ABSTRACT

PAN, YOUWEN. A Comparative Study of Serotype 1/2a and Serotype 4b Strains of *Listeria monocytogenes* in Biofilms Using a Simulated Food Processing System. (Under the direction of Dr. Fred Breidt, Jr.)

The majority of *Listeria monocytogenes* isolates from foods and the environment are serotype 1/2a strains. However, serotype 4b strains cause the majority of human listeriosis outbreaks. The purpose of the research has been to compare the growth of serotype 1/2a strains and serotype 4b strains in biofilms. A method to enumerate viable *L. monocytogenes* cells of each serotype in mixed culture biofilms was developed using real-time PCR with propidium monoazide. To determine the competitive fitness of strains of serotype 1/2a and 4b, cocktails of each serotype were mixed to form biofilms. The biofilms were treated with a simulated food processing (SFP) system composed of repeated cycles of growth, sanitation treatment, and starvation. Data show that the serotype 1/2a strains were generally more efficient than the 4b strains at forming biofilms and predominated in the mixed culture biofilms. The growth of 4b strains was not inhibited in mixed culture biofilms compared to the single serotype (4b) biofilms in the SFP system. To compare the density of biofilms formed by strains of the two serotypes, 18 strains of each serotype were examined for biofilm formation under a variety of conditions, including varying concentrations of glucose, sodium chloride and ethanol at different temperatures using a microplate assay. Results indicate that the serotype 1/2a strains formed higher density biofilms than the 4b strains under most conditions. The data from this project support the hypothesis that *L. monocytogenes* serotype

1/2a strains are more efficient in biofilm production than 4b strains and may help to explain the higher percentage of 1/2a isolates from foods and the environment.

A Comparative Study of Serotype 1/2a and Serotype 4b Strains of *Listeria monocytogenes* in Biofilms Using a Simulated Food Processing System

by
Youwen Pan

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APPROVED BY:

_____	_____
Dr. Fred Breidt Committee Chair	Dr. James W. Brown
_____	_____
Dr. Eric Miller	Dr. Sophia Kathariou

BIOGRAPHY

Youwen Pan was borne and grew up in Dongtai, Jiangsu, China. He got his bachelor's degree in the Department of Biology at Lanzhou University (Lanzhou, China). He worked as a research assistant for 2 years at Jiangsu Institute of Microbiology (Wuxi, China) after graduation. He worked as a manager at the Microbiology Laboratory, Quality Assurance Department in Sino-Swed Pharmaceutical Corp. Ltd. (Wuxi, China) for 7 years before he came to Raleigh for his graduate study. He got his Master's degree in Food Science in North Carolina State University.

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LITERATURE REVIEW

GENUS OF *LISTERIA* AND *LISTERIA MONOCYTOGENES*

Listeria monocytogenes, a facultative intracellular food-borne pathogen, is responsible for approximately 2,500 listeriosis cases and 500 deaths in each year in the United States (Mead *et al.*, 1999). Almost 99% of human listeriosis has resulted from consumption of contaminated foods (Mead *et al.*, 1999). Due to the high fatality rate (approximately 25%) of the infection by *L. monocytogenes*, the U.S. Food and Drug Administration (FDA) and the Food Safety and Inspection Service (FSIS) of the U.S. Department of Agriculture (USDA) have set up a "zero tolerance" policy for this bacterium in ready-to-eat (RTE) foods [9 CFR 430. 4(a)]. Despite the zero tolerance policy there have been several outbreaks of listeriosis since the 1980s with multiple contaminated foods, including: coleslaw, unpasteurized cheese, butter, raw milk, pasteurized milk, and processed meat and poultry products (Dalton *et al.*, 1997; Headrick and Tollefson, 1998; Maijala *et al.*, 2001; Ryser, 1999; Schlech *et al.*, 1983; and Linnan *et al.*, 1988). A recent outbreak with 22 deaths out of 57 cases was due to the consumption of contaminated ready-to-eat meat in Canada in the summer/fall of 2008 (The Public Health Agency of Canada, 2009). Therefore, it is not surprising that *L. monocytogenes* has been considered an important opportunistic human food-borne pathogen.

The genus *Listeria* represents a group of bacteria that are Gram-positive, facult atively anaerobic, motile, non-sporeforming, rod-shaped, about 0.5 μm in width and 1-1.5 μm in length, and have a low genorm G + C content (39%) (Seelinger & Jones, 1986). A total of seven species have been included in the genus *Listeria* (*L. monocytogenes, L. ivanovii, L. innocua, L. seeligeri, L. welshimeri, L. grayii, and L. murrayi*). Among these

species, only *L. monocytogenes* and *L. ivanovii* are pathogenic. *L. monocytogenes* causes disease in humans and animals; whereas, *L. ivanovii* causes disease predominantly in animals (sheep, cattle, etc.) (Roberts and Wiedmann, 2003; Swaminathan, 2001; Vazquez-Boland *et al*., 2001). *Listeria* species share not only common morphological characteristics but also biochemical traits. For all *Listeria* species, the catalase test is positive whereas indole and oxidase tests are negative. All members can hydrolyze aesculin but not urea. These common features are often used to differentiate *Listeria* from other microorganisms. Nonetheless, each species of *Listeria* has its own specific biochemical properties that can be used for species-specific identification. It has also been demonstrated that there is significant variation among species in terms of their ability to lyse horse or sheep red blood cells and generate acid from L-rhamnose, D-xylose and α-methyl-D-mannoside (Robinson *et al*., 2000).

Listeria species are tolerant to extreme pH, temperature, and salt conditions (Miller, 1992; Sleator *et al*., 2003; Liu, 2006). *Listeria* species are widely spread in the environment and have been isolated from a variety of sources, including: soil, vegetation, meat, silage, fecal material, sewage, and water. *L. monocytogenes* is relatively more resistant to harsh conditions than are most other non-sporeforming foodborne Bacteria (Ryser, 1999). *Listeria* is able to grow in a range of conditions, including: temperatures from 1^{o}C to 45^{o}C (optimum around 37^{o}C), pH from 4.4 to 9.4, and water activities $\geq$0.92 (Miller, 1992).

Specific surface proteins and polysaccharides in *Listeria* species, such as somatic (O) and flagellar (H) antigens, are useful targets for serological identification with corresponding monoclonal and polyclonal antibodies. A total of fifteen somatic (O) antigen subtypes (I -

XV) and four flagellar (H) antigens (A-D) have been detected in *Listeria* species. The serotypes of individual *Listeria* strains are determined by the unique combinations of O and H antigens (Seeliger and Jones, 1986; Liu, 2006). At least 12 serotypes of *L. monocytogenes* (1/2a, 1/2b, 1/2c, 3a, 3b, 3c, 4a, 4b, 4c, 4d, 4e and 7) have been detected by examining O and H antigens using slide agglutination. This has revealed at minimum seven serotypes in *L. seeligeri* (1/2a, 1/2b, 3b, 4a, 4b, 4c and 6b), one in *L. ivanovii* (e.g. 1/2b), and a few in *L. innocua*, *L. welshimeri* and *L. grayi* (e.g. 1/2b, 6a and 6b) (Seeliger and Jones, 1986; Liu, 2006). The major drawbacks to slide agglutination include cost, antibody availability, standardization of reagents, and technical requirements for testing. An ELISA (Enzyme-Linked Immunosorbent Assay) serotyping format has been developed, that is less expensive and more efficient (Palumbo *et al*., 2003). For increased ease of strain serotyping, PCR (polymerase chain reaction) methods with specific primers have been developed for four important food-borne serotype strains (1/2a, 1/2b, 1/2c, and 4b) (Borucki and Call, 2003; Doumith *et al*., 2004b; Chen and Knabel, 2007). It has been found that the serotypes of 1/2a, 1/2b, and 4b of *L. monocytogenes* are responsible for approximately 98% of documented human listeriosis cases (Wiedmann *et al*., 1996; Jacquet *et al*., 2002; Tappero *et al*., 1995). Serotype 4b strains are isolated mostly from epidemic outbreaks of listeriosis, and serotypes 1/2a and 1/2b appear linked to sporadic human listeriosis (Wiedmann *et al*., 1996; Kathariou, 2002). Although *L. monocytogenes* serotype 1/2c strains rarely causes human illness, 1/2c strains are common in food-processing plants (Kathariou, 2002).

Although serotyping is a universal method used for differentiation, the significance of serotyping in *L. monocytogenes* epidemiological investigations is limited because there are only three serotypes (1/2a, 1/2b, and 4b) that are primarily associated with human listeriosis (Wiedmann *et al*., 1996; Jacquet *et al*., 2002; Kathariou, 2002). Because *L. monocytogenes* has a variety of strains, the lack of differentiation by serotyping can be overcome by alternative methods for phenotypic and genetic subtyping. Other phenotypic subtyping methods such as phage typing, multilocus enzyme electrophoresis (MLEE), and esterase typing have been used to discriminate among *L. monocytogenes* strains (Loessner, 1991; Bibb *et al*., 1989; Piffaretti *et al*., 1989; Gilot and Andre, 1995; Harvey and Gilmour, 1996). The genetic subtyping methods that are utilized in *L. monocytogenes* epidemiological investigations consist of pulsed-field gel electrophoresis (PFGE) (Brosch *et al*., 1994; Graves *et al*., 1994; Kerouanton *et al*., 1998), ribotyping, PCR-based subtyping (e.g. random amplification of polymorphic DNA [RAPD]) (Franciosa *et al*., 2001), amplified fragment length polymorphism (AFLP) (Ripabelli *et al.*, 2000; Guerra *et al.*, 2002; Keto-Timonen *et al.*, 2003), and DNA sequencing based subtyping methods (e.g. multilocus sequence typing [MLST]) (Wiedmann *et al.*, 1997; Jersek *et al.*, 1999; Ward *et al.*, 2004). Multiple-locus variable-number tandem-repeat analysis (MLVA or VNTR) was recently proven to be a reliable subtyping method for *L. monocytogenes* (Sperry *et al.*, 2008). Although each of the subtyping methods has unique features for identifying *L. monocytogenes* strains, the combination of two or more approaches may be more powerful and discriminatory than are applied individually.

L. monocytogenes strains are generally divided into three genotypic groups. These groups are designated lineages I, II and III, as shown by molecular subtyping methods, including ribotyping, MLEE, PFGE, and virulence gene sequencing (Wiedmann, 2002). Strains of serotypes 1/2b, 3b, 3c, and 4b are in lineage I; serotypes 1/2a, 1/2c, and 3a strains are in lineage II (Piffaretti *et al*., 1989; Rasmussen *et al.*, 1995; Nadon *et al*., 2001), and 4a, 4c, and a subset of 4b are in lineage III (Cai *et al*., 2002; Nadon *et al*., 2001; Ward *et al*., 2004). However, in different typing and population genetic studies, other genetic divisions or lineages were designated (Bibb *et al*., 1989; Brosch *et al*., 1994; Graves *et al*., 1994; Doumith *et al*., 2004a; 2004b). Doumith *et al.* designated serovar 1/2a, 1/2c, and 3c strains as lineage I strains; serovar 4b, 1/2b, and 3b strains as lineage II strains; and serovar 4a and 4c strains as lineage III strains (Doumith *et al*., 2004a). Based on variable gene content, the three divisions I, II, and III of *L. monocytogenes* were further divided into five phylogenetic groups, each of which could be associated with serovars: I.1 (1/2a & 3a), I.2 (1/2c & 3c), II.1 (4b, 4d, and 4e), II.2 (1/2b, 3b, and 7), and III (4a & 4c) (Doumith *et al*., 2004a). Lineage specific marker genes were identified using comparative genomics and DNA arrays, which provided new insights into the development of rapid typing methods (Doumith *et al*., 2004a) as well as for functional analysis of species- and serovar-specific genes to unveil their roles in pathogenicity. Several studies reported that *L. monocytogenes* subtypes and lineages differ in their association with specific hosts and environments (Jaradat *et al.*, 2002; Nadon *et al.*, 2001; Norton *et al.*, 2001; Graves *et al.*, 1999). The majority of sporadic human listeriosis cases appear to be caused by serotypes 1/2a, 1/2b, and 4b, while most human listeriosis outbreaks have been caused by serotype 4b strains (Kathariou, 2002; Wiedmann, 2002).

Lineage III strains are common in animal listeriosis (Michael *et al.*, 2004; Jeffers *et al.*, 2001).

PATHOGENICITY OF *L. MONOCYTOGENES*

Listeriosis in humans occurs primarily in two forms, including a mild noninvasive gastrointestinal illness (GI) and an invasive disease. The noninvasive GI form of listeriosis mostly occurs in otherwise healthy adults. Typical symptoms for noninvasive disease are gastroenteritis, fever, diarrhea and vomiting, and the incubation time is approximately 18–20 h. Because this pathogen can be carried by humans without symptoms, the frequency of noninvasive gastrointestinal listeriosis is difficult to estimate (Slutsker and Schuchat, 1999).

Invasive listeriosis primarily takes place in adults who are immuno-compromised, including the elderly, pregnant women, cancer patients, organ transplant recipients, and patients with acquired immunodeficiency syndrome (AIDS), or anyone getting immunosuppressive therapy. The typical clinical symptoms of listeriosis are meningitis, meningoencephalitis, septicemia, abortion, perinatal infections, and gastroenteritis (Vazquez-Boland *et al.*, 2001).

Following ingestion of contaminated food, *L. monocytogenes* gets through the acidic environment in the stomach, and disseminates from the intestinal lumen to the central nervous system and the placenta in pregnant woman. *L. monocytogenes* is capable of crossing the intestinal barrier, the blood-brain barrier, and the fetoplacental barrier in humans. E-cadherin, a glycoprotein in humans, serves as a receptor for the surface protein internalin-A

(InlA) in *L. monocytogenes*. The interaction between the receptor and the internalin-A contributes to the capability of *L. monocytogenes* to target and cross human intestinal, neural, and placental barriers (Lecuit *et al.*, 2001; 2004).

There are several molecular determinants, besides InlA, that have been found to be involved in adhesion and invasion of eukaryotic cells. InlB, a GW (Gly−Trp) protein that functions both as a growth factor and as an invasion, is located in the same operon as InlA and is involved in the entry of *Listeria* into a broad range of cell lines, including hepatocytes and nonepithelial cells (Dramsi *et al.*, 1995). Ami, an amidase anchored in bacterial cell walls, is an autolysin involved in adhesion (Milohanic *et al.*, 2001). Listeriolysin O (LLO), a member of the pore-forming, cholesterol-dependent cytolysin family, is involved in *Listeria* escape from primary and secondary vacuoles (Portnoy *et al.*, 1988). FbpA, a multifunctional surface protein, contributes to cell adherence, and modulates the levels of listeriolysin O and InlB at a posttranscriptional stage (Dramsi *et al.*, 2004). ActA, a surface actin-polymerizing protein, is responsible for the intracellular actin-based motility of *Listeria*. It was also found that expression of ActA in *L. monocytogenes* was sufficient to promote bacterial entry in epithelial cell lines (Suarez *et al.*, 2001). In contrast to the role of flagella in virulence of many other bacteria, the flagella of *L. monocytogenes* hardly have an effect on the virulence because biosynthesis of flagella is repressed at physiological temperatures (37°C and above) (Shen *et al.*, 2006). A brief review of flagella regulation in *L. monocytotenes* is in the section below on biofilm formation of *L. monocytogenes*.

As a facultative intracellular parasite, *L. monocytogenes* can invade and replicate in both epithelial cells and macrophages. After entry into a host cell, the bacterium lyses the internalized vacuole and escapes into the cytoplasm, a process that is promoted by expression of LLO in concert with two phospholipases C (PLCs) (Dramsi and Cossart, 2002). The bacterium replicates when it is in the cytoplasm. The bacterium uses the actin of the host cell, associating with its ActA protein, to propel itself intracellularly (Cossart, 2000). The bacterium invades neighboring cells by the formation of double membrane protrusions called pseudopods, resulting in the formation of a secondary vacuole that is engulfed by an adjacent host cell. After uptake by adjacent cells, the bacterial cell lyses the vacuole by means of the hemolysin (listeriosin O, LLO) and phospholipases, allowing a new intracellular infection cycle in adjacent cells (Dramsi and Cossart, 1998; Tilney and Portnoy, 1989).

Neonates are also at particular risk of acquiring invasive listeriosis from an infected mother. The fetus is infected in the uterus by transplacental transmission of bacteria from an infected mother. In neonatal listeriosis, the mortality rate ranges from 20 to 30% (Roberts and Wiedmann, 2003).

BACTRERIAL BIOFILMS

Introduction

It is well accepted that bacteria growing in a free swimming planktonic state are significantly different from those in surface-associated biofilms communities (Hall-Stoodley *et al.*, 2004). The study of microbial biofilms started from observations of environmental

microbes that adhere to a variety of surfaces, including everything from river rocks, hulls of ships to teeth and medical devices (Costerton *et al*., 1987, 1995). Scientists from various disciplines including microbial ecology, medical bacteriology and chemical engineering utilized a variety of approaches to examine attached bacteria and study their behavior. Biofilms are generally characterized by dense, clusters of bacterial cells embedded in highly hydrated extracellular polymeric substances (EPS) that is secreted by cells and hold the cells together on the surface (Costerton *et al.*, 1987, 1994). The surface could either be a solid-liquid interface or an air-water interface (called floating biofilms or pellicles). The physical properties of the surfaces to which microorganisms adhere, including roughness, hydrophobicity and hydrophilicity, and conditioning films, were an early important focus of biofilm studies (Costerton *et al*., 1987, 1995). In addition to exopolysaccharides, proteins, lipids, nucleic acids, and lipopolysaccharide (LPS) been identified in biofilms (Branda *et al.*, 2005; Friedman and Kolter, 2004; Costerton *et al.*, 1981). Membrane vesicles (MVs) derived from the outer membrane of Gram-negative Bacteria were also reported to be a component of the matrix of *Pseudomonas aeruginosa* biofilms (Schooling and Beveridge, 2006). The EPS holds the biofilm cells together and serves other functions, such as nutrient trapping and protection from antimicrobial challenges (Stewart and Franklin, 2008; Zhang and Mah, 2008). Protease was found to inhibit biofilm formation (Longhi et al., 2008) and disrupt biofilms formed by *L. monocytotenes* (Gamble and Muriana, 2007). Cellulase and lipase have also been found to be effective in dispersing *L. monocytotenes* biofilms (Gamble and Muriana, 2007). These data suggest that protein, polysaccharides and lipid molecules all play important roles in the integrity of biofilms.

The structure, composition and function of a biofilm formed by a specific strain varies dramatically with environmental conditions, including the growth phase of bacterial cells, availability of nutrients, shear forces, and the characteristics of the surfaces to which the bacteria attach (Chandra *et al.*, 2001; Costerton *et al.*, 1995; Klausen *et al.*, 2003; Stoodley *et al.*, 1998, 2001). Some organisms may have multiple genetic pathways to be used to regulate and form a biofilm (O'Toole and Kolter, 1998).

Once biofilms are formed the organisms are extremely difficult to eradicate because the biofilm protects them from antimicrobials and host defenses. The biofilm matrix provides a protective barrier to antimicrobials, and antibodies fail to penetrate biofilms (de Beer *et al.*, 1997). Furthermore, catalase protects aggregated bacteria by preventing full penetration of hydrogen peroxide into the biofilm (Stewart *et al.*, 2000). Compared with organisms in planktonic state, biofilm cells have low metabolic activity and are more resistant to antimicrobials. Heterogeneity in biofilms also influences the antimicrobial susceptibility of biofilm-embedded bacteria (Anderl *et al.*, 2003; Walters *et al.*, 2003).

There are many physical, biological, and chemical processes involved in biofilm formation. Once a solid surface is exposed to a liquid, the surface chemistry is changed by macromolecule adsorption (Verran and Whitehead, 2005; Strevett and Chen, 2003). Cells in the surrounding area may be transported to the substratum: liquid interface. Attachment is controlled by diffusion, convection, sedimentation, and motility. Once bacterial cells attach to a surface, secretion of signal molecules is up-regulated to orchestrate community-wide phenotypic responses via quorum sensing (Camilli and Bassler, 2006; Harraghy *et al.*, 2007; Rumbaugh, 2007). These responses may include the up-regulation of virulence factors

(Branda *et al.*, 2005; Otto, 2006) and secretion of EPS (Hall-Stoodley *et al.*, 2004). Biofilms continue to develop and mature by metabolizing soluble nutrients and recruiting other bacterial species (Brunstedt *et al.*, 1995). Other quorum sensing signals may regulate detachment and shedding of bacterial cells from biofilms (Xavier *et al.*, 2005).

Physiological heterogeneity in biofilms

Most bacteria have a tendency to form biofilms (Davey and O'Toole, 2000). Bacterial cells within a biofilm are in a wide range of physiological states (Stewart and Franklin, 2008). Since the environmental conditions in a biofilm are not homogeneous, the physiological response of the bacteria cells to the environment varies. Cells within a biofilm display significant structural, chemical and biological heterogeneity. Mature biofilms undergo gradient concentrations of metabolic substrates and products. Oxygen gradients are formed from the fluid above the biofilm to the attachment surface (Ramsing and Jorgensen, 1993; De Beer *et al.*, 2004; Schramm *et al.*, 1996; Xu *et al.*, 1998; Okabe *et al.*, 1999; and Rani *et al.*, 2007). In contrast to the distribution of oxygen or nutrients within a biofilm, metabolic products have a higher concentration in the biofilm and lower concentration outside the biofilm, forming a concentration gradient deccreasing from the interior to the exterior of the biofilm. Damgaard *et al.* (2001) used a microscale biosensor for methane to measure methane concentration in a 3.5-mm-thick sewage outlet biofilm and found that the methane concentration at the surface of the biofilm was approximately 10% of that measured at a depth of 2 mm. For solutes that are both produced and consumed in a biofilm, the solute concentration profiles may display a maximum within the biofilm. In a denitrifying biofilm,

nitrate is reduced to nitrogen gas via nitrite. As an intermediate solute, the nitrite concentration in the biofilm could reach to four times higher than that in the fluid outside the biofilm (De Beer *et al.*, 1997). In addition the solutes mentioned above, several other solutes have also been reported to form gradients within biofilms, such as nitrate and ammonium (Okabe *et al.*, 1999), hydrogen sulphide (Ramsing *et al.*, 1993; Okabe *et al.*, 1999), carbon dioxide (Beyenal *et al.*, 2004), hydrogen peroxide (Liu *et al.*, 1998), chlorine (De Beer et al., 1994), and chlorine dioxide (Jang *et al.*, 2006). The profiles of pH within biofilms have also been measured (Ramsing *et al.*, 1993; De Beer *et al.*, 1997). Steward (2003) proposed a mathematical method named reaction-diffusion theory for biofilms to analyze the distribution of a chemical solute in space and time resulting from the interaction of two processes: metabolism of the solute and its transport by diffusion.

The chemical heterogeneity within a biofilm result in biofilm cells with diversified physiological status for adaptation to a specific local environment. Gradients in specific respiratory activity were observed and quantified within a biofilm formed by *Klebsiella pneumoniae* and *Pseudomonas aeruginosa* on stainless steel surfaces by using a fluorogenic redox indicator 5-cyano-2,3- ditolyl tetrazolium chloride (CTC) and a DNA stain 4',6-diamidino-2- phenylindole (DAPI), which can differentiate between respiring and nonrespiring cells in biofilms (Huang *et al.*, 1995). The expression of alkaline phosphatase in response to phosphate starvation was demonstrated to be spatially and temporally heterogeneous in biofilms formed either by *Klebsiella pneumoniae* or *Pseudomonas aeruginosa* (Huang *et al.*, 1998). Rani *et al.* (2007) demonstrated the spatial patterns of DNA replication and protein synthetic activity within *Staphylococcal* biofilms using

immunofluorescent detection of pulse-labeled DNA and also an inducible green fluorescent protein (GFP) construct.

In addition to chemical heterogeneity and physiological adaptation to the local environment in biofilms, genetic variation and non-uniform gene expression have also been demonstrated to contribute to the phenotypic heterogeneity through mutation or recombination. Variant subpopulations have been frequently observed during the growth of bacteria in biofilms (Allegrucci and Sauer, 2007**;** Hansen *et al.*, 2007; Valle *et al.*, 2007; Koh *et al.*, 2007). Following the growth of a given strain, small percentages of isolates exhibit colony morphologies that are distinct from the parent. A small rough or wrinkly colony subpopulation was observed in biofilms formed by *P. aeruginosa* (Kirisits *et al.*, 2005; Nguyen and Singh, 2006). Small non-mucoid variants developed in *Streptococcus pneumoniae* biofilms (Allegrucci and Sauer, 2007); and rough and wrinkled colonies emerged in *Vibrio cholerae* biofilms (Yildiz and Schoolnik, 1999). Most of the morphological variations have been mapped to changes in specific genetic regions. For example, the wrinkled colony variant of *Vibrio cholerae* O1 (biotype El Tor), was found to produce an exopolysaccharide, EPS^{ETr} and exhibit chlorine resistance. EPS^{ETr} production requires a chromosomal locus, *vps*, which contains sequences homologous to carbohydrate biosynthesis genes of other bacterial species (Yildiz and Schoolnik, 1999). Variants of *Pseudomonas putida* isolated from mixed culture biofilms with an *Acinetobacter sp*. were found with mutations in a *wapH* gene homolog involved in core lipopolysaccharide biosynthesis (Hansen *et al.*, 2007). These phenotypic variations in biofilms may not have necessarily resulted from the physiological regulation of gene expression in response to the

local environmental conditions, but may be due to mutations in DNA sequences. Mutation and generic rearrangement might first generate variants and then the variants may be enriched through natural selection in the local micro-environment in a biofilm. In a mixed-species biofilm, genetic changes during biofilm formation can result in the evolution of species interactions (Hansen *et al.*, 2007). It has been proposed that genetic alteration and diversification of bacteria in biofilms may help protect cells from future antimicrobial challenges or environmental changes (Boles *et al.*, 2004; and Cooper *et al.*, 2005). It was suggested that phenotypic variation generates cells that are more robust and adept at surface colonization than their parent strain (Yildiz and Schoolnik, 1999).

Stochastic gene expression may be a cause of diversified phenotypes in a biofilm in which the cell population is independent of the prevailing environmental conditions (Stewart and Franklin, 2008; McAdams, H. H. and Arkin, 1997; Elowitz *et al.*, 2002). The exopolysaccharide of *Bacillus subtilis* biofilms is produced by enzymes encoded by the *eps*A-O operon and the TasA protein (Branda *et al.*, 2001; 2006). The gene clusters encoding EPS are regulated by a repressor protein SinR and its anti-repressor SinI. Chai *et al.* used different colored fluorescent reporter genes that were fused to the promoters for *sinR*, *sinI* and the EPS operon and found that while the repressor *sinR* was expressed in almost all cells, the *sinI* anti-repressor was expressed in only a subset of cells (Chai *et al.*, 2008). The expression of *sinI* is generally associated with cells in which the matrix production was induced. The authors proposed that EPS production in *B. subtilis* is under the control of a bistable switch, and only a subset of cells are involved in EPS production (Chai *et al.*, 2008). The mechanism of the stochastic switch might not be dependent on sensing local

environmental conditions. Bay *et al.* examined the expression of the *chiA* gene (chitinase) in a marine *Pseudoalteromonas spp*. biofilms, and found that individual cells in which the *chiA* was strongly expressed could be adjacent to the cells that were not expressing *chiA*. The authors proposed that the differential expression reflects a "division of labor strategy" by subset cells (Baty *et al.*, 2000a, 2000b). In summary, the generation of physiological heterogeneity within biofilms may be associated with several processes including: adaptation to the spatially microscale environmental conditions, the generation of variants having phenotypically distinct characteristics due to mutation and gene rearrangement; a stochastic gene-expression process that could be independent of the prevailing environmental conditions.

L. MONOCYTOGENES BIOFILMS

Introduction

The food-processing environment has been considered to be a primary niche of *L. monocytogenes* (Kathariou, 2002). Some strains of *L. monocytogenes* are known to persist in the food-processing equipment for more than 10 years, and these strains have been demonstrated to be responsible for outbreaks. However strain types isolated from food-processing facilities only partially overlap with those involved in human outbreaks (Kathariou, 2002; Tompkin, 2002). *L. monocytogenes* exists in food-processing facilities primarily in the form of biofilms, which form on materials that are commonly used for food-processing equipment. The *L. monocytogenes* biofilms on either stainless steel or Teflon surfaces have been found to confer tolerance to sanitizers, heat treatment, and other stresses,

compared to planktonic cells (Chmielewaski and Frank, 2004; Mosteller and Bishop, 1993; Ren and Frank, 1993; Stopforth *et al*., 2002).

Most studies that have been performed on the biofilms of *L. monocytogenes* focus on comparing attachment and biofilm formation with different isolated strains under varying conditions (media, temperature, food-processing surfaces), or on the resistance of specific strains in biofilms to stressful conditions (sanitizers, antimicrobial agents, and high temperatures). *L. monocytogenes* strains vary in adherence and biofilm formation. Norwood and Gilmour (1999) and Lunden *et al.* (2000) reported that strains corresponding to serotype 1/2c, and strains identified as persistent in food-processing systems, are more capable of attaching to stainless steel surfaces. Additionally, a report by Djordjevic *et al*. (2002) suggests that strains belonging to lineage I (including serotypes 4b, 1/2b, 3c, and 3b) have a relatively higher ability to form biofilms on PVC microtiter plates than either lineage II strains (including serotype 1/2a, 1/2c, and 3a) or lineage III strains [including serotype 4a and 4c (Nadon *et al.*, 2001; Rasmussen *et al.*, 1995)]. Consequently, a hypothesis that the ability to form biofilms by lineage I strains correlated with prevalence in human outbreaks. Other work, however, has shown contradictory results (Borucki *et al.*, 2003), suggesting that there may not be a relationship between biofilm formation, outbreaks, and strain subtype (Kalmokoff *et al.,* 2001). Furthermore, Folsom *et al.* reported recently that a change of growth medium concentration (10 fold dilution of culture medium) could alter biofilm formation on stainless steel by 1/2a strains and 4b strains (Folsom *et al.*, 2006).

Listeria monocytogenes isolates vary in the capacity to form biofilms (Borucki *et al.*, 2003; Djordjevic *et al.*, 2002). Some strains of *L. monocytogenes* have been shown to form

multi-layer biofilms (Chavant *et al.*, 2002; Marsh *et al.*, 2003; Møretrø and Langsrud, 2004). In contrast to the biofilm structure of other bacteria, in which the bacterial cells are embedded in extracellular substances, *L. monocytogenes* biofilm cells stack on each other with little extracellular material, and are surrounded by holes and narrow slots (Marsh *et al.*, 2003 and Rieu *et al.*, 2008). However, little information is available about the detailed components of extracellular material in *L. monocytogenes* biofilms. Adherence to surface confers resistance to sanitizers and other stresses (Frank and Koffi, 1990; Lee and Frank, 1991; Mosteller and Bishop, 1993; Ronner and Wong, 1993; Norwood and Gilmour, 2000; Stopforth *et al.*, 2002; Pan *et al.*, 2006). Biofilm formation is a complex process. Biofilm formation can be affected by the efficiency of cell attachment at the initiation stage and by various factors involved in both microcolonization and development stages. Briandet *et al.* (1999) found the cell surface charge and the electron donor and acceptor properties of *L. monocytogenes* cells influence their attachment at different temperatures. Chavant *et al.* (2002) examined *L. monocytogenes* LO28 and found that cell surface physicochemical properties play a role in adhesion and surface colonization at the early stages of biofilm formation. The physiochemical properties of the physical surface to which the cells attach may influence on cell adhesion (Krysinski *et al.*, 1992; Smoot and Pierson, 1998a, 1998b). It was observed that a variety of different density biofilms were formed by *L. monocytogenes* on different materials commonly used for food processing equipment (Blackman and Frank, 1996).

Nutrient profile is another factor influencing biofilm formation of *L. monocytogenes*. Kim and Frank found that biofilm development varied with different phosphate levels in a

chemically defined minimal medium, and was affected by glucose levels ranging from 0.1% to 2% (Kim and Frank, 1995). Alternating amino acid concentrations could affect initial attachment but had no influence on the biofilm development (Kim and Frank, 1995). The concentrations of ammonium chloride and iron were also critical to the efficiency of initial attachment (Kim and Frank, 1994).

Biofilm formation of *L. monocytogenes* can also be influenced by the presence of other bacteria in the environment. Biofilms in food processing areas may be composed of mixed types of bacterial cells. Laboratory studies reported that *L. monocytogenes* occupied 1-10% of the total biofilm cell population in mixed cultures with *Pseudomonas* and *Staphylococcus* (Jeong and Frank, 1994; Norwood and Gilmour, 2000). Due to the complexity of mixed cell biofilms, there is limited information about the interaction and behavior of biofilms composed of *L. monocytogenes* and other species of microorganisms. The attachment of *L. monocytogenes* cells to a glass surface was observed to be enhanced in the presence of *P. fragi* (Sasahara and Zottola, 1993). However, biofilm formation of *L. monocytogenes* could be constrained by other organisms producing antagonistic compounds and competing for nutrients. It has been found that the presence of *Bacillus* spp. and *Staphylococcus* spp. decreased adhesion and biofilm formation by *L. monocytogenes* (Jeong and Frank, 1994; Leriche and Carpentier, 2000; Norwood and Gilmour, 2000, 2001). Lactic acid bacteria including *Enterococcus durans*, *Lactococcus lactis* subsp. *lactis*, and *Lactobacillus plantarum* have been shown to be able to inhibit the growth and even eliminate *L. monocytogenes* in mixed culture biofilms by producing antimicrobial compounds such as nisin and organic acids (Zhao *et al.*, 2004, 2006). To control *L. monocytogenes* effectively

and strategically in the food processing environment, more research is needed to determine the interaction between *L. monocytogenes* and other microorganisms existing in the same environment.

Molecular mechanisms controlling adhesion and biofilm formation of *L. monocytogenes*

Understanding the molecular mechanisms of *L. monocytogenes* biofilm formation may help optimize traditional control procedures and develop new strategies for preventing cell attachment and biofilm formation in the food processing environment. Compared to the extensive molecular studies on biofilm formation of several pathogenic bacteria such as *P. aeruginosa*, *Escherichia coli*, *Bacillus subtilis*, *S. aureus*, detailed knowledge about the molecular mechanisms involved in *L. monocytogenes* biofilm formation is limited. The flagella of *L. monocytogenes* were implicated in initial attachment and biofilm formation, similar to gram-negative bacteria such as *E. coli*, *Vibrio cholerae*, and *P. aeruginosa* (Vatanyoopaisarn *et al.*, 2000; Gorski *et al.*, 2003; Lemon *et al.*, 2007). Adhesion of *L. monocytogenes* serotype 1/2c was reported to be greater than that of other serotypes, suggesting differences in flagella among the serovars (Norwood and Gilmour, 1999; Lunden *et al.*, 2000). Vatanyoopaisarn *et al.* compared the efficiency of initial attachment between nutrient deprived nonmotile, but viable, cells with flagella-minus mutant cells and found that the flagellated wild type cells attached 10-times better to stainless steel surface in the first 4 h than the mutant, suggesting that flagella have a role as adhesions in initial attachment (Vatanyoopaisarn *et al.*, 2000). Gorski *et al.* found three Tn*917*-LTV3 mutants that were defective in attachment to radish tissue and lacked motility at 30^{o}C (Gorski *et al.*, 2003).

However, this phenomenon was not observed in the study of Lemon *et al.* who compared the initial attachment among three types of cells, wild type, flagellated but non-motile, and aflagellated cells. They found that flagella-mediated motility is significant for both initial attachment and subsequent biofilm formation; and the role of flagella as an adhesion seemed to be minimal in initial attachment (Lemon *et al.*, 2007). On the other hand, flagellin protein was observed to be down regulated in biofilm-grown cells (Trémoulet *et al.*, 2002; Hefford *et al.*, 2005). It is worth noting that the flagella in *L. monocytogenes* are regulated differently from those in other gram negative bacteria. As a facultative intracellular pathogen, transcription of flagellar motility genes (*flaA*) at physiological temperatures (37°C and above) is repressed by MogR, a DNA binding protein required for virulence (Gründling *et al.*, 2004). MogR repression at low temperatures (30°C and below) is mediated by two response regulators, DegU and GmaR (Shen *et al.*, 2006). GmaR is an anti-repressor of MogR, and it is temperature dependent due to DegU-dependent transcriptional activation of *gmaR* at low temperatures. GmaR also functions as a glycosyltransferase exhibiting O-linked N-acetylglucosamine transferase (OGT) activity for flagellin (FlaA). The flagellin protein of *L. monocytogenes* has been demonstrated to be posttranslationally modified with O-linked *N*-acetylglucosamine (GlcNAc) (Schirm *et al.*, 2004). GmaR is involved in a glycosylation process that is required for flagella motility. GmaR-negative bacteria are nonmotile even though FlaA is secreted and observed on the *GmaR* mutant (Shen *et al.*, 2006). These findings suggest that glycosylation of flagellin is critical to the motility of flagella in *L. monocytogenes*. Even though flagella mediate or facilitate the adhesion and invasion of

eukaryotic host cells by *L. monocytogenes* (Bigot *et al.*, 2005; Dons *et al.*, 2004), the role of flagella in vivo might be minimal.

Researchers have investigated the differences in protein expression in *L. monocytogenes* between cells in biofilms and the planktonic state (Trémoulet *et al.*, 2002; Hefford *et al.*, 2005). Thirty one proteins that were differently between the two growth conditions were found in one study (Trémoulet *et al.*, 2002), and nineteen up-regulated proteins in biofilm-grown cells were found in another study (Hefford *et al.*, 2005). Some up-regulated proteins in biofilm-grown cells were found to be involved in stress response (YvyD, rpsB, CysK, GroES, GroEL, UspA, and EF-Tu). These findings suggest that biofilm cells are in a stressed state while growing at a very slow rate. The discrepancies found in the proteomic studies on *L. monocytogenes* biofilms may be due to the heterogeneity and complexity of cells in biofilms.

Quorum sensing (cell-cell signaling, QS) has been considered to be one of the factors regulating biofilm development (Kjelleberg and Molin, 2002). The role of quorum sensing in biofilm formation has been a focus of study with several bacteria including *Pseudomonas aeruginosa* (Patriquin *et al.*, 2008), *Aeromonas hydrophila* (Lynch *et al.*, 2002), *Burkholderia cepacia* (Simões *et al.*, 2007), *Serratia liquefaciens* (Labbate *et al.*, 2004), *Escherichia coli* (Li *et al.*, 2007), *Vibrio cholerae* (Liu *et al.*, 2007), *Staphylococcus aureus* (Balaban *et al.*, 2007), *Streptococcus mutans* (Wen *et al.*, 2002**),** *Bacillus subtilis* (Lombardía *et al.*, 2006), and *L. monocytogenes* (Challan Belval *et al.*, 2006). A precursor of the autoinducer 2 (AI-2) in *L. monocytogenes*, *S*-ribosyl homocysteine (SRH), is encoded by lmo1288 (*luxS*), and was found to play a role in biofilm formation. The function of SRH in

Table 1-1. List of the genes that are up-regulated in *Listeria monocytogenes* biofilm formation.

Genes	Functions of gene products	Reference
relA	RelA, primary biosynthetic enzyme for 5'-pppGpp-3' in stringent response.	Taylor *et al.*, 2002
hpt	Hgprt, required in purine salvage pathway that is necessary for pppGpp synthesis.	Taylor *et al.*, 2002
yvyD	YvyD, a stress response ribosomal protein controlled by both σ^B and σ^H.	Trémoulet *et al.*, 2002
rpsB	30S ribosomal protein S2, a stress response ribosomal protein.	Trémoulet *et al.*, 2002
lmsod	SOD, detoxification of free oxygen radicals	Trémoulet *et al.*, 2002; Helloin *et al.*, 2003
cysK	CysK, O-acetylserine (thiol)-lyase, involved in oxidative stress response.	Trémoulet *et al.*, 2002
luxS (lmo1288)	*S*-ribosylhomocysteinase, catalyzes the hydrolysis of *S*-ribosylhomocysteine to homocysteine and 4,5-dihydroxy-2,3-pentadione (DPD), which serves as a precursor of autoinducer 2 (AI-2)	Belval *et al.*, 2006; Sela *et al.*, 2006
lmo0842	LPXTG (Leu-Pro-X-Thr-Gly) protein, involved in biofilm formation and virulence.	Cabanes *et al.*, 2002
codY	CodY, a GTP activated global regulator that suppresses the expression of a wide variety of genes induced by nutrient limitation and the entry into stationary phase.	Helloin *et al.*, 2003; Hefford *et al.*, 2005; Helloin *et al.*, 2003
Gapdh	GAPDH (Glyceraldehyde 3-phosphate dehydrogenase), glycolytic enzyme, highly expressed in biofilm grown cells. Besides glycosis, it is implicated in several non-metabolic processes, including *transcription* activation, initiation of apoptosis.	Hefford *et al.*, 2005
PGAM	PGAM (glycolytic enzyme phosphoglycerate mutase), highly expressed in biofilm grown cells.	Hefford *et al.*, 2005
tim	TIM (Triosephosphate isomerase), a central enzyme in the glycolytic pathway, highly expressed in biofilm grown cells.	Hefford *et al.*, 2005

cell-to-cell communication, however, has yet to be demonstrated (Challan Belval *et al.*, 2006). The Agr peptide-sensing system is one of the best-studied QS system in low G+C Gram-positive bacteria (Lyon and Novick, 2004). The *agr* locus consists of four genes encoding four proteins; AgrB is responsible for transportation and proteolytic processing of the QS peptide AgrD, AgrC (histidine kinase) and AgrA comprise a two-component signal transduction system. Deletion of *agrD* in *Listeria monocytogenes* resulted in significantly reduced biofilm formation (Riedel *et al.*, 2009).

Although much work has been done, little is known about how a persistent strain of *L. monocytogenes* survives in a food-processing environment. The primary factors affecting persistence of *Listeria* in food-processing environments may include inadequate nutrition, dehydration, unfavorable temperature, cleaning and sanitation, interaction with other microflora existing in the environment, shear forces, etc. A persistent strain should be able to adapt to, and survive in varying environmental conditions.

GENOMICS OF *L. MONOCYTOGENES*

Among the 13 serotypes of *L. monocytogenes*, serotype 1/2a, 1/2b and 4b constitute 95% of human infections (Kathariou, 2002). Serotype 1/2a strains are frequently isolated from foods or food processing environments, whereas serotype 4b strains are typically responsible for outbreaks and sporadic cases of listeriosis when compared with other serotypes (Barbour *et al.*, 2001**;** WHO, 2001). These two serovars have been found to differ by molecular subtyping data (Zhang *et al.*, 2003; Piffaretti *et al.*, 1989; Brosch *et al.*, 1994). In order to better understand the molecular mechanisms of *L. monocytogenes* virulence in humans and

survival of this bacterium in food and in the environment, several studies have been performed to compare the gene content and transcriptional signatures between strains of different serovars, origins and phylogenetic lineages (Glaser *et al.*, 2001; Nelson *et al.*, 2004; Zhang *et al.*, 2003; Doumith *et al.*, 2004; Call *et al.*, 2004; and Severino *et al.*, 2007). Based on the whole-genome comparison data for *L. monocytogenes* EGD-e (serovar 1/2a, isolated from animal illness cases in 1924) and *L. innocua* strain CLIP11262 (serovar 6a), *Listeria* has a close relationship to *Bacillus subtilis*, and 9.5% of genes are specific to *L. monocytogenes* while 5% are specific to *L. innocua*. The species-specific genes of *L. monocytogenes* encoded virulence associated proteins and proteins implicated in adaptation to different environments (Glaser *et al.*, 2001). Nelson *et al.* (2004) did a comparative analysis of the genomes of three strains of *L. monocytogenes* representing the two major phylogenetic lineages. They found that *L. monocytogenes* genomes are very similar in gene content and structure, with the majority of genomic differences were in phage insertions, transposable elements, scattered unique genes, and islands encoding proteins of mostly unknown functions, as well as single nucleotide polymorphisms (SNP) in many virulence genes. The completed genome of *L. monocytogenes* strain F2365 (serotype 4b, epidemic clone I, cheese isolate implicated in California outbreak in 1985) is a single, circular chromosome of 2.905M bp with a G+C content of 38%. This genome contains only 51 strain specific genes. Another serotype 4b strain H7858 (meat isolate, epidemic clone II, involved in the 1998-1999 multistate outbreak in the USA) had 69 specific genes; while the serotype 1/2a strain F6854 (epidemic clone III, turkey frankfurter isolate implicated in human illness in 1988) had 97 specific genes, although the genome sequence was not completed. The

genome of *L. monocytogenes* strain EGD-e (serovar 1/2a) contains a circular chromosome of 2.944 Mbp with an average G+C content of 39% (Glaser *et al.*, 2001). The overall similarities in metabolic and transportation systems between the two different serotypes (4b via 1/2a) suggest that metabolism is similar between the different *L. monocytogenes* serovars. The unique genes of *L. innocua* CLIP 11262, excluding prophage genes, were absent from the genomes of the other sequenced *L. monocytogenes* strains, implying that gene loss from a lineage ancestral to *L. monocytogenes* and *L. innocua* preceded the genomic diversification of *L. monocytogenes* into genomic divisions I and II (Nelson *et al.*, 2004). The genomes of different *L. monocytogenes* strains are very similar and apparently stable. Major virulence determinants such as LLO (listeriolysin), phospholipases PlcA and PlcB (escape from the host vacuole), ActA (movement within the host cell cytoplasm), PrfA (transcriptional regulator of the virulence genes), InlA, and InlB (internalization) are conserved in four sequenced *L. monocytogenes* strains. *L. monocytogenes* strains that are prevalent in human and animal illness rely on a relatively small number of unique regions for antigenic diversity and epidemiologically relevant attributes (Nelson *et al.*, 2004). Comparative genomic analysis and DNA microarrays have shown that most of the strain specific genes typically fall into four major categories: synthesis of surface protein, cell wall and teichoic acids, transcriptional regulation of virulence gene expression, and transport proteins (Zhang *et al.*, 2003). Different subgroups of each lineage contain different sets of surface proteins, which may result in varying potential to cause illness or to adapt to different environments.

Comparative transcriptome analysis revealed differences between the two major lineages (lineage I and lineage II) with respect to cell wall synthesis, the stress-related sigma B

regulon, and virulence-related genes (Severino *et al.*, 2007). To uncover the strain- and lineage-specific differences in *L. monocytogenes*, Severino *et al.* (2007) compared global gene expression profiles with two different in vivo infection models (chick embryo model and mouse model of infection). Six *L. monocytogenes* strains with different epidemiological backgrounds (epidemic, carrier, and environmental isolates) belonging to the two major disease-related lineages [lineage I (1/2a) and lineage II (1/2b and 4b)] were used. Interestingly, motility genes were found to be over expressed in lineage II (1/2b and 4b) *L. monocytogenes* strains. Differences in the expression of cell wall associated genes were found between the two lineages. The serotype designations of *Listeria* are related to teichoic acids (polyribitol phosphate covalently linked to peptidoglycan) present in the cell wall. Glycosidic replacements of the ribitol phosphate units provide the teichoic acid with variability in structure and antigenic features (Lei *et al.*, 2000; Kamisango *et al.*, 1983). *L. monocytogenes* serotype 1/2 and 4 strains show structural differences in the cell wall due to distinct genome content (Fiedler, 1988). However, the gene expression of cell wall related genes was shown to be different between the two lineages, suggesting that there are structural differences in the teichoic acid composition and differential regulation of teichoic acid biosynthesis between the two lineages. Differences were also found in the expression of *prfA* and PrfA-regulated genes. *prfA, plcB* and *plcA* (phospholipase C), *hly* (listeriosin O precursor), *actA* (actin-assembly inducing protein precursor), and *inlAB* (internalin A and B) were more highly expressed in 1/2a strains compared to expression in 1/2b and 4b strains (Severino *et al.*, 2007). Differential expression of *sigB* and SigB-regulated genes was also detected for the two lineages. In *L. monocytogenes*, Sigma B, acts as a global regulator,

confering stress resistance and contributing to pathogenesis by affecting prfA (transcriptional regulator of the virulence genes) transcription (Kazmierczak *et al.*, 2003). *sigB* was found to be over expressed in 1/2a strains compared to expression in 1/2b and 4b strains (Severino *et al.*, 2007). A virulence study showed that six of these strains varied in infection models (chicken embryo and mouse infection), suggesting the diverse interactions between *L. monocytogenes* strains and their hosts (Severino *et al.*, 2007).

Comparison of cell wall-associated proteins and surface antigens between *L. monocytogenes* serotype 4b and 1/2a strains

A total of 133 surface proteins were identified in *L. monocytogenes* EGD-e (serovar 1/2a, isolated from animal illness cases in 1924), including 41 LPXTG (Leucine-proline-X-threonine-glycine) proteins, nine GW (glycine-tryptophan) proteins with a signal peptide, 11 hydrophobic tail proteins, four P60-like proteins (P60 is a major 60-kDa surface protein that has a murein hydrolase activity and is implicated in cell division) and 68 lipoproteins (Cabanes et al., 2002). Thus, at least 4.7% of the coding capacity of the genome is dedicated to surface proteins (Cabanes et al., 2002). Further, 22.6% of genes encoding putative cell surface proteins are absent from *L. innocua*, suggesting that the primary differences between these two species can be attributed to their surface protein composition (Cabanes *et al.*, 2002).

A genome comparison of four *L. monocytogenes* strains [F2365 (serotype 4b), F6854 (serotype 1/2a), H7858 (serotype 4b), and EGD-e (serotype 1/2a)] showed that that the serotype 1/2a-specific genes comprise an operon. The operon includes genes encoding the

biosynthetic pathway for the antigenic rhamnose constituents that decorate the cell wall-associated teichoic acid polymers in serotype 1/2a strains, five glycosyltransferases, and an adenine-specific DNA methyltransferase (Nelson *et al.*, 2004), which was confirmed by a microarray analysis (Zhang *et al.*, 2003). Teichoic acids are a major determinant of the somatic antigens in *L. monocytogenes* strains (Lei *et al.*, 2001; Promadej *et al.*, 1999; Uchikawa *et al.*, 1986). Two 4b specific genes (*gtcA* and *gltAB*) have been identified that are involved in galactose decoration of cell wall teichoic acids in *L. monocytogenes* serotype 4b strains (Promadej *et al.*, 1999, Lei *et al.*, 2001). Strain-specific genes associated with cell wall and teichoic acid biosynthesis, as well as lycosyltransferases, might be related to differences in somatic antigens and involved in virulence and immuno-genicity of strains of different serotypes.

As a notably invasive bacterium associated with life-threatening food-borne disease in humans, *L. monocytogenes* has been a concern for food safety, and for clinical and medical research, but the solutions to prevent infection of this bacterium have yet to be identified. Preventing contamination during and after processing prior to consumption would be an ideal solution. Once present, however, *L. monocytogenes* may persist for a long period of time as in the form of biofilms in food processing facilities. Although there is some evidence those persistent strains of *L. monocytogenes* have an increased ability to form biofilms, the data have not clear cut. The cause for abnormal distribution of different setoypes in environment and outbreaks has yet to be explained. Further research with conditions reflective of the food

processing environment, and additonal ecological and epidemiologic studies may help prevent outbreaks of listeriosis.

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CHAPTER 2

Enumeration of Viable *Listeria monocytogenes* Cells by Real-Time PCR with Propidium Monoazide and Ethidium Monoazide in the Presence of Dead Cells

ABSTRACT

Propidium monoazide (PMA) and ethidium monoazide (EMA) have been reported to be useful for quantifying viable bacteria with real-time PCR. The purpose of this study was to compare the effectiveness and compatibility of these two fluorescent dyes in enumerating viable cells of *Listeria monocytogenes* in combination with real-time PCR. EMA was observed to have lethal effect on *L. monocytogenes* during incubation and light exposure, presumably by penetrating through the cytoplasmic membrane of viable cells. However, PMA, at its used concentration, had no apparent affect on the growth or survival of *L. monocytogenes*. Viable *Listeria* was subjected to a range of temperatures for two hours prior to EMA or PMA treatment. Increase of temperature resulted in a decrease in cell viability and the amplification of DNA from EMA treated viable cells. Cell viability and amplification of DNA from viable cells was not affected by PMA regardless of temperature. When quantifying viable cells of *Listeria* in biofilms by real-time PCR, PMA treatment to a viable/dead cell mixture was optimized by doubling the treatment and increasing light intensity. Compared to EMA, PMA is a more reliable and useful chemical in enumerating viable *L. monocytogenes* cells in the presence of dead cells with real-time PCR. Viable cell counts were linearly related to real-time PCR Ct values for PMA treated cells of *L. monocytogenes*, over a 5 log range.

INTRODUCTION

Propidium monoazide (PMA) and ethidium monoazide (EMA) have been reported to be useful for quantifying viable bacteria with real-time PCR (Nocker and Camper, 2006; Nocker *et al*., 2006; Nogva *et al*., 2003; and Rudi *et al*., 2005). Both EMA and PMA can intercalate into double stranded DNA or RNA and then irreversibly crosslink to the nucleic acids following photoactivation (Bolton and Kearns, 1978). The dyes have been reported to be excluded from viable bacterial cells because of the inability to cross intact biological membranes. The viable microorganisms could therefore be enumerated by real-time PCR detection methods once free DNA or DNA from dead cells is inactivated (Nocker *et al*., 2006; Nogva *et al*., 2003; and Rudi *et al*., 2005).

Real-time PCR methods have been developed to detect and quantify *Listeria monocytogenes* in food products such as milk, cabbage, and cheese, as well as in biofilms (Doumith *et al.,* 2004; Guilbaud *et al*., 2005; Hough *et al*., 2002; Nogva *et al*., 2000; Rodriguez-Lazaro *et al*., 2004 and 2005). The combination of EMA and real-time PCR (EMA-PCR) has been developed for selective analysis of DNA from live cells of *L. monocytogenes* (Rudi *et al*., 2005). However, Nocker et al. (2006) observed EMA, but not PMA was able to penetrate into viable cells of *L. monocytogenes* using fluorescence microscopy. In this report we provided solid data in comparison of the efficiencies of PMA and EMA for enumeration of viable cells in the presence of DNA from dead cells, and determined an optimized procedure. EMA, but not PMA was potentially toxic to *L. monocytogenes* cells than PMA at their used concentrations. Furthermore, we demonstrated

the use of PMA in combination with real-time PCR for enumerating viable cells in *L. monocytogenes* from biofilms.

MATERIALS AND METHODS

Bacterial strains and growth conditions. Four strains of *L. monocytogenes* with different resources and serotypes were used, SK1386 (serotype 1/2b, clinic, Canada, 2002), SK1389 (serotype 1/2c, clinic, England, 2002), SK1403 (serotype 4b, Food, USA, 2002), and SK1420 (serotype 1/2a, Food, USA, 2002). These strains were obtained from the culture collection of Dr. Sophia Kathariou in the Department of Food Science, North Carolina State University. Storage and transfer of the strains were described in previous study (Pan et al, 2006). To determine the effectiveness of EMA and PMA in quantitatively differentiating viable and killed *L. monocytogenes* cells, each strain was individually incubated in Bacto™ tryptic soy broth containing 0.6% yeast extract (TSB-YE, pH 7.2, BD Biosciences, San Jose, CA) at 25°C for 11-12 h after inoculating 1-2 colonies from a culture agar plate. The exponential stage cultures were centrifuged at 3,000 × g for 10 min at 10°C and re-suspended in saline (NaCl, 0.85%). The optical density at 630 nm (OD_{630}) of each cell suspension in saline was adjusted to OD_{630} = 0.5 (ca. 10^9 CFU/ml). Equal volume of the four cell suspensions were pooled together to make a mixture. Half volume of the mixture was transferred to another tube and subjected to heat treatment for 10 min at 80°C. The heat treated cell suspension represented killed cell samples.

Preparation and Treatment of EMA and PMA. Both EMA ($C_{21}H_{18}BrN_5$, Phenanthridium, 3-amino-8-azide-5-ethl-6-phenyl, bromide; Molecular probes, Inc., OR) and PMA ($C_{27}H_{33}Cl_2N_6$, Biotium, Inc., CA) were prepared and stored as in previous studies (Nocker et al, 2006; Nocker and Camper, 2006; Nogva et al, 2003). EMA and PMA were dissolved in 20% DMSO to make stock solutions in 5,000 μg/ml and 2,500 μM, respectively. The stock solutions were kept at -20^{o}C in the dark. Ten micro liters of either EMA or PMA stock solution were added to 490 μl of each test culture in a micro-centrifuge tube (1.5ml) to make a final concentration of 100 μg/ml (237.9 μM) for EMA or 50 μM (25.6 μg/ml) for PMA, which were reported to be sufficient for use in both planktonic bacterial suspensions and biofilms (Nocker et al, 2006; Nocker and Camper, 2006; Nogva et al, 2003, Rudi et al., 2005a,b). Following addition of the dyes into bacterial suspensions, the suspensions were vortexed for two to three seconds and incubated in the dark for 5 min at room temperature unless otherwise specified. The tubes were then placed in chipped ice, with their lids off, and subjected to light exposure for 5 min using a 600-W halogen light source placed 20 cm directly above the samples. After light exposure for cross-linking of the dyes with DNA, the cell suspensions were centrifuged at 6,000 ×g for 10 min. The cell pellets were either resuspended by saline for more treatments with PMA or finally subject to DNA extraction.

Examination of light intensity on PMA treatment using different light source. A different halogen light with 650W (GE Lighting, General Electric Co. Cleveland, OH) was used to examine and compare the effects of light intensity on PMA treatment. The procedure for culture preparation and PMA treatment was the same as that described above.

DNA extraction and real-time PCR. Genomic DNA was isolated with a DNeasy® Blood & Tissue kit (QIGEN) by following the supplier's protocol for gram-positive bacteria with minor modifications. Each cell pellet from either planktonic cells or biofilm detached cells was mixed with 180µl freshly prepared lysis buffer containing 40mg/ml lysozyme, and incubated for over 6h at 37°C. To get maximum DNA from each sample, the final elution (100µl) was repeated three times. The extracted DNA was stored at -20°C and ready for real-time PCR assay.

A genus-specific gene of *Listeria*, *prs*, was selected for primer design (Doumith et al, 2004). The primers (For: 5'-GCGGATGTGATTGATTTAC-3'; Rev: 5'-AAACTGCACTAACTCTTGAAT-3') were design by a real-time PCR assay design software RealTimeDesign™ (Biosearch technologies, CA) and synthesized by Biosearch Technologies, Inc. (Novato, CA). The real-time amplifications were performed in a 96-well thermocycler (iCycler, Bio-Rad Laboratories). Amplification reaction mixture (total volume 25 µl) was consisted of the primers at a concentration of 300nM, 4.0 µl of template, and 12.5 µl of 2 ×iQ SYBR Green Supermix (Catalog # 170-8882, Bio-Rad Laboratories, CA). Real-time PCR cycling conditions were as follows: 95 °C for 3 min followed by 40 cycles of 95 °C for 20s, 55 °C for 30s, and 72 °C for 25s, followed by a final step of 72 °C for 10min after cycling was completed.

Real-time PCR and data analysis were performed with MyiQ™ Single-Color Real-Time PCR Detection system (Bio-Rad Laboratories). Values of threshold cycle (Ct) were automatically generated by the MyiQ software.

Effects of PMA/EMA on *Listeria* cell survival and on DNA amplification. Aliquots of 490 µl of both viable cell suspension and killed cell suspension (ca.10^7CFU/ml) were put in water baths with different temperatures: 5, 15, 25, and 37^{o}C for 2h prior to PMA/EMA treatment and viable cell plating. The procedures for PMA/EMA treatment, real-time PCR assay, and plating were strictly followed as described above.

Antimicrobial effects of EMA/PMA. The antimicrobial activities of EMA and PMA against *L. monocytogenes* were tested by a microtitre assay. Two hundred micro liters of the four-strain culture mix in fresh TSB-YE (ca. 10^5 CFU/ml) was added into each well of 96-well plates containing 2-fold serial dilutions of either PMA or EMA. Control wells contained bacterial cultures in TSBYE (Positive control) or the media only (Negative control). Following incubation at 37^{o}C in the dark for 16 h, bacterial growth was measured in optical density at 630 nm with a microtitre plate reader (SAFIRE, TECAN, Austria). OD_{630} value in each well was measured before and after incubation. The minimal inhibitory concentration (MIC) was defined as the lowest concentration of each specific dye at which no bacterial growth was detected.

To determine the killing kinetics of each dye in used concentration, a mixture (ca. 10^8 CFU/ml) composed of equal amount of each of the four overnight cultures in saline (NaCl, 0.85%) was prepared. Either PMA (50 µM) or EMA (100 µg/ml) was added and vortexed in the dark, and then divided into two groups followed by different incubation conditions. One group was continuously incubated at room temperature (22.5 ± 1.5^{o}C) in the dark. The other group was subjected to the same condition as that in the dye treatment for PCR assay. At

designated intervals, samples were removed, serially diluted and plated onto agar media TSAYE for colony count. Plating and colony counting procedure was followed as described previous (Pan et al, 2006).

Establishment of standard curves for viable cell quantification from viable/dead cell mixtures. A total of seven groups of serial dilutions of viable cell suspension were prepared. A viable cell suspension (ca. 10^8 CFU/ml) composed of four different strains at post exponential stage was serially diluted in 10 fold with saline, heat killed cell suspensions in 10^3 CFU/ml, 10^4 CFU/ml, 10^5 CFU/ml, 10^6 CFU/ml, 10^7 CFU/ml, 10^8 CFU/ml, respectively. Each group was composed of seven serial diluted viable cell suspensions (10^8 CFU/ml, 10^7 CFU/ml, 10^6 CFU/ml, 10^5 CFU/ml, 10^4 CFU/ml, 10^3 CFU/ml, and 10^2 CFU/ml) (Table 1). Four hundred and ninety micro liters of each cell suspension was transferred to a 1.5 ml micro-centrifuge tube. Each tube containing the specified cell suspension was subjected to PMA treatment (incubation and light exposure) twice, DNA extraction, and subsequent rea-time PCR. The methods of PMA treatment, DNA extraction, and real-time PCR reactions were strictly followed as described above. The mean Ct values of three replicates from each sample were used to make a standard curve of Ct value versus viable cell count.

Biofilm formation, sanitizer treatment and detachment. The procedures for strain preparation and biofilm formation were followed as described in our previous study (Pan et al, 2006) with small modifications. Briefly, following incubation at 25°C for 18 to 20h, the cultures were centrifuged and the cell pellets were suspended in saline to make a cell

suspension (ca. 10^8 CFU/ml). The stainless steel coupons (T-316, no. 7 finish, 82.5mm by 25mm by 1.6 mm, M.G. Newell Corp., Greensboro, NC) positioned in pipette tip plastic boxes (120mm by 88mm by 50mm; Rainin, Woburn, MA) were fully submerged in the mixed cell suspension, and incubated at 37 °C for 3 h for attachment. The coupons were then sequentially washed three times to remove loosely attached cells by aspiration. Ten fold diluted rich medium (TSB-YE/10) was added into the boxes until the coupons were completely submerged. The boxes containing coupons in the medium were incubated at 37°C for 48h allowing biofilm formation. The medium was refreshed every 12 h during incubation.

Following biofilm formation, the biofilms were rinsed with sterile deionized water for three times and then exposed to a hydrogen peroxide based sanitizer (Matrixx™, 100ppm of total product, pH3.8, Ecolab, St. Paul, MN). The coupons were taken out and immediately neutralized by being submerged in 0.1% sodium thiosulfate-phosphate solution (pH 7.0) for at least 1 min at specific intervals. After neutralization with sodium thiosulfate, bacteria cells were removed from the surface of the coupons using sterile cotton tipped swabs (Puritan Medical Products Company LLC, Maine). Prior to swabbing, swabs were soaked in a 0.1% peptone solution containing 0.1% Tween 80. A selected area (55mm by 25mm) on the surface of each coupon was sequentially swabbed three times with separate swabs to remove the cells from the surface. The swab tips from each surface were snapped and combined in a plastic screw-cap tube (50ml; corning) containing 20 ml of saline. Upon completion of stirring with a Vortex mixer at full speed for 2 min, fifteen milliliters of cell suspension from each tube were transferred to another tube for spinning at 6,000 ×g for 10 min. The

supernatant was removed by aspiration and the pellet was resuspended in 1.5 ml of saline. Two aliquots of 490 µl of the concentrated cell suspension from each sample were transferred into 1.5 ml microcentrifuge tubes for PMA treatment, and real-time PCR assay. Each aliquot was repeatedly treated with PMA (50uM) and light exposure for two times, followed by DNA extraction and real-time PCR assay as described above. The remaining cell suspensions were subject to plating for viable cell count. The plating and counting procedures were performed as described in the previous study (Pan et al, 2006).

Experimental design and statistical analysis. All trials were performed in triplicate, and the data are presented as the means ± standard deviations obtained for the multiple replications. Statistical analysis was performed for multiple comparisons of the means and standard deviations for different treatments. Analysis of variance (ANOVA) was carried out to determine the significant difference ($P < 0.05$) using the SAS program (SAS institute Inc., NC).

RESULTS AND DISCUSSION

EMA, not PMA, is lethal on *L. monocytogenes* at their working concentrations. To obtain representative data and minimize the difference among strains in experiments, the four-strain mixture *L. monocytogenes* was used in the study. Since temperature plays a role in microbial cell membrane permeability (Konings *et al*., 2002; and Vossenberg *et al*., 1995), the effect of temperature on lethality of EMA (or PMA) to *L. monocytogenes* cells was investigated. The lethal effects of 240 µM (100 µg/ml) EMA increased with incubation temperatures from 5^{o}C to 37^{o}C (Fig. 1A, shaded columns). However, 50 µM PMA did not

show any lethal effect (Fig. 1A, blank columns). Following incubation with the dyes for 5 min in the dark, similarly prepared cells were exposed for 5 min to a 600-W halogen light source placed 20 cm directly above the 500 µl samples in open microcentrifuge tubes on chipped ice (to prevent heating). This resulted in greater than a 6 $\log_{10}$ reduction with EMA (Fig. 1B, shaded columns), but no reduction was seen with PMA treated cells (Fig. 1B, blank columns). The minimum inhibitory concentrations (MIC) for EMA and PMA were determined using 2-fold serial dilutions of the dyes, with cells were held at 37^{o}C in TSB-YE in the dark for 16h. The MICs of EMA and PMA were 240 µM and 1600 µM respectively.

Rudi *et al* (2005) investigated the exclusion of EMA from viable *Campylobacter jejuni* by using inhibitors of efflux systems. They found no evidence that efflux pump systems were responsible for excluding EMA from viable cells (2005). The effects of temperature we observed for EMA toxicity may reflect changes in cell membrane fluidity or permeability. PMA apparently can not penetrate through the viable cell membrane of *L. monocytogenes*. This may be due to the higher positive charge of the PMA molecule than EMA (Nocker *et al*., 2006).

EMA, not PMA, interferes with DNA amplification during PCR from viable cells at different temperatures. The Ct values for EMA (240 µM) treated viable cells increased by 0.8 cycles to 4.5 cycles as the preconditioning temperature was raised from 5^{o}C to 37^{o}C (Fig. 1C, shaded columns). However, there was no difference in Ct values for PMA (50 µM) treated cells (Fig. 1C, blank columns). The Ct values for killed cell aliquots treated with EMA or PMA did not change with different preincubation temperatures (Fig. 1D). The

concentrations of the two dyes used in this study are reported to be the optimal concentrations in viable cell enumeration (Nocker and Camper, 2006; Nogva *et al*., 2003; and Rudi *et al*., 2005). A lower concentration of EMA (50 μM) was tested against its higher concentration (240 μM), as well as the same concentration of PMA (50 μM) in the efficiency of viable cell enumeration. There was no statistically significant difference of EMA in its efficiency of viable cell enumeration of *L. monocytogenes* between the higher concentration (240 μM) and the lower concentration (50 μM) (Data not shown).

Optimization of PMA treatments for *L. monocytogenes*. To optimize the PMA treatments, viable and killed cell aliquots were treated with different concentrations of PMA or repeated PMA treatments. The Ct values for the killed cell aliquots (approximately 2.4×10^7 cells/ml) treated with PMA (50μM) two or three times were similar, 10.7 ± 0.35, and averaged 2.8 cycles more than the aliquots treated only once. Varying PMA concentrations (50, 100, 200μM) or incubation temperature (23^oC, 40^oC) did not contribute a significant difference in Ct values for either viable or killed cell samples. Based on these data, repeating the PMA (50μM) treatment twice at room temperature was considered as an optimal condition for quantifying viable cells of *L. monocytogenes* with the PMA-PCR assay.

Increase of light intensity contributes to the effectiveness of PMA treatment. To determine the effects of light intensity on the PMA treatment, a different halogen light with 650W was used against the light with 600W. The light of 650W could provide a 5 log difference when the sample was treated twice with PMA, but provide 4 log difference if the

sample was treated only once that was the same effectiveness as provided by the 600W light for twice treatment (Table 2).

Determination of dynamic range of killed/viable cell ratio in which viable *L. monocytogenes* could be quantified by real-time PCR. Three replicates of each mixture were used to determine correlation coefficients (R^2) for Ct values and viable cell counts (CFU/ml) with varying concentrations of dead cells (Table 1, Figure 2A). A linear relationship between Ct and viable cells (R^2 value of 0.9816) was observed as long as the ratio of dead cells to viable cells was no greater than 10^4, and the minimum number of viable cells was not less than 10^3 (Fig. 2A, Table 1). This is similar to the results for EMA-PCR with *Campylobacter jejuni* reported by Rudi et al (2005). We found that the minimum amount of DNA copies that are available for PCR analysis and the faction of dead cells in a viable/dead cell mixture are two critical factors that could limit the range of PMA-PCR assay.

Quantification of viable cells of *L. monocytogenes* in sanitizer treated biofilms by Real-Time PCR. Stainless steel coupons (T-316, no. 7 finish, 82.5 mm by 25 mm by 1.6 mm, M.G. Newell Corp., Greensboro, NC) were used to form biofilms of the four-strain mixture of *L. monocytogenes* as described (Pan *et al*., 2006). Briefly, following 3h of cell attachment in a prepared cell suspension of the four-strain mixture (ca. 10^8 CFU/ml), the stainless steel coupons (40 total) were submerged in 10 fold diluted rich medium (TSB-YE/10) and incubated at 37°C for 48 h allowing biofilm formation. After biofilm formation, 3 sets of 10 coupons were then treated with a peroxide based sanitizing agent (Matrixx, 100

pm, pH 3.8, Ecolab, St. Paul MN) for 1 min, 2 min, and 3 min, respectively. A control group (10 coupons) was treated with saline (0.85% NaCl) in place of the sanitizer. Following neutralization with 0.1% sodium thiosulfate-phosphate solution (pH 7.0), biofilms were then detached from the surface of each coupon using sterile cotton tipped swabs (Pan *et al*., 2006). The detached biofilm cell suspension from each coupon was concentrated into 1.5 ml by centrifugation. Two aliquots of the concentrated cell suspension from each coupon were transferred into 1.5 ml microcentrifuge tubes for PMA treatment (50 μM, twice) in 500 μl total volume followed by DNA extraction, and real-time PCR. The remaining cell suspension from each sample was used to determine the viable cell count. The viable cell density in non-sanitizer treated biofilms on stainless steel surface was approximately 10^7 CFU/cm^2. The viable cell count decreased with increased exposure to the sanitizer. A linear relationship (R^2 = 0.96) could be established between Ct value and viable biofilm cells, when the estimated ratio of dead cells and viable cell was no more than 10^4 (Fig. 2B).

We found that PMA, in combination with real-time PCR, could be used for the quantification of viable cells of *L. monocytogenes* in suspensions in which the ratio of dead cells versus viable cells is no more than 10^5 and the live cells is no less than 10^3 CFU/ml given the light provides enough light intensity in PMA treatment. Cell suspensions prepared from broth culture or biofilms gave similar results. Compared with EMA, PMA was not found to penetrate into live cells, as determined by the toxicity of the two dyes to bacterial cells. PMA application with real-time PCR may be useful for quantifying viable cells of *L. monocytogenes* in food, pharmaceutical and environmental applications. Further studies on

the refinement of PMA treatment with real-time PCR are needed so as to overcome the limitations and maximize the utilization of the method in DNA-based quantitative analysis.

Table 2 .1 Antimicrobial activity of PMA/EMA in 2-fold serial dilutions [µM (µg/ml)] against *Listeria monocytogenes*.

PMA	50 (25.6)	100 (51.1)	200 (102.2)	400 (204.4)	800 (408.8)	1600 (817.6)	3200 (1635.2)
EMA	14.9 (6.25)	29.75 (12.5)	59.5 (25)	119.0 (50)	237.9 (100)	475.8 (200)	951.6 (400)

Listeria monocytogenes growth was detected in the concentrations of PMA/EMA listed in the shaded boxes.

Table 2.2 Comparison of effects of light intensity on PMA-PCR assay.

Light intensity	Treatment	C_T value with a viable cell density (log10CFU) in the presence of dead cells (7.9 $\log_{10}$CFU)*							R^2
		7.9	6.9	5.9	4.9	3.9	2.9	1.9	
600W	Once	13.03 ± 0.1	15.4 ± 0.2	17.87 ± 0.2	18.91 ± 0.1	**19.95 ± 0.3**	**19.08 ± 0.3**	**19.59 ± 0.2**	0.9713
	Twice	12.855 ± 0.1	15.94 ± 0.1	18.76 ± 0.2	21.2 ± 0.1	22.74 ± 0.0	**23.2 ± 0.3**	**23.7 ± 0.0**	0.9846
650W	Once	12.83 ± 0.1	15.65 ± 0.1	18.86 ± 0.2	20.97 ± 0.3	22.39 ± 0.1	**22.37 ± 0.2**	**22.21 ± 0.5**	0.9785
	Twice	12.52 ± 0.3	15.62 ± 0.2	18.98 ± 0.1	21.93 ± 0.2	23.52 ± 0.1	25.65 ± 0.3	**25.4 ± 0.4**	0.9832

* The boldface values were outliers and were not included in the linear regression analysis

Table 2.3 Dynamic range test for the dead cell/viable cell ratio of *L. monocytogenes* with the PMA-PCR assay

Dead cell density (log_{10} CFU/ml)	C_T value with a viable cell density (log_{10} CFU/ml) of *:							R^2
	8	7	6	5	4	3	2	
0	12.85 ± 0.07	16.03 ± 0.14	19.78 ± 0.12	23.19 ± 0.35	25.38 ± 0.81	28.57 ± 0.14	**30.69 ± 1.98**	0.9952
3	12.83 ± 0.17	16.02 ± 0.26	19.17 ± 0.52	22.95 ± 0.17	25.12 ± 0.17	28.22 ± 0.39	**29.65 ± 1.62**	0.9964
4	12.77 ± 0.33	16.42 ± 0.42	19.33 ± 0.21	23.19 ± 0.43	25.42 ± 0.53	29.01 ± 0.85	**29.24 ± 2.30**	0.9967
5	12.74 ± 0.38	15.68 ± 0.33	18.94 ± 0.28	22.64 ± 0.32	24.99 ± 0.70	28.32 ± 0.84	**29.68 ± 2.89**	0.9978
6	12.56 ± 0.27	15.68 ± 0.45	18.91 ± 0.31	23.06 ± 0.60	25.28 ± 0.56	28.47 ± 0.98	**30.02 ± 1.74**	0.9953
7	12.57 ± 0.36	15.56 ± 0.28	19.09 ± 0.34	22.32 ± 0.47	24.26 ± 0.82	**25.79 ± 1.34**	**27.75 ± 1.82**	0.9918
8	12.58 ± 0.32	15.26 ± 0.52	18.08 ± 0.35	21.05 ± 0.76	23.34 ± 0.46	**24.28 ± 1.24**	**24.38 ± 1.91**	0.9986

* The boldface values were outliers and were not included in the linear regression analysis

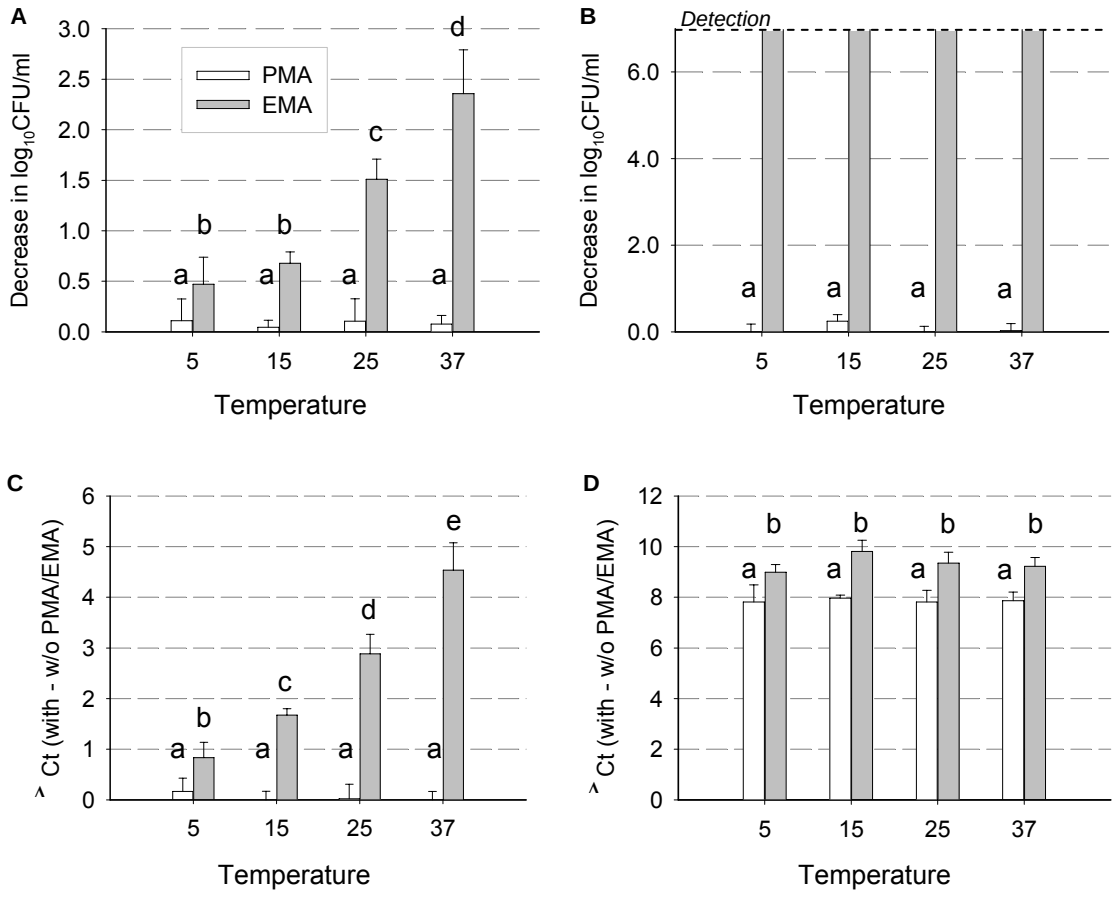

Figure 2.1. Viable cell density measured by PMA/EMA.

The viable cell density was determined following 5 min incubation in the dark (A), and after 5 min of exposure to light (B). Effects of PMA/EMA on DNA amplification from viable cells (C) and heat-killed cells (D). The bars indicate the mean values, and the error bars indicate the standard deviations (n = 3). Different letters indicate a significant difference ($p < 0.05$).

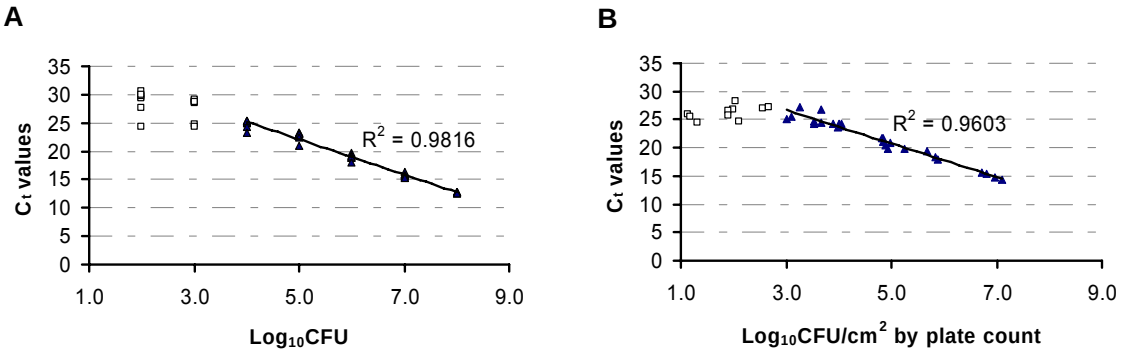

Figure 2.2. Standard curves for quantifying viable *Listeria monocytogenes* in a viable cell/dead cell mixture (A) and in stressed biofilms (B).

The solid standard line and the linear regression coefficient factor (R2) were generated using the solid triangles. The open squares are outlier data points not included in the linear regression analysis

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CHAPTER 3

Competition of *Listeria monocytogenes* Serotype 1/2a and 4b Strains in Mixed Culture Biofilms

ABSTRACT

The majority of *Listeria monocytogenes* isolates recovered from foods and the environment are strains of serogroup 1/2a. However, serotype 4b strains cause the majority of human listeriosis outbreaks. Our investigation of *L. monocytogenes* biofilms used a simulated food processing system that consisted of repeated cycles of growth, sanitation treatment, and starvation to determine the competitive fitness of strains of serotype 1/2a and 4b in pure and mixed culture biofilms. Selective enumeration of strains of a certain serotype in mixed culture biofilms on stainless steel coupons was accomplished using serotype-specific quantitative PCR and propidium monoazide treatment to prevent amplification of extracellular DNA or DNA from dead cells. The results showed that the serotype 1/2a strains tested were generally more efficient at forming biofilms, and predominated in the mixed culture biofilms. Growth and survival of strains of one serotype was not inhibited by strains of the other serotype in mixed culture biofilms. However, we found that a cocktail of serotype 4b strains survived and grew significantly better in mixed culture biofilms containing a specific strain of serotype 1/2a, with final cell densities averaging 0.5 $\log_{10}$ CFU/cm^2 higher, than without the serotype 1/2a strain. The methodology used in this study allowed unique insights in determining how environmental stresses and microbial competition influence survival and growth of *L. monocytogenes* in pure and mixed culture biofilms.

INTRODUCTION

A prominent foodborne pathogen, *Listeria monocytogenes* can cause severe infections in humans, primarily in high-risk populations, though the disease (listeriosis) is relatively rare (Farber and Peterkin, 1991; McLauchlin, 1997; Ryser and Marth, 2006). Outbreaks of listeriosis have resulted from contamination of a variety of foods by *L. monocytogenes*, especially meat and dairy products (Lianou and Sofo, 2007). *L. monocytogenes* is ubiquitous in the environment, able to grow at refrigeration temperature, and tolerant of low pH (pH 3 to 4) typical of acidified foods (Miller, 1992; Sleator *et al*., 2003; Liu, 2006). The capacity to produce biofilms confers to this bacterium further protection against stresses common in the food processing environment (Moretro and Langsrud, 2004; Gandhi and Chikindas, 2007).

Biofilms are characterized by dense clusters of bacterial cells embedded in extracellular polymeric substances which are secreted by cells to aid in adhesion to surfaces and to other cells (Costerton et al., 1987, 1994). Strains of *L. monocytogenes* have been known to persist for years in food processing environments, presumably in biofilms. Of the 13 known serotypes of *L. monocytogenes*, three (1/2a, 1/2b, and 4b) account for >95% of the isolates from foods and human outbreaks (Kathariou, 2002). Serotype 1/2a accounts for >50% of the *L. monocytogenes* isolates recovered from foods and the environment, while most major outbreaks of listeriosis have been caused by serotype 4b strains (Chemaly et al., 2008; Tresse et al., 2007; Guerini et al., 2007a; Gilbreth et al., 2005; Revazishvili et al., 2004; Aarnisalo et al., 2003; Gilot et al, 1996; Wallace et al., 2003; Lukinmaa et al., 2003; Kathariou et al.,

2006; Mead et al., 2006). No correlation has been identified between strain fitness and serotype of *L. monocytogenes* (Gorski et al., 2006; Jensen et al, 2007). Some studies have reported that repeatedly isolated strains (defined as persistent strains) had higher adherence capacity than sporadic isolates (Norwood and Gilmour, 1999; Borucki et al. 2003), while this phenomenon was not observed by others (Djordjevic et al., 2002). Serotype 4b strains exhibited higher capacity for biofilm formation than did serotype 1/2a strains (Norwood and Gilmour, 1999), whereas this was not observed by Di Bonaventura and colleagues (2008). It has been suggested that serotype 1/2a strains could be more robust in biofilm formation under a variety of environmental conditions compared to serotype 4b strains. Furthermore, strains of these serotypes differ in terms of the medium that promotes biofilm formation. Biofilm formation with serotype 4b strains was higher in full-strength tryptic soy broth than in diluted medium, whereas the opposite was observed with serotype 1/2a strains, which produced more biofilm in diluted medium (Folsom et al., 2006).

There is limited information in microbial competition between strains of different serotypes in biofilms or on how the environmental stresses present in food processing environments may affect biofilm formation and survival of *L. monocytogenes* of different serotypes. In food processing plants the environmental stresses encountered by bacteria are more complex and variable than most laboratory systems used for microbial ecology and biofilm studies. A simulated food processing system (SFP) has been developed to address this issue (Pan et al., 2006). The SFP system incorporates several stresses that may affect bacteria in biofilms in the food processing environment, including exposure to sanitizing agents, dehydration and starvation. By subjecting biofilms to the SFP regimen over a period of several weeks, the cell

numbers of *L. monocytogenes* strains in biofilms initially were reduced, then increased as the culture adapted (Pan et al., 2006). The development of resistance to sanitizing agents was specific to the biofilm-associated cells, and was not maintained once the cells were detached (Pan et al., 2006). It was subsequently found that real-time PCR, in combination with propidium monoazide (PMA) treatment of samples prior to DNA isolation, is an effective method for enumerating viable cells in biofilms (Pan and Breidt., 2007).

The objective of this study was to determine if either 1/2a or 4b strains of *L. monocytogenes* had a competitive advantage in biofilm formation and if strains of one serotype had a selective advantage under stress conditions. We investigated and compared the capability of the two serotype strains *L. monocytogenes* in initial attachment, biofilm formation, and analyzed the survival and growth of each serotype in mixed serotype biofilms in the SFP system using PMA with quantitative PCR.

MATERIALS AND METHODS

Strains and growth conditions. Eight *L. monocytogenes* serotype 4b strains and eight serotype 1/2a strains from different sources were used in the study (Table 1). The bacterial cultures were prepared as described by Pan et al. (2006), using tryptic soy agar supplemented with yeast extract (TSA-YE) or tryptic soy broth supplemented with yeast extract (TSB-YE) (Difco Laboratories, Detroit, MI). Overnight cultures were diluted (1:100) in TSB-YE and re-incubated either at 37^{o}C for 12 h or at 30^{o}C for 18 h to prepare cells for biofilm formation as described below. Each culture was harvested and washed with sterile saline (0.85% NaCl) by centrifugation (3,500 × g for 10 min at 10°C) and resuspended in saline or in TSB-YE

diluted ten-fold with sterile water (TSB-YE/10). The cell density of each strain was adjusted to OD (Optical Density)$_{600}$ = 0.5 (Ca. 10^8 CFU/ml). An equal volume of each cell suspension for each serotype was pooled to make the cocktails of serotype 4b or 1/2a cells (strain SK1387 was used separately) as described below.

Biofilm formation of individual strains. The biofilm formation capacity of each strain was determined using a microtiter plate assay as described previously (Djordjevic et al., 2002) with a few modifications. Briefly, 200 µl of each overnight culture diluted with TSBYE (1:50) was transferred into 96-well microplates (Nunclon™ Delta, Cat. No. 163320; Denmark). Replicate plates were sealed with parafilm and incubated statically for 40 h at 22.5°C, 30°C, and 37°C, respectively. Upon completion of incubation, the well contents were removed and the wells were rinsed three times with sterile purified water to remove unattached bacterial cells. The wells were filled with 200 µl of 0.8% crystal violet (CV; Acros Organics, NJ) and kept for 15 min at room temperature, followed by being flushed with tap water and air dried for 30 min in a Biological Safety Cabinet (Class II type A2). Both the medium used for biofilm formation and CV solution were filtered using 0.22 µm pore size filters prior to use. The air dried wells were filled with 200 µl of 95% ethanol for 15 min with shaking (100 rpm) to solubilize the dye. To determine the CV density in microplate wells, 100 µl from each well was transferred into a new 96-well flat-bottom microplate (BD Falcon™, Cat. No. 353075) and OD_{580} of the well content was assayed in a 96-well microplate reader (TECAN Safire, Austria) with the software Magellan 6.5 (TECAN, Austria). The mean OD_{580} of the wells containing sterile media only was used as a blank measurement to adjust the OD_{580} of all other wells.

Cell attachment, biofilm formation and treatment in SFP regime. The procedure for cell attachment and biofilm formation was as described previously (Pan et al., 2006), with minor modifications. All cultures were incubated at 30oC. The cell suspensions were added (230 ml/box) into sterile Rainin pipette tip boxes (without the tip holding grid) in which the stainless steel coupons were positioned separately by ridges on side walls of the box. Biofilms were prepared on stainless steel coupons (T-304L, no. 4 finish; 780mm by 25mm by 1.2mm) using the 1/2a and 4b cocktails separately (230 ml/box of each cocktail), and mixed using equal volumes of each cocktail (115 ml/box of each cocktail). Similarly, biofilms were prepared from the 1/2a strain SK1387 singly, and in combination with the 4b cocktail. Following 3 h of incubation at 30^{o}C, the cell suspension was removed from the boxes and the coupons were rinsed three times with sterile saline to remove loosely attached cells. Sterile boxes were then filled with TSB-YE/10 and placed at 22.5°C for 7 days. The TSB-YE/10 was replaced every 24 h. The coupons were subjected to a 24 h cycle with three steps: 1) sanitation with a hydrogen peroxide-based sanitizer (MatrixxTM, 100 ppm, pH 3.8; Ecolab) followed by neutralization using 0.1% sodium thiosulfate-phosphate solution (pH 7.0) and rinsing at room temperature; 2) dehydration (no aqueous medium) for 15 h at 22.5°C; and 3) incubation with TSB-YE/10 for 8 h at 22.5°C. Sample coupons (4 replicates/box) were removed at the indicated times to determine viable cell density (mean ± STDEV log CFU/cm^2) at the start of the SFP regimen, and at 14 and 28 days of continuous SFP cycles.

Measurement of viable cell density of strains with the same serotype in single or mixed biofilms in the SFP regime. To determine the viable cell density of bacteria in pure

and mixed serotype biofilms, cells were detached from the surface of the stainless steel coupons using sterile cotton-tipped swabs as described (Pan et al., 2006). A designated area (55 mm by 25 mm) on the surface of each coupon was swabbed with six separate swabs to remove cells from the surface. The swab tips from each sample were placed in a plastic screw-cap tube (50 ml; Corning) containing 20 ml of sterile saline and 6-8 solid glass beads (4 mm in diameter). The bead-cell mixture was vortexed for 60 s. Total viable cell density of each sample was enumerated by plating as described previously (Pan et al., 2006). The viable cell density of serotype 1/2a and 4b bacteria was analyzed by quantitative real-time PCR with serotype specific primers (Table 2). To prevent DNA amplification from dead cells or cell free DNA, the cell suspension was treated with propidium monoazide (PMA) prior to DNA isolation as described by Pan and Breidt (2007). Ten milliliters of cell suspension from each sample was concentrated by centrifugation (3,500 × g for 10 min at 10°C) and cells were resuspended in 490 µl of sterile saline. PMA was added (10 ul of a 2.5 mM solution) and exposed to a 650-W light prior to DNA extraction (Pan and Briedt, 2007). DNA was extracted using a DNeasy Blood & Tissue Kit, following the procedure recommended for bacteria by the supplier (#69506; QIAGEN Sciences, MD). Each PCR reaction mixture (25 µl) was composed of the primers (300 nM, Table 2), probe (250 nM, Table 2), 6 µl of template DNA, and 12.5 µl of 2× iQ Supermix (#170-8862; Bio-Rad Laboratories, CA). The PCR reaction consisted of 94°C for 3 min, followed by 35 cycles of 94°C for 20 s, 58°C for 30 s, and 72°C for 25 s and then a final step of 72°C for 10 min., using a quantitative PCR machine (iCycler, BioRad Laboratories). The relationship between threshold cycle times (using the mean of duplicates for each sample) and CFU/ml was determined using a standard

curve with 10^3 CFU/ml to 10^8 CFU/ml live cells spiked with dead cells (up to 10^8 cells/ml; data not shown) as described (2007).

To determine the cell density of serotype 1/2a and 4b strains in the medium following 8 h incubation in TSB-YE/10 in each cycle of the SFP treatment, aliquots of cell suspension in the box for serotype-mix biofilms were sampled. The total cell density was determined by plating and PMA-qPCR analysis was employed to determine the concentration of bacterial cells of each serotype as described above.

Attachment and motility. The culture of each strain was prepared in TSB-YE at 30°C for 18 h and 37°C for 12 h. Cells were harvested by centrifugation (3,500 × g for 10 min at 10°C), re-suspended in fresh TSB-YE/10, and adjusted to OD_{600} = 0.5 (Ca. 10^8 cells/ml). Multi-strain mixtures of each serotype were prepared as described above. Cell suspensions of serotype 1/2a and 4b cultures grown at 37°C and at 30°C were added (230 ml) into the Rainin pipette tip boxes containing stainless steel coupons. Following 3 h incubation at 30°C, the cell suspension was removed from the boxes and the coupons were rinsed with saline three times. Six replicate stainless steel coupons were used for each cell suspension. The cell density of each suspension was determined by plating. Cell motility in each suspension was determined by visual inspection under a microscope. Attached cells were enumerated using 6 replicate coupons from each mixture as described above. To determine motility of individual *L. monocytogenes* strains, stationary phase cell suspensions were inoculated into 5 ml of TSBYE with 0.3% agar in a 10 ml glass tube, by stabbing down through the center of the agar cylinder with an inoculation needle. The tubes were incubated at 30°C and 37°C for 48 h, respectively, and motility determined by visual inspection of the growth of cells

surrounding the stab. Growth of non-motile cells was evident only on along the stab, while motile cells spread through the soft agar.

Statistical analysis. The cell densities (log_{10} CFU/cm^2) of seven biofilm treatments (serotype 1/2a, 4b in single and mixed serotype biofilms, strain SK1387 in pure culture biofilms, and 4b and SK1387 in their mixed biofilms) were measured at three time points (day 0, 14 and 28) in the SFP regime. The experiment was repeated three times. All data were statistically analyzed using the General Linear Models Procedure of SAS version 9.1 (SAS Inc., Cary, NC). The significant differences among the treatment means were computed by the Turkey's method.

RESULTS

Cell motility and initial attachment. To optimize the conditions for biofilm formation, each of the sixteen strains was examined for motility and attachment to stainless steel coupons when grown at 30°C and 37°C. As expected, the motility of all strains was suppressed at 37°C regardless of serotype, while at 30°C motility was evident. Cell cocktails with eight strains of each serotype (~ 5×10^8 CFU/ml total) were prepared from cells grown at 30°C and 37°C and examined for attachment to stainless steel coupons at 30°C. The attached cell densities of the serotype 1/2a group grown at 30°C and 37°C were 5.1 (± 0.35) log CFU/cm^2 and 5.5 (± 0.42) log CFU/cm^2, respectively. Similarly, the attached cell density of the serotype 4b group was 5.2 (± 0.12) log CFU/cm^2 and 5.5 (± 0.22) log CFU/cm^2 for the cultures grown at 30°C and 37°C, respectively. These data show that there was no significant

difference in cell densities between the serotype 1/2a vs. 4b strains on stainless steel surfaces ($P > 0.05$) or for a given serotype with cells prepared at 30°C or 37°C ($P > 0.05$).

Biofilm formation of single strains. Biofilm density was determined for each strain using the microtiter plate assay at 22.5°C, 30°C, and 37°C. A direct correlation was observed between increasing temperature and increasing density, which is in accordance with the results of Di Bonaventura et al. (2008). Strain SK600 formed the highest density biofilm at 37°C as measured by the optical density of the extracted CV dye, whereas strain SK1387 formed the highest density biofilm at 30°C. This strain was therefore selected for use in mixed culture biofilm experiments (below) which were carried out at 30°C (Figure 1).

Mixed culture biofilms with 1/2a and 4b strain cocktails. Following cell growth and attachment to stainless steel coupons at 30°C and subsequent biofilm formation at 22.5°C, the mixture of the seven serotype 1/2a strains formed higher density biofilms on stainless steel compared to the serotype 4b strain mixture ($P < 0.05$). The serotype 1/2a strain mixture had a mean cell density of 6.07 (± 0.45) log CFU/cm^2 vs. 5.40 (± 0.47) log CFU/cm^2 for the 4b strain mixture (Figure 2, day 0 of the SFP regimen). After 14 days of the SFP regimen, all cell densities in the biofilms were reduced, although the cell density of the serotype 1/2a group remained higher than the serotype 4b group (4.03 ± 0.6 log CFU/cm^2 vs. 3.34 ± 0.33 log CFU/cm^2). The observed differences in biofilm formation between the serotype 1/2a and 4b strain mixtures were small (ca. 0.6 log) but were consistently observed and statistically significant ($P < 0.05$). By the end of the SFP regimen at 28 days, cell numbers for strain mixtures increased, with cell densities of 5.34 (± 0.25) log CFU/cm^2 for 1/2a strains, and 4.71 (± 0.38) log CFU/cm^2 for 4b strains. It was not possible to determine if individual strains of a

given serotype had grown preferentially. Interestingly, the difference between the log of the cell numbers for the two serotype mixtures remained constant (0.6 to 0.7) during the 28 day experiment. These results indicated that the 4b strains experienced the same trend as that of the 1/2a strains in mixed biofilms during the 28 day SFP regimen. Similar results were observed for the release of biofilm cells into the TSB-YE/10 during the SFP treatment. After each 8 h incubation period in TSB-YE/10, the cell density of each serotype mixture in the liquid media was determined (Figure 3). The cell density of serotype 1/2a strains was consistently higher (0.3~0.6 log CFU/ml) than the serotype 4b strains during the 28 day experiment.

Mixed culture biofilms with serotype 4b strains and the serotype 1/2a strain SK1387. *L. monocytogenes* strain SK1387 (serotype 1/2a) was found to form the most biofilm mass in pure culture at 30^{o}C among the strains used in this study (Figure 1). Biofilms of the serotype 1/2a strain cocktail (excluding SK1387), strain SK1387 alone, strain SK1387 with the serotype 4b strain cocktail, and the serotype 4b strain cocktail alone were prepared and subjected to the SFP regimen. The results are shown in Figure 4. The initial biofilm densities for the 1/2a cocktail (excluding SK1387), SK1387 with the 4b cocktail, and SK1387 alone were 6.2 (± 0.19) log CFU/gm^2, 6.9 (± 0.45) log CFU/gm^2, 7.2 (± 0.3) log CFU/gm^2, respectively. However, after 14 or 28 days, there was no significant difference in biofilm cell density ($P > 0.05$) among the 1/2a cocktail (1/2a), strain SK1387 (SK1387), and SK1387 in mixed biofilms with the 4b cocktail (SK1387 in mix) (Figure 4A). The presence of 4b strains (4b cocktail) in mixed biofilms with strain SK1387 or 1/2a cocktail (excluding SK1387) did not affect the biofilm density when compared to SK1387 alone or the 1/2a

cocktail alone. The were no significant differences in biofilm density among the 1/2a cocktail and strain SK1387 irrespective of the presence or absence of the 4b cocktail after 14 days in the SFP system. Interestingly, the biofilm density of the 4b strains was initially greater when biofilms were prepared using the mixture with strain SK1387 compared to the 4b cocktail alone ($P < 0.05$; Figure 4B, day 0). Similarly, the biofilm density of the serotype 4b strain cocktail with SK1387 was significantly higher than without SK1387 after 28 days of the SFP regimen ($P < 0.05$; Figure 4B, day 28).

DISCUSSION

Research on *L. monocytogenes* biofilms has focused on attachment and biofilm formation with strains of different serotypes, but consistent trends have not emerged. The inconsistent results could be due to differences in strains, media and environmental conditions during biofilm formation (Stewart and Franklin, 2008). It is interesting to investigate the ability of serotype 1/2a and 4 b strains to form biofilms because serotype 1/2a strains are frequently isolated from food processing environments but serotype 4b strains are commonly implicated in foodborne outbreaks of listeriosis (Chemaly et al., 2008; Tresse et al., 2007; Guerini et al., 2007b; Gilbreth et al., 2005; Revazishvili et al., 2004; Aarnisalo et al., 2003; Gilot et al, 1996; Wallace et al., 2003; Lukinmaa et al., 2003**;** Kathariou et al., 2006; Mead et al., 2006)

The formation of a biofilm has been considered as a stress response of bacterial cells to defense against unfavorable conditions so to withstand starvation, changes of osmosis and pH, oxygen radicals, disinfectants and antibiotics (Jefferson, 2004). General experimental

conditions utilized for biofilm research in a laboratory are quite different from that outside of the laboratory in which bacteria survive and grow in a less-than-idea environment. Further, bacteria generally exist in a multi species community in environment other than a pure culture in the laboratory. The data from experiments using pure culture in a full strength condition may not reflect the behavior of bacteria existing in other than laboratory environment.

We used the SFP system that consists of several stresses to determine if *L. monocytogenes* strains of serotype 1/2a or 4b would have a selective advantage in the presence of environmental stresses in pure and mixed culture biofilms. The use of quantitative PCR with serotype specific primers and PMA treatment made it possible to selectively enumerate viable cells of each serotype in mixed culture biofilms. Our results confirm the observations of Folsom et al. (2006) that 1/2a strains form higher density biofilms than 4b strains in diluted medium. The cell counts of each serotype released to the media from biofilms during the growth phase of the SPF system paralleled the cell counts in the biofilms, suggesting the more cells in a biofilm, the more cells could release to the environment. These data suggest that 1/2a strains may have a competitive advantage over 4b strains due to the ability of 1/2a strains to form higher density biofilms once attached to stainless steel surfaces. Once biofilms were formed, however, there was no evidence for the inhibition of growth due to competition between strains of the two serotypes in mixed culture biofilms. Both 1/2a strains and strain SK1387 formed and maintained similar density biofilms regardless of whether they were in the presence or absence of 4b strains at each time point throughout the SFP system. Given that serotype 1/2a strains and serotype 4b strains

exhibited equal capability for initial attachment, the different biofilm densities of the two serotypes might be attributed to increased production of extracellular polymeric material or differences in the growth rates of attached cells. No difference in the growth rates of serotype 1/2a strains and serotype 4b strains was observed for planktonic cells by Gorski et al., (2006). The average growth rate of the 4b strains were even little higher than were the 1/2a strains used in this study (separate publication). Surprisingly, we found that the mixed culture 4b strains formed higher density biofilms in the presence of the serotype 1/2a strain SK1387 in mixed culture biofilms. This may indicate that the extracellular matrix produced by strain SK1387 conferred greater protection from the stresses, allowing better growth of the serotype 4b cells in these mixed culture biofilms.

Motility has been demonstrated to be essential for initial cell attachment and biofilm formation of several bacteria, e.g., *E. coli* (Pratt and Kolter**,** 1998), *Campylobacter jejuni* (Kalmokoff et al., 2006), *Vibrio cholerae* (Watnick et al., 2001), and *Yersinia enterocolitica* (Kim et al., 2008). On the other hand, some studies show that flagella were not necessary for initial attachment or biofilm formation of *Pseudomonas aeruginosa* (Klausen et al, 2003) and *P. fluorescens* (Robleto et al., 2003). It has been observed that *L. monocytogenes* does not generate flagella and are non-motile at 37^{o}C, but motile at 30^{o}C and lower temperatures, although there is variation from strain to strain (Peel et al., 1988; Way et al., 2004). The relationship between the presence of flagella and attachment and biofilm formation has been studied (Todhanakasem and Young, 2008; Gorski et al., 2003; Lemon et al., 2007; Djordjevic et al., 2002; Di Bonaventura et al., 2008; Vatanyoopaisarn et al., 2000). Mutagenesis studies revealed that flagellum-mediated motility was essential for initial attachment (Todhanakasem

and Young, 2008; Gorski et al., 2003; Lemon et al., 2007; Vatanyoopaisarn et al., 2000), although other studies showed that motility was not required for attachment (Djordjevic et al., 2002; Di Bonaventura et al., 2008). We found no significant difference in attachment to stainless steel for serotype 1/2a and 4b strains regardless of growth temperature (30 vs 37C). These data suggest that flagella and/or flagella associated motility of *L. monocytogenes* may not be a critical factor in initial attachment for biofilm formation. However, even though motility was not detected at 37^{o}C, there may still be some expression of motility-associated genes at this temperature, as the studies with flagellin mutants suggest (Lemon et al., 2007; Vatanyoopaisarn et al., 2000).

The use of PMA prior to DNA isolation enabled us to enumerate viable cells in biofilms by quantitative PCR. Using this method we were able to enumerate cells of a specific serotype detached from the SFP treated biofilm coupons. The resulting data from these experiments and data from a related study (Pan et al., 2006) support the hypothesis that biofilms may provide a shield and control adaptation and survival of *L. monocytogenes* on stainless steel surfaces in the presence of environmental stresses. Strain SK1387 was first isolated from a sporadic outbreak associated with the consumption of contaminated turkey franks in 1988, and then the same genotype 1/2a strains were isolated from several cases in the following ten more years, suggesting strain SK1387 is a persistent strain in turkey deli meat processing facilities (Kathariou, 2003). Out data suggest that the distinctive capacity in biofilm formation of strain SK1387 at low temperature may contribute to the persistence of this strain in deli meat processing environment. The enhanced growth seen with the 4b cocktail in the presence of strain SK1387 may be attributed to the benefit provided by strain SK1387, which

supports the hypothesis that selfless behavior may occur in a community mode of growth as in biofilms (Jefferson, 2004). The biochemical composition of the extracellular matrix of SK1387 and other 1/2a and 4b strains of *L. monocytogenes* is the subject of future investigation. The methods used in this manuscript may also be applied for the study of other mixed culture biofilms, if appropriate primers are available.

Table 3 .1 *Listeria monocytogenes* strains used in the study

Strain ID[a]	Serotype	Persistence[b]	Source[c]	Reference / other IDs
SK1450	4b	N/A	Hot dog outbreak, 1998-1999.	(Kathariou et al, 2006) / H7550
SK1403	4b	N/A	Food, USA (California outbreak), 1985.	(Nelson et al, 2004) / F2365, G3990
2140	4b	NP	2001.	(Borucki et al., 2003);
SK1495	4b	NP	Turkey processing environment, 2003.	(Eifert et al, 2005) / L0315
SK1277	4b	NP	Turkey processing environment, 2003.	(Eifert et al, 2005) / 82-2a
M35402A	4b	NP	Bulk milk, 2001.	(Borucki et al., 2003)
M33027A	4b	NP	Bulk milk, 2001.	(Borucki et al., 2003)
SK1463	4b	P	Turkey processing environment, 2002.	(Kathariou et al, 2006) / J1815
SK1387	1/2a	P	Food (Frankfurter), 1988.	(Nelson et al, 2004) / G3965, F6854
SK90	1/2a	P	Turkey processing environment, 2004.	(Kim et al, 2008) / 90
SK1637	1/2a	NP	Turkey processing environment, 2005.	(Kim et al, 2008) / 1637
SK600	1/2a	P	Turkey processing environment, 2004.	(Mullapudi et al, 2008) / 600
M39503A	1/2a	P	Bulk milk. 2001.	(Borucki et al., 2003)
SK754	1/2a	NP	Turkey processing environment, 2004.	(Mullapudi et al, 2008) / 754
SK2642	1/2a	P	Turkey processing environment, 2006.	(Mullapudi et al, 2008) / 2642
SK2508	1/2a	NP	Turkey processing environment, 2004.	(Kim et al, 2008) / 2508

a. The strains which IDs begin with SK are culture collection of S. Kathariou; others are culture collection of D. Call's lab at the Department of Veterinary Microbiology and Pathology, Washington State University, Pullman, Washington 99164.

b. The strains were designated as persistent (P) or non-persistent (NP) based on the frequency of the same PFGE patterns of the isolates from the same source. Others are not available (N/A). Strains with the same source might be isolated from different facilities.

Table 3.2 Oligonucleotides used in this study

Serotype	Gene	Oligonucleotide	Target fragment size	Reference
4b	ORF0799F ORF0799R Probe	5'-GCTGGGTTTCTTACGA-3' 5'-CAACCGTTCATTTAGCTCAT-3' FAM-TCTGCTGTTCAGTTATTGGAGTGGGA-BHQ-1	83bp	Doumith et al., Infect. Immun. 72:1072-1083.2004
1/2a	lmo0737F lmo0737R Probe	5'-GCGGATGTGATTGATTTAC-3' 5'-AAACTGCACTAACTCTTGAAT-3' FAM-TGCTCCAGGATCAAGACACGGTA-BHQ-1	78bp	Doumith et al., J. Clin. Microbiol. 42: 3819-3822.2004

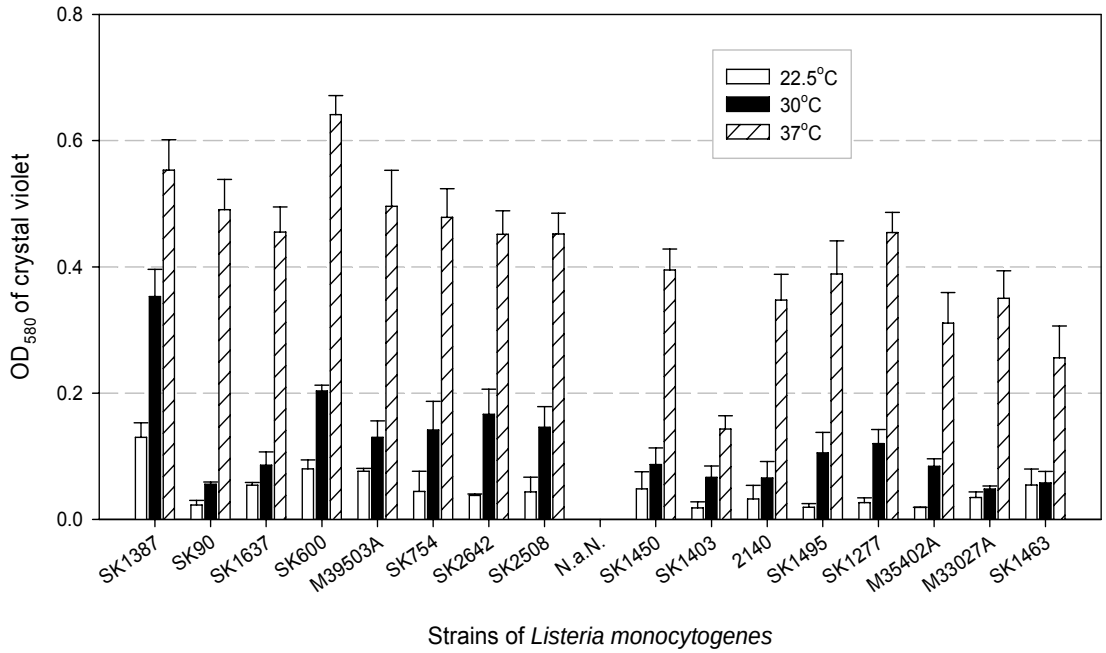

Figure 3.1. Biofilm production of strains of *L. monocytogenes* using microtiter plate assay. The bars are presented as the mean of triplicate replications, and error bars represent the standard deviation.

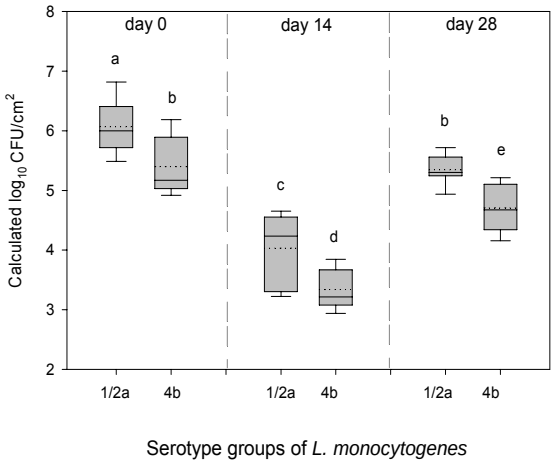

Figure 3.2. Calculated cell density of *Listeria monocytogenes* serotype 1/2a and serotype 4b strains in mixed biofilms on stainless steel in a simulated food processing system.

Each box represents the range of cell density of the indicated serotype from twelve replicate samples. The boundary of each box closest to zero indicates the 25th percentile; a solid line and a dotted line within a box mark the median and the mean, respectively (n = 12); and the boundary of the box farthest from zero indicates the 75th percentile. The error bars above and below the box indicate the 95th and 5th percentile. Boxes labeled with same letters on the top have no significant difference ($P > 0.05$).

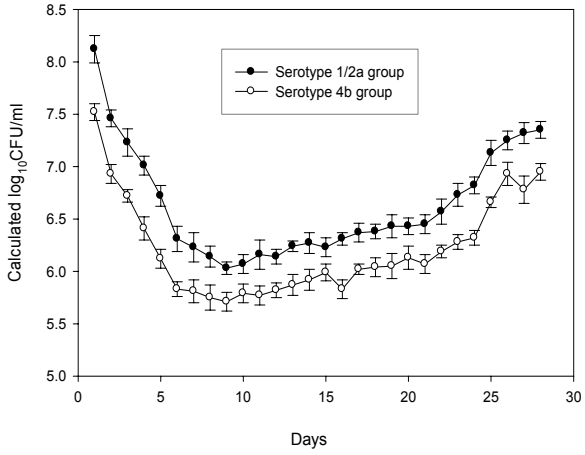

Figure 3.3. Planktonic cell density of *L. monocytogenes* serotype 1/2a and serotype 4b strains in diluted media (TSB-YE/10).

The filled symbols represent 1/2a strains, the open symbols represent 4b strains. The error bars represent the mean and standard deviation (n = 3).

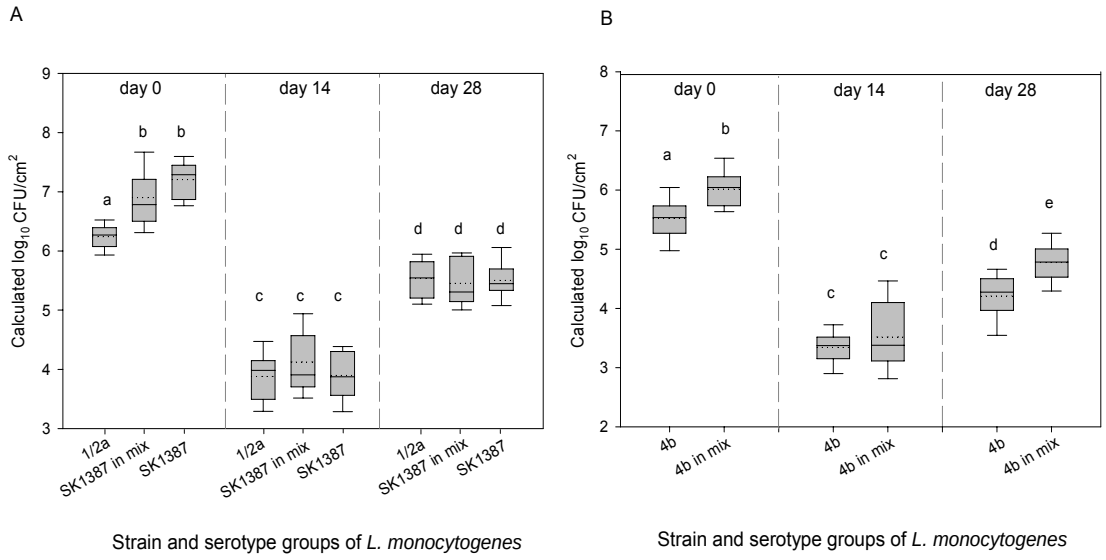

Figure 3.4. Calculated cell density of *Listeria monocytogenes* in biofilms on stainless steel in a simulated food processing system.

Serotype 1/2a cocktail (1/2a) (excluding SK 1387), strain SK1387 in the presence (SK1387 in mix) or absence (SK1387) of 4b cocktail are grouped in panel A. Serotype 4b cocktail along with (4b in mix) strain SK1387 or absence of strain SK 1387 (4b) are shown in panel B. Each box represents the range of cell density of a certain serotype from twelve replicate samples at a specific time. The boundary of each box closest to zero indicates the 25th percentile; a solid line and a dotted line within a box mark the median and the mean, respectively (n = 12); and the boundary of the box farthest from zero indicates the 75th percentile. The error bars above and below the box indicate the 95th and 5th percentile. Boxes labeled with same letters have no significant difference ($P > 0.05$).

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CHAPTER 4

Listeria monocytogenes serotype 1/2a strains form higher density biofilms than serotype 4b strains under a variety of environmental conditions

ABSTRACT

Biofilm formation in *Listeria monocytogenes* is generally associated with its persistence in food processing environment. Serotype 1/2a strains make up more than 50% of the total isolates recovered from food and environment, while serotype 4b strains are most often associated with major outbreaks of human listeriosis. Using a microplate assay, we examined biofilm formation by 18 strains of each serotype under a variety of conditions, including increased concentration of glucose, sodium chloride and ethanol at different temperatures. The addition of glucose, and salt, and increasing temperature stimulated biofilm formation. A positive synergistic effect on biofilm formation was displayed by these three factors. The serotype 1/2a strains formed higher density biofilms than the 4b strains under different conditions. The growth rate for each strain was measured under different conditions. The 4b strains grew relatively faster than the 1/2a strains, suggesting that growth rate may not be directly related to the capacity to form biofilms. The data suggest that the enhancement in biofilm formation by environmental factors was primarily due to the production of extracellular polymeric substances, instead of accumulating biofilm cells. Enzymes including protease, cellulase and lipase were successfully used to detach viable cells from biofilms. Crystal violet, used in a microplate assay, was found to stain both bacterial cells and biofilm matrix material.

INTRODUCTION

Listeria monocytogenes, a gram-positive bacterium, is capable of causing severe foodborne infections in both humans and animals. The organism is ubiquitous in the environment, and can grow in a wide variety of foods, including those stored at refrigeration temperatures. It is particularly difficult to eliminate this bacterium from ready-to-eat foods and food processing equipment (Kathariou, 2002). The ability to form biofilms protects the bacterium from stresses in food processing environment (Moretro and Langsrud, 2004; Gandhi and Chikindas, 2006). Among the 13 different serotypes described, serotypes 1/2a, 1/2b, and 4b are involved in the majority of human cases of listeriosis. Serotype 4b strains have accounted for most human outbreaks, whereas the majority of *L. monocytogenes* isolated from foods or food-processing plants are serotype 1/2a (Kathariou, 2002).

Comparative studies of bacterial growth of serotypes 1/2a and 4b have revealed variable results. Buncic *et al* (2001) have shown that 1/2a isolates were more resistant to antilisterial bacteriocins than 4b strains at 4^{o}C. They also found that 4b isolates exhibited more resistance to heat treatment at 60^{o}C, and were easier to recover than 1/2a strains immediately following cold storage. Bruhn *et al* (2005) observed that 1/2a strains (lineage 2) grew faster than 4b and 1/2b (lineage 1) strains in a commonly used enrichment broth media (University of Vermont medium I and II). Other studies have indicated that similar differences could not be linked to serotype (Gorski *et al*., 2006).

Some *L. monocytogenes* strains have consistently been isolated from food processing plants over many years (Norwood and Gilmour, 1999; Borucki et al., 2003). Although

several studies have been carried out to identify differences in cell adherence and biofilm formation among different serotypes, conflicting results were obtained from different labs. Lineage I isolates (including serotypes 4b, 1/2b, 3c, and 3b) were found to produce biofilms with higher density than lineage II isolates (including serotypes 1/2a, 1/2c, and 3a) (Djordjevic et al., 2002; Norwood and Gilmour, 1999). However, this conclusion was not supported by other studies (Borucki et al. 2003; Di Bonaventura et al., 2008; and Kalmokoff et al., 2001). For serotype 4b strains, the capacity to form biofilms was reduced when nutrient level in a medium decreased, while serotype 1/2a strains maintained their capacity in biofilm formation (Folsom et al., 2006).

It has been suggested that the formation of a biofilm is a stress response by bacterial cells (Jefferson, 2004). The findings from biofilm research under laboratory conditions may not reflect the traits of biofilms in the environment. In order to unveil the behavior of *Listeria monocytogenes* in bioiflms in the environment, a simulated food processing (SFP) system including several stresses was designed (Pan et al., 2006). The SFP system was used for a study of 1/2a and 4b strain in mixed culture biofilms (Chapter 3). The bacterial cells of the 1/2a cocktail were predominant over the 4b strains when exposed to the SFP system for four weeks, but no competitive inhibition was observed (Chapter 3). The objective of this study was to investigate and compare biofilm formation by *L. monocytogenes* serotype 1/2a strains and serotype 4b strains under a variety of environmental conditions including different temperatures, varying concentrations of salt, sugar, and ethanol, which are common in foods and the food processing environment.

MATERIALS AND METHODS

Strains and growth conditions. Eighteen *L. monocytogenes* serotype 1/2a strains and 18 serotype 4b isolates from diverse sources were used in the study (Table 1.). The methods for storage and preparation of strains were described previously (Pan et al., 2006). Briefly, each strain was transferred from a frozen stock (- 80°C) onto a plate of tryptic soy agar containing 0.6% yeast extract (TSAYE; BD Biosciences) and incubated at 37°C for 20 to 24 h. One or two typical colonies from the recovery plate were inoculated into 8 ml of tryptic soy broth containing 0.6% yeast extract (TSBYE) and incubated statically for 8 h at 30^{o}C to generate late exponential stage cultures.

Microplate assays. The capacity of each strain to form biofilms was measured by microplate assay as described previously (Chapter 3). Four microliters of each late exponential stage culture (OD_{600} = 0.5) was diluted 1:50 with of TSBYE (196 µl) supplemented with either glucose (1.0%, 5.0%, and 10.0%), sodium chloride (2.0%, 5.0%, and 7%), or ethanol (1.0%, 2.0%, 3.0%, and 5.0%) in a 96-well microplate (Cat. No. 163320; Nunclon^{TM} Delta, Denmark). The plates were incubated statically for 40 h at 22.5^{o}C, 30 ^{o}C, and 40 ^{o}C. The planktonic cell suspension in each well was removed and the wells were rinsed twice with sterile water, followed by staining with 0.8% crystal violet solution (CV, Acros Organics, NJ) (200 µl/well) for 15 min at room temperature. The microplates were flushed with tap water five times and air dried. The dye bound to the biofilms in each well was resolubilized with 95% of ethanol (200 µl/well) for 15 min at room temperature. The ethanol-dye solution (100 µl/well) was transferred to an second microtiter plate, and the

absorbance of the each well was measured at 580 nm (A_{580}) using a microplate reader (TECAN Safire, Austria) with Magellan version 6.5 software (TECAN, Austria). The mean absorbance at 580 nm from the original wells containing only media was subtracted from the other wells. The absorbance for each serotype strains was represented as mean ± STDEV (standard deviation; n = 18).

Factors affecting biofilm formation. Biofilm formation was analyzed using the microplate assay with various concentrations of sodium chloride (2 to 7%, w/v), glucose (1 to 10%, w/v), and ethanol (1 to 5%, v/v). To determine the effect of temperature on biofilm formation, triplicate microplates were prepared and incubated at 22.5^{o}C, 30^{o}C, and 37^{o}C for 40 h, respectively. To determine the synergistic effects of glucose, salt and temperature on biofilm formation, bacteria cells of each strain were grown in TSBYE containing additional glucose or sodium chloride or both at their optimal concentrations for biofilm formation at 22.5^{o}C, 30^{o}C, and 37^{o}C for 40 h, respectively.

Growth rate analysis. The growth rate of each strain was determined using microplate assay. The culture preparation was carried out as described above. Following inoculation of the bacterial cells into microplate wells at approximately 1×10^{7} CFU/ml (200 µl/well of final volume) from late exponential phase cells, the optical density in each well was monitored at 600nm by periodic measurements using a 96-well microplate reader. To determine the growth rate, the slope of the linear part ($R^2 > 0.98$) for at least five data points of the semilogarithmic plot of optical density [$Ln(OD_{600nm})$] *vs*. incubation time (hour) was

calculated. The maximum growth rate of each serotype strains was expressed as mean ± STDEV (standard deviation) h^{-1} (n = 18).

Biofilm cell detachment. Strain SK1387, selected for this assay because of it's superior ability to form biofilms, was used to form biofilms in TSBYE or $TSBYE_{gluc1\%+NaCl2\%}$ in six replicate microplate wells at different temperatures as stated above. Following 40 h of incubation, the medium was removed and the wells were washed twice with saline. Biofilms in three replicate wells were detached by swabbing, followed by dilution and plating as described previously (Pan et al., 2006). The remaining three replicate wells with intact biofilms were filled with 200 µl of a mixed enzyme solution containing lipase (100 U/ml; Cat. No. 62285, Sigma-Aldrich, Switzerland) and cellulase (100 U/ml; Cat. No. C0615, Sigma-Aldrich, Japan) in Tris-HCl buffer (20mM, pH 7.8) with 150 mM NaCl, 1 mM $CaCl_2$ and 2 mM $MgCl_2$. The wells were incubated at 37°C for 30 min followed by the addition of 10 µl /well of protease K stock solution (2000 µg/ml; Cat. No. 19131, QIAGEN, CA) with continuing incubation for 30 min at 37°C. The well contents were diluted and plated to determine biofilm cell density as described (Pan et al., 2006). Bacterial cell counts were determined by the swabbing method, and plotted against the logarithmic values of cell counts by enzymatic treatment from corresponding replicate wells. The relationship between the data from the two methods was analyzed using linear regression.

CV staining of fresh bacterial cells. A six-strain cocktail prepared as described above was washed twice by centrifugation at 3,500 × g for 10 min at 10°C, and resuspended in saline (8.5 g/liter of NaCl). The cell suspension was then serially diluted with saline. The cell

count was determined by plating on TSAYE. One milliliter of cell suspension from each dilution was filtered using a 13 mm syringe filter (0.2 μm; Cat. No. 09-720-5, Fisher, Ireland). The filter with bacterial cells was stained with CV solution for 15 min at room temperature. The stained filters were then flushed with deionized water until the filtrate was clear, followed by drying in air overnight in a biosafety cabinet. Five milliliters of 95% ethanol was used to solubilize CV bound to the filter and bacterial cells. The absorbance at 580nm (A_{580nm}) for 100 μl of ethanol containing destained CV from each filter was assayed using the 96-well microplate reader. The A_{580nm} value from the filter without bacterial cells was used as a blank to subtract the A_{580nm} values for the other filters. The relationship between cell mass (CFU) and adjusted A_{580nm} value for CV was analyzed using linear regression.

Statistics and reproducibility of results. At least three replicates for each of three independent repeats were performed for each experiment. The data presented are the means of data generated from three independent repeats. The comparisons of multiple means were done using a Student's t-test.

RESULTS

The influence of salt concentration on biofilm production. Serotype 1/2a strains and 4b strains formed similar density biofilms when they were grown in TSBYE with 0.5% salt at 22.5°C for 40h ($P > 0.05$; Figure 1). Salt was found to enhance biofilm formation in growth media (Jensen et al., 2007). We asked whether the two serotype groups were stimulated differently in biofilm formation in response to salt in the medium. As expected, almost all strains had enhanced biofilm formation when the salt concentration was increased from 0.5% to up to 7% at 30°C and below, and up to 2% at 37°C (Figure 1). The optimal salt concentration for biofilm formation was 5% at 22.5°C, and 2% at 30°C and 37°C. Most of the 1/2a strains formed significantly higher density bioiflms than the 4b strains in TSBYE supplemented with 2 to 5 percent of sodium chloride at 22.5°C and 30°C ($P \leq 0.04$). Nearly 90% of the 1/2a strains formed significantly higher density bioiflms than did most 4b strains in $TSBYE_{NaCl\ 2\%}$ at 37°C ($P < 0.005$) with the exception of 4b strains RM2387, RM4504, and RM3013 (Figure 1.3). With few exceptions (in base medium at 22.5°C, and in TSBYE with ≥5% sodium chloride at 37°C), *L. monocytogenes* serotype 1/2a strains normally formed higher density biofilms than serotype 4b strains when the salt concentration was in a range from 2% to 7% at 30°C and below, or below 2% at 37°C.

Influence of glucose concentration on biofilm formation. Compared to the absorbance values for biofilms formed in TSBYE which contained 0.25% glucose, most strains formed higher density biofilms at a high concentration of glucose (1 to 10%) at 22.5°C, 30°C, and 37°C except the 4b strain RM3013 which formed the highest density biofilms in TSBYE at

37°C. At a given temperature, however, each serotype group formed comparable density biofilms ($P > 0.05$), but the serotype 1/2a strains consistently formed higher density biofilms than the serotype 4b strains ($P < 0.05$) at different concentrations of glucose (from 1% to 10%), except in $TSBYE_{gluc10\%}$ at 37°C (Figure 2). Compared to the absorbance values (0.14 ± 0.12 and 0.06 ± 0.03 for 1/2a and 4b, respectively) for the biofilms grown in $TSBYE_{gluc1\%}$ at 22.5 °C, the average absorbance values for both 1/2a (0.26 ± 0.14) and 4b (0.17 ± 0.10) strains were increased more than two fold at 30°C and more than seven folds at 37°C (0.94 ± 0.16 and 0.60 ± 0.20 for 1/2a and 4b, respectively) (Figure 2). Similar to the salt effect, serotype 1/2a strains generally formed higher density biofims than serotype 4b strains in the presence of high percentage of glucose.

Effect of ethanol on biofilm formation. *L. monocytogenes* was only enhanced in biofilm formation by ethanol at 22.5 °C (Figure 3). Approximately 60% (11/18) of the 1/2a strains produced higher density biofilms in the presence of ethanol, whereas only about 20% (4/18) of the 4b strains formed higher density biofilms when exposed to ethanol at 22.5°C (Figure 3 and Figure 3.1). The optimal concentration of ethanol for biofilm formation was 3.0%. In the presence of ethanol in TSBYE, the 1/2a strains consistently formed higher density biofilms than the 4b strains at 22.5°C, 30°C and 37°C ($P \leq 0.01$), unless the ethanol concentration was 5.0% (v/v).

Synergistic effect of salt, glucose and temperature on biofilm formation. The addition of either glucose or sodium chloride in TSBYE stimulated *L. monocytogenes* strains to form higher density biofilms (Figure 1 and 2). The combination of salt and glucose

resulted in higher density biofilms at all three temperatures (Figure 4). The CV absorbance (A_{580nm}) for the biofilms formed by the 1/2a strains was three times more in $TSBYE_{gluc1\% + NaCl2\%}$ (0.38 ± 0.29) than either in $TSBYE_{gluc1\%}$ (0.14 ± 0.13) or in $TSBYE_{NaCl2\%}$ (0.12 ± 0.06) at 22.5°C (Figure 4A). The same trend was observed at 30°C (Figure 4B) and 37°C (Figure 4C). Although the serotype 4b strains were also enhanced in biofilm formation in $TSBYE_{gluc1\% + NaCl2\%}$, their biofilm density was less than that of the serotype 1/2a group ($P < 0.01$). These data suggest that temperature, glucose, and salt have synergistic effects on biofilm formation. The 1/2a strains formed higher density biofilms than the 4b strains in $TSBYE_{gluc1\%+NaCl\ 2\%}$ at all three temperatures.

Growth rates in TSBYE. Since the serotype 1/2a strains generally formed higher density biofilms than the 4b group, we asked whether the growth rate of each serotype was related to the difference in biofilm formation between the two groups. The mean growth rate of the 1/2a strains was 0.42 ± 0.04 h^{-1} in TSBYE at 22.5°C, which was similar to that of the 4b strains (0.45 ± 0.04 h^{-1}) ($P = 0.05$). The addition of glucose in TSBYE stimulated the growth of several 4b strains, making the growth rate of the 4b strains higher than that of the 1/2a strains (0.48 ± 0.06 h^{-1} *vs.* 0.43 ± 0.04 h^{-1}, $P < 0.01$) at 22.5°C (Figure 5A). Increasing salt concentration (from 0.5% to 2%) in TSBYE did not significantly affect the growth of either serotype at all three temperatures, and growth rates were similar to the growth rate of each serotype in $TSBYE_{gluc1\%+NaCl\ 2\%}$ ($P > 0.1$). Unexpectedly, more than 75% of 4b strains had higher growth rates than the 1/2a strains in all conditions ($P < 0.01$) except in TSBYE. The growth rate of all strains was increased by approximately 0.2 h^{-1} on average when the temperature increased from 22.5°C to 30°C in each medium ($P < 0.001$). The average growth

rate of all strains was approximately 0.12 h^{-1} higher at 37°C than at 30°C ($P < 0.001$). A few of the 4b strains including SK1403 (1.31 ± 0.05 h^{-1}), RM2992 (1.33 ± 0.02 h^{-1}), and RM4515 (1.19 ± 0.02 h^{-1}) grew significantly faster than the average for the 4b strains (average rate 0.81 ± 0.1 h^{-1}) in $TSBYE_{gluc1\%}$ at 37°C ($P < 0.001$; Supplement figure S5.3). Interestingly, the slowest growing strain in the 4b strains was RM3013 (0.58 ± 0.04 h^{-1}), although it formed the highest density biofilms in TSBYE at 37°C (Figure S2.3). These data suggest that 4b strains generally grow faster than 1/2a strains and indicate growth rate is not directly related to formation of biofilms.

Time course of biofilm formation. In order to know the progress of biofilm mass and biofilm cells during biofilm formation, a six-strain cocktail was used to form biofilms in microplate wells for 40 h in two media (TSBYE, $TSBYE_{gluc1\%+NaCl\ 2\%}$) at 22.5°C, 30°C and 37°C. To monitor biofilm formation, the absorbance (A_{580nm}) of CV, viable cell density in biofilms, and the viable cell density and pH in the corresponding planktonic cell suspensions were determined at each time point. The CV absorbance values (A_{580nm}) increased during 40 h incubation for biofilm formation for all treatments. The viable cell density in biofilms increased from 4.8 ± 0.05 log_{10}CFU/well at 8 h to 6.3 ± 0.07 log_{10}CFU/well at 40 h in TSBYE at 22.5°C (Figure 6A). Compared to the viable cell density at 22.5°C, higher biofilm cell density was observed at 8h at 30°C (6.4 ± 0.12 log_{10}CFU/well, $P < 0.01$) and increased to 6.9 ± 0.13 log_{10}CFU/well at 40 h in TSBYE (Figure 7A). At 37°C, the biofilm cell density was (6.6 ± 0.07 log_{10}CFU/well) at 8 h and remained at the same density in the following 32 h in TSBYE (Figure 8A). In contrast to the biofilms formed in TSBYE, the biofilm cell density increased from 4.9 ± 0.08 log_{10}CFU/well at 8 h to 6.6 ± 0.1 log_{10}CFU/well at 32 h in

$TSBYE_{gluc1\% + NaCl\ 2\%}$ at 22.5°C and declined afterwards (Figure 6C). The biofilm cell density increased from 6.5 ± 0.1 log_{10}CFU/well at 8 h to 7.2 ± 0.17 log_{10}CFU/well at 16 h in $TSBYE_{gluc1\% + NaCl\ 2\%}$ and then declined to 4.5 ± 0.2 log_{10}CFU/well at 40 h at 30 °C (Figure 7C). A similar trend was found at 37°C (Figure 8C). The viability of planktonic cells and the pH declined faster in $TSBYE_{gluc1\% + NaCl\ 2\%}$ than in TSBYE. In TSBYE, the pH decreased to 5.2 within the first 16 h and then was constant at 22.5°C (Figure 6B), and the planktonic cell density increased from 6.8 ± 0.08 log_{10}CFU/ml to 9.2 ± 0.05 log_{10}CFU/ml in the first 16h (Figure 6B). In $TSBYE_{gluc1\% + NaCl\ 2\%}$, the pH declined from 6.9 at the beginning to 4.3 at the end of incubation. The viable planktonic cell density also decreased after 16 h, and cell numbers were 7.2 ± 0.1 log_{10}CFU/ml at 40 h (Figure 6D). Similar trends were found at 30°C and 37°C, but the pH and planktonic cells declined faster at higher temperature in $TSBYE_{gluc1\% + NaCl\ 2\%}$ (Figure 7B, 7D, 8B, and 8D). With the decrease of pH, the viability of both planktonic cells and biofilm cells declined accordingly. Unexpectedly, the CV values increased while the viable cell counts in the biofilms declined.

Cell counts and crystal violet staining. A fresh culture cocktail of six *L. monocytogenes* strains was prepared to determine the relationship between the absorbance of CV extracted from bacterial cells and the number (CFU/ml) of bacterial cells stained using a filtration method. Since the crystal violet adsorbed onto the filter during cell staining, the adjusted absorbance (A_{580nm}) was close to zero until the amount of the bacterial cells retained on a filter membrane was more than 2.8E+07 CFU. A curve representing the relationship between OD values and corresponding cell counts was plotted and analyzed using linear regression. A linear relationship was found between the cell counts and the corresponding

absorbance values when the cell numbers were in the range from 2.8E + 07 CFU to 4.5E + 08 CFU (Figure 9).

Cell counts from two detachment methods. Protease, lipase and cellulose have been reported to be effective in disrupting *L. monocytogenes* biofilms (Gamble and Muriana, 2007). We asked whether the enzymatic treatment was able to replace the traditional swabbing method used in previous study (Chapter 3). The swabbing method utilized in previous studies (Pan et al., 2006 and Chapter 3) was compared to the enzymatic treatment. A linear relationship ($R^2 = 0.9867$) between the cell counts was obtained from the two methods (Figure 10). When the cell counts were between 10^7 to 10^8 CFU, enzymatic treatment resulted in 10-30% more CFU than swabbing methods, suggesting that part of viable cells may be lost during the swabbing procedure. The enzymatic treatment did not show any antimicrobial effects at their working concentrations when tested with planktonic cells (Appendix). The results indicate that protease, lipase and cellulose are potentially useful for enumerating cells in biofilms, and possibly in controlling and eliminating *Listeria* biofilms in food processing facilities.

DISCUSSION

The objective of this research was to determine if there is a difference in biofilm formation between serotypes 1/2a and 4b of *L. monocytogenes* under a variety of conditions. Previous research by Djordjevic et al (2002) and Borucki et al (2003) has shown the capacity to form biofilms varied among individual strains in each serotype. In the current study, eighteen strains from a variety of sources were examined for each serotype (1/2a and 4b).

Despite the variation among individual strains in each serotype, consistent differences were observed in biofilm formation between the two subpopulations of *L. monocytogenes*. Serotype 1/2a strains generally formed higher density biofilms than serotype 4b strains under a variety of conditions. However, the 4b strains exhibited higher growth rates than the 1/2a strains (Figure 5), suggesting that growth rate may not have a direct effect on bacterial biofilm formation. Djordjevic *et al* (2002) also reported that there was no correlation between growth rate and biofilm formation in modified Welshimer's broth (MWB).

Various extrinsic factors affect cell attachment and subsequent biofilm formation of *L. monocytogenes* (Moretro and Langsrud, 2004). With the increase of temperature from 22.5^{o}C to 37^{o}C, we found bacterial cells generally have greater tendency to attach to surface and form higher density biofilms (Figure 1, 2, and 3), which is commensurate with the findings from previous studies (Di Bonaventura et al., 2008; Moltz and Martin, 2005; Chavant et al., 2002; Briandet et al., 1999). It has been suggested that the increased hydrophobicity at high temperature (e.g. 37 ^{o}C) may enhance the initial cell adherence contributing to a higher biofilm density (Di Bonaventura et al., 2008). Here, it was observed that biofilm mass (indicated as absorbance value of destained CV) increased as viability of corresponding biofilm cells decreased (Figure 6C, 7C and 8C). The biofilm density was greater at 37 ^{o}C than that at 30^{o}C even though the viable cell densities were similar (Figure 7C and 8C). The results suggest that biofilm cells may generate and secret more extracellular polymeric substances (EPS) at 37 ^{o}C than that at 30^{o}C.

Higher density biofilms were observed when bacterial cells were grown in TSB or LB medium with heightened glucose levels (Jensen et al., 2007), which has also been demonstrated in several species in the genus of *Staphylococcus* (Lim et al., 2004; Frank and Patel, 2007; and Dobinsky et al., 2003). In the current study, TSBYE supplemented with a range of glucose concentrations (1% to 10%) was tested, and the 1/2a strains formed higher density biofilms than the 4b strains although the individual strains varied (Figure 1). Growth medium enriched with sodium chloride has also been reported to significantly enhance biofilm formation in *L. monocytogenes* (Jensen et al., 2007) and other species (Lim et al., 2004; Frank and Patel, 2007; Møretrø et al., 2003). Here it was demonstrated that the 1/2a strains formed higher density biofilms than the 4b strains in the presence of sodium chloride (2%~ 5%) at 30^{o}C and below, or 2% of salt at 37^{o}C in TSBYE (Figure 2). Ethanol was an additional factor reported to be able to stimulate *L. monocytogenes* biofilm production at low temperature (Gravesen et al., 2005). In the current study, only a small percentage of the 4b strains responded positively to the presence of ethanol in relation to biofilm formation, compared to most of the 1/2a strains at 22.5^{o}C. Particularly, serotype 1/2a strain SK1387 formed the highest density biofilms in the presence of different concentrations of glucose, salt, and ethanol at low temperature. Strain SK1387 was involved in several sporadic outbreaks taking place in the period of more than ten years, suggesting strain SK1387 is a persistent strain in turkey deli meat processing facilities (Kathariou, 2003). The superior ability of this strain (compared to the other strains tested) to form biofilms at low temperature may contribute to its persistence in food processing plants.

The microplate assay has been widely used in biofilm research due to the convenience, rapidness, simplicity, and the reproducibility of the assay. It is an indirect method for measuring the density of biofilms by measuring the absorbance of destained CV in organic solvents. Time course monitoring for biofilm formation revealed that absorbance values did not correspond to the growth of bacterial cells in biofims. In some cases, the total biofilm mass as measured by CV increased while the viable cell density decreased. Absorbance values for biofilms formed in $TSBYE_{gluc1\% + NaCl\ 2\%}$ were always higher than that grown in TSBYE, suggesting the addition of glucose and sodium chloride stimulated bacterial cells to produce more extracellular matrix material. These data also support previously studies showing that the EPS production is stimulated at 37^{o}C (Di Bonaventura et al., 2008), and that biofilms are largely composed of bacterial cells and relatively little EPS at temperatures of 25^{o}C and below (Rieu et al., 2008). The data also indicate that stresses from starvation, toxic metabolite accumulation, and low pH may provide bacterial cells with extra stimuli to generate EPS.

Moderate levels of salt, sugar, ethanol, and temperature are commonly associated with foods and food processing environment, which may provide a priority niche for serotype 12/a strains over 4b strains to attach to surface and form biofilms on food processing facilities. In our previous competition study using a simulated food processing system (SFP), a seven-1/2a-strain cocktail maintained their dominance over 4b strains in mixed biofilms. Strain SK1387 exhibited a protective effect on an eight strain cocktail of 4b cells in the mixed biofilms in the SFP system. The 1/2a strain biofilms were also found to release more cells into the surrounding environment than the 4b strains (Chapter 3). The data from this study

support the hypothesis that serotype 1/2a strains may inherently form higher density biofilms than serotype 4b strains, and may help to explain the higher percentage of 1/2a isolates from foods and the environment.

Table 4.1 *Listeria monocytogenes* strains used in the study

Serotype 1/2a strains			Serotype 4b strains		
Strain no.	Origin	Reference/other IDs	Strain no.	Origin	Reference/other IDs
SK1387	Food (Frankfurter), 1988.	(Nelson et al, 2004) / G3965/F6854	RM4573	Patient, Canada; 2004.	TS79 (L4738)
SK90	Turkey processing, 2004.	(Kim et al, 2008) / 90	RM 2387	Mint, 2000.	
SK1637	Turkey processing, 2005.	(Kim et al, 2008) / 1637	RM2992	Cucumber, 2002.	2223
SK600*	Turkey processing, 2004.	(Mullapudi et al, 2008) / 600	RM2998	Human, 2002.	2207
M39503A*	Bulk milk. 2001.	(Borucki et al., 2003)	RM3013	Human, 1998.	ATCC19115
SK754	Turkey processing, 2004.	(Mullapudi et al, 2008) / 754	RM4503*	Food, Canada; 2004.	TS9 (L4707)
SK2642	Turkey processing, 2006.	(Mullapudi et al, 2008) / 2642	RM4504	Food, USA; 2004.	TS10 (F8353)
SK2508	Turkey processing, 2004.	(Kim et al, 2008) / 2508	RM 3302	Cow brain, 2002.	CWD874
RM3023	Poultry, England; 1998.	ATCC19111	RM3817	Oyster, 1999.	33007
RM3316	Silage, 2002.	CWD243	RM4515	Food, switzerland; 2004.	TS21 (L4486j)
RM3349	Turkey Frank, 2002.	F6854	SK1450*	Hot dog, 1998-1999.	(Kathariou et al, 2006)
RM3354	Pork plant; 2002.	JL1-6, MFS-1	SK1403	Food, USA, 1985.	F2365, G3990
RM3373	Human, USA; 2002.	1155	2140	2001.	(Borucki et al., 2003);
RM3834*	Cooked cornbeef, 2000.	33034	SK1495*	Turkey processing, 2003.	(Eifert et al, 2005)
RM3835	Sausage, 2000.	33035	SK1277	Turkey processing, 2003.	(Eifert et al, 2005)
RM4527	Patient, England; 2004.	TS33 (L745)	M35402A	Bulk milk, 2001.	(Borucki et al., 2003)
RM4543	Food, USA; 2004.	TS49 (F7273)	M33027A	Bulk milk, 2001.	(Borucki et al., 2003)
RM4561	Patient, USA; 2004.	TS67 (F6953)	SK1463	Turkey processing, 2002.	(Kathariou et al, 2006)

Continued from table 4.1

Comments

- The strains which IDs begin with SK are culture collection of S. Kathariou. The strains which IDs begin with RM are culture collection of L. Gorski at the Produce Safety and Microbiology Research Unit, USDA-ARS, 800 Buchanan st. Albany, CA 94710. Others are culture collection of D. Call's lab at the Department of Veterinary Microbiology and Pathology, Washington State University, Pullman, Washington 99164.
- Strains with the same source might be isolated from different facilities.
- The strains marked with "*" were used for time course monitoring study.

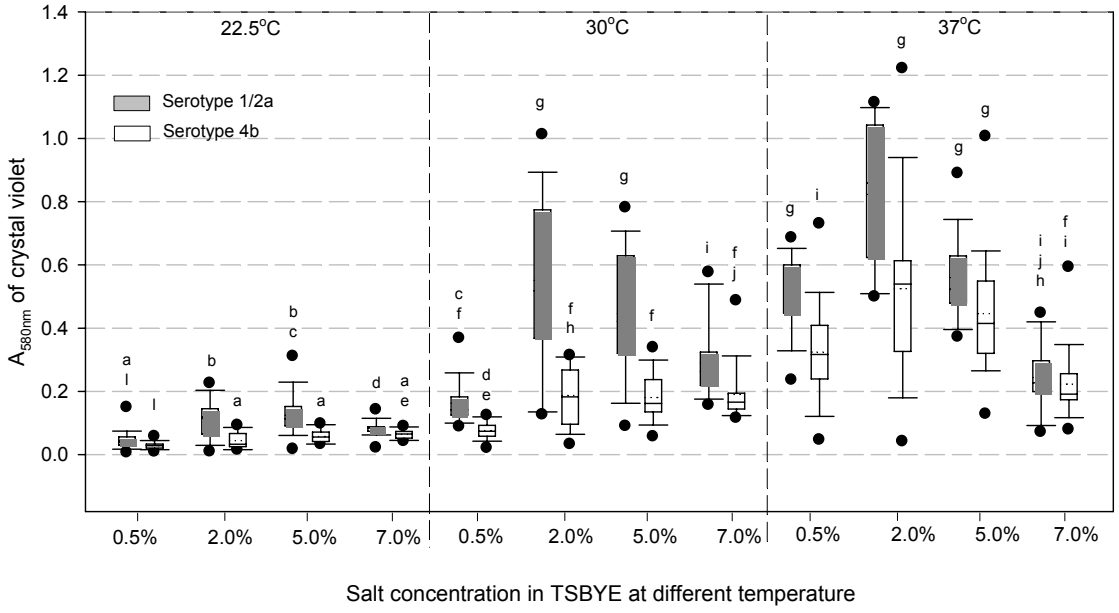

Figure 4.1. Absorbance (A_{580nm}) of crystal violet destained from *Listeria monocytogenes* biofilms.

Absorbance (A_{580nm}) of crystal violet destained from *Listeria monocytogenes* biofilms formed by 18 serotype 1/2a strains and 18 serotype 4b strains in TSBTE containing increased concentration of sodium chloride at different temperature. Each box represents the range of absorbance values for the eighteen strains of indicated serotype. The boundary of each box closest to zero indicates the 25th percentile; a solid line and a dotted line within a box mark the median and the mean, respectively (n = 18); and the boundary of the box farthest from zero indicates the 75th percentile. The error bars above and below the box indicate the 95th and 5th percentile. Solid dots represent the data beyond the 5th and 95th percentiles. Boxes labeled with same letters on the top have no significant difference ($P > 0.05$).

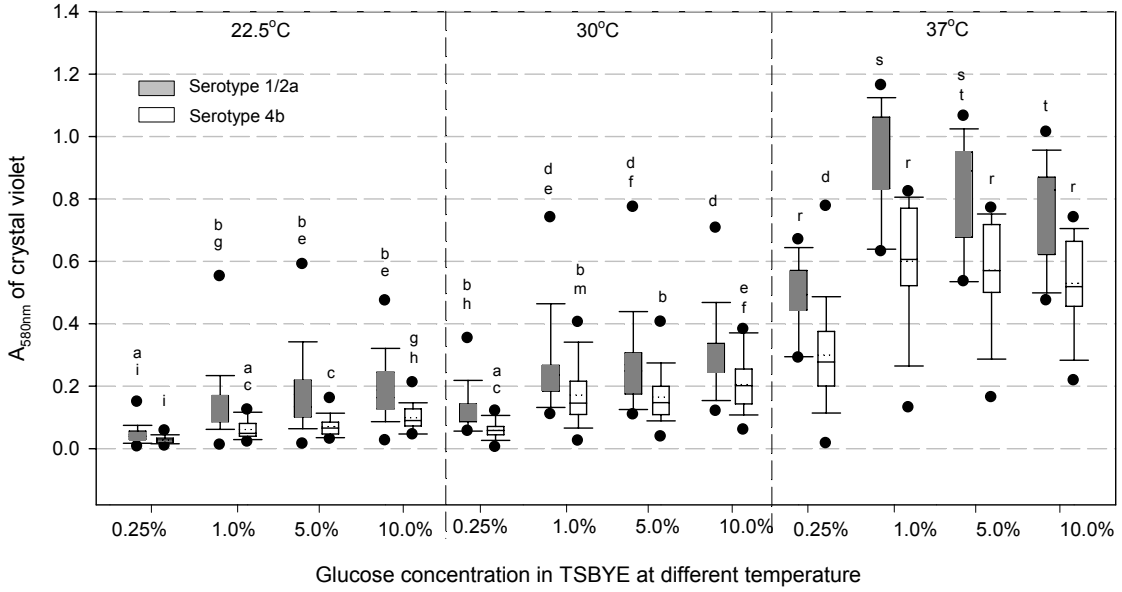

Figure 4.2. Absorbance (A_{580nm}) of crystal violet destained from *Listeria monocytogenes* biofilms in different concentration of glucose.

Absorbance (A_{580nm}) of crystal violet destained from *Listeria monocytogenes* biofilms formed by 18 serotype 1/2a strains and 18 serotype 4b strains in TSBTE containing increased concentration of glucose at different temperature (Data for individuals are in figure 2.1, figure 2.2, and figure 2.3). Each box represents the range of absorbance values for the eighteen strains of indicated serotype. The boundary of each box closest to zero indicates the 25th percentile; a solid line and a dotted line within a box mark the median and the mean, respectively (n = 18); and the boundary of the box farthest from zero indicates the 75th percentile. The error bars above and below the box indicate the 95th and 5th percentile. Solid dots represent the data beyond the 5th and 95th percentiles. Boxes labeled with same letters on the top have no significant difference ($P > 0.05$).

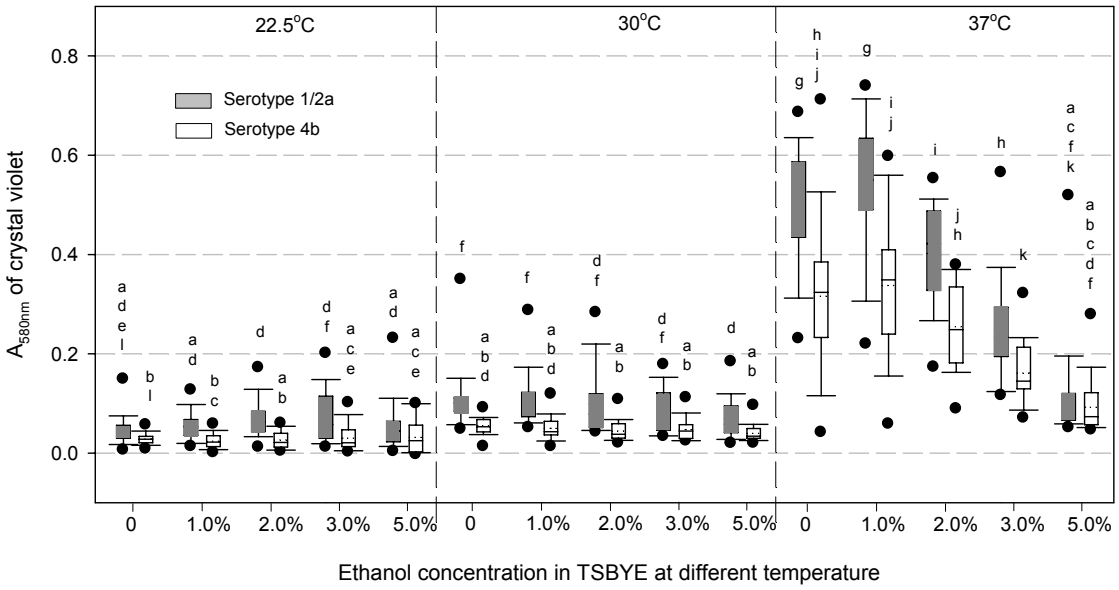

Figure 4.3. Absorbance (A_{580nm}) of crystal violet destained from *Listeria monocytogenes* biofilms formed concentration of ethanol.

Absorbance (A_{580nm}) of crystal violet destained from *Listeria monocytogenes* biofilms formed by 18 serotype 1/2a strains and 18 serotype 4b strains in TSBTE containing increased concentration of ethanol at different temperature (Data for individuals are in figure 3.1, figure 3.2, and figure 3.3). Each box represents the range of absorbance values for the eighteen strains of indicated serotype. The boundary of each box closest to zero indicates the 25th percentile; a solid line and a dotted line within a box mark the median and the mean, respectively (n = 18); and the boundary of the box farthest from zero indicates the 75th percentile. The error bars above and below the box indicate the 95th and 5th percentile. Solid dots represent the data beyond the 5th and 95th percentiles. Boxes labeled with same letters on the top have no significant difference ($P > 0.05$).

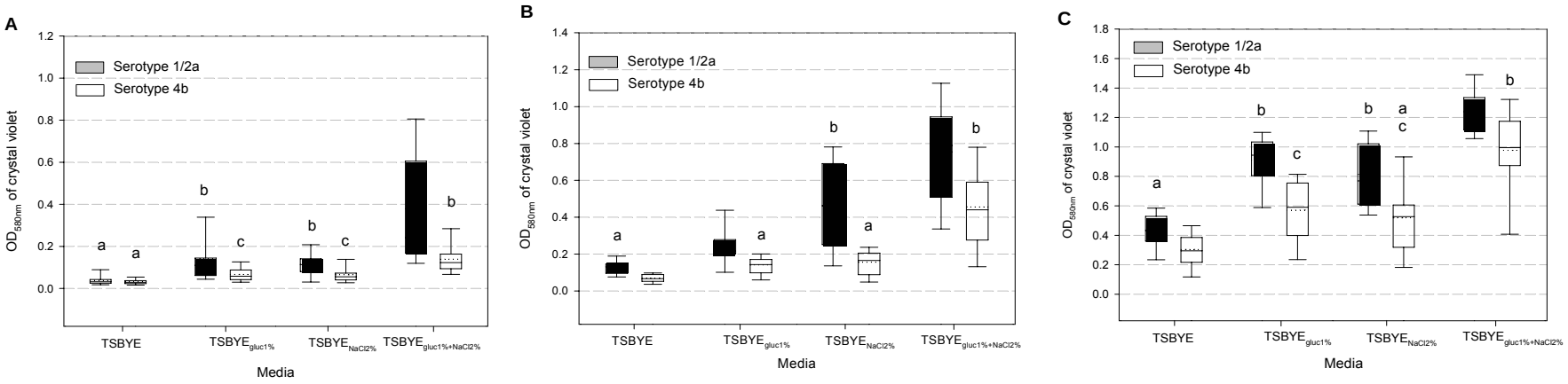

Figure 4.4. Synergistic effects of glucose, sodium chloride on *Listeria monocytogenes* biofilm formation .

Synergistic effects of glucose, sodium chloride on *Listeria monocytogenes* biofilm formation in TSBYE at 22.5^{o}C (A), 30^{o}C (B), and 37^{o}C (C). Absorbance (A_{580nm}) of crystal violet destained from *Listeria monocytogenes* biofilms formed by 18 serotype 1/2a strains and 18 serotype 4b strains in TSBTE containing increased concentration of glucose at different temperature. Each box represents the range of absorbance values for the eighteen strains of indicated serotype (Data for individuals are in figure 4.1, figure 4.2, and figure 4.3). The boundary of each box closest to zero indicates the 25th percentile; a solid line and a dotted line within a box mark the median and the mean, respectively (n = 18); and the boundary of the box farthest from zero indicates the 75th percentile. The error bars above and below the box indicate the 95th and 5th percentile. Boxes labeled with same letters on the top have no significant difference (P > 0.05).

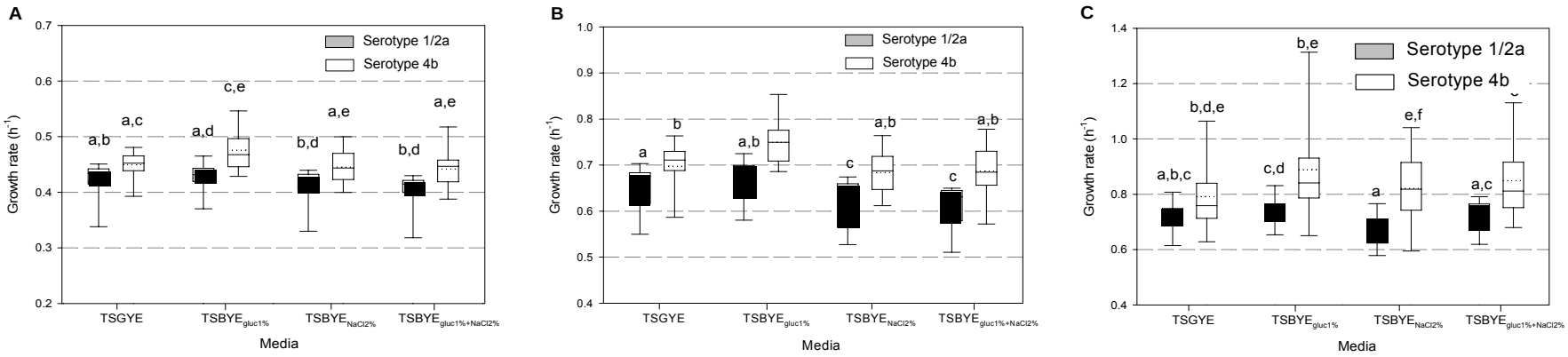

Figure 4.5. Growth rate (h^{-1}) of *L. monocytogenes*.

Growth rate (h^{-1}) of *L. monocytogenes* in TSBYE supplemented with extra glucose and/or sodium chloride at 22.5°C (A), 30°C (B), and 37°C (C). Each box represents the range of absorbance values for the eighteen strains of indicated serotype (Data for individuals are in figure 5.1, figure 5.2, and figure 5.3). The boundary of each box closest to zero indicates the 25th percentile; a solid line and a dotted line within a box mark the median and the mean, respectively (n = 18); and the boundary of the box farthest from zero indicates the 75th percentile. The error bars above and below the box indicate the 95th and 5th percentile. Boxes labeled with same letters on the top have no significant difference (P > 0.05).

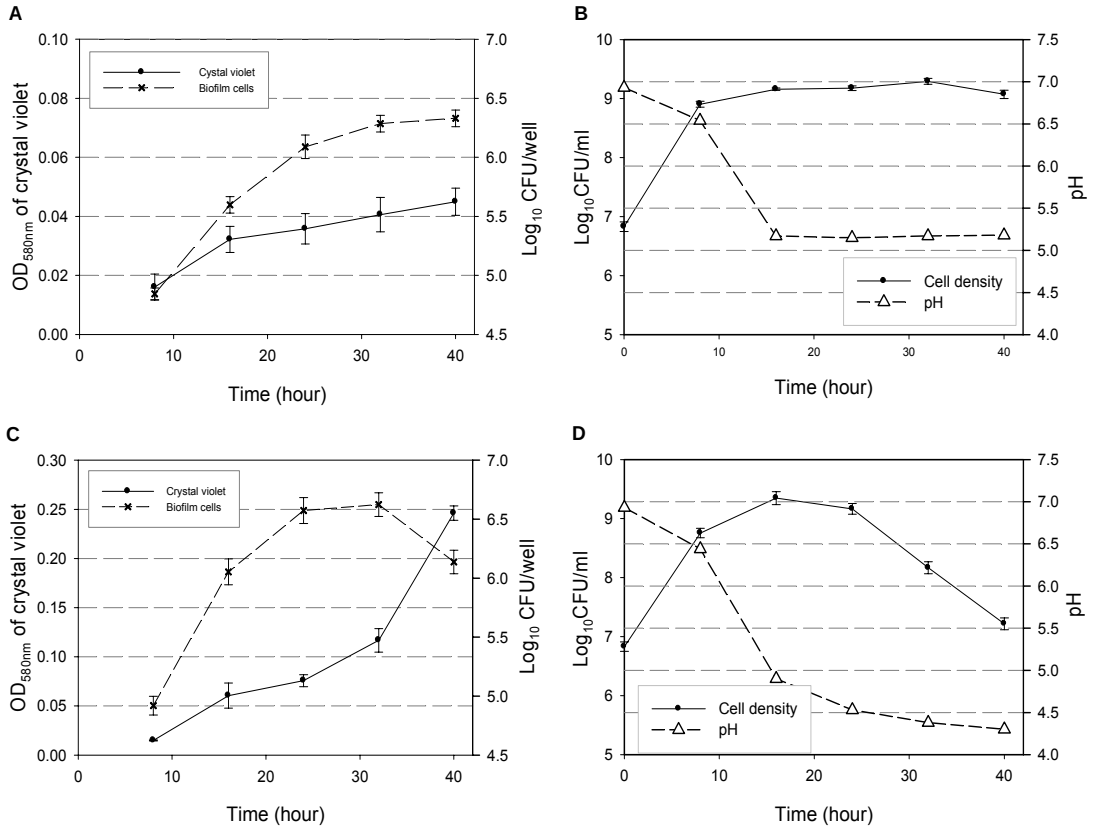

Figure 4.6. Time course monitoring of biofilm development at 22.5°C.

Time course monitoring of cell vitality in biofilms and absorbance values of crystal violet destained from biofilms (A, C), and cell vitality and pH in cell suspension (B, D) during biofilm formation in TSBYE (A, B) and $TSBYE_{gluc1\% + NaCl\ 2\%}$ (C, D) for 40 h at 22.5°C. Each data point is presented as the mean of six replicates. Error bars indicate the standard deviation of mean (n = 6).

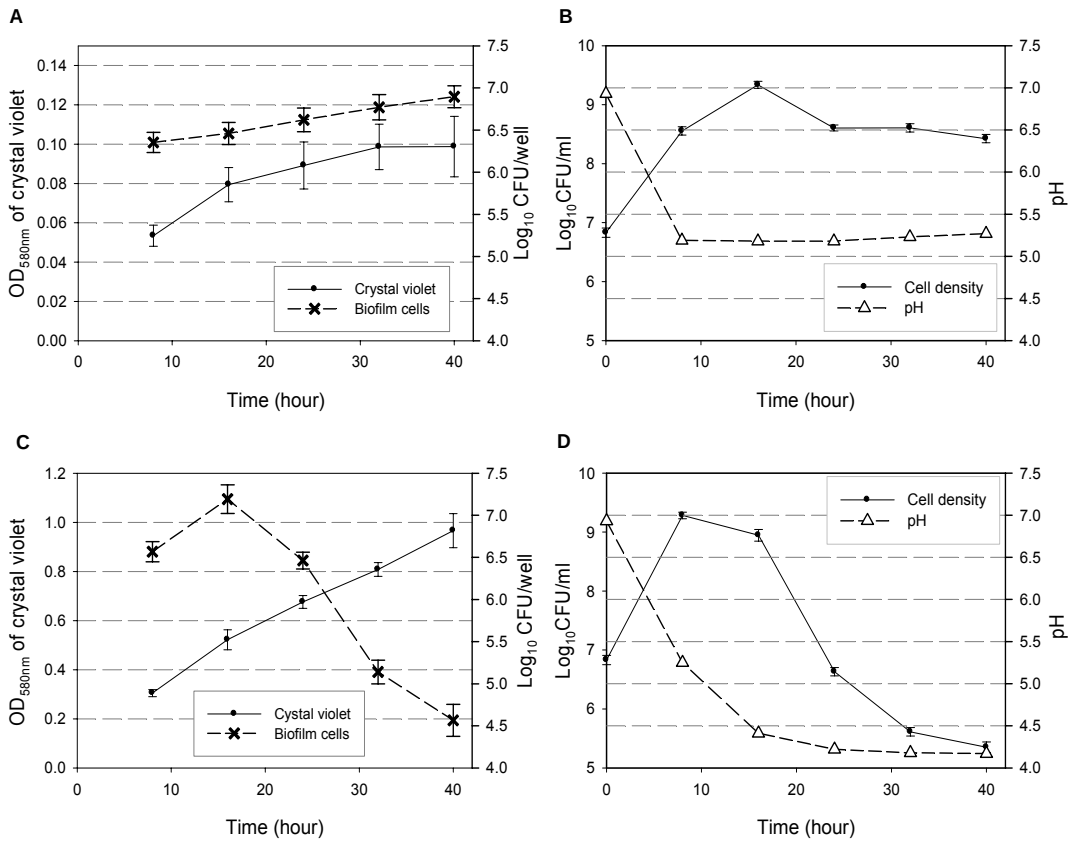

Figure 4.7. Time course monitoring of biofilm development at 30°C.

Time course monitoring of cell vitality in biofilms and absorbance values of CV destained from biofilms (A, C), and cell vitality and pH in cell suspension (B, D) during biofilm formation in TSBYE (A, B) and $TSBYE_{gluc1\% + NaCl\ 2\%}$ (C, D) for 40 h at 30°C. Each data point is presented as the mean of six replicates. Error bars indicate the standard deviation of mean (n = 6).

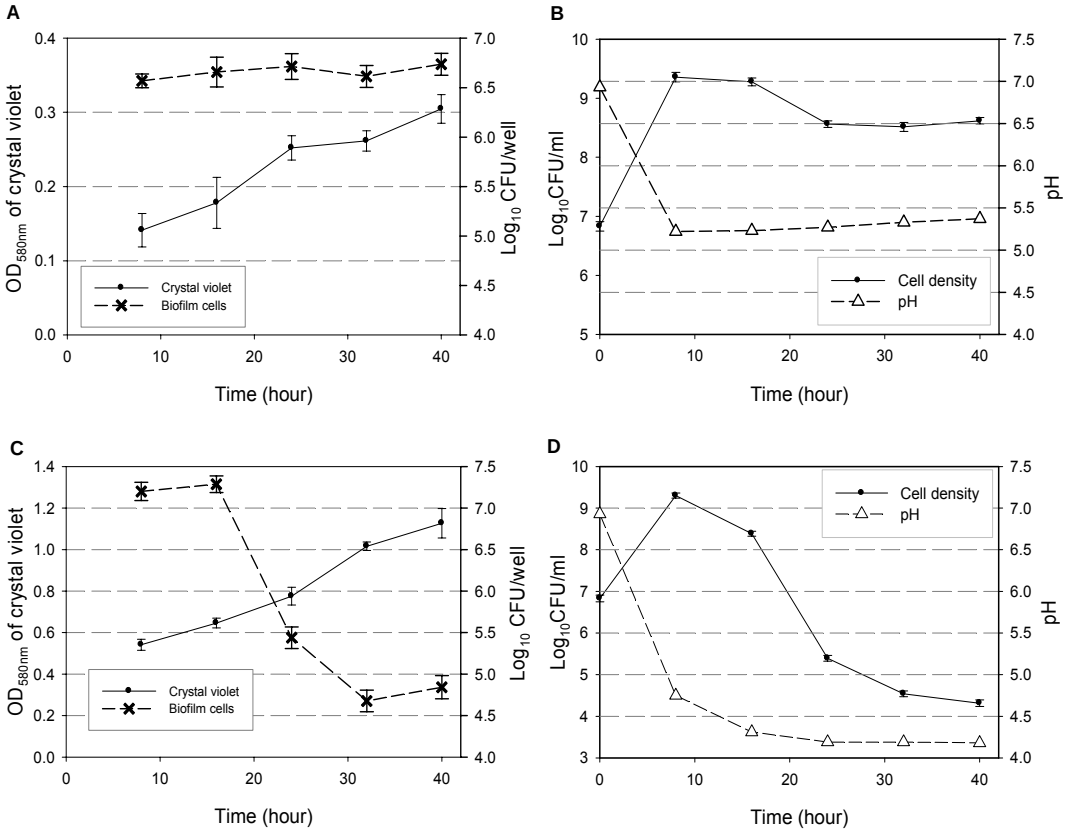

Figure 4.8. Time course monitoring of biofilm development at 37°C.

Time course monitoring of cell vitality in biofilms and absorbance values of CV destained from biofilms (A, C), and cell vitality and pH in cell suspension (B, D) during biofilm formation in TSBYE (A, B) and $TSBYE_{gluc1\% + NaCl\ 2\%}$ (C, D) for 40 h at 37°C. Each data point is presented as the mean of six replicates. Error bars indicate the standard deviation of mean (n = 6).

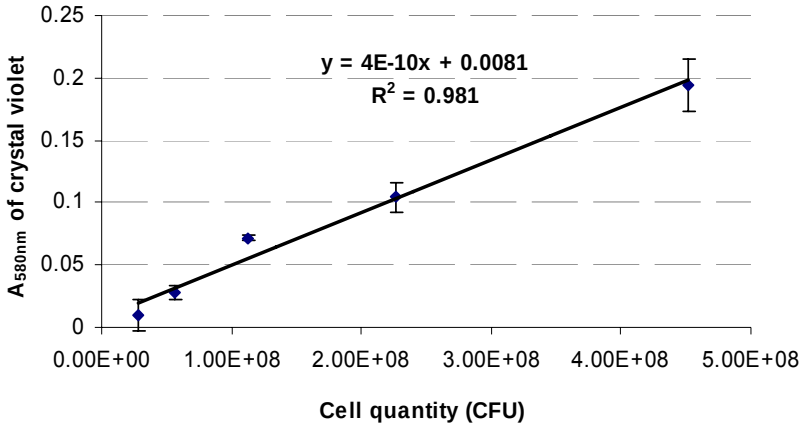

Figure 4.9. Relationship between bacterial cell counts and absorbance values of crystal violet destained from corresponding replicate samples. Each data point is presented as the mean of four replicates. Error bars indicate the standard deviation of mean (n = 4).

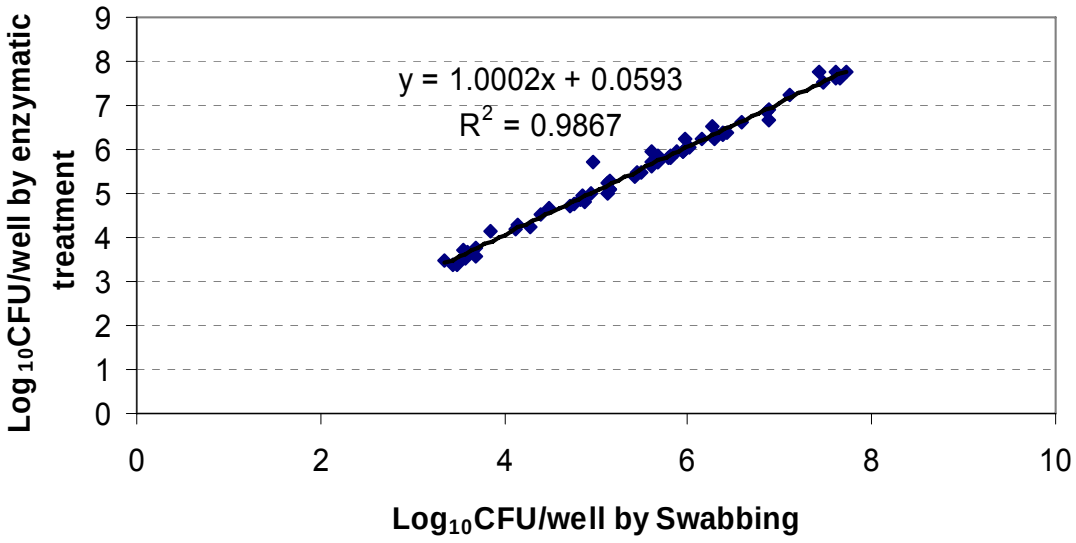

Figure 4.10. Relationship between *Listeria monocytogenes* biofilm cell counts obtained using swabbing method and corresponding cell counts obtained using enzymatic (protease, lipase and cellulase) treatment.

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