INFLUENCE OF ENVIRONMENTAL FACTORS AND THE HOST'S SUSCEPTIBILITY ON THE DEVELOPMENT OF *LISTERIA MONOCYTOGENES* INFECTION IN THE GUINEA PIG MODEL

by

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ABTRACT OF THE DISSERTATION

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Listeria monocytogenes is an intracellular pathogen commonly associated with ready-to-eat foods. Using the guinea pig model, the impact of environmental factors on the infectivity of *L. monocytogenes*, as well as the impact of a host's immunity, on the development of listerial infection was investigated. Experiments were conducted to determine whether exposure to food environment and improper handling conditions impact the infectivity of *L. monocytogenes*. Results indicated that the virulence of *L. monocytogenes* was not influenced by exposure of the pathogen to food or improper handling conditions. In young adult guinea pigs orally challenged with 10^2 *L. monocytogenes*, a 27% infection rate occurred.

Further studies were conducted to determine the impact of immunomodulation of a host's immunity on the development of listerial infection using aged guinea pigs. Flow cytometry analysis showed that daily supplementation with vitamin E significantly

increased the level of $CD8^+$ T cells, while cyclosporine A (CsA) treatment resulted in a 25% decrease of $CD8^+$ T cells. HPLC analysis showed that short-term supplementation with vitamin E resulted in an overall 1-fold increase in the plasma alpha-tocopherol concentration. In low-dose (10^2 CFU) challenged animals, 50% of the control-group animals became infected. Only 22% of animals receiving the orthomolecular dose of vitamin E became infected, whereas animals that were immunosuppressed had an infection rate of 89%. In the immunosuppressed group three animals (16%) developed listerial infection with a quantifiable bacterial level of 0.3-3 log CFU g(-1) of organ in the spleen and liver. In the high-dose study, the population of *L. monocytogenes* was consistently 1 log CFU lower in the spleen and liver of animals in the vitamin E group as compared to the control and CsA-treated groups. The level of *L. monocytogenes* was not significantly different between the control and CsA-treated groups. The results suggest that immunomodulation of the host can influence listerial infection within an aged population of guinea pigs. From a public health perspective, the research indicates that a low oral dose of *L. monocytogenes* may pose a human health risk.

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CHAPTER I INTRODUCTION

A. *LISTERIA MONOCYTOGENES*: A FOODBORNE PATHOGEN

Listeria monocytogenes is a Gram-positive bacillus, ubiquitous in the environment. An intracellular pathogen of humans and animals, this psychrotroph is also the etiologic agent of the disease listeriosis. During the early eighties, it became a well-known foodborne pathogen after it was implicated in a series of foodborne outbreaks in North America (FDA/CFSAN, USDA/FSIS, 2003). Listeriosis is a severe illness. Compared to other foodborne pathogens, *L. monocytogenes* infection is responsible for a higher hospitalization rate (88%), compared to *Yersinia* (36%), *E. coli* O157:H7 (37%), and *Salmonella* (22%) (Swartz, 2002).

Although the incidence of listeriosis is low, at an approximate 2500 cases per year in the US, the associated mortality rate (25% - 40%) is the highest among foodborne pathogens. Of the 13 known serovars of *L. monocytogenes*, serovars 4b, 1/2a, and 1/2b are implicated in 96% of cases of human illness (Vazquez-Boland *et al*., 2001). In North America and Europe, epidemiological evidence associated with cases of human listeriosis showed that serovar 4b is most commonly linked to human illness, while serovar 1/2a is commonly isolated from food (Vazquez-Boland *et al*., 2001, Buncic *et al.,* 2000, Datta, 1994). A number of studies showed that variation in virulence exists among serotypes (Barbour *et al*., 2001,1996, Buncic *et al*., 2000). Brosch *et al.* (1993) found no differences in virulence of *L. monocytogenes* strains (n=125) based on typing characteristics and animal models (Brosch *et al.*, 1993). Kim *et al.* (2004) found that *L. monocytogenes* strains isolated from epidemics were more invasive than strains isolated

from the environment, regardless of serotypical differences. However, *L. monocytogenes* isolated from invasive and febrile gastroenteritis outbreaks were evaluated (n=27), and the results showed no significant differences in virulence properties based on pulsed-field gel electrophoresis and a mouse virulence assay (Franciosa *et al.*, 2005). Recently, a study by Takeuchi *et al*. (2006) evaluated 10 *L. monocytogenes* strains isolated from various foods, clinical, and human origins, and found no differences in the virulence and pathogenicity of *L. monocytogenes*.

Human listeriosis can be classified as invasive or non-invasive. Invasive listeriosis predominates in the young, the elderly, pregnant women, and the immunocompromised; those groups are largely responsible for the high mortality rate (Wing and Gregory, 2002). Exposure to *L. monocytogenes* can lead to two main forms of clinical syndromes: invasive listeriosis and gastrointestinal listeriosis (Schlech, 1997). The manifestations of invasive listeriosis include spontaneous abortions in pregnant women, meningitis, or bacteremia in adults. Recent evidence of epidemiological investigations suggests that ingestion of *L. monocytogenes* at a high dose, at levels of at least 10^6 CFU, can lead to febrile gastroenteritis without progression to invasive listeriosis. (Danielsson-Tham *et al*., 2004, Sim *et al*., 2002, Aureli *et al*., 2000). Clinical symptoms for listerial infections are diverse including fever, headache, stiff neck, nausea, flu-like symptoms, and vomiting (Schlech, 1997). Typically, patients experience diarrhea prior to development of bacteremia or meningitis (Ooi and Lorber 2005). The onset of a listerial infection can be sudden, from 9-16 hours in healthy individuals manifesting as

gastroenteritis, or gradual from 11-70 days, which is typical for invasive listeriosis (Ooi and Lorber, 2005, Donnelly, 2001, Schlech 1997).

B. TRANSMISSION OF *L. MONOCYTOGENES*

The usual mode of acquiring a *L. monocytogenes* infection is through the consumption of ready-to-eat foods. Common vehicles of *L. monocytogenes* include fresh produce, dairy products, and ready-to-eat (RTE) foods such as frankfurters and deli meats (FDA/CFSAN, USDA/FSIS, 2003). Since *L. monocytogenes* infections can lead to severe human health consequences with an associated high mortality rate, a zero tolerance approach was implemented for all ready-to-eat foods in the United States. Other countries, such as Germany, Canada, Denmark, and France adopted a risk-based approach (Donnelly 2001, FDA/CFSAN, USDA/FSIS, 2003).

B.1. Outbreaks leading to invasive listeriosis

One of the first epidemiological reports that established *L. monocytogenes* as a foodborne pathogen responsible for human listeriosis came from an outbreak where pasteurized milk was implicated as the vehicle. This outbreak occurred during the summer of 1983 in a hospital in Massachusetts. Various serotypes of *L. monocytogenes* were isolated from the farm, however, 32 out of 40 of the isolates tested were serotype 4b. This outbreak affected 49 patients, with an associated mortality rate of 29% (Fleming *et al*., 1985). In 1985, one of the largest outbreaks occurred in Los Angeles and it was linked to Mexican-style soft cheese. This outbreak resulted in 142 cases of illnesses and caused 48 deaths (Linnan *et al*., 1988). During the period of 1998 to 1999, a major

outbreak involving frankfurters occurred. Although the level of contamination was extremely low, <0.3 CFU/g of frankfurters, 101 cases of listeriosis and 21 fatalities occurred (Donnelly, 2001). More recently, a multi-state outbreak of listeriosis in the Northeast was linked to consumption of *L. monocytogenes* contaminated turkey deli-meat. This outbreak resulted in one of the largest food recalls in the United States (27.4 millions lbs). There were 46 cases of illness, 7 deaths and 3 stillbirths/miscarriages reported in 8 states (MMWR, 2002).

B.2. Outbreaks leading to gastroenteritis

A series of epidemiological reports of foodborne disease indicated that oral ingestion of *L. monocytogenes* can cause febrile gastroenteritis without progression to invasive listeriosis. In 1994, chocolate milk was implicated as the vehicle of transmission where 45 people were affected, and no deaths were reported. This was the first documented report of gastroenteritis caused by *L. monocytogenes* infection. The chocolate milk was heavily contaminated with 1.2×10^9 CFU/ml *L. monocytogenes*; the average dose ingested was 10^{11} CFU/person. There were no deaths, rather symptoms were self-limiting and febrile gastroenteritis with no progression to invasive disease occurred (Dalton *et al*., 1997). An outbreak was reported from Italy where corn was implicated as the vehicle of transmission causing 1566 cases of gastroenteritis. Of the 1566 cases, 1473 involved children aged between 6-10 years. The symptoms that were reported were mainly diarrhea, headache, nausea and abdominal pain. This outbreak was the result of mishandling and cross-contamination, where the corn from sterile cans was left at room temperature for 10 h, leading to a high level of *L. monocytogenes* in the food.

The *L. monocytogenes* isolated was serotype 4b. The dose ingested could not be determined, however the epidemiological investigation indicated a high level of contamination (Aureli *et al*., 2000). Although outbreaks of non-invasive listeriosis (gastroenteritis) are relatively uncommon, recent outbreaks leading to febrile gastroenteritis have included foods such as corn and tuna salad (Aureli *et al*., 2000), ready-to-eat meats (Sim *et al*., 2002) and cheese (Makino *et al*., 2005, Danielsson-Tham *et al*., 2004) worldwide. Interestingly, in all those cases of gastroenteritis, the dose of *L. monocytogenes* consumed was high, at least at 10^6 CFU (Ooi and Lorber, 2005).

Most epidemic or sporadic outbreaks of listeriosis were a consequence of post-processing contamination. Often, a low level of contamination of ready-to-eat foods can increase to high levels by the time the product reaches the consumer. The ubiquitous nature of *L. monocytogenes* and the ability of the microbe to survive and grow at refrigeration temperatures, make it a challenge to effectively eliminate *Listeria* from food processing facilities. Therefore, additional studies are needed to more effectively address issues in preventing cross- and post-processing contamination of ready-to-eat foods. It is important to identify potential microbial hazards during food processing especially as the incidence of foodborne outbreaks and the associated illnesses could have a significant economic and public health impact.

C. FACTORS CONTRIBUTING TO HUMAN ILLNESS

The clinical outcome of a listerial infection varies with respect to incubation time, infectious dose, virulence properties of the strain, and the immunological status of the host (Schlech 1997, Vazquez-Boland *et al*., 2001). Risk factors associated with a host include changes in the immune status, underlying predisposing conditions such as chronic diseases, malnutrition, and medications. Collectively, these factors can interplay and increase the propensity to developing infectious diseases (Thom and Forrest, 2006).

Over the course of infection, *L. monocytogenes* may invade a variety of cell types and tissues (Vazquez-Boland *et al*., 2001, Conte *et al*., 2000). For *L. monocytogenes* to mount a successful infection, it has to first overcome the host's cell-mediated immunity (MacDonald and Carter, 1980). The outcome of a listerial infection is highly dependent upon the underlying factors of the host, and in large part the host's cell-mediated immunity (Cossart and Lecuit, 1999, Lane and Unanue, 1972). *L. monocytogenes* can cross the blood brain barrier and subsequently lead to meningitis and/or encephalitis (Hertzig *et al*., 2002, Greiffenberg *et al*., 1998). Severe listeriosis resulting in central nervous system infections or spontaneous abortions predominates among a population consisting of the elderly, pregnant women, or those with impaired T-cell functions (CDC 2004, Hof 2004). Epidemiological evidence and recent findings suggest that *L. monocytogenes* has the ability to cross the intestinal, placental, and blood-brain barrier resulting in gastroenteritis, materno-fetal infections, and central nervous system infections, respectively (CDC, USDA, 2003, Barkardjiev *et al*., 2005, 2004, Lecuit *et al*., 2005, 2001).

C.1. Susceptibility to listerial infection

A major deciding factor in determining whether exposure to *L. monocytogenes* will lead to manifestation of clinical symptoms is the immune response. For decades, *L. monocytogenes* has been used as the model intracellular microorganism for delineating different cellular immune responses (Mackaness, 1968, Lane and Unanue, 1972, Acheson and Luccioli, 2004). Research demonstrated that *L. monocytogenes* can evade hosts' responses and it is able to survive and multiply in macrophages (Hanawa *et al*., 1995, Mackaness, 1968). It has been established that cell mediated immunity is responsible for combating intracellular pathogens such as *L. monocytogenes*, and cytotoxic T cells (CD8+) are largely responsible for clearance of the microbe (Lane and Unanue, 1972, Lara-Tejero and Pamer, 2004). Epidemiological reports, coupled with studies using animals models collectively showed that individuals with an impaired T cell function are at greater risk for developing listerial infections (Center for Disease Control and Prevention, 2003, Hof, 2003, 2004). Specifically, rates of listerial infection are the highest in pregnant women, immuno-compromised individuals, and the aged population (FDA/CFSAN, USDA/FSIS, 2003).

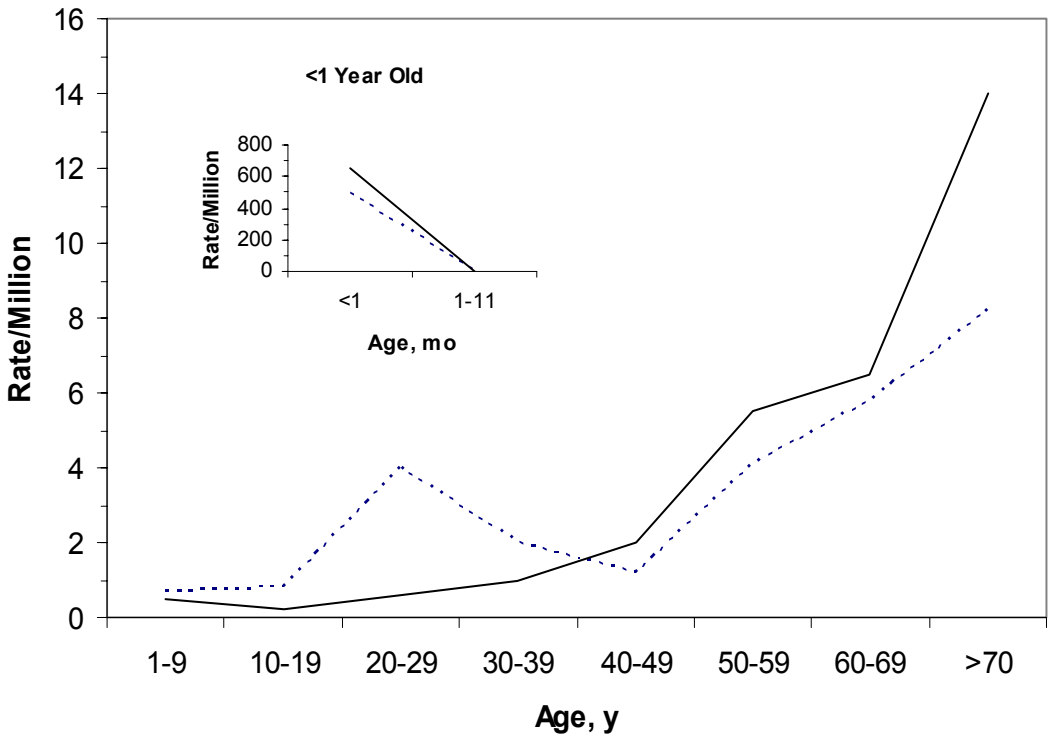

FIGURE 1. Age-specific incidence of listeriosis, by sex. Solid line indicates male patients; dashed line, female patients. Adapted from Lorber 1996.

Figure 1 shows the age-specific incidence of listeriosis (Lorber, 1997). An increase in the incidence of listeriosis was seen in women between 20-35 years of age, corresponding to the child-bearing age of women, representing one of target populations of *L. monocytogenes*. Beginning at around age 40-49, a precipitous increase was seen in both males and females, indicating the age-associated risk in developing listeriosis following consumption of a contaminated food.

C.2. Immunocompromised individuals

Immunocompromised individuals often include hospitalized patients, persons with HIV or cancer, and people taking glucocorticosteroid medication (Georgia Epidemiology Report, 1999). Owing to its intracellular nature and the ability to cross the blood-brain barrier, invasive listeriosis leading to meningitis is the most common clinical symptom in immunocompromised persons (Lorber, 1997). The cure rate for listeriosis in these individuals is only approximately 70% (Hof, 2004). To simulate an immunocompromised state, researchers have used nude or pathogen-free murine models. Studies also employed the use of substances (pharmaceutical agents) such as carrageenan, cyclophosphamide, cyclosporine A, and antibiotics to alter intestinal microflora, to selectively suppress the immune system, and to investigate the severity of listerial infections (Okamoto *et al*., 1994, Schelch *et al*., 1993, Lammerding *et al*., 1992, Baker *et al*., 1987, Hugin *et al*., 1986, Schaffner *et al*., 1983).

C.3. The pregnant women

Pregnant women were often asymptomatic during listerial infection, however clinical outcomes often included spontaneous abortion or neonates presenting with septicemia or meningitis (Abram *et al*., 2003, Vazquez-Boland *et al*., 2001). Recent research using pregnant guinea pigs provided the mechanistic explanation for the fetoplacental transfer of *L. monocytogenes* (Lecuit *et al*., 2004, Barardjiev *et al*., 2004, 2005).

C.4. The elderly

During the aging process, a decline in T-lymphocyte production and proliferation, presence of chronic diseases such as diabetes, cancer, and systemic inflammatory infections can occur (Yoshikawa, 2004). These factors could influence one another and necessitate the use of medications that may further suppress innate or acquired immune responses. One of the newer treatments for systemic inflammatory conditions such as rheumatoid arthritis is the use of anti alpha-tumor necrosis factor (TNF) therapy. However, anti alpha-TNF therapy has led to cases of patients from a long-term health care facility to develop listeria-associated infections, indicating an increased propensity for listeria infections associated with such therapy (Schelt *et al*., 2005, Slifman *et al*., 2003).

In the United States, census data indicate that 12% of the US population is 65 years and older (The AGS foundation for health in aging, 2002). Incidence of listerial infection steadily increases from age 40 to 64 with a more dramatic increase noted at age 65 onwards, indicating that age or age-related reasons is a predisposing factor to developing listeriosis (CDCP, 2004, Lorber, 1996). Circumstantial evidence indicates that pneumonia and septicemia remain among the top 10 causes of death in the elderly (CDCP 2004). *L. monocytogenes* infection is responsible for bacterial meningitis in the elderly, ranked as the second most common cause of mortality among adults 50 years of age and older (CDCP, 2001). Nagel *et al*. (1981) found a decreased level of cytotoxic T cells (CD8+) in aged humans. Such age-associated decline in T cell-mediated immunity can lead to higher risks in acquiring infectious diseases in the aged (Han and Meydani,

2000, Serafini, 2000). It is widely established that cell mediated immunity is essential in combating listeriosis or a listerial infection (Hof, 2003, Schaffner *et al*., 1983). Apart from epidemiological evidence, as well as clinical reports of nursing homes and hospitals, little is known about listerial infection within the elderly population.

D. VITAMIN E AND THE HUMAN HEALTH

There are eight isoforms of vitamin E in nature, each consisting of a chromanol ring with an aliphatic side chain. Vitamin E is divided into two groups based upon saturated and unsaturated side chains, known as tocopherols and tocotrienols, respectively. Vitamin E has a high antioxidant activity and acts as a free radical scavenger by inhibiting the propagation of free-radical reactions within membrane phospholipids and plasma lipoproteins (DRI, 2000). Of these eight isoforms, only alpha-tocopherol is maintained in human plasma (DRI, 2000, Traber, 1999). All the tocopherol forms (alpha, beta, gamma, and delta) occur naturally in foods and upon ingestion, they are emulsified and absorbed into the small intestines as micelles as with other types of dietary fat-soluble compounds (Hathcock *et al*., 2005). Retention of vitamin E is solely under the control of the alpha-tocopherol transfer protein (α-TTP) (Meier *et al*., 2003). However, only the alpha-tocopherol form is recognized by the α-TTP, and therefore, is responsible for the transport and storage of alpha-tocopherol in the tissues of the body (Meier *et al*., 2003, DRI, 2000). This also provides the basis for using only alpha-tocopherol to set the nutritional requirements such as Dietary Reference Intakes (DRI) and Estimated Average Requirements (EARs) (DRI, 2000).

Antioxidants, such as vitamin E, or C, have been shown to exhibit immunostimulatory effects in both animals and humans (Moriguchi and Muraga, 2000). Research showed that vitamin E positively affects the proliferation and stimulation of the cytotoxic T cells in humans and animals. Specifically, this positive immunostimulatory effect of vitamin E is attributed to its ability to regulate production of cytokines such as IL-2 and IFN-gamma, which in turn positively influenced cytotoxic T cells proliferation (Adolfsson *et al*., 2001, Moriguchi *et al*., 1998, Meydani *et al*., 1986, 1990).

Based on review of the literature it has been established that the decline in several aspects of immune functions is age-associated. There is a plethora of data showing that the protective properties of vitamin E can enhance the immune system of the elderly (Meydani *et al*., 1990, 1997, 2004). Short-term daily supplementation of vitamin E has been reported to enhance the immune system in the elderly, and served to mitigate viral and bacterial infections (Andreone *et al*., 2000, Meydani *et al*., 1998, 2004). In vivo measures of cell-mediated immunity indicate that ingestion of orthomolecular levels of vitamin E can significantly increase responsiveness to concavalin A, a mitogen, and enhance the T cell responses to IL-2 and delayed type hypersensitivity tests (Serafini, 2000, Meydani *et al*., 1990). In aged mice, vitamin E was found to restore cell division and the IL-2 production in T cells (Adolfsson *et al*., 2001).

Research also indicated that vitamin E supplementation beyond recommended daily requirements must be at a dose high enough (100mg/day) to elicit a pronounced cellular response (Pallast *et al*., 1999). The safety of orthomolecular supplementation with vitamin E was studied in 88 elderly individuals receiving vitamin E at doses of 60,

200, and 800mg/day over a period of 4 months. No adverse effects were observed (Meydani *et al*., 1998). Ingestion of larger doses of alpha-tocopherol acetate was positively correlated to an increase the alpha-tocopherol levels in the tissues, and this was shown to be more effective than ingesting lower doses over a longer period of time (DRI, 2000). A few reports have addressed the issue of toxicity associated with ingestion of high doses of vitamin E, and found that ingestion of vitamin E up to 1600 IU is safe (Kappus and Diplock, 1992, Hathcock *et al*., 2005). Collectively, vitamin E is a potent antioxidant and could be used specifically as an immuno-modulator to restore the impaired functions of the immune system associated with aging.

E. IMMUNOSUPPRESSIVE DRUGS USED IN ANIMAL STUDIES

Drugs that act to selectively reduce the defense mechanisms of the immune system in patients undergoing for example, organ transplant or cancer therapy have also been used to study development of bacterial and viral infections. However, commonly used drugs employed in animal studies to investigate the development of listerial infections include antibiotics to inhibit the intestinal microflora, gastric acid suppressants, immunosuppressive drugs such as carrageenan, cyclosporine A, and hydrocortisone acetate (Takeuchi *et al*., 2006, Lammerding *et al.*, 1992, Okamoto *et al*., 1994, Golnazarian *et al*., 1989, Stelma *et al*., 1987, Hugin *et al*., 1986). The immunosuppressants were shown to have a selective effect on various components of the immune system such as macrophages, polymorphonuclear cells, and/or T lymphocytes; their pharmacological actions vary depending on the period of suppression, dose applied, and the method of delivery. (Vessie *et al*., 2005, Stelma *et al*., 1989, Baker *et al*., 1987,

Schaffner *et al*., 1983). Considering the varied responses to those agents, the methods used to administer the agents, and the animal model used, it is difficult to compare those studies.

In murine models designed to investigate listerial infection, carrageenan has been used as a macrophage-suppressing agent and was shown to have a long lasting effect of approximately 2 weeks; this is commonly used in achieving an effective immunocompromised mouse model (Takeuchi *et al*., 2006, Stelma *et al*., 1987, Tatsukawa *et al*., 1979). Other agents such as hydrocortisone acetate suppress cell-mediated immunity, however, its immunosuppressive effect depletes after 24 hr (Golnazarian *et al*., 1989).

Cyclosporine A (CsA) is a fungal metabolite, that initially targets and more specifically suppresses T cells following peritoneal or oral administration in rats, mice, and guinea pigs (Borel *et al*., 1977). It was first used clinically in 1978, and because of its specific suppression of T cells, CsA is now commonly used in transplantation surgeries such as skin-grafting and organ transplant (Freitas, 2003). Animal studies demonstrate that the immuno-suppressive effect of CsA is not dose-dependent and it is a potent immuno-suppressant (Hugin *et al*., 1986, Schaffner *et al*., 1983). Schaffner *et al.* (1983) demonstrated that CsA suppresses T cell-mediated immunity without significant inhibition of nonimmune macrophages. It was also found that administering a low dose (10mg/kg) of CsA in isolation could lead to total suppression of the immune system (Freitas, 2003). The mechanism by which CsA inhibits T cell activation is by targeting a

phosphatase, calcineurin, which is an intracellular receptor of T cells. This in turn leads to downregulation of IL-2 production, a soluble cytokine that could otherwise induce the proliferation of cytotoxic T cells (Ho *et al*., 1996). Due to its specificity in targeting T lymphocytes, CsA is now used as a tool by immunologists for studying the cellular responses of the immune system.

F. USE OF ANIMAL MODELS IN INVESTIGATING LISTERIAL INFECTIONS

The murine model has been traditionally used for investigating the different aspects of a listerial infection. The infecting dose used often differed depending on route of delivery (intraperitoneal, intragastric, or intravenous), but was generally extremely high ($\geq 10^9$ CFU) with respect to numbers of cells that may be ingested during a foodborne outbreak (Barbour *et al*., 2001, 1996, Lammerding *et al*., 1992, Pine *et al*., 1990). Intravenous inoculation of mice with *L. monocytogenes* can induce a dose-dependent lethality, but not following oral challenge (Lecuit, 2005). Research indicates that the lack of a specific amino acid in the e-cadherin receptor of rats and mice renders the receptor inappropriate for interaction with *L. monocytogenes*, and therefore the subsequent entry into specific host cells (Lecuit *et al*., 1999). This may have precipitated the need to use a high dose (10^9 CFU) in rats and mice, hence explaining in part the inconsistent results among previous studies (Hamrick *et al*., 2003; Mahoney and Henriksson, 2002; Pine *et al*., 1990). Moreover, when a pregnant rat model was used to investigate listeriosis, results showed that healthy pups were delivered suggesting that

rats were resistant to listerial infection, and likely not the appropriate model for investigating listerial infection (Czuprynski and Balish, 1981).

During the early stages of an oral infection, production of internalin A and interaction with e-cadherin, a mammalian receptor, is required for the entry of *L. monocytogenes* (Racz *et al*., 1972). The specificity lies in the amino acid, proline, and interaction between internalin and e-cadherin (Lecuit *et al*., 1999). Lecuit *et al.* (2001) in a seminal study demonstrated that e-cadherin expressed by guinea pigs is essentially the same as that in humans. Furthermore, researchers have used in vivo and in vitro methods to investigate the dissemination of *Listeria* in the placenta (Bakardjiev *et al*., 2004, Lecuit *et al*., 2004). Using placental villous explants and trophoblastic cell lines, Lecuit *et al*. (2004) provided mechanistic evidence that crossing of the feto-placental barrier is dependent on the interaction between internalin A and e-cadherin, therefore making guinea pigs perhaps the more appropriate model for studying human listeriosis. Using flow cytometry analysis, Takizawa *et al*. (2004) evaluated the values of leukocyte fractions in blood and lymphoid tissues in guinea pigs. Results indicated that the guinea pig model was immunologically more similar to humans, as compared to other rodents. Collectively, these studies support the finding that the guinea pig is the more appropriate surrogate for investigating various disease conditions associated with humans.

II. HYPOTHESIS AND AIMS

Food contaminated with *L. monocytogenes* continues to be linked to illness and death worldwide. Post-processing contamination of a food is a common route of contamination based on epidemiology of listeriosis outbreaks (Donnelly, 2001). Reports of outbreaks in the past decade indicate that the dose of *L. monocytogenes* consumed ranged from 0.3 CFU/g to as high as 10^9 CFU/ml (Donnelly, 2001, Dalton *et al*., 1994). The infectious dose remains an enigma. Risk factors to the development of a listerial infection include the interplay of factors such as virulence of a strain, environment factors (processing environments, pH, food, etc.), dose consumed, and hosts' susceptibility. There have been several animal models used in investigating the different clinical aspects associated with listerial infection (Smith *et al*., 2002; Lammerding *et al*., 1992; Czuprysnki and Balish, 1981). As previously stated, research has established that the guinea pig is the appropriate surrogate for investigating listerial infection (Takizawa *et al*., 2004; Lecuit *et al*., 2001)

Hypothesis

The exposure of *L. monocytogenes* to environmental factors and the immuno-modulation of the host can influence the development of listerial infection.

The hypothesis is addressed by the following objectives:

1. Determine whether the exposure to food environment and improper handling conditions influence the infectivity of *L. monocytogenes* in the young adult guinea pig model.

2. Investigate the impact of immonomodulation in an aged guinea pig model on the development of listerial infection.

a) Demonstrate the immunoenhancing effect of an orthomolecular dose of vitamin E.

b) Demonstrate the effect of CsA as an immunosuppressant.

c) Examine the severity of listerial infection in the immunomodulated aged animals following oral challenge with a high and low dose of *L. monocytogenes.*

To address the influence of a food environment (exposure to food and improper handling condition) prior to consumption on the infectivity of *L. monocytogenes,* young adult guinea pigs were selected to represent the general population, and *L. monocytogenes* was delivered in chocolate milk, BHI, and water at 10^2 and 10^8 CFU. (Pang and Matthews, 2006). Our research findings suggest that exposure to a food environment have little or no impact on the outcome of a *L. monocytogenes* infection. The research does demonstrate however, that a low challenge dose (10^2 CFU) can result in listerial infection. Research is now focused on the outcome of listerial infection in a susceptible population. A comprehensive description of the research is presented in Chapter 2.

In chapter 3, we focused on the impact of immunomodulation in aged guinea pigs on the outcome of a listerial infection. In short, we investigated the influence of immunoenhancement (vitamin E supplementation) and immunosuppression (cyclosoporine A treatment) on the outcome of listerial infection following challenge with a high and low dose of *L. monocytogenes*.

REFERENCES

Abram, M., D. Schluter, D. Vuckovic, B. Wraber, M. Doric, and M. Deckert. 2003 Murine model of pregnancy-associated *Listeria monocytogenes* infection. FEMS Immunol Med Microbiol. **35**:177-82.

Acheson, D.W., and S. Luccioli. 2004. Microbial-gut interactions in health and disease. Best Pract Res Clin Gastroenterol. **18**: 387-404.

Adolfsson, O., B.T. Huber, and S.M. Meydani. 2001. Vitamin E-enhanced IL-2 production in old mice: Naïve but not memory T cells show increased cell division cycling and IL-2-producing capacity. J. Immuno.**167**: 3809-3817.

Andreone, P., S. Fiorino, C. Cursaro, A. Gramenzi, M. Margotti, L. Di Giammarino, M. Biselli, R. Miniero, G. Gasbarrini, and M. Bernadi. 2000. Vitamin E as treatment for chronic hepatitis B: results of a randomized controlled pilot trial. Antiviral Res. **49**: 75-81.

Aureli, P., G.C. Fiorucci, D. Caroli, G. Marchiaro, O. Novara, L. Leone, and S. Salmaso. 2000. An outbreak of febrile gastroenteritis associated with corn contamination by *Listeria monocytogenes.* New Engl. J. Med. **342:** 1236-1241.

Barbour, H.A., A. Rampling, and C. Hormaeche. 2001. Variation in the infectivity of *Listeria monocytogenes* isolates following intragastric inoculation of mice. Infect. Immun. **69**:4657-4660.

Barbour, H.A., A. Rampling, and C. Hormaeche. 1996. Comparison of the infectivity of isolates of *Listeria monocytogenes* following intragastric and intravenous inoculation in mice. Micro. Patho. **20**:247-253.

Bakardjiev, A.I, B.A. Stacy, S.J. Fisher, and D. Portnoy. 2004. Listeriosis in the pregnant guinea pig: a model of vertical transmission. Infect. Immun. **72**: 489-497.

Bakardjiev, A.I, B.A. Stacy, and D. Portnoy. 2005. Growth of Listeria monocytogenes in the guinea pig placenta and the role of cell-to-cell spread in fetal infection. J. Infect. Dis. **191**:1889-1897.

Baker, D., K. Karcher, A.V. Antouniou, J.L. Turk, B.T.G. Tan, and R.J. Scheper. 1987. Changes in lymphocyte subsets after treatment with cyclophosphamide and during the development of contact sensitivity in the guinea pig. Int. J. Immunopharmac. **9**: 175-183.

Brosch, R., B. Catimel, G. Milon, C. Buchrieser, E. Vindel, and J. Rocourt. 1993. Virulence heterogeneity of *Listeria monocytogenes* strains from various sources (food,

human, animal) in immunocompromised mice and its association with typing characteristics. J. Food Prot. **56**:296-301.

Borel, J.F., C. Feurer, C. Magnee, and H. Stahelin. 1977. Effects of the new anti-lymphocytic peptide cyclosporin A in animals. Immunology. **32**: 1017-1025

Centers for Disease Control and Prevention. 2004. Summary of notifiable diseases, United States, 2002. Morb. Mortal. Wkly. Rep. 51(53):1-78.

Centers for Disease Control and Prevention. 2004. Deaths: final data for 2002. National Vital Statistics Report, vol. 53, no.5. Available at: http://www.cdc.gov/nchs/data/nvsr/nvsr53/nvsr53_05acc.pdf. Accessed November, 2, 2005.

Ciesielski, C.A, A.W. Hightower, S.K. Parsons, and C.V. Broome. 1988. Listeriosis in the United States: 1980-1982. Arch Intern Med. **148**: 1416-1419.

Czuprynski, C.J., and E. Balish. 1981. Pathogenesis of *Listeria monocytogenes* for gnotobiotics rats. Infect. Immun. **32**:323-331.

Dalton, C.B., C.C. Austin, J. Sobel, P.S.Hayes, W.F. Bibb, L.M. Graves, B. Swaminathan, M.E. Proctor, and P.M. Griffin. 1997. An outbreak of gastroenteritis and fever due to *Listeria monocytogenes* in milk. N. Engl. J. Med. **336**: 100-105.

Danielsson-Tham, M.L., E. Eriksson, S. Helmersson, M. Leffler, L.Ludtke, M. Steen, S. Sorgjerd, and W. Tham. 2004. Causes behind a human cheese-borne outbreak of gastrointestinal listeriosis. Foodborne pathogens and disease. **1:** 153-159.

Dietary Reference Intakes. In. Vitamin E. National Academies Press. **2000**: 186-269.

Donelly C.W. 2001. *Listeria monocytogenes*: A continuing challenge. Nutri Rev. **59**: 183-194.

Farber, J.M., and P.I. Peterkin. 1991. *Listeria monocytogenes*, a foodborne pathogen. Microbiol. Rev. **55**:476-511.

FDA/CFSAN, USDA/FSIS. 2003. Interpretive Summary: Quantitative assessment of the relative risk to public health from foodborne *Listeria monocytogenes* among selected categories of ready-to-eat foods. Available at http://vm.cfsan.fda.gov/~dms/lmr2buch.html. Last assessed on April 16th, 2004.

FDA/CFSAN, CDCP. 2003. Reducing the risk of *Listeria monocytogenes* FDA/CDC 2003 update of the *Listeria* action plan. Available at http://www.cfsan.fda.gov/~dms/lmr2plan.html. Last assessed on April 16th, 2004.

Franciosa, G., A. Maugliani, F. Floridi, and P. Aureli. 2005. Molecular and experimental virulence of *Listeria monocytogenes* strains isolated from cases with invasive listeriosis and febrile gastroenteristis. FEMS Immun. Med. Micro. **43**: 431-439.

Freitas, R.A. 2003. Nanomedicine, Volume IIA: Biocompatibility. (http://www.nanomedicine.com/NMIIA/15.2.3.4.htm) Accessed 10 October 2005.

Georgia Epidemiology Report. March 1999. Division of Public Health. Volume 15, no. 3 (http://health.state.ga.us).

Golnazarian, C.A., C.W. Donelly, S.J. Pintauro, and D.B. Howard. 1989. Comparison of infectious dose of *Listeria monocytogenes* F6817 as determined for normal versus compromised C57B1/6J mice. J. Food Prot. **52**: 696-701.

Greiffenberg, L., W. Goebel, K.S. Kim, I. Weiglein, A. Bubert, F. Engelbrecht, M. Stins, and M. Kuhn. 1998. Interaction of *Listeria monocytogenes* with human brain microvascular endothelial cells: InlB-Dependent invasion, long-term intracellular growth, and spread from macrophages to endothelial cells. Infect. Immun. **66**:5260-5267.

Grundling, A., M.D. Gonzalez, and D.E. Higgins. 2003. Requirement of the *Listeria monocytogenes* broad-range phospholipase PC-PLC during infection of human epithelial cells. J Bacteriol. **185**:6295-6307.

Hamrick, S.T., J.R. Horton, P.A. Spears, E.A. Havell, I.W. Smoak, and P.E. Orndorff. 2003. Influence of pregnancy on the pathogenesis of listeriosis in mice inoculated intragastrically. Infect. Immun. **71**:5202-5209.

Hanawa, T., T. Yamamoto, and S. Kamiya. 1995. *Listeria monocytogenes* can grow in macrophages without the aid of proteins induced by environmental stresses. Infect. Immun. **63**: 4595-4599.

Hertzig, T., M.Weber, L. Greiffenberg, B.S. Holthausen, W.Goebel, K.S. Kim, and M. Kuhn. 2002. Antibodies present in normal human serum inhibit invasion of human brain microvascular endothelial cells by *Listeria monocytogenes*. Infect. Immun. **71**:95-100.

Ho, S., N. Clipstone, L. Timmermann, J. Northrop, I. Graef, D. Fiorentino, J. Nourse, and G.R. Crabtree. 1996. The mechanism of action of cyclosporin A and FK506. Clin. Immuno. and Immunopath. **80** (3): S40-S45.

Hof, H. 2004. An update on the medical management of listeriosis. Expert Opin. Pharmacother. **5**: 1727-1735.

Hof, H. 2003. History and epidemiology of listeriosis. FEMS Immun. Med. Microbiol. **35**: 199-202.

Hugin, A.W., A. Cerny, M. Wrann, H. Hengartner, and R.M. Zinkeragel. 1986. Effect of cyclosporin A on immunity to *Listeria monocytogenes*. Infect Immun. **52** :12-7.

Kim, S.H., M.K. Bakko, D. Knowles, and M.K. Borucki. 2004. Oral inoculation of A/J mice for detection of invasiveness differences between *Listeria monocytogenes* epidemic and environmental strains. Infect and Immun. **72**: 4381-4321.

Lammerding, A.M., K.A. Glass, A. Gendron-Fitzpatrick, and M.P. Doyle. 1992. Determination of virulence of different strains of *Listeria monocytogenes* and *Listeria innocua* by oral inoculation of pregnant mice. Appl. Environ. Microbiol. **58**:3991-4000.

Lane F.C., and E.R. Unanue. 1972. Requirement of thymus (T) lymphocytes for resistance to listeriosis. J. of Experiment. Med. **135:** 1104-1112.

Lecuit, M. 2005. Understanding how *Listeria monocytogenes* targets and crosses host barriers. Clin. Microbiol. Infect. **11**: 430-436.

Lecuit, M., D.M. Nelson, S.D. Smith, H. Khun, M. Huerre, M. Vacher-Lavenu, J.I. Gordon, and P. Cossart. 2004. Targeting and crossing of the human maternofetal barrier by *Listeria monocytogenes*: Role of internalin interaction with trophoblast E-cadherin. PNAS. **101**: 6152-6157.

Lecuit, M., S. Vandormeal-Pournin, J. Lefort, M. Huerre, P. Gounon, C. Dupuy, C. Babinet, and P. Cossart. 2001. A transgenic model for listeriosis: Role of internalin in crossing the intestinal barrier. Science. **292**:1172-1725.

Lecuit, M., S.Dramsi, C. Gottardi, M. Fedor-Chaiken, B. Gumbiner, and P. Cossart. 1999. A single amino acid in E-cadherin responsible for host specificity towards the human pathogen *Listeria monocytogenes*. EMBO J. **18**:3956-3963.

Linnan, M.J., L. Mascola, X.D. Lou, V. Goulet, S. May, C. Salminen, D.W. Hird, M.L. Yonekura, P. Hayes, R. Weaver, A. Audurier, B.P. Plikaytis, S.L. Fannin, A. Kleks, and C.V. Broome. 1988. Epidemic listeriosis associated with Mexican-style cheese. N. Engl. J. Med. **319**:823-828

Lorber, B. 1996. Listeriosis. Clin. Infect. Dis. **24**:1-11.

MacDonald, T.T., and P.B. Carter. 1980. Cell mediated immunity to intestinal infection. Infect. and Immun. **28**: 516-523.

Makino, S.I., K. Kawamoto, K. Takeshi, Y. Okada, M. Yamasaki, S. Yamamoto, S. Iqimi. 2005. An outbreak of food-borne listeriosis due to cheese in Japan, during 2001. Int. J Food Microbiol. **104**: 189-196.

Meier, R., T. Tomizaki, C. Schulze-Briese, U. Baumann, and A. Stocker. 2003. The molecular basis of vitamin E retention: Structure of human alpha-tocopherol transfer protein. J. Mol. Biol. **331**: 725-734.

Meydani S.M., S.N. Han, and D.H. Hamer. 2004. Vitamin E and respiratory infection in the elderly. Ann. N.Y. Acad. Sci. **1031**: 214-222.

Meydani, N.S., M. Meydani, J.B. Blumberg, L.S. Leka, M. Pedrosa, R. Diamond, and E.J. Schaefer. 1998. Assessment of the safety of supplementation with different amounts of vitamin E in healthy older adults. Am J Clin. Nutr. **68**:311-8.

Meydani, S.N., M.P. Barklund, S. Liu, M. Meydani, R.A. Miller, J.G. Cannon, F.D. Morrow, R. Rocklin, and J.B. Blumberg. 1990. Vitamin E supplementation enhances cell-mediated immunity in healthy elderly subjects. Am. J. Clin. Nutr. **52**:557-563.

Moriguchi, S. 1998. The role of vitamin E in T– cell differentiation and the decrease of cellular immunity aging. Biofactor. **7**:77-78.

Moriguchi, S., and M. Muraga. 2000. Vitamin E and immunity. Vitam Horm. **59**: 305-336.

Nagel, J.E., F.J. Chrest, and W.H. Adler. 1981. Enumeration of T lymphocyte subsets by monoclonal antibodies in young and aged humans. J. Immunol. **127**: 2086-2088.

Okamoto, M., A. Nakane, and T., Minagawa. 1994. Host resistance to an intragastric infection with *Listeria monocytogenes* in mice depends on cellular immunity and intestinal bacterial flora. Infect. Immun. **62**:3080-3085.

Ooi, S-T. , and B. Lorber. 2005. Gastroenteritis due to *Listeria monocytogenes.* Clin. Infect. Dis. **40**: 1327-1332.

Pang, H. E., and K. Matthews. 2006. Influence of a food environment on listerial infection in the guinea pig model. Journal of Food Safety **26**: 313-324

Pallast, E.G., E.G. Schouten, F.G. de Waart, H.C. Fonk, G. Doekes, B.M. von Blomberg, and F. J. Kok. 1999. Effect of 50- and 100-mg vitamin E supplements on cellular immune function in noninstitutionalized elderly persons. Am. J. Clin. Nutr. 69: 1273-1281.

Pine, L., G.B. Malcom, and B.D. Plikaytis. 1990. *Listeria monocytogenes* intragastric and intraperitoneal approximate 50% lethal doses for mice are comparable, but death occurs earlier by intragastric feeding. Infect. Immun. **58**:2940-2945.

Racz, P., K. Tenner, and E. Mero. 1972. Experimental *Listeria* enteritis. I. An electron microscopic study of the epithelial phase in experimental *Listeria* infection. Lab. Invest. **26**: 694-700.

Sahyoun, N.R., H. Lentzner, D. Hoyert, and K.N. Robinson. 2001. Trends in causes of death among the elderly. Aging Trends No. 1. Page 1-9. CDCP: National Center for Health Statistics.

Schlech, W.F. 1997. *Listeria* gastroenteritis. Old syndrome, new pathogen. New Engl. J. Med. **336**: 130-132

Schaffner, A., H. Douglas, and C.E. Davis. 1983. Models of T cell deficiency in listeriosis: The effects of cortisone and cyclosporin A on normal and nude BALB/c mice. The J. of Immun. **131**: 450-453.

Schett, G., P. Herak, W. Graninger, J. Smolen, and M. Aringer. 2005. Listeria-associated arthritis in a patient undergoing etanercept therapy: case report and review of the literature. J. Clin. Microbiol. **43**: 2537-2541.

Schlech, W.F., D.P. Chase, and A. Bradley. 1993. A model of food-borne *Listeria monocytogenes* infection in the Sprague-Dawley rat using gastric inoculation: development and effect of gastric acidity on infective dose. Int. J. Food Microbiol. **18**: 15-24.

Serafini, M. 2000. Dietary vitamin E and T cell-mediated function in the elderly: effectiveness and mechanism of action. Int. J. Devl. Neuroscience. **18**: 401-410.

Sim, J., D. Hood, L. Finnie, M. Wilson, C. Graham, M. Brett, and J.A. Hudson. 2002. Series of incidents of Listeria monocytogenes non-invasive febrile gastroenteritis involving ready-to-eat meats. Lett. in Appl. Microbiol. **35**: 409-413.

Slifman, N.R., S.K. Gershon, J.H. Lee, E.T. Edwards, and M.M.Braun. 2003. *Listeria monocytogenes* infection as a complication of treatment with tumor necrosis factor alpha-neutralizing agents. Arthritis Rheum. **48**: 319-324.

Smith, M. A., K. Takeuchi, R.E. Brackett, H.M. McClure, R.B. Raybourne, K.M. Williams, U.S. Babu, G.O. Ware, J.R. Broderson, and M.P. Doyle. 2002. Nonhuman primate model for *Listeria monocytogenes*-induced stillbirths. Infect. Immun. **71**: 1574-1579.

Stelma, G.N., A.L. Reyes, J.T. Peeler, D.W. Francis, J.M. Hunt, P.L. Spaulding, C.H. Johnson, and J. Lovett. 1987. Pathogenicity test for *Listeria monocytogenes* using immunocompromised mice. J. Clin. Microbiol. **25**: 2085-2089.

Swartz, N.M. 2002. Human Diseases caused by foodborne pathogens of animal origin. Clin. Infect. Diseases. **34**:S111-122.

Takeuchi, K., N. Mytle, S. Lambert, M. Coleman, M.P. Doyle, and M.A. Smith. 2006. Comparison of *Listeria monocytogenes* virulence in a mouse model. J. Food Prot. **69**: 842-846.

Takizawa, M., J. Chiba, S. Haga, T. Asano, N. Yamamoto, and M. Honda. 2004. The normalization of guinea pig leukocyte fractions and lymphocyte subsets in blood and lymphoid tissues using a flow cytometric procedure. Exp. Anim. **53**: 321-329.

Tatsukawa, K., M. Mitsuyama, K. Takeya, and K. Nomoto. 1979. Differing contribution of polymorphonuclear cells and macrophages to protection of mice against *Listeria monocytogenes* and *Pseudomonas aeruginosa.* J. Gen. Micro. **115**: 161-166.

Traber M.G. 1999. Utilization of vitamin E. Biofactors. **10**: 115-20.

The decade for health in aging. The AGS foundation for health in aging. 2000. Available at: http://www.healthinaging.org/public_policy/decade_challenge.php Accessed on November, 2. 2005

Vazquez-Boland, J.A., M. Kuhn, P. Berche, T. Chakraborty, G. Dominguez-Bernal, W. Goebel, B. Gonzalez-Zorn, J. Wehland, and J. Kreft. 2001**.** *Listeria* pathogenesis and molecular virulence determinants. Clin. Microbiol. Rev. **14**:584-640.

Vessie, E.L., G.M. Hirsch, T.D.G. Lee. 2005. Aortic allograft vasculopathy is mediated by CD8+ T cells in cyclosporin A immunosuppressed mice. Transplant Immun. **15**: 35-44.

Wing, E.J., and S.H. Gregory. 2002. *Listeria monocytogenes*: Clinical and experimental update. J. Infect. Dis. **185** (Suppl 1): S18-24.

Yoshikawa, T. 2000. Epidemiology and unique aspects of aging and infectious diseases. Clin. Infect. Dis. **30**: 931-33.

CHAPTER 2

INFLUENCE OF A FOOD ENVIRONMENT ON *LISTERIA MONOCYTOGENES* INFECTION IN THE GUINEA PIG MODEL

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I. ABSTRACT

Listeria monocytogenes is a widespread foodborne pathogen associated with severe illness in humans. Food composition, processing, storage, distribution, and handling conditions are all factors that may individually or collectively contribute to the virulence of a pathogen. Using the guinea pig as a human surrogate, we investigated the impact of carrier vehicle and improper food handling practices on the infectivity of *L. monocytogenes*. The chocolate milk was artificially contaminated with *L. monocytogenes* prior to exposure to improper handling conditions. Improper handling of chocolate milk included leaving the milk at an elevated temperature (37^{o}C) and then placing in the refrigerator (4^{o}C). Guinea pigs were orally challenged with either 10^2 or 10^8 CFU of *L. monocytogenes* in 1 ml of nutrient broth or chocolate milk that had been exposed to improper handling conditions. A third group was challenged with *L. monocytogenes* suspended in water. On day 2 and 4, upon enrichment of organ samples, presence of *L.*

monocytogenes was detected in 27 % of the animals receiving the low dose regardless of the carrier vehicle and improper handling conditions. Animals fed the high dose (10^8 CFU) of *L. monocytogenes* had similar populations of the pathogen in the spleen and liver, regardless of the carrier vehicle. These results are significant in that a low dose (10^2 CFU) could lead to listerial infection. Under conditions tested in this study, the carrier vehicle, and exposure of the pathogen to improper handling practices had no detectable effect on the establishment of listerial infection in the guinea pig model.

II. INTRODUCTION

The status of *Listeria monocytogenes* as a foodborne pathogen in the United States was elevated during the early 1980s, after it was implicated in a series of outbreaks (FDA/CFSAN, USDA/FSIS, 2003; Hof 2003; Donnelly, 2001). Although only approximately 2500 cases occur per year in the US, the mortality rate (ca. 25%) is the highest among foodborne pathogens (Swartz, 2002; Mead *et al*., 1999). Frequent vehicles associated with *L. monocytogenes* transmission include unpasteurized milk, soft cheeses, deli-meats, and fresh produce (FDA/CFSAN, USDA/FSIS, 2003). Due to the severe consequences of listeriosis a zero tolerance approach was implemented for all RTE foods in the United States (FDA/CFSAN, USDA/FSIS, 2003). However, a risk-based approach for *L. monocytogenes* has been adopted in countries such as Canada, Germany, Denmark and France (FDA/CFSAN, USDA/FSIS, 2003; Donnelly, 2001).

As an intracellular pathogen transmitted primarily through contaminated foods, *L. monocytogenes* faces the challenge of a food environment (i.e. pH, temperature, NaCl, water activity) before encountering a host (Datta, 1994; Mekalanos, 1992). From a microbial safety perspective, foods are complex environments consisting of factors that can interplay and eventually impact the survival and virulence of *L. monocytogenes*. Changing the physiology of the pathogen could potentially result in unintended consequences, e.g., leading to enhanced survival in a host. Studies have demonstrated that food components (acids, salts, carbohydrates) and food processes (Maillard reaction, temperature shifts) can alter the virulence of *L. monocytogenes* (Koutsoumanis *et al*., 2003; Conte *et al*., 2000; O'Driscoll *et al*., 1996; Sheehan *et al*., 1995; Sheikh-Zeinoddin *et al*., 1995, Leimester-Wachter *et al.*, 1992). In animal challenge studies, the culture medium and carrier used to deliver the pathogen to an animal could be of the utmost importance. Previous animal studies have used foods such as salami batter, milk, and whipping cream as carriers when investigating the virulence of *L. monocytogenes* (Mytle *et al*., 2006; Mahoney and Henriksson, 2002; Smith *et al*., 2002; Pine *et al.,* 1990). Depending on the study, *L. monocytogenes* was inoculated into sterile foods or delivered directly to animals without exposure to adverse handling conditions (Mahoney and Henriksson, 2002; Smith *et al*., 2002). Unlike previous research, the aim of our study was to investigate the effect of food and improper handling conditions in food on the virulence of *L. monocytogenes* prior to oral challenge using the guinea pig model.

In the present study, using conventionally reared outbred guinea pigs as an experimental animal model; we investigated listerial infection using *L. monocytogenes*

contaminated chocolate milk that was improperly handled. Chocolate milk was selected as a carrier for reasons including the ease of administration and that it supports the growth of *L. monocytogenes* (data not shown). We simulated a possible outbreak scenario by mimicking the natural route of infection by oral gavage of guinea pigs with *L. monocytogenes* suspended in improperly handled food. The study was designed to address the use food and handling conditions as mitigating factors to investigate listerial infection in the guinea pig model, rather than to mimic a specific outbreak.

III. MATERIALS AND METHODS

A. BACTERIAL STRAIN

L. monocytogenes serotype 4b used in this study was isolated from a patient linked to an outbreak of foodborne illness (ribotype DUP-1044A, Cornell University; (http://www.pathogentracker.net). Before use in this study, a specific PCR assay was conducted to confirm that the isolate was *L. monocytogenes*. *L. monocytogenes* was cultured overnight in 10 ml of brain heart infusion (BHI) broth (Difco, Detroit, MI, USA) at 37^{o}C with aeration for 18 h, centrifuged (5500 × g, 10 min, 4^{o}C), suspended in phosphate buffered saline (PBS; Sigma, St. Louis, MO, USA) and serially diluted to achieve desired doses of bacteria.

B. PREPARATION OF FOOD VEHICLE AND BACTERIAL INOCULA

Chocolate milk (1% low fat) was purchased from a local supermarket. Aerobic bacteria were determined by spread-plating onto plate count agar (Difco, USA). Resuspended overnight culture of *L. monocytogenes* was serially diluted to achieve final concentrations of 10^8 and 10^2 CFU ml^{-1} in chocolate milk, BHI, and sterile water, respectively. Bacterial numbers were confirmed by plating (1:10 serial dilutions) of the inocula onto Rapid L'mono (Bio-Rad Laboratories, Hercules, CA, USA) plates. Plates were incubated at 37°C for 24-48 h, and *L. monocytogenes* colonies enumerated.

C. TREATMENT OF FOOD VEHICLE

L. monocytogenes was inoculated into chocolate milk or BHI to achieve concentrations of ~10^2 CFU ml^{-1} and ~10^8 CFU ml^{-1}, then exposed to the following handling conditions. Chocolate milk and BHI were held at 37°C for 2 h, and then cooled for 2 h at 4°C. The final temperature of BHI and chocolate milk was 4°C. This treatment was designed to simulate improper handling of individual servings (250 ml) of chocolate milk. There was limited bacterial growth during the treatment period, hence bacterial numbers were adjusted appropriately using improperly handled pathogen-free chocolate milk to achieve approximately ~10^2 and ~10^8 CFU/ml of bacteria. The control for improperly handled chocolate milk and BHI was an overnight (37°C, 18 h) culture of *L. monocytogenes* grown in BHI and then suspended in sterile water and per-orally administered to guinea pigs. Therefore, the overnight culture of *L. monocytogenes* suspended in sterile water served as a control for the improper handling scheme, while *L. monocytogenes* delivered in the improperly handled BHI served as a control for the

improperly handled chocolate milk. Experiments demonstrated no loss in viability of *L. monocytogenes* while suspended in sterile water (data not shown).

D. ANIMALS

Outbred female Hartley guinea pigs weighing between 300-350 g were purchased (Elm Hills Labs Inc., Chelmsford, MA, USA), and housed under standard conditions (50 % humidity and 12-h dark-12-h light cycle) at the Rutgers University animal facility. Guinea pigs, between 3-4 weeks old were kept in individual cages and fed guinea pig chow and water ad libitum. All animal experiments were conducted in accordance with federal guidelines and approved by the Rutgers University Animal Care and Facilities Committee.

E. INTRAGASTRIC CHALLENGE OF GUINEA PIGS

Guinea pigs were starved 12 h prior to intragastric challenge to prevent regurgitation during oral administration of *L. monocytogenes* (Mahoney and Henriksson, 2002). Using a 15-in. nasogastric feeding tube (Jorgensen Laboratories, Inc., Loveland, CO, USA) fitted into a 3 ml syringe, guinea pigs were intragastrically challenged with either a low dose (ca. 10^2 CFU) or a high dose (10^8 CFU) of *L. monocytogenes* in 1 ml of either: chocolate milk, BHI, or sterile water. Therefore, in animals receiving the 10^8 dose, three groups of guinea pigs received *L. monocytogenes* suspended in each of the carriers (i.e., chocolate milk, BHI, sterile water). On day 2 and day 4 animals were sacrificed. The same experimental approach was utilized for the animals receiving 10^2 *L. monocytogenes*. The experiment was conducted twice: in the first experiment, 36

animals were used, and in the second experiment 24 animals were used. In total 60 animals were used, with collectively 5 animals per group (i.e., chocolate milk, BHI, sterile water). The high and low doses were included in each set of experiments. Animals were not treated to alter stomach acidity or the intestinal flora prior to per-oral administration of the challenge inoculum. Data from both experiments were combined and reported.

F. ENUMERATION OF *L. MONOCYTOGENES* FROM TISSUES OF CHALLENGED GUINEA PIGS.

Guinea pigs were sacrificed on day 2 (48 h) and day 4 (96 h) post-challenge. The spleen, liver, mesenteric lymph nodes, and small intestines were aseptically removed *in toto*. Each organ was individually weighed, placed into an equal volume of cold sterile PBS + 0.001% Triton-X (vol/vol), and homogenized using a tissue homogenizer (Polytron®, Kinematica, Switzerland). Intestines, after weighing, were suspended in an equal volume of cell culture medium (Earls Minimal Essential Medium [EMEM] supplemented with gentamicin sulfate (40 ug/ml; Cellgro, Herndorn, VA, USA) and incubated at room temperature for 1 h. Following incubation the intestines were removed, washed twice in EMEM, rinsed once with PBS, placed into an equal volume of cold sterile PBS + 0.001% Triton-X (vol/vol), and homogenized. This procedure results in killing of extracellular bacteria permitting the enumeration of intracellular bacteria (Lecuit *et al.* 2001). Organ homogenates were serially diluted (1:10) in PBS+ 0.001% Triton-X (vol/vol), and spread plated in duplicate on Rapid L'mono plates (Leclercq

2004). Plates were incubated at 37°C for 48-72 h. *L. monocytogenes* colonies were enumerated and numbers expressed as mean log/ g of organ.

G. ENRICHMENT OF ORGAN SAMPLES

All organ samples from animals receiving 10^2 CFU *L. monocytogenes* were enriched with an equal volume of 2X BHI. Organ samples were incubated for 24 hr, and 100 µl of each sample was spread plated onto Rapid L'mono in duplicate. The minimum detection limit for the plating method was 10 CFU/ g organ sample. The plates were incubated in the dark at 37°C for 48-72 hr, and the presence of *L. monocytogenes* on the plates was recorded. Samples that were negative for *L. monocytogenes* were subjected to enrichment using the entire organ sample (liver, spleen, small intestines, and lymph nodes). Animals whose liver or spleen samples were positive for *L. monocytogenes* were scored positive for listerial infection. The percent of infected animals was calculated by dividing the number of animals whose spleen or liver samples were positive by the total number of animals receiving 10^2 *L. monocytogenes*.

H. STATISTICAL ANALYSIS

Means and standard errors were calculated using Microsoft Excel's SEM function. To determine whether means differed among carriers used, data were analyzed using the Student's T- test. The level of statistical relevance for all comparisons was set at $P<0.05$.

IV. RESULTS AND DISCUSSION

In this study, chocolate milk was subjected to improper handling by holding the milk at 37^{o}C for 2 h and then refrigerating (4^{o}C, 2 h). This scenario could potentially occur during an outdoor event in the summer where milk is left out in the open, returned to the refrigerator, and consumed afterwards. Throughout the duration of the study, all the animals were observed daily for signs of distress. The animals exhibited no adverse signs (diarrhea, loose stool, ruffled coat, arched back, loss of appetite) following challenge. At the 10^2 dose, *L. monocytogenes* was not recovered from the spleen, liver, mesenteric lymph nodes, or small intestines harvested from test animals using conventional plating (data not shown). In an effort to detect low numbers of *L. monocytogenes,* samples were enriched (BHI added to organ homogenates, followed by incubation at 37^{o}C for 24 h) and plated onto Rapid L'mono plates. We found that 27% of the animals were positive for *L. monocytogenes* (Table 2.1). The percentage of positive animals was determined by dividing the number of organ samples positive for *L. monocytogenes* by the total number of animals orally challenged with 10^2 *L. monocytogenes*. Specifically, only the presence of *L. monocytogenes* in the liver or spleen of an animal was counted as positive for infection. Results suggested that a dose of 10^2 CFUcould lead to infection in the guinea pig model under conditions evaluated regardless of carrier vehicle. However, the immune system of a healthy host seems to be able to effectively clear low numbers of the pathogen since the target microbe was only detected following enrichment of the organ samples. During the early stages of listerial infection, an increase in neutrophils and natural killer cells leading to an up-regulation of

T-cell induced cytokines such as TNF alpha can occur and may be sufficient to control low numbers of *L. monocytogenes* (Conlan, 1997; Takada *et al*., 1996; Conlan and North, 1994; Schlech *et al*., 1993).

All animals receiving 10^8 CFU *L. monocytogenes* developed infection based on microbiological analysis of the liver and spleen (Fig. 2.1A and B). *L. monocytogenes* delivered in sterile water was recovered at 0.5 log CFU/ g of liver whereas the levels recovered in animals receiving the chocolate milk or BHI was at approximately 1.5 log CFU/ g of liver. However, only 1 out of 5 animals receiving *L. monocytogenes* delivered in sterile water had quantifiable levels of *L. monocytogenes* (Fig. 1A). By day 4 however, *L. monocytogenes* delivered in all three carriers i.e., BHI, chocolate milk, and water reached similar populations of *L. monocytogenes* in the liver and spleen**.** Regardless of the delivery vehicle or improper handling conditions, populations of *L. monocytogenes* recovered on day 2 or day 4 from the liver (Fig. 1A) or spleen (Fig. 1B) were similar. In general, all animals exhibited an approximate 0.5 log decrease in *L. monocytogenes* populations in the liver that occurred from day 2 to day 4.

TABLE 2.1. ENRICHMENT OF ORGAN SAMPLES FROM GUINEA PIGS THAT RECEIVED 10^2 *L. MONOCYTOGENES* SUSPENDED IN CHOCOLATE MILK, BHI, OR STERILE WATER.

		Day post-challenge					
		Day 2			Day 4		
		Chocolate milk[c]	BHI	Water	Chocolate milk	BHI	Water
Organs	Liver[a]	1/5[b]	0/5	2/5	0/5	0/5	2/5
	Spleen	1/5	0/5	0/5	0/5	1/5	1/5
	Lymph Nodes	0/5	0/5	3/5	1/5	0/5	0/5
	Small Intestines	3/5	4/5	0/5	2/5	3/5	0/5
Total no. of animals infected		8					
% of infected animals		27% [d]					

[a]Each organ was collected *in toto*. Organ samples were enriched for 24 h using BHI at 37°C, and plated onto Rapid L'mono plates in duplicate.

[b]Values indicate number of organ samples found positive for *L. monocytogenes* / total number of animals tested.

[c]Groups of 5 guinea pigs were orally challenged with 10^2 *L. monocytogenes* suspended in chocolate milk, BHI, or water. Animals were sacrificed on day 2 (48 h) and day 4 (96 h) post orally challenge with *L. monocytogenes* serotype 4b. Data presented are from two experiments using a total of 30 animals.

[d] % of infection was calculated based upon the presence of *L. monocytogenes* in the liver or spleen. Eight out of 30 animals were found to be positive for listerial infection. % value was obtained by dividing 8 out of 30 and multiplying the value by 100%.

For the animals receiving a high dose (10^8) of *L. monocytogenes*, a similar trend was also observed in the mesenteric lymph nodes and small intestines. On day 2, the animals that received *L. monocytogenes* delivered in sterile water had significantly lower (1 log CFU/g of organ) bacterial populations as compared to those animals receiving chocolate milk (4.4 log CFU/g of organ) or BHI (3.4 log CFU/g of organ) (Fig. 2.1C). The populations of *L. monocytogenes* (10^3 to 10^4 CFU/ g of organ) were greatest in the mesenteric lymph nodes (Fig. 2.1C) compared to all organs evaluated (Fig. 2.1). *L. monocytogenes* populations were approximately 1 log greater in the mesenteric lymph nodes compared to the small intestines (Fig. 2.1D). *L. monocytogenes* populations remained relatively constant from day 2 to day 4 in the mesenteric lymph nodes. In animals administered with *L. monocytogenes* using water as the delivery vehicle, populations were approximately 2–3 log lower in the mesenteric lymph nodes (Fig. 2.1C) and 1 – 2 log lower in the small intestines (Fig. 2.1D) on day 2. Perhaps in our study the conditions of improper handling of the contaminated food positively impacted the pathogen, thereby enhancing the initial infection process. It is also likely that bacteria ingested in a carrier such as water are generally more susceptible to insult during passage through the gastrointestinal tract compared to carrier vehicles such as chocolate milk and BHI. The differences between chocolate milk and BHI were not statistically significant ($P>0.05$). Regardless of the delivery vehicle, day 4 populations of *L. monocytogenes* within the mesenteric lymph nodes and small intestine were similar (Fig. 2.1C and D). Overall, the carrier vehicle and improper handling condition did not have an impact of the infectivity of *L. monocytogenes*.

The intent of our study was to maintain normal physiology of the animals; therefore they were not dosed with antibiotics, sedated with acepromazine or sodium pentobarbital, or given acid blockers prior to challenge. Those treatments may enhance the disease process and therefore lead to over predictions of virulence (Czuprynski *et al*., 2003; Elenkov *et al*., 1998; Okamoto *et al*., 1994; Schlech *et al*., 1993). Guinea pigs were used since they express e-cadherin, a mammalian receptor required for entry of *L. monocytogenes* into the intestinal epithelium (Lecuit *et al*., 2001). We designed experiments to simulate consumption of *L. monocytogenes* contaminated food that had been handled improperly and consumed by the general population (not specifically targeting the young, elderly, or immunocompromised). The aim was to represent the physiological behavior of a host capable of mounting a normal immune response. If improper handling of a contaminated food influences virulence or survival of the organism: or if the carrier significantly impacts infection (i.e., survival during passage through the gastrointestinal tract thereby influencing numbers in the liver or spleen), then the use of immunocompromised (i.e., elderly or pregnant) or young animals may confound the results. The oral challenge method was used to mimic the natural route of infection and to provide an assessment of the role of a representative food carrier (chocolate milk).

Studies linking bacterial culture, improper handling practices, and delivery of a target pathogen in a given foodstuff to an animal are limited. In a mouse model, *L. monocytogenes* cultured in salami batter alone was more pathogenic compared to *L. monocytogenes* cultured in salami batter containing starter cultures (Mahoney and Henriksson, 2002). Pine *et al*. (1990) used milk as a growth medium and as a carrier, but

the study found no impact on virulence of *L. monocytogenes*. Acid adapted strains of *L. monocytogenes* were reported to be more virulent in vitro and in vivo compared to the non-acid-adapted strains (Conte *et al*., 1998; O'Driscoll *et al*., 1996).

Comparing results of studies investigating listerial infection is difficult since animal model (mice, rat, guinea pig), treatment of animals prior to challenge, bacterial culture conditions, and carrier may differ. Such differences could affect the dissemination and survival of bacteria and hence influence the pathogenesis of *L. monocytogenes* during passage through the GI tract (Barkardjiev *et al*., 2004; Smith *et al*., 2002; Mahoney and Henriksson, 2002; Lecuit *et al*., 2001; Okamoto *et al.*, 1994; Schlech *et al*., 1993; Pine *et al*., 1990). The guinea pig model used in this study was to better represent the effects of listerial infections in the general population. In this study, the carrier vehicle and improper handling of the contaminated carrier did not ultimately influence the final infection outcome. Based on the design of this study, we showed that *L. monocytogenes* at a 10^2 dose can lead to listerial infection based on culture positive liver or spleen samples. Experiments investigating different serotypes, foods, handling conditions, health status (young, immunocompromised: elderly or pregnant), dose of *L. monocytogenes*, and study duration are the variables that must be evaluated to determine the significance of low levels of *L. monocytogenes* in foods.

REFERENCES

Barbour, H.A., A.Rampling, and C. Hormaeche. 2001. Variation in the infectivity of *Listeria monocytogenes* isolates following intragastric inoculation of mice. Infect. Immun. **69**: 4657-4660.

Barbour, H.A., A. Rampling, and C. Hormaeche. 1996. Comparison of the infectivity of isolates of *Listeria monocytogenes* following intragastric and intravenous inoculation in mice. Micro. Patho. **20**: 247-253.

Bakardjiev, A.I., B.A. Stacy, S.J. Fisher, and D. Portnoy. 2004. Listeriosis in the pregnant guinea pig: a model of vertical transmission. Infect. Immun. **72**: 489-497.

Conlan, J.W. 1997. Neutrophils and tumour necrosis factor-a are important for controlling early gastrointestinal stages of experimental murine listeriosis. J. Med. Microbiol. **46**: 239-250.

Conlan, J.W., and R.J. North. 1994. Neutrophils are essential for early anti-listeria defense in the liver, but not in the spleen or peritoneal cavity, as revealed by a granulocyte-depleting monoclonal antibody. J. Exp. Med. **179**: 25-268.

Conte, M.P., G. Petrone, A.M. Di Biase, M.G. Ammendolia, F. Superti, and L. Seganti. 2000. Acid tolerance in *Listeria monocytogenes* influences invasiveness of enterocyte-like cells and macrophage-like cells. Microbial. Patho. **20**: 137-144.

Czuprynski, C.J., N.G. Faith, H. Steinberg, and B. Neudeck. 2003. Sodium pentobarbital anesthesia transiently enhances the severity of infection following intragastric, but not intravenous, inculation of Listeria monocytogenes in mice. **23**: 81-86.

Dalton, C.B., C.C. Austin, J. Sobel, P.S. Hayes, W.F. Bibb, L.M. Graves, B. Swaminathan, M.E. Proctor, and P.M. Griffin. 1997. An outbreak of gastroenteritis and fever due to *Listeria monocytogenes* in milk. N. Engl. J. Med. **336**: 100-105.

Datta, A.R. 1994. Factors controlling expression of virulence genes in *Listeria monocytogenes.* Food Microbiol. **11**: 123-129.

Donnelly, C.W. 2001. *Listeria monocytogenes*: A continuing challenge. Nutri Rev. **59**: 183-194.

Elenkov, I.J., E. Webster, D.A. Papanicolaou, T.A. Fleisher, G.P. Chrousos, and R. L. Wilder. 1998. Histamine potently suppresses human IL-2 and stimulates IL-10 production via H2 receptors. J. Immunol. **161**: 2586-2593.

FDA/CFSAN, USDA/FSIS. 2003. Interpretive Summary: Quantitative assessment of the relative risk to public health from foodborne *Listeria monocytogenes* among selected categories of ready-to-eat foods.

Hamrick, S.T., J.R. Horton, P.A. Spears, E.A. Havell, I.W. Smoak, and P.E. Orndorff. 2003. Influence of pregnancy on the pathogenesis of listeriosis in mice inoculated intragastrically. Infect. Immun. **71**: 5202-5209.

Hof, H. 2003. History and epidemiology of listeriosis. FEMS Immun. Med. Microbiol. **35:** 199-202.

Koutsoumanis, K.P., P.A. Kendall, and J.N. Sofos. 2003. Effect of food processing related stresses on acid tolerance of *Listeria monocytogenes*. Appl. Environ. Microbiol. **12:** 7514-7516.

Lammerding, A.M., K.A. Glass, A. Gendron-Fitzpatrick, and M.P. Doyle. 1992. Determination of virulence of different strains of *Listeria monocytogenes* and *Listeria innocua* by oral inoculation of pregnant mice. Appl. Environ. Microbiol. **58**: 3991-4000.

Leclercq, A. 2004. Atypical colonial morphology and low recoveries of *Listeria monocytogenes* strains on Oxford, PALCAM, Rapid'L.mono and ALOA solid media. J. Micro. Methods. **57**: 251-258.

Lecuit, M., S. Vandormeal-Pournin, J. Lefort, M. Huerre, P. P. Gounon, C. Dupuy, C. Babinet, and P. Cossart. 2001. A transgenic model for listeriosis: Role of internalin in crossing the intestinal barrier. **292**: 1172-1725.

Lecuit, M., S. Dramsi, C. Gottardi, M. Fedor-Chaiken, B. Gumbiner, and P. Cossart 1999. A single amino acid in E-cadherin responsible for host specificity towards the human pathogen *Listeria monocytogenes*. EMBO J. **18**: 3956-3963.

Lemeister-Wachter, M., E. Domann, and T. Chakraborty. 1992. The expression of virulence genes in *Listeria monocytogenes* is thermoregulated. J. Bacteriol. **174**: 947-952.

Mahoney, M., and A. Henriksson. 2002. The effect of processed meat and meat starter cultures on gastrointestinal colonization and virulence of *Listeria monocytogenes* in mice. Int. J. Food Microbiol. **2619**: 1-7.

Mead P.S., L. Slutsker, V. Dietz, L.F. McCaig, J.S. Bresee, C. Shapiro, P.M. Griffin, and R.V. Tauxe. 1999. Food-related illness and death in the United States. Emerg. Infect. Dis. **5**: 607-25.

Mekalanos, J.J. 1992. Environmental signals controlling expression of virulence determinants in bacteria. J. Bacteriol. **174**: 1-7.

Mytle, N., G.L. Anderson, S. LAMBERT, M.P. DOYLE, and M.A. SMITH. 2006. Effect of fat content on infection by *Listeria monocytogenes* in a mouse model. J. of Food Protection. **69**:660-665.

O'Driscoll, B., C.G.M. Gahan, and C. Hill. 1996. Adaptive acid tolerance response in *Listeria monocytogenes*: Isolation of an acid-tolerant mutant which demonstrates increased virulence. Appl. Environ. Microbiol. **62**: 1693-1698.

Okamoto, M., A. Nakane, and T. Minagawa. 1994. Host resistance to an intragastric infection with *Listeria monocytogenes* in mice depends on cellular immunity and intestinal bacterial flora. Infect. Immun. **62**: 3080-3085.

Pine, L., G.B. Malcom, and B.D. Plikaytis. 1990. *Listeria monocytogenes* intragastric and intraperitoneal approximate 50% lethal doses for mice are comparable, but death occurs earlier by intragastric feeding. Infect. Immun. **58**: 2940-2945.

Schlech, W. F., D.P. Chase, and A. Badley. 1993. A model of food-borne *Listeria monocytogenes* infection in the Sprague-Dawley rat using gastric inoculation: development and effect of gastric acidity on infective dose. Int. J. Food Microbiol. **18**: 15-24.

Sheikh-Zeinoddin, M., T.M. Perehinec, S.E. Hill, and C.E.D. Rees. 2000. Maillard reaction causes suppression of virulence gene expression in *Listeria monocytogenes*. Int. J. Food Microbiol. **61**: 41-49.

Smith, M. A., K. Takeuchi, R.E. Brackett, H.M. McClure, R.B. Raybourne, K.M. Williams, U.S. Babu, G.O. Ware, J.R. Broderson, and M.P. Doyle. 2002. Nonhuman primate model for *Listeria monocytogenes*-induced stillbirths. Infect. Immun. **71**: 1574-1579.

Swartz, N.M. 2002. Human Diseases caused by foodborne pathogens of animal origin. Clin. Infect. Diseases. **34**: S111-122.

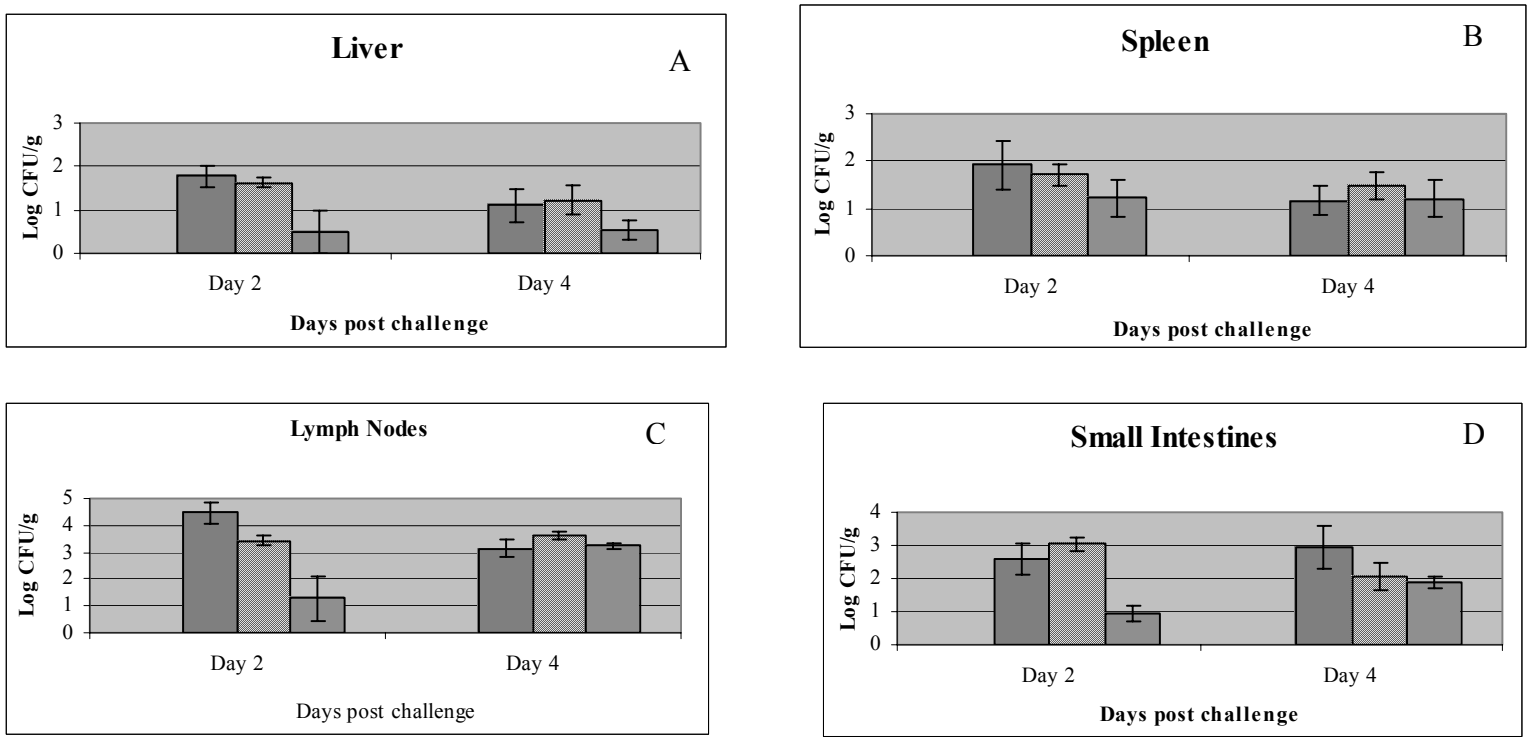


FIGURE 2.1. INFLUENCE OF IMPROPERLY HANDLED CARRIER ON INFECTIVITY OF *L. MONOCYTOGENES.* (A) Liver, (B) Spleen, (C) Mesenteric lymph nodes, and (D) Small Intestines. *L. monocytogenes* was suspended in chocolate milk (▩), BHI (▨), and water (▦) to achieve 10^8 CFU ml^{-1} and guinea pigs orally challenged with 1 ml of the suspension. *L. monocytogenes* was suspended in chocolate milk and BHI and held at 37°C for 2 h, and then cooled for 2 h at 4°C. An 18 h culture was suspended in sterile water and used as a control for improper handling conditions. A total of 30 animals were used in two experiments; 15 were sacrificed on day 2 and 15 were sacrificed on day 4. Results for all animals receiving the 10^8 dose are reported. Each bar represents the mean $\log_{10}$ CFU g^{-1} organ (n=5). An asterisk indicates a significantly lower (P<0.05) level of *L. monocytogenes.*

IMPACT OF IMMUNOMODULATION ON THE DEVELOPMENT OF LISTERIOSIS IN AGED GUINEA PIGS

I. ABSTRACT

L. monocytogenes continues to be a significant source of foodborne illness and deaths worldwide. We investigated the impact of immunomodulation on the development of listeriosis within an aged population after an oral challenge with *L. monocytogenes*. During the immunoregime, animals were immunoenhanced with daily supplementation of vitamin E for a period of 35 days or immunosuppressed with cyclosporine A (CsA), a fungal metabolite that selectively suppressed the T lymphocyte population. An untreated control group was included. Daily supplementation with vitamin E significantly increased the level of $CD8^+$ T cells, while CsA treatment resulted in a 25% decrease of $CD8^+$ T cells. HPLC analysis showed that short-term supplementation with vitamin E led to an overall 1-fold increase in the plasma alpha-tocopherol concentration. In the animals receiving the low-dose (10^2 CFU) of *L. monocytogenes*, an infection rate of 50% occurred in the control group. Only 22% of animals that received the orthomolecular dose (400 IU) of vitamin E became infected; 2.5-fold lower compared to the control group. Animals that were immunosuppressed had an infection rate of 89%; moreover, three animals (16%) developed listerial infection with a quantifiable bacterial level of 0.3-3 log CFU/g found in the spleen and liver. In the high dose challenge study, population of *L. monocytogenes* was consistently 1 log

CFU lower in the spleen or liver of the vitamin E-supplemented group, as compared to the control and CsA-treated groups. No significant differences in the level of *L. monocytogenes* were seen between the control and CsA-treated groups. At day 4, a significant increase in the levels of cytotoxic T cells ($CD8^+$) during listerial infection occurred in vitamin E-supplemented animals, suggesting an increased ability to produce $CD8^+$ T cells. Results suggest that immunomodulation of a host can influence the outcome of a listerial infection within an aged population of guinea pigs. Experiments demonstrated a pronounced effect of vitamin E in mitigating listerial infection.

II. INTRODUCTION

L. monocytogenes is an intracellular pathogen of humans and animals. This psychrotroph is also the etiologic agent of the disease listeriosis. The mode of acquisition is commonly through the consumption of contaminated ready-to-eat foods. Although *L. monocytogenes* is responsible for approximately 2500 cases of foodborne illnesses in the United States, it results in 91% of hospitalizations attributed to foodborne pathogens with an associated high mortality rate [25 -40%] (Swatz *et al*., 2002; Wing and Gregory, 2002). The infectious dose of *L. monocytogenes* is not yet known. However, reports of outbreaks in the past decade indicate that levels of *L. monocytogenes* consumed range from as low as 0.3 CFU/g to as high as 10^9 CFU/ml (Sim *et al*., 2002, Donnelly, 2001, Dalton *et al*., 1994). There are two main forms of human listeriosis, invasive or non-invasive. Invasive listeriosis predominates in the young, the elderly, pregnant women, and the immunocompromised (Wing and Gregory, 2002, Donnelly, 2001). Although

outbreaks of non-invasive listeriosis (gastroenteritis) are relatively uncommon, recent outbreaks leading to febrile gastroenteritis have included foods such as corn and tuna salad (Aureli *et al*., 2000), ready-to-eat meats (Sim *et al*., 2002) and cheese (Makino *et al*., 2005, Danielsson-Tham *et al*., 2004) worldwide. Notably, in all those cases of gastroenteritis, the dose of *L. monocytogenes* consumed was high, at least at 10^6 CFU (Ooi and Lorber, 2005).

Risk factors attributing to the severity of a listerial infection include the virulence of a strain, the dose consumed, and the target population (Schlech, 1997). Epidemiological outbreaks of foodborne listeriosis indicated that individuals with an impaired T cell-mediated immunity such as the pregnant women, immunocompromised people, and the elderly were more likely to develop the disease (Vazquez-Boland *et al*., 2001). The aged are at high risk for listerial infection. In the United States, 12% of the US population is 65 years and older (The AGS foundation for health in aging, 2002). Over the next 30 years, it is estimated that the current aged population of ~34 million persons will double to 21% of all Americans (Projections of the Population of the United States: 1982 to 2050 (Advance report)). Epidemiological data indicates that the incidence of listerial infection dramatically increases from age 40 (CDCP, 2004; Lorber, 1997). Nagel *et al*. (1981) found a decreased level of cytotoxic T cells ($CD8^+$) in aged humans. Such age-associated decline in T cell-mediated immunity can lead to higher risks in acquiring infectious diseases in the aged (Han and Meydani, 2000; Serafini 2000).

Researchers have established that cell mediated immunity is essential in combating listeriosis or a listerial infection (Hof, 2003; Schaffner *et al*., 1983; Lane and

Unanue, 1972). There is a plethora of evidence showing that supplementation with vitamin E stimulates the immune system of the elderly (Meydani *et al*., 1990). Vitamin E can positively affect the proliferation and stimulation of the cytotoxic T cells in humans and animals; the positive immunostimulatory effect of vitamin E is associated with modulation in the regulation of cytokines such as interleukin-2 and interferon-gamma (Meydani *et al*., 1990, 1986; Moriguchi *et al*., 1998). Short-term daily supplementation with vitamin E was reported to enhance the immune system in the elderly, and helped mitigate viral and bacterial infections (Andreone *et al*., 2000; Meydani *et al*., 2004, 1998). Based on those studies, an orthomolecular dose of vitamin E was administered to elicit cellular immune responses, specifically enhancing the T cell response. Cyclosporine A (CsA), is a fungal metabolite shown to suppress the entire immune system and have a selective effect against T cells (Freitas, 2003; Borel *et al*., 1977).

The intent of the present study was to investigate the susceptibility to listerial infection of an aged guinea pig population receiving imunomodulating treatment. Aged animals were selected based on life expectancy or age, currently the most accepted bio-marker (Flurkey and Currer, 2004, Takizawa *et al.*, 2004). Aged guinea pigs that were immuno-enhanced with daily supplementation of vitamin E or immunosuppressed with CsA were studied. The animals were intragastrically challenged with a high or low dose of *L. monocytogenes* and listerial infection determined.

III. MATERIALS AND METHODS

A. BACTERIAL CELLS AND MEDIA

Three serotype 4b strains of *L. monocytogenes*; N1-225 (Human epidemic), J1-110 (food epidemic), and MMS97-1 (raw beef) were used in the present study (Table 1). Stock cultures were maintained in BHI-glycerol (1:1) and stored at -70^{o}C until use. Each isolate was cultured in 10 ml of brain heart infusion (BHI) broth (Difco, Detroit, USA) at 37^{o}C with aeration for 18 h, centrifuged at 5500 × g at 4^{o}C for 10 min, and suspended in 10 ml of phosphate buffered saline (PBS; Sigma, St. Louis, MO, USA). A three-strain cocktail was prepared by mixing equal volumes of the bacterial suspensions, serially diluted to achieve doses of 10^2 and 10^8 CFU/ mL. Prior to use in animal experiments, the cocktail was serially diluted in PBS, plated onto BHI agar in duplicate, and the plates were incubated at 37^{o}C for 24 h to confirm bacterial numbers.

TABLE 3.1. **SUMMARY OF *L. MONOCYTOGENES* ISOLATES.**

Strains	Serotype	Ribotype	Source Information
N1-225	4b	DUP-1044A	Human, Epidemic, 1998
J1-110	4b	DUP-1038	Food, Epidemic, 1985
MMS97-1	4b	MMS 97-1	Raw Beef, 1989

B. ANIMALS

Outbred female Dunkin-Hartley guinea pigs (retired breeders) weighing approximately 1000 g were purchased (Charles River Lab. Inc, MA or Elm Hill Breeding Lab) and housed under standard conditions (50% humidity and a diurnal 12 h

cycle) at the Rutgers animal facility. Guinea pigs were weighed upon arrival, and then randomly assigned to three experimental groups. These groups were; vitamin E-supplemented (immuno-enhanced), CsA-treated (immunosuppressed), and control. Two independent experiments were conducted; 54 animals (18 animals/group) were used in each experiment. Guinea pigs were kept in individual cages, and received fresh guinea pig chow and water ad libitum. Animals were allowed to acclimate to the environment for 5-7 days before being used in the experiments. All animal experiments were conducted in accordance with federal guidelines and approved by the Rutgers University Animal Care and Facilities Committee.

C. REGIMEN FOR IMMUNOMODULATION: VITAMIN E SUPPLEMENTATION AND CsA

A group (n=36) of animals was fed daily 400 IU of vitamin E (DL-alpha-tocopherol acetate) for a period of 35 days. For the immunosuppressed group (n=36), CsA was dissolved in ethanol and a dose of 25 mg/kg was injected intraperitoneally into the guinea pigs 48 h before oral challenge with *L. monocytogenes*. The control group (n=36) of animals was fed regular guinea pig chow and fresh water daily.

D. BLOOD AND PLASMA COLLECTION

In each experiment, blood was collected from 6 animals randomly selected from each group. Guinea pigs were anesthetized using ketamine/xylazine at a concentration of 20mg/kg and 80mg/kg, respectively. Blood samples were collected from animals prior to, and once weekly during vitamin E supplementation through day 35.

For the immunosuppressed group, blood was collected prior to and 48 h after CsA treatment for analysis to determine changes in $CD8^+$ T-cell counts. To establish a baseline prior to listeria challenge, blood was drawn from animals in all three groups (vitamin E, CsA, and control) prior to, and on day 2, 4, and 6 post-challenge. From each animal, one ml of peripheral blood was drawn from the jugular vein into syringes containing 5% K_2 EDTA. The sample was aliquoted and used within 4 h for indirect immunostaining and blood plasma was collected by centrifugation (1800 × g, 10 min, 4°C). Aliquots of plasma were frozen immediately at -70°C for further analysis.

E. DIRECT AND INDIRECT IMMUNOSTAINING

Blood (100 µl) was incubated with an equal volume of IQ lyse (Biotest Diagnostics Corporation, NJ) for 10 min at room temperature to lyse erythrocytes. Cells were washed with PBS + 1% FBS. Monoclonal antibodies, $CD45^+$, and $CD8^+$ directly conjugated to FITC, $CD3^+$ directly conjugated APC (Serotec Ltd., Kidlington, Oxford, UK), were incubated with the cells that remained, the leukocyte, at room temperature for 30 min. Cells were washed and when necessary, incubated with $F(ab')_2$ rabbit anti-mouse IgG:RPE-labeled secondary antibody. Working concentrations of primary and secondary antibodies were used according to manufacturer's recommendations. Cells were washed, and then fixed in PBS + 1 % paraformaldehyde. Samples were stored in the dark at 4°C for flow cytometry analysis. Single stain cells were used as negative controls. Samples were analyzed in a Coulter Cytomics FC500 Flow cytometer (Becton-Dickinson).

F. HPLC ANALYSIS OF ALPHA-TOCOPHEROL CONCENTRATION IN THE PLASMA

Plasma samples stored at -80°C were thawed at room temperature and used for HPLC analysis. Forty-eight plasma samples from the guinea pigs receiving vitamin E supplementation were analyzed in this study. After vigorous agitation, 150μL of plasma was transferred to a microcentrifuge tube followed by adding 150μL of Internal Standard (IS) (0.1 mg/mL alpha-tocopherol acetate; ethanol : acetonitrile = 1:1 (v:/v)) and 700μL hexane). Alpha-tocopherol extraction was performed by vortex-mixing for 20 s followed by centrifugation for 6 min at 14,000 × g. The alpha-tocopherol extracts were obtained from 700μL of supernatant and transferred into a new tube, where the alpha-tocopherol extract samples were placed in a vacuum dryer for 30 min. Each sample was reconstituted with 150μL methanol and quantified using a Shimadzu LC-2010A HPLC (Tokyo, Japan) equipped with a ternary pump delivery system. Each extract sample (20 μL) was injected into the column (Luna, 150 mm x 4.6 mm, 3 μm, Phenomenex, Torrance, CA, USA). Mobile phase was conducted with methanol as the eluent at a flow rate of 0.85 mL min^{-1}. The alpha-tocopherol and internal standard were monitored using a UV detector at 295 nm.

G. PER-ORAL CHALLENGE OF THE GUINEA PIGS

Prior to oral challenge, food was withheld for 12 h from all guinea pigs to prevent regurgitation during oral challenge of *L. monocytogenes* (Mahoney and Henriksson, 2002; Lecuit *et al*., 2001; Pine *et al*., 1990). In each group, nine animals received a low dose of 10^2 CFU and nine animals received a high dose of 10^8 CFU of *L.*

monocytogenes. Using a 38-cm nasogastric feeding tube (Jorgensen Laboratories, Inc., Loveland, CO, USA) fitted into a 3-ml syringe, guinea pigs were intragastrically challenged with either a low dose (ca. 3×10^2 CFU) or a high dose (ca. 2.5×10^8 CFU) of *L. monocytogenes* in 1 ml of saline for the vitamin E supplemented, CsA-treated, and the control groups.

H. ENUMERATION OF *L. MONOCYTOGENES* IN THE ORGAN SAMPLES

Guinea pigs were euthanized via intraperitoneal injection using sodium pentobarbital (euthanasia grade) at a dose of 390 mg/kg on day 2, day 4, and day 6. The protocol for organ processing was based on Pang and Matthews (2006). In brief, the spleen, liver, lymph nodes and small intestine were aseptically removed in toto. Each organ was aseptically weighed, placed into an equal volume of cold sterile PBS + 0.001% Triton-X (vol/vol), and homogenized using a tissue homogenizer (Polytron®, Kinematica, Switzerland). The intestines were suspended in an equal volume of cell culture medium (Earls Minimal Essential Medium [EMEM] supplemented with gentamicin sulfate (40 µg ml^{-1}; Cellgro, Herndorn, VA, USA) after weighing and incubated at room temperature, with gentle shaking, for at least 40 min. Following incubation the intestines were washed twice in EMEM, rinsed once with PBS, placed into an equal volume of cold sterile PBS + 0.001% Triton-X (vol/vol), and homogenized. The procedure results in killing of extracellular bacteria permitting the enumeration of only intracellular bacteria. Organ homogenates were serially diluted (1:10) in PBS+ 0.001% Triton-X (vol/vol), and 100 µl was spread plated in duplicate on Rapid L'mono or modified oxford plates (Leclercq, 2004). Plates were incubated at 37^{o}C for 48-72 h. *L. monocytogenes* colonies were

enumerated and numbers expressed as mean log/g of organ. Remaining organ samples are stored at 4^{o}C. When no *L. monocytogenes* was detected based on colony formation, organ samples were enriched and plated onto Rapid L'mono plates or modified oxford agar in duplicate.

I. ENRICHMENT OF ORGAN SAMPLES

All organ samples from animals receiving 10^2 CFU *L. monocytogenes* were enriched with an equal volume of 2× BHI media. Samples were incubated for 24 h, and 100 µl of each sample was plated onto Rapid L'mono or modified oxford media in duplicate. Plates were incubated at 37^{o}C for 48-72 h, and samples identified positive for presence of *L. monocytogenes* colonies were recorded.

J. STATISTICAL ANALYSIS

Means and standard deviation of means were calculated using Microsoft Excel's STDEV function. Standard error is calculated by taking the standard deviation and dividing it by the square root of experimental samples (n). Data are presented as means ± SEM. The statistical differences between the treatment means were determined using the T-TEST function in Microsoft Excel. Specifically, the results were analyzed using unpaired student's T-test. For HPLC analysis, paired t-test was used to determine the significance differences of alpha-tocopherol concentration in the plasma prior to, and 1, 3, and 5 weeks post vitamin E supplementation. The level of statistical relevance for all comparisons was set at $P<0.05$.

IV. RESULTS

A. EFFECT OF IMMUNO-MODULATION ON THE LEVELS OF $CD8^+$ T CELLS IN AGED GUINEA PIGS

The effect of daily supplementation with vitamin E, and CsA treatment in the respective groups of guinea pigs were assessed by specifically detecting levels of $CD8^+$ T cells. Figures 3.1 and 3.2 show the effect of vitamin E supplementation on the $CD8^+$ T cell levels in guinea pigs over a 5-week period. Daily supplementation with 400 IU of vitamin E resulted in an increase of $CD8^+$ T cells during the 35-day period (Figure 3.1). The animals exhibited no adverse signs (diarrhea, loose stool, ruffled coat, arched back, loss of appetite) following supplementation with vitamin E. There was a significant ($P<0.05$) increase in the level of $CD8^+$ T cells population during the first week of vitamin E supplementation, from 36.1% at week 0, to 43.2% at week 1. Levels of $CD8^+$ T cells remained relatively constant from week 1 to week 5. By week 5, the level of $CD8^+$ T cells increased to 46.5%. In addition, Fig. 3.2 shows an increase in maturation of $CD8^+$ T cells, a shift from $CD8^-$ to $CD8^+$ T cells. Results demonstrate that vitamin E supplementation leads to enhancement of $CD8^+$ T cells in aged guinea pigs. In contrast, animals receiving the CsA treatment exhibited a decline in the number of $CD8^+$ T cells. A significant 25% decrease ($P=0.002$) in the level of $CD8^+$ T cells compared to total lymphocytes at 48 h post administration of CsA was observed (Fig. 3.3). The overall T cell population (indicated by $CD3^+$ marker) decreased approximately 40% (data not shown) with respect to total leukocyte levels; other leukocyte fractions such as neutrophils and monocytes remained unchanged (data not shown). The levels of $CD8^+$ T

cells in the control animals were also determined, however, no significant changes in cell population were observed (data not shown).

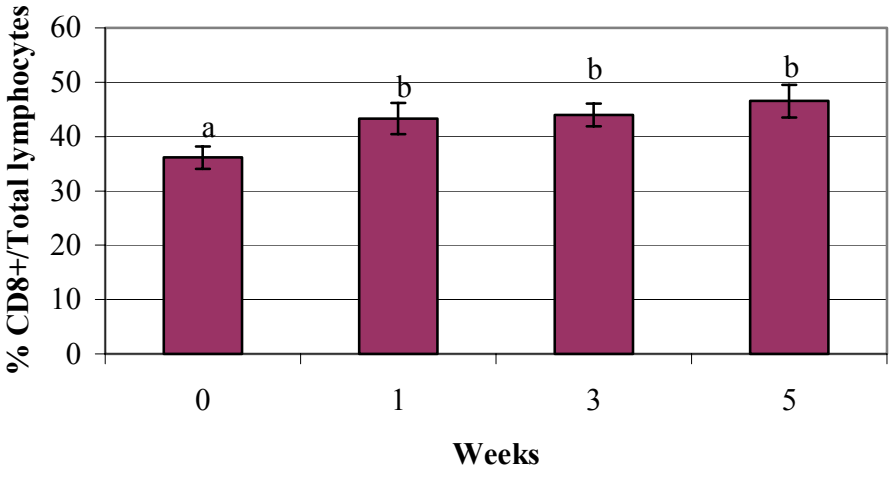


FIGURE 3.1. EFFECT OF DAILY SUPPLEMENTATION WITH VITAMIN E ON THE LEVEL OF CD8$^+$ T CELLS IN GUINEA PIGS. The level of CD8$^+$ T cells was significantly higher in guinea pigs supplemented with vitamin E for a period of 35 days. Averages were obtained from 2 independent experiments (n=10). Values were expressed as means ± SEM. Statistical significance was set at $P<0.05$.

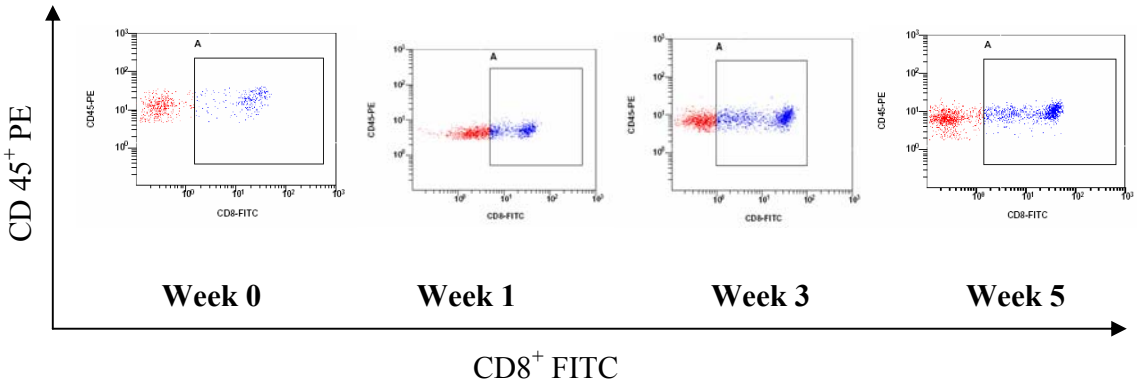


FIGURE 3.2. REPRESENTATIVE FLOW CYTOMETRIC DOT PLOTS OF TOTAL LYMPHOCYTES AND CD8$^+$ T CELLS SUB-POPULATIONS. Maturation process of CD8$^+$ T cells within the total lymphocytes population. Blood samples were collected from aged guinea pigs before daily supplementation with vitamin E at week 0, and at weeks 1, 3, and 5 after supplementation with vitamin E. A maturation of the CD8$^+$ T cells population, based on a shift from left to right (within the box), was noted.

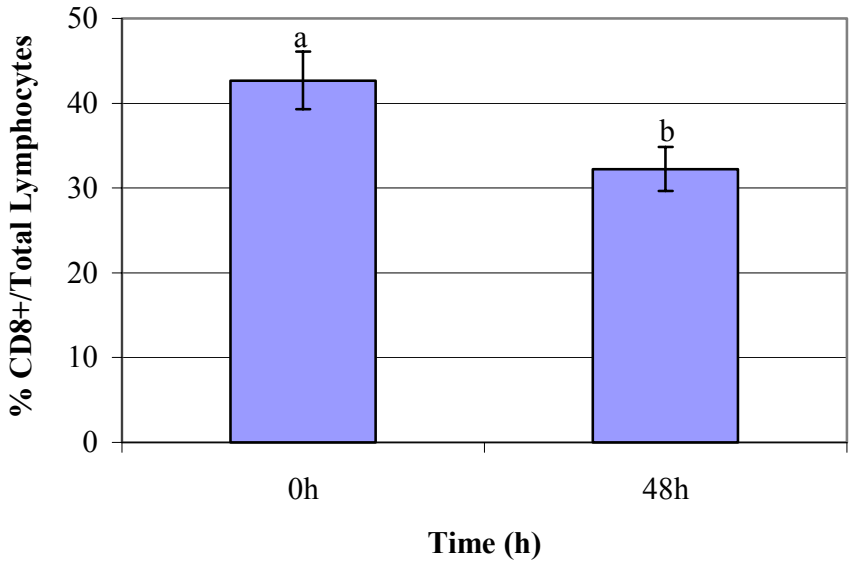


FIGURE 3.3. EFFECT OF CsA ON THE LEVEL OF $CD8^+$ T CELLS IN AGED GUINEA PIGS.

A 25% decrease (P=0.002) in the levels of $CD8^+$ T cells was observed at 48 h post treatment with cyclosporin A. Flow cytometry was used to analyze changes in the level of $CD8^+$ T cells with respect to total lymphocytes. Bars represent mean values ± SEM. Data were obtained from 2 independent experiments (n=10). The level of statistical significance was set at $P<0.05$.

TABLE 3.2. CONCENTRATION OF PLASMA ALPHA-TOCOPHEROL IN AGED GUINEA PIGS BEFORE AND DURING VITAMIN E SUPPLEMENTATION

Weeks	Average ± SD (μM)	% increase compared to week 0
0	34.81±15[a]	
1	90.85±22[b]	**62%**
3	73.94±13[b]	**53%**
5	77.56±15[b]	**55%**

[ab] Values were all significantly higher than the value obtained on Week 0 (P<0.05). Data from two independent experiments were pooled and values expressed as means ± SEM. Paired student's t test was used in the analysis of results.

B. HPLC ANALYSIS OF PLASMA ALPHA-TOCOPHEROL CONCENTRATIONS IN AGED GUINEA PIGS

Analysis of plasma alpha-tocopherol concentration prior to and following vitamin E supplementation was determined on week 0, and week 1, 3, and 5. The concentration of alpha-tocopherol before vitamin E supplementation was 4.8 μM (week 0), corresponding to the baseline level of alpha-tocopherol level in the plasma. At week 1, 3, and 5 of daily supplementation with vitamin E, the concentration of alpha-tocopherol in the plasma significantly increased to 90.6 μM (P= 0.008), corresponding to a ~62%

increase from week 0; and 73.9 μM (P= 0.001), and 77.6 μM (P=0.003), corresponding to a 53% and 55% increase from week 0, respectively. The slight decrease from week 3 (73.9 μM) to 5, (77.6 μM) was not significant (P> 0.05). There was an overall 1-fold increase in the concentration of alpha-tocopherol during the 35-day supplementation. These results confirmed the presence of alpha-tocopherols in the plasma, and correlated with the increase of $CD8^+$ T cells (Table 3.2).

C. LOW DOSE (10^2) CHALLENGE OF *L. MONOCYTOGENES* CAN LEAD TO LISTERIAL INFECTION

Subsequent to immuno-enhancing a group of guinea pigs with vitamin E, and immuno-suppression with CsA, the guinea pigs were intragastrically challenged with a low (10^2) and high (10^8) dose of *L. monocytogenes*. Based upon conventional culture of the organ samples, three animals from the immunosuppressed group developed listerial infection following an oral challenge with 10^2 CFU of *L. monocytogenes*. The percentage of positive animals was determined by dividing the number of organ samples positive for *L. monocytogenes* by the total number of animals in each group (18 animals per group) intragastrically challenged with 10^2 *L. monocytogenes*. Specifically, only the presence of *L. monocytogenes* in the liver or spleen of an animal was counted as positive for infection. All other organ samples from animals regardless of treatment group were enriched, since no *L. monocytogenes* was detected except for three animals from the CsA treated group. Following enrichment, results indicated that 22% of animals that were fed vitamin E were positive for *L. monocytogenes* (Table 3.3.A). In contrast, 89% of CsA-treated animals were infected, while 50% of animals in the control group were infected.

No *L. monocytogenes* was detected in the organ samples of the animals based on conventional culture of the organ samples, except for day 4 and day 6 of CsA-treated animals (Table 3.3.B). During experiment 2 (the repeat experiment), 2 CsA-treated animals on day 4 (dose of 10^2) had quantifiable numbers of *L. monocytogenes* in the liver, spleen and lymph nodes. On day 6, 0.3 log CFU/g of organ *L. monocytogenes* was detected in the spleen of an additional CsA-treated animal. Based on microbiological results, these three animals developed listerial infection even at a low dose of 10^2 CFU *L. monocytogenes*.

TABLE 3.3.A. ENRICHMENT OF ORGAN SAMPLES FOLLOWING ORAL CHALLENGE WITH A LOW DOSE (10^2) OF *L. MONOCYTOGENES*

	Organs	Vitamin E	Control	Cyclosporin A
Day 2[a]	Liver[d]	2/6[e]	6/6	6/6
	Spleen	0/6	1/6	3/6
	Lymph Nodes	0/6	0/6	1/6
	Small Intestines	0/6	4/6	3/6
Day 4[b]	Liver	0/6	1/6	5/6*
	Spleen	0/6	0/6	2/6*
	Lymph Nodes	0/6	0/6	1/6*
	Small Intestines	3/6	2/6	3/6
Day 6[c]	Liver	1/6	1/6	4/6
	Spleen	2/6	1/6	5/6*
	Lymph Nodes	1/6	2/6	5/6
	Small intestines	0/6	0/6	3/6
Total no. of animals infected		4	9	16
% of animals infected		22%[f]	50%	89%

[a,b,c] indicate respectively 24 h, 48 h and 72 h post-inoculation

[d]Each organ was collected in toto. Organ samples were enriched for 24 h using BHI at 37^{o}C, and plated onto Rapid L'mono or modified oxford plates in triplicate.

[e] Values indicate number of organ samples found positive for *L. monocytogenes* / total number of animals tested.

[f] % infection was calculated based upon the presence of *L. monocytogenes* in the liver or spleen. In each treatment group, % value was obtained by dividing the total of no. of animals infected by 18, and multiplying the value by 100%.

* These animals were found to have quantifiable levels of *L. monocytogenes* in the spleen, liver, and lymph nodes prior to the enrichment of organ samples. Values of bacterial load in the organ samples are presented in Table 3.3.B.

TABLE 3.3.B. BACTERIAL LOAD IN THE INFECTED ORGANS OF CsA-TREATED ANIMALS ORALLY CHALLENGED WITH A LOW DOSE OF (10^2) *L. MONOCYTOGENES*

		Organs (CFU/g of organ)			
	Animal I.D.	Liver	Spleen	Lymph Nodes	Small Intestines
Day 4	CsA-18	1.95[a]	2.75	0	0
	CsA-22	2.3	3.31	4.07	0
Day 6	CsA-16	0	0.3	0	0

[a] Values represent log CFU/g of organ.

HIGH DOSE (10^8) INFECTION WITH *L. MONOCYTOGENES*

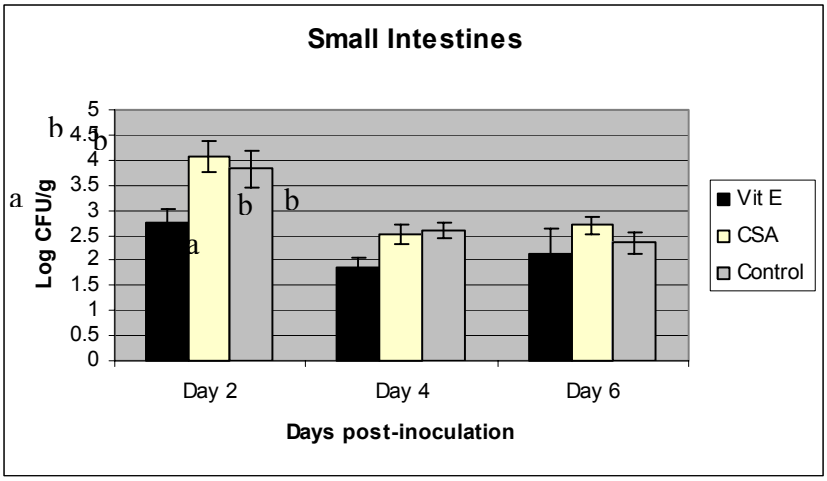
All the guinea pigs challenged with 10^8 CFU of *L. monocytogenes* developed listerial infection in the spleen and liver, however, no deaths occurred. The small intestines and lymph nodes of all animals receiving the high dose were positive for *L. monocytogenes*. Animals receiving orthomolecular doses of vitamin E for 5 weeks had statistically significant lower levels of *L. monocytogenes* in the small intestines on day 2 and day 4 post-challenge ($P<0.05$). Compared to the control animals, the level of *L. monocytogenes* in the vitamin E group was consistently ~1 log lower. *L. monocytogenes* populations in target organs of the CsA-treated and control animals were not statistically different ($P>0.05$).

To examine the effects of immuno-modulation on the severity of listerial infection, we compared the levels of *L. monocytogenes* in the liver and spleen samples. On day 2, the level of *L. monocytogenes* in the liver of the vitamin E-supplemented animals were at 1.3 log CFU/g of organ, a significantly lower level compared to levels of 3 log CFU/g of organ for the control and CsA-treated animals ($P<0.05$). Bacterial level in the spleen was

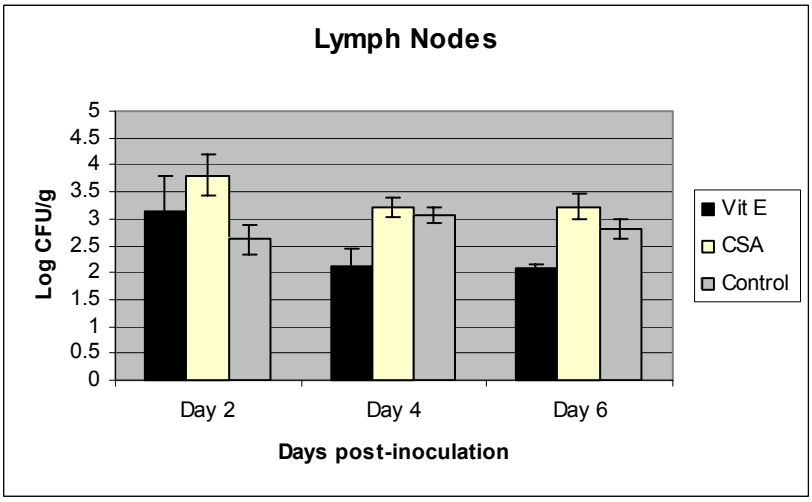
lower in the vitamin E-supplemented animals (0.5-1 log CFU/g of organ) compared to the CsA-treated and control group. In the spleen, bacterial load in the vitamin E-supplemented animals on day 4 was at 1 log CFU/g of organ ($P<0.05$), a significantly lower level compared to the bacterial load in CsA and control group remained at approximately 2 log CFU/g of organ. At day 6, bacterial load of all three treatment groups was at approximately 2 log CFU/g of organ in the spleen. In all cases, there were no significant differences in the level of *L. monocytogenes* between control and CsA-treated groups.

D. CHANGES IN THE LEVEL OF $CD8^+$ T CELLS DURING LISTERIAL INFECTION PHASE

During the 6 day infection period changes in $CD8^+$ T cells were noted in all three treatment groups (Fig. 3.5). At day 0 of the oral challenge, the level of $CD8^+$ T cells was the highest in the vitamin E-supplemented group at 43.7%, compared to 39.8%, and 32.17% in the control and CsA-treated groups, respectively. However, it was not significantly higher than the $CD8^+$ T cell level of the control group, at 39.8% ($P=0.08$). In the CsA-treated group, the $CD8^+$ T cell level was significantly suppressed (32.17%) compared to the control group (39.8%). Animals receiving vitamin E mounted a response based on a significant increase in $CD8^+$ T cells levels on day 4 (at 59.6%) and day 6 (50%). In the control group, the production of $CD8^+$ T cells (trend) was not as pronounced as compared to the vitamin E group, indicating an inability to mount an immune response. The CsA-treated group exhibited a similar level of response to the control group.


Small Intestines
Log CFU/g
5
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
b
b
a
b
b
Day 2
Day 4
Day 6
Days post-inoculation
Vit E
CSA
Control

b


Lymph Nodes
Log CFU/g
5
4.5
4
3.5
3
2.5
2
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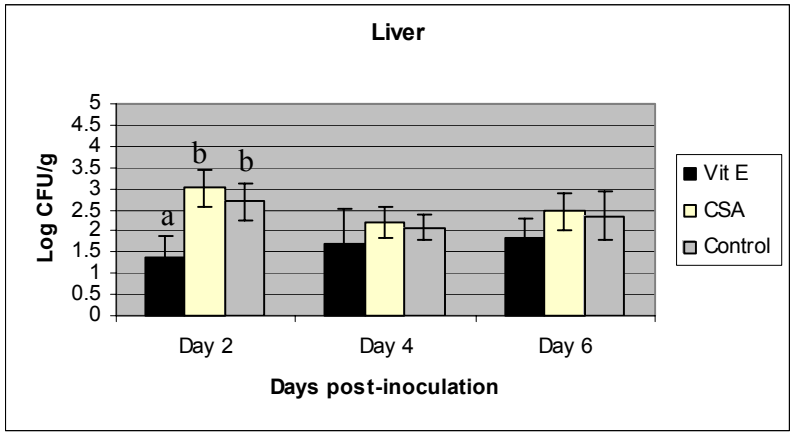
Days post-inoculation
Vit E
CSA
Control



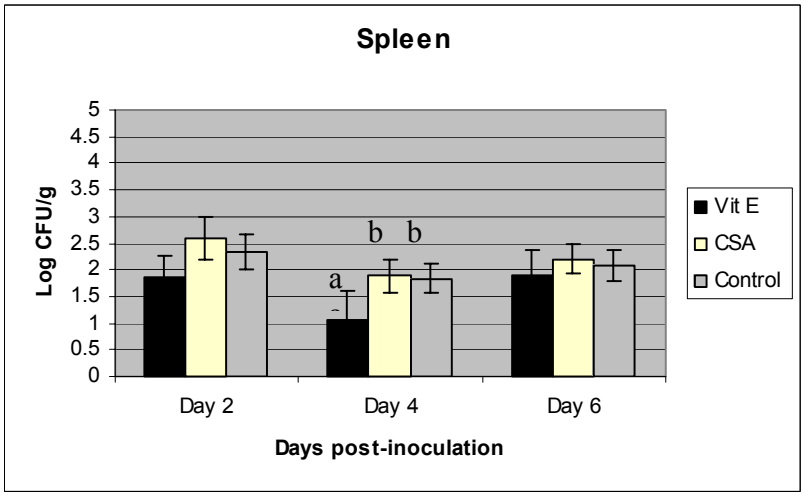


FIGURE 3.4. POPULATIONS OF VIABLE BACTERIA IN THE LIVER, SPLEEN, LYMPH NODES AND SMALL INTESTINES OF AGED GUINEA PIGS AFTER INTRAGASTRIC CHALLENGE WITH 10^8 CFU OF *L. MONOCYTOGENES.* The bacterial numbers within each organ were quantified on days 2, 4 and 6 post-challenge with 10^8 CFU of *L. monocytogenes*. Data were pooled from two independent experiments and expressed as log CFU of *L. monocytogenes* per organ. Each column represents means ± SEM (n=6) from two independent experiments. Student's *t* test was used in the analysis of results.

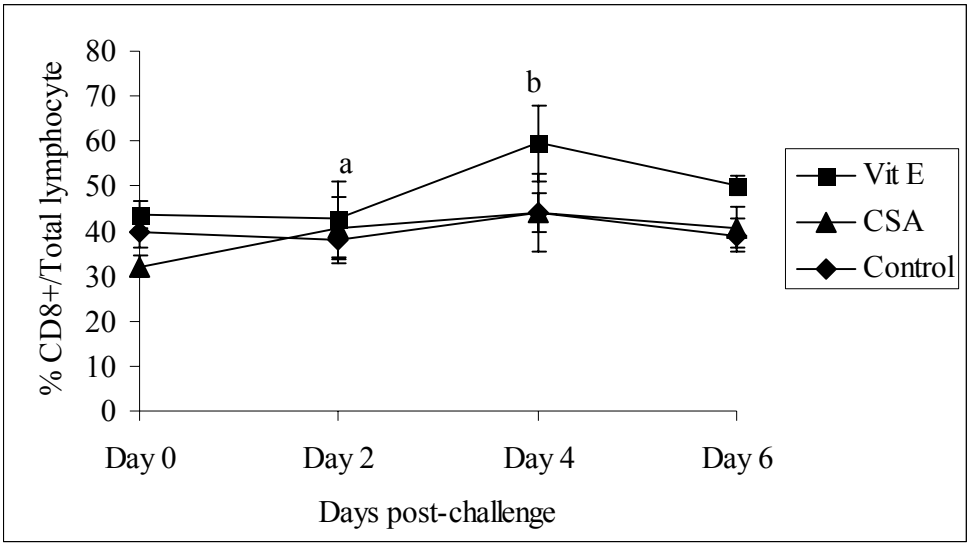


FIGURE 3.5. CHANGES IN THE LEVEL OF CD8$^+$ T CELLS IN THE VITAMIN E-SUPPLEMENTED (—■—), CsA-TREATED (--▲--), AND CONTROL ANIMALS (—♦—) POST-CHALLENGE WITH *L. MONOCYTOGENES*. Blood samples were collected from animals of all three groups on days 0, 2, 4, and 6 to for analysis to determine change in CD8$^+$ T cell levels during listerial infection. Each curve represents means and standard errors of two independent experiments (n=4). The level of statistical significance is set at $P<0.05$.

V. DISCUSSION

We investigated the effects of immuno-modulation on the outcome of a listerial infection using the guinea pig model as a human surrogate. Orthomolecular doses of vitamin E and treatment with CsA to enhance and suppress, respectively, the immune system of aged animals were used. It is widely established that function of the immune system declines with age (Lorber, 1997, Nagel *et al*., 1981), hence increasing susceptibility of the elderly population to listeriosis. Listerial infection evokes a T cell-mediated response, specifically, the critical T cells for clearing listerial infection are $CD8^+$ T cells (Harty and Bevan, 1996, Lane and Unanue, 1972). In this study, the levels of $CD8^+$ T cells were used to gauge the outcome of the immuno-modulation regimen.

The guinea pig has emerged as the animal model of choice to study listerial infection due to a functional e-cadherin that is specific for the entry of *L. monocytogenes* during an intragastric inoculation (Lecuit *et al*., 1999, 2001). Compared to other rodents, the guinea pigs was found to be immunologically similar to humans based on values of leukocyte fractions as determined in the blood and lymphoid tissues (Takizawa *et al*., 2004). In the present study, guinea pigs of approximately 2 years old (retired breeders) were chosen to represent an aged human population. Life span is the most widely accepted biomarker. Specific biomarkers for guinea pigs have not been defined, and therefore, age is used as the exclusive biomarker (Flurkey and Currer, 2004, Takizawa *et al*., 2004).

Previous studies have established that vitamin E, a known immune potentiator, stimulates T-cell proliferation and differentiation in rats and guinea pigs (De la Fuente *et al*., 2000, Moriguchi *et al*., 1998). Specifically, vitamin E acts by modulating the production of cytokines such as IL-2, which in turn affects the production of cytotoxic T ($CD8^+$) cells (Adolfsson *et al*., 2001). Meydani *et al*. (1998) showed that daily supplementation of 60, 200, and 800 IU of vitamin E for 4 months had no adverse impact on healthy elderly adults. Moreover, ingestion of larger doses of alpha-tocopherol acetate was positively correlated to an increase in the alpha-tocopherol levels in the tissues, and this was shown to be more effective than ingesting lower doses over a longer period of time (DRI, 2000). We showed that short-term daily supplementation with vitamin E resulted in a positive immunostimulatory effect on the aged guinea pigs based on the increase in the $CD8^+$ T cells population (Fig. 3.1). Results showed a significant increase in level of $CD8^+$ T cells (Fig. 3.1). Cytometric analysis revealed that a maturation of $CD8^+$ T cells occurred within the lymphocyte population during the period of vitamin E supplementation in the aged guinea pigs. Our results support the findings that short-term daily supplementation with vitamin E at 400 IU can enhance the immune system through maturation of CD8+ T cells (Fig. 3.1 and 3.2). HPLC analysis of the plasma samples showed an overall 1-fold increase in the concentration of alpha-tocopherol confirming absorption of alpha-tocopherol by the guinea pigs (Table 3.2). These results support that the increase in $CD8^+$ T cells was associated with vitamin E supplementation and absorption of alpha-tocopherol. .

The number of infected animals receiving the orthomolecular dose regime of vitamin E was 2.5-fold lower than the number of control animals (Table 3.3.A). Post challenge with the low dose 50% of the control (aged) animals became infected. Within an aged population of animals challenged with the low dose, there was a pronounced effect of vitamin E in mitigating listerial infection. This conclusion is supported by our results that demonstrated that vitamin E induces a positive immuno-stimulatory response in the aged guinea pigs.

The immuno-suppressive drug, CsA, is widely used in organ transplant patients (Freitas, 2003; Ho *et al*., 1996; Schaffner *et al*., 1983). Cs A is an immunosuppressor that specifically targets the T cell mediated response. CsA was administered to suppress the immune system of the aged animals to simulate or represent an elderly population taking glucocortico-steriods or those with underlying disease conditions. Other agents such as carrageenan have been used as immuno-suppressive agent in mice models studying the effects of listerial infections. (Takeuchi *et al*., 2006, Lammerding *et al*., 1992, Stelma *et al.*, 1987). Subsequent to CsA administration, all animals appeared lethargic and were physically less active. However, no significant decrease in weight was observed. No animals died as a result of CsA treatment. At 48 h post-admintration of CsA, an approximately 25% decrease in the CD8+ levels (Fig. 3.3) and an overall decline in T lymphocytes population (~40%; data not shown), indicating the specificity of CsA in suppressing T cells. Our results agreed with the findings of others, where they showed that it specifically suppresses T cells following peritoneal or oral administration in rats, mice, and guinea pigs (Schaffner *et al*., 1983, Borel *et al*., 1977). The decrease in $CD8^{+}$

T cells in the CsA treated group correlated with the outcome of listerial infection. Within animals challenged with the low dose the highest infection rate (89%) occurred in the CSA-treated animals, which translates to a 1.6 fold greater number of animals infected compared to the control animals. Moreover, quantifiable numbers of *L. monocytogenes* were obtained from CSA-treated animals on day 4 and day 6. Viable *L. monocytogenes* was found in the spleen or the liver of three animals, indicating a positive listerial infection (Brosch *et al*., 1983).

In the high dose challenge study, all animals became infected with *L. monocytogenes.* However, no animals died as a result of receiving the high dose challenge. Populations of *L. monocytogenes* in the liver, spleen and small intestines on days 2, 4 and 6 were consistently lower in animals that received vitamin E compared to control and CsA-treated animals (Fig. 3.4. A, C, and D). Significant differences in numbers of *L. monocytogenes* occurred on day 2 in the liver. Levels of *L. monocytogenes* in CsA-treated guinea pigs were not consistently higher compared to levels in the control guinea pigs. Therefore, based on microbiological analysis of the liver, vitamin E given at an orthomolecular dose appeared to enhance the immune system having positive effect in mitigating the initial severity of listerial infection. The high dose challenge likely overwhelmed the host's immune system limiting even the immuno-enhancing effect of vitamin E supplementation. By day 6, the level of *L. monocytogenes* in the liver did not decline. In contrast, Lecuit *et al*. (2001) reported that numbers of *L. monocytogenes* began to decrease by day 6. Perhaps the age of the animals used in the

present study, and therefore lack of vigor of the immune system, impaired the removal of the pathogen permitting greater numbers to remain for an extended period.

During the 6-day infection period, the level of $CD8^+$ T cells was higher and a more pronounced trend was noted in the vitamin E supplemented animals compared to the control and CsA-treated animals (Fig. 3.5). At day 4 of infection, the magnitude of the response in the vitamin E-supplemented group (~43%), compared to the control (~16%) and CsA-treated group (<10%), may correspond to the clonal expansion of $CD8^+$ T cells (Fig. 3.5). This relates to a host's immunological response triggered during an acute infection with intracellular pathogens such as influenza and *L. monocytogenes* (Busch *et al*., 1998, Butz and Bevan, 1998). Using aged mice, Aldofsson *et al*. (2001) showed that vitamin E acts by modulating the production of cytokines such as IL-2, which in turn affects the production of $CD8^+$ T cells. Although this experiment is not designed to directly determine whether the proportion of $CD8^+$ T cells is due to presence of memory T cells in the aged guinea pigs or the stimulated naïve T cells, Badovinac *et al*. (2003) determined that both groups of $CD8^+$ T cells conferred the same level of protection. The overall <10% change observed in $CD8^+$ T cells of the CsA-treated animals indicated no significant immune response occurred in this group of animals.

Throughout the study, behavior, physical appearance, movements, and diet of animals in all three groups (vitamin E supplemented, CsA treated, and control) was monitored twice daily. Short-term daily supplementation with 400 IU of vitamin E did not result in any adverse effects to the animals. In fact, a positive notable difference was observed in the animals physical appearance by the end of the 35-day supplementation

period. Moreover, an approximately 5-10% increase in weight was observed during the first 3 weeks. Fecal samples were in pelleted-form and no signs of loose stools were observed. Overall, the orthomolecular dose of vitamin E appeared to invigorate the health of the animals.

Based on the experiments conducted in this study, we showed that the immuno-regime of the aged guinea pigs led to a change in the immuno- responses based on $CD8^+$ T cells count, which impacted the outcome of a listerial infection. Within an aged population, the results support the concept that modulation in the host's immune system either through diet or medical therapy can influence the severity of infection or whether infection occurs. Additional studies are necessary to develop a more robust assessment of the impact of vitamin E supplementation on mitigation of listerial infection in the elderly.

VI. FUTURE WORK

As previously stated, research by Lecuit *et al.*(1999, 2001) showed that the guinea pig possesses a functional receptor , e-cadherin, that is exactly the same as that in humans. Moreover, the guinea pig was shown to be immunologically similar to humans, and hence a better animal model than rats and mice (Takizawa et al., 2004). Those studies served as the basis to investigate the human form of listeriosis in a guinea pig model. Since then, a series of studies emerged using the pregnant guinea pig model to investigate mechanisms behind the feto-placental transfer of *L. monocytogenes* (Barkardjiev *et al*., 2005, 2004, Lecuit *et al*., 2004).

Over the next 30 years, it is estimated that the current aged population of ~34 million persons will double to 21% of all Americans (Projections of the Population of the United States: 1982 to 2050 (Advance report)). The elderly are at increased risk for foodborne illnesses which is in part associated to an age-related decline in immune function. Based on the collective information research should address factors that relate to the opportunity for infection, the mechanisms of immunity, and the target population for prevention of listerial infection. For future studies, we would like to further develop the aged guinea pig as an animal model for investigating food-related illness and assessment of health promoting compounds that target the immune response.

- Characterize and compare the leukocyte population, and specific immune components, in the blood and the lymphoid organs in the guinea pigs of various ages (young, adult, elderly)

- Develop the animal model for use in evaluating different health-promoting nutraceuticals, or in studying the virulence of other foodborne pathogens or pathogens that negatively impact human health (viruses)

In animal studies, weight is typically used as an approximate representation of an animal's physiological age. Findings of these studies would establish a link between the age, weight, and immunological status of the animal. It would also provide insight into how novel nutraceuticals may play a role in stimulating the health of an elderly individual.

REFERENCES

Adolfsson, O., B.T. Huber, and S.M. Meydani. 2001. Vitamin E-enhanced IL-2 production in old mice: Naïve but not memory T cells show increased cell division cycling and IL-2-producing capacity. **167**: 3809-3817.

Andreone, P., S. Fiorino, C. Cursaro, A. Gramenzi, M. Margotti, L. Di Giammarino, M. Biselli, R. Miniero, G. Gasbarrini, and M. Bernadi. 2000. Vitamin E as treatment for chronic hepatitis B: results of a randomized controlled pilot trial. Antiviral Res. **49**: 75-81.

Badovinac, V.P., K.A. Messingham, S.E. Hamilton, and J.T. Harty. 2003. Regulation of CD8+ T cells undergoing primary and secondary responses to infection in the same host. J. Immunol. **170**: 4933-4942.

Bakardjiev, A.I, B.A. Stacy, S.J. Fisher, and D. Portnoy. 2004. Listeriosis in the pregnant guinea pig: a model of vertical transmission. Infec. Immun. **72**: 489-497.

Bakardjiev, A.I, B.A. Stacy, and D. Portnoy. 2005. Growth of Listeria monocytogenes in the guinea pig placenta and the role of cell-to-cell spread in fetal infection. J. Infect. Dis. **191**:1889-1897.

Borel, J.F., C. Feurer, C. Magnee, and H. Stahelin. 1977. Effects of the new anti-lymphocytic peptide cyclosporin A in animals. Immunology. **32**: 1017-1025

Brosch, R., B. Catimel, G. Milon, C. Buchrieser, E. Vindel, and J. Rocourt. 1993. Virulence heterogeneity of *Listeria monocytogenes* strains from various sources (food, human, animal) in immunocompromised mice and its association with typing characteristics. J. Food Prot. **56**:296-301.

Busch, D.H., I. Pilip, and E.G.Pamer. 1998. Evolution of a complex T cell receptor repertoire during primary and recall bacterial infection. J. Exp. Med. **188**:61-70.

Butz, E.A., and M.J. Bevan. 1998. Massive expansion of antigen-specific CD8+ T cells during an acute virus infection. Immunity **8**:167-175.

Centers for Disease Control and Prevention. 2004. Summary of notifiable diseases, United States, 2002. Morb. Mortal. Wkly. Rep. 51(53):1-78.

Centers for Disease Control and Prevention. 2004. Deaths: final data for 2002. National Vital Statistics Report, vol. 53, no.5. Avaliable at: http://www.cdc.gov/nchs/data/nvsr/nvsr53/nvsr53_05acc.pdf. Accessed November 2005.

Dalton, C.B., C.C. Austin, J. Sobel, P.S.Hayes, W.F. Bibb, L.M. Graves, B. Swaminathan, M.E. Proctor, and P.M. Griffin. 1997. An outbreak of gastroenteritis and fever due to *Listeria monocytogenes* in milk. N. Engl. J. Med. **336**: 100-105.

Decennial censuses of Population, 1900-1980 and projections of the population of the United States: 1982-2050 (advance report). Current population report series P-25, no. 922. Washington, CS: US Bureau of the Census, October 1982.

Dietary Reference Intakes. In. Vitamin E. National Academies Press. **2000**: 186-269.

De la Fuente, M., M. Carazo, R. Correa, and M. Del Rio. 2000. Changes in the macrophage and lymphocyte functions in guinea-pigs after different amounts of vitamin E ingestion. Brit. J. Nutr. **84**:25-29.

FDA/CFSAN, USDA/FSIS. 2003. Interpretive Summary: Quantitative assessment of the relative risk to public health from foodborne *Listeria monocytogenes* among selected categories of ready-to-eat foods. http://www.cfsan.fda.gov~dms/lmr2-toc.html

Flurkey, K., and J.M. Currer. 2004. Pitfalls of animal model systems in ageing research. Best Prac. Res. Clin. Endo. Met. **18**:407-421.

Hathcock, J.N., A. Azzi, J. Blumberg, T. Bray, A. Dickinson, B. Frei, I. Jialal, C. S. Johnston, F.J. Kelly, K. Kraemer, L. Packer, S. Parthasarathy, H. Sies, and M.G. Traber. 2005. Vitamins E and C are safe across a broad range of intakes. Am J Clin. Nutr. **81**: 736-745.

Han, S.N., and S.M. Meydani. 2000. Antioxidants, cytokines, and influenza infection in aged mice and elderly humans. J. Infect. Dis. **182** (Suppl 1): S74-80.

Ho, S., N. Clipstone, L. Timmermann, J. Northrop, I. Graef, D. Fiorentino, J. Nourse, and G.R. Crabtree. 1996. The mechanism of action of cyclosporin A and FK506. Clin. Immuno. and Immunopath. **80** (3): S40-S45.

Hof, H. 2003. History and epidemiology of listeriosis. FEMS Immun. Med. Microbiol. **35**: 199-202.

Hugin, A.W., A. Cerny, M. Wrann, H. Hengartner, and R.M. Zinkeragel. 1986. Effect of cyclosporin A on immunity to *Listeria monocytogenes*. Infect Immun. **52** :12-7.

Kappus H., and A.T. Diplock. 1992. Tolerance and safety of vitamin E: a toxicological position report. Free Radic. Biol. Med. **13**(1): 55-74.

Lane F.C., and E.R. Unanue. 1972. Requirement of thymus (T) lymphocytes for resistance to listeriosis. J. of Experiment. Med. **135:** 1104-1112.

Lara-Terejo, M., and E.G. Pamer. 2004. T cell responses to Listeria monocytogenes. Curr. Opin. Microbiol. **7**:45-50.

Leclercq, A. 2004. Atypical colonial morphology and low recoveries of Listeria monocytogenes strains on Oxford, PALCAM, Rapid'L.mono and ALOA solid media. J. Micro. Methods. **57**:251-258.

Lecuit, M., S. Vandormeal-Pournin, J. Lefort, M. Huerre, P. P. Gounon, C. Dupuy, C.Babinet, and P. Cossart. 2001. A transgenic model for listeriosis:
Role of internalin in crossing the intestinal barrier. **292**: 1172-1725.

Lecuit, M., S. Dramsi, C. Gottardi, M. Fedor-Chaiken, B. Gumbiner, and P. Cossart. 1999. A single amino acid in E-cadherin responsible for host specificity towards the human pathogen *Listeria monocytogenes*. EMBO J. **18**: 3956-3963.

Lorber, B. 1996. Listeriosis. Clin. Infect. Dis. **24**:1-11.

Meier, R., T. Tomizaki, C. Schulze-Briese, U. Baumann, and A. Stocker. 2003. The molecular basis of vitamin E retention: Structure of human alpha-tocopherol transfer protein. J. Mol. Biol. **331**: 725-734.

Messaoudi, I., J. Warner, D. Nikolich-Zugich, M. Fischer, and J. Nikolich-Zugich. 2006. Molecular, cellular, and antigen requirements for development of age-associated T cell clonal expansions in vivo. J. Immunol. **176**: 301-308.

Meydani, N.S., M. Meydani, J.B. Blumberg, L.S. Leka, M. Pedrosa, R. Diamond, and E.J. Schaefer. 1998. Assessment of the safety of supplementation with different amounts of vitamin E in healthy older adults. Am J Clin. Nutr. **68**:311-8.

Meydani S.M., S.N. Han, and D.H. Hamer. 2004. Vitamin E and respiratory infection in the elderly. Ann. N.Y. Acad. Sci. **1031**: 214-222.

Meydani, S.N., M.P. Barklund, S. Liu, M. Meydani, R.A. Miller, J.G. Cannon, F.D. Morrow, R. Rocklin, and J.B. Blumberg. 1990. Vitamin E supplementation enhances cell-mediated immunity in healthy elderly subjects. Am. J. Clin. Nutr. **52**:557-563.

Moriguchi, S. 1998. The role of vitamin E in T– cell differentiation and the decrease of cellular immunity aging. Biofactor. **7**:77-78.

Nagel, J.E., F.J. Chrest, and W.H. Adler. 1981. Enumeration of T lymphocyte subsets by monoclonal antibodies in young and aged humans. J. Immunol. **127**: 2086-2088.

Sahyoun, N.R., H. Lentzner, D. Hoyert, and K.N. Robinson. 2001. Trends in causes of death among the elderly. Aging Trends No. 1.CDCP: National Center for Health Statistics.

Serafini, M. 2000. Dietary vitamin E and T cell-mediated function in the elderly: effectiveness and mechanism of action. Int. J. Devl. Neuroscience. **18**: 401-410.

Schaffner, A., H. Douglas, and C.E. Davis. 1983. Models of T cell defiency in listeriosis: The effects of cortisone and cyclosporin A on normal and nude BALB/c mice. The J. of Immun. **131**: 450-453.

Schlech, W.F. 1997. Listeria gastroenteritis. Old syndrome, new pathogen. New Engl. J. Med. **336**: 130-132

Sim, J., D. Hood, L. Finnie, M. Wilson, C. Graham, M. Brett, and J.A. Hudson. 2002. Series of incidents of Listeria monocytogenes non-invasive febrile gastroenteritis involving ready-to-eat meats. Lett., in Appl. Microbiol. **35**: 409-413.

Stelma, G.N., A.L. Reyes, J.T. Peeler, D.W. Francis, J.M. Hunt, P.L. Spaulding, C.H. Johnson, and J. Lovett. 1987. Pathogenicity test for *Listeria monocytogenes* using immunocompromised mice. J. Clin. Microbiol. **25**: 2085-2089.

Takeuchi, K., N. Mytle, S. Lambert, M. Coleman, M.P. Doyle, and M.A. Smith. 2006. Comparison of *Listeria monocytogenes* virulence in a mouse model. J. Food Prot. **69**: 842-846.

Takizawa, M., J. Chiba, S. Haga, T. Asano, N. Yamamoto, and M. Honda. 2004. The normalization of guinea pig leukocyte fractions and lymphocyte subsets in blood and lymphoid tissues using a flow cytometric procedure. Exp. Anim. **53**: 321-329.

The decade for health in aging. Available at: http://www.healthinaging.org/public_policy/decade_challenge.php. Accessed on April. 11, 2006.

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Vazquez-Boland, J.A., M. Kuhn, P. Berche, T. Chakraborty, G. Dominguez-Bernal, W. Goebel, B. Gonzalez-Zorn, J. Wehland, and J. Kreft. 2001**.** *Listeria* pathogenesis and molecular virulence determinants. Clin. Microbiol. Rev. **14**:584-640.

APPENDIX 1

THE INFLUENCE OF FOOD AND IMPROPER HANDLING PRACTICES ON THE VIRULENCE OF *LISTERIA MONOCYTOGENES*

Previous research conducted in Dr. Matthews' laboratory has addressed the impact of food and temperature abuse on virulence of *L. monocytogenes* based on the ability of the pathogen to enter and multiply in an intestinal cell line (Caco-2). We found that carrier vehicle and improper handling has little or no impact on the virulence of *L. monocytogenes* as determined by multiplication within Caco-2 cells (Pang and Matthews, 2004). In this section, results from reverse-transcriptase PCR showed that *groEL*, a stress response gene, was up-regulated during the exposure to food and mishandling practices. The RNA isolation and RT-PCR protocols are presented. For all other protocols, please refer to Pang and Matthews (2004).

I. ABSTRACT

The influence of exposure of *Listeria monocytogenes* to foods, followed by improper handling conditions, was investigated based upon its ability to enter into and multiply within Caco-2 cells in a previous study. In addition, the expression of *groEL* was determined. *L. monocytogenes* inoculated into Brain Heart Infusion broth (control), chocolate milk, or frankfurter slurry was held at 4^{o}C for 24 h to simulate short-term exposure to each food. The *L. monocytogenes* contaminated food was then exposed to a

series of temperature shifts to simulate improper handling. The stress response gene, *groEL*, was only induced in the control cells (suspended in BHI) that had been exposed to improper handling conditions. Expression of *groEL* was evident in cells exposed to foods and improper handling conditions suggesting that the foods used in this study were a stressful environment for the cells.

II. INTRODUCTION

Listeria monocytogenes, an intracellular pathogen, has been linked to foodborne illnesses following consumption of contaminated meat, dairy products, and produce (Donnelly, 2001; Dalton et al., 1997). Of the 13 known serovars of *L. monocytogenes,* serovars 4b, 1/2a and 1/2b have been implicated in 98% of cases of human listeriosis (Datta, 1994, Farber and Peterkin, 1991). Studies have also shown that variation in virulence exists among serotypes (Buncic *et al*., 2000; Brosch *et al*., 1993). Serovar 4b seems to exhibit greater virulence than serovars 1/2a and 1/2b based on epidemiological evidence associated with cases of human listeriosis and based on animal studies (Farber and Peterkin, 1991).

Factors contributing to listerial illness include the carrier food, virulence of the strain, and susceptibility of the consumer (FDA/CFSAN, USDA/FSIS 2003; FDA/CFSAN, CDCP. 2003). A number of parameters have been shown to influence the virulence gene expression of a microorganism prior to or upon encountering a host (Mekalanos, 1992). Studies have demonstrated that food components (acids, salts,

carbohydrates), food processing and handling (temperature shifts), and chemical reactions in a food (Maillard reaction) can alter the virulence of *L. monocytogenes* (Becker *et al*., 2000, 1998; Sheikh-Zeinoddin *et al*., 2000; Duche *et al*., 2002; Brehm *et al.*, 1999; Huillet *et al*.,1999; Sampathkumar *et al*., 1999; Behari and Youngman, 1998; O'Driscoll *et al*., 1996; Ripio *et al*., 1996). Acid-adapted and acid tolerant mutants of *L. monocytogenes* became more invasive based on in vitro cell culture assays (Conte *et al.*, 2000) and displayed greater virulence in vivo (O'Driscoll *et al*., 1996). The exposure to refrigeration temperature influences the ability of the pathogen to survive in the presence of antimicrobial agents and heat treatment. Following exposure to 4^{o}C, *L. monocytogenes* serovar 1/2a survived the effects of bacteriocins better than serovar 4b whereas, serovar 4b survived post-cold storage heat treatment better than serovar 1/2a (Buncic *et al*., 2001). Research has shown that environmental stresses including low pH, low or high temperature, and salt can influence the expression of an array of genes including *hly* (listerolysin O), *dnaK, groES, groEL*, (heat shcok proteins) and *csp* (cold shock protein) (Hanawa *et al*., 2002; Gahan *et al*., 2001; Bayles *et al*., 2000, 1996; Abee and Wouters, 1999; Sampathkumar *et al*., 1999). Expression of these proteins by *L. monocytogenes* might enhance the survival of a pathogen in food and increase the likelihood of human infection.

An aim of the present study was to investigate the role of food and improper handling of the food on the stress response gene, *groEL*, of *L. monocytogenes*. Foods used in this study, frankfurters and chocolate milk, have been associated with large *L. monocytogenes* outbreaks in the U.S. Presence of mRNA from *groEL* was determined using reverse transcriptase PCR.

III. MATERIALS AND METHODS

A. TOTAL RNA EXTRACTION AND REVERSE TRANSCRIPTION-PCR

Cells (1×10^8 CFU; strains H4b, H1/2a, and H1/2b) from dialysis tubing supernatants (chocolate milk, frankfurter slurry, and BHI) held at 4°C for 24 h were harvested as where cells following exposure to improper handling conditions. Total RNA was extracted using RNAwiz (Ambion Inc., Austin TX) according to manufacturer's recommendations. RNA samples were DNase-treated using DNA-*free*™ (Ambion Inc., Austin TX) to remove contaminating genomic DNA. DNase treated samples were resuspended and stored in 30 µl of nuclease-free water at –20°C. RNA samples were examined spectrophotometrically using SmartSpec™ 3000 (Bio-Rad, CA) to calculate yield and for determining RNA purity.

For reverse transcriptase PCR analysis, 4 µL of total RNA of each strain was used in each RT-PCR reaction. RT-PCR reactions (25µL volume per reaction) were set up using the Maximquick™-RT-PCR kit (Promega) according to manufacturer's instructions. The cycling parameters were set at 48°C for 2 min, 94°C for 30 min, 55°C for 45 sec, 72°C for 2 min for a total of 35 cycles using GeneAmp®PCR system 2400 (Perkin-Elmer). To ensure that the RNA samples were free of DNA contamination, control reactions with no reverse transcriptase enzyme were set up together with the RT-PCR reactions. The size and integrity of RT-PCR samples were checked by 1.2% agarose gel stained with ethidium bromide and then detected using UV light. cDNA samples were stored at –20°C for use as negative controls in RT- PCR.

B. PRIMER DESIGN

The Primer 3 output program (http://www.broad.mit.edu/cgi-bin/primer/primer3.cgi) was used to design gene-specific primers suitable for RT-PCR (Table 1). All primers were tested using a gradient thermal cycler and had a calculated annealing temperature of 55°C. To check for the presence of primer dimers, a control was run in which cDNA was omitted from the RT-PCR reaction.

TABLE 1 Gene-specific primers for reverse-transcriptase PCR used in this study

Target gene (Accession Number)	Primer Name	Sequence (5'→3')	Size (bp)
prs (AF497214)	prs-F	cacgtcaagaccgtaaaggc	494
	prs-R	aagaactgggtgcgaacaac	
groEL (AF335323)	groEL-F	gtagtagccgtgaaagc	721
	groEL-R	gtagagcggaacgtgttac	

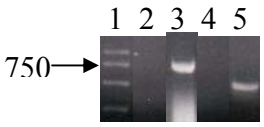
IV. RESULTS AND DISCUSSION

The influence of food matrix and improper handling practices on gene expression (*prs, groEL*) by *L. monocytogenes* was determined using RT-PCR. The intent of the RT-PCR was used as a qualitative assay was to determine if transcripts of interest (*groEL* and *prs*) occurred, not as a quantitative assay to determine the number of transcripts. Moreover, a no reverse-transcriptase control reaction (PCR control) was included in the experiments to ensure that the RNA isolated was free of genomic DNA contamination. RNA isolated from *L. monocytogenes* strain H4b exposed to BHI, chocolate milk, and frankfurter slurry dialysates, and having undergone improper

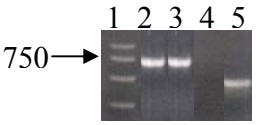
handling conditions was utilized. BHI dialysate served as the control treatment. The housekeeping gene, *prs* encoding for phosphoribosyl synthetase was used as a control for gene expression. The heat shock gene, *groEL*, is responsible for production of a heat shock protein in response to general stress (Gahan *et al*., 2001). Expression of *groEL* is elevated during infection and required for maximum virulence potential (Gahan *et al*., 2001). In this study, expression of *groEL* was used as an indicator of stress response. Expression of *groEL* was not detected in *L. monocytogenes* suspended in BHI that was held at 4^{o}C, but was expressed by cells after exposure of the BHI to improper handling conditions (Fig. 1A). However, *groEL* was expressed by *L. monocytogenes* exposed to each food and following exposure to improper handling (Fig. 1B and 1C). Results suggest that the exposure of *L. monocytogenes* to food (chocolate milk and frankfurter) environments used in this study can result in stress to the bacterium. Even exposure of *L. monocytogenes* to relatively moderate shifts in temperature (4^{o}C to 22^{o}C or 30^{o}C) resulted in increased expression of *groEL* (Fig. 1A). However, the RT-PCR assay was used to indicate the expression of *groEL*, not to quantitate the level of *groEL* expression. An extension of this research would be to use northern blot or quantitative real time PCR assay to quantify the transcripts of interest. Expression of *groEL* by a bacterium prior to entering a host may provide a survival advantage (e.g., passage through the acidic environment of the stomach) for the cell since shock proteins synthesized in response to one stress may provide protection against other stressors including increased tolerance to acidic conditions.

The findings of this study add new information about the influence that chocolate milk, frankfurter, and improper handling of those foods have on *L. monocytogenes* virulence. Serovar 4b is more commonly linked to cases of foodborne listeriosis and serovars 1/2a and 1/2b are more commonly isolated from foods (Donnelly, 2001; ICMSF, 1996). Only results for the human strain 4b were reported since all strains from each serovar used in this study behaved similarly. These results are not unexpected since a previous study demonstrated that *L. monocytogenes* regardless of origin or serovar were virulent in a mouse model (Brosch *et al*., 1993). However, considering foods are complex environments and strains of an organism may behave differently in such environments, extrapolating results to all foods and all strains of *L. monocytogenes* would not be prudent.

A) BHI dialysate



B) Chocolate milk dialysate



C) Frankfurter slurry dialysate

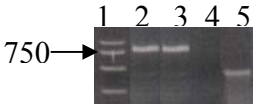


FIGURE 1. RT-PCR analysis of *groEL* (stress response), and *prs* (phosphoribosyl pyrophosphate synthetase, a house-keeping gene) in *L. monocytogenes* H4b that were exposed to and had undergone improper handling conditions. A) BHI dialysate, B) Chocolate milk dialysate, and C) Frankfurter slurry dialysate. Lane 1, molecular weight marker; lane 2, expression of *groEL* by cells after exposure to dialysates at 4^{o}C; lane 3, expression of *groEL* by cells after exposure to improper handling conditions; lane 4, negative control; lane 5, positive control (*prs*).

REFERENCES

Abee, T., and J.A., Wouters. 1999. Microbial stress response in minimal processing. Int. J. of Food Microbiol. **15**: 65-91.

Bayles, D.O., M.H. Tunick, T.A. Foglia, and A.J. Miller. 2000. Cold shock and its effect on ribosomes and thermal tolerance in *Listeria monocytogenes*. Appl. and Environ. Microbiol. **66**: 4351-4355.

Bayles, D.O., B.A. Annous, and B.J. Wilkinson. 1996. Cold stress proteins induced in *Listeria monocytogenes* in response to temperature downshock and growth at low temperatures. Appl. and Environ. Microbiol. **62**: 116-1119.

Becker, L.A., S. N. Evans, R.W. Hutkins, and A. Benson. 2000. Role of σ^B in adaptation of *Listeria monocytogenes* to growth at low temperature. J. of Bact. **182**: 7083-7087.

Becker, L.A., M.S. Cetin, R.W. Hutkins, and A. Benson. 1998. Identification of the gene encoding the alternative sigma factor σ^B from *Listeria monocytogenes* and its role in osmotolerance. J.of Bact. **180**: 4547-4554.

Behari, J., and P.Youngman. 1998. Regulation of *hly* expression in *Listeria monocytogenes* by carbon sources and pH occurs through separate mechanisms mediated by PrfA. Infect. and Immun. **66**: 3635-3642.

Brehm,K., M-T. Ripio, J. Kreft, and J.A.Vazquez-Boland, 1999. The *bvr* locus of *Listeria monocytogenes* mediates virulence gene repression by β–glucosides. J. of Bact. **181**: 5024-5032.

Brosch, R., B. Catimel, G. Milon, C. Buchrieser, E. Vindel, and J. Rocourt. 1993. Virulence heterogeneity of *Listeria monocytogenes* strains from various sources (food, human, animal) in immunocompromised mice and its association with typing characteristics. J. of Food Prot. **56**: 296-301.

Bruhn, C., and H. Schutz. 1999. Consumer food safety knowledge and practices. J. Food Safety. **19**: 73-87.

Buncic, S., S.M. Avery, J. Rocourt, and M. Dimitrijevic, 2000. Can food-related environmental factors induce different behavior in two key serovars, 4b and 1/2a, of *Listeria monocytogenes*? Int. J. of Food Microbiol. **65**: 201-212.

Conte, M.P., G. Petrone, A.M. Di Biase, M.G.Ammendolia, F. Superti, and L. Seganti. 2000. Acid tolerance in *Listeria monocytogenes* influences invasiveness of enterocyte-like cells and macrophage-like cells. Microbial Pathogenesis. **20**: 137-144.

Dalton, C.B., C.C. Austin, J. Sobel, P.S.Hayes, W.F. Bibb, L.M. Graves, B. Swaminathan, M.E. Proctor, and P.M. Griffin. 1997. An outbreak of gastroenteritis and fever due to *Listeria monocytogenes* in milk. New Engl. J. of Med. **336**: 100-105.

Datta, A.R. 1994. Factors controlling expression of virulence genes in *Listeria monocytogenes.* Food Microbiol. **11**: 123-129.

Donnelly C.W. 2001. *Listeria monocytogenes*: A continuing challenge. Nutrit. Rev. **59**: 183-194.

Duché, O., F. Trémoulet, P. Glaser, and J. Labadie. 2002. Salt stress proteins induced in *Listeria monocytogenes*. App. and Environ. Microbiol. **68**: 1491-1498.

Farber, J.M., and P.I. Peterkin. 1991. *Listeria monocytogenes*, a foodborne pathogen. Microbiol. Rev. **55**: 476-511.

FDA/CFSAN, USDA/FSIS. 2003. Interpretive Summary: Quantitative assessment of the relative risk to public health from foodborne *Listeria monocytogenes* among selected categories of ready-to-eat foods. http://www.cfsan.fda.gov/~dms/lmr2-toc.html

FDA/CFSAN, CDCP. 2003. Reducing the risk of *Listeria monocytogenes* FDA/CDC 2003 update of *Listeria* action plan. http://www.cfsan.fda.gov/~dms/lmr2plan.html

Gahan, C.G.M., J. O'Mahony, and C. Hill. 2001. Characterization of the *groESL* operon in *Listeria monocytogenes*: Utilization of two reporter systems (*gfp* and *hly*) for evaluating *in vivo* expression. Infect. and Immun. **69**: 3924-3932.

Hanawa, T., S. Yamanishi, S. Murayama, T. Yamamoto, and S. Kamiya. 2002. Participation of DnaK in expression of genes involved in virulence of *Listeria monocytogenes*. FEMS Microbiol. Lett. **214**: 69-75.

Huillet, E., S. Larpin, P. Pardon, P. Berche. 1999. Identification of a new locus in *Listeria monocytogenes* involved in cellobiose-dependent repression of *hly* expression. FEMS Microbiol. Lett. **174**: 265-272.

International Commission on Microbiological Specifications for Foods. 1996. *Listeria monocytogene.* In Roberts, T.A., Baird-Parker, A.C., and Tompkin, R.B. (Eds.), Microorganisms in Foods 5, Microbiological Specifications of Food Pathogens. Blackie Academic and Professional, New York, NY, pp. 141-143.

Mekalanos, J.J. 1992. Environmental signals controlling expression of virulence determinants in bacteria. J. of Bacteriol. **174**:1-7.

O'Driscoll, B., C.G.M. Gahan, and C. Hill. 1996. Adaptive acid tolerance response in *Listeria monocytogenes*: Isolation of an acid-tolerant mutant which demonstrates increased virulence. App. and Environ. Microbiol. **62**: 1693-1698.

Pang, H.E., and K. Matthews. 2004. Impact of food matrix and temperature abuse on the virulence of *Listeria monocytogenes*. (Master's Thesis)

Ripio, M.-T., G. Dominguez-Bernal, M. Suarez, K. Brehm, P. Berche, J.-A. Vazquez-Boland, 1996. Transcriptional activation of virulence genes in wild-type strains of *Listeria monocytogenes* in response to a change in the extracellular medium composition. Res. in Microbiol. **147**: 371-384.

Roche, S.M., P. Velge, E. Bottreau, C. Durier, N., Marquet-vander Mee, and P. Pardon. 2001. Assessment of the virulence of *Listeria monocytogenes* : agreement between a plaque-forming assay with HT-29 cells and infection of immunocompetent mice. Int. J. of Food Microbiol. **68**: 33-44.

Sampathkumar, B., I.J. Xavier, L.S.L. Yu, and G.G. Khachatourians. 1999. Production of listeriolysin O by *Listeria monocytogenes* (Scott A) under heat-shock conditions. Int. J. of Food Microbiol. **48**: 131-137.

Sheikh-Zeinoddin, M., T.M.Perehinec, S.E. Hill, and C.E.D. Rees. 2000. Maillard reaction causes suppression of virulence gene expression in *Listeria monocytogenes*. Int. J. of Food Microbiol. **61**: 41-49.

APPENDIX 2

TABLE 1. Pre- and post-infection antibody titers to *L. monocytogenes* in aged guinea pigs.

Sample collection	Antibody titer by Treatment groups		
	Vitamin E	CsA	Control
Pre-infection [b] (Avg ± SD)	0.440 ± 0.086[a]	0.486 ± 0.087	1.055 ± 0.119
Day 6 Post-Infection (Avg ± SD)	0.360 ± 0.110[c]	0.513 ± 0.395	1.274 ± 0.589

[a] Value is avg ± SD, an average of six determinations and are representative of two independent experiments.

[b] Pre- infection sera of animals from each treatment group were drawn prior to oral challenge.

[c] Statistical significance was set at $P< 0.05$. No statistical significance was found in the antibody titers between the pre-infection and post-infection phase.

In brief, enzyme linked immunosorbant assay (ELISA) was conducted to establish antibody titers in the adult guinea pigs prior to and during listerial infection. Protocol used was modified from Kolling and Matthews, 1999. Briefly, a cocktail of 18 h cultures of the *L. monocytogenes* strains N1-225, J1-119, MMS 97-1 was be washed twice in PBS, dispensed into 96 well plates at a concentration of 10^8 CFU/ml. Cells were bound to the plates using 0.5% glutaldehyde in PBS and incubated at 37^{o}C for 30 min or overnight. Wells were washed three times with wash buffer (0.85% NaCl; 0.05% Tween 20). Blocking solution (3% BSA in PBS; 0.05% Tween) was used to block non-specific binding for 1 h at 37^{o}C. Plasma (100 μl) was added to the wells. Bound antibodies were

detected addition of 1/2000 dilution of alkaline phosphatase conjugated rabbit anti-guinea immunoglobulin G (Sigma-Aldrich, St. Louis, MO). Plates were incubated at 37^{o}C for 1 h, washed, and 4-nitrophenyl phosphate disodium salt, hexahydrate at 1 mg/ml, 0.2M Tris, pH 9 added for color development. The reaction was stopped with 0.1 N NaOH and reactions read at 405 nm.

The antibody titer to *L. monocytogenes* was established in all the aged guinea pigs. The antibody titer was determined to provide insight into the magnitude of the immune response elicited in animals that underwent different immuno-modulation regimes (orthomolecular doses of vitamin E and CsA treatment). The humoral immune response in guinea pigs to listerial infection was measured using an enzyme-linked immunosorbent assay designed to detect IgG antibody to the 3 strain cocktail of *L. monocytogenes* serotype 4b. Results showed that there was no difference ($P<0.05$) in the IgG titer pre- and post-infection with *L. monocytogenes* within each treatment group (Table 1). However, those results are not totally unexpected since it takes about 2 weeks to precipitate an IgG response. Results suggest that animals did not previously have listerial infection, since the challenge administered in this study would have acted as a "booster" and the IgG titer would have increased significantly.

REFERENCES

Kolling, G.L., and K.R. Matthews. 1999. Functional characterization of membrane vesicles released by *Escherichia coli* O157:H7. (Master's Thesis).

Curriculum Vita

Hoan-Jen Eunice Pang

Education / Certification

Ph.D., Food Science, Rutgers University, May 2007

M.Sc., Food Science, Rutgers University, Aug 2001- May 2004

B.Sc., Biochemistry, State University of New York at Stony Brook, Jan 1997-May 2001

HACCP: A basic concept for food protection 15th-17th, Nov 2004, 01.8 CEUs, Rutgers University

Research Experience

Visiting Scientist

Microbial Food Safety Unit, Eastern Regional Research Center of the U.S. Department of Agriculture, Wyndmoor, Pennsylvania (Feb 2006- July 2006)

- Independently developed and troubleshoot 2D gel electrophoresis protocol; analyzed and identified global protein changes in *E. coli* O157:H7 using Z3 compugen program; performed in-gel protein digestion and sample preparation for MALDI-Tof analysis
- Involved in the identification of proteins involved in the formation and regulation of biofilm by *E. coli* O157:H7
- Trained and supervised technician and college-level intern in molecular and microbiological techniques, specifically in 2D gel electrophoresis and in conducting *L. monocytogenes* growth curve studies
- Involved in examining the biofilm formation of *Salmonella enteritidis* to stainless steel in various media and temperature conditions

Graduate Research Assistant

Department of Food Science, Rutgers University, New Jersey (2001- present)

- Influence of environmental factors and the host's susceptibility on the development of *Listeria monocytogenes* infection in the guinea pig model (Ph.D. Dissertation)
- Impact of food matrix and temperature abuse on the virulence of *L. monocytogenes*. (Masters' Thesis)

Publications

H.E. Pang, C-Y Lo, and K. Matthews. 2007. Influence of immuno-modulation on the development of *Listeria monocytogenes* infection in aged guinea pigs. FEMS Immunology & Medical Microbiology 49: 415-424.

Hoan-Jen E. Pang, Catherine Potenski, Karl Matthews. 2007. Exposure of *Listeria monocytogenes* to food and temperature abuse using a dialysis culture method. Journal of Food Safety (Accepted)

Hoan-Jen E. Pang and Karl Matthews. 2006. Influence of a food environment on listerial infection in the guinea pig model. Journal of Food Safety 26: 313-324.

H.E. Pang and K.R. Matthews. 2006. Impact of immuno-modulation on the development of listerial infection in aged guinea pigs. American Society for Microbiology 106th General meeting, May 22nd, 2006. (Abstract)

Gunther, N.W. IV, **Pang, H.-J.**, Uhlich, G.A., and Fratamico, P.M. 2006. A proteomic-based approach to understanding the responses of food-borne pathogens to environmental factors, p. FS1-FS5. Proceedings of the United States-Japan Cooperative Program in Natural Resources - Food and
Agriculture Panel Meeting, Rohnert Park, CA. (Publication in Proceedings)

Nereus W. Gunther IV, **Hoan-Jen Pang**, Alberto Nunez, Gaylen A. Uhlich. 2006. Comparing techniques for comparative proteomics: Two-dimensional gel electrophoresis and two-dimensional liquid chromatography. Society for Industrial Microbiology. Baltimore, Maryland. (Abstract)

Hoan-Jen E. Pang. 2004. Impact of food matrix and temperature abuse on the virulence of *Listeria monocytogenes* (Masters Thesis)

Ethan B. Solomon, **Hoan-Jen E. Pang**, Karl Matthews. 2003. Passage of *Escherichia coli* O157:H7 from contaminated water to lettuce is dependent on irrigation methodology. Journal of Food Protection 66:2198-2202.

John Allen and ***Hoan-Jen Pang**. 2003. Hot topics in seafood quality and safety. Food Protection Trends Vol. 23, No. 11 (session summary)
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H.E. Pang and K. Matthews. 2004. Evaluation of *Listeria monocytogenes* virulence post exposure to foods and temperature abuse using an in vitro Caco-2 cell assay. American Society for Microbiology, 104th General meeting. New Orleans, LA. (Abstract)

H.E. Pang, C.J. Potenski, K.R. Matthews. 2003. Impact of Food Matrix on *L. monocytogenes* gene response and virulence in a guinea pig model. American Society for Microbiology, 103rd General meeting. Washington, D.C. (Abstract)

Presentations

H.E. Pang and K.R. Matthews. 2006. The influence of vitamin E supplementation on the development of listeriosis in aged guinea pigs. Institute of Food Technologist (IFT) National meeting, Orlando, June 27th, 2006. (Oral)

H.E. Pang and K.R. Matthews. 2006. Impact of immuno-modulation on the development of listerial infection in aged guinea pigs. American Society for Microbiology 106th General meeting, May 22nd, 2006. (Poster)

H.E. Pang, V. Gomes, K.R. Matthews. *Listeria monocytogenes* infection in the guinea pig model: Influence of the carrier vehicle or the dose consumed? IFT National meeting, New Orleans, July 18th, 2005. (Oral)

H.E. Pang. Singapore: Cultures and Gastronomy. Mini-Symposium on International Perspectives, Cook College, Rutgers University. Nov 18th, 2004. (Invited Speaker)

H.E. Pang and K.R. Matthews. Evaluation of *L. monocytogenes* virulence post-exposure to foods and temperature abuse using an in vitro Caco-2 cell assay. American Society for Microbiology 104th General meeting, New Orleans. May 23rd, 2004 (Poster)

H.E. Pang and K.R. Matthews. Exposure of *L. monocytogenes* to food matrices and temperature abuse using a novel system. IFT National meeting, Las Vegas, July 12th, 2004 (Poster)

H.E. Pang, C.J. Potenski, and K.R. Matthews. Impact of food matrix on *L. monocytogenes* gene response and virulence in a guinea pig model. American Society for Microbiology, 103rd General meeting. Washington, D.C., May 20, 2003 (Poster)

Solomon, Ethan B., **Hoan-Jen E. Pang**, and Karl Matthews. Fate of salmonellae on/in tomato fruits as affected by method of application of contaminated irrigation water. Theobald Smith Society Meeting in Miniature, April 25, 2002. (Poster)

E.Chen, D.Rosa, **H.E. Pang**, C. Brathwaite, S.Maitra. Effects of Sepsis on the Expression of SAPK/JNK Signaling Pathway. FASEB-Experimental Biology 2001. (Poster)

Honors, Awards, Scholarships

- Albert Kleinman Scholarship in Food Science, 2006-2007
- First Place Award in Institute of Food Technologist (IFT) – Food Microbiology Division Graduate Research Paper Oral Competition, 2006
- First Place in Chinese-American Microbiology Society (CASM) Graduate Student Poster Competition, 2006
- New York Regional Section of the Institute of Food Technologist scholarship, 2005, 2006
- Student Travel Award to Attend Annual Meeting of the American Society (ASM) for Microbiology, 2005

- Outstanding Student Achievement Award of the Institute of Food Technologist Student Association, 2005
- Theobald Smith Society (TSS) Graduate Student Scholarship, 2004
- Certificate of Commendation from the Cook/NJAES International Mini-Symposium Series, Rutgers University, 2004
- Hachnasarian Hamo Scholarship, Rutgers University, 2002

Professional Development

- Selected participant of the American Society of Microbiology (ASM) Robert J. Kadner Institute, 2006
- Team leader of the product development team: Development of a probiotic ice cream for the Danisco knowledge award competition, 2006
- Coordinator and organizer of Rutgers Night 2005; Team with faculty members, New York IFT members, and students to address the details involved, 2004-2005
- President of the Food Science Graduate Student Association, Rutgers University, March 2004 - 2005
- Spearheaded an ice-cream fundraiser and initiated an ice-cream flavor development project for Rutgers University, 2004-2005
- Student Representative to New York and Central New Jersey Institute of Food Technologist, 2004-2005
- Reviewed chapter of textbook, Food Microbiology, An Introduction, ASM Press, TJ Montville and K.R. Matthews

Professional Affiliations

- American Society for Microbiology (2002-present)
- Theobald Smith Society, New Jersey Branch of the American Society of Microbiology (2002-present)
- Institute of Food Technologist (National, New York/Central New Jersey Sections) (2001-present)