THESIS

INVESTIGATION OF NICHE ADAPTATION IN *LISTERIA MONOCYTOGENES* SUBPOPULATIONS AND SMALL MOLECULE INHIBITORS OF *ESCHERICHIA COLI* O157:H7

Submitted by

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WE HEREBY RECOMMEND THAT THE THESIS PREPARED UNDER OUR SUPERVISION BY JESSICA LEE CORRON ENTITLED INVESTIGATION OF NICHE ADAPTATION IN *LISTERIA MONOCYTOGENES* SUBPOPULATIONS AND SMALL MOLECULE INHIBITORS OF *ESCHERICHIA COLI* O157:H7 BE ACCEPTED AS FULFILLING IN PART REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE.

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ABSTRACT OF THESIS

INVESTIGATION OF NICHE ADAPTATION IN *LISTERIA MONOCYTOGENES* SUBPOPULATIONS AND SMALL MOLECULE INHIBITORS OF *ESCHERICHIA COLI* O157:H7

Listeriosis is a severe and often fatal disease caused by the foodborne pathogen *Listeria monocytogenes*. At least two distinct subpopulations of this pathogen have been observed, including (i.) Epidemic clone (EC) strains, which have been responsible for numerous outbreaks worldwide, as well as the majority of sporadic listeriosis cases in the U.S., and (ii.) strains carrying a premature stop codon mutation (PMSC) in a key virulence gene *inlA*, leading to natural virulence attenuation. The latter population is prevalent in foods, with strains harboring an *inlA* PMSC comprising approximately 50 percent of food isolates. We thus hypothesized that these two distinct subpopulations have undergone niche adaptation, where EC strains have become adapted to a human host niche while *inlA* PMSC strains have become adapted to a food niche and additionally alternative mammalian hosts (e.g. mice, rats, etc.). In order to test this hypothesis we assembled a strain set of eight strains, four EC strains and four *inlA* PMSC strains, and subjected these strains to various assays, which mimic a host or food environment. Specifically we performed intracellular growth assays and cytotoxicity assays in macrophage like-cells. Additionally we performed invasion assays in mouse L929 fibroblast cells to determine the ability of these cells to invade cells other than

human cells. InlA, a surface protein, binds to the receptor E-cadherin and mice possess a different isoform which does not allow for efficient InlA binding; therefore, these assays should provide insight on invasion of cells with different non-human isoforms of E-cadherin. Lastly, we performed cold growth assays in BHI broth at 7˚C as well as a growth study on deli turkey in order to evaluate growth in food-like or food conditions. Overall, we found that although all strains grew similarly in all broth cold growth conditions; EC and *inlA* PMSC strains showed differences in terms of virulence phenotype as well as the ability to grow in RTE deli turkey. Although all strains grew similarly at all time points during intracellular growth experiments, differences between EC and *inlA* PMSC strains were observed at later time points in the cytotoxicity assays. Specifically, the *inlA* PMSC strains exhibited higher cytotoxicity 9, and 12 hours following inoculation ($P = 0.0028$, and $P < 0.0001$ respectively). All *inlA* PMSC strains invaded mouse L929 cells better than a standard laboratory control strain, in comparison to only one EC strain which showed the same trend ($P<0.05$). Lastly, *inlA* PMSC strains had higher exponential phase growth rates ($P = 0.0243$) when compared to EC strains when grown on deli turkey for 28 days. These data support the conclusion that EC strains are better adapted for pathogenesis in a human host while *inlA* PMSC strains are better adapted to survival in foods as well as in non-human hosts where *inlA* mediated invasion is not necessary for internalization.

Escherichia coli O157:H7 causes an estimated 73,480 cases of illness each year, a small percentage of these cases progress to a potentially fatal disease called hemolytic uremic syndrome or HUS, sometimes leading to kidney failure or even death in serious cases. The majority of *E. coli* O157:H7 cases are food related, and cattle are implicated as the

major reservoir host for this pathogen. Antibiotics are not recommended for the treatment of *E. coli* O157:H7 infections, thus treatment options are limited. Therefore, it is critical to minimize the load of *E. coli* O157:H7 that reaches the consumer. In order to identify compounds to be used for the control and treatment of this deadly pathogen, we designed a high throughput, small molecule, turbidometric growth assay for the identification of bactericidal or bacteriostatic compounds. Through this screening we investigated the potential of nearly 65,000 compounds, which were assayed in duplicate, and identified 43 which inhibited *E. coli* O157:H7 growth. Of the 43 compounds, 38 were known bioactive compounds, while the other five were from libraries of commercially available chemical compounds. Many of the known bioactive compounds were known antibiotics, specifically cephalosporins (n=13), fluorquinolones (n=12), and tetracyclines (n=9), as well as one carbapenem and two other antibiotics. Additionally, an antiviral agent, an inhibitor of the tricarboxylic acid cycle, as well as two structurally related disinfectants, which are used in a number of non-clinical applications, were also discovered. Specifically, the non-clinical disinfectants show promise for spray-wash and dip type treatments to be implemented in the food supply, due to their antimicrobial activities against *E. coli* O157:H7, and their cytotoxicity, which is similar to other widely used disinfectants.

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DEDICATION

I dedicate this thesis to my grandmother, Geraldine Schaefer. You have always served as a role model to me. Your strength in the face of adversity has always encouraged me to keep going, even when things seemed too hard to see through. Your encouragement and love have been what kept me going in the face of seemingly insurmountable challenges. I love you and I wish you were here during this important and difficult time in my life. I miss you.

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CHAPTER 1

OBJECTIVES OF THESIS

The objectives of the studies reported in this thesis were:

Overall Objective:

To elucidate the niche adaptation of two subpopulations within *Listeria monocytogenes*, including Epidemic clone (EC) strains and strains containing a virulence attenuating mutation in *inlA*, as well as, to identify small molecule compounds that inhibit the growth of *Escherichia coli* O157:H7 for use clinically or in the food supply.

Specific Objectives:

1. To assess the virulence phenotype of select EC and *inlA* PMSC strains by subjecting them to intracellular growth assays, cytotoxicity assays, and invasion assays.
2. To assess the ability of select EC and *inlA* PMSC strains to grow in food-like conditions and on a food matrix.
3. To design a high throughput small molecule screening assay to detect compounds that inhibit the growth of *E. coli* O157:H7.

4. To identify compounds which inhibit the growth of *E. coli* O157:H7 through the use of a high throughput small molecule screening assay.

5. To determine the cytotoxicity of compounds found to inhibit the growth of *E. coli* O157:H7 as part of examining their suitability for use as potential therapeutic agents or for use in the food supply.

CHAPTER II: Foodborne Illness in the United States

2.1 Overview

Foodborne pathogens are responsible for an estimated 76 million cases of disease in the U.S. each year. It is estimated that of these, 323,000 result in hospitalization and 5,200 result in death. Foodborne diseases are caused by numerous bacterial, parasitic, and viral agents, and of these, *Listeria monocytogenes* and *Escherichia coli* O157:H7 are among the top bacterial concerns for the food industry. *Listeria monocytogenes* accounts for nearly 30% of deaths associated with foodborne pathogens, while *E. coli* O157:H7 is associated with 3% (Mead et al., 1999). These represent two of the most severe pathogens contaminating the food supply, and government agencies have enacted rules and regulations to increase the safety of our food supply from the threat of these pathogens. *Listeria monocytogenes* is subject to a strict zero tolerance policy in ready-to-eat (RTE) foods in the U.S. (Shank et al., 1996), whereas *E. coli* O157:H7 is considered an adulterant in non-intact beef products (FSIS Directive 10,010.1, 2009).

Listeria monocytogenes is the etiologic agent of listeriosis, a severe invasive disease. Symptoms of listeriosis include septicemia, meningitis and encephalitis in susceptible individuals as well as still births in pregnant women (Schlech, 2000). *L. monocytogenes* results in about 2,500 cases of disease each year, and approximately 500 of these result in

death. *Escherichia coli* O157:H7 infects about 73,000 individuals in the U.S. each year and the vast majority of these cases are foodborne (>80%). Approximately 4% of these cases result in Hemolytic Uremic Syndrome (HUS), which can result in kidney failure and even death (Mead et al., 1999). In fact, HUS is the leading cause of kidney failure in children (Karmali et al., 1989).

While many foodborne pathogens cause substantially more cases of disease (e.g., *Salmonella* spp., *Campylobacter* spp.), *Listeria monocytogenes* demonstrates an exceptionally high mortality rate, while *E. coli* O157:H7 is associated with severe and chronic sequelae such as HUS. Due to the severe nature of the diseases caused by these bacteria, both pathogens are targets of the Healthy People 2010 initiative, and the food safety objectives are a joint venture of the Food and Drug Administration and the Food Safety and Inspection Service of the U.S. Department of Agriculture. Goals set for the year 2000 were not met, so new goals were made for 2010. The specific aim is to decrease *Listeria monocytogenes* to 0.25 cases per 100,000, *Escherichia coli* O157:H7 infections to 1 case per 100,000. Both numbers are approximately half of the 1997 baseline (Healthy People 2010, 2010).

Based on 1993 figures, the cost of illness for *L. monocytogenes* is estimated to be approximately 0.2-0.3 billion dollars, and for *Escherichia coli* O157:H7, this figure is estimated to be between 0.2 and 0.6 billion dollars (Economic Research Service/USDA, 2009). Due to their severe impact on public health, and cost to the food industry, these two pathogens are of particular concern.

2.2 *Listeria monocytogenes*

Ecology of *Listeria monocytogenes*

Listeria monocytogenes has been commonly isolated from the environment. Early studies found that *L. monocytogenes* was present in 20.3% of samples from soil and plants, 15.7% of deer and stag feces, as well as 17.3% of feces from birds (Weis and Seeliger, 1975). Furthermore, *Listeria* has been isolated from plant and soil samples, and serotyping has indicated that samples from nearby soil and plants are not always identical. This trend was observed in both farm and non-farm environments. These data suggest a saprophytic life style for *Listeria* (Welshimer and Donker-Voet, 1971).

These studies were performed before it was known that there are multiple species in the genus *Listeria*. More recent studies indicate that *L. monocytogenes* can be found in 0.72-2.1% of samples from natural environments, with prevalence being highest in the summer (Sauders et al., 2005). A recent study, which models isolation of *Listeria* spp., identified freeze-thaw cycling, the presence of loam soil, water storage, slope, and cardinal direction to the north as factors which influence the isolation of *Listeria* from the environment (Ivanek et al., 2009).

Many different animal species can harbor this pathogen and are prone to disease. Numerous ruminant species (bovine, ovine, and caprine) as well as horses, pigs, cats, dogs, rabbits, numerous types of rodents, birds and fish are susceptible to disease caused by *L. monocytogenes*. Frogs, toads, and reptiles may harbor this pathogen, but do not shown signs of disease (Lecuit, 2007). Farm animals, particularly ruminants, are

susceptible to colonization and subsequent infection by *L. monocytogenes* and are thus hypothesized to play an important role in the spread and transmission of this pathogen. A case control study found that about 20% of samples from farms, both those with and without cases of listeriosis, were positive for *L. monocytogenes*. Specifically, the prevalence of *L. monocytogenes* was found to be 24.4% on cattle case farms, 20.2% on cattle control farms, 32.9% on sheep and goat case farms, and 5.9% on sheep and goat control farms (Nightingale et al., 2004).

Listeria monocytogenes has been shown to persist in the processing plant environment. Work done in smoked fish processing plants prior to the onset of specific intervention strategies found that 19.2% of raw samples, 26.1% of samples from the processing plant environment, and 8.7% of samples from finished product were positive for *Listeria spp.* Ribotyping of isolates showed that *L. monocytogenes* in the raw product was not always the source of contamination in the final product, and that the processing environment can be a source of *L. monocytogenes* found in the finished product (Lappi et al., 2004). Work by Orsi et al. (2008b) demonstrates that a single strain of *L. monocytogenes* was able to persist in the same food processing plant for over 12 years, and over this span of time was able to cause at least one sporadic case and one major outbreak. Thus, long term persistence has significant implications on the transmission and spread of this organism through food vehicles to the consumer.

Listeria monocytogenes in Foods

It is estimated that over 99 % of all listeriosis cases are attributable to exposure through contaminated foods (Mead et al., 1999). *Listeria monocytogenes* is easily inactivated by

cooking and pasteurization (Van der Veen et al., 2009). However, *Listeria monocytogenes* thrives in temperatures from 0-45˚C, which includes temperatures encompassing refrigerated storage conditions (Farber et al., 1988). *Listeria monocytogenes* also has a well documented ability to survive carbon starvation, acid, and oxidative stress, mainly due to the contributions of Sigma B, an alternative sigma factor, responsible for stress response (Ferreira 2001 et al., Kazmierczak et al., 2003, Sue et al., 2004, Abram et al., 2008). Many of these stresses are used to control pathogens in foods and in the food processing environment. Therefore, ready-to-eat foods that are refrigerated and not heated prior to consumption have the greatest likelihood of harboring *L. monocytogenes*.

In a 2003 *Listeria monocytogenes* risk assessment, predicted median cases of listeriosis for 23 food categories were calculated on per annum and per serving basis and deli meats topped the list for cases on both a per annum and per serving basis. These foods were estimated to account for over 85% of listeriosis cases. In addition to deli meats, frankfurters (not reheated), pate, unpasteurized milk, smoked seafood, and RTE crustaceans were considered high risk on a *per serving* basis (FDA/USDA/CDC, 2003).

A recent risk assessment involving deli meat suggests that reformulation of deli meats with growth inhibitors will decrease cases caused by these foods 2.5 – 7.8 fold depending on the specific food product (Pradhan et al., 2009). However, it is also possible that there are foods that have not yet been linked to this illness. The >30 day incubation period for this pathogen is a hindrance to investigations, and often makes it difficult to pinpoint a food source (Swaminathan and Gerner-Smidt, 2007). A recent recall of sprouts

highlights the need to examine other food sources as potential food vehicles for this pathogen (FDA, 2009).

Virulence Factors of *Listeria monocytogenes*

An increasing body of evidence has begun to elucidate the genetic mechanisms through which *Listeria monocytogenes* mediates the switch between a saprophytic lifestyle and virulent pathogen. An RNA thermosensor regulates expression of Primary Regulatory Factor A (*prfA)*, which modulates the expression of a number of virulence genes. This thermosensor allows for the expression of the *prfA* regulon at 37°C and suppression of these genes at 30°C, a temperature more characteristic of the environment (Johansson et al., 2002).

L. monocytogenes utilizes Internalin A (*inlA*) to induce its own uptake into non-professional phagocytes through its interaction with host cell E-cadherin (Lecuit et al., 1997). Once inside the cell, *L. monocytogenes* uses a suite of genes clustered in a pathogenicity island designated LIPI-1 to survive and replicate intracellularly. These genes are necessary for the intracellular life cycle of this pathogen, which allow it to evade the humoral arm of the immune system. In *L. monocytogenes*, this locus consists of 6 genes: *prfA*, *plcA*, *plcB*, *hly*, *mpl*, and *actA*. Hly (or LLO) is a pore forming toxin, which in conjunction with phosopholipases *plcA and plcB* is responsible for disruption of the vacuole (Camilli et al., 1993, Gedde et al., 1999). After *L. monocytogenes* escapes to the cytosol, where it can replicate freely, it recruits host cell actin using *actA*, in order to become motile and spread into adjacent cells (Tilney and Portnoy, 1989).

Numerous studies have identified premature stop codon mutations (PMSCs) caused by a single nucleotide polymorphism (SNP) in the virulence gene *inlA* (Olier et al., 2002, Rousseaux et al., 2004, Nightingale et al., 2005, Felicio et al., 2007, Handa-Miya et al., 2007, Van Stelten and Nightingale, 2008). These mutations occur upstream of the LPXTG membrane anchoring motif and result in a truncated and secreted InlA protein (Nightingale et al., 2005). This results in a decreased ability to invade non-phagocytic cells, and thus, attenuated mammalian virulence (Nightingale et al., 2008). Application of a single nucleotide polymorphism (SNP) genotyping assay developed in our laboratory has demonstrated that nearly 50% of food isolates carry these mutations (Van Stelten and Nightingale, 2008). To date, thirteen different mutations, which cause PMSCs in *inlA* have been discovered, suggesting selective pressure for the loss of a functional InlA.

Listeriosis

Listeria monocytogenes causes less severe gastroenteritis in healthy individuals, and listeriosis, a severe and invasive disease, in immunocompromised individuals (Drevets et al., 2008). Those that are particularly susceptible to listeriosis include the elderly, children, pregnant women, HIV infected individuals, those with autoimmune disease, or individuals undergoing immunosuppressive treatments. *L. monocytogenes* can cause septicemia, meningitis, encephalitis, as well as still births and spontaneous abortions in pregnant women. The fatality rate for listeriosis is around 20%. *L. monocytogenes* has an astonishingly long incubation period, frequently greater than 30 days, which is a hindrance to traditional epidemiological investigation (Swaminathan and Gerner-Smidt,

2007). However, with the advent of molecular methods, and subsequent molecular epidemiology, much has been elucidated on the transmission of this organism.

Epidemiology of Listeriosis

For *Listeria monocytogenes,* only three serotypes (1/2a, 1/2b, and 4b) of 13 are responsible for the vast majority of disease (>90%), suggesting varied pathogenic potential amongst serotypes (McLauchlin, 1990). Specifically, serotype 4b appears to be responsible for many outbreaks worldwide, as well as sporadic cases in the U.S. Within this serotype are two highly clonal groups responsible for numerous outbreaks. Specifically, the epidemic clone I group contains strains responsible outbreaks in Nova Scotia (1981), Massachusetts (1983), California (1985), Swizerland (1983 to 1987), Denmark (1985 to 1987) and France (1992). Epidemic clone group II (ECII) contains strains responsible a 1998 multistate hot dog outbreak in the United States. Lastly, a third epidemic clone group (ECIII) exists and differs from the other two epidemic clone groups in that it is serotype 1/2a. ECIII was responsible for an outbreak associated with sliced turkey in 2000 in the U.S (Kathariou, 2002).

Data on serotypes common in foods is scarce, although serotype 4b is not the most prevalent (Kathariou, 2002). This information suggests that many of the strains in foods may be distinct in comparison to those responsible for human clinical cases. Work by Grey et al. (2004) further illustrates this point. EcoRI ribotyping performed on 492 human isolates and 502 food isolates determined that many ribotypes are significantly associated with either a human or a food origin. Specifically, 1030B, 1038B, 1039A, 1039B, 1042B, 1044A, 1044B, and 1053A are all more frequently associated with

clinical cases than isolation from foods, while 1043A, 1052A, 1062A, and 1062D are more frequently from foods than clinical cases (Grey et al., 2004). It is noteworthy that 1038B, 1044A, and 1053A, which contain epidemic clones I, II, and III respectively are among those ribotypes more associated with isolation from clinical samples than foods.

Furthermore, dose response data have highlighted differences between different subtypes and lineages of *Listeria monocytogenes* in their ability to cause disease. Work by Chen et al. (2006) utilized a conditional probability model to examine differences amongst subtypes in terms of their dose response. This work found that ribotypes 1039C and 1062A were present at the highest levels in foods, with an average dose of $1.29X10^6$ and $2.04X10^3$ respectively. Using probability data, virulence parameters (r-value) were computed for each subtypes. Ribotype 1039C has the lowest r-value while 1042B has the highest. This data indicates that there is variability in a particular subtype's ability to cause disease and that the most common strains in food are not the most virulent (Chen et al., 2005).

Genetic Diversity of *Listeria*

Six species comprise the genus *Listeria*, and includes both pathogens and non-pathogens. *L. monocytogenes* and *L. invanovii* are the only pathogens, with *L. monocytogenes* being the only one that affects humans. *L. ivanovii* primarily infects animals, particularly ruminants. The non-pathogenic species are *L. innocua, L. welshimeri, L. seeligeri,* and *L. grayi* (Hain et al., 2007). A seventh proposed species, *L. marthii,* has been discovered in environmental samples from the Finger Lakes National Forest in New York (Graves et al., 2009). Notably, a few hemolytic *L. innocua* strains have been discovered, which

contain the LIPI-1 pathogenicity island (normally absent in *L. innocua*). These, however, are avirulent, likely due to their lack of internalins (Johnson et al., 2004). Two major lineages exist in *Listeria monocytogenes*. Lineage I is overrepresented in human listeriosis cases, and Lineage II is overrepresented among food isolates. A third lineage is described, containing three distinct subgroups: IIIA, IIIB, and IIIC (Roberts et al., 2005). However, recent evidence suggests that lineage IIIB constitutes its own lineage: lineage IV (Ward et al., 2008, Orsi et al., 2008a).

2.3 *Escherichia coli* O157:H7

Ecology

Cattle are the major reservoir for *E. coli* O157:H7, and in contrast to humans, they do not show signs of disease when carrying this pathogen. Early reports indicated that this was due to the lack of globotriaosylceramide (Gb3) receptor in cattle, but a more recent report found Gb3 receptors in cattle. It appears that the distribution of the Gb3 receptors in the epithelium and the kidney (but not in the endothelium) lead to the localization of the bacterium in the intestine and the lack of systemic disease (Hoey et al., 2002). Other ruminants (sheep, goats, wild deer) are known to harbor this bacterium asymptomatically and may also play a role in the transmission of this organism (Shukla et al., 1995, Kudva et al., 1996, Dunn et al., 2004). *E. coli* O157:H7 has also been isolated from feral pigs surrounding the spinach fields implicated in a 2006 outbreak (Jay et al., 2007).

E. coli O157:H7 in foods

Following a large outbreak of foodborne illness attributed to *E. coli* O157:H7 in 1993, regulatory agencies such as the United States Department of Agriculture (USDA) enacted a number of new policies aimed at reducing foodborne illness and death. There is currently a zero tolerance policy for visible fecal contamination on carcasses (9 CFR Part 304), and *E. coli* O157:H7 is considered an adulterant in raw non-intact beef products. These regulations have led to the use of steam vacuuming and knife trimming of carcasses, which help decrease visible fecal contamination and thus enteric bacterial levels, including *E. coli* O157:H7 (Koohmaraie et al., 2005) .

Due to the asymptomatic carriage of this pathogen in cattle, complete pre-harvest elimination of *E. coli* O157:H7 presents quite a challenge. Source tracking data has indicated that transportation and lairage at the processing plant are major sources of contamination. In one study, 50.3% of animals had *E. coli* O157:H7 on their hides, and when sampled during processing, this number jumped to 94.4%, with *E. coli* O157:H7 isolated from 9.8% of carcasses (Arthur et al., 2006). These data suggest that interventions should be targeted primarily at the post-harvest level, particularly at the hide removal step, which appears to be a critical source of contamination. Chemical dehairing and rinses are often employed prior to hide removal to reduce pathogen loads. Following removal of the hide, steam vacuuming, hot water and/or organic acid rinses are frequently implemented (Koohmaraie et al., 2005).

None of the intervention steps are aimed at the specific reduction of *E. coli* O157:H7, and thus kill many pathogens and non-pathogens. To date, there are no specific antimicrobials approved for use in the food supply to decrease levels of *E. coli* O157:H7.

In 2004, the Food and Drug Administration amended 21 CFR part 173 to approve the use of cetylpyridinium chloride (CPC) for use as a spray treatment on poultry carcasses in order to reduce levels of *Salmonella* spp. present. Much research has been done on the feasibility of the use of CPC in the beef industry. Drastic reductions of *E. coli* O157:H7 can be achieved on carcasses using CPC (Bosilevac et al., 2004; Stopforth et al., 2004), but this chemical is not currently approved for use in beef. Furthermore, nearly all the antimicrobials approved for use in foods have been used for many decades, and little research has been performed to study the development of resistance to these compounds (Davidson et al., 2002).

Virulence Factors in *E. coli* O157:H7

Escherichia coli O157:H7 was first recognized as a pathogen in 1982 when it was associated with 2 outbreaks (Riley et al., 1983). Genome comparison between non pathogenic *E. coli* K-12 and *E. coli* O157:H7 strain EDL 933 (involved in an outbreak associated with ground beef), revealed many differences between this *E. coli* O157:H7 strain and non-pathogenic *E. coli*. Specifically, 1,387 new genes were found in *E. coli* O157:H7. A number of these genes encode for virulence factors, which play a role in the ability of this microorganism to cause disease, unlike its related ancestral strains. These genes include shiga-like toxins, multiple adhesions, and a type III secretion system (T3SS) (Perna et al., 2001). The type III secretion apparatus has been elucidated in microorganisms other than *E. coli.* Its function involves the secretion of effector molecules directly into the cytosol of host cells. In *E. coli* O157:H7, the T3SS is encoded in the Locus of Enterocyte Effacement (LEE). A number of effector proteins are encoded

in this locus, including Tir (transolcated intimin receptor). As its name implies, Tir serves as a receptor for the adhesion intimin, and in conjunction these two molecules are responsible for tight adherence to host cells (Roe et al., 2003). *E. coli* O157:H7 is also equipped with a large virulence plasmid. Sequencing of this plasmid identified a putative toxin belonging to the family of large clostridial toxins and confirmed the existence of other previously identified virulence genes, such as hemolysin (Burland et al., 1998).

The *stx* genes that encode shiga-like toxins are key to this organism's virulence. Shiga toxins are encoded on a prophage. They are AB5 toxins, and the pentameric B subunit is responsible for binding to the globotriaosylceramide receptor, Gb3, in susceptible hosts. The toxin is internalized into host cells, where it inactivates the 60S ribosomal subunit and halts proteins synthesis (O'Laughlin and Robins-Browne, 2001).

Hemolytic Uremic Syndrome

After ingestion of *E. coli* O157:H7, these cells can produce toxins in the intestinal mucosa and damage the intestines. This damage results in diarrhea, which may or may not be bloody. Some research has noted the ability of Stx to be transported across the epithelium, which may play a role in entry into circulation and systemic disease (O'Loughlin and Roins-Browne, 2001). In the majority of patients, the infection resolves itself, but in a small percentage of individuals, Hemolytic Uremic Syndrome (HUS) may develop. Symptoms of HUS include hemolytic anemia, thrombocytopenia, and kidney abnormalities. HUS can resemble thrombotic thrombocytopenic purpura (TTP), but TTP typically occurs without bloody diarrhea. HUS is found predominately in children, who have increased expression of the Gb3 receptor in the glomeruli of the kidney. This

syndrome can result in kidney failure and even death in 5-10% of cases (Ray and Liu., 2001).

Epidemiology

A survey of *E. coli* O157:H7 outbreaks from 1982-2002 indicated that 52% of outbreaks during this time were linked to foods. Of food-associated outbreaks, 41% were associated with ground beef and 6% were linked to other beef products (Rangel et al., 2005). Exposure to *E. coli* O157:H7 through this particular vehicle is highest during the summer months (June through September), and lower throughout the rest of the year. Young children, despite their smaller consumption of ground beef (decreased serving size) in comparison to other age groups, are overrepresented among *E. coli* O157:H7 cases. Specifically, individuals aged 0-5 years account for 7% of ground beef consumption, and 15% of illnesses associated with this food (USDA/FSIS, 2001).

Other foods have been found to transmit *E. coli* O157:H7. In particular, 21% of cases were attributable to produce, 4% due to dairy products, 10% other foods, and in 23% of food associated outbreaks, the food vehicle was unknown (Rangel et al., 2005). This pathogen has also been implicated in outbreaks involving unpasteurized juice (Cody et al., 1999). Food aside, water serves as a major vehicle through which this organism is transmitted, as well as person-to-person and person-to-animal contact. For instance, petting zoos have been found to be an important source of exposure to this deadly pathogen (Rangel et al., 2005).

Genetic Diversity of *Escherichia coli* O157:H7

While *E. coli* O157:H7 is generally considered to be a genetically conserved pathogen, evidence is emerging that this pathogen may be more diverse than originally believed, particularly in virulence genes. Some of the first observed differences between *E. coli* O157:H7 strains were variation of the genes responsible for production of the shiga toxins. Two major variants of the shiga-like toxin producing genes exist within *E. coli* O157:H7. Stx-1 is most similar to Stx from *S. dysenteriae*, sharing about 98% homology, while Stx-2 is much less similar, with approximately 60% homology (O'Loughlin and Robins-Browne, 2001). Furthermore, Stx-2 has been shown to be more toxic in both cell and animal models (Tesh et al., 1993, Louise and Orig, 1995, Siegler et al., 2003). Strains of *E. coli* O157:H7 can harbor both major shiga toxin variants, one, or neither. A recent study by Carlson et al. in 2009, which examined genetic diversity of *E. coli* O157:H7 in feedlot cattle, demonstrated that 45.5% and 58.7% (during collections 1 and 2 respectively) of feed lot cattle excreted feces containing an *E.* coli O157:H7 organism possessing at least one *stx* gene. It was observed that 41.4% and 38.9% of cattle shedding *E. coli* O157:H7 had both *stx-1* and *stx-2* present, while 4.1% and 19.8% had *stx-1* only, for collections 1 and 2 respectively (Carlson et al., 2009). These data suggest that shiga toxin variation is a significant source of genetic diversity within the organism.

More recently, certain tir polymorphisms have been associated with a human vs. bovine origin. Five polymorphisms were discovered upon sequence examination of 22 sequences of different origins. Screening of 187 isolates revealed that a *tir* 255 T>A T polymorphism was found in 99% of human isolates and 55% of bovine isolates, while a RRI-RU3 repeat was found in 57% of bovine isolates and was absent in nearly all human

isolates. The authors of this study postulated that these mutations may make the strains more fit to harbor or colonize a specific niche (Bono et al., 2007).

SNP genotyping of 96 loci in *E. coli* O157:H7 strains from clinical cases identified at least 9 clades, some more frequently associated with disease. Clade 8 was found to be associated with the 2006 outbreaks associated with spinach and lettuce, which had high hospitalization rates and rates of HUS. Pyrosequencing identified 192 genes missing from the Clade 8 spinach outbreak strain that appear in *E. coli* O157:H7 Sakai (isolated from a 1996 outbreak in Japan). Clade 8 also appears to be more common among HUS patients (Manning et al., 2008). These data suggest that through evolution, some *E. coli* O157:H7 strains have become particularly virulent and better human pathogens. Further analysis of the spinach outbreak genome has revealed approximately 70 KB of new genetic information when compared to strains Sakai and EDL 933. Included in these new genes are T3SS effector proteins as well as a nitric oxide reductase gene, which may contribute to the enhanced virulence of this particular strain (Kulasekara et al., 2009). These data support the conclusion that *E. coli* O157:H7 demonstrates some genetic diversity, but more importantly that strains are diverse in their ability to cause disease in humans.

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CHAPTER III:

Listeria monocytogenes subpopulations, including epidemic clone strains and strains carrying unique virulence-attenuating mutations in *inlA*, show evidence for niche adaptation

3.1 ABSTRACT:

Listeria monocytogenes is a human foodborne pathogen that may cause a potentially fatal disease known as listeriosis in susceptible populations such as the elderly, pregnant women and their fetuses or neonates and otherwise immonocompromised individuals. Epidemic clone (EC) strains have been linked to the majority of listeriosis outbreaks worldwide and are overrepresented among sporadic listeriosis cases in the U.S. On the other hand, approximately 45% of *L. monocytogenes* isolates from ready-to-eat foods harbor premature stop codon (PMSC) mutations in the key virulence gene *inlA*, which has been shown to be responsible for attenuated mammalian virulence. The purpose of this study was to probe the hypothesis that EC strains are better adapted to infect human hosts, while strains harboring a PMSC in *inlA* are better adapted to (i) survive and grow under food-associated stresses and in a food matrix or (ii) infect other hosts where InlA-mediated invasion is not required. A set of four EC and four *inlA* PMSC strains were characterized through a battery of experiments. These experiments consisted of *in vitro*

virulence phenotype assays, growth assays simulating food-associated stresses, and ready-to-eat deli meat inoculation studies. Our data support that *inlA* PMSC strains demonstrated significantly higher exponential growth rates than EC strains on inoculated deli meat surfaces ($P = 0.0243$). Our data also show that EC strains exhibited a significantly lower level of cytotoxicity in comparison to *inlA* PMSC strains at 9, and 12 h following infection with *L. monocytogenes* ($P = 0.0028$, and $P < 0.0001$ respectively). Additionally, all *inlA* PMSC strains demonstrated an increased ability to invade mouse fibroblast cells (a mechanism that does not involve InlA) when compared to a standard laboratory control strain. These data suggest that EC strains may be better adapted as pathogens in a human host; whereas strains harboring a PMSC in *inlA* may be better adapted for growth in foods and to infect non-human hosts where InlA-mediated invasion does not contribute to pathogenesis.

3.2 INTRODUCTION:

Listeria monocytogenes is the etiologic agent of listeriosis; a human foodborne disease that primarily affects individuals with definite immunocompromising circumstances (Schlech, 2000). Invasive listeriosis is a rare but severe and often fatal disease associated with a hospitalization rate of 90% and a mortality rate of 20-30% (Mead et al., 1999). Clinical manifestations of invasive listeriosis include septicemia, encephalitis, and meningitis, as well as still births and spontaneous abortions in pregnant women (Schlech, 2000). *L. monocytogenes* is ubiquitous in nature, which consequently makes eradication impractical, and as a result this pathogen represents a significant concern for the ready-to-eat (RTE) food industry (Weis and Seeliger, 1975). Although *L. monocytogenes* present in raw materials is easily inactivated by cooking and

pasteurization, cross-contamination by *L. monocytogenes* present in the food-processing plant environment after the lethality step represents the primary route of finished RTE product contamination (Farber and Peterkin, 1991). Furthermore, *L. monocytogenes,* unlike many other foodborne pathogens, can survive and grow at refrigeration temperature (Farber et al., 1988). Risk assessments indicate that ready-to-eat deli meats and frankfurters that are not reheated prior to consumption are 'high-risk" food vehicles and are predicted to be responsible for the majority of listeriosis cases in the U.S. (U.S. FDA/FSIS/CDC, 2003).

L. monocytogenes is comprised of four genetic lineages, including two major lineages (termed lineages I and II) that are common at the population level along with two minor lineages (termed lineages III and IV) that are more rare in general (Orsi et al., 2008; Ward et al., 2008). Molecular epidemiology studies suggest that these genetic lineages differ in their pathogenic potential as well as host and tissue preferences (Grey et al., 2004; Nightingale et al., 2005; Nightingale et al., 2007; Pohl et al., 2007). More specifically lineage I strains are overrepresented among human listeriosis cases despite a more frequent exposure to lineage II strains in contaminated RTE foods (Grey et al., 2004; Ward et al., 2004; Nightingale et al., 2005). Additionally, mouse cell plaquing assays, a common *in vitro* virulence phenotype assay used to measure cell-to-cell spread within a host, demonstrated that lineage I strains form significantly larger plaques than lineage II strains, suggesting that lineage I strains may have enhanced pathogenic potential (Grey et al., 2004; Nightingale et al., 2007).

Nearly 95% of human listeriosis cases appear to be caused by three (i.e., serotypes 1/2a, 1/2b and 4b) of the thirteen serotypes that *L. monocytogenes* isolates may be

grouped into, where serotype 4b strains are responsible for the majority of listeriosis outbreaks worldwide (McLauchlin et al., 1990, Kathariou, 2002). Serotype 4b includes highly clonal strains (termed epidemic clone strains), which have been implicated in listeriosis outbreaks on multiple continents and are overrepresented among sporadic listeriosis cases in some countries including the U.S. (Kathariou, 2002). On the other hand, 18 distinct single nucleotide polymorphisms (SNPs) leading to a premature stop codon (PMSC) in the key virulence gene *inlA* have been described worldwide (Van Stelten et al., 2010). Internalin A (InlA; encoded by *inlA*) is a bacterial cell wall anchored virulence factor that binds certain isoforms (i.e., human and guinea pig but not mouse and rat) of the cellular receptor E-cadherin, facilitating the subsequent uptake of the bacterium by intestinal epithelial cells during the initial stages of an infection (Lecuit et al., 1997). Strains harboring a PMSC in *inlA* produce a truncated and secreted form of InlA and have been associated with attenuated invasion of intestinal and hepatic cell lines *in vitro* as well as attenuated mammalian virulence as demonstrated through intragastric guinea pig infection experiments (Nightingale et al., 2008). A recently described *inlA* SNP genotyping assay showed that 45% of *L. monocytogenes* isolates from RTE foods but < 5% of human clinical isolates, carry a PMSC mutation in *inlA*. Collectively, these studies support that *L. monocytogenes* contains two subpopulations including (i) EC strains, which have been linked to multiple listeriosis outbreaks and commonly associated with sporadic cases, and may thus be characterized by enhanced pathogenic potential, and (ii) strains carrying unique virulence-attenuating mutations in *inlA* that are common in RTE foods but only associated with human disease on rare occasions.

Based on these previous molecular epidemiology and pathogenesis studies, we hypothesized that these two subpopulations adapted to different niches, where EC strains evolved to become better adapted to infect humans and strains harboring a PMSC in *inlA* adapted to survive in foods or infect alternative hosts where interactions between InlA and E-cadherin are not important for pathogenesis (e.g., mice and rats). In order to test this hypothesis, we assembled a strain set comprised of four EC strains and four strains carrying a PMSC in *inlA*. We subsequently subjected this strain set to a number of experiments simulating both a human host and food environments to probe the hypothesis that EC and *inlA* PMSC strains are adapted to a human host or environmental associated niches, respectively. Results from this study provide important insight into pathogenic potential along with survival and growth characteristics of *L. monocytogenes* strains commonly found in foods as well as those commonly associated with human disease.

3. 3 MATERIALS AND METHODS:

Bacterial Strains. Epidemic clone strains (CSUFSL N1-074, CSUFSL N1-072, CSUFSL N1-064, and CSUFSL N1-054) were chosen because these four strains were previously shown to be highly invasive across two cell lines, including HepG2 and CaCo-2 under two different bacterial growth conditions (Roberts, et al., 2009). Strains harboring a PMSC in *inlA* (CSUFSL N1-021, CSUFSL N1-040, CSUFSL N1-061, and CSUFSL N1-080) were selected to represent the most common PMSC mutation types observed among *L. monocytogenes* isolates from RTE foods in the U.S. (Nightingale et al., 2005; Orsi et al., 2007). The standard laboratory control strain (10403S) was

included in all experiments (Table 1). Frozen stocks of all strains were maintained in 15% glycerol and stored at -80°C.

37°C Growth Curves. Growth curves were first performed at 37°C, which corresponds to the human body temperature and represents a typical incubation temperature for *L. monocytogenes*, in order to detect any overall growth deficiencies. Strains were streaked out from frozen stocks on to Brain Heart Infusion agar (BHI; Benton Dickson, Sparks, MD). A single well-isolated colony was used to inoculate a 5 ml tube of BHI broth, and this culture was grown overnight (12 to 18 h) at 37°C with shaking (250 rpm). Following overnight incubation, a 1% volume of the culture was transferred to a new 5 ml BHI tube. This tube was incubated at 37°C and grown to a target optical density $(O.D.)_{600}$ of 0.4 as determined by a spectrophotometer (Spectronic 20D). An adjusted 1% inoculum (based on actual O.D. reading) was transferred to side arm flasks containing 50 ml of BHI. $O.D._{600}$ measurements of the 50ml cultures were taken every hour for 12 h, and bacterial populations were enumerated by plating appropriate serial dilutions in duplicate at the following time-points: T=0, time where $O.D._{600}$ reached approximately 0.4 (mid-log phase), 8, 10, and 12 h after inoculation of the side arm flask. Plates were incubated at 37°C for 24 h and resultant colony forming units (CFU) were counted. At least two independent growth curves were performed for each strain.

Intracellular Growth Assays. Intracellular growth experiments were performed to determine the ability of EC and PMSC *L. monocytogenes* strains to grow intracellularly. The J774 mouse macrophage cell line was used for intracellular growth assays, as macrophages are phagocytic cells, and do not require a functional InlA protein for uptake. Semi-confluent J774 monolayers grown in Dulbecco's Modified Eagle Medium

(DMEM) were infected with approximately 6.5 $\log_{10}$ CFU of each respective *L. monocytogenes* strain in duplicate and after 30 minutes of incubation at 37˚C and 5% CO_2 growth media was replaced with media containing 150 μg/ml gentamicin. At 1.5, 5, 9, and 12 h post-inoculation cell monolayers were washed three times with phosphate buffered saline (PBS), and then lysed with cold deionized water. Lysed cell suspensions and overnight cultures used for inoculation were serially diluted and spread plated in duplicate in order to enumerate the intracellular bacterial and actual inoculation populations, respectively. The intracellular growth of each strain was characterized in at least three independent experiments. The numbers of intracellular bacteria determined for these four time points were averaged across duplicate wells infected with a single strain for a given experiment and log transformed. The amount of intracellular bacteria was calculated by subtracting the log CFU bacteria in the inoculum minus the log CFU recovered as intracellular bacteria. These values were then normalized to the control strain 10403S.

Cytotoxicity Assays. In order to characterize the cytotoxicity of the isolates in our strain set, EC and PMSC strains were characterized by an *in vitro* cytotoxicity assay. Lactate dehydrogenase (LDH), an enzyme released upon lysis of cells, is often used as an indicator of cytotoxicity (Promega, Technical Bulletin #TB 163). Semi-confluent J774 monolayers were inoculated with approximately 6.5 $\log_{10}$ CFU of each *L. monocytogenes* strain of interest. Thirty minutes after inoculation, media was removed and replaced with media containing 150 μg/ml gentamicin. We quantified LDH levels in the cell supernatant at 1.5, 5, 9, and 12 h post inoculation using a commercial cytoxicity assay (Cytotox 96 Non-Radioactive cytotoxicity assay; Promega, Madison, WI) according to

manufacturer instructions and absorbance was read at 490 nm. Duplicate wells were inoculated with each strain and cytotoxicity of each strain was characterized in at least two independent experiments.

Invasion Assays. Invasion assays with a non-phagocytic mouse fibroblast cell line (L929), which does not express an isoform of E-cadherin that binds InlA, were performed to provide insight into invasion where InlA-mediated invasion is not a factor. Duplicate wells of semi-confluent monolayers of L929 cells were inoculated with approximately 7.0 $\log_{10}$ CFU of stationary phase *L. monocytogenes* strains, grown at 30°C without shaking (a condition shown to promote invasion facilitated by other Internalins; Kim et al., 2004). At 30 min post-inoculation, old media was removed from L929 cells and replaced with fresh growth media, and adherent bacteria were allowed an additional 15 minutes to invade L929 cells before media with 150 μg/ml gentamicin was added to kill all extracellular bacteria. After 90 minutes following infection, media was aspirated, L929 cells were washed with PBS, and lysed with cold deionized water. Intracellular bacterial populations and overnight cultures were enumerated by plating appropriate serial dilutions on BHI in duplicate. Percent invasion was calculated by dividing intracellular bacteria population by the population of bacteria used to inoculate L929 monolayers, and multiplying by 100. The L929 invasion efficiency of each strain was assayed in at least three independent experiments.

Low Inoculum Cold Growth Curves. A number of cold growth curves were performed at 7°C under various conditions (i.e., three different pH levels, with or without the addition of NaCl) to simulate food-associated environments. Initially growth curves were performed in BHI broth at a typical growth medium pH of 7.4, and this pH is typical of

what the bacterium encounters in the bloodstream. To simulate food environments we also performed growth curves in BHI broth, where the pH was adjusted to 5.5 and 6.5 to mimic to pH of RTE meats. Sodium chloride is commonly used to preserve and add flavor to food, so we performed growth curves with 2.2% (w/v) added NaCl in BHI broth under the three pH levels listed above.

Overnight cultures were prepared as described previously, for 37˚C growth curves, and a 1% inoculum of these overnight cultures were transferred to tubes containing 9.9 mls of BHI broth, and grown to O.D. $_{600}$ 0.4. An adjusted 25% inoculum was then transferred to tubes containing 7.5 mls of pre-chilled BHI broth. Cultures were serially diluted in pre-chilled BHI broth tubes to yield cultures in BHI with approximately 2.0 $\log_{10}$ CFU/ml. These tubes were then incubated at 7˚C until bacterial populations reached stationary phase and began to die off (18 d for pH 7.4 and 6.5 conditions, and 24 d for the pH 5.5 conditions). Aliquots were removed every three days throughout each set of cold growth experiments, serially diluted, and spread onto BHI plates in duplicate in order to enumerate resultant bacterial populations. Exponential growth rates were computed as previously described (Madigan et al., 2003) using $\log_{10}$ CFU/ml values observed at each time point. Two independent experiments were performed to characterize the growth characteristics of each strain under each condition.

Inoculation Studies with Deli Turkey. In order to assess the ability of these strains to grow in an actual food matrix, we performed inoculation studies for each EC and PMSC strain using deli turkey. Deli turkey meat (formulated without antimicrobials to control *L. monocytogenes* growth during refrigerated storage) was provided by a large commercial RTE meat processor for inoculation studies. Turkey was frozen upon receipt

at -20˚C, and thawed for 5 days at 4˚C prior to inoculation. Turkey was sliced (15mm thickness) and cut into square pieces (5 cm by 5 cm). Overnight cultures were prepared as previously described for 37°C growth curves. Following incubation overnight, a 1% inoculum was transferred to a fresh BHI tube, and following growth to O.D. $_{600}$ 0.4, an adjusted 1% inoculum was transferred to a new BHI tube, and grown for 10 hours at 37˚C. Next, cells were pelleted in a microcentrifuge at maximum speed for 10 minutes, and cell pellets were re-suspended in 1ml PBS. Cultures were subsequently diluted and applied to both sides of individual turkey slices, in a biological safety cabinet to yield approximately $1x10^3$ to $1x10^4$ CFU/cm^2. After inoculation of each side of the turkey piece, pieces were allowed to dry for 5-10 minutes. Two slices of turkey were placed on top of each other and vacuum-sealed in one bag, and bags were then incubated at 7˚C for 28 d. Two pieces of un-inoculated turkey were stacked and sealed in a vacuum bag as a negative control, and a sufficient number un-inoculated controls were prepared for each sampling day. On each individual sample collection (0, 2, 4, 6, 8, 12, 20, and 28 days post inoculation), one vacuum bag inoculated with each strain was removed and microbiologically analyzed. Briefly, turkey pieces were placed individually into whirl-pack bags containing 100 mls of PBS. These bags were shaken vigorously by hand for 30 seconds and then appropriate serial dilutions were performed and spread plated onto Modified Oxford plates (Oxoid, Cambridge, UK) for enumeration and plates were incubated at 37°C for 48 h. Additionally, on each sampling day an un-inoculated bag of deli turkey was opened and one piece of turkey was microbiologically analyzed as described above, while the other piece of turkey was subjected to chemical analysis. The pH of un-inoculated control samples on each collection day was determined as per the

procedure recommended by the American Association of Analytical Chemists (981.12), and water activity (A_w) was measured using an AquaLab water activity meter (Decagon Devices, Pullman, WA). Resultant bacterial populations were analyzed by fitting growth data for individual strains to the Gompertz equation, and computing Exponential Growth Rates (EGR) and Lag Phase Duration Time (Lag) (Whiting et al., 2001). At least two individual experiments were performed for each strain.

Statistical Analyses. The mixed model procedure as implemented in Statistical Analyses Software (SAS; Cary, NC) was used to analyze data from intracellular growth and cytotoxicity assays. Experimental replicate was included in the model as a random variable, while strain, genotype (EC or PMSC), time point, and the interactions genotype*time point and strain*timepoint were included as fixed variables. The least square means statement was used to generate *P*-values for the genotype*time point interaction, to elucidate differences between EC and PMSC strains within each time point. One-way analysis of variance (ANOVA) was used to analyze differences in invasion efficiencies of EC and PMSC strains for L929 cells. Dunnett's test was then performed to compare the L2 invasion efficiency of each EC and PMSC strains to that of the standard laboratory control strain 10403S. One-way ANOVA was performed to compare EC and PMSC strain exponential growth rates within each cold growth condition (i.e., pH level and salt). For deli turkey meat inoculation experiments, EGR and Lag estimate for PMSC and EC strains did not satisfy the assumption of normality and were thus compared using the Krusikal-Walis Test, a non-parametric alternative to the T-test. All comparisons where $P < 0.05$ were considered to be statistically significant.

3.4 RESULTS AND DISCUSSION:

L. monocytogenes carrying a mutation leading to a PMSC in *inlA* accounted for approximately 45% isolates from >30,000 RTE food samples, suggesting this subpopulation may be better adapted to the food-associated environments (Gray et al., 2004; Van Stelten et al., 2010). On the other hand, EC strains have been implicated in most listeriosis outbreaks worldwide to date and are overrepresented among sporadic cases in the U.S. (Gray et al., 2004; Kathariou, 2002). We thus hypothesized that EC and PMSC strains may have preferentially adapted to different niches, and we probed this hypothesis by characterizing the survival, growth and *in vitro* virulence phenotypes of these two *L. monocytogenes* subpopulations in various host, food-associated and food environments. We subjected a representative set of *L. monocytogenes* strains (i.e., four EC, four PMSC, and a standard laboratory control strain) to a battery of assays that mimic host and food niches. We performed intracellular growth and cytotoxicity assays in a macrophage cell line to evaluate the virulence phenotype of these strains. In addition we performed invasion assays in mouse fibroblast cells, to evaluate the ability of these strains to invade cells of a potential alternative host, where InlA does not contribute to virulence. Additionally, broth culture growth studies were performed to evaluate the effects of some intrinsic properties of food (temperature, pH and salt) on the growth of PMSC and EC strains. Lastly, we performed inoculation studies in deli turkey to evaluate the ability of our strains to grow in an actual food matrix. Results from this study support that (i) *L. monocytogenes* EC and PMSC strains show similar growth characteristics in broth culture, under all conditions tested, (ii) *L. monocytogenes* EC and

PMSC strains demonstrate significant differences in some *in vitro* virulence phenotype assays, and (iii) *L. monocytogenes* PMSC strains demonstrate accelerated exponential growth rates on RTE turkey deli meat, while maintaining similar lag phase duration times. Overall, our findings suggest that *L. monocytogenes* subpopulations adapted to different niches, where (i) PMSC strains appear to be better suited for survival and growth in RTE foods and to infect alternative host species that do not express an isoform of E-cadherin that binds InlA and (ii) EC strains demonstrate *in vitro* virulence phenotypes consistent with enhanced pathogenic potential.

L. monocytogenes EC and PMSC strains show similar _in vitro_ growth characteristics.

Growth curves in BHI broth at 37°C were conducted to probe any overall deficiencies among the four EC and four PMSC strains included in this study, relative to the standard laboratory control strain 10403S. Any general deficiency in growth could complicate interpretation of downstream experiments to probe *in vitro* virulence phenotypes, conditions to simulate food-associated stresses and food matrix inoculation studies. Notable differences in growth were not observed based on $O.D._{600}$ measurements collected every hour (Figure 1) or by resultant bacterial cell counts obtained from five time points over the course of this assay (data not shown).

Cold growth curves were conducted in BHI broth formulated to represent a variety of pH conditions with or without additional salt to simulate concentrations in RTE foods. The EGR of each strain grown under each condition was calculated and compared amongst strains within a given cold growth condition. Not surprisingly there were differences in EGRs observed among conditions (Table 2; Overall F-Test, $P < 0.001$).

However, differences in EGRs between strains within each cold growth condition were not observed (Overall F-Test, $P > 0.05$ within each cold growth condition).

Results presented by van der Veen and co-workers (2008) support that *L. monocytogenes* strains belonging to serotype 4b, which includes EC strains linked to multiple listeriosis outbreaks, may be less well suited for growth in environments simulating food-associated stresses (i.e., acidic pH, salt, and refrigeration). However, *L. monocytogenes* strains carrying a PMSC in *inlA* (serotype 1/2a, 1/2b, and 1/2c) and EC strains (serotype 4b) characterized in this study did not show discernable differences in cold growth conditions designed to simulate environments associated with food stresses.

***L. monocytogenes* EC and PMSC strains demonstrate significant differences in some *in vitro* virulence phenotype assays.** Intracellular growth assays in mouse macrophage cells were conducted to evaluate the abilities of PMSC and EC strains to survive and grow intracellularly, a critical component in *Listeria monocytogenes'* intracellular life cycle and pathogenesis of listeriosis. Both *L. monocytogenes* subpopulations (i.e., PMSC and EC strains) demonstrated similar intracellular populations at each time point assayed throughout intracellular growth experiments. For example, at 1.5 hours after inoculation EC strains showed $\log_{10}$ transformed percent intracellular populations that ranged from 1.96 to 2.00, while PMSC strain populations that ranged 1.97-2.02 (Figure 2), supporting that both subpopulations were internalized by this mouse macrophage cell line. At 12 h following inoculation $\log_{10}$ transformed percent intracellular populations ranged from 2.00 to 2.04 for EC strains and 2.03 to 2.22 for PMSC strains. Upon statistical analyses of intracellular growth assay data, main effects and interactions were tested in a mixed model, where experimental replicate was included as a random variable. The class

variables, genotype (with or without a PMSC in *inlA*) and time point (1.5, 5, 9, and 12 h after inoculation) were included in the mixed model as fixed effects. Strain was marginally significant ($P < 0.0969$) and there was a marginally significant interaction between genotype and time point ($P < 0.0927$); however, none of the comparisons between PMSC and EC strain groups within each time point were statistically significant (Figure 2; $P > 0.05$). These data suggest that EC and PMSC groups survive and grow similarly within mouse macrophage cells. The *Listeria* Pathogenicity Island-1 (LIP-1) in *Listeria monocytogenes* encodes genes involved in *L. monocytogenes'* intracellular life cycle. This pathogenicity island encodes hemolysin (encoded by *hly*), phospholipases (encoded by *plcA* and *plcB*), a metalloprotease (encoded by *mpl*), ActA (encoded by *actA*), for the polymerization of actin, and positive regulatory factor A (PrfA; encoded *prfA*) the global virulence gene transcriptional regulator (Vásquez-Boland et al., 2001). Our results showed that EC and PMSC strains survived and grew similarly intracellularly in macrophage cells, and as a result, there is no evidence supporting additional defects within the LIPI-1 pathogenicity island for *L. monocytogenes* strains carrying a PMSC in *inlA*. Retention of a functional LIPI-1 may in part explain the ability of strains carrying a PMSC in *inlA* to cause human disease, albeit on very rare occasions (Nightingale et al., 2005; Van Stelten et al., 2010). *L. monocytogenes* strains encoding a truncated and secreted form of InlA have been implicated in bacterimia and central nervous system infections but never pregnancy cases, suggesting that a fully functional InlA may be necessary for this route of infection (Jacquet et al., 2004).

Cytotoxicity assays were performed in order to determine if there were differences among PMSC strains and EC strains with respect to toxicity for mouse

macrophage cells. The first time point showed evidence of similar levels of cytotoxicity in EC and PMSC groups. Specifically, EC strains showed LDH release levels ranging from 0.258-0.523, while PMSC strains had values ranging from 0.245-.504 at 1.5 h after inoculation. However, by the end of the experiment, 12 h following inoculation with *L. monocyotogenes* strains, PMSC strains showed higher LDH release levels, suggesting greater cytotoxicity, where LDH release values ranged from 0.0.836 to 2.177 for PMSC strains and EC strain LDH levels were between 0.621 to 1.324.

Statistical analyses showed that all main effects and interactions were significant individually (genotype: $P<0.001$, strain: $P<0.001$, timepoint: $P<0.001$) and in the multivariate model (genotype*timepoint: $P<0.001$ and strain*timepoint: $P=<0.0001$). *L. monocytogenes* strains carrying a PMSC in *inlA* demonstrated significantly higher cytotoxicity at 9, and 12 h following inoculation (Figure 3; F-Test, $P = 0.0028$, and $P < 0.0001$, respectively). Previous studies showed that genetically engineered mutants designed to over-express listeriolysin O (LLO; expressed by *hly*), the chief virulence factor involved in cytotoxicity, were avirulent in a mouse model, upon injection through the tail vein (Glomski et al., 2003). The observation in the current study that PMSC strains demonstrated greater cytoxicity as compared to EC strains is likely explained by the intracellular lifestyle of *L. monocytogenes*. As an intracellular pathogen, killing off the host cell is a disadvantage, because the bacterial cell becomes exposed to the extracellular host immune defenses. Therefore, a reduced level of cytotoxicity as demonstrated by EC strains may be advantageous.

Invasion assays using the mouse L2929 fibroblast cell line, were conducted to assess the abilities of PMSC and EC strains to invade an alternative host, where the

interaction between InlA and E-cadherin does not contribute to pathogenesis as the murine isoform of E-cadherin does not bind InlA. Overall, PMSC strains demonstrated higher invasion efficiencies for L929 cells as compared to EC strains, where PMSC showed an average invasion efficiency of 0.047% (standard deviation of 0.016) and EC strains showed an average invasion efficiency of 0.027% invasion (standard deviation of 0.018; Figure 4). A one-way ANOVA using percent invasion as the response variable was performed to test for differences among PMSC and EC strains to actively invade murine fibroblast cells. A significant strain effect was observed (Overall F-test, $P < 0.001$). The invasion efficiencies for each strain were then compared to the laboratory control strain 10403S and all four PMSC strains demonstrated significantly higher invasion of murine L2 cells when compared to the control. Contrastingly, one EC strain (CSUFSL N1-074) showed significantly higher invasion of L2 cells than the control (Figure 4; Dunnett's Test, $P < 0.05$), while all other EC strains demonstrated L2 cell invasion efficiencies similar to the control strain.

L. monocytogenes strains carrying a mutation leading to a PMSC in *inlA* thus show potential to efficiently infect alternative host species such as mice and rats that encode an isoform of E-cadherin that does not bind InlA. It is possible that InlA, may be camouflaging other proteins on the cell surface (e.g., Internalin B), which may be responsible for the increased invasion of PMSC strains in these L929 cells. Evidence of protein camouflaging has been documented in other bacterial pathogens such as *Streptococcus*. *Streptococcus pyogenes* and *Streptococcus agalactiae* express leucine-rich repeat proteins (LRRs), Slr and Blr, respectively which are related to *Listeria monocytogenes* InlA. Slr binding antibodies react weakly with *S. pyogenes*, which

encodes the proteins M protein and F protein but this antibody binding is much higher in the absence of M or F protein, suggesting that these proteins may camouflage the Slr protein. The Blr protein in *S. agalactae*, on the other hand appears to be camouflaged by the capsule (Waldermarsson et al., 2006). Our results suggest that expression a truncated and secreted form of InlA may represent a molecular mechanism that facilitates recognition of other virulence factors that may contribute to invasion of alternative host species where InlA mediated invasion is not relevant.

***L. monocytogenes* carrying a PMSC in *inlA* demonstrate accelerated exponential growth rates on RTE turkey deli meat as compared to EC strains.** Observed bacterial populations for each strain at each time point for turkey deli meat inoculation experiments were fitted to the Gompertz equation, thus allowing calculating exponential growth rates (EGRs) and lag phase duration time for each strain at each time point. A Kruskal-Wallis test revealed a marginally significant overall difference in EGRs attributed to the eight *L. monocytogenes* strains evaluated ($P = 0.0519$) in a turkey deli meat matrix. To further test the differences between PMSC and EC strain groupings, another test was performed where genotype (with or without a PMSC in *inlA*) was included as the class variable. Furthermore, *L. monocytogenes* strains carrying a PMSC in *inlA* demonstrated significantly higher EGR in a food matrix overall as compared to EC strains (Figure 5; $P = 0.0243$). These findings are consistent with a previous report describing that strains associated with a PMSC in *inlA* (i.e, ribotypes DUP-1062A and DUP-1039C) were present at significantly higher levels in RTE foods (CFU/serving) of 2.04×10^3 and 1.29×10^6, respectively, as compared to EC strain ribotypes 1042B and 1038B, which were found at a combined level of 8.92×10^1 (Chen et al., 2006). Our work,

which indicates that PMSC strains have higher EGRs than EC strains, may explain the previously observed increased concentrations of *L. monocytogenes* strains carrying a PMSC in *inlA* in RTE foods as compared to EC strains.

Similar tests were performed where lag phase duration time was used as the response variable and the effect of strain was marginally significant ($P = 0.0661$), while the effect of genotype (with or without PMSC) was not significant (Figure 6; $P = 0.3938$). This suggests that variability among the lag times exists; however, these differences are not associated with the presence of a PMSC in *inlA* or belonging to an EC strain grouping. Observed pH and water activity measurements did not show notable changes over the course of the 28-day experiment. Specifically, pH values across three independent experiments averaged 6.39, over the entire sampling period, with a standard deviation of 0.15, while water activity across three independent experiments averaged 0.974 with a standard deviation of 0.004.

3.5 CONCLUSIONS.

In summary, *L. monocytogenes* EC strains and strains carrying a PMSC in *inlA* demonstrated some evidence of niche adaption. More specifically, EC strains and PMSC strains show some differences in terms of their *in vitro* virulence characteristics, where EC strains demonstrated decreased cytotoxicity, suggesting that they may be better adapted to sustaining an intracellular life cycle. *L. monocytogenes* strains carrying a PMSC in *inlA*, on the other hand, showed increased active invasion of murine fibroblast cells, indicating that while they may not be suited to infect and cause disease in a human host, they may be better apt to infect mice, and potentially other hosts where a full-length InlA is not required for invasion. *L. monocytogenes* EC and PMSC strains showed

similar growth rates in broth culture growth experiments simulating food-associated stresses (i.e., refrigeration, reduced pH, and salt); however, strains carrying virulence-attenuating PMSC mutations in *inlA* as a whole showed accelerated exponential growth rates on deli turkey meat as compared to EC strains, suggesting that PMSC strains may be better adapted to utilize of nutrients in this particular matrix and thus may be adapted to grow on RTE foods. Further studies to elucidate the relationship between *L. monocytogenes* strains in a broader array of environments will be necessary in order to come to a more comprehensive understanding of *L. monocytogenes* niche adaptation. More virulence phenotype assays, such as plaque assays, and invasion assays in other cell lines, will be necessary to further probe the virulence characteristics of these strains as well as their host preference. Growth on food matrices other than deli turkey meat should be investigated, to determine if the trends we see in growth on turkey holds true for other foods. Future studies on the global transcriptional analyses for EC and PMSC strains grown in host- and food-associated conditions will provide additional insight in the molecular mechanisms that facilitate niche adaptation of these two *L. monocytogenes* subpopulations.

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Table 3.1: Description of strains used in this study

Strain	Identification	Lineage	Serotype	Ribotype	Description	Isolated From
CSUFSL N1-021	FSL F2-563	I	1/2 b	DUP-1052A	Invasion Attenuated, mutation type 1	CSF Human
CSUFSL N1-040	FSL F2-515	II	1/2 a	DUP-1062A	Invasion Attenuated, mutation type 3	RTE Turkey
CSUFSL N1-061	FSL R2-074	I	1/2 b	DUP-1025A	Invasion Attenuated, mutation type 2	Deli Salad
CSUFSL N1-080	FSL F2-640	II	1/2 c	DUP-1039C	Invasion Attenuated, mutation type 4	Pork Sausage
CSUFSL N1-074	FSL J1-012	I	4b	DUP-1038B	Enhanced Invasion, Epidemic Clone I	Human Outbreak
CSUFSL N1-072	FSL J1-123	I	4b	DUP-1038B	Enhanced Invasion, Epidemic Clone I	Human Outbreak
CSUFSL N1-	FSL J1-220	I	4b	DUP-	Enhanced Invasion,	Human Outbreak

064				1042B	Outbreak Strain	
CSUFSL N1-054	FSL N1-225	I	4b	DUP-1044A	Enhanced Invasion, Epidemic Clone II	Human Outbreak
CSUFSL N1-051	10403S	II	1/2 a	DUP-1030A	Standard Laboratory Control	

Information on strains was obtained via queries of the PathogenTracker database (Fugett et al, 2006).

Table 3.2: Table 2: Mean exponential growth rates of *Listeria monocytogenes* strains at 7°C.

pH	NaCl (%)	Exponential Growth Rate (Log CFU/day)
7.4	0.0	0.704^{a}
7.4	2.2	0.586^{b}
6.5	0.0	0.569^{b}
6.5	2.2	0.547^{b}
5.5	0.0	0.422^{c}
5.5	2.2	0.366^{c}

Mean exponential growth rates are an average of data all eight *Listeria monocytogenes* strains over two replicate experiments (n=16). Means lacking common superscripts are different ($P < 0.05$)

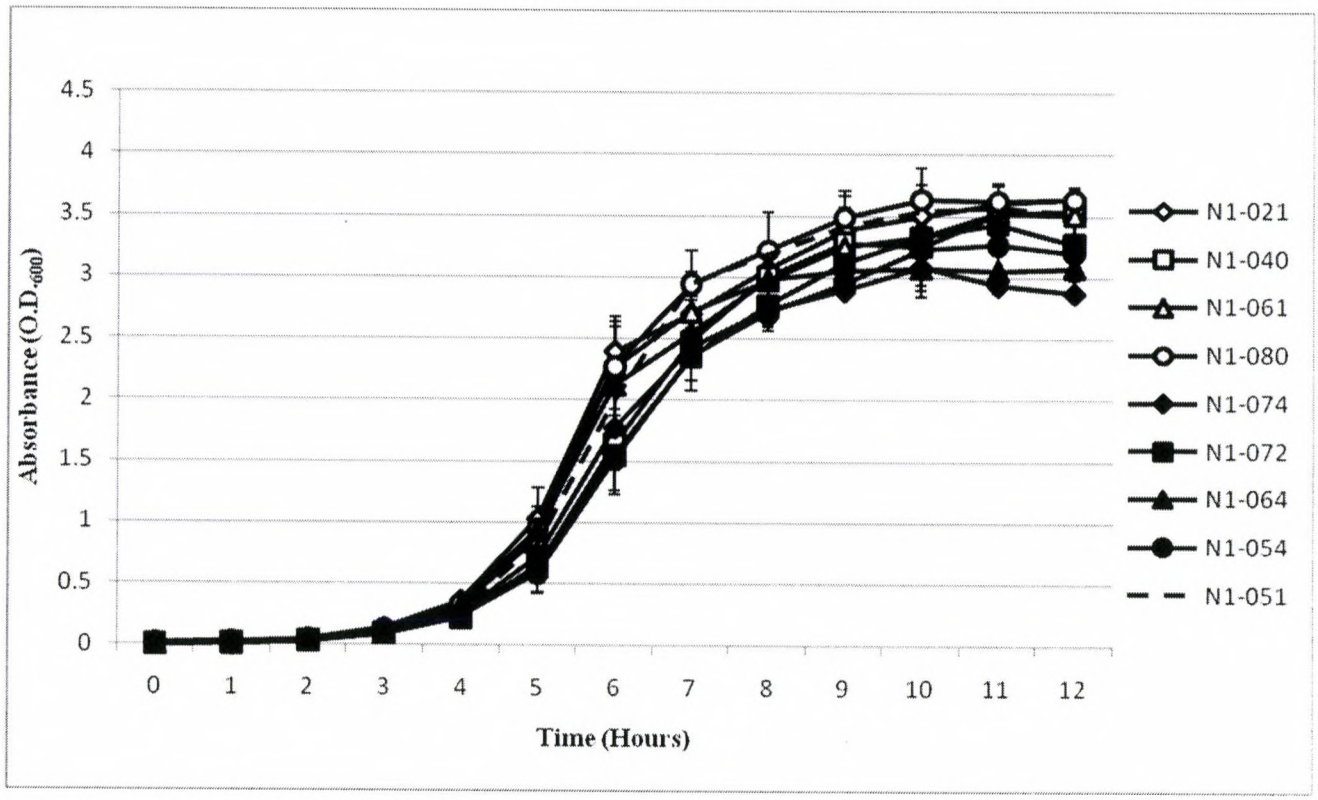

FIG 3.1. This figure demonstrates the growth of all strains at 37°C with shaking in BHI. Optical Density measurements were taken each hour over the course of a 12 hour experiment. The X-axis denotes the hours following inoculation of the flask. Each dot represents the level of *Listeria* monocytogenes for that strain at that particular day, which was the average of at least three independent experiments. Error bars indicte standard deviations around these averages. PMSC strains are denoted by open symbols, while EC strains are denoted by filled in symbols. The dashed line corresponds to N1-051, the control strain used in this study. All strains were found to grow similarly over the course of the 12 hour experiment.

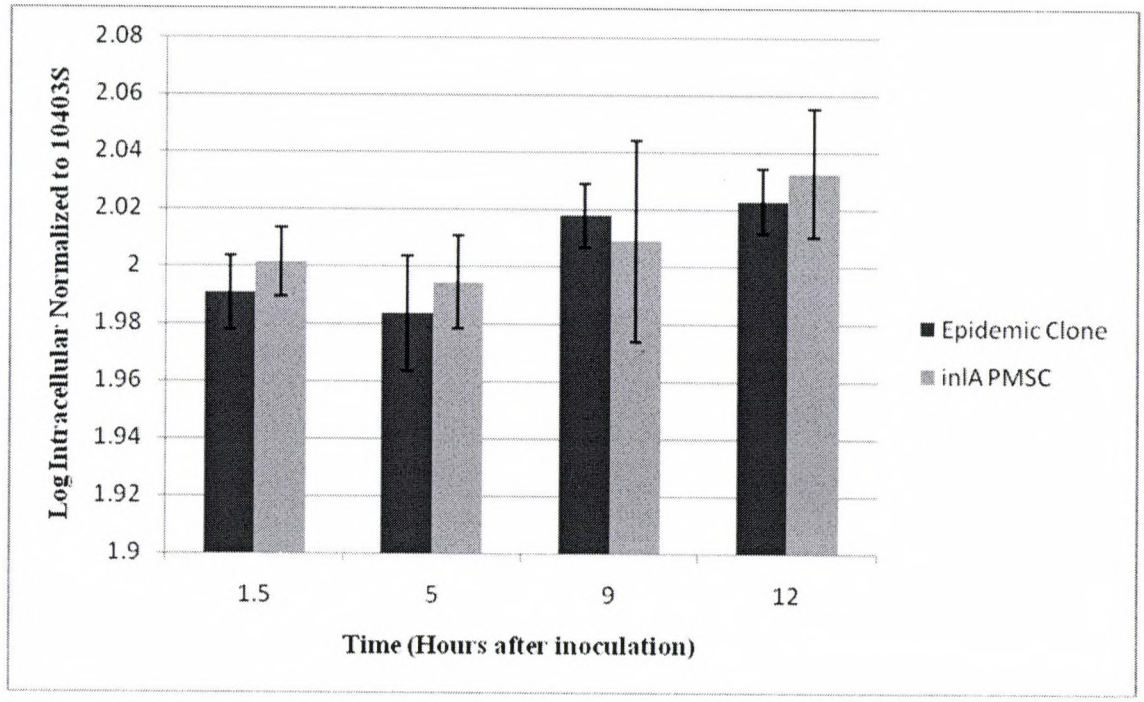

FIG 3.2. Intracellular survival and growth for *L. monocytogenes* epidemic clone strains (EC; indicated by black columns) and strains carrying a premature stop codon (PMSC; indicated by gray columns) in *inlA* for the murine J774 macrophage cell line. The Y-axis indicates $\log_{10}$ transformed percent intracellular populations for each EC and PMSC strain normalized to the laboratory control strain 10403S. The X-axis denotes each post-inoculation time point where intracellular *L. monocytogenes* populations were enumerated. Each strain was assayed in duplicate well over at least three independent experiments. Columns indicate mean $\log_{10}$ transformed intracellular populations for EC and PMSC strains each post-inoculation time point and error bars indicate standard deviations around the mean. Statistical analyses by the mixed model procedure showed that intracellular survival and growth in J774 murine macrophage cells was similar ($P > 0.05$) between EC and PMSC strains across all time points.

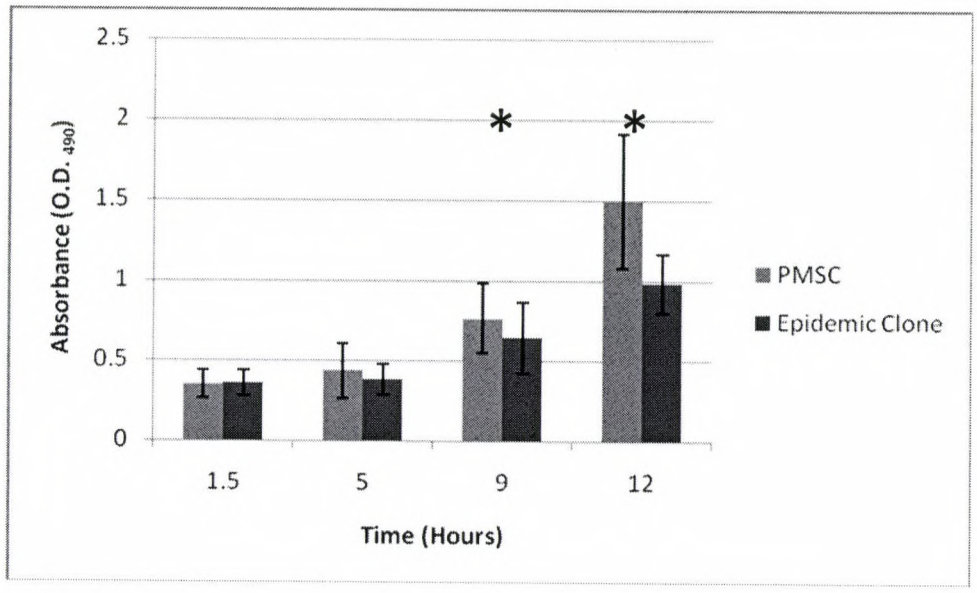

FIG 3.3. Cytotoxicity of *L. monocytogenes* epidemic clone strains (EC; indicated by black columns) and strains carrying a premature stop codon (PMSC; indicated by gray columns) in *inlA* for the murine J774 macrophage cell line. The Y-axis indicates O.D. $_{490}$ which corresponds to the cytotoxicity of EC and PMSC strains. The X-axis denotes each post-inoculation time point where intracellular *L. monocytogenes* cytotoxicity was evaluated. Each strain was assayed in duplicate wells over at least two independent experiments. Columns indicate mean O.D. $_{490}$ for EC and PMSC strains each post-inoculation time point and error bars indicate standard deviations around the mean. Statistical analyses by the mixed model procedure showed that cytotoxicity in J774 murine macrophage cells were different ($P< 0.05$) between EC and PMSC at 9, and 12 hours post inoculation.

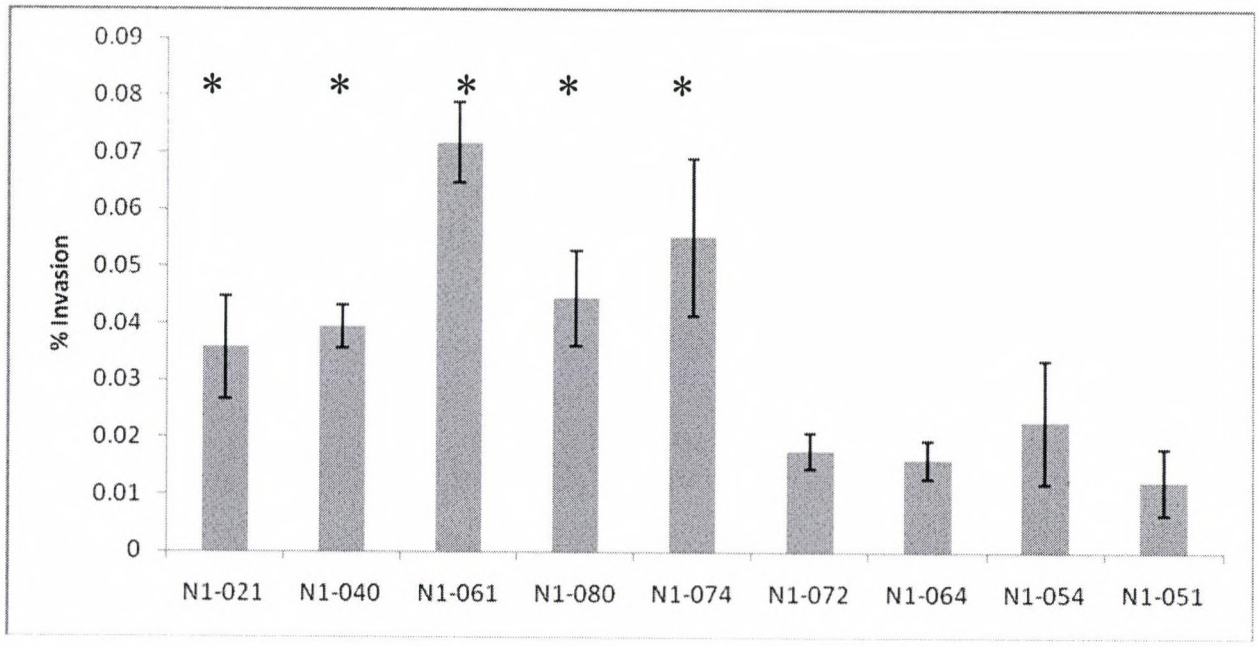

FIG 3.4. Invasion efficiencies of *L. monocytogenes* epidemic clone strains and strains carrying a premature stop codon in *inlA* for the murine L929 macrophage cell line. The Y-axis indicates invasion efficiencies ([intracellular/inoculum] *100) for EC and PMSC strains. The X-axis denotes each strain used in this experiment. Each strain was assayed in duplicate well over at least three independent experiments. Columns indicate mean % invasion for each strain and error bars indicate standard deviations around the mean. Statistical analyses, including a one-way ANOVA followed by a Dunnett's test demonstrated that CSUFSL N1-021, CSUFSL-N1-040, CSUFSL N1-061, CSUFSL N1-080, and CSUFSL N1-074 all demonstrated higher invasion efficiencies than the control strain CSUFSL N1-051 ($P<0.05$).

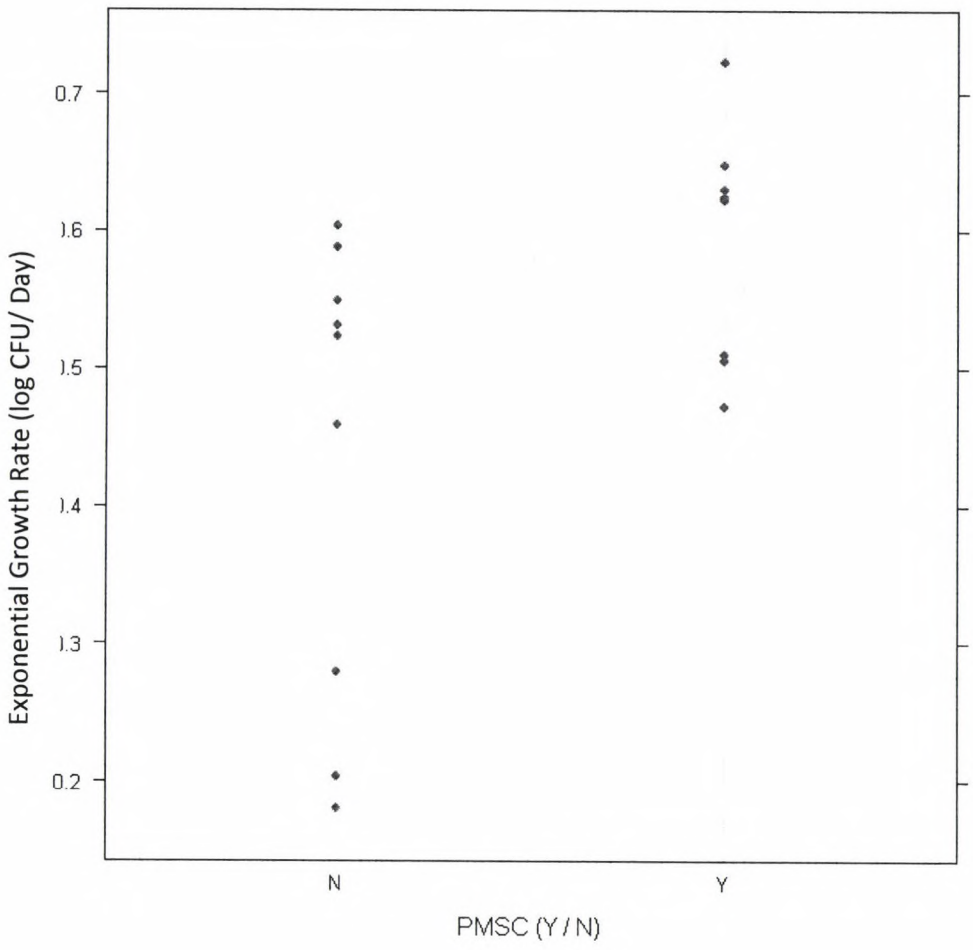

FIG 3.5. Dot plot of Exponential Growth Rates (EGR), for both epidemic clone strains and strains carrying a premature stop codon in *inlA*, on deli turkey. The x-axis denotes the *inlA* PMSC or epidemic clone group. Epidemic clones were denoted with an "N", while *inlA* PMSC strains were denoted with a "Y". Each dot represents the EGR for a given strain in an independent experiment. EGR was computed for each strain in at least two independent experiments. Using the Krusikal-Walis test, differences were found between the *inlA* PMSC and Epidemic Clone groups (P=0.0243).

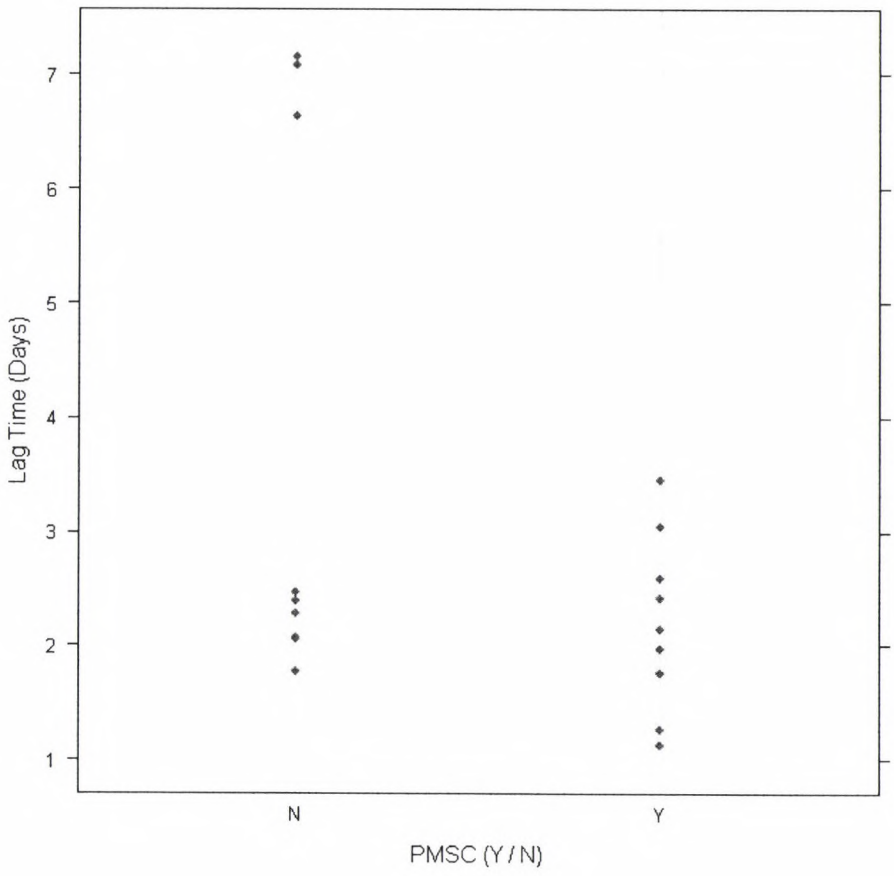

FIG 3.6. Dot plot of lag phase times for both epidemic clone strains and strains carrying a premature stop codon in *inlA*, on deli turkey. The x-axis denotes the *inlA* PMSC or epidemic clone group. Epidemic clones were denoted with an "N", while *inlA* PMSC strains were denoted with a "Y". Each dot represents the lag phase time for a given strain in an independent experiment. Lag was computed for each strain in at least two independent experiments. Using the Krusikal-Walis test, no differences were found between *inlA* PMSC and Epidemic Clone groups (P=0.2332).

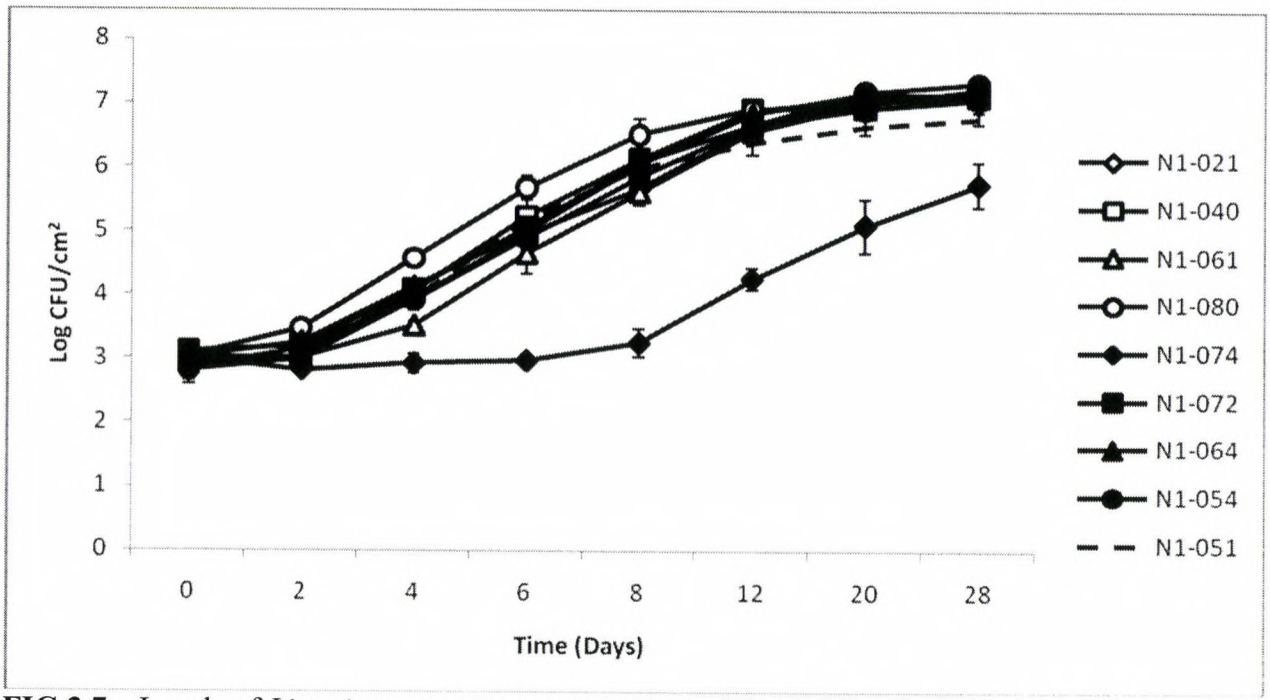

FIG 3.7. Levels of *Listeria monocytogenes* enumerated from deli turkey slices over the course of the 28 day expermiment. The Y-axis indicates the level of *Listeria monocytogenes* on the surface of turkey slices. The X-axis denotes the days following inoculation to turkey slices, on which *Listeria monocyotogenes* strains were enumerated. Each dot represents the level of *Listeria* monocytogenes for that strain at that particular day, which was the average of at least two independent experiments. Error bars indicate the minimum and maximum levels observed each strain at inidivdual time points. *inlA* PMSC strains are denoted by open symbols, while Epidemic Clone strains are denoted by filled in symbols. The dashed line corresponds to N1-051, the control strain used in this study.

CHAPTER IV:

Small Molecule Screening Reveals Structurally Diverse Compounds that Inhibit the Growth of *Escherichia coli* O157:H7 *In Vitro*

4.1 ABSTRACT:

Escherichia coli O157:H7 causes approximately 62,500 foodborne illnesses in the U.S. each year (Mead et al., 1999). A fraction of individuals infected by *E. coli* O157:H7 develop hemolytic uremic syndrome (HUS), a life-threatening condition that can result in kidney failure and death. Treating individuals infected by *E. coli* O157:H7 with certain antibiotics may lead to an increased incidence of HUS; as a result, therapeutics are limited. This demonstrates a clear need to identify novel antimicrobials to reduce the load of this pathogen entering the human food supply and as therapeutics to treat clinical infections. We thus developed a high-throughput turbidometric assay to identify novel chemical compounds that inhibit *E. coli* O157:H7 growth. Specifically, pin transfers were performed to introduce small molecule libraries into 384-well plates, where each well contained approximately 5 $\log_{10}$ CFU of an *E. coli* O157:H7 strain isolated from cattle feces. Plates were incubated for 18 h at 37°C and OD_{600} was measured to determine the effect of each small molecule on *E. coli* O157 growth. Altogether 64,562

compounds were screened in duplicate and 43 compounds inhibited *E. coli* O157:H7 growth. Thirty-eight of the 43 compounds are known bioactive compounds and the other five are novel commercially available compounds. Bioactive compounds that inhibited *E. coli* O157:H7 growth were most frequently classified as cephalosporin (n=13), fluorquinolone (n=12) and tetracycline antibiotics (n=6). One carbapenem, two other antibiotics and an anti-viral and a compound which disrupts the citric acid cycle, also inhibited *E. coli* O157:H7 growth. Lastly, two structurally related known bioactives, both common disinfectants used in a number of non-clinical applications, inhibited *E. coli* O157:H7 growth. In conclusion, we identified a number of antimicrobials, which are both diverse structurally as well as in their modes of action, that effectively inhibit *E. coli* O157:H7 growth. Known bioactive compounds used for non-clinical applications that inhibited *E. coli* O157 growth have potential to be applied as dipping or spray-wash interventions to reduce *E. coli* O157:H7 populations during food processing.

4.2 INTRODUCTION:

Escherichia coli O157:H7 causes roughly 73,480 illnesses each year in the United States. Food serves as a major vehicle for the transmission of *E. coli* O157:H7 to humans; in fact, approximately 85% of all cases are attributed to a foodborne route (Mead et al., 1999). Approximately 4% of reported cases of *Escherichia coli* O157:H7 progress to a very serious disease, Hemolytic Uremic Syndrome (HUS), which can result in kidney failure and death. Cattle are the major natural reservoir for this pathogen and *E. coli* O157:H7 is primarily introduced into the human food supply by fecal contamination

of raw agricultural commodities at the pre-harvest level and during processing (Armstrong et al., 1996). The first major outbreak of illness associated with *E. coli* O157:H7 (in 1982) was linked to ground beef, which remains the most commonly associated food vehicle, implicated in 41% of foodborne outbreaks. Between 1982 and 2002, 52% of outbreaks associated with *E. coli* O157:H7 were of foodborne origin, other major modes of transmission include person-to-person contact, exposure to contaminated water, and direct contact with animals (Rangel et al., 2005).

The shiga toxins encoded by *stx*1 and *stx*2 are central to the pathology of disease associated with *E. coli* O157:H7. Shiga toxins exert their action through inactivation of the 60S subunit of the ribosome through a blockade of aminoacyl-tRNA binding, thus halting protein synthesis (O'Loughlin et al., 2001). The shiga toxins are located on λ phages, as lysogens. It is well documented that when the operator of these phages is bound, repression of the genes encoded by prophage results. Therefore, whatever disrupts this repressor may increase production of these phage genes. There is much evidence that agents inducing the SOS response to DNA damage in bacteria disrupt these repressors and hence increases production of phage, and shiga toxins (Waldor et al., 2005).

This information suggests that treatment with antibiotics may worsen the effects of an infection caused by Shiga toxin producing *E. coli*. Furthermore decreased outer membrane permeability has been demonstrated in *E. coli* O157:H7 as compared to a non-pathogenic *E. coli* strain (*E. coli* K-12), which could result in antimicrobial resistance in *E. coli* O157:H7 (Martinez et al., 2001). A study by Wilkerson et al. in 2004 found that

tetracycline resistance is the most common type of antibiotic resistance in a panel of 901 *E. coli* isolates from humans and cattle. Streptomycin and ampicillin resistance were also common among this set of *E. coli* isolates. These studies support the clear need to identify alternatives to antibiotics as clinical therapeutics to treat individuals with an *E. coli* O157:H7 infection. Shiga toxin binders were ineffective in clinical trials at providing therapeutic relief for patients with an *E. coli* O157:H7 infection, and it has been suggested that these toxin binders cannot be administered early enough in the development of disease to yield beneficial effects (MacConnachie et al., 2004). Due to the inherent difficulties in treating disease caused by *E. coli* O157:H7, it is imperative that effective intervention strategies are developed to reduce bacterial loads in food prior to consumption by the consumer and to probe alternatives for therapeutic treatment of clinical infections.

Most antimicrobials approved for utilization in the food supply have been in use for at least 50 years. Furthermore, the majority of food antimicrobials are used to inhibit broad range bacterial growth, rather than combat specific microorganisms. Little is known about resistance to antimicrobials approved for use in food; however, the widespread nature of bacterial resistance to antimicrobials and modes of action are well documented (Davidson et al., 2002). Furthermore, it is possible that the use of organic acids in meat processing may lead to the expansion and growth acid-resistant *E. coli* O157:H7, as these acids may not completely eliminate all *E. coli* O157:H7 present (Samelis et al., 2001). The possibility of acquired resistance to both antimicrobials used in foods and antibiotics used as clinical therapeutics paired with the lack of specific inhibitors of *E. coli* O157:H7 for use in the food supply demonstrate a need for the

discovery, evaluation, approval, and utilization of novel antimicrobial compounds that are bactericidal against *E. coli* O157:H7.

Small molecules (typically < 500 Daltons) have been useful to probe biological function at the molecular and cellular levels as well as for treating disease, as most therapeutic drugs fall within this category of molecules. Chemical biology uses "diversity-orientated synthesis" to create large structurally diverse collections of complex and structurally diverse small molecules, which are derived from splitting and pooling reactions. Small molecules may be coupled with basically any biological assay to test the ability of these compounds to induce defined biological phenotypes (Schreiber, 2000). Here we present a novel application of high throughput small molecule screening to identify novel chemicals that inhibit the growth of *E. coli* O157:H7 for potential future use in foods and as therapeutics. The objectives of this study include (i) develop and validate a high-throughput turbidometric growth assay for *E. coli* O157:H7 for screening purposes, (ii) perform a high-throughput small molecule screen to identify novel chemicals that inhibit the growth of this pathogen and (iii) initially evaluate the cytotoxic effects of selected small molecules, which demonstrated inhibitory activity against *E. coli* O157:H7, on an immortal human intestinal cell line. We identified three lead compounds that demonstrate potential for application as spray-washing or dipping treatments to be used in food production to reduce to load of *E.coli* O157:H7 in foods along with a number of antibiotics or structural derivatives to be evaluated as therapeutics.

4. 3 MATERIALS AND METHODS:

Bacterial Strains. An *E.* coli O157:H7 isolate (CSUFSL C1-057) from a cattle fecal sample was selected for screening purposes. This isolate was characterized by a previously described multiplex PCR , which revealed that this isolate was devoid of both *stx*1 and *stx*2 responsible for the production of Shiga toxins (Hu et al., 1999). This isolate was selected for use in our screening experiments due to the obvious safety concerns of working with large volumes of Shiga toxin producing *E. coli* O157:H7.

Small Molecule Libraries. A total of 64,562 compounds were tested during the screen. This screen took place at The National Screening Laboratory for the Regional Centers of Excellence in Biodefense and Emerging Infectious Diseases (Boston, MA). Small molecule libraries screened belonged to three broad categories: commercially available compounds, known bioactives, and natural products. We screened 59,055 compounds from libraries of commercially available compounds, including ActiMol TimTec 1, Asinex 1, Bionet 1 and 2, ChemBridge 3, ChemDiv 3 and 4, Enamine 1, Life Chemicals 1, Maybridge 1, 4, and 5, and Peakdale 2 libraries. In addition 2,640 small molecules were screened from the following libraries of known bioactive compounds: Biomol ICCB 2, National Institute of Neurological Disorders and Stroke (NINDS) Custom Collection 2, and Prestwick 1. Lastly we screened 2,867 small molecules from the following natural products libraries, International Cooperative Biodiversity Groups (ICBG) Extracts 4 and 6 as well as the Starr Foundation 2 library. Small molecules are suspended in Dimethyl Sufoxide (DMSO) and are stored in 384 well plates at -80°C. The concentration of the compounds in these libraries varied according to the library of interest. More information on chemical libraries, including compound concentrations in library plates, can be found

on the screening facility's website (http://iccb.med.harvard.edu/screening/compound_libraries/index.htm).

Small Molecule Screening. Validation of the fitness of our small molecule assay was determined utilizing the Z' statistic (Zhang et al., 1999). A Z' value of 0.706 was obtained during optimization at screening facilities, and this value constitutes a 'good' assay per facility guidelines. Briefly, overnight cultures of *E. coli* O157:H7 (CSUFSL C1-057) were grown in brain heart infusion broth (BHI; Benton Dickson, Sparks, MD) for 12-18 hours at 37°C with shaking. A 50 µl aliquot was transferred to a fresh BHI tube and incubated with shaking for 37°C for 2 h. A 1:100 dilution of this mid-log phase culture was dispensed into 384-well microplates (Corning, Corning, NY) utilizing an automated wellmate (Thermo Scientific, Waltham, MA). Each well was filled with 80 ul of this inoculated broth culture, which was determined to contain approximately 5 $\log_{10}$ CFU of *E. coli* O157:H7. Pin transfers using a robot (Epson Robotics, Carson, CA) were then completed to transfer 100 nl of small molecules from library plates to columns 1-10 of 384 well assay plates prefilled with inoculated BHI broth in duplicate. Gentamicin (150 µg/ml final concentration) was added to the last column (column 12) of 384 well assay plates to serve as a positive control in the screening process, demonstrating a complete inhibition of bacterial growth. One column (column 11) of inoculated BHI broth without the addition of small molecules or gentamicin served as a negative control, demonstrating uninhibited growth. Microplates were then incubated for 18 h at 37°C and then optical density was read at a OD_{600} with an automated Envision plate reader (Perkin Elmer, Waltham, MA).

Data Analysis and Hit Determination. As consistent with small molecule terminology, "hits" are compounds which elicit the desired biological response. In this study a hit was defined as a compound which inhibited the growth of *E. coli* O157:H7 strain CSUFSL C1-057 used in our screen. Absorbance data was converted to percent inhibition scores in order to best interpret the data generated in these experiments. Scores were obtained by dividing the absorbance of the positive control well for each row in a given plate by the absorbance of each individual experimental well in the same row and multiplied by 100 to express scores as a percentage of the positive gentamicin control. Compounds demonstrating at least 80% inhibition were defined as a "strong hit" compounds demonstrating 65-80% inhibition constituted a "medium strength hit."

Follow-up Screening. The screening facility provided 1µl aliquots of the five commercial compounds identified as hits in the primary screen (Table 1). Selection of small molecules for follow up study is commonly referred to as the "cherry pick" Only the five commercially available compounds deemed as 'hits' in our high throughput screen were provided for follow-up studies, as known bioactive compounds are readily available from vendors. Cherry pick compounds were diluted 1:10 upon receipt and follow-up screen was performed according to the protocol described above to achieve to same concentrations used in the high-throughput screen.

Cytotoxicity Assays. Alexidine (Toronto Research Chemicals, North York, Ontario), Chlorhexidine (Sigma, St. Louis, MO), and Dioxidine (ChemDiv, San Diego, CA) were selected for initial evaluation of cytotoxicity, since these three compounds demonstrated the greatest potential for use to control *E. coli* O157:H7 in foods. Upon receipt, 10 mM

working stocks of these compounds were prepared by suspending them in DMSO. A commercial assay (Cytotox 96 Non-Radioactive Cytotoxicity Assay, Promega, Madison, WI) was used to assess cytotoxicity by lactate dehydrogenize release. Twenty-four well plates (Corning, Corning, NY) were seeded with approximately $1.0X10^5$ Caco-2 cells per well and plates were incubated to allow cell growth to approximately 90% confluence. Prior to addition of small molecules, DMEM with 20% Fetal Bovine Serum (FBS) (Gibco, Invitrogen) was removed and then replaced with DMEM lacking FBS. A major component of FBS is bovine serum albumin (BSA), and chlorhexidine exhibits reduced antibacterial activity towards *Actinobacter spp.* in the presence bovine serum albumin, therefore we avoided the use FBS during our small molecule assay (Kawamura-Sato et al., 2008). Aliquots of small molecule stocks were transferred to the 24 well plates, and final concentrations equaled those used in the screen (Alexidine:12.5μM, Chlorhexidine: 5μM, and Dioxidine: 28μM). Two wells were inoculated with each chemical compound during each experiment and each experiment was performed in triplicate. Two wells per experiment were inoculated with 11.2μl DMSO to serve as a control. This volume of DMSO was chosen as it was the largest volume of small molecules suspended in DMSO added to achieve the desired concentrations above. Following incubation with chemicals for 18 h at 37˚C, 50 μl aliquots of cell supernatant were removed and transferred to a 96 well plate (Corning, Corning, NY), and assayed according to the instruction provided by the kit manufacturer (Promega, Madison, WI). Absorbance values of duplicate wells exposed to each chemical compound were averaged within each independent experiment. The general linear model procedure as implemented in Statistical Analysis Software

(SAS v 9.2; Cary, NC) was utilized to compare the cytotoxicity of chemicals to DMSO control with Dunnett's test to correct for multiple comparisons.

4.4 RESULTS AND DISCUSSION:

We conducted a high throughput small molecule screen to identify compounds that inhibit the growth of *E. coli* O157:H7. Altogether 64,562 compounds were screened and initial analysis revealed 49 hits from our small molecule screen; however, redundancy between the NINDS Custom Collection 2 and Prestwick 1 libraries resulted in duplicate screening of six compounds that were similar or identical molecules. Thus, 43 structurally diverse compounds were found to inhibit the growth of approximately $1x10^5$ CFU of *E. coli* O157:H7. Known bioactives accounted for 38 of the 43 compounds identified in this screen, and the other five compounds belonged to libraries of commercially available compounds. Two structurally related biguanides from known bioactive libraries, which have been used in a number of non-clinical applications, showed effective inhibition of *E. coli* O157:H7 *in vitro* and these two antimicrobials may have potential application as spray-washing or dipping treatments to control this pathogen in foods. Specifically, these two biguanide compounds have been used as disinfectants in mouthwash and contact lens solutions, suggesting they may be safe for use in foods.

Most hits demonstrating bactericidal activity against *E. coli* O157:H7 belonged to libraries of known bioactive compounds. Thirty-eight compounds with known bioactive properties inhibited the growth of *E. coli* O157:H7 at a minimum of 65.2% to a maximum of 111.9% as compared to the positive control gentamicin (Table 1).

Antibiotics typically function by interfering with the cell wall, inhibiting protein synthesis, or inhibiting DNA replication or mismatch repair (Walsh, 2000). Overall, fourteen β-lactam antibiotics inhibited *E.* coli O157:H7 growth, including 13 cephalosporins and 1 carbapenam. β-lactam antibiotics weaken the cell wall by blocking bacterial enzymes responsible for cross-linking peptidoglycan. Our screen also identified 12 flouroquinolones that inhibited growth of *E. coli* O157:H7. Flouroquinolones, a synthetic sublcass of quinolone antibiotics, exhibit broad-spectrum activity. Quinolones interfere with DNA synthesis though interactions with DNA gyrase and topoisomerase IV. Tetracyclines work by inhibiting protein biosynthesis (Walsh, 2003), and six hits identified through this small molecule screen grouped into this class of antibiotics. Mitomycin C and polymyxin B sulfate are mechanistically and structurally distinct from the antibiotic classes discussed above. The mitomycins were first discovered in *Streptomyces caespitousus*, and mitomycin C has been found to be the most active form of mitomycin. The mitomycins covalently bind DNA, forming cross-links between the two strands, thus halting DNA synthesis (Arora, 1979). Interestingly, sub-inhibitory concentrations of mitomycin C have been found to be useful in the isolation of non-O157 Shiga toxin producing *E. coli* (STEC). Specifically, the addition of this antibiotic to washed sheep blood agar, increases the number of STEC strains demonstrating enterohemolysis on this media (Sugiyama et al., 2001). Lastly, polymyxin B sulfate is a cationic peptide, which non-specifically inserts into cell membranes leading to altered membrane permeability (Walsh 2000).

Four other hits from known bioactive libraries including zidovudine, diphenyleneiodonium, chlorhexidine, and alexidine hydrochloride were not classified as

antibiotics used for clinical treatments (http://www.accessdata.fda.gov/Scripts/cder/DrugsatFDA/). These four compounds are structurally diverse and demonstrate wide ranging functions. Zidovudine, also known as 3'-azido-3'deoxythymidine or AZT, is a nucleoside analog, and was the first drug approved for the treatment of HIV (Pastor-Anglada et al., 1998). This drug has also proven effective in reducing vertical transmission of HIV from an infected mother to her fetus (Connor et al., 1994). This compound has demonstrated anti-bacterial activity against a wide range of Gram-negative bacteria, including *E. coli* as well as other foodborne pathogens including *Salmonella* Typhimurium, *Salmonella* Enteritidis, and *Yersinia enterocolitica* (Sandrini et al., 2007). Dipehnyleneiodonim was shown to inhibit both the pentose phosphate pathway and the tricarboxylic acid cycle in microglial cells (Riganti et al., 2004). Should diphenyleneiodonim have a similar mode of action in bacterial cells, this would explain the inhibition of growth we observed in our screen. Chlorhexidine is a disinfectant commonly used in mouthwashes and alexidine hydrochloride is a structurally similar compound (Figure 1) used in contact lens solution. Early research showed that *Providencia stuartii* strains possess innate resistance to chlorhexidine; however, these strains are not resistant to alexidine, suggesting that these two compounds have different modes of action (Chawner and Gilbert, 1989).

Of the all known bioactive compounds found to inhibit growth, chlorhexidine and alexidine hydrochloride demonstrate the most potential for application as spray-washing or dipping treatments to control pathogen populations in foods, since these compounds are already safely included in solutions that come into contact with the mouth and eyes. In 2004, the U.S. Food and Drug Administration approved the use of cetylpyridinium

chloride (CPC) as an antimicrobial for use during poultry processing (21 CFR Part 173); however, this compound is not considered to be GRAS (FDA,2000). CPC is a quaternary ammonium compound that has traditionally been used in mouthwashes as an antiseptic to decrease dental plaque and gingivitis (Silva et al., 2009). Cetylpyridinium chloride inhibits bacterial growth through its binding of acidic groups on the surface of bacteria. Several studies have shown that CPC treatment of poultry skins can reduce adherent *Salmonella* in either by spray-wash or dip type application (Kim and Slavik, 1995, Xiong et al., 1998, and Yang et al., 1998). Although CPC is only approved for use in poultry, several studies have demonstrated that this compound also effectively controls a variety of foodborne pathogens in foods other than poultry. For example, CPC has been shown to be effective against *Listeria monocytogenes* in cooked roast beef (Singh et al., 2005); *Campylobacter jejuni* in chicken meat and skin (Riedel et al., 2009); *E. coli* O157:H7, *Listeria monocytogenes*, and *Staphylococcus aureus* in sliced roast beef (Lim et al., 2006); and *Shigella sonnei,* and *Salmonella* Gaminara in lettuce (Osman et al., 2006).

Chlorhexidine is used in the formulation of antiseptic products (e.g., mouthwashes) and is a cationic biguanide that binds the negatively charged bacterial cell wall, thereby altering the osmotic pressure of the cell (McDonnell et al., 1999). However, more recent work demonstrated that both chlorhexidine and alexidine bind specifically to lipopolysaccharide (LPS) and lipoteichoic acid (LTA) from the cell walls of Gram-negative and Gram-positive bacteria, respectively (Zorko et al., 2008). There has been little indication of resistance to chlorhexidine, only small changes in minimum inhibitory concentration (MIC) (Gilbert et al., 2005). Work by Aarestrup et al. (2003) found no evidence of resistance to chlorhexidine when they examined a total of 569

bacterial isolates, including 202 *E. coli* isolates. Chlorhexidine and alexidine thus show potential for application as antimicrobials in spray-washing or dipping treatments to reduce pathogens in foods.

Screening libraries of commercially available compounds revealed additional hits, including compounds that are structurally similar to existing antibiotics.

Commercially available compounds used in small molecule screening are often synthetic, created by a process called "diversity oriented synthesis" or DOS. Diversity oriented synthesis aims to create diverse compounds through various chemical modifications that are drug-like in nature (Spandl et al., 2008). Diversity oriented synthesis starts with a single chemical structure or a group of related structures followed by treatment of these structures with different reagents to achieve a few chemical transformations yielding many diverse compounds related to the parent (Schreiber et al., 2003). Five compounds from commercial libraries were shown to inhibit the growth of *E. coli* O157:H7 in our high-throughput screen. Three these five compounds, including dioxidine, (4S,4aR,5R,5aS,6S,12aS) - 4- (dimethylamino) - 3,5,10,12,12a- pentahydroxy- 6- methyl- 1- oxo- 1,4,4a,5,5a,6,11,12a- octahydrotetracene- 2- carboxamide, and 1- cyclopropyl-6-fluoro-4-oxo-7-(4-sulfidocarbothioylpiperazin-1-yl) quinoline-3- carboxylate were confirmed as hits upon follow-up screening in our laboratory (Table 1).

The compound (4S,4aR,5R,5aS,6S,12aS) - 4- (dimethylamino) - 3,5,10,12,12a- pentahydroxy- 6- methyl- 1- oxo- 1,4,4a,5,5a,6,11,12a- octahydrotetracene- 2- carboxamide was structurally similar to tetracycline antibiotics (Sun et al., 2008), while,1-cyclopropyl-6-fluoro-4-oxo-7-(4-sulfidocarbothioylpiperazin-1-yl) quinoline-3-

carboxylate was structurally similar to ciprofloxacin and thus other fluoroquinolones (Lipsky and Barker, 1999). Lastly, dioxidine is a drug that has been used in Russia for over nearly 30 years to treat "purulent infection" and can be applied topically or injected intravenously into body cavities, and was the only well characterized commercial hit compound found to inhibit *E. coli* O157:H7 growth. Dioxidine's mode of action involves interference with DNA synthesis (Pushkina et al., 2008). Recent research has investigated potential applications of dioxidine, ranging from an anti-tuberculosis cocktail to antimicrobial topical ointments (Glushkov et al., 2007, Pushkina et al., 2008).

Cytotoxicity of compounds of interest for use in the food supply. Alexidine, chlorhexidine, and dioxidine were selected for follow-up evaluation to assess potential cytotoxicity. Alexidine and chlorhexidine were selected for further evaluation because they are safely used as disinfectants and antiseptics but not clinical therapeutics. Dioxidine was also selected because this was the only commercially available compound confirmed to inhibit *E. coli* O157:H7 growth upon follow-up screening that was not structurally similar to antibiotics currently used in the U.S. An overall F-test on the variable chemical yielded a *P*-value of <0.001, indicating that significant differences exist between the three chemicals of evaluated and the DMSO control included in the study (Figure 1). A Dunnett's test revealed that alexidine demonstrated significantly higher cytotoxicity ($P = 0.0003$) than the control, while the dioxidine and chlorhexidine showed no such differences ($P = 0.4135$ and $P = 0.0863$, respectively). A study examining the cytotoxicity of antiseptic agents, found chlorhexidine digluconate to demonstrate higher cytotoxicity than CPC, yet lower cytotoxicity when compared to triclosan, both widely used biocides (Müller and Kramer, 2008). Further work is needed

to identify the minimum inhibitory concentrations (MIC) of each compound and to determine if cytotoxicity is observed at these levels.

Our work and the work of others suggest that these three compounds, alexidine, chlorhexidine, and dioxidine may be suitable for use for use in the food supply, especially in conjunction with interventions that are already in-place. These compounds have the potential to be used as one of many possible hurdles to decrease pathogenic bacteria loads in the food supply. The multiple hurdle approach refers to concept where many "hurdles" or interventions are incorporated throughout a given process to reduce pathogen populations (e.g., a series of carcass washes) for a maximal synergistic inhibition of pathogens (Leistner et al., 2000). Previous work has shown that chlorhexidine in particular may be an effective intervention strategy. A study involving beef carcass contamination with *E. coli* O157:H7 and the use of a chlorhexidine solution (0.1%) to decontaminate fat and lean tissues achieved a 5 log CFU/cm^2 reduction (Delazari et al., 1998). Chlorhexidine initially was examined as part of a screen to identify compounds suitable to be fed to broiler chickens. Although chlorhexidine was shown to inhibit *Salmonella* at a 10% concentration, it was ineffective at lower concentrations *in vitro* (Barnhart et al., 1999). Future work to address the potential use of alexidine and dioxidine, may yield promising results as well.

4.5CONCLUSIONS:

In the current study, we performed a high throughput small molecule screen to identify novel compounds that inhibit the growth of *Escherichia* coli O157:H7. We screened over 64,000 chemical compounds and identified 43 diverse compounds that inhibit the growth

of *E. coli* O157:H7, many of which show promise both as drugs in the clinical setting, but also as important intervention steps to be implemented in various points along the food chain. Future work is needed to assess minimum inhibitory concentrations of these compounds, their cytotoxicity at these levels, and their efficacy to inhibit *E. coli* O157:H7 in food matrices. Further studies to assess the abilities of these chemical agents to elicit Shiga toxin production is also necessary, to determine feasibility of use in a clinical setting. This study is only the first step in the identification of antimicrobials to mitigate the burden of this dangerous foodborne pathogen. In particular, we have identified three compounds, alexidine, chlorhexidine, and dioxidine, which are promising lead chemicals, which may have the potential to be used as interventions in the food supply.

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Table 4.1. Chemicals identified as strong or medium hits to inhibit *E. coli* O157:H7 growth *in vitro*

Library Plate Number[a]	Well	Percent Inhibition	Library	Identity	Library Type (Antibiotic Subclass)	Molecular Weight (g/mol)	Final Concentration (uM)
PL-1536	P09	94.49	ActiMolTimTec1	(4S,4aR,5R,5aS,6S,12aS) - 4-(dimethylamino) - 3,5,10,12,12a-pentahydroxy- 6- methyl- 1- oxo-1,4,4a,5,5a,6,11,12a- octahydrotetracene-2- carboxamide	Commercial	466.91	13.4
PL-1543	J06	98.22	ActiMolTimTec1	1-cyclopropyl-6-fluoro-7-[4-(mercaptocarbonothioyl)piperazin-1-yl]-4-oxo-1,4-dihydroquinoline-3-carboxylic acid	Commercial	405.47	15.4
PL-1615	P12	99.83	ChemDiv4	2-(1H-imidazo[4,5-b]pyridin-2-ylsulfanyl)-N-(4-methylphenyl)acetamide	Commercial	298.36	20.9
PL-1613	L20	94.91	ChemDiv4	5-(1,3-dioxoisoindol-2-yl)benzene-1,3-dicarboxylic acid	Commercial	311.25	20.1
PL-1922	C13	103.59	NINDS Custom Collection 2	Alexidine dihydrochloride	Known Bioactive (Disinfectant)	580.42	12.5
PL-1570	E16	65.24	Prestwick1 Collection	Cefamandole sodium	Known Bioactive (Cephalosporin)	484.48	5.2
PL-1922	L19	109.67	NINDS Custom Collection 2	Cefdinir	Known Bioactive (Cephalosporin)	395.41	12.5
PL-1571	O18	90.95	Prestwick1 Collection	Cefepime hydrochloride	Known Bioactive (Cephalosporin)	535.04	4.7
PL-1569	M06	94.18	Prestwick1 Collection	Cefixime	Known Bioactive (Cephalosporin)	453.45	5.5
PL-1570	K21	66.23	Prestwick1 Collection	Cefmetazole sodium salt	Known Bioactive (Cephalosporin)	493.52	5.1
PL-1569	A15	93.00	Prestwick1 Collection	Cefoperazone dihydrate	Known Bioactive (Cephalosporin)	681.70	3.7

PL-1920 [PL-1568]	K09 [K20]	94.90 [89.03]	NINDS Custom Collection 2 [Prestwick1 Collection]	Cefotaxime Sodium [Cefotaxime sodium salt]	Known Bioactive (Cephalosporin)	477.45 [477.45]	12.5[5.2]
PL-1569	O08	95.89	Prestwick1 Collection	Cefotetan	Known Bioactive (Cephalosporin)	575.62	4.3
PL-1569	B05	109.20	Prestwick1 Collection	Cefotiam hydrochloride	Known Bioactive (Cephalosporin)	562.09	4.4
PL-1569	B19	82.15	Prestwick1 Collection	Ceftazidime pentahydrate	Known Bioactive (Cephalosporin)	636.65	3.9
PL-1922	L17	104.92	NINDS Custom Collection 2	Ceftibuten	Known Bioactive (Cephalosporin)	410.42	12.5
PL-1922 [PL-1922]	G03 [F14]	98.54 [99.63]	NINDS Custom Collection 2 [NINDS Custom Collection 2]	Ceftriaxone sodium [Ceftriaxone sodium trihydrate]	Known Bioactive (Cephalosporin)	652.59 [652.59]	12.5 [12.5]
PL-1568	M08	.	Prestwick1 Collection	Chlorhexidine	Known Bioactive (Disinfectant)	505.45	4.9
PL-1568	M12	75.81	Prestwick1 Collection	Chlortetracycline hydrochloride	Known Bioactive (Tetracycline)	515.34	4.9
PL-1568 [PL-1922]	G08 [E18]	92.88 [94.15]	Prestwick1 Collection [NINDS Custom Collection 2]	Ciprofloxacin [Ciprofloxacin hydrochloride]	Known Bioactive (Flouroquinolone)	367.80 [331.34]	6.8
PL-1570	G08	80.56	Prestwick1 Collection	Demeclocycline hydrochloride	Known Bioactive (Tetracycline)	501.31	5.0
PL-1473	C16	103.40	ChemDiv3	Dioxidine	commercial	222.20	28.1
PL-1792	C09	97.83	BIOMOL ICCB Known Bioactives 2	Diphenyleneiodonium	Known Bioactive (NADPH oxidase inhibitor)	279.10	22.4
PL-1569 [PL-1922]	G07 [I17]	111.89 [103.24]	Prestwick1 Collection [NINDS Custom Collection 2]	Enoxacin [Enoxacin]	Known Bioactive (Flouroquinolone)	320.32 [320.32]	7.8 [12.5]
PL-1921	O10	104.20	NINDS Custom Collection 2	Flumequine	Known Bioactive (Flouroquinolone)	261.25	12.5

PL-1922	J15	100.94	NINDS Custom Collection 2	Gatifloxacin	Known Bioactive (Flouroquinolone)	375.39	12.5
PL-1922	H08	91.81	NINDS Custom Collection 2	Gemifloxacin mesylate	Known Bioactive (Flouroquinolone)	485.49	12.5
PL-1922	J07	98.54	NINDS Custom Collection 2	Levofloxacin	Known Bioactive (Flouroquinolone)	361.37	12.5
PL-1921	F20	100.00	NINDS Custom Collection 2	Lomefloxacin hydrochloride	Known Bioactive (Flouroquinolone)	387.81	12.5
PL-1921 [PL-1569]	F17 [K14]	96.04 [82.40]	NINDS Custom Collection 2 [Prestwick1 Collection]	Meclocycline sulfosalicylate [Meclocycline sulfosalicylate]	Known Bioactive (Tetracycline)	695.05 [695.05]	3.6 [12.5]
PL-1571	M14	70.35	Prestwick1 Collection	Meropenem	Known Bioactive (Carbapenam)	383.46	6.5
PL-1571	A09	72.74	Prestwick1 Collection	Methacycline hydrochloride	Known Bioactive (Tetracycline)	478.88	5.2
PL-1792	O09	80.03	BIOMOL ICCB Known Bioactives 2	mitomycin C	Known Bioactive (DNA Cross Linking Antibiotic)	334.33	18.7
PL-1570	D19	82.80	Prestwick1 Collection	Moxalactam disodium salt	Known Bioactive (Cephalosporin)	564.44	4.4
PL-1922	L03	103.90	NINDS Custom Collection 2	Moxifloxacn hydrochloride	Known Bioactive (Flouroquinolone)	465.95	12.5
PL-1920	B16	106.93	NINDS Custom Collection 2	Norfloxacin	Known Bioactive (Flouroquinolone)	319.33	12.5
PL-1921	H12	104.69	NINDS Custom Collection 2	Ofloxacin	Known Bioactive (Flouroquinolone)	361.37	12.5
PL-1920	F04	82.49	NINDS Custom Collection 2	Oxytetracycline	Known Bioactive (Tetracycline)	496.12	12.5

PL-1922	B14	99.44	NINDS Custom Collection 2	Prefloxacine mesylate	Known Bioactive (Flourouqinolone)	429.46	12.5
PL-1920	J04	97.61	NINDS Custom Collection 2	Polymyxin B sulfate	Known Bioactive (Cationic Peptide Antibiotic)	1301.56	12.5
PL-1922	D04	91.13	NINDS Custom Collection 2	Sarafloxacin hydrochloride	Known Bioactive (Flouroquinolone)	421.83	12.5
PL-1568	K22	74.29	Prestwick1 Collection	Tetracycline hydrochloride	Known Bioactive (Tetracycline)	480.90	5.2
PL-1921 [PL-1569]	J20 [C07]	89.00/ [68.00]	NINDS Custom Collection 2 [Prestwick1 Collection]	Zidovudine [Zidovudine]	Known Bioactive (Nucleoside Analog)	267.24 [267.24]	12.5 [9.4]

[a][ ] Indicate identical compounds from more than one library identified as a hit.

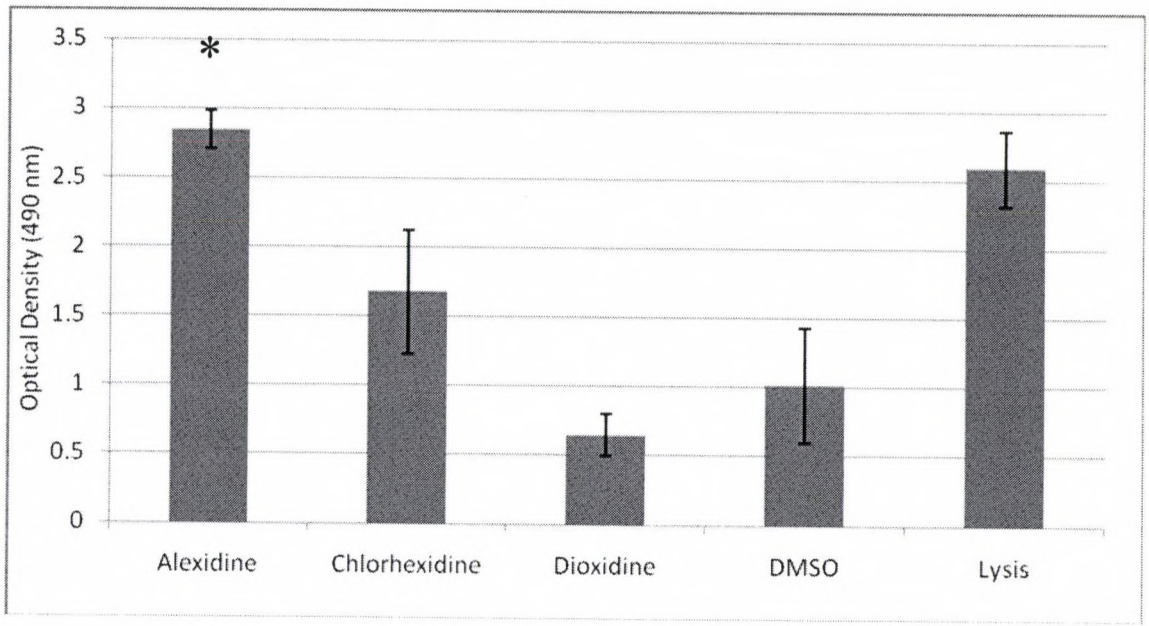
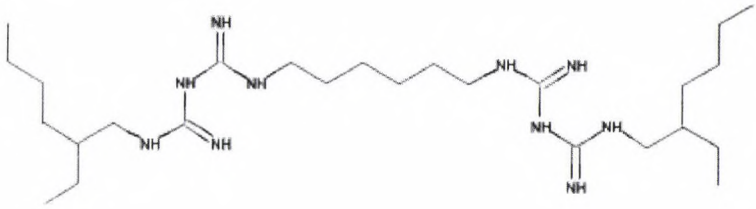
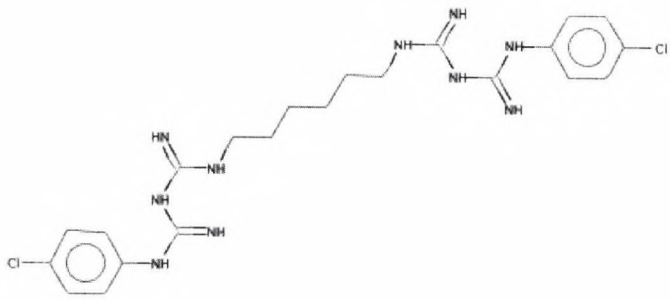


FIG. 4.1. Cytotoxicy of select compounds which inhibited *E. coli* O157:H7 growth in the small molecule screen. The X-axis denotes the compound of interest, while the Y-axis denotes the Optical Density measured at 490 nm, which is an indicator of cytotoxicity. An asterisk denotes significance of a Dunnett's test comparing that chemical to the DMSO control with an alpha level of 0.05.

A



B



C

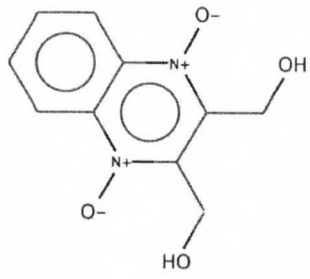


FIG. 4.2. Structures of novel compounds exhibiting inhibition of growth of *E. coli* O157:H7. A) Alexidine B) Chlorhexidine C) Dioxidine. (PubChem).

APPENDIX: Data used in statistical analysis.

37°C Growth Curves

Averages

Hour following inoculation	0	1	2	3	4	5	6	7	8	9	10	11	12
N1-021	0.005	0.012	0.046	0.142	0.365	1.030	2.395	2.700	3.065	3.390	3.505	3.605	3.650
N1-040	0.004	0.012	0.034	0.095	0.240	0.630	1.640	2.483	3.007	3.287	3.253	3.520	3.543
N1-061	0.004	0.009	0.034	0.112	0.309	0.877	2.265	2.720	2.975	3.250	3.330	3.530	3.515
N1-080	0.008	0.011	0.042	0.127	0.334	0.950	2.275	2.945	3.220	3.490	3.635	3.625	3.635
N1-074	0.006	0.013	0.037	0.101	0.262	0.727	1.775	2.430	2.730	2.895	3.088	2.940	2.880
N1-072	0.010	0.012	0.036	0.092	0.223	0.647	1.550	2.355	2.770	3.115	3.330	3.438	3.275
N1-064	0.009	0.016	0.047	0.130	0.314	0.930	2.118	2.545	2.980	3.050	3.075	3.055	3.080
N1-054	0.019	0.026	0.044	0.100	0.233	0.585	1.515	2.365	2.710	2.950	3.233	3.270	3.203
N1-051	0.007	0.010	0.036	0.108	0.282	0.824	2.003	2.953	3.185	3.413	3.546	3.574	3.523

Min/Max Values

Hour following inoculation	0	1	2	3	4	5	6	7	8	9	10	11	12
N1-021	0.003	0.006	0.005	0.011	0.002	0.095	0.285	0.120	0.035	0.180	0.255	0.145	0.080
N1-040	0.001	0.001	0.002	0.003	0.011	0.061	0.056	0.106	0.015	0.050	0.188	0.062	0.075
N1-061	0.002	0.000	0.004	0.012	0.048	0.203	0.335	0.140	0.075	0.130	0.430	0.230	0.205
N1-080	0.002	0.004	0.002	0.009	0.009	0.065	0.165	0.095	0.050	0.180	0.255	0.145	0.105
N1-074	0.006	0.001	0.001	0.006	0.042	0.219	0.345	0.270	0.130	0.035	0.238	0.060	0.030
N1-072	0.003	0.003	0.004	0.005	0.033	0.209	0.320	0.085	0.200	0.235	0.340	0.063	0.275
N1-064	0.006	0.002	0.003	0.020	0.058	0.350	0.523	0.205	0.070	0.140	0.135	0.175	0.190
N1-054	0.015	0.018	0.008	0.011	0.039	0.165	0.255	0.285	0.090	0.000	0.293	0.280	0.223
N1-051	0.005	0.003	0.010	0.019	0.042	0.176	0.268	0.267	0.347	0.298	0.137	0.096	0.088

Intracellular Growth Assays

Strain	PMSC	Time Point	Percent Log Intracellular Normalized to 10403S
N1-054	N	1.5	1.995
N1-054	N	1.5	1.981
N1-054	N	1.5	1.992
N1-064	N	1.5	2.004
N1-064	N	1.5	2.001
N1-064	N	1.5	1.989
N1-072	N	1.5	1.998
N1-072	N	1.5	1.962
N1-072	N	1.5	1.985
N1-074	N	1.5	2.004
N1-074	N	1.5	1.977
N1-074	N	1.5	2.002
N1-021	Y	1.5	1.998
N1-021	Y	1.5	2.010
N1-021	Y	1.5	2.017
N1-040	Y	1.5	2.005
N1-040	Y	1.5	2.003
N1-040	Y	1.5	2.008
N1-061	Y	1.5	1.999
N1-061	Y	1.5	2.008
N1-061	Y	1.5	1.997
N1-080	Y	1.5	1.968
N1-080	Y	1.5	2.003
N1-080	Y	1.5	2.000
N1-054	N	5	1.983
N1-054	N	5	1.995
N1-054	N	5	1.995
N1-064	N	5	1.977
N1-064	N	5	1.991
N1-064	N	5	2.005

N1-072	N	5	1.986
N1-072	N	5	1.938
N1-072	N	5	1.976
N1-074	N	5	1.994
N1-074	N	5	1.956
N1-074	N	5	2.007
N1-021	Y	5	1.993
N1-021	Y	5	1.987
N1-021	Y	5	1.997
N1-040	Y	5	1.981
N1-040	Y	5	2.010
N1-040	Y	5	1.986
N1-061	Y	5	2.016
N1-061	Y	5	1.993
N1-061	Y	5	2.029
N1-080	Y	5	1.978
N1-080	Y	5	1.985
N1-080	Y	5	1.977
N1-054	N	9	2.024
N1-054	N	9	2.024
N1-054	N	9	2.026
N1-064	N	9	2.028
N1-064	N	9	2.001
N1-064	N	9	2.027
N1-072	N	9	2.014
N1-072	N	9	1.995
N1-072	N	9	2.021
N1-074	N	9	2.022
N1-074	N	9	2.007
N1-074	N	9	2.027
N1-021	Y	9	2.027
N1-021	Y	9	1.999
N1-021	Y	9	2.028
N1-040	Y	9	2.009
N1-040	Y	9	2.040
N1-040	Y	9	2.014
N1-061	Y	9	2.041
N1-061	Y	9	2.012
N1-061	Y	9	2.024

N1-080	Y	9	1.907
N1-080	Y	9	1.994
N1-080	Y	9	2.012
N1-054	N	12	2.031
N1-054	N	12	2.022
N1-054	N	12	2.013
N1-064	N	12	2.014
N1-064	N	12	2.035
N1-064	N	12	2.022
N1-072	N	12	2.032
N1-072	N	12	2.023
N1-072	N	12	2.015
N1-074	N	12	2.016
N1-074	N	12	2.044
N1-074	N	12	2.005
N1-021	Y	12	2.006
N1-021	Y	12	2.050
N1-021	Y	12	2.066
N1-040	Y	12	2.004
N1-040	Y	12	2.019
N1-040	Y	12	2.011
N1-061	Y	12	2.024
N1-061	Y	12	2.051
N1-061	Y	12	2.055
N1-080	Y	12	2.015
N1-080	Y	12	2.032
N1-080	Y	12	2.059

Cytotoxicity Assays

Strain	PMSC	Passage	Time Point	Rep	O.D. $_{490}$
N1-051	.	14	1.5	1	0.273
N1-051	.	14	1.5	1	0.239
N1-051	.	16	1.5	2	0.308
N1-051	.	16	1.5	2	0.315
N1-051	.	27	1.5	5	0.36

N1-051	.	27	1.5	5	0.388
N1-054	N	14	1.5	1	0.262
N1-054	N	14	1.5	1	0.307
N1-054	N	16	1.5	2	0.321
N1-054	N	16	1.5	2	0.321
N1-054	N	27	1.5	5	0.373
N1-054	N	27	1.5	5	0.432
N1-064	N	14	1.5	1	0.278
N1-064	N	14	1.5	1	0.259
N1-064	N	16	1.5	2	0.336
N1-064	N	16	1.5	2	0.454
N1-064	N	27	1.5	5	0.352
N1-064	N	27	1.5	5	0.377
N1-072	N	14	1.5	1	0.273
N1-072	N	14	1.5	1	0.27
N1-072	N	16	1.5	2	0.453
N1-072	N	16	1.5	2	0.488
N1-072	N	27	1.5	5	0.397
N1-072	N	27	1.5	5	0.523
N1-074	N	14	1.5	1	0.261
N1-074	N	14	1.5	1	0.258
N1-074	N	16	1.5	2	0.433
N1-074	N	16	1.5	2	0.46
N1-074	N	27	1.5	5	0.352
N1-074	N	27	1.5	5	0.352
N1-021	Y	14	1.5	1	0.277
N1-021	Y	14	1.5	1	0.256
N1-021	Y	16	1.5	2	0.504
N1-021	Y	16	1.5	2	0.475
N1-021	Y	27	1.5	5	0.28
N1-021	Y	27	1.5	5	0.369
N1-040	Y	20	1.5	1	0.276
N1-040	Y	20	1.5	1	0.297
N1-040	Y	20	1.5	2	0.334
N1-040	Y	20	1.5	2	0.297
N1-040	Y	20	1.5	5	0.359
N1-040	Y	20	1.5	5	0.311
N1-061	Y	14	1.5	1	0.252
N1-061	Y	14	1.5	1	0.254

N1-061	Y	16	1.5	2	0.45
N1-061	Y	16	1.5	2	0.464
N1-061	Y	27	1.5	5	0.431
N1-061	Y	27	1.5	5	0.382
N1-080	Y	14	1.5	1	0.245
N1-080	Y	14	1.5	1	0.248
N1-080	Y	16	1.5	2	0.465
N1-080	Y	16	1.5	2	0.446
N1-080	Y	27	1.5	5	0.385
N1-080	Y	27	1.5	5	0.35
N1-051	.	14	5	1	0.289
N1-051	.	14	5	1	1.023
N1-051	.	16	5	2	0.447
N1-051	.	16	5	2	0.522
N1-051	.	27	5	5	0.394
N1-051	.	27	5	5	0.47
N1-054	N	14	5	1	0.241
N1-054	N	14	5	1	0.258
N1-054	N	16	5	2	0.465
N1-054	N	16	5	2	0.49
N1-054	N	27	5	5	0.369
N1-054	N	27	5	5	0.401
N1-064	N	14	5	1	0.292
N1-064	N	14	5	1	0.291
N1-064	N	16	5	2	0.524
N1-064	N	16	5	2	0.368
N1-064	N	27	5	5	0.409
N1-064	N	27	5	5	0.403
N1-072	N	14	5	1	0.271
N1-072	N	14	5	1	0.293
N1-072	N	16	5	2	0.469
N1-072	N	16	5	2	0.498
N1-072	N	27	5	5	0.402
N1-072	N	27	5	5	0.367
N1-074	N	14	5	1	0.257
N1-074	N	14	5	1	0.27
N1-074	N	16	5	2	0.521
N1-074	N	16	5	2	0.502
N1-074	N	27	5	5	0.425

N1-074	N	27	5	5	0.374
N1-021	Y	14	5	1	0.299
N1-021	Y	14	5	1	0.265
N1-021	Y	16	5	2	0.694
N1-021	Y	16	5	2	0.554
N1-021	Y	27	5	5	0.454
N1-021	Y	27	5	5	0.399
N1-040	Y	20	5	1	0.367
N1-040	Y	20	5	1	0.305
N1-040	Y	20	5	2	0.384
N1-040	Y	20	5	2	0.4
N1-040	Y	20	5	5	0.401
N1-040	Y	20	5	5	0.437
N1-061	Y	14	5	1	0.279
N1-061	Y	14	5	1	0.323
N1-061	Y	16	5	2	0.581
N1-061	Y	16	5	2	0.672
N1-061	Y	27	5	5	0.46
N1-061	Y	27	5	5	0.959
N1-080	Y	14	5	1	0.263
N1-080	Y	14	5	1	0.264
N1-080	Y	16	5	2	0.597
N1-080	Y	16	5	2	0.304
N1-080	Y	27	5	5	0.403
N1-080	Y	27	5	5	0.361
N1-051	.	14	9	1	0.859
N1-051	.	14	9	1	1.057
N1-051	.	16	9	2	1.074
N1-051	.	16	9	2	1.38
N1-051	.	27	9	5	1.016
N1-051	.	27	9	5	1.044
N1-054	N	14	9	1	0.352
N1-054	N	14	9	1	0.405
N1-054	N	16	9	2	0.565
N1-054	N	16	9	2	0.58
N1-054	N	27	9	5	0.715
N1-054	N	27	9	5	0.839
N1-064	N	14	9	1	0.446
N1-064	N	14	9	1	0.353

N1-064	N	16	9	2	0.794
N1-064	N	16	9	2	0.817
N1-064	N	27	9	5	0.847
N1-064	N	27	9	5	0.768
N1-072	N	14	9	1	0.254
N1-072	N	14	9	1	0.382
N1-072	N	16	9	2	0.812
N1-072	N	16	9	2	1.216
N1-072	N	27	9	5	0.59
N1-072	N	27	9	5	0.619
N1-074	N	14	9	1	0.457
N1-074	N	14	9	1	0.514
N1-074	N	16	9	2	0.721
N1-074	N	16	9	2	0.799
N1-074	N	27	9	5	0.842
N1-074	N	27	9	5	0.784
N1-021	Y	14	9	1	0.524
N1-021	Y	14	9	1	0.545
N1-021	Y	16	9	2	0.779
N1-021	Y	16	9	2	0.946
N1-021	Y	27	9	5	1.015
N1-021	Y	27	9	5	0.83
N1-040	Y	20	9	1	0.571
N1-040	Y	20	9	1	1.006
N1-040	Y	20	9	2	0.425
N1-040	Y	20	9	2	0.665
N1-040	Y	20	9	5	1.204
N1-040	Y	20	9	5	0.627
N1-061	Y	14	9	1	0.558
N1-061	Y	14	9	1	0.597
N1-061	Y	16	9	2	1.075
N1-061	Y	16	9	2	0.976
N1-061	Y	27	9	5	0.881
N1-061	Y	27	9	5	1.029
N1-080	Y	14	9	1	0.569
N1-080	Y	14	9	1	0.44
N1-080	Y	16	9	2	0.835
N1-080	Y	16	9	2	0.754
N1-080	Y	27	9	5	0.788

N1-080	Y	27	9	5	0.737
N1-051	.	14	12	1	1.478
N1-051	.	14	12	1	1.622
N1-051	.	16	12	2	1.453
N1-051	.	16	12	2	1.834
N1-051	.	27	12	5	1.304
N1-051	.	27	12	5	1.639
N1-054	N	14	12	1	0.854
N1-054	N	14	12	1	0.749
N1-054	N	16	12	2	0.81
N1-054	N	16	12	2	1.074
N1-054	N	27	12	5	0.774
N1-054	N	27	12	5	0.85
N1-064	N	14	12	1	0.941
N1-064	N	14	12	1	0.621
N1-064	N	16	12	2	1.036
N1-064	N	16	12	2	0.821
N1-064	N	27	12	5	1.274
N1-064	N	27	12	5	1.024
N1-072	N	14	12	1	0.753
N1-072	N	14	12	1	0.857
N1-072	N	16	12	2	1.13
N1-072	N	16	12	2	1.177
N1-072	N	27	12	5	1
N1-072	N	27	12	5	1.324
N1-074	N	14	12	1	0.935
N1-074	N	14	12	1	1.028
N1-074	N	16	12	2	1.094
N1-074	N	16	12	2	1.108
N1-074	N	27	12	5	1.183
N1-074	N	27	12	5	1.222
N1-021	Y	14	12	1	1.242
N1-021	Y	14	12	1	1.389
N1-021	Y	16	12	2	1.657
N1-021	Y	16	12	2	1.145
N1-021	Y	27	12	5	1.471
N1-021	Y	27	12	5	1.349
N1-040	Y	20	12	1	2.087
N1-040	Y	20	12	1	1.858

N1-040	Y	20	12	2	2.014
N1-040	Y	20	12	2	2.177
N1-040	Y	20	12	5	2.127
N1-040	Y	20	12	5	2.172
N1-061	Y	14	12	1	1.452
N1-061	Y	14	12	1	1.133
N1-061	Y	16	12	2	1.522
N1-061	Y	16	12	2	1.867
N1-061	Y	27	12	5	1.609
N1-061	Y	27	12	5	1.569
N1-080	Y	14	12	1	1.016
N1-080	Y	14	12	1	0.836
N1-080	Y	16	12	2	1.029
N1-080	Y	16	12	2	1.06
N1-080	Y	27	12	5	1.209
N1-080	Y	27	12	5	0.989

Invasion Assays

Strain	PMSC	rep	% invasion
N1-021	Y	1	0.044
N1-021	Y	3	0.026
N1-021	Y	4	0.037
N1-040	Y	1	0.042
N1-040	Y	3	0.035
N1-040	Y	4	0.041
N1-061	Y	1	0.075
N1-061	Y	3	0.064
N1-061	Y	4	0.076
N1-080	Y	1	0.044
N1-080	Y	3	0.036
N1-080	Y	4	0.053
N1-074	N	1	0.042
N1-074	N	3	0.055
N1-074	N	4	0.069
N1-072	N	1	0.021

N1-072	N	3	0.017
N1-072	N	4	0.015
N1-064	N	1	0.016
N1-064	N	3	0.013
N1-064	N	4	0.020
N1-054	N	1	0.020
N1-054	N	3	0.035
N1-054	N	4	0.014
N1-051	.	1	0.017
N1-051	.	3	0.006
N1-051	.	4	0.014

Low Inoculum Cold Growth Curves

Strain	pH	Salt Level	rep	PMSC	EGR
N1-021	7.4	0	1	Y	0.806
N1-040	7.4	0	1	Y	0.738
N1-061	7.4	0	1	Y	0.711
N1-080	7.4	0	1	Y	0.723
N1-072	7.4	0	1	N	0.678
N1-074	7.4	0	1	N	0.691
N1-064	7.4	0	1	N	0.748
N1-054	7.4	0	1	N	0.789
N1-051	7.4	0	1	.	0.703
N1-021	7.4	0	2	Y	0.715
N1-040	7.4	0	2	Y	0.719
N1-061	7.4	0	2	Y	0.676
N1-080	7.4	0	2	Y	0.702
N1-072	7.4	0	2	N	0.619
N1-074	7.4	0	2	N	0.656
N1-064	7.4	0	2	N	0.656
N1-054	7.4	0	2	N	0.643
N1-051	7.4	0	2	.	0.609
N1-021	6.5	0	1	Y	0.584
N1-040	6.5	0	1	Y	0.506
N1-061	6.5	0	1	Y	0.577

N1-080	6.5	0	1	Y	0.591
N1-074	6.5	0	1	N	0.580
N1-072	6.5	0	1	N	0.577
N1-064	6.5	0	1	N	0.555
N1-054	6.5	0	1	N	0.562
N1-051	6.5	0	1	.	0.589
N1-021	6.5	0	2	Y	0.565
N1-040	6.5	0	2	Y	0.518
N1-061	6.5	0	2	Y	0.583
N1-080	6.5	0	2	Y	0.578
N1-074	6.5	0	2	N	0.581
N1-072	6.5	0	2	N	0.580
N1-064	6.5	0	2	N	0.588
N1-054	6.5	0	2	N	0.583
N1-051	6.5	0	2	.	0.581
N1-021	5.5	0	1	Y	0.450
N1-040	5.5	0	1	Y	0.417
N1-061	5.5	0	1	Y	0.407
N1-080	5.5	0	1	Y	0.312
N1-051	5.5	0	1	.	0.462
N1-074	5.5	0	1	N	0.433
N1-072	5.5	0	1	N	0.416
N1-064	5.5	0	1	N	0.433
N1-054	5.5	0	1	N	0.442
N1-021	5.5	0	2	Y	0.456
N1-040	5.5	0	2	Y	0.414
N1-061	5.5	0	2	Y	0.448
N1-080	5.5	0	2	Y	0.345
N1-051	5.5	0	2	.	0.448
N1-074	5.5	0	2	N	0.448
N1-072	5.5	0	2	N	0.423
N1-064	5.5	0	2	N	0.450
N1-054	5.5	0	2	N	0.460
N1-021	7.4	2.2	1	Y	0.671
N1-040	7.4	2.2	1	Y	0.572
N1-061	7.4	2.2	1	Y	0.717
N1-080	7.4	2.2	1	Y	0.680
N1-074	7.4	2.2	1	N	0.690
N1-072	7.4	2.2	1	N	0.578

N1-064	7.4	2.2	1	N	0.664
N1-054	7.4	2.2	1	.	0.689
N1-051	7.4	2.2	1	Y	0.630
N1-021	7.4	2.2	2	Y	0.531
N1-040	7.4	2.2	2	Y	0.574
N1-061	7.4	2.2	2	Y	0.525
N1-080	7.4	2.2	2	Y	0.493
N1-074	7.4	2.2	2	N	0.502
N1-072	7.4	2.2	2	N	0.459
N1-064	7.4	2.2	2	N	0.525
N1-054	7.4	2.2	2	N	0.500
N1-051	7.4	2.2	2	.	0.459
N1-021	6.5	2.2	1	Y	0.646
N1-040	6.5	2.2	1	Y	0.536
N1-061	6.5	2.2	1	Y	0.626
N1-080	6.5	2.2	1	Y	0.605
N1-074	6.5	2.2	1	N	0.567
N1-072	6.5	2.2	1	N	0.560
N1-064	6.5	2.2	1	N	0.620
N1-054	6.5	2.2	1	N	0.635
N1-051	6.5	2.2	1	.	0.561
N1-021	6.5	2.2	2	Y	0.515
N1-040	6.5	2.2	2	Y	0.555
N1-061	6.5	2.2	2	Y	0.529
N1-080	6.5	2.2	2	Y	0.484
N1-074	6.5	2.2	2	N	0.489
N1-072	6.5	2.2	2	N	0.412
N1-064	6.5	2.2	2	N	0.501
N1-054	6.5	2.2	2	N	0.474
N1-051	6.5	2.2	2	.	0.433
N1-021	5.5	2.2	1	Y	0.441
N1-040	5.5	2.2	1	Y	0.408
N1-061	5.5	2.2	1	Y	0.430
N1-080	5.5	2.2	1	Y	0.314
N1-074	5.5	2.2	1	N	0.382
N1-072	5.5	2.2	1	N	0.397
N1-064	5.5	2.2	1	N	0.416
N1-054	5.5	2.2	1	N	0.420
N1-051	5.5	2.2	1	.	0.406

N1-021	5.5	2.2	2	Y	0.345
N1-040	5.5	2.2	2	Y	0.400
N1-061	5.5	2.2	2	Y	0.272
N1-080	5.5	2.2	2	Y	0.313
N1-074	5.5	2.2	2	N	0.290
N1-072	5.5	2.2	2	N	0.329
N1-064	5.5	2.2	2	N	0.354
N1-054	5.5	2.2	2	N	0.347
N1-051	5.5	2.2	2	.	0.330

Deli Turkey Inoculation Studies

Strain	PMSC	Rep	Lag Time	EGR
N1-021	Y	1	2.583632	0.472
N1-021	Y	2	1.123699	0.510
N1-040	Y	1	2.408535	0.647
N1-040	Y	2	2.145935	0.625
N1-061	Y	1	3.451381	0.505
N1-061	Y	2	3.046573	0.625
N1-080	Y	1	1.970402	0.630
N1-080	Y	2	1.757034	0.623
N1-080	Y	3	1.258929	0.723
N1-074	N	1	7.157198	0.279
N1-074	N	2	7.08438	0.203
N1-074	N	3	6.634494	0.180
N1-072	N	1	2.469423	0.588
N1-072	N	2	2.276405	0.531
N1-064	N	1	2.394807	0.549
N1-064	N	2	1.775308	0.523
N1-054	N	1	2.065279	0.604
N1-054	N	2	2.051609	0.458
N1-051	.	1	2.084858	0.551
N1-051	.	2	2.786239	0.657
N1-051	.	3	2.674856	0.738
N1-051	.	4	2.863136	0.709

Small Molecule Cytotoxicity Experiment Data

Chemical	Concentration (μM)	Rep	Time Point	OD_{490}
alexidine	12.5	1	18	2.9895
alexidine	12.5	2	18	2.709
alexidine	12.5	3	18	2.838
chlorhexidine	5	1	18	1.556
chlorhexidine	5	2	18	1.302
chlorhexidine	5	3	18	2.176
dioxidine	2.8	1	18	0.5045
dioxidine	2.8	2	18	0.6315
dioxidine	2.8	3	18	0.808
dmso	.	1	18	0.54
dmso	.	2	18	1.213
dmso	.	3	18	1.295
lysis		1	18	2.341
lysis		2	18	2.8755
lysis		3	18	2.56