ATTACHMENT AND BIOFILM FORMING ABILITIES OF PERSISTENT AND NON-PERSISTENT *LISTERIA MONOCYTOGENES* ISOLATES

by

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(Under the Direction of JOSEPH F. FRANK)

ABSTRACT

Attachment and biofilm formation by 27 selected strains of *Listeria monocytogenes* that had been isolated from a chicken further processing plant were determined. One objective was to determine whether the persistent *L. monocytogenes* isolates demonstrated different attachment and biofilm formation than the non-persistent isolates. Second objective was to establish the microtiter plate assay as a rapid screening method for the attachment and biofilm forming ability. Persistent and non-persistent *Listeria* isolates showed similar attachment to the hydrophilic and hydrophobic microtiter plates as well as on stainless steel coupons ($p<0.05$). Only one persistent *Listeria* isolate exhibited significantly greater ($p<0.05$) biofilm formation than the non-persistent genotypes in both high and low nutrient media and on hydrophobic and hydrophilic surfaces. The results indicate that significant phenotypic variation occurs within persistent genotypes. Data obtained using the microtiter plate assay highly correlated ($p<0.0001$) with the data for the bacterial attachment and biofilm formation on stainless steel.

INDEX WORDS: *Listeria monocytogenes*, persistence, attachment, biofilm.

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# CHAPTER 1

# INTRODUCTION

The genus *Listeria* includes six different species (*L. monocytogenes, L. ivanovii, L. innocua, L. welshimeri, L. seegligeri,* and *L. grayi*). Only *Listeria monocytogenes* is consistently associated with human illness *(70, 93)*. Moreover, *L. monocytogenes* is widely distributed in soil, plants and water, and survives in or on food for long periods over a wide range of temperatures *(78)*. *L. monocytogenes* can attach to the inert surfaces in the processing plants and may contaminate the processed product *(83)*. The raw material is one of the significant sources of contamination at the processing plants, and the risk is further exacerbated by the survivors in the processing environment (e.g., food contact surfaces, floors, and drains) *(60, 80)*. However, recontamination is the primary source of *L. monocytogenes* in many commercially prepared ready-to-eat foods *(99)*.

Most of the incidences linked to human listeriosis are foodborne. *L. monocytogenes* has been recognized as a foodborne pathogen with major public health consequences to all food manufacturing companies because of the potentially life-threatening illness. Although the incidence of listeriosis is low (2,500 cases of people seriously ill with listeriosis per year), the fatality rate is high (approximately 500 of these individuals die) *(71)*. *L. monocytogenes* was first isolated from a meat product (oven-ready poultry) in 1976 by Gitter. *(50)*. In 2002, a multistate outbreak of listeriosis was associated with seven deaths and three miscarriages. *L. monocytogenes* was isolated from one intact food product and 25 environmental samples from a poultry processing plant related to this outbreak (3).

*L. monocytogenes* has the ability to attach to inert surfaces found in the food processing environments and form biofilms; however, there are differences in both the extent and rate of adsorption depending on the surface selected *(54)*. Previous research has shown evidence that formation of biofilms by *L. monocytogenes* can form unique microniches which allows their extended survival in food-processing plants *(9, 104)*.

*L. monocytogenes* shows higher prevalence in poultry (fresh chicken carcasses with contamination levels up to 62% have been reported) than in other meats *(74)*. Studies have indicated presence of *L. monocytogenes* strains in the poultry products at the abattoir, processing plant and even at the retail level in products such as undercooked chicken and uncooked hotdogs. These incidences of occurrence might be due to antimicrobial resistance *L. monocytogenes* strains or cross contamination of *Listeria* at the processing level *(4, 13)*. Although the incidence of *L. monocytogenes* in cooked ready-to-eat and raw sliced meats has declined significantly over the last 10 years, the continued presence of *Listeria* in the raw and cooked environmental sites highlights the potential for post-processing contamination *(61)*. This suggests that some *L. monocytogenes* isolates may attach better than others and may persist in the plant for years due to their ability to survive in the production lines or other plant environmental sites *(63, 74, 78)*.

The present study was designed to compare the adherence and biofilm forming capabilities of persistent and non-persistent strains of *L. monocytogenes* isolated from a single, chicken further processing plant over a period of 1 year. The selected persistent and non-persistent isolates represented a variety of genotypes, source, and evidence of persistence in the food-processing environment. The attachment and biofilm forming ability of the *Listeria* isolates were examined on different surfaces under the influence of physico-chemical factors such as hydrophobicity, temperature, pH and nutrient composition of the suspension media. Further,

since the attachment and biofilm forming ability of the *L. monocytogenes* isolates was determined using microtiter plates and stainless steel coupons, the results would determine if microtiter plates can be used as a rapid screening method for observing the attachment and biofilm ability.

CHAPTER 2

LITERATURE REVIEW

## General information

**1. History** (*L. monocytogenes* and listeriosis)

*L. monocytogenes* is a member of the Family *Corynebacteriaceae*, order *Eubacteriales*. Pirie, in 1940, chose *Listeria* as the generic name in honor of Lord Lister, the well-known pioneer in the field of bacteriology *(38)*. The species name *Listeria monocytogenes* suggested by Murray et al. *(75)* is derived from the fact that large numbers of monocytes are often found in the peripheral blood of some infected monogastric animals, although not in ruminants. *L. monocytogenes* belongs to the *Clostridium* sub-branch and has been listed in the *Bergey's Manual of Determinative Bacteriology* in a section entitled Regular, Nonsporing Gram-Positive Rods *(41)*.

The first human foodborne transmission of listeriosis was detected in coleslaw in 1981 in Nova Scotia, Canada *(64)*. This was followed by evidence of *L. monocytogenes* and the disease listeriosis for foodborne transmission in humans in several countries in 1980s *(60)*. Listeriosis mainly affects neonates followed by the elderly, pregnant women and immunosuppressed individuals *(27)*. In a case–control study in the USA consumption of undercooked chicken and inadequately re-heated hotdogs were identified as risk factors for sporadic listeriosis, and in a similar study in England and Wales ready-cooked chicken and undercooked chicken were associated with sporadic cases of *Listeria* infections *(75)*.

## 2. General characteristics

The smooth, pathogenic form *L. monocytogenes* is a small gram-positive, catalase-positive, oxidase negative, non-sporeforming, non-capsulating, non-acid fast, diphtheroid-like rod with rounded ends measuring 1.0 to 2.0 μ , by 0.5μ. They are facultative anaerobes and are motile at 20-25°C by means of peritrichous flagella but non-motile at 37°C *(41)*. The colonies of *L. monocytogenes* demonstrate a finely textured, characteristic blue-green sheen under a transmitted oblique light on colorless medium viz. nutrient agar. The colonies after 24 hours of incubation at 37°C are round with an entire margin, translucent, watery in consistency and bluish-gray under normal illumination. The colonies are 0.3 to 1.5mm in diameter which may reach up to 3 to 5mm or larger size on incubation after 5 to 10 days *(33)*.

## 3. Genetic Lineages of *Listeria* and their significance

*L. monocytogenes* has been classified into three distinct lineages based on ribotype patterns and virulence gene polymorphisms. Of these three lineages, only lineage I and II have been isolated from the food-processing environment. Lineage I includes all strains associated with epidemic outbreaks of listeriosis, while no human isolates have been documented in lineage III *(66, 81)*. Hence Lineage I may be especially important while considering the sporadic and epidemic outbreaks in humans as a result of transmission of *L. monocytogenes* through the food environment. Mean biofilm production of lineage I strains is significantly greater than that observed for lineage II and lineage III strains *(19)*.

## 4. Growth and survival characteristics

*L. monocytogenes* has an optimum growth temperature of 30-37°C but they are also able to grow at very low temperatures (-1.5°C) *(41, 43)*. Moreover, freezing and storage at -18°C and even repeated freezing are more likely to injure *L. monocytogenes* cells rather than inactivate

them and has little effect on reducing the size of the bacterial population. Hence, ready-to-eat and stored foods at refrigeration temperatures for a long period of time are considered high risk foods, especially when the contamination risk of the foods with *L. monocytogenes* is high (>100 cfu/g). However, *L. monocytogenes* are substantially injured or killed by heating to 54°C or above *(23, 74)*. Epidemiological and laboratory studies indicate that processed and ready-to-eat meats can cause listeriosis outbreaks since the organism is capable of growing in refrigerated processed foods before use by the consumer. This lead to the institution of a zero tolerance policy for *L. monocytogenes* (less than one cell per 25 g of the product, <0.04 cfu/g) in ready-to-eat products by the U.S. Department of Agriculture (USDA) in 1989 *(2, 26, 76)*.

The Department of Health and Human Services (HHS), the U.S. Food and Drug Administration (FDA) and the USDA have identified ready-to-eat meat (RTE) products as high risk foods in terms of *Listeria* contamination. This is because of the ability of *L. monocytogenes* to grow at refrigeration temperature, combined with the fact that all RTE meats carry an extended shelf life at refrigeration temperature. This situation presents a challenge because *Listeria* can establish itself in the food processing area and some of the isolates can persist in the plant environment for months or years even in the presence of a well-organized sanitation program *(22)*.

Water activity ($a_w$) of ≥ 0.97 is optimal for the growth of *L. monocytogenes* strains. But the heat resistance of the bacterium increases as the water activity decreases which leads to a greater chance of surviving cells in a processed food commodity. The problem escalates further since *L. monocytogenes* is capable of growing at refrigeration during the storage period of the food in household refrigerators *(64)*. Also, considerable adaptive acid tolerance response has

been observed for *L. monocytogenes* cells. Adaptation to mild acidic conditions can increase the acid tolerance at all stages of the life cycle to pH as low as 3.5 *(68)*.

**Attachment/adherence**

Attachment, which is initially facilitated by the bacterial cell surface properties, is necessary in the natural environment as it permits the microorganisms to procure nutrients and protects the cells from environmental stresses *(14)*. Attached cells secrete extracellular polysaccharide material which extends the strength of the attachment bond. *L. monocytogenes* grows on surfaces commonly used in the food processing equipment and may survive for long periods. However, the survival is affected by pH, relative humidity, temperature, attachment surface and soil. Among all different types of meats analyzed, chicken breast fascia allowed the greatest attachment with cut chicken muscle, which is of concern in the refrigerated retail packages. Even freshly slaughtered chicken skin surface has many crevices and feather follicles which allow the cells to physically entrap, directly adhere or float in the water film coating the skin. This initial attachment is merely a physical entrapment and does not involve the use of attachment mechanism such as flagella and fimbriae *(30)*.

*L. monocytogenes* is capable of attaching to different contact surfaces such as stainless steel, glass, polypropylene and rubber after contact times as short as 20 minutes *(57)*. *L. monocytogenes* strains have a varied attachment ability on a broad temperature range (4°C to 21°C ) based on the type of nutrient available in the attachment suspension *(42, 46)*. Attachment is dependent on the rate of nutrient depletion which can lead to an increase in the attachment because of the starvation stress created. Attachment has a bearing on the virulence of the pathogens depending on the availability of metallic ions which can alter the physicochemistry of the bacterial surface and hence its interaction with the host tissue *(49)*. The extent and rate of cell

attachment is also influenced by cell surface hydrophobicity, lipopolysaccharide (LPS), presence of fimbriae and flagella, and production of extracellular polymeric substance (EPS) *(20)*.

*L. monocytogenes* attaches readily to hydrophobic and hydrophilic surfaces commonly used in the food industry, when suspended in phosphate buffer saline (PBS). The cells subsequently form biofilms and the surface adherent microcolonies become more resistant to the commonly used sanitizers in the food industry viz. those containing chlorine, iodine, quaternary ammonium compounds, and anionic acid *(31, 84)*.

Although *L. monocytogenes* cannot multiply in frozen products, it can survive and remains viable to a greater extent than most bacterial species during prolonged periods of storage at sub-zero temperatures *(51)*. *Listeria* generally re-enters the product through post-processing contamination, since it is unable to survive the processing treatment. Contaminated processed meat and poultry tend to have a higher numbers/g of *L. monocytogenes* as compared to the fresh meats contributing to the foodborne outbreaks of listeriosis. This suggests that *L. monocytogenes* may be unable to efficiently compete with other microorganisms or that cooked meat is a better growth medium for it *(44)*.

Some isolates of *L. monocytogenes* have the ability to persist in the food processing environment. This may be due to the fact that sublethal stresses such as pH, temperature and low water activity usually encountered by *Listeria* in the food processing environment have a positive influence on its attachment to the common food contact surfaces *(78)*. Moreover, *L. monocytogenes* shows significantly higher attachment and survival on unclean environmental surfaces leading to its enhanced persistence in the food processing facility *(7)*.

Attachment studies on *L. monocytogenes* have used both hydrophobic (Buna-N rubber) and hydrophilic surfaces (stainless steel). Basic food processing environmental conditions can

cause sub-lethal stress and result in the persistence of *L. monocytogenes*. This influences the attachment of the cells to the food contact surfaces by changes in the regulatory mechanisms that determine the cell surface properties. Subsequent growth of the cells leads to the biofilm formation once the attachment has occurred *(6, 78)*.

**Biofilm formation**

The biofilm formation is a regulated process that often results in the formation of a complex community of organisms on a surface. Biofilm formation consists of four distinct steps, viz., conditioning of a surface, adhesion of cells on the conditioned surface, formation of microcolonies by the adhering cells, and finally the formation of biofilm *(52, 58, 71)*. The formation of biofilms in food systems is undesirable as it causes hygienic problems and economic losses resulting from food spoilage due to the shedding of the cells into the surrounding food. Biofilm consists of microbes and their extracellular polymeric substances (EPS), usually polysaccharides, which provides protection to the microbes from environmental stress and causes contamination through individual microorganisms on detaching from the surface *(63, 73)*.

The formation of biofilm by bacteria provides a protective surface which is efficient in channeling nutrients to the cells and protecting them against stressful environment *(15, 59, 65)*. On detaching, the biofilm can release non-sessile planktonic bacteria which can rapidly disperse in an industrial environment and are capable of causing chronic bacterial infections *(16)*. Biofilm accumulation is affected by a combination of three factors, viz. attachment, growth at the surface, and detachment. In food processing industries biofilms formed by pathogenic bacteria such as *Listeria*, *Pseudomonas, Klebsiella, Campylobacter, Escherichia. coli* and *Salmonella* have been reported. *L. monocytogenes* has been isolated from chicken further processing plants,

and it has been established that various isolates of *L. monocytogenes* in the environmental sites in food processing facilities can coexist *(5)*.

The presence of microbial flora that can enhance *L. monocytogenes* colonization in food processing environment is of great concern. It is the nature of the EPS rather than its quantity that may affect the *L. monocytogenes* biofilm population. This is because no correlation exists between EPS production by the different strains comprising the house flora of food processing premises and their impact on *L. monocytogenes* biofilm population *(9)*. Biofilms, once formed on the contact surfaces in the processing environment, could be a continuous source of contamination, especially to the processed foods that come in contact with them *(47)*.

The nutrient medium also has a major role to play in the attachment and biofilm accumulation. Some researchers have stated that *L. monocytogenes* forms better biofilm on nutrient-rich medium when grown on hydrophobic surfaced microtiter plates, indicating a significant influence on biofilm formation by the growth medium *(79)*. However, it has been documented that *L. monocytogenes* also produces biofilms under low nutrient conditions. *L. monocytogenes* has been shown to survive in media containing low nutrients (PBS) and limited nutrients (diluted milk) *(37)*. This is of much concern because biofilm accumulation may be difficult to control just by limiting the supply of nutrients. Hence biofilm control at ambient temperature in the food processing environment can be controlled by reducing the water availability and cleaning the surfaces at frequent intervals *(50)*.

Even during poultry processing it is not practical to control the nutrient accumulation, which can support extensive biofilm formation under favorable conditions of water activity and ambient temperature. Biofilm control can be primarily done by implementing effective cleaning to remove food soils which can provide *L. monocytogenes* with harborages for survival and

potential growth. However, equipment surfaces and equipment exterior are often not equipped for efficient removal of food residues and can act as a harborage when moisture is supplied *(29)*.

Biofilm formation could be stable and favorable at 22°C on hydrophilic and hydrophobic surfaces since it has been published that flagellation and motility have a positive influence on the different stages of biofilm formation *(72)*.The incubation temperature for the attachment, and biofilm forming ability was selected as 25°C as flagellated *L. monocytogenes* has exhibited significantly higher attachment at 22°C than the non-flagellated *L. monocytogenes (80)*. Thus, 25°C is an ideal temperature for assessing the differences in the attachment and biofilm forming abilities among the *L. monocytogenes* isolates since it is known that *L. monocytogenes* have motility by means of a few peritrichous flagella at 20-25°C, even though the optimum temperature for their growth is 30-37°C *(41)*. Although flagella play a role in the initial attachment, by bringing the cells in close proximity to the surface and spreading them across the surface, it delays the biofilm formation. It is only after the bacteria adjust to the immobility on the attachment surface, that they loose their flagella and increase the production of the EPS for an enhanced rate of biofilm formation *(17)*.

Previous research has shown that stress-induced changes in cells during long-term chilled storage will negatively influence the ability of those cells to attach to meat surfaces and thereby may change the listeriogenic potential of long-term stored chilled meat *(13)*. Moreover, it has been shown that the hydrophilicity of the *L. monocytogenes* cells increased significantly along with a decrease in the growth rate and the electrophoretic mobility as the growth temperature decreased towards 8°C, which considerably lowers the colonization ability of the cells *(8)*. However, since, *L. monocytogenes* can persist for long periods in the food processing environment, cells with a range of attachment ability may be present in such habitats. This might

be one of the reasons for the persistence of certain strains in and their recovery from food processing environments *(24)*.

**Methods used to study attachment and biofilm formation**

Bacterial attachment and biofilm formation have been determined using a variety of direct and indirect methods. Some of the indirect methods for *in situ* estimation for the number of attached organisms include radiolabeled bacteria, enzyme-linked immunosorbent assay, biological assays, stained bacterial films and microtiter plate procedures. Direct observation methods to observe the biofilm formation include microscopy techniques (light, epifluorescence microscopy, laser-scanning confocal, transmission electron and scanning electron microscopy) *(19, 85)*.

**1. Direct methods**

Light microscopy is the oldest and a relatively inexpensive method for studying bacterial attachment and colonization. However, low resolution and the lack of information on microbial viability or the three dimensional structure of the biofilm are some of the disadvantages of using a light microscope. Laser scanning confocal microscopy, on the other hand provides higher resolution, and three dimensional images of the biofilm using fluorescent stains such as DAPI and acridine orange (DNA and RNA binding), and Texas Red-labeled lectin (polysaccharide binding dye). Live and non-viable cells can be differentiated using the confocal microscope by differentiating fluorescent dyes such as propidium iodide and 5-cyano-2,3-ditolyl tetrazolium chloride *(21)*. Transmission electron microscopy offers additional utility in the direct observation of bacterial biofilm structures. However, artifact formation from sample preparation and the lack of information on the bacterial viability are some of the problems associated with transmission electron microscopy. Scanning electron microscopy is a preferred tool for observing the nature of

attachment, but the field of focus is small making the procedure tedious and giving greater variability in the results than other direct methods *(85)*.

Epifluorescent microscopy, with image analysis, although only two-dimensional has been widely used to study biofilm development by foodborne bacteria *(83)*. Traditional cultivation and swab method though practical in testing the presence of microorganisms on the surface, have been reported to give false results in case of growing biofilms. This is mainly due to the fact that as the biofilm matures, the difficulty of detaching the cells in the biofilm from the surfaces to the swab increases *(82)*. However, it has been reported that the quantitative analysis of the cell coverage may lead to overestimates of the area covered, since some extracellular polymer is stained *(6)*. Epifluorescence microscopy, like confocal microscopy involves the use of fluorescent dyes. Hoechst dye is another cell-membrane permeant stain that has been used to study biofilms. It fluoresces bright blue upon binding to DNA *(19, 36)*.

**2. Indirect *in situ* methods**

Radiolabeled bacteria are widely used as it is a sensitive method to study the adhesion of microorganisms to surfaces. However, full biofilm development is difficult to study using this method as the counts-per-minute-to-microbe is unstable. Further, discarding the scintillating fluid is expensive and the analysis requires expensive, sophisticated instrumentation *(85)*. On the other hand, enzyme linked immunosorbent assay (ELISA) involves the use of enzyme-linked antibody. However, ELISA fails to detect the attached cells embedded in a dense biofilm, under a layer of microorganisms or covered with EPS material. Biological assays do not require much expertise and do not involve the use of hazardous materials. This method is sensitive for studying the microbial adhesion on a wide range of objects and under varying conditions of attachment. However, biological assays require measuring the biological product produced by the

microorganisms and co-relating the same with the number of microbes attached on to the surface. This can lead to inaccuracy because normally the tests are conducted taking into account the biological products produced by the planktonic cells which may not co-relate with the sessile cells on a biofilm *(85)*.

Another simple and rapid method for estimation of the bacterial density and the extracellular matrix production uses stained bacterial films. The two approaches for the stained bacterial films are “the tube” and “the microtiter plate” methods. Both methods are quantitative assays to determine the level of microbial colonization and the extracellular slime production by spectrophotometric determination of the optical density. The test tube method involves the staining of bacterial film on the test tubes containing broth cultures of bacteria. It is a simple and expedient “presence or absence” test. However, with narrow tubes the low oxygen content in the bottom of the tubes can interfere with the slime production by the bacteria, leading to a false negative test.

The microtiter assay involves exposing microtiter plates to bacterial suspensions followed by rinsing off the unattached cells, staining the cells and finally de-staining. The adsorption of crystal violet in the de-staining solution gives an indirect indication of the level of biofilm produced by use of an automatic spectrophotometer. Microtiter plate assay is a quick and corrects many of the deficiencies encountered in test tube method for studying bacterial biofilm formation *(19)*. Microtiter plate assay provides precise quantitative data but it fails to indicate a low level of bacterial attachment to the substratum. It can be used to study the effects of hydrophobicity on the cell attachment using commercially available surfaces *(77)*.

In microtiter plate assay, the biofilm formation as influenced by nutrients was determined using trypticase soy broth (TSB) and chemically defined minimal medium (D10). The cell

suspensions were incubated at 25°C for 24 hours and 10°C for 5 days. Twenty five degrees Celsius is the environmental temperature in a processing plant and 10°C was chosen as this is the temperature encountered by the chicken carcasses while de-boning and packaging, while cooling after the processing treatment. Moreover, many moist surfaces in meat plants where *Listeria* spp. are found are at cold temperatures *(45)*.

**Persistence**

Similar genetic subtypes of *L. monocytogenes* have been recurrently recovered in the same product of the same producer or products of several producers of different countries over several years. This suggests a possible persistence of the house-flora strains in the processing plant, which may not always be producer-specific *(4)*. A number of strains of *L. monocytogenes* were seen to recur in the environmental sites from the same food processing establishment over a period of years and were isolated repeatedly. These isolates were referred to as persistent strains. The other isolates from the environmental sites inside this processing plant were found sporadically or only once throughout the sampling period and were termed as non-persistent isolates *(19, 54)*.

Investigations of *L. monocytogenes* isolated from a turkey processing plant in Denmark indicated that none of the flocks examined before slaughter sampled positive for *L. monocytogenes* and that the prevalence of *L. monocytogenes* in the processed product increased during processing. This suggests that the outbreak of listeriosis connected with the turkey meat was dependent on the processing plant environmental sanitation and the removal of the established *L. monocytogenes* strains in the factory rather than the initial contamination of the raw turkeys *(69)*.

Some persistent *L. monocytogenes* strains exhibit a higher minimum inhibitory concentration (MIC) to disinfectants (quaternary ammonium compounds and tertiary alkylamine), when subjected to disinfectants at sublethal concentrations *(53)*. The MICs for some persistent strains were also found to be higher than those for the non-persistent isolates when they were exposed to progressively increasing disinfectant concentrations after the sublethal exposure to the disinfectants. Moreover, persistent *L. monocytogenes* showed a more recurrent production of bacteriocin (monocin type E) and heavy metal salts (cadmium) resistance as compared to the non-persistent strains *(35)*. This resistance to cleaning and disinfectants may be a factor among others influencing the ability of a strain to persist in the food processing facility during processing, after cleaning and before the start of the process, as prevailing ribotypes have been found in the food processing environment by some researchers *(32, 61)*. Aase et al. found that the prevailing ribotypes of *L. monocytogenes* are superior in their ability to develop resistance against disinfectants due to their marked ability to persist and form biofilm, consequently leading to contamination risks in the processing facility *(1)*.

Other researchers have found that the nature of persistence among *L. monocytogenes* is not due to ineffectively designed cleaning programs *(40)*. It was observed that the environmental samples of persistent *L. monocytogenes* were not significantly different than the laboratory strains of *L. monocytogenes* as far as the resistance to the commercial disinfectants is concerned. Moreover the resistance of the persistent *L. monocytogenes* strains (isolated from a processing plant) to commercial disinfectants was not found to be a plasmid conferred resistance *(25)*. It was observed that the strains carrying the plasmids were equally resistant to the commercial disinfectants as those lacking the specific plasmids and no correlation could be established

between the persistent and the sporadic strains with respect to the size and the incidence of the plasmids.

The persistent *L. monocytogenes* strains are initially introduced into the food processing plants via the constant influx of the raw materials, and the persistence of the strains may be due to the continuous flow of the raw materials in the plants *(28, 56)*. However, persistence may also be exhibited by other strains due to the strain-specific properties that influence their survival and colonization *(62)*. Some researchers have observed different plasmid profiles for the persistent *Listeria* spp. isolated from the same raw product over a period of time. The presence of a few dominating clones of *L. monocytogenes* primarily localized to the abattoirs might explain their endemic occurrence in the broiler production *(70)*.

Other scientists have found that some *L. monocytogenes* strains are able to persist in the meat processing environment for several months while some others were found in the processing line from time to time but were not detected during cleaning operations *(12)*. This study suggested that some isolates of *Listeria* collected were able to survive the cleaning and sanitization in the processing line while other isolates continuously gained entry into the processing plant area and established there by the means of the contaminated live poultry and pork on which they were adapted. The *Listeria* strains established in the microniches in the plant environment subsequently form biofilms which provides the cells added advantage towards disinfectants and cleaning agents *(32)*. It was observed that the food handlers did not play a major role in the dissemination of *L. monocytogenes* strains throughout the processing plant. Rather, the predominant profile groups found to recur in the food industries were able to survive because of their particular adaptation to a specific niche or habitat *(18)*.

Some persistent *L. monocytogenes* strains exhibit significantly higher attachment to the stainless steel surfaces than the non-persistent strains *(67)*. Thus persistent strains can become established on equipments and machines in the areas of the processing industry with high hygienic standards (areas receiving effective cleaning and disinfection treatment), which can cross-contaminate and transfer the persistent strains from one part of the plant to the other, and also contaminate other plants while relocation of the processing machines from one plant to the other. Strains of *L. monocytogenes* which are persistent in the food-processing environment do not loose their pathogenic potential *(34)*. In fact some clones that were persistent and prevalent in the processing facilities have been associated with human listeriosis *(48)*. Researchers have demonstrated that the variations in the growth rates of planktonic cells are different from those observed in case of the growth of *Listeria* in biofilms. This difference in the biofilm growth rates again varied when grown under static conditions and in continuous flow systems. The variations in the mature biofilm growth as influenced by the growth conditions could be a significant cause for the prevalence of persistent strains of *Listeria* within the food processing environment *(10)*. The problems exacerbate if machine parts cannot be dismantled frequently due to economic reasons. Thus some machine parts are hard-to-reach and not easily cleaned mechanically *(55)*. Hence development of more economical, consistent and rapid methods in the food industry would help monitor and control the *Listeria* contamination in the processing environment more effectively.

The presence of persistent *L. monocytogenes* strains, however, does not always indicate a high contamination level in the processing plant and cannot always be attributed to poor manufacturing practices. Persistent strains have been found in a plant with a low overall incidence (<1%), together with the incidence levels for the individual sites, of *L. monocytogenes*.

The low incidence level in this case indicates that the factories under examination were achieving their cleaning and sanitation goals and were hence 'in control'. It can be inferred that persistent strains are more likely to be found in large and old environmental sites, those places associated with the reception of raw material, sites with the largest number of process lines and environmental niches, and those areas referred to as 'high risk' areas. The persistence of the strains depends on a number of factors affecting physical adaptation such as surface attachment, biofilm formation, attachment strength, reduced growth rate, quiescence, and cleaning and disinfection resistance. In addition, environmental conditions in the plant viz. low temperature, wide pH range, fluctuating nutrient supply and moisture levels, and the frequency of cleaning and disinfection also have a role to play in the persistence of strains *(39)*. Effective cleaning and sanitization practices viz. alternative use of two different cleaning products have been successful in eradicating the persistent *L. monocytogenes* strains from the processing plant *(3, 11, 62)*.

# CHAPTER 3

# MATERIALS AND METHODS

## Storage of cultures

The 27 *L. monocytogenes* isolates used in the experiments were obtained from USDA, Russell Research Center, Athens, GA (Table 1). The isolates recovered from one of the three processing lines of a chicken further processing plant were classified by PFGE and grouped into 10 genotypes based on the sequence on the *actA* gene. The strains that were re-isolated from multiple sites from the plant over the 12 month sampling period were classified as persistent isolates (sampling was carried out every 6 weeks over the course of the 1 year sampling period) and the strains that were isolated only once from the plant over the 12 month sampling period were classified as non-persistent isolates (sampling was carried out every 6 weeks over the course of the 1 year sampling period) *(7)*.

The stock cultures were activated by inoculating them into 10 ml trypticase soy broth-yeast extract [TSBYE] (Becton, Dickinson and Company, Sparks, MD) at 25°C for 24 hours. The culture was then streaked on to *Listeria* Selective Agar [LSA] and incubated at 32°C for 24 hours (Becton, Dickinson and company, Sparks, MD) for isolation and identification. Representative colonies were picked up from the LSA plates and suspended in cryovial containing beads (Microbank™, Prolab Diagnostics, Ontario, Canada). The cryovials were subsequently stored at -80°C.

## Culture Preparation

The working cultures were activated by transferring single beads of the *L. monocytogenes* strains into 10 ml TSBYE for 18 h at 32°C. Optical density of the cultures was adjusted (Ultraspec Spectrophotometer LKB Instruments, Inc., Houston, TX) to 0.45 nm (ca. $10^6$ cfu/ml) with phosphate buffer saline [PBS; pH: 7.2] (Becton, Dickinson and Company, Sparks, MD) for the biofilm study. Similarly the optical density at 600 nm wavelength was adjusted to 0.678-0.783 nm (average ca. $7.5x10^8$ cfu/ml) with PBS; pH: 7.2 for the attachment study. The optical density was determined using a spectrophotometer (Beckman DU® 530, Beckman Instruments, Inc., Fullerton, CA). The inoculum cell population was enumerated by serial dilution of the strains using PBS and spiral plating (Autoplate® 4000, Exotech, Inc., Gaithersburg, MD) the appropriate dilutions on plate count agar (Becton, Dickinson and Company, Sparks, MD) incubated at 32°C for 24 hours. Colonies were counted using Darkfield Quebec® Colony Counter (Model 3325, American Optical, Scientific Instrument Division, Buffalo, NY) after incubation at 25°C for 24 h.

**Microtiter plate assay for the assessment of *L. monocytogenes* biofilm formation**

The screening assay used for determining the attachment and biofilm formation by the *L. monocytogenes* isolates was a modification of a previously reported protocols *(89, 95)*. Each *L. monocytogenes* strain was grown in 10 ml of rich undefined medium, TSBYE, at 32°C for 18 h. Cultures in TSBYE were then transferred (0.1 ml) to 10 ml each of 1:10 dilution of TSB (using sterile distilled water) and TSB (Becton, Dickinson and Company, Sparks, MD), and vortexed. One hundred and fifty microliter volume of cell suspensions were then transferred into 96-well sterilized PVC microtiter plates, (Costar®, Corning Incorporated, Corning, NY) eight wells per isolate. Each plate included eight wells of media without *L. monocytogenes* as controls. Plates were incubated at 25°C for 24 h. The biofilm formation was also determined at 10°C for 5 days

using the same protocol. To minimize evaporative loss and edge effects, the outermost rows and columns of each plate were filled with 150 µl of sterile water. The edges of the plate were then sealed with Parafilm. After 24 h, the cell suspension from each of the wells was aspirated and unattached cells were removed by rinsing three times in 150 µl of sterile deionized water (Aqua Solutions, Inc., Jasper, GA) using a hand held multichannel pipettor (Labnet International, Inc., Woodbridge, N.J.) The rinse was removed by inverting the plates over paper towels in a placed in a small tub and the plates were subsequently dried in an inverted position for 30 min. Biofilms were stained by adding 150 µl of a 1% crystal violet solution, aqueous (LabChem Inc., Pittsburgh, PA), to each well and the plates were then incubated for 45 min at room temperature. Unbound dye was removed by rinsing three times in 150 µl of sterile water. At this point, biofilms were visible as purple rings formed on the side of each well. The crystal violet was solubilized by the addition of 150 µl of 95% ethanol and incubating the plates at 4°C for 30 min. From the contents of each well, 100 µl were transferred to a new microtiter plate, and the optical density of the cell turbidity of each well was recorded using a microtiter plate reader (Spectramax Plus 384; Molecular Devices, Sunnyvale, Calif.) at 595 nm ($OD_{595}$). The quantitative measurement of biofilm was obtained by subtracting the average OD of the control wells from the OD of all test wells. The microtiter plate biofilm assay was performed three times for all *L. monocytogenes* strains, and the averages and standard deviations were calculated for all replications of the experiment.

**Microtiter plate assay for the assessment of *L. monocytogenes* attachment**

Each *L. monocytogenes* strain was grown in 10 ml of TSBYE (high nutrient medium), at 32°C for 18 h. The culture was then streaked on LSA, and 1ml inoculum from each of the defined cultures was inoculated in 10 ml TSB, which was subsequently incubated at 25°C for 24

h. At the end of the incubation period, the culture was centrifuged; the supernatant was removed, and the concentrated cells were added to 10 ml of PBS [pH:7.2] or 10 ml PBS enriched with calcium chloride ($CaCl_2$) solution (2.2mM solution) and vortexed. The pH of the $CalCl_2$ enriched PBS was adjusted to 6.8 (Model 720A, Orion Research Inc., Boston, MA) using 3N HCl (J.T. Baker, Phillipsburg, NJ). The OD was standardized for all the suspensions. Attachment of *Listeria* isolates was determined using a cell population with an $OD_{595}$ of 0.678 - 0.783 which is equivalent to a count of $6x10^8$ to $10^9$ cfu/ml cells. One hundred and fifty microliter volume of cell suspensions were transferred into 96-well sterilized PVC microtiter plates, eight wells per isolate. Each plate included eight wells of media without *L. monocytogenes* as controls. Plates were incubated at 25°C for 2 h. After 2 h the cell suspension from each of the wells was aspirated, and the quantitative analysis of biofilm production was conducted by the protocol as described in case of microtiter plate assay for the biofilm study. This microtiter plate assay was performed three times as well for all *L. monocytogenes* isolates, and the averages and standard deviations were calculated.

**Biofilm and attachment study for *L. monocytogenes* strains on stainless steel using epifluorescent microscope**

***Cleaning of stainless steel coupons*** SS coupons 10 $cm^2$ (5 cm x 2 cm, type 304, No.4 finish) were placed in individual 25 ml test tubes, containing 10% Microsoap® solution [alkali based detergent] (Micro™, International Products Corporation, Burlington, NJ). The test tubes containing the coupons were subjected to sonication at 75ºC for 60 min (Aquasonic, model 550HT, VWR Scientific, Atlanta, GA). The Microsoap® solution was then poured off and the coupons were rinsed with deionized water. Acid cleaner [or 15% phosphoric acid] (Zep formula 3586, Zep Manufacturing, Atlanta, GA) was added to test tubes and the sonication was

conducted at 75ºC, allowing exposure to acid for no more than 20 min. The coupons inside the test tubes were then rinsed with deionized water three times. The coupons were then drip dried by flipping the test tube rack, upside-down onto the rack holder. Finally the test tubes (15 cm x 2.5 cm) containing the coupons were autoclaved for 15min at 121ºC.

***Stain preparation:*** Hoechst stain was prepared by add 1 ml of 0.5 mg/ml 2, 2 (4 hydroxyphenyl) 6 benzimidazolyl-trihydrochloride (Hoechst 33258), (Sigma Chemical Co., St. Louis, MO) to 9 ml of sterile /ml water. This 0.05mg/ml concentration of stain served as the working solution.

***Attachment of Listeria isolates to stainless steel:*** All the 27 strains of *L. monocytogenes* were activated from frozen beads (Microbank™, Prolab Diagnostics, Austin, TX) by inoculating them in 10 ml TSBYE for 18 h at 32ºC. The culture was then streaked on LSA; and 1 ml inoculum from each of the cultures was inoculated into 10 ml TSB at 25°C for 24 h. At the end of the incubation period, the culture was centrifuged, the supernatant was removed, and the concentrated cells were added to 40 ml of PBS [pH: 7.2]. The OD (absorptivity) was adjusted for all the culture solutions to 0.2 at λ (wavelength) = 600nm (actual average OD was 0.211). The average cell count for the 40 ml PBS suspension containing the concentrated cells was 2.63 x $10^8$ cfu/ml. The SS coupons, in duplicate, were then immersed in the culture suspension in individual test tubes, followed by incubation for 20 min at 25ºC. The coupons were then rinsed with sterile deionized water to remove the unattached cells. After rinsing, the SS coupons were stained with 50 ug/ml concentration of Hoechst 33258 (DNA binding fluorescent stain) and incubated in dark for 20 min.

The coupons were then rinsed with sterile deionized water to wash off the residual stain and air dried in the dark. The attachment of *Listeria* cells on SS coupons was observed under

Nikon Eclipse 600E fluorescent microscope at 400X magnification [area of the microscopic field under observation was 0.03872mm$^2$]. The images were captured using Optronics CCD camera (Southern Micro Instruments, Marietta, GA). Magnafire software was used to capture the microscopic field on to the monitor of the computer [10 fields per coupon]. Images of the attached cells were subsequently processed using the software program, Image-Pro® plus (Media Cybernetics®, Silver Spring, MD). After the contrast enhancement the cells were counted using the automated counter in the software. The cells were colored, representing the individual cells attached to the black background representing the SS. Images were analyzed for the attached cells for each field observed. The observations for each isolate were obtained using duplicate SS samples and the experiment was repeated thrice for each *L. monocytogenes* isolate for a total of 60 images per culture. The averages and standard deviations were calculated for all replications of the experiment. The cell count obtained for each field area was calculated in terms of cell count per cm$^2$ of the SS coupon by multiplying the average cell count for each replication with a magnification factor. Statistical analysis for the attachment data by different isolates using GLM by 1 way ANOVA and Duncan's Multiple Range Test with statistically significant differences ($\alpha$=0.05) was performed using statistical analysis software 8.2 [SAS®] (SAS Institute Inc., Cary, NC).

***Biofilm formation on stainless steel*** All 27 strains of *L. monocytogenes* were activated from frozen beads (Microbank™, Prolab Diagnostics, Austin, TX) by inoculating them in 20 ml of TSBYE (sufficient to completely immerse the SS coupons) for 18 h at 32ºC. The SS coupons were then immersed in the activated culture, followed by incubation for 4 h at 25ºC. The coupons were then rinsed with sterile deionized water to remove the unattached cells, followed by further incubation of the coupons in diluted TSB (1:10) for 48 h at 25ºC to allow biofilm

development. The coupons were then rinsed with sterile deionized water to remove the unattached cells. After rinsing, the SS coupons were stained with 50 ug/ml concentration of Hoechst 33258 (DNA binding fluorescent stain) and incubated in dark for 20 min.

The coupons were then rinsed with sterile deionized water to wash off the residual stain and air dried in the dark. The biofilm formed by the *Listeria* isolates on the SS coupons was observed and the data was collected as previously described in the determination of attachment of *Listeria* isolates on SS coupons. Images of the biofilms were subsequently converted into black and white pixels using Adobe Photoshop 7.0 software (Adobe Photoshop, Adobe System Inc, San Jose, CA). The cells were white, representing the percent area covered by the biofilm and SS was represented by black pixels. Images were analyzed for the percentage area covered by the biofilm using the software program, image tool (University of Texas Health Science, San Antonio, TX). Each observation for each culture isolate used at least duplicate SS samples and the experiment was repeated thrice for each *L. monocytogenes* isolate for a total of 60 images per culture treatment. All work except for the microscopic observation was done in a class II biological cabinet. Statistical analysis on the data obtained was conducted as previously described in the analysis for attachment of *Listeria* isolates on SS surface.

# CHAPTER 4

# RESULTS

## Attachment of *L. monocytogenes* isolates to microtiter plates

The attachment all the *L. monocytogenes* isolates under investigation was similar on hydrophobic and hydrophilic surfaced microtiter plates and in phosphate buffer saline and calcium chloride enriched phosphate buffer saline used as the suspension media. The attachment for the *L. monocytogenes* isolates on hydrophobic surfaced microtiter plates expressed in terms of O.D. at 595nm ranged from 0.064 to 0.509 with a standard deviation of 0.013 to 0.225 while using PBS as the suspension medium (Fig. 1) and O.D. of 0.057 to 0.564 with a standard deviation of 0.008 to 0.252in case of $CaCl_2$ enriched PBS as the suspension medium (Fig. 2). In case of hydrophilic microtiter plates, the O.D. when using PBS as the suspension medium ranged from 0.014 to 0.167with standard deviation from 0.012 to 0.104 (Fig. 3) and an O.D. range of 0.035 to 0.22with standard deviation ranging from 0.011 to 0.062when using $CaCl_2$ enriched PBS as the suspension medium (Fig. 4). Supplementing PBS with $CaCl_2$ did not affect attachment on hydrophobic and hydrophilic surfaces.

## Attachment of *L. monocytogenes* isolates to stainless steel surface

Attachment for all the *L. monocytogenes* isolates on stainless steel surface using PBS as the suspension medium was similar as observed by quantitative epifluorescent microscopic analysis. The attachment of the *L. monocytogenes* isolates on the SS surface expressed in terms of cell counts ranged from $1.74x10^5$ to $1.47x10^6$ cells per $cm^2$ area of the SS surface with standard deviation ranging from $4.41x10^4$ to $7.67x10^5$ (Fig. 5).

**Biofilm formation by *L. monocytogenes* isolates on microtiter plates**

The biofilm formation on microtiter plates was similar for all isolates on both hydrophobic and hydrophilic microtiter plates except for one persistent isolate, 311 from genotype J which showed significantly high biofilm formation ($p<0.05$) in low (diluted TSB) as well as high nutrient conditions (TSB) and on hydrophobic and hydrophilic surfaces. The biofilm formation by the isolates on hydrophobic surface was higher as compared to that on hydrophilic surface in both low nutrient (1:10 dilution of TSB) and nutrient rich medium (TSB). The biofilm formation by the highest biofilm forming isolate (O.D.=1.622) was 5.5 times that of the next highest biofilm forming isolate (O.D.=0.297) on hydrophobic plates using 10%TSB. On hydrophilic plates using 10%TSB and under the same incubation conditions the biofilm formation of the same highest biofilm former (in this case O.D.= 0.177) was just 1.2 times that of the next highest biofilm forming isolate (O.D.=0.153) (Fig. 5+6). The biofilm formation for the *L. monocytogenes* isolates on hydrophobic microtiter plates expressed in terms of O.D. at 595nm ranged from 0.057 to 1.622 with a standard deviation of 0.009 to 0.369 while using 1:10 dilution of TSB as the growth medium (Fig. 6) and O.D. of 0.031 to 0.424 with a standard deviation of 0.003 to 0.383 in case of TSB as the growth medium (Fig. 7). In case of hydrophilic microtiter plates, the O.D. when using 1:10 dilution of TSB as the growth medium ranged from 0.037 to 0.177 with standard deviation from 0.002 to 0.029 (Fig. 8) and an O.D. range of 0.047 to 0.269 with standard deviation ranging from 0.002 to 0.039 when using TSB as the growth medium (Fig. 9).

This indicates that hydrophobic surfaces and the use of low nutrient media are more efficient in depicting the biofilm forming abilities of *L. monocytogenes* strains on a wider scale. This is consistent with the results obtained by Mafu et al. *(67)* who found that even though *L.*

*monocytogenes* has a hydrophilic surface when grown in TSB, it still attaches in greater numbers to some hydrophobic surfaces as compared to hydrophilic ones.

The validity of the microtiter plate assay was assessed using General Linear Model (GLM) procedure by 1 way ANOVA which showed that the cultures were significantly different but the repetitions were not ($p>0.05$) in case of the attachment as well as the biofilm study.

**Biofilm formation by *L. monocytogenes* isolates on stainless steel surface**

Quantitative epifluorescent microscopic analysis of the biofilm formation by the isolates on SS surface using 1:10 dilution of TSB as the growth medium revealed that the biofilm formation for all the *L. monocytogenes* isolates was similar with the exception of one persistent isolate, 311 which formed significantly higher ($p<0.05$) biofilm as compared to the rest of the isolates. The percentage area covered by the biofilm ranged from 0.16% to 3.38% of the SS surface area with standard deviation ranging from 0.03 to 2.08 (Fig. 10).

**Attachment of persistent vs. non-persistent *L. monocytogenes* isolates**

Persistent and non-persistent isolates showed similar attachment ($p>0.05$) on hydrophobic as well as hydrophilic surfaced microtiter plates using PBS and $CaCl_2$ enriched PBS as the suspension medium. Also similar attachment was observed for the persistent and non-persistent isolates by microscopic analysis on SS surface using PBS as the suspension medium (Fig 11, micrographs A and B).

**Biofilm formation by persistent vs. non-persistent *L. monocytogenes* isolates**

Persistent and non-persistent isolates showed similar biofilm formation on hydrophobic as well as hydrophilic surfaced microtiter plates using TSB and 1:10 dilution of TSB as the growth medium except for one persistent isolate, 311 which formed significantly higher ($p<0.05$) biofilm on both surfaces and using TSB as well as 1:10 dilution of TSB. Also similar results

were observed for the persistent and non-persistent isolates by microscopic analysis on SS surface using 1:10 dilution of TSB as the growth medium. Persistent *L. monocytogenes* isolate (311) formed significantly higher ($p<0.05$) biofilm in terms of percentage area covered on the SS surface (Fig 11, micrographs C and D).

**Biofilm formation by *L. monocytogenes* isolates based on genotypes**

Persistent genotype J was significantly higher biofilm former as compared to the rest of the genotypes in low and nutrient rich medium, on hydrophobic as well as hydrophilic surface. Since persistent isolate, 311 from genotype J was an extremely high biofilm former it was treated separately and the mean biofilm formation for the genotypes were compared after excluding isolate 311. After 311 was removed for the analysis, biofilm formation by persistent Genotype J was still significantly higher ($p<0.05$) on hydrophobic surfaced microtiter plates when using 1:10 dilution of TSB as the growth medium. However the biofilm formation for the genotypes was similar on hydrophobic microtiter plates using TSB as the growth medium and on hydrophilic surfaced microtiter plates while using 1:10 dilution of TSB as well as TSB as the growth medium. The mean biofilm formation for the *L. monocytogenes* genotypes on hydrophobic surfaced microtiter plates expressed in terms of O.D. at 595nm ranged from 0.285 to 0.093 with a standard deviation of 0.037 to 0.014 while using 1:10 dilution of TSB as the growth medium (Fig. 12) and O.D. of 0.176 to 0.058 with a standard deviation of 0.093 to 0.012 in case of TSB as the growth medium (Fig. 13). In case of hydrophilic microtiter plates, the O.D. when using 1:10 dilution of TSB as the growth medium ranged from 0.125 to 0.048 with standard deviation from 0.032 to 0.006 (Fig. 14) and an O.D. range of 0.103 to 0.053 with standard deviation ranging from 0.023 to 0.005 when using TSB as the growth medium (Fig. 15). Even while observing the biofilm formation on SS surface, isolate 311 was a significantly high biofilm

former and hence was excluded from the genotype J while calculating the mean biofilm formation by the genotypes. The percentage area covered by the biofilm for the genotypes after excluding 311 ranged from 0.2% to 1.51% of the SS surface area with standard deviation ranging from 0.12 to 1.53 (Fig. 16).

**Correlation of attachment and biofilm production data obtained using microtiter plate assay vs. data from microscopic analysis**

The correlation of the data obtained using the microtiter plate assay vs. the data for the bacterial attachment and biofilm formation on stainless steel was calculated (Table 2). The correlation coefficient for the OD (obtained from the microtiter analysis on hydrophobic plates) was more significant [$p < 0.0001$]) (Fig. 17) than the correlation coefficient for the OD (obtained from the microtiter analysis on hydrophilic plates [$p<0.006$]) (Fig. 18), when correlated with the percentage area covered observations of the biofilm formed on the stainless steel coupons. However, the correlation coefficient was correspondingly statistically significant for both the hydrophilic ($p<0.0002$) (Fig. 19) and hydrophobic plates ($p<0.0001$) (Fig. 20) when the OD values obtained from the microtiter analysis were correlated with the observations from the microscopic analysis for the values obtained in terms of the cell count in case of the bacterial attachment.

**Biofilm formation by *L. monocytogenes* isolates at 10ºC**

The biofilm formation for all the isolates was evaluated for a growth time period of 5 days at 10ºC, but even for the extended incubation time period the biofilm formation, in general, was less than the $OD_{595}$ observed in case of 25ºC. The biofilm formation for one of the isolates from genotype J, 311 was significantly higher ($p<0.05$) than the rest of the isolates as was observed in case of the microtiter analysis of biofilm using TSB and 1:10 dilution of TSB on

hydrophobic and hydrophilic surfaced microtiter plates at 25 ºC. The biofilm formation by the isolates at 10ºC using 1:10 dilution of TSB as the growth medium expressed in terms of O.D. at 595nm ranged from 0.193 to 0.008 and the standard deviation varied from 0.107 to 0.000 (Fig. 21).

**Correlation of the data for attachment vs. biofilm production by *L. monocytogenes* isolates on stainless steel surface as observed by microscopic analysis**

Correlation of the data for attachment vs. biofilm production by *L. monocytogenes* isolates on stainless steel surface as observed by microscopic analysis was significant ($p < 0.008$; $R^2=0.50$). This correlation indicates that there is a weak relationship between the attachment and the degree of biofilm formation by *L. monocytogenes*.

# CHAPTER 5

# DISCUSSION

## Attachment and biofilm formation by persistent and non-persistent *L. monocytogenes* isolates

Cell attachment was determined on microtiter plates over a 2 hour attachment period. Persistent and non-persistent isolates showed similar attachment ($p>0.05$) on both hydrophobic and hydrophilic surfaced microtiter plates using either PBS or $CaCl_2$ enriched PBS as the suspension medium. Also no significant difference ($p>0.05$) in the attachment was observed between persistent and non-persistent isolates on stainless steel surface as observed by quantitative epifluorescent microscopy. This is inconsistent with the results reported by Lunden et al. *(63)* who found that most of the persistent strains (obtained from poultry plants and an ice-cream plants throughout several years) demonstrated higher adherence during short contact times on stainless steel surfaces observed using epifluorescent microscope.

However, wide range of biofilm formation was observed within the persistent isolates. One of the persistent strains, 311 formed significantly higher biofilm ($p<0.05$) on TSB as well as 1:10 dilution of TSB on both, hydrophilic and hydrophobic surfaces. After the microscopic analysis and microtiter assay were completed, *L. monocytogenes* isolates were arranged from the highest to the lowest biofilm formers (Table 3). It was observed that only persistent isolate 311 was a significantly high biofilm former as compared to other persistent and non-persistent isolates. Hence, if 311 is excluded these results are in accordance with those found by some other researchers that persistent isolates are similar in their biofilm forming ability as non-persistent

strains of *Listeria* on PVC microtiter plates and SS surface *(24)*. However some researchers have reported differences in the biofilm forming abilities of persistent and non-persistent strains *(10, 63)*. It was also observed that one of the Lineage I isolates formed statistically significant biofilm formation ($p<0.05$) as compared to the rest of the isolates. However, if the high biofilm forming isolate, 311 was excluded, the mean biofilm formation by Lineage I would not be significantly greater than the mean biofilm formation by Lineage II. Hence it cannot be concluded that Lineage I strains are better biofilm formers than the Lineage II strains some of the Lineage II isolates formed better biofilms than that formed by the Lineage I strains *(24)*. Moreover other researchers have reported that Lineage II strains are better biofilm formers than Lineage I strains *(10)*. The problem of contamination is further exacerbated since persistent isolates may transfer from one plant to another despite stringent hygienic practices followed in the plant area *(64)*.

**Influence of nutrient level on the biofilm formation**

This research focused on the biofilms of pure *L. monocytogenes* isolates. It was observed that the biofilm formation was higher in low nutrient environment (1:10 dilution of TSB). This is consistent with the results obtained by Kim et al *(56)* who found 50-fold higher attachment in 4 hours, on stainless steel using chemically defined minimal medium as compared to TSB (nutrient rich medium). Hence if *L. monocytogenes* isolates find harborage on walls, ceilings, or within condensate (on the exterior of the equipment or lines), it is likely that they would grow under these reduced nutrient conditions and form biofilm. In a food processing environment, *L. monocytogenes* may be exposed to fluctuating levels of nutrients depending upon the location. Most of the isolates (good as well as poor biofilm formers) were obtained from nutrient rich or nutrient variable environments such as raw product, cooked side of equipment and drain of the plant (which will be exposed to high level of nutrients during pre-rinsing of equipment prior to

cleaning and sanitizing). Hence the biofilm and attachment assays were also performed using a nutrient rich medium, TSB. Biofilm formation was found to be substantially less in TSB as compared to that in diluted TSB. However, no significant differences were observed in the attachment of *L. monocytogenes* isolates when the PBS, used as the suspension medium was supplemented with calcium chloride. This is in accordance with the results reported by other researchers, who observed similar attachment of *L. monocytogenes* on hydrophobic and hydrophilic surfaces *(42)*.

**Microtiter plate assay as a screening tool**

Microtiter plates have been used in various attachment and biofilm assays to evaluate a variety of aspects related to bacterial physiology viz. effect of a range of nutrients and osmolarity of the medium on biofilm formation, effect of temperature, and the importance of protein synthesis and their role during biofilm initiation phase *(63, 77, 89, 95)*. These assays are based on the extraction of dye bound to the attached cell mass. The hydrophobic microtiter plate biofilm assay revealed greater differences in the biofilm formation between the *L. monocytogenes* isolates than those observed by hydrophilic microtiter plate biofilm assay. Differences in biofilm formation among the isolates observed by microscopic analysis as well as microtiter assay were statistically significant. However, the greater differences in the amount of biofilm observed between the *Listeria* isolates by microtiter plate assay than those observed my microscopic analysis could be due to the fact that the surface areas observed by each method are different. In the microtiter plate assay the area covered by the cells in an individual well is approximately 192 $mm^2$ while the total area of the 10 fields examined by microscopy on each slide represents only about 0.3872 $mm^2$. Hence microtiter plate assay appeared to detect differences in the biofilm forming ability of *L. monocytogenes* isolates on a larger scale. More consistent results were obtained

(lower standard deviations between replications within an experiment and between experiments) by microtiter plates than with direct microscopy because of the much larger surface area in case of microtiter plates. The large amount of cells stained and extracted minimized the errors, but the microscopic findings are direct and accurate even though they are less precise because of the factors that contribute to the higher standard deviation such as variations in the intensity of the rinsing off of unattached cells on the SS coupons, staining, and field selection on the slides containing the biofilm *(104)*. Significant Pearson correlation coefficients were observed when the attachment and biofilm formation of *L. monocytogenes* isolates on microtiter plates was compared with the attachment and biofilm formation on SS surface. This is concurrent with the results obtained by Djordjevic et al *(24)* who observed significant correlation between microtiter plate assay and microscopic coverage on PVC as well as stainless steel. In the present experiment, it was also found that attachment and biofilm formation on hydrophobic (non-coated) microtiter plates was much greater as compared to the attachment and biofilm formation on the hydrophilic microtiter plates (tissue culture treated). Previous findings have their results for attachment and biofilm formation based just on hydrophobic PVC microtiter plates *(24, 63)*. In conclusion, microtiter plate assay using hydrophobic microtiter plates and low nutrient medium can provide rapid and reproducible screening for the differences between biofilm forming abilities of strains.

Table 1: Description of *L. monocytogenes* isolates[a] detected in a chicken further processing plant.

| Isolate | Genotype | Source of detection | Lineage | Persistent or Not |
|---|---|---|---|---|
| 235 | A | Drain of the plant | I | Persistent[b] |
| 398 | A | Drain of the plant | I | Persistent |
| 233 | B | Raw product | I | Non-persistent[c] |
| 239 | C | Drain of the plant | II | Persistent |
| 395 | C | Drain of the plant | II | Persistent |
| 52 | C | Cooked side of the equipment | II | Persistent |
| 85 | C | Cooked side of the AC drain | II | Persistent |
| 99 | C | Shipping and receiving condensate squeegee | II | Persistent |
| 102 | C | Cooked side squeegee | II | Persistent |
| 105 | C | Shipping and receiving drain | II | Persistent |
| 279 | C | Drain of the plant | II | Persistent |
| 309 | C | Cooked side of equipment | II | Persistent |
| 341 | C | Drain of the plant | II | Persistent |
| 381 | C | Raw product | II | Persistent |
| 251 | D | Raw product | I | Persistent |
| 377 | D | Drain of the plant | I | Persistent |
| 254 | F | Drain of the plant | II | Persistent |
| 386 | F | Drain of the plant | II | Persistent |
| 288 | H | Drain of the plant | II | Persistent |
| 406 | H | Puddle on floor | II | Persistent |
| 267 | I | Raw product | II | Non-persistent |
| 311 | J | Raw product | I | Persistent |
| 80 | J | Drain of the plant | I | Persistent |
| 82 | J | Floor line | I | Persistent |
| 83 | J | Floor line | I | Persistent |
| 370 | M | Raw product | II | Non-persistent |
| 369 | S | Raw product | -- | Non-persistent |

**a-** Isolates were obtained from Dr. Mark E. Berrang, USDA ARS, Russell Research Center, Athens, GA.

**b-** Strains that were re-isolated from multiple sites from the chicken further processing plant over the 12 month sampling period were termed as persistent isolates (sampling was conducted every 6 weeks over the course of the 1 year sampling period).

**c-** Strains that were isolated only once from the chicken further processing plant over the 12 month sampling period were termed as non-persistent isolates (sampling was conducted every 6 weeks over the course of the 1 year sampling period).

Table 2: Correlation of data from microtiter plate assay with that obtained by microscopic analysis for determining the difference in the degree of attachment[a] and biofilm formation[b] by *L. monocytogenes* isolates.

| Description | Correlation coefficient ($R^2$) | Significance level |
|---|---|---|
| Biofilm analysis : Hydrophobic microtiter plates vs. SS[c] surface | 0.76 | $p<0.0001$ |
| Biofilm analysis: Hydrophilic microtiter plates vs. SS surface | 0.51 | $p<0.006$ |
| Attachment analysis: Hydrophobic microtiter plates vs. SS surface | 0.87 | $p<0.0001$ |
| Attachment analysis: Hydrophilic microtiter plates vs. SS surface | 0.81 | $p<0.0002$ |

**a-** Attachment of *L. monocytogenes* isolates in microtiter plates was allowed in phosphate buffer saline (pH: 7.2) after incubation for 2 hours at 25ºC. Microscopic examination of the attachment of *L. monocytogenes* isolates on SS coupons was conducted in phosphate buffer saline (pH: 7.2) after incubation for 20 minutes at 25ºC.

**b-** Biofilm formation by *L. monocytogenes* isolates in microtiter plates was allowed in 1:10 dilution of TSB after incubation for 24 hours at 25ºC. Microscopic examination of the biofilm production by *L. monocytogenes* isolates on SS coupons was conducted in 1:10 dilution of TSB after incubation for 48 hours 25ºC.

**c-** Stainless steel

Table 3: Ranking of *L. monocytogenes* isolates by the degree of biofilm formation as measured by the microtiter plate assay[a] and the microscopic examination[b] of SS coupons based on the Duncan grouping.

| Ranking | 1:10 dilution of TSB using SS[c] coupons | TSB using TCT[d] Microtiter plates | 1:10 dilution of TSB using TCT Microtiter plates | TSB using UT[e] Microtiter plates | 1:10 dilution of TSB using UT Microtiter plates |
|---|---|---|---|---|---|
| 1 | 311 | 311 | 311 | 311 | 311 |
| 2 | 82 | 239 | 83 | 80 | 83 |
| 3 | 398 | 395 | 82 | 83 | 82 |
| 4 | 267 | 52 | 233 | 267 | 80 |
| 5 | 288 | 80 | 370 | 369 | 406 |
| 6 | 239 | 82 | 80 | 395 | 288 |
| 7 | 406 | 105 | 254 | 251 | 233 |
| 8 | 235 | 381 | 235 | 82 | 395 |
| 9 | 369 | 83 | 395 | 377 | 267 |
| 10 | 83 | 341 | 105 | 370 | 235 |
| 11 | 80 | 309 | 406 | 386 | 239 |
| 12 | 102 | 102 | 341 | 233 | 105 |
| 13 | 254 | 275 | 386 | 235 | 251 |
| 14 | 105 | 406 | 99 | 406 | 377 |
| 15 | 309 | 267 | 275 | 239 | 398 |
| 16 | 251 | 85 | 288 | 102 | 369 |
| 17 | 279 | 370 | 239 | 309 | 254 |
| 18 | 341 | 377 | 398 | 341 | 102 |
| 19 | 233 | 369 | 381 | 52 | 341 |
| 20 | 395 | 233 | 102 | 398 | 370 |
| 21 | 381 | 386 | 267 | 105 | 386 |
| 22 | 386 | 251 | 251 | 381 | 381 |
| 23 | 377 | 99 | 369 | 85 | 275 |
| 24 | 370 | 235 | 377 | 275 | 85 |
| 25 | 52 | 254 | 309 | 288 | 52 |
| 26 | 99 | 398 | 85 | 99 | 309 |
| 27 | 85 | 288 | 52 | 254 | 99 |

**a-** Time allowed for biofilm formation in microtiter plates was 24 hours at 25°C.
**b-** Time allowed for biofilm formation in microtiter plates was 24 hours at 25°C.
**c-** stainless steel
**d-** Tissue culture treated (hydrophilic)
**e-** Untreated (hydrophobic)

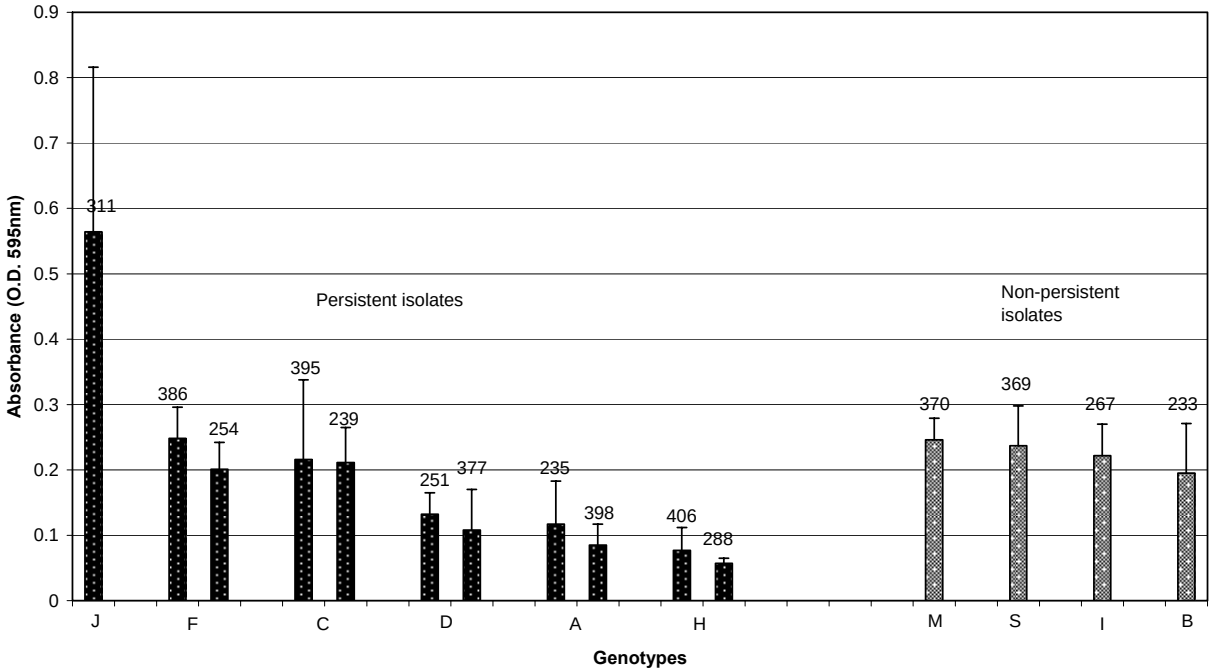


Figure 1: Attachment of *L. monocytogenes* isolates in phosphate buffer saline (pH: 7.2) incubated for 2 hours at 25ºC on hydrophobic microtiter plates. Isolate numbers are given on the bars. Degree of attachment (cell mass) was determined by the absorbance of crystal violet dye.

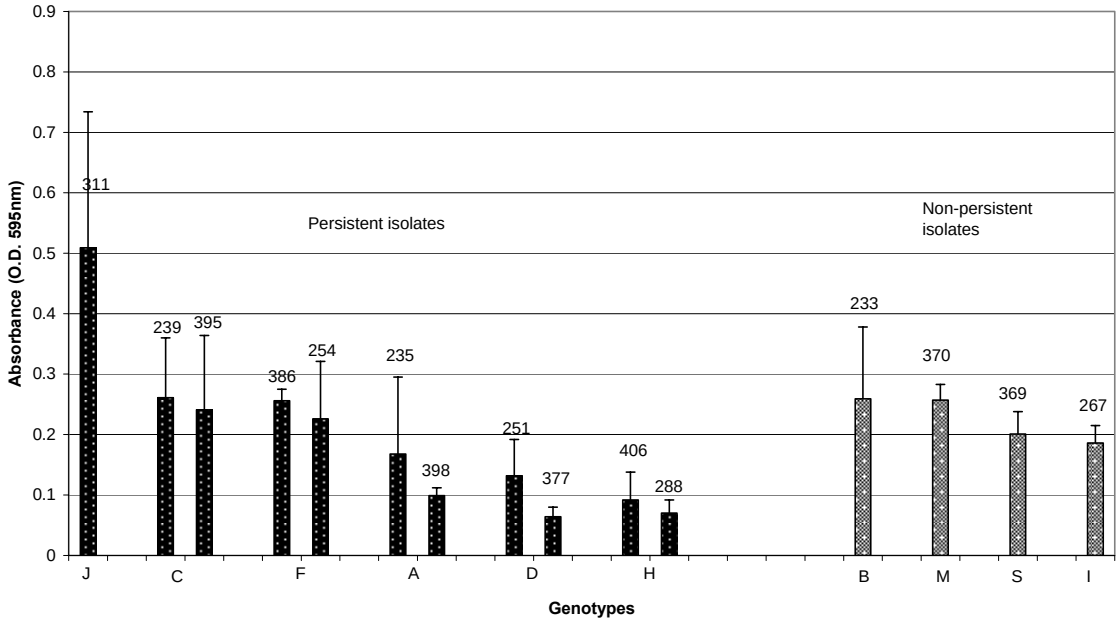


Figure 2: Attachment of *L. monocytogenes* isolates in 2.2mM $CaCl_2$ enriched phosphate buffer saline (pH: 6.8) incubated for 2 hours at 25ºC on hydrophobic microtiter plates. Isolate numbers are given on the bars. Degree of attachment (cell mass) was determined by the absorbance of crystal violet dye.

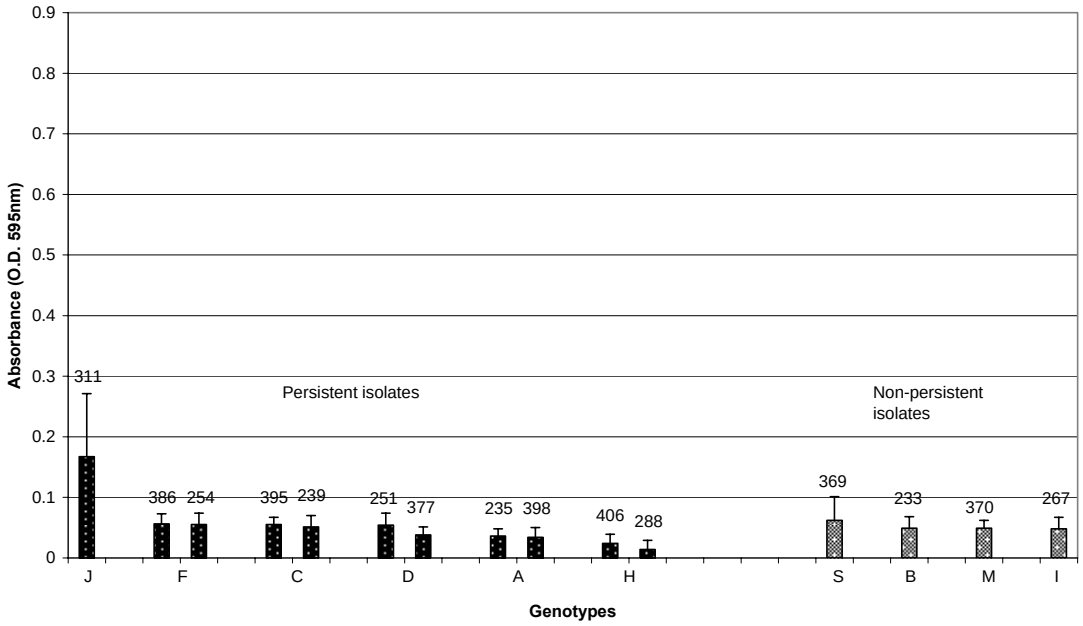


Figure 3: Attachment of *L. monocytogenes* isolates in phosphate buffer saline (pH: 7.2) incubated for 2 hours at 25ºC on hydrophilic microtiter plates. Isolate numbers are given on the bars. Degree of attachment (cell mass) was determined by the absorbance of crystal violet dye.

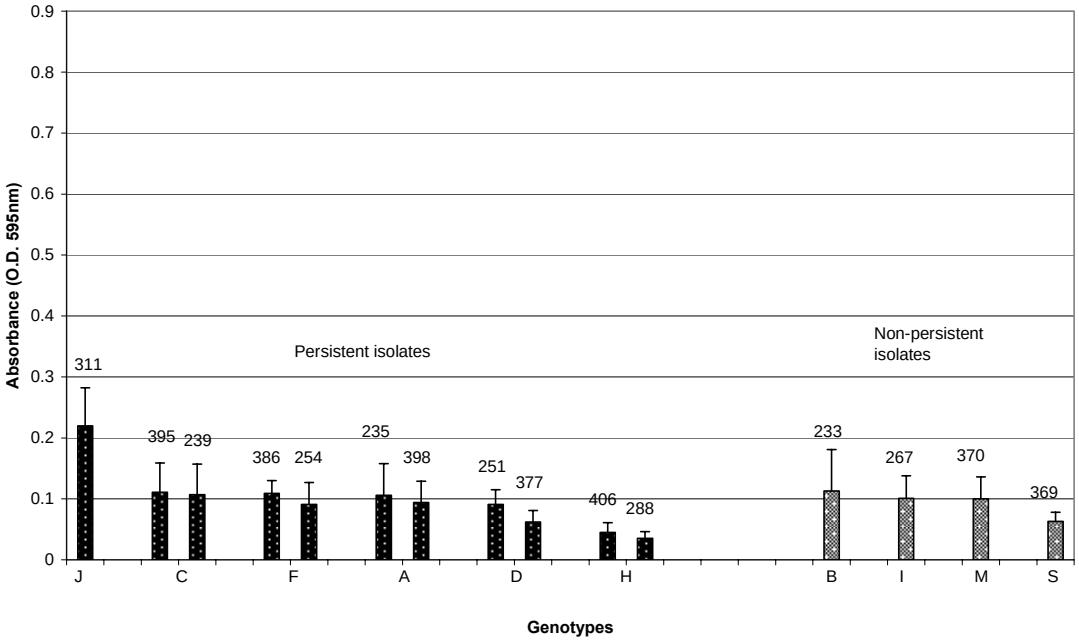


Figure 4: Attachment of *L. monocytogenes* isolates in 2.2mM $CaCl_2$ enriched phosphate buffer saline (pH: 6.8) incubate for 2 hours at 25ºC on hydrophilic microtiter plates. Isolate numbers are given on the bars. Degree of attachment (cell mass) was determined by the absorbance of crystal violet dye.

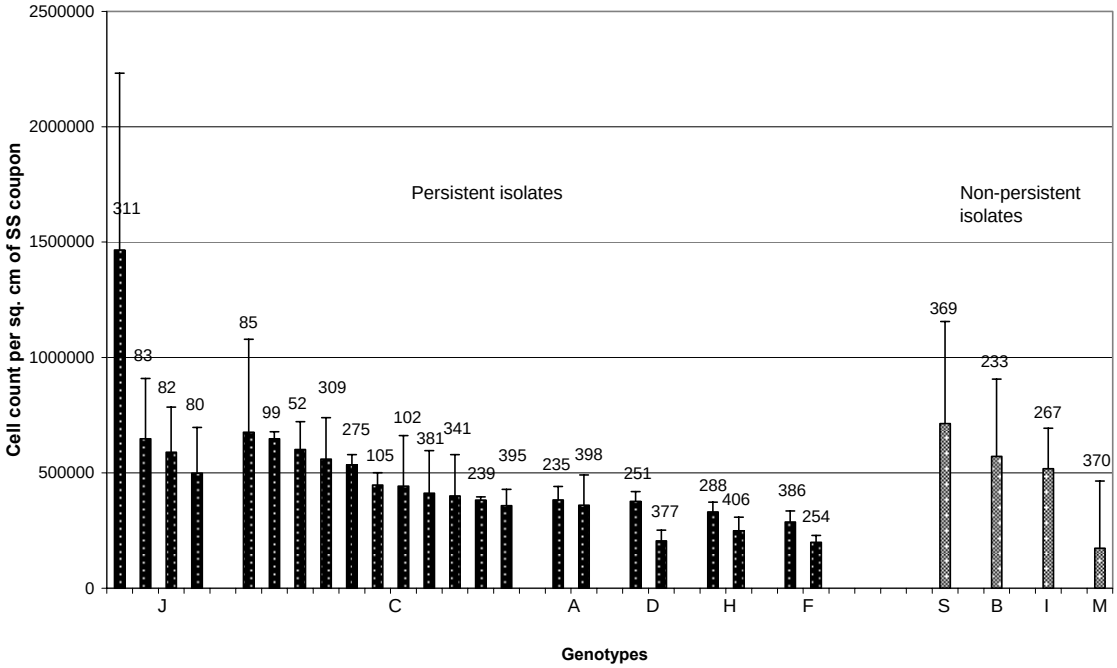


Figure 5: Attachment of *L. monocytogenes* isolates in phosphate buffer saline (pH: 7.2) incubated for 20 minutes at 25ºC on stainless steel coupons. Isolate numbers are given on the bars. Degree of attachment (cell mass) was determined by quantitative epifluorescent microscopy.

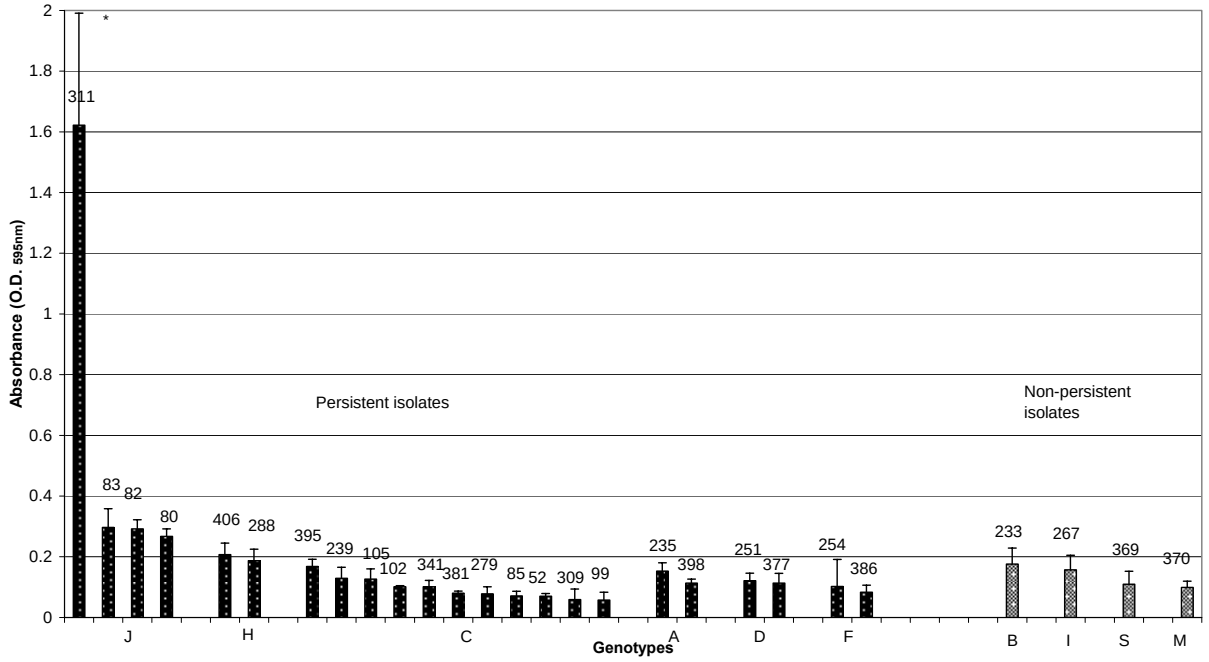


Figure 6: Biofilm formation by *L. monocytogenes* isolates on hydrophobic microtiter plates in 1:10 dilution of TSB incubated at 25ºC for 24 hours. Isolate numbers are given on the bars. Amount of biofilm (cell mass) was determined by the absorbance of crystal violet dye.

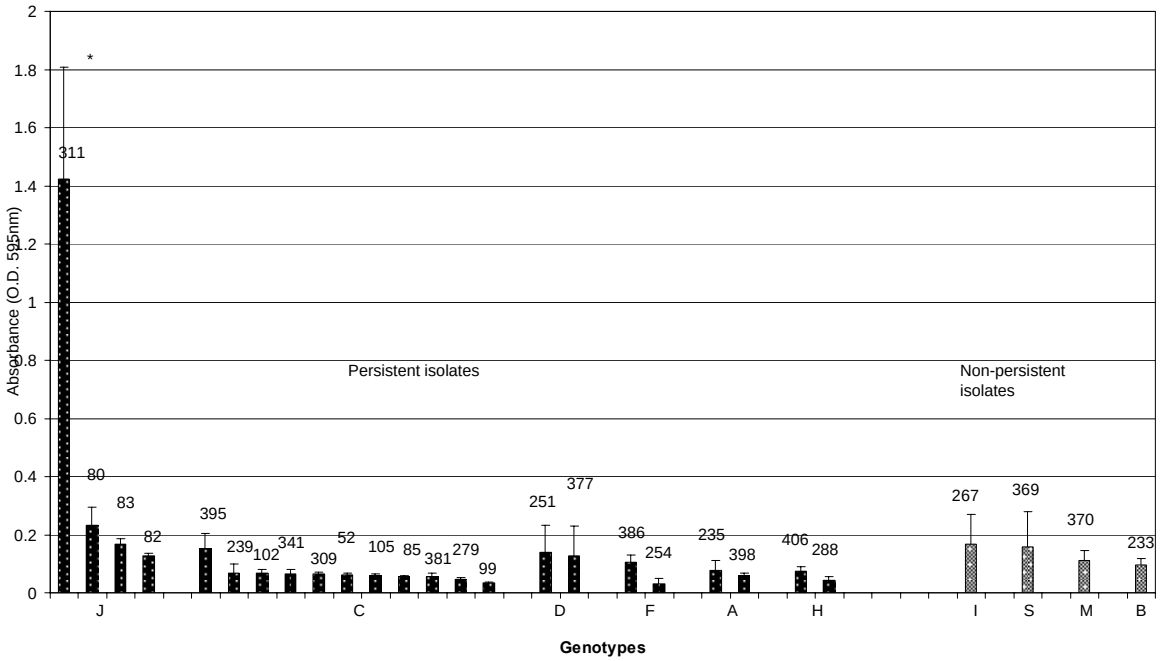


Figure 7: Biofilm formation by *L. monocytogenes* isolates on hydrophobic microtiter plates in TSB incubated at 25ºC for 24 hours. Isolate numbers are given on the bars. Amount of biofilm (cell mass) was determined by the absorbance of crystal violet dye.

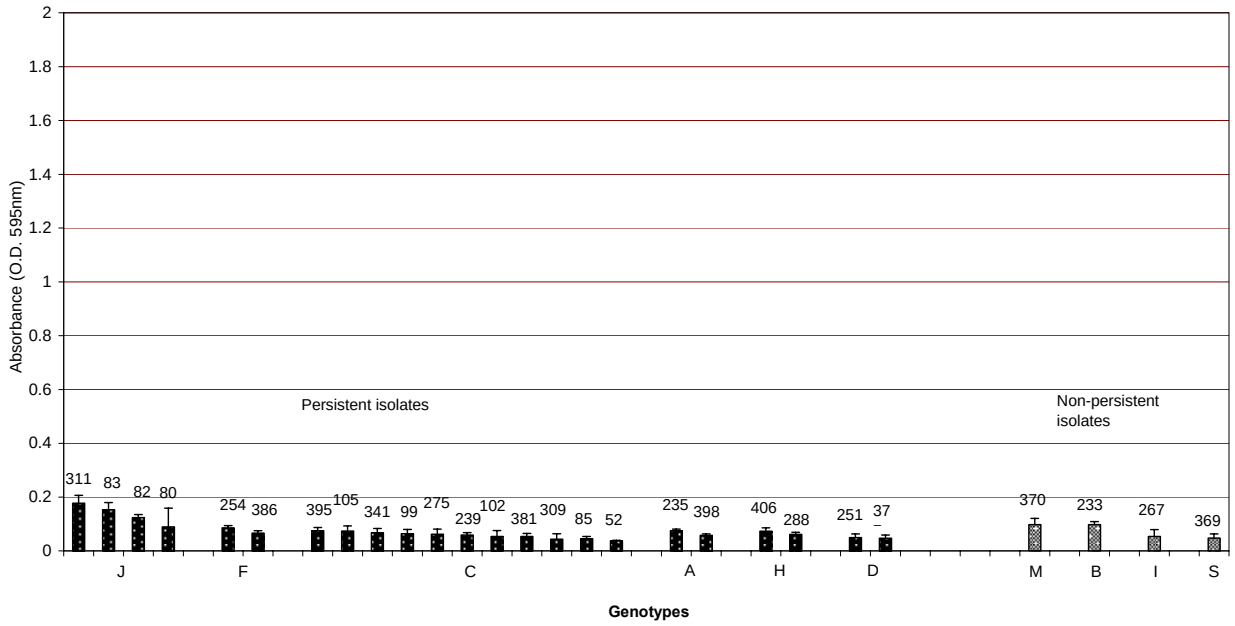


Figure 8: Biofilm formation by *L. monocytogenes* isolates on hydrophilic microtiter plates in TSB incubated at 25ºC for 24 hours. Isolate numbers are given on the bars. Amount of biofilm (cell mass) was determined by the absorbance of crystal violet dye.

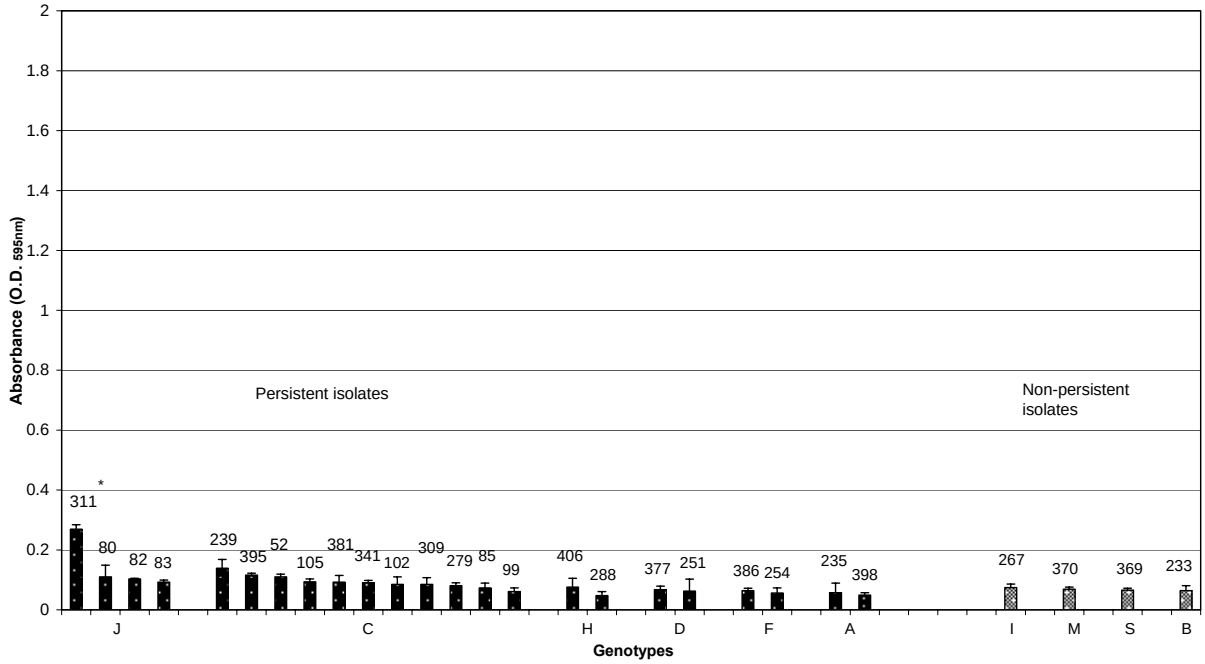


Figure 9: Biofilm formation by *L. monocytogenes* isolates on hydrophilic microtiter plates in 1:10 dilution of TSB incubated at 25ºC for 24 hours. Isolate numbers are given on the bars. Amount of biofilm (cell mass) was determined by the absorbance of crystal violet dye.

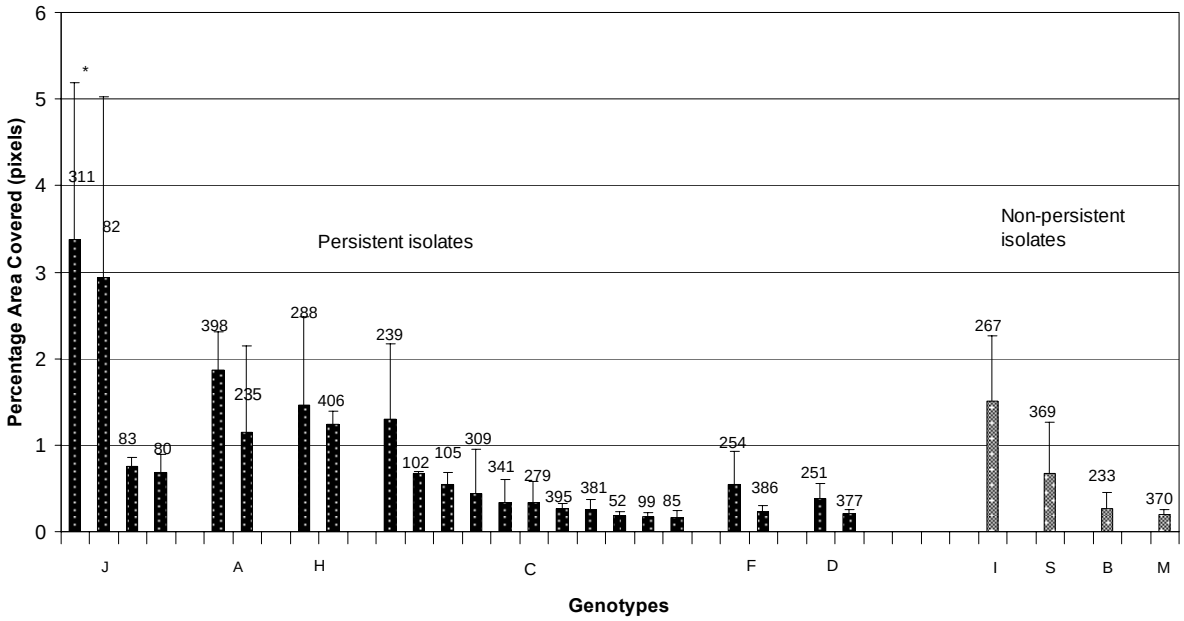


Figure 10: Biofilm formation by *L. monocytogenes* isolates on stainless steel surface in 1:10 dilution of TSB incubated at 25ºC for 48 hours. Isolate numbers are given on the bars. Amount of biofilm (cell mass) was determined by epifluorescent microscopy.

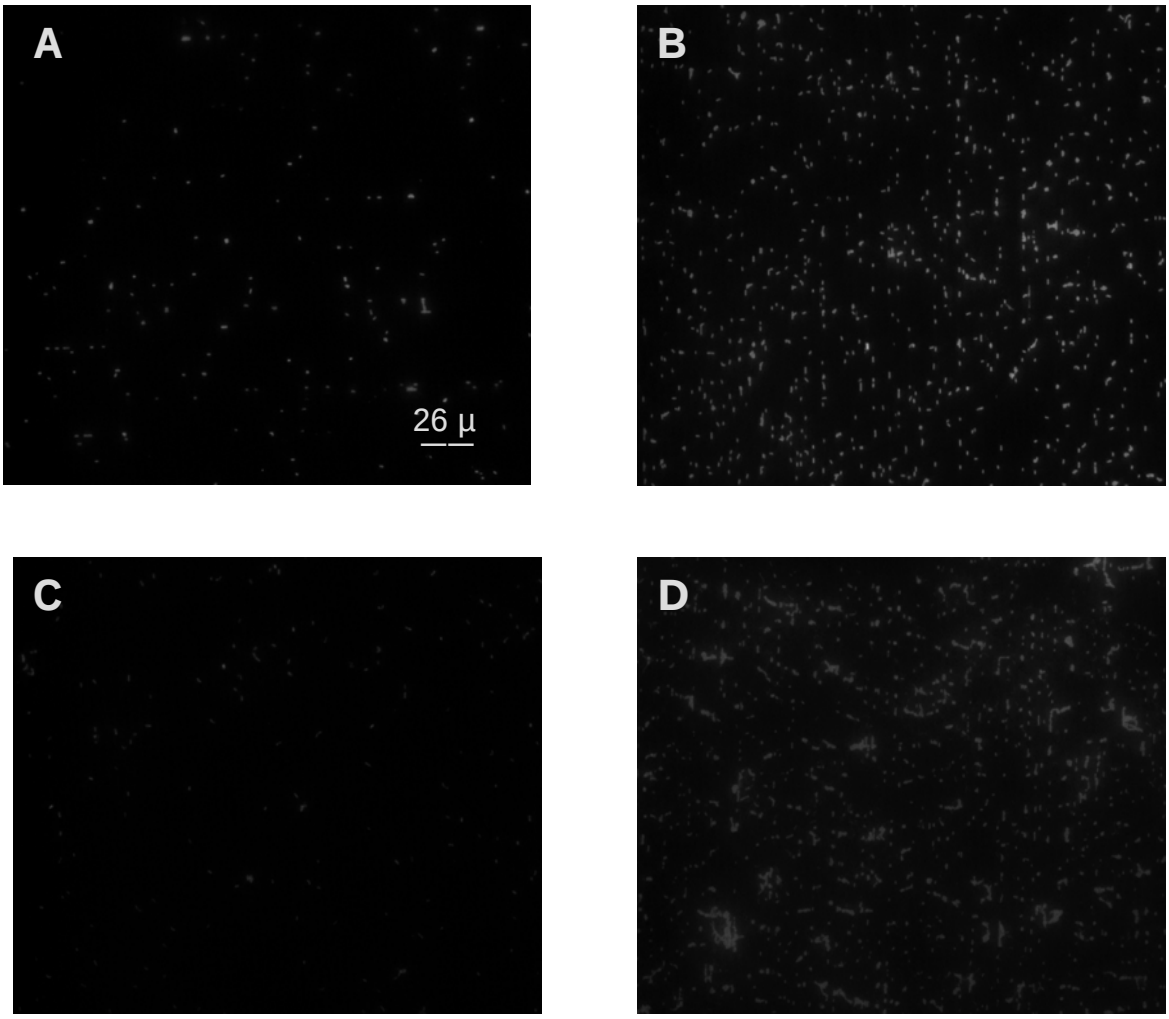


Figure 11: Photomicrographs of attachment and biofilm formation by *L. monocytogenes* isolates on stainless steel surface. Cells were stained with Hoescht. A represents attachment of *L. monocytogenes* isolate 288; B represents attachment of *L. monocytogenes* isolate 311; C represents biofilm formation by *L. monocytogenes* isolate 288 and D represents biofilm formation by *L. monocytogenes* isolate 311

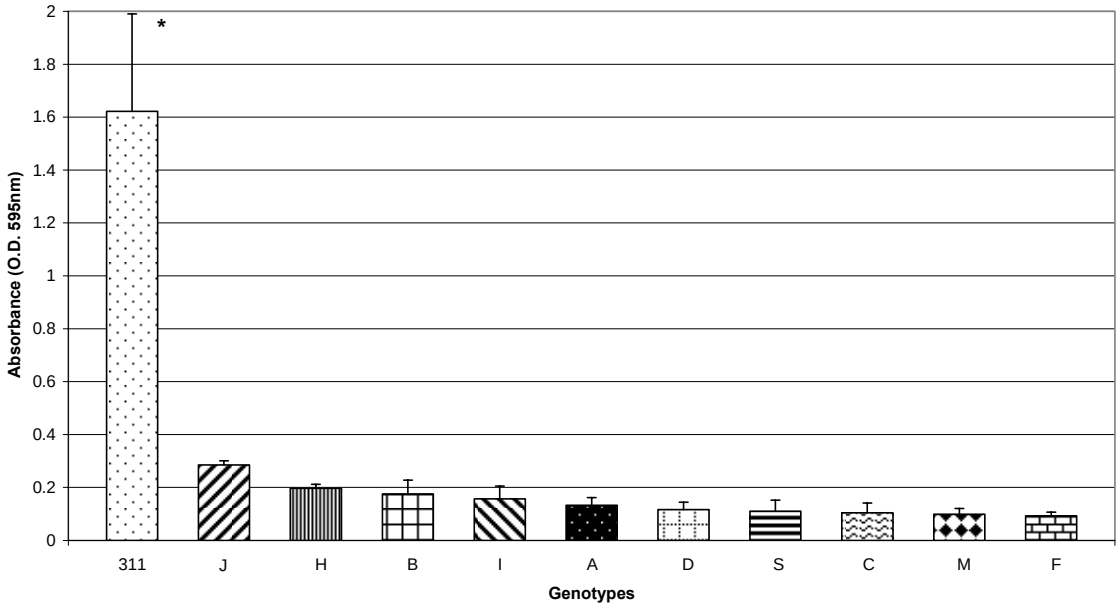


Figure 12: Mean biofilm production by *L. monocytogenes* isolates on hydrophobic microtiter plates in 1:10 dilution of TSB incubated at 25ºC for 24 hours based on genotypes. Amount of biofilm (cell mass) was determined by the absorbance of crystal violet dye. {311 is a high biofilm forming isolate from Genotype J}

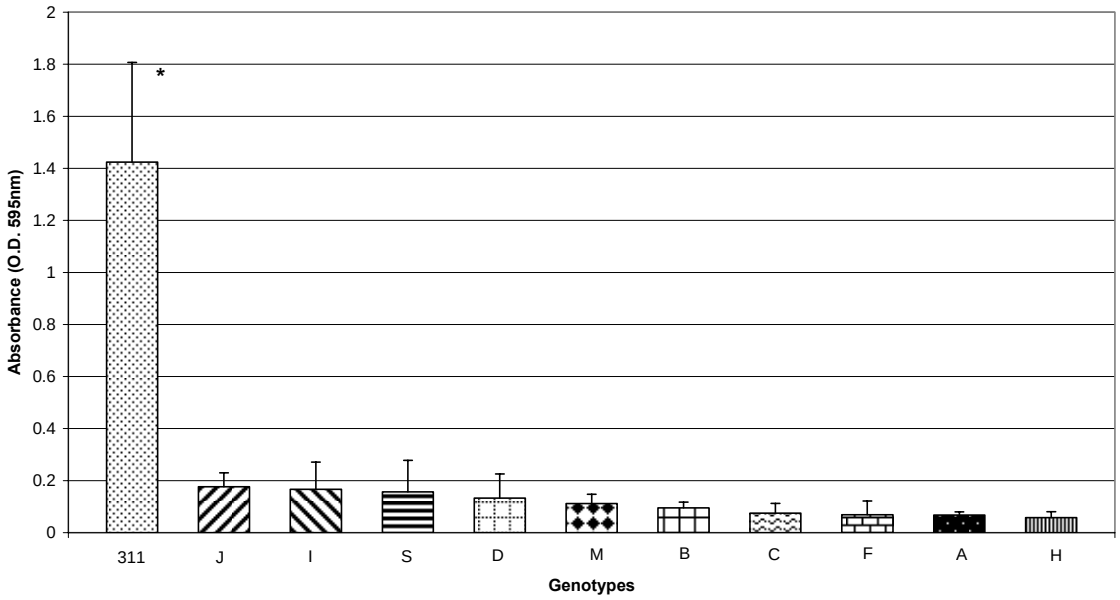


Figure 13: Mean biofilm production by *L. monocytogenes* isolates on hydrophobic microtiter plates in TSB incubated at 25ºC for 24 hours based on genotypes. Amount of biofilm (cell mass) was determined by the absorbance of crystal violet dye. {311 is a high biofilm forming isolate from Genotype J}

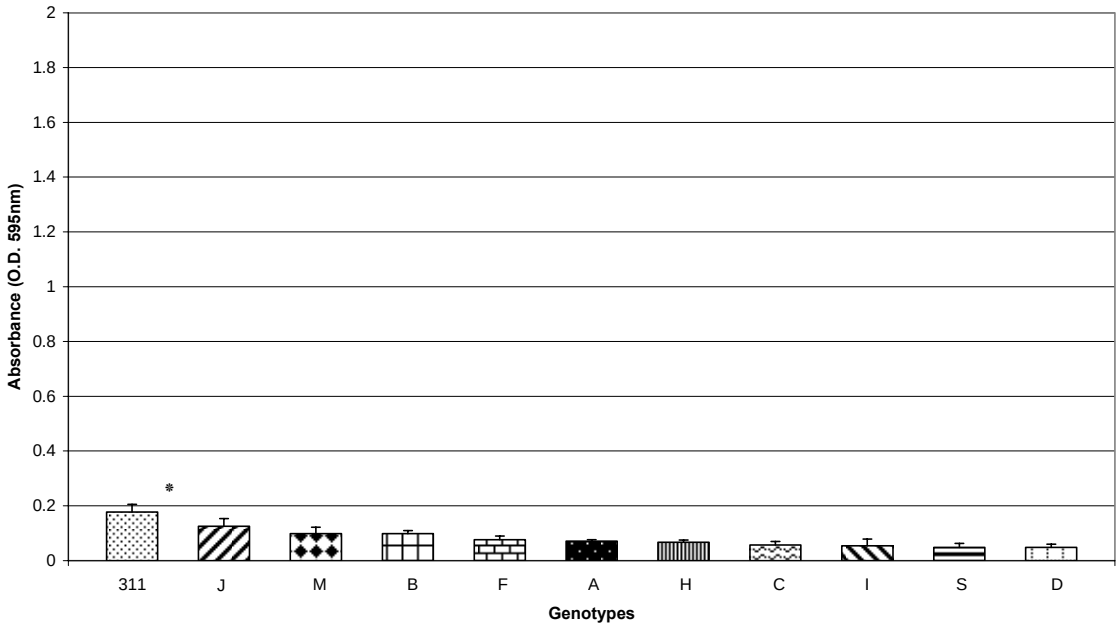


Figure 14: Mean biofilm production by *L. monocytogenes* isolates on hydrophilic microtiter plates in 1:10 dilution of TSB incubated at 25ºC for 24 hours based on genotypes. Amount of biofilm (cell mass) was determined by the absorbance of crystal violet dye. {311 is a high biofilm forming isolate from Genotype J}

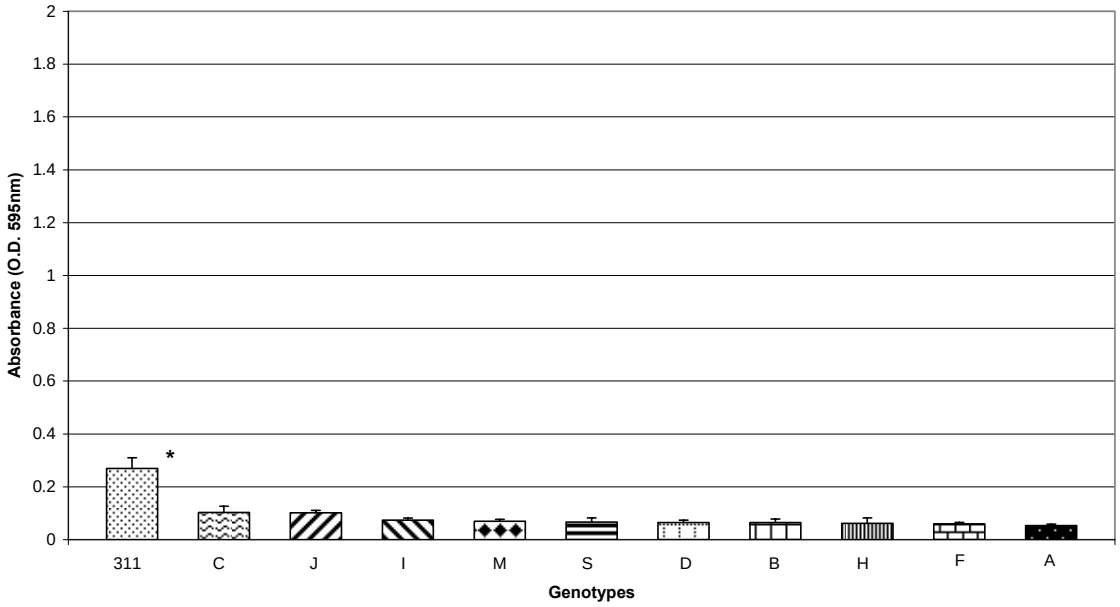


Figure 15: Mean biofilm production by *L. monocytogenes* isolates on hydrophilic microtiter plates in TSB incubated at 25ºC for 24 hours based on genotypes. Amount of biofilm (cell mass) was determined by the absorbance of crystal violet dye. {311 is a high biofilm forming isolate from Genotype J}

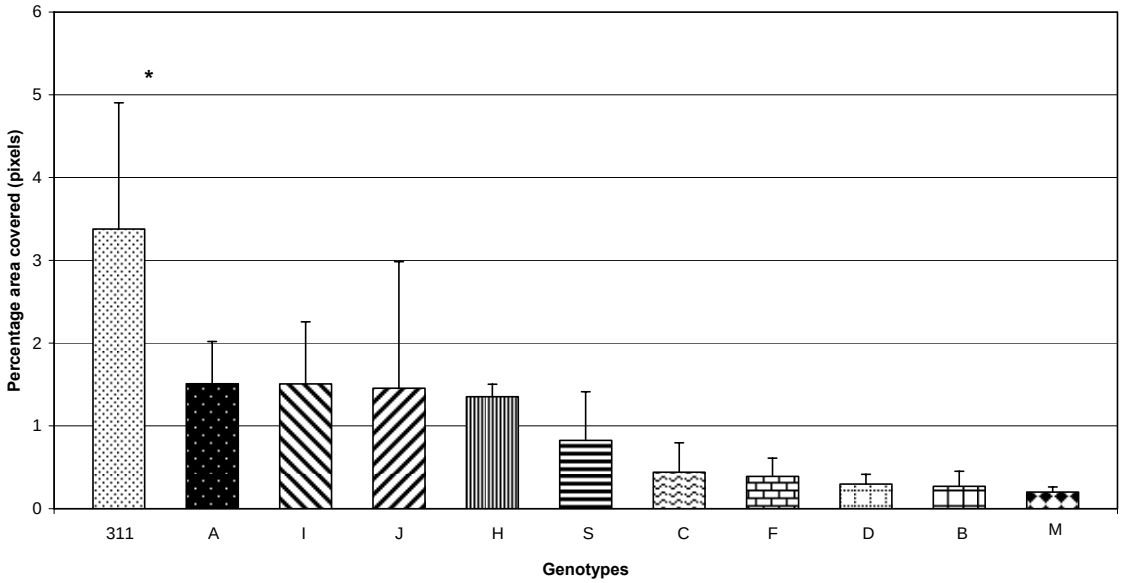


Figure 16: Mean biofilm production by *L. monocytogenes* isolates on stainless steel surface in TSB incubated at 25ºC for 48 hours based on genotypes. Amount of biofilm (cell mass) was determined by epifluorescent microscopy. {311 is a high biofilm forming isolate from Genotype J}

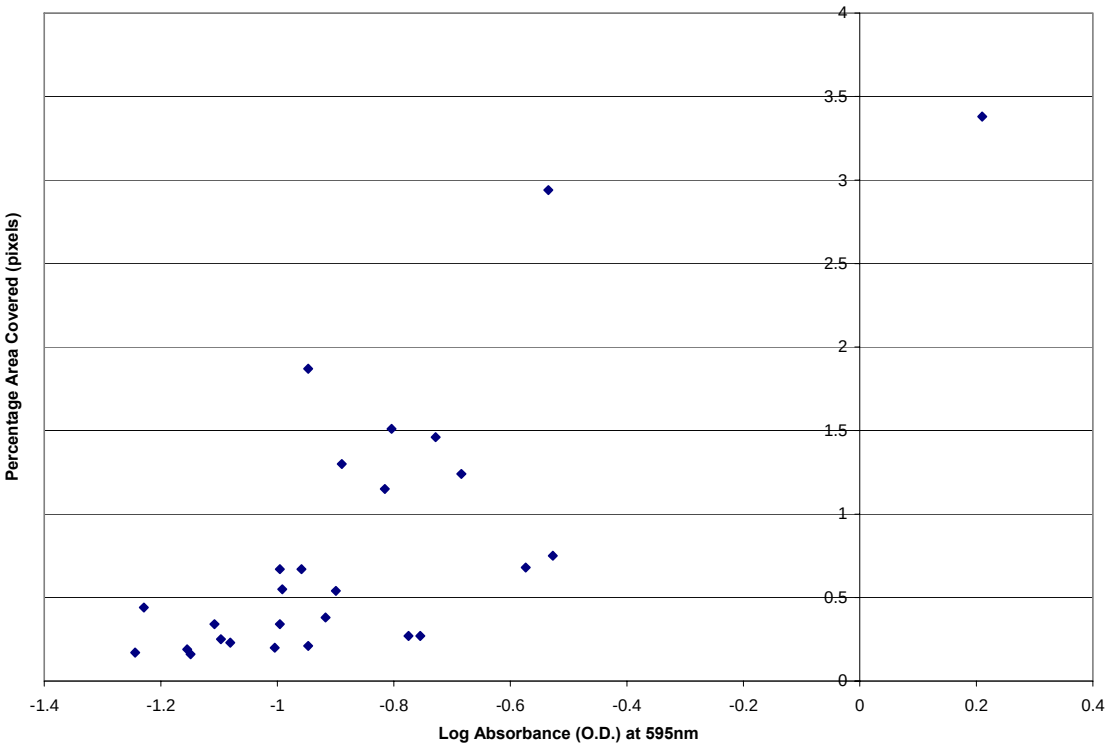


Figure 17: Scatter plot of biofilm formation by *L. monocytogenes* isolates as determined by hydrophobic microtiter plate assay and microscopic analysis using 1:10 dilution of TSB as the growth medium.

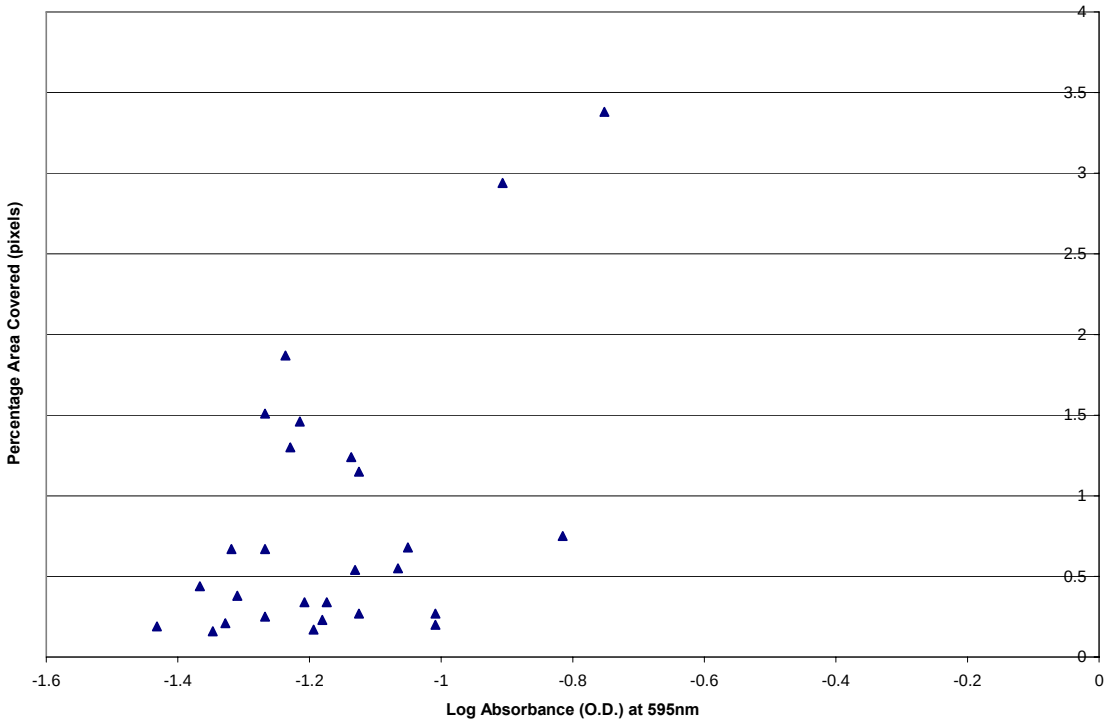


Figure 18: Scatter plot of biofilm formation by *L. monocytogenes* isolates as determined by hydrophilic microtiter plate assay and microscopic analysis using 1:10 dilution of TSB as the growth medium.

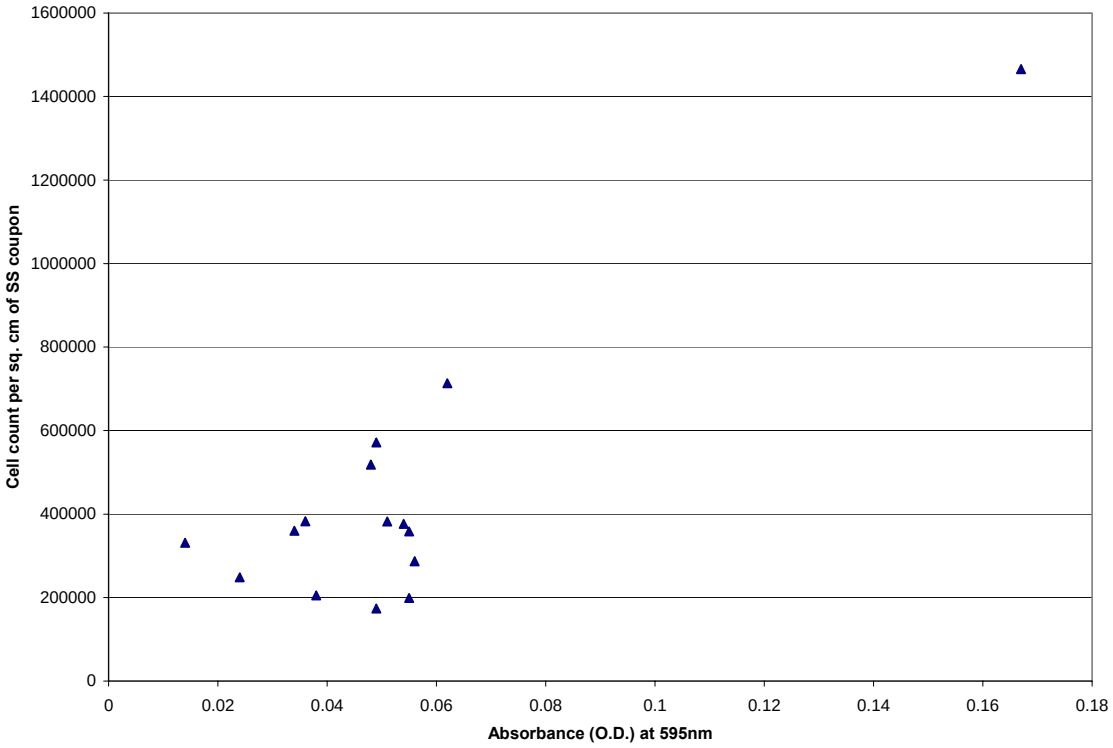


Figure 19: Scatter plot of attachment by *L. monocytogenes* isolates as determined by hydrophilic microtiter plate assay and microscopic analysis using phosphate buffer saline (pH: 7.2) as the suspension medium.

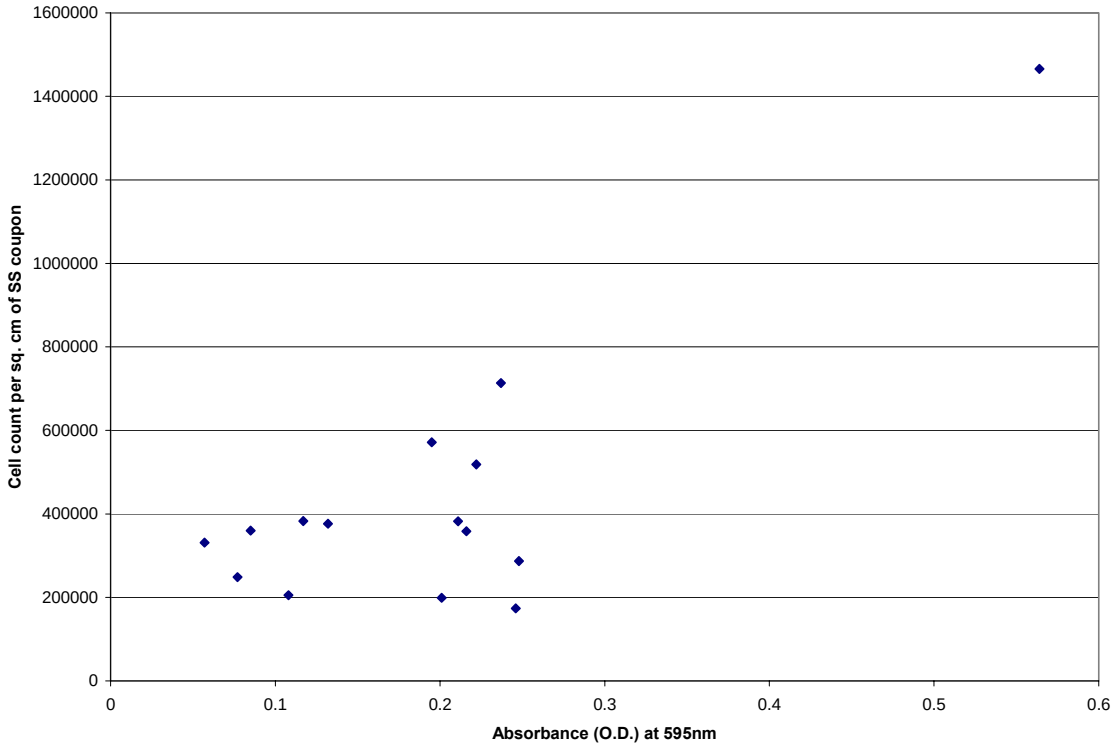


Figure 20: Scatter plot of attachment by *L. monocytogenes* isolates as determined by hydrophobic microtiter plate assay and microscopic analysis using phosphate buffer saline (pH: 7.2) as the suspension medium.

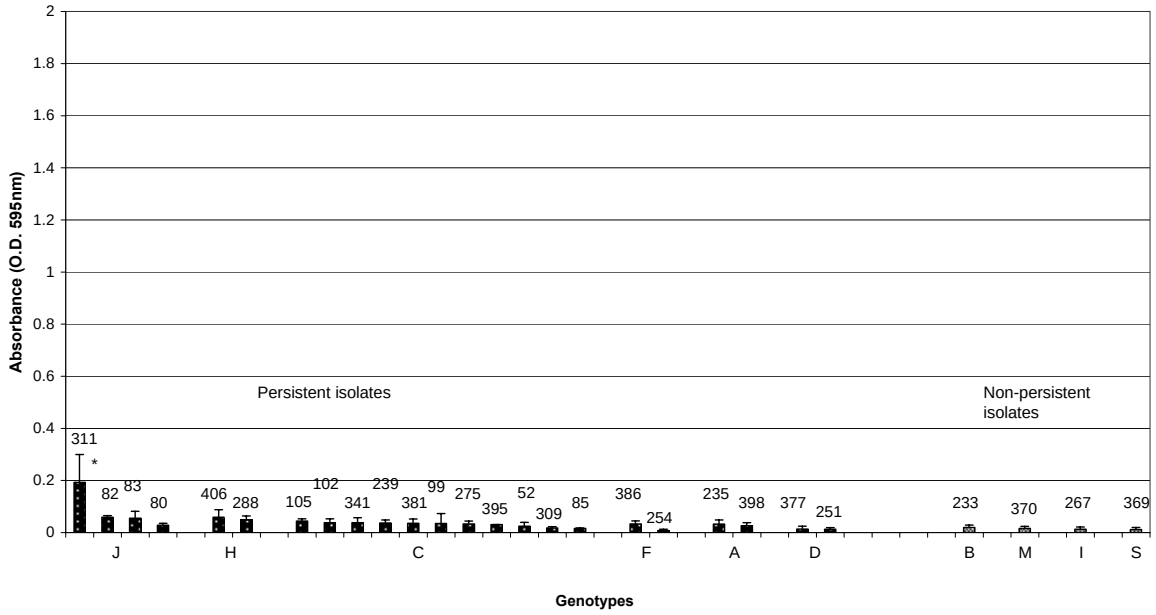


Figure 21: Biofilm formation by *L. monocytogenes* isolates on hydrophobic microtiter plates in TSB incubated at 10ºC for 120 hours. Isolate numbers are given on the bars. Amount of biofilm (cell mass) was determined by the absorbance of crystal violet dye.

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