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Invasion and Survival of Salmonella in the Environment: The Role of Biofilms

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1. Introduction

Bacteria compose the majority of living biomass on Earth and play a vital role in the recycling of elements critical to sustaining life. We are discovering that they often exist as interlinked, multispecies colonies termed biofilms. They are all around us, on us, and in us. In fact, over 99% of microorganisms on Earth live as biofilms. They play a critical role in the ecology of the earth and the sustainability of life. For many years, studies of bacterial physiology focused primarily on the planktonic state neglecting the bacteria within the biofilm. The biofilm state is now recognized as the predominant form in which bacteria endure the stresses of the environment (An and Parsek, 2007; Hall-Stoodley et al., 2004; Hoffman et al., 2005; Karatan and Watnick, 2009; Stoodley et al., 2001)

Bacterial biofilms have long been recognized as participants in tooth decay, slippery rock surfaces, and contaminated water. Now these colonies are being investigated as perpetrators of persistent low-level food contamination which threaten animal and human health. Bacteria existing as biofilms are capable of surviving for extended periods in various environments, such as water, animal manure, and a range of agricultural soil types. For example, human pathogens can attach to and colonize the surfaces of plants and form biofilms on plant tissues (Annous et al., 2006). These biofilms are problematic because they are extremely hearty and difficult to remove by simple washing techniques. Causing, foodborne illnesses associated with human consumption of contaminated fresh fruits and vegetables (Fett and Cooke, 2003; Sivapalasingam et al., 2004). Living in biofims is advantageous for bacteria as it increases survival chances when confronted with unpredictable environmental stresses such as: temperature changes, desiccation, ultraviolet rays, etc.

In recent years, bacterial biofilms have been increasingly linked to food safety issues worldwide. The culprits of three recent foodborne illness outbreaks in cantaloupe melons, apples, and leafy greens have been identified as pathogenic bacteria existing in biofilms (Annous et al., 2009). They have also been implicated as the cause of many chronic infections in humans and are frequently associated with implanted devices, such as catheters, prosthetics, and contact lenses (Prouty et al., 2002). There is increasing interest in biofilms found on mucosal surfaces, such as the colon, particularly with respect to their role in disease processes (Macfarlane and Macfarlane, 2006).

There are numerous definitions of biofilms but all share the common threads of a concept involving an assemblage of microorganisms in which some of the bacteria adhere to the surface and exude an extracellular polymeric substance (EPS) that forms a matrix for further cellular attachment. The matrix is comprised of proteins, polysaccharides, extra-cellular DNA, and the various organisms involved. Biofilms can range from simple single species monolayer matrices, to complex multi-organism communities and sometimes even involve higher level organisms such as nematodes and larvae (Cloete et al., 2009).

Initially, the term "biofilm" was used informally among scientists for many years. It first appeared in a scientific journal in 1977 (Montana State University, http://www.biofilm.montana.edu/node/2930). Early researchers examining the phenomenon of microbes attaching to surfaces include Windogradsky, Cholodny, and Conn in the 1930's (Lappin-Scott, 1999). An important observation made by these scientists, was that bacteria which grew attached to a surface (in this case glass slides immersed in soil slurry) were phenotypically different from those cultured from the water phase of soil slurry (Lappin-Scott, 1999). Henrici studying freshwater bacteria observed that "for the most part water bacteria are not free floating but grow attached to the surfaces" (Lappin-Scott, 1999). These early researchers described how bacteria that were attached to surfaces exhibited diverse populations and developed into "microbial films". ZoBell's research from the early 1930's, focused on the role of bacteria in biofouling (the unwanted accumulation of microorganisms on surfaces) (Lappin-Scott, 1999). In fact ZoBell & Allen (1935), report the first apparatus specifically designed to examine bacterial attachment to surfaces. It was a carrier that held 16 glass slides and was designed to be lowered into the ocean where marine microbes could attach to the glass. Using this apparatus, ZoBell & Allen found a greater diversity of bacteria in the biofilm "lawn" on the slide than that which could be cultured from the sea water.

The bacteria found in biofilms are phenotypically distinct from their planktonic form. These changes include alterations in the regulation of large suites of genes (Hall-Stoodley et al., 2004; Karatan and Watnick, 2009). The transformation from planktonic existence to biofilm formation is a complex process, often triggered by various alterations in the surrounding environment. Bacteria in biofilms exhibited: protein profiles that more closely resemble those of exponentially growing planktonic cells (Mikkelsen et al., 2007); significant differences in the genes that are expressed (Teplitski et al., 2006; Trevors, 2011); and significant differences in the degree of resistance to antibiotics and disinfectants (Brooun et al., 2000; Ryu and Beuchat, 2005).

Bacteria living within biofilms can exhibit 1000 times more resistance to antimicrobials than their planktonic peers. The close proximity of fellow bacteria within this community allows for the increased incidence of gene transfer; resulting in increased genetic diversity, including augmented antimicrobial resistance. Biofilms impart increased levels of protection against environmental stresses, such as depleted nutrient, moisture and oxygen levels; inhospitable surrounding pH and salinity; excessive shear forces and UV exposure, and even metal toxicity. Additionally, life in a biofilm protects against attacks by a host immune system's protective proteins and signaling molecules, phagocytes, antibiotics and disinfectants (Jefferson, 2004; Mara and Horan, 2002).

Even after more than 80 years of research, there are still many unanswered question about the formation, function, maturation and eventual death of biofilms. Biofilms are typically attached and sessile. However, they have become ubiquitous in the environment because,

portions can detach and relocate to other hospitable surroundings. There is widespread scientific interest in investigating the molecular mechanisms underlying life in these intriguing bacterial communities that are able to inhabit such diverse environments.

2. Biofilm development

Despite the years of research into the mechanism of bacterial attachment, there remain many basic facets of the process that are still a mystery. The nuances of the attachment are difficult to elucidate. What is known is that the multifaceted process involves a complete alteration in life style of the bacteria involved. A generalized model for bacterial transformation from a planktonic to biofilm existence can be made (Lemon et al., 2008). This model contains five major phases: attachment; formation; micro-colony development; maturation; and finally detachment/dispersal of the biofilm. Each phase can be described by key features and triggers unique to that phase of development and will be discussed in the remainder of this section.

Cell attachment occurs in five stages. The first stage is a reversible stage where cells lightly attach to the surfaces. It is followed by a second, more permanent stage, where the cells affix themselves securely by forming an adhesive exopolymeric compound. Then in stage three, the biofilm begins to expand by the recruitment of cells into micro-colonies. In stage four, the mature biofilm is characterized by the development of a three-dimensional structure containing cells packed in clusters with channels forming to aid in the movement of nutrients and molecules to cells beneath the colony surface. In the fifth and final stage, the cells detach which facilitates dispersal and the initiation of new similar biofilms at more favorable locations. It is important to note that cell division is uncommon in mature biofilms, and energy is used predominantly to produce exopolysaccharides (Watnick and Kolter, 2000).

Bacteria within biofilms exhibit a range of phenotypes; some of these do not exist in the planktonic phase. These phenotypes include: freely suspended naked cells (resuming their planktonic state); cells reversibly attached to a surface; cells irreversibly attached to a surface and not encapsulated by EPS; embedded attached cells surrounded by EPS matrix or deeply embedded attached cells within a the three dimensional microbial stack; embedded cells sloughed into suspension; and planktonic daughter cells (Parry, 2004).

Quorum sensing allows bacterial cells to communicate resulting in a cohesiveness of function that benefits an entire population and allow the community to operate as a living system (Smith and Chapman, 2010). The channels between cell clusters deliver water and nutrients to each cell and facilitate waste removal. These structures combined with strong adhesive properties and sophisticated cell-cell communication make biofilms highly resistant to conventional cleansing agents such as biocides and disinfectants. Not surprisingly, once biofilms form, they are difficult to eliminate.

2.1 Attachment

Surface attachment offers distinct advantages for bacteria which depend on the diffusion of nutrients and wastes for their well-being. Most natural aqueous environments contain only dilute substances which can be used for metabolism and growth. On the other hand, natural surfaces tend to collect and concentrate nutrients by charge-charge or hydrophobic interactions; which provide bacteria exposure to more concentrated foodstuffs. Biofilms are

initiated when individual motile bacteria localize onto a surface and begin major physiological alterations. This initial attachment is reversible but encourages aggregation and attachment of more planktonic bacteria and other organisms. During this phase the attraction is mediated by weak forces, such as van der Waals, acid-base and simple electrostatics processes.

2.2 Formation

Permanent formation and expansion of the biofilm occurs when the initial transient attachment is reinforced by the production of cell surface adhesive compounds, pilli and fimbriae (Kaplan, 2010). The complex transition from transient to permanent attachment is associated with the formation of a monolayer via the up-regulation of genes responsible for the production of an extracellular matrix composed of exopolysaccharides, and extracellular DNA. Bacterial motility is lost by removal of cell flagellum by protease and replacement with a holdfast protrusion composed of oligomers of *N*-acetylglucosamine. The holdfast is composed of a strong adhesive polysaccharide that ensures a tight bond to the surface (Karatan and Watnick, 2009). In some strains of bacteria cell wall bound surface proteins called biofilm-associated protein (BAP) begin to be expressed and promote cell to cell interactions and the development of the extracellular matrix (Lasa and Penades, 2006).

Further development of the biofilm is promoted by the production of molecules which cause potassium leakage and trigger the activation of a membrane kinase (Lopez et al., 2009). In addition, the transcription of flagellar genes is repressed when the monolayer stage is achieved. Transcription of a large number of methyl-accepting chemotaxis genes are activated in the monolayer stage. Studies suggest that chemotaxis proteins influence monolayer formation. One possibility is that flagellar rotation pausing, which plays a role in the response to chemoattractants, also enhances the transition to permanent attachment (Karatan and Watnick, 2009). Some of the different components involved in the formation of the matrix include pilli and extracellular DNA (Banas and Vickerman, 2003; Kachlany et al., 2001; Petersen et al., 2005).

2.3 Micro-colony development

Now that the bacteria are sessile and biofilm formation is initiated, the bacteria actively multiply and communicate via quorum sensing signals. Once the quorum sensing threshold is achieved, exopolysaccharide production begins and micro-colonies develop through a variety of mechanisms. *Pseudomonas aeruginosa* use flagella and pili-mediated twitching motility to redistribute across the surface. *Escherichia coli* utilize fimbriae, flagella and pili for the same purpose. Others spread and generate micro-colonies through cell division, where the daughter cells spread outward and upward (Cloete et al., 2009).

2.4 Maturation

Maturation results in the formation of pillars and masses of tightly packed cells intermixed with fluid filled channels allowing for the exchange of nutrients, oxygen, and waste products between the biofilm and the surrounding liquid (Cloete et al., 2009). EPS is a key component of the biofilm matrix and may be composed of a number of sugar monomers such as glucose, galactose, mannose and xylose and some non-carbohydrate substitutes

(such as acetate, pyruvate, succinate, and phosphate). Most EPS molecules are neutral or polyanionic in nature, which aids in immune evasion and tolerance toward antibacterial agents. Enzymatic alteration of EPS is thought to significantly change its physicochemical properties and consequently the entire structure. Some examples of polymeric biofilm matrix constituents include the glucan polysaccharides produced by *Streptococcus mutans* (Banas and Vickerman, 2003), proteinaceous fimbriae produced by *Aggregatibacter actinomycetemcomitans* and *Porphyromonas gingivalis* (Kachlany et al., 2001; Lamont et al., 2002); extracellular, double-stranded DNA in biofilms produced by *A. actinomycetemcomitans*, *S. mutans,* and *Streptococcus intermedius* (Inoue et al., 2003; Petersen et al., 2004; Petersen et al., 2005) and a wide variety of proteins, glycoproteins, glycolipids, and enzymes.

Mature biofilms are intricate structures containing sectors with distinctive microenvironments that differ in cell densities, oxygen and nutrient levels, and pH ranges. As a result, the metabolic and reproductive functionality of the bacteria located in these distinct sectors are quite divergent (Kaplan, 2010). Metabolically dormant cells located in the interior of the colony are often more resistant to the actions of antimicrobial agents that target actively growing cells near the exterior (Fux et al., 2005).

2.5 Detachment and dispersal

The fifth and final phase of a biofilm lifecycle is detachment and dispersal. Growth and detachment are interdependent. Under robust conditions, the detachment rate has been shown to increase with increasing growth rates (Gjaltema et al., 1997). This phase leads to colonization of new areas offering fresh resources, which is critical for long-term survival. This phase is also important in the dissemination of infection and therefore, disease transmission in clinical and public health settings. As with all aspects of the biofilm lifecycle, the processes surrounding detachment and dispersal are very intricate; involving a wide variety of environmental and physiological triggers and signal transduction pathways (Karatan and Watnick, 2009). Individual bacteria employ somewhat different methods of dispersal, which can be divided into three discrete stages: (a) detachment of cells from the colony; (b) relocation of cells to an alternative site; and (c) re-attachment of the cells to a new substrate site (Kaplan, 2010). Rochex et al. (2009) found that one dominant species often comprises most of the weakly cohesive, thick top layer of the biofilm; while a more diverse population comprises the strongly cohesive, thin basal layer. These findings suggest that determining species diversity may be an important parameter in understanding detachment and dispersal.

2.5.1 Key factors for detachment

Both biochemical and physical factors participate in the major processes facilitating biofilm detachment; those being erosion; sloughing; abrasion, grazing, and human intervention Numerous biochemical factors involved in detachment are: the production of EPS-degrading enzymes; lytic bacteriophage activation; expression of phosphodiesterases; and quorum-sensing signaling. Physical detachment factors are : microbiologically generated gas bubbles; the presence of cross-linking cations; nutrient limitations; metabolite accumulations; changes in osmolarity; high cell density growth; and fluidic shear factors (Thormann et al., 2006).

2.5.2 Erosion, sloughing and abrasion

Erosion and sloughing are two mechanisms of spontaneous biofilm cellular detachment. The distinction between erosion and sloughing has a considerable effect on bacterial species competition within biofilms and thus morphology (Telgmann et al., 2004). Erosion is the continual detachment of single cells or small fragments from the biofilm at low levels over the course of formation. Researchers have noted that the rate of erosion from the biofilm increases with increased matrix thickness and fluidic shear forces at the cell-liquid interface. An increase in the flow velocity causes the hydrodynamic boundary layer to decrease, resulting in amplified turbulence at the biofilm surface. Sloughing is the swift, massive loss of large chunks of biofilm greater than or equal to the overall thickness. Sloughing is a more random occurrence than erosion and is thought to result from nutrient or oxygen depletion within the structure and is more commonly observed in thicker systems (Donlan, 2002).

Erosion and sloughing occur when local shear forces overwhelm the cohesiveness of the biofilm. Overall cohesiveness is strongly influenced by the composition and the structure of the polymeric matrix, which is dependent on the formation history, the environmental growth conditions and the developmental stage of the biofilm. The resulting strength of biofilm attachment is contingent on cell density, composition of extracellular polymeric substances, and levels of specific compounds, such as the calcium. Fast growing organisms with high initial cell growth rates favor the development of protrusions and the formation of a heterogeneous biofilm structure. Shear forces more easily erode these protrusions (Telgmann et al., 2004).

Abrasion is the loss of biofilm due to collision of particles from the surrounding fluid with the exposed surface. Biofilms in fluidized beds, filters, and particle-laden environments such as surface waters are often subjected to abrasion (Donlan, 2002). Work by Rochex et al. (2009) demonstrated that abrasion characteristics, such as particle collision frequency and pressure strongly affect biofilm detachment rates. Experiments by Gjaltema et al. (1997) have shown that inter-particle collisions cause an on-going abrasion of the biofilm

2.5.3 Grazing and human intervention

A key mortality factor in the control of bacteria within biofilms is grazing. Grazing is the uptake and killing of bacteria by phagocytic protozoa and metazoa in close association with biofilms. These biofilm-associated protozoa exhibit three modes of predation: 1) planktonic, but swimming close to the biofilm surface; 2) surface attachment on biofilm, but feeding on suspended prey; and 3) feeding directly on biofilm as prey. Protozoans benefit from this association as demonstrated by their increased numbers and taxa diversity when associated with a biofilm community compared to the surrounding plankton environment (Boenigk and Arndt, 2000).

Protozoa exhibit a sizeable diversification of morphologies consequent to developing a variety of means to capture and engulf their bacterial prey. However, they are commonly grouped into flagellates, ciliates, and amoebae. All three free-living groups efficiently graze on bacteria exposed on the biofilm surface. Flagellates and ciliates contain feeding types primarily focused on suspended bacteria with only a few that preferably feed on surface-bound bacterial prey (Parry, 2004). For instance, the flagellate *Rhynchomonas nasuta* feed on attached *Pseudomonas* spp. at rates between 13 and 120 bacteria per

flagellate per hour (Boenigk and Arndt, 2000). Sibille et al. (1998) found that a mixed population of flagellates could consume on average 12 suspended bacteria per flagellate per hour. The ciliate *Euplotes* spp. grazes on adherent *Vibrio natriegens* and *Pseudomonas fluorescens* at rates of 120 and 882 bacteria per ciliate per hour, respectively (Lawrence and Snyder, 1998), while Ayo et al. (2001) found that in general ciliates showed a grazing rate of ≤20 free swimming bacteria per ciliate per hour.

Amoebae protozoans feed almost exclusively on surface-bound bacteria (Parry, 2004). Amoebae species such as *Hartmanella cantabrigiensis*, *Platyamoeba placida*, *Saccamoeba limax*, *Vahlkampfia avara* eat attached *Escherichia coli* at rates of 15 to 440 bacteria per amoeba per hour (Heaton et al., 2001).

Many predators are selective and remove only a subset of the microbial community thus altering the biofilm community structure (Parry, 2004). Morphological differences in biofilm structure correlate with predation. Without the pressure of predation a flat, compact structure results. Conversely in the presence of predators, an open and heterogeneous structure results.

Metazoa (rotatoria, nematoda, and oligochaeta) are the main group of higher level predators responsible for grazing. Their grazing on biofilms initially decreases microbial biomass, and unless grazing pressure is severe, the secondary microbial community that develops will have increased rates of metabolic activity and growth. Total microbial biomass will be greater and the turnover rates of both the substrates and microorganisms will increase. The diversity of the community structure will decrease as the biofilm community shifts towards faster growing organisms.

Bacterial predators, such as *Bdellovibrio bacteriovorus; Micavibrio* spp.; and *Hyphomicrobium* spp. also play a vital role in the life and death of biofilms. *Bdellovibrio bacteriovorus* is a gram-negative, aerobic bacterium that preys upon a wide variety of other gram negative bacteria, including *E. coli*; which, in simple biofilms, can devastate a community altogether (Dashiff et al., 2010). Additionally, *Micavibrio* spp. is also a gram-negative, aerobic bacterium that also preys on bacteria and biofilm structures. Unlike *Bdellovibrio* which penetrate their prey, *Micavibrio* attach to the outside surface and eventually lyse their host bacteria. *Bdellovibrio* and *Micavibrio* spp. have been shown to be extremely host specific; for example, *Micavibrio aeruginosavorus* strain ARL-13 preys only on *Pseudomonas aeruginosa*. In static and flow cell experiments, *M. aeruginosavorus* not only modified *P. aeruginosa* biofilm structure, but also decreased bacterial viability. The alterations were likely caused by increased cell-cell interactions brought about by the presence of the predator (Donlan, 2002).

Human intervention involves both mechanical action and the use of disinfectants. Any type of brush or scouring pad provides the agitation required to disrupt the biofilm structure. Once the community has been physically disrupted the addition of a surfactant and disinfectant is required to complete the destruction process. In the case of contact lens , Wu et al. (2011) found that *Staphylococcus aureus* or *Pseudomonas aeruginosa* biofilms required rubbing and rinsing with multipurpose disinfecting solutions followed by tissue-wiping and air-drying to remove them from the surface. *Listeria monocytogenes,* an important foodborne pathogen, has the ability to form persistent biofilm matrices in food processing environments. Soni & Nannapaneni, (2010) determined that a cocktail of different bacteriophages may be essential for their removal. Lequette et al. (2010) found that

solubilization of polysaccharidases and proteases in a buffer containing surfactants, along with dispersing and chelating agents, enhanced their efficiency of removing biofilms by targeting several components of EPS of *Bacillus* spp. and *Pseudomonas* spp..

Biofilms have been extensively studied in the dental industry. Periodontitis is a chronic bacterial infectious disease whose hallmark is the presence of a bacterial biofilm at the gum line. The condition necessitates thorough removal of the biofilm for therapy. However, debridement using hand instruments or oscillating scalers is both technically demanding and time consuming, and may lead to severe root damage over time (Petersilka, 2011). Air-polishing with glycine powder proved to be an easy, safe and effective means of biofilm removal from teeth (Petersilka, 2011).

3. Quorum sensing

For many years, bacteria were believed to exist as individual cells that existed to find nutrients and multiply. The discovery of intercellular communication among bacteria led to the realization that bacteria are capable of coordinated activity that was once thought to be restricted to higher organisms (reviewed in (Waters and Bassler, 2005). The ability to behave collectively has obvious advantages, for example, the ability to migrate to a more suitable environment or better nutrient supply and to adopt new modes of growth, such as biofilm formation, which may afford protection from harmful environments. This intercellular communication is called quorum sensing. The mechanism used for quorum sensing is the process of recognition of and response to small molecules, called autoinducers, secreted by the bacteria themselves. The process of biofilm creation in a variety of bacteria has been shown to specifically involve quorum sensing. These autoinducers are used by bacteria to regulate their behavior according to population density. The phenomenon relies on the principle that when a single bacterium releases autoinducers into the environment, the concentration is too low to be detected. However, when sufficient bacteria are present, autoinducer concentrations reach a threshold level that allows the bacteria to sense a critical mass and respond by the activation or repression of target genes (de Kievit and Iglewski, 2000). Quorum sensing manifests itself as a synchronization of individual behavior into cooperative group activity, often resulting in a change of phenotype within a population once bacterial densities have reached a threshold level. The specific threshold level can be different for each population. Examples of density-dependent changes include the turning on of bioluminescence within *Vibrio fischeri*, conjugal transfer in *Agrobacterium tumefaciens*, swarming in *Serratia liquefacians*, production of virulence factors in *Burkholderia cepacia* and *Pseudomonas aeruginosa*, and biofilm formation in numerous species including *Pseudomonas aeruginosa*, *Pantoea stewartii* and *Vibrio cholera* (Bottomley et al., 2007; Davies et al., 1997; Nadell et al., 2008; Ward et al., 2004).

4. Biofilm and virulence

Many bacterial pathogens including *Listeria monocytogenes, Salmonella* spp., *Shigella* spp., *Staphylococcus aureus, Escherichia coli,* and *Enterobacter* spp., utilize a biofilm strategy to survive inhospitable conditions and to cause disease. Tamayo et al. (2010) found that pathogenic *Vibrio cholera* in both dispersed and intact biofilms vastly out-competed planktonic populations. Huang et al. (2008) found that that *Streptococcus mutans* utilizes the

general secretory pathway to secrete virulence factor proteins and the level of SecA, the key factor in the general secretory pathway, was influenced significantly by biofilm formation. PrfA is the critical virulence transcription factor that regulates the switch from extracellular, flagellum-propelled bacterium to intracellular pathogen in *L. monocytogenes*. Lemon et al. (2010) reported the first evidence that PrfA has a significant positive impact on extracellular biofilm development. Mutants lacking *prfA* were defective in surface-adhered biofilm indicating that PrfA positively regulates biofilm establishment and has a role in modulating the life-style of *L. monocytogenes*. This could provide selective pressure to maintain this critical virulence regulator when *L. monocytogenes* is outside host cells. The human-enteropathogenic species *Yersinia enterocolitica* and *Y. pseudotuberculosis* and the highly virulent plague bacillus *Y. pestis*, represent ideal species to study how bacteria adapt from different environments and evolve to be highly virulent. The work of Hinchliffe et al. (2008) found that several alleged virulence determinants of the *Yersinia* species, regulated by a phosphorelay, also regulated proteins involved in biofilm formation, motility, mammalian cell adhesion and stress survival. *Escherichia coli* are one of the first colonizers of the gastrointestinal tract of newborns and a normal component of the gastrointestinal flora of almost every human being. Found in concentrations up to 10^8 cells ml^{-1} it is a major source for the spread of potentially pathogenic *E. coli* to susceptible sites via the fecal route. Adherence and invasion of intestinal epithelial cells mediated by type 1 fimbriae is a feature of *E. coli* strains isolated from lesions of Crohn's disease.

Salmonella enterica serovar Enteritidis has emerged as one of the most important foodborne pathogens for humans. It is often associated with consumption of contaminated produce, poultry meat and eggs. The spiA gene within S. enterica serovar Typhimurium encodes an outer-membrane component of the SPI-2 type III secretion system that is essential for virulence in host cells. Dong et al. (2011) found that that the spiA gene is also critical to biofilm formation. Biofilm cells, from *Listeria monocytogenes* and *S. enterica* serovar Typhimurium , which survived disinfection, seem to develop a stress response and become more virulent, which may compromise food safety and increase public health risk (Rodrigues et al., 2011a). Legendre et al. (2011) showed that adhered *S. enterica* serovar Enteritidis bacteria were more resistant to antibacterial agents than their planktonic counterparts. Xu et al. (2010) found that the enterotoxin production and invasion ability of biofilm *S. enterica* serovar Typhimurium cells is enhanced under acidic stress conditions. Further, cells of *S. enterica* serovar Typhimurium, collected from a biofilm, showed increased adhesive ability within the spleens of mice. The invasion of *S. enterica* serovar Typhimurium into the intestinal epithelial cells is the crucial step in pathogenesis. Wilson et al. (2007) reported that *S. enterica* serovar Typhimurium samples grown during the weightlessness of space flight exhibited enhanced virulence in a mouse infection model, along with extracellular matrix accumulation consistent with a biofilm.

5. Biofilm development in Salmonella

Scientific understanding of the formation process of biofilms by Salmonella is insufficient and replete with opportunities for further exploration. While some generalities can be made, each species has its own idiosyncrasies relating to the influence of local environmental conditions, gene expression and protein production and secretion. Some of these differences will be discussed in the following section.

In recent years, outbreaks Salmonellosis have often been traced back to contaminated plant sources (CDC, 2011). Lately it has been determined that contamination of plants with Salmonella is not superficial, but due to specific attachment of the bacteria to plant tissues by surface molecules (Barak et al., 2005; 2002). Salmonella uses extracellular matrix components, such as thin aggregative fimbriae and polymers (cellulose and O-antigen capsules) to colonize the plants, forming a biofilm, which is ultimately consumed by and causes illness in humans (Barak et al., 2007). The determination that Salmonella specifically attaches with biofilm formation, challenges the public concept that cleaning vegetables by simply rinsing with water is adequate for bacterial removal. These surface molecules appear to aid this pathogen in the utilization of plants as vectors for spreading and increase the risk of contamination of fresh produce.

Iturriaga, et al. (2007) found that during growth of tomatoes in greenhouses or during postharvest handling, higher humidity promotes biofilm development on the surface of the fruit. These biofilms provide a protective environment for pathogens and reduce the effectiveness of sanitizers and other inhibitory agents used to clean the fruit prior to consumption. *S. enterica* serovar Montevideo was shown to grow on tomato surfaces under a wide range of temperature and relative humidity combinations even when external nutrients were scarce. These findings reinforce the importance of maintaining fruits and vegetables under proper storage conditions to reduce the incidence of Salmonella biofilm development.

Fifteen *S. enterica* serotypes, Anatum; Baidon; Caracase; Cubana; Give; I 13,23,d-; Isangi; Montevideo; Muenchen; Newport; Onderstepoort; Senftenberg; Teko; Wandsbek and Weltevideo, found to form biofilms, were identified from various foods, spices and water sample (Xia et al., 2009). Pulse Field Gel Electrophoresis showed that eight out of the 15 serotypes had patterns indistinguishable from patterns of strains from human clinical samples or foods (US PulseNet National database); indicating that the isolates could potentially infect humans and cause salmonellosis.

Patel & Sharma, (2010) investigated the ability of five *S. enterica* serovars to attach to and colonize intact and cut lettuce (Iceberg, Romaine) and cabbage surfaces. They found that biofilm formation was significantly affected by the serovars used. Generally, *S. enterica* serovars Tennessee and Thompson showed significantly more biofilm formation than serovars Braenderup, Negev, and Newport; and were thus classified as strong biofilm producers according to the criteria suggested by Stepanovic et al. (2004). The criteria states, that strong biofilm producer had four times the optical density (OD) cutoff, which is three standard deviations above the mean OD of the negative control. Understanding the attachment mechanisms of Salmonella to vegetables may be useful in developing new intervention strategies to prevent contamination.

Kim and Wei, (2009) demonstrated that the knockout of the *yjc*C gene, encoding putative diguanylate cyclase/phosphodiesterase, in *S. enterica* serovar Typhimurium DT104 enhanced biofilm formation by the mutant in meat and poultry broths and on contact surfaces. This work also showed that biofilm formation by *S. enterica* serovar Typhimurium DT104 could be affected by the type of food products, since the *yjc*C mutant produced greater biofilms in meat and poultry broths than in vegetable broths. Therefore, the prevention of bacterial biofilm formation on food contact surfaces is critical for controlling cross-contamination of *S. enterica* serovar Typhimurium DT104 in food processing.

Bhowmick, et al. (2011)found the existence of an alternative biofilm regulatory pathway in *S. enterica* serovar Weltevreden from seafood isolates. This is the most prevalent serovar associated with seafood. While human illness caused by this serovar is rare in Europe and United States, it has been reported in Asia. In *S. enterica* serovar Typhimurium the gcpA gene plays a critical role in biofilm formation under low nutrient conditions. In *S. enterica* serovar Weltevreden deletion of the gcpA gene resulted in its inability to produce cellulose and failure to produce biofilm on polystyrene substrate. This indicated that in the case of *S. enterica* serovar Weltevreden, gcpA is critical for activating cellulose synthesis and biofilm formation. The characterization of genes involved in biofilm formation will help in defining critical control points within the process that may be manipulated to control for or possible eliminate the development of biofilms in certain environments.

Small RNAs (sRNA) are non-coding RNA molecules, 50-250 nucleotides in length, produced by bacteria. Kint et al. (2010) showed that biofilm formation is influenced by the sRNA molecule in various *S. enterica* serovar Typhimurium mutants. The sRNA was encoded in the same region as the quorum sensing synthase luxS. Quorum sensing represents a coordinated gene expression response in bacteria, stimulated by local population density. Autoinducer-2 (AI-2) is considered a universal signaling molecule in quorum sensing that is widespread in bacteria, and the LuxS enzyme is required for AI-2 synthesis. Quorum sensing plays an important role in biofilm formation and survival (see section 3). MicA is a family of small RNA molecules highly conserved in several Enterobacteriaceae. These sRNA's are reported to be a regulatory mechanism necessary for biofilm formation in many bacterial species and whose balanced expression level is essential for mature Salmonella biofilm formation.

The *ydcI* gene is differentially regulated in response to conditions of low fluid shear force that increase bacterial virulence and alter other phenotypes in *S. enterica* serovar Typhimurium (Jennings et al., 2011). They found that the *S. enterica* serovar Typhimurium strain in which *ydcI* expression is induced; invaded cells at a level 2.8 times higher than that of the wild type strain. Further, induction of *ydcI* resulted in the formation of a biofilm in stationary cultures, indicating that the *ydcI* gene encodes a conserved DNA binding protein involved with aspects of prokaryotic biology related to stress related biofilm production and possibly virulence. Further, these studies indicate that the *S. enterica* serovar Typhimurium *ydcI* gene is conserved across genera and has auto-regulated expression. When induced, it alters the interactions of *S. enterica* serovar Typhimurium host cells and expedites biofilm formation.

Human-to-human transmission of *S. enterica* serovar Typhimurium makes this a pathogen of global concern. Random transposon mutants of this serovar were screened for impaired adherence and biofilm formation on cholesterol-coated surfaces; 49 mutants with this phenotype were found (Crawford et al., 2010). It was determined that genes involved in flagellum biosynthesis and structure primarily mediated the attachment to cholesterol. In addition, the presence of the flagellar filament enhanced binding and biofilm formation in the presence of bile. This improved understanding of the early events during biofilm development, specifically how Salmonella bind to cholesterol, provides potential therapeutic targets for alleviating asymptomatic gallbladder carriage of *S. enterica* serovar Typhimurium.

6. Biofilm and Salmonella survival

Salmonella enterica serovar Enteritidis is a significant biofilm-forming pathogen. The survival of Salmonella on equipment and instruments in the food industry might be one of the most important contributing factors to food contamination and the subsequent foodborne infection. Further, the biofilm formation ability of foodborne pathogens has attracted much attention in the medical field and food industry due to its potential risks, including transfer of antimicrobial resistance and virulence factors (Xu et al., 2010).

Hasegawa et al. (2011) found that the ability of Salmonella strains to survive in the presence of acetic acid and rice vinegar paralleled their ability to form biofilms. Thus, Salmonella with a high biofilm-formation capability might be more difficult to kill in a food production setting. Salmonella cells embedded in these matrices show reduced susceptibility to trisodium phosphate, desiccation, and chlorination. Further, the connection between biofilm-forming ability and risk of foodborne outbreaks has been suggested in Salmonella. The work of Vestby et al. (2009) showed a correlation between persistence and biofilm establishment of Salmonella thus this may be an important factor for its longevity in the factory environment. These Salmonella strains appear to be a greater risk to human health via food contamination by surviving for longer periods (Iibuchi et al., 2010).

Mangalappalli-Illathu et al. (2008) found significant differences in the pattern and degree of resistance between planktonic and biofilm *S. enterica* serovar Enteritidis cells to benzalkonium chloride (BC). They established that the biofilm phenotype resulted in an early, more efficient adaptive response, and produced a higher proportion of adapted individuals than the planktonic phenotype. Once adapted, these cells were better able to survive BC than the planktonic cells. It is worth mentioning that disrupted BC adapted biofilm cells seem to have a better likelihood to attach, multiply, and form biofilms in BC-containing environments if the concentration is sublethal. The presence of these BC adapted *S. enterica* serovar Enteritidis biofilm cells presents a potential problem in environments such as health care facilities, the food industry, and households.

The presence of *S. enterica* serovars in animal feed ingredients is a well-known problem, resulting in contamination that vectors Salmonella infections in livestock farms. Dual-species biofilms favored Salmonella growth compared to Salmonella in mono-species biofilms, where biomass increased 2.8-fold and 3.2-fold in the presence of Staphylococcus and Pseudomonas, respectively (Habimana et al., 2010). Thus contamination with Salmonella in the presence of other bacteria will only exacerbate the problem of dissemination of Salmonella.

Fresh fruits and vegetables have been increasingly associated with outbreaks of foodborne illness. Salmonella contamination was higher on members of the Brassicaceae family (radish, turnip, and broccoli) than on lettuce, tomatoes, and carrots when sown and grown in contaminated soil. Vegetables that had soft rot exhibited twice the Salmonella contamination as did healthy produce. This could be stress related or possibly because the vegetables are already immunocompromised (Barak and Liang, 2008). Biofilm formation on plant tissue enabled foodborne pathogens to survive in the harsh phyllosphere and decreased the efficacy of commonly used sanitizers (Critzer and Doyle, 2010). Lapidot and Yaron, (2009) demonstrated that *S. enterica* serovar Typhimurium could be transferred from irrigation water to the edible parts of parsley plants. This work also revealed that *S. enterica* serovar

Typhimurium formed aggregates at a depth of 8 to 32 µm beneath the leaf surface. Penetration was most likely achieved through the roots or the phyllosphere. They further determined that, curli and cellulose, both components involved in the formation of biofilms, play a major role in the transfer or survival of *S. enterica* serovar Typhimurium in the plant. Incidences of salmonellosis caused by eating fresh produce continue to increase. This appears to be the result of *S. enterica* serovar Typhimurium attaching to and colonizing plants, rather than incidental contamination. *S. enterica* serovar Typhimurium that preferentially colonize roots use a hydrolase for swarming or biofilm production on plants; this multicellular behavior of *S. enterica* serovar Typhimurium has emerged as central to plant colonization (Barak et al., 2009).

A series of studies from our lab provided a molecular-based characterization of both the biofilm and planktonic populations from continuous-flow culture community. These studies examined the ability of *S. enterica* serovar Typhimurium to colonize a defined microfloral community established to model chicken ceca at day-of-hatch, 7 and 14 days old. The bacterial communities were allowed to equilibrate biofilm and planktonic populations for 3 weeks prior to introduction of *S. enterica* serovar Typhimurium. The one common factor relating to successful invasion of the community was the presence of *S. enterica* serovar Typhimurium within the biofilm. If the introduced *S. enterica* serovar Typhimurium could invade and sequester within the biofilm, then colonization appeared long-term. However if it only invaded the planktonic portion, then it was unable to gain a foothold and did not persist within the community (Crippen et al., 2008; Sheffield et al., 2009a, b).

7. Salmonella biofilms in the environment

Salmonella causes an estimated 93.8 million human infections and 155,000 deaths annually worldwide (Majowicz et al., 2010). The U.S. Centers for Disease Control and Prevention (CDC) have estimated that over 1.4 million cases of infection and 600 deaths related to salmonellosis may occur every year, accounting for about 31% of all food-related deaths in the USA (Wang et al., 2010). Poultry, poultry products, red meat, pork, wild game, and vegetables are all possible vehicles of transmission to humans.

7.1 Poultry

There are many avenues for Salmonella persistence in large scale poultry houses; one is to develop biofilms. Poultry feed has been demonstrated to be a leading source of Salmonella introduction into a poultry production facility (Park et al., 2011). Further, containers used in transporting live poultry between production and processing units have also been incriminated as primary sources of contamination for processed poultry products (Ramesh et al., 2002).

7.2 Non-poultry food animals

In developed countries, the production of food animals (i.e. cattle and hogs) is often limited to highly concentrated rearing facilities, also known as concentrated animal feeding operation (CAFO). This provides a conduit for the spread of Salmonella serovars to a large number of individuals within the herd, as demonstrated by the *S. enteritidis* pandemic in the 1990s, which affected both developed and developing countries (Hendriksen et al., 2011).

Additionally, where wild game is still a key food source, the incidence of Salmonella in feces is upwards of 22% of the wild boar and 48% of the wild rabbit populations in some areas (Vieira-Pinto et al., 2011). This demonstrates the potential for the exchange of bacterial pathogens between wild and domestic animals, which is cause of concern for the welfare of both the wild and the domestic populations, as well as for the humans in contact with them.

Antimicrobial resistance gene-bearing organisms that move from nutritionally rich to more dilute environments, such as when inadvertently washed from CAFO's into the surrounding watershed, survive longer in biofilms (Engemann et al., 2008). Additionally, antimicrobial resistance genes readily transfer into biofilms, which can then be transferred into the surrounding environment, in particular aquatic systems. These organisms are then accessible to wild fauna also utilizing the environment. Many studies have been performed investigating wild animals acting as reservoirs of disease for domestic animals. However, the influence of domestic animals serving as a reservoir of diseases transferable to wildlife is rarely considered. Domestic stock, particularly ungulates, have introduced many diseases into wildlife populations, sometimes with catastrophic results for that population and wildlife conservation on the whole (Mathews, 2010).

7.3 Processed foods

Food processing or handling equipment may provide a niche in which pathogenic bacteria such as *S. enterica* can grow rapidly into highly hydrated biofilms resulting in cross-contamination from food processing surfaces to food products. This cross-contamination can potentially lead to foodborne illnesses. Such cross-contamination of food products has been observed from the use of inadequately cleaned/sanitized processing equipment. Some examples include pumps, containers, or tanks first used for handling raw food materials and subsequently used for processed food products without first undergoing proper sanitation procedures (Jun et al., 2010). Predictably, the food industry has increased interest in chemical, physical, and biological interventions that mitigate food-borne pathogens on these products (Ha and Ha, 2011). *Salmonella* spp. is one of the most commonly isolated pathogens associated with fresh produce (Wong et al., 2011). Penteado & Leitao, (2004) demonstrated that low acid fruits are good substrates for the survival and growth of *S. enterica* serovar Enteritidis, a known biofilm forming pathogen.

8. Salmonella biofilm control measures

Salmonella enterica is a major cause of bacterial food-borne diseases worldwide, and serovars, such as Typhimurium, can cause a localized self-limiting gastroenteritis in humans. In immunocompromised people, Salmonella infections are often fatal if they are not treated promptly with antibiotics (Janssens et al., 2008). While Salmonella infections are most commonly treated using fluoroquinolones (e.g., ciprofloxacin) and extended spectrum cephalosporins (e.g., cefotaxime), there are disturbing reports regarding the development of resistance against these antimicrobials. Further, Salmonella is able to form biofilms on a variety of biotic and abiotic surfaces, where they are a double threat in that they allow the Salmonella to survive and spread in the environment outside the host (Janssens et al., 2008). The Salmonella found in these biofilms show an even higher tolerance to antibiotics than most Salmonella and according to the National Institutes of Health; approximately 80% of persistent bacterial infections in the United States are caused by biofilms (NIH, 1997).

Therefore, the need for alternative strategies to combat the spread of bacterial biofilm related infections is emerging (Janssens et al., 2008).

8.1 Chemical control

Salmonella in biofilms is less susceptible to disinfectants than planktonic Salmonella (Wong et al., 2010); therefore the eradication of biofilm sequestered pathogens is more challenging. *S. enterica* can itself form biofilms that are relatively resistant to chemical sanitizing treatments. The use of glutaraldehyde, formaldehyde, and peroxygen at a concentration of 1.0% in field conditions is insufficient to eradicate Salmonella biofilms (Marin et al., 2009). However, Rodrigues et al. (2011a) and Wong et al. (2011) showed sodium hypochlorite to be one of the most effective disinfectants against biofilms; with the ability to eradicate biofilms at concentrations as low as 3.125 mg per ml. Rodrigues et al. (2011a) also found that bacterial cells from biofilms, which survived disinfection, appeared to develop a stress response and/or become more virulent. The main finding of this work is the worrying fact that, even at concentrations that lead to significant reduction in biofilm biomass, disinfectants may actually enhance virulence within the surviving cells. Adding to this is the fact that the biofilm forms of Salmonella have significantly increased antibiotic resistance properties compared to their planktonic forms (Papavasileiou et al., 2010). These studies confirm that the biofilm form of Salmonella is not only more difficult to remove during sanitation procedures, but has an increased potential to compromise food safety and potentiate public health risk.

In further work, Rodrigues et al. (2011b) examined the adhesion, formation and viability within biofilms of *S. enterica* serovar Enteritidis on regular (granite, marble, stainless steel) and triclosan-impregnated kitchen bench stones (Silestones). Triclosan is a polychlorophenoxy phenol compound with broad spectrum antimicrobial activity that works by targeting lipid biosynthesis and inhibiting cell growth. Salmonella cells adhered equally well (4 to 5 log CFU per cm^2) to all surfaces, with the exception of silestone, which exhibited a potential for bacteriostatic activity. Less *S. enterica* serovar Enteritidis biofilms formed on impregnated silestones and cell viability was one to two logs lower than on other materials (Rodrigues et al., 2011b).

Hasegawa et al. (2011) observed a positive relationship between acid tolerance and biofilm-formation capability in Salmonella by examining the ability of strains to survive and form biofilms in the presence of acetic acid and rice vinegar. It has been suggested that a positive relationship exists between biofilm formation and increased risk of foodborne outbreaks. Therefore, when developing strategies for the prevention of Salmonella contamination of foods it is important to consider the biofilm-formation capability of each particular strain (Hasegawa et al., 2011).

Rosenberg et al. (2008) demonstrated that biofilm formation can be prevented through controlled release of nature-derived antimicrobials, such as salicylate-based poly (anhydride esters). The inhibition of the biofilm appeared to be caused by the irreversible interaction of salicylic acid molecules with the cells. The inhibition was not caused by interference with attachment but rather, via another mechanism essential for biofilm development that remains to be elucidated.

Another promising area of biofilm control is the use of essential oils from a variety of plants. The efficacy of essential oils from the leaves of *Myrcia ovata* Cambess for antimicrobial

activity and prevention of the formation of microbial biofilms by *Enterococcus faecalis* was examined (Candido et al., 2010). The essential oil from this plant is commonly used in Brazil for the treatment of gastric illnesses. This oil showed antimicrobial activity against *E. faecalis, E. coli, P. aeruginosa, S. choleraesuis, Staphylococcus aureus, Streptococcus pneumoniae* and *Candida parapsilosis.* Further, at a concentration as low as 0.5 % it appreciably reduced the formation of biofilm by *E. faecalis* (Candido et al., 2010).

8.2 Predation

Protozoa are important participants within microbial food webs; however protozoan feeding preferences and their effects with respect to bacterial biofilms are not very clear. Work by Chabaud et al. (2006) demonstrated that protozoan grazing had a substantial effect on the removal of pathogenic coliforms in septic effluent and in the presence of a biofilm. Coliform survival was 10 times lower in a septic effluent with protozoa than without them. Further, removal of the bacteria within the biofilm was 60% higher in the presence of protozoa.

A landmark study examined the predatory range of *Myxococcus virescens* and *Myxococcus fulvus*, on a variety of human pathogens, including *Staphylococcus aureus*, *Mycobacterium phlei*, *Shigella dysenteriae*, *Vibrio cholerae*, *Proteus* X, and several Salmonella isolates (Mathew and Dudani, 1955). With the exception of M. phlei, all of the examined pathogenic species were completely or partially lysed, indicating that deciphering the predatory mechanism utilized by *Myxobacteria* species is of practical importance to improve our understanding of how to treat bacterial infectious diseases.

In 1983 Lambina and colleagues (Lambina et al., 1983) isolated a new species (*Micavibrio* spp.) of exoparasitic bacteria with an obligatory parasitic life cycle. They are gram negative, small curved rod shaped (0.5 x 1.5 mm), bacteria with a single polar flagellum. A titer as low as 10 plaque forming units per well of *M. aeruginosavorus* was sufficient to produce a 78% reduction in a *P. aeruginosa* biofilm after 30 min exposure in a static assay (Kadouri et al., 2007).

Dopheide et al. (2011) examined the grazing interactions of two ciliates, the free-swimming filter feeder *Tetrahymena* spp. and the surface-associated predator *Chilodonella* spp., on biofilm-forming bacteria. They found that both ciliates readily consumed cells from both *Pseudomonas costantinii* and *Serratia plymuthica* biofilms. They also found that both ciliates used chemical cues to locate biofilms. Further, using confocal microscopy they discovered that *Tetrahymena* spp. had a major impact on biofilm morphology, forming holes and channels throughout *S. plymuthica* biofilms and reducing *P. costantinii* biofilms to isolated, grazing-resistant microcolonies. Grazing by *Chilodonella* spp. resulted in the development of less-defined trails through *S. plymuthica* biofilms and caused *P. costantinii* biofilms to become homogeneous scatterings of cells (Dopheide et al., 2011).

Bdellovibrio spp. are small, predatory bacteria that invade and devour other gram-negative bacteria. Under dilute nutrient conditions, bdellovibrio prevented the formation of simple bacterial biofilms and destroyed established biofilms (Nunez et al., 2005). During the active prey-seeking period of its life cycle, it moved through water or soil searching for prey. Once it encountered a prey cell, bdellovibrio attached to the prey bacterium's surface, broke the outer membrane, and killed the prey cell by halting its respiration and growth. During the growth period, this predator utilized the prey's macromolecules for fuel and the carcass

provided a protected, nutrient-rich habitat for development. Once the prey resource was exhausted, bdellovibrio divided into multiple progeny that lyse the remains of the prey and swim away to pursue new prey. Depending on the prey and the environmental conditions, its life cycle takes roughly 3–4 h (Berleman and Kirby, 2009; Nunez et al., 2005). While many predatory bacteria have been identified, most have been studied only superficially. Predation behavior has evolved a number of times. Examples of predatory bacteria are found in diverse genera, within the *Proteobacteria*, *Chloroflexi*, and *Cytophagaceae* (Berleman and Kirby, 2009). Dashiff et al. (2010) has demonstrated that predatory bacteria, *Bdellovibrio bacteriovorus* and *Micavibrio aeruginosavorus*, are able to attack bacteria from a variety of genus, including *Acinetobacter, Aeromonas, Bordetella, Burkholderia, Citrobacter, Enterobacter, Escherichia, Klebsiella, Listonella, Morganella, Proteus, Pseudomonas, Salmonella, Serratia, Shigella, Vibrio* and *Yersinia*. Further, predation occurred on single and multispecies planktonic cultures, as well as on monolayer and multilayer biofilms. Finally, *Bdellovibrio bacteriovorus* and *Micavibrio aeruginosavorus* have the ability to reduce many of the multidrug-resistant pathogens associated with human infection (Dashiff et al., 2010).

8.3 Radiation

Niemira & Solomon, (2005) found that while the radiation sensitivity of Salmonella is isolate specific, the biofilm associated cells of *S. enterica* serovar Stanley were significantly more sensitive to ionizing radiation than the respective planktonic cells. The dose of radiation value required to reduce the population of *E. coli* O157:H7 by 90% (D10) was highly dependent on the isolate. One isolate exhibited significantly ($P < 0.05$) higher D10 values for planktonic cells than those observed for biofilm cells indicating a significantly increased sensitivity to irradiation for cells in the biofilm habitat. However, for another isolate of *E. coli* O157:H7 exhibited exactly the opposite results. It appears that culture maturity had a more significant influence on the irradiation efficacy of planktonic cells than on biofilm-associated cells of *E. coli* O157:H7 (Niemira, 2007).

9. Future outlook

Current research investigating Salmonella biofilms covers efforts to fully understand the multifaceted process of biofilm development and the intricate relationships between biofilms and virulence, and to develop more effective and environmentally friendly control methods. In the following section we will discuss some of the most recent work reported in these areas.

Shah et al. (2011) have found an association between the pathogenicity of *S. enterica* serovar Enteritidis strains and the differential production of type III secretion system proteins during the production of biofims. In addition several factors including motility, fimbriae, biofilm production, and the presence of large molecular mass plasmids can augment pathogenicity. Such research will provide more insights into molecular basis of *S.* Enteritidis virulence and thus delineate a new direction for the reduction of virulence in *S.* Enteritidis. Based on recent finding, solid murine tumors might represent a unique model to study biofilm formation *in vivo*. Crull et al. (2011) found that systemic administration of *S. enterica* serovar Typhimurium to tumor bearing mice resulted in preferential colonization of the tumors by Salmonella and retardation of tumor growth. Ultrastructural analysis of these tumors did not detect the Salmonella intracellularly, but revealed that the bacteria had

formed biofilms. This model could provide the means for further clarification of the biofilm development process. Research by Sha et al. (2011) utilized the high resolution tool, Rep-PCR, to differentiate closely related microbial strains among Salmonella. This methodology could provide more discriminatory information essential to pin pointing bacterial sources, which is critical to maintaining food safety and public health in the future.

Perez-Conesa et al. (2011) tested eugenol and carvacrol delivered within surfactant micelles at concentrations of 0.9 and 0.7%, respectively. Eugenol is a component of essential oils primarily from clove, nutmeg, cinnamon, and bay leaf; and carvacrol is a predominant phenol found in wild oregano oil. These oils decreased viable counts of 48 hr biofilms of pure *E. coli* O157:H7 or *L. monocytogenes* on stainless steel surfaces by 3.5 to 4.8 logs of CFU per cm2, respectively, within 20 minutes of exposure. Thus, micelle-encapsulated eugenol and carvacrol appear to be good vehicles to deliver hydrophobic antimicrobials through the exopolymeric structure to cells embedded within biofilms. Potentially, these oils could be used in combination with other treatments to diminish biofilm formation on food and food contact surfaces.

The pathogenicity of several significant human pathogens has been linked to the activity of AI-2 quorum sensing signaling, which is also involved with the development of biofilms (Roy et al., 2011). The ubiquitous nature of AI-2 makes it an excellent target as a potential antimicrobial therapy against a broad spectrum of pathogens. Additionally, as AI-2 is not essential for cell growth or survival, interference with its synthesis and processing will probably not stimulate development of resistance. However, as with any single piece of the biofilm pathogenicity puzzle, it is unlikely that quorum sensing quenching drugs will be the "magic bullet" for the treatment of bacterial infections. Therefore, according to Roy et al. (2011*)* a mixed therapy of quorum sensing quenchers and traditional antibiotics appears to be a promising approach for the future. Finally, it is important that our understanding of signaling molecules be increased, thereby allowing the identification of potential new antimicrobial therapies.

Many questions remain to be answered on the path to understanding the complicated processes involved in the development and expansion of biofilms in human, animal and environmental settings. What specific factors, both biotic and abiotic, govern the initiation and continuation of the biofilm process? What impact does quorum sensing have on the initiation and differential development of the unique biofilm characteristics? What influences the ability of Salmonella to form biofilms and the development of virulence and antibiotic resistance? The final question is how to use this knowledge to manage the environment, and components involved in the biofilm development process to reduce their negative impact on human and animal health.

10. References

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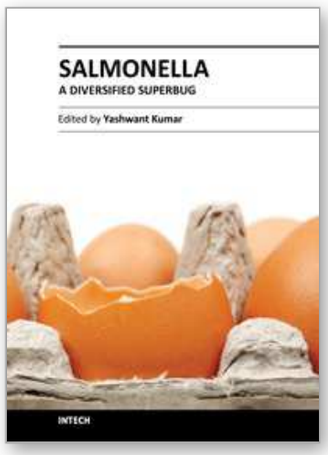

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Salmonella is an extremely diversified genus, infecting a range of hosts, and comprised of two species: enterica and bongori. This group is made up of 2579 serovars, making it versatile and fascinating for researchers drawing their attention towards different properties of this microorganism. Salmonella related diseases are a major problem in developed and developing countries resulting in economic losses, as well as problems of zoonoses and food borne illness. Moreover, the emergence of an ever increasing problem of antimicrobial resistance in salmonella makes it prudent to unveil different mechanisms involved. This book is the outcome of a collaboration between various researchers from all over the world. The recent advancements in the field of salmonella research are compiled and presented.

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