CONTROL STRATEGIES FOR *LISTERIA MONOCYTOGENES* IN READY-TO-EAT FOODS AND ON FOOD CONTACT SURFACES

by

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B.S., Punjab Agricultural University, 2005
M.S., Kansas State University, 2008

AN ABSTRACT OF A DISSERTATION

submitted in partial fulfillment of the requirements for the degree

DOCTOR OF PHILOSOPHY

Food Science

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Abstract

The ubiquitous nature and continued presence in food processing environments makes *Listeria monocytogenes* a significant threat in ready-to-eat (RTE) food products. This study was performed in two phases; Phase 1 studied lauric arginate (LAE) as an antimicrobial on food contact surfaces and shredded mozzarella cheese, and use of glucose oxidase (GOX), sodium lactate (SL), and acidified calcium sulfate (ACS) as preservatives in mozzarella cheese; Phase 2 evaluated efficacy of Photohydroionization (PHI) technology to control *L. monocytogenes* on food contact surfaces, sliced American cheese, and ready-to-eat turkey. Stainless steel coupons, mozzarella cheese, American cheese, and turkey were surface inoculated with a three- or five-strain cocktail of *L. monocytogenes.* Coupons were treated with 100 and 200 ppm solution of lauric arginate for 5 and 15 min. Mozzarella cheese was treated with different combinations of treatments comprising LAE, GOX, SL, ACS, dextrose, and anticaking agents (free flow 1031 and cellulose). Results indicated up to 2.5 log CFU/coupon reductions and it was concluded that LAE was effective in controlling low levels of contamination of *L. monocytogenes* on food contact surfaces. In mozzarella cheese, results indicated that lauric arginate provided no additional antimicrobial effect ($P > 0.05$) as compared to GOX + dextrose. The antimicrobial blends with GOX, SL, and ACS were different ($P < 0.05$) from the controls but showed no differences ($P > 0.05$) in their effect in controlling bacterial populations. Results from treatment with PHI unit showed significant ($P < 0.05$) reduction in bacterial populations. *L. monocytogenes* populations reduced by 4.37 log CFU/coupon on stainless steel surfaces after 15 min of treatment; 2.16 and 2.52 log CFU/sample reduction on American cheese and ready-to-eat turkey, respectively, after short treatment time of 5 min. Lipid oxidation analyses performed on cheese and turkey samples indicated that the PHI treatment did not affect ($P > 0.05$) TBAR values. These studies suggest that LAE and GOX as antimicrobials and PHI treatment can be used as intervention strategies in an integrated process to ensure safe production of food. Further research is needed to evaluate applicability of SL and ACS in mozzarella cheese.

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Chapter 1 - Introduction

Listeria monocytogenes is a Gram positive, rod shaped, non sporeforming, ubiquitous organism found naturally in the environment, air, water, soil and is carried by healthy humans and animals (Jay and others 2005). It is a bacterium of public health significance, which is causative of agent of serious foodborne illness, called Listeriosis; and is a major challenge to the food safety of the food supply globally. A number of widespread outbreak of Listeriosis have been reported in the past few decades that have established *L. moncytogenes* as a pathogen of high priority for foodborne disease. The invasive illness caused by *L. monocytogenes* is mainly seen in a well-defined high-risk group that includes elderly, immunocompromised, pregnant women, newborns, and infants (Rebagliati 2009).

Foods that have been commonly implicated with *L. monocytogenes* contamination include ready-to-eat (RTE) foods, meat and poultry products, dairy products, and seafood (Ryser and Marth 1991). Extensive research indicates that recontamination is the primary source of *L. moncytogenes* in commercially processed ready-to-eat (RTE) foods (Tompkin 2001). *Listeria monocytogenes* has the ability to grow at a wide temperature range, 1 to 45ºC (ICMSF 1996), including refrigerated temperatures; has a tolerance to high salt concentrations (Farber and Peterkin 1991); and has an ability to grow at relatively low pH (Bell and Kyriakides 2005); making *L. monocytogenes* difficult to control in food. In addition, food processing environments provide ideal conditions for growth and multiplication of this organism.

Several factors contribute to the growth of microorganisms in food processing environments, which include moisture, nutrients, pH, oxidation-reduction potential, temperature, presence or absence of inhibitors, microbial interactions, and time. *Listeria monocytogenes* is also known to be frequently associated with raw materials used in food processing facilities, possibly constantly reintroducing the organism to the plant environment (Zotolla 1994). *Listeria monocytogenes* can be destroyed by heat treatment, so it is well established that contamination of ready-to-eat foods occurs during post processing procedures such a peeling, slicing, and packaging (Farber and Peterkin 1991). Therefore, effective post-processing antimicrobial interventions to prevent, control or inhibit the growth of this pathogen are essential.

Based on the characteristics of the organism and the nature of the disease caused, the U.S. Food and Drug Administration (FDA) and the United States Department of Agriculture Food Safety and Inspection Service (USDA FSIS) maintain a zero tolerance policy for detection of *L. monocytogenes* in RTE foods (Klontz and others 2008; Brandt and others 2010).

Saini (2008) evaluated the potential for translocation of *L. monocytogenes* in drains onto food contact surfaces in the processing environment, using *Listeria innocua* as a surrogate. The results showed that bacterial cells present in the drain can translocate into the surrounding environment and food contact surfaces which may result in contaminated product along the production and processing line even though the food is adequately processed. The study also suggested the scope for increasing importance of the cleaning phase in terms of removal of attached bacteria. Optimization is required in terms of efficacy of removal and limitation of the generation of viable aerosols. Higher degree of mechanical action, scrubbing, and the use of detergents may play a role in the reduction in the spread of contamination by aerosols.

Various intervention strategies have focused on controlling *L. monocytogenes* in ready-to-eat food products and food processing environments. Previous research has shown bactericidal activity of lauric arginate, in combination with other antimicrobials, in milk and Queso Fresco cheese (Soni and others 2010), and with lactates in commercially produced frankfurters (Martin and others 2009). The advanced oxidation process (AOP), referred to as Photohydroionization is a patented technology of RGF Environmental Group Inc. (West Palm Beach, FL), that uses ultraviolet (UV) light lamps at different wavelengths to produce ozone and vapor hydrogen peroxide at low levels which brings about the bactericidal effect. This AOP generates high reaction hydroxyl radicals and several other reactive oxygen species (ROS) which attack the microbial contaminant on its surface and disrupt cell growth and proliferation.

The bactericidal effect of ultraviolet (UV) and ozone is well documented (Jin-Kab and others 1999; Khadre and others 2001). Ozonation followed by the use of UV has been used for treatment of drinking water and wastewater applications to improve the efficiency of inactivation of microbiological contamination (White 1992; Amirsardi and others 2001; Oh and others 2003). Food Safety and Inspection Service (FSIS) in its guidance document for control of *L. monocytogenes* in post-lethality exposed ready-to-eat meat and poultry product specifies that depending on whether it eliminates, reduces or suppresses growth of *L. monocytogenes,* ultraviolet treatment can be used either as a post-lethality treatment or antimicrobial agent or

process (FSIS 2006). The anti-listerial activity of both ozone and UV radiation has been demonstrated with an added advantage of leaving no harmful residues or by-products (Clifford 1999).

Given the widespread nature of *L. monocytogenes*, ability to grow at refrigerated temperatures, high acid and salt concentrations, and its common occurrence in food processing environments; it becomes necessary to continually evaluate methods and technologies that may be applied to food products or in the production facilities to control this pathogen, avoid contamination and re-contamination, and ensure safety of food. The main objective of this research was to evaluate control strategies for *L. monocytogenes* in ready-to-eat (RTE) foods and on food contact surfaces.

Two different kinds of interventions for control were evaluated. The first study was on the use of antimicrobial, lauric arginate, for control of *L. monocytogenes* on food contact surfaces and shredded mozzarella cheese. The study was further extended to evaluate glucose oxidase, sodium lactate, and acidified calcium sulfate as preservatives in *L. monocytogenes* susceptible food products such as mozzarella cheese. The objectives of this study were: 1) to validate the efficacy of Mirenat- N (lauric arginate) antimicrobial against *L. monocytogenes* on stainless steel coupon surfaces; 2) to study the microbiological activity and applicability of lauric arginate (LAE® 200 ppm) combined with anticaking ingredients for controlling *L. monocytogenes* in shredded mozzarella cheese stored under refrigerated conditions; and 3) to evaluate the microbiological activity of glucose oxidase and different ingredients applied in shredded mozzarella cheese for controlling *L. monocytogenes* in the presence of a Gram-negative enteric bacterium, *Salmonella* spp.

The second study was on the use of advanced oxidation process to control *L. monocytogenes* on food contact surfaces in processing environments as well ready-to-eat foods (sliced American cheese and turkey). The objective of this study was to evaluate the efficacy and applicability of Advanced Oxidation Technology with Photohydroionization as a surface treatment for control of *L. monocytogenes* on stainless steel coupon surfaces, sliced American cheese, and ready-to-eat turkey.

Chapter 2 - Literature Review

Listeria monocytogenes

Listeria moncytogenes is a Gram positive bacterium first described by Murray and others (1926), and has been recognized as human pathogen since 1929 (Ryser and Marth 1991). It is a motile bacterium with diptheroid-like rod shaped cells measuring 1.0 to 2.0 μm by 0.5 μm (Gray and Killinger 1966). It is non-sporeforming, highly motile, and facultative anaerobic (Farber and Peterkin 1991). It can grow over a wide temperature range from 1 to 45ºC; with optimum being 30 to 37ºC (ICMSF 1996). When grown at 20 to 25ºC, Listeriae exhibits characteristic 'tumbling motility' predominantly by means of peritrichous flagella (Gary and Killinger 1966). The pH range for growth is between 4.5 and 9.6 (Seelinger and Jones 1986) and water activity is ≥ 0.92 (ICMSF 1996). *Listeria* spp. can multiply in high salt concentrations (up to 10% sodium chloride) or bile (Seelinger 1961). It grows readily on blood agar, where it produces incomplete β – hemolysis (Farber and Peterkin 1991). *Listeria* spp. is catalase positive, oxidase negative, methyl red positive, and Voges Proskauer positive (Schuchat and others 1991).

Six species of *Listeria* are currently recognized: *L. monocytogenes, L. innocua, L. seeligeri, L. welshimeri, L. ivanovii* and *L. grayi.* Table 1 shows the differentiation of different species of *Listeria* based on hemolysis, sugar fermentation, and virulence. The species further give rise to 17 serovars which are characterized by possession of specific antigens. *Listeria monocytogenes* is the primary pathogenic species, and a number of cellular O and flagellar H antigens have been identified and are used to divide it into 13 serovars (Varnam and Evans 1996). Serotypes 4b, 1/2a and 1/2b account for 95% of all isolates and have been linked with most of all disease (Bucholz and Mascola 2001). The CAMP test (Christine Atkins Munch-Pertersen; Table 2) is a useful test to differentiate between different species of *Listeria*, specifically between *L. monocytogenes* and *L. innocua* as they produce similar biochemical tests. It detects synergistic reaction of hemolysins produced by *Listeria* spp. against beta toxin of *Staphylococcus aureus* and an exofactor of *Rhodococcus equi* (Schuchat and others 1991).

Table 1 Differentiation of *Listeria* species (Anthony and Jinneman 2011)

		Acid produced from			
Species	**β-Hemolysis[a]**	**Mannitol**	**Rhamnose**	**Xylose**	**Virulence[b]**
L. monocytogenes	+	-	+	-	-
***L. ivanovii*[c]**	+	-	-	+	+
L. innocua	-	-	v[d]	-	-
L. welshimeri	-	-	v[d]	+	-
L. seeligeri	+	-	-	+	-
***L. grayi*[e]**	-	+	v[d]	-	-

[a] Sheep blood agar stab.
[b] Mouse test.
[c] Ribose fermenting strains are classified as *L. ivanovii* subsp. *ivanovii* and ribose non-fermenters as *L. ivanovii* subsp. *londiniensis*.
[d] v, variable biotypes
[e] Includes two subspecies - *L. grayi* subsp. *murrayi* reduces nitrate *L. grayi* subsp. *grayi* does not reduce nitrate.

Table 2 CAMP[a] test hemolytic enhancement of *Listeria* species (Anthony and Jinneman 2011)

	Hemolytic enhancement with	
	Staphylococcus aureus (S)	***Rhodococcus equi (R)***
L. monocytogenes	+	-[b]
L. ivanovii	-	+
L. innocua	-	-
L. welshimeri	-	-
L. seeligiri	+	-

[a]CAMP – Christine Atkins Munch Peterson
[b] Rare strains are S+ and R+. The R+ reaction is less pronounced than that of *L. ivanovii*.

Sources of *Listeria monocytogenes* Contamination

The ubiquitous nature of *Listeria monocytogenes* and its ability to grow at refrigerated temperatures makes it a significant threat to the safety of global food supply. It associates commonly with animals and has been commonly isolated from plants, soil, air, silage, feces, sewage, and water (Cox and others 1989). It is known to exist widely in food processing environments (Ryser and Marth 1991) and can survive for extended periods of time in production areas and environments. Although raw plant and animal foods are commonly found to be contaminated with *L. monocytogenes*, it is also commonly associated with cooked foods which can be become contaminated during post-processing steps. It has been isolated in foods such as raw meat, fermented and cooked sausages, raw and pasteurized fluid milk, cheeses, ice cream, vegetables, raw and cooked poultry, and raw and smoked seafood (Ryser and Marth 1991). Table 3 shows the incidence *L. monocytogenes* reported in some raw and processed foods in the U.S. Due to the ability of *L. monocytogenes* to survive, grow, and proliferate at wide range of environmental conditions such as low temperatures, high salt concentrations, and low pH, it poses a significant risk to food safety barriers and is a threat to human health (Gandhi and Chikindas 2007).

Table 3 Incidence of *Listeria monocytogenes* reported in some raw and processed foods (Bell and Kyriakides 2005)

Source of Samples	Incidence (%)	Reference
Raw cow's milk from farm bulk tanks	4.1	Rohrbach and others 1992
Vegetable salads	1.6	Lin and others 1996
Raw ground beef and steak	30	Amoril and Bhunia 1999
Raw poultry	35	
Raw ground pork	50.2	Kanuganti and others 2002
Ready-to-eat meat and poultry products:		
Sausage	1.8	Anonymous 2000
Cooked poultry	1.4	Bell and Kyriakides 2005
Sliced ham/pork	4.6	Bell and Kyriakides 2005
Cooked/roast/corned beef	2.7	Bell and Kyriakides 2005

Listeriosis and Pathogenesis

Listeria monocytogenes is the causative agent of serious foodbone illness called Listeriosis which accounts for about 1,600 cases of illness every year in the U.S. (CDC 2011). Listeriosis, an infection with high hospitalization rates of those who become ill, primarily affects the very young, elderly, and immunocompromised populations, including pregnant women, diabetics, transplant recipients, and cancer patients, and 260 of these 1,600 cases result in death (CDC 2011). Acquisition of listeriosis in humans is typically through ingestion of food contaminated with this organism. It also may be transmitted from mother to child through placenta or an infected birth canal, and cross infection in neonatal nurseries. At the beginning, Listeriosis often manifests influenza-like symptoms, and sometimes diarrhea, which may progress to include high fever, severe headache, and neck stiffness. However, the manifestations are host dependent. The infection occurs in several steps: a. entry of bacterium into the host; b. lysis of phagosomal vacuole; c. multiplication in cytosol; and d. direct cell-to-cell spread using actin-based motility (Jemmi and Stephan 2006).

Listeriosis can lead to septicemia, meningitis, and spontaneous abortion. The overall mortality rate for listeric meningitis is 70%; septicemia 50%; while in case of perinatal/neonatal, it may be as high as 80% or greater. When infected during pregnancy, however, the mother usually survives (USFDA 2007). The disease seems to resemble mononucleosis as cervical and generalized lymphadenopathy is associated with adults (Malinverni and others 1985).

The pathogenicity of *L. monocytogenes* is evident as invasive and non-invasive disease. The nature of the disease is influenced by virulence of the infecting organism, susceptibility of the host, and amount of the inoculum (Schuchat and others 1991). For transmission through food, the organism requires to first penetrate the host through the intestine (Berche and others 1988). Intracellular multiplication can occur in various types of cells. The intracellular functionality of *L. monocytogenes* that can occur in various types of cells is due to several virulence factors. The bacterial cell possesses internalin, a surface protein; which reacts with a receptor on macrophages and E-cadherin, the cells lining the intestine; to induce its own ingestion. Once the organism penetrates the lining of gastrointestinal tract, phagocytosis occurs (Gaillard and others 1987). The major virulence factor, Listeriolysin O along with phospholipases, enables listeriae to escape from the phagosome and avoid intercellular killing. Once free in the cytoplasm, the bacterium can divide and propel itself to the cell membrane by inducing polymerization in host cell and subsequently

invading adjacent macrophages. Thus, this kind of cell life cycle allows *L. monocytogenes* to move from cell to cell, evading exposure to antibodies, complement, or neutrophils. The organism is transported to different parts of the body through phagocytes. Host susceptibility to *L. monocytogenes* primarily depends on cell-mediated immunity, and listeriosis mostly occurs in individuals with impaired cell-mediated immunity, the immunocompromised. Disease manifestation of invasive listeriosis may include meningitis, septicemia, meningoencephalitis, abscesses of brain and spinal cord, osteomyelitis, septic arthritis, fever, ataxia, seizures, depressed consciousness, and altered mental status. Neonates are at particular risk of acquiring this kind of listeriosis from an infected mother. Neonatal listeriosis may show early or late onset. Early onset occurs as a result of intrauterine infection leading to clinical illness of the newborn or shortly after birth while late onset may take several days to weeks after birth. Although the infectious dose of listeriosis in humans is unknown, amount of inoculum that can cause the infection is most likely influenced by host susceptibility (Schuchat and others 1991). In susceptible persons, fewer than 1,000 total organisms may cause the disease (FDA 2012).

The second form, non-invasive listeriosis results in febrile gastroenteritis with fever, diarrhea, and vomiting. The median incubation period prior to onset of symptoms is short, typically 18 to 20 h (Dalton and others 1997). It occurs in otherwise healthy adults but severity of infection is unclear as it is influenced by host and bacterial characteristics (Schuchat and others 1991).

The virulence factors that are considered important in the pathogenicity of *L. monocytogenes* have been characterized as follows (Temmi and Stephan 2006). The internalines, which are encoded by different internaline genes (*inl*), take part in the invasion of epithelial cells and also seem to be responsible for the tissue tropism of the pathogen (Dramsi and others 1997; Schubert and others 2002). Listeriolysin O is encoded by gene *hylA*, and phosphatidylinositol-specific phospholipase C (PI-PLC) encoded by the genes *plcA*. These take part in the lysis of the phagosomes of the host cell, making the intracellular growth of the bacterium possible (Marquis and others 1995; Sibelius and others 1999). Act A-protein is involved in the motility (Domann and others 1992). Enzymes such as lecithinase, zinc metal protease, and serine protease are involved (Gaillot and others 2000; Raveneau and others 1992; Vazquez-Boland and others 1992). FbpA, a fibronectin-binding protein seems to be involved in intestinal and liver colonization processes (Dramsi and others 2004).

Outbreaks of Listeriosis

Listeriosis is a disease with a high case fatality rate at an average of 20 to 30%, in spite of the availability of adequate antimicrobial treatment (Swaminathan and Smidt 2007). Several widespread outbreaks of Listeriosis have occurred in the past few decades that have established *L. moncytogenes* as a pathogen of high priority for foodborne disease.

One of the earliest reported outbreak due to *Listeria monocytogenes* occurred in 1980-81 in Canada involving coleslaw that affected 41 people and caused 18 deaths (Bell and Kyriakides 2005). In 1983, an outbreak of Listeriosis in Massachusetts involving pasteurized milk killed 14 people. This raised questions about the efficacy of the pasteurization treatment in inactivating *L. monocytogenes.* Pasteurization studies indicate that the intercellular nature of *L. monocytogenes* in milk as a Polymorphonuclear Leucocyte (PMNL) offers thermal resistance to the organism (Doyle and others 1987). A survey indicated that this pathogen is present in about 4% of U.S. raw milks. It also grows well in liquid dairy products at 4 to 35ºC and the population numbers increase favorably in flavored milks such as chocolate milk as compared to unflavored milks (Pearson and Marth 1990).

Listeria monocytogenes contamination at levels 10^4-10^6 cfu/g was reported in surface ripened cheese, Vacherin Mont d'Or, in Switzerland, that caused an outbreak in 1983-87 affecting 122 persons and 34 deaths. Another outbreak in 1987-89 that lead to >350 cases and >90 deaths occurred in United Kingdom due to *L. monocytogenes* contamination in Belgian pâté (Bell and Kyriakides 2005). A large outbreak involving Mexican-style cheese, Queso Blanco, in 1985 in Southern California affected 142 people, caused 48 deaths, and 94 perinatal cases in Hispanic women (Swaminathan and Smidt 2007). In 1992, another outbreak of *L.* monocytogenes, involving pork tongues in France affected 279 people, caused 63 deaths, and 22 abortions (Bell and Kyriakides 2005).

From 1998-2008, on an average 2.4 *Listeria* outbreaks per year were reported to Centers for Disease Control and Prevention (CDC 2011). In 1998-99, a multistate outbreak involving contamination of frankfurters and deli meats with *L. monocytogenes* was reported to CDC which caused 101 cases and 21 deaths (CDC 1999). In the year 2000, another multistate outbreak linked to deli-turkey meat resulted in 29 cases, 4 deaths, and 3 miscarriages or still births (CDC 2000). The second largest outbreak of *L. monocytogenes* contamination in food was reported in

2002, where consumption of contaminated turkey deli-meat led to 54 illnesses, 8 deaths, and 3 fetal deaths in 9 states in the U.S. (CDC 2002; CDC 2011).

A large outbreak in Switzerland between 1983-1987 with soft cheese, the 2000 outbreak in North Carolina involving home-made Mexican-style cheese, the 2002 outbreak in Quebec, Canada with cheese made from raw milk, and the 2003 outbreak in Texas involving Mexican-style cheese highlighted the risk posed by use of raw milk in the production of soft, unripened cheeses. The outbreaks clearly indicate that raw milk and dairy products must not be consumed by susceptible populations (Swaminathan and Smidt, 2007). Also, in case of ready-to-eat food products, contamination of the product occurs post-heat-processing, or during packaging steps. The most recent and the largest by far multistate outbreak of Listeriosis reported has been linked to whole cantaloupes, where 146 persons were infected, and 30 deaths were reported (CDC, 2011).

Listeriosis is a disease that commonly affects persons with impaired immune systems. From a public health standpoint, identification of the vehicle of contamination and its removal from the food distribution channels is very important to reduce the number of cases and deaths resulting from the illness, and also establish measures to prevent cases in the future (Swaminathan and Smidt 2007).

Listeria monocytogenes and Ready-To-Eat (RTE) Foods

Listeria monocytogenes presents a significant concern as a number of widespread outbreaks involving processed meats have been reported in the past decade (CDC 1999; Olsen and others 2005; Gottlieb and others 2006). By definition, RTE meat and poultry products are the products that are in a form that is edible without additional preparation to achieve food safety and may receive additional preparation for palatability or aesthetic, gastronomic or culinary purposes (9 CFR Part 430). *Listeria monocytogenes* relative resistance to acid and high salt concentrations, ability to grow at low temperatures (down to freezing point) indicates its ability to grow in refrigerated foods, making it different than most foodborne bacterial pathogens (Swaminathan and Smidt 2007). Although *Listeria monocytogenes* can be easily inactivated with heat treatment, contamination of ready-to-eat (RTE) cooked foods occurs during post-processing steps (Zhu and others 2005). The ubiquitous nature and common occurrence in processing environments makes post-cooking contamination during packaging a major concern. *Listeria*

monocytogenes contamination in cured and non-cured RTE cooked meats is a major safety issue that is of significant concern (Zhu and others 2005) as RTE cooked meats are consumed without further heating and have a long shelf life. *Listeria monocytogenes* is also known for its ability to grow and proliferate at refrigerated temperatures for extended periods of time even in the presence of curing salts (Lou and Yousef 1999). Furthermore, multiple resistance strains of *Listeria* spp. are emerging due to acquisition of a replicon from staphylococci (Lemaitre and others 1998).

Listeria monocytogenes in Food Processing Environments

It is well established that bacteria can enter foods via contact with contaminated surfaces (Edington and others 1995). Outbreak investigations provide evidence that indicates commercially processed food products do not become contaminated with *L. monocytogenes* and other *Listeria* spp. as a result of survival of the organism in the processing operation but because of its continued presence in post-processing environments. Moreover, its ubiquitous nature and association with raw materials in food processing plants allows constant reintroduction in the plant environments (Doyle 1988). Senczek and others (2000) used pulsed-field gel electrophoresis (PFGE) to type different strains *Listeria* spp. and showed the persistence of closely related *Listeria* strains in the environment of a meat processing plant for a 2 year period.

A number of factors contribute to the growth of microorganisms in food processing environments, including moisture, nutrients, pH, oxidation-reduction potential, temperature, presence or absence of inhibitors, microbial interactions, and time. Moisture plays an increasingly important role and considerably helps in survival of bacterial cells on different surfaces. Processing plant structures, including equipment, as well as maintenance, repair, and practices that entrap moisture often result in microbial niche development (Faust and Gabis 1988). A niche is a site in processing environment that is normally difficult to reach and clean by normal cleaning and sanitizing procedures and becomes a reservoir of the pathogen from where it is continuously introduced into the processing operation, onto food contact surfaces and the food itself (Tompkin 2002).

Biofilms

In an aqueous environment when bacteria start to attach to a surface, colonization occurs and they begin to excrete a slimy substance that helps them to attach to different kinds of surfaces in food production environments. This mucilaginous, slimy coating that entraps bacterial cells is called a biofilm (Zotolla 1994).

Due to the existence of favorable conditions such as flowing water, nutrients, attachment surfaces, raw materials, and presence of microorganisms itself; attachment of organisms and biofilm formation is commonly reported in food processing environments. *Listeria* spp. posses this unique capability of forming a biofilm and is known to attach and grow on different kinds of surfaces. The cleaning of surfaces in food processing facilities is rendered difficult due to the ability of *L. monocytogenes* to form biofilms (Zotolla 1994).

A biofilm consists of microorganisms colonizing on the surface and associated polymers. The matrix of biofilm consists of microbial cells that are associated with a surface and are enclosed in hydrated extracellular polymeric substances (EPS). The main components of EPS include polysaccharides, proteins, phospholipids, teichoic acids, and nucleic acids. The EPS matrix is the key component in development of the complex three dimensional structures of attached communities of microbes in biofilms that are formed by allowing the cells to stick together (Sauer and others 2007).

The mucilaginous matrix is known to protect the bacterial cells against environmental stress, offer resistance to cleaning and disinfection activities, and makes their eradication or removal difficult. Several studies have indicated that sessile microorganisms have an advantage over planktonic forms as they are more resistant to disinfectants and are difficult to remove from food contact surfaces by mechanical means (Gilbert and others 2003; Gandhi and Chikindas 2007). Studies have also shown that microorganisms in biofilms have increased resistance to heat (Dhir and Dodd 1995; Frank and Koffi 1990).

A number of studies have evaluated the attachment of cells and biofilm formation of *Listeria* spp. on stainless steel, rubber, plastic, ultra high molecular weight polyethylene, and glass (Chae and others 2006; Hood and Zotolla 1997; Mafu and others 1990; Sinde and Carballo 2000; Stepanovic and others 2004). The adhesion of bacterial cells depends on both the nature of inert surface and the properties of the bacterial surface (Carballo 1992; Chamberlain and Johal, 1988; Hood and Zotolla 1995; Teixeira and Oliveira 1999). Chae and Schraft (2000) showed that

all 13 strains of *L. monocytogenes* formed biofilms under static conditions at 37°C within first 24 h. Blackman and Frank (1996) found that *Listeria* grew on stainless steel, teflon, nylon, and polyester for 7 to 18 d, whereas its biofilm formation was supported at 21ºC but was reduced at 10°C.

Intervention of *Listeria monocytogenes* Contamination

The U.S. Food and Drug Administration (FDA) and USDA Food Safety and Inspection Service (FSIS) in consultation with CDC have quantified the relative risk (FDA 2003) associated with some RTE foods that have had history of contamination with *L. moncytogenes* or have been implicated in an outbreak or linked with sporadic cases of listeriosis (FDA 2008). Studies have shown that foods that have been implicated with listeriosis contained at least 1,000 CFU/g or ml of the pathogen (CDC 1999; ICMSF 1994; ICMSF 1996; Tompkin 2002). The risk assessment estimates that the foods that pose the highest risk are the ones that support the growth of *L. moncytogenes* while the foods that pose the lowest risk of being associated with listeriosis possess intrinsic and extrinsic factors that to prevent growth of *L. moncytogenes,* or are processed in a way that changes the normal characteristics of that food such as pH, water activity or post-processing treatments. It is well known that *L. monocytogenes* does not grow in foods with a pH of ≤4.4, water activity of ≤0.92, or in the food that is frozen (IFT 2001; Slutsker and Schuchat 1999; Barnes and others 1989; Shank and others 1986; Sorrells and others 1986).

Given the food safety threat of *L. monocytogenes* in association with RTE foods, the FDA and FSIS maintain a zero tolerance policy for *L. monocytogenes* in RTE foods (Klontz and others 2008; Brandt and others 2010). This means if ≥1 CFU of *L. monocytogenes* in a 25 g sample of food is detected, a recall is required for RTE foods. The Food Safety and Inspection Service (FSIS) published a Federal Register notice comprising guidelines for establishment producing processed products that are susceptible to *L. monocytogenes* contamination and sampling plans. The LM Final Rule lays down compliance guidelines to control *L. monocytogenes* in post-lethality exposed ready-to-eat meat and poultry products. This rule was published in 9 CFR part 430 (Code of Federal Regulations) on June 6, 2003 and became effective on October 6, 2003. The rule underlines compliance with one of the three alternatives in post-lethality exposed ready-to-eat products (e-CFR 2012, FSIS 2006, Barry 2007):

1. Alternative 1: Post-Lethality Treatment
    - An antimicrobial agent or process that reduces or eliminates *L. monocytogenes* and an antimicrobial agent or process to suppress or limit the growth of the pathogen.
    - It must be a critical control point (CCP) in the Hazard Analysis Critical Control Point (HACCP) plan.
    - Plant requires documentation that shows that the desired bactericidal effect has been achieved before the product goes for distribution.
    - Food contact surface testing is recommended but not required.

The post lethality treatments that may be used are steam pasteurization, hot water pasteurization, radiant heating, and high pressure processing.

2. Alternative 2: Post-Lethality Treatment or Antimicrobial Process
    - To control or restrict the growth of *L. monocytogenes.*
    - It could be a CCP, Sanitation Standard Operating Procedures (SSOP) or prerequisite program.
    - Documentation is required that water activity is met but does not need to be a CCP.
    - Food contact surface testing is required at least quarterly with a provision to hold the product if positive tests are seen.
3. Alternative 3: Control by Sanitation
    - Application of a post lethality treatment or antimicrobial process to control *L. monocytogenes* is not required in the post-lethality exposed product.
    - Rigid sanitation control measures, which may be incorporated in the production establishment's Hazard Analysis Critical Control Point (HACCP) plan, Sanitation Standard Operating Procedures (SSOP) or pre-requisite program.
    - Product contact surface testing is required and is established bases on product type and the size of the production plant.
    - Documentation outlining procedures for holding the product, if samples test positive, are needed.

Food Preservatives

Various salts and acids have been used for years as preservatives and control the growth of microorganisms in foods. Nisin is one of the commonly used bacteriocins, approved for its use as an antimicrobial in 1969 by Joint FAO/WHO Expert Committee on Food Additives, to control growth of several different pathogenic microorganisms including *L. monocytogenes.* Nisin is a polypeptide that is produced by certain strains of lactic acid bacteria, *Lactococcus lactis* subsp. *Lactis* shows antimicrobial effect against a number of Gram-positive bacteria (Broughton 2005), including sporeformers (Davies and others 1996). It is approved for use in meat and poultry products at 250 mg/kg in finished product, 6.30 mg/kg in finished product when casings are used, and 5.0 mg/kg in cooked meat and poultry products (USDA 2008).

Several studies have demonstrated the sensitivity of *L. monocytogenes* to nisin. Davies and others (1996) indicated nisin levels at 2.5 mg/l effectively controlled 10^2-10^3 CFU/g of inoculated *L. monocytogenes,* during storage period of 8 weeks or more in ricotta-type cheeses. Another study showed 4 log reductions of total aerobic counts and *L. monocytogenes* in ready-to-eat turkey and ham when treated with 0.2, 0.3, 0.4, 0.5% nisin solutions , vacuum packaged, and stored at 4ºC for 63 d (Ruiz and others 2008). Antilisterial effect of nisin, 0.7 to 2.2 log reductions, was observed in traditional Greek whey cheese (Samelis and others 2003).

Another bacteriocin, Pediocin PA-1 produced by some strains of pediococci generally of meat origin, class II, which has shown strong antilisterial activity (Bhunia and others 1988; Rodriguez and others 1997). Reuterin is another bacteriocin which has shown inhibitory effect on *L. monocytogenes* (Zhu and others 2005). Research showed that reuterin produced by *Lactobacillus reuteri* strain 12002 at a concentration of 250 activity units/g reduced *L. moncytogenes* by 3.0 logs in raw ground pork after 1 week of storage at 7°C (El-Ziney and others 1999). Another bacteriocin, purified sakacin P, produced by *Lactobacillus sakei* has showed listercidal effect in cold chicken cuts (Zhu and others 2005). Studies have also shown that bacteriocins produced by enterococci, enterocins, may be a promising alternative to traditional chemical preservatives for control of pathogens in meat products (Hugas and others 2003; Leroy and others 2003).

Salts of lactate and diacetate are also known for their inhibitory effect on pathogenic microorganisms in meat products. Several researchers have evaluated the antimicrobial effects of the combination of sodium lactate and sodium diacetate against *L. monocytogenes* and have

shown significant reductions over the storage period of different ready-to-eat meat products (Glass and others 2002; Mbandi and Shelef 2002; Samelis and others 2002; Stekelenburg 2003). Trisodium phosphate is another chemical antimicrobial that has shown inhibitory effect against *L. monocytogenes* in chicken meat after several days of refrigerated storage (Capita and others 2001). A short-chain peptide, chemically synthesized, containing six leucine and eight lysine residues; was found to be biocidal against several foodborne organisms, *Escherichia coli* O157:H7, *Listeria monocytogenes*, *Pseudomonas fluorescens*, and *Kluyveromyces marxianus*; suspended in phosphate buffer at concentrations of 5-50 µg/ml (Appendini and Hotchkiss 2000).

Organic acids have also been studied for their inhibitory effect against *L. monocytogenes* in ready-to-eat meat products. Based on equal molar concentration, organic acids showed following order of effectiveness against *L. monocytogenes*: citric > malic > lactic > acetic (Samelis and Sofos 2003; Campos and others 2011). Murphy and others (2006) evaluated combination of organic acids and steam pasteurization to eliminate *L. monocytogenes* in fully cooked frankfurters. Results showed that when frankfurters were dipped in solution containing 2% acetic acid, 1% lactic acid, 0.1% propionic acid, and 0.1% benzoic acid and then steam pasteurized, *L. monocytogenes* growth was prevented for 14 weeks at 7ºC.

Hurdle Technology

Hurdle technology is based on the combined use of preservative factors to achieve microbial reductions in food to improve safety, shelf life and stability of foods (Leistner 1978). Several hurdles are applied for RTE meat products that include thermal processing, vacuum packaging, refrigerated storage, and use of nitrite and bacteriocins (Zhu and others 2005). However, these hurdles are sometimes insufficient to control *L. monocytogenes* due to its ubiquitous nature (Beresford and others 2001), ability to grow at refrigerated temperatures, and under reduced oxygen atmospheres, and resistance to salt and nitrites (Lou and Yousef 1999). Additional hurdles such as high pressure processing, irradiation, and steam pasteurization are needed to overcome post-processing contamination that can occur. Table 4 shows some uses of antimicrobials and hurdle technologies to control *L. monocytogenes* in food (Campos and others 2011).

Table 4 Uses of antimicrobials and hurdle technologies to control *Listeria monocytogenes* in foods

Antimicrobial	Additional Hurdle	Food Application	Reference
Enterocins A and B	High Hydrostatic Pressure	Cooked ham, Low acid dry fermented sausages	Marcos and others 2008 Jofre and others 2009
Nisin	EDTA[a] and lysozyme	Ham and bologna	Gill and Holley 2000
Nisin	Heat (60-65˚C)	Cold packed lobster meat Milk	Budu-Amoako and others 1999 Maisenier-Patin and others 1995
Nisin	Low dose irradiation (2.3 kGy)	Frankfurters	Chen and others 2004
Sodium Lactate and refrigeration	Nisin	Cold-smoked rainbow trout	Nykanen and others 2000
Sorbate and refrigeration	Nisin	Ricotta-type cheeses	Davies and others 1996
Organic acid solutions	Steam surface pasteurization	Fully cooked frankfurters	Murphy and others 2006

[a]EDTA- Ethylenediamine tetracetic acid

High Pressure Processing

High pressure processing has emerged as a powerful tool to control pathogenic microorganisms and has also shown effectiveness against spores and viruses (Zhu and others 2005). It is a novel non-thermal method of food processing in which food is subjected to different pressure levels. Since there is little or no heat treatment, changes in color, texture or nutritional value are minimal (Hugas and others 2002; Ross and others 2004). A research study focusing on the effect of mild heat treatment followed by high pressure processing (550 MPa for 5 min at 25ºC) showed inhibition of *L. monocytogenes* as a result of the treatment (Koseki and others 2008). Nine strains of *L. monocytogenes* were tested and significant variability in response to the high pressure treatment was seen (Tay and others 2003). Simpson and Gilmour (1997) found a wide range of sensitivities between three different strains of *L. monocytogenes.* Scott A strain was not eliminated at 450 MPa for 30 min at ambient temperature, another strain (poultry isolate) was eliminated at 400 MPa pressure treatment after 15 min while a third strain,

NTC11994 was completely eliminated after 450 MPa treatment for 30 min. Several factors such as level of pressure, pH, time and temperature of treatment, water activity, and food composition are known to affect the efficacy of the high pressure processing treatment (Hugas and others 2002). Lucore and others (2000) showed rapid inactivation of *L. monocytogenes* at 700 MPa in packaged frankfurters. Another study showed at 400 MPa, D-values of *Listeria innocua*, a surrogate microorganism for *L. monocytogenes*, was 7.35 min and 8.23 min at 2ºC and 20ºC, respectively (Ponce and others 1998). Styles and others (1991) illustrated that *L. monocytogenes* at 6 log CFU/ml level was inactivated within 20 min at 345 MPa pasteurization treatment in a buffer at 23ºC. Furthermore, Stewart and others (1997) found that pressure in combination with low pH between 3 and 4 in citrate buffer destroyed 7 log CFU/ml populations of *L. monocytogenes* in 30 min.

Irradiation

Irradiation is a physical treatment that can be used as an effective method to control *Listeria monocytogenes* in food when used in conjunction with chemical preservatives and antimicrobials. Ionizing radiation; that includes gamma rays, electron beams, and X-rays; is a process in which food products are exposed to radiant energy (Andrews and others 1998) that inactivates or controls growth of foodborne pathogens. Several studies have determined the effect of irradiation on *L. monocytogenes* in different food products. Irradiation of ground pork at 2.5 kGy reduced *L. moncytogenes* by approximately 6 log units (Mendonca and others 2004). Cooked pork chops were inoculated with *L. monocytogenes* and were irradiated at low (0.75 to 0.90 kGy, 2.5 M/min conveyor speed) and medium (1.8 to 2.0 kGy, 5.4 meter/min) doses, reduced the bacterial population by more than 2 log with low dose treatment (Fu and others 1995). Tarte and others (1996) studied sensitivity of five Listerial strains to electron beam irradiation in ground pork and established that irradiation of beef and pork at levels of 1.5 to 4.5 kGy were adequate to eliminate *L. monocytogenes* from pork. In another study, hams inoculated with *L. monocytogenes* were treated with 2 kGy electron beam irradiation that resulted in 2.42 log CFU/g reduction in bacterial populations (Song and others 2011).

The calculated D-values for *L. monocytogenes* was 1.38 kGy in soft whey cheese, Anthotyros, which was inoculated with Scott A strain of *L. monocytogenes* and gamma radiation treated (Tsiotsias and others 2002). The effect of irradiation against *L. monocytogenes* depends

on the substrate in which the organism grows; presence of other chemical preservatives, time and temperature of treatment. Sommers and others (2003) showed that addition of citric acid to frankfurters enhanced the lethality effect of ionization radiation. The presence of oxygen is also known to significantly enhance the lethal effect of the irradiation dose in vacuum packaged and modified atmosphere packaged food products (Thayer and Boyd 1999). Since the irradiation effect is determined by a combined mix of several key determinants (dosage, time, substrate) it is important to consider these factors and the type of product to achieve the desired effect of controlling or eliminating pathogens (Zhu and others 2005).

In-Package Thermal Pasteurization

It is well established that contamination of ready-to-eat food products with *L. monocytogenes* occurs post processing. Although, the organism is easily inactivated by heat treatment, the ubiquitous nature and its continued presence in food processing environments makes it necessary to evaluate in-package thermal pasteurization processes. The effectiveness of this process in inactivating pathogenic microorganisms is dependent on the package size and roughness of the product surface (Muriana and others 2002; Murphy and others 2003). Several studies suggest when fully cooked meat products (weighing up to 9 kg) were pasteurized post-packaging by submersion in water (96ºC) for about 10 min, a 2 to 4 log destruction of *L. monocytogenes* was seen on the product surface (Houben and Eckenhausen 2006). Muriana and others (2002) evaluated the efficacy of post-package pasteurization of deli-style RTE against *L. monocytogenes*. RTE foods (turkey, ham and roast beef) vacuum sealed in shrink wrap packaging bags and processed by minimal heating for 2 min at 90.5 to 96.1ºC, resulted in 2 log reductions of *L. monocytogenes*. A 7 log reductions of *L. monocytogenes* in inoculated vacuum packaged fully cooked sliced chicken was seen as a result of post-cook hot water pasteurization and steam pasteurization (Murphy and others 2003). Muriana and others (2004) showed effect of pre-package and post-package pasteurization on post-process elimination of *L. monocytogenes* on turkey deli products. Various deli-style RTE food products were evaluated for radiant heat pre-package pasteurization, submersed water post-package pasteurization, and combinations of the two techniques. A 2 to 2.8 log and 2.8 to 3.8 log reductions were seen prepackage pasteurization when samples were processed for 60 s and 75 s, respectively. Radiant heat package pasteurization resulted in 3.53 log reduction in 60 s and 4.76 log reductions with 75 s

treatment. Submerged water post-package samples showed 1.95 to 3.0 log reductions in bacterial populations when they were processed for up to 5 min.

Packaging

Growth inhibitor packaging is another intervention used to control *L. monocytogenes* in ready-to-eat foods. The product is thermally processed in a casing that is coated with an active antimicrobial agent on the internal surface. The antilisterial treatment is transferred to the surface of the product on processing and remains there even after the removal of the casing providing effective protection against *L. monocytogenes* during subsequent packaging process. Packaging has known also to perform a lethality hurdle to *L. monocytogenes* in several meat products. NOJAX AL^{TM} (Viskase Corp., Chicago, IL, USA) is one such casing package that is approved for use in U.S. by FDA and FSIS for its lethality impact, its key component being nisin. This technology is mostly applied to sausages and hotdogs that are enclosed in cellulose casings. Edible zein coatings are another effective packaging alternative for *L. monocytogenes* control in RTE foods (FSIS 2006). Janes and others (2002) showed 4.5 to 5 log CFU/g reductions in *L. moncytogenes* surface inoculated on cooked chicken samples, with zein film coatings with nisin, after 16 d at 4ºC.

Modified atmosphere packaging along with antimicrobial treatments has also been effective against *L. monocytogenes*. Modified atmosphere packaging (MAP) (100% CO_2, 80% CO_2 + 20% air)/nisin (10^3, 10^4 IU/ml) combination system used in a study decreased growth of *L. moncytogenes* and *Pseudomonas fragi*, and this inhibitory effect was higher at 4ºC than at 20ºC (Fang and others 1994).

Chitosans are another form of edible films extensively investigated for their antimicrobial properties. The positively charged chitosan molecules interact with the negatively charged bacterial cells and produce permeability changes, interact with microbial DNA with mRNA and protein synthesis, act as water and metal binding agent, and can also inhibit enzymes (Shahidi and others 1999; No and others 2007). A number of research studies have established the ability of chitosan edible films to inhibit growth of *L. monocytogenes* in different kinds of foods such as ham steaks (Ye and others 2008), cold smoked salmon (Ye and others 2008) and red meat (Beverly and others 2008).

Competitive Inhibition and Control in Processing Environments

In order to control microbial hazards linked with food products, some traditional, common approaches followed in food processing environments include frequent inspection and monitoring of facilities and operations, employee education, training programs, and extensive microbiological testing of raw ingredients and unfinished and finished products. An effective cleaning and sanitation program in place in food processing plants helps to reduce contamination post-processing in food products. Research studies have evaluated the use of sodium hypochlorite, quaternary ammonium compound, and peroxyacetic acid as sanitizers in meat processing plants to effectively eliminate *L. monocytogenes* (Romanova and others 2002; Stopforth and others 2002; Zhu and others 2005).

One of the novel methods to control *Listeria* spp. is by competitive inhibition. Research has been done to evaluate control of *Listeria* spp. by competitive exclusion bacteria in floor drains. A research study evaluated the anti- *L. monocytogenes* activity of two lactic acid competitive exclusion bacterium isolates, *Lactococcus lactis* subsp. *Lactis* C-1-92 and *Enteroccocus durans* 152, which were originally obtained from biofilms in the floor drains. Results revealed high bactericidal effect and control of *L. monocytogenes* in biofilms 4 to 37ºC (Zhao and others 2004). Another study conducted at a poultry processing plant showed similar results. The two lactic acid bacterium isolates, *Lactococcus lactis* subsp. *lactis* C-1-92 and *Enterococcus durans* 152 greatly reduced the number of *Listeria* spp. in floor drains at 3 to 26ºC (Zhao and others 2006). Metabolites of *Enterococcus* spp. that are used as starter cultures for meat fermentations, have been documented to have a bactericidal effect on *L. monocytogenes* (Siragusa 1992). Leriche and others (1999) showed antilisterial activity of *Lactococcus lactis* within first 6 hours of growth of *L. monocytogenes* in biofilms. *Enterococcus faecium* efficiently reduced *L. monocytogenes* biofilm formation, suggesting a potential control strategy for the pathogen (Minei and others 2008).

To control *L. monocytogenes* in food processing facilities, six strategies may be followed, (i) establishment and growth of listeriae in niches or other sites that can lead to the contamination of RTE foods should be prevented; (ii) sampling programs that can assess in a timely manner if the environment in which RTE foods are exposed is compromised, should be implemented; (iii) a rapid and effective response strategy when a positive product contact sample is detected; (iv) verification procedures in place to ensure adequate steps have been taken to correct the problem

detected; (v) a short-term assessment (e.g., involving the last four to eight samplings) to facilitate the detection of problems and trends; and (vi) a longer-term assessment (e.g., quarterly, annually) to assess widely distributed positive sites throughout the production lines and to analyze overall progress toward continuous improvement (Tompkin 2002).

Advanced Oxidation Technology

Advanced Oxidation Technology (AOT) or Advanced Oxidation Processes (AOPs) can be broadly defined as aqueous or gaseous phase oxidation methods that are based on the generation of highly reactive species, commonly referred to as Reactive Oxygen Species (ROS), and hydroxyl radicals in the mechanisms leading to the destruction of target contaminant (Comninellis and others 2008; Vogelpohl and Kim 2004). The AOPs are a set of specific processes that involve ozone, hydrogen peroxide, ultraviolet light or their combinations. These high energy oxidants are used to generate the highly reactive intermediates, the hydroxyl radicals (Munter 2001). The hydroxyl radical is a powerful, non selective chemical oxidant, illustrated in Table 5, that acts very rapidly with most organic compounds. Compared to conventional ozone or UV or hydrogen peroxide, AOPs can often achieve oxidative destruction of compounds faster. They have the potential to completely oxidize organic contaminants to carbon dioxide and water and mineral salts depending on the type of substrate (Vogelpohl and Kim 2004). The type of AOP depends on its application based in the specific target.

Table 5 Relative oxidation power of some oxidizing species (Glaze and others 1987; Oppenlander 2003)

Oxidizing Species	Relative Oxidation Power (Chlorine = 1)
Hydroxyl Radical	2.05
Atomic Oxygen	1.78
Ozone	1.52
Hydrogen Peroxide	1.31
Permanganate	1.24
Chlorine	1.00

Advanced Oxidation Technology offers several advantages such as rapid reaction rates, potential to reduce toxicity, complete oxidation of organic contaminants and effectiveness. However, it does involve understanding of complex chemical reactions to be tailored to a specific target and is cost and capital intensive.

Some commonly available advanced oxidation processes used for generating the hydroxyl radicals include: ozone + hydrogen peroxide, ozone/UV, ozonation at elevated pH (>8.5), hydrogen peroxide/UV, ozone/hydrogen peroxide/UV, photocatalytic oxidation (UV/TiO_2), and photo-fenton/fenton-like systems. The knowledge about the exact mechanism of action of the AOP is still incomplete and there is an ever growing need to evaluate their effectiveness, costs, and possible side effects.

Ultraviolet/Ozone Advanced Oxidation Process

The UV/Ozone process is a kind of advanced oxidation process that utilizes the combined effect of UV and ozone (O_3) to oxidize the substrate. The ozone readily absorbs the UV radiation at 254 nm wavelength in the presence of water to produce hydrogen peroxide as an intermediate, which then decomposes to produce hydroxyl radical (Munter 2001). The oxidation of organic substrates occurs due to the reaction with hydroxyl radicals, molecular ozone, and direct photolysis. The synergistic oxidation effect allows enhanced microbial reductions or destruction of dissolved organic compounds (Langlais and others 1991). Ozonation followed by the use of UV has been used for treatment of drinking water and wastewater applications to improve the efficiency of inactivation of microbiological contamination (White 1992; Amirsardi and others 2001; Oh and others 2003). A study performed in a commercial poultry processing plant to evaluate the use of UV-enhanced ozonation to control pathogenic microorganisms in chiller water indicated bactericidal effects with optimum treatments providing greater than 99.9% control of pathogenic microorganisms. The study also documented additional synergistic reduction effect $\geq$ 0.8 log CFU/ml of aerobic plate counts as compared to the cumulative effect of ozone and UV acting in series (Diaz and others 2001). Another study evaluating the kinetics of *Escherichia coli* inactivation using combined ozone and UV radiation established that synergism improved effectiveness and was attributed to the generation of hydroxyl radicals (Magbanua and others 2006). Similarly, Sharrer and Summerfelt (2007) showed that combining ozone and UV dosages, total heterotrophic bacterial (1.6 to 2.7 log CFU/ml) and total coliform

(2.5-4.3 CFU/ml) bacterial reductions were higher as compared to when the two treatments were individually applied in freshwater recirculating system.

Mechanism of Microbicidal Action of Ozone

Ozone is one of the most potent, broad spectrum antimicrobial agent that is known to be effective against bacteria, fungi, viruses, protozoa, bacterial and fungal spores at relatively low concentrations. Bacterial inactivation by ozone is a complex process that involves attack of ozone on numerous cell components such as proteins, unsaturated lipids, enzymes, cell membranes, peptidoglycans in cells walls, enzymes and nucleic acids in the cytoplasm, proteins in spore coats and capsids (Khadre and others 2001). Several researchers have studied the microbicidal effect of ozone, some concluded that ozone was the main inactivator of microorganisms while number of others emphasized on the reactive oxygen species produced as a result of ozone decomposition such as $^{\cdot}OH$, O_2^- , and HO_3^- that bring about the microbicidal action (Chang 1971; Harekh and Butler 1985; Glaze and Kang 1989; Bablon and others 1991; Hunt and Marinas 1997). Ozone may bring about oxidation of various components of the cell envelope including polyunsaturated acids, membrane-bound enzymes, glycoproteins, and glycolipids that can lead to leakage of cell content and eventually cell lysis or death (Scott and Lesher 1963, Murray and others 1965).

Disruption of normal cellular activity including cell permeability occurs when the double bonds of unsaturated lipids and the sulfhydryl groups of the enzymes are oxidized by ozone and rapid cell death follows (Khadre and others 2001). Ozone has also shown effect on bacterial sporecoats. Foegeding (1985) found that *Bacillus cereus* spores without coat proteins were inactivated rapidly as compared to intact spores. Although, it is known that spore coats are the main protection barriers for the cells against ozone but Khadre and Yousef (2001) showed heavy disruption of spore coats of *Bacillus subtilis* when treated with aqueous ozone. Several researchers have studied the mechanism of action of ozone on the enzymes on microbial cells. Sykes (1965) concluded that ozone acts as a general protoplasmic oxidant while Ingram and Haines (1949) in their studies found that ozone kills by interfering with the respiratory system of the cell. Chang (1971) established that the inactivation of enzymes by ozone as a result of oxidation of sulfhydryl group in cysteine residues. Studies have also found that ozone brought about *in vitro* modification of nucleic acids, opened the circular plasmid DNA and reduced its

transforming ability, produced double and single strand breaks in the plasmid DNA, and decreased transcription activity (Scott 1975; Ishizaki and others 1981; Hamelin 1985; Mura and Chung 1990; Khadre and others 2001).

Table 6 Inactivation of bacteria by ozone (Jin-Gab and others 1999)

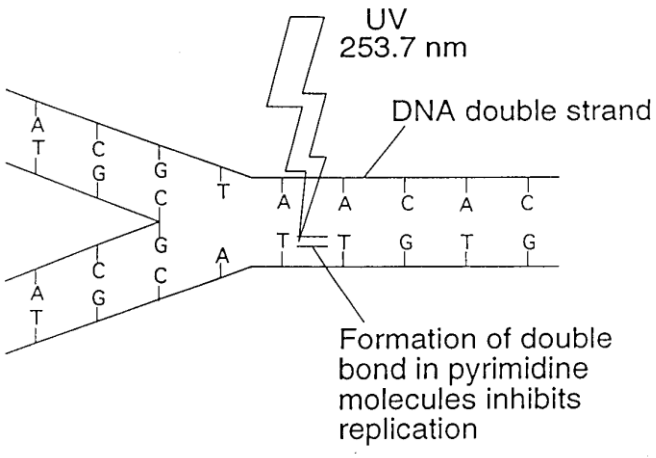
Bacterium	Inactivation (Log_{10})	Treatment time (min)	Concentration (mg/l)	pH	Temp (^oC)	Medium	Reactor type
Bacillus cereus	>2.0	5	0.12		28	O_3 demand-free water	
B. cereus (spores)	>2.0	5	2.29		28	O_3 demand-free water	
Escherichia coli	4.0	1.67	0.23-0.26	7	24	O_3 demand-free water	Continuous flow
Escherichia coli	3.0	19	Initial 2.2, residual 0.06	7.5	16	Raw wastewater	Continuous flow
Escherichia coli	2.0	0.1	0.53	6.8	1	Phosphate buffer	Batch
Legionella pnemophila	>4.5	20	0.32	7	24	Distilled water	Batch
Mycobacterium fortuitum	1.0	1.67	0.23-0.26	7	24	O_3 demand-free water	Continuous flow
Pseudomonas fluorescens	>2.0	0.25					
Salmonella Enteriditis	1.0	0.25	8% (wt/wt)		25	Broiler carcass	Ozone gas
Salmonella Typhimurium	4.3	1.67	0.23-0.26	7	24	O_3 demand-free water	Continuous flow
Staphylococcus aureus	>2.0	0.25		7	25	Phosphate buffer	Batch (bubbling)

Mechanism of Microbicidal Action of Ultraviolet

The efficacy of UV radiation against vegetative and spore forms of bacteria, viruses and other pathogenic microorganisms is well established. The photoinactivation process of UV light is a physical method in which energy is the germicidal medium and it does not produce any undesirable by-products (Chang and others 1985) that may affect the overall quality of the final product after treatment (Guerrero-Beltran and Barbosa-Canovas 2004). Depending on whether it eliminates, reduces or suppresses growth of *L. monocytogenes*, ultraviolet treatment can be used either as a post-lethality treatment or antimicrobial agent or process (FSIS 2006). Inactivation of microorganisms due to irreparable damage to their nucleic acids is brought about by electromagnetic radiation in the wavelength ranging from 240-280 nm, the most potent wavelength for damaging DNA being 254nm (Wolfe 1990). The germicidal effect of UV is due to the photochemical damage caused to RNA and DNA within the microorganisms. The nucleic acids in microorganisms are the important absorbers of light energy in the wavelength range 240-

280 nm (Jagger 1967). Damage to DNA and RNA, the genetic material of the microorganisms, resulting in dimerization of pyrimidine molecules (cystosine, thymine, and uracil), can effectively kill the organisms. Once the DNA helical structure is distorted due to UV radiation, pyrimidine molecules bond together, which makes the replication of nucleic acids difficult (Snider and others 1991). Figure 1 shows the germicidal inactivation observed with UV radiation (EPA 1999).

Figure 1 Germicidal Inactivation by UV radiation (Tachobanglous 1997; EPA 1999)



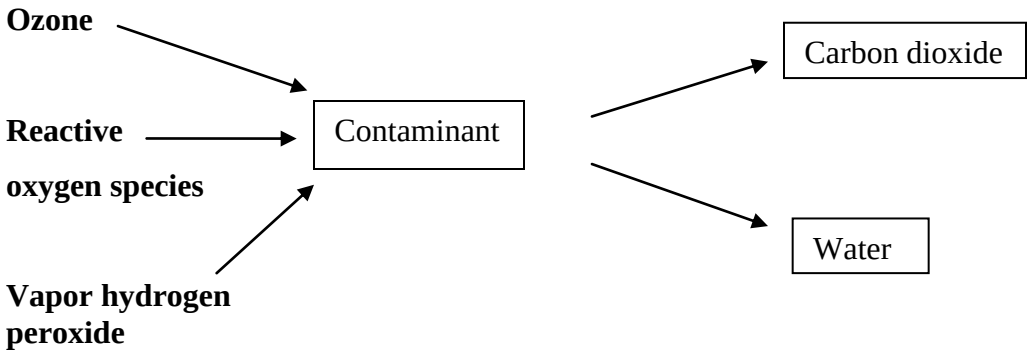
Photohydroionization™ (PHI™) Technology

Photohydroionization™ is an advanced oxidation technology developed and owned by RGF Environmental Group (West Palm Beach, FL, USA). In this process, a broad spectrum UV light is targeted on a quad metallic catalyst ultraviolet target in a low level ozone atmosphere and moist atmosphere. As a result, advanced oxidation process friendly oxidizers, reactive oxygen species (ROS), and vapor hydrogen peroxide, are created. The ROS are aggressive oxidizers but safe, and revert back to oxygen and hydrogen as part of the process. The ROS, low levels of ozone and vapor hydrogen peroxide are also called the plasma. By definition, plasma is a quasineutral gas formed by neutral and/or charged particles (ions and electrons) which are produced when ionization occurs as a result of an electron gaining sufficient energy to overcome the attractive force of atom nuclei (Scholtz 2007). The plasma may contain reactive forms of oxygen, under certain conditions, which have much higher reactivity than the stable oxygen

molecule. These reactive forms include atomic oxygen, singlet oxygen, ozone, superoxide anion, and hydroxyl radical. Non-thermal plasmas are increasing finding their application as an intervention to effectively inactivate microorganisms (Kelly-Wintenber and others 2000; Thiyagarajan 2005). The electric field and plasma products, such as ultraviolet (UV) radiation, charged particles, ozone, vaporized hydrogen peroxide (H_2O_2), and other reactive oxygen species (ROS) are generally considered the bactericidal agents (Deng and others 2006; Gaunt and others 2006; and Gallagher and others 2007). The low temperature or non-thermal plasmas as a decontamination agent has several advantages: it does not raise the temperature of the contaminant, purchase costs are low and operational costs are negligible, no chemical residue is on the surface, no closed chamber is required, surfaces and products are immediately ready to use, and the process does not generate any side effects.

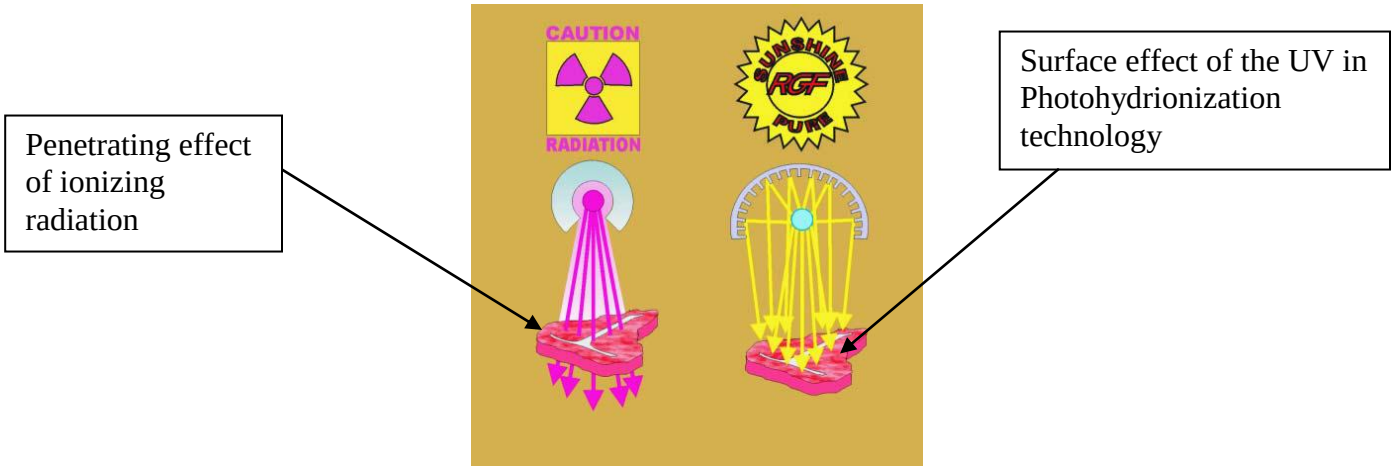
Photohydrionization is finding successful applications in air, water, food, laundry and grease industry (Fink 2004). This system uses UV-C ozone lamps to potentially reduce pathogens such as *L. monocytogenes* in ready-to-eat foods and on food contact surfaces. The lamps are not just the conventional 254 nm germicidal UV lamps but broad spectrum PHI^{TM} lamps that produce different wavelengths of UV light, which include 366, 310, 287 and 185 nm. These wavelengths utilize water vapor in the air and as a result of the advanced oxidation processes; low levels of ozone and vapor hydrogen peroxide are produced. Furthermore, the AOP result in generation of hydroxyl radicals, hydroperoxides and several other highly reactive species (Fink 2004). These reactive intermediates then attack the contaminants and water and carbon dioxide is produced as a result of the process (Figure 2).

Figure 2 Illustration of advanced oxidation process



Photohydroionization technology can be used as a surface treatment to control contamination of food product or product contact surface. It can be incorporated as a part of the overall hurdle effect to control foodborne pathogens in processing environments. It particularly seems to be an effective intervention strategy for control of *L. monocytogenes* in food processing environments as it is well established that contamination occurs during post processing steps. Figure 3 shows the surface effect of the UV radiation in this technology. One of the advantages the UV-C ozone lamps offer is that the ozone which is produced as a part of the ongoing process can travel through the air and bring about the desired germicidal effect as opposed to conventional 254 nm germicidal UV in which air that passes only a few inches close to the UV lamps is treated.

Figure 3 Surface effect of ionizing radiation in the Photohydroionization Technology (Fink 2004)



Furthermore, due to the high oxidation potential of ozone, bacterial cells do not develop resistance to ozone, which has been a problem posed for years by other chemical decontamination agents such as chlorine (Anonymous 2012). The advanced oxidation process which produces these plasmas, ozone, ROS, UV, and vapor hydrogen peroxide, does not leave any residue to the short half life (Table 7) of these agents of decontamination.

Table 7 Half life of the plasmas

Species	Half Life	Reference
Hydroxyl radical	10^{-9} seconds	Sies 1993
Atomic Oxygen (O)	Microseconds	Bentor 2012
Singlet oxygen (with displaced electron)	Microseconds	Barbalace 2012
Superoxide	Microseconds	Opperdoes 1997
Hydrogen Peroxide	Variable	Block 2001
Ozone	20 min max, carbon load dependent	Lenntech 2012

Role of Vapor Hydrogen Peroxide

Vapor hydrogen peroxide produced as a part of the advanced oxidation process plays a role in the antimicrobial efficacy of the process. It is a registered sterilant by Environmental Protection Agency (EPA) that causes inactivation of bacterial spores on environmental surfaces in an enclosed area; and is used to decontaminate or sterilize sealed enclosures such as isolators, workstations, and pass through rooms used in commercial, institutional, and industrial settings (EPA 2011). A number of research studies have shown the broad-spectrum efficacy of vapor hydrogen peroxide against bacteria, bacterial spores, viruses, and fungi. Extensive research has been done to evaluate the use of vapor hydrogen peroxide in hospital settings to prevent nosocomial infections caused by methicillin resistant *Staphylococcus aureus* and *Clostridium difficile* (Dryden and others 2008; French and others 2004; Boyce and others 2008). Vapor peroxide has also been used in the fumigation process, >6 log reduction was observed, due to its ability to safely and reproducibly decontaminate enclosed areas (Meszaros and others 2005).

The combined treatment of ozone and vapor hydrogen peroxide produced as a result of advanced oxidation process in the presence of UV light increases the efficacy of the process. Small amount of hydrogen peroxide is effective in initiating ozone decomposition by production of hydroperoxide ions as a result of the following process:

1. $H_2O_2 + H_2O \longrightarrow H_2O^- + H_3O^+$
(hydroperoxide anion)

2. $O_3 + OH^- \rightarrow HO_2^- + O_2$

3. $O_3 + HO_2\text{-} \rightarrow {}^{\bullet}OH + O_2^{\bullet -} + O_2$

Hydroperoxide ions are highly reactive with ozone. These are consumed by ozone quickly which shifts the equilibrium of the reaction 1 to the right (Taube and Bray 1940).

Recovery Media

For detection of specific microorganisms, various chemical compounds, selective agents, antimicrobials, and nutritive supplements are added to different media to make them selective. However, these chemical compounds or selective agents may only be able to target healthy cells cells for growth while inhibiting the injured ones. Modified oxford (MOX) medium is the most widely used selective media for *L. monocytogenes.* Curtis and others (1989) found that almost complete inhibition of the unwanted organisms was achieved using this media and *Listeria* spp. colonies could be detected possibly after 24 h with the aesculin-ferric ammonium citrate indicator system. The ingredients including Columbia agar base combines pantone, bitone, and tryptic digest of beef heart as a source of nitrogen, carbon, amino acids and vitamins, agar as solidifying agent, sodium chloride to maintain osmotic balance, ferric ammonium citrate for differentiation of *Listeria* spp. Selectivity is obtained due to the presence of lithium chloride which inhibits the growth of enterococci. The selectivity is increased by addition of moxlactam and colistin methane sulfonate or colistin sulfate as the oxford antimicrobic supplement.

When cells are exposed to environmental stresses, sublethal structural and physiological changes may occur which makes it harder to detect them with traditional culture methods (Besnard and others 2000). *Listeria monocytogenes* is susceptible to injury as a result of exposure to heat, freezing temperature, drying, irradiation, and chemicals. Sublethally injured cells have reduced ability to grow on selective media, although they can be recovered on a non selective media. Kang and Fung (1999) developed the thin agar layer (TAL) method to recover injured cells, and also improve selectivity. In this method, a non selective media, tryptic soy agar (TSA), is overlaid on top of the solidified selective agar MOX. The injured *L. monocytogenes* cells repair and start to grow on TSA during the first few hours of incubation. During the

resuscitation of injured cells, selective agents from MOX diffuse to the TSA top layer to inhibit other microorganisms and show typical black colonies of *L. monocytogenes* after 24 h of incubation at 37ºC.

To summarize, the ubiquitous nature of *Listeria monocytogenes* makes it an environmental contaminant. It has been commonly implicated in several outbreaks with ready-to-eat foods due to its ability to grow at refrigerated temperatures, tolerance to high acid and salt concentrations, and low infective dose in susceptible populations. A number of control interventions for *L. monocytogenes* have been continually developed, studied, evaluated, and validated for their effectiveness in ready-to-eat food products. The food safety threat of this bacterium and the risk posed to public health establishes the need to develop and evaluate effective control interventions.

Chapter 3 - Phase 1: Study 1- Validating the Efficacy of Mirenat- N (Lauric Arginate) Antimicrobial against *Listeria monocytogenes* on Stainless Steel Coupons

Abstract

Contamination of commercially processed food products with *Listeria monocytogenes* occurs due to contact with post processing environments where the bacterium survives due to persistence of favorable conditions for its growth. Lauric Arginate (LAE) is a novel antimicrobial that has been approved as Generally Recognized as Safe (GRAS) within the U.S. in certain food applications. The objective of this study was to evaluate the efficacy of Mirenat-N, LAE dissolved in foodgrade propylene glycol, against *L. monocytogenes* on food product contact surfaces. A three-strain cocktail of *L. monocytogenes* was used to inoculate 24 polished stainless steel coupons, divided into three treatment groups of 100 ppm solution LAE, 200ppm solution of LAE, and water (control); and two subtreatments of high (6 log CFU/ml) and low (3 log CFU/ml) inoculum levels. Populations of *L. monocytogenes* were calculated by dislodging attached cells by vortexing coupons for 1 min with 20 g of 3 mm solid glass beads in 10 ml of 0.1% peptone diluent and plating onto modified oxford medium (MOX), and thin agar layer MOX (TALMOX) agar. The 100 ppm treatment showed average reductions of 1.38 Log CFU/coupon after 5 min exposure and 2.57 log CFU/coupon after 15 min at low inoculum level, and 0.37 and 0.62 log CFU/coupon reductions were observed for 5 and 15 min exposure, respectively at high inoculum levels. The 200 ppm treatment at high inoculum level showed 1.23 and 1.88 log CFU/coupon reductions for 5 and 15 min, respectively while at low inoculum level 1.4 and 1.58 log CFU/coupon reductions for 5 and 15 min, respectively. This study shows that lauric arginate was effective in controlling low levels of contamination of *L. monocytogenes* on food contact surfaces and may be used as a part of integrated cleaning and sanitation program.

Introduction

Bacteria have been shown to enter foods as a result of contact with contaminated surface (Egintonand and others 1995). Pathogenic bacteria can survive the cleaning and disinfection activities on the equipment surfaces in food processing environments, thus increasing the risks associated with transmission of disease (Austin and Bergeron 1995; Dunsmore and others 1981). There is clear evidence that suggests contamination of commercially processed food products occurs with *L. monocytogenes* not because the organism is able to survive the processing operation, but due to contact with post processing environments that include food and non-food contact surfaces. Moisture plays an increasing important role in survival and attachment of bacterial cells to different surfaces. Processing plant structures may easily becomes niches for bacterial cell development (Faust and Gabis 1988).

A number of sanitizers and cleaners are continuously being evaluated for their use in food processing environments and *L. monocytogenes* has shown sensitivity to several sanitizing agents. Research has shown that chlorine-based, iodine-based, acid anionic, and quaternary ammonium-based sanitizers when used at concentrations, 100 ppm, 25-45 ppm, 200 ppm, and 100-200 ppm, respectively, were effective against *L. monocytogenes* (Lopes 1986; Orth and Mrozek 1989). 21 CFR 178.1010-Sanitizing solutions defines the use of these sanitizers on finished product contact surfaces at 200 ppm levels without the requirement of subsequent water rinse, except iodine-based sanitizers where the maximum level is 25 ppm.

Lauric arginate (LAE) is a novel antimicrobial that has been approved as Generally Recognized as Safe (GRAS) within U.S. in certain food applications (Bakal and Diaz 2005; Benford and others 2009). Lauric arginate acts on cytoplasmic membranes of microorganisms disrupting the normal metabolic processes without causing cell lysis (Rodriguez and others 2004). Several studies have indicated reduction in bacterial populations of *L. moncytogenes* on the surface upon contact with LAE (Porto-Fett 2009; Taormina and Dorsa 2009; Martin and others 2009). This study was designed to evaluate the efficacy of Mirenat-N, LAE dissolved in foodgrade propylene glycol, against *L. monocytogenes* on food product contact surfaces.

Materials and Methods

Bacterial cultures and inoculum preparation

The bacterial cultures used in this study were obtained from the American Type Culture Collection (Rockville, MD, USA) and included three strains of *Listeria monocytogenes* (ATCC 19115, 19113, 19112). The source of ATCC 19112 was also spinal fluid from a man in Scotland. ATCC 19115 (serotype 4b) and ATCC 19113 were human isolates. To prepare the inoculum, cultures were grown individually in 9 ml of tryptic soy broth (TSB, Difco; Franklin Lakes, NJ, USA) for 24 h at 35ºC. For inoculation purposes, each strain was combined into a single mixed culture suspension in phosphate buffer. The combined suspension was serially diluted to prepare two inoculum levels to inoculate the stainless steel surfaces. Targeted low and high levels of inoculum were prepared and plated onto modified oxford medium agar (MOX, Oxoid, UK), and thin agar layer MOX agar (TALMOX) incubated at 35ºC for 24 h to confirm target levels.

Preparation of Food Contact Surfaces

Twenty four polished stainless steel coupons (#316 finish, 6.4 x 1.9 x 0.7 cm) were initially cleaned using Sparkleen detergent (pH 9.5-10 in solution; Fisher Scientific, Hampton, New Hampshire, USA), and then autoclaved.

Inoculation of Samples

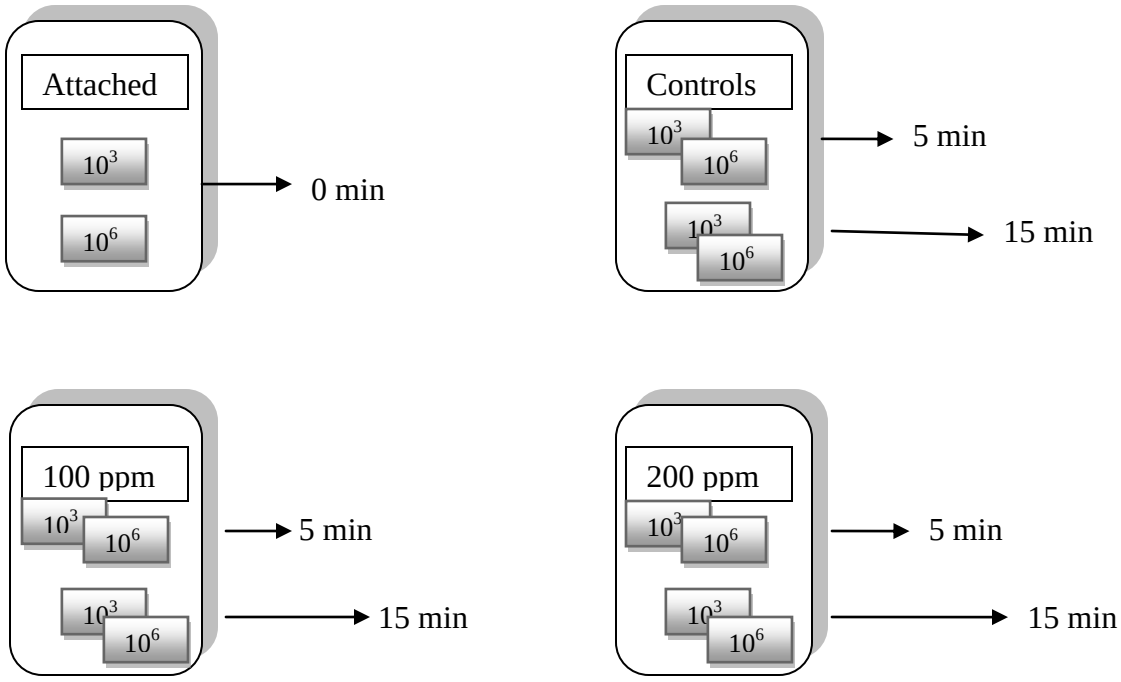
Two groups of 8 coupons were inoculated with two different levels of inoculum (6 coupons to a level of attachment of 10^3 and 6 coupons to a level of attachment of 10^6) by dipping the coupons in the three-strain cocktail of *L. monocytogenes* for 1 min and allowing to drip dry on racks for 30 min.

Treatment of Samples

After inoculation, one coupon from each group of 6 coupons was randomly assigned to each treatment as shown in Figure 4. Treatment application for both the hanging coupons (1 from each group) was performed simultaneously by spraying the antimicrobial solution using a spray bottle on both sides of the coupon. Solutions, 100 ppm aand 200 ppm, were prepared according to the labeled instructions of the manufacturer (Vedeqsa, Inc., Spain). The first paired coupon was removed from each treatment or control solution after 5 min of exposure to the

antimicrobial. The second paired coupon was removed after 15 min of exposure to the antimicrobial.

Figure 4 Schematic of treatment of stainless steel coupons with lauric arginate (LAE)



Recovery and Enumeration

Each coupon was individually placed in a 50 ml conical tube with 20g of 3 mm sterile solid glass beads and 15 ml sterile 0.1% phosphate buffer. Bacteria attached to the coupon were dislodged by vortexing for 1 min. Serial dilutions were prepared from the coupon wash suspension in 0.1% peptone diluent and spread plated (0.1 ml) onto MOX agar (Oxoid, UK), and thin agar layer MOX agar (TALMOX), these plates were incubated at 35ºC for 48 h. Recovered populations of *L. monocytogenes* were calculated and reported as log CFU/coupon. Three replications of the experimental set were performed and data was compiled in Microsoft excel 2007.

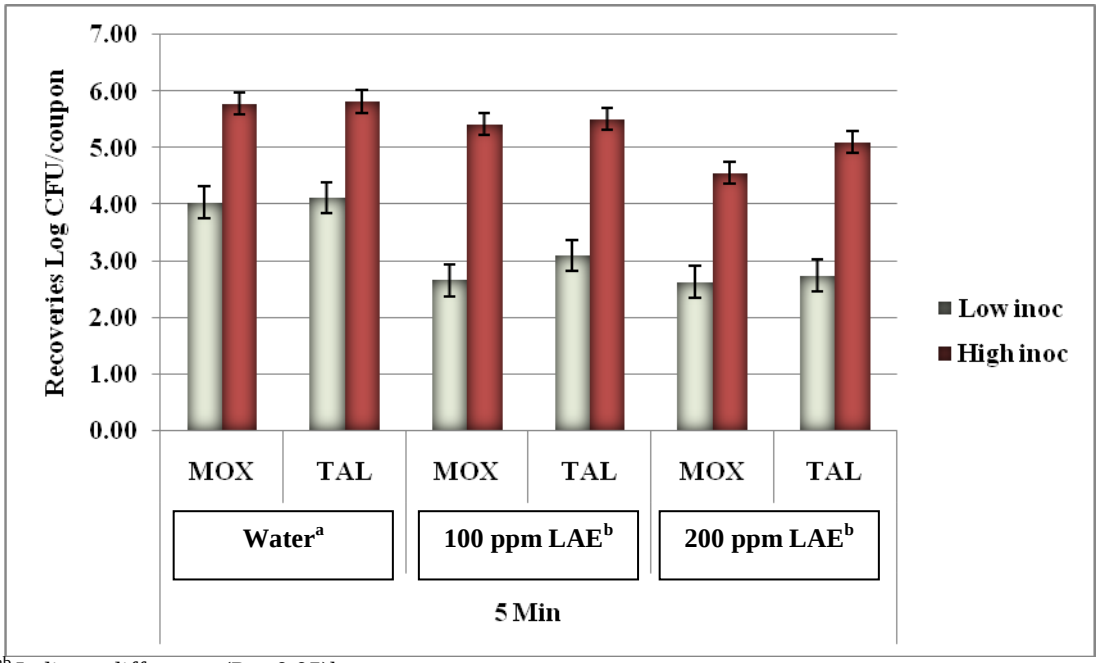
Statistical Analysis

Listeria monocytogenes population data obtained was analyzed using PROC MIXED in SAS version 9.0 (SAS Institute, Cary, NC, USA). Least square means ($P < 0.05$) were used to compare interactions between treatment, inoculum level, and time of treatment.

Results

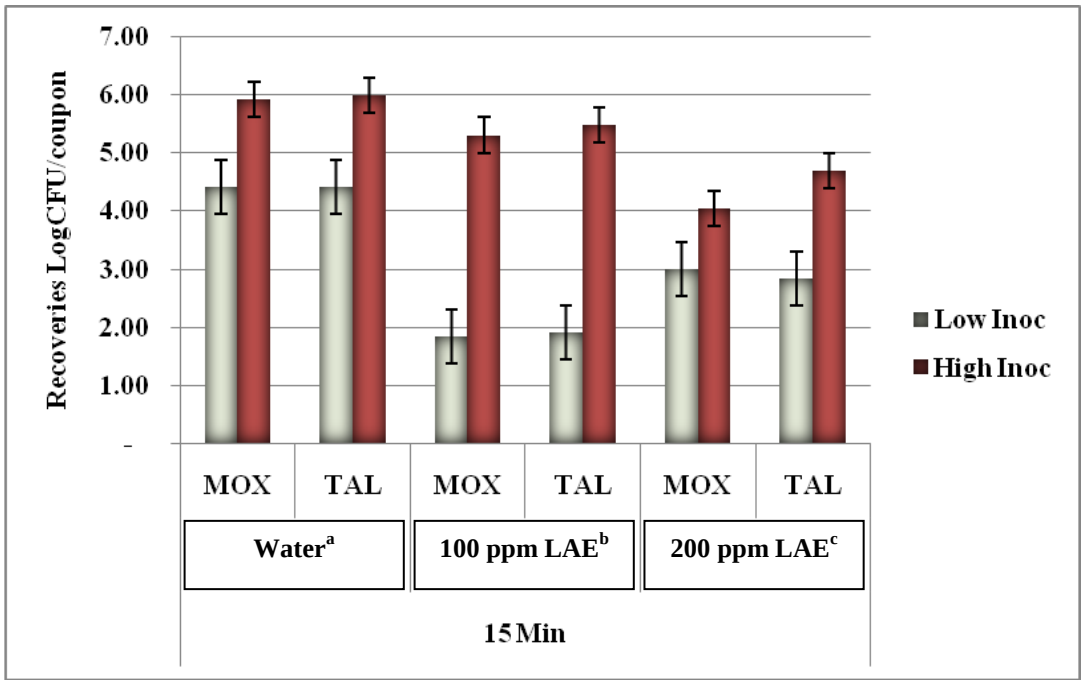
The initial levels of attachment of bacterial cells on the stainless steel coupons for the high and low levels of inoculums, as enumerated on MOX plates were approximately 6 log CFU/coupon and 4 log CFU/coupon, respectively. Figure 5 shows recovered *L. monocytogenes* populations at high and low inoculums levels for 5 min treatment with water (positive control), 100 ppm LAE, and 200 ppm LAE. Similarly, Figure 6 shows *L. monocytogenes* populations recovered from stainless steel coupons after 15 min treatment with water, 100 ppm LAE, and 200 ppm LAE, for high and low inoculums levels. The treatments, 100 ppm LAE and 200 ppm LAE, showed reduction in bacterial populations (Figure 7) computed with the positive control treatment, water.

Figure 5 Mean and standard error of *Listeria monocytogenes* populations recovered on modified oxford medium (MOX) and thin agar layer MOX (TALMOX) from stainless steel coupons at high and low inoculum levels for 5 min treatment with water (positive control), 100 ppm lauric arginate (LAE), and 200 ppm LAE solution



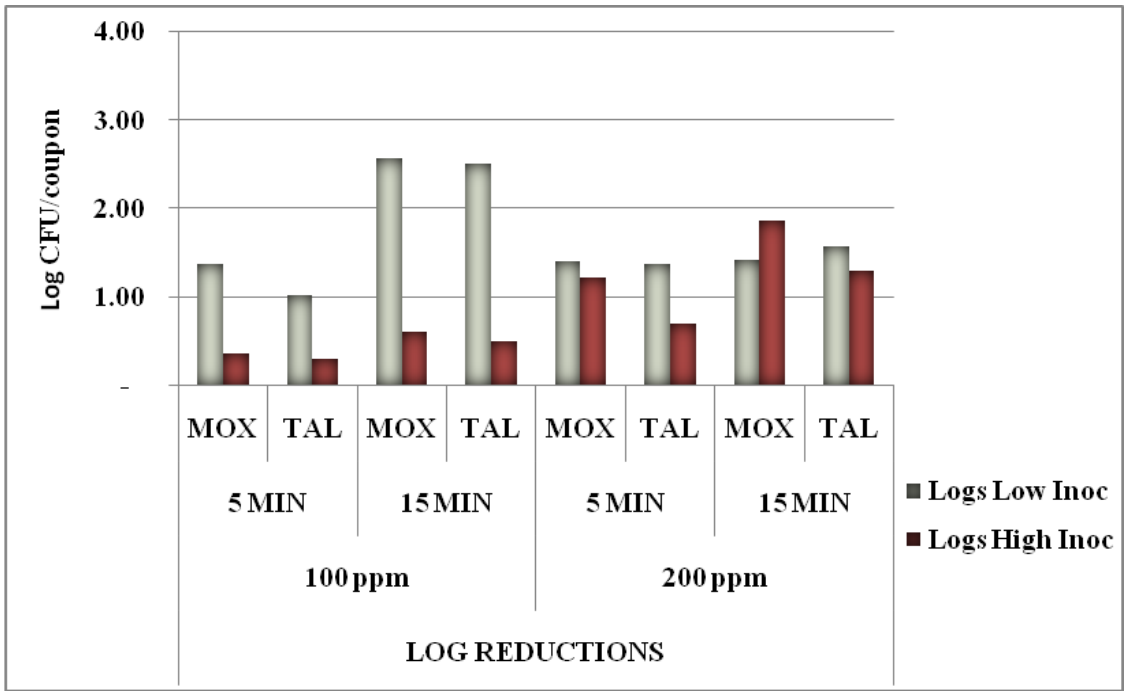
[ab] Indicate difference ($P < 0.05$)between treatments

Figure 6 Mean and standard error of *Listeria monocytogenes* populations recovered on modified oxford medium (MOX) and thin agar layer MOX (TALMOX) from stainless steel coupons at high and low inoculum levels for 15 min treatment with water (positive control), 100 ppm lauric arginate (LAE), and 200 ppm LAE solution



[abc] Indicate difference ($P < 0.05$)between treatments

Figure 7 Reductions in *Listeria monocytogenes* populations on modified oxford medium (MOX) and thin agar layer MOX (TALMOX) on stainless steel coupons due to treatment with 100 ppm and 200 ppm lauric arginate



Listeria monocytogenes populations differed significantly ($P < 0.05$) due to treatment x inoculum interaction. The media used for enumeration of bacterial populations had no effect on the recovery of *L. monocytogenes*. No difference ($P > 0.05$) was observed between bacterial growth on MOX and TALMOX media, which indicated that the reductions observed were due to a full lethality effect of LAE. The damage to the cell was complete and no injured cells, expected to be recovered on TALMOX, were left after the treatment. No differences ($P > 0.05$) were observed when comparing the two treatments (100 ppm LAE versus 200 ppm LAE) tested after 5 min exposure. For 15 min contact time, 100 ppm LAE and 200 ppm LAE differed from each other ($P < 0.05$) and the control.

The 100 ppm LAE treatment showed average reductions of 1.38 log CFU/coupon after 5 min exposure and 2.57 log CFU/coupon after 15 min. This treatment appeared to be more effective for low contamination levels (low inoculum) than high concentration levels, where only 0.37 and 0.62 log CFU/coupon reductions in bacterial populations were observed for 5 and 15 min exposure, respectively.

Furthermore, at high inoculum levels, the 200 ppm LAE treatment showed better lethality than the 100 ppm LAE treatment with 1.23 log CFU/coupon reductions after 5 min and 1.88 log CFU/coupon reductions in 15 min. This interaction was expected because as the concentration of the antimicrobial increases with the exposure time, lethality also increases.

Discussion

Under the LM Final rule, Alternative 3 is control by sanitation, in which rigid sanitation control measures are to be incorporated in the production establishment's Hazard Analysis Critical Control Point (HACCP) plan, Sanitation Standard Operating Procedures (SSOP) or prerequisite program. An effective cleaning and sanitation program is required to be in place. Cleaning is complete removal of food soil with the use of chemical compounds with appropriate detergent properties under recommended conditions while sanitizing is the act of maintaining clean environment by means of a physical or chemical agent. Sanitizer on food contact surface by definition is required to bring about a 5 log reduction after 30 s contact time (Parker 2007). Lauric arginate tested at 100 and 200 ppm concentration showed a maximum of 2.5 log CFU/coupon at 100 ppm concentration with exposure time of 15 min at low bacterial concentration levels of 3 log CFU/coupon. Therefore, it does not meet the requirements of being either a cleaner or sanitizer by itself.

The antimicrobial activity of LAE is attributed to its ability to alter cytoplasmic membranes of microorganisms (Rodriguez and others 2004). In addition, LAE has low oil-water equilibrium partition coefficient which means it tends to concentrate in the aqueous phase of the products where most of the bacterial action occurs (Bakal and Diaz 2005). Being a cationic surfactant, it may interact strongly with other anionic or hydrophobic groups on a contact surface, this defining its practical applications. Given its GRAS nature, LAE may be successfully used as a part of the integrated cleaning and sanitation program.

Conclusion

Lauric arginate cannot be used as a sanitizer by itself in a food sanitation program. This research shows the potential use of lauric arginate as an antimicrobial against *L. monocytogenes* on food contact surfaces as a part of the integrated cleaning and sanitation program in a food production establishment. Further research is needed to determine its efficacy against biofilms formed by *L. moncytogenes* in food processing environments.

Chapter 4 - Phase 1: Study 2 – Antimicrobial Effect and Applicability of Lauric Arginate (LAE) to Control *Listeria monocytogenes* in Shredded Mozzarella Cheese with a Blend of Anticaking Agents and Other Ingredients

Abstract

Listeria monocytogenes has been commonly identified as the cause of contamination of milk and milk products in several dairy related outbreaks. The ability of this bacterium to grow at refrigerated temperatures makes it a high risk factor in soft, white, fresh cheeses that are minimally processed and have short shelf-life. This study was performed with an objective to evaluate the antimicrobial activity and applicability of the novel antimicrobial, lauric arginate, in controlling *L. monocytogenes* in shredded mozzarella cheese with a blend of anticaking agents and other ingredients. A three-strain cocktail of *L. monocytogenes*, high (10^8 CFU/ml) and low (10^4 CFU/ml) level was used to inoculate 100 g bags of shredded mozzarella cheese, divided into 7 treatment groups of control, Free Flow 1031 (FF), FF + 200 ppm lauric arginate (LAE), FF + glucose oxidase (GOX), FF + GOX + dextrose, FF + LAE + GOX, and FF + LAE + GOX + dextrose; and 2 subtreatments of high (8 log CFU/g) and low (4 log CFU/g) inoculum levels. Sampling was conducted at 0, 1, 3, 5, and 8 d of the refrigerated storage period. A 10 g portion of cheese sample was taken from the 100 g treatment bag and suspended in 90 ml of peptone and then stomached for 1 min. Populations of *L. monocytogenes* were calculated by plating appropriate dilutions onto modified oxford medium (MOX), and thin agar layer MOX (TALMOX) agar. Results obtained and statistical analysis indicated that only FF + GOX + dextrose and FF + LAE + GOX + dextrose were effective in controlling *L. monocytogenes* populations in shredded mozzarella cheese. Reductions obtained by both treatments ranged from 2.5 (Day 1) to 4.0 (Day 8) log CFU/g, but there was no difference ($P > 0.05$) observed between them (as FF + GOX + dextrose alone showed similar antimicrobial effects as FF +LAE + GOX + dextrose). In summary, LAE 200 ppm provided no additional antimicrobial effects to that obtained with the application of GOX + dextose.

Introduction

A large number of Listeriosis outbreaks have been known to be linked with contamination of dairy products. Contamination from environmental sources post-processing has been identified as the leading cause (WHO 1988) and with the cheese production industry, it is most likely due to the open nature of the processing. Soft, white, fresh cheeses are minimally processed and have short shelf-life even at refrigerated temperatures. The ability of *L. monocytogenes* to grow at low temperatures makes it a high risk factor for contamination in these types of cheeses (Davies and others 1996).

Mozzarella cheese is a mild, soft white cheese made by a special pasta filata process (Altieri and others 2005). More than 3 billion pounds of mozzarella cheese is consumed and it is the second most popular cheese in the U. S. (USDA 2006). High moisture content makes the cheese very perishable in nature with an estimated shelf-life of approximately 30 days at 2ºC (USDA 1980). Mozzarella cheese receives heat treatment during curd stretching, however post-processing contamination with a number pathogens or spoilage organisms may occur (Spano and others 2003). Common sites for post-processing contamination of mozzarella cheese with *L. monocytogenes* include filling and packing lines, conveyors, racks for transporting products, handtools, gloves, freezers, and gaskets (Tompkin and others 1999).

There has always been a strong demand in the food industry for antimicrobials that can be successfully used in *L. monocytogenes* susceptible food products as preservatives or to control contamination (Soni and others 2010). Several research studies have evaluated intervention strategies for control of *L. monocytogenes* in soft white cheeses, such as use of bacteriocin, nisin in ricotta-type cheese, lauric arginate in queso fresco cheese, chitosan-lysozyme film and coatings in mozzarella cheese and use of pediocin producing lactic acid bacteria in cheese manufacturing process (Davies and others 1996; Soni and others 2010; Duan and others 2007; Rodriguez and others 2004). Lauric arginate (LAE, ethyl N-lauroyl- L- arginate hydrochloride) is an FDA approved GRAS food preservative that can be used at concentrations up to 200 ppm (FDA 2005). Studies have demonstrated listericidal effect of LAE in ready-to-eat ham and frankfurters during early storage period (Luchansky and others 2005; Martin and others 2009).

Presently there are no studies on use of lauric arginate as an antimicrobial in mozzarella cheese. The objective of this study was to evaluate the usefulness of LAE for the control of *L. monocytogenes* in shredded mozzarella cheese. Microbiological activity of the anticaking ingredients was studied separately and in combination with LAE applied in shredded mozzarella cheese for control of *L. monocytogenes*.

Materials and Methods

Bacterial Cultures and Inoculum Preparation

Bacterial cultures used in this study, were obtained from the American Type Culture Collection (Rockville, MD, USA), included three strains of *Listeria monocytogenes* (ATCC 19115, 19113, 19112). The source of ATCC 19112 was spinal fluid from a man in Scotland. ATCC 19115 (serotype 4b) and ATCC 19113 were human isolates. To prepare inoculum, cultures were grown individually in 9 ml of tryptic soy broth (TSB, Difco; Franklin Lakes, NJ, USA) for 24 h at 35ºC. For inoculation purposes, each strain was combined into a single mixed culture suspension. The combined suspension was serially diluted to prepare two inoculum levels to inoculate the shredded cheese. Targeted low (4 log CFU/ml) and high levels (8 log CFU/ml) of inoculum were prepared and plated onto modified oxford medium (MOX, Oxoid, UK), and thin agar layer MOX (TALMOX) agar incubated at 35ºC for 24 h to confirm target levels.

Experimental design

Shredded mozzarella cheese (Table 8) was treated with combinations (Table 9) of lauric arginate, glucose oxidase, dextrose, and anticaking agent (Free Flow 1031) at various levels (Table 10). The experimental design included controls (non-inoculated shredded cheese). Each treatment that was inoculated was subjected to two different inoculum levels as subtreatments: subtreatment 1: high level inoculum of *L. monocytogenes* suspension (10^8 CFU/ml) and subtreatment 2: low level inoculum of *L. monocytogenes* suspension (10^4 CFU/ml). Three replications of the experiment were performed.

Table 8 Proximate analysis (n=3) for mozzarella cheese

Sample	% Moisture	% Protein*	% Fat	% Sodium	pH
Mozzarella cheese	46.96±1.00	26.81±0.68	21.21±0.57	0.55±0.05	5.63±0.03

*Crude protein is calculated using a 6.25 conversion factor (%N x 6.25)

Table 9 List of ingredients

Ingredient	Description
Mozzarella Cheese	In blocks, commonly found in the global market with ingredients, milk, starter culture and rennet
Free Flow 1031*	Anticaking (Potato starch, Corn starch, Calcium sulfate)
LAE® Dehydrated*	ethyl-Nα-dodecanoyl-L-arginate·HCl, CAS number 60372-77-2. Purity 89% and particle size ≤ 300 μm
Glucose Oxidase (GOX)*	Enzyme, powdered form
Dextrose*	Monosaccharide, powdered form

*Ingredients obtained from Allied Blending, Inc. CA, USA

Table 10 Different treatment combinations applied to evaluate the applicability of lauric arginate in shredded mozzarella cheese to control *Listeria monocytogenes*

Treatment	Combination
Treatment 1	Non Inoculated – Shredded Cheese
Treatment 2	Inoculated - Shredded Cheese
Treatment 3	Inoculated - Shredded Cheese + Free Flow 1031
Treatment 4	Inoculated - Shredded Cheese + (Free Flow 1031 + *LAE® 200 ppm)
Treatment 5	Inoculated - Shredded Cheese + (Free Flow 1031 + *GOX)
Treatment 6	Inoculated - Shredded Cheese + (Free Flow 1031 + *GOX + Dextrose)
Treatment 7	Inoculated - Shredded Cheese + (Free Flow 1031 + *LAE® 200 ppm +GOX)
Treatment 8	Inoculated - Shredded Cheese + (Free Flow 1031 + *LAE® 200 ppm + *GOX + Dextrose)

*LAE® - Lauric Arginate, GOX – Glucose Oxidase

Table 11 Blends of anticaking with antimicrobials and other ingredients

TREATMENT	ANTIMICROBIAL BLEND							
	Free Flow 1031		LAE 200ppm		GOX		Dextrose	
	Grams	%	Grams	%	Grams	%	Grams	%
Treatment 1	0		0	0.00%	0	0	0	0.00%
Treatment 2	0		0	0.00%	0	0	0	0.00%
Treatment 3	4.00	100.00%	-	0.00%	-	0.00%	-	0.00%
Treatment 4	3.98	99.50%	0.02	0.50%	-	0.00%	-	0.00%
Treatment 5	3.99	99.75%	-	0.00%	0.01	0.25%	-	0.00%
Treatment 6	3.19	79.75%	-	0.00%	0.01	0.25%	0.80	20.00%
Treatment 7	3.97	99.25%	0.02	0.50%	0.01	0.25%	-	0.00%
Treatment 8	3.17	79.25%	0.02	0.50%	0.01	0.25%	0.80	20.00%

Sample Preparation

Blocks of low moisture part skim mozzarella cheese (900 g), obtained from local grocery store was shredded using Black and Decker food blender (Model no. FP1600B; New Britain, CT, USA). A 100 g portion of shredded cheese was then weighed into sterile polyethylene bags for treatment application. All bags were heat sealed and stored at 4ºC until inoculation.

Inoculation of Samples

For targeted high inoculum, 10 ml of a 24 h grown culture suspension, combined into a cocktail of three strains of *L. monocytogenes* was used. For targeted low inoculum, three serial dilutions of the original cocktail were made and 10 ml of the third serial dilution was used to inoculate the 100 g bags of shredded cheese.

Treatment inoculations were then performed. Each treatment (Treatments 2 to 8) consisted of 100 g of shredded cheese in individual heat sealed bags (5 for each subtreatment). Samples for each treatment were inoculated with a low level and the other seven paired samples were inoculated with a high level of inoculum using corresponding combined suspensions of *L. monocytogenes* respectively, as subtreatments 1 and 2. Five additional non-inoculated samples were stored under same conditions as a control (Treatment 1).

Treatment was applied by adding 4 g of the antimicrobial blend into each designated bag of a treatment. After inoculation and treatment, bags were closed and shaken by hand for 2 min to allow even distribution of the antimicrobial blend and culture suspension in the shredded cheese.

Enumeration

Each treatment was enumerated for *L. monocytogenes* populations just after inoculation (0 day), and after 1, 3, 5, and 8 d of refrigerated storage conditions at 4ºC. An aliquot of 10 grams of sample was aseptically obtained and placed in a stomacher bag. To this 90 ml of sterile 0.1% peptone diluent was added and then stomached (Seward Stomacher 400, UK) for 1 min. After homogenizing, samples were then serially diluted and spread plated (0.1 ml) onto modified oxford medium (MOX, Oxoid, UK), and thin agar layer MOX agar (TALMOX), these plates were incubated at 35ºC for 48 h. Recovered populations of *L. monocytogenes* were calculated and reported as log CFU/g.

Statistical Analysis

Listeria monocytogenes population data obtained was analyzed using PROC MIXED in SAS version 9.0 (SAS Institute, Cary, NC, USA). Fixed effects for statistical analysis were treatment, day, inoculum, media, treatment by day, treatment by inoculum, day by inoculum, treatment by media, inoculum by media, treatment by day by inoculum, treatment by day by media, treatment by inoculums by media, and day by inoculum by media, and treat by day by inoculums by media. The random effect was replication. Least square means ($P < 0.05$) were used to compare the interactions.

Results

The average initial attached populations of *L. monocytogenes* recovered on MOX and TALMOX agar for high and low inoculum were 7.5 log CFU/g and 5.2 log CFU/g, respectively. Treatment 1, the non-inoculated shredded mozzarella cheese, showed no growth of *L. monocytogenes* throughout the 8 d storage period. Figure 8 shows mean recoveries of *L. monocytogenes* on MOX in shredded mozzarella cheese during the 8 days of refrigerated storage with different antimicrobial blends at high inoculum levels. Similarly, Figure 9 shows average recoveries of *L. monocytogenes* on MOX in shredded mozzarella cheese during the eight days of refrigerated storage with different antimicrobial blends at low inoculum levels. Figures 10 and 11 shows average recoveries of *L. moncytogenes* on the resuscitating media, TALMOX, in shredded mozzarella cheese during 8 days of storage under refrigerated conditions. There was no significant reduction ($P > 0.05$) in bacterial populations immediately after the treatments were applied to the shredded mozzarella cheese samples at day 0. The antimicrobial effect, i.e.

reduction in the population of *L. monocytogenes*, in the cheese due to various treatments applied was seen after 1 day of refrigerated storage. However, significant reductions over time, ranging from 2.5 (day 1) to 4.0 (day 8) log CFU/g, were only observed in treatments 6 and 8 while treatments 3, 4, 5, and 7 showed no additional reductions over time.

Figure 8 Mean and standard error (n=3) of *Listeria monocytogenes* populations recovered on modified oxford medium (MOX) in shredded mozzarella cheese at day 0, 1, 3, 5, and 8 of refrigerated storage with different antimicrobial blends consisting of free flow 1031 (FF), lauric arginate (LAE), glucose oxidase (GOX), and dextrose (Dex) at high inoculum levels

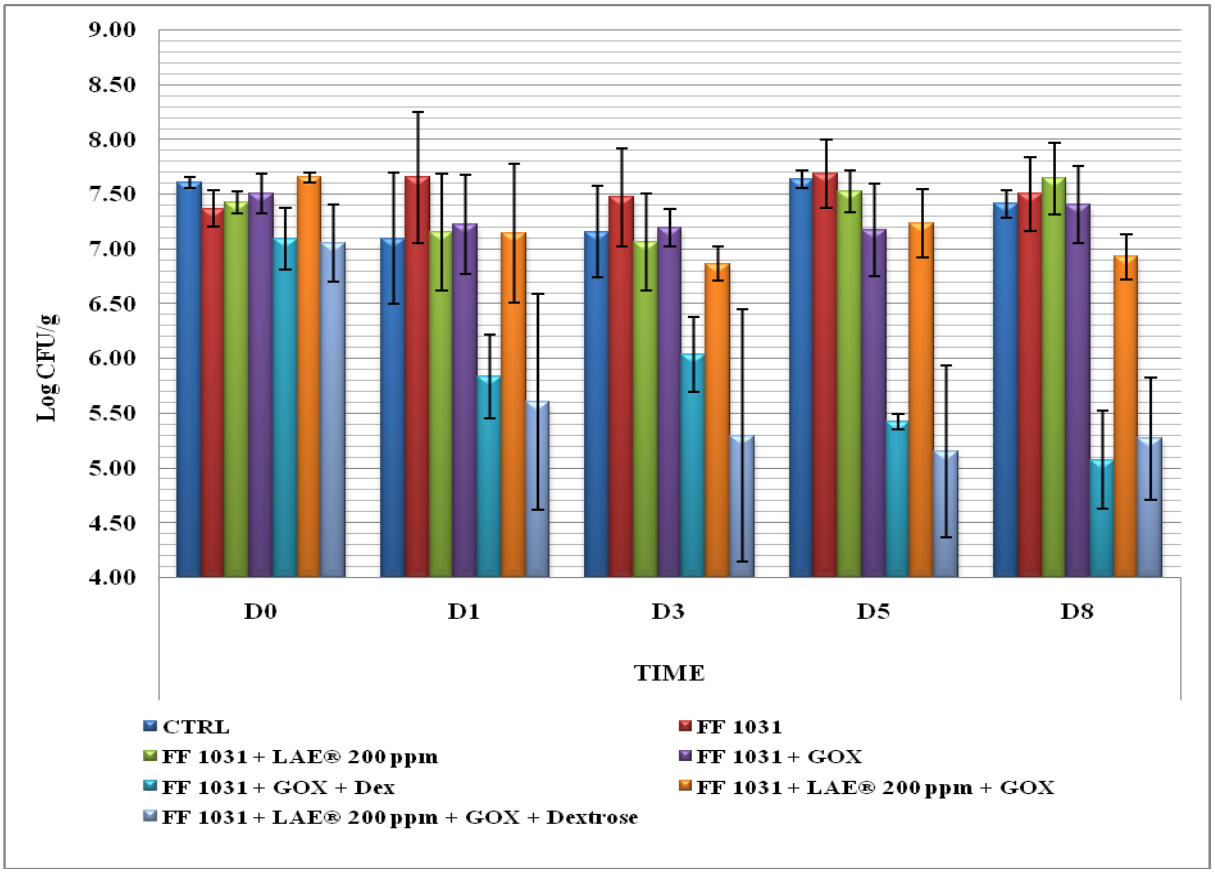


Figure 9 Mean and standard error (n=3) of *Listeria monocytogenes* populations recovered on modified oxford medium (MOX) in shredded mozzarella cheese at day 0, 1, 3, 5, and 8 of refrigerated storage with different antimicrobial blends consisting of free flow 1031 (FF), lauric arginate (LAE), glucose oxidase (GOX), and dextrose (Dex) at low inoculum levels

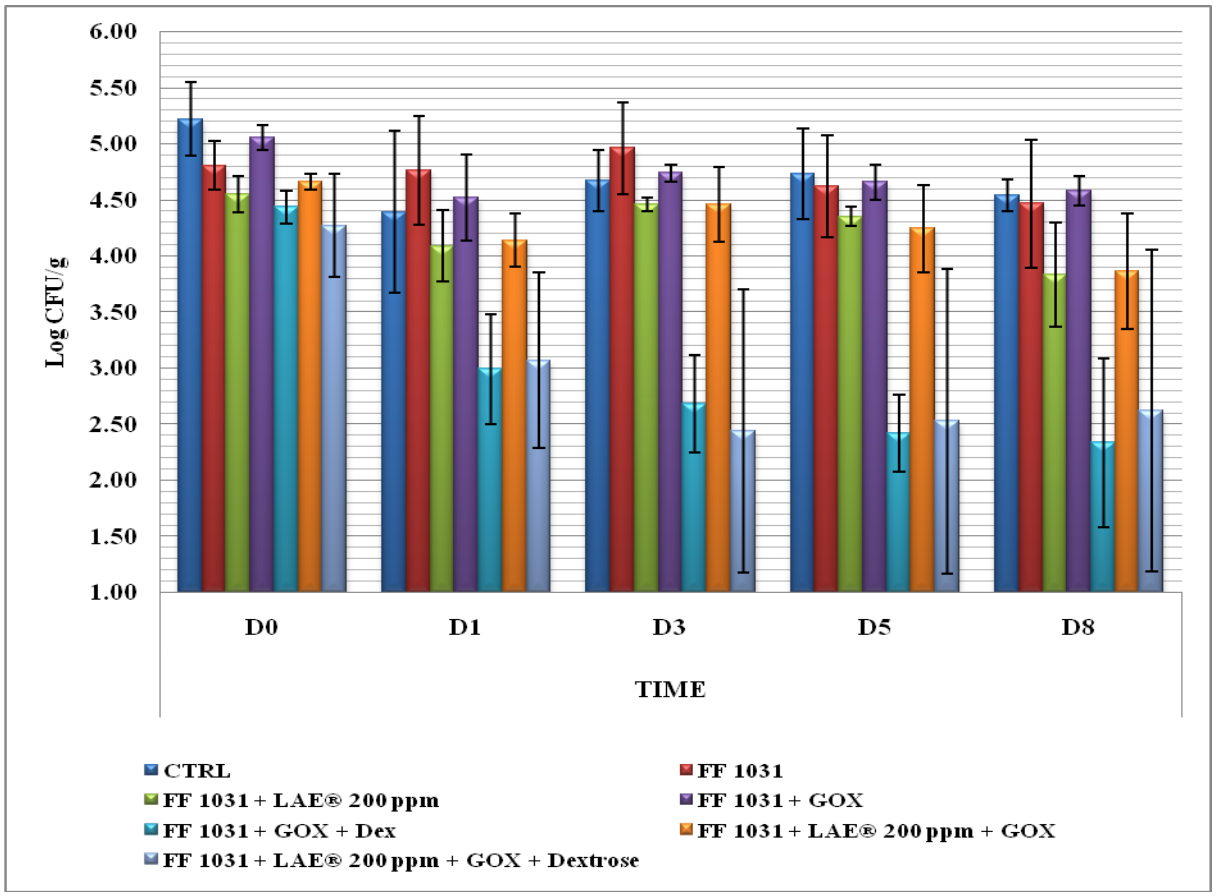


Figure 10 Mean and standard error (n=3) of *Listeria monocytogenes* populations recovered on thin agar layer modified oxford medium (TALMOX) in shredded mozzarella cheese at day 0, 1, 3, 5, and 8 of refrigerated storage with different antimicrobial blends consisting of free flow 1031 (FF), lauric arginate (LAE), glucose oxidase (GOX), and dextrose (Dex) at high inoculum levels

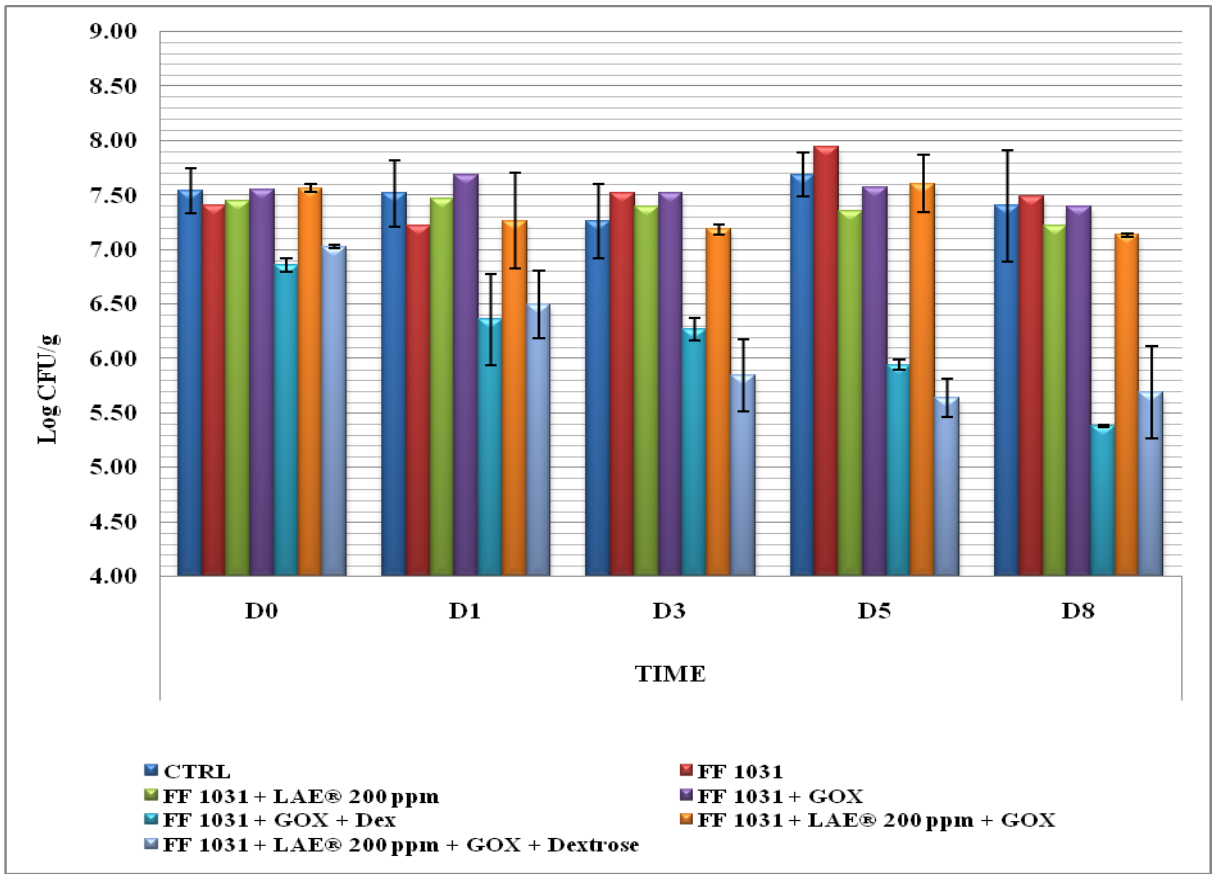
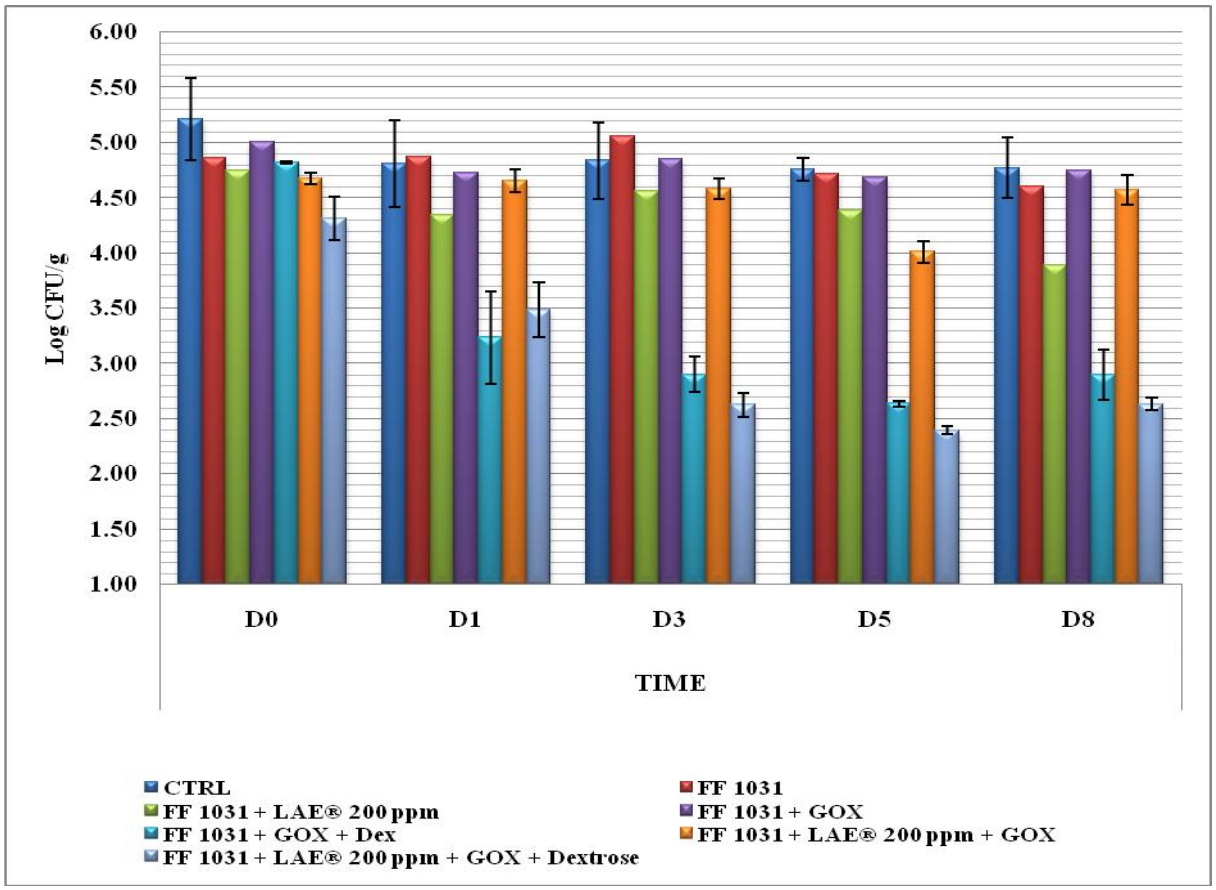


Figure 11 Mean and standard error (n=3) of *Listeria monocytogenes* populations recovered on thin agar layer modified oxford medium (TALMOX) in shredded mozzarella cheese at day 0, 1, 3, 5, and 8 of refrigerated storage with different antimicrobial blends consisting of free flow 1031 (FF), lauric arginate (LAE), glucose oxidase (GOX), and dextrose (Dex) at low inoculum levels



The two treatments that differed significantly ($P < 0.05$) from control were treatment 6 and 8. Treatments 6 (FF 1031+GOX+Dex) and 8 (FF 1031+LAE 200 ppm+GOX+Dex) showed reduction ($P > 0.05$) in bacterial population during the 8 day refrigerated storage; hence were effective in controlling *L. monocytogenes.* The inoculum had no effect ($P > 0.05$) on the efficacy of the treatments; both treatment 6 and 8 performed equally effectively at both high and low levels of inoculum. Furthermore, treatment 6 and 8 behaved similarly in the microbiological action against *L. monocytogenes*. Statistical analysis clearly indicates that there was no difference ($P > 0.05$) between treatment 6 and 8; FF 1031 + GOX + Dextrose alone showed similar antimicrobial effects as FF 1031+LAE 200 ppm + GOX + Dextrose. Hence, in this study LAE 200 ppm provided no additional antimicrobial effect to that obtained with the application of GOX + Dextrose alone.

Another observation made from the results obtained was that the variability of bacterial populations recovered also was much higher for the FF 1031+LAE® 200 ppm + GOX + Dextrose (treatment 8) when compared to FF 1031 + GOX + Dextrose (treatment 6) which implies that lauric arginate does not produce a consistent antimicrobial effect. Recoveries observed on resuscitating media (TALMOX) were slightly higher than those obtained in selective media (MOX), showing that both treatments (6 and 8) left close to 0.5 logs CFU/g of injured bacterial populations when *L. monocytogenes* was present at high levels. Lethality appeared to be more effective at low level, where almost no additional injured cells were recovered.

Discussion

Lauric arginate has surface active properties. Its positively charged nature facilitates association with bacteria but it also has measurable antimicrobial effects on microbial cells (Toarmina and Dorsa 2009). Studies have also revealed that its mechanism of action is different for Gram-negative *Salmonella* Typhimurium and Gram-positive *Staphylococcus aureus*. In Gram-negative cells, the alterations are in cytoplasmic membrane and external membrane while in Gram-positive cells, changes were observed in cell membrane and cytoplasm (Rodriguez and others 2004).

A limited amount of work has been done to assess the activity of LAE in controlling *L. monocytogenes* in foods. A research study evaluating the effectiveness of LAE showed that a 5% solution of LAE reduced *L. monocytogenes* by ≥ 6 logs on hams within 24 h at 4ºC (Luchansky

and others 2005). Research has been done to study the bactericidal activity of LAE in milk and queso fresco cheese (QFC). *Listeria monocytogenes* populations reduced by 1 log CFU/ml after 24 h with 200 ppm LAE while in QFC bacterial populations reduced by 0.3 to 2.6 log CFU/g with 200 ppm LAE treatment over 28 d storage at 4ºC (Soni and others 2010). Although our study also showed the greater antimicrobial effect of 200 ppm LAE with reductions up to 4 log CFU/g during the 8 day storage of shredded mozzarella cheese under refrigerated conditions, solid conclusions cannot be drawn from it. The results indicated that the antimicrobial effect was only seen in treatments where GOX and dextrose were used together. Glucose oxidase catalyse the oxidation of D-glucose to produce D-gluconolactone and hydrogen peroxide (Wong and others 2008). The generation of hydrogen peroxide may have the potential antimicrobial effect that is observed in this study. Furthermore, the applicability of LAE in mozzarella cheese has not been studied at all. More work is justified to explore possible applications of LAE and GOX in ready-to-eat food products.

Conclusion

Our results obtained, it clearly shows that glucose oxidase by itself has similar antimicrobial effect (up to 4 log CFU/g reduction) as lauric arginate and glucose oxidase together in controlling *Listeria monocytogenes* in shredded mozzarella cheese during 8 d of refrigerated storage.

Chapter 5 - Phase 1: - Study 3 - Microbiological Activity of Glucose Oxidase and Other Raw Ingredients applied in Shredded Mozzarella Cheese for Controlling *Listeria monocytogenes* in the Presence of a Gram-Negative Enteric Bacteria, *Salmonella* spp.

Abstract

This study was performed with an objective to evaluate the microbiological activity of glucose oxidase and different raw ingredients applied in shredded mozzarella cheese for controlling *L. monocytogenes* in a matrix with presence of Gram-negative enteric bacteria, *Salmonella* spp. A three-strain cocktail of *L. monocytogenes* and *Salmonella* spp. (high and low level), was used to inoculate 100 g bags of shredded mozzarella cheese, divided into 6 treatment groups of cellulose, cellulose + glucose oxidase (GOX) + dextrose + mineral oil (MO), cellulose + acidified calcium sulfate (ACS) + dextrose + MO, cellulose + sodium lactate (SL) + dextrose + MO, cellulose + Enzyme 2 + dextrose + MO, and control; and two subtreatments of high (8 log CFU/ml) and low (4 log CFU/ml) inoculum levels. Sampling was conducted at day 0, 1, 3, 5, and 8 of the refrigerated storage period. A 10 g portion of cheese sample was taken from the 100 g treatment bag and suspended in 90 ml of peptone water and then stomached for 1 min. Populations of *L. monocytogenes* and *Salmonella* spp. were calculated by plating appropriate dilutions onto modified oxford medium (MOX), and xylose lysine desoxycholate (XLD) agar. Results obtained and statistical analysis indicated reductions up to 4.0 log CFU/g over the 8 day storage period. Cellulose + SL+ GOX + dextrose + MO showed the greatest reduction, at both high and low inoculums levels. Treatment with cellulose was different ($P < 0.05$) from all others; and, it did not produce any reductions ($P > 0.05$) in bacterial populations. No difference ($P > 0.05$) among others treatments was seen indicating that although GOX has an antimicrobial effect on *L. monocytogenes* and *Salmonella* spp. in shredded mozzarella cheese, the use of additional preservatives such as sodium lactate and acidified calcium sulfate would require further research.

Introduction

Bacterial pathogens can contaminate cheese during post-processing steps and if sanitation and others measures employed in cheese processing plants post-processing are not adequate to prevent re-contamination (Linnan and others 1988). The characteristics of the specific variety of cheese govern the potential for growth and survival of pathogenic microorganisms. Soft fresh cheeses are more susceptible than hard ripened cheeses where combination of factors such as pH, salt, water activity, interact to render hard cheese microbiologically safer. Pathogens such as *Listeria monocytogenes*, *Salmonella enterica*, *Staphylococcus aureus* and enteropathogenic *Escherichia coli* are known to pose a great risk to the safety of cheese (Donnelly 2004).

Glucose oxidase (GOX) is commercially used food additive that removes traces of glucose and oxygen in different types of food products such as beer, cheese, carbonated beverages, dried eggs, and fruit pies; and prevent deterioration during storage by oxidation or browning (Shakuntala and Manay 2001). GOX, produced from *Aspergillus niger*, is Generally Recognized as Safe (GRAS) and is used in several industrial applications. It is a well characterized glycoprotein consisting of two identical 80-kDa subunits bound with two FAD (flavin adenine dinucleotide) co-enzymes. Glucose oxidase catalyse the oxidation of D-glucose to produce D-gluconolactone and hydrogen peroxide (Wong and others 2008). It is the generation of hydrogen peroxide which may have a potential antimicrobial effect in the food products. The continued catalytic activity of GOX has been reported in literature (Tiina and Sandholm 1989; Dobbenie and others 1995; Wu and others 1995) and has been shown to be very effective against both bacteria and fungi, especially if the microorganism does not produce catalase that scavenges hydrogen peroxide (Wong and others 2008).

As established from the previous study in which lauric arginate (LAE) showed no additional effect in controlling *L. monocytogenes* with dextrose and glucose oxidase, this research study was performed to evaluate the microbiological activity of glucose oxidase and other preservatives, sodium lactate and acidified calcium sulfate, applied to shredded mozzarella cheese for controlling *L. monocytogenes* in the presence of a Gram-negative enteric bacteria, *Salmonella* spp.

Materials and Methods

Bacterial Cultures and Inoculum Preparation

The bacterial cultures used in this study, were obtained from the American Type Culture Collection (Rockville, MD, USA), included three strains of *Listeria monocytogenes* (ATCC 19115, 19113, 19112) and three strains of *Salmonella* spp. (ATCC 13311, 13314, 43845). The source of ATCC 19112 was spinal fluid from a man in Scotland. ATCC 19115 (serotype 4b) and ATCC 19113 were human isolates. ATCC 13311 was *Salmonella enterica* subsp. *enterica* serovar Typhimurium isolated from human feces of a food poisoning case; ATCC 13314 was *Salmonella enterica* subsp. *arizonae*; and ATCC 43845 was *Salmonella enterica* subsp. *enterica* serovar Senftenberg. To prepare the inoculum, cultures were grown individually in 9 ml of tryptic soy broth (TSB, Difco; Franklin Lakes, NJ, USA) for 24 h at 35ºC. For inoculation purposes, each strain was combined into a single mixed culture suspension. The combined suspension was serially diluted to prepare two inoculum levels to inoculate the shredded cheese. Targeted low (10^4 CFU/ml) and high (10^8 CFU/ml) levels of inoculum were prepared and plated onto modified oxford medium (MOX, Oxoid, UK), and xylose lysine desoxycholate agar (XLD, Difco; Franklin Lakes, NJ, USA) incubated at 35ºC for 24 h to confirm target levels.

Experimental design

Shredded mozzarella cheese (Table 11) was treated with combinations (Table 12) of glucose oxidase, acidified calcium sulfate, sodium lactate, dextrose, enzyme 2, mineral oil, and anticaking agent (cellulose) at various levels (Table 13). The experimental design included control (non-inoculated shredded cheese and inoculated shredded cheese). Each treatment that was inoculated was subjected to two different inoculum levels as subtreatments: subtreatment 1: high level inoculum of *L. monocytogenes* and *Salmonella* spp. suspension (10^8 CFU/ml) and subtreatment 2: low level inoculum of *L. monocytogenes* and *Salmonella* spp. suspension (10^4 CFU/ml). Three replications of the experiment were performed.

Table 12 List of ingredients

Ingredient	Description
Mozzarella Cheese	In blocks, commonly found in the global market with ingredients, milk, starter culture and rennet
Cellulose (CL)*	Anticaking
Sodium Lactate (SL)*	Preservative
Acidified Calcium Sulfate (ACS)*	Preservative
Glucose Oxidase (GOX)*	Enzyme
Dextrose (Dex)*	Monosaccharide
Enzyme 2 (Enzy 2)*	For enzyme modified formulation
Mineral Oil (MO)*	Food grade, to avoid cheese shreds from sticking to each other

***Ingredients obtained from Allied Blending, Inc. CA, USA

Table 13 Different treatment combinations applied to evaluate microbiological activity of glucose oxidase and other ingredients in shredded mozzarella cheese to control *Listeria monocytogenes* and *Salmonella* spp.

Treatment	Specification[a]
Treatment 1	Inoculated – Shredded Cheese + Cellulose
Treatment 2	Inoculated - Shredded Cheese + Cellulose + GOX + Dex + MO
Treatment 3	Inoculated - Shredded Cheese + Cellulose + ACS + GOX + Dex+ MO
Treatment 4	Inoculated - Shredded Cheese + Cellulose + SL + GOX + Dex + MO
Treatment 5	Inoculated - Shredded Cheese + Cellulose + Enzy 2 + GOX + Dex + MO
Control	Inoculated - Shredded Cheese

[a]Abbreviations: GOX – Glucose Oxidase, ACS – Acidified Calcium Sulfate, SL – Sodium Lactate, MO – Mineral Oil, Enzy 2 – Enzyme 2, Dex - Dextrose

Table 14 Blends of anticaking with antimicrobials and other ingredients

TREATMENT 1		
Ingredient	**Grams**	**%**
Cellulose	100	100
TREATMENT 2		
Cellulose	79.85	79.85
Glucose oxidase	0.10	0.10
Dextrose	20.00	20.00
Mineral oil	0.045	0.045
TREATMENT 3		
Cellulose	79.85	79.85
Acidified calcium sulfate	0.002	0.002
Glucose oxidase	0.10	0.10
Dextrose	20.00	20.00
Mineral oil	0.045	0.045
TREATMENT 4		
Cellulose	79.85	79.85
Sodium lactate	0.002	0.002
Glucose oxidase	0.10	0.10
Dextrose	20.00	20.00
Mineral oil	0.045	0.045
TREATMENT 5		
Cellulose	79.85	79.85
Glucose oxidase	0.10	0.10
Enzyme 2	0.0002	0.002
Dextrose	20.00	20.00
Mineral oil	0.045	0.045

Sample Preparation

Blocks of low moisture part skim mozzarella cheese (900 g), obtained from local grocery store was shredded using food blender (Black and Decker, Model no. FP1600B; New Britain, CT, USA). A 100 g portion of shredded cheese was then weighed into sterile polyethylene bags for treatment application. All bags were heat sealed and stored at 4ºC until inoculation.

Inoculation of Samples

Each treatment (Treatments 1 to 5) consisted of 10 samples of 100 g of shredded cheese in individual sterile zip lock bags (5 for each inoculum level or subtreatment). Samples were inoculated by adding 10 ml of inoculum inside the bag. Bags were closed, shaken for 2 min by hand, and then sealed closed. Five samples for each treatment were inoculated with a low level (10^4) and the other five paired samples were inoculated with a high level (10^8) of inoculum using

corresponding combined suspensions of *Listeria monocytogenes* and *Salmonella* spp. inoculum respectively, as subtreatments 1 (low inoculum) and 2 (high inoculum).

After inoculation, blends were added to the inoculated cheese samples by adding the recommended amounts of each blend (Table 14) into the bag. Bags were closed and manually shaken for 2 min to allow for even distribution of the antimicrobial blend and the culture suspension. After homogenizing, all 10 bags from each treatment were stored under refrigeration conditions (4ºC). Controls were run parallel to the experimental set.

Enumeration

Each treatment was enumerated for population of *L. monocytogenes* and *Salmonella* spp. immediately after inoculation (0d), and after 1 (1d), 3 (3d), 5 (5d), and 8 days (8d) of refrigerated storage conditions at 4ºC. An aliquot of 10 g of sample was aseptically obtained from each bag. The 10 g portion of the cheese sample was added to 90 ml of sterile 0.1% peptone diluent and homogenized by blending in a stomacher (Seward Stomacher 400, UK) for 1 min. After homogenizing, samples were serially diluted and spread plated (0.1 ml) onto MOX agar for *Listeria monocytogenes*, and XLD agar for *Salmonella* spp. The plates were incubated at 35ºC for 48 h. Recovered populations were calculated and reported as log CFU/g.

Statistical Analysis

Listeria monocytogenes population data obtained was analyzed using PROC MIXED in SAS version 9.0 (SAS Institute, Cary, NC, USA). Fixed effects for statistical analysis were treatment, day, inoculum, media, treatment by day, treatment by inoculum, day by inoculum, treatment by media, inoculum by media, treatment by day by inoculum, treatment by day by media, treatment by inoculums by media, and day by inoculum by media, and treat by day by inoculums by media. The random effect was replication. Least square means ($P < 0.05$) were used to compare the interactions.

Results

The average initial populations of *L. monocytogenes* recovered on MOX for high and low inoculum were 7.3 log CFU/g and 5.5 log CFU/g, respectively. For XLD agar, the average initial recoveries of *Salmonella* spp. were 6.8 log CFU/g and 5 log CFU/g, respectively, for high and low inoculums levels. Figures 12 and 13 show the mean recoveries and standard error of *L.*

monocytogenes populations on MOX from shredded mozzarella cheese for various treatments at day 0, 1, 3, 5, and 8 of refrigerated storage with different antimicrobial blends at high and low inoculum levels. The mean recoveries and standard error of *Salmonella* spp. populations on XLD from shredded mozzarella cheese for various treatments at day 0, 1, 3, 5, and 8 of refrigerated storage with different antimicrobial blends at high and low inoculums levels are presented in Figures 14 and 15, respectively.

No reductions ($P > 0.05$) in bacterial populations were seen immediately after treatment. The antimicrobial effect of different treatments applied was seen after 1 day of refrigerated storage and additional reductions were observed over time. All the treatments 1-5 differed significantly ($P < 0.05$) from control, indicating bacterial reductions due to treatments with time. Reductions up to 4.0 log CFU/g were seen over the 8 day storage period. Overall, for both *Listeria monocytogenes* and *Salmonella* spp. when they were present together as a cocktail, treatment 4, Cellulose + SL+ GOX + Dextrose + MO, showed the greatest reduction, at both high and low inoculums levels. Further analysis of the data indicates that lethality appears to be more effective at low levels of bacterial populations.

All the treatments produced an antimicrobial effect on the bacterial populations. However, it is interesting to note that there was no difference ($P > 0.05$) among them except treatment 1 (cellulose only). Statistical analysis indicated a variability where treatment 1 differed significantly ($P < 0.05$) from all other treatments overall but did not produce any significant ($P > 0.05$) bacterial log reductions.

Figure 12 Mean and standard error (n=3) of *Listeria monocytogenes* populations recovered on modified oxford medium (MOX) in shredded mozzarella cheese at day 0, 1, 3, 5, and 8 of refrigerated storage with different antimicrobial blends consisting of cellulose, acidified calcium sulfate (ACS), sodium lactate (SL), glucose oxidase (GOX), dextrose (Dex), enzyme 2 (Enzy 2), and mineral oil (MO) at high inoculum levels

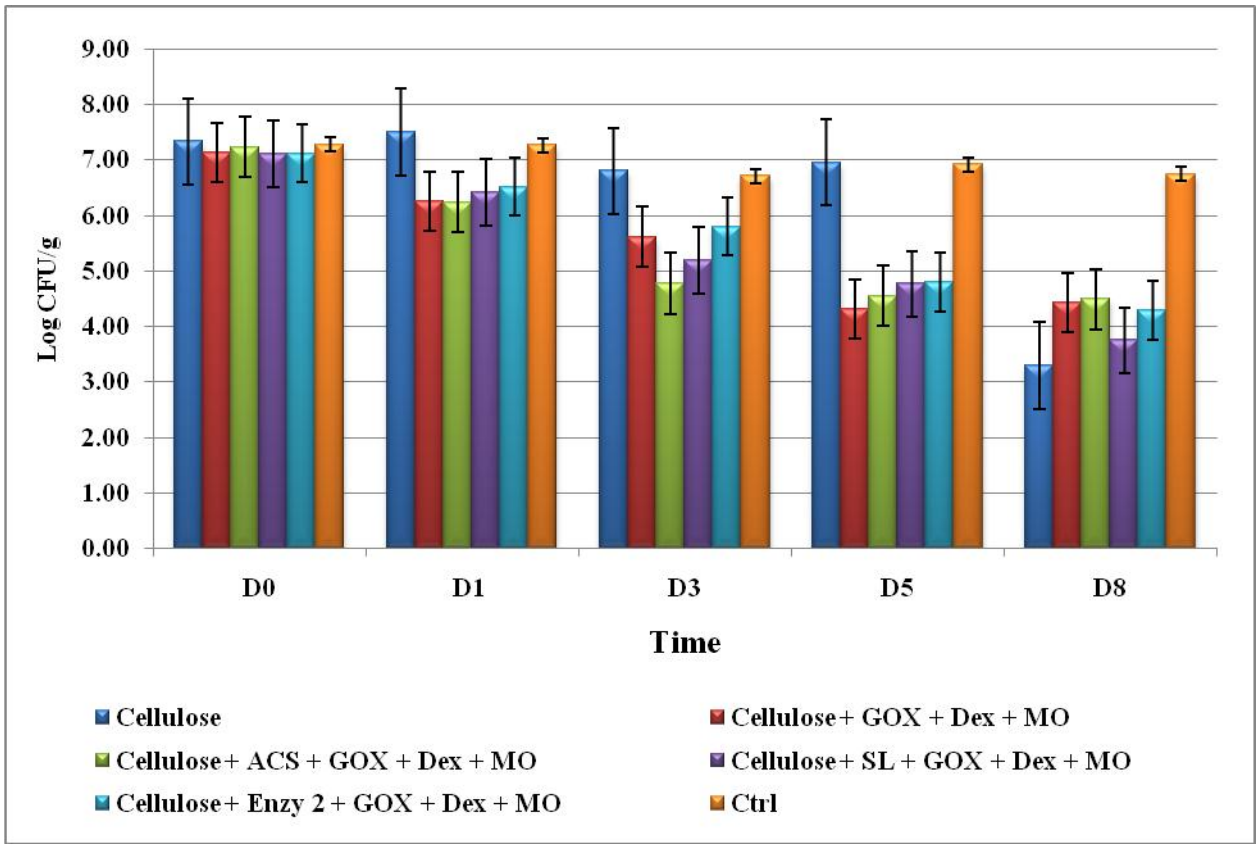


Figure 13 Mean and standard error (n=3) of *Listeria monocytogenes* populations recovered on modified oxford medium (MOX) in shredded mozzarella cheese at day 0, 1, 3, 5, and 8 of refrigerated storage with different antimicrobial blends consisting of cellulose, acidified calcium sulfate (ACS), sodium lactate (SL), glucose oxidase (GOX), dextrose (Dex), enzyme 2 (Enzy 2), and mineral oil (MO) at low inoculum levels

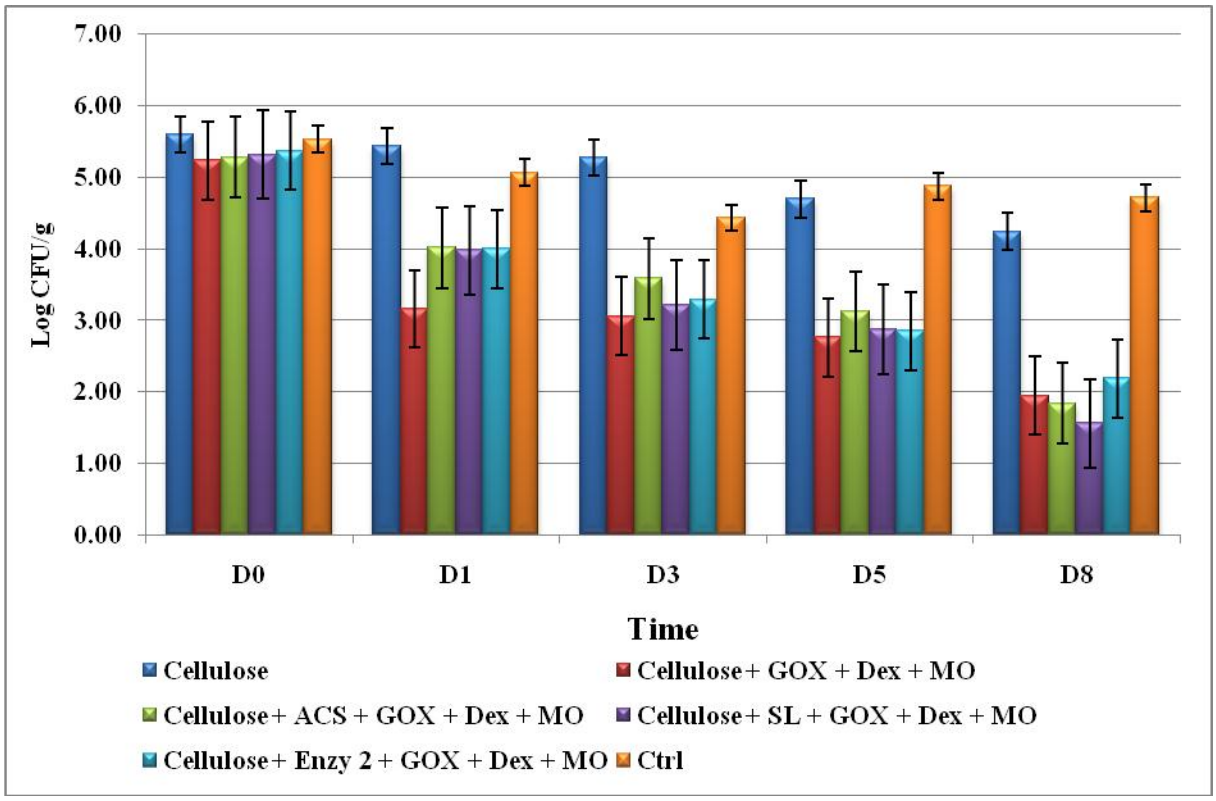


Figure 14 Mean and standard error (n=3) of *Salmonella* populations on xylose lysine desoxycholate (XLD) agar in shredded mozzarella cheese at day 0, 1, 3, 5, and 8 of refrigerated storage with different antimicrobial blends consisting of cellulose, acidified calcium sulfate (ACS), sodium lactate (SL), glucose oxidase (GOX), dextrose (Dex), enzyme 2 (Enzy 2), and mineral oil (MO) at high inoculum levels

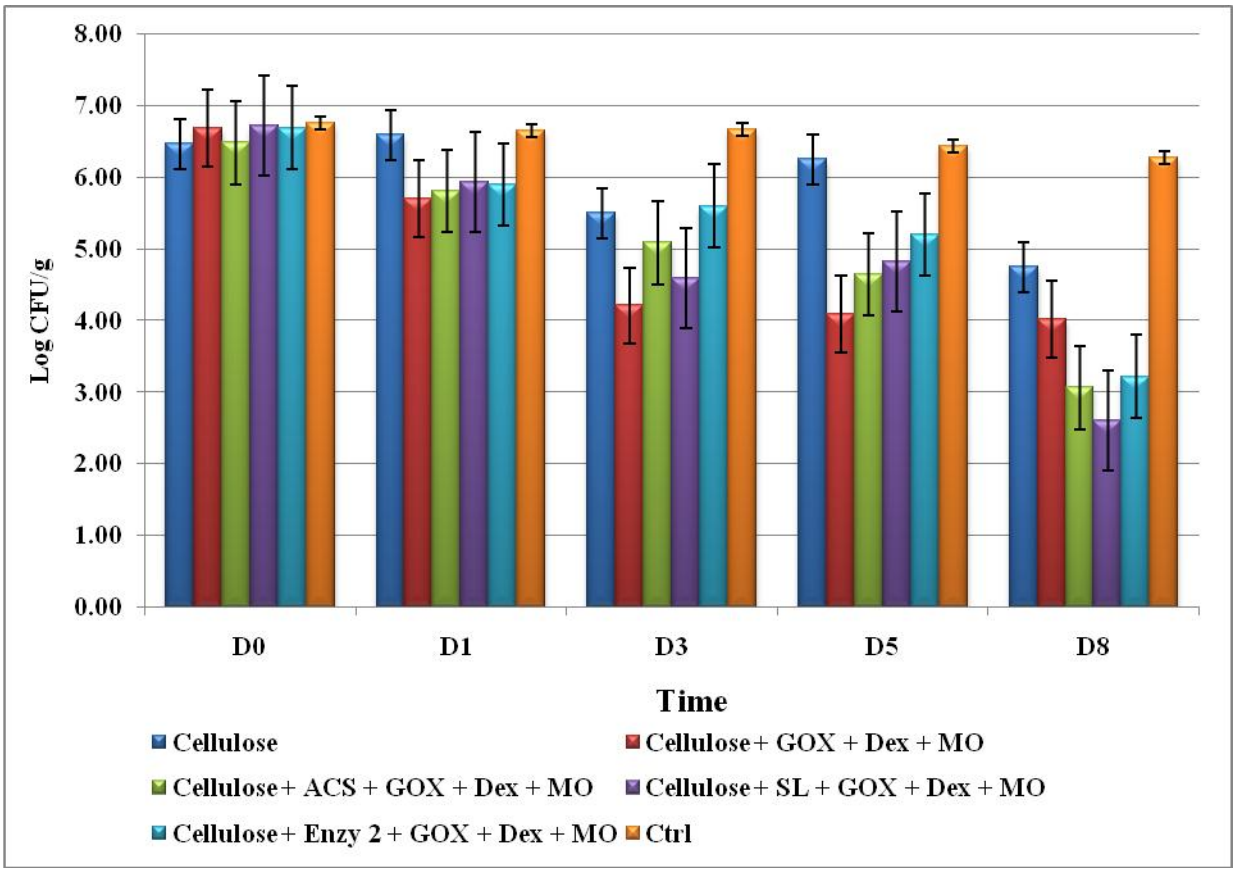
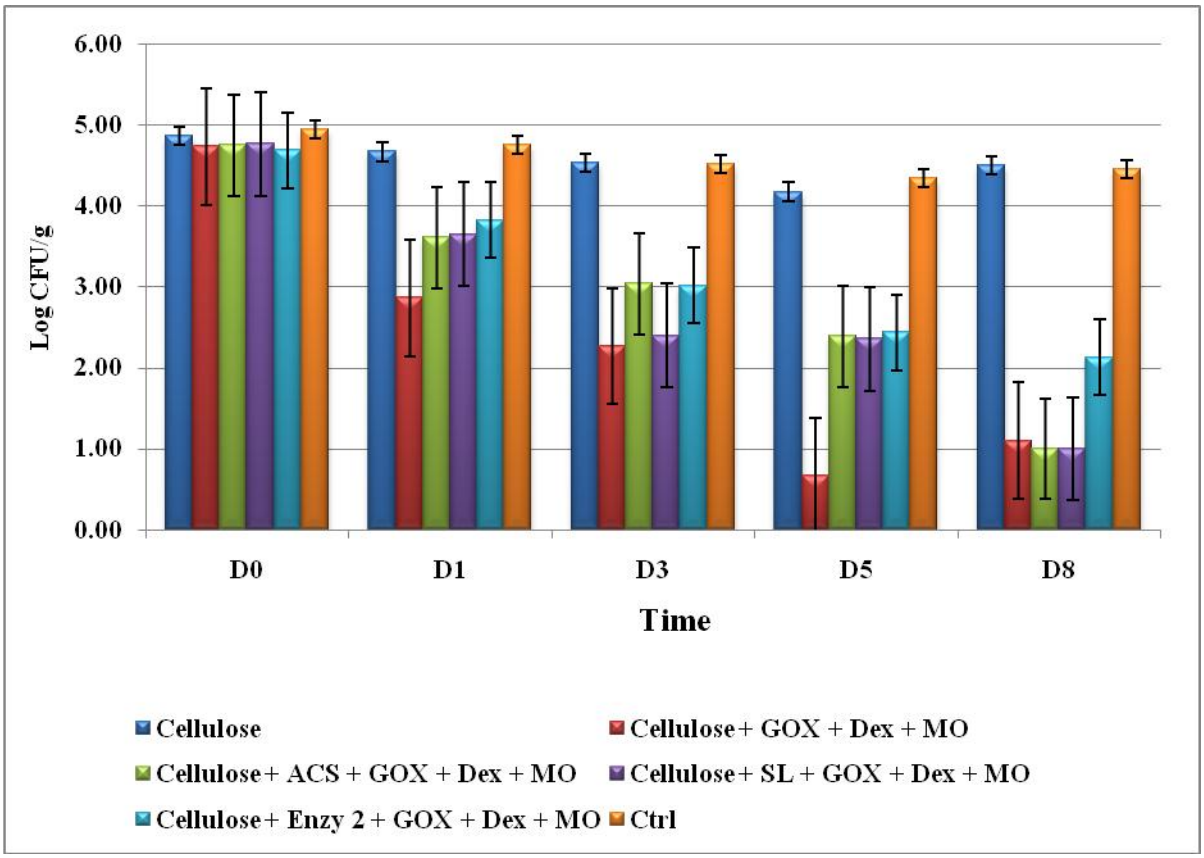


Figure 15 Mean and standard error (n=3) of *Salmonella* populations recovered on xylose lysine desoxycholate (XLD) agar in shredded mozzarella cheese at day 0, 1, 3, 5, and 8 of refrigerated storage with different antimicrobial blends consisting of cellulose, acidified calcium sulfate (ACS), sodium lactate (SL), glucose oxidase (GOX), dextrose (Dex), enzyme 2 (Enzy 2), and mineral oil (MO) at low inoculum levels



An interesting observation was made in high inoculum cellulose only treated samples for both *Listeria monocytogenes* and *Salmonella* spp., where up to 2 log CFU/g reductions were seen at the end of 8 day refrigerated storage. Cellulose is primarily used in shredded mozzarella cheese as an anticaking agent; absorbs moisture from the product. This absorption of moisture may have possible lowered the water activity or moisture content of the cellulose treated mozzarella cheese over the period of 8 days and could potentially be the reason for observed antimicrobial effect. However, further testing such as proximate analysis may be required to substantiate this observation.

Based on the results obtained, although treatment 4 (cellulose + GOX + SL + Dex + MO) showed highest bacterial reductions, it is difficult to conclude which treatment performed the best. Glucose oxidase alone may be an effective antimicrobial in shredded mozzarella cheese. Further research is needed to study interactions between each individual constituent of the treatments.

Discussion

Acidified calcium sulfate (ACS) is a food-approved highly acidic lactic acid/calcium sulfate complex containing lactic acid, sulfuric acid, and calcium hydroxide (Kemp and others 2005; USDA-FSIS 2009), which acts by disrupting the internal pH homeostasis and leading to eventual bacterial cell death (Kemp and others 2003). ACS has shown significant bactericidal effects in ready-to-eat meat and poultry products such as commercially prepared hams (Luchansky and others 2005), combined with lactic acid in ground beef (Zhao and others 2004), and with lactic acid or propionic acid in hot dogs (Nunez and others 2004). However, its use in cheese has not been studied so far.

Sodium lactate is commonly used as synergist for antioxidants in cheese and a preservative replacement for salt. In ready-to-eat meat products, up to 2% sodium lactate is added as a preservative to prevent post-processing outgrowth of bacterial populations (Campos and others 2011). Again, its application in cheese has not been studied so far.

Glucose oxidase serves as an important component of the lactoperoxidase system which is useful antimicrobial agent in dairy. As an antimicrobial, GOX continuously produces and replenishes hydrogen peroxide in the food system (Seifu and others 2005). Both *Listeria monocytogenes* and *Salmonella* spp. are catalase positive (Schuchat and others 1991; Todar 2012). So these bacteria may be intrinsically protected from hydrogen peroxide's antibacterial effect. However, if hydrogen peroxide is continually produced by GOX, the antibacterial effect is increased (Wong and others 2008). Measurement of the amount of hydrogen peroxide may be an important observation that is required to be made before the antimicrobial effect of GOX in this study is ascertained.

Conclusions

Our results obtained show that GOX has an antimicrobial effect on *L. monocytogenes* and *Salmonella* spp.in shredded mozzarella cheese. Glucose oxidase can be used as a potential antimicrobial agent (as an intervention in the hurdle technology) to control *Listeria monocytogenes* and *Salmonella* spp. in shredded mozzarella cheese. However, the use of additional preservatives such as sodium lactate and acidified calcium sulfate would require further research.

Chapter 6 - Phase 2: Efficacy and Applicability of Advanced Oxidation Technology with Photohydroionization as a Surface Treatment for Control of *Listeria monocytogenes* on Stainless Steel Coupon Surfaces, Sliced American Cheese, and Ready-To-Eat Turkey

Abstract

Major outbreaks of Listeriosis have been related to ready-to-eat meat and poultry products and dairy products. The widespread nature in food processing environments and the ability to survive a range of adverse conditions including acidic pH, low temperatures, and high salt concentrations makes *Listeria monocytogenes* difficult to control in food. The objective of this study was to evaluate the efficacy of a novel advanced oxidation technology called Photohydroionization (PHI) as a surface treatment for control of *L. monocytogenes* on food contact surfaces, sliced American cheese, and ready-to-eat turkey. A five-strain cocktail of *L. monocytogenes* was used to inoculate the surface of the three types of samples which were then treated in a closed chamber with a PHI unit. Food contact surfaces were exposed to UV and other oxidative gases produced by the PHI system for 10, 20, 30, 45, 60, and 120 s, and 5, 10, and 15 min; while cheese and turkey samples were treated for 30 s, 60 s, 120 s and 5 min. At each time point, 7 samples of each type of matrice were treated and enumeration of populations was done by plating appropriate dilutions onto modified oxford medium (MOX) and thin agar layer MOX (TALMOX). Results showed significant ($P < 0.05$) reductions; *L. monocytogenes* populations reduced by 4.37 log CFU/coupon on stainless steel surfaces after 15 min of treatment; 2.16 and 2.52 log CFU/sample reduction on American cheese and ready-to-eat turkey, respectively, after short treatment time of 5 min. Lipid oxidation analyses performed on cheese and turkey samples indicated that the PHI treatment did not significantly affect ($P > 0.05$) TBAR values. This study demonstrates the efficacy of the PHI treatment to reduce *L. monocytogenes* on stainless steel and ready-to-eat foods.

Introduction

The production of wholesome and safe food has been a continuous challenge for the food and beverage industry. Foodborne pathogens such as *Listeria monocytogenes, Escherichia coli* O157:H7, *Salmonella*, *Clostridium perfringens*, *Staphylococcus aureus* and others are a serious concern due to their continuous re-emergence in the global food supply over the years. For food processors, spoilage microorganisms are another major concern as they shorten the shelf-life of food products and also cost millions of dollars to food companies every year in spoiled product. An estimated 6.5 to 33 million cases of human illness and up to 9,000 deaths are reported in the United States (U.S.) each year due to microbial foodborne pathogens. The United States Department of Agriculture (USDA) annually estimates costs $2.9 to $6.7 billion associated with foodborne illnesses (Buzby 1996). Better control of microbiological contamination, prevention of cross contamination and post-processing contamination, and effective disinfection are continually needed in the food industry.

The advanced oxidation process (AOP) is an increasingly important tool that is being employed to control and prevent environmental contamination and also for surface decontamination of various foods. It utilizes the microbicidal action of the plasmas, ozone, ultraviolet (UV), vapor hydrogen peroxide and reactive oxygen species (ROS) produced in the process. Photohydroionization is a type of AOP that has been studied as a potential antimicrobial intervention for environmental and surface decontamination of foods. It utilizes a broad-spectrum high-intensity UV light targeted on a quad metallic catalyst ultraviolet target in a low-level ozone and moist atmosphere. After utilization, ozone decomposes into oxygen, leaving no harmful by-products (Purofist 2000). The oxidation potential of ozone is 2.07 compared to 1.36 for chlorine, and ozone can be used to disinfect water three to four times more effectively than chlorine. Upon oxidation, ozone disrupts the substance molecule and leaves no residue (Fink 1994). In June 2001, the Food and Drug Administration (FDA) approved the use of ozone as a sanitizer for food contact surfaces and for direct application on food products (USDA 2001; FDA 2001, 2003). The FDA acknowledged ozone's ability to reduce levels of *E. coli* and *Cryptosporidium* in juice and has approved ozone as an antimicrobial food additive (FDA, 2004; Ortega and others 2007). Studies have shown 96.4 to 99.9% reduction of ten most pathogenic forms of mold, fungi, bacteria, and virus which included methicillin-resistant *Staphylococcus aureus* MRSA, *E. coli*, *Bacillus* spp., and *Stachybotrys chartarum*, using photocatalysis and

ozone treatment for 24 h (Ortega and others 2007). Research has shown that use of high intensity UV can reduce *E. coli* O157:H7 and *Salmonella* spp. on surface inoculated sub primal meat cuts by 2 log CFU/cm^2 after 20 and 30 s treatments (Saini and Marsden 2010).

Listeria monocytogenes is an important foodborne pathogen that has gained considerable attention in the food industry due to its widespread existence in food processing environments and low infective dose. Foods can become contaminated by contact with surface contaminated by *Listeria* spp. Furthermore, due to its ability to survive and grow at refrigerated temperatures, packaged ready-to-eat (RTE) foods become prime carriers of *L. monocytogenes* if the product gets contaminated post-processing or during packaging because these foods are consumed without further cooking (Saulo 2005). Therefore, the objective of this study was to determine the efficacy and applicability of Advanced Oxidation Technology with Photohydroionization as a surface treatment for control of *L. monocytogenes* on stainless steel coupon surfaces, sliced American cheese, and ready-to-eat turkey.

Materials and Methods

Preliminary Testing

In order to establish the efficacy of the advanced oxidation technology with photohydroionization (PHI) process, some preliminary tests were done. Modified oxford medium (MOX, Oxoid, UK) plates were streaked with 24 h grown culture of *L. monocytogenes.* These plates were covered in half with aluminum foil and then treated for different time periods, 30, 45, 60, and 120 s, 5 and 10 min, in an enclosed chamber with broad spectrum PHI lamps producing UV rays and other oxidative gases as a result of the process. The ozone and hydrogen peroxide measured during the treatment were 0.15 to 0.4 ppm and 0.05 to 0.2 ppm. These plates were then incubated at 35ºC for 24 h and growth after incubation was observed. Preliminary test results (Figure 16) clearly indicate that the treatment was successful in inhibiting the growth of *Listeria monocytogenes* on the surface of the petri plate even at short exposure time of 30 s.

Figure 16 Effect on *Listeria monocytogenes* before and after the treatment of 30 s in Photohydroionization (PHI) Chamber

Bacterial Cultures and Inoculum Preparation

The bacterial cultures used in this study were obtained from the American Type Culture Collection (Rockville, MD, USA), included five strains of *L. monocytogenes* (ATCC 19112, 19115, 19113, 13932 and SLR-2249). ATCC 13932 (serotype 4b) was isolated from the spinal fluid of child suffering from meningitis in Germany. The source of ATCC 19112 also was spinal fluid from a man in Scotland. SLR-2249 is a laboratory developed strain in which the *ActA* gene is removed. ATCC 19115 (serotype 4b) and ATCC 19113 were human isolates. To prepare the inoculum, cultures were grown individually in 9 ml of tryptic soy broth (TSB, Difco; Franklin Lakes, NJ, USA) for 24 h at 35ºC. For inoculation purposes, each strain was combined into a single mixed culture suspension to obtain a five-strain bacterial cocktail for inoculating food and food contact surfaces. The cell density of the suspension was determined by plating appropriate dilutions onto selective agar, modified oxford medium (MOX, Oxoid, UK) for enumerating *L. monocytogenes.* Plates were incubated at 35ºC for 24 h. Cultures were then confirmed for their purity by biochemically analysts using BBL Crystal Gram Positive (Becton and Dickinson, Frankin Lakes, NJ, USA) rapid test kits.

Sample Preparation

Three matrices were studied: stainless steel coupons (food contact surface), American cheese, and deli-style turkey. Polished stainless steel coupons of #316 finish were initially cleaned using Sparkleen detergent (pH 9.5-10 in solution (Fisher Scientific, Hampton, New Hampshire, USA), and then autoclaved. Individually wrapped sliced American cheese was obtained from a local grocery store (Table 15, 17). For ready-to-eat turkey, low sodium oven roasted turkey was obtained from a local grocery store and sliced at 40 barr thickness and cored to obtain 15.9 cm^2 surface area (Table 16, 17).

Table 15 List of ingredients in sliced American cheese

Ingredient name	
Cultured milk	**Calcium Phosphate**
Water	**Salt**
Cream	**Sorbic Acid (preservative)**
Whey	**Sodium Phosphate**
Sodium Citrate	**Citric Acid**
Milk Protein Concentrate	**Lactic Acid**
Modified Food Starch	**Enzymes**
Whey Protein Concentrate	**Vitamin D_3**

Table 16 List of ingredients in ready-to-eat turkey

Ingredient name
Turkey Breast Meat
Turkey Broth
2 % or less Salt
Carageenan
Potassium Phosphate

Table 17 Proximate analysis (n=3) for sliced American cheese and ready-to-eat turkey samples

Sample	% Moisture	% Protein*	% Fat	% Sodium	pH
Sliced American cheese	44.90±0.61	13.98±0.39	23.40±0.27	0.99±0.07	6.10±0.02
RTE turkey	75.19±0.78	21.19±0.66	0.67±0.07	0.50±0.02	6.41±0.03

*Crude protein is calculated using a 6.25 conversion factor (%N x 6.25)

Sample Inoculation

Stainless steel coupons were inoculated by dipping the coupons in the five-strain cocktail of *L. monocytogenes* for 1 min and allowed to drip dry on racks for 30 min. For inoculating the sliced American cheese, 0.1 ml of the five-strain bacterial cocktail was pipetted on to the top surface of the cheese slice and spread across the area using a sterile loop. Slices were left to dry for 10 min in a biosafety cabinet. Cored turkey slices were inoculated similar to sliced cheese,

0.05 ml of inoculum was pipetted onto the surface of the turkey and spread across the area using a sterile loop. These were also left to dry for 10 min in a biosafety cabinet.

Sample Treatment

Samples were treated in an enclosed chamber equipped with the Photohydroionization unit manufactured by RGF Environmental Group Inc. (West Palm Beach, FL, USA). As per the manufacturer, the 6 lamp hood, at a distance of 15.24 cm, delivers an average 16.65 mJ/cm^2 germicidal 254 nm UV energy, and requires lamp replacement after 8,000 h or annually.

Stainless steel coupons were not treated 0 h (control) or treated for 10, 20, 30, 45, 60, and 120 s, and 5, 10, and 15 min. Sliced American cheese and turkey samples were not treated 0 h (control) or treated inside the chamber at 30, 60, and 120 s, and 5 min. At each time point, 7 samples were treated and analyzed making a total of n=21 for three replications per time point.

Ozone and Hydrogen Peroxide Measurements

The Photohydroionization unit was stabilized by running it for 30 min prior to treatment of samples. The ozone and hydrogen peroxide measurements were taken using the Draeger Tube System (Drager Safety Inc., PA, USA; Figure 17). Draeger-Tubes® are glass vials filled with a chemical reagent that reacts to a specific chemical or family of chemicals. A Dreager accuro pump is used to draw a calibrated 100 ml sample of air through the tube. If the targeted chemical(s) is present the reagent in the tube changes color and the length of the color change typically indicates the measured concentration. Draeger tubes were used separately for ozone and hydrogen peroxide measurements.

Figure 17 Draeger Tube System

Sampling and Enumeration

Stainless Steel Coupons – After treatment, each stainless steel coupon was individually placed in a 50 ml conical tube with 15 ml of 0.1% peptone water. Bacteria attached to the coupon were dislodged by vortexing for 1 min. Serial dilutions were prepared from the coupon wash suspension in 0.1% peptone diluent and spread plated (0.1 ml) onto modified oxford medium agar (MOX, Oxoid, UK), and thin agar layer MOX agar (TALMOX).

Sliced American Cheese – Each slice of cheese was individually placed in a stomacher (Seward Stomacher 400, UK) bag after the treatment. To this 50 ml of 0.1% peptone water was added and then stomached for 1 min. Serial dilutions were prepared and spread plated (0.1 ml) onto modified oxford medium (MOX,Oxoid, UK), and thin agar layer MOX agar (TALMOX).

Ready-to-eat oven roasted Turkey – After treatment, each turkey sample was individually placed in a stomacher bag. To this 50 ml of 0.1% peptone water was added and then stomached (Seward Stomacher 400, UK) for 1 min. Serial dilutions were prepared and spread plated (0.1 ml) on to modified oxford medium agar (MOX, Oxoid, UK), and thin agar layer MOX agar (TALMOX).

All plates were incubated at 35ºC for 48 h. Recovered populations of *L. monocytogenes* were calculated and reported as log CFU/individual sample unit.

Thiobarbituric acid reactive substances (TBAR) Analysis

Since the reactive oxygen species (ROS) are the key components in this advanced oxidation process, oxidative changes in the foods, if any, were studied by performing thiobarbituric acid reactive substances (TBARS) analysis. TBARS values are widely accepted as evidence of oxidation and they reflect the oxidation of polyunsaturated fatty acids. For each replication of research, one sample per time point, 120 s and 5 min was analyzed for each set of sample analyzed per time point (n=3). The following methodology was used to analyze cheese and turkey samples:

Principle - In the presence of thiobarbituric acid (TBA), malonaldehyde and other aldehyde products of lipid oxidation (TBA reactive substances; TBARS) form pink chromogens with maximum absorbance at 532-535 nm.

Reagents - TBA stock solution - 0.375% thiobarbituric acid, 15% trichloroacetic acid, and 0.25N HCl. Stock solutions (100 ml) are sufficient for 20 individual tests. Stock solution may be stored at room temperature in the dark (foil-wrapped container).

Procedure - The treated cheese and turkey samples were finely chopped or minced and weighed out in duplicate 0.5 g samples. To each of these samples, 2.5 ml of the TBA stock solution was added, making it a dilution factor of 6. Samples were then heated in boiling water for 10 min placed in a water bath in round bottom Pyrex centrifuge tube with loose fitted caps. Tubes were then cooled under cold water and centrifuged at 5,000 × g for 10 min to obtain a clear supernatant. A portion of the supernatant was carefully pipetted into the spectrophotometer cuvette and supernatant absorption was measured at 532 nm against a blank that contains all the reagents minus the food sample. The TBA value was calculated and expressed as ppm malonaldehyde, using 1.56×10^5/M/cm as the extinction coefficient of the pink TBA chromogen (Sinnhuber and Yu 1958), as follows:

TBARS number (mg MDA/kg) = sample A_{532} × (1 M TBA chromagen/156,000) × [(1mole/L/M] × (0.003 L/0.5 g meat) × (72.07 g MDA/mole MDA) × 1000 mg/g) × 1000 g/kg)

Or **TBARS value (ppm)** = sample A_{532} × 2.77

Statistical Analysis

Listeria monocytogenes population data obtained was analyzed using PROC MIXED in SAS version 9.0 (SAS Institute, Cary, NC, USA). Fixed effects for statistical analysis were time, media, and time by media. The random effect was replication. Least square means ($P < 0.05$) were used for analysis.

Results

The ozone and hydrogen peroxide readings taken during the treatment with the draeger tube system ranged from 0.2 to 0.3 ppm and 0.15 to 0.2 ppm, respectively. The unit was stabilized by letting it run for 30 min before the treatment of samples. The ozone and hydrogen peroxide levels peaked during this level at 0.6 and 0.5 ppm, respectively, during this period but stabilized later. This stabilization period was determined based on repeated readings of ozone and hydrogen peroxide concentrations during preliminary studies.

Reduction in *L. monocytogenes* populations on stainless steel coupons after treatment with the Photohydroinization unit for 10, 20, 30, 45, 60, and 120 s, 5, 10, and 15 min compared to the controls is presented in Figure 18. Figure 18 also shows the mean recoveries of *L. monocytogenes* populations from the stainless steel coupons after the treatment. The treatment showed reductions ($P < 0.05$) in bacterial populations with time. Microbial reductions on MOX

were 0.60 , 0.71, 1.08, 1.59, 1.40, 2.00, 2.48, 3.91, and 4.30 log CFU/coupon for 10, 20, 30, 45, 60, and 120 s, 5, 10, and 15 min, respectively. Microbial reductions on TALMOX were 0.64 , 0.78, 1.16, 1.49, 1.38, 2.02, 2.48, 3.89, and 4.37 log CFU/coupon for 10, 20, 30, 45, 60, and 120 s, 5, 10, and 15 min, respectively. There was no significant difference ($P > 0.05$) between bacterial populations recovered on MOX and TALMOX indicating that there was no injury to the cells as a result of the treatment.

Figure 18 Mean (n=21) log recoveries and bacterial reductions of *Listeria monocytogenes* on stainless steel coupons on modified oxford medium (MOX) and thin agar layer MOX (TALMOX) due to Photohydroionization treatment

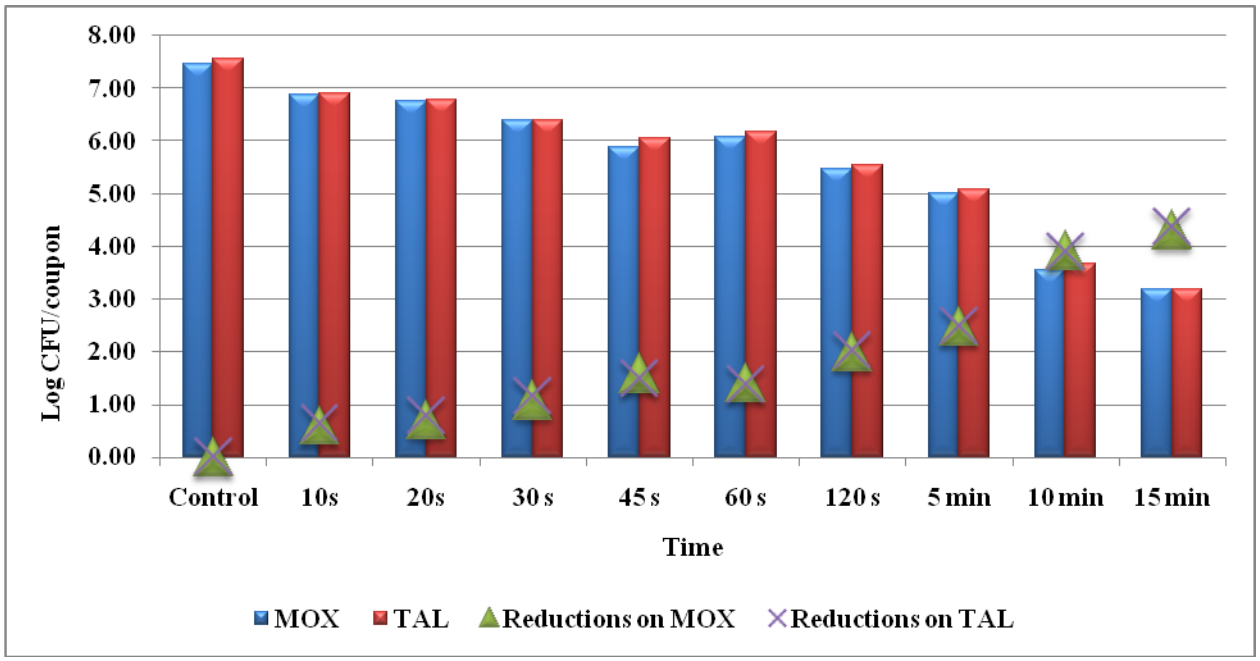
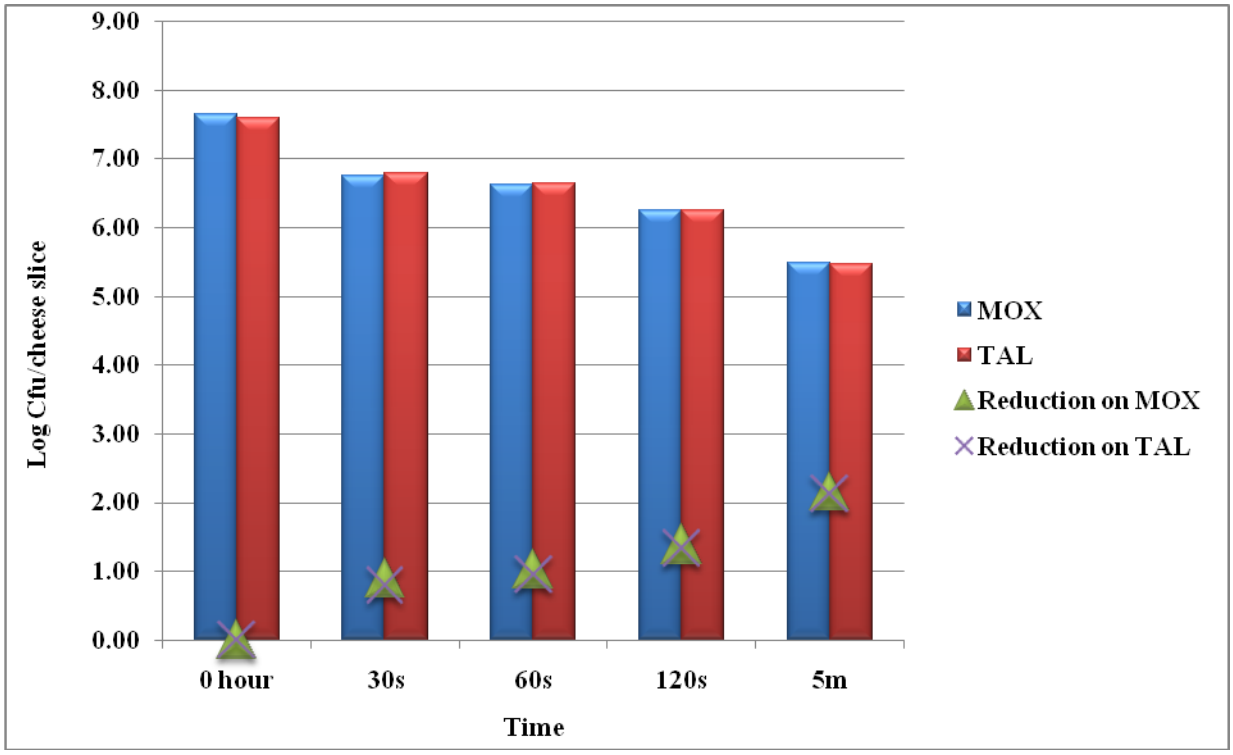


Figure 19 shows the mean recoveries of *L. monocytogenes* and reduction in populations on sliced American cheese after treatment with the Photohydroinization UV Ozone unit for 30 s, 60 s, 120 s, and 5 min compared to the controls. The treatment showed significant reductions ($P < 0.05$) in bacterial populations with time. However, there was no significant difference ($P > 0.05$) between bacterial populations recovered on MOX and TALMOX indicating that there was no injury to the cells as a result of the treatment. Reductions seen as a result of 30 s, 60 s, 120 s

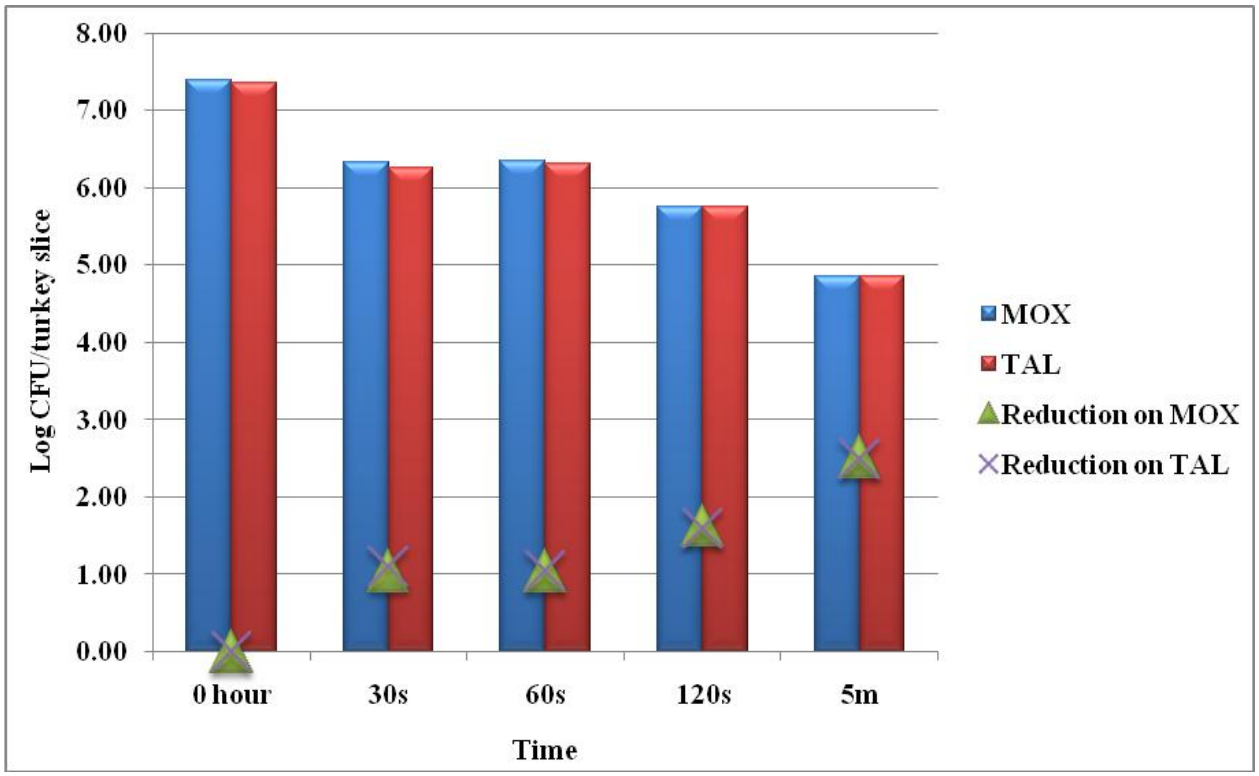
and 5 min treatment of cheese with the Photohydroionization unit were 0.9, 1.03, 1.39, 2.16 log CFU/sample, respectively.

Figure 19 Mean (n=21) log recoveries and bacterial reductions of *Listeria monocytogenes* from sliced American cheese on modified oxford medium and thin agar layer MOX (TALMOX) due to Photohydroionization treatment



The mean recoveries and reductions in *L. monocytogenes* populations due to Photohydroionization UV Ozone treatment of sliced oven roasted ready-to-eat turkey samples for 30 s, 60 s, 120 s and 5 min are presented in Figure 20. The ready-to-eat turkey samples showed similar results to American cheese due to the treatment. Significant bacterial reductions ($P < 0.05$) were seen over time with the treatment. Reductions up to 2.5 log CFU/sample were observed during the 5 min treatment time.

Figure 20 Mean (n=21) log recoveries and bacterial reductions of *Listeria monocytogenes* populations from ready-to-eat turkey on modified oxford medium (MOX) and thin agar layer MOX (TALMOX) due to Photohydroionization treatment for 30s, 60s, 120s, and 5m



Lipid oxidation analyses performed for cheese and turkey samples for the two longest treatment times, 120 s and 5 min, indicates that the Photohydroionization treatment did not significantly affect ($P > 0.05$) TBAR values. The TBAR values for turkey samples for both 120 s and 5 min treatment were significantly lower than the controls (Table 14). Similarly, TBAR values for cheese samples for 120 s and 5 min treatment were not significantly different from the controls.

Table 18 Mean and standard deviation of TBAR values (ppm) in treated cheese and turkey samples at 120 s and 5 min time

Sample	TBAR Value (ppm)		
	Control	120 s Treatment	5 min Treatment
Turkey	3.443 ± 0.049	1.751 ± 0.689	1.627 ± 0.510
Cheese	0.302 ± 0.011	0.273 ± 0.038	0.328 ± 0.026

Discussion

Ultraviolet light (UV) and ozone have continued to receive attention due to their antimicrobial effects. Due to its oxidative ability, ozone has been used for many years in the food industry for odor control, disinfection, and water treatment and has also been used with mixed success to inactivate contaminant microflora on meat, poultry, eggs, fish, fruits and vegetables, and dry foods (Jin and others 1999). A study by Katara and others (2008) showed that UV light can have efficient inactivation of bacteria up to 4 logs up to a distance of eight feet on either side and an exposure time of 30 min was adequate. Studies have evaluated the combined effect of UV and ozone. UV treatment followed by ozonation showed a reduction in total heterotropic bacteria and total coliform count by 1 and 2 log CFU/ml, respectively, in recirculating aquaculture system (Sharrer and Summerfelt 2007). UV-C is applied to fresh fruits and vegetables before storage to reduce microbial contamination on the surface. The U.S. FDA has approved UV treatments for pathogen reduction in water. Ultraviolet treatment has shown effectiveness at reducing pathogens in apple cider (Hanes and others 2002; Quintero-Ramos and others 2004) and goat milk (Matak and others 2005).

Photohydroionization with UV and ozone is a novel technology which is finding increasing food use application. To our knowledge, this is the first time has been evaluated as a treatment to control surface contamination of food contact surface and food products like sliced American cheese and ready-to-eat turkey. Considerable reductions were seen as a result of use of this kind of advanced oxidation process. A 4.37 log CFU/coupon reduction in *L. monocytogenes* populations were seen on stainless steel surfaces only after 15 min of treatment. American cheese and ready-to-eat turkey showed 2.16 and 2.52 log CFU/sample reduction in bacterial populations after short treatment time of 5 min without producing any undesirable effects on the

product. This technology works due to combined action of the plasmas, UV, ozone and vapor hydrogen peroxide produced as part of the process. Short exposure times and low levels of ozone allow an added advantage to the process. Ozone attacks bacterial cells causing lysis within few seconds. Therefore, microorganisms cannot develop resistance to ozone, thus eliminating the need to change biocides periodically (Pope and others 1984). Our study has demonstrated the potential of this technology as a surface decontaminant for food applications hence establishes the need for further research of this technology and its future applications.

Conclusion

The Photohydroionization UV Ozone system was found to be effective in controlling surface contamination of food contact surfaces and ready-to-eat products (American cheese and oven roasted turkey). It can be applied as a potential post-lethality treatment (produced >1 log reduction in sliced American cheese and RTE turkey) in the integrated process to control *Listeria monocytogenes* contamination and ensure safe production of food. Further research is needed for increased applications in the food industry.

Chapter 7 - Conclusions

Listeria monocytogenes is a widespread contaminant in food processing environment. There is an ever-growing need for control strategies for this bacterium in ready-to-eat food products and food production areas. Control strategies should be aimed at preventing contamination of foods with *L. monocytogenes* and preventing growth of *L. monocytogenes* to high numbers in foods.

Our study showed that lauric arginate (LAE) can be used as an antimicrobial treatment for *L. moncytogenes* for low contamination levels on stainless steel surfaces. It cannot be used as a sanitizer by itself as it does not produce 5 log reductions after 30 s contact contact time and must be integrated as a part of food sanitation program of a food establishment. Glucose oxidase is effective in controlling *Listeria monocytogenes* and it can be applied as a post-lethality treatment (> 1 log reduction) in shredded mozzarella cheese. Further research needs to be conducted to evaluate applicability of glucose oxidase as an antimicrobial treatment in various ready-to-eat food products. The use of acidified calcium sulfate and sodium lactate in mozzarella cheese would also require more research. The Photohydroionization (PHI) technology can be successfully used; as a post-lethality treatment due to its ability to produce greater than 1 log reduction of *L. monoctytogenes*; as a part of the integrated process to control contamination and ensure safe production of food. Research is needed to study its use as a post-lethality treatment or antimicrobial process to provide an additive effect to surface application of control agents such as citric acid and lactic acid in foods to obtain a more effective kill.

To conclude, *Listeria monocytogenes* is is common throughout the food chain, a single intervention strategy is not sufficient or adequate for its control. An integrated approach from processing to packaging to control *Listeria monocytogenes* needs to be adopted in food processing establishments.

Chapter 8 - References

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