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IMPACT OF BIOFILM FORMATION AND SUBLETHAL INJURY OF *LISTERIA MONOCYTOGENES* ON TRANSFER TO DELICATESSEN MEATS

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IMPACT OF BIOFILM FORMATION AND SUBLETHAL INJURY OF *LISTERIA MONOCYTOGENES* ON TRANSFER TO DELICATESSEN MEATS

By

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ABSTRACT

IMPACT OF BIOFILM FORMATION AND SUBLETHAL INJURY OF *LISTERIA MONOCYTOGENES* ON TRANSFER TO DELICATESSEN MEATS

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Presence of *Listeria monocytogenes* strains endemic to food processing environments is presumably related to biofilm formation. Following exposure to various environmental stresses, *Listeria* cells may be more prone to attach to surfaces. Due to concerns regarding the potential impact of biofilm formation on *Listeria* cross-contamination of ready-to-eat meats in delicatessens, a series of studies was conducted to: (1) determine the ability of *L. monocytogenes* to form biofilms under various temperatures and stress conditions present in food processing and retail environments, (2) determine the effects of biofilm-forming ability on direct and sequential transfer rates for *L. monocytogenes* from delicatessen slicers to ready-to-eat meats, (3) determine the effects of environmental stress on direct and sequential transfer rates for *L. monocytogenes* from delicatessen slicers to ready-to-eat meats, and (4) develop one or mathematical models that can be used to predict the transfer rates for *L. monocytogenes* during retail slicing of ready-to-eat meats. A total of 196 *L. monocytogenes* isolates were assessed for biofilm formation at 22 and 4°C in Modified Welshimer's Broth, as measured by optical density (OD) of stained biofilms, while a subset of 26 food, environmental and human clinical isolates were further assessed for biofilm formation after exposure to common environmental stressors (starvation, cold-shock, chlorine injury and acid injury). Only 5% of all isolates were strong biofilm-formers, forming biofilms with OD values two standard deviations above the mean, with 81% of strains

failing to produce detectable biofilms at 4°C. Prior injury of *L. monocytogenes* by starvation and cold resulted in enhanced biofilm formation, while exposure to acid and chlorine diminished subsequent biofilm formation. Cold- and chlorine-shock produced statistically similar levels of injury, however the cultures were significantly different in their abilities to form biofilms (mean OD chlorine-shock = 0.309, mean OD cold-shock = 1.457), showing that non-oxidative stresses common in the environment increase likelihood of biofilm formation.

Thereafter, six of the identified strong and weak biofilm-forming strains were combined into two 3-strain cocktails. The cocktails (healthy, cold-shocked or chlorine-injured) were used to inoculate stainless steel delicatessen slicer blades (10^6 CFU/blade). After incubation for 6 and 24 h (22°C/~78% RH), the inoculated blades were attached to a gravity-fed delicatessen slicer and used to generate 30 slices from retail chubs of roast turkey breast or Genoa salami. Biofilm-forming ability, length of incubation on stainless steel, and prior injury had no significant affect on transfer. *Listeria* was able to survive physiological stress and contaminate at statistically similar levels to healthy cells. Overall, significantly greater cumulative transfer to turkey (cumulative transfer = 4.2 log CFU) than salami (cumulative transfer = 3.5 log CFU) was observed. Under all conditions, *L. monocytogenes* was still present on the slicer after slicing.

These findings were then used to validate a predictive model in the form [CFU (X) = ka^X] along with a program written in GWBasic. This model can be used if any two of the following three values are known: (a) initial inoculum, (b) total bacteria transfer, (c) bacteria fraction remaining on the blade after consecutive slicing, solving for each model parameter CFU (X), k, or a. The fit of the model ranged from $R^2 = 0.65 - 0.94$.

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KEY TO SYMBOLS AND ABBREVIATIONS

a_w	Water activity
AISI	American Iron and Steel Institute
ANOVA	Analysis of Variance
ASTM	American Society for Testing and Materials
BATH	Bacterial Adhesion to Hydrocarbons
BPB	Butterfield's Phosphate Buffer
CDC	Centers for Disease Control and Prevention
CFSAN	Center for Food Safety and Applied Nutrition
CFU	Colony Forming Unit(s)
CLSM	Confocal scanning laser microscopy
cm	centimeter(s)
CT	Composite tissue
CY	Calendar Year
d	day(s)
DNA	Deoxyribonucleic Acid
FAO	Food and Agriculture Organization of the United Nations
FDA	Food and Drug Administration
FSIS	Food Safety Inspection Service
g	gram(s)
GLM	General Linear Model

h	hour(s)
HACCP	Hazard Analysis and Critical Control Point
HIC	Hydrophobic Interaction Chromatography
min	minute(s)
ml	milliliter(s)
mm	millimeter(s)
MOX	Modified Oxford Agar
MWB	Modified Welshimer's Broth
mTPA	Modified Tryptose Phosphate Agar
mTPAN	Modified Tryptose Phosphate Agar with 4.5% Sodium Chloride (NaCl)
OD	Optical Density
PBS	Phosphate Buffered Saline
PFGE	Pulsed-field Gel Electrophoresis
ppm	parts per million
RAPD	Random Amplicification of Polymorphic DNA
RH	Relative Humidity
RTE	Ready-to-eat
s	second(s)
SAS	Statistical Analysis Systems
TPA	Tryptose Phosphate Agar
TPAN	Tryptose Phosphate Agar with 4.5% Sodium Chloride (NaCl)
TSA-YE	Trypticase Soy Agar with 0.6% Yeast Extract

TSB-YE	Trypticase Soy Broth with 0.6% Yeast Extract
US	United States of America
US-DHHS	United States Department of Health and Human Services
USDA	United States Department of Agriculture
WHO	World Health Organization
µl	microliter(s)
µm	micron(s)

INTRODUCTION

Listeria monocytogenes is the leading microbiological cause of Class I recalls of cooked or ready-to-eat (RTE) meat products in the United States. Contamination of these products usually occurs after processing, prior to packaging (Levine et al., 2001). Delicatessen-sliced RTE turkey meat has been involved in 3 outbreaks of listeriosis since 2000, resulting in a total of 92 cases of illness, including 11 deaths and 6 miscarriages (CDC, 2001; CDC, 2002; Olsen et al., 2005). In a subsequent survey of 31,705 RTE products sampled from eight RTE product categories (fresh soft cheeses, bagged salads, blue-veined cheeses, mold-ripened cheeses, seafood salads, smoked seafood, luncheon meats and deli salads), 577 samples were positive for *L. monocytogenes* (Gombas et al., 2003). Of the 9,199 luncheon meat samples taken as part of the study, 82 were *L. monocytogenes*-positive, giving a prevalence rate of 0.89% (Gombas et al., 2003). Most positive samples (75.6%) contained less than 1 CFU/g (Table 1.2). Most importantly, luncheon meats that were store-packaged were more frequently contaminated with *L. monocytogenes* (6.8 times as likely to be contaminated) than manufacturer-packaged meats.

The higher prevalence of *L. monocytogenes* in delicatessen meat sliced at retail strongly suggests that the delicatessen slicer is an important vehicle for cross-contamination of products. In order for cross-contamination to occur, *L. monocytogenes* must survive on the surface for a period of time between the slicing of various products. Prior to its introduction to the slicer surface, *L. monocytogenes* may be exposed to refrigeration temperatures, low pH (fermented meats and cheeses), limited available

water, and sanitizers. While on the slicer surface, *L. monocytogenes* exposure to desiccation and sanitizers is likely. These stresses have been shown to alter the sensitivity of *L. monocytogenes* to other subsequent stresses, sometimes making it more difficult to eradicate from the environment (Lou and Yousef, 1997; Koutsoumanis et al., 2003; Koutsoumanis and Sofos, 2004; Gravesen et al., 2005; Moorman et al., 2005).

Listeria monocytogenes is able to become established in niches in food processing environments, where certain strains have been found to persist for years (Tompkin, 2002; Lunden et al., 2001). Equipment such as peelers, slicers, dicers, and conveyor belt lines are not always designed in a way that facilitates effective cleaning and sanitizing. In a survey of *L. monocytogenes* contamination in poultry processing environments, several food contact surfaces were persistently contaminated with the same strains, including slicer blades and blade covers, dicing machine blades and blade covers, a conveyor belt, and a spiral conveyor in a freezer (Lunden et al., 2003). This inability to adequately clean surfaces allows *L. monocytogenes* to persist in the environment and form biofilms on food contact surfaces where the pathogen can be potentially transferred to RTE foods.

Persistent strains play an important role in contamination of RTE foods with such strains 8 times more likely to contaminate finished product than transient strains (Lunden et al., 2003). Lunden et al. (2000) reported that the same pulsed-field gel electrophoresis (PFGE) type of *L. monocytogenes* was transferred to three different processing plants in a dicing machine used in the three plants. The persistent strain was then tested for adherence to stainless steel in broth culture at 25°C for 1, 2, and 72 h, along with three non-persistent strains of *L. monocytogenes* isolated from the third plant. The persistent strain was significantly more adherent than the non-persistent strains, a trend that has also

been correlated with biofilm formation in other studies (Lunden et al., 2000; Norwood and Gilmour, 1999; Borucki et al., 2003).

Increased awareness of the potential cost and risk of multi-state listeriosis outbreaks spurred the development of risk assessments for *Listeria* by United States government agencies (FDA/USDA/CDC, 2003). Although 3 categories of RTE foods showed a higher prevalence of *L. monocytogenes* contamination (pâtés, smoked seafoods, and fresh, soft cheeses), deli meats ranked first in relative risk, due to their higher per capita consumption which, in turn, leads to a wider exposure of the public to *L. monocytogenes* (FDA/USDA/CDC, 2003). Overall, 14 cases of listeriosis are predicted to occur for every 100 million servings of deli meat consumed (FDA/USDA/CDC, 2003). While this may seem like a small number of cases, the mortality rate for listeriosis is high, and listeriosis is the second most costly foodborne illness in the United States, with an estimated annual cost of $2.3 billion in medical expenses and lost productivity, including death (Frenzen, 2003). Listeriosis has the highest hospitalization rate and the second highest number of fatalities of any foodborne illness tracked by the Centers for Disease Control and Prevention in FoodNet (FDA/FSIS/CDC, 2003). The Healthy People 2010 national health objective for listeriosis was to reduce the number of cases to 2.5 per 1,000,000 people by 2005. However, the number of listeriosis cases was 3.0 per 1,000,000 people in 2005 with the targeted goal not yet achieved (Reuters, 2006).

The research presented in this dissertation was conducted in response to *Listeria* transfer rates being identified as a key informational gap in the *Listeria* Risk Assessment published by the US federal government (FDA/USDA/CDC, 2003). Data obtained from the research was used to validate the utility of a model developed by Vorst et al. (2005—

Ch.5) in predicting transfer of *L. monocytogenes* after exposure to bacterial stress (cold-injury and chlorine-injury) and prolonged (6 and 24 h) desiccation on stainless steel to turkey and salami. Additionally, the model was also tested for its ability to predict *L. monocytogenes* transfer based on strain persistence and biofilm formation. The underlying hypothesis for this study was that strain persistence would have an affect on the survival and transfer of *L. monocytogenes* to delicatessen meats, particularly after prolonged desiccation on stainless steel.

CHAPTER 1

LITERATURE REVIEW

1.1 *Listeria* and Listeriosis

Listeria is a genus of Gram-positive, non-spore forming, short rod-shaped, facultatively anaerobic bacteria that are catalase positive, oxidase negative, methyl red positive, and Voges-Proskauer positive (Swaminathan, 2001). The genus is comprised of six species: *L. monocytogenes*, *L. innocua*, *L. ivanovii*, *L. seeligeri*, *L. welshimeri*, and *L. grayi*. Species can be differentiated by hemolytic activity—*L. ivanovii* is strongly beta-hemolytic, *L. monocytogenes* and *L. seeligeri* are weakly beta-hemolytic; and *L. innocua*, *L. welshimeri* and *L. grayi* are non-hemolytic. *Listeria monocytogenes* can be differentiated from *L. ivanovii* and *L. seeligeri* by its positive result for the CAMP-test with *Staphylococcus aureus* and negative reaction with *Rhodococcus equi* on sheep blood agar, and its inability to produce acid from D-xylose (Rocourt, 1999; Swaminathan, 2001). *Listeria monocytogenes* is the primary human pathogen within the genus and is of concern to food processors due to its high fatality rate, growth at refrigeration temperatures, resistance to salt and acid, and ability to persist in food processing environments for up to 12 years (Lunden et al., 2001).

Listeriosis is the disease caused by infection with *L. monocytogenes*. Groups at particular risk for listeriosis include the elderly, immunocompromised adults, pregnant women, and neonates. Listeriosis is rare—out of an estimated 76 million cases of foodborne illnesses per year in the United States, only about 2500 are caused by *L. monocytogenes*. However, the mortality rate for listeriosis is high, with those 2500 cases

resulting in an estimated 500 deaths every year. Nearly 90% of all reported cases of listeriosis result in hospitalization (Mead et al., 1999). Due to its severity, invasive listeriosis is the second most costly foodborne illness in the United States, with an estimated annual cost of $2.3 billion in medical expenses and lost productivity, including death (Frenzen, 2003). Listeriosis has the highest hospitalization rate and the second highest number of fatalities of any foodborne illness tracked by the Centers for Disease Control and Prevention in FoodNet (FDA/FSIS/CDC, 2003). The Healthy People 2010 national health objective for listeriosis was to reduce the number of cases to 2.5 per 1,000,000 people by 2005. Partly as a result of new regulations, the number of cases dropped from 4.7 per 1,000,000 people in 1997 to 2.6 cases per 1,000,000 in 2002, nearly reaching the stated goal (US-DHHS, 2004). However, the number of listeriosis cases has since increased to 3.0 per 1,000,000 people in 2005 with the targeted goal not yet achieved (Reuters, 2006).

Humans acquire listeriosis through ingestion of contaminated food in 90% of listeriosis cases (Mead et al., 1999). Listeriosis results in flu-like symptoms, meningitis, spontaneous abortion, fetal death, or neonatal septicemia (Slutsker and Schuchat, 1999). Febrile gastroenteritis, a less common and poorly characterized form of listeriosis, usually occurs in previously healthy adults who ingest unusually large quantities of the pathogen (Schlech, 2000). The incubation period for listeriosis ranges from 24-48 hours for febrile gastroenteritis, to 14 to 70 days for the more typical invasive form of listeriosis (Schlech, 2000).

Listeria monocytogenes contains 13 serotypes based on somatic (O) and flagellar (H) antigens. Four of these serotypes—1/2a, 1/2b, 1/2c and 4b, account for over 95% of

human listeriosis cases with serotype 4b strains predominating (Graves et al., 1999; Nightingale et al., 2005). Conventional strain typing methods, such as serotyping and phage typing, result in poor discrimination and reproducibility between strains. Serotyping of *L. monocytogenes* strains is difficult due to the limited availability of high quality antisera and the number of antigens shared by different serotypes. For instance, serotypes 4a, 4b, 4c, 4d, 1/2b, and 3b, all contain the same H antigens, and multiple common O antigens are present in different serotypes (Liu et al., 2006). Molecular typing methods, such as multilocus enzyme electrophoresis, ribotyping, random amplicification of polymorphic DNA (RAPD), and pulsed-field gel electrophoresis (PFGE) result in better reproducibility and discrimination between strains. PFGE, which is the basis for CDCs PulseNet System, is now used throughout the United States, Canada and elsewhere to identify potential common source outbreaks of listeriosis and other foodborne illnesses (Graves et al., 1999).

Listeria monocytogenes can be divided into three distinct genetic lineages: Lineage I (serotypes 1/2b, 3b, 3c and 4b), Lineage II (serotypes 1/2a, 1/2c, and 3a), and Lineage III (serotypes 4a, 4b, and 4c) (Nightingale et al., 2005; Roberts et al, 2006). Lineage I strains are responsible for most human listeriosis cases, while Lineage II strains are common environmental isolates that are infrequently implicated in human listeriosis (Nightingale et al., 2005; Saunders et al., 2006). Lineage III strains are rare, with one survey showing that fewer than 3% of 1800 *L. monocytogenes* strains belonged to Lineage III (Roberts et al., 2006). While strains of any lineage have the potential to cause listeriosis, most research to date indicates that Lineage I strains are better adapted to survive and multiply in foods and have greater pathogenic potential (Nightingale et al.,

2005). In contrast, Lineage II strains are better adapted to survive in the environment, and can outcompete Lineage I strains during selective enrichment (Bruhn et al., 2005).

1.2 Listeriosis Outbreaks from Ready-to-Eat Meats

In the United States, transmission of *L. monocytogenes* from ready-to-eat (RTE) meat products was first documented in a 1988 when a breast cancer patient in Oklahoma developed listeriosis after consuming turkey frankfurters. The opened frankfurter package recovered from the patient's refrigerator contained over 10^3 CFU/g of *L. monocytogenes*. After tracing the product back to the manufacturer, the initial contamination level (as determined by most probable number) was < 0.3 CFU/g (Barnes et al., 1989).

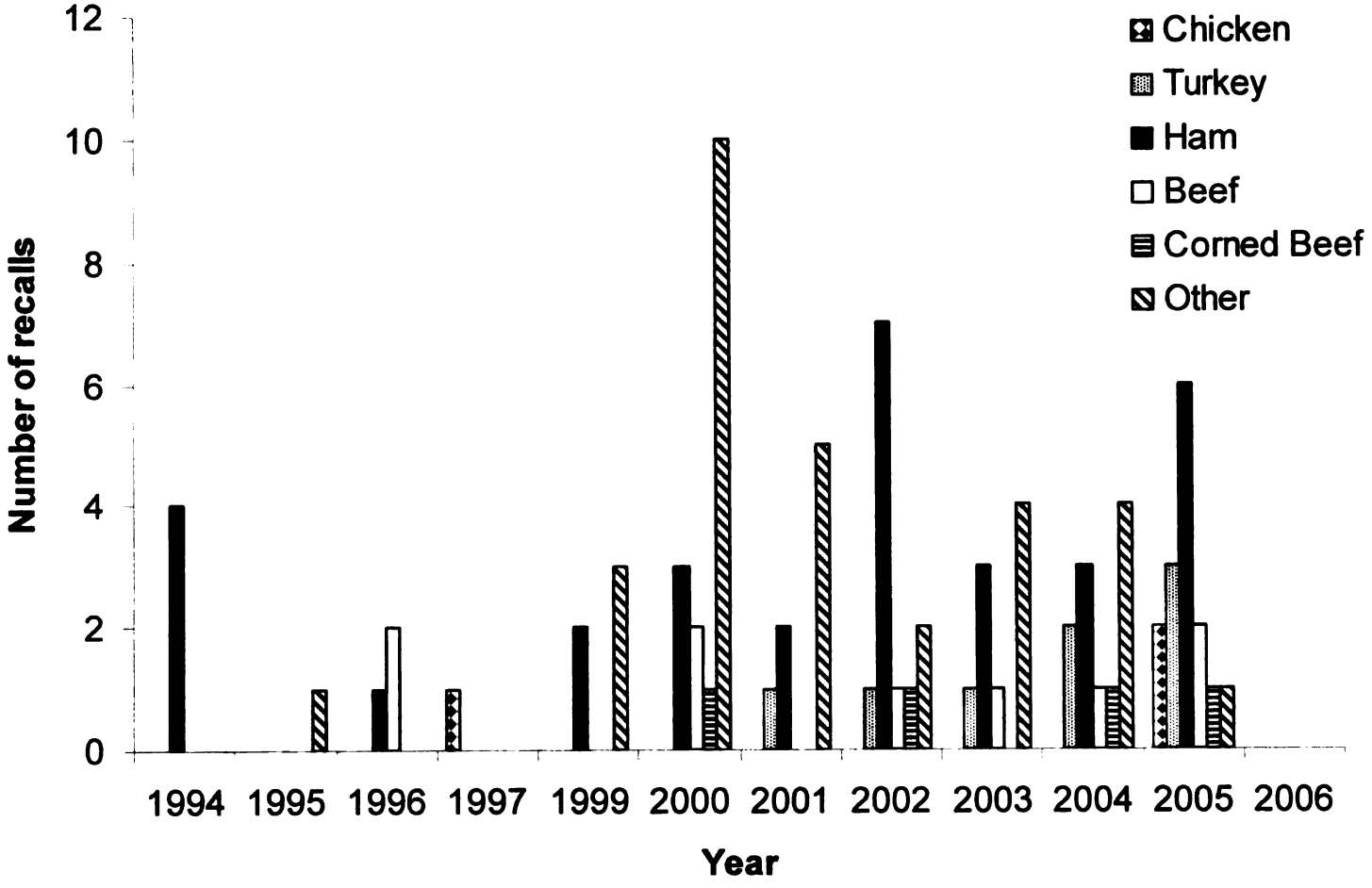
A number of listeriosis outbreaks have since been linked to consumption of fully cooked RTE meat products. These outbreaks involved multiple states in the US and consequently attracted considerable public attention. The first of these multi-state outbreaks, in 1998, involved turkey frankfurters contaminated with *L. monocytogenes* serotype 4b—this outbreak caused 108 cases of listeriosis, 14 deaths and 4 miscarriages or stillbirths in 24 states (Graves et al., 2005). Two major multi-state outbreaks of listeriosis linked to the consumption of delicatessen-sliced RTE turkey meat occurred in 2000 and 2002, and involved 11 and 9 states, respectively. Both outbreaks resulted from the consumption of turkey delicatessen meat contaminated with *L. monocytogenes* serotype 4b. The 2000 outbreak was responsible for 30 cases of listeriosis, 4 deaths, 3 miscarriages/stillbirths and the recall of 16.9 million pounds of turkey (Olsen et al., 2005). The outbreak in 2002 caused 46 cases of listeriosis, 7 deaths, and 3 miscarriages/stillbirths in 9 primarily northeastern states. This outbreak prompted the

recall of 27.4 million pounds of delicatessen turkey meat (CDC, 2002). A third outbreak of listeriosis linked to delicatessen-sliced turkey meat occurred in 2001 in Los Angeles County, California, but was different from the others insofar as it caused 16 cases of acute febrile gastroenteritis, resulting in no fatalities. It was caused by *L. monocytogenes* serotype 1/2a, found at levels of 1.6×10^9 CFU/g in the implicated turkey meat (Frye et al., 2002).

1.3 *Listeria* Recalls

Listeria monocytogenes is the leading microbiological cause of Class I recalls of cooked or RTE products. Contamination usually occurs after processing, prior to packaging (Levine et al., 2001). From 1994 to April 2006, 85 recalls were issued for deli meats containing *L. monocytogenes* with ham most frequently implicated (31 recalls), followed by luncheon meats and sausages (category labeled as "other"—30), followed by beef (13 recalls), turkey (8 recalls), and chicken (3 recalls) (Figure 1.1; USDA-FSIS, 2006).. Products were recalled if *L. monocytogenes* was present in a 25 g sample of the meat product. Although most frequently contaminated, ham has not yet been linked to any listeriosis outbreaks, whereas the infrequently recalled turkey has been involved in 3 outbreaks since 2000, resulting in a total of 92 cases of illness, including 11 deaths and 6 miscarriages (CDC, 2001; CDC, 2002; Olsen et al., 2005). Studies have shown that growth conditions on RTE poultry are more favorable to *L. monocytogenes* than ham (Glass and Doyle, 1989; Beumer et al., 1996; Burnett et al., 2005). This may explain why, despite the higher number of ham recalls, only turkey has been linked to large outbreaks of listeriosis.

Figure 1.1. ***Listeria monocytogenes*-related Class I recalls of delicatessen meat products, 1994-2004 (USDA-FSIS, 2006)**



1.4 *Listeria monocytogenes* in RTE Meats

Given the likelihood for the presence of *L. monocytogenes* in the processing environment, contamination of RTE meat products is a concern for both the meat industry and regulatory agencies. A monitoring program for *Listeria* in cooked beef products has been in place in the United States since 1987 and in 1993 the sampling program was expanded to include meat/poultry products and meat/poultry spreads (USDA-FSIS, 2003). Based on government survey data from 1990-2000, *L. monocytogenes* was more prevalent in ham and luncheon meats compared to the other categories of RTE products (Table 1.1; Levine et al., 2001). In 1999, Hazard Analysis and Critical Control Point plans (HACCP) were completely phased in for all meat and poultry establishments, in accordance with the Pathogen Reduction: Hazard Analysis and Critical Control Points final rule, also known as the "Mega-Reg" (USDA-FSIS, 1998).

In a subsequent survey of 31,705 RTE products sampled from eight RTE product categories (fresh soft cheeses, bagged salads, blue-veined cheeses, mold-ripened cheeses, seafood salads, smoked seafood, luncheon meats and deli salads), 577 samples were positive for *L. monocytogenes* (Gombas et al., 2003). Of the 9,199 luncheon meat samples taken as part of the study, 82 were *L. monocytogenes*-positive, giving a prevalence rate of 0.89% (Gombas et al., 2003). Most positive samples (75.6%) contained less than 1 CFU/g (Table 1.2). Luncheon meats that were store-packaged were more frequently contaminated with *L. monocytogenes* (6.8 times as likely to be contaminated) than manufacturer-packaged meats. However, the samples contaminated at levels higher than 10^2 CFU/g were more likely to be manufacturer-packaged (Gombas et al., 2003).

Table 1.1. Prevalence (%) of *L. monocytogenes* in RTE meat and poultry products, CY 1990-2000 (Levine et al., 2001)

Year	Cooked, Roast, Corned Beef	Sliced Ham & Luncheon Meats	Small Cooked Sausages	Large Cooked Sausages	Jerky	Cooked Poultry Products	Salads/ Spreads/ Pâtés	Fermented Sausages	Other
1990	6.38 (345)[a]	7.69 (13)	4.21 (309)	5.32 (94)	0.00 (25)	2.79 (430)	5.48 (347)	ND[b]	ND
1991	4.02 (498)	5.48 (73)	7.24 (387)	4.60 (261)	0.00 (39)	2.62 (649)	3.17 (473)	ND	ND
1992	3.86 (492)	7.89 (114)	6.03 (348)	0.42 (239)	0.00 (19)	2.01 (349)	3.32 (241)	ND	ND
1993	3.04 (428)	8.05 (149)	5.30 (472)	2.13 (328)	0.00 (39)	1.91 (314)	2.19 (274)	ND	ND
1994	2.09 (479)	5.46 (238)	4.81 (603)	1.14 (438)	2.22 (45)	2.37 (549)	2.41 (580)	ND	ND
1995	2.68 (560)	5.00 (100)	4.09 (611)	1.14 (438)	0.00 (50)	2.25 (889)	4.69 (597)	ND	ND
1996	3.35 (507)	7.69 (91)	3.74 (561)	0.95 (420)	0.00 (43)	3.17 (883)	2.17 (554)	ND	ND
1997	2.08 (530)	4.20 (286)	2.74 (621)	1.62 (371)	0.00 (40)	0.95 (946)	2.43 (206)	9.26 (108)	ND
1998	2.15 (511)	4.18 (263)	3.49 (746)	1.19 (506)	1.56 (192)	2.22 (857)	3.11 (225)	2.87 (244)	ND
1999	2.71 (922)	4.58 (960)	1.76 (2162)	0.43 (1167)	0.00 (278)	1.44 (970)	1.15 (435)	2.09 (478)	ND
2000	2.24 (NR)[c]	3.05 (NR)	1.26 (NR)	0.51 (NR)	0.75 (NR)	1.24 (NR)	0.98 (NR)	1.49 (NR)	0.00 (NR)
Cumulative %	**2.95**	**4.47**	**2.97**	**1.09**	**0.58**	**1.97**	**2.83**	**2.67**	**0.00**

[a] (Total number of samples tested)
[b] ND = Not Determined
[c] NR = Total number of samples not reported

Table 1.2. *L. monocytogenes*-positive luncheon meat samples from the Gombas et al., survey (2003)

Contamination Level (CFU/g)	Number of Positive Samples	Percent of Positive Samples
0.04 – 0.1	42	51.2
>0.1 – 1	20	24.4
>1 – 10	10	12.2
>10 – 10^2	2	2.4
>10^2 – 10^3	7	8.5
>10^3 – 10^4	1	1.2

In 2003, the *Listeria monocytogenes* final rule was put into effect, mandating three alternative *Listeria* control strategies that are required for manufactures of RTE meats. Currently, a multi-state survey is being conducted to determine the prevalence of *L. monocytogenes* in RTE meat in the wake of the new regulation. In this study, 8,000 samples of RTE delicatessen meats from four FoodNet states (Georgia, California, Minnesota, and Tennessee) are being purchased at retail and examined for both presence and numbers of *L. monocytogenes.* Overall prevalence of *L. monocytogenes* in delicatessen meats since the implementation of the final rule has decreased slightly to 0.77%, as opposed to the 0.89% observed by Gombas et al. (Draughon et al., 2006). Delicatessen-sliced meats were again more likely to be *L. monocytogenes*-positive (1.4%) than manufacturer-sliced meats (0.17%) (Draughon et al., 2006). Prior to the *L. monocytogenes* final rule, 0.4% of manufacturer-sliced and 2.7% of delicatessen-sliced meats were *L. monocytogenes*-positive (Gombas et al., 2003). In the current study, pork

products had the highest prevalence (0.89%), followed by beef (0.79%), and poultry (0.67%) (Draughon et al., 2006).

Numerous studies have shown that *L. monocytogenes* can grow on RTE meat at refrigeration temperatures (Glass and Doyle, 1989; Grau and Vanderlinde, 1992; Beumer et al., 1996; Burnett et al., 2005). *Listeria* contamination of RTE meats is typically low-level (<0.03 CFU/g) (Gombas et al., 2003; Draughon et al., 2006). However, certain products have been shown to support growth to higher levels during extended refrigerated storage. In one study involving naturally contaminated RTE meats (luncheon meat, ham, and chicken breast), *L. monocytogenes* populations increased to 10^4 CFU/g after 4-6 weeks in products above pH 5 (Beumer et al., 1996). Burnett et al. (2005) studied growth of *L. monocytogenes* on RTE turkey breast (uncured, pH 6.2, a_w 0.98) and RTE ham (cured, pH 6.2, a_w 0.98) at 5, 7, and 10°C, and found that growth rates in RTE turkey were higher than in ham (Table 1.3). These results are similar to earlier findings of Glass and Doyle (1989) who showed that *L. monocytogenes* grew to 10^3-10^5 CFU/g on vacuum-packaged processed poultry during 4 weeks of storage at 4.4°C, while populations increased 10^3-10^4 CFU/g over 6 weeks on ham, bologna and bratwurst. Foods having a pH at or below 5 are generally unable to support growth of *L. monocytogenes*. However, *L. monocytogenes* can survive in fermented meats including hard salami, for at least three months, with greater survival observed in products as water activity increases (Johnson et al., 1988).

Table 1.3. Growth of *L. monocytogenes* on RTE delicatessen meat (Burnett et al., 2005)

Product	Temperature (°C)	Log CFU/g growth per day
Turkey (turkey breast meat, turkey broth, dextrose, salt, sodium phosphate, garlic, flavoring)	5	0.45
	7	0.83
	10	1.53
Ham (ham, water, salt, sugar, dextrose, sodium phosphate, monosodium glutamate, sodium erythorbate, sodium nitrite)	5	0.42
	7	0.58
	10	0.98

In addition to low pH or lower a_w, certain additives can also suppress growth of *L. monocytogenes* in RTE meat including nitrite at levels of 140-200 ppm (Grau and Vanderlinde, 1992). A combination of sodium lactate (1.8%) and sodium diacetate (0.25%) also completely inhibited *L. monocytogenes* growth in vacuum-packaged pork frankfurters during 40 days of storage at 10°C, with an initial population reduction if the contaminated frankfurters were dipped in 2.5% lactic or acetic acid prior to storage (Barmpalia et al., 2004).

1.5 *Listeria* Risk Assessments for RTE Meats

Increased awareness of the potential cost and risk of multi-state listeriosis outbreaks spurred the development of several risk assessments for *Listeria* by United States government agencies (FDA/USDA/CDC, 2003). The Office of Management and the Budget requires that risk assessments be conducted by U.S. federal government agencies in order to assess the costs and benefits of planned regulations which will ensure that an equally effective and equally beneficial alternative is not being overlooked (Buchanan et al., 2004). As defined by the Codex Alimentarius, microbial risk assessments should include the following four elements (Barraj and Petersen, 2004):

1) Hazard identification: The identification of biological, chemical and physical agents that are capable of causing adverse health effects and that may be present in a particular food or group of foods.

2) Hazard characterization: The qualitative or quantitative evaluation of the nature of the adverse health effects associated with biological, chemical and physical agents that may be present in food.

3) Exposure assessment: The qualitative or quantitative evaluation of the likely intake of biological, chemical and physical agents via food and exposures from other sources if relevant.

4) Risk characterization: The qualitative or quantitative estimation, including attendant uncertainties, of the probability of occurrence and severity of known or potential adverse health effects in a given population based on hazard identification, hazard characterization and exposure assessment.

Initially, an assessment of the relative risk to public health from foodborne *L. monocytogenes* was conducted, in which the risks of contracting listeriosis from 23 categories of RTE foods were ranked (Table 1.4). Although 3 categories of RTE foods showed a higher prevalence of *L. monocytogenes* contamination (pâtés, smoked seafoods, and fresh, soft cheeses), deli meats ranked first in relative risk, due to their higher per capita consumption which, in turn, leads to a wider exposure of the public to *L. monocytogenes*. Exposure can be high if the pathogen concentration in the food is high or if large quantities are consumed, even if overall pathogen concentration is low (Barraj and Petersen, 2004), as is the case for *L. monocytogenes* in deli meats. Overall, 14 cases of listeriosis were predicted to occur for every 100 million servings of deli meat consumed (FDA/USDA/CDC, 2003). Based on this initial risk assessment, another risk assessment was carried out specifically for *Listeria* in RTE meat and poultry products (USDA-FSIS, 2003). This risk assessment found that combined interventions including various combinations of increased testing of food contact surface and sanitation, pre- and post-packaging microbial reduction strategies, and product reformulation to include growth inhibitors would be the most effective means of controlling the risk of listeriosis (USDA-FSIS, 2003). These strategies were incorporated into the *Listeria monocytogenes* final rule implemented in 2003.

Table 1.4. Predicted Relative Risk Rankings for Listeriosis among Food Categories for Three U.S. Age-Based Subpopulations Using Median Estimates of Relative Predicted Risks for Listeriosis on a Per Annum Basis (USDA-FSIS, 2003)

Food Categories[a]	Subpopulation		
	Intermediate Age[b]	Elderly[b]	Perinatal[b]
SEAFOOD			
Smoked Seafood	6	6	7
Raw Seafood	17	20	17
Preserved Fish	13	13	13
Cooked Ready-to-Eat Crustaceans	9	8	9
PRODUCE			
Vegetables	11	9	11
Fruits	16	14	14
DAIRY			
Soft Mold-Ripened and Blue-Veined Cheese	14	15	15
Goat, Sheep, and Feta Cheese	18	17	18
Fresh Soft Cheese (e.g., queso fresco)[c]	7	11	6
Heat-Treated Natural Cheese and Processed Cheese	10	10	10
Aged Cheese	19	18	19
Fluid Milk, Pasteurized[d]	3	2	2
Fluid Milk, Unpasteurized[d]	15	16	16
Ice Cream and Frozen Dairy Products	20	19	20
Miscellaneous Dairy Products	5	4	5

Table 1.4 (Cont'd)

MEATS			
Frankfurters[e]	4	5	4
Dry/Semi-Dry Fermented Sausages	12	12	12
Deli Meats	1	1	1
Pâté and Meat Spreads	8	7	8
COMBINATION FOODS			
Deli Salads	2	3	3

[a] Food categories are grouped by type of food but are not in any particular order.

[b] A ranking of 1 indicates the food category with the greatest predicted relative risk of causing listeriosis and a ranking of 20 indicates the lowest predicted relative risk of causing listeriosis.

[c] Data from soft ripened cheese made from unpasteurized milk were used in the modeling to define the shape of the distribution of contamination data for fresh soft cheese.

[d] All available data for this food category were used in the modeling to define the shape of the distribution for this food category but only contamination data from North America were used to determine the frequency of contamination. Also see text for discussion of the effects of uncertainty on the ranking for pasteurized milk and other foods that are consumed in high amounts.

[e] This ranking is based on the assumption that 1% to 14% of frankfurters are consumed without reheating and the remainder are adequately heated before consumption.

1.6 Strategies and Regulations for Decreasing the Incidence of Listeriosis

In the wake of the multi-state listeriosis outbreaks linked to consumption of delicatessen-sliced RTE turkey meat, the USDA implemented new regulations for the control of *L. monocytogenes* in RTE meat processing facilities, in addition to the "zero-tolerance" regulation, and the requirement that HACCP plans address potential *L. monocytogenes* contamination problems. The major requirement instituted was that facilities producing high-risk RTE meat products must develop scientifically validated *L. monocytogenes* control programs. Meat processing facilities are currently required to choose from the following three alternative control strategies (USDA-FSIS, 2003):

- **Alternative 1** – Employ both a post-lethality treatment and a growth inhibitor for *L. monocytogenes* on RTE products. Establishments opting for this alternative will be subject to FSIS verification activity that focuses on the post-lethality treatment's effectiveness. Sanitation is important but is built into the degree of lethality necessary for safety.

- **Alternative 2** – Employ either a post-lethality treatment or a growth inhibitor for the pathogen on RTE products. Establishments opting for this alternative will be subject to more frequent FSIS verification activity than those in Alternative 1, and will be required to test for the presence of *L. monocytogenes* or *Listeria spp.* on food contact surfaces.

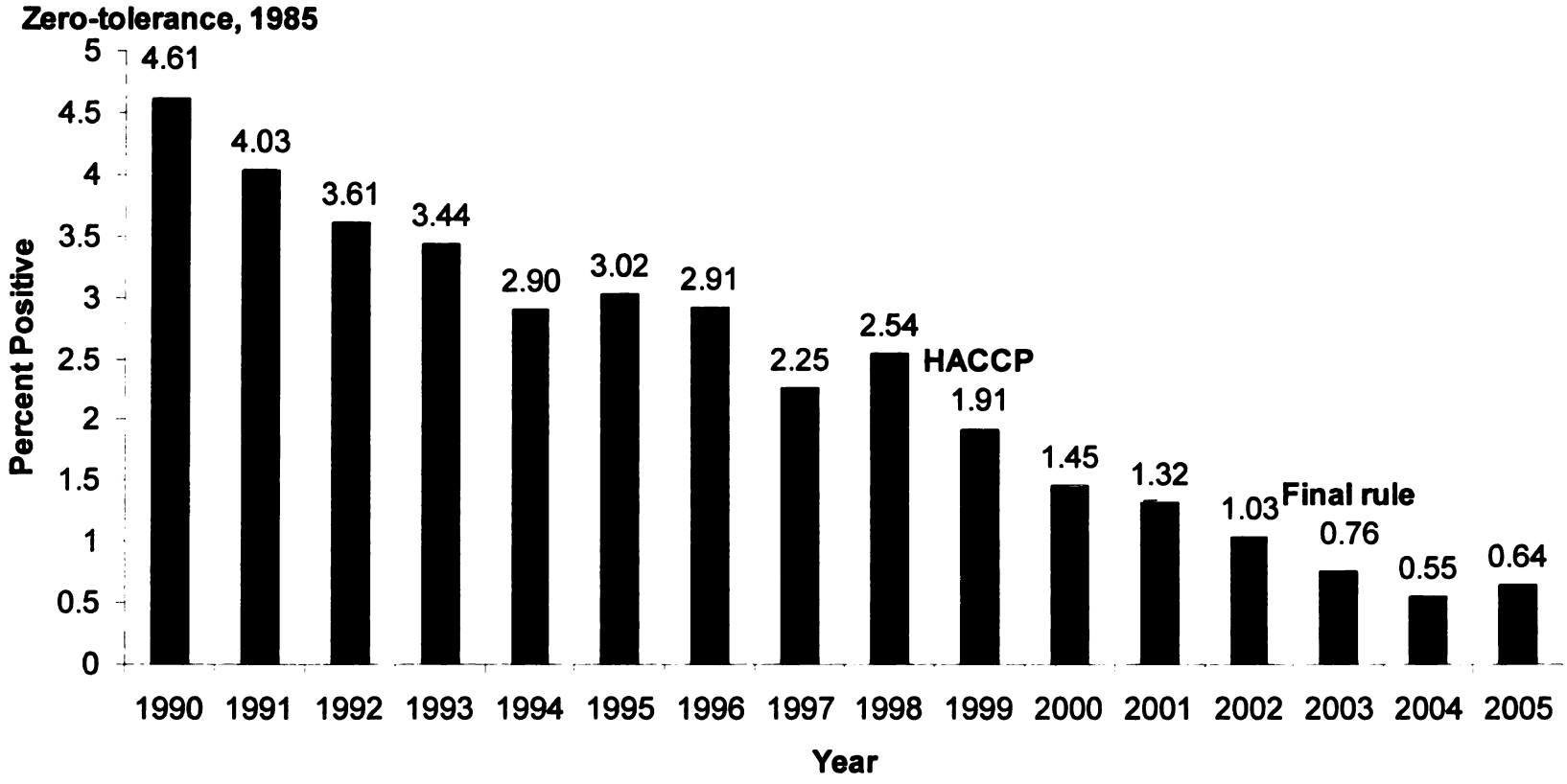
- **Alternative 3** – Employ sanitation measures only. Establishments opting for this alternative will be targeted with the most frequent level of FSIS verification activity. Within this alternative, FSIS will place increased scrutiny on operations

that produce hotdogs and deli meats. In a 2001 risk ranking, FSIS and FDA identified these products as being high-risk products for listeriosis.

Within one year of the 2002 listeriosis outbreak traced to delicatessen turkey meat, USDA-FSIS reported a 25% decrease in the number of regulatory samples testing positive for *L. monocytogenes* (between January 2003-September 2003, compared with the number of positive samples detected in 2002; Gottlieb et al., 2006) (Figure 1.2). Preliminary national surveillance data showed a decrease of 40% in cases of human listeriosis, compared with the average yearly number of cases detected between 1996 and 1998. By the beginning of 2004, there were 2.7 cases of listeriosis per million, which was nearly at the national goal of 2.5 cases/million by 2005 (Gottlieb et al., 2006). Unfortunately, in 2005 the number of cases increased to 3.0 per million, short of the targeted goal (Reuters, 2006).

At retail, the FDA Food Code provides the only requirements and guidelines for preventing contamination with *L. monocytogenes*, and its implementation is taught to retail and restaurant managers via the ServSafe Food Safety Training and Certification Program. Largely, these requirements are specific to sanitation of food contact surfaces, particularly central contact points, such as delicatessen slicers, knives, countertops and coolers. The most recent version of the Food Code specifies that any product which is stored under a controlled temperature and for a specified length of time for safety reasons is a potentially hazardous food and more frequent cleaning of contact surfaces is required (at least once every 4 h, as opposed to once every 10 h for non-hazardous foods; FDA, 2005).

Figure 1.2. Regulatory testing for *Listeria monocytogenes* in RTE products by calendar year, 1990-2005 (USDA-FSIS, 2006)



1.7 *Listeria* survival in the environment and on delicatessen slicers

The higher prevalence of *L. monocytogenes* in delicatessen meat sliced at retail strongly suggests that the delicatessen slicer is an important vehicle for cross-contamination of products. In order for cross-contamination to occur, *L. monocytogenes* must survive on the surface for a period of time between the slicing of various products. Prior to its introduction to the slicer surface, *L. monocytogenes* may be exposed to refrigeration temperatures, low pH (fermented meats and cheeses), limited available water, and sanitizers. While on the slicer surface, *L. monocytogenes* exposure to desiccation and sanitizers is likely. These stresses have been shown to alter the sensitivity of *L. monocytogenes* to other subsequent stresses, sometimes making it more difficult to eradicate from the environment (Lou and Yousef, 1997; Koutsoumanis et al., 2003; Koutsoumanis and Sofos, 2004; Gravesen et al., 2005; Moorman et al., 2005).

Metal may have certain bactericidal properties. Stainless steel in contact with the air will expose bacteria to desiccation, but food soils often prolong bacterial survival in this otherwise inhospitable environment (Robine et al., 2002; Kusumaningram et al., 2003). Composition of the metal alloy and its surface finish may also impact the availability of harborage sites for bacteria and their subsequent survival. Massive copper (99.99% copper) resulted in poorer survival of *Enterococcus faecalis* over 96 h than 304 AISI stainless steel and copper-rich stainless steel (AISI 211 stainless steel plus copper; Robine et al., 2002). However, applying the inhibitory metal to stainless steel (as in the case of the copper-rich stainless steel) did not result in significantly better inhibition of *E. faecalis* than was seen with 304 AISI stainless steel (Robine et al., 2002). Silver ion-

treated stainless steel can reportedly inhibit biofilm growth of *Staphylococcus spp.* on catheters of kidney transplant recipients; however it has not been studied for its effectiveness in food processing environments (Loertzer et al., 2006).

A typical stress that *L. monocytogenes* may encounter on a delicatessen slicer is desiccation. Depending on temperature and RH of the environment, *L. monocytogenes* can survive 82 (22°C, 0% RH) to more than 151 days (10°C, 88% RH) on sand without a nutrient source (DeRoin et al., 2003). Survival under desiccated conditions is better at lower temperatures (10 vs. 22°C) and higher RH (88 vs. 40 or 0%; DeRoin et al., 2003). Under osmotic stress, *L. monocytogenes* will try to accumulate osmolytes, particularly glycine betaine and carnitine, from the growth medium (Gardan et al., 2003). *Listeria monocytogenes* increases the expression of 12 different stress proteins when exposed to salt, and suppress the production of 21 proteins (Esvan et al., 2000). In the absence of available osmolytes, *Listeria* also produces a general stress protein, *ctc* that promotes osmotic stress tolerance (Gardan et al., 2003).

Listeria is able to grow at 4°C, by altering its membrane composition in order to maintain membrane fluidity and increase passive permeability. This is achieved through changes in fatty acid composition (Neunlist et al., 2005). In addition, when exposed to cold starvation conditions, *L. monocytogenes* undergoes shrinkage of the cytoplasm, eventually resulting in holes in the cytoplasm (Dykes, 1999). Carnitine, which *L. monocytogenes* uses as an osmoprotectant, is also thought to aid in tolerance to cold-stress (Dykes and Moorhead, 2000).

Acid tolerance of *L. monocytogenes* has been studied by several researchers. *Listeria* is able to better withstand lethal acid concentrations (pH 3.5) after habituation to

sublethal acid stress (pH 5 - 6), with maximum acid tolerance induced by habituation to pH 5.5 (Koutsoumanis and Sofos, 2004; Koutsoumanis et al., 2005). *Listeria monocytogenes* can also remain viable for at least 20 h at pH 4.0, and also at pH 3.5 in the presence of glucose (Shabala et al., 2002) by maintaining a higher intracellular pH of 7.0-7.5. However, in the absence of glucose, the ability to maintain a higher intracellular pH at pH 5.5 is lost (Shabala et al., 2002). Other physiological changes in response to acid stress include changes in protein synthesis and fatty acid composition of the cell membrane (Koutsoumanis and Sofos, 2004), the latter of which increases the surface hydrophobicity of acid adapted cells (Lou and Yousef, 1997).

Exposure to the aforementioned stresses can alter *Listeria* sensitivity to quaternary ammonium sanitizers. After exposure to acid or starvation stress, *L. innocua* is less sensitive to the quaternary ammonium sanitizer, cetrimide (Moorman et al., 2005). This cross-protection does not occur after exposure to cold and heat stress, which increases *L. innocua* sensitivity to cetrimide (Moorman et al., 2005). *Listeria monocytogenes* strains that are resistant to quaternary ammonium compounds, other sanitzers and antibiotics have been found to contain a gene (*mdrL*) that encodes for an efflux pump (Romanova et al., 2002). However, some resistant isolates do not appear to rely on efflux pumps, and instead alter their cell membrane fatty acid profile in response to sanitizer stress, which can prevent entry of foreign chemicals into the cytoplasm (To et al., 2002). The same study found that upon initial (30 h) exposure to sublethal levels of benzalkonium chloride, biofilm growth was favored (To et al., 2002). Exposure to sublethal concentrations of ethanol and isopropanol can also increase *L. monocytogenes* attachment at 10, 20 and 30°C (Gravesen et al., 2005) with ethanol-adaptation increasing

L. monocytogenes resistance to lethal levels of acid, ethanol, hydrogen peroxide, and sodium chloride (Lou and Yousef, 1997).

Elimination of *Listeria* biofilms from food contact surfaces is particularly difficult. In general, a high degree of mechanical action and friction is required to remove biofilms (Gibson et al., 1999). Hydrogen peroxide is effective at eliminating biofilms, particularly since its efficacy is unaffected by high organic loads (Robbins et al., 2005). Exposure to a pH of 12 through the addition of sodium hydroxide, is also an effective means to eliminate *L. monocytogenes*, either as a biofilm or planktonic cells, particularly when used prior to exposure to quaternary ammonium santizers (Chavant et al., 2004a; Chavant et al., 2004b).

1.8 Prevalence of *Listeria monocytogenes* in Food Processing Environments

Tompkin (2002) identified three scenarios for the occurrence of foodborne listeriosis:

1) Isolated, sporadic cases in which no food origin is identified.
2) Outbreaks involving a single lot of contaminated food, as the result of errors in handling that lead to contamination and growth of *L. monocytogenes* in the product.
3) Outbreaks involving a few to several hundred cases scattered over time and location as the result of contamination of the food processing environment by a persistent and virulent strain of *L. monocytogenes*, which then contaminates multiple lots of product for days or even months.

In order for the third scenario to occur, *L. monocytogenes* must find a niche in the processing environment that is protected from normal cleaning and sanitizing procedures (Tompkin, 2002). Unfortunately, there is no shortage of possible niches in the typical food-processing environment, and *L. monocytogenes* has been found to persist in processing environments for up to 12 years (Lunden et al., 2001). Equipment such as peelers, slicers, dicers, and conveyor belt lines are not always designed in a way that facilitates effective cleaning and sanitizing. In a study of 13 dried sausage-processing plants, effective cleaning and sanitizing was prevented by the complexity of processing lines and machines (Thevenot et al., 2005). Organic residues remaining on these pieces of equipment were associated with samples positive for *L. monocytogenes* when the equipment was "clean" prior to the beginning of the processing shift (Thevenot et al., 2005). Another study of meat, poultry and seafood processing facilities found that the cleaning procedures used were ineffective in eliminating *Listeria* spp. in certain areas of the processing environment, particularly conveyor belts, carts, floors and drains, with these areas accounting for nearly 95% of *L. monocytogenes*-positive samples. Following sanitation, 23.4% of samples from these areas were still *L. monocytogenes*-positive (Godbjornsdottir et al., 2004). In a survey of *L. monocytogenes* contamination in poultry processing environments, several food contact surfaces were persistently contaminated with the same strains, including slicer blades and blade covers, dicing machine blades and blade covers, a conveyor belt, and a spiral conveyor in a freezer (Lunden et al., 2003). This inability to adequately clean surfaces allows *L. monocytogenes* to persist in the environment and form biofilms on food contact surfaces where the pathogen can be potentially transferred to RTE foods.

Persistent strains play an important role in contamination of RTE foods with such strains 8 times more likely to contaminate finished product than transient strains (Lunden et al., 2003). While transient strains were likely to be found in both the incoming raw product and environment prior to any lethal cooking process, they were not found in any of the post-cooking processing lines (Lunden et al., 2003). Other studies have also reported that it is unusual to have the same strain of *L. monocytogenes* contaminating both incoming raw product and the RTE final product, leading to the conclusion that persistent environmental strains are frequently responsible for recontamination of RTE products (Nesbakken et al., 1996; Lappi et al., 2004).

It has been calculated that ideally, a food and environmental *Listeria* control program in a processing facility may be able to keep the prevalence of product contamination of a cooked RTE product at < 0.5%, with post process pasteurization yielding a contamination rate that is essentially zero. A single lot of product contaminated at this level may still be accepted despite the zero-tolerance regulation, since there is a 61% statistical likelihood of *L. monocytogenes* contamination at the 0.5% level going undetected by end product testing (Tompkin, 2002). Intervention strategies can be effective in controlling the prevalence of *L. monocytogenes* in meat processing environments, but appropriate intervention strategies vary between facilities. In some plants, increased compartmentalization of raw and cooked processing areas is required to decrease *L. monocytogenes* prevalence in the environment, which often requires structural changes to the facility (Lunden et al., 2003; Lappi et al., 2004). Changes in equipment design in order to eliminate harborage sites, such as difficult to clean areas in mechanical slicers, interlocking conveyor belts and hollow rollers in conveyors may also

play an important role in improving *Listeria* control (Tompkin, 2002; Lappi et al., 2004). In some cases, changes in sanitation programs are required including the addition of sanitizing footbaths and trench drains (Lappi et al., 2004). Employee training in the importance of certain procedures to reduce the risk of cross-contamination may also be effective in reducing *Listeria* transfer to different areas and products in the plant (Lappi et al., 2004).

1.9 Bacterial transfer during food processing

Meat processing equipment is typically constructed out of stainless steel, which shares similarities with nonporous plastic cutting boards such as the ability to become scratched and scarred with use and the inability to irreversibly absorb bacteria. When beef trim was surface-inoculated with *E. coli* O157:H7 at a level of ~6.0 log CFU/g and ground using a Hobart model 84142 grinder, populations of 3-4 log CFU/cm^2 were recovered from the stainless steel auger housing during grinding (Farrell et al., 1998). Following cleaning and sanitizing the grinder with chlorine or peroxyacetic acid sanitizers, *E. coli* O157:H7 was still recoverable from the stainless steel surface by enrichment (Farrell et al., 1998). In a similar study involving the distribution of contamination by in a table-top bowl chopper used to process beef inoculated with *E. coli* O157:H7 (2 log CFU/g), the pathogen was always transferred to subsequent batches of beef processed in the bowl chopper with *E. coli* O157:H7 also present on the comb/knife guard and the knife after processing (Flores, 2004). These findings emphasize the importance of thorough cleaning and sanitizing to minimize cross-contamination in the processing environment.

Lin et al. (2006) conducted a study in which the blade of a commercial-scale meat slicer used to slice roast turkey breast, salami and bologna was inoculated to contain *L. monocytogenes* at levels of 1, 2, or 3 log CFU/blade (1 and 2 log CFU/blade inoculum used with turkey only). The slices of meat were subsequently deposited on a conveyor belt (material unspecified), which were then tested in pooled samples of five slices, and quantified via MPN. More slices of meat tested positive by enrichment at the 3 log CFU/blade inoculum level than at 1 or 2 log CFU/blade (Lin et al., 2006). Of the 20 equipment samples (10 under the blade housing, 6 blade samples, and 4 on conveyor belts) taken after each of two slicing replicates per product, 5 blade surface samples were positive, 6 positives were found from the blade housing, and 2 conveyor belt samples were positive (Lin et al., 2006). Additionally, Lin et al. (2006) found that more equipment samples were positive for *L. monocytogenes* after slicing salami (8 samples) than turkey (3 positive samples) or bologna (1 positive sample), which supports a longer residence time for *L. monocytogenes* on fat-coated slicers as suggested by Vorst et al. (2006).

A study was conducted of *L. monocytogenes* transfer from biofilms (both pure culture and mixed with processing plant isolates) developed in raw beef exudate pipetted onto a stainless steel surface (AISI grade 304, 2RB finish) at 15 and 25°C/100% RH to mimic meat industry biofilms (Midelet et al., 2006). Transfer to a trypticase soy agar cylinder used as a model food product was quantified and a transfer rate of 55% was observed from pure culture biofilms, while the presence of *Kocuria varians*, a gram-positive organism isolated from a dairy processing environment, increased the *L. monocytogenes* transfer rate to 78% (Midelet et al., 2006). In this study, transfer

increased throughout the first three contacts, and then declined with each successive contact beyond the third contact (Midelet et al., 2006). Exposure to chlorine shock increased the adhesiveness of *L. monocytogenes* to the stainless steel surface, resulting in a smaller transfer coefficient. This led the authors to conclude that while cleaning and sanitizing may mitigate immediate risk of high initial levels of *Listeria*, reducing the delayed risk of *Listeria* contamination due to prolonged attachment to surfaces could only be achieved by lowering the attachment strength of *L. monocytogenes* to the food contact surface, which none of the tested sanitizers was able to do (Midelet et al., 2006).

In a study in which volunteers' hands were inoculated with wild-type *E. coli* (at levels of 4-6 log CFU recovered from the hands), it was found that flock-lined rubber gloves (20 mil thickness) largely prevented transfer to subsequently handled raw beef cuts (Gill and Jones, 2002). Knitted polyester or cotton gloves reduced transfer (0.30 – 1.6 log CFU/beef sample) as compared to transfer observed from bare hands (2.5 – 3.5 log CFU/beef sample), but were not as effective in inhibiting transfer as rubber gloves ($\leq$ 0.90 CFU/beef sample; Gill and Jones, 2002).

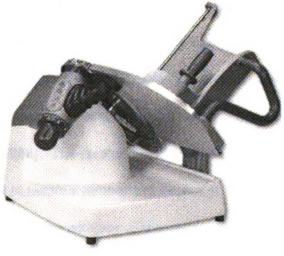
1.10 Bacterial transfer during retail food handling

Delicatessen slicer designs vary, but all of them contain the following basic components (Figure 1.3): a circular, stainless steel blade; a built-in blade sharpener; a stainless steel blade cover, typically grooved; a table to hold the meat, also grooved for drainage; a grooved back plate to hold the meat in position for slicing; a collection area for sliced product; and a motor covered by a stainless steel case. Based on this design, several areas of the slicer are difficult to clean and sanitize effectively (e.g., the blade, the

area behind the blade, non-removable components), thus providing several potential niches for bacterial pathogens including *L. monocytogenes*. However, as slicer manufacturers have become more aware of the increased difficulty in cleaning these areas, delicatessen slicers are now being re-designed for easy disassembly, cleaning and sanitizing with most of these microbial niche areas being eliminated. (Figure 1.4).

Figure 1.3. Example of Delicatessen meat slicer (Chefmate, 10" manual slicer)

Figure 1.4. Example of delicatessen meat slicer designed for easier sanitation (Berkel Company; South Bend, IN; X13 Slicer)



In food retail food handling environments, bacterial contaminants including *Listeria* are most often found in difficult to clean areas that contain food particulates and adequate moisture. Bacteria within these harborage sites are typically exposed to stressful conditions including sanitizers, dehydration, starvation, and extremes in both temperature and pH. Under these extreme conditions, *L. monocytogenes* can become sublethally injured with the pathogen then unable to grow on many commonly used selective plating media. Complex substrates are needed for growth with many of same stresses present in the human host also present in food, such as lack of iron, oxidative stress, pH extremes and starvation (Archer, 1996). This may have the effect of triggering the expression of virulence genes, increasing the ability of pathogens to induce illness (Archer, 1996).

Even under these unfavorable environmental conditions, bacterial foodborne pathogens can remain viable on common food contact surfaces for days or weeks and go on to cross-contaminate other products. In one early report, 469 cases of typhoid fever were traced to a single can of corned beef sliced at a delicatessen with *Salmonella* Typhi transferred from the delicatessen slicer to other deli meats that were subsequently sold and consumed (Howie, 1968). The greater prevalence of *L. monocytogenes* in delicatessen- as opposed to manufacturer-sliced meat is at least partly due to cross-contamination in the delicatessen with one of the most obvious contact points being the delicatessen slicer (Gombas et al., 2003). A study of *L. monocytogenes* contamination routes in smoked salmon processing plants also found that slicers were harborage sites in both plants in the study (Vogel et al., 2001).

In recent bacterial transfer work with mechanical delicatessen slicers, *L. monocytogenes* has been shown to readily transfer both to and from slicer blades and deli meats. Based on the work of Vorst et al., (2006), *L. monocytogenes* transferred from a blade inoculated at 8 log CFU/blade to 30 successive slices of roast turkey breast with transfer decreasing logarithmically to 2 log CFU/slice by the 30th slice. At lower inoculum levels (5 log CFU/blade and 3 log CFU/blade), transfer was not quantifiable beyond the 5th slice, with negative enrichments after 27 and 15 slices, respectively (Vorst et al., 2006). In the same study, transfer to salami was more continuous throughout the 30 slices than to turkey or bologna, both of which were higher in moisture and lower in fat than the salami. The difference in transfer between the products was attributed to the layer of fat that accumulated on the slicer blade during slicing of salami, which was not seen for the other two products (Vorst et al., 2006). These findings resemble those of Ak et al. (1994) which showed that chicken fat protected bacteria from desiccation and removal from cutting boards. Consequently, fat from the salami may help protect *L. monocytogenes* from desiccation and dispersal from the slicer in a similar fashion. Furthermore, this corroborates the aforementioned results obtained by Lin et al. (2006) who found that commercial-scale slicer equipment used to slice salami yielded more positive samples than that used to slice turkey or bologna.

A study was conducted using *Enterobacter aerogenes* as a surrogate for *Salmonella* spp., and rates for bacterial transfer were compared for transfer to and from hands with or without polyethylene, food service grade gloves during cutting of chicken and lettuce (Montville et al., 2001). The chicken was inoculated with ~ 8 log/CFU per portion, and participants were instructed to cut the chicken into cubes, transfer it to a

bowl and then slice lettuce. Participants were not instructed in the proper way to put on gloves, in order to better simulate real-world conditions of use (Montville et al., 2001). The resulting transfer rate from chicken to lettuce was 0.01% to and from gloved hands, while the transfer rate between ungloved hands and food was 10% (Montville et al., 2001). In another study evaluating transfer of *E. aerogenes* from chicken to hands (using the aforementioned inoculation method), and then to hand-washing areas and lettuce, the reported transfer rates were highly variable (Chen et al., 2001). Transfer rates ranged from 0.3 – 100% from chicken to hands, 0.003 – 12.3% from hands to the spigot on a handwashing sink, 0.001 – 45.7% from dirty hands to clean hands after handwashing (implying that hands were improperly washed), and 0.01 – 100% from "clean" hands to lettuce (Chen et al., 2001).

1.11 Bacterial transfer in the home

Food handling in home kitchens can also lead to multiple routes of cross contamination. Several studies have assessed bacterial transfer between food contact surfaces, cloths and sponges used for cleaning, hands and food products (Scott and Bloomfield, 1990; Montville et al., 2001; Sattar et al., 2001; Gill and Jones, 2002). Two of these studies found that moist surfaces, cloths and hands transferred higher numbers of bacteria to one another than was observed when the aforementioned surfaces were dry (Scott and Bloomfield, 1990; Sattar et al., 2001). According to Sattar et al., (2001) transfer of *Staphylococcus aureus* between moist cloths and moist fingertips was always higher than transfer between the two if they were dry, and that the addition of friction by rubbing the cloth with the hands for 10 s resulted in a five-fold increase in transfer.

However, even in the scenario with the greatest amount of bacterial transfer (moist cloths to moist hands with friction), only 2.5% of the *S. aureus* (5 log CFU/ piece of fabric) inoculum was transferred (Sattar et al., 2001). Furthermore, a study by Scott and Bloomfield (1990) found that *S. aureus*, *E. coli* and *Salmonella* spp. (2 log CFU/cloth inoculum) were able to survive on soiled cloths for up to 24 h and transferred to laminate surfaces and fingertips, which allows for possible recontamination of a domestic kitchen during cleaning.

Several studies have attempted to quantify transfer between food contact surfaces and food in the absence of friction common to food processing scenarios. According to Kusumaningram et al. (2003), 21-43% of the *S. aureus*, *Campylobacter jejuni* and *Salmonella enteriditis* populations on inoculated (6.7 – 9.4 log CFU/sponge) wet sponges were transferred to stainless steel (AISI grade 304), with no significant differences seen between the organisms in their rate of transfer to stainless steel or to subsequently applied food products. Subsequently, 25-100% of the available population transferred to roast chicken when applied to the stainless steel for 10 s with greater transfer observed when a 500-g weight was added to the chicken. Increasing the product weight did not have the same effect on transfer to cucumber slices, which was found to occur at 50-100% of the available bacterial population (Kusumaningram et al., 2003). However, when compared to several other existing studies, it seems unlikely that 100% transfer can be achieved by simply placing a food on a surface with the unusually high transfer levels achieved in this study likely caused by inaccurate estimation of the surface inoculum. In the aforementioned study, an unorthodox contact plate method was used to quantify the bacterial population on stainless steel with the agar from the contact plate then suspended

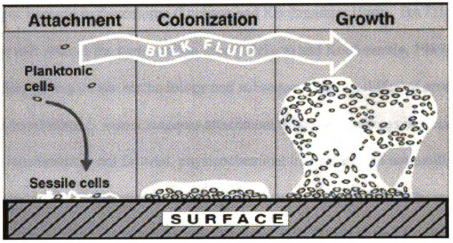
and homogenized in a peptone saline solution, and diluted to a countable level prior to plating (Kusumaningram et al., 2003). This undermines the validity of the transfer rates.

Another study found that ground beef (75-100 g patties) with an average bacterial load of 6.7 log CFU/g transferred 2.5-3.0 log CFU/cm^2 of *E. coli* O157:H7 to cutting boards (polyethylene and wood laminate, no significant difference) after a 30 minute contact time (Miller et al., 1996). Although transfer to the different cutting board types was not significantly different, subsequent cross-contamination from cutting boards to other surfaces was different. In an earlier study that examined the survival of *L. monocytogenes*, *Salmonella typhimurium* and *Escherichia coli* O157:H7 on cutting boards (cut into 5 cm^2 blocks), porous wooden cutting boards more effectively inhibited cross-contamination due to the capillary action of the wood (Ak et al., 2004). Both wooden (species included: ash, basswood, beech, birch, butternut, cherry, hard maple, oak, and American black walnut) and plastic (polyacrylic, polyethylene, foamed polypropylene, polystyrene, and hard rubber) were examined. Using wooden boards, bacteria were internalized in the board to a depth of at least 15 μm within 3-10 min and were unavailable for subsequent transfer to other foods or knives when the boards were later used for food preparation (Ak et al., 1994). However, bacteria were able to persist and even multiply on the surface on nonporous plastic boards over a 12 h period at 22°C, particularly if the ambient humidity was sufficient to prevent the surface from drying, with the greatest persistence and survival in knife-scarred areas of the board containing chicken fat (Ak et al., 1994).

1.12 *Listeria* persistence on surfaces and biofilm formation

Up to this point, virtually all bacterial transfer work has been conducted using bacterial cultures that have been inoculated onto a surface, briefly dried and then placed in contact with other surfaces or foods to simulate various transfer scenarios. However, in reality, bacterial transfer is far more complex with the extent of bacterial attachment to these surfaces ranging from loosely attached cells to biofilms. Bacterial attachment is defined as an affiliation between a bacterium and a surface (Notermans et al., 1991). Many types of bacteria, including *L. monocytogenes*, can attach and persist on equipment over extended periods of time. *Listeria monocytogenes* has been shown to adhere to 17 approved food contact surfaces, consisting of rubbers, polymers and metals, including stainless steel (Beresford et al., 2001). Attachment is the first step in biofilm formation (Figure 1.5).

Figure 1.5. Stages in biofilm development (MSU-CBE, 2002)



Initial attachment occurs via electrostatic or hydrophobic interactions between the bacterial surface and the contact surface (Arnold and Bailey, 2000). The literature is contradictory concerning the nature of the *L. monocytogenes* cell surface as well as food contact surfaces and their subsequent interactions. *Listeria monocytogenes* has a negative surface charge (Dickson and Koohmaraie, 1989; Chavant et al., 2002) and some studies indicate that the surface is hydrophobic (Dickson and Koohmaraie, 1989; Ukuku and Fett, 2002), while others indicate that the surface is hydrophilic (Mafu et al., 1991; Briandet et al., 1999; Chavant et al., 2002). These discrepancies in the measurement of cell surface hydrophobicity or hydrophilicity may be due to surface variations between strains or differences in the methods used to measure hydrophobicity. Three commonly used methods for determining hydrophobicity of microorganisms — hydrophobic interaction chromatography (HIC), bacterial adherence to hydrocarbons (BATH), and contact angle measurement—often produce very different results for the same bacterial strain (Dickson and Koohmaraie, 1989). HIC and BATH are used more frequently since they require less specialized equipment than contact angle measurement, but there are

disadvantages to both methods. HIC may produce inconsistencies due to nonspecific binding of bacteria to the column (Dickson and Koohmaraie, 1989). BATH results may vary due cell lysis or the hydrocarbon used (Dickson and Koohmaraie, 1989).

Depending on the methodology and subsequent results, different predictions are made as to whether *L. monocytogenes* attachment to various surfaces is favored or not. Even if attachment is not favored, physicochemical interactions do not totally prevent attachment with attachment still occurring, albeit at lower levels on surfaces that would otherwise be predicted to naturally repel bacterial cells (Dickson and Koohmaraie, 1989; Mafu et al., 1991; Cunliffe et al., 1999). Smoot and Pierson (1998) reported a faster *L. monocytogenes* attachment rate to stainless steel than to Buna-N rubber, although cell surface hydrophobicity and surface free energies predicted that adhesion to Buna-N rubber would be favored. Another study also found that hydrophilic and negatively charged *L. monocytogenes* cells adhered better to stainless steel, than to polytetrafluoroethylene (Chavant et al., 2002). Briandet et al. (1999) reported that although *L. monocytogenes* tended to be hydrophilic, strains that were slightly more hydrophobic than others adhered better to stainless steel. However, Midelet and Carpentier (2002) observed stronger attachment of *L. monocytogenes* biofilms to polymers (polyvinyl chloride and polyurethane) than to stainless steel, and also noted that all of these surfaces were hydrophobic, with these same surfaces becoming hydrophilic after exposure to meat exudate based on contact angle measurement. Cunliffe et al. (1999) found that hydrophilic uncharged surfaces were slightly repellent to *L. monocytogenes*. Another study reported that *L. monocytogenes* Scott A was more attracted to polypropylene and rubber surfaces than to glass and stainless steel (Mafu et

al., 1991). When studying the physicochemical characteristics of *L. monocytogenes* and its attachment to glass, hydrophobicity and surface charge had no correlation to the degree of cell attachment (Chae et al., 2006). The trends suggested by many of these physicochemical measurements are influenced by conditions of the individual experiment and the methods used to determine surface hydrophobicity. Studies have found that surface soil (meat exudate, skim milk, various proteins) will enhance attachment, and alter the contact surface physicochemical properties (Barnes et al., 1999; Midelet and Carpentier, 2002). Physicochemical properties of the cell surface and contact surface may enhance or inhibit the rate of initial attachment, but attachment was not completely inhibited in any of the aforementioned studies.

After the reversible step of initial attachment, irreversible attachment can follow. This is the process in which bacteria secrete exocellular polymeric substances or exopolysaccharides (EPS) that function as a glue to hold the bacterial cell to the surface and retain the organism in subsequently formed biofilm communities (Stoodley et al., 2002). Development of biofilms requires water or a relative humidity (RH) above 84%, with optimal biofilm growth at 100% RH (Else et al., 2003). Level and rate of *L. monocytogenes* attachment can be used to predict the ability of strains to form biofilms and persist in the environment. Lunden et al. (2000) reported that the same pulsed-field gel electrophoresis (PFGE) type of *L. monocytogenes* was transferred to three different processing plants in a dicing machine used in the three plants. The persistent strain was then tested for adherence to stainless steel in broth culture at 25°C for 1, 2, and 72 h, along with three non-persistent strains of *L. monocytogenes* isolated from the third plant. The persistent strain was significantly more adherent than the non-persistent strains, a

trend that has also been observed in other studies (Lunden et al., 2000; Norwood and Gilmour, 1999; Borucki et al., 2003). Kalmokoff et al. (2001) also reported variation in the ability of different *L. monocytogenes* strains to adhere to stainless steel, and subsequent biofilm formation by the strains varied as well, with only one strain capable of forming a biofilm consisting of bacterial aggregates. *Listeria* was able to adsorb to stainless steel within 2 h, and enhanced attachment was observed in strains that produced extracellular fibrils. Enhanced attachment also has been observed by *L. monocytogenes* grown at 20 - 25°C, which is optimal for flagella production (Vatanyoopaisarn et al., 2000).

Given the predominance of Lineage II *L. monocytogenes* strains in the environment, these strains would be expected to show increased biofilm formation, leading to greater persistence in the environment. However, results vary, with one study reporting better biofilm formation by Lineage I strains (Djordjevic et al., 2002), while another noted better biofilm formation by Lineage II strains (Borucki et al., 2003).

Mutations and environmental stress have an affect on *L. monocytogenes* biofilm formation. The ability to respond to nutrient deprivation seems to be a requirement for survival in a biofilm and also to cause illness. Mutants lacking in the ability to mount a stringent response to amino acid deprivation were inhibited in their ability to attach to surfaces, and were also avirulent in a mouse model (Taylor et al., 2002). Cell surface proteins are also important for surface growth — the addition of 0.01% trypsin to growth media can reduce the adherent cell population by 99.9%, as compared to control cultures without trypsin (Smoot and Pierson, 1998). However, excess protein in growth media inhibits cell attachment to surfaces, thus chemically defined minimal media results in

better biofilm formation by *L. monocytogenes* (Kim and Frank, 1994). This provides further evidence that biofilm formation is a survival strategy for bacteria in stressful environments, along with the observation that biofilm formation aids subsequent survival upon exposure to sanitzers (Chavant et al., 2004; Lomander et al., 2004; Somers et al., 2004; Robbins et al., 2005), and also improves resistance to dessication, possibly due to retention of water by EPS or by limiting the surface area available to air, thus slowing evaporation of water from the microbial community (Flemming and Wingender, 2002).

The presence of other microorganisms on a surface can also influence the level of *L. monocytogenes* colonization. *Listeria monocytogenes* will attach in significantly higher numbers (> 3 log CFU/cm^2) to a condensate-covered surface containing a pre-existing *Pseudomonas putida* biofilm (Hassan et al., 2004). The effect of interactions with other resident flora can also be inhibitory to *L. monocytogenes*. In a study of 31 strains of resident microflora from a food processing environment, 16 strains inhibited growth of *L. monocytogenes* on the surface, 11 strains had no effect, and 4 strains enhanced *L. monocytogenes* biofilm formation, as opposed to pure culture (Carpentier and Chassaing, 2004). Furthermore, one of the 4 synergistic strains, *Comamonas testosteroni* CCL 24, a gram-negative organism which was isolated from a food processing environment, released a metabolite into its biofilm growth medium supernatant, which was sufficient to enhance *L. monocytogenes* biofilm growth in pure culture (Carpentier and Chassaing, 2004). This interaction is similar to that from quorum-sensing molecules, which have not been characterized in *L. monocytogenes*. Quorum-sensing is the regulation of gene expression in response to changes in cell population density via chemical signaling using oligopeptides produced by Gram-positive

bacteria, and N-acyl-homoserine lactones in Gram-negative bacteria (Miller and Bassler, 2001; Lazazzera, 2000). Thus, the problem of *Listeria* transfer becomes very complex if strongly adhering cells in biofilms are to be studied under the most realistic scenarios.

1.13 Predictive modeling

Within the last decade, risk assessments have necessitated the development of dynamic models that provide estimates of bacterial survival, growth, and distribution throughout food processing and storage. Microbiological risk assessments depend upon exposure assessments; however, these exposure assessments rely on existing data for presence of bacteria in food products, the accuracy of which is limited by sample size and test methods in existing prevalence surveys (Gardner, 2004). Predictive modeling can account for variations in sample size and test method in existing prevalence surveys, and can allow for estimations of microbial contamination levels, distribution and rate of transfer in the environment.

1.14 Predictive modeling of bacterial growth

Predictive models for the growth and distribution of microorganisms can be divided into three types as defined by Bernaerts et al. (2004):

1) Empirical models, which are derived from experimental data and are essentially curve-fitting models;
2) Mechanistic models, which are a precise mathematical translation of underlying biological mechanisms;

3) Semimechanistic models, which take elements of both empirical and mechanistic models due to the complexity and knowledge gaps about all possible underlying biological mechanisms resulting in the difficulty in development of purely mechanistic models.

Numerous predictive growth models have been developed and compared by researchers, including the Pathogen Modeling Program developed by the United States Department of Agriculture (USDA; Buchanan and Phillips, 1990; Tamplin, 2002). The more recent version of the USDA Pathogen Modeling Program has a pre-programmed graphical user interface and generates graphs and tables for various growth parameters, which can provide input based on the needs of each user (Tamplin, 2002). These models have been developed to predict the growth of foodborne pathogens, including *L. monocytogenes*, in foods based on pH, salt and sodium nitrite content, as well as storage temperature (Houtsma et al., 1996; LeMarc et al., 2002; Tamplin, 2002). Recently, an attempt has been made to cross-reference the raw data used to develop models with the resultant predictive models, in order to increase transparency for the derivation of models. This has resulted in the development of ComBase, which allows users to access the growth curves upon which predictive models are based (Baranyi and Tamplin, 2004).

1.15 Predictive modeling of bacterial transfer

Schaffner (2004) has described the basic mathematical framework for modeling *L. monocytogenes* cross-contamination in food processing plants, using the following equations:

1) Raw product CFU x Cross-contamination rate = Environmental CFU
2) Environmental CFU x Persistence rate = Environmental reservoir CFU
3) Environmental reservoir CFU x Cross-contamination rate = Product contact surface CFU
4) Product contact surface CFU x Persistence rate = Product contact surface reservoir CFU
5) Product contact surface reservoir CFU x Cross-contamination rate = Finished product CFU

Due to a lack of data for quantitative transfer of *L. monocytogenes*, the two models Schaffner (2004) developed around this framework using a Monte Carlo simulation were only able to track *L. monocytogenes* numbers and prevalence or *L. monocytogenes* prevalence alone, but not *L. monocytogenes* concentrations within raw and finished products. The model illustrates the additive effect, in which each fraction of transfer "f_x" is an additive function of the previous fraction or $f_x = f_a * f_b$ where

"f_a" = raw product and

"f_b" = cross contamination rate

Using the resultant models can help a processor determine whether an overall greater reduction in *L. monocytogenes* prevalence in a plant could be achieved by requiring better raw material quality or by improved sanitation efforts (Schaffner, 2004). Furthermore,

these models predict that low numbers of incoming persistent strains of *L. monocytogenes* strains will eventually predominate in the finished product (Schaffner, 2004). In a model developed to determine the risk of cross-contamination of salads by *Salmonella* spp. or *Campylobacter* spp. from chicken in domestic kitchens, a higher probability of *Campylobacter* spp. entering salads was predicted, due to its higher prevalence and level in chicken (Kusumaningrum et al., 2004).

A model with similar benefits, in terms of determining the best testing sites to minimize contamination of ground beef produced using a commercial grinder, was developed by Flores and Stewart (2004). According to the model, rather than random sampling of a ground beef lot to determine *E. coli* O157:H7 contamination, a more accurate determination of contamination of the lot could be obtained by testing the collar that fixes the grinder die and blade to the meat grinder (Flores and Stewart, 2004). This was based on the fact that samples from a lot made with a randomly selected piece of beef trim contaminated with 2 log CFU of *E. coli* O157:H7 would test negative for E. coli O157:H7. However, in each case, the collar tested positive for *E. coli* O157:H7 (Flores and Stewart, 2004).

In models that have been specifically developed to assess transfer of *L. monocytogenes*, one model was developed to determine the risk of *L. monocytogenes* transfer and subsequent growth due to contact with bare hands or gloved hands (Perez-Rodriguez et al., 2006). This model predicted that the highest risk of contamination comes from handling raw and ready-to-eat meats with the same gloves. This risk was higher than the risk of cross-contamination from bare, washed hands (Perez-Rodriguez et al., 2006). According to calculations obtained from this model, *L. monocytogenes* on

hands would have to be reduced 80% by washing in order to achieve a 50% reduction in *L. monocytogenes* on subsequently handled slices of ham with *L. monocytogenes* counts exceeding the European Union Food Safety Objective for *L. monocytogenes* (2 log CFU/g at consumption) by the end of the potential storage period (Perez-Rodriguez et al., 2006).

1.16 Goals of the current study

Thus far, Vorst (2005b) has developed the only model to predict *L. monocytogenes* transfer during slicing of RTE delicatessen meats on a commercial delicatessen slicer. The model is a linear model that predicts the number of CFU transferred to any given slice, as well as the number CFU lost to the environment through aerosols and bacterial death. Under the conditions tested by Vorst (2005b), this model had a correlation coefficient varying from $R^2 = 0.40$ when slicing salami, to over 0.90 when slicing turkey or bologna with a slicer blade inoculated at 8 log CFU/blade (Vorst, 2005b).

Empirical data obtained from four years of laboratory research in the current study was used to validate the utility of the Vorst (2005b) model in predicting transfer of *L. monocytogenes* after exposure to bacterial stress (cold-injury and chlorine-injury) and prolonged (6 and 24 h) desiccation on stainless steel to turkey and salami. Additionally, the model was also tested for its ability to predict *L. monocytogenes* transfer based on strain persistence and biofilm formation. The underlying hypothesis for this study was that strain persistence would have an affect on the survival and transfer of *L. monocytogenes* to delicatessen meats, particularly after prolonged desiccation on stainless

steel. This research was conducted in response to *Listeria* transfer rates being identified as a key informational gap in the *Listeria* Risk Assessment published by the US federal government (FDA/USDA/CDC, 2003). Additional research is required in this area in order to refine existing assessments of the risk to the public for contracting listeriosis from the consumption of delicatessen-sliced RTE meats.

CHAPTER 2

VARIATION IN BIOFILM FORMATION BY *LISTERIA MONOCYTOGENES* STRAINS AT 4°C AND 22°C

2.1 ABSTRACT

Potential biofilm formation by *Listeria monocytogenes* on food contact surfaces can lead to cross-contamination and further spread of *Listeria* in commercial and home settings. Additional research on *Listeria* biofilm formation is needed to help better define the impact of food preparation practices on listeriosis estimations being developed in current risk assessments. This study characterized biofilm-forming capabilities of a diverse set of 196 *L. monocytogenes* strains at 4 and 22°C. *Listeria monocytogenes* isolates from food, environmental, veterinary and clinical sources comprised of 16 different ribotypes were assessed for biofilm formation in Modified Welshimer's Broth using 96-well untreated polystyrene microtiter plates (3 wells/strain x 3 replicates). Following 4 and 60 days of incubation at 22 and 4°C, respectively, the microtiter plate wells were emptied, rinsed and air-dried. After staining fixed cells with crystal violet, the optical density (OD) of the resolubilized dye was read at 570 nm. At 22°C, 83% and 95% of the OD values were within one and two standard deviations of the mean -- 0.53 $\pm$ 0.38 and 0.76, respectively. At 4°C, 92% and 97% of the OD's were within one and two standard deviations of the mean—0.12 $\pm$ 0.10 and 0.20, respectively, with 109 of 196 (55%) strains failing to produce detectable biofilms at 4°C. Significant differences in biofilm formation were observed between strains of the same ribotype. While most *L. monocytogenes* strains formed biofilms at room temperature, appreciable biofilm formation was typically absent at 4°C, thus suggesting the inability of most *L. monocytogenes* strains to produce significant biofilms in otherwise clean cold storage areas.

2.2 INTRODUCTION

Many bacteria, including *Listeria monocytogenes*, have the ability to attach and persist on equipment over extended periods of time. Persistent strains may play an important role in the contamination of ready-to-eat (RTE) foods. In one processing facility, persistent *L. monocytogenes* strains were 8 times more likely to contaminate finished product than transient strains (Lunden et al., 2003). According to Lunden et al. (2003) transient strains were prevalent in both incoming raw product and the environment before processing, but were not found in any post-cooking processing lines. Other studies have reported that the same strain of *L. monocytogenes* are infrequently recovered from both incoming raw products and final RTE products, leading to the conclusion that persistent environmental strains are most often responsible for recontaminating fully-cooked RTE products (Nesbakken et al., 1996; Lappi et al., 2004).

Level and rate of *L. monocytogenes* attachment have been used to predict the ability of strains to form biofilms and persist in the environment. Lunden et al. (2000) reported that a persistent strain of *L. monocytogenes* was transferred to three different processing plants via a dicing machine and was significantly more adherent than non-persistent strains, a trend which also has been reported previously (Lunden et al., 2000; Norwood and Gilmour, 1999; Borucki et al., 2003). However, Djordjevic et al. (2002) found no significant difference in biofilm-forming ability of *L. monocytogenes* strains according to genetic lineage or environmental persistence. Kalmokoff et al. (2001) reported variation in the ability of *L. monocytogenes* strains to adhere to stainless steel with the extent of subsequent biofilm formation also varying. However, only one of 36 strains was capable of forming a biofilm containing bacterial aggregates. Enhanced

attachment has been observed by *L. monocytogenes* grown at 20-25°C, which is optimal for flagella production (Vatanyoopaisarn et al., 2000).

Initial bacterial attachment to surfaces occurs via electrostatic or hydrophobic interactions between the bacterial cell surface and the contact surface (Arnold and Bailey, 2000). Despite having a negative surface charge (Dickson and Koohmaraie, 1989; Chavant et al., 2002), some studies claim that the surface of *L. monocytogenes* is hydrophobic (Dickson and Koohmaraie, 1989; Ukuku and Fett, 2002), while others indicate that the surface is hydrophilic (Mafu et al., 1991; Briandet et al., 1999; Chavant et al., 2002). These discrepancies in cell surface hydrophobicity may be due to strain-to-strain variation as well as differences in the methods used to measure hydrophobicity.

The objective of this study was to first assess biofilm-forming ability by a diverse collection of 196 *L. monocytogenes* strains comprised of veterinary, clinical, food and environmental isolates. From this collection, a subset comprised of the weakest and strongest biofilm formers was evaluated for differences in cell surface hydrophobicity via hydrophobic interaction chromatography (HIC) to help identify the role of cell surface hydrophobicity in biofilm formation.

2.3 MATERIALS AND METHODS

2.3.1 *Listeria monocytogenes* strains.

A diverse set of 196 *L. monocytogenes* strains, partially characterized by lineage, serotype, ribotype and isolation source (Table 2.1), was assayed for biofilm formation at 22 ± 2°C and 4 ± 2°C. Surface hydrophobicity was tested on a subset of 6 strains of *L. monocytogenes* (CWD 33, CWD 182, CWD 205, CWD 578, CWD 730, and CWD 845). All strains were maintained at -80°C in trypticase soy broth containing 0.6% (w/v) yeast extract (TSB-YE; Becton Dickinson, Sparks, MD) and 10% (v/v) glycerol (Sigma Chemical Company, St. Louis, MO).

2.3.2 Culture preparation.

All strains were grown in TSB-YE for 18 h at 37°C, and then streaked to plates of trypticase soy agar containing 0.6% yeast extract (TSA-YE) (Becton Dickinson) to obtain confluent growth after 18 h of incubation at 37°C. Thereafter, *L. monocytogenes* cells were harvested from TSA-YE plates by flooding the agar surface with 10 ml of 0.1% sterile peptone, with the concentration of the resulting cell suspension estimated from MacFarland Turbidity Standards (Acuff, 1992). The resuspended culture was serially diluted to a final concentration of 10^2 CFU/ml in Modified Welshimer's Broth (MWB), which contained the following ingredients per liter: KH_2PO_4 (6.56 g), $Na_2HPO_4 \cdot 7H_2O$ (30.96 g), $MgSO_4 \cdot 7H_2O$ (0.41 g), ferric citrate (0.088 g), glucose (10.0 g), L-leucine (0.1 g), L-isoleucine (0.1 g), L-valine (0.1 g), L-methionine (0.1 g), L-arginine (0.1 g), L-cysteine (0.1 g), L-glutamine (0.6 g), riboflavin (0.5 mg), thiamine (1.0 mg), biotin (0.5

mg) and thioctic acid (0.005 mg) (Premaratne et al., 1991). All components of MWB were obtained from Sigma Chemical Company.

2.3.3 Microtiter plate assay for biofilm formation.

A modification of the assay described by Stepanovic et al. (2000) was used to assess biofilm formation by *L. monocytogenes.* After vortexing, 200 µl of the diluted cell suspension containing 10^2 CFU/ml was pipetted into three wells of a 96-well untreated polystyrene microtiter tissue culture plate (BD Falcon Microtest™ Flat Bottom; Becton Dickinson, Franklin Lakes, NJ). Three wells per plate containing 200 µl of MWB served as negative controls. Biofilm assays were carried out at 22 ± 2°C for 4 d and at 4 ± 2°C for 60 d. At the end of incubation, the microtiter plate wells were emptied, rinsed three times with 0.85% physiological saline while being gently shaken to remove unattached cells, and then allowed to air-dry. The remaining *Listeria* cells were fixed to the well by adding 200 µl of 99% methanol (Fisher Chemicals, Fair Lawn, NJ) with the methanol decanted 15 min later. After allowing the plates to air-dry, the microtiter wells were stained with 200 µl of 2% crystal violet (Biochemical Sciences, Inc., Swedesboro, NJ) for 5 min. After decanting the crystal violet, the wells were rinsed five times with deionized water and air-dried. The remaining dye was resolubilized in 160 µl of 33% (v/v) glacial acetic acid (EM Science, Gibbstown, NJ) and optical densities were read at 570 nm using a Vmax™ Kinetic Microplate Reader (Molecular Devices, Menlo Park, CA).

2.3.4 Measurement of cell surface hydrophobicity by hydrophobic interaction chromatography.

After subculturing from the frozen stock cultures twice in TSB-YE, cultures were centrifuged (9740 × *g*, 10 min) at 4°C. The resulting cell pellets were washed twice in a salt peptone solution containing 0.85% NaCl and 0.05% Bacto Peptone (Becton Dickinson). For hydrophobic interaction chromatography, capillary pipettes (16 cm long, 5 mm diameter) (Corning Labware, Corning, NY) were plugged with glass wool and washed sequentially with 5 ml of 75% ethanol and 10 ml of 0.02 M $NaPO_4$ buffer (pH 6.8) (Sigma). Columns were packed with octyl-sepharose CL-4B (Sigma) and equilibrated overnight at 4°C in 12 ml of $NaPO_4$ buffer. Washed bacterial cell suspensions (0.1 ml) were loaded onto the surface of the column followed by 12 ml of $NaPO_4$ buffer and the eluent was collected as described by Dickson and Koohmaraie (1989). Eluted solution was plated on Modified Oxford Agar (MOX; Becton Dickinson) and incubated at 35°C for 24 h. The experiment was replicated three times for each strain. Relative hydrophobicity was calculated according to Dickson and Koohmaraie (1989) using following formula:

Relative hydrophobicity = (CFU retained by the column) / (CFU eluted from the column).

When the log value of relative hydrophobicity was < 0, the cell surface was considered hydrophilic.

2.3.5 Statistical Analysis.

All experiments were replicated three times. Statistical analysis of the OD values from the complete set of 196 strains was performed using a general linear model with a general randomized complete block design using SAS (SAS, Version 8, SAS Institute, Inc. Cary, NC). Surface hydrophobicity data were analyzed using a general linear model. Significance was determined at $P < 0.05$. Statistical significance for biofilm formation by lineage, serotype, ribotype and source was determined using a linear mixed effects model (significance at $P < 0.05$).

2.4 RESULTS

2.4.1 Biofilm formation by *Listeria monocytogenes.*

Overall, significant variations in biofilm forming ability were observed for this diverse set of *L. monocytogenes* isolates. OD values at 22 and 4°C ranged from 0.061 to 2.61 and 0.05 to 0.92, respectively, (Table 2.1) and were skewed to the left at both temperatures (Figures 2.1 and 2.2), indicating that most strains were relatively weak biofilm formers, with a few outlying strains yielding higher optical densities indicative of stronger biofilms. At 22°C, 83% and 95% of the OD values were within one and two standard deviations of the mean—0.53 $\pm$ 0.38 and 0.76, respectively. Based on this analysis, 64, 31, and 5% of the strains were classified as weak (OD < 0.53), medium (OD 0.53 - 1.28) and strong (OD > 1.28) biofilm formers at 22°C. At 4°C, 92% and 97% of the OD values were within one and two standard deviations of the mean—0.12 $\pm$ 0.10 and 0.20, respectively, with 159 of 196 (81%) strains failing to produce detectable

biofilms, defined as an OD value not significantly different from the MWB negative control.

Table 2.1. ***L. monocytogenes*** **strain information (strains listed in descending order by biofilm forming ability at 22°C)**

Strain ID	Source	Serotype	Ribotype	Lineage	Mean OD	
					4°C	22°C
CWD 845[a]	dairy plant	1/2b	54081	NA[b]	0.10	2.61
CWD 730	dairy plant	1/2a	19092	NA	0.13	1.94
CWD 33	unknown	4b	19167	NA	0.14	1.91
CWD 1368	ground beef	NA	54183	NA	0.08	1.80
CWD 1734	pork sausage	3b	54081	NA	0.05	1.62
CWD 338	dairy plant	1/2a	19092	NA	0.16	1.49
CWD 1440	unknown	NA	NA	NA	0.08	1.37
CWD 764	hot dog	1/2b	28643	NA	0.15	1.31
CWD 1258	pork sausage	NA	28623	NA	0.22	1.30
CWD 1632	ground beef	NA	54184	NA	0.05	1.29
CWD 1032	pork sausage	NA	54081	NA	0.42	1.28
CWD 600	dairy plant	1/2b	54081	NA	0.08	1.27
CWD 603	dairy plant	NA	54081	NA	0.10	1.11
CWD 1520	ground turkey	NA	19236	NA	0.07	1.10
CWD 1429	unknown	NA	NA	NA	0.09	1.08
CWD 1733	pork sausage	NA	54132	NA	0.06	1.07
CWD 1430	unknown	NA	NA	NA	0.17	1.06
CWD 766	hot dog	3a	19092	NA	0.13	1.02
CWD 580	dairy plant	1/2b	54081	NA	0.07	1.01
CWD 1011	pork sausage	NA	NA	NA	0.06	0.97
CWD 25	unknown	NA	19075	NA	0.09	0.97
CWD 831	dairy plant	NA	19231	NA	0.12	0.97
CWD 1078	chicken	NA	19161	NA	0.12	0.96

Table 2.1. (Cont'd)

CWD 1369	ground beef	NA	19071	NA	0.09	0.96
CWD 1728	pork sausage	NA	19071	NA	0.09	0.93
CWD 1760	raw goat milk	NA	NA	NA	0.05	0.93
CWD 1634	ground beef	1/2b	54081	NA	0.05	0.88
CWD 1742	pork sausage	NA	19231	NA	0.05	0.88
CWD 1038	pork sausage	NA	19071	NA	0.06	0.87
CWD 1667	pork sausage	NA	19071	NA	0.06	0.87
CWD 1157	ground beef	NA	54132	NA	0.14	0.84
CWD 371	dairy plant	NA	NA	NA	0.16	0.84
CWD 1278	pork sausage	NA	54081	NA	0.10	0.83
ETR-6-3[c]	pork processor	NA	NA	NA	0.16	0.83
CWD 1061	pork sausage	NA	19231	NA	0.12	0.81
ETR-7-1	pork processor	NA	NA	NA	0.07	0.81
ETR-2-4	pork processor	NA	NA	NA	0.20	0.79
ETR-7-2	pork processor	NA	NA	NA	0.16	0.79
CWD 1205	ground turkey	NA	19192	NA	0.11	0.78
CWD 372	dairy plant	1/2a	NA	NA	0.07	0.78
ETR-6-1	pork processor	NA	NA	NA	0.32	0.77
CWD 602	dairy plant	1/2a	54183	NA	0.05	0.76
CWD 1118	ground beef	NA	54081	NA	0.09	0.75
CWD 1305	chicken	NA	19071	NA	0.06	0.75
CWD 1648	chicken	NA	19161	NA	0.05	0.72
CWD 701	cheese	1/2b	54135	NA	0.06	0.72
FSL J1-116[d]	human, epidemic, UK, 1988-1990	4b	DUP-1042	I	0.14	0.70
CWD 1094	chicken	NA	54081	NA	0.07	0.69

Table 2.1. (Cont'd)

CWD 271	dairy plant	4b	19161	NA	0.05	0.69
ETR-7-3	pork processor	NA	NA	NA	0.34	0.68
CWD 1664	pork sausage	NA	54132	NA	0.05	0.67
CWD 1724	chicken	NA	19231	NA	0.06	0.67
CWD 1768	raw goat milk	NA	NA	NA	0.05	0.67
ETR-2-5	pork processor	NA	NA	NA	0.07	0.65
ETR-7-5	pork processor	NA	NA	NA	0.15	0.65
CWD 2087	unknown	NA	NA	NA	0.06	0.64
CWD 1461	unknown	NA	NA	NA	0.11	0.63
CWD 1776	raw goat milk	NA	NA	NA	0.07	0.62
CWD 878	human clinical	NA	19161	NA	0.08	0.62
FSL J1-094	human, sporadic case	1/2c	DUP-1030	II	0.12	0.62
CWD 102	silage	NA	19075	NA	0.08	0.61
FSL J2-064	animal, cow	1/2b	DUP-1052/ dd 1962	I	0.10	0.61
CWD 1120	ground beef	NA	19071	NA	0.26	0.60
CWD 1176	ground turkey	1/2b	19192	NA	0.16	0.59
CWD 1438	unknown	NA	NA	NA	0.05	0.58
CWD 210	raw milk	NA	19092	NA	0.05	0.58
ETR-6-2	pork processor	NA	NA	NA	0.16	0.58
CWD 1298	chicken	NA	19161	NA	0.06	0.57
CWD 1706	ground beef	NA	19071	NA	0.05	0.54
CWD 273	dairy plant	NA	19161	NA	0.05	0.54
CWD 811	dairy plant	NA	19092	NA	0.08	0.53

Table 2.1. (Cont'd)

CWD 939	dairy plant	NA	19186	NA	0.05	0.53
FSL C1-122	human, sporadic case	4b	DUP-1038B	I	0.15	0.53
CWD 1427	unknown	NA	NA	NA	0.15	0.52
CWD 725	cow brain	NA	NA	NA	0.11	0.51
FSL J1-177	human sporadic case	1/2b	DUP-1024	I	0.13	0.51
CWD 1002	pork sausage	1/2c	19071	NA	0.08	0.50
CWD 1424	unknown	NA	NA	NA	0.07	0.50
CWD 1528	ground turkey	NA	54183	NA	0.05	0.50
CWD 1624	unknown	NA	19231	NA	0.05	0.50
CWD 1198	ground turkey	NA	19231	NA	0.08	0.49
CWD 680	cow udder	NA	19071	NA	0.15	0.49
CWD 897	dairy plant	NA	19103	NA	0.10	0.49
ETR-3-3	pork processor	NA	NA	NA	0.69	0.49
ETR-1-1	pork processor	NA	NA	NA	0.22	0.46
CWD 1191	ground turkey	NA	19157	NA	0.23	0.45
CWD 1318	chicken	NA	18647	NA	0.15	0.45
CWD 1433	unknown	NA	NA	NA	0.06	0.45
CWD 184	raw milk	NA	19092	NA	0.20	0.45
CWD 224	dairy plant	NA	19167	NA	0.10	0.44
CWD 317	dairy plant	NA	19092	NA	0.09	0.44
CWD 631	unknown	NA	54081	NA	0.08	0.44
ETR-2-3	pork processor	NA	NA	NA	0.17	0.44
FSL J2-035	animal, goat	1/2b	dd 3581	I	0.12	0.44
CWD 685	cow udder	NA	19078	NA	0.08	0.43

Table 2.1. (Cont'd)

CWD 1092	chicken	NA	54183	NA	0.15	0.42
CWD 1224	pork sausage	NA	19071	NA	0.07	0.42
CWD 1281	pork sausage	NA	19071	NA	0.09	0.42
CWD 1566	unknown	NA	19074	NA	0.08	0.41
CWD 1603	unknown	NA	19161	NA	0.06	0.41
CWD 1709	ground beef	NA	54132	NA	0.05	0.41
ETR-5-3	pork processor	NA	NA	NA	0.92	0.41
FSL R2-500	food, epidemic, North Carolina (2000)	4b	DUP-1042B	I	0.13	0.41
CWD 1677	pork sausage	NA	19161	NA	0.05	0.39
CWD 1807	raw goat milk	NA	NA	NA	0.15	0.39
CWD 531	dairy plant	NA	19092	NA	0.11	0.39
ETR-1-4	pork processor	NA	NA	NA	0.25	0.39
FSL J2-020	animal, cow	1/2a	DUP-1039C	II	0.10	0.38
CWD 1223	pork sausage	1/2b	28647	NA	0.09	0.37
ETR-3-2	pork processor	NA	NA	NA	0.35	0.37
ETR-5-5	pork processor	NA	NA	NA	0.18	0.37
FSL R2-501	human, epidemic, North Carolina (2000)	4b	DUP-1042B	I	0.13	0.37
CWD 1420	unknown	NA	NA	NA	0.05	0.36
CWD 1436	unknown	NA	NA	NA	0.06	0.36
ETR-1-2	pork processor	NA	NA	NA	0.21	0.36

Table 2.1. (Cont'd)

FSL J1-049	human, sporadic case	3c	DUP-1042	I	0.09	0.36
CWD 1790	raw goat milk	NA	NA	NA	0.10	0.35
ETR-3-5	pork processor	NA	NA	NA	0.28	0.35
ETR-6-4	pork processor	NA	NA	NA	0.06	0.35
FSL J1-169	human, sporadic	3b	DUP-1052	I	0.11	0.35
CWD 1287	pork sausage	NA	19161	NA	0.06	0.34
ETR-3-4	pork processor	NA	NA	NA	0.24	0.34
FSL R2-502	food, epidemic, Illinois (1994)	1/2b	DUP-1051B	I	0.11	0.34
ETR-5-2	pork processor	NA	NA	NA	0.22	0.33
FSL J1-119	human, epidemic, L.A., 1985	4b	DUP-1038	I	0.06	0.33
CWD 1108	chicken	NA	54132	NA	0.08	0.32
CWD 1326	chicken	NA	19192	NA	0.05	0.32
CWD 1448	unknown	NA	NA	NA	0.11	0.32
ETR-2-1	pork processor	NA	NA	NA	0.12	0.32
ETR-2-2	pork processor	NA	NA	NA	0.23	0.32
ETR-7-4	pork processor	NA	NA	NA	0.14	0.32
FSL J1-126	human, epidemic, Switzerland, 1987	4b	DUP-1038	I	0.13	0.32
FSL J2-063	animal, sheep	1/2a	DUP-1047/ dd 1153	II	0.12	0.32

Table 2.1. (Cont'd)

FSL N1-225	human, epidemic (US 1998-99)	4b	DUP-1044A	I	0.13	0.32
CWD 30	French Brie	NA	19106	NA	0.08	0.31
CWD 431	cow udder	NA	19071	NA	0.12	0.31
CWD 1656	chicken	NA	54084	NA	0.08	0.30
CWD 180	human clinical	NA	19161	NA	0.07	0.30
CWD 1817	raw goat milk	NA	NA	NA	0.06	0.30
CWD 575	dairy plant	NA	54081	NA	0.07	0.30
ETR-5-1	pork processor	NA	NA	NA	0.11	0.30
CWD 1066	pork sausage	NA	54135	NA	0.06	0.29
CWD 1418	unknown	NA	NA	NA	0.20	0.29
CWD 1573	unknown	NA	19074	NA	0.06	0.29
ETR-5-4	pork processor	NA	NA	NA	0.49	0.29
FSL N3-031	food (hot dog), sporadic, US	1/2a	DUP-1053	II	0.11	0.29
CWD 1814	raw goat milk	NA	NA	NA	0.05	0.28
CWD 554	dairy plant	NA	54081	NA	0.16	0.28
ETR-1-3	pork processor	NA	NA	NA	0.14	0.28
CWD 1313	chicken	NA	19231	NA	0.11	0.27
CWD 852	dairy plant	NA	19092	NA	0.13	0.27
ETR-3-1	pork processor	NA	NA	NA	0.26	0.27
FSL R2-503	human, epidemic, Illinois (1994)	1/2b	DUP-1051B	I	0.13	0.27
CWD 246	silage	NA	19193	NA	0.15	0.26
ETR-6-5	pork processor	NA	NA	NA	0.18	0.26
CWD 1525	ground turkey	NA	54084	NA	0.05	0.25

Table 2.1. (Cont'd)

CWD 1723	chicken	NA	54084	NA	0.05	0.25
CWD 95	silage	NA	19071	NA	0.16	0.25
FSL J1-031	human, sporadic case	4a	DUP-1059A	III	0.12	0.25
CWD 1521	ground turkey	NA	19071	NA	0.09	0.24
CWD 1789	raw goat milk	NA	NA	NA	0.05	0.24
CWD 1794	raw goat milk	NA	NA	NA	0.05	0.24
CWD 475	dairy plant	NA	19071	NA	0.05	0.24
FSL N3-013	food, epidemic, UK, 1988-1990	4b	DUP-1042	I	0.07	0.24
FSL R2-499	human, epidemic (sliced turkey) (2000)	1/2a	DUP-1053	II	0.11	0.24
CWD 1332	chicken	NA	54084	NA	0.06	0.23
FSL C1-115	human, sporadic	3a	DUP-1039C	II	0.12	0.23
FSL N3-022	food, epidemic, Switzerland, 1987	4b	DUP-1038	I	0.08	0.23
FSL W1-112	unknown (formerly X1-010)	4a	dd 6824	III	0.07	0.23
CWD 561	dairy plant	1/2a	19071	NA	0.09	0.22
CWD 909	dairy plant	NA	19092	NA	0.10	0.22
FSL W1-110	unknown (formerly X1-008)	4c	dd 3823	III	0.15	0.22
CWD 24	unknown	NA	19071	NA	0.10	0.21
ETR-1-5	pork processor	NA	NA	NA	0.09	0.21

Table 2.1. (Cont'd)

FSL J1-108	human, epidemic, Halifax, 1981	4b	DUP-1038	I	0.10	0.21
FSL J1-225	human, epidemic (Mass., 1983, Scott A)	4b	DUP-1042	I	0.11	0.21
FSL J2-054	animal, sheep	1/2a	DUP-1045/dd 1067	II	0.12	0.20
CWD 570	dairy plant	NA	19092	NA	0.12	0.19
FSL J1-158	animal, goat	4b	DUP-10142	III	0.15	0.19
FSL J1-168	human, sporadic case	4a	DUP-1061	III	0.13	0.19
CWD 179	cow brain	NA	19075	NA	0.06	0.18
CWD 923	dairy plant	NA	54184	NA	0.08	0.18
FSL C1-056	human, sporadic case	1/2a	DUP-1030	II	0.08	0.18
FSL J1-023	unknown	3a	DUP-10143	III	0.09	0.18
FSL J2-031	animal, bovine	1/2a	DUP-1039/dd 6362	II	0.19	0.18
FSL M1-004	human, sporadic case	N/A	DUP-1039B	II	0.07	0.18
CWD 182	unknown	4b	19078	NA	0.06	0.17
CWD 578	dairy plant	4d	19161	NA	0.08	0.17

Table 2.1. (Cont'd)

FSL N1-227	food, epidemic (US 1998-99)	4b	DUP-1044A	I	0.08	0.17
FSL J1-101	human (hot dog), sporadic, US	1/2a	DUP-1053	II	0.16	0.16
ATCC 19115	unknown	4b	NA	NA	0.06	0.14
CWD 205	unknown	4c	19078	NA	0.21	0.14
FSL J2-066	animal, sheep	1/2a	DUP-1054/ dd 3075	II	0.14	0.13
FSL J1-110	food, epidemic, L.A. 1985	4b	DUP-1038	I	0.10	0.12
FSL N3-008	food, epidemic, Halifax, 1981	4b	DUP-1038	I	0.10	0.08
FSL W1-111	unknown (formerly X1-009)	4c	dd 6821	III	0.08	0.08

[a] CWD strains provided by Dr. Catherine Donnelly, University of Vermont

[b] NA = Not available

[c] ETR strains from Michigan State University, Department of Food Science and Human Nutrition Culture Collection

[d] FSL strains provided by Dr. Martin Wiedmann, Cornell University

Figure 2.1. Distribution of optical densities at 4°C for 196 *L. monocytogenes* isolates

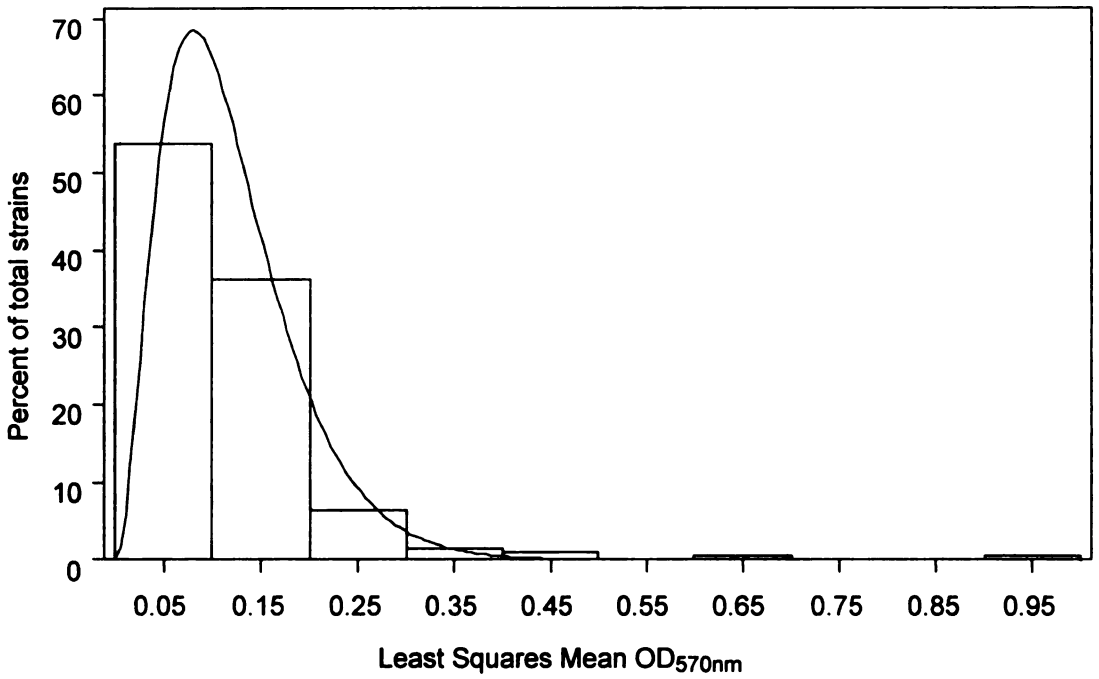
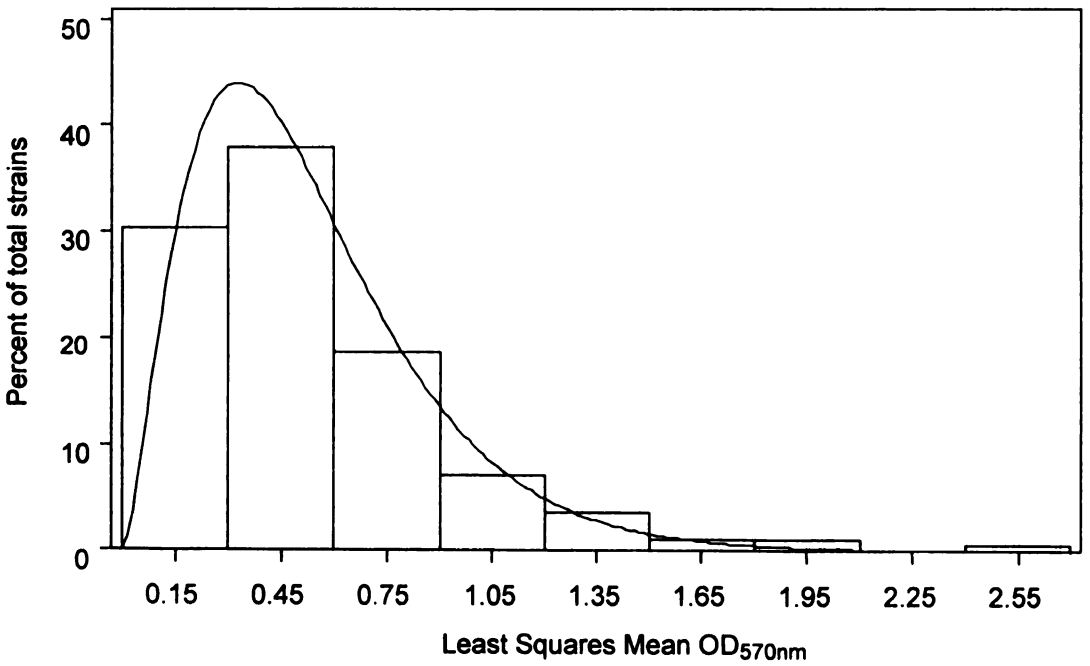


Figure 2.2. Distribution of optical densities at 22°C for 196 *L. monocytogenes* isolates



Significant differences in biofilm-forming ability were observed based on source (Table 2.2) and lineage (Table 2.3) of the isolate. Food and environmental isolates were significantly better at forming biofilms than clinical and veterinary isolates. Lineage I strains were significantly better at forming biofilms than Lineage III strains. Significant differences were also observed based on serotype, with serotype 1/2b forming significantly stronger biofilms than serotypes 1/2a and 4b (Table 2.4). However for some serotypes no significant differences were observed, possibly due to the small sample size and large standard deviation. No significant differences were observed in biofilm forming ability by ribotype when analyzing ribotypes of which there were three or more strains.

Table 2.2. *L. monocytogenes* biofilm formation at 22°C by source

Source	*n*	Mean OD_{570}	Std. D.	Range
Clinical	22	0.34^{a}	0.16	0.16 – 0.70
Environment	58	0.56^{bc}	0.44	0.17 – 2.61
Food	74	0.61^{b}	0.36	0.08 – 1.80
Veterinary	13	0.34^{ac}	0.15	0.13 – 0.61

Means with different letters are significantly different ($P < 0.05$).

Table 2.3. *L. monocytogenes* biofilm formation at 22°C by lineage*

Lineage	*n*	Mean OD_{570}	Std. D.	Range
I	21	0.34^{a}	0.16	0.08 – 0.70
II	12	0.26^{ab}	0.14	0.13 – 0.62
III	7	0.19^{b}	0.05	0.08 – 0.25

* FSL strains provided by Dr. Martin Wiedmann, Cornell University

Means with different letters are significantly different ($P < 0.05$).

Table 2.4. *L. monocytogenes* biofilm formation at 22°C by serotype

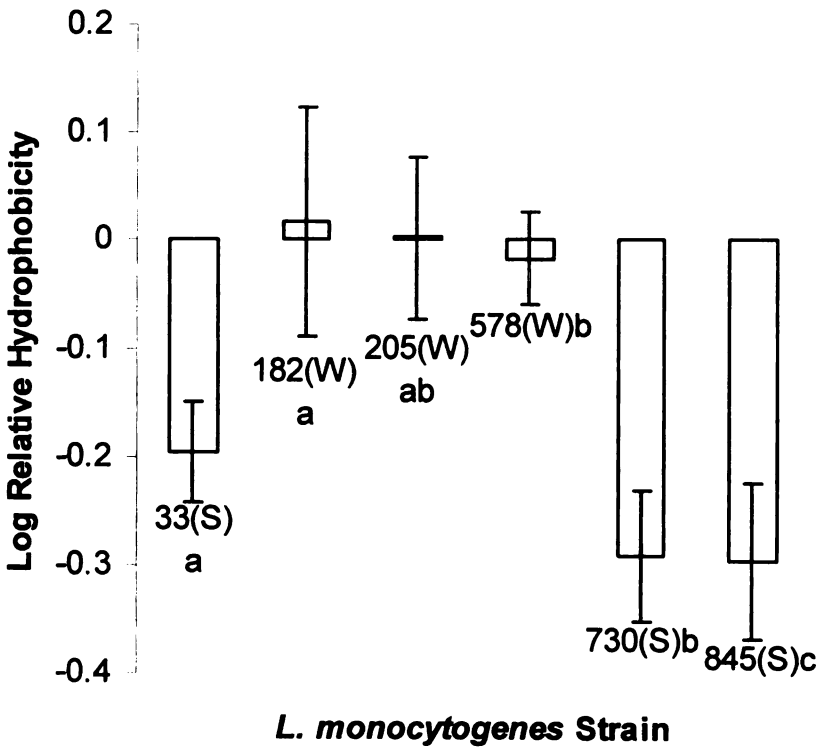
Serotype	*n*	Mean OD_{570}	Std. D.	Range
1/2a	14	0.52^{ad}	0.55	0.13 – 1.94
1/2b	13	0.84^{be}	0.63	0.27 – 2.61
1/2c	2	0.56^{ae}	0.09	0.50 – 0.62
3a	3	0.48	0.47	0.18 – 1.02
3b	2	0.99^{de}	0.90	0.35 – 1.62
3c	1	0.36	NA	NA
4a	3	0.22^{a}	0.03	0.19 – 0.25
4b	15	0.39^{ad}	0.46	0.08 – 1.91
4c	3	0.15^{c}	0.07	0.08 – 0.22
4d	1	0.17	NA	NA

Means with different letters are significantly different ($P < 0.05$).

2.4.2 Surface hydrophobicity of weak and strong biofilm formers.

Based on these results, the three strongest and three of the weakest biofilm formers were further evaluated for hydrophobicity using hydrophobic interaction chromatography. The three weakest biofilm forming strains (CWD 182, CWD 205, and CWD 578) were significantly more hydrophobic than the three strongest biofilm forming strains (CWD 33, CWD 730, and CWD 845) (Figure 2.3). However, while one of the weakest biofilm formers (CWD 578) was slightly hydrophilic, its cell surface hydrophobicity was not significantly different from the other more hydrophobic weak biofilm forming strains.

Figure 2.3. Log relative hydrophobicity of weak and strong biofilm forming strains of *L. monocytogenes*



(S) = Strong biofilm forming *L. monocytogenes* strain
(W) = Weak biofilm forming *L. monocytogenes* strain
Means with different letters (a, b, or c) are significantly different ($P < 0.05$).

2.5 DISCUSSION

Microtiter plate assays provide an indirect means to screen many different strains for quantitative differences in biofilm formation (Stepanovic et al., 2000; Djordjevic et al., 2002; Borucki et al., 2003). While unable to precisely mimic conditions encountered in food processing environments, these biofilm assays can be used to rapidly screen and identify specific bacterial strains for further use in more labor-intensive studies. Researchers have previously used microtiter plate assays to evaluate biofilm formation by different strains of *L. monocytogenes* on polyvinyl chloride (PVC) at 32°C after 20 and 40 h (Djordjevic et al., 2002) and at 30°C after 40 h (Borucki et al., 2003). Both studies compared biofilm formation by persistent and non-persistent strains. According to Borucki et al. (2003), persistent strains were better able to form biofilms. However, Djordjevic et al. (2002) observed no such difference between persistent and non-persistent strains. Additionally, while Borucki et al. (2003) reported greater biofilm formation by *L. monocytogenes* strains belonging to Lineage II, Djordjevic et al. (2002) observed greater biofilm formation in Lineage I strains. Our results differed from both of these studies. While we found no significant difference in biofilm formation between Lineages I and II, significant differences were observed between Lineage I and III strains, with the latter producing significantly weaker biofilms. Given the predominance and increased persistence of Lineage II strains in food processing environments, these strains would be expected to produce stronger biofilms as shown from our data. In general, significantly better biofilm formation was observed among environmental and food as opposed to clinical and veterinary isolates, however, lineage data were only available for the 40 strains obtained from Wiedmann. Unlike the study by Borucki et al. (2003),

significant differences in biofilm formation were observed between the three major serotypes – 1/2a, 1/2b and 4b. In addition to the use of different strain sets varying according to size, isolation source and genetic diversity, considerable strain-to-strain variation in biofilm formation leading to large standard deviations complicates any direct comparison of results from other studies. In this study, the same strains used by Djordjevic et al. (2002) were analyzed, with different results seen in their biofilm forming ability in relation to each another. In addition, the OD values were generally far lower than those reported by Djordjevic et al. (2002), with this outcome likely due to procedural differences in the microtiter plate assay and incubation temperatures. However, even though the identical strains used by Djordjevic et al. (2002) generally yielded lower OD values in our study, a far greater range in biofilm formation was observed at 22°C for all 197 strains, as measured by OD (0.078 – 2.605).

For those strains at either extreme in biofilm formation, differences were observed in cell surface hydrophobicity. Isolates that were particularly strong biofilm formers in this study were significantly more hydrophobic than those strains forming extremely weak biofilms. Reports from the literature vary greatly concerning hydrophobicity of the *L. monocytogenes* cell surface, and the pathogen's subsequent interactions with food contact surfaces. Smoot and Pierson (1998) found that the rate of *L. monocytogenes* attachment to stainless steel was faster than attachment to Buna-N rubber, although cell surface hydrophobicity and surface free energies predict that adhesion to Buna-N rubber is favored. Another study also found that hydrophilic, negatively-charged cells of *L. monocytogenes* adhered better to stainless steel, than to polytetrafluoroethylene (Chavant et al., 2002). Briandet et al. (1999) found that although *L. monocytogenes* tends to be

hydrophilic, strains that are slightly more hydrophobic than others adhere better to stainless steel. However, Midelet and Carpentier (2002) observed stronger attachment of *L. monocytogenes* biofilms to polymers (polyvinyl chloride and polyurethane) than to stainless steel, and also noted that meat exudates changed these surfaces from hydrophobic to hydrophilic as determined by contact angle measurement. According to Cunliffe et al. (1999), hydrophilic uncharged surfaces were slightly repellent to *L. monocytogenes*. In other work, *L. monocytogenes* Scott A was more attracted to polypropylene and rubber surfaces than to glass and stainless steel (Mafu et al., 1991). In a study of the physicochemical characteristics of *L. monocytogenes* and its attachment to glass, hydrophobicity and surface charge had no correlation to the extent of cell attachment (Chae et al., 2006).

Differences seen in the previous studies may be due to the choice of strains tested. If these strains were randomly chosen rather than because they frequently appear in the existing literature (e.g., *L. monocytogenes* LO28, and Scott A), interpretation of these findings may be difficult without first knowing how the strains behave relative to one another on surfaces. In this study, Scott A was not a strong biofilm former, which may be correlated to a more hydrophilic cell surface compared to the strong biofilm formers. However, extrapolating the impact of cell surface hydrophobicity to biofilm-forming ability by all *L. monocytogenes* based on a few well-studied strains may lead to inaccurate assumptions about *L. monocytogenes* interactions with food contact surfaces.

CHAPTER 3

VARIATION IN BIOFILM FORMATION BY HEALTHY AND COLD-, STARVE-, ACID-, AND CHLORINE-STRESSED *LISTERIA MONOCYTOGENES*

3.1 ABSTRACT

Presence of *Listeria monocytogenes* strains endemic to food processing environments is presumably related to biofilm formation. Following exposure to various environmental stresses, *Listeria* cells may be more prone to attach to surfaces. This study quantified the degree of biofilm formation in a defined set of *L. monocytogenes* strains when uninjured, cold-starved, cold-shocked, acid-shocked and chlorine-shocked. Twenty-six *L. monocytogenes* strains (including clinical, food and dairy plant isolates) were selected from a set of 196 strains previously characterized for biofilm formation. *L. monocytogenes* (10^2 CFU/ml) was subjected to the previously mentioned stresses. Uninjured cultures were used as controls. Biofilm formation by the uninjured and injured bacteria was quantified in Modified Welshimer's Broth (MWB) using 96-well untreated polystyrene microtiter plates (3 wells/strain x 3 replicates). Following 4 days of incubation at 22°C, the microtiter plate wells were emptied, rinsed and air-dried. After staining cells that were fixed in 99% methanol with crystal violet, biofilm formation as measured by optical density (OD) of the resolubilized dye was read spectrophotometrically at 570 nm. Prior injury of *L. monocytogenes* by starvation (28.5% injured) and cold (39.2% injured) lead to enhanced biofilm formation. Acid injured (60.2% injured) and chlorine injured (55.1% injured) cultures showed a diminished ability to form biofilms. Strains that comprised the extreme OD values in biofilm formation remained consistent between all three treatments. Dairy plant isolates did not predominate as strong biofilm formers.

3.2 INTRODUCTION

Prior to coming in contact with food processing surfaces, *L. monocytogenes* may be exposed to various environmental stresses including nutrient deprivation, refrigeration temperatures, low pH, limited available water, and sanitizers. These stresses can alter the sensitivity of *L. monocytogenes* to other subsequent stresses, sometimes making it more difficult to eradicate *Listeria* from the environment (Lou and Yousef, 1997; Koutsoumanis et al., 2003; Koutsoumanis and Sofos, 2004; Gravesen et al., 2005; Moorman et al., 2005).

Listeria is able to grow at refrigeration temperatures by altering its membrane composition in order to maintain membrane fluidity and increase passive permeability. This is achieved through changes in fatty acid composition (Neunlist et al., 2005). When exposed to cold starvation conditions, *L. monocytogenes* undergoes shrinkage of the cytoplasm, eventually resulting in holes in the cytoplasm (Dykes, 1999). The ability of *L. monocytogenes* to respond to nutrient deprivation is a likely requirement for survival in a biofilm. Mutants lacking the ability to mount a stringent response to amino acid deprivation showed decreased attachment to surfaces, and were also avirulent in a mouse model (Taylor et al., 2002).

Acid tolerance by *L. monocytogenes* has been studied by several researchers. *Listeria* is able to better withstand lethal acid concentrations (pH 3.5) after habituation to sublethal acid stress (pH 5-6), with maximum acid tolerance induced by habituation to pH 5.5 (Koutsoumanis and Sofos, 2004; Koutsoumanis et al., 2005). *Listeria monocytogenes* can survive without loss of viability for at least 20 h at pH 4.0, and also at pH 3.5 in the presence of glucose (Shabala et al., 2002). In order to survive, *L.*

monocytogenes maintains a higher intracellular pH than the surrounding acidic environment. While able to maintain an intracellular pH of 7.0-7.5 in glucose-containing environments pH as low as 4.0, in the absence of glucose, *L. monocytogenes* is unable to maintain a higher intracellular pH at pH 5.5 (Shabala et al., 2002). Other physiological changes in response to acid stress include changes in protein synthesis and fatty acid composition of the cell membrane (Koutsoumanis and Sofos, 2004). Changes to the cell membrane result in increased surface hydrophobicity of acid adapted *L. monocytogenes* (Lou and Yousef, 1997) with such changes having the potential to impact initial attachment of *Listeria* to surfaces. For example, exposure to sublethal concentrations of ethanol and isopropanol increased *L. monocytogenes* attachment at 10, 20 and 30°C (Gravesen et al., 2005). Other oxidative stresses, such as chlorine and sanitizer stress likely affect cellular proteins as evidenced by the fact that protein synthesis is essential for subsequent cell repair and growth (Flanders et al., 1995).

Exposure to the aforementioned stresses can alter *Listeria* sensitivity to quaternary ammonium sanitizers. After exposure to acid or starvation stress, *Listeria innocua* was less sensitive to the quaternary ammonium sanitizer, cetrimide (Moorman et al., 2005). This cross-protection did not occur after exposure to cold and heat stress, which increased *L. innocua* sensitivity to cetrimide (Moorman et al., 2005). *Listeria monocytogenes* strains that are resistant to quaternary ammonium compounds, other sanitzers and antibiotics have been found to contain a gene (*mdrL*) that encodes an efflux pump (Romanova et al., 2002). However, some resistant isolates do not appear to rely on efflux pumps, and instead alter their cell membrane fatty acid profile in response to sanitizer stress, which can prevent entry of foreign chemicals into the cytoplasm (To et

al., 2002). The same study also found that upon initial (1st 30 h) exposure to sublethal levels of benzalkonium chloride, biofilm growth was favored (To et al., 2002).

Many types of bacteria, including *L. monocytogenes*, have the ability to attach and persist on equipment over extended periods of time. Initial attachment occurs via electrostatic or hydrophobic interactions between the bacterial surface and the contact surface (Arnold and Bailey, 2000). It is thought that the rate of initial attachment is predictive of ultimate biofilm forming ability—level and rate of *L. monocytogenes* attachment have been correlated to the ability of strains to form biofilms and persist in the environment (Lunden et al., 2000). Several studies have found that persistent strains of *L. monocytogenes* are significantly more adherent than non-persistent strains (Lunden et al., 2000; Norwood and Gilmour, 1999; Borucki et al., 2003). If biofilm-forming ability is affected by hydrophobic or electrostatic interactions, then alterations to the cell membrane from exposure to environmental stress suggest that biofilm formation should be different from that seen in unstressed healthy cells. Hence, the objective of this study was to determine whether exposure to common environmental stresses would alter biofilm formation by a set of 26 *L. monocytogenes* isolates of varying biofilm-forming abilities.

3.3 MATERIALS AND METHODS

3.3.1 *Listeria monocytogenes* strains.

A subset of 26 *L. monocytogenes* strains (Table 3.1) were selected from a larger subset of 196 strains so that the distribution of their biofilm forming abilities as measured by optical density reflected that of the entire set of 196 strains. The strains were assayed for biofilm formation at 22 ± 2°C after being subjected to injury. All strains were maintained at -80°C in trypticase soy broth containing 0.6% (w/v) yeast extract (TSB-YE; Becton Dickinson, Sparks, MD) and 10% (v/v) glycerol (Sigma Chemical Company, St. Louis, MO).

3.3.2 Culture preparation.

All strains were subcultured in TSB-YE (Difco), incubated 18 h at 37°C, and streaked to plates of trypticase soy agar containing 0.6% yeast extract (TSA-YE; Difco) to obtain confluent growth after 18 h of incubation at 37°C. *L. monocytogenes* was harvested from TSA-YE plates by flooding the agar surface with 10 ml of 0.1% sterile peptone and suspending the cells using a sterile 10 µl inoculating loop (Becton Dickinson). Cells were pipetted into a test tube and the concentration of the resuspended culture was estimated from MacFarland Turbidity Standards (Acuff, 1992). The resuspended culture was serially diluted to a final concentration of 10^2 CFU/ml in Modified Welshimer's Broth (MWB), which contained the following ingredients per liter: KH_2PO_4 (6.56 g), $Na_2HPO_4 \cdot 7H_2O$ (30.96 g), $MgSO_4 \cdot 7H_2O$ (0.41 g), ferric citrate (0.088g), glucose (10.0 g), L-leucine (0.1 g), L-isoleucine (0.1 g), L-valine (0.1 g), L-

methionine (0.1 g), L-arginine (0.1 g), L-cysteine (0.1 g), L-glutamine (0.6 g), riboflavin (0.5 mg), thiamine (1.0 mg), biotin (0.5 mg) and thioctic acid (0.005 mg) (Premaratne et al., 1991). All components of MWB were obtained from Sigma Chemical Company.

Table 3.1. *L. monocytogenes* strains and sources

Strain ID	Source	Serotype
CWD 1002[a]	Pork Sausage	1/2c
CWD 1176	Ground Turkey	1/2b
CWD 1223	Pork Sausage	1/2b
CWD 1634	Ground Beef	1/2b
CWD 1734	Pork Sausage	3b
CWD 182	Unknown	4b
CWD 205	Unknown	4c
CWD 271	Dairy Plant Environment	4b
CWD 33	Unknown	4b
CWD 338	Dairy Plant Environment	1/2a
CWD 372	Dairy Plant Environment	1/2a
CWD 561	Dairy Plant Environment	1/2a
CWD 578	Dairy Plant Environment	4d
CWD 580	Dairy Plant Environment	1/2b
CWD 600	Dairy Plant Environment	1/2b
CWD 602	Dairy Plant Environment	1/2a
CWD 701	Cheese	1/2b
CWD 730	Dairy Plant Environment	1/2a
CWD 764	Hotdog	1/2b
CWD 766	Hotdog	3a
CWD 845	Dairy Plant Environment	1/2b
FSL J1-119[b]	Human, epidemic, L.A., 1985	4b
FSL J1-225	Human epidemic (Mass., 1983; Scott A)	4b
FSL N1-225	Human epidemic (US 1998-99)	4b
FSL R2-499	Human, epidemic (sliced turkey) (2000)	1/2a
FSL R2-501	Human, epidemic, North Carolina (2000)	4b

[a] CWD strains provided by Dr. Catherine Donnelly, University of Vermont

[b] FSL strains provided by Dr. Martin Wiedmann, Cornell University

3.3.3 Acid injured.

L. monocytogenes cells were harvested from TSA-YE into 10 ml of sterile TSB-YE (pH 5.3) by flooding the agar surface with 10 ml of acidified TSB-YE and suspending the culture using a sterile inoculating loop. After 4 h of incubation at 4°C the cultures were serially diluted to contain 10^2 CFU/ml in MWB, as determined by comparison to MacFarland Turbidity Standards.

3.3.4 Cold shocked.

L. monocytogenes cells were harvested from TSA-YE into 10 ml of sterile 0.1% peptone broth and serially diluted to a final concentration of 10^2 CFU/ml (as determined by comparison to MacFarland Turbidity Standards) in pre-chilled MWB. The cells were then incubated for 2 h in a 4°C water bath.

3.3.5 Cold starved.

L. monocytogenes cells were harvested from TSA-YE into 10 ml of sterile Butterfield's Phosphate Buffer (BPB) and then centrifuged (Super T21, Sorvall Products, Newtown, CT) at 9740 × *g* for 15 min at 4°C in sterile 50 ml polypropylene centrifuge tubes (Corning Inc., Corning, NY). After resuspending the pellet in 10 ml of BPB, starvation was achieved by holding the cells for 10 d at 4°C. Following incubation, the cultures were serially diluted to a final concentration of 10^2 CFU/ml (as determined by comparison to MacFarland Turbidity Standards) in MWB.

3.3.6 Chlorine injured.

L. monocytogenes cells were harvested from TSA-YE into 10 ml of sterile phosphate buffered saline (PBS), then serially diluted into PBS containing 100 ppm chlorine (Clorox; The Clorox Company, Oakland, CA) for 1 minute. Following

exposure, each strain was serially diluted into Neutralizing Buffer (Difco) to inactivate the chlorine and then serially diluted in MWB to contain 10^2 CFU/ml, as determined by comparison to MacFarland Turbidity Standards.

3.3.7 Quantification of injury.

After each of injury treatment, injured cultures were spread-plated on Tryptose Phosphate Agar (TPA; Difco) and TPA with 4.5% NaCl (TPAN). Percent injury was determined according to the following equation: % Injury = {(Count on non-selective medium – count on selective medium) / (count on non-selective medium)} × 100 (Mathew and Ryser, 2002). Following the injury treatment, cultures were used in the microtiter plate assay for biofilm formation.

3.3.8 Microtiter plate assay for biofilm formation.

A modification of the assay described by Stepanovic et al. (2000) was used to assess biofilm formation by *L. monocytogenes.* After vortexing, 200 µl of the diluted (10^2 CFU/ml) culture was pipetted into three wells of a 96-well untreated polystyrene microtiter tissue culture plate (BD Falcon Microtest™ Flat Bottom; Becton Dickinson and Company, Franklin Lakes, NJ). Three wells per plate containing 200 µl of MWB served as negative controls. Assays of injured cultures were carried out at 22 ± 2°C for 4 d. At the end of incubation, the microtiter plate wells were emptied and rinsed three times with physiological saline. The plates were gently shaken while rinsing to remove unattached cells and were then allowed to air-dry. The remaining bacterial cells were fixed to the well with 200 µl of 99% methanol (Fisher Chemicals, Fair Lawn, NJ). The methanol was decanted 15 min later and the plates were allowed to air-dry. The microtiter wells were stained with 200 µl of 2% crystal violet (Biochemical Sciences,

Inc., Swedesboro, NJ) for 5 min. After decanting the crystal violet, the wells were rinsed five times with deionized water and were allowed to air-dry. The remaining dye was resolubilized in 160 µl of 33% (v/v) glacial acetic acid (EM Science, Gibbstown, NJ) and optical densities were read at 570 nm using a Vmax™ Kinetic Microplate Reader (Molecular Devices, Menlo Park, CA).

3.3.9 Statistical Analysis.

All experiments were replicated three times. Statistical analysis was performed using a general linear model procedure (SAS, Version 8, SAS Institute, Inc. Cary, NC). Significance was determined at $P < 0.05$.

3.4 RESULTS

Overall, cold injury and cold starvation enhanced biofilm formation, while acid injury and chlorine injury inhibited subsequent biofilm formation (Table 3.2). Cold injured cells and cold starved cells were significantly better at forming biofilms than uninjured cells ($P < 0.05$).

Within treatments, greater variation in biofilm formation was observed for uninjured *L. monocytogenes* and strains subjected to cold injury and cold starvation (Figures 3.1, 3.4, and 3.5), with a higher overall OD seen for cold injured (OD 0.12 – 3.73) and cold starved (OD 0.10 – 3.84) as opposed to uninjured bacteria (OD 0.14 – 2.61). Acid injured (OD 0.09 – 1.27) and chlorine injured (OD 0.05 – 1.09) cultures of *L. monocytogenes* exhibited less variability in biofilm formation by strain (Figures 3.2 and 3.3). Due to the criteria used to select isolates for this study (that the distribution of OD_{570} at 22°C for the isolates follow the overall distribution for the complete collection

of 196 strains; Keskinen et al., 2006a), large standard deviations in mean OD_{570} were observed for each treatment.

Table 3.2. Overall differences in injury and biofilm formation by 26 *L. monocytogenes* strains following various treatments

Treatment	OD_{570}	Injury (%)
Uninjured	0.83 ± 0.66^{a}	0 ± 0^{a}
Cold injured	1.28 ± 1.12^{b}	39.2 ± 15.3^{b}
Cold starved	1.14 ± 1.12^{b}	28.5 ± 14.0^{c}
Acid injured	0.32 ± 0.24^{c}	60.2 ± 26.7^{d}
Chlorine injured	0.26 ± 0.25^{c}	55.1 ± 22.3^{d}

$n = 26$
Means with different letters are significantly different ($P < 0.05$).

Figure 3.1. *L. monocytogenes* biofilm formation by uninjured cells

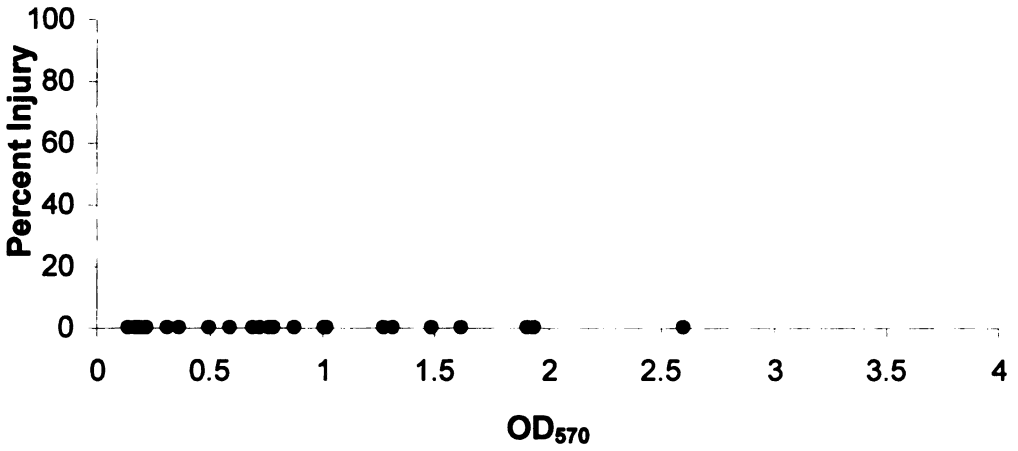


Figure 3.2 *L. monocytogenes* biofilm formation by acid-injured cells

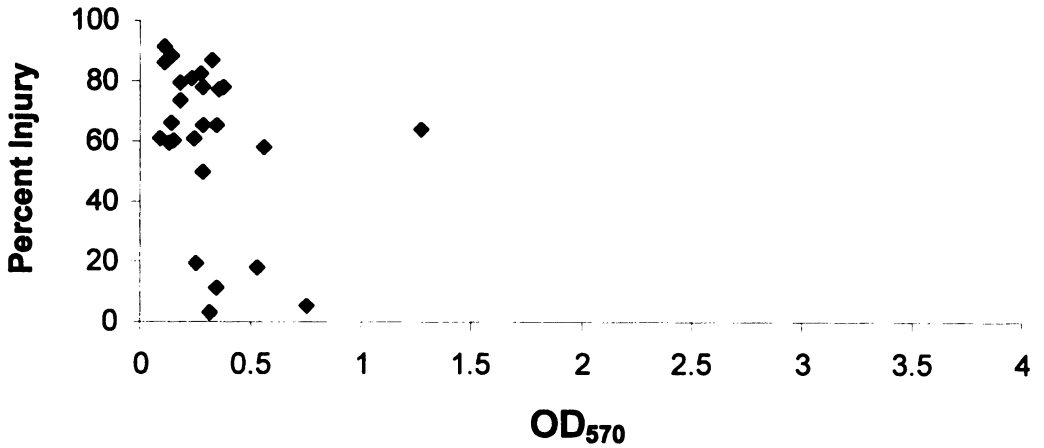


Figure 3.3. ***L. monocytogenes*** **biofilm formation by chlorine-injured cells**

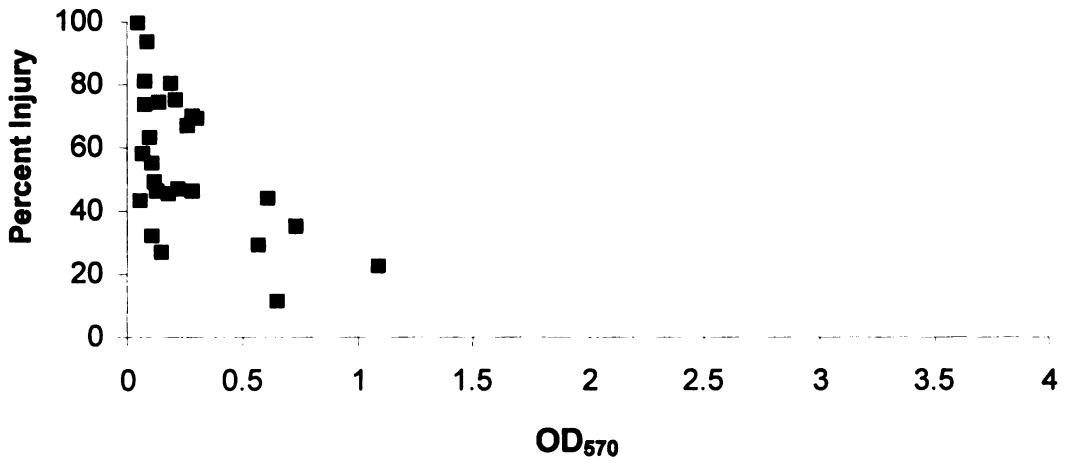


Figure 3.4. ***L. monocytogenes*** **biofilm formation by cold-injured cells**

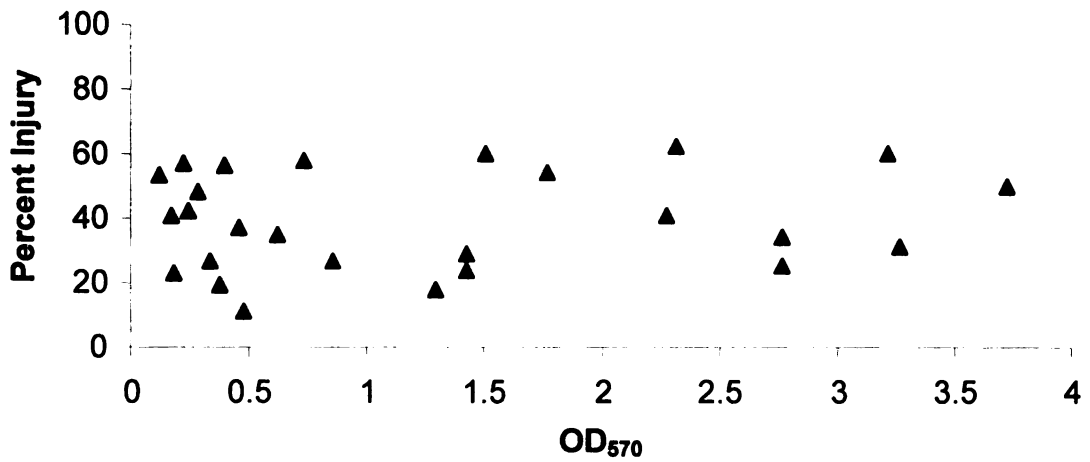
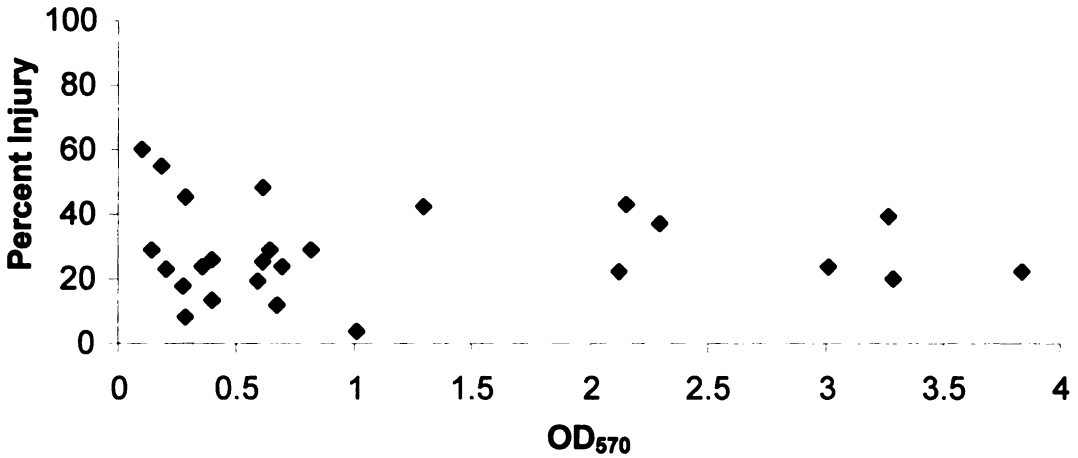


Figure 3.5. ***L. monocytogenes*** **biofilm formation by cold-starved cells**



When the strains were ranked according to biofilm forming ability in relation to each other (Table 3.3), the same strains consistently emerged as the strongest biofilm formers, while different strains consistently predominated as the weakest biofilm formers. Exposure to injury, even cold injury and cold starvation, did not significantly ($P < 0.05$) enhance biofilm formation by the weakest biofilm forming strains in comparison to the strong biofilm formers Additionally, no trends were observed in relative biofilm formation by strains of the same serotype or strains from similar sources (clinical, food, or environment; Table 3.1).

Table 3.3. Relative rankings of *L. monocytogenes* strains according to biofilm forming ability (1 = strongest, 26 = weakest)

Strain ID	Uninjured	Acid-injured	Chlorine-injured	Cold-injured	Cold-starved
CWD 845	1	1	1	6	5
CWD 730	2	17	22	1	1
CWD 33	3	5	4	7	4
CWD 1734	4	9	5	2	2
CWD 338	5	7	8	5	6
CWD 764	6	3	3	3	3
CWD 600	7	19	13	10	11
CWD 766	8	6	2	4	7
CWD 580	9	26	19	12	10
CWD 1634	10	11	6	9	8
CWD 372	11	12	14	11	13
CWD 602	12	25	21	18	14
CWD 701	13	14	24	13	12
CWD 271	14	18	17	15	20
CWD 1176	15	16	10	14	15
CWD 1002	16	23	20	8	9
FSL R2-501	17	4	7	17	17
CWD 1223	18	21	23	21	22
FSL J1-119	19	15	26	16	16
FSL N1-225	20	2	11	19	18
CWD 561	21	13	16	23	19
FSL J1-225	22	10	9	22	23
FSL R2-499	23	8	12	20	21
CWD 578	24	22	25	24	24
CWD 182	25	24	15	25	25
CWD 205	26	29	18	26	26

3.5 DISCUSSION

Based on these results, environmental stresses to which *L. monocytogenes* may be exposed in meat processing environments and delicatessens (cold, low nutrient concentration) may lead to increased persistence and biofilm formation. However, this only occurred after inoculation into MWB followed by incubation at ambient temperature, which may explain why cold injury enhanced biofilm formation while uninjured *L. monocytogenes* did not appear to form biofilms when incubated at 4°C (Keskinen et al., 2006a).

Initial bacterial attachment to surfaces occurs via electrostatic or hydrophobic interactions between the bacterial surface and the contact surface (Arnold and Bailey, 2000). Cell surface hydrophobicity can be affected by anything that will result in changes to the cell surface. Therefore, changes in membrane fluidity that occur from exposure to refrigeration temperatures are due to changes in the fatty acid profile of the cell surface (Neunlist et al., 2005). Under these conditions, cell surface hydrophobicity may have been sufficiently altered so that the hydrophobic interactions between injured *L. monocytogenes* cells and the polystyrene surface were more favorable than for uninjured cells. If initial attachment is more favorable, ultimate biofilm formation is stronger compared to cells that exhibit poorer initial attachment (Lunden et al., 2000). Acid and chlorine injury inhibited subsequent biofilm formation by *L. monocytogenes*. Similar to cold injury, acid and sanitizer injury often decrease cell permeability by altering the cell membrane fatty acid composition (Lou and Yousef, 1997; To et al., 2002). Acid injury increases cell surface hydrophobicity (Lou and Yousef, 1997), which affects initial attachment. Denaturation of proteins in the cell membrane by acid or

chlorine may also cause inhibition of biofilm formation. Cell surface proteins are important for surface growth—the addition of 0.01% trypsin to growth media can reduce adherent cell populations by 99.9%, as compared to control cultures without trypsin (Smoot and Pierson, 1998). However, further research is required to determine whether changes in surface hydrophobicity are indeed the cause for enhanced biofilm formation by injured cells, or whether there are other factors that are more influential.

CHAPTER 4

IMPACT OF BIOFILM FORMING ABILITY ON TRANSFER OF SURFACE-DRIED *LISTERIA MONOCYTOGENES* FROM KNIFE BLADES TO ROAST TURKEY BREAST

4.1 ABSTRACT

Listeria contamination of food contact surfaces can lead to cross-contamination of ready-to-eat foods in delicatessens. In the present study, six previously identified strong and weak biofilm-forming strains of *L. monocytogenes* were grown at 22°C for 48 h on Trypticase soy agar containing 0.6% yeast extract and harvested in 0.1% peptone. Thereafter, the strains were combined to obtain two 3-strain cocktails and resuspended in turkey slurry to inoculate flame-sterilized grade 304 stainless steel knife blades at concentrations of 10^8 and 10^6 CFU/blade. After incubation at ~78% relative humidity for 6 and 24 h, retail roast turkey breast was cut into 16 slices using the knives mounted on an Instron Universal Testing Machine. In the evaluation of *Listeria* transfer from knife blades to turkey breast, *Listeria* populations decreased 3-5 log CFU/slice after 16 slices. Overall, total transfer to turkey was significantly greater for strong (4.4 log CFU total) as opposed to weak biofilm formers (3.5 CFU total; $P < 0.05$). In addition, significantly more listeriae were transferred at 6 h (4.6 log CFU total) than at 24 h (3.3 log CFU total; $P < 0.05$). For both inoculum levels, transfer was observed out to the 16^{th} slice. Greater transfer was seen for the strong biofilm cocktail with increased survival of the strong biofilm cocktail as observed via viability staining suggesting that these strains are better adapted to survive stressful conditions than weak biofilm formers.

4.2 INTRODUCTION

The level and rate of *Listeria monocytogenes* attachment and biofilm formation are useful predictors of persistence in the environment (Lunden et al., 2000; Norwood and Gilmour, 1999; Borucki et al., 2003). In a recent study of *L. monocytogenes* transfer from biofilms on stainless steel to a model food product, a transfer rate of 55% was observed from pure culture biofilms with the presence of *Kocuria varians* (a Gram-positive environmental isolate) increasing the *L. monocytogenes* transfer rate to 78%, suggesting that this difference in transfer is related to differences in the adhesiveness of *L. monocytogenes* in pure versus mixed culture (Midelet et al., 2006).

Food handling in home kitchens can lead to multiple routes of cross contamination. Several studies have attempted to quantify transfer between food contact surfaces and food in domestic kitchen-type scenarios. According to Kusumaningram et al. (2003), 21-43% of the *S. aureus*, *Campylobacter jejuni* and *Salmonella enteriditis* populations on inoculated (6.7 – 9.4 log CFU/sponge) wet sponges transferred to stainless steel (AISI grade 304), with no significant differences seen between organisms in their rate of transfer to stainless steel or to food products. Subsequently, 25-100% of the available population transferred to roast chicken when applied to stainless steel for 10 s with greater transfer observed when a 500-g weight was added to the chicken. Increasing the product weight did not have the same effect on transfer to cucumber slices, which was found to occur at 50-100% of the available bacterial population (Kusumaningram et al., 2003). However, when compared to several other existing studies, a transfer rate of 100% is highly improbable with this overestimation of transfer likely due to inaccuracies in estimating the surface inoculum. In the aforementioned study, an unorthodox contact

plate method was used to quantify the bacterial population on stainless steel with the agar from the contact plate then suspended and homogenized in a peptone saline solution, and diluted to a countable level before plating (Kusumaningram et al., 2003). In another study, ground beef (75 - 100 g patties) with an average bacterial load of 6.7 log CFU/g transferred 2.5-3.0 log CFU/cm^2 of *E. coli* O157:H7 to polyethylene and wood laminate cutting boards after 30 min of contact, with no significant differences in transfer based on cutting board material (Miller et al., 1996).

Given the lack of quantitative data for *Listeria* transfer in the existing literature, the primary objective of this study was to determiner the transfer rate for *L. monocytogenes* from knife blades to delicatessen turkey meat. The specific goals of the study were to (a) determine whether differences exist in *L. monocytogenes* transfer based on biofilm forming ability, particularly after desiccation on a stainless steel knife blade for extended periods of time and (b) assess the viability of these same strains following desiccation on stainless steel by viability staining.

4.3 MATERIALS AND METHODS

4.3.1 *Listeria monocytogenes* strains

Six strains of *Listeria monocytogenes* (obtained from Dr. Catherine W. Donnelly, University of Vermont, Burlington, VT) were selected due to their ability to form weak or strong biofilms in a microtiter plate assay (Keskinen, et al., 2003). Strong biofilm forming strains included CWD 33 (unknown source, serotype 4b), CWD 730 (dairy plant environmental isolate, serotype 1/2a), and CWD 845 (dairy plant environmental isolate, serotype 1/2b); whereas the weak biofilm forming strains included CWD 182 (unknown source, serotype 4b), CWD 205 (unknown source, serotype 4c), and CWD 578 (dairy

plant environmental isolate, serotype 4d). All strains were maintained at -80°C in trypticase soy broth containing 0.6% yeast extract (TSB-YE; Becton Dickinson, Sparks, MD) containing 10% (v/v) glycerol.

4.3.2 Preparation of turkey slurry

A turkey slurry was prepared for inoculation of knife blades by diluting 25 g of retail restructured roast turkey breast (Gordon Food Stores, Lansing, MI) 1:10 in sterile deionized water and homogenizing in a model DIFP2 blender (General Electric, Bridgeport, CT) at high speed for 1 min. The resulting slurry was filtered through five layers of cheesecloth into sterile 50 ml conical polypropylene centrifuge tubes (Corning, Corning, NY), heated in an 80°C water bath for 20 min, cooled, and stored at -20°C. Before use, the turkey slurry was thawed overnight at 4°C.

4.3.3 Culture preparation

All frozen stock cultures were subcultured separately in TSB-YE (Becton Dickinson) for 18 h at 37°C, and then streaked to plates of trypticase soy agar containing 0.6% yeast extract (TSA-YE; Becton Dickinson) to obtain confluent growth after 18 h of incubation at 37°C. *Listeria monocytogenes* was harvested from the TSA-YE plates by flooding and suspending the cells in 10 ml of 0.1% sterile peptone (Becton Dickinson). Each *Listeria* suspension was then combined in equal volumes to produce two separate cocktails containing three weak and three strong biofilm formers. The concentration of each 3-strain cocktail was determined by optical density at 600 nm using a spectrophotometer (Genesys20 Spectrophotometer, Thermo Electron Corp., Waltham, MA) and by spiral plating (Autoplate® 4000 Spiral Plater, Spiral Biotech Inc., Norwood,

MA) on TSA-YE (Becton Dickinson) followed by 48 h of incubation at 35°C. Each cocktail was then serially diluted in the turkey slurry to a concentration of 10^7 or 10^9 CFU/ml in turkey slurry for inoculation.

4.3.4 Knife blades

A set of six medium sharp electropolished grade 304 stainless steel knife blades measuring 12 cm x 5 cm (product contact area of 60 cm^2 on each side of the blade) with a thickness of 1.4 mm were manufactured by ProAxis, Inc., (Lafayette, IN). Medium sharp blades were machined to allow for a slightly dull blade by milling at a 45° angle 10 mm from the end of the blade, and then machined with a blunt end 0.5 mm from the tip. The end result was a blade meant to mimic a knife slightly dulled by routine usage.

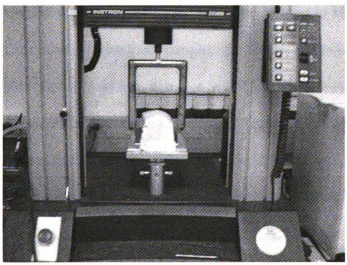
4.3.5 Knife blade inoculation

After flame sterilizing in 95% ethanol, a set of 6 identical knife blades were inoculated on one side with 100 µl of a 3-strain cocktail so as to contain 10^6 or 10^8 *L. monocytogenes* CFU/blade. The inoculum was uniformly spread over the 60 cm^2 product contact area with a 1 µl inoculating loop, allowed to dry on the blade for 5 min under ambient conditions (~22°C and ~40% relative humidity), and then incubated at ambient temperature (~22°C) at 78% relative humidity (R. H.; ASTM Standard Method E104) for 6 or 24 h before surface sampling or slicing. Relative humidity was monitored with a hygrometer (Fisher Scientific; Hampton, NH).

4.3.6 Standardization of cutting force and speed

An Instron 5565 electromechanical compression analyzer (Instron; Canton, MA) was used to standardize cutting force at a cutting speed of 8.3 mm/s. Each knife blade was manufactured with a 1 cm x 2 cm flange at each end so that the blade could be attached to a specially made support bracket and used with an Instron electromechanical compression analyzer. A custom-made knife support bracket to which all knife blades were attached was secured to the upper load cell (1124 lb) for cutting delicatessen turkey meat (Figure 4.1).

Figure 4.1. Instron 5565 electromechanical compression analyzer with modified upper load cell for knife blades



4.3.7 Restructured roast turkey breast

A retail brand of restructured roast turkey breast (2.5 to 2.9 kg each) was purchased in chub-form from a local retailer (Gordon Food Service, Lansing, MI), stored at 4°C, and used within 30 d of purchase. According to the package label, composition of the roast turkey breast was as follows: turkey breast, turkey broth, < 2% each of salt,

dextrose and sodium phosphate. The restructured roast turkey breast averaged 78% moisture, < 1% fat, and 19% protein (Vorst et al., 2006).

4.3.8 Transfer of *L. monocytogenes* from inoculated grade 304 stainless steel knife blades to uninoculated restructured roast turkey breast

Whole chubs of turkey were sliced using a knife blade inoculated at 10^8 or 10^6 CFU/blade to obtain sixteen slices. Each slice was diluted 1:5 (w/v) in either Phosphate Buffered Saline (PBS; 10^8 CFU/blade) or University of Vermont Medium (UVM; Becton Dickinson; 10^6 CFU/blade) for subsequent enrichment at 30°C, and homogenized in a Stomacher (Seward, Norfolk, UK) for 1 minute. Samples obtained using blades inoculated at 10^8 CFU/blade were spread-plated to Modified Oxford Agar (MOX; Becton Dickinson). In contrast, samples sliced with blades inoculated at 10^6 CFU/blade were pour-plated (5 ml into 25 ml of MOX) in duplicate in 150-mm diameter disposable Petri dishes (Fisher Scientific; Chicago, IL) and counted after 2 d of incubation at 35°C to determine the number of listeriae transferred to each slice. When *L. monocytogenes* could not be detected by direct plating, the UVM enriched samples were plated to MOX after 2 d of incubation at 35°C to determine presence or absence of *Listeria*. Each experiment was replicated three times.

4.3.9 Quantification of *L. monocytogenes* on used and unused knife blades

Two inoculated knife blades were surface sampled using the 1-ply composite tissue (CT) method developed by Vorst et al. (2004) after 6 and 24 h of incubation at ~22°C/78% R.H. as a positive control for each experiment. All knife blades were sampled to determine numbers of *L. monocytogenes* remaining on the blade after 16

slices. The CT was rehydrated with 10 ml of PBS in a Whirl-Pak™ bag (Nasco, Inc., Fort Atkinson, WI) and then used to swab the blade, after which the blade was dried using a dry CT. After returning both CT to the original Whirl-Pak™ bag, 40 ml of PBS was added. The sample was then homogenized in a Stomacher for 1 min. Duplicate samples were spread- or pour-plated, as previously described, and incubated at 35°C for 2 d before counting.

4.3.10 Cleaning and decontamination of knife blades

Knife blades were removed from the support bracket after use and soaked in an activated 32% alkaline glutaraldehyde solution (CIDEX®; Advanced Sterilization Products, Irvine, CA). Sanitized knife blades were washed in Tergizyme (Alconox, Inc., New York, NY), rinsed six times in tap water, followed by six rinses in deionized water. The components were then dried using a CT. To prevent surface oxidation during storage, the knife blades were coated with a thin layer of mineral oil. After storage, all components were cleaned again with 70% ethanol and rinsed with sterile deionized water immediately before use in order to remove the mineral oil film.

4.3.11 Evaluation of survival of *L. monocytogenes* on knife blades using confocal scanning laser microscopy

A confocal scanning laser microscope (CSLM; Zeiss LSM 5 Pascal; Carl Zeiss, Inc., Thornwood, NY) was used to evaluate the survival of *L. monocytogenes* on knife blades after incubation for 1, 6 and 24 h at ~22°C/78% R.H. Listeriae were grown on TSA-YE, harvested into 5 ml of 0.1% peptone and combined to form the two 3-strain cocktails as previously described. The cocktails were then centrifuged (Super T21,

Sorvall Products, Newtown, CT) at 9740 × *g* for 10 min at 4°C in sterile 50 ml polypropylene centrifuge tubes. After decanting the supernatant, a 10 µl loop was used to transfer the resulting pellet into 10 µl of turkey slurry on a flame-sterilized piece of grade 304 electropolished stainless steel measuring 2.5 cm x 7.5 cm. Following 6 or 24 h of incubation at ~22°C/78% R.H. in a humidity chamber, as previously described, the bacteria were stained for viability using a LIVE/DEAD® *Bac*Light™ Bacterial Viability Kit (Molecular Probes, Inc., Eugene, OR) prepared as recommended by the manufacturer, with propidium iodide and SYTO 9 mixed together for the staining stock solution. Samples were stained by depositing 10 µl of stain on the sample followed by covering with a glass coverslip. After 1 h incubation under ambient conditions, the stained cells were observed using a CSLM equipped with a 100× oil immersion objective (numerical aperature = 1.3, Carl Zeiss, Inc., Thornwood, NY) and an argon-ion laser. Fluorescence was detected using an excitation wavelength of 488 nm and emitted light was separated through a 488 nm neutral density filter and a 545/635 nm secondary dichroic beam splitter. Simultaneous dual-channel imaging was used to create computer-generated pseudocolor images of the live and dead bacterial cells. One channel was equipped with a 505-530 nm band pass filter for detection of SYTO 9 stained cells (emission wavelengths: 510-540 nm) whereas the second channel was equipped with a 560 nm long pass filter for detection of propidium iodide stained cells (emission wavelengths: 620-650 nm). Five randomly chosen fields of view for each combination of time and cocktail (n = 45 micrographs) were printed and individual cells were counted by hand to determine the percentage of live and dead bacteria were present under each set of conditions.

4.3.12 Statistical analysis

All experiments were replicated three times, except for the microscopy experiments, which were replicated until 5 micrographs with countable fields of view for each treatment were obtained. The resulting data were analyzed using SAS (SAS Version 8; SAS Institute, Cary, NC) software with a general linear mixed effects model and analysis of variance (ANOVA) for least significant differences among the combinations of treatments ($P < 0.05$). For analysis of transfer by biofilm forming ability, time and inoculation level, results were analyzed based on the average transfer to all 16 slices in a replicate.

4.4 RESULTS

4.4.1 *Listeria* transfer from knife blades to product

In the evaluation of *Listeria* transfer from knife blades to turkey breast, *L. monocytogenes* populations decreased 3-5 log CFU/slice after 16 slices with a general decrease observed in transfer to successive slices. (Figures 4.2 and 4.3). Significantly greater overall transfer ($P < 0.05$) of *Listeria* was seen for strong (4.4 log CFU total) as opposed to weak biofilm formers (3.5 log CFU) and after 6 (4.6 log CFU) as opposed to 24 h (3.3 log CFU) of desiccation on the blade. Greater total numbers of cells transferred from knife blades inoculated at 8.9 log (6.4 log CFU transferred; Figure 4.2) than 6.9 log CFU/blade (1.5 log CFU transferred; Figure 4.3) All two- and three-way interactions (e.g., strong biofilm forming cocktail at 6 h vs. weak biofilm forming cocktail at 6 h) were not significantly different for overall transfer.

Figure 4.2. Transfer of weak and strong biofilm forming cocktails of *L. monocytogenes* from an inoculated knife blade (8 log CFU/blade; incubation = 6 and 24 h, 78 ± 2% RH/22°C) to roast turkey breast (n = 3)

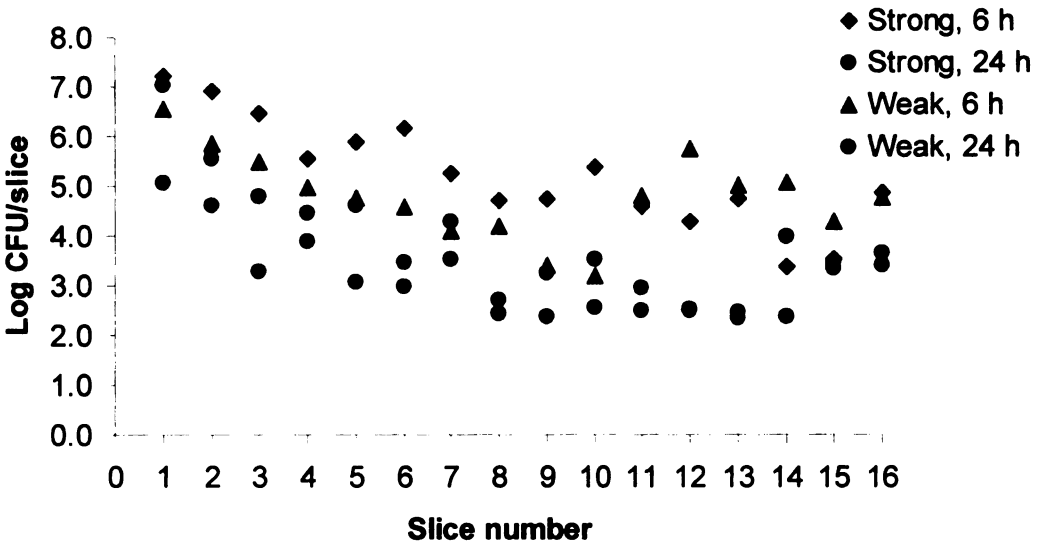


Figure 4.3. Transfer of weak and strong biofilm forming cocktails of *L. monocytogenes* from an inoculated knife blade (6 log CFU/blade; incubation = 6 and 24 h, 78 ± 2% RH/22°C) to roast turkey breast (n = 3). Open symbols not quantifiable by direct plating, but were positive by enrichment.

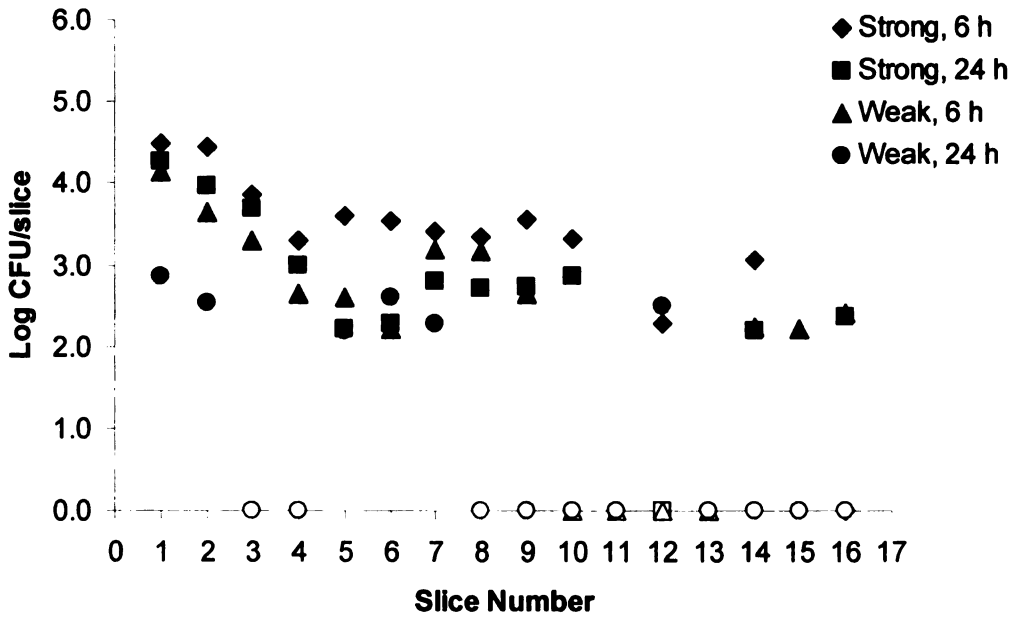


Table 4.1. Number of direct counts and positive enrichments for roast turkey breast sliced with *L. monocytogenes*-contaminated knife blades after 6 and 24 h

	10^6 CFU/Blade				10^8 CFU/Blade			
	Strong cocktail		Weak cocktail		Strong cocktail		Weak cocktail	
Slice	6 h	24 h	6 h	24 h	6 h	24 h	6 h	24 h
1	3/3[a]	3/3	3/3	1/3	3/3	3/3	3/3	3/3
2	3/3	2/3	3/3	2/3	3/3	3/3	3/3	3/3
3	3/3	2/3	2/3	0/3	3/3	3/3	3/3	3/3
4	3/3	3/3	3/3	0/3	3/3	3/3	3/3	3/3
5	3/3	1/3	3/3	1/3	3/3	2/3	3/3	2/3
6	2/3	1/3	2/3	1/3	3/3	2/3	3/3	1/3
7	2/3	2/3	2/3	1/3	3/3	2/3	3/3	1/3
8	2/3	2/3	1/3	0/3	3/3	2/3	3/3	0/3
9	1/3	1/3	1/3	0/3	3/3	1/3	3/3	1/3
10	2/3	1/3	0/3	0/3	3/3	1/3	3/3	2/3
11	NT[b]	NT	0/1[c]	0/1[c]	3/3	1/3	3/3	2/3
12	2/3	0/3	3/3	1/3	3/3	1/3	3/3	2/2
13	NT	NT	0/1[c]	0/1[c]	2/3	1/3	3/3	2/3
14	1/3	1/3	1/3	0/3	3/3	1/2	3/3	2/3
15	NT	NT	1/1[c]	0/1[c]	3/3	2/3	3/3	2/3
16	0/3	1/3	1/3	0/3	3/3	1/2	3/3	2/2

[a]Direct counts / enrichment results for 3 replicates
[b]NT = Not tested
[c]Direct counts / enrichment result for 1 plated replicate

4.4.2 Survival of *L. monocytogenes* on knife blades over time

Strong biofilm formers survived drying on knife blades in significantly greater numbers ($P < 0.05$) than weak biofilm formers, with survival rates of 51.4% ± 23.2 and 38.7% ± 24.9, respectively. *Listeria* viability was significantly greater after 1 h (53.0% ± 17.5; $P < 0.05$) as opposed to 6 (38.9% ± 28.8) or 24 h (43.9% ± 25.1) of incubation on stainless steel.

4.5 DISCUSSION

In a related *Listeria* transfer study by Vorst (2005) using knife blades inoculated with *L. monocytogenes* at 8 log CFU/blade a 2 log CFU decrease in transfer was seen over 12 slices of roast turkey breast with the pathogen quantifiable out to 30 slices. However, when the inoculum level was decreased to 5 log CFU/blade, transfer was only quantifiable for the first 20 slices with slices 26 through 30 negative by enrichment. In this study, transfer was only quantifiable to 16 slices of turkey using a similar a knife blade initially inoculated at 6 log CFU/blade. However, given the extended incubation times of 6 and 24 h on the knife blade as opposed to 1 h in the Vorst (2005) study, the surviving population on the blade after surface drying was likely ~5 log CFU/blade, making the initial inoculum levels roughly similar. This reduction in number of *L. monocytogenes* was confirmed by viability staining (Table 4.2), which showed that significantly lower numbers of *Listeria* survived 6 and 24 h of incubation, as opposed to 1 h.

According to Vorst (2005), greater transfer was seen using AISI grade 304 than grade 316 stainless steel knife blades reinforcing increased cleanability of the latter (Arnold and Bailey, 2000; Leclercq-Pelat and Lalande, 1994). Therefore, in this study, AISI grade 304 stainless steel blades were used in order to mimic the worst-case scenario for harboring *Listeria* during extended incubation times on a surface in the absence of water.

The two sets of *L. monocytogenes* strains used in this study were among the strongest and weakest biofilm formers from a set of 122 strains that was previously characterized for biofilm formation using a microtiter plate assay. According to Borucki

et al. (2003), persistent strains were better able to form biofilms, as measured by a microtiter plate assay for biofilm formation. Lunden et al. (2000) reported that a persistent strain of *L. monocytogenes* was transferred to three different processing plants via a dicing machine and was significantly more adherent than non-persistent strains, a trend which also has been previously observed (Lunden et al., 2000; Norwood and Gilmour, 1999; Borucki et al., 2003). Kalmokoff et al. (2001) reported variation in the ability of *L. monocytogenes* strains to adhere to stainless steel with the extent of subsequent biofilm formation also varying. Therefore, the relationship between assumed persistence of *L. monocytogenes* according to biofilm formation and extent of transfer from stainless steel to roast turkey breast was also assessed.

Significantly higher total transfer was observed by strong (and assumedly persistent) biofilm forming strains of *L. monocytogenes* as opposed to weak biofilm formers. This would run counter to some of the assumptions one might make regarding persistence—if persistence predicts strength of adhesion to a surface, one would predict greater transfer of the less persistent strains. Since the opposite proved to be the case, it was decided to examine survival by strong and weak biofilm forming strains dried on the stainless steel. Although viability staining showed that strong biofilm formers survived in greater numbers than weak biofilm formers, the ability of some *L. monocytogenes* strains to form stronger biofilms may enhance survival to various environmental stresses including lack of moisture and sanitizer exposure. Thus, greater transfer of strong biofilm formers to turkey during slicing may be at least partially due to their increased survival as compared to the weak biofilm forming strains.

Greater transfer of strong biofilm formers may also be related to differences in attachment to stainless steel due to cell surface hydrophobicity. In a previous study (Keskinen et al., 2003), same weak biofilm forming strains used here were significantly more hydrophobic than the strong biofilm forming strains, however the literature is contradictory as to whether hydrophobic or hydrophilic cells will attach more strongly to stainless steel. While hydrophilic and negatively charged *L. monocytogenes* cells adhered better to stainless steel, than to polytetrafluoroethylene (Chavant et al., 2002), Briandet et al. (1999) observed better adherence to stainless steel among *L. monocytogenes* strains that were slightly more hydrophobic. Finally, Midelet and Carpentier (2002) reported stronger attachment of *L. monocytogenes* biofilms to polyvinyl chloride and polyurethane than to stainless steel, and also noted that all of these surfaces were hydrophobic, with these same surfaces becoming hydrophilic after exposure to meat exudate based on contact angle measurement. Hence, further research into the surface characteristics of both *L. monocytogenes* and common food contact surfaces is required to more fully determine whether attachment influences persistence and transfer of *L. monocytogenes* during slicing of delicatessen meats

CHAPTER 5

IMPACT OF BACTERIAL STRESS AND BIOFILM FORMING ABILITY ON TRANSFER OF SURFACE-DRIED *LISTERIA MONOCYTOGENES* DURING SLICING OF DELICATESSEN MEATS

5.1 ABSTRACT

Listeria contamination of delicatessen slicer blades can lead to cross-contamination of luncheon meats. In the present study, six previously identified strong and weak biofilm-forming strains of *L. monocytogenes* were grown at 37°C/18-24 h on trypticase soy agar containing 0.6% yeast extract, harvested in 0.1% peptone and then combined to obtain two 3-strain cocktails. The cocktails were resuspended in turkey slurry with or without prior cold-shock at 4°C/2h and then used to inoculate flame-sterilized stainless steel delicatessen slicer blades at a concentration of 10^6 CFU/blade. After incubation at 22°C/78 ± 2% relative humidity for 6 and 24 h, the inoculated blades were attached to a gravity-fed delicatessen slicer and used to generate 30 slices from retail chubs of roast turkey breast or Genoa salami. Slices (~25 g) were diluted 1:5 in phosphate buffered saline or University of Vermont Medium and then pour-plated (5 ml) into 150-mm dia. Petri plates using 20 ml of Tryptose Phosphate Agar containing esculin and ferric ammonium citrate with the transfer results reported as the average of 30 slices. Overall, more strong biofilm-formers transferred (3.62 log CFU) than weak biofilm-formers (3.12 log CFU), cumulatively. Significantly greater transfer to turkey (3.61 log CFU) than to salami (3.12 log CFU) was observed. Previous cold-shock significantly increased subsequent *Listeria* transfer (3.69 log CFU) compared to healthy (3.30 log CFU) and chlorine-injured cells (3.12 log CFU). Length of desiccation on the blade also significantly affected overall transfer, with greater transfer after 6 h of desiccation. These results are likely due to differences in both product composition and survival of *L. monocytogenes* that were observed via viability staining.

5.2 INTRODUCTION

In retail food handling environments, bacterial contaminants including *Listeria* are most often found in difficult to clean areas that contain food particulates and adequate moisture. Bacteria within these harborage sites are typically exposed to stressful conditions including sanitizers, dehydration, starvation, and extremes in both temperature and pH. Under these extreme conditions, *L. monocytogenes* can become sublethally injured with the pathogen then unable to grow on many commonly used selective plating media. Even under these unfavorable environmental conditions, bacterial foodborne pathogens can remain viable on common food contact surfaces for days or weeks and cross-contaminate other products. In one early report, 469 cases of typhoid fever were traced to a single can of delicatessen-sliced corned beef with *Salmonella* Typhi transferred from the delicatessen slicer to other deli meats that were subsequently sold and consumed (Howie, 1968). The greater prevalence of *L. monocytogenes* in delicatessen- as opposed to manufacturer-sliced meat is at least partly due to cross-contamination in the delicatessen with one of the most obvious contact points being the delicatessen slicer (Gombas et al., 2003).

In recent bacterial transfer work with mechanical delicatessen slicers, *L. monocytogenes* was shown readily transfer both to and from slicer blades and deli meats. Based on the work of Vorst et al., (2006), *L. monocytogenes* transferred from a blade inoculated at 8 log CFU/blade to 30 successive slices of roast turkey breast with transfer decreasing logarithmically to 2 log CFU/slice by the 30^{th} slice. At lower inoculums (5 log and 3 log CFU/blade), transfer was not quantifiable beyond the 5^{th} slice, with negative enrichments after 27 and 15 slices, respectively (Vorst et al., 2006). In the same

study, transfer to salami was more continuous throughout the 30 slices than to turkey or bologna, both of which were higher in moisture and lower in fat than salami. The difference in transfer between the products was attributed to the layer of fat that accumulated on the slicer blade during slicing of salami, which was not seen for the other two products (Vorst et al., 2006).

Lin et al. (2006) conducted a study in which the blade of a commercial-scale meat slicer used to slice roast turkey breast, salami and bologna was inoculated to contain *L. monocytogenes* at levels of 1, 2, or 3 log CFU/blade (1 and 2 log CFU/blade inoculum used with turkey only). More slices tested positive by enrichment using 3 log CFU/blade than at 1 or 2 log CFU/blade (Lin et al., 2006). Additionally, Lin et al. (2006) found that more equipment samples were positive for *L. monocytogenes* after slicing salami (8 samples) than turkey (3 samples) or bologna (1 sample), which supports a longer residence time for *L. monocytogenes* on fat-coated slicers as suggested by Vorst et al. (2006).

In a study of *L. monocytogenes* transfer from meat industry biofilms, transfer to a trypticase soy agar cylinder used as a model food product was quantified and a transfer rate of 55% was observed from pure culture biofilms, while the presence of *Kocuria varians* (a Gram-positive environmental isolate) increased the *L. monocytogenes* transfer rate to 78% (Midelet et al., 2006). Exposure to chlorine shock increased the adhesiveness of *L. monocytogenes* to the stainless steel surface, resulting in less transfer (Midelet et al., 2006).

Given these previous findings, the specific goal of this study was to determine whether differences exist in the transfer of *L. monocytogenes* strains based on their ability

to persist in the environment (as determined by biofilm forming ability), particularly after desiccation on a stainless steel slicer blade for extended periods of time. Strong and weak biofilm forming cocktails of *L. monocytogenes* were also subjected to sublethal cold- and chlorine-injury before desiccation on slicer blades, to assess the impact of injury on subsequent transfer while slicing turkey or salami. These same cocktails were also compared in regards to their ability to survive desiccation, with and without prior sublethal cold- and chlorine-injury, as measured by viability staining.

5.3 MATERIALS AND METHODS

5.3.1 *Listeria monocytogenes* strains

Six strains of *Listeria monocytogenes* (obtained from Dr. Catherine W. Donnelly, University of Vermont, Burlington, VT) were selected due to their ability to form weak or strong biofilms in a microtiter plate assay (Keskinen et al., 2003). Strong biofilm forming strains included CWD 33 (unknown source, serotype 4b), CWD 730 (dairy plant environmental isolate, serotype 1/2a), and CWD 845 (dairy plant environmental isolate, serotype 1/2b); whereas the weak biofilm forming strains included CWD 182 (unknown source, serotype 4b), CWD 205 (unknown source, serotype 4c), and CWD 578 (dairy plant environmental isolate, serotype 4d). All strains were maintained at -80°C in trypticase soy broth containing 0.6% yeast extract (TSB-YE; Becton Dickinson, Sparks, MD) containing 10% (v/v) glycerol.

5.3.2 Preparation of turkey slurry

A turkey slurry was prepared for inoculation of delicatessen slicer blades by diluting 25 g of retail restructured roast turkey breast (Gordon Food Stores, Lansing, MI) 1:10 in sterile deionized water and homogenizing in a model DIFP2 blender (General Electric, Bridgeport, CT) at high speed for 1 min. The resulting slurry was filtered through five layers of cheesecloth into sterile 50 ml conical polypropylene centrifuge tubes (Corning, Corning, NY), heated in an 80°C water bath for 20 min, cooled, and stored at -20°C. Prior to use, the turkey slurry was thawed overnight at 4°C.

5.3.3 Culture preparation, uninjured cocktails

All frozen stock cultures were subcultured separately in TSB-YE (Becton Dickinson) for 18 h at 37°C, and then streaked to plates of trypticase soy agar containing 0.6% yeast extract (TSA-YE; Becton Dickinson) to obtain confluent growth after 18 h of incubation at 37°C. *Listeria monocytogenes* was harvested from TSA-YE by flooding the plates and suspending the cells in 10 ml of 0.1% sterile peptone (Becton Dickinson). Each individual *Listeria* suspension was then combined in equal volumes to form two 3-strain cocktails consisting of weak and strong biofilm formers. The concentration of each 3-strain cocktail was determined by optical density at 600 nm using a spectrophotometer (Genesys20 Spectrophotometer, Thermo Electron Corp., Waltham, MA) and by spiral plating (Autoplate® 4000 Spiral Plater, Spiral Biotech Inc., Norwood, MA) on TSA-YE followed by 48 h of incubation at 35°C. The cocktail was then serially diluted to a concentration of 10^7 CFU/ml in turkey slurry for inoculation.

5.3.4 Culture preparation, cold-injured cocktail

Listeria monocytogenes strains were grown as previously described, individually harvested from TSA-YE plates by flooding the surface with 10 ml of Butterfield's Phosphate Buffer, and then combined in equal volumes to form two 3-strain cocktails of weak and strong biofilm formers. After determining the cell concentration by optical density at 600 nm each cocktail was incubated for 2 h in an ice water bath and then serially diluted to a concentration of 10^7 CFU/ml in turkey slurry. Injury was quantified by spiral plating (Spiral Biotech Inc.) to tryptose phosphate agar (TPA; Becton Dickinson) and tryptose phosphate agar containing 4.5% sodium chloride (TPAN; Becton Dickinson) followed by 48 h of incubation at 35°C. Percent injury was determined using the following equation:

Percent injury = [(TPA count – TPAN count)/TPA count] * 100

5.3.5 Culture preparation, chlorine-injured cocktail

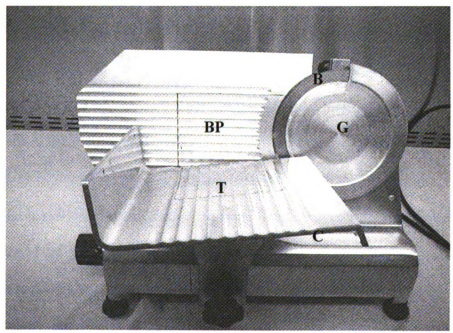
Listeria monocytogenes strains were grown as previously described, individually harvested from TSA-YE plates by flooding the surface with 10 ml of Phosphate Buffered Saline, and then combined in equal volumes to form two 3-strain cocktails of weak and strong biofilm formers. After determining the cell concentration by optical density at 600 nm, each cocktail was then injured by exposure to 100 ppm chlorine (Clorox; The Clorox Company, Oakland, CA) for 1 minute. Following exposure, each cocktail was serially diluted in Neutralizing Buffer (Becton Dickinson) to inactivate the chlorine and then

serially diluted to a concentration of 10^7 CFU/ml in turkey slurry. Injury was quantified by plating on TPA and TPAN as previously described.

5.3.6 Delicatessen slicer inoculation

A commercial gravity-fed delicatessen slicer (Model 220F, Omcan Manufacturing; Niagara Falls, NY) was used for slicing with eight additional electropolished grade 304 stainless steel slicer blades also obtained from the same manufacturer. None of the remaining slicer components were electropolished. After flame sterilizing in 95% ethanol, the product contact surface of four slicer blades, as determined using Glo-Germ™ powder (Vorst et al., 2005), were inoculated with 300 µl of turkey slurry so as to contain 10^6 CFU/blade and then incubated for 6 or 24 h at ambient temperature (~22°C) and 78% R. H. (ASTM standard method E104) before surface sampling or slicing.

Figure 5.1. Contact areas of gravity fed delicatessen slicer



(T) = table, (BP) = back plate, (B) = blade, (G) = guard, (C) = collection area

5.3.7 Delicatessen meats

One retail brand each of restructured roast turkey breast and Genoa hard salami (5.5 to 6.5 lbs each) was purchased in chub-form from a local retailer (Gordon Food Service, Lansing, MI), stored at 4°C, and used within 30 d of purchase. According to the package label, composition of the roast turkey breast was as follows: turkey breast, turkey broth, < 2% each of salt, dextrose and sodium phosphate. The stated product composition of the Genoa salami was as follows: pork, beef, salt < 2% each of dextrose, water, natural spices, sodium ascorbate, lactic acid starter culture, garlic powder, sodium nitrite, BHA, BHT, and citric acid. The restructured roast turkey breast averaged 78% moisture, < 1% fat, and 19% protein, while the Genoa hard salami contained 43% moisture, 36% fat, and 17% protein (Vorst et al., 2005).

5.3.8 *L. monocytogenes* transfer from an inoculated delicatessen slicer blade to uninoculated product

Whole chubs of turkey and salami were sliced using an inoculated slicer blade to obtain 30 2- to 3- mm thick slices weighing approximately 25 g each. Each slice was diluted 1:5 (w/v) in University of Vermont Medium (UVM; Becton Dickinson), homogenized in a Stomacher (Seward; Norfolk, UK) for 1 minute, and pour-plated (5 ml into 25 ml of agar) in duplicate in 150 mm diameter disposable Petri dishes (Fisher Scientific; Chicago, IL) in modified TPA (mTPA) and TPAN (mTPAN) containing ferric ammonium citrate (0.5 g/L), esculin (1 g/L), and lithium chloride (3.75 g/L). Ferric ammonium citrate and esculin allow for the differentiation of *L. monocytogenes* from background microflora. Lithium chloride was added at one-quarter of the strength that is found in Modified Oxford Agar, which was found to be adequate to select against the

lactic acid bacteria found in salami which can also react with ferric ammonium citrate and esculin (data not shown). After 4 d of incubation at ambient temperature (~22°C), the plates were counted to determine the number of listeriae and percent injury per slice, with percent injury determined as follows:

Percent injury = [(TPA count – TPAN count)/TPA count] * 100

When *L. monocytogenes* could not be detected by direct plating, the UVM enriched samples were plated to Modified Oxford Agar (MOX; Becton Dickinson) after 4 d of incubation at ambient temperature (~22°C) to determine presence or absence of *Listeria*. Each experiment was replicated three times.

5.3.9 Quantification of *L. monocytogenes* on used and unused slicer blades

An inoculated slicer blade was surface sampled using the 1-ply composite tissue surface sampling (CT) method developed by Vorst et al. (2004) after 6 and 24 h of incubation at ambient temperature (~22°C) and 78% R.H. as a positive control for each experiment. All slicer blades were also similarly sampled to determine numbers of *L. monocytogenes* remaining on the blade after 30 slices. The CT was rehydrated with 10 ml of PBS in a Whirl-Pak™ bag (Nasco, Inc., Fort Atkinson, WI) and then used to swab the blade, after which the blade was dried using a CT. After returning both CT to the original Whirl-Pak™ bag, 40 ml of UVM was added. The sample was then homogenized in a Stomacher for 1 min. Duplicate samples were pour-plated using mTPA and mTPAN and incubated at ambient temperature (~22°C) for 4 d, as previously described. Percent injury was calculated as previously described.

5.3.10 Cleaning and decontaminating the slicer

After use and disassembly, the slicer table, guard and blade (Figure 5.1) were wiped with a CT and soaked for 30 min in a pan containing an activated 32% alkaline glutaraldehyde solution (CIDEX®; Advanced Sterilization Products, Irvine, CA). Non-removable components of the slicer were wiped with a CT, disinfected with a 32% alkaline glutaraldehyde solution, and then air-dried for 30 min. After disinfection, non-removable components were wiped with a CT soaked in 70% ethanol (v/v), followed by a CT soaked in deionized water, and dried using a CT. Sanitized removable slicer components were washed in Tergizyme (Alconox, Inc., New York, NY), rinsed six times in tap water, followed by six rinses in deionized water. The components were then dried using a CT. After storage, all components were cleaned again with 70% ethanol and rinsed with sterile deionized water immediately before use.

5.3.11 Evaluation of survival of *L. monocytogenes* on knife blades using confocal scanning laser microscopy

A confocal scanning laser microscope (CSLM; Zeiss LSM 5 Pascal; Carl Zeiss, Inc., Thornwood, NY) was used to evaluate the survival of *L. monocytogenes* on knife blades after 1, 6 and 24 h of incubation at ~22°C/78% R.H. Listeriae were grown on TSA-YE, harvested into 5 ml of 0.1% peptone and combined to form the two 3-strain cocktails, as previously described. The cocktails were then centrifuged (Super T21, Sorvall Products, Newtown, CT) at 9740 × *g* for 10 min at 4°C in sterile 50 ml polypropylene centrifuge tubes (Corning Inc., Corning, NY). After decanting the supernatant, a 10 µl loop was used to transfer the resulting pellet into 10 µl of turkey slurry on a flame-sterilized piece of grade 304 electropolished stainless steel measuring

2.5 cm x 7.5 cm. Following 6 or 24 h of incubation at ~22°C/78% R.H. in a humidity chamber, as previously described, the bacteria were stained for viability using a LIVE/DEAD® *Bac*Light™ Bacterial Viability Kit (Molecular Probes, Inc., Eugene, OR) prepared as recommended by the manufacturer, with propidium iodide and SYTO 9 combined to obtain the staining stock solution. Samples were stained by depositing 10 µl of the stock solution of on the sample followed by a glass coverslip. After 1 h of incubation under ambient conditions, the stained bacteria were observed using a CSLM equipped with a 100× oil immersion objective (numerical aperature = 1.3, Carl Zeiss, Inc., Thornwood, NY) and an argon-ion laser. Fluorescence was detected using an excitation wavelength of 488 nm with transmitted light separated through a 488 nm neutral density filter, a 545/635 nm secondary dichroic beam splitter. Simultaneous dual-channel imaging was used to create computer-generated pseudocolor images. One channel was equipped with a 505-530 nm band pass filter for detection of SYTO 9 stained cells (emission wavelengths: 510-540 nm) and the second channel was equipped with a 560 nm long pass filter for detection of propidium iodide stained cells (emission wavelengths: 620-650 nm). Five randomly chosen fields of view for each combination of time and cocktail (n = 45 micrographs) were printed and individual cells were counted by hand to determine the percentage of live and dead bacteria were present under each set of conditions.

5.3.12 Statistical analysis

All experiments were replicated three times, except for the microscopy experiments, which were replicated until 5 micrographs with countable fields of view for each treatment were obtained. The resulting data were analyzed using SAS (SAS

Version 8; SAS Institute, Cary, NC) software with a general linear mixed effects model and analysis of variance (ANOVA) for least significant differences among the combinations of treatments ($P < 0.05$). For analysis of transfer by biofilm forming ability, injury, time and inoculation level, results were analyzed based on the average transfer to all 30 slices in a replicate.

5.4 RESULTS

5.4.1 Transfer of surface-dried *L. monocytogenes* from an inoculated delicatessen slicer blade to uninoculated product

Listeria monocytogenes transfer from an inoculated slicer blade containing 10^6 CFU/blade to Genoa salami and roast turkey breast differed depending on the biofilm-forming ability of the inoculum and the injury to which the inoculum was exposed prior to inoculation on the slicer blade. While there was an overall decrease in the amount of transfer to each successive slice (Figures 5.2 – 5.13), transfer was not generally linear ($R^2 < 0.70$) or logarithmic ($R^2 < 0.70$).

Figure 5.2. Transfer of healthy, strong biofilm forming *L. monocytogenes* from an inoculated slicer blade (6 log CFU/blade; 6 and 24 h/ 78 ± 2% RH/22°C) to uninoculated turkey (n = 3). Open symbols not quantifiable by direct plating, positive by enrichment.

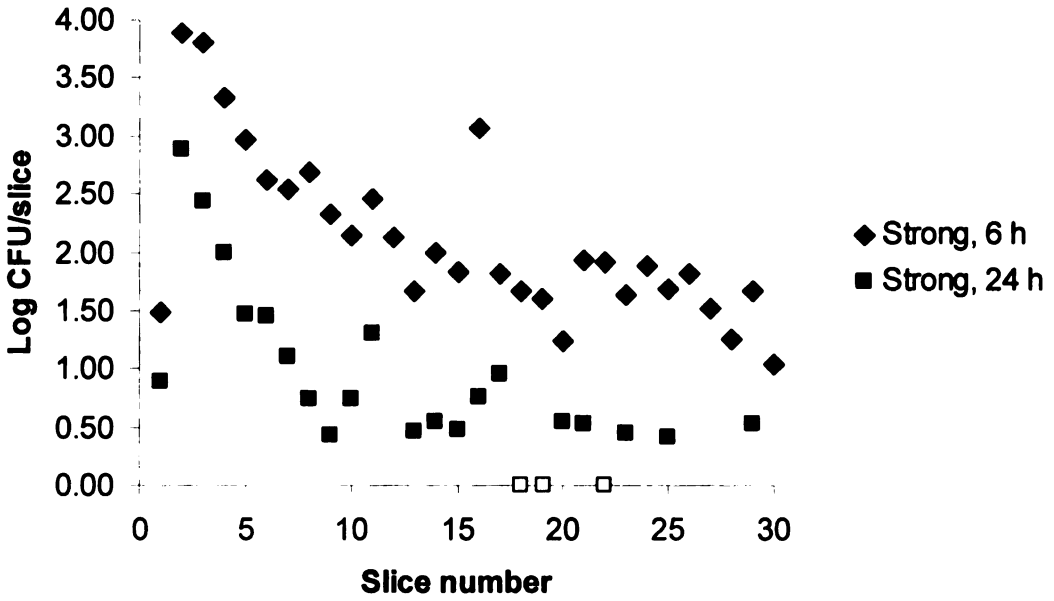


Figure 5.3. Transfer of healthy, weak biofilm forming *L. monocytogenes* from an inoculated slicer blade (6 log CFU/blade; 6 and 24 h/ 78 ± 2% RH/22°C) to uninoculated turkey (n = 3). Open symbols not quantifiable by direct plating, positive by enrichment.

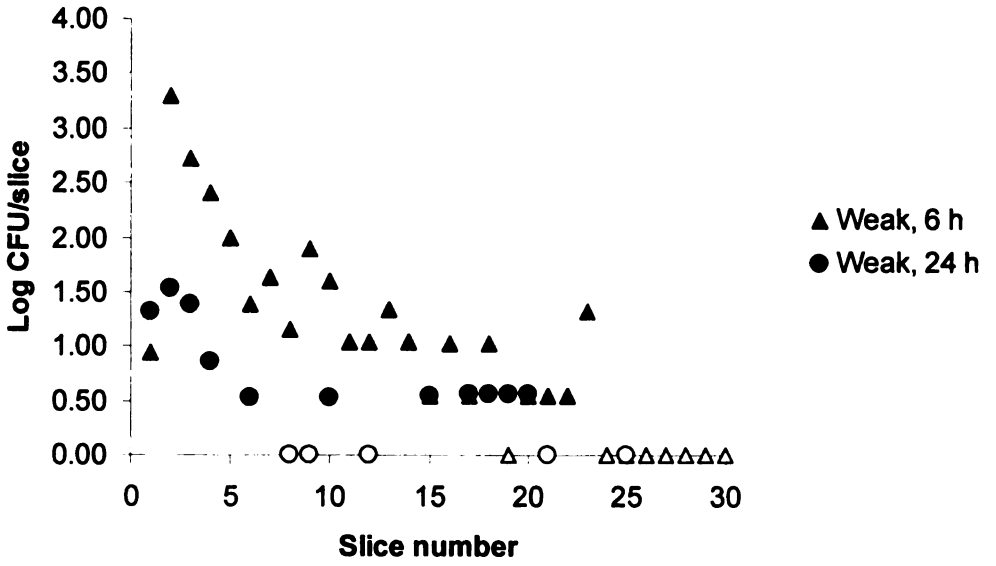


Figure 5.4. Transfer of healthy, strong biofilm forming *L. monocytogenes* from an inoculated slicer blade (6 log CFU/blade; 6 and 24 h/78 ± 2% RH/22°C) to uninoculated salami (n = 3). Open symbols not quantifiable by direct plating, positive by enrichment.

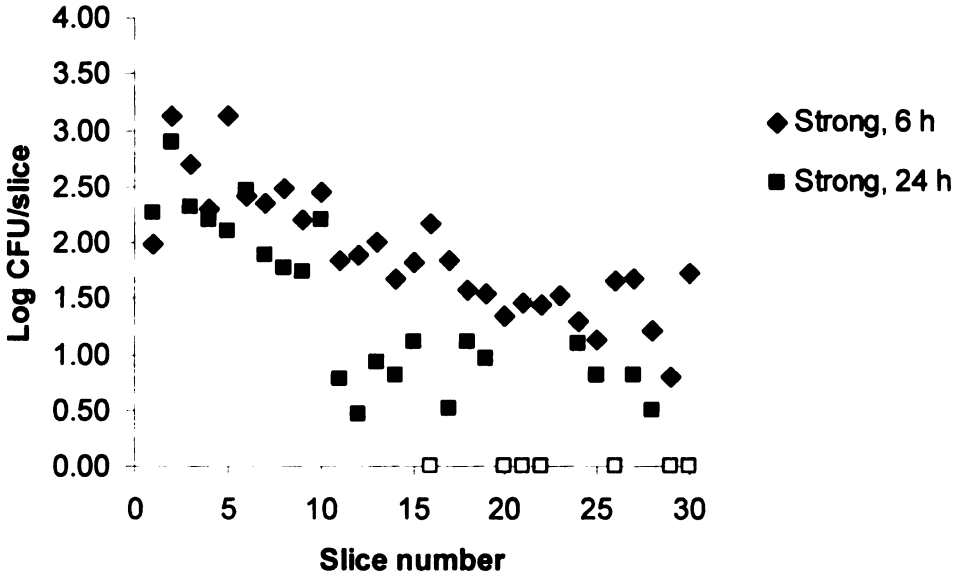


Figure 5.5. Transfer of healthy, weak biofilm forming *L. monocytogenes* from an inoculated slicer blade (6 log CFU/blade; 6 and 24 h/ 78 ± 2% RH/22°C) to uninoculated salami (n = 3). Open symbols not quantifiable by direct plating, positive by enrichment.

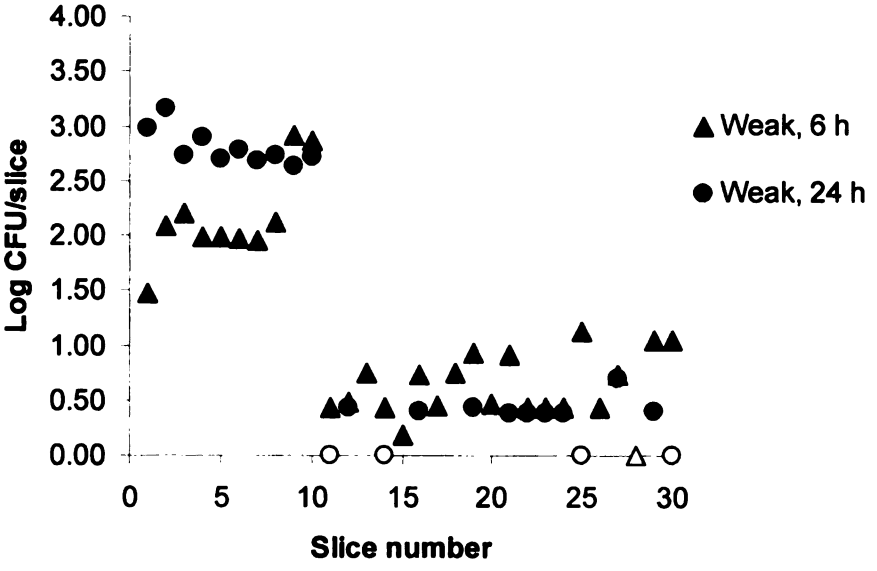


Figure 5.6. Transfer of chlorine-injured, strong biofilm forming *L. monocytogenes* from an inoculated slicer blade (6 log CFU/blade; 6 and 24 h/ 78 ± 2% RH/22°C) to uninoculated turkey (n = 3). Open symbols not quantifiable by direct plating, positive by enrichment.

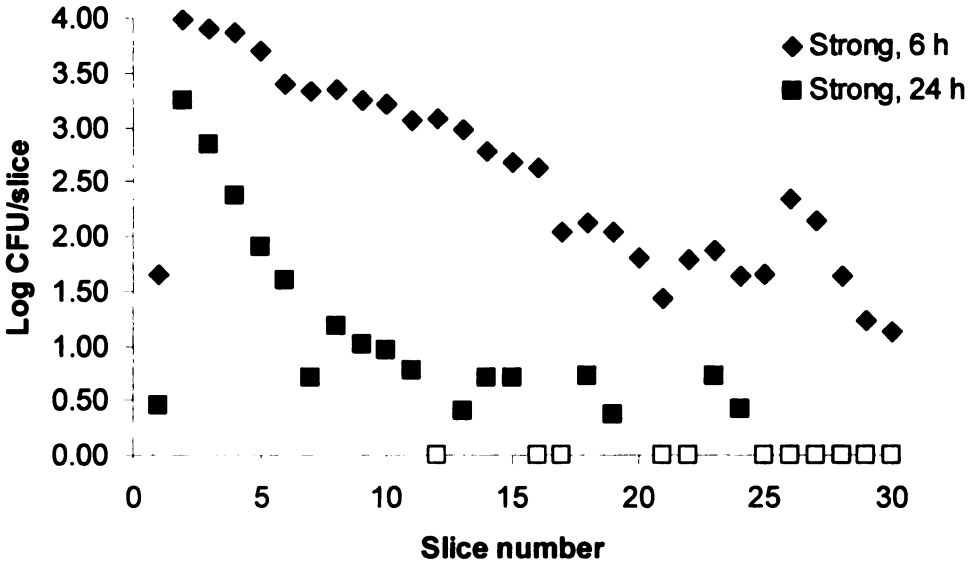


Figure 5.7. Transfer of chlorine-injured, weak biofilm forming *L. monocytogenes* from an inoculated slicer blade (6 log CFU/blade; 6 and 24 h/ 78 ± 2% RH/22°C) to uninoculated turkey (n = 3). Open symbols not quantifiable by direct plating, positive by enrichment.

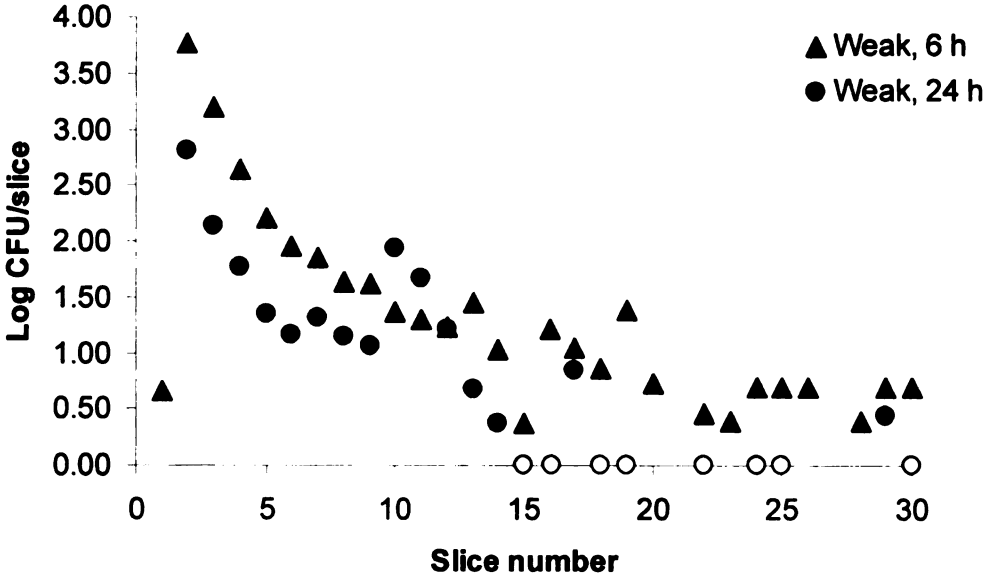


Figure 5.8. Transfer of chlorine-injured, strong biofilm forming *L. monocytogenes* from an inoculated slicer blade (6 log CFU/blade; 6 and 24 h/ 78 ± 2% RH/22°C) to uninoculated salami (n = 3). Open symbols not quantifiable by direct plating, positive by enrichment.

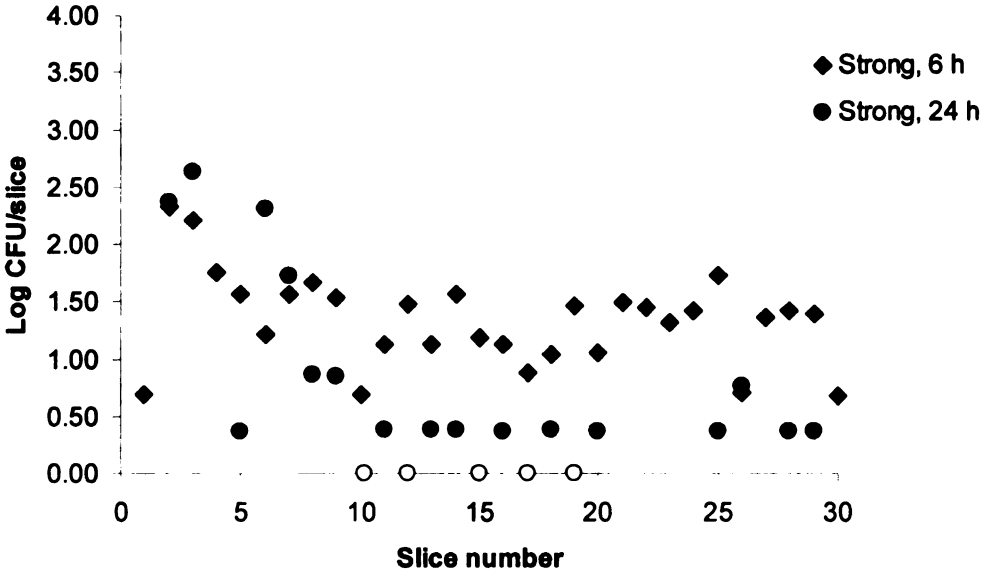


Figure 5.9. Transfer of chlorine-injured, weak biofilm forming *L. monocytogenes* from an inoculated slicer blade (6 log CFU/blade; 6 and 24 h/ 78 ± 2% RH/22°C) to uninoculated salami (n = 3). Open symbols not quantifiable by direct plating, positive by enrichment.

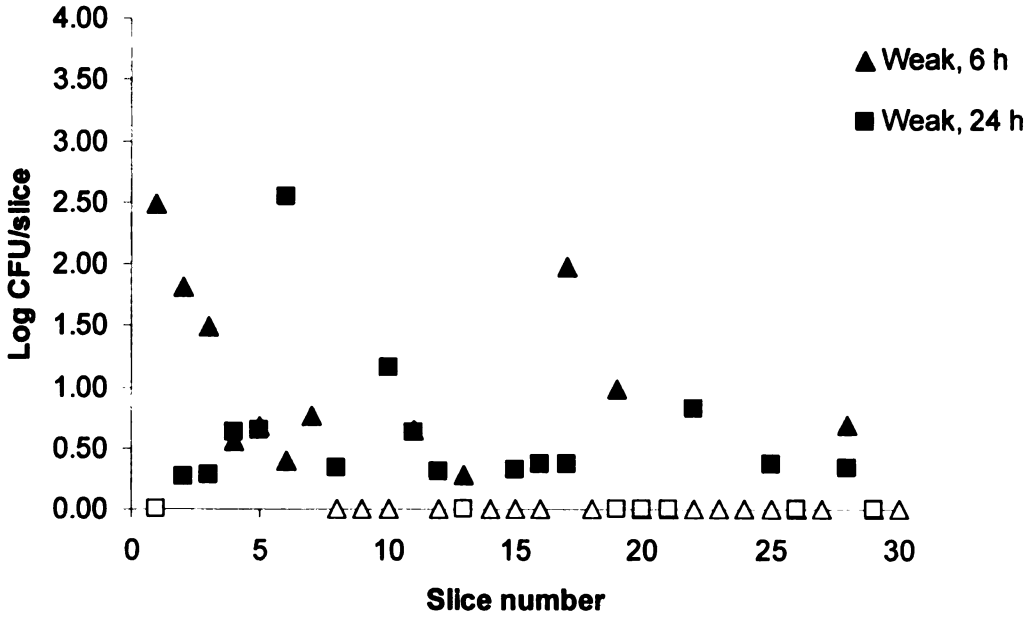


Figure 5.10. Transfer of cold-injured, strong biofilm forming *L. monocytogenes* from an inoculated slicer blade (6 log CFU/blade; 6 and 24 h/ 78 ± 2% RH/22°C) to uninoculated turkey (n = 3).

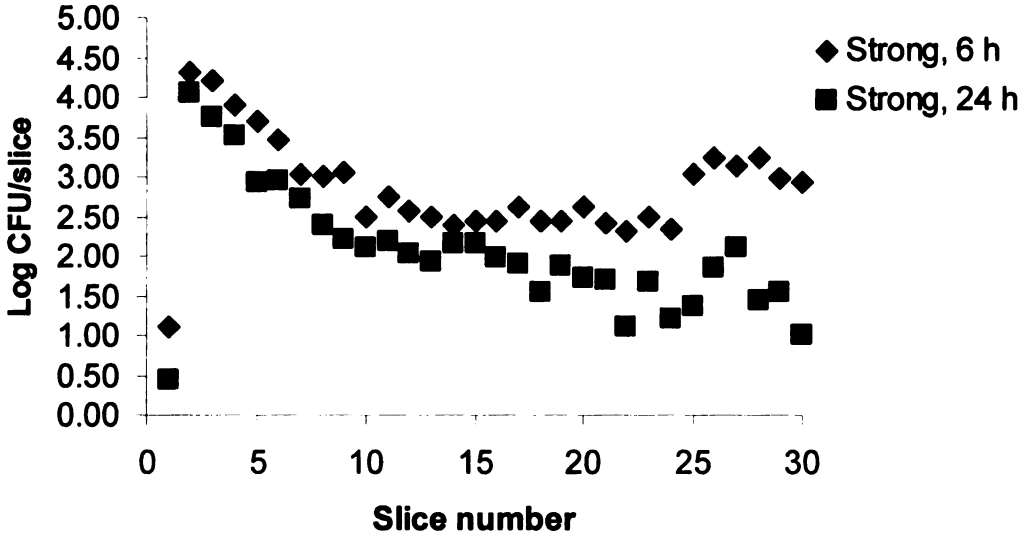


Figure 5.11. Transfer of cold-injured, weak biofilm forming *L. monocytogenes* from an inoculated slicer blade (6 log CFU/blade; 6 and 24 h/ 78 ± 2% RH/22°C) to uninoculated turkey (n = 3). Open symbols not quantifiable by direct plating, positive by enrichment.

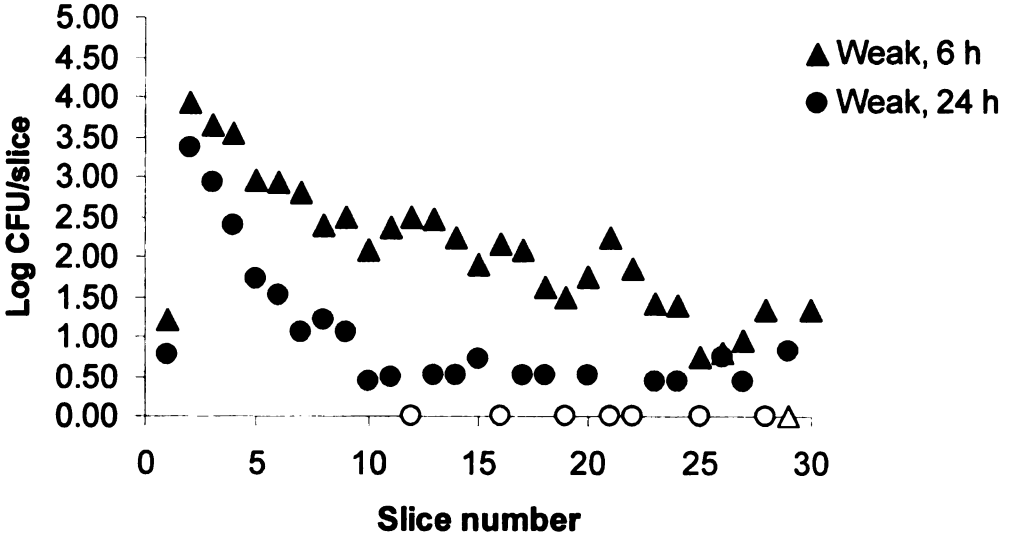


Figure 5.12. Transfer of cold-injured, strong biofilm forming *L. monocytogenes* from an inoculated slicer blade (6 log CFU/blade; 6 and 24 h/ 78 ± 2% RH/22°C) to uninoculated salami (n = 3).

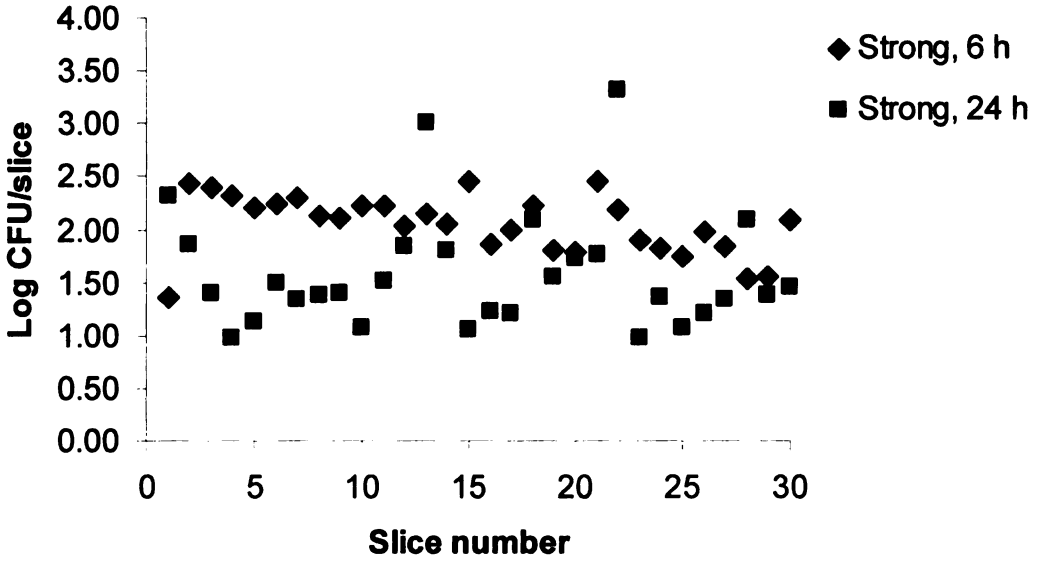
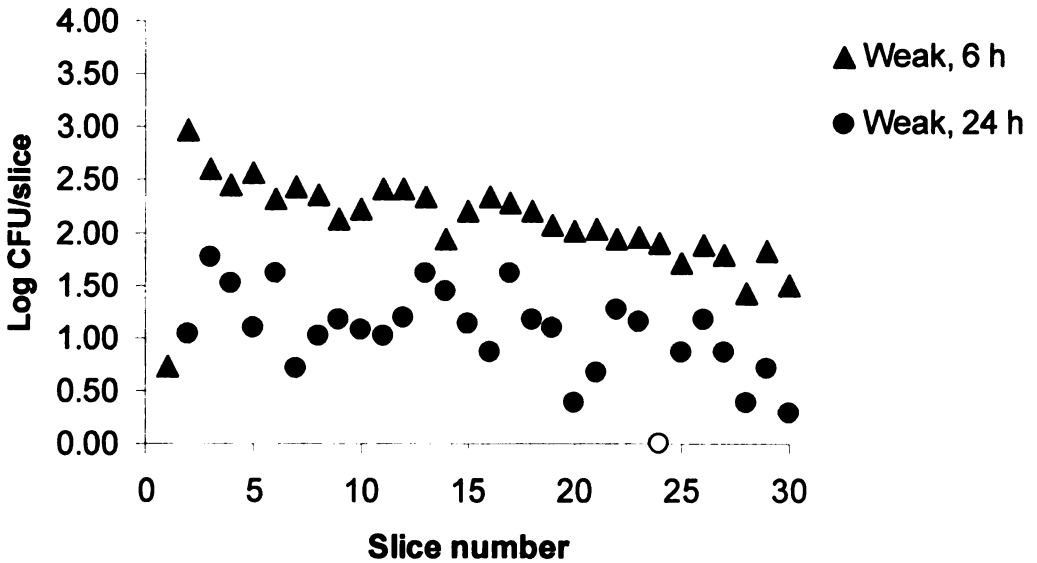


Figure 5.13. Transfer of cold-injured, weak biofilm forming *L. monocytogenes* from an inoculated slicer blade (6 log CFU/blade; 6 and 24 h/ 78 ± 2% RH/22°C) to uninoculated salami (n = 3). Open symbols not quantifiable by direct plating, positive by enrichment.



Overall, less transfer was observed to the first slice for each combination of conditions. This is likely due to *Listeria* drying and adhering to the blade since once moisture and friction are introduced to the surface, a larger number of cells subsequently transfer from the blade to the product. Transfer was observed out to 30 slices for most cocktail/injury/incubation time/product combinations. However, direct counts could not be obtained for every slice, with some slices negative for *L. monocytogenes* in all three replicates for specific treatment combinations (Tables 5.1 and 5.2).

Table 5.1. Number of direct counts and positive enrichments for salami sliced with slicer blades contaminated with healthy, cold- or chlorine-injured *L. monocytogenes* (6 log CFU/blade)

Slice	Strong Biofilm Former						Weak Biofilm Former					
	Healthy		Cold		Chlorine		Healthy		Cold		Chlorine	
	6 h	24 h	6 h	24 h	6 h	24 h	6 h	24 h	6 h	24 h	6 h	24 h
1	3/3[a]	3/3	2/3	3/3	1/1	0/0	3/3	3/3	1/3	0/0	1/1	0/1
2	3/3	3/3	3/3	3/3	3/3	2/2	3/3	3/3	3/3	2/3	2/3	1/1
3	3/3	3/3	3/3	2/2	3/3	1/1	3/3	3/3	3/3	3/3	3/3	1/2
4	3/3	3/3	3/3	3/3	2/2	0/0	3/3	3/3	3/3	2/3	2/3	1/1
5	3/3	3/3	3/3	3/3	2/2	1/1	3/3	3/3	3/3	2/2	1/2	2/2
6	3/3	3/3	3/3	3/3	2/2	2/2	3/3	3/3	3/3	2/3	1/2	1/1
7	3/3	2/3	3/3	3/3	3/3	2/2	2/2	3/3	3/3	1/2	2/3	0/0
8	3/3	3/3	3/3	2/3	3/3	1/1	3/3	3/3	3/3	2/3	0/2	1/1
9	3/3	2/2	3/3	3/3	3/3	3/3	3/3	3/3	3/3	2/2	0/2	0/0
10	3/3	2/3	3/3	3/3	2/2	0/0	3/3	3/3	3/3	2/2	0/3	1/1
11	3/3	2/3	3/3	3/3	1/1	1/1	2/3	0/1	3/3	2/2	2/3	2/2
12	3/3	2/3	3/3	2/3	3/3	0/1	2/3	1/1	3/3	2/3	0/2	1/1
13	3/3	2/3	3/3	3/3	2/2	1/1	2/3	0/0	3/3	2/2	1/2	0/1
14	3/3	2/3	3/3	3/3	3/3	1/1	1/3	0/1	3/3	2/2	0/1	0/0
15	3/3	1/3	3/3	2/3	1/1	0/0	1/3	0/0	3/3	2/2	0/2	1/1
16	3/3	3/3	3/3	3/3	1/3	1/1	1/3	1/2	3/3	2/3	0/1	1/2
17	3/3	1/3	3/3	3/3	2/2	0/1	2/2	0/0	3/3	2/3	1/2	1/1
18	3/3	2/2	3/3	3/3	2/2	1/1	1/3	0/0	3/3	2/2	0/2	0/0
19	3/3	2/2	3/3	2/3	3/3	0/0	2/3	1/1	3/3	2/3	1/3	0/1
20	3/3	0/2	3/3	3/3	2/2	1/1	1/3	0/0	3/3	1/2	0/2	0/1
21	3/3	0/3	3/3	3/3	3/3	0/0	2/3	1/1	3/3	1/2	0/2	0/1
22	3/3	0/2	3/3	3/3	3/3	0/0	1/3	1/1	3/3	2/2	0/1	3/3
23	3/3	1/2	3/3	2/3	2/2	0/0	1/2	1/1	3/3	1/2	0/2	0/0
24	2/3	1/3	3/3	3/3	2/2	0/0	2/3	1/1	3/3	1/2	0/2	0/0
25	3/3	1/2	3/3	2/3	3/3	1/1	1/3	0/2	3/3	1/2	0/1	1/2
26	3/3	0/3	3/3	2/2	2/2	1/2	1/3	0/1	3/3	1/2	0/2	0/1
27	3/3	1/2	3/3	3/3	2/3	0/0	1/1	1/1	3/3	1/2	0/2	0/0
28	3/3	1/2	3/3	3/3	3/3	1/1	0/0	0/0	3/3	1/2	1/1	1/1
29	3/3	0/2	3/3	2/3	2/2	1/1	2/3	1/1	2/3	2/2	0/1	0/2
30	3/3	0/2	3/3	1/3	1/2	0/0	2/3	0/2	2/3	1/2	0/0	0/0

[a]Direct counts / enrichment results for 3 replicates

Table 5.2. Number of direct counts and positive enrichments for roast turkey breast sliced with slicer blades contaminated with healthy, cold- or chlorine-injured *L. monocytogenes* (6 log CFU/blade)

Slice	Strong Biofilm Former						Weak Biofilm Former					
	Healthy		Cold		Chlorine		Healthy		Cold		Chlorine	
	6 h	24 h	6 h	24 h	6 h	24 h	6 h	24 h	6 h	24 h	6 h	24 h
1	3/3[a]	1/1	1/1	1/3	3/3	1/3	2/2	3/3	3/3	1/2	2/2	0/0
2	3/3	2/2	3/3	3/3	3/3	3/3	3/3	2/2	3/3	3/3	3/3	3/3
3	3/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3
4	3/3	2/2	3/3	3/3	3/3	3/3	3/3	1/1	3/3	3/3	3/3	2/3
5	3/3	2/2	3/3	3/3	3/3	3/3	3/3	0/0	3/3	2/3	3/3	2/3
6	3/3	2/2	3/3	3/3	3/3	3/3	2/2	1/1	3/3	3/3	3/3	2/3
7	2/2	2/2	3/3	3/3	3/3	2/2	3/3	0/0	3/3	2/3	3/3	2/3
8	2/2	2/2	3/3	3/3	3/3	3/3	2/2	0/1	3/3	2/3	3/3	2/3
9	2/2	1/1	3/3	3/3	3/3	2/2	3/3	0/1	3/3	2/3	2/2	2/2
10	2/2	1/1	3/3	3/3	3/3	1/3	2/2	1/1	3/3	1/3	2/2	2/3
11	2/3	1/1	3/3	3/3	3/3	1/2	1/2	0/0	3/3	1/3	2/2	2/3
12	2/2	0/1	3/3	3/3	3/3	0/2	2/2	0/1	3/3	0/3	3/3	1/1
13	1/2	1/1	3/3	3/3	3/3	1/2	1/2	0/0	3/3	1/3	2/2	1/1
14	1/2	0/1	3/3	3/3	3/3	2/3	2/3	0/0	3/3	1/3	2/2	1/2
15	2/2	0/0	3/3	2/3	3/3	1/2	1/2	1/1	3/3	1/2	1/2	0/2
16	2/3	1/1	3/3	2/3	3/3	0/2	1/2	0/0	3/3	1/1	2/2	0/1
17	2/2	1/1	3/3	3/3	3/3	0/2	1/2	1/2	3/3	1/3	2/2	1/2
18	2/2	0/1	3/3	2/3	3/3	2/3	1/1	1/1	3/3	0/2	2/3	0/1
19	2/2	0/1	3/3	2/3	3/3	1/3	0/1	1/1	2/3	1/2	2/2	1/1
20	2/2	0/0	3/3	2/3	3/3	0/2	1/1	1/1	2/2	1/1	2/2	0/0
21	1/2	0/0	3/3	2/3	3/3	0/2	1/1	0/1	2/3	0/2	0/0	0/0
22	2/3	0/1	3/3	3/3	3/3	0/2	1/1	0/0	2/2	0/3	1/1	0/1
23	2/2	1/1	3/3	3/3	3/3	1/2	2/2	0/0	2/3	1/2	1/2	0/0
24	1/2	0/0	3/3	3/3	3/3	1/2	0/1	0/0	2/3	1/2	1/1	0/1
25	1/2	2/2	3/3	2/3	3/3	0/2	0/1	0/1	2/3	0/1	1/1	0/1
26	2/2	0/0	3/3	3/3	3/3	0/2	0/2	0/0	2/3	1/2	1/1	0/0
27	2/2	0/0	3/3	3/3	3/3	0/2	0/1	0/0	1/3	1/3	0/0	0/0
28	2/2	0/0	3/3	3/3	3/3	0/2	0/2	0/0	2/3	0/3	1/1	0/0
29	2/2	0/1	3/3	3/3	2/3	0/2	0/1	0/0	2/3	1/2	1/1	1/1
30	1/2	0/0	3/3	2/3	2/3	0/2	0/1	0/0	2/3	0/0	2/3	0/1

[a]Direct counts / enrichment results for 3 replicates

5.4.2 Affect of biofilm forming ability, injury, incubation time and product on transfer of *L. monocytogenes*

When the results for all 30 slices are summed for each treatment, significantly greater cumulative transfer ($P < 0.05$) can be seen to turkey (3.61 ± 0.89 CFU) than to salami 3.12 ± 0.64 log CFU Furthermore, significantly greater cumulative transfer was seen for cold-injured as opposed to chlorine-injured or healthy *L. monocytogenes* cells. (Table 5.3). More *Listeria* were transferred after being dried on the slicer blade for 6 h (3.72 ± 0.69 log CFU) than 24 h (3.01 ± 0.78 log CFU), however, no significant difference in percent injury of the transferred bacteria was seen between the two desiccation times. Strong biofilm formers transferred to meat in significantly higher ($P < 0.05$) numbers (3.62 ± 0.79 log CFU) than weak biofilm formers (3.12 ± 0.76 log CFU.) with the biofilm formers (73.2 ± 23.1% injury) being significantly more injured at the time of transfer than strong biofilm formers(54.3 ± 34.2% injury). Significant differences were also observed for overall transfer (Figures 5.14 – 5.16) and injury at the time of transfer (Figure 5.17) based on combined affects of product, time, cocktail and injury.

Table 5.3. Cumulative log transfer of previously injured and uninjured *L. monocytogenes* to delicatessen meat and percent injury at the time of transfer.

	Cumulative Log CFU	Injury (% of total CFU transferred)
Healthy	3.30 ± 0.71^{a}	50.7 ± 27.1^{a}
Cold	3.69 ± 0.79^{b}	47.8 ± 26.2^{a}
Chlorine	3.12 ± 0.85^{a}	92.8 ± 11.0^{b}

Means with different superscripts are significantly different ($P < 0.05$)

Figure 5.14. Cumulative log transfer of *L. monocytogenes* to delicatessen meat by product and incubation time (n = 18). Means with different superscripts are significantly different for total transfer ($P < 0.05$).

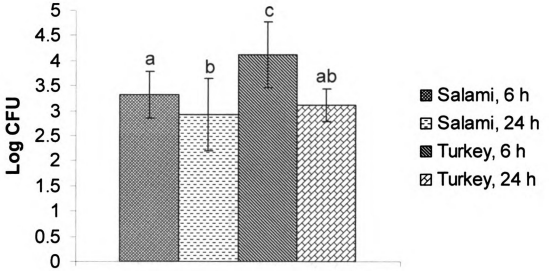


Figure 5.15. Cumulative log transfer of *L. monocytogenes* to delicatessen meat by product and injury treatment (n = 12). Means with different superscripts are significantly different for total transfer ($P < 0.05$).

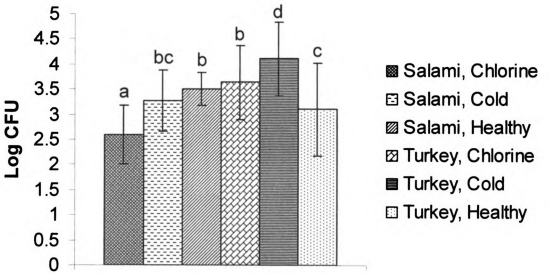


Figure 5.16. Cumulative log transfer of *L. monocytogenes* to delicatessen meat by product and cocktail (n = 18; Strong = Strong biofilm-former cocktail; Weak = Weak biofilm-former cocktail). Means with different superscripts are significantly different for total transfer ($P < 0.05$).

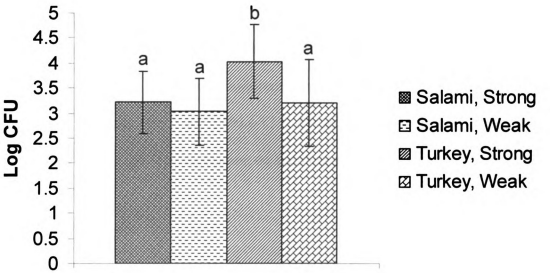
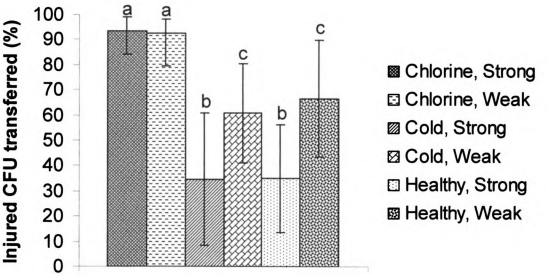


Figure 5.17. Percent injury of *L. monocytogenes* at the time of transfer to delicatessen meat by previous injury treatment and cocktail (n = 12; Strong = Strong biofilm-former cocktail; Weak = Weak biofilm-former cocktail). Means with different superscripts are significantly different from percent injury ($P < 0.05$).



5.4.3 Survival of *L. monocytogenes* on slicer blades over time

Strong biofilm formers survived desiccation on slicer blades in significantly greater numbers than weak biofilm formers, with survival rates of 51.4% ± 23.2 and 38.7% ± 24.9, respectively. Listeriae that were cold-injured before desiccation on stainless steel exhibited significantly greater survival (55.8% ± 20.9; $P < 0.05$) compared to uninjured (40.1% ± 28.2) and chlorine-injured cells (39.3% ± 21.7). *Listeria* viability was significantly greater after 1 h (53.0% ± 17.5; $P < 0.05$) as opposed to 6 (38.9% ± 28.8) or 24 h (43.9% ± 25.1) of incubation on stainless steel.

5.5 DISCUSSION

While there was an overall decrease in the amount of transfer to each successive slice, transfer was not generally linear ($R^2 < 0.70$) or logarithmic ($R^2 < 0.70$). In this respect, the transfer observed after 6 and 24 h of desiccation on the slicer blade resembled the transfer from slicer blades inoculated at 10^3 CFU/blade previously described by Vorst et al. (2006) Using an initial inoculum of 6 log CFU/blade, *L. monocytogenes* transfer was quantifiable to all 30 slices, particularly for both cold-injured cocktails. However, Vorst et al. (2006) were unable to quantify transfer beyond the 5th slice using inoculum levels of 5 and 3 log CFU/blade. Greater overall transfer of cold-injured as opposed to healthy and chlorine-injured cells of *L. monocytogenes* may be related to the higher overall survival of the former as determined by viability staining. Healthy and chlorine-injured cells showed no difference in overall transfer. However, the chlorine-injured cells that transferred exhibited significantly greater injury at the time of transfer than the uninjured and cold-injured cells, indicating that refrigeration extended the persistence of *L. monocytogenes* in the environment for as long as 24 h following exposure.

To the contrary, exposure to chlorine at sublethal levels will not significantly impair cell survival and transfer as compared to their uninjured counterparts. Furthermore, in contrast to the findings by Midelet et al (2006), in which chlorine increased the adherence of *L. monocytogenes* to stainless steel, under the conditions used in this study, no significant difference was seen in the ability of uninjured and chlorine-injured cells to be transferred from stainless steel. However, in this study, exposure to chlorine occurred before *Listeria* was inoculated on the stainless steel surface, suggesting that a greater reduction in transfer and a corresponding increase in adhesiveness to the

surface may have resulted if *Listeria* had been exposed to chlorine after inoculation onto stainless steel.

Strong biofilm forming strains of *L. monocytogenes* transferred to delicatessen meats in greater numbers than weak biofilm formers. The strains chosen for this study consisted of *L. monocytogenes* that formed either the strongest biofilms (as determined by a microtiter plate assay for biofilm formation), or the weakest biofilms out of 122 strains that were previously tested for biofilm-forming ability (Chapter 2). According to Borucki et al. (2003), persistent strains were better able to form biofilms, as measured by a microtiter plate assay for biofilm formation with other studies showing that persistent strains are significantly more adherent to surfaces (Lunden et al., 2000; Norwood and Gilmour, 1999). Therefore, it was decided to see whether this assumed persistence of *L. monocytogenes* according to biofilm formation would impact transfer from delicatessen slicers to delicatessen meats.

Significantly higher total transfer was observed for strong biofilm forming strains of *L. monocytogenes* as opposed to weak biofilm formers. Since this was the opposite of what would be expected if biofilm formation is a predictor of persistence, survival of strong and weak biofilm forming strains was examined during desiccation. Viability staining showed that strong biofilm formers survived in greater numbers under desiccated conditions than weak biofilm formers, suggesting that the ability to form thick biofilms is advantageous for survival of *Listeria* in stressful environments. Greater overall transfer of the strong biofilm-forming strains to turkey may be partially due to their increased ability to survive desiccation. In a previous study (Chapter 2), the weak biofilm forming strains used here were significantly more hydrophobic than the strong biofilm forming

strains, however the literature is contradictory as to whether hydrophobic cells or hydrophilic cells will attach more strongly to stainless steel (Chavant et al., 2002; Briandet et al., 1999; Midelet and Carpentier, 2002). Hence, further research into the surface characteristics of both *L. monocytogenes* and common food contact surfaces is needed to better assess the impact of attachment and persistence on *L. monocytogenes* transfer.

Significantly greater transfer was seen after 6 h as opposed to 24 h, however, this cannot be explained by survival differences between the two time points since no significant difference in survival was seen by viability staining. In the aforementioned Vorst et al. (2006) study, transfer to salami was more continuous throughout the 30 slices than to turkey or bologna, both of which were higher in moisture and lower in fat than salami. Similar trends were observed in this study, which showed that *L. monocytogenes* had lower overall cumulative transfer to salami than to turkey. This further reinforces the conclusions drawn by Lin et al (2006) and Vorst et al. (2006) that the fat deposited by the salami may help *L. monocytogenes* remain on equipment longer, and therefore result in continual transfer of low numbers.

Various combinations of factors including time and product, injury and product, and biofilm forming ability and product all significantly impacted *Listeria* transfer during slicing. The greatest overall transfer was generally observed to turkey under conditions that allowed significantly greater survival, whether by strong biofilm formers, or cold-injured cells. Hence, survival appears to play a large role in transfer of *L. monocytogenes* over extended periods of time, thereby reinforcing the importance of proper cleaning and

sanitizing of food contact surfaces to prevent the establishment of persistent *L. monocytogenes* strains in niches within food processing and retail environments.

CHAPTER 6

VALIDATION OF A PREDICTIVE MODEL FOR *LISTERIA MONOCYTOGENES* TRANSFER DURING SLICING OF DELICATESSEN MEATS

6.1 ABSTRACT

Despite careful attention to cleaning and sanitizing, *Listeria monocytogenes* can persist for weeks or months on difficult-to-clean stainless steel surfaces including delicatessen slicer blades. Consequently, improperly cleaned slicers can contain small numbers of cells that can potentially be transferred to deli meats during slicing. In response to these concerns, transfer of healthy, chlorine-injured and cold-shocked *L. monocytogenes* cells of weak and strong biofilm-forming ability was assessed after 6 and 24 hours of drying on delicatessen slicer blades. These data were then used to test a previously developed predictive model for *Listeria* transfer to and from mechanical delicatessen slicers and kitchen knives.

The model and subsequent computer program in GWBasic are based on the following two assumptions: 1) the number of *Listeria* cells transferred from the blade to the meat during slicing is a fraction of the number of *Listeria* cells on the blade just before each sequential slice, and 2) the number of *Listeria* cells transferred to surrounding areas is a different fraction of the number of cells on the blade just before each sequential slice. The model predicts an exponential decay in the number of cells versus slice number. Observed and predicted values were similar for the transfer of healthy cells to salami and roast turkey breast ($R^2 = 0.88$). However, the model was least accurate when used to predict transfer of previously cold-injured cells. Greater variance was seen between the observed values and the predicted values ($R^2 = 0.65$). For all other scenarios, which included transfer from strong and weak-biofilm forming cells to salami and turkey, and transfer after 6 and 24 h of desiccation on a slicer blade, the model was an accurate predictor of transfer ($0.77 \leq R^2 \leq 0.94$).

6.2 INTRODUCTION

In the federal *Listeria* Risk Assessment (FDA/USDA/CDC, 2003), delicatessen meats were ranked as posing the highest relative risk of exposing the public to *L. monocytogenes* out of 23 categories of ready-to-eat (RTE) foods. In the same risk assessment, quantitative transfer to and from commercial meat slicers, knives and cutting boards in delicatessens was identified as a key informational gap for assessing exposure (FDA/USDA/CDC, 2003). In a survey of *L. monocytogenes* in RTE foods, luncheon meats that were store-packaged were more frequently contaminated with *L. monocytogenes* (6.8 times as likely to be contaminated) than manufacturer-packaged meats (Gombas et al., 2003). However, the samples contaminated at levels higher than 10^2 CFU/g were more likely to be manufacturer-packaged, with most positive samples containing less than 1 CFU/g (Gombas et al., 2003). The higher prevalence of *L. monocytogenes* in delicatessen meat sliced at retail strongly suggests that the delicatessen slicer is an important vehicle for cross-contamination of products.

Within the last decade, risk assessments have necessitated the development of dynamic models that provide estimates of bacterial survival, growth, and distribution throughout food processing and storage. Microbiological risk assessments depend upon exposure assessments, however, these exposure assessments rely on existing data for presence of bacteria in foods, the accuracy of which may be limited by sample size and different test methods (Gardner, 2004).

Predictive modeling can be used to estimate microbial contamination levels, distributions and rates of transfer in the environment. Schaffner (2004) has described the basic mathematical framework for modeling *L. monocytogenes* cross-contamination in

food processing facilities. Using the resultant models can help a processor determine whether an overall greater reduction in *L. monocytogenes* prevalence in a production facility could be achieved by requiring better raw material quality or by improved sanitation efforts.

A model with similar benefits, in terms of determining the best testing sites to minimize contamination of ground beef produced using a commercial grinder, was developed by Flores and Stewart (2004). According to their model, rather than random sampling of a ground beef lot to determine *E. coli* O157:H7 contamination, testing the collar that fixes the grinder die and blade to the meat grinder was shown to be a more accurate predictor of contamination.

In models that have been specifically developed to assess transfer of *L. monocytogenes*, one assessed the risk of *L. monocytogenes* transfer and subsequent growth due to contact with bare or gloved hands (Perez-Rodriguez et al., 2006). This model predicted that the highest risk of contamination comes from handling raw and ready-to-eat meats with the same gloves. This risk was higher than the risk of cross-contamination from bare, washed hands (Perez-Rodriguez et al., 2006).

Thus far, Vorst (2005b) has developed the only model to predict *L. monocytogenes* transfer during slicing of RTE delicatessen meats on a commercial delicatessen slicer. Their exponential decay for direct CFU model predicts the number of CFU transferred to any given slice, as well as the number CFU lost to the environment through aerosols and bacterial death. Under the conditions tested by Vorst (2005b), this model had a correlation coefficient varying from $R^2 = 0.40$ when slicing salami, to over

0.90 when slicing turkey or bologna with a slicer blade inoculated at 8 log CFU/blade (Vorst, 2005b).

The objective of this study was to verify the predictive model developed by Vorst, 2005b) for *Listeria* transfer based on quantitative data obtained from slicing of turkey and salami with an inoculated delicatessen slicer blade with additional variables including the physiological state (healthy vs. injured), and biofilm-forming ability (strong vs. weak) of *L. monocytogenes* and length of desiccation on the blade before slicing (6 vs. 24 h).

6.3 MATERIALS AND METHODS

6.3.1 Transfer coefficients for surface-dried, uninjured and injured *L. monocytogenes* during slicing of turkey and salami

Transfer data were obtained from slicing roast turkey breast and salami with a delicatessen slicer that was inoculated with an uninjured, cold-injured or chlorine-injured cocktail of *L. monocytogenes* and then held for 6 or 24 h at 78% RH before slicing (Chapter 5). The previously developed model of Vorst (2005) was used to determine transfer coefficients for *L. monocytogenes* from contaminated knife and slicer blades to uncontaminated product.

6.3.2 Predictive modeling of *L. monocytogenes* transfer during slicing of roast turkey breast and salami

A model based on the following three assumptions was developed to predict the previously calculated transfer coefficients by Vorst (2005b): a) the number of *Listeria* cells transferred from the blade to the meat during slicing is a fraction (f_1) of the number of *Listeria* cells on the blade just before each sequential slice, b) the number of *Listeria*

cells transferred to surrounding areas is a different fraction (f_2) of the number of cells on the blade just before each sequential slice, and c) the CFU on the blade before any slicing begins is N_0.

The consequences of these assumptions are as follows (Vorst, 2005b):

1st Slice

CFU on Meat = $f_1 N_0$ (1a)

CFU to Surroundings = $f_2 N_0$ (1b)

CFU left on Blade = $N_0 - f_1 N_0 - f_2 N_0 = (1 - f_1 - f_2) N_0$ (1c)

2nd Slice

CFU on Meat = $f_1(1 - f_1 - f_2) N_0$ (2a)

CFU to Surroundings = $f_2(1 - f_1 - f_2) N_0$ (2b)

CFU left on Blade = $(1 - f_1 - f_2) N_0 - f_1(1 - f_1 - f_2) N_0 - f_2(1 - f_1 - f_2) N_0$

$= (1 - f_1 - f_2)^2 N_0$ (2c)

3rd Slice

CFU on Meat = $f_1(1 - f_1 - f_2)^2 N_0$ (3a)

CFU to Surroundings = $f_2(1 - f_1 - f_2)^2 N_0$ (3b)

CFU left on Blade = $(1 - f_1 - f_2)^2 N_0 - f_1(1 - f_1 - f_2)^2 N_0 - f_2(1 - f_1 - f_2)^2 N_0$

$$= (1 - f_1 - f_2)^3 N_0 \quad (3c)$$

X^{th} Slice

CFU on Meat = $f_1(1 - f_1 - f_2)^{X-1} N_0$ (4a)

CFU to Surroundings = $f_2(1 - f_1 - f_2)^{X-1} N_0$ (4b)

CFU left on Blade = $(1 - f_1 - f_2)^{X-1} N_0 - f_1(1 - f_1 - f_2)^{X-1} N_0 - f_2(1 - f_1 - f_2)^{X-1} N_0$

$$= (1 - f_1 - f_2)^X N_0 \quad (4c)$$

6.3.3 Predicting CFU on meat as a function of slice number (X)

The model predicts that the number of CFU transferred to slice X is:

CFU (X) = $f_1 (1 - f_1 - f_2)^{X-1} N_0$ (5a)

This can be arranged as:

CFU (X) = $\frac{f_1 N_0}{1 - f_1 - f_2} (1 - f_1 - f_2)^X$ (5b)

This can be rewritten as:

CFU (X) = ka^X (5c)

Where "k" and "a" are constants related to the model parameters f_1, f_2, and N_0.

6.3.4 Fitting the equation to experimental data (finding "k" and "a")

Taking the natural log of the predictive equation gives the general equation for a straight line.

$$y = b + mx \tag{6a}$$

This equation can then be fitted to the data to find the slope (m) and intercept (b), where:

$y = \ln(CFU)$

$b = \ln(k)$

$m = \ln(a)$

x = slice number

It then follows from equations (5b) and (5c) that:

$$a = 1 - f_1 - f_2 = e^m \tag{6b}$$

$$k = f_1 N_0 (1 - f_1 - f_2) = e^b \tag{6c}$$

6.3.5 Interpretation of fit results

Given that m and b are known from the straight line fit to $y = ln\ (CFU)$ vs. $x = slice\ number$, equations (6b) and (6c) can be used to find f_1 and f_2 if the original inoculum level, N_0, is known. The parameter "a" is the fraction of CFU remaining on the blade after any slice. The slope "m" from the fit will always be negative, so $(1 - f_1 - f_2) < 1$. The number of CFU transferred from the blade to the first slice is $f_1 N_0$. From the relationships between the fit parameters "m" and "b" and the model parameters "f_1", "f_2" and "N_0" it follows that:

Fraction remaining on blade = e^m

CFU transferred to 1st slice = e^{m+b}

Given the inoculation level or original number of CFU on the blade, " f_1 ", " f_2 " and " N_0 " can be found as follows using these previous equations:

$$f_1 = \frac{e^{m+b}}{N_0} \tag{7a}$$

$$f_2 = 1 - f_1 - e^m \tag{7b}$$

6.4 RESULTS

Listeria cumulative transfer amounts based on the experimental data for each mechanical slicer scenario are presented in Figures 6.1 – 6.4. A similar trend was seen for all scenarios, with 99% of the total *Listeria* transfer occurring within the first 10 slices. Significantly more transfer to turkey than to salami had been observed previously (Chapter 5). This resulted in smaller transfer coefficients f_1 and f_2 for salami than for turkey. This is reflected in the fact that cumulative transfer for salami does not reach a plateau, with small numbers of listeriae continually transferred over more slices than what is observed for transfer to turkey. For the scenarios studied—biofilm forming ability, time, and injury—a plateau was reached in the transfer of each, with 99% of the total transfer generally occurring within the first 10 slices.

Figure 6.1. Cumulative *L. monocytogenes* transfer from an inoculated slicer blade (6 log CFU/blade) to turkey and salami

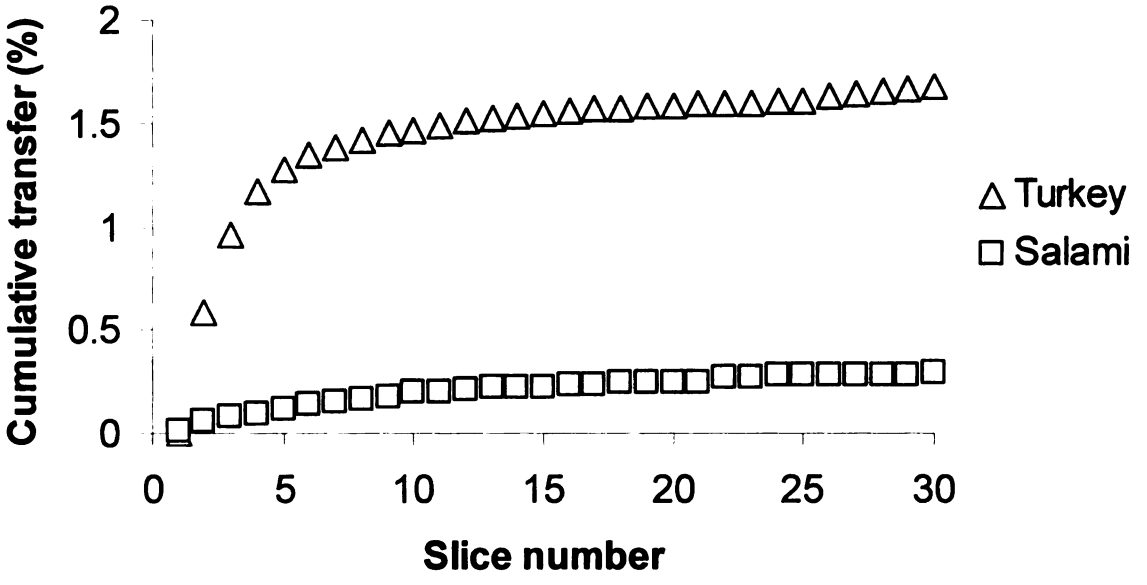


Figure 6.2. Cumulative transfer by strong and weak biofilm forming *L. monocytogenes* from an inoculated slicer blade (6 log CFU/blade) to turkey and salami

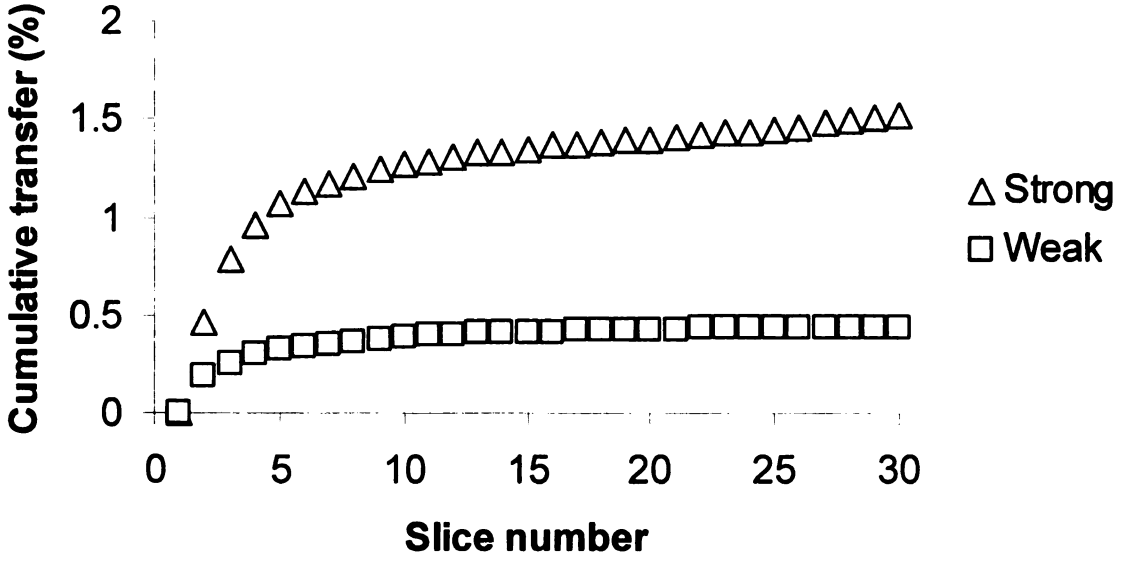


Figure 6.3. Cumulative *L. monocytogenes* transfer from an inoculated slicer blade (6 log CFU/blade; 6 and 24 h incubation, 22°C/78% RH) to turkey and salami

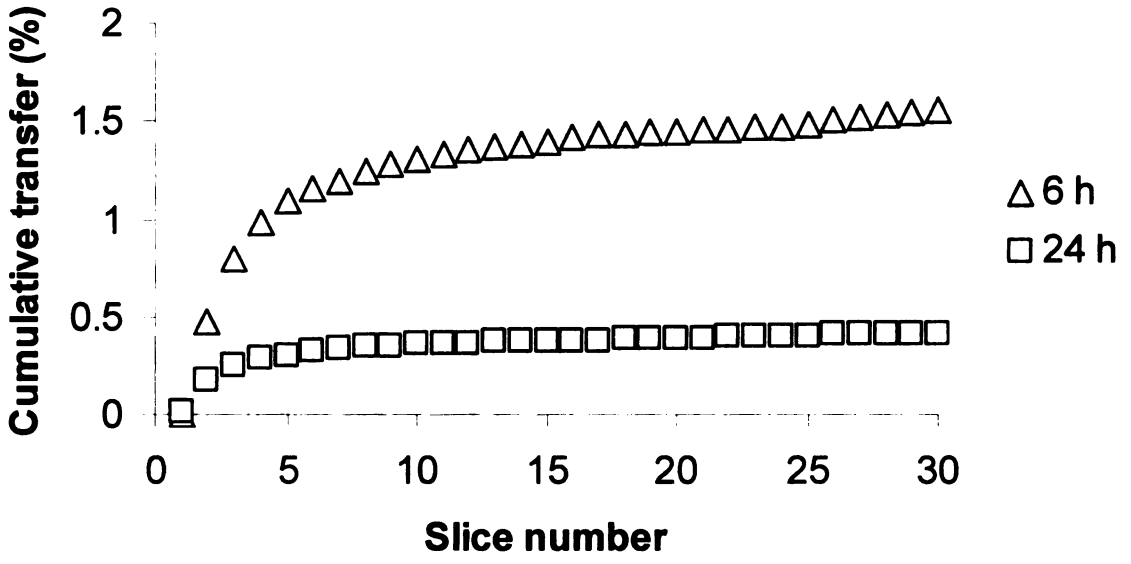
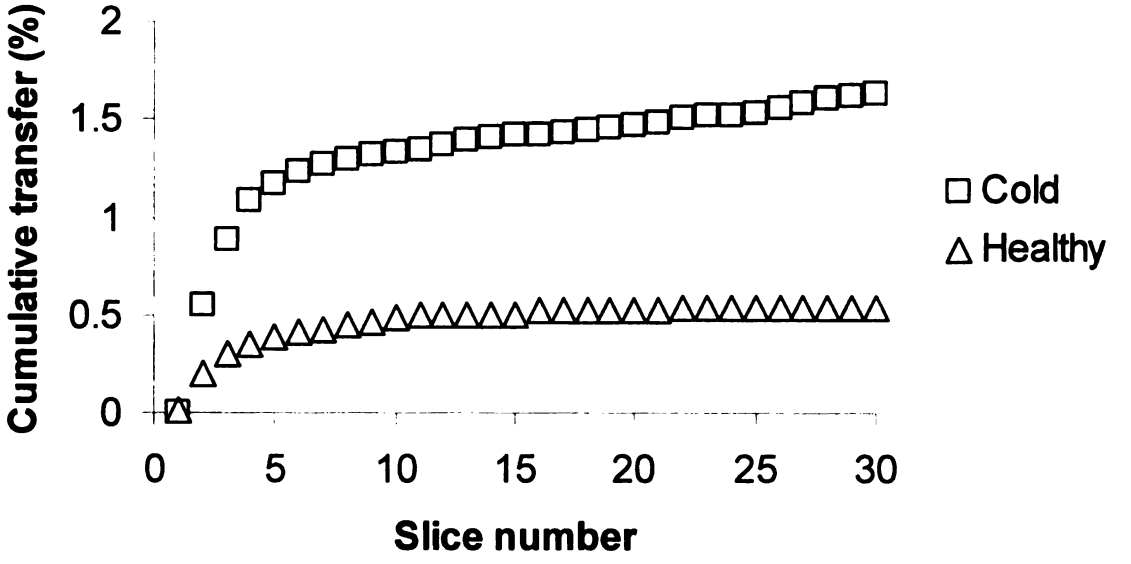


Figure 6.4. Cumulative transfer by uninjured and cold-injured *L. monocytogenes* from an inoculated slicer blade (6 log CFU/blade) to turkey and salami



6.4.1 Predictive model for *L. monocytogenes* transfer during slicing of turkey and salami using a mechanical slicer

Using the aforementioned predictive model (Sections 6.3.2 – 6.3.3), a program was developed using GWBasic to find the transfer coefficients f_1 and f_2 for the different slicer scenarios, including experimental variables; product (turkey and salami), incubation time on the stainless steel blade (6 and 24 h), bacterial injury (healthy and cold-injured), and biofilm-forming ability (strong and weak) which had been identified in previous studies as being significantly different in total cumulative transfer (Chapter 5). One inoculum level was used for all scenarios ($N_0 = 10^6$ CFU/blade). All data replicates were averaged with regards to the aforementioned scenarios, resulting in a minimum of 24 (healthy vs. cold-injured) and a maximum of 36 (all other scenarios) averaged replicates for use in the GWBasic model.

The first slice for each replicate was not modeled. Due to the length of incubation on the blade, the first slice generally had less transfer than the second slice. This is an artifact of the experiment, it is unlikely that *L. monocytogenes* would completely dry on a slicer blade for 6 or 24 h without disturbance. Therefore, modeling began with the second slice, which followed the output of the program shown in Figure 6.5.

Transfer of weak biofilm-forming *L. monocytogenes* strains to turkey and salami resulted in the lowest variance ($R^2 = 0.94$) for observed vs. predicted values for all models tested (Figures 6.6 and 6.13). Transfer of cold-injured *L. monocytogenes* to turkey and salami showed the greatest deviation from the predicted values ($R^2 = 0.65$; Figure 6.11). Since the transfer to the first slice was typically less than 100 CFU, it was not necessary to modify the N_0 used in the program—the starting number of bacteria

available to transfer to the second slice was still approximately 10^6 CFU. Likewise, when percent survival (~40 – 60% of initial inoculum, Chapter 5) at the time of transfer was input into the model, it simply resulted in a 0.009 - 0.01 decrease for the f_1 calculated by the model, with no affect on the predicted transfer to each slice or the correlation coefficient (data not shown). Again, this is due to the fact that ~40 – 60% of an initial inoculum of 10^6 CFU is still approximately 10^6 CFU. In all possible combinations of variables, the fraction transferred to the surroundings (f_2) always exceeded the fraction transferred to each slice of delicatessen meat (f_1). This is shown in Table 6.1, which summarizes the model results for all of the scenarios tested.

Figure 6.5. Example: GWBasic output for turkey and salami sliced using a knife blade inoculated with weak biofilm forming *L. monocytogenes* (10^6 CFU/blade).

Fraction left on blade during each slice = .8541745

CFUs transferred to 1st slice = 403.5263

Above results are independent of N_0

If initial CFUs on the blade = 1E+06, then

Fraction transferred to the product during each slice = 4.035263E-04

Fraction transferred to surroundings during each slice = .145422

Fitted equations (all equivalent) are:

1) ln CFU(s) = -.1576198 *s + 6.157861

2) CFU(s) = 472.4167 * .8541745^s

3) CFU(s) = 472.4167 * e^(-.1576198 *s)

4) CFU(s) = 472.4167 * 10^(-6.845341E-02 *s)

Correlation coefficient for fit is R^2 = .9410404

Table 6.1. Model predicted fraction of transfer of *Listeria monocytogenes* from delicatessen slicers to delicatessen meat (f_1) and environment (f_2) by product, biofilm forming ability, injury, and incubation time on stainless steel blade

Product	Biofilm forming ability	Type of Injury	Time (h)	f_1	f_2	R^2
Turkey	Weak	Cold	6	2.51×10^{-3}	0.18	0.93
			24	1.42×10^{-4}	0.15	0.70
		Chlorine	6	4.45×10^{-4}	0.21	0.74
			24	1.85×10^{-4}	0.24	0.74
		Uninjured	6	1.68×10^{-4}	0.15	0.83
			24	3.63×10^{-6}	0.16	0.47
	Strong	Cold	6	2.37×10^{-3}	0.07	0.47
			24	1.66×10^{-3}	0.15	0.85
		Chlorine	6	9.43×10^{-3}	0.19	0.95
			24	7.49×10^{-5}	0.11	0.66
		Uninjured	6	1.57×10^{-3}	0.15	0.83
			24	2.68×10^{-4}	0.28	0.84
Salami	Weak	Cold	6	4.78×10^{-4}	0.08	0.90
			24	2.67×10^{-5}	0.04	0.53
		Chlorine	6	2.11×10^{-5}	0.08	0.47
			24	3.24×10^{-6}	0.09	0.26
		Uninjured	6	1.92×10^{-4}	0.14	0.65
			24	3.00×10^{-4}	0.24	0.58
	Strong	Cold	6	2.37×10^{-4}	0.05	0.69
			24	3.07×10^{-5}	0.02	0.11
		Chlorine	6	4.60×10^{-5}	0.04	0.43
			24	1.48×10^{-5}	0.18	0.49
		Uninjured	6	5.34×10^{-4}	0.12	0.87
			24	1.71×10^{-4}	0.13	0.82

Figure 6.6. Plotted output using GWBasic for assessing *L. monocytogenes* transfer from an inoculated slicer blade (6 log CFU/blade) to salami

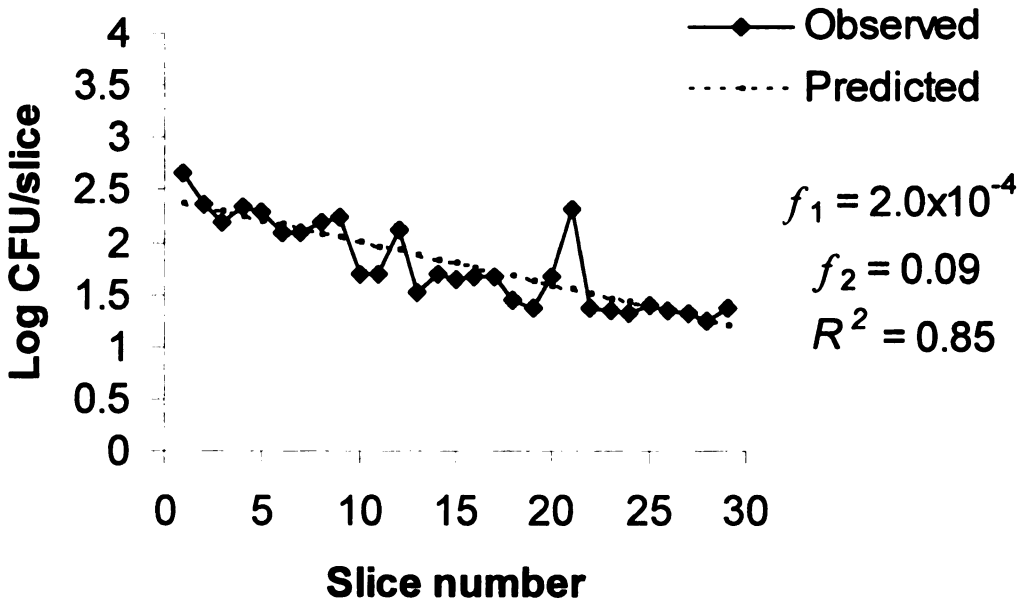


Figure 6.7. Plotted output using GWBasic for assessing *L. monocytogenes* transfer from an inoculated slicer blade (6 log CFU/blade) to turkey

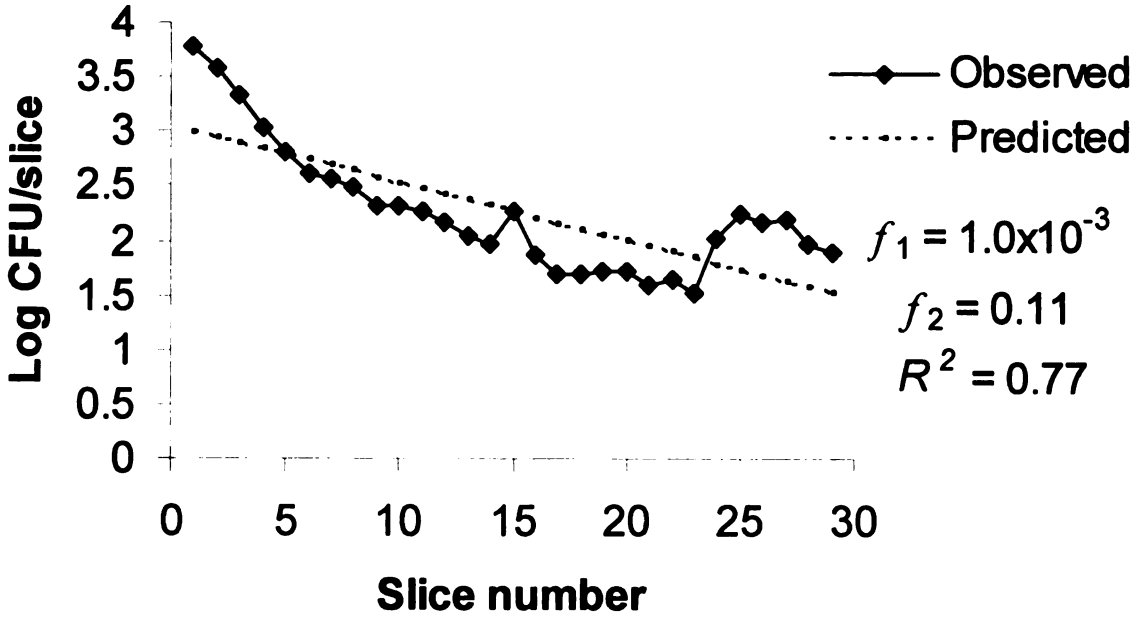


Figure 6.8. Plotted output using GWBasic for assessing transfer of strong biofilm-forming *L. monocytogenes* from an inoculated slicer blade (6 log CFU/blade) to turkey and salami

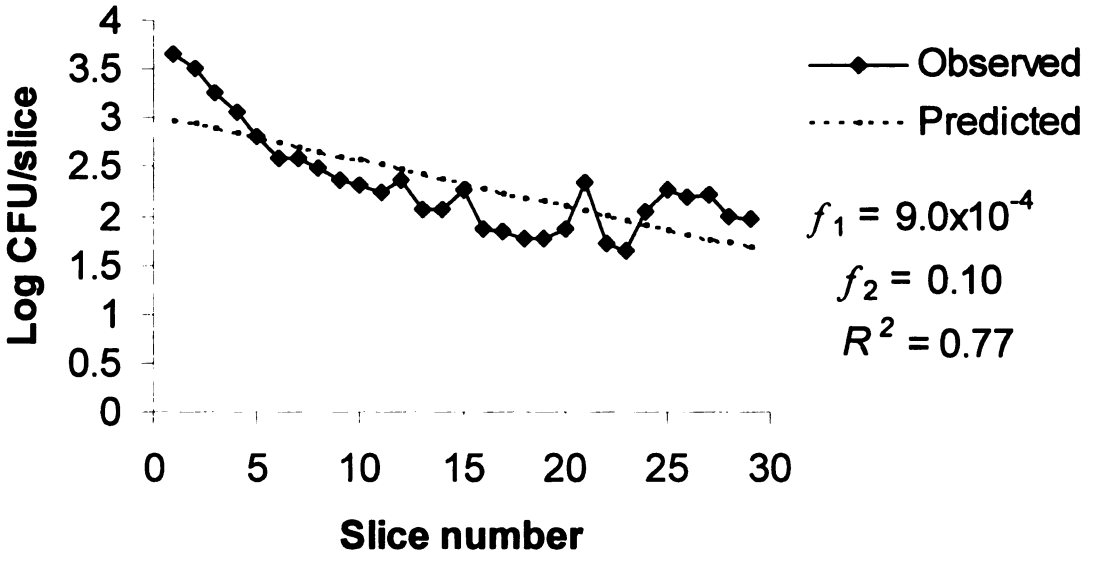


Figure 6.9. Plotted output using GWBasic for assessing transfer of weak biofilm-forming *L. monocytogenes* from an inoculated slicer blade (6 log CFU/blade) to turkey and salami

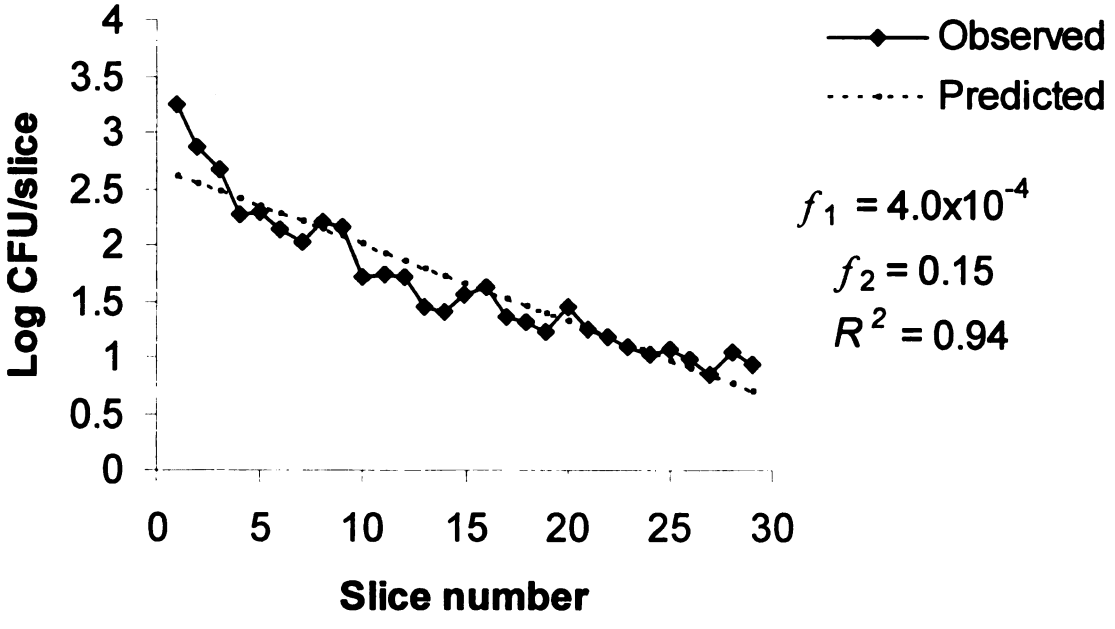


Figure 6.10. Plotted output using GWBasic for assessing transfer of uninjured *L. monocytogenes* from an inoculated slicer blade (6 log CFU/blade) to turkey and salami

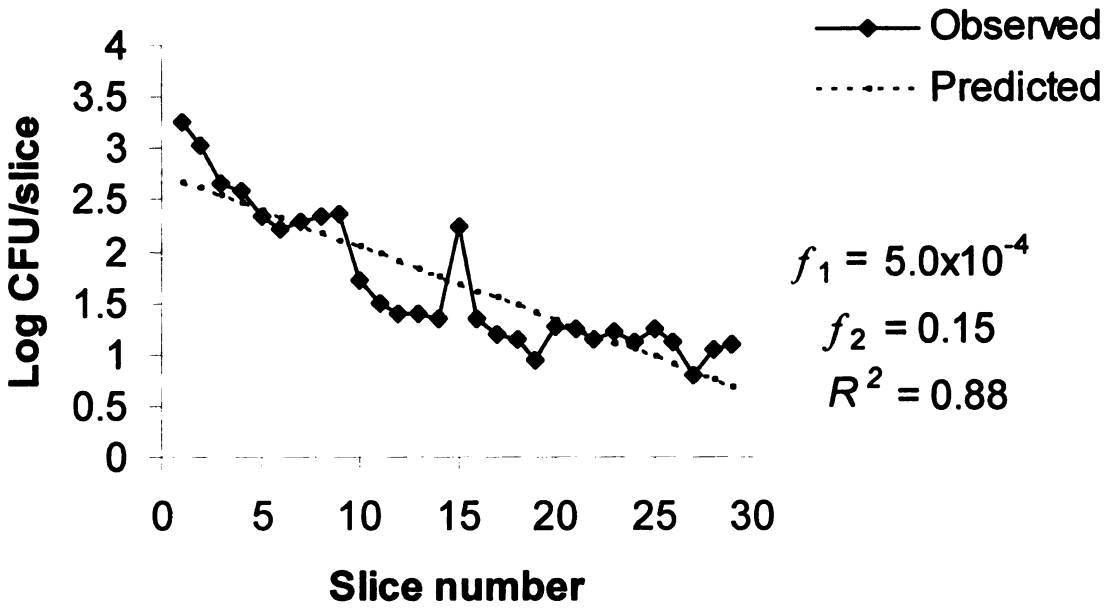


Figure 6.11. Plotted output using GWBasic for assessing transfer of cold-injured *L. monocytogenes* from an inoculated slicer blade (6 log CFU/blade) to turkey and salami

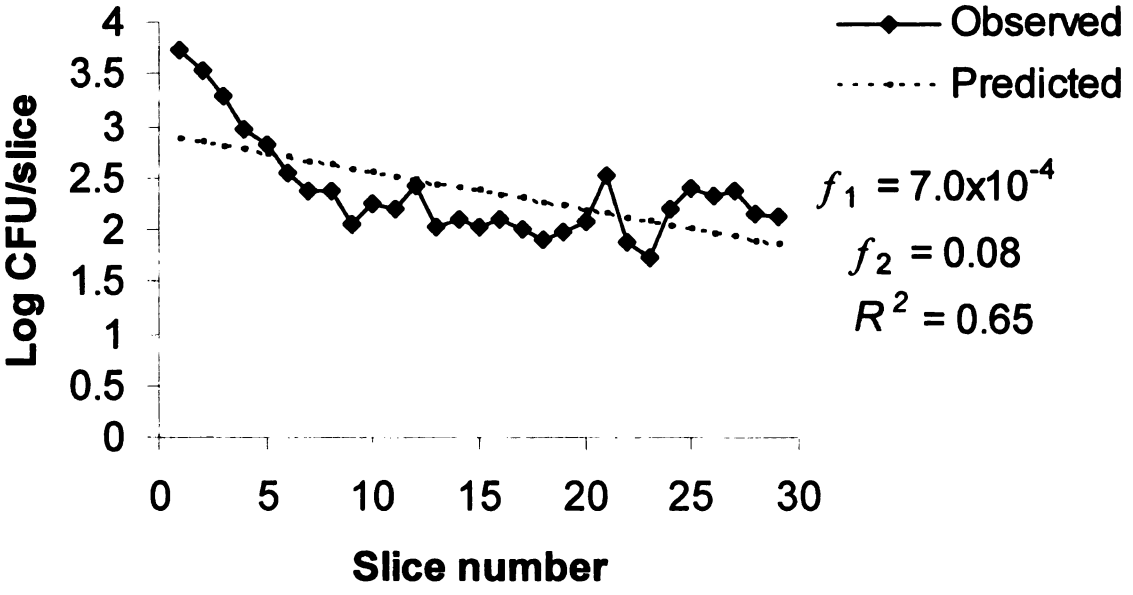


Figure 6.12. Plotted output using GWBasic for assessing transfer of *L. monocytogenes* from an inoculated slicer blade (6 log CFU/blade; 6 h incubation, 22°C/78% RH) to turkey and salami

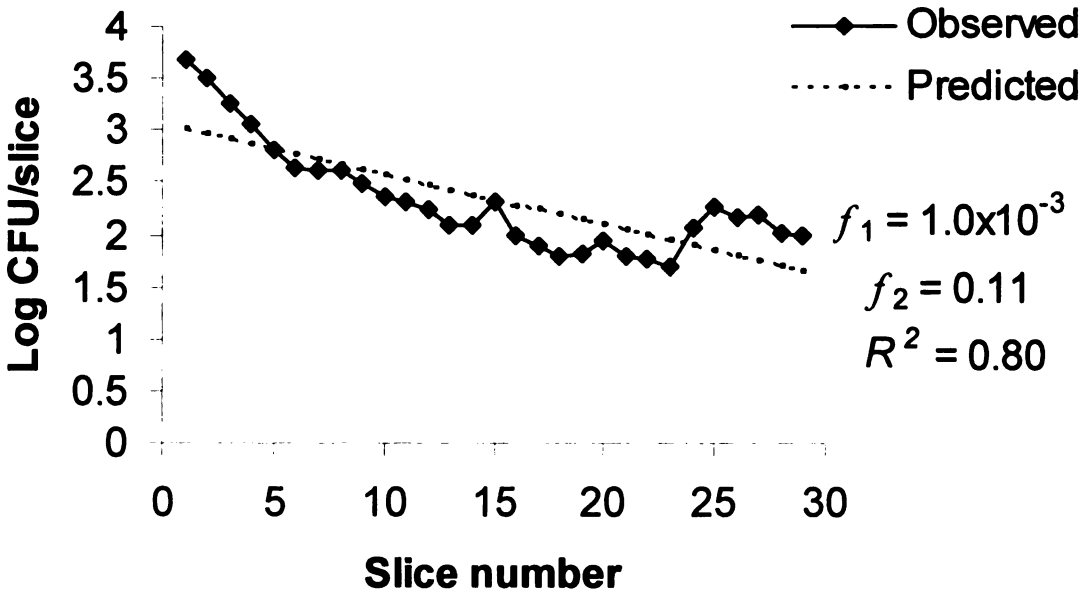
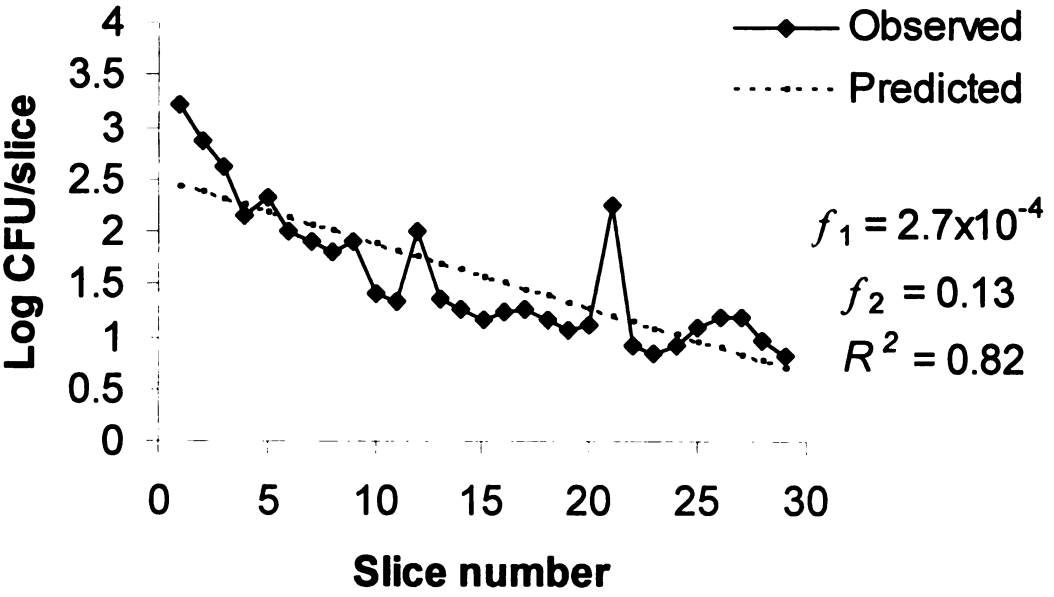


Figure 6.13. Plotted output using GWBasic for assessing transfer of *L. monocytogenes* from an inoculated slicer blade (6 log CFU/blade; 24 h incubation, 22°C/78% RH) to turkey and salami



6.5 DISCUSSION

Predictive modeling of microbial pathogens during food production and storage has been approached using various mathematical models and methods, including empirical modeling or mechanistic mathematical translation of various factors including attachment properties and metabolic functions (Bernaerts et al., 2004). To date, Vorst (2005b) has developed the only model to predict *L. monocytogenes* transfer during slicing of RTE delicatessen meats on a commercial delicatessen slicer. The model predicts the number of CFU transferred to any given slice, as well as the number CFU lost to the environment through aerosols and bacterial death. Assumptions made in this model were: a) the number of *Listeria* transferred (CFU) to any particular slice is a fraction "f_1" of the number of *Listeria* on the blade just before each slice, b) the number of *Listeria* transferred to the surrounding areas during the slicing of each slice is a different fraction, "f_2" of the number of *Listeria* on the blade just before slicing, and c) "N_0," the number of *Listeria* cells on the blade that are available for transfer before any slicing begins is known. The fractions "f_1" and "f_2" are expected to be constant because the degree of adhesion between *Listeria* and the blade/meat surface stabilizes after the first slice. Under the conditions tested by Vorst (2005b), this model had a correlation coefficient varying from $R^2 = 0.40$ when slicing salami, to over 0.90 when slicing turkey or bologna with a slicer blade inoculated at 8 log CFU/blade (Vorst, 2005b).

In this study, which tested the validity of the aforementioned model, a better correlation coefficient was obtained for transfer to salami due to the larger number of salami replicates resulting in an increased number of data points. Salami seems to represent a different transfer scenario than turkey or bologna, due to being a high-fat,

low-moisture product. Lin et al. (2006) conducted a study in which the blade of a commercial-scale meat slicer used to slice roast turkey breast, salami and bologna was inoculated to contain *L. monocytogenes* at levels of 1, 2, or 3 log CFU/blade (1 and 2 log CFU/blade inoculum used with turkey only). In their study, more equipment samples were positive for *L. monocytogenes* after slicing salami (8 samples) than turkey (3 samples) or bologna (1 sample), which supports a longer residence time for *L. monocytogenes* on fat-coated slicers as suggested by Vorst et al. (2006; Lin et al., 2006). Although fat caused *Listeria* to remain on the surface longer when slicing salami, the model was accurate when more replicates were analyzed.

Furthermore, the model was fairly accurate in predicting transfer under all of the tested conditions ($0.77 \leq R^2 \leq 0.94$), despite the fact that the transfer data for all of the modeled conditions were significantly different (Chapter 5). The only exception was for the prediction of previously cold-injured cells ($R^2 = 0.65$), in which the predicted values showed a greater deviation from the observed values. In all scenarios modeled, the transfer of *L. monocytogenes* to the surroundings (f_2), was much greater than the amount of *L. monocytogenes* transferred to the slices of meat (f_1, Table 6.1). This indicates that there is a risk of *L. monocytogenes* remaining in the delicatessen environment on surfaces and equipment surrounding the slicer.

The model had previously been used to predict transfer of *L. monocytogenes* from a slicer blade inoculated at levels of 3, 5 and 8 log CFU/blade (Vorst, 2005b). However, in this study, the slicer blade was inoculated at 6 log CFU/blade, and *L. monocytogenes* was desiccated to the stainless steel surface for either 6 or 24 h prior to slicing turkey or salami. Despite these additional variables that greatly affected the numbers of *L.*

monocytogenes that remained viable and able to be transferred, the model was still remarkably accurate.

Empirical (curve fitting) models predict populations based on previously obtained experimental data, which offers more accurately predicted environmental populations. However, many empirical models based on empirical data do not account for underlying factors that influence the results and in some cases may be dependent on specific environmental or laboratory conditions.

This work confirms the findings by Vorst et al. (2005) that the greatest number of *Listeria* (> 90%) will be found in the first 15 slices of delicatessen meats after mechanical or knife slicing. Despite the Vorst (2005b) model being an empirical model, it appears to be accurate for certain underlying microbiological mechanisms that may affect survival (cold-injury and desiccation over time) and may affect attachment and persistence on surfaces (biofilm forming ability). However, in order to be truly applicable to real-world situations, the model needs to be improved and refined to predict low-level *Listeria* transfer, as would be expected with more realistic inoculum levels (< 3 log CFU/blade), especially in a long-term continuous transfer scenario, such as one would expect when slicing salami.

CONCLUSIONS AND FUTURE RECOMMENDATIONS

Cross-contamination of RTE meats by *Listeria monocytogenes* poses a health risk to the public, and a safety and financial concern to food processors and food retail establishments. Results from this research demonstrate that *L. monocytogenes* can survive under desiccated conditions on a delicatessen slicer or knives for up to 24 h and contaminate product sliced during this time period. Prior injury of *L. monocytogenes* due to exposure to refrigeration temperatures or chlorine does not significantly inhibit this transfer, and in the case of exposure to cold, can actually enhance subsequent transfer to delicatessen meats.

The first objective of this research, to assess the biofilm formation by *L. monocytogenes*, demonstrated that considerable variation exists in the ability of different *L. monocytogenes* isolates to form biofilms. Differences were also observed in the biofilm forming ability of strains of different serotypes and isolates from different sources. Food and environmental isolates were significantly better at forming biofilms than clinical and veterinary isolates. Lineage I strains were significantly better at forming biofilms than Lineage III strains. Significant differences were also observed based on serotype, with serotype 1/2b forming significantly stronger biofilms than serotypes 1/2a and 4b. Further research needs to be conducted as to the genetic traits of strong biofilm forming strains of *L. monocytogenes*, to determine whether this is an evolutionary advantage and how it may relate to virulence of strains.

The second objective of this research, to evaluate biofilm formation on polystyrene by selected strains of *L. monocytogenes* following cold injury, cold

starvation, acid injury and chlorine injury, demonstrated that certain types of injury (cold injury and cold starvation) enhance biofilm formation as compared to uninjured cells. However, oxidative injuries (acid injury and chlorine injury) inhibit subsequent biofilm formation. Further research is required to better understand the mechanism of injury induced by exposure to chlorine, and any subsequent effects it may have on cell physiology. In addition, the nature of *L. monocytogenes* cell surface hydrophobicity and the role that injury may play in altering this hydrophobicity needs to be determined along with the potential of attachment and subsequent biofilm formation on surfaces. Current methods yield widely variable and contradictory results with new methods for the determination of cell surface characteristics needed if the role of attachment in biofilm formation is ever to be truly understood.

The third and fourth objectives of this research were quite similar, in that they both required the determination of sequential transfer of *L. monocytogenes* from stainless steel knives or slicer blades to product sliced with contaminated blades. Overall, biofilm-forming ability had a significant effect on *L. monocytogenes* transfer, with strong biofilm forming strains transferring to products in higher numbers than weak biofilm formers. Additionally, cold-injured cells of *L. monocytogenes* transferred in higher numbers to delicatessen meats than uninjured or chlorine-injured strains. In both cases, enhanced survival of strong biofilm formers and cold-injured listeriae were thought to explain the higher levels of transfer observed. However, as in the second objective, cell surface hydrophobicity and its effect on interactions with stainless steel may play a role in enhancing or inhibiting bacterial transfer to products. Higher numbers of *L. monocytogenes* transferred to delicatessen meats after 6 h of desiccation, however,

transfer still occurred after 24 h, thus raising concern that even under unfavorable environmental conditions, *L. monocytogenes* can survive in environmental niches to later contaminate products. Product composition had a significant effect on transfer, with higher numbers of listeriae transferred to turkey than to salami. Fat from the salami seemed to retain *L. monocytogenes* to the slicer surface, and further research into the protective effect of fat on localization and survival of listeriae on stainless steel surfaces under desiccated conditions may provide insight into specific risks associated with cross-contamination in the processing and handling of products with different fat and moisture contents.

The final objective of this research was to validate a model developed by Vorst et al. (2005) to predict *L. monocytogenes* transfer during slicing of delicatessen meats. The model performed well, with correlation coefficients ranging from 0.65 – 0.94. However, the model has only been tested and shown to perform accurately when used to predict the transfer of unreasonably high inoculum levels (10^3 CFU/blade and greater). Research has shown that contaminated delicatessen meats generally contain < 1 CFU/g of *L. monocytogenes* (Gombas et al., 2003). New methods must be developed to detect and quantify low levels of *L. monocytogenes* in order to determine whether the models developed can provide accurate predictions for transfer of *L. monocytogenes* under these more realistic levels. Alternatively, it may be possible to compensate for the difficulty of quantifying low levels of *L. monocytogenes* by pooling samples and replicating experiments a numerous times to obtain enough data to generate reasonably accurate transfer curves.

Overall, this research shows that food processors and retailers need to be aware that *L. monocytogenes* is able to survive unfavorable conditions and persist in the environment. Biofilm formation can be used as a predictor for survival of *L. monocytogenes* in the environment with weak biofilm-producing strains being most vulnerable to environmental stress. Many processors are aware and concerned about issues with biofilms, but this research shows that sites associated with biofilm development are not the only sites that could be colonized by persistent strains of *L. monocytogenes*—these strong biofilm forming strains can still survive longer than their weak biofilm forming counterparts when present in niches that will not support biofilm development. Furthermore, the practice of storing delicatessen slicers at refrigerator temperatures overnight to prevent the development of unpleasant odors may be indicative of inadequate sanitation of delicatessen slicers. The odor-causing product residues may harbor *L. monocytogenes*, and the exposure to lower temperatures may enhance *L. monocytogenes* survival and subsequent transfer to product. Equipment designed to facilitate cleaning and sanitizing is vital in combating persistence of *L. monocytogenes* in the environment, and adequate and frequent sanitation is the only certain means to reduce *L. monocytogenes* post-processing contamination. Given the risk of *L. monocytogenes* transfer to a variety of products from delicatessen slicers, it may be prudent for operators of delicatessens to only use products obtained from facilities operating under UDSA *L. monocytogenes* control strategies Alternative 1 and Alternative 2, since the measures in both of these alternatives are more stringent than Alternative 3.

APPENDIX I

KNIFE TRANSFER DATA

Table A1.1. *Listeria monocytogenes* transfer from knife blades to turkey (6 log CFU/blade)

slice	biofilm forming ability	CFU per slice	time (h)	rep
1	strong	112424	6	1
2	strong	152396.16	6	1
3	strong	93089.28	6	1
4	strong	28072	6	1
5	strong	12506.56	6	1
6	strong	3946.16	6	1
7	strong	3898.2	6	1
8	strong	894	6	1
9	strong	11236.96	6	1
10	strong	5530.8	6	1
11	strong	281.32	6	1
12	strong	210.4	6	1
13	strong	174.8	6	1
14	strong	153.92	6	1
15	strong	103.8	6	1
16	strong	444.72	6	1
1	strong	116724	6	2
2	strong	9430	6	2
3	strong	74358	6	2
4	strong	8301.48	6	2
5	strong	2311.96	6	2
6	strong	953.16	6	2
7	strong	1005.72	6	2
8	strong	162.4	6	2
9	strong	4188.8	6	2
10	strong	351.12	6	2
11	strong	19.96	6	2
12	strong	154.56	6	2
13	strong	813.6	6	2
14	strong	599.2	6	2
15	strong	5110.56	6	2
16	strong	451.36	6	2
1	strong	163254	6	3
2	strong	29760	6	3
3	strong	4985.76	6	3
4	strong	3528.32	6	3
5	strong	376	6	3
6	strong	367.2	6	3
7	strong	3154.56	6	3
8	strong	870.24	6	3
9	strong	0	6	3
10	strong	131.6	6	3
11	strong	1438.4	6	3
12	strong	39.12	6	3

Table A1.1. (Cont'd)

13	strong	224.4	6	3
14	strong	225.12	6	3
15	strong	131.6	6	3
16	strong	5953.76	6	3
1	strong	35616	24	1
2	strong	59462.4	24	1
3	strong	1468.8	24	1
4	strong	37712.64	24	1
5	strong	13319.6	24	1
6	strong	3337.32	24	1
7	strong	3030.2	24	1
8	strong	4919.2	24	1
9	strong	424.2	24	1
10	strong	154	24	1
11	strong	46.8	24	1
12	strong	50.16	24	1
13	strong	153.6	24	1
14	strong	54.64	24	1
15	strong	23.24	24	1
16	strong	36.4	24	1
1	strong	16944	24	2
2	strong	11340	24	2
3	strong	1431.36	24	2
4	strong	2592.8	24	2
5	strong	1718.72	24	2
6	strong	989.4	24	2
7	strong	83.04	24	2
8	strong	851.44	24	2
9	strong	16.88	24	2
10	strong	35.44	24	2
11	strong	22.68	24	2
12	strong	21.12	24	2
13	strong	159.36	24	2
14	strong	19.68	24	2
15	strong	0	24	2
16	strong	41.84	24	2
1	strong	1407.44	24	3
2	strong	1350.72	24	3
3	strong	365.76	24	3
4	strong	169.2	24	3
5	strong	0	24	3
6	strong	0	24	3
7	strong	57.24	24	3
8	strong	0	24	3
9	strong	0	24	3
10	strong	17.76	24	3
11	strong	17.28	24	3
12	strong	22	24	3

Table A1.1. (Cont'd)

13	strong	190.4	24	3
14	strong	65.04	24	3
15	strong	20.48	24	3
16	strong	24.52	24	3
1	weak	78820	6	1
2	weak	6725.92	6	1
3	weak	4380.8	6	1
4	weak	1747.2	6	1
5	weak	564.96	6	1
6	weak	250.08	6	1
7	weak	104	6	1
8	weak	46.72	6	1
9	weak	0	6	1
10	weak	0	6	1
11	weak	20.6	6	1
12	weak	0	6	1
13	weak	0	6	1
14	weak	15.84	6	1
15	weak	17.2	6	1
16	weak	15.12	6	1
1	weak	12620	6	2
2	weak	2646.68	6	2
3	weak	2125.92	6	2
4	weak	623.2	6	2
5	weak	313.04	6	2
6	weak	376.2	6	2
7	weak	145.6	6	2
8	weak	88.16	6	2
9	weak	148.68	6	2
10	weak	70.8	6	2
11	weak	40.8	6	2
12	weak	20.4	6	2
13	weak	74.4	6	2
14	weak	154.56	6	2
15	weak	17.44	6	2
16	weak	14.96	6	2
1	weak	145632	6	3
2	weak	9525.6	6	3
3	weak	2461.76	6	3
4	weak	6369.56	6	3
5	weak	2311.92	6	3
6	weak	1362.4	6	3
7	weak	3056.4	6	3
8	weak	1531.6	6	3
9	weak	1364.56	6	3
10	weak	186.48	6	3
11	weak	78.8	6	3
12	weak	342.68	6	3

Table A1.1. (Cont'd)

13	weak	22.92	6	3
14	weak	25.84	6	3
15	weak	25.6	6	3
16	weak	22.88	6	3
1	weak	14800	24	1
2	weak	2880	24	1
3	weak	2427.8	24	1
4	weak	3605.12	24	1
5	weak	1444	24	1
6	weak	1009.8	24	1
7	weak	1167	24	1
8	weak	1065	24	1
9	weak	712.92	24	1
10	weak	1320	24	1
11	weak	369.6	24	1
12	weak	223.2	24	1
13	weak	13.48	24	1
14	weak	98.84	24	1
15	weak	214.4	24	1
16	weak	35.52	24	1
1	weak	224.64	24	2
2	weak	1313.2	24	2
3	weak	21.2	24	2
4	weak	22.4	24	2
5	weak	22.08	24	2
6	weak	0	24	2
7	weak	0	24	2
8	weak	0	24	2
9	weak	0	24	2
10	weak	0	24	2
11	weak	52.56	24	2
12	weak	27.44	24	2
13	weak	52	24	2
14	weak	0	24	2
15	weak	61.68	24	2
16	weak	56.76	24	2
1	weak	27260	24	3
2	weak	1784.88	24	3
3	weak	896	24	3
4	weak	1940	24	3
5	weak	1363.2	24	3
6	weak	178.56	24	3
7	weak	259.2	24	3
8	weak	22.28	24	3
9	weak	125.28	24	3
10	weak	518.4	24	3
11	weak	37.36	24	3
12	weak	187.2	24	3

Table A1.1. (Cont'd)

13	weak	100.32	24	3
14	weak	20.72	24	3
15	weak	20.16	24	3
16	weak	18.6	24	3

Table A1.2. *Listeria monocytogenes* transfer from knife blades to turkey (8 log CFU/blade)

slice	biofilm forming ability	CFU per slice	time (h)	rep
1	strong	22070160	6	1
2	strong	2560000	6	1
3	strong	611900.8	6	1
4	strong	509081.076	6	1
5	strong	1247129.6	6	1
6	strong	1113763.2	6	1
7	strong	13416	6	1
8	strong	35046	6	1
9	strong	78336	6	1
10	strong	194392.8	6	1
11	strong	27121.6	6	1
12	strong	161.2	6	1
13	strong	11520	6	1
14	strong	1729.2	6	1
15	strong	3984.8	6	1
16	strong	7387.6	6	1
1	strong	21517200	6	2
2	strong	15916080	6	2
3	strong	5940933.12	6	2
4	strong	404088	6	2
5	strong	207452.8	6	2
6	strong	644504	6	2
7	strong	356011.2	6	2
8	strong	102810	6	2
9	strong	70676.8	6	2
10	strong	495232	6	2
11	strong	85248	6	2
12	strong	56358.4	6	2
13	strong	148238.4	6	2
14	strong	4727.2	6	2
15	strong	4658.8	6	2
16	strong	207721.6	6	2
1	strong	3973320	6	3
2	strong	5529600	6	3
3	strong	1820145.6	6	3
4	strong	152493.6	6	3
5	strong	748417.6	6	3
6	strong	2596800	6	3
7	strong	141198	6	3
8	strong	15690	6	3
9	strong	10416	6	3
10	strong	3338.8	6	3
11	strong	777.6	6	3
12	strong	182.4	6	3
13	strong	217.2	6	3
14	strong	432	6	3

A1.2. (Cont'd)

15	strong	1468.8	6	3
16	strong	1302	6	3
1	strong	716352	24	1
2	strong	20736	24	1
3	strong	12672	24	1
4	strong	22924	24	1
5	strong	2270.4	24	1
6	strong	194	24	1
7	strong	1346.4	24	1
8	strong	269.2	24	1
9	strong	323.6	24	1
10	strong	336	24	1
11	strong	212.8	24	1
12	strong	179.6	24	1
13	strong	234	24	1
14	strong	249.6	24	1
15	strong	236.4	24	1
16	strong	205.6	24	1
1	strong	31524800	24	2
2	strong	1059100	24	2
3	strong	167411.2	24	2
4	strong	59889.2	24	2
5	strong	121082.4	24	2
6	strong	8377.6	24	2
7	strong	54810.4	24	2
8	strong	1067.2	24	2
9	strong	4826	24	2
10	strong	560.8	24	2
11	strong	2224.8	24	2
12	strong	546.4	24	2
13	strong	243.6	24	2
14	strong	450.4	24	2
15	strong	6260	24	2
16	strong	7134	24	2
1	strong	10438.884	24	3
2	strong	160	24	3
3	strong	176.8	24	3
4	strong	440	24	3
5	strong	202.8	24	3
6	strong	204.4	24	3
7	strong	191.6	24	3
8	strong	189.2	24	3
9	strong	177.2	24	3
10	strong	164.8	24	3
11	strong	245.6	24	3
12	strong	176.8	24	3
13	strong	181.2	24	3
14	strong	0	24	3

Table A1.2. (Cont'd)

15	strong	0	24	3
16	strong	0	24	3
1	weak	2138704.488	6	1
2	weak	215654.796	6	1
3	weak	4716	6	1
4	weak	100805.76	6	1
5	weak	3612	6	1
6	weak	82820	6	1
7	weak	4552.4	6	1
8	weak	18792	6	1
9	weak	204.4	6	1
10	weak	1170	6	1
11	weak		6	1
12	weak	73008	6	1
13	weak		6	1
14	weak	203.6	6	1
15	weak		6	1
16	weak	140697.2	6	1
1	weak	4062024.576	6	2
2	weak	1016966.016	6	2
3	weak	152191.656	6	2
4	weak	71870.4	6	2
5	weak	74157.6	6	2
6	weak	11037.6	6	2
7	weak	167.2	6	2
8	weak	4084.8	6	2
9	weak	383.2	6	2
10	weak	1422	6	2
11	weak		6	2
12	weak	770.4	6	2
13	weak		6	2
14	weak	2851.2	6	2
15	weak		6	2
16	weak	231.2	6	2
1	weak	4277456.928	6	3
2	weak	818070.4	6	3
3	weak	753347.2	6	3
4	weak	114646	6	3
5	weak	97006	6	3
6	weak	18422.8	6	3
7	weak	32126.4	6	3
8	weak	21708	6	3
9	weak	7000	6	3
10	weak	2129.6	6	3
11	weak	63369.6	6	3
12	weak	1502300.8	6	3
13	weak	103329.2	6	3
14	weak	342092.8	6	3

Table A1.2. (Cont'd)

15	weak	18701.6	6	3
16	weak	27434.4	6	3
1	weak	38169.792	24	1
2	weak	14728.8	24	1
3	weak	2102.4	24	1
4	weak	1865.6	24	1
5	weak	415.2	24	1
6	weak	219.6	24	1
7	weak	9652	24	1
8	weak	305.6	24	1
9	weak	277.2	24	1
10	weak	8481.6	24	1
11	weak		24	1
12	weak	627.6	24	1
13	weak		24	1
14	weak	20908.8	24	1
15	weak		24	1
16	weak	12506.4	24	1
1	weak	90032.544	24	2
2	weak	13270.4	24	2
3	weak	1332	24	2
4	weak	2532	24	2
5	weak	268.8	24	2
6	weak	209.6	24	2
7	weak	230.8	24	2
8	weak	263.6	24	2
9	weak	186.8	24	2
10	weak	253.6	24	2
11	weak	233.2	24	2
12	weak	0	24	2
13	weak	186.4	24	2
14	weak	264.8	24	2
15	weak	192.8	24	2
16	weak	0	24	2
1	weak	209347.776	24	3
2	weak	91566.4	24	3
3	weak	2178	24	3
4	weak	17971.2	24	3
5	weak	2766.4	24	3
6	weak	2467.2	24	3
7	weak	261.6	24	3
8	weak	218.8	24	3
9	weak	228.8	24	3
10	weak	859.2	24	3
11	weak	396.8	24	3
12	weak	366.4	24	3
13	weak	383.2	24	3
14	weak	7488	24	3

Table A1.2. (Cont'd)

15	weak	5588.8	24	3
16	weak	402.4	24	3

APPENDIX II

SLICER TRANSFER DATA

"10" CFU in "Rep" column indicates negative count, positive by enrichment

Table A2.1. *Listeria monocytogenes* transfer from slicer to turkey (strong biofilm former/uninjured/6 h incubation)

Strong, 6 h
Turkey

Slice		Rep 1	Rep 3	Rep 4	Average	Rep 1 Healthy	Rep 3 Healthy	Rep 4 Healthy
	1	10	29.4	61.2	3.35E+01	13.28	0	40.8
	2	1924	7992.32	13299.12	7.74E+03	1027	3166.6	10417.12
	3	1044	2200.08	15755.52	6.33E+03	198.36	1100.04	12946.56
	4	234.36	1371.6	4734.56	2.11E+03	52.92	702	3850.64
	5	65.88	1232.84	1464.12	9.21E+02	14.64	476.56	1195.2
	6	67.68	395.2	789.36	4.17E+02	30.08	156	526.24
	7	62.4	459.2	504	3.42E+02	23.4	324.8	403.2
	8	16.8	1064	386.08	4.89E+02	25.2	560	243.84
	9	48.48	402.56	177.84	2.10E+02	0	152.32	168.48
	10	41.2	159.6	213.36	1.38E+02	8.24	148.2	111.76
	11	32.8	514.8	304.8	2.84E+02	8.2	251.68	274.32
	12	17.04	183.04	203.2	1.34E+02	0	68.64	152.4
	13	10	68.88	71.12	5.00E+01	0	103.32	60.96
	14	10	183.04	111.32	1.01E+02	0	91.52	141.68
	15	25.2	91.84	82.24	6.64E+01	16.8	80.36	20.56
	16	2920.92	57.4	491.2	1.16E+03	2814.88	11.48	417.52
	17	10.2	45.92	135.52	6.39E+01	0	11.48	135.52
	18	10.32	34.44	92.16	4.56E+01	0	0	102.4
	19	10.36	78.96	31.2	4.02E+01	31.08	11.28	72.8
	20	10	11.28	41.12	2.08E+01	10.6	11.28	41.12
	21	10	22.88	227.92	8.69E+01	0	0	145.04
	22	10	116.8	124.8	8.39E+01	10.48	23.36	156
	23	10.52	57.4	61.2	4.30E+01	0	11.48	40.8
	24	10	162.4	62.88	7.84E+01	0	46.4	62.88
	25	10	80.92	62.64	5.12E+01	0	46.24	41.76
	26	10.36	114.4	72.8	6.59E+01	0	0	62.4
	27	20.64	46.08	30.96	3.26E+01	0	23.04	51.6
	28	10.64	22.96	20.32	1.80E+01	0	57.4	50.8
	29	21.52	66.96	50.6	4.64E+01	10.76	22.32	20.24
	30	10	22.08	10.24	1.41E+01	0	0	0
Total CFU transferred		6695.32	17289.28	39673.4		4295.92	7657.8	31954.64
					Rep % Injury	35.836972 69	55.707814 32	19.455756 25

Table A2.2. *Listeria monocytogenes* transfer from slicer to turkey (weak biofilm former/uninjured/6 h incubation)

Weak, 6 h, Turkey Slice		Rep 1	Rep 2	Rep 3	Average	Rep 1 Healthy	Rep 2 Healthy	Rep 3 Healthy
	1	11.3 2	15.2	0	8.84E+00	0	0	0
	2	191. 84	4447. 52	1304. 16	1.98E+03	52.32	2332.44	364.8
	3	48.6 4	796.4 8	696.3 2	5.14E+02	24.32	356.32	152.32
	4	20.6 4	371.4 8	380.1 6	2.57E+02	0	251	52.8
	5	20.7 2	182.8 8	88	9.72E+01	0	50.8	8.8
	6	0	52.4	21.36	2.46E+01	0	20.96	0
	7	10.7 2	96.12	20.96	4.26E+01	0	32.04	0
	8	0	42.4	0	1.41E+01	0	53	9.92
	9	10.8	216.8	10.48	7.94E+01	0	75.88	0
	10	0	107.6	10.52	3.94E+01	0	32.28	0
	11	10	32.28	0	1.41E+01	0	32.28	0
	12	11.2 4	21.44	0	1.09E+01	0	21.44	0
	13	0	53.6	10	2.12E+01	0	0	0
	14	10	21.36	10.8	1.41E+01	0	10.68	0
	15	10	10.56	0	6.85E+00	0	0	0
	16	0	31.8	10	1.39E+01	0	0	0
	17	0	10.52	10	6.84E+00	0	0	0
	18	0	31.32	0	1.04E+01	0	0	0
	19	0	10	0	3.33E+00	0	0	0
	20	0	10	0	3.33E+00	0	10.52	0
	21	0	10.48	0	3.49E+00	0	0	0
	22	0	10.4	0	3.47E+00	0	0	0
	23	0	51	10.84	2.06E+01	0	51	0
	24	0	10	0	3.33E+00	0	0	0
	25	0	10	0	3.33E+00	0	0	0
	26	0	10	10	6.67E+00	0	0	0
	27	0	10	0	3.33E+00	0	0	0
	28	0	10	10	6.67E+00	0	0	0
	29	0	10	0	3.33E+00	0	0	0
	30	0	10	0	3.33E+00	0	0	0
Total CFU transferred		355. 92	6703. 64	2603. 6		76.64	3330.64	588.64
					Rep % Injury	78.467071 25	50.315947 75	77.391304 35

Table A2.3. *Listeria monocytogenes* transfer from slicer to turkey (strong biofilm former/uninjured/24 h incubation)

Strong, 24 h, Turkey								
Slice		Rep 1	Rep 3	Rep 4	Average	Rep 1 Healthy	Rep 3 Healthy	Rep 4 Healthy
	1	0	0	22.8	7.60E+00	0	0	0
	2	1867.92	145.04	286	7.66E+02	984.64	0	549.12
	3	639.84	70	107.2	2.72E+02	309.6	60	96.48
	4	272	10.6	8.2	9.69E+01	104	10.6	24.6
	5	55.44	31.44	0	2.90E+01	55.44	10.48	0
	6	73.8	10.48	0	2.81E+01	16.4	0	0
	7	16.8	10.4	10.12	1.24E+01	25.2	10.4	0
	8	16.72	0	0	5.57E+00	0	0	0
	9	8.12	0	0	2.71E+00	16.24	0	0
	10	16.24	0	0	5.41E+00	8.12	0	0
	11	61.04	0	10	2.37E+01	8.72	0	0
	12	10	0	10	6.67E+00	0	0	0
	13	8.8	0	10	6.27E+00	8.8	0	0
	14	10	0	10.32	6.77E+00	0	0	0
	15	0	0	8.88	2.96E+00	0	0	0
	16	17.36	0	10	9.12E+00	0	0	0
	17	16.96	0	10.2	9.05E+00	8.48	0	0
	18	10	0	0	3.33E+00	0	0	0
	19	10	0	10	6.67E+00	0	0	0
	20	0	0	10	3.33E+00	0	0	10.36
	21	0	0	10.28	3.43E+00	0	0	0
	22	10	0	0	3.33E+00	0	0	0
	23	0	0	0	0.00E+00	8.36	0	0
	24	0	0	0	0.00E+00	0	0	0
	25	0	0	0	0.00E+00	7.8	0	0
	26	0	0	0	0.00E+00	0	0	0
	27	0	0	0	0.00E+00	0	0	0
	28	0	0	0	0.00E+00	0	0	0
	29	0	0	10	3.33E+00	0	0	10.44
	30	0	0	0	0.00E+00	0	0	0
Total CFU transferred		3121.04	277.96	544		1561.8	91.48	691
					Rep % Injury	49.95898803	67.08878975	0

Table A2.4. *Listeria monocytogenes* transfer from slicer to turkey (weak biofilm former/uninjured/24 h incubation)

Weak, 24 h, Turkey Slice		Rep 1	Rep 2	Rep 3	Average	Rep 1 Healthy	Rep 2 Healthy	Rep 3 Healthy
		26.7		23.0				
	1	6	11.76	4	2.05E+01	0	0	15.36
	2	0	91.56	9.16	3.36E+01	0	39.24	9.16
				18.3				
	3	20	34.44	2	2.43E+01	10	0	18.32
	4	0	21.36	0	7.12E+00	0	0	0
	5	0	0	0	0.00E+00	0	0	0
				10.1				
	6	0	0	6	3.39E+00	0	0	0
	7	0	0	0	0.00E+00	0	0	0
	8	0	10	0	3.33E+00	0	0	0
	9	0	10	0	3.33E+00	0	0	0
				10.2				
	10	0	0	8	3.43E+00	0	0	0
	11	0	0	0	0.00E+00	0	0	0
	12	0	10	0	3.33E+00	0	0	0
	13	0	0	0	0.00E+00	0	0	0
	14	0	0	0	0.00E+00	0	0	0
	15	0	10.4	0	3.47E+00	0	0	0
	16	0	0	0	0.00E+00	0	0	0
	17	10	10.8	0	6.93E+00	0	0	0
	18	0	10.96	0	3.65E+00	0	0	0
	19	0	10.88	0	3.63E+00	0	0	0
	20	0	11	0	3.67E+00	0	0	0
	21	0	10	0	3.33E+00	0	0	0
	22	0	0	0	0.00E+00	0	0	0
	23	0	0	0	0.00E+00	0	0	0
	24	0	0	0	0.00E+00	0	0	0
	25	0	10	0	3.33E+00	0	0	0
	26	0	0	0	0.00E+00	0	0	0
	27	0	0	0	0.00E+00	0	0	0
	28	0	0	0	0.00E+00	0	0	0
	29	0	0	0	0.00E+00	0	0	0
	30	0	0	0	0.00E+00	0	0	0
Total CFU		56.7	263.1	70.9				
transferred		6	6	6		10	39.24	42.84
					Rep %	82.381959	85.088919	39.627959
					Injury	13	29	41

Table A2.5. *Listeria monocytogenes* transfer from slicer to turkey (strong biofilm former/cold-injured/6 h incubation)

Strong, 6h, Cold,
Turkey

Slice	Rep 1	Rep 2	Rep 3	Average	Rep 1 Healthy	Rep 2 Healthy	Rep 3 Healthy
1	0	38	0	12.66667	0	26.6	0
2	16450.56	32284.8	11652.48	20129.28	11880.96	26309.28	10357.76
3	11992.16	27953.28	7666.56	15870.67	9717.12	25767.36	4820.64
4	3483.36	18422.4	1581.84	7829.2	2180.64	15061.12	1469.52
5	691.44	13432.32	520.56	4881.44	627.12	13780.8	616.96
6	699.48	7808.24	375.96	2961.227	546.72	6888.64	424.16
7	480	2466.2	245	1063.733	344	2758.8	254.8
8	133.28	2600.64	226.32	986.7467	148.96	2151.36	147.6
9	130.56	3015.36	180.72	1108.88	145.92	2047.68	110.44
10	249.24	572.88	80	300.7067	241.2	616.28	90
11	787.64	630.48	202.4	540.1733	373.52	562.32	101.2
12	563.04	361.2	200	374.7467	408	395.6	180
13	484.8	216	246	315.6	323.2	190.08	108.24
14	444.4	77.76	205.6	242.5867	218.16	86.4	133.64
15	88.88	537.6	204.8	277.0933	113.12	385.28	122.88
16	408	101.76	295.8	268.52	336	144.16	183.6
17	784	221.52	247.68	417.7333	560	178.92	144.48
18	424	140.8	237.36	267.3867	360	132	123.84
19	678.72	86	41.6	268.7733	597.92	103.2	62.4
20	1042.76	42.8	137.28	407.6133	1416.88	34.24	73.92
21	650.72	78.12	53.2	260.68	564.48	43.4	21.28
22	335.4	60.48	231.44	209.1067	288.6	43.2	178.84
23	595.84	68.16	246.72	303.5733	486.08	25.56	205.6
24	572.32	25.32	41.6	213.08	431.2	16.88	10.4
25	3056	77.04	20.72	1051.253	1360	77.04	0
26	4968.72	17.04	30.96	1672.24	5145.6	34.08	0
27	4032	10	51.6	1364.533	1680	25.56	41.28
28	5008	17.04	10.16	1678.4	3072	8.52	0
29	2811.84	10	51.6	957.8133	2763.36	8.68	0
30	2516.52	10	31.2	852.5733	2138.64	16.96	10.4
Total CFU transferred	64563.68	111383.24	25317.16		48469.4	97920	19993.88
				Rep % Injury	24.9277 6124	12.08731 224	21.02637 105

Table A2.6. *Listeria monocytogenes* transfer from slicer to turkey (weak biofilm former/cold-injured/6 h incubation)

Weak, 6h, Cold, Turkey

Slice		Rep 1	Rep 2	Rep 3	Average	Rep 1 Healthy	Rep 2 Healthy	Rep 3 Healthy
	1	34.88	7.56	5.6	16.01333	17.44	0	5.6
	2	1046 6.4	5258. 16	8923.2	8215.92	2724.4	2042.16	6138.08
	3	3948. 16	868.3 2	8256.4 8	4357.653	783.68	393.96	6386.08
	4	1086. 8	95.04	8951.0 4	3377.627	79.04	47.52	3594.24
	5	200	128	2412	913.3333	20	40	1602
	6	257	80.8	2214	850.6	61.68	40.4	1323
	7	210.8 4	72.72	1630.7 2	638.0933	20.08	48.48	716.8
	8	156.6	56.84	519.68	244.3733	20.88	48.72	224
	9	135.7 2	48.72	762.72	315.72	41.76	24.36	390.44
	10	10	33.12	325.44	122.8533	10.56	16.56	207.92
	11	95.4	48.96	526.64	223.6667	10.6	0	463.08
	12	42.72	16.4	866.4	308.5067	32.04	16.4	665.76
	13	43.04	81.6	726.4	283.68	10.76	40.8	553.88
	14	31.8	8.08	479.12	173	0	0	171.76
	15	10	8.2	229	82.4	10.8	0	164.88
	16	82.24	10	334.08	142.1067	10.28	8.24	287.68
	17	21.36	8.28	338.92	122.8533	10.68	0	109.92
	18	21.28	16.64	82.44	40.12	21.28	0	137.4
	19	10	8.44	83.16	33.86667	0	0	83.16
	20	0	8.36	157.08	55.14667	0	0	101.64
	21	10.56	10	503.8	174.7867	0	0	311.44
	22	42.4	0	162.72	68.37333	0	0	117.52
	23	42.72	10	36	29.57333	0	0	18
	24	21.28	10	53.28	28.18667	0	0	44.4
	25	10	8.04	8.96	9	0	0	26.88
	26	10.56	8.24	10	9.6	0	0	0
	27	10	10	26.52	15.50667	0	0	0
	28	10.4	10	54.48	24.96	0	0	18.16
	29	10	10	10	10	10.28	0	9
	30	20.64	10	44.8	25.14667	10.32	0	53.76
Total CFU transferred		1705 2.8	6950. 52	38734. 68		3906.56	2767.6	23926.48
					Rep % Injury	77.091386 75	60.181396 5	38.229824 02

Table A2.7. *Listeria monocytogenes* transfer from slicer to turkey (strong biofilm former/cold-injured/24 h incubation)

Strong, 24h, Cold, Turkey

Slice		Rep 1	Rep 2	Rep 3	Average	Rep 1 Healthy	Rep 2 Healthy	Rep 3 Healthy
	1	10	8.04	10	9.346667	0	0	0
	2	12932.24	19972.8	1770.16	11558.4	9642.64	16293.6	985.36
	3	4351.2	10016.64	2059.72	5475.853	2817.92	8560.32	1166.88
	4	2377.44	6420.48	1222.08	3340	1067.04	4798.64	848.16
	5	157.76	1970.36	439.04	855.72	139.2	2308.88	268.8
	6	419.4	2152.28	110.88	894.16	382.12	1679.56	120.12
	7	158.44	1185.92	199.76	514.7067	83.88	854.56	118.04
	8	75.2	497.04	153.68	241.9733	9.4	427.28	90.4
	9	56.64	371.28	64.12	164.0133	0	176.8	54.96
	10	9.52	142.08	233	128.2	9.52	177.6	93.2
	11	76.48	268.8	95.2	146.8267	19.12	116.48	28.56
	12	28.56	133.8	150.4	104.2533	0	107.04	103.4
	13	9.6	151.64	94.8	85.34667	0	107.04	37.92
	14	9.48	63	344.16	138.88	9.48	54	133.84
	15	10	45.4	375.96	143.7867	0	45.4	144.6
	16	10	172.52	113.28	98.6	0	208.84	66.08
	17	19.12	63	161.16	81.09333	0	18	37.92
	18	10	10	104.72	41.57333	0	46.6	95.2
	19	10	36.8	188	78.26667	0	27.6	56.4
	20	10	36.48	116.16	54.21333	9.64	0	29.04
	21	10	119.08	28.44	52.50667	0	64.12	18.96
	22	9.76	9.2	19.12	12.69333	0	36.8	38.24
	23	19.68	45.4	76.16	47.08	0	18.16	9.52
	24	9.76	9.04	30	16.26667	0	0	0
	25	9.8	10	58.56	26.12	9.8	0	0
	26	184.68	10	29.88	74.85333	97.2	18.16	9.96
	27	286.8	9.08	77.76	124.5467	105.16	9.08	29.16
	28	35.68	9	38.88	27.85333	35.68	18	0
	29	9.48	18.08	77.12	34.89333	9.48	0	57.84
	30	10	10	29.4	16.46667	19.04	0	0
Total CFU transferred		21326.72	43967.16	8471.6		14466.32	36172.56	4642.56
					Rep % Injury	32.168097 11	17.728231 71	45.198545 73

Table A2.8. *Listeria monocytogenes* transfer from slicer to turkey (weak biofilm former/cold-injured/24 h incubation)

Weak, 24h, Cold, Turkey

Slice		Rep 1	Rep 2	Rep 3	Average	Rep 1 Healthy	Rep 2 Healthy	Rep 3 Healthy
	1	10	0	18.16	9.386667	0	0	9.08
	2	425.04	59.04	6316.8	2266.96	151.8	19.68	3033.6
	3	228.48	60.48	2157.84	815.6	76.16	0	1010.88
	4	178.56	9.32	533.32	240.4	69.44	0	238.8
	5	18.4	10	138.24	55.54667	0	0	38.4
	6	27.24	9.32	61.44	32.66667	9.08	0	23.04
	7	18.56	10	15.36	14.64	9.28	0	15.36
	8	9.44	10	38.4	19.28	0	0	30.72
	9	10	9.52	23.28	14.26667	0	0	7.76
	10	10	10	8.04	9.346667	0	0	8.04
	11	9.4	10	10	9.8	0	0	0
	12	10	10	10	10	0	0	0
	13	9.52	10	10	9.84	0	0	0
	14	9.52	10	10	9.84	0	0	0
	15	10	0	16.08	8.693333	0	0	8.04
	16	0	0	10	3.333333	0	0	7.96
	17	9.84	10	10	9.946667	0	0	0
	18	10	0	10	6.666667	0	0	0
	19	0	10	10	6.666667	0	0	8
	20	9.84	0	0	3.28	0	0	0
	21	10	0	10	6.666667	0	0	0
	22	10	10	10	10	0	0	0
	23	10	0	8.16	6.053333	0	0	0
	24	10	0	8.2	6.066667	0	0	8.2
	25	0	0	10	3.333333	0	0	0
	26	10	0	10	6.666667	0	0	16.32
	27	10	10	8.12	9.373333	0	0	8.12
	28	10	10	10	10	0	0	0
	29	19.6	0	10	9.866667	0	0	0
	30	0	0	0	0	0	0	0
Total CFU transferred		1103.44	277.68	9491.44		315.76	19.68	4472.32
					Rep % Injury	71.384035 38	92.912705 27	52.880490 21

Table A2.9. *Listeria monocytogenes* transfer from slicer to turkey (strong biofilm former/chlorine-injured/6 h incubation)

Strong, 6h, Chlorine,
Turkey

Slice		Rep 1	Rep 2	Rep 3	Average	Rep 1 Healthy	Rep 2 Healthy	Rep 3 Healthy
	1	105.8	8.36	17.36	43.84	0	0	2.48
	2	12636.56	5019.68	10794.4	9483.547	382	586.24	3798.64
	3	13672.96	3017.12	7420.56	8036.88	580.16	340.08	1281.84
	4	10480	1738.24	9521.28	7246.507	264	170.24	1176.24
	5	12378.56	264	2444	5028.853	428.24	35.2	142.88
	6	5901.36	450.84	1090.56	2480.92	96.48	26.52	53.76
	7	5635.28	253.12	509.52	2132.64	194.88	36.16	15.44
	8	6215.6	165.6	323.08	2234.76	131.2	27.6	0
	9	5030.24	18.64	188.16	1745.68	135.04	0	15.68
	10	4664	55.92	70.92	1596.947	101.76	18.64	0
	11	3247.84	83.16	122.88	1151.293	84.8	9.24	15.36
	12	3358.08	46.6	126.08	1176.92	59.36	0	15.76
	13	2654.24	27.96	117.6	933.2667	59.36	0	0
	14	1721.44	9.48	63.36	598.0933	8.48	0	0
	15	1305.92	47.6	72.36	475.2933	0	9.52	0
	16	1104.24	47.2	88.88	413.44	0	0	0
	17	291.04	9.44	24.12	108.2	0	0	0
	18	346.04	38.24	15.92	133.4	0	0	0
	19	224.64	28.32	70.92	107.96	8.32	0	0
	20	144.84	18.96	23.88	62.56	0	0	0
	21	25.32	10	55.44	30.25333	0	9.32	7.92
	22	73.8	47.6	56.84	59.41333	8.2	0	0
	23	101.76	58.08	57.12	72.32	8.48	0	8.16
	24	42.4	38.08	48.72	43.06667	0	0	0
	25	50.16	19.12	64.96	44.74667	8.36	0	0
	26	50.4	9.6	586.92	215.64	0	0	112.56
	27	42	19.36	346.08	135.8133	0	0	49.44
	28	25.2	19.6	81.2	42	0	0	0
	29	33.28	10	16.48	19.92	0	0	0
	30	16.64	10	24.12	16.92	0	0	0
Total CFU transferred		91579.64	11589.92	34443.72		2559.12	1268.76	6696.16
					Rep % Injury	97.205579 76	89.052901 14	80.559126 6

Table A2.10. *Listeria monocytogenes* transfer from slicer to turkey (weak biofilm former/chlorine-injured/6 h incubation)

Weak, 6h, Chlorine,
Turkey

Slice		Rep 1	Rep 2	Rep 3	Average	Rep 1 Healthy	Rep 2 Healthy	Rep 3 Healthy
	1	7.24	0	6.48	4.573333	0	0	0
	2	10677.12	759.92	5751.68	5729.573	40.2	392.84	89.44
	3	3044.8	170.56	1524.24	1579.867	8.8	52.48	6.96
	4	616.2	61.2	605.44	427.6133	0	0	0
	5	290.88	13.92	167.04	157.28	0	0	0
	6	152	6.92	113.28	90.73333	0	6.92	0
	7	130.56	0	84	71.52	0	6.96	7
	8	65.28	14.08	49	42.78667	0	0	0
	9	40.8	0	84	41.6	0	0	0
	10	40.8	0	28.96	23.25333	0	0	0
	11	16.16	0	42.24	19.46667	0	0	0
	12	16.16	7.2	27.84	17.06667	0	7.2	0
	13	49.92	0	35.2	28.37333	0	0	0
	14	24.84	0	7.28	10.70667	0	0	0
	15	10	0	7.12	5.706667	0	0	0
	16	42	7.4	0	16.46667	0	0	0
	17	25.32	0	7.16	10.82667	0	0	0
	18	10	7.44	14.32	10.58667	0	0	0
	19	33.92	37.6	0	23.84	0	0	0
	20	8.4	0	7.32	5.24	0	0	0
	21	0	0	0	0	0	0	0
	22	8.44	0	0	2.813333	0	0	0
	23	10	7.4	0	5.8	0	0	0
	24	0	0	14.48	4.826667	0	0	0
	25	0	0	14.88	4.96	0	0	0
	26	0	0	14.8	4.933333	0	0	0
	27	0	0	0	0	0	0	0
	28	0	7.4	0	2.466667	0	0	0
	29	0	0	14.48	4.826667	0	0	0
	30	10	7.4	7.28	8.226667	0	0	0
Total CFU transferred		15330.84	1108.44	8628.52		49	466.4	103.4
					Rep % Injury	99.680382 81	57.922846 52	98.801648 49

Table A2.11. *Listeria monocytogenes* transfer from slicer to turkey (strong biofilm former/chlorine-injured/24 h incubation)

Strong, 24h, Chlorine,
Turkey

Slice		Rep 1	Rep 2	Rep 3	Average	Rep 1 Healthy	Rep 2 Healthy	Rep 3 Healthy
	1	8.48	10	10	9.493333	0	0	0
	2	1902.8	979.04	2400.24	1760.693	0	33.76	759.2
	3	1033.68	350.8	686.72	690.4267	0	0	153.92
	4	335.16	118.8	250.32	234.76	6.84	0	65.56
	5	108.16	67.2	65.16	80.17333	0	0	14.48
	6	55.36	57.68	7.4	40.14667	0	0	7.4
	7	0	8.04	7.48	5.173333	0	0	14.96
	8	13.84	8.2	22.56	14.86667	0	0	7.52
	9	0	8.44	22.56	10.33333	0	0	0
	10	6.96	10	10	8.986667	0	0	0
	11	0	10	7.52	5.84	0	0	7.52
	12	0	10	10	6.666667	0	0	0
	13	0	10	7.56	5.853333	0	0	0
	14	6.84	8.6	10	8.48	0	0	0
	15	0	10	15.28	8.426667	0	0	0
	16	0	10	10	6.666667	0	0	0
	17	0	10	10	6.666667	0	0	0
	18	7	8.64	10	8.546667	0	0	0
	19	7	10	10	9	0	0	0
	20	0	10	10	6.666667	0	0	0
	21	0	10	10	6.666667	0	0	0
	22	0	10	10	6.666667	0	0	0
	23	0	10	15.68	8.56	0	0	0
	24	0	10	7.84	5.946667	0	0	0
	25	0	10	10	6.666667	0	0	0
	26	0	10	10	6.666667	0	0	0
	27	0	10	10	6.666667	0	0	0
	28	0	10	10	6.666667	0	0	0
	29	0	10	10	6.666667	0	0	0
	30	0	10	10	6.666667	0	0	0
Total CFU transferred		3485.28	1815.52	3686.32		6.84	33.76	1030.56
					Rep % Injury	99.80374604	98.14047766	72.04366414

Table A2.12. *Listeria monocytogenes* transfer from slicer to turkey (weak biofilm former/chlorine-injured/24 h incubation)

Weak, 24h, Chlorine,
Turkey

Slice	Rep 1	Rep 2	Rep 3	Average	Rep 1 Healthy	Rep 2 Healthy	Rep 3 Healthy
1	0	0	0	0	0	0	0
2	227.92	26.08	1659.68	637.8933	0	0	0
3	52.16	6.92	353.28	137.4533	0	0	0
4	81.64	10	95.68	62.44	0	0	0
5	6.48	10	59.2	25.22667	0	0	0
6	6.52	10	37.4	17.97333	0	0	7.48
7	46.48	10	15.12	23.86667	0	0	0
8	26.88	10	15.2	17.36	0	0	0
9	26.88	0	7.72	11.53333	0	0	0
10	244.8	10	7.68	87.49333	0	0	0
11	132.24	10	7.68	49.97333	0	0	0
12	48.44	0	0	16.14667	6.92	0	0
13	13.92	0	0	4.64	0	0	0
14	7	10	0	5.666667	0	0	0
15	10	10	0	6.666667	0	0	0
16	10	0	0	3.333333	0	0	0
17	21	10	0	10.33333	0	0	0
18	10	0	0	3.333333	0	0	0
19	10	0	0	3.333333	0	0	0
20	0	0	0	0	0	0	0
21	0	0	0	0	0	0	0
22	0	10	0	3.333333	0	0	0
23	0	0	0	0	0	0	0
24	0	10	0	3.333333	0	0	0
25	0	10	0	3.333333	0	0	0
26	0	0	0	0	0	0	0
27	0	0	0	0	0	0	0
28	0	0	0	0	0	0	0
29	0	0	8.12	2.706667	0	0	0
30	0	10	0	3.333333	0	0	0
Total CFU transferred	982.36	173	2266.76		6.92	0	7.48
				Rep %	99.295573		99.670013
				Injury	92	100	59

Table A2.13. *Listeria monocytogenes* transfer from slicer to salami (strong biofilm former/uninjured/6 h incubation)

Strong, 6 h
Salami

Slice	Rep 1	Rep 2	Rep 3	Average	Rep 1 Healthy	Rep 2 Healthy	Rep 3 Healthy
1	44.28	167.28	78	9.65E+01	11.48	118.08	54.6
2	1555.2	1190.64	1192.32	1.31E+03	1728	1064.8	665.28
3	510.72	716.8	240	4.89E+02	468.16	918.4	201.6
4	220.92	252.56	115.2	1.96E+02	136.76	298.48	38.4
5	1935.68	1906.96	132.72	1.33E+03	231.44	1877.92	94.8
6	257.28	364.8	142.8	2.55E+02	160.8	288	95.2
7	266	190.4	218.04	2.25E+02	42.56	180.88	180.12
8	313.2	197.4	379.2	2.97E+02	108	75.2	208.56
9	97.92	199.92	171.36	1.56E+02	54.4	85.68	95.2
10	419.64	157.08	247.52	2.75E+02	86.08	64.68	285.6
11	21.36	120.12	66.08	6.92E+01	42.72	73.92	28.32
12	10.76	36.96	186.4	7.80E+01	10.76	46.2	111.84
13	84.48	62.44	149.76	9.89E+01	63.36	53.52	84.24
14	73.36	45.6	18.96	4.60E+01	52.4	45.6	18.96
15	116.6	64.12	19.12	6.66E+01	95.4	36.64	28.68
16	302.4	44.4	84.96	1.44E+02	356.4	35.52	66.08
17	53.8	45.2	106.04	6.83E+01	21.52	18.08	28.92
18	84.8	17.76	9.56	3.74E+01	53	44.4	0
19	30.96	61.32	9.52	3.39E+01	41.28	61.32	28.56
20	10.6	17.68	37.76	2.20E+01	21.2	0	0
21	51.6	16.96	18.24	2.89E+01	30.96	8.48	9.12
22	10.48	26.52	45.2	2.74E+01	10.48	17.68	36.16
23	40.16	48	9.56	3.26E+01	20.08	38.4	28.68
24	30.48	28.32	10	2.29E+01	40.64	18.88	0
25	20.48	9.24	9.52	1.31E+01	51.2	9.24	19.04
26	41.6	17.76	77.12	4.55E+01	31.2	8.88	57.84
27	63.12	9.28	66.08	4.62E+01	0	9.28	18.88
28	20.8	10	27.72	1.95E+01	20.8	9.52	0
29	10	18.96	10	1.30E+01	31.44	28.44	18.56
30	51	66.64	38.4	5.20E+01	0	47.6	38.4
Total CFU transferred	6749.68	6111.12	3917.16		4022.52	5583.72	2541.64
				Rep % Injury	40.40428583	8.630169265	35.11523655

Table A2.14. *Listeria monocytogenes* transfer from slicer to salami (weak biofilm former/uninjured/6 h incubation)

Weak, 6h Salami

Slice	Rep 1	Rep 2	Rep 3	Average	Rep 1 Healthy	Rep 2 Healthy	Rep 3 Healthy
1	18.08	33	36.6	2.92E+01	0	0	14.64
2	52.8	116.28	192.56	1.21E+02	21.12	24.48	99.6
3	10.88	255.36	201.76	1.56E+02	10.88	114.24	124.16
4	0	163.68	123.6	9.58E+01	27.72	148.8	65.92
5	9.32	154.4	123	9.56E+01	0	38.6	41
6	36.8	117	123	9.23E+01	27.6	15.6	49.2
7	0	125.44	141.44	8.90E+01	0	39.2	24.96
8	54.48	175.12	158.08	1.29E+02	9.08	63.68	49.92
9	1874.56	358.2	198.72	8.10E+02	18.56	71.64	57.96
10	1474.76	485.56	217.36	7.26E+02	0	119.4	58.52
11	10	8	10	9.33E+00	9.24	0	0
12	9	0	10	6.33E+00	0	8	0
13	8.44	8.08	10	8.84E+00	0	0	0
14	10	8.12	10	9.37E+00	0	0	0
15	4.56	10	10	8.19E+00	4.56	0	0
16	10	16.16	10	1.21E+01	0	0	0
17	0	10	8.48	6.16E+00	0	16.32	0
18	10	10	16.8	1.23E+01	0	0	0
19	10	16.4	8.44	1.16E+01	0	0	8.44
20	8.72	10	10	9.57E+00	0	0	0
21	10	16.4	8.4	1.16E+01	0	0	0
22	10	8.08	10	9.36E+00	0	0	0
23	0	8.04	10	6.01E+00	0	0	0
24	10	8.12	10	9.37E+00	0	0	8.32
25	10	40.6	10	2.02E+01	0	0	0
26	10	10	8.16	9.39E+00	0	0	0
27	0	16.08	0	5.36E+00	0	0	0
28	0	0	0	0.00E+00	0	0	0
29	10	8.12	24.84	1.43E+01	0	0	0
30	10	16.08	16.56	1.42E+01	8.48	8.04	0
Total CFU transferred	3682.4	2212.32	1717.8		137.24	668	602.64
				Rep % Injury	96.27308277	69.8054531	64.91791827

Table A2.15. *Listeria monocytogenes* transfer from slicer to salami (strong biofilm former/uninjured/24 h incubation)

Strong, 24h
Salami

Slice	Rep 1	Rep 2	Rep 3	Average	Rep 1 Healthy	Rep 2 Healthy	Rep 3 Healthy
1	460	34.76	60.48	1.85E+02	441.6	18.96	20.16
2	132.6	1796.76	445.76	7.92E+02	70.2	1689.12	382.08
3	118.8	430.08	73.08	2.07E+02	129.6	174.08	52.2
4	75.6	313.72	84.8	1.58E+02	54	445.28	0
5	71.96	199.2	105.16	1.25E+02	71.96	139.44	9.56
6	119.52	58.08	684.44	2.87E+02	49.8	19.36	38.56
7	206.64	19.04	10	7.86E+01	78.72	19.04	0
8	88.56	66.64	19.6	5.83E+01	59.04	19.04	9.8
9	98.4	67.2	0	5.52E+01	68.88	57.6	0
10	354.24	124.28	10	1.63E+02	354.24	38.24	0
11	9.68	8.36	10	9.35E+00	0	0	0
12	10	8.72	10	9.57E+00	0	8.72	9.6
13	19.44	6.16	10	1.19E+01	38.88	3.08	0
14	10	10	19.12	1.30E+01	9.72	0	0
15	10	10	38.24	1.94E+01	0	0	28.68
16	0	10	10	6.67E+00	19.44	9.48	18.64
17	10	9.64	10	9.88E+00	0	0	0
18	28.8	0	9.04	1.26E+01	0	0	45.2
19	18.4	0	8.52	8.97E+00	9.2	0	8.52
20	10	0	10	6.67E+00	0	0	0
21	10	10	10	1.00E+01	0	0	0
22	10	0	10	6.67E+00	0	0	0
23	10	0	10	6.67E+00	0	0	9.6
24	10	36.48	10	1.88E+01	0	18.24	0
25	0	19.04	10	9.68E+00	0	0	0
26	10	10	10	1.00E+01	0	0	0
27	0	10	19.2	9.73E+00	8.16	0	9.6
28	9.44	0	10	6.48E+00	0	0	0
29	10	10	0	6.67E+00	0	0	0
30	10	0	10	6.67E+00	0	0	0
Total CFU transferred	1932.08	3268.16	1727.44		1463.44	2659.68	642.2
				Rep % Injury	24.2557244	18.61842749	62.82360024

Table A2.16. *Listeria monocytogenes* transfer from slicer to salami (weak biofilm former/uninjured/24 h incubation)

Weak, 24h
Salami

Slice	Rep 1	Rep 2	Rep 3	Average	Rep 1 Healthy	Rep 2 Healthy	Rep 3 Healthy
1	2653.28	48.6	167.16	9.56E+02	1880.48	64.8	31.84
2	4132.8	74.8	156.56	1.45E+03	2394.4	13.6	16.48
3	1397.28	49.2	148.32	5.32E+02	1175.76	29.52	8.24
4	1944.8	120.32	247.2	7.71E+02	1343.68	112.8	32.96
5	1261.44	84	158.84	5.01E+02	1103.76	14	16.72
6	1611.84	73.2	106.6	5.97E+02	1296.48	43.92	0
7	1060.8	118.4	254.2	4.78E+02	884	74	0
8	901.68	127.84	549.4	5.26E+02	601.12	52.64	41
9	792	127.16	369.84	4.30E+02	739.2	67.32	8.04
10	1088.24	218.08	254.72	5.20E+02	892	255.68	55.72
11	0	0	10	3.33E+00	0	0	0
12	0	0	8.16	2.72E+00	0	0	0
13	0	0	0	0.00E+00	0	0	0
14	0	10	0	3.33E+00	0	0	0
15	0	0	0	0.00E+00	0	0	0
16	10	7.48	0	5.83E+00	0	0	0
17	0	0	0	0.00E+00	0	0	0
18	0	0	0	0.00E+00	0	0	0
19	0	0	8.12	2.71E+00	0	0	0
20	0	0	0	0.00E+00	0	0	0
21	0	7.28	0	2.43E+00	0	0	0
22	0	7.28	0	2.43E+00	0	0	0
23	0	7.28	0	2.43E+00	0	0	0
24	0	7.32	0	2.44E+00	0	0	0
25	10	0	10	6.67E+00	0	0	0
26	0	10	0	3.33E+00	0	0	0
27	0	14.64	0	4.88E+00	0	0	0
28	0	0	0	0.00E+00	0	0	0
29	0	7.4	0	2.47E+00	0	0	0
30	0	10	10	6.67E+00	0	0	0
Total CFU transferred	16864.16	1130.28	2459.12		12310.88	728.28	211
				Average % Injury	26.999743 84	35.566408 32	91.419694 85

Table A2.17. *Listeria monocytogenes* transfer from slicer to salami (strong biofilm former/cold-injured/6 h incubation)

Strong, 6h, Cold, Salami

Slice	Rep 1	Rep 2	Rep 3	Average	Rep 1 Healthy	Rep 2 Healthy	Rep 3 Healthy
1	8.96	60.2	10	26.38667	0	8.6	0
2	23.4	310.08	463.68	265.72	23.4	218.88	463.68
3	72	198.88	468.48	246.4533	64.8	90.4	387.96
4	28.64	200	395.2	207.9467	35.8	128	608
5	300.12	31.52	142.12	157.92	29.28	39.4	224.4
6	44.16	162.12	324.24	176.84	0	108.08	270.2
7	66.24	131.92	399.36	199.1733	22.08	77.6	384
8	36	185.28	183.36	134.88	14.4	30.88	168.08
9	21.72	133.96	220.4	125.36	14.48	39.4	205.2
10	194.4	163.8	148.96	169.0533	50.4	62.4	188.16
11	36.8	118.2	338.84	164.6133	29.44	70.92	236.4
12	10	176	138.24	108.08	7.32	96	115.2
13	42.96	198	185.28	142.08	28.64	55.44	200.72
14	50.68	118.8	165.48	111.6533	50.68	134.64	189.12
15	709.52	63.04	85.8	286.12	101.36	70.92	124.8
16	14.72	78.8	124.8	72.77333	14.72	23.64	156
17	7.32	93.6	192	97.64	7.32	15.6	107.52
18	313.04	31.36	160.44	168.28	101.92	0	61.12
19	65.88	62.72	61.76	63.45333	87.84	23.52	30.88
20	80.52	70.92	30.88	60.77333	65.88	23.64	77.2
21	596.16	39.4	216.16	283.9067	272.32	15.76	123.52
22	294.4	47.76	112.56	151.5733	176.64	23.88	136.68
23	10	10	234.32	84.77333	14.8	16	145.44
24	7.36	15.76	176.88	66.66667	0	0	176.88
25	50.96	23.76	93.6	56.10667	0	7.92	78
26	58.88	23.52	193	91.8	29.44	0	162.12
27	37	23.88	144.72	68.53333	29.6	7.96	136.68
28	10	23.76	79.6	37.78667	7.36	23.76	111.44
29	7.36	23.76	77.6	36.24	0	15.84	69.84
30	296	23.88	39	119.6267	199.8	0	39
Total CFU transferred	3495.2	2844.68	5606.76		1479.72	1429.08	5378.24
				Average % Injury	57.66422522	49.7630665	4.07579422

Table A2.18. *Listeria monocytogenes* transfer from slicer to salami (weak biofilm former/cold-injured/6 h incubation)

Weak, 6h, Cold,
Salami

Slice	Rep 1	Rep 2	Rep 3	Average	Rep 1 Healthy	Rep 2 Healthy	Rep 3 Healthy
1	10	10	15.84	11.94667	0	0	2.64
2	1965.4	630.08	163.2	919.56	1128.4	372.32	95.2
3	589	478.72	141.36	403.0267	279	359.04	156.24
4	443.52	307.44	101.92	284.2933	246.4	226.92	87.36
5	892.32	180	30.24	367.52	511.68	216	30.24
6	416.16	163.68	37.8	205.88	220.32	59.52	15.12
7	682	87.36	45.12	271.4933	384.4	36.4	0
8	371.2	163.76	150.4	228.4533	145	156.64	105.28
9	297.04	93.08	14.96	135.0267	158	28.64	7.48
10	363.56	115.2	22.08	166.9467	154.96	57.6	44.16
11	378.2	287.04	115.84	260.36	148.8	191.36	28.96
12	407.04	364	14.88	261.9733	184.44	182	14.88
13	211.2	360.64	83.16	218.3333	57.6	228.16	60.48
14	119.32	118.4	14.88	84.2	25.12	88.8	22.32
15	243.2	220.8	21.84	161.9467	108.8	125.12	14.56
16	109.48	529.92	7.36	215.5867	45.08	272.32	7.36
17	195.92	360.64	14.56	190.3733	44.24	147.2	0
18	129.36	321.64	29.76	160.2533	49.28	209.44	14.88
19	190.96	142.88	14.88	116.24	49.28	112.8	14.88
20	107.44	164.56	36.4	102.8	56.88	74.8	14.56
21	189.6	120.32	14.8	108.24	25.28	120.32	7.4
22	82.68	148	29.44	86.70667	25.44	74	7.36
23	56.52	75.2	139.08	90.26667	56.52	67.68	65.88
24	171.36	57.6	7.4	78.78667	42.84	50.4	7.4
25	62.4	82.72	7.36	50.82667	6.24	37.6	14.72
26	171.36	37.6	15.28	74.74667	116.28	30.08	0
27	54.72	59.52	67.32	60.52	12.16	29.76	7.48
28	25.12	37.8	14.72	25.88	37.68	60.48	0
29	49.28	145.92	10	68.4	30.8	30.72	0
30	24.64	69.12	10	34.58667	18.48	53.76	0
Total CFU transferred	9010	5933.64	1391.88		4369.4	3699.88	846.84
				Rep % Inj	51.5049 9445	37.645694 72	39.158548 15

Table A2.19. *Listeria monocytogenes* transfer from slicer to salami (strong biofilm former/cold-injured/24 h incubation)

Strong, 24h, Cold,
Salami

Slice	Rep 1	Rep 2	Rep 3	Average	Rep 1 Healthy	Rep 2 Healthy	Rep 3 Healthy
1	32.76	203.36	372	202.7067	214.2	0	570.4
2	169.36	14	32.6	71.98667	40.88	7	32.6
3	0	35.6	39.36	24.98667	0	21.36	19.68
4	10	7.12	20.88	12.66667	7.2	14.24	13.92
5	7.2	6.88	25.76	13.28	7.2	13.76	25.76
6	28.8	49.28	13.6	30.56	0	21.12	0
7	50.68	13.76	10	24.81333	7.24	13.76	13.52
8	36.8	10	34.2	27	0	0	20.52
9	29.12	10	46.76	28.62667	29.12	14.4	33.4
10	7.4	7.24	20.64	11.76	0	0	6.88
11	74.4	21.48	10	35.29333	37.2	7.16	7.08
12	177.6	10	28.16	71.92	118.4	0	21.12
13	2977.04	13.76	48.44	1013.08	635.8	0	55.36
14	89.28	34.8	61.2	61.76	89.28	0	81.6
15	10	6.88	27.52	14.8	0	0	6.88
16	7.48	42.48	10	19.98667	14.96	7.08	27.84
17	10	28.16	20.76	19.64	15.04	0	13.84
18	338.4	7.16	13.68	119.7467	112.8	7.16	0
19	82.72	21.48	10	38.06667	0	0	0
20	110.4	20.52	27.84	52.92	7.36	6.84	0
21	141.36	14	20.64	58.66667	66.96	0	13.76
22	6118.64	7.12	20.76	2048.84	1540.88	0	6.92
23	7.4	20.76	10	12.72	0	13.84	0
24	7.44	54.72	6.8	22.98667	0	0	0
25	10	35.4	10	18.46667	14.72	0	0
26	20.28	27.84	0	16.04	27.04	0	0
27	6.24	53.76	6.8	22.26667	0	0	0
28	327.6	26.72	6.84	120.3867	226.8	0	6.84
29	51.24	21.24	10	27.49333	7.32	7.08	0
30	86.4	10	10	35.46667	21.6	0	0
Total CFU transferred	11026.04	835.52	975.24		3242	154.8	977.92
				Rep % Inj	70.596877 94	81.472615 86	0

Table A2.20. *Listeria monocytogenes* transfer from slicer to salami (weak biofilm former/cold-injured/24 h incubation)

Weak, 24h, Cold,
Salami

Slice	Rep 1	Rep 2	Rep 3	Average	Rep 1 Healthy	Rep 2 Healthy	Rep 3 Healthy
1	0	0	0	0	0	0	0
2	10	14.4	8.52	10.97333	0	0	8.52
3	113.92	52.64	8.76	58.44	64.08	22.56	0
4	83.52	14.96	10	36.16	13.92	7.48	0
5	30.08	7.6	0	12.56	22.56	0	0
6	106.4	15.52	10	43.97333	83.6	7.76	0
7	15.2	10	0	8.4	22.8	0	0
8	15.28	15.2	10	13.49333	15.28	7.6	0
9	44.4	10	0	18.13333	7.4	7.6	0
10	28	7.64	0	11.88	14	0	0
11	14.8	15.68	0	10.16	7.4	0	0
12	29.6	15.68	10	18.42667	7.4	0	0
13	14.88	108.08	0	40.98667	14.88	46.32	0
14	7.36	75.6	0	27.65333	22.08	45.36	0
15	10	30.24	0	13.41333	7.32	7.56	0
16	7.52	14.64	10	10.72	15.04	0	0
17	80.96	38.2	10	43.05333	14.72	0	0
18	7.2	37.6	0	14.93333	14.4	7.52	0
19	22.32	15.2	10	15.84	0	0	0
20	7.24	10	0	5.746667	0	0	0
21	14.08	10	0	8.026667	7.04	0	0
22	39.36	15.36	0	18.24	19.68	0	0
23	41.76	10	0	17.25333	20.88	0	0
24	10	10	0	6.666667	14.24	0	0
25	22.08	10	0	10.69333	14.72	15.52	0
26	44.4	10	0	18.13333	0	0	0
27	21.84	10	0	10.61333	0	0	0
28	7.28	10	0	5.76	14.56	0	0
29	7.4	7.88	0	5.093333	7.4	7.88	0
30	5.76	10	0	5.253333	0	0	0
Total CFU transferred	862.64	612.12	87.28		445.4	183.16	8.52
				Rep % Inj	48.3678 0117	70.0777625 3	90.2383134 7

Table A2.21. *Listeria monocytogenes* transfer from slicer to salami (strong biofilm former/chlorine-injured/6 h incubation)

Strong, 6h, Chlorine,
Salami

Slice		Rep 1	Rep 2	Rep 3	Average	Rep 1 Healthy	Rep 2 Healthy	Rep 3 Healthy
	1	0	0	9.76	4.88	0	0	0
	2	316.8	405.6	19.2	212.4	40.32	0	0
	3	216.3 2	248.6 4	7.36	157.44	13.52	0	0
	4	124.3 2	45.08	0	56.46667	8.88	0	0
	5	8	102.4	0	36.8	0	0	0
	6	15.84	32.6	0	16.14667	7.92	0	0
	7	24.12	78.24	7.4	36.58667	16.08	0	0
	8	48.24	66.4	22.3 2	45.65333	0	0	0
	9	40.2	46.2	14.6 4	33.68	0	0	0
	10	8	6.6	0	4.866667	0	0	0
	11	0	39.6	0	13.2	0	0	0
	12	7.96	73.04	7.36	29.45333	0	0	0
	13	0	33.2	7.24	13.48	0	0	0
	14	16	80.16	14.5 6	36.90667	0	0	0
	15	0	45.36	0	15.12	0	0	0
	16	10	39.84	10	19.94667	0	0	0
	17	0	19.92	2.64	7.52	0	0	0
	18	0	26.4	5.8	10.73333	0	0	0
	19	8.08	71.72	7.48	29.09333	0	0	0
	20	8	26.08	0	11.36	0	0	0
	21	24	47.04	22.3 2	31.12	0	0	0
	22	16	60.84	7.44	28.09333	0	0	0
	23	8.08	54.4	0	20.82667	0	0	0
	24	64.64	13.52	0	26.05333	0	0	0
	25	32.16	54.4	73.6	53.38667	0	0	0
	26	8.04	6.88	0	4.973333	0	0	0
	27	7.96	60.84	10	26.26667	0	0	0
	28	7.88	33.8	36.2 4	25.97333	0	0	0
	29	0	53.12	19.6 8	24.26667	0	0	0
	30	0	13.92	10	7.973333	0	0	0
Total CFU transferred		1020. 64	1885. 84	315. 04		86.72	0	0
					Rep % Inj	91.5033 7043	100	100

Table A2.22. *Listeria monocytogenes* transfer from slicer to salami (weak biofilm former/chlorine-injured/6 h incubation)

Weak, 6h, Chlorine,
Salami

Slice		Rep 1	Rep 2	Rep 3	Average	Rep 1 Healthy	Rep 2 Healthy	Rep 3 Healthy
	1	0	896	0	298.6667	0	0	0
	2	21.1	10	171.6	67.57333	0	5.72	28.6
	3	21.6	54	17.28	30.96	0	0	0
	4	10	5.12	5.8	6.973333	0	0	0
	5	14.0	0	10	8.026667	0	0	0
	6	7.32	0	10	5.773333	0	0	0
	7	10	11.52	5.68	9.066667	0	0	0
	8	10	0	10	6.666667	0	0	0
	9	10	10	0	6.666667	0	0	0
	10	10	10	10	10	0	0	0
	11	7.6	5.68	10	7.76	0	0	0
	12	10	0	10	6.666667	0	0	0
	13	10	0	5.72	5.24	0	0	0
	14	10	0	0	3.333333	0	0	0
	15	10	0	10	6.666667	0	0	0
	16	10	0	0	3.333333	0	0	0
	17	0	284	10	98	0	0	0
	18	10	0	10	6.666667	0	0	0
	19	10	28.6	10	16.2	0	0	0
	20	10	0	10	6.666667	0	0	0
	21	10	10	0	6.666667	0	0	0
	22	0	0	10	3.333333	0	0	0
	23	10	10	0	6.666667	0	0	0
	24	10	10	0	6.666667	0	0	0
	25	10	0	0	3.333333	0	0	0
	26	10	10	0	6.666667	0	0	0
	27	10	0	10	6.666667	0	0	0
	28	14.4	0	0	4.826667	0	0	0
	29	10	0	0	3.333333	0	0	0
	30	0	0	0	0	0	0	0
Total CFU transferred		286.2	1354.92	336.08		0	5.72	28.6
					Rep % Inj	100	99.577834 85	91.490121 4

Table A2.23. *Listeria monocytogenes* transfer from slicer to salami (strong biofilm former/chlorine-injured/24 h incubation)

Strong, 24 h,
Chlorine, Salami

Slice	Rep 1	Rep 2	Rep 3	Average	Rep 1 Healthy	Rep 2 Healthy	Rep 3 Healthy
1	0	0	0	0	0	0	0
2	20.88	662.72	0	227.8667	0	82.84	0
3	0	1286.56	0	428.8533	0	172	0
4	0	0	0	0	0	0	0
5	6.92	0	0	2.306667	0	0	0
6	0	598.4	7.44	201.9467	0	0	0
7	0	136.04	22.44	52.82667	0	0	0
8	0	0	21.96	7.32	0	0	0
9	6.92	7.08	7.4	7.133333	0	0	0
10	0	0	0	0	0	0	0
11	0	7.16	0	2.386667	0	0	0
12	0	0	10	3.333333	0	0	0
13	0	7.2	0	2.4	0	0	0
14	0	7.24	0	2.413333	0	0	0
15	0	0	0	0	0	0	0
16	6.92	0	0	2.306667	0	0	0
17	10	0	0	3.333333	0	0	0
18	0	7.2	0	2.4	0	0	0
19	0	0	0	0	0	0	0
20	6.88	0	0	2.293333	0	0	0
21	0	0	0	0	0	0	0
22	0	0	0	0	0	0	0
23	0	0	0	0	0	0	0
24	0	0	0	0	0	0	0
25	6.84	0	0	2.28	0	0	0
26	0	7.16	10	5.72	0	0	0
27	0	0	0	0	0	0	0
28	0	7.04	0	2.346667	0	0	0
29	0	7.04	0	2.346667	0	0	0
30	0	0	0	0	0	0	0
Total CFU transferred	65.36	2740.84	79.24		0	254.84	0
				% Injury		90.702120	
				Rep	100	52	100

Table A2.24. *Listeria monocytogenes* transfer from slicer to salami (weak biofilm former/chlorine-injured/24 h incubation)

Weak, 24h, Chlorine,
Salami

Slice		Rep 1	Rep 2	Rep 3	Average	Rep 1 Healthy	Rep 2 Healthy	Rep 3 Healthy
	1	10	0	0	3.333333	0	0	0
	2	0	5.52	0	1.84	0	0	0
	3	10	5.76	0	5.253333	0	5.76	0
	4	0	12.5 6	0	4.186667	0	0	0
	5	6.92	6.36	0	4.426667	0	0	0
	6	1045. 76	0	0	348.5867	220.16	0	0
	7	0	0	0	0	0	0	0
	8	0	6.4	0	2.133333	0	0	0
	9	0	0	0	0	0	0	0
	10	0	0	42	14	0	0	0
	11	0	6.4	6.16	4.186667	0	0	0
	12	0	0	6.04	2.013333	0	0	0
	13	0	0	10	3.333333	0	0	0
	14	0	0	0	0	0	0	0
	15	0	0	6.28	2.093333	0	0	0
	16	6.84	0	10	5.613333	0	0	0
	17	6.88	0	0	2.293333	0	0	0
	18	0	0	0	0	0	0	0
	19	0	0	10	3.333333	0	0	0
	20	0	0	10	3.333333	0	0	0
	21	0	0	10	3.333333	0	0	0
	22	6.88	6.48	6.16	6.506667	0	0	0
	23	0	0	0	0	0	0	0
	24	0	0	0	0	0	0	0
	25	6.88	0	10	5.626667	0	0	0
	26	0	0	10	3.333333	0	0	0
	27	0	0	0	0	0	0	0
	28	0	6.48	0	2.16	0	0	0
	29	10	10	0	6.666667	0	6.4	0
	30	0	0	0	0	0	0	0
Total CFU transferred		1110. 16	65.9 6	136. 64		220.16	12.16	0
					% Injury Rep	80.168624 34	81.564584 6	100

APPENDIX III

SAMPLE MICROGRAPHS

Sample micrographs from viability staining using CSLM. Images in this dissertation are presented in color.

Figure A3.1. Live/Dead micrograph of *Listeria monocytogenes* (strong biofilm formers, cold-injured) after 6 h of incubation on dry stainless steel

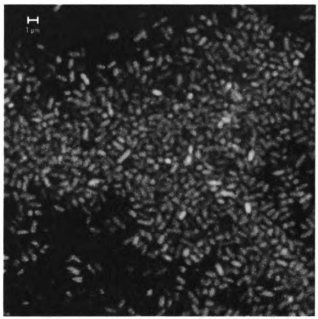


Figure A3.2. Live/Dead micrograph of *L. monocytogenes* (strong biofilm formers, chlorine-injured) after 1 h of incubation on dry stainless steel

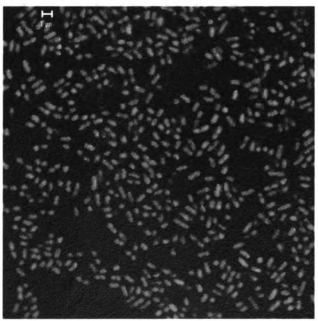


Figure A3.3. Live/Dead micrograph of *L. monocytogenes* (strong biofilm formers, cold-injured) after 6 h of incubation on dry stainless steel

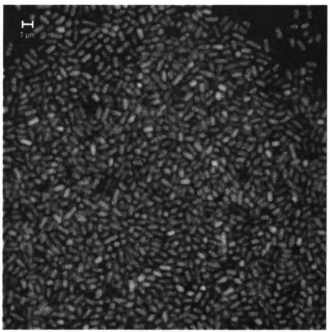
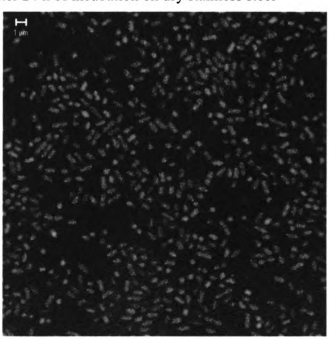


Figure A3.4. Live/Dead micrograph of *L. monocytogenes* (weak biofilm formers, cold-injured) after 24 h of incubation on dry stainless steel



APPENDIX IV

Figure A4.1. An example of the GWBasic modeling program

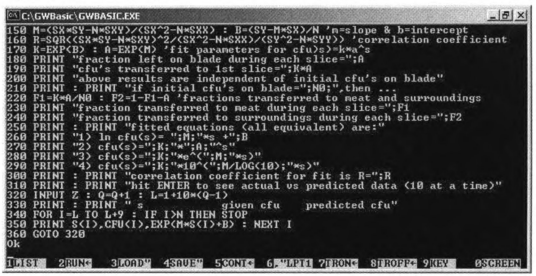
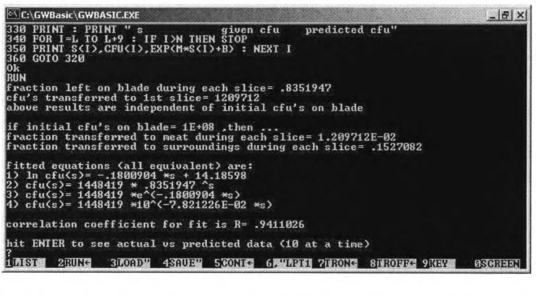


Figure A4.2. GWBasic modeling program output when used to model transfer of *Listeria monocytogenes* (10^8 CFU/blade initial inoculum level) to delicatessen meat



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