al.* 2013) comparisons of resistance between persistent and transient strains have typically employed planktonic cultures, thus preventing assessments of associations between persistence and sanitizer tolerance in biofilms (Earnshaw and Lawrence, 1998; Heir *et al.* 2004; Holah *et al.* 2002; Lundén *et al.* 2003, 2008). This can be problematic, as in at least one study, the differences in resistance were minor when planktonic cultures were used, but pronounced when biofilms were employed (Nakamura *et al.* 2013). On another disconcerting note, conclusions from many studies are frequently drawn without taking into account the potential impacts of biofilm maturity, other microbiota, media and other factors that may play out in actual food processing environments. Thus, it is difficult to adequately identify correlations (or lack thereof) between sanitizer resistance and attributes such as serotype, lineage, persistence or virulence, even though current data indeed suggest strain-specific differences in sanitizer resistance of biofilm-associated *L. monocytogenes* (Kastbjerg and Gram, 2009; Nakamura *et al.* 2013; Pan *et al.* 2009; Robbins *et al.* 2005; Skovager *et al.* 2013). Differences in amount and composition of EPS and the microscopic structure of biofilm (Combrouse *et al.* 2013; Kalmokoff *et al.* 2011; Nakamura *et al.* 2013; Saá-Ibusquiza *et al.* 2012) may contribute to the observed strain-specific differences in resistance.

Most comparative studies have employed pure cultures of individual strains, but a few investigations used multi-strain biofilms to assess relative fitness in the context of biofilm formation and sanitizer treatment. Multiple strains of different lineages (serotypes 1/2a and 4b) were combined in biofilms using the "simulated food processing environment" (SFP) biofilm model system discussed previously (Pan *et al.* 2006). The relative prevalence of the serotype groups was monitored by serotype-specific primers using qPCR and propidium monoazide treatment for the selective enumeration of live cells (Pan *et al.* 2006, 2009). In such mixed-culture biofilms, the cell density of serotype 1/2a was originally higher than that of 4b, with the differences in relative numbers remaining constant at later time points (days 14 and 28), suggesting that strains of serotype 1/2a were more likely to adhere but that subsequent survival and growth in the SFP system was similar between 1/2a and 4b strains. Interestingly, when serotype 4b strains were mixed with one particular strain of serotype 1/2a, their total numbers after 28 days in the SFP model system were higher than when present alone (Pan *et al.* 2009).

Two other studies have examined sanitizer resistance of three different strains of *L. monocytogenes* together with one other bacterial species (*Salmonella enterica* and *Pseudomonas putida*, respectively) in multi-strain, mixed-species biofilms on stainless steel (Giaouris *et al.* 2013; Kostaki *et al.* 2012). Strain-specific differences were reported in both studies, even though the observed strain ratios differed. Strain ratios were determined based on pulsed-field gel electrophoresis profiles, which limited the number of colonies that could be genotyped, possibly resulting in sampling bias. This may be the reason for the observed major differences in strain ratios

in the untreated or treated single-species biofilms between the two studies (Giaouris *et al.* 2013; Kostaki *et al.* 2012). Employment of alternative strain identification formats such as propidium monoazide qPCR using strain-specific markers (Pan and Breidt, 2007; Pan *et al.* 2009) will be needed to further elucidate persistence and resistance-related fitness components of *L. monocytogenes* in complex biofilms.

3.3.5 Impact of other microbial species in multi-species biofilms

Multi-species biofilms are the norm in nature as well as in food processing plants and other environments relevant to foodborne transmission of *L. monocytogenes* (Elias and Banin 2012; Davey and O'Toole 2000; Ghannoun, 2004; Giaouris *et al.* 2014; James *et al.* 1995; Parsek and Greenberg 2005; Rao *et al.* 2005; Rendueles and Ghigo 2012; Rickard *et al.* 2003; Tolker-Nielsen and Molin 2000; Wimpenny 2009; Wimpenny *et al.* 2000; Yang *et al.* 2011). Stability and sanitizer resistance in multi-species biofilms may exceed those encountered in monoculture biofilms such as those typically investigated in the laboratory (Behnke *et al.* 2011; Burmølle *et al.* 2006; Giaouris *et al.* 2014; Habimana *et al.* 2009; Klayman *et al.* 2009; Leriche *et al.* 2003; Lindsay *et al.* 2002; Marouani-Gadri *et al.* 2009; Moons *et al.* 2006; Simões *et al.* 2007, 2011). There is thus interest in assessing the impact of other microbes on abundance, persistence and sanitizer resistance of *L. monocytogenes* in biofilms. To facilitate monitoring of the microbial partners, the majority of such studies have involved dual-species biofilms with *L. monocytogenes* and one additional bacterial species.

In dual-species biofilms formed at 25°C on stainless steel and polypropylene with *L. monocytogenes* and *P. putida*, resistance of *L. monocytogenes* to benzalkonium chloride was significantly higher than in mono-species biofilms (Saá-Ibusquiza *et al.* 2012). In contrast, another study of *L. monocytogenes-P. putida* biofilms (18°C) on stainless steel indicated no impact on benzalkonium chloride resistance of *L. monocytogenes* following exposure to sublethal concentrations of benzalkonium chloride (50 ppm), although, interestingly, benzalkonium chloride resistance of *P. putida* was noticeably enhanced (Giaouris *et al.* 2013). In these mixed-species biofilms *L. monocytogenes* was noticeably more susceptible to benzalkonium chloride than *P. putida*, accounting for the majority of benzalkonium chloride-killed cells (Giaouris *et al.* 2013; Saá-Ibusquiza *et al.* 2012).

Variable results have been obtained in studies involving other types of mixed-species biofilms. No impact of mixed- versus mono-species biofilms was noted for resistance of *L. monocytogenes* to benzalkonium chloride (50 ppm), sodium hypochlorite (10 ppm) or peracetic acid (10 ppm) in *L. monocytogenes-Salmonella enterica* biofilms formed on stainless steel at 15°C (Kostaki *et al.* 2012). However, susceptibility of *L. monocytogenes* to a mixture of hydrogen peroxide (5 ppm) and peracetic acid (5 ppm) appeared to be higher in the mixed-species biofilms than in mono-species biofilms. In such biofilms, *L. monocytogenes* was significantly more

tolerant to the disinfectants than *Salmonella*; even though the two species reached similar proportions in the mixed-species biofilms prior to disinfection, *L. monocytogenes* accounted for the majority of the survivors (Kostaki *et al.* 2012).

In contrast, in *L. monocytogenes-Lactobacillus plantarum* biofilms, *L. monocytogenes* exhibited enhanced (>2 log increase) resistance to benzalkonium chloride and peracetic acid (van der Veen and Abee 2011a), in comparison to single-species biofilms; noticeably increased resistance was also observed for the *L. plantarum* partner (van der Veen and Abee 2011a). Analyses of *L. monocytogenes* resistance to sodium hypochlorite using a steady-state multi-species (*L. monocytogenes*, *P. fragi*, and *Staph. xylosus*) biofilm showed that long (20 min) exposures to high levels of sodium hypochlorite (1000 ppm free chlorine) were required for 2 log reduction of *L. monocytogenes* (Norwood and Gilmour 2000). As in other studies, *L. monocytogenes* constituted a small proportion (at steady state, 1.8%) of the bacterial population in these mixed-species biofilms (Norwood and Gilmour 2000). The observed tolerance of *L. monocytogenes* in these multi-species biofilms was noticeably higher than observed with single-species *L. monocytogenes* biofilms on stainless steel (Lee and Frank 1991; Mosteller and Bishop 1993).

Enhanced sanitizer resistance of *L. monocytogenes* in mixed-species biofilms was also reported in a study of *L. monocytogenes-P. aeruginosa* PAO1 biofilms formed at 12 and 37°C using the Calgary Biofilm Device (Lourenco *et al.* 2011). In the mixed-species biofilms *L. monocytogenes* generally exhibited reduced susceptibility to the sanitizers, which included different commercial compounds based on alkyl-amine-acetate, chlorine, and phosphoric acid. Furthermore, reductions in susceptibility of *L. monocytogenes* in either single or mixed-species biofilms were greater in biofilms formed at 12°C than at 37°C (Lourenco *et al.* 2011). Such findings suggest the potential usefulness of assessing sanitizer efficacy for *L. monocytogenes* under diverse conditions, including relevant food contact surfaces and reduced temperatures (Somers and Wong 2004) as well as in the presence of other species.

L. innocua was used as surrogate for *L. monocytogenes* in assessments of disinfectant efficacy for mixed *L. innocua-P. aeruginosa* biofilms on various food contact surfaces, including stainless steel and rubber (Bourion *et al.* 1996). Mixed *L. innocua-E. coli* biofilms formed on the pegs of the CBD were also investigated for disinfectant efficacy in another study (Ali *et al.* 2006). However, the utility of *L. innocua* and other non-pathogenic *Listeria* spp. as surrogates in such assessments remains to be fully exploited. Furthermore, the impact of other *Listeria* spp. on *L. monocytogenes*' resistance to sanitizer and persistence in biofilms remains to be elucidated. In harborage sites of food processing plants, *L. monocytogenes* is likely to occur together with other *Listeria* spp., especially *L. innocua* and *L. welshimeri* (Barbalho *et al.* 2005; Hofer *et al.* 2000; Kathariou 2002).

Mixed-species biofilms on stainless steel involving *L. monocytogenes* and four other bacterial species (*Staphylococcus simulans*, *Lactobacillus fermentum*, *P. putida*, and *Salmonella enterica*) were employed to assess efficacy of the essential oil of *Satureja thymbra* (winter savory of the *Labiatae* family) in comparison to

three standard acid and alkaline chemical sanitizers; in these investigations, the essential oil-based preparations proved significantly more effective than the chemical sanitizers (Chorianopoulos *et al.* 2008). Further assessments of the efficacy of essential oils and other plant-based antimicrobial compounds in mixed-species biofilms are clearly warranted.

As mentioned earlier, most studies of mixed species biofilms have employed only a few (usually two) different species. Daily treatments of a complex mixed-species biofilm to a chlorinated alkaline solution suggested that certain species may benefit from protective effects stemming from the spatial organization of different taxa in the biofilm (Leriche *et al.* 2003). Little understanding is currently available on how *L. monocytogenes* may respond to sanitizer treatment when present as member in biofilms comprised of complex microbial communities that would include not only multiple species of bacteria but also other microorganisms. Phages, fungi, protozoa, and other microscopic eukaryotes (nematodes and rotifers) can play important roles in establishment and population dynamics of *L. monocytogenes* in biofilms in nature, the food processing environment and equipment, and foods themselves (e.g., Addis *et al.* 2001; Corsetti *et al.* 2001; Gori *et al.* 2013; Guillier *et al.* 2008; McBain *et al.* 2003; Roth *et al.* 2011; Schuppler 2014). In the case of protozoa, the impact for *L. monocytogenes* can be protective, promoting growth via excreted compounds (Fieseler *et al.* 2014; Schuppler 2014) and possibly shielding the bacteria from disinfectants, as reported for other systems (Snelling *et al.* 2005, 2006) or inhibitory, if a predator-prey relationship is primarily involved.

Further studies utilizing model biofilms comprised of complex communities representative of those in the food processing ecosystem (as well as other ecosystems frequently exposed to sanitizers, such as healthcare facilities) will be needed to elucidate the impacts of sanitation and disinfectants on biofilm-associated *L. monocytogenes* in a microbial ecology context. The advent of next generation sequencing and accompanying bioinformatics tools can now facilitate the implementation of such studies with focus not only on model systems such as stainless steel biofilms involving a selected panel of relevant microbes, but also on intact ecosystems, via elucidation of the microbiome, monitoring of community composition trends, transcriptome analysis, and other approaches.

3.4 SANITIZER RESISTANCE OF BIOFILM-ASSOCIATED *L. MONOCYTOGENES*: CONSISTENT FINDINGS AND TRENDS

Generally, *L. monocytogenes* grown as biofilms on solid surfaces exhibit greater tolerance to various sanitizers than planktonic cells of the same strains (Folsom and Frank, 2006; Norwood and Gilmour 2000; Oh and Marshall 1996; Robbins *et al.* 2005; Romanova *et al.* 2007; van der Veen and Abee 2010a, b; Yun *et al.* 2012). Differences were especially pronounced in certain studies, with resistance to

benzalkonium chloride and sodium hypochlorite being more than 1000-fold greater for biofilm cells than for their planktonic counterparts (Norwood and Gilmour 2000; Romanova *et al.* 2007). However, in some model systems differences in resistance between planktonic and sessile populations were modest or not noted at all (Chavant *et al.* 2004, for several disinfectants on static biofilms; Robbins *et al.* 2005, for resistance to chlorine in static biofilms; van der Veen and Abee 2010a, b, for benzalkonium resistance of continuous-flow biofilms).

Differences in biofilm age, type (i.e., static vs continuous-flow systems), material type and surface texture, nutrients, temperature, strains, type and concentration of the sanitizer, and duration of the treatment are among the multitude of factors that can contribute to different findings, making it difficult to compare results from different studies. Nonetheless, certain consistent trends have emerged: resistance tends to be greater as biofilms mature and appears to be impacted by surface features, with rough surfaces and surfaces soiled with food residues affording greater resistance to various sanitizers (Chavant *et al.* 2004; Nilsson *et al.* 2011; Norwood and Gilmour, 2000; Oh and Marshall, 1996; Somers and Wong 2004; Yang *et al.* 2009). Interestingly, resistance of *L. monocytogenes* in biofilms to desiccation also was enhanced with increasing biofilm maturity and in the presence of salt or fat during desiccation (Hingston *et al.* 2013). Such data are intriguing and relevant for the food processing environment. Desiccation can become incorporated in cleaning and sanitation regimes (e.g., via drying of surfaces following cleaning and use of alcohol-based sanitizers) and has been a component of biofilm systems aiming to simulate the food processing environment (Bae *et al.* 2012; Pan *et al.* 2006, 2009).

3.5 MECHANISMS OF DISINFECTANT RESISTANCE IN BIOFILM-ASSOCIATED *L. MONOCYTOGENES*

Resistance of biofilm-associated *L. monocytogenes* to disinfectants can be mediated by diverse mechanisms, several of which remain poorly understood. These include the protective impact of the extracellular matrix and limited access of the disinfectant to the cells; the specialized physiological state and dedicated stress responses of the cells in the biofilms; expression of genes (including those acquired via horizontal gene transfer) mediating efflux of the disinfectant; and the presence of a sub-population of dormant cells (persisters), which tolerate otherwise lethal disinfectant exposures.

Analysis of the response of multiple strains to chlorine following planktonic or biofilm growth on stainless steel has revealed little or no correlation between resistance profiles when the strains were grown planktonically and on biofilms, suggesting that different mechanisms mediate resistance in planktonic versus biofilm cells (Folsom and Frank 2006). Similar conclusions were reached following analysis of resistance to hydrogen peroxide treatment of planktonic and biofilm cultures (Yun *et al.* 2012). The mechanisms mediating these differences to sanitizer resistance

between planktonic cells and biofilms are complex, as numerous aspects of the physiological and structural state of *L. monocytogenes* in planktonic cultures differ markedly from biofilms (Hefford *et al.* 2005; Helloin *et al.* 2003; Lourenco *et al.* 2013; Renier *et al.* 2011; Rieu *et al.* 2008; Trémoulet *et al.* 2002). Key attributes that have been further characterized include the potential role of EPS and of specific genes of *L. monocytogenes*.

3.5.1 EPS and its role in sanitizer resistance of *LISTERIA MONOCYTOGENES* in biofilms

EPS acts as a first barrier between the biofilm cells and their surrounding environment and provides mechanical stability and protection to the underlying biofilm cells. The exact composition of any given EPS can vary dramatically based on multiple factors, including the constituent bacterial species, the environmental conditions under which the biofilm is produced and the maturity of the biofilm (Ghannoun 2004; Sutherland 2001).

L. monocytogenes biofilms do not form a mushroom-shaped structure but instead involve a dense network of knitted chains composed of elongated cells under continuous-flow conditions, or rod-shaped microcolonies morphologically similar to planktonic cells under static conditions (Chae *et al.* 2000; Kalmokoff 2001; Renier *et al.* 2011; Rieu *et al.* 2008; Rodriguez *et al.* 2008). These attributes may contribute to the ability of *L. monocytogenes* cells to join existing biofilms established by other microorganisms (Bremer *et al.* 2001; Habimana *et al.* 2009; Sasahara and Zottola, 1993), as discussed earlier in the context of multi-species biofilms. Biofilm microstructures (cloud-type biofilms) were found to be correlated with relative resistance to benzalkonium chloride (Saá-Ibusquiza *et al.* 2012). The polyanionic nature of EPS has been hypothesized to be an effective tool against molecules of cationic detergents such as quaternary ammonium compounds (Stopforth 2002). Size attributes may also be involved, and the small size of peracetic acid may contribute to its high efficacy against biofilm-associated *L. monocytogenes* (Saá-Ibusquiza *et al.* 2012). Direct evidence for the role of biofilm microstructure on the penetration profile of different sanitizers in *L. monocytogenes* biofilms is still missing, but relevant data have been obtained from other systems (de Beer 1994; Suci *et al.* 1994; Szomolay *et al.* 2005). Studies measuring the structural, chemical and pH heterogeneity across *L. monocytogenes* biofilms could prove helpful in understanding the direct and indirect contribution of EPS to sanitizer tolerance of biofilm-associated *L. monocytogenes*.

Mature (11-days) biofilms on polystyrene or stainless steel had a cloud structure and were noticeably more resistant to benzalkonium chloride, peracetic acid, and nisin than immature (4-day) biofilms, which also lacked the cloud structure (Saá-Ibusquiza *et al.* 2012). Peracetic acid was found to be the most effective sanitizer for the *L. monocytogenes* biofilms in these studies, which also noted cross-resistance among different sanitizers for some of the strains (Saá-Ibusquiza *et al.* 2012).

Tentative associations have been noted between strain persistence in the food processing plant environment, production of EPS, biofilm forming potential, and tolerance of the biofilm-associated *L. monocytogenes* to benzalkonium chloride (Nakamura *et al.* 2013). Use of ATP bioluminescence showed that the concentration of the disinfectant resulting in 50% reduction in ATP bioluminescence was around 150-fold higher in persistent than in the transient strains, and the EPS amounts were also noticeably greater in the former (Nakamura *et al.* 2013). The association of these differences with the biofilm state was supported by the findings of much lower difference (~2-fold) in ATP bioluminescence when planktonic cells of persistent and transient strains were analyzed (Nakamura *et al.* 2013). The protective role of EPS towards sanitizers is also suggested by the finding that sanitizer resistance of cells from disrupted *L. monocytogenes* biofilms is frequently similar to that of their planktonic counterparts (Kastbjerg and Gram 2009; Pan *et al.* 2006; Stopforth *et al.* 2002; van der Veen and Abee 2010a, b).

Major knowledge gaps remain on the composition of EPS from *L. monocytogenes* biofilms. Analysis of microtiter plate biofilms formed at 37°C by a panel of *L. monocytogenes* strains revealed that the most common EPS component was proteins followed by extracellular DNA (eDNA) and polysaccharides, with the relative quantities dependent on strain and medium (Combrouse *et al.* 2013). High molecular weight eDNA has been proposed to play a crucial role in overall morphology and surface attachment of *L. monocytogenes* biofilms (Harmsen *et al.* 2010; Renier *et al.* 2011). Indeed, *lmo1386*, a gene encoding a putative DNA translocase, was one of 24 loci identified in high-throughput screens of a transposon mutant library for biofilm forming potential (Chang *et al.* 2012). However, further studies are needed to determine whether eDNA is also a major component of biofilms formed using surfaces and conditions highly relevant to food contamination and whether it may be related to sanitizer resistance of biofilm-associated *L. monocytogenes*.

Recent studies with *L. monocytogenes* EGD-e revealed that accumulation of the second messenger cyclic dimeric GMP (c-di-GMP) resulted in production of an exopolysaccharide (of yet undetermined composition), auto-aggregation of the cells in liquid cultures and reduced motility on soft agar, even though no impacts were noted on biofilm formation (Chen *et al.* 2014). Accumulation of c-di-GMP occurred through genetic inactivation of phosphodiesterases that normally degrade the second messenger. Interestingly, such enhanced levels of c-di-GMP were also accompanied but markedly higher tolerance of *L. monocytogenes* EGD-e to several disinfectants, as well as to desiccation (Chen *et al.* 2014). Biosynthesis of the c-di-GMP-dependent exopolysaccharide was mediated by *pssA-E* (*lmo0527–0531*), but it remains unclear whether this exopolysaccharide contributes to the EPS of *L. monocytogenes* biofilms. The potential role of c-di-GMP in disinfectant resistance of biofilm-associated cells remains to be determined, as are environmental conditions and signals that may lead to accumulation of c-di-GMP.

3.5.2 Inherent antimicrobial resistance attributes of biofilm-associated *L. MONOCYTOGENES*

Unless a sanitizer rapidly equilibrate across the biofilm, the delay in sanitizer diffusion would limit access of lethal levels of sanitizer to at least some of the cells in the biofilm, with those further away from the exposed biofilm surfaces being most protected, and would provide an opportunity for the biofilm cells to develop responses to the sanitizer. Broadly, such responses involve direct and indirect mechanisms. While direct mechanisms such as cell membrane permeability and efflux pumps directly counter the adverse effects of the sanitizers, indirect mechanisms would encompass physiological changes in the biofilm cells including activation of cell stress responses and survival of resistant sub-populations of dormant cells (persisters).

Drug efflux is a crucial determinant contributing to both the intrinsic and/or acquired form of antimicrobial resistance and may involve flexible and overlapping drug specificities (Li and Nikaido, 2009). Among transporters harbored by all tested *L. monocytogenes* strains, energy-dependent major facilitator super family (MFS) efflux system transporter proteins such as MdrL and Lde have been implicated in disinfectant resistance (Rakic-Martinez *et al.* 2011; Romanova 2007; To *et al.* 2002). In addition, the small multidrug resistance (SMR) transporter cassette *bcrABC* confers resistance to benzalkonium chloride and other quaternary ammonium disinfectants and is frequently harbored on large plasmids (e.g., pLM80) present in strains from the 1988–1999 hotdog outbreak and the 2001 turkey deli meats outbreak (Elhanafi *et al.* 2010; Kuenne *et al.* 2013; Nelson *et al.* 2004). Certain strains also appear to harbor *bcrABC* chromosomally and this cassette has also been identified in non-pathogenic *Listeria* spp. such as *L. innocua* and *L. welshimeri*, from which it can be transferred via conjugation to other non-pathogenic listeriae as well as to *L. monocytogenes* (Dutta *et al.* 2013; Katharios-Lanwermeyer *et al.* 2012). In addition, a transposon (Tn*6188*) harboring a different SMR transporter system conferring resistance to benzalkonium chloride has been identified in certain strains of *L. monocytogenes*, primarily of serotype 1/2a (Muller *et al.* 2013). Further studies are needed to elucidate the roles of these systems in resistance of *L. monocytogenes* in biofilms exposed to quaternary ammonium compounds and other sanitizers, especially in industry-relevant settings.

Adverse environmental stimuli act as an impetus for the planktonic cells to initiate biofilms and mount physiological responses marked by the activation of cell stress modulators (Costerton *et al.* 1995; O'Toole and Stewart 2005). The role of the acid tolerance response via which sublethal acid exposure of planktonic cells leads to enhanced tolerance of sessile cells in biofilms to otherwise lethal levels of acid was discussed earlier. Well known stress modulators, such as alternative sigma factors (*sigB*), molecular chaperones (*hrcA* and *dnaK*), superoxide dismutase, and the SOS response have been implicated in *L. monocytogenes* biofilm formation (van der Veen and Abee 2010 a, b, c; Suo *et al.* 2012).

Of special interest is *sigB*, a major stress response modulator in *L. monocytogenes* that regulates multiple stress responses at the transcriptional and translational level (Hain *et al.* 2008; Kazmierczak *et al.* 2003; Oliver *et al.* 2009). Oxidative stress is markedly induced during biofilm formation with multiple downstream effects (Boles and Singh 2008; Poole 2012). *sigB* mutants are impaired in biofilm forming capacity in static and continuous-flow biofilms; furthermore, *sigB* was required for resistance of both planktonic cells and cells detached from the biofilms to benzalkonium chloride and peracetic acid (van der Veen and Abee 2010a). However, the impact of *sigB* on sanitizer resistance of cells still embedded in the biofilms remains to be determined.

Similar to *sigB*, class I heat-shock proteins (DnaK and HrcA) have also been associated with antimicrobial resistance in *L. monocytogenes* biofilms, with cells detached from biofilms of *dnaK* and *hrcA* mutants being less resistant to benzalkonium chloride and peracetic acid than their wildtype counterparts; resistance patterns were restored upon genetic complementation (van der Veen and Abee 2010b). As with the *sigB* mutants described earlier, roles of *dnaK* and *hrcA* in resistance of cells in the actual biofilm cannot be fully assessed from these studies, which employed detached cells for the resistance determinations.

Proteomic analysis suggests an intricate involvement of stress response mechanisms during *L. monocytogenes* biofilm formation (Hefford *et al.* 2005; Helloin *et al.* 2003; Renier *et al.* 2011; Trémoulet *et al.* 2002). In addition, numerous genes and proteins implicated in biofilm formation have been identified via mutant screens, analysis of specific targeted genes and inter-strain comparisons (e.g., Chang *et al.* 2012; Lemon *et al.* 2007, 2010; Lourenco *et al.* 2013; Renier *et al.* 2011). However, the potential roles of biofilm-implicated stress modulators and other genes in the response of biofilm-associated *L. monocytogenes* to sanitizers remain largely uncharacterized.

Persisters are a sub-population of dormant cells that are resistant to otherwise lethal levels of biocides due to their specialized physiological state (Lewis 2007). Persisters were indeed identified in *L. monocytogenes* exposed to antibiotic, with their frequency tending to be higher in surface-adherent cultures than planktonic cells (Knudsen *et al.* 2013). The contribution of persisters to survival of *L. monocytogenes* following sanitizer treatment of biofilms remains to be characterized.

3.5.3 Sanitizer-resistant variants emerging in response to sanitizer exposure of biofilms

Limited data are available on emergence of stable sanitizer-resistant variants of *L. monocytogenes* following sanitizer treatment of biofilms. Studies of sanitizer-adapted strains have primarily involved laboratory model systems with agar-grown or (mostly) broth cultures exposed to benzalkonium chloride. Benzalkonium chloride-adapted variants were shown to have a higher minimum inhibitory concentration (MIC) for benzalkonium chloride than their non-adapted counterparts and in certain

studies they also had increased MICs to unrelated toxic compounds, including certain antibiotics (Aase *et al.* 2000; Lundén *et al.* 2003; Rakic-Martinez *et al.* 2011; Romanova *et al.* 2006; To *et al.* 2002). Enhanced expression of multi-drug efflux pumps such as *mdrL* and *lde* may contribute to the enhanced resistance attributes of the adapted variants (Rakic-Martinez *et al.* 2011; Romanova *et al.* 2006). Further studies are needed to elucidate the mechanisms involved, which may include spontaneous mutations inactivating transporter repressors or enhancing expression of the transporter genes.

Investigations of biofilms formed by strain Scott A on stainless steel identified cultural variants (rough variants and secondary smooth derivatives thereof) following exposure to hypochlorous acid (Folsom and Frank 2007). These variants not only produced more biofilm on stainless steel but also yielded biofilms that were more chlorine-tolerant than those of the Scott A parental strain. Proteomic analysis of one of these variants revealed differential expression of several proteins, including a sugar binding protein (encoded by *lmo0181*) that was upregulated in the biofilm of the variant in comparison to the parental strain (Folsom and Frank 2007). Such data reveal the potential for biofilms exposed to sanitizer to yield stable variants with altered bacteriological attributes and sanitizer tolerance profiles. Mutagenic (SOS system and RecA-mediated) repair of DNA damage induced by reactive oxygen species in biofilms, especially during exposure to sanitizers, may underlie the emergence of such mutants. As discussed earlier, evidence has been indeed provided for SOS responses and RecA-mediated mutagenesis being involved in continuous-flow biofilms (van der Veen and Abee 2010c, 2011b). However, these studies did not assess frequency of mutants with enhanced sanitizer tolerance or in the presence/absence of sanitizer treatment.

Worthy of note is also the fact that numerous *L. monocytogenese* strains harbor *comK* prophage, the junction fragments of which exhibit pronounced recombination-driven sequence diversity. *comK* presence impacted biofilm formation on meat-conditioning films, suggesting roles in rapid adaptation to biofilm growth (Verghese *et al.* 2011). It remains to be determined whether prophage content and sequence type impact the resistance of biofilm-associated *L. monocytogenes* strains to sanitizers.

In addition to mutations and other events (e.g., prophage junction sequence diversification) in the genome of a single strain, horizontal gene transfer (HGT) of determinants mediating resistance may take place in biofilms. Transfer of the benzalkonium chloride resistance cassette *bcrABC* was documented in laboratory systems involving direct donor-recipient contacts on agar media (Katharios-Lanwermeyer *et al.* 2012). Even with potentially low frequency of such HGT events within biofilms, selection pressure from exposure to sanitizers would promote survival and growth of transconjugants, which acquire resistance determinants from other listeriae or other bacterial species in the biofilm. HGT via conjugation or phage may operate effectively between *L. monocytogenes* and other genera such as *Staphylococcus aureus* (Bertsch *et al.* 2013; Charpentier *et al.* 1995; Chen and

Novick, 2009; Lemaitre *et al.* 1998; Winstel *et al.* 2013). Such transfers would be expected to be promoted in multi-species biofilms that constitute the norm in the food processing ecosystem.

Other mechanisms such as quorum sensing and physiological heterogeneity of cells within a biofilm have also been reported to contribute towards sanitizer resistance (Giwercman *et al.* 1991; Huang *et al.* 1995; Lewis 2001; Mah and Toole 2001; Maira-Litran *et al.* 2000; Skandamis and Nychas, 2012). Possible contributions of these mechanisms towards resistance of biofilm-associated *L. monocytogenes* have not been directly explored yet.

3.5.4 Resistance in adapted variants or acquired via HGT: How relevant is it in actual food processing environments?

With few exceptions (Folsom and Frank, 2007), the relative fitness of adapted variants in actual biofilm systems with and without sanitizers remains to be determined. As discussed previously, the majority of studies involving adapted strains have utilized monocultures in liquid laboratory media or, less commonly, on agar. It is worthy of note that benzalkonium chloride-resistant variants are readily obtained on agar media in the laboratory, to the point where benzalkonium chloride is not effective as selective agent in conjugation experiments (Katharios-Lanwermeyer *et al.* 2012). Yet, *L. monocytogenes* strains from the food processing environment appear to be resistant to benzalkonium chloride only if they harbor dedicated efflux systems such as *bcrABC* or Tn*6188* (Dutta *et al.* 2013; Muller *et al.* 2013). Such findings suggest that adapted variants are not commonly obtained in the food industry due to yet unidentified issues, such as limitations associated with type and concentration of disinfectant as well as frequency or duration of treatment or that they have impaired fitness in processing plant ecosystems. Direct evidence for emergence of such adapted derivatives in the food processing environment is lacking, in spite of potential suggestive data (Ortiz *et al.* 2014).

Investigations of the response of strain EGD to three industrial disinfectants (based on chlorine, combination of peracetic acid/hydrogen peroxide and quaternary ammonium, respectively) failed to yield evidence for derivatives with tolerance to in-use levels, leading the authors to conclude that industrial disinfectants do not select for resistance under long-term exposures (Kastbjerg and Gram 2012). However, as with many other studies such data need to be viewed with caution as they involved planktonic cells of monocultures, both for selection and for assessment of possibly altered resistance (Kastbjerg and Gram 2012). It is worthy of note that that several recent outbreaks have indeed involved *bcrABC*-harboring strains with resistance to quaternary ammonium disinfectants (Elhanafi *et al.* 2010; Kuenne *et al.* 2013; Nelson *et al.* 2004), and that such strains are frequently highly prevalent in food processing plants (Dutta *et al.* 2013; Mullapudi *et al.* 2008). Based on

available data, it is tempting to hypothesize that selection for sanitizer resistance in food processing ecosystems operates less at the level of endogenous mutations and more so at the level of resistance acquired via HGT, with the latter being more likely within multi-species biofilms. Experimental biofilm systems for assessments of relative fitness under conditions relevant to the food processing ecosystem will make major contributions in elucidating this issue.

3.6 CONCLUDING REMARKS AND FUTURE TRENDS

In spite of a plethora of investigations on the response of biofilm-associated *L. monocytogenes* to sanitizers, the issue remains poorly understood and challenging for a number of reasons. It is clear that the impacts of biofilms on sanitizer resistance depend on a complex network of cellular and extracellular mechanisms, which we are just now beginning to appreciate and elucidate. Our understanding of the genetic and environmental factors that contribute to biofilm formation and sanitizer resistance remains limited. Limitations of the existing model systems only add to the challenge. Major differences in experimental design and methodology, including methods to assess resistance of biofilm-associated bacteria, have often led to conflicting results and compromise efforts for adequate interpretations and a meaningful synthesis. Nonetheless, certain consistent trends have emerged and a useful methodological toolkit has been assembled, setting the ground for the next generation of studies to further elucidate the responses of *L. monocytogenes* in biofilms and better simulate ecosystems of interest, including harborage sites in food processing plants or other environments.

The data reviewed here are consistent with the hypothesis that the ratio of extracellular polymeric substances to viable cell count in a biofilm is an important (and not well characterized) factor in the survival and persistence of *L. monocytogenes* in biofilms. Mixed or pure cultures in biofilms presumably contain growing, viable cells on the biofilm surface, where nutrients are plentiful. These cells would consume most of the incoming nutrients, and subsurface cells would therefore have limited nutrients and much lower growth rates. However, sanitizer treatments would likely kill the surface organisms, and their biomass, including lipids, protein and nucleic acids would then add to the protective EPS and enhance survival of subsurface cells. In this view, sanitizer treatment may decrease the total cell count in the biofilm, but would effectively increase the EPS/CFU ratio for the surviving cells. Sanitizers such as chlorine or peroxide-based agents function by free radical oxidation, and the exposed cell contents from dead cells would be interfering targets by adsorbing the sanitizer, thus reducing the efficacy of the agent for subsurface surviving cells. Support for this argument can be seen in the results from Pan *et al.* (2006) and Nakamura *et al.* (2013), as described previously.

It is also possible that the differences in cell survival for pure and mixed culture biofilms can be attributed to the EPS/CFU ratio, with some cell populations

producing more (or less) EPS material. In mixed cultures, cells that are more susceptible to a sanitizer or preferentially located on the biofilm surface would be killed, contributing biomass EPS. This would enhance the EPS/CFU ratio for the more resistant species or species that are relegated to the biofilm subsurface in the mixed species community. These hypotheses are testable, and future work will be needed to clarify the effects of the EPS/CFU ratio on survival of *Listeria* in pure and mixed culture biofilms.

It is becoming increasingly evident that assessments of sanitizer or sanitizing treatment efficacy need to include not only standard assays utilizing planktonic monocultures but also cells grown in biofilms under conditions relevant to the food industry. Identifying these conditions, including biotic attributes, will require a systems-based understanding of the processing plant ecosystem and other relevant environments. Elucidation of the microbiomes of harborage sites via well-designed metagenomic analyses will make major contributions to our understanding of the ecology of *L. monocytogenes* biofilms and their responses to cleaning and sanitation. Such understanding will be critical for effective design of experimental studies of sanitizer resistance of biofilm-associated *L. monocytogenes* and for adequate interpretation of the findings. In addition, incorporation of novel and optimized approaches to assess survival of *L. monocytogenes* in sanitizer-treated biofilms will need to be a major thrust in such efforts.

There is tremendous potential for development and application of novel sanitizing reagents and treatments. In the near future growth in this direction will involve multiple angles, including (1) novel and improved methodologies, (e.g., electrolyzed water, ozonation, nanoparticles, and novel modes of application such as aerosols, some of which have already begun to be investigated) (Ammendolia *et al.* 2014; Ayebah *et al.* 2006; Chorianopoulos *et al.* 2011b; Hagens and Loessner 2014; Park *et al.* 2012; Skovager *et al.* 2013) and (2) *Listeria*-specific reagents such as listeriaphage (and derivatives thereof such as endolysins), novel bacteriocins and competitive exclusion cultures, some of which have already begun to be developed and tested (Hagens and Loessner 2014; Ganegama Arachchi *et al.* 2014; Soni and Nannapaneni 2010; Zhao *et al.* 2004, 2013).

Phages are of special interest, since their potential usefulness for biocontrol of adherent *L. monocytogenes* has been recognized for some time (Roy *et al.* 1993) but the advent of phage-derived reagents such as endolysins greatly expands venues for control (Hagens and Loessner 2014). This can be especially relevant for *L. monocytogenes* strains with attributes (e.g., special restriction-modification systems) that render them resistant to phage, as has been described for Epidemic Clone II (Kim *et al.* 2009, 2012).

Essential oils and other anti-listerial compounds derived from GRAS sources hold great promise for control of biofilm-associated *L. monocytogenes* (Chorianopoulos *et al.* 2008; Giaouris *et al.* 2014; Weiss *et al.* 2011) and will likely be the subject of intense attention in the near future. Strategically designed combinations among novel and existing tools via synergistic combinations and expanded

applications of the hurdle concept are expected to enhance efficacy while reducing likelihood for emergence of resistance.

Transcriptomics, proteomics, metabolomics and other "Omics" tools are expected to greatly strengthen not only our understanding of the molecular ecology of *L. monocytogenes*-harboring biofilms in food industry ecosystems, but also to help elucidate the mechanisms underlying the responses of biofilm-associated *L. monocytogenes* to sanitizer treatments. Application for these tools will open new vistas for viewing *L. monocytogenes* in the context of the food processing environment and will undoubtedly lead to novel reagents, approaches, and tools for improved control.

In this chapter, we have focused on sanitizer resistance of *L. monocytogenes* in biofilms most likely to be encountered in food industry ecosystems, especially those in harborage sites of food processing plants. However, the issues involved are highly relevant for *L. monocytogenes* biofilms in other ecosystems as well, including those on or in foods themselves, food service and domestic settings, healthcare environments, pathogenesis-related sites in infected individuals, and the still-poorly characterized niches that serve as *L. monocytogenes*' reservoirs in nature. Some efforts in these directions have been made in regard to food contamination (Bae *et al.* 2011, 2013; Formato *et al.* 2007; Peterson *et al.* 2007; Rieu *et al.* 2010; Stopforth *et al.* 2005). This is an area with major knowledge gaps, for which special attention is warranted.

In the future, control of *L. monocytogenes* in biofilms is likely to benefit greatly from the increasingly rich toolkit concerning natural antimicrobials and biocontrol strategies identified from other biological systems. These are likely to contribute to efforts to not only disinfect and remove biofilms but to also prevent them in the first place. Innovative tools and applications will accrue from advances with *Listeria* and other food-related microbes (de la Fuente-Núñez *et al.* 2012; Laitman *et al.* 2014; Nguyen *et al.* 2014; Sandasi *et al.* 2010; Upadhyay *et al.* 2013; Yun *et al.* 2014) but also (and perhaps especially so) from inter-disciplinary perspectives derived from other systems. Of special promise are compounds identified in efforts to reduce the burden of nosocomical infections by other pathogens (e.g., *Acinetobacter baumannii* and methicillin-resistant *Staph. aureus*) with pronounced environmental persistence attributes (e.g., Peng *et al.* 2011; Yeagley *et al.* 2013).

Our presentation, together with our conclusions and prospects for future studies, has been based on our assessment of the relevant peer-reviewed literature and emerging themes and trends. We have focused on selected topics, findings, and emerging trends that we consider not only informative but also useful in guiding future innovative studies and tool development. However, our coverage was not intended by any means to be exhaustive or encyclopedic; we trust and hope that this will be understood for relevant reports and investigations that may not have been discussed in our chapter, and we offer in advance our apologies for inadvertent omissions.

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