Effect of Ozone and Ultraviolet Irradiation Treatments on *Listeria monocytogenes* Populations in Chill Brines.

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ABSTRACT

The efficacy of ozone and ultraviolet light, used in combination, to inactivate *Listeria monocytogenes* in fresh (9% NaCl, 91.86% transmittance at 254 nm) and spent chill brines (20.5% NaCl, 0.01% transmittance at 254 nm) was determined. Preliminary studies were conducted to optimize parameters for the ozonation of "fresh" and "spent" brines. These include diffuser design, comparison of kit to standard methods to measure residual ozone, studying the effect of ozone on uridine absorbance and determining presence of residual listericidal activity post ozonation.

An ozone diffuser was designed using 3/16 inch PVC tubing for the ozonation of brines. The sparger was designed to facilitate better diffusion and its efficiency was tested. The modified sparger diffused 1.44 ppm of ozone after 30 minutes of ozonation and the solution had an excess of 1 ppm in 10 minutes of ozonating fresh brine solution (200ml). Population levels of *L. monocytogenes* were determined at various time intervals post-ozonation (0, 10, 20, 60 min) to determine the presence of residual listericidal activity. The population post ozonation (0 minutes) was 5.31 Log CFU/ml and was 5.08 Log CFU/ml after a 60 minute interval. Therefore, residual antimicrobial effect was weak. Accuracy of the Vacu-vial Ozone analysis kit was evaluated by comparing the performance of the kit to the standard indigo colorimetric method for measuring residual ozone. The kit was inaccurate in determining residual ozone levels of spent brines and 1% peptone water. Uridine was evaluated as a UV actinometric tool for brine solutions

that were ozonated before UV treatment. The absorbance of uridine (A_{262}) decreased after ozonation from 0.1329 to 0.0512 for standard 10 minutes UV exposure duration. Absorbance of uridine was influenced by ozone indicating that the presence of ozone may hamper UV fluence determination accuracy in ozone-treated solutions.

Upon completion of diffuser design and ozone/UV analysis studies, the effect of ozone-UV combination on *L. monocytogenes* in fresh and spent brines was evaluated. Ozonation, when applied for 5 minutes, caused a 5.29 mean Log reduction while 5 minutes of UV exposure resulted in a 1.09 mean Log reduction of *L. monocytogenes* cells in fresh brines. Ten minutes of ozonation led to a 7.44 mean Log reduction and 10 minutes of UV radiation caused a 1.95 mean Log reduction of *Listeria* in fresh brine.

Spent brines required 60 minutes of ozonation for a 4.97 mean Log reduction in *L. monocytogenes* counts, while 45 minutes resulted in a 4.04 mean Log reduction. Ten minutes of UV exposure of the spent brines resulted in 0.30 mean Log reduction in *Listeria* cells. A combination of 60 minutes ozonation and 10 minute UV exposure resulted in an excess of 5 log reduction in cell counts. Ozonation did not cause a sufficient increase in the transmittance of the spent brine to aid UV penetration but resulted in apparent color change as indicated by change in L*a*b* values. Ozonation for sufficient time had considerable listericidal activity in fresh brines and spent brines and when combined with UV treatment, is effective reducing *L. monocytogenes* to undetectable levels in fresh brines.

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Dedication

Dedicated to my parents, Mrs. Padmini Dev Kumar and Mr. Dev Kumar Bala for their love, support and encouragement.

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CHAPTER I

INTRODUCTION

Listeria monocytogenes is a Gram-positive, non-sporulating, facultative intracellular foodborne pathogen capable of causing serious complications in pregnant women, neonates and immunocompromised adults. In 2000, the CDC reported that of all the foodborne pathogens tracked by the agency, *L. monocytogenes* had the second highest case fatality rate (21%) and the highest hospitalization rate (90.5%) (CDC, 2000; Ryser and Marth, 2007).

Almost all cases of human listeriosis are caused by contaminated food (Mead et al., 1999). Foods associated with listeriosis include dairy products such as cheeses, ice cream, chocolate milk, meats such as sausages, frankfurters, ham and pate, poultry, seafood and vegetables such as radishes and salad mixes. (Shank et al,1996; Ryser and Marth 2007). In 1999, frankfurters and deli meats from a processing plant in Michigan were implicated in an outbreak covering 22 states. There were more than 100 illnesses as well as 16 deaths and 6 miscarriages. More than 35 million lbs of the products were recalled at a cost of $75 million (CDC, 1999, Ryser and Marth, 2007). A risk assessment study conducted by the U.S. Department of Health and Human Service, Food and Drug Administration's Center for Food Safety and Applied Nutrition (DHHS/FDA/CFSAN) in collaboration with the U.S. Department of Agriculture's Food Safety and Inspection Service (USDA/FSIS) and in consultation with the DHHS Centers for Disease Control and Prevention to examine systematically the available scientific data and information to estimate the relative risks of serious illness and death associated with consumption of

different types of ready-to-eat (RTE) foods that may be contaminated with *L. monocytogenes* rated non-reheated frankfurters both on a per serving basis and on a per annum basis as high risk.

The FDA and USDA, based on the known characteristics of the organism have a "Zero tolerance policy" for *L. monocytogenes* in ready-to-eat (RTE) foods in the United States. Hence contaminated frankfurters could not only cause harm to human life but also cost millions of dollars because of recalls.

Temperatures above 56ºC cause protein unfolding, denaturation and ribosomal damage in *Listeria monocytogenes* (Anderson et al., 1991). It is known that *Listeria monocytogenes* cannot survive the cooking process. Therefore, contamination of RTE meats could occur in the post processing environments. Recirculating chill brines used for the cooling of the product could be a potential habitat for *Listeria monocytogenes* and other contaminants (Ryser and Marth, 2007). Research has demonstrated that the low temperatures and high salinity of chill brines are often not listeriostatic let alone listericidal and that the chill brines could serve as a vehicle of *L. monocytogenes* contamination. It is important to develop new hurdle technology that could reduce numbers of *L. monocytogenes* cells keeping in mind the physiochemical nature of the brine, cost of treatment and environmental considerations.

UV radiation has been used for the disinfection of surfaces, fluids and drinking water (Yuan et al., 2003; Yousef and Marth, 1988**)** because it is germicidal to bacteria, yeasts viruses, algae and protozoa. Ozone is an allotropic modification of oxygen that has been used for the disinfection of bottled water and waste water treatment. Both ozone and

UV radiation have demonstrated anti-listerial activity and do not leave harmful residual byproducts (Clifford, 1999).

Previous research has shown ozone and UV treatment to be beneficial in reducing bacterial content of poultry chill water and red meat processing waste water when used individually and in combination. Ozonated water has also shown anti-listerial activity on produce.

More information is required on the potential of UV radiation and ozone against *L. monocytogenes* in fresh chill brines and on turbid spent chill brines rich in organic content. An ideal technique would be one that has no impact on the sensory characteristics of the frankfurters but has the ability to completely eliminate high levels of *L. monocytogenes* in chill brine. Another aspect that should be taken into account is the recycling and disposal costs of chill brine. The hurdle technique should increase the longevity of the chill brine and render the chill brine environmentally safe for disposal and easy to treat apart from primarily eliminating *Listeria*. Research is required to determine the listericidal activity of ozone and UV radiation in fresh and spent chill brines. This research study aims to explore the potential of these treatments and parameters that maximize efficiency of these treatments.

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CHAPTER II

LITERATURE REVIEW

Listeria monocytogenes

Listeria monocytogenes has been recognized as a foodborne pathogen rather recently in human history as compared to other pathogenic microorganisms that have impacted human civilizations through large outbreaks. Though the isolation of *L. monocytogenes* from human tissue sections may have occurred as early as 1891, it was not until 1926 that the organism was fully described (McCarthy, 1990; Reed, 1958). Murray, Webb and Swann in 1926 isolated the small, Gram-positive rod bacterium responsible for an epizootic outbreak among rabbits and guinea pigs and named the organism *Bacterium monocytogenes.* The organism was named *Listerella hepatolytica* by Pirie in 1927 when he isolated the same organism from gerbils in South Africa. In 1940 the name *L. monocytogenes* was proposed by Pirie (Donnelly, 1994).

The first reported case of human listeriosis was in 1929 and the first perinatal case was reported in 1936 (Gray and Killinger, 1966). Human listeriosis is now recognized as a foodborne disease though the first foodborne case was not reported until 1953, when the stillbirth of twins was linked to the consumption of raw milk from a cow with listerial mastitis (Potel, 1953). Food was recognized as a primary route of transmission for human exposure after several large outbreaks in North America and Europe in the 1980's (Gellin and Broome, 1989). Coleslaw made from cabbages in fields fertilized with raw manure from sheep affected by fatal listeriosis, pasteurized whole or 2% milk, Mexican style soft cheese, Vacherin soft cheese were some of the foods implicated in those outbreaks. One

of the first microbiological food isolates from an Ready-to-Eat(RTE) product associated with sporadic clinical listeriosis in the United States was a microwave reheated turkey frank, consumed by an immunocompromised woman in 1988 (FDA and USDA, 2003; Lianou and Sofos, 2007).

The microorganism has been reported to cause disease in a wide range of wild and domestic animals, birds, crustaceans, fish, insects and reptiles (McCarthy, 1990). Modes of *Listeria* transmission include vertical (mother to child), zoonotic (contact between animal and man) and nosocomial (Hospital acquired), though most cases of *Listeria* transmission are believed to be foodborne (Mead et al., 1999; Roberts and Wiedman, 2003).

General Characteristics

Of the eight *Listeria* species (*L. monocytogenes, L. innocua, L. grayii, L. murrayi, L. welshimeri, L. seeligeri, L. innocua, L. ivanovii*), only *monocytogenes* is considered a pathogenic species. *Listeria monocytogenes* causes diseases in both humans and animals while *Listeria ivanovii* causes diseases predominantly in sheep. *Listeria seeligeri* and *L. ivanovii* have been implicated in human listeriosis on rare occasion (Cummins et al., 1994; Lessing et al., 1994). Only the hemolytic species of *Listeria* have been associated with human pathogenicity (Roberts and Wiedman, 2003). At least 13 serotypes exist for *Listeria monocytogenes* based on the cellular (O) and flagellar (H) antigens. Almost all disease is caused by 4b, 1/2a and 1/2b (Schuchat et al., 1991).

L. monocytogenes is a Gram-positive, non-sporulating, facultative intracellular foodborne pathogen. Cells are small (0.5 μm in diameter and 1-2 μm in length) rounded rods that are found singly or in short chains. In clinical specimens, *L. monocytogenes*

may appear as diptherioid, cocci or diplococci. The organism has polar flagella and expresses a typical tumbling motility at 20-25^{o}C but not at 35^{o}C (Bell and Kyriakides, 1998; Broadwater et al., 1973; Farber and Peterkin, 1991; Finch et al., 1988). *L. monocytogenes* is catalase-positive and oxidase-negative, slightly β-hemolytic and ferments a number of sugars, producing acid without gas. When viewed with Henry's illumination (light transmitted at a 45 degree angle) the colonies appear blue. Most strains are Methyl Red (MR) and Voges Proskauer (VP) positive. All strains grow under aerobic conditions on glucose forming lactate, acetate and acetoin as the main end products (Ryser and Marth, 2007).

L. monocytogenes is a psychrotroph and grows over a temperature range of 1^{o}C to 45^{o}C with an optimum around 30^{o}C - 37^{o}C (Schuchat et al., 1991). Growth can occur at pH levels between 4.4 to 9.4 and water activities ≥0.92 with NaCl as the solute (Petran and Zotolla, 1989). *Listeria monocytogenes* is salt tolerant and has been known to survive in commercial cheese brines (23.8% NaCl, pH 4.9) at 4ºC for 229 days (Larson et al., 1999) and grow in media containing up to 10% NaCl. Temperatures above 56ºC cause ribosomal damage and unfolding of proteins (Ryser and Marth, 2007). The robustness of *L. monocytogenes* makes it a post-processing contamination threat in food processing environments.

Environmental Occurrence and Distribution

L. monocytogenes is commonly found in soil, water, and plant material. The pathogen is wide spread in its existence and inhabits a wide ecological niche. It can survive longer under adverse environmental conditions than many non-sporeforming bacteria of importance in foodborne disease (Ryser and Marth, 2007; Schlech, 2000).

Listeria has been isolated from a variety of animals such as sheep, cattle, goats, pigs, chickens, turkeys and pheasants, fishes, crustaceans, mice, rats, rabbits, dogs, cats, deer, pigeons, parrots, frogs and insects (Ryser and Marth, 2007). Mead et al (1999) suggested that the consumption of contaminated food products is the cause of 99% of human listeriosis. Foods from which *L. monocytogenes* has been isolated include many dairy products such as cheeses, ice cream, and chocolate milk. Meats such as sausages, frankfurters, ham, pate, poultry, seafood and vegetables such as potatoes, radishes and salad mixes are also potential sources (Bell and Kyriakides, 1998).

The robustness and the ability to survive in diverse environmental niches make *L. monocytogenes* a post processing contaminant threat. Its presence is an indicator of poor processing plant hygiene. *L. monocytogenes* has been isolated from floors, drains, HVAC, coolers, condensate, conveyors, peelers, mops, sponges and even dust particles (Ryser and Marth, 2007).

When human disease is caused by *L. monocytogenes,* it more commonly occurs in the first 30 days of life (infants) and in patients older than 60 years. The high risk group for listeriosis includes pregnant women, neonates and immunocompromised adults, but the disease may occur in individuals with no predisposing underlying condition. The annual rate of listeriosis is 0.31 cases per million individuals but the rate is three times higher for persons over the age of 70 and is 17 times higher for pregnant women (Ryser and Marth 2007; Shank et al., 1996).

Approximately 500 of the 1250 estimated annual deaths from the top five pathogens (all serotypes of *Campylobacter, Escherichia coli* O157:H7, *E coli* non

O157:H7 STEC and *L. monocytogenes*) are due to *L. monocytogenes* (Gellin and Broome, 1989; Lianou and Sofos, 2007; Ryser and Marth, 2007).

L. monocytogenes is responsible for the highest hospitalization rates (91%) among foodborne pathogens in the United States with an overall fatality of 33% for recent outbreaks (CDC, 2000; Gellin and Broome 1989; Ryser and Marth, 2007; Schuchat et al., 1991; Tasara and Stephan, 2000). Listeriosis may occur as a mild gastrointestinal illness or as an invasive disease. The noninvasive disease occurs in healthy humans and the symptoms include fever, vomiting and diarrhea. Invasive disease can manifest itself as a neuropathic disease or septicemia. Invasive disease mostly occurs in adults with imunosupression. Listeriosis can also manifest as a febrile gastroenteritis syndrome (Schlech , 2000; Ryser and Marth, 2007).

Virulence genes and virulence related genes influence the outcome of human infection in *L. monocytogenes*. The organism has six virulence genes on an 8.2 kb pathogenicity island. These along with intracellular virulence genes are responsible for the intracellular infectious cycle (Roberts and Wiedmann, 2003).

L. monocytogenes enters the host primarily through the intestine. The liver is thought to be their first target organ after intestinal translocation. The listeriae multiply in the liver until controlled by cell mediated immune response. This subclinical step is fairly common due to listerial contamination of food with normal individuals maintaining antilisterial memory T cells, but in debilitated and immunocompromised individuals the unrestricted proliferation in the liver could result in prolonged low level bacterimia leading to invasion of the brain and the gravid uterus (the preferred secondary target organs) and to overt clinical disease (Farber and Peterkin, 1999; Ryser and Marth, 2007).

L. monocytogenes is facultative intracellular parasite able to survive in macrophages and able to invade a variety of nonphagocytic cells such as epithelial cells, hepatocytes and endothelial cells. In all these cell types, pathogenic listeriae go through an intracellular life cycle involving early escape from phagocytic vacuole, rapid intracytoplasmic multiplication, bacterially induced actin based motility, and direct spreading to neighboring cells in which they reinitiate their life cycle. In this way *L. monocytogenes* disseminates into the host tissue causing abortions, meningoencephalitis, sepsis and febrile gastroenteritis in immunocompromised, debilitated individuals, pregnant women, neonates and in healthy individuals in rare instances (Farber and Peterkin, 1991; Gellin and Broome, 1989; Ryser and Marth 2007).

Clinical manifestations of invasive listeriosis are usually severe and include abortion, sepsis, endophthalmitis, hepatitis, rhomboencephalitis and meningoencephalitis. Listeriosis can also manifest as a febrile gastroenteritis syndrome (Schlech, 2000; Ryser and Marth, 2007).

Listeria and Frankfurters

Listerial contamination not only causes harm to human lives but, also costs companies millions of dollars due to recalls. In 1999, frankfurters and deli meats from a processing plant in Michigan were implicated in an outbreak covering 22 states. There were more than 100 illnesses as well as 16 deaths and 6 miscarriages. More than 35 million lbs of the products were recalled at a cost of $75 million (CDC, 1999). Recent Class 1 recalls occurred in Utah 2004 where 1470 lbs of frankfurters were recalled (USDA, 2004).

It is estimated that 20% of the 1600 cases of listeriosis in the United States annually result from the consumption of non-reheated frankfurters and undercooked chicken (Schwartz et al., 1988). Palumbo and Williams (1991) showed that *Listeria* was quite resistant to killing or injury when suspended in ground beef, ground turkey and frankfurters, survived freezing to -18°C with a reduction of <1 log over a 14 week period. *Listeria monocytogenes* can survive refrigeration for long periods of time and it is imperative in preventing post processing contamination of RTE foods.

The U.S. Department of Health and Human Service, Food and Drug Administration's Center for Food Safety and Applied Nutrition (DHHS/FDA/CFSAN) conducted a risk assessment of foods capable of capable of harboring *L. monocytogenes* and causing listeriosis in collaboration with the U.S. Department of Agriculture's Food Safety and Inspection Service (USDA/FSIS) and in consultation with the DHHS Centers for Disease Control and Prevention (CDC). Uncooked frankfurters and deli meats were rated as "very high risk foods" for listerial contamination (USDA, 2004).

Frankfurter production process and possible routes of contamination

Frankfurters are a popular ready to eat food .They are typically prepared from a finely ground mixture (emulsion) of beef, pork or other meats, which is stuffed into artificial or natural casings. Large scale, commercial production of frankfurters is a mechanized process where meat batter is pumped into a stuffer/ linker machine. The batter is put into a tube like casing and the casings are then twisted at precise points to produce a long linked strand of equally sized frankfurters. After twisting the casings in approximately 6 inch intervals, the links are hung for smoking and cooked until an internal temperature of 65°C to 77°C is achieved. Once the casings are thoroughly

cooked, they are then showered with chill brine to help equalize their internal temperature. Skinless frankfurters have their artificial casing mechanically removed from the congealed meat mixture (Aberle, 2001).

Frankfurters are vulnerable to contamination with *Listeria* and other microorganisms during the peeling process. In addition, brine chillers have also been cited as potential sources of *L. monocytogenes*, leading to contamination of casing and product surfaces (Ryser and Marth, 2007).

Chill Brine and Listerial contamination

Chill brines are used to cool frankfurters once they are thoroughly cooked. The cooling is done to reduce time at which the temperature is conducive to bacterial growth. Chill brines are often recycled to reduce water usage and lower waste water treatment costs.

The objective of the cooling process is to increase the shelf life of the product. The salinity, low temperature and the infusion of nutrients from the product create an environment that could aid in the survival of *L. monocytogenes* (Ryser and Marth, 2007). Recycled brine harboring *L. monocytogenes* used to chill cooked frankfurters could contaminate the cooked frankfurters resulting in post processing contamination. The USDA states that brines may be reused to chill cooked products for various lengths of time based on salinity and temperature.

Table 2.1. Limits for Recycled Chill Brine Use (USDA, 1983)

	Solution Maintenance Conditions	
Duration of Brine Use	Minimum NaCl Concentration (% w/v)	Maximum Brine Temperature
One production shift	None	Undefined
Up to 24 h	5	40°F (4.4°C)
Up to 1 week	9	28°F (-2.2°C)
Up to 4 weeks	20	10°F (-12.2°C)

Numerous studies have been performed to explore the survival of *Listeria monocytogenes* in high salt concentrations. Larson et al. (1999) demonstrated that the pathogen can survive in commercial cheese brines (23.8% NaCl, pH 4.9) stored at 4ºC for 259 days. *L. monocytogenes* survived longer in brines held at 4°C than at 12°C.

A study by Gailey et al. (2003), using 20% salt brine in a test tube system with an initial cell count of 8 Log CFU, revealed that at pH values of 5, 6, 7 with chlorine concentrations of 2 and 3 ppm, an immediate drop of 0.28 Log CFU/ml with no significance between treatments ($P>0.05$) occurred. This was followed by a steady survival phase with a destruction slope near zero. At the inoculation concentration of 2 Log CFU in a test tube system (pH values of 5 and 7 with 0, 10 ppm of chlorine), the average initial drop for all treatments was 0.1 Log CFU/ml, followed by a steady survival

phase. It was observed that the destruction of cells was not drastic with these measures and that the recovery rate was less for *L. monocytogenes*.

Kim et al. (2005) studied *L. monocytogenes* survival and growth patterns in refrigerator dill pickles at 1.3, 3.8, and 7.6% salt concentrations. Pickling cucumbers were dipped into an inoculum of *L. monocytogenes*, brine mixtures were added, and cucumbers were held at room temperature for 1 week and then refrigerated for up to 3 months. The internal tissues of pickles with 1.3, 3.8, or 7.6% salt concentrations were presumptively positive for *L. monocytogenes* by the enrichment method for up to 49 days. At 91 days the surfaces of such pickles were still positive for *L. monocytogenes*.

Ye et al. (2001) found an increase in *Listeria* population in brine (sodium phosphate, sodium chloride, and lemon juice solids) used to enhance the moisture of pork. The cell count at the end of the process was significantly higher than the initial listerial population. *Listeria monocytogenes* is able to survive in chill brines and often have increases in cell numbers at ambient temperatures because of their wide range of growth temperatures (1–45°C) and their resistance to high salt concentrations. Extended survival of *Listeria* occurs at a wide range of salt concentrations. *L. monocytogenes* can persist for 150 days in pure salt and for 545 days in 0.85% NaCl (Pomanskaya, 1961).

There may be many factors that lead to the osmotolerance of *Listeria monocytogenes*. Osmoprotectants such as glycine, proline, betaine, carnitine and potassium ions may be involved in the osmotic shock response (Patchett et al., 1996). Processed meats (bologna, frankfurters, wieners, ham, bratwurst, salami, etc.) contain betaine at 0.34-0.48 nmol/mg and carnitine at 0.23-0.95 nmol/mg (Smith, 1996). The composition of the food may aid survival in high salt environments.

Osmoprotectant uptake of glycine, carnitine, and betaine has also been attributed to increased resistance to cold environments serving as cryoprotective osmolytes. Complex molecular mechanisms such as the production of cold shock proteins and other intracellular processes to maintain fluidity may also take place (Ryser and Marth 2007; Smith, 1996).

High salt concentrations reduce water activity (a_w), decrease turgor pressure in bacterial cells. Reduction of turgor pressure inhibits bacterial growth. Like most bacteria, *Listeria monocytogenes* grows optimally at a water activity (a_w) of > 0.97. However, the bacterium has the ability to multiply at water activity values as low as 0.90. Although *Listeria monocytogenes* does not grow at water activities less than 0.90, it survives in these environments particularly under refrigeration for long periods (Ryser and Marth, 2007).

Low water activity (a_w) may be listeriostatic, but rapid growth may resume as a_w increases (Farber and Peterkin, 1991; Gardan et al., 2003; Kim et al., 2006; Tasara and Stephan, 2000). Tolerance of *L. monocytogenes* to low temperature and low water activity should be considered during food applications that use brines.

Ozone

Ozone was discovered by C.F Schonbein in 1839, deriving its name from the Greek word *ozein*, which means" to smell" (Zeynep, 2003). Ozone has been used for the disinfection of bottled water and waste water treatment, but its applications are diverse. Hill and Rice (1982) have noted that the powerful oxidizing capacity of ozone has been used for purification and artificial aging of alcoholic beverages, odor control and medical therapy.

Ozone was granted generally regarded as safe (GRAS) status by the Food and Drug Administration for use in bottled water in 1982 (FDA, 1982) following which the USDA permitted ozone use for reconditioning recycled poultry chilling water (USDA, 1984). Ozone is formally approved by the FDA for use as a disinfectant or sanitizer in the gas or liquid phase on food including meat and poultry (21 CFR, Part 173).

Characteristics of Ozone

Ozone is an allotropic modification of oxygen with a pungent characteristic odor. It is produced in the earth's atmosphere photochemically. Commercially, ozone is produced by activating oxygen in air with radiation of 185 nm emitted by high transmission UV lamps. The corona discharge method has been used most widely to produce higher concentrations of ozone (Khadre et al., 2001; Zeynep, 2003)

Ozone is relatively unstable in aqueous solutions decomposing continuously to oxygen. Various parameters affect the disassociation of ozone in water. Solubility of ozone in water increases with a decrease in temperature and smaller bubble size. Purity and pH also greatly affect the solubility of ozone. Organic matter consumes ozone and may compete with microorganisms reducing efficiency of ozone (Kim et al., 1999a; Khadre et al., 2001; Zeynep et al., 2003).

The association of microorganisms with suspended matter or the presence of organic matter hampers impact of ozone with microorganisms. Restanio et al. (1995) reported that presence of 20 ppm of bovine serum albumin in solution significantly affected the death rate of Gram-positive (*i.e.*, *Staphylococcus aureus, Listeria monocytogenes*) and Gram-negative (i.e., *Escherichia coli* and *Salmonella* Typhimurium) microorganisms in ozonated water.

In solution ozone decomposes in a sequential fashion producing in turn hydroperoxyl, hydroxyl and superoxide radicals. Free radicals formed from ozone decomposition in aqueous solutions have high oxidizing power and may react with impurities (Khadre et al., 2001; Kim et al., 1999; Zeynep et al., 2003).

Detection of Ozone

Ozone detection methods may be classified into physical, physiochemical and chemical methods. These consist of UV absorption, acid chrome violet method, membrane electrode, the iodide method and the indigo method. The iodometric methods were not recommended for residual ozone determination because of potential errors resulting from air, hydrogen peroxide oxidation of ozone as well as pH affecting the air-iodide ratios (Straka et al., 1985). The indigo colorimetric method is a chemical method approved by the Committee on Standard Methods for the Examination of Water and Waste Water. It is used to measure residual ozone. Ozone adds across the carbon-carbon double bond of the sulfonated indigo dye resulting in its decoloration causing a change in absorbance. The change in absorbance can be measured spectrophotometrically. The method is fast and sensitive (Bader and Hoigene, 1981; Khadre et al., 2001).

Biological effects of ozone

Ozone is a powerful antimicrobial because of its progressive oxidation of vital cellular components. Ozone may cause damage to the unsaturated lipids and peptidoglycan in cell envelopes, to respiratory enzymes, to lipopolysaccharide layer of Gram-negative bacteria, to intracellular enzymes and to nucleic acids present in the cytoplasm. It may act as a protoplasmic oxidant leading to cell lysis and leakage (Broadwater et al., 1973; Khadre et al., 2001; Moore et al., 2000; White, 1999).

Chang (1971) reported that enzymes could be inactivated by ozone because of the oxidation of sulphydryl groups in cysteine residues. Unsaturated lipids in the cytoplasmic membrane form ozonide when the olefinic bonds are attacked upon exposure to ozone. The ozonide has high oxidation potential. It is unstable and exerts its own antimicrobial effect by attacking enzymes, sulfhydryl groupings and peroxides, releasing peroxyl compounds that are also antimicrobial. Thus the action of ozone is characterized by many other substances which can compete with or supplement the action of ozone to destroy crucial sites within the cell or to oxidize the protoplasm. This effect might be unique to ozone and its decomposition products (Chang, 1971; Kim et al., 1999; Komanapalli, 1996; Moore et al., 2000).

Genetic material might also be a potential site of damage. Studies by Scott (1975) on the DNA of *Escherichia coli* have shown a higher sensitivity to ozone by thymine in comparison to uracil and cytosine. Ozone has also been shown to produce single and double stranded breaks in DNA and decrease transcription activity (Hamelin, 1985; Ishizaki et al., 1981; Scott, 1975).

Bactericidal effects of ozone on Listeria monocytogenes *in aqueous media*.

Ozone is effective against a broad range of Gram-positive and Gram-negative bacteria and viruses, fungi and parasites. The sensitivity of *Listeria monocytogenes* to ozone has been indicated in many studies though data comparing the sensitivity of Gram positive bacteria to Gram negative bacteria is at times inconsistent.

Restaino et al. (1995) studied the antimicrobial effects of ozonated water against four Gram-positive and four Gram-negative bacteria in a recirculating concurrent reactor. Gram-negative bacteria were more sensitive than Gram-positive bacteria to ozone in pure

water. *Listeria monocytogenes* showed greater sensitivity than *Staphylococcus aureus* and *Enterococcus faecalis* to ozonated water. More than 5 log CFU/ml of *L. monocytogenes* cells were killed at time zero by ozonated deionized water. The death rates of *L. monocytogenes* and *Staphylococcus aureus* were comparable in presence of organic matter (20 ppm bovine serum albumin).

The effectiveness of ozone against food borne microorganisms was studied by Kim and Yousef (2000) in a batch type reaction system. The study revealed that *Listeria monocytogenes* was the least resistant to ozone. Similar results were observed in experiments by Kim and Yousef (2000) during treatment of foodborne spoilage bacteria and pathogenic bacteria to ozone suggesting sensitivity of *Listeria monocytogenes* to ozone.

Wade et al. (2002) studied the efficiency of aqueous ozone treatment in killing *Listeria monocytogenes* on inoculated alfalfa seeds and sprouts. The continuous sparging of seeds with ozonated water (initial concentration 21.3 ± 0.2μg/ml) for 20 minutes was effective in significantly reducing *Listeria monocytogenes* populations by 1.48 $\log_{10}$ CFU/g.

Difference in ozone sensitivity at 0.25 ppm, 0.40 ppm and 1.00 ppm in distilled water and phosphate buffer saline was found in all the six strains of *Listeria monocytogenes* examined by Fisher et al. (2000). Greater cell death was observed at lower temperatures (4ºC). Cells in the early stationary phase cells were less sensitive to ozone than mid-exponential and late stationary phase cells

Ozone for chill brines

Ozone is an effective disinfectant for treating recycled red meat processing waste water and for the treatment of poultry chill water. Waldroup et al. (1993) demonstrated that ozone can be successfully used to treat poultry chill water to allow for maximum recycling. A reduction in excess of 99% for total aerobes, *E. coli* and presumptive coliforms, post ozonation, was achieved. After ozonation, light transmission ranged from 88 to 99% (540 nm) as compared to fresh water.

Ozone proved to be a potent disinfectant for red-meat processing water (Wu and Doan, 2005). A 99% inactivation of aerobic bacteria, total coliforms and *E. coli* was observed. Ozone level of 23.9 mg/min/L was applied for eight minutes. Reductions in biological oxygen demand and chemical oxygen demand were observed though the light transmission and the level of total suspended solids did not vary significantly post treatment.

Ultraviolet Radiation

The germicidal effects of UV radiation have been known since the 1800s. The UV range of the electromagnetic spectrum is 100 to 400 nm and is categorized into UVA, UVB, UVC and vacuum UV. Wavelengths ranging between 220 and 300 nm are considered to be germicidal to bacteria, yeasts viruses, algae and protozoa. The highest germicidal effect is observed between 250-270 nm; hence a wavelength of 254 nm (generated by low pressure mercury lamps) is used for the disinfection of surfaces, fluids and drinking water (Yaun et al., 2003, Yousef and Marth, 1988). Antimicrobial effect of UV radiation occurs due to the photochemical changes that take place in proteins and nucleic acids when the UV radiation is absorbed. Death occurs due to the disruption of transcription and replication of DNA due to mutations. Mutations are caused by the

excitation of electrons in DNA molecules resulting in the formation of pyrimidine dimers in the chromosome.

In 1971, Collins determined the susceptibility of *Listeria monocytogenes* on Trypticase Soy Agar plates to UV radiation emitted from a 14 W cold cathode mercury vapor lamp. A radiation output of 40 W/cm^2 was chosen and was at a distance of 40 cm from the source. Time of exposure was 30, 60, 90 and 120 seconds. A ten-fold population decrease was found after the first 60 seconds of irradiation.

Various foods and beverages have been treated with UV radiation to decrease bacterial content and eliminate pathogens such as *Listeria monocytogenes*. A study that involved the effect of UV radiation on pathogens present in peptone water by Kim et al., (2006) revealed that intensities of 250 to 600μW/cm^2 reduced all suspended pathogen cells in peptone water by 5 log cycles after 2 min. *Listeria monocytogenes* was completely reduced after 3 min treatment; a reduction of 8.39 Log cycles.

Matak et al. (2005) studied the efficacy of UV light in the reduction of *Listeria monocytogenes* in goat's milk. A CiderSure 3500 apparatus was used to expose 10^7 cells of *Listeria monocytogenes* to UV light. More than a 5 log reduction was achieved when the milk received a cumulative UV dose of 15.8 mj/cm^2. The results indicate that UV radiation could be used to reduce listerial contamination of non-transparent (colloidal) fluids.

Listeria. innocua inoculated into apple cider was more resistant to UV than *E. coli* K-12. *L. innocua* population was reduced by 2.5 ± 0.1 log after being exposed for 58 seconds while *E. coli* had a 3.4 ± 0.3 log reduction after being exposed for 19 seconds (Geveke, 2005).

The efficiency of UV radiation in reducing levels of *Listeria monocytogenes* might depend on factors such as the type of strain, food source the organism is present in, opacity of the fluid and the presence of suspended solids. UV radiation has many advantages and has been used in dairy, meat and fish processing plants. It is less expensive than chlorination, leaves no residue and does not have legal restrictions (Yousef and Marth, 1988).

Combined UV- ozone treatment

The disinfection potential of ozone, UV-C radiation and ozone/UV-C combination was investigated for reducing microbial flora of fresh cut onion, escarole, carrot and spinach wash waters collected from food manufacturing operations. Ozone, UV and their combined use were effective disinfection treatments for vegetable wash water. A 6.6 CFU/ml reduction was the maximum achieved after 60 minutes of treatment. Turbidity of the wash water was reduced considerably after O_3 and O_3/UV treatments while not affecting the physiochemical properties of the water. The results suggest that O_3 and O_3/UV have potential as alternate sanitizers that are cost effective and environmentally friendly as they might promote water reuse (Selma et al., 2008).

The combination of UV radiation and ozone proved effective in lowering *E. coli* O157:H7 in poultry chiller water. UV and ozone treatment combined yielded an additional inactivation effect that was a sum of the individual hurdles. UV enhanced ozonation of unscreened overflow chiller water from a commercial poultry had a significant effect on microbiological parameters with possible synergistic effect when used in combination. The process improved the microbiological quality; turbidity and

water reuse efficiency while resulting in greater than 99.9 % reduction of pathogenic microorganisms (Diaz et al., 2002; Waldroup et al., 1993; Wu and Doan, 2005)

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Chapter III

Factors affecting efficiency and measure of Ozone and UV irradiation treatments on *Listeria monocytogenes* populations in chill brines.

Keywords: *Listeria monocytogenes*, brine, ozone, UV radiation

ABSTRACT

The parameters influencing optimal ozonation and measure of ozone- UV dosage of fresh (9% NaCl, 91.86%. transmittance) and spent chill brine (20.5% NaCl, 0.01% transmittance) were evaluated. These included diffuser design, comparison of kit to standard methods for measure of residual ozone, studying the effect of ozone on uridine absorbance and determining presence of residual listericidal activity post ozonation. An ozone diffuser was designed using clear PVC tubing with perforations for better ozonation of brines and its efficiency was tested. The perforated tube sparger diffused 1.44 ppm of ozone after 30 minutes of ozonation and the solution had an excess of 1 ppm in 10 minutes of ozonating fresh brine solution (200ml). Accuracy of the Vacu-vial Ozone analysis kit was evaluated by comparing the performance of the kit to the standard indigo colorimetric method for measuring residual ozone. The kit was inaccurate in determining residual ozone levels of spent brines and 1% peptone water. Uridine was evaluated as a UV actinometric tool for brine solutions that were ozonated before UV treatment. The absorbance of uridine (A_{262}) changed with times of ozonation from 0.1329 to 0.0512 for standard 10 minutes UV exposure duration, indicating that the variability in absorbance of uridine might hamper fluence determination accuracy. No residual listericidal activity was found in spent brines after ozonation was stopped. The population of *L.monocytogenes* in the brine (0 minutes) was 5.31 Log CFU/ml and was 5.08 Log CFU/ml after a 60 minutes, indicating a lack of significant reduction in population.

INTRODUCTION

Ozone is an allotropic modification of oxygen. It is used for the disinfection of bottled water and waste water treatment because of its powerful oxidizing capacity (Hill and Rice, 1982). Commercially, it is produced by activating oxygen in air with radiation of 185 nm emitted by high transmission UV lamps or by the Corona Discharge method (Khadre et al., 2001; Zeynep, 2003).

Ozonation is usually accomplished by dissolving gaseous ozone into a fluid being treated in order to react with target pathogens. Ozone is relatively unstable in aqueous solutions decomposing continuously to oxygen (Kim et al., 1999; Zeynep, 2003). Various parameters affect the disassociation of ozone in water such as the temperature of the liquid, bubble size, agitation of the fluid, organic content of the liquid medium (Khadre et al, 2001). The association of microorganisms with suspended matter or the presence of organic matter hampers association of the microorganisms with ozone. Organic matter consumes ozone and may compete with microorganisms reducing efficiency of ozone Purity and pH also greatly affect the solubility of ozone (Khadre et al., 2001; Kim et al 1999a; Zeynep et al, 2003). Ozonation has been used to treat poultry chiller water and red meat processing water and has been effective in pathogen elimination (Diaz et al., 2001; Wu and Doan, 2005).

Advanced oxidation processes are treatments that generate highly reactive by-intermediates such as hydroxyl ions ($^{\cdot}OH$). UV enhanced ozonation of unscreened overflow chiller water from a commercial poultry had a significant effect in reducing microbiological counts. A possible synergistic effect was suggested when treatments were used in combination (Diaz et al., 2001; Waldroup et al., 1993). The presence of a

residual antimicrobial effect after ozonation in spent chill brines needs to be explored when ozone and UV are used as separate treatments.

Ozone detection methods can be classified into physical, physiochemical and chemical methods. The DPD method is an iodometric technique. Iodometric methods were not recommended for residual ozone determination because of potential errors resulting from air, hydrogen peroxide oxidation of ozone as well as pH affecting the air-iodide ratios (Straka et al., 1985). The remaining methods for ozone detection include direct UV absorption, acid chrome violet method, membrane electrode and the indigo method (Straka et al., 1985). The indigo colorimetric method is a chemical method that is used to measure residual ozone. It has been recommended for measuring residual ozone in water and in aqueous ozone samples that contain organic and inorganic matter. The method is approved by the Committee on Standard Methods for the Examination of Water and Waste Water (APHA, 2005)

When using the indigo colorimetric method to measure residual ozone, ozone adds across the carbon-carbon double bond of the sulfonated indigo dye resulting in its decoloration causing a change in absorbance. The change in absorbance can be measured spectrophotometrically. The indigo method is rapid and sensitive (Bader and Hoigene, 1981; Khadre et al., 2001).

CHEMetrics Vacu-Vials (K7403, Calverton, VA) kit uses a methyl substituted form of the DPD (diethyl-p-phenylenediamine) reagent for the determination of ozone levels. The method has been used to determine levels of residual ozone in water used to wash produce (Garcia et al., 2003). The stability of the method for measuring ozone levels in spent brine was investigated.

Uridine is a uracil with a ribose ring attached. When uridine is exposed to UV radiation it undergoes a 5', 6' photohydration reaction (Swenson and Setlow, 1963). A water molecule replaces the 5', 6' double bond forming a photohydrate product. The change in absorption is used to measure the UV absorbed by the sample. Genetic material might also be a potential site of damage during the ozonation process. The effect of ozone on the absorbance of uridine needs to be evaluated as ozone is a strong oxidizing agent and could affect the effectiveness of urdine as an actinometric tool (Zeynep et al, 2003).

The objectives of this study were to (a) compare the efficiency of a modified sparger design and commercial sparger to introduce ozone into a brine solution, (b) to assess the use of uridine as an actinometric tool during ozone and UV treatment of brines, (c) to compare the efficiency of a modified DPD method in a commercial testing kit (CHEMetrics Vacu-Vials , ozone K-7403, Calverton, VA) for measuring residual ozone in spent brines and fresh brines containing peptone, and (d) to assess residual antimicrobial activity of ozone after ozonation.

MATERIALS AND METHODS

Fresh Brine Preparation. Fresh brine was prepared by dissolving 90 g of Top-Flo evaporated salt (7559 Cargill, Minneapolis, MN) in 1000 ml distilled, autoclaved water in a volumetric flask. After dissolving, the brine was filter sterilized using 0.45 μM pore size filter (Whatman International Limited, Maidstone, England) and stored at 2-4°C until use. Fresh brine was prepared within 24 hours of use.

Spent Brines. Recycled spent brine was obtained from a frankfurter processor after its maximum usage. Each batch of spent brine (approximately 1000 L) was shipped via refrigerated truck and stored at 4°C until use. Twenty-four hours prior to treatment, the

spent brine was filter sterilized using nylon membrane filters, 0.45μM pore size (Whatman International Limited, Maidstone, England) to remove existing microbial flora and the filtered brine was stored at 2-4°C until use.

Sparger. The CD-3KB (Ozone solutions, Iowa. U.S.A) sparger was used for comparison with the perforated tube sparger. The CD-3KB sparger had a maximum pore size of 80 microns and nominal particle retention of 25 microns. The dimensions of the sparger were 1.5-in x 0.75-in x 0.75-in with 1/4-in barb kynar (PVDF) connection. The "perforated tube" sparger was designed in the Department of Food Science and Technology at Virginia Polytechnic and State University. Clear PVC tubing (Nalgene 180 clear plastic tubing,I.D.,1/8 inch x O.D.3/16 inch x Wall 1/32 inch, Nalgene Nunc Int. Corp; Rochester, N.Y., U.S.A.) was perforated using a 12 gauge insulin syringe (Kroger). Maximum perforations were created in the middle of the tube with the concentration of perforations tapering towards the edges to provide even distribution of ozone bubbles.

Comparison of modified sparger to commercial sparger for efficiency in ozone diffusion. 200 ml of fresh brine (prepared as described previously) was added to a pre-sterilized container (Nalgene 300 filter receiver, Nalgene Nunc Int. Corp; Rochester, N.Y., U.S.A.).The container housed the CD-3KB sparger (Ozone solutions, Iowa. U.S.A) which was placed in the bottom center, immersed in the brine. Brines were ozonated using the ozonation unit (Golden Buffalo; Orange, Calif., U.S.A.) for treatment times of 10, 20 and 30 min.The ozonated brine was sampled to determine the residual ozone after

each treatment using the indigo colorimetric method. For comparison, the "perforated tube" sparger was fitted to the bottom inside circumference of a sterilized container .The container was filled with 200 ml of fresh brine solution. The container was placed on top of a stir plate and the stir bar was spun at medium speed throughout the ozone application. The ozonation unit was used to ozonate the brine samples for 10, 20 and 30 min. Samples were collected post treatment and analyzed for residual ozone using the indigo colorimetric method.

Effect of ozonation on the absorbance of uridine. To observe the effect of ozone on the absorbance of uridine (A15227 Alfa Aesar, WardHill, MD), a 10^{-4}M uridine working solution was prepared by adding 1 ml of the 10^{-2}M stock solution to a 100 ml volumetric flask and bringing it up to volume with fresh brine. The brine containing the uridine was ozonated for time intervals of 1, 10, 20 and 40 minutes and then treated with UV for 10 minutes. The absorbance of the sample was measured (Ab_{262}) using a Shimadzu spectrophotometer (Model UV-2101PC).

Comparison of Chemetrics vacu-vials to standardized protocol for residual ozone measurement. Spent brine, 200 ml, was added to a sterilized container (Nalgene 300 filter receiver, Nalgene Nunc Int. Corp; Rochester, N.Y., U.S.A.) housing the perforated tube sparger fitted to the bottom inside circumference. A stir bar was placed in the center of the container. The container was placed on top of a stir plate and the stir bar was spun at medium speed throughout the ozone application. The ozonation unit was used to ozonate the brine samples for 15, 30, 45 and 60 min. Residual ozone of the brine samples

were measured using CHEMetrics Vacu-Vials (ozone K-7403, Calverton, VA) and the Indigo Colorimetric Method (Standard methods for the examination of waters and waste waters, 20th edition) (APHA, 2005).

Residual antimicrobial effect of Ozone. A 200 ml portion of *Listeria*-brine suspension was ozonated for 60 minutes. After 60 minutes of ozonation the unit was switched off. Bacteriological enumeration of the ozonated *Listeria*-brine suspension was done at 0, 10, 20 and 60 minutes post ozonation. Enumeration of *L. monocytogenes* was done by serially diluting the ozonated brine sample in 0.1% peptone (211677 Becton, Dickinson and Co, Sparks, MD) and spread plating 0.1 ml onto duplicate MOX plates , prepared with Oxford Medium Base (222530, Becton, Dickinson and Co, Sparks, MD) and incubating at 37°C for 48 hours.

RESULTS AND DISCUSSION

Comparison of modified sparger to commercial sparger for efficiency in ozone diffusion (Chart 3.2). The modified sparger generated 1.10 ppm of ozone in 200 ml of fresh brine solution and the concentration of ozone increased to 1.44 ppm after 30 minutes of ozonation. The CD-3KB ozone diffuser (Ozone solutions, Iowa, U.S.A) produced 0.38 ppm of ozone after 10 minutes and the concentration reduced to 0.27 ppm after 30 minutes. Both experiments were carried out with 200 ml of fresh brine solution. The small amount of liquid used (200ml) and the lack of depth for proper diffusion of the bubbles in the bench-top set up could have caused off gassing and less diffusion into the liquid. Solubility of ozone in water increases with a decrease in temperature and smaller

bubble size (Kim et al., 1999a). The pores caused by insulin syringe perforation and the fitting of the tubing to the internal diameter could have resulted in the availability of more depth and thus more bubble dispersion volume. These factors could have resulted in enhanced performance of the perforated tube sparger.

Effect of ozonation on the absorbance of uridine (Table 3.4). Uridine is an actinometric tool used to calculate the dose of UV absorbed by the treated liquid. The dose is calculated based on the change in absorbance of uridine when it is exposed to UV light. Uridine is a uracil with a ribose ring attached, which exposed to UV radiation undergoes a 5', 6' photohydration reaction. A water molecule replaces the 5', 6' double bond forming a photohydrate product causing a change in absorbance at 262 nm (Swenson and Setlow, 1963). Ozone has been known to effect nucleotide bases in bacteria. Studies by Scott on the DNA of *E. coli* have shown that higher sensitivity of thymine in comparison to uracil and cytosine. Ozone has also been shown to produce single and double stranded breaks in DNA and decrease transcription activity (Hamelin, 1985; Ishizaki et al., 1981; Scott, 1975).When the absorbance of uridine was measured after different ozonation times followed by a constant UV exposure time of 10 minutes, a varied absorbance was observed. The absorbance dropped from 0.1329 after 1 minute of ozonation to 0.0512 after 40 minutes of ozonation. A constant UV exposure time of 10 minutes was maintained throughout the experiment. The results suggest that ozone might interact with uridine as it has the propensity to react with genetic material and might cause differences in absorbance leading to miscalculation of ozone dose (Hamelin, 1985;

Ishizaki et al., 1981; Scott, 1975). The stability of uridine at higher doses and for longer periods of UV exposure needs to be evaluated.

Comparison of Chemetrics vacu-vials to standardized protocol for residual ozone measurement (Table 3.1). The CHEMetrics Vacu-Vials (ozone K-7403, Calverton, VA) kit detects iodine that is produced when potassium iodide reacts with ozone by using a methyl substituted form of the DPD (Diethyl-p- phenylene diamine) reagent. The color change is determined spectrophotometrically to measure the residual ozone in water. CHEMetrics Vacu-Vials have been used to determine the residual level of ozone in water used to wash lettuce (Garcia et al., 2003). The kits indicated that the presence of halogens or halogenating agents could produce color with the reagent. The indigo colorimetric method is a chemical method used to measure residual ozone and is approved by the Committee on Standard Methods for the Examination of Water and Waste Water. The method is fast and sensitive and has been recommended for use in water with organic and inorganic load (Bader and Hoigne, 1981; Khadre et al., 2001). The test was conducted to study the accuracy of the kit in solutions with high organic content, such as spent brines. The accuracy was determined based on comparison to residual ozone levels determined by the indigo colorimetric method. A surrogate 1% peptone solution was also used to model a solution with an organic load.

When ozonated spent brine was tested using CHEMetrics Vacu-Vials, the residual ozone in the spent brine after 15 minutes of ozonation was 1.26 ppm and 2.38 ppm after 30 minutes. Brine analyzed using the indigo colorimetric method showed no detectable levels of ozone after 30, 45 and 60 minutes of ozonation. Distilled water with 1% peptone

showed high levels of ozone (> 2.38 ppm) after 30 minutes of ozonation when measured using CHEMetrics Vacu-Vials. Organic matter consumes ozone and hence liquids with high levels of organic content would usually tend to show lower levels of residual ozone. Disparity between measurements in comparison to the standard accepted indigo colorimetric technique occurred when CHEMetrics Vacu-Vials were used to measure residual ozone in spent brines.

Residual antimicrobial effect of Ozone (Table 3.3). Ozonated spent brines were tested for the presence of a residual listericidal effect post ozonation. The population of *Listeria monocytogenes* cells was determined at 0, 10, 20 and 60 min after the ozonation treatment of the brine ended. The population of the brine at the end of the ozonation treatment was 5.31 Log CFU/ml. When the brine population was enumerated 10 minutes later, the population of cells was 5.27 Log CFU/ml. The population did not have a considerable drop or increase as indicated by the table. The population of *L. monocytogenes* 60 minutes post-treatment was 5.08 Log CFU/ml. The absence of a considerable drop in population indicated a lack of residual antimicrobial activity. The high organic content of the brines could have increased the ozone demand. The residual level of ozone in chill brines after 60 minutes was below detectable levels.

CONCLUSIONS

Optimal ozone diffusion requires the production of small size bubbles with depth of the liquid to facilitate diffusion. From experimental evaluation it was determined that, for measuring residual ozone, the Indigo Colorimetric test was most stable and that the

CHEMetrics Vacu-Vials (ozone K-7403, Calverton, VA) was inaccurate in measuring dissolved ozone content in spent brines and in 1% peptone water. It is important to note that CHEMetrics Vacu-Vials kit is not marketed for use in solutions that contain substances that may interfere with interpretation of results. The absorbance of uridine in solution treated for 10 minutes with UV irradiation dropped with increase of ozonation time. The drop in absorbance indicates that uridine might be unsuitable for UV actinometric measurements when ozonation is used in combination with UV treatment. No residual ozone was found on spent brines. The population after 60 minutes of ozonation was 5.31 Log CFU/ml and remained at 5.08 Log CFU/ml. The lack of change in population of *listera* cells co-related to the lack of a residual antimicrobial effect in the brines post ozonation. These results were useful for the design of experiments to determine the duration of ozonation and UV exposure to reduce *Listeria monocytogenes* in chill brines.

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TABLES

Table 3.1 Evaluation of the accuracy of CHEMetrics Vacu-vials in measuring the residual ozone level in spent brine by comparing values measured using standard indigo colorimetric method.

Ozonation time Minutes	Ozone level Vacu-vials[a] ppm	Ozone level Indigometric method[b] ppm
15	1.26	ND[d]
30	2.38	ND[d]
45	AR[c]	ND[d]
60	AR[c]	ND[d]

.

c Above the range of detection

d Not detectable, below the range of detection

Table 3.2 Comparison of perforated tube sparger performance vs CD 3KB ozone diffuser to ozonate 200 ml of fresh brine.

Time of Ozonation Min	Perforated tube sparger Residual ozone level(ppm)	CD 3KB Residual ozone level(ppm)
10	1.10	0.38
20	1.29	0.35
30	1.44	0.27

Table 3.3 Enumeration of *Listeria monocytogenes* population in spent brines post ozonation at 0, 10, 20 and 60 min intervals for indication of residual listericidal activity.

Time interval post ozonation (Minutes)	Listeria population[a] (Log CFU/ml)
0	5.31
10	5.27
20	5.25
60	5.08

Table 3.4 Absorbance of uridine in standard 10 min treated uridine-brine solution (10^{-4}) after different ozonation times.

Time of Ozonation Min	Absorbance (262 nm) after 10 minutes UV exposure[a]
1	0.1329
10	0.0746
20	0.0554
40	0.0512

a Absorbance of the sample measured spectrophotometrically at 262 nm.

Chapter IV

Effect of Ozone and Ultraviolet radiation on *Listeria monocytogenes* populations in chill brines

Keywords: Listeria monocytogenes, brine, ozone, UV radiation, uridine
Note: This chapter has been formatted for submission to the Journal of Food Protection

ABSTRACT

This study was conducted to explore the efficacy of Ozone and Ultra Violet light treatment as hurdles against *Listeria monocytogenes* in fresh and spent brines. The duration of ozone-UV radiation treatments to decrease levels of *Listeria* were determined. Fresh brines had 9% NaCl concentration with a transmittance of 91.86%. Spent brine (received from national RTE meat manufacturer) had 20.5% NaCl concentration with 0.01% transmittance. The effects of ozone on the physical characteristics of spent brines such as color and transmittance were also evaluated. Ozonation for 5 min caused a 5.29 mean log reduction while 5 minutes of UV exposure resulted in a 1.09 mean log reduction of *L. monocytogenes* cells in fresh brines. Ten minutes of ozonation led to a 7.44 mean log reduction and 10 minutes of UV radiation caused a 1.95 mean log reduction of *Listeria*. Ten minutes of Ozonation and UV exposure in combination resulted in greater than 8 log reduction in *L .monocytogenes* counts. Spent brines required 60 minutes of ozonation for a 4.97 mean log reduction in *L. monocytogenes* counts, while 45 minutes resulted in a 4.04 mean log reduction. Ten minutes of UV exposure of the spent brines resulted in 0.30 mean log reduction in *Listeria* cells. A combination of 60 minutes ozonation and 10 minute UV exposure resulted in an excess of 5 log reduction in cell counts. Ozonation did not cause a sufficient increase in the transmittance of the spent brine to aid UV penetration. Ozonation for sufficient time had considerable listericidal activity in fresh brines and spent brines .Ozonation in combination with UV treatment is an effective hurdle and aided in reducing *L. monocytogenes* to undetectable levels in fresh brines.

INTRODUCTION

Listeria monocytogenes is a foodborne pathogen that causes illness ranging from mild gastroenteritis to severe invasive disease. Those at high risk for listeriosis include pregnant women, neonates, the elderly and the immunocompromised (Ryser and Marth, 2007). Clinical manifestations of invasive listeriosis are usually severe and include abortion, sepsis, meningoencephalitis, and death. Listeriosis can also manifest as a febrile gastroenteritis syndrome (S). The Centers for Disease control and prevention (CDC) in 2000, reported that amongst foodborne pathogens, *L. monocytogenes* has the second highest case fatality rate (21%) and the highest hospitalization rate (90.5%).

Non-reheated frankfurters and deli meats have been classified as "high risk foods" for *Listeria* contamination and a "Zero tolerance policy" for *L. monocytogenes* in RTE (ready-to-eat) foods exists in the United States (Shank et al., 1996). Clearly *L.monocytogenes* contamination of frankfurters is a threat to human health, but it should also be noted that *Listeria*-associated recalls cost millions of dollars annually (CDC, 2000).

L.monocytogenes is a post processing contaminant threat capable of existing in various niches of the food production environment. (Ryser and Marth, 2007). It can survive longer under adverse environmental conditions than many non-spore forming bacteria of importance in food borne disease (Schlech, 2000). The halotolerance and pshychrotolerance of *L. monocytogenes* highlight its potential as a contaminant of brines used to chill RTE foods post heat processing (Larson et al, 1999; Petran and Zotolla, 1989).

Chill brines, used to cool frankfurters once they are thoroughly cooked, are often recycled to cut down on production, waste water treatment costs and reduce water usage. The United States Department of Agriculture (USDA) requires chill brines to have a minimum NaCl concentration of 9% at -2.2°C in order for reuse of up to a week and 20% NaCl at -12.2°C for 4 weeks of reuse (USDA, 2000). *L. monocytogenes* has been known to survive in cheese brines (23.8% NaCl, pH 4.9) stored at 4ºC for 259 days (Larson et al, 1999). Palumbo and Williams, (1991), demonstrated that *Listeria* was quite resistant to inactivation or injury when suspended in ground frankfurters, surviving freezing to -18°C with a reduction of < 1 log over a 14 week period. Thus the use of additional hurdles to prevent post processing contamination is necessary.

Ozone (O_3) is an allotrope of oxygen used for disinfection of bottled water and waste water treatment (Hill and Rice, 1982). In bacteria, it may act as a protoplasmic oxidant causing progressive oxidation of vital cellular components (Khadre et al., 2001). It is approved by the United States Food and Drug Administration for use as a disinfectant or sanitizer in the gas or liquid phase on food including meat and poultry and has GRAS status (FDA, 1982; USDA, 1984). Ozone is effective against a broad range of Gram-positive and Gram-negative bacteria with *Listeria* showing high sensitivity to ozone (Khadre et al., 2001; Zeynep et al., 2003)

UV radiation has been used for the disinfection of surfaces, fluids and drinking water because it is germicidal to bacteria, yeasts viruses, algae and protozoa (Yaun et al., 2003; Yousef and Marth, 1988**)**. The antimicrobial effect of UV radiation occurs due to the photochemical changes that take place in proteins and nucleic acids when UV radiation is absorbed by the cell (Lopez-Malo and Palou, 2005). Various foods and

beverages have been treated with UV radiation to decrease bacterial content and eliminate pathogens such as *L. monocytogenes.*

Both ozone and UV radiation have demonstrated anti-listerial activity and do not leave harmful residue (Clifford, 1999). Previous research has shown ozone and UV treatment to be beneficial in reducing bacterial content of poultry chill water and red meat processing waste water when used individually and in combination (Diaz et al., 2002; Waldroup et al., 1993; Wu and Doan, 2005).

The purpose of this study was to (a) determine the time of ozonation and UV treatment to reduce *L. monocyotgenes* in fresh brine, (b) determine ozonation and UV treatment time to cause decrease in *L. monocytogenes* populations in spent brine, and (c) determine the effects of these treatments on the color and UV transmittance of spent brines.

MATERIALS AND METHODS

Ozone Equipment. A bench top activated oxygen generator was used for ozonating the brines (Golden Buffalo; Orange, Calif., U.S.A.) generating 0.9 g ozone/h at a flow rate of 2.4 L/min. Ozone was produced by passing room air over a UV lamp (Voltarc, GPH287T5/VH-S400/CB) having a UV output of 3W and a spectral output of 185 NM and pumped into the solution from the system through a delivery tube (0.25-inch inner diameter; Nalgene 180 PVC; Nalgene Nunc Int. Corp; Rochester, N.Y., U.S.A.) Ozone was sparged through a perforated tubing ring (Nalgene 180 clear plastic tubing, I.D.,1/8 inch x O.D.3/16 inch x Wall 1/32 inch, Nalgene Nunc Int. Corp; Rochester, N.Y., U.S.A.) fitted to the bottom inside circumference of a pre-autoclaved container (Nalgene 300 filter receiver (Nalgene Nunc Int. Corp; Rochester, N.Y., U.S.A.). The Nalgene

container was placed on a magnetic stirrer/ hotplate (Fisher scientific) and a stir bar was placed in the center of the receiver to facilitate further diffusion of the gas into the solution. All ozonation experiments were carried out in a chemical hood.

UV Equipment. For UV treatment an Oriel photoreactor (Model 66901, Newport Stratford, Stratford, CT) was used. The photoreactor consisted of an aluminum arc lamp housing unit, 350-Watt mercury bulb (Model 6286, Newport Stratford, Stratford, CT), and a digital power supply unit. A reflector located parallel to the bulb focused the beam onto a condensing lens, producing a collimated (straight) beam directed horizontally into the UV chamber. A two inch, 253.7±10 nm directional filter (Model 56501, Newport Stratford, Stratford, CT) was attached to the end of the sample container to exclude all other wavelengths (250 to 2500 nm) produced by the mercury bulb. Maximum intensity (350–400 mW/cm²) of the mercury bulb and was measured using a radiant power meter with thermopile sensor (Models 70260, 70263, Newport Stratford, Stratford, CT). Thermopile detectors are sensitive to environmental temperatures because they work by absorbing radiation and measuring the resultant temperature increase. A fused silica filter (Model 70185, Newport Stratford, Stratford, CT) was mounted to the sensor to minimize drift in measurements, because of the sensitivity of the thermopile sensors to environmental temperatures. Constant power to the bulb was provided by a digital power supply unit that displayed the wattage, and approximated the bulb hours (rated at 1000 hrs maximum). A 24x12x12 in polystyrene chamber with an opening for the condensing lens on one side and a pocket for the sensor directly opposite, lined with particle board to

prevent light penetration, was constructed to house the 4x4x2 in sample stand during UV exposure.

Before use the quartz cylinders were rinsed with acetone (42324-0040 Fisher Scientific, Suwanee, GA), followed by sterile, ditilled water and autoclaved along with a 6x3x2 mm stir bar placed within the container. During UV treatments, a stirrer housed inside the sample stand was used to continuously stir the test liquid. Sample temperature was maintained at 10–15°C by re-circulating a 50/50 mix of water and ethylene glycol through the UV chamber and through the sample stand for one hour before and during UV application.

Culture maintenance. *Listeria monocytogenes* strains N1-227, serotype 4b isolated from hotdog batter implicated in a 1998 outbreak; N3-031, serotype 1/2a, isolated from turkey franks; and R2-499, serotype 1/2a, isolated from a RTE meat product were obtained from Dr. Kathryn Boor and Dr. Martin Wiedmann of Cornell University (Fugett et al., 2006). These were stored in cryovials of Nutrient Broth (23300,Becton, Dickinson and Co, Sparks, MD) supplemented with 15% glycerol (G33-1 FisherScientific, Suwanee, GA), and were obtained from storage at -70°C from the culture collection at the Department of Food Science and Technology, Virginia Polytechnic Institute and State University. Monthly, one cryovial of each strain was thawed at room temperature. Using a sterile prepackaged inoculating loop (13-070-3 Fisher Scientific, Suwanee, GA), 10µl of inoculum was transferred into 10 ml of Tryptic Soy Broth (Becton, Dickinson and Co, Sparks, MD) + 0.6% yeast extract (TSBYE) and incubated at 32°C for 48 hrs. Next, 10 µl was streaked onto Modified Oxford Agar plates (MOX) prepared with Oxford Medium

Base (Becton, Dickinson and Co, Sparks, MD) supplemented with Modified Oxford Antimicrobic Supplement (Becton, Dickinson and Co, Sparks, MD) and incubated at 35°C for 48 hrs. Strains were confirmed, using *Listeria* API (Biomerieux, l'Etoile, France). A confirmed colony was then transferred into 10 ml of TSBYE and incubated at 35°C for 48 hrs. After incubation, a loopful of culture was streaked onto multiple TSAYE slants, incubated at 35°C for 48 hrs, and stored at 2-4°C for up to one month as stock cultures.

Fresh Brine Preparation. Fresh brine was prepared by dissolving 90 g of Top-Flo evaporated salt (7559 Cargill, Minneapolis, MN) in 1000 ml distilled, autoclaved water in a volumetric flask. After dissolving the brine was filter sterilized using 0.45 μM pore size filter (Whatman International Limited, Maidstone, England) and stored at 2-4°C until use. Fresh brine was prepared within 24 hours of use.

Spent Brines. Recycled spent brine was obtained from a frankfurter processor after its maximum usage. Each batch of spent brine (approximately 1000 L) was shipped via refrigerated truck and stored at 4°C until use. Twenty-four hours prior treatment, the spent brine was filter sterilized using nylon membrane filters, 0.45μM pore size (Whatman International Limited, Maidstone, England) to remove existing microbial flora and the filtered brine was stored at 2-4°C until use.

Inoculum Preparation. Each strain of *L. monocytogenes* was activated from stock cultures by three successive 24 hrs transfers in 25 ml TSBYE and incubated with shaking

at 35°C for 24 hrs. After the final incubation, the cultures were centrifuged for 5 min at 10,000 x g, the supernatant decanted, and the pellet washed with sterile, distilled water. This process was repeated twice before the pellet was re-suspended in 10 ml of the fluid being treated (fresh brine or spent brine). Equal portions of each strain (6.6 ml) were added to a sterile 500 ml volumetric flask and brought up to 200 ml using the fluid being treated (fresh brine or spent brine). Each preparation was made the day ozonation and UV treatment exposure and stored at 2-4°C.

Ozone Treatment. 200 ml of the *Listeria* brine suspension was added to a sterilized Nalgene container (Nalgene 300 filter receiver, Nalgene Nunc Int. Corp; Rochester, N.Y., U.S.A.) with the sparger fitted to the bottom inside circumference. A stirbar was placed inside the container and was activated following which the Ozonation unit was switched on. On achieving the required time of ozonation, the stirring of the brine suspension was stopped and the unit switched off. Samples were aseptically collected to measure residual ozone values, for further UV treatment and for bacteriological analysis. In spent brines, samples for bacteriological analysis were obtained once the foam settled. Fresh brine samples were ozonated for 5 minutes and 10 minutes. Spent brines were ozonated for 30 minutes, 45 minutes and 60 minutes.

UV treatment. One hour before treatment, the re-circulating refrigeration system was adjusted to 4–10°C and allowed to stabilize. Thirty minutes before treatment, the photoreactor was turned on, allowing the bulb to reach maximum intensity and for output to stabilize output. UV intensity was recorded before and after UV exposure (without the

quartz cylinder). After ozonation, 14 ml of the ozone treated brine was aseptically transferred into a sterile quartz cylinder (14-385-930E Fisher Scientific, Suwanee, GA). Samples were exposed for 5 minutes and 10 minutes for fresh brine and for 10 minutes for spent brine. After the UV treatment, brine samples were collected for bacteriological analysis.

Microbiological evaluation. *Listeria. monocytogenes* was enumerated before and after ozonation and UV treatment from brine solutions by serial dilution in 0.1% peptone (211677 Becton, Dickinson and Co, Sparks, MD) and spread plating 0.1 ml onto duplicate MOX and TSAYE plates, and incubating at 37°C for 48 hrs. A dual plating system was employed in order to determine the extent of cell injury. Additionally, 3 ml of each ozone-UV treated sample was enriched in 45 ml of Brain Heart Infusion broth (BHI, 237500 Becton, Dickinson and Co, Sparks, MD) and incubated at 37°C for 48 hrs. If turbid after 48 hrs, the BHI sample was streaked onto TSAYE and incubated at 37°C for 24–48 hrs for confirmation with *Listeria* API (10-300 Biomerieux, l'Etoile, France).

Residual Ozone Measurement. Residual ozone in fresh and spent brines was measured spectrophotometrically for a concentration range of 0.01 to 0.1 mg O3/L using the Indigo Colorimetric Method (Standard methods for the examination of waters and waste waters, 20^{th} edition) (APHA, 2005).

Analysis of Color and Transmittance. The transmittance of fresh brine, untreated spent brine and spent brine ozonated for 30, 45 and 60 minutes was measured at 262 nm using

a Shimadzu spectrophotometer (Model UV-2101PC). Colorimetric analysis of spent brine ozonated for 30 , 45 and 60 minutes was performed using a Minolta Chromometer (CR 300 series, Ramsey, NJ, USA) to determine the L*(lightness), a* (redness), b* (yellowness). Measurements were carried out for triplicates.

Statistical analysis. All ozonation and UV treatment experiments of fresh and spent brines were carried out in triplicates. The experiment for the studies was a randomized blocked design factorial treatment arrangement, repeated measures with sampling, blocked on replication. Means were separated using Least Square Means, SAS version 9.1 (SAS Institute,Cary, NC); significant differences are defined at $P < 0.05$ and highly significant differences are defined as $P<0.0001$.

RESULTS

Ozone (O3) and Ultra Violet treatment of fresh brines (9% NaCl). Fresh brines (9% NaCl) were treated with ozone (O3) and Ultra Violet radiation (UV) for different time intervals. Combinations tested were 5 minutes ozone/ 5 minutes UV, 5 minutes ozone / 10 minutes UV, 10 minutes ozone /5 minutes UV and 10 minutes ozone /10 minutes UV. Sparged air was used as a control.

Treatment with Air. Sparging of air for 5 minutes and 10 minutes through the fresh brine caused a 0.04 and 0.09 mean log reduction of *L. monocytogenes* respectively ($P > 0.05$).

O3/ UV treatment. Combination of 5 minutes of ozonation and 5 minutes of UV treatment resulted in a 5.95 CFU/ ml mean log reduction of *L. monocytogenes* MOX and a 5.7 mean log reduction on TSAYE ($P<0.0001$) . 5 minutes of ozone treatment and 10 minutes of UV irradiation caused a 7.32 mean log reduction on MOX and 5.82 mean log reduction on TSAYE ($P<0.0001$). Populations decreased to undetectable levels (<1 CFU/ ml) with treatments involving 10 minutes of ozonation. Ozonation for 10 minutes in combination with 5 minutes UV exposure caused a 8.79 mean log reduction of *L. monocytogenes* populations on MOX and 9.02 mean log reduction on TSAYE ($P<0.0001$). Treatment of brines with ozone and UV for 10 minutes respectively resulted in a 9.32 mean log reduction in MOX and 9.37 mean log reduction on TSAYE.

A 5.29 mean log reduction *L. monocytogenes* was observed after 5 minutes of ozonation and a 7.44 mean log reduction was observed after 10 minutes of ozonation ($P< 0.001$). Five minutes of UV treatment caused a 1.09 mean log reduction and 10 minutes of UV exposure caused a 1.95 mean log reduction in *Listeria monocytogenes* cells suspended in the brine ($P<0.0001$).

Level of Ozone in Brine. Residual level of ozone in the *L. monocytogenes*- brine suspension after 5 minutes ozonation was 0.03 ppm and after 10 minutes ozonation was 0.07 ppm.

Ozone (O3) and Ultra Violet treatment of spent brines (20.5 % NaCl). Spent brine (20.5 NaCl) with suspended *L. monocytogenes* cells was treated with ozone for 30 minute, 45 minute and 60 minute time intervals followed by 10 minutes of UV irradiation

for all three treatments. Air was sparged through the spent brine for 30 minutes, 45 minutes and 60 minutes as a control.

Treatment with air. Sparging of air for 30 minutes, 45 minutes and 60 minutes caused a 0.12 mean log reduction in the spent brines after each treatment.

O3/ UV treatment. Ozonation for 60 minutes with 10 minutes of UV treatment resulted in 5.53 mean log reduction on MOX and a 5.39 mean log reduction on TSAYE of *Listeria monocytogenes* in spent brine ($P<0.0001$). UV exposure for 10 minutes following 45 minutes ozonation resulted in a 4.03 mean log reduction on MOX and 3.54 mean log reduction ($P<0.0001$). A 0.29 mean log reduction and 0.17 mean log reduction ($P>0.05$) was observed after 30 minutes of ozonation followed by 10 minutes of UV treatment on MOX and TSAYE respectively. Thirty minutes ozonation of spent brine- *L. monocytogenes* suspension caused a 0.15 mean log reduction in cells ($P>0.05$). Treatments of 45 minutes and 60 minutes caused a significant reduction in *L. monocytogenes* with 3.2 mean log reduction and 4.97 mean log reduction respectively. UV treatment of 10 minutes post ozonation resulted in a 0.37 mean log reduction of the *L. monocytogenes* cocktail in spent brine ($P<0.0001$) .**Level of ozone in brine**. Dissolved ozone was not detected in the spent brine solution after 30 minutes, 45 minutes and 60 minutes of ozone treatment.

Change in transmittance of spent brine. Untreated spent brine had a transmittance of 0.01%. 30 minutes of ozonation resulted in a transmittance of 0.16 %. The transmittance of spent brine was 0.18% after 30 and 45 minutes ozonation.

DISCUSSION

Several studies highlight the effectiveness of ozone and UV treatment combinations. UV-enhanced ozonation of unscreened overflow chiller water resulted in greater than 99.9 % reduction of pathogenic microorganisms. (Diaz et al., 2002; Waldroup et al., 1993; Wu and Doan, 2005). Sensitivity of *L. monocytogenes* to ozonation was observed in experiments by Kim and Yousef (2000) during treatment of food spoilage and pathogenic bacteria. A 5 to 6 log reduction was observed when bacteria exposed to 2.5 ppm ozonated water for 40 seconds. *L. monocytogenes* showed lower resistance to the treatment than all the other pathogens and food spoilage organisms tested (*E. coli* O157:H7, *Pseudomonas fluorescens, Leuconostoc mesenteroides*).

A 9 % NaCl fresh brine solution and spent brine received from a national RTE meat manufacturer was used to simulate brine salinity levels recommended by the USDA for 1 week and 4 week reuse. As indicated by the tables (1 and 2) , ozone and UV hurdle was an effective treatment of fresh brines in eliminating *L. monocytogenes* counts beyond detectable levels. Reduction exceeding 8 log CFU/ml of *L.monocytogenes* cells was observed when combined O_3/UV treatment was used. The efficiency of the treatments primarily depended on the time of treatments and the physical nature of the brine used. Brine sample were inoculated into in Brain Heart Infusion Broth (BHIB), after treatment, as growth and turbidity in BHIB would indicate presence of damaged cells that were not

killed. After, 10 minutes Ozone/10 minute UV treatment, no turbidity was observed in BHIB, indicating the absence of resuscitation of injured or damaged cells. A dual plating (MOX and TSAYE) system was used for enumeration to determine the extent of cell damage.

Ozonation effects on Brine- *Listeria* suspension. The indigometric method was used to measure residual ozone in the *Listeria*-brine suspension. Fresh brines (200ml) had a residual ozone level of 0.03 ppm after 5 minutes of ozonation and 0.07 ppm after 10 minutes of ozone treatment. Spent brines (200ml) did not have any detectable residual ozone after 30, 45 or 60 minutes of measurement. This could have resulted from the higher organic load of spent brines resulting in higher ozone demand. As indicated by the graph, 60 minutes of O_3/UV treatments were required to achieve an excess of 5 log reduction of *L. monocytogenes* cells indicating presence of ozone consuming compounds and lesser accessibility of ozone to target pathogen in spent brines. While transmittance was not dramatically affected by the ozonation treatment, the color of the brine was considerably lighter after treatment. When colorimetrically analyzed, the redness (a*) of the brine reduced from +0.40 to +0.27. The change in color apart from the microbicidal activity might be advantageous during recycling or treatment of the spent brines.

A 5.29 mean log reduction was observed after 5 minutes of ozonation ($P< 0.0001$) in fresh brines while it required 60 minutes of ozonation in spent brines to achieve a 4.97 mean log reduction ($P< 0.0001$). Efficacy of the ozonation treatment in reducing microorganisms might be affected by the presence of organic molecules as they increase the ozone demand of the medium. Restanio et al.(1995) reported that the presence of 20

ppm of Bovine Serum Albumin in solution significantly affected the death rate of gram-positive (*Staphylococcus aureus, L. monocytogenes*) and gram negative (*Escherichia coli* and *Salmonella Typhimurium*) bacteria in ozonated water. Exposure to ozonated water or ozonated water containing soluble starch caused instantaneous death of more than 5 log units each of *S. typhimurium* and *E. coli* cells per ml (time zero) , whereas equivalent levels of death for *S. typhimurium* and *E.coli* in the presence of BSA (bovine serum albumin) occurred only after 2- and 5-min exposures, respectively. Thus the type of organic contaminant in the medium being ozonated also affects the efficiency of the treatment.

The infusion of debris, particulate matter, nutrients and other substances during the repeated use of the chill brines could increase the organic content, resulting in a higher ozone demand of the spent brine in comparison to the fresh brine solution.

Efficiency of UV radiation in Brine treatment. Fresh brines (9% NaCl) had a transmittance of 91.86% while spent brines (20.6% NaCl) had 0.01% transmittance. The color compounds, debris and other byproducts from the frankfurters, infusing into the brine due to continuous could have caused the decreased transmittance. The effect of UV treatment for 10 minutes in fresh brines resulted in a 1.95 ($P<0.0001$) mean log reduction while UV treatment is spent brine caused only a 0.37 ($P<0.0001$) mean log reduction. The transmittance of the brine is an important factor for the effective microbicidal action of UV radiation. UV radiation can not penetrate into food surfaces. Typically used for the treatment of drinking water and surface treatments, transmittance and turbidity of the solution might be important factors that affect the efficiency of the treatment.

Studies by Waldroup et al. (1993) showed an 88% to 99% transmittance in poultry chiller water as compared to fresh water while ozonation of red meat recycling brines did not show an increase in transmittance (Wu and Doan, 2005). The transmittance of the spent brines increased from 0.01% to 0.18% after 45 minutes of ozonation and remained at 0.18% after 60 minutes of ozone treatment. The lack of an increase in transmittance of the brine might have hampered the performance of the UV treatment in the fresh brines.

UV treatment units for semi opaque liquids have been used. A 5 log reduction in *Listeria monocytogenes* counts was observed in goat's milk when treated with UV irradiation (15.8 ±1.6 mJ/cm^2) using the CiderSure 3500 A apparatus (Matak et al., 2005). UV treatment has also been used to treat liquids like apple cider (Geveke, 2005). Apparatus design that facilitates UV penetration efficiently throughout the liquid might improve the efficiency of UV in semi-opaque brine treatment. UV enhanced ozonation processes may aid in better dose transmittance of UV radiation.

CONCLUSIONS

Treatment of brines with ozone and UV is an effective method to reduce *Listeria* populations. The efficiency of the process depends on the quality of brine used and dose or intensity of the treatments based on brine parameters. Spent brines required higher ozonation times. The low transmittance of spent brines stunted UV treatment efficiency. The combination of ozone and UV treatments resulted in an excess of 9 log reduction in *Listeria monocytogenes* cells after a 10 minute ozone-UV treatment. An excess of a 5 log reduction was achieved after 60 minutes of ozonation and 10 minute UV exposure in spent brine. The reduction in redness of the brine and *Listeria counts* along with the

absence of residual byproducts that are damaging to the environment suggests the applicability of the process in treating frankfurter chill brines.

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TABLES

Table 4.1. Effect of ozone-UV treatment on *Listeria monocytogenes* populations in fresh brines (9% NaCl, 91.86%. transmittance).

Treatment time (Min)			*L.monocytogenes* decrease post treatment. Enumeration on MOX Log CFU/ml	*L.monocytogenes* decrease post treatment. Enumeration on TSAYE Log CFU/ml	Ozone concentration ppm	UV intensity[e] (mW/cm^2)
O3	Air	UV				
	5 (Control)		0.06	0.03		
	10(Control)		0.25	0.03		
5		5	6.02	5.70	0.02	11.31
5		10	7.33	5.83	0.04	10.89
10		5	8.83	9.04	0.08	11.36
10		10	9.03	9.09	0.07	10.85

Table 4.2. Effect of ozone-UV treatment on *Listeria monocytogenes* populations in spent brines (20.5% NaCl, 0.01% transmittance).

Treatment time(Min)			*L.monocytogenes* decrease post treatment. Enumeration on MOX Log CFU/ml	*L.monocytogenes* decrease post treatment. Enumeration on TSAYE Log CFU/ml	Ozone concentration ppm	UV intensity[e] (mW/cm^2)
O3	Air	UV				
	(Control)		0.13	0.11		
30		10	0.29	0.17	Not Detected	11.44
45		10	4.03	3.54	Not Detected	10.36
60		10	5.53	5.39	Not Detected	11.73

Appendix

Equipment used for ozonation and UV treatment and the effect of ozonation on spent brine

Figure A.1 Ultraviolet light treatment system

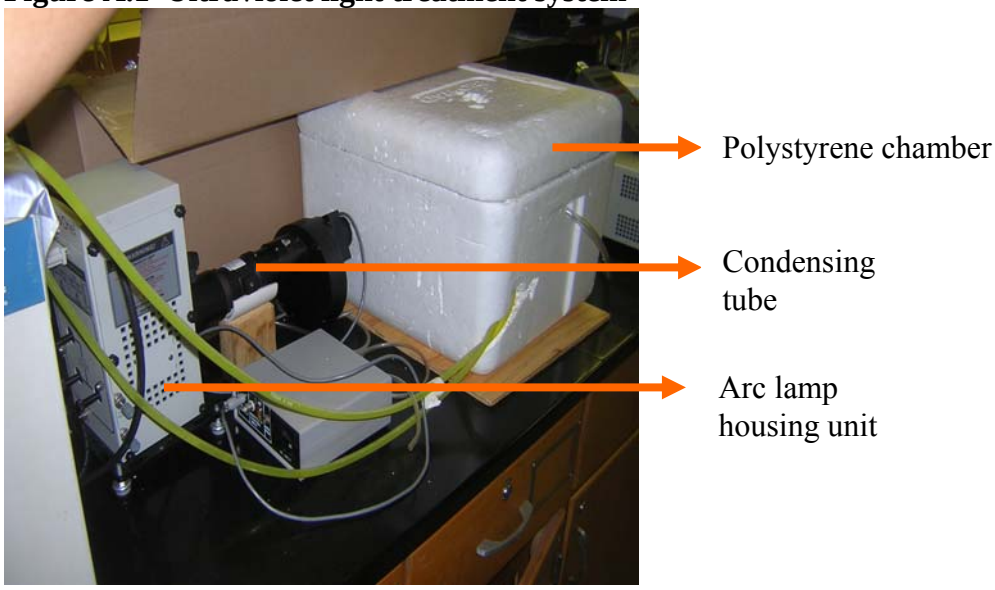

Image by Govindaraj Dev Kumar

Figure A.2 Ouartz cylinder with *Listeria*-brine suspension under UV light exposure.

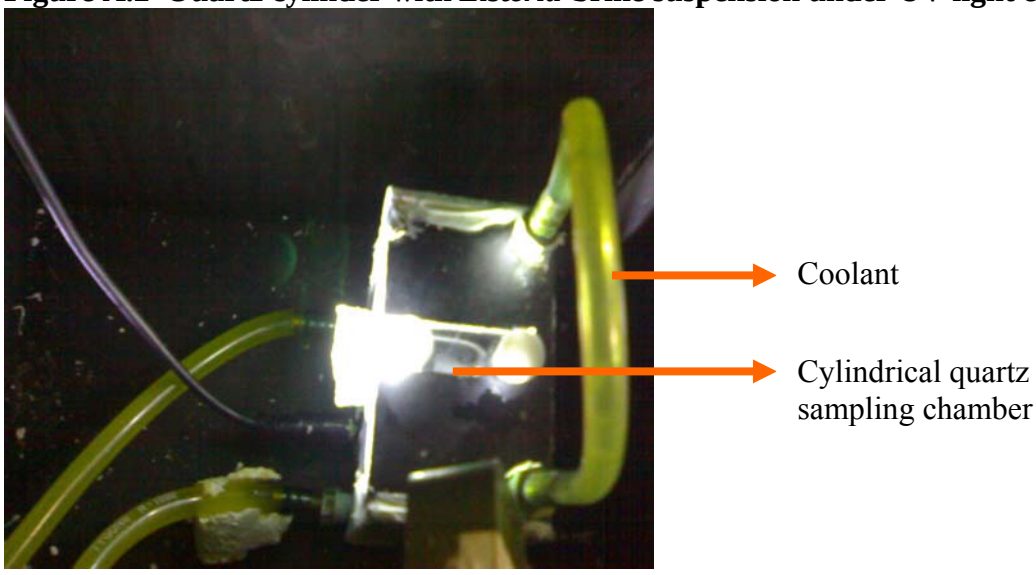

Image by Govindaraj Dev Kumar

Figure A.3 Golden Buffalo ozonation unit.

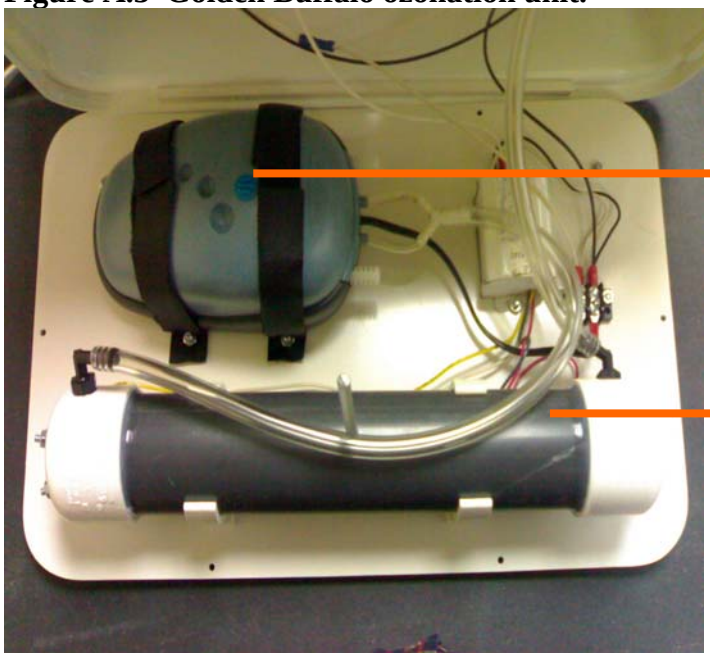

Image by Govindaraj Dev Kumar

Figure A.4 Spent brine after and before ozonation.

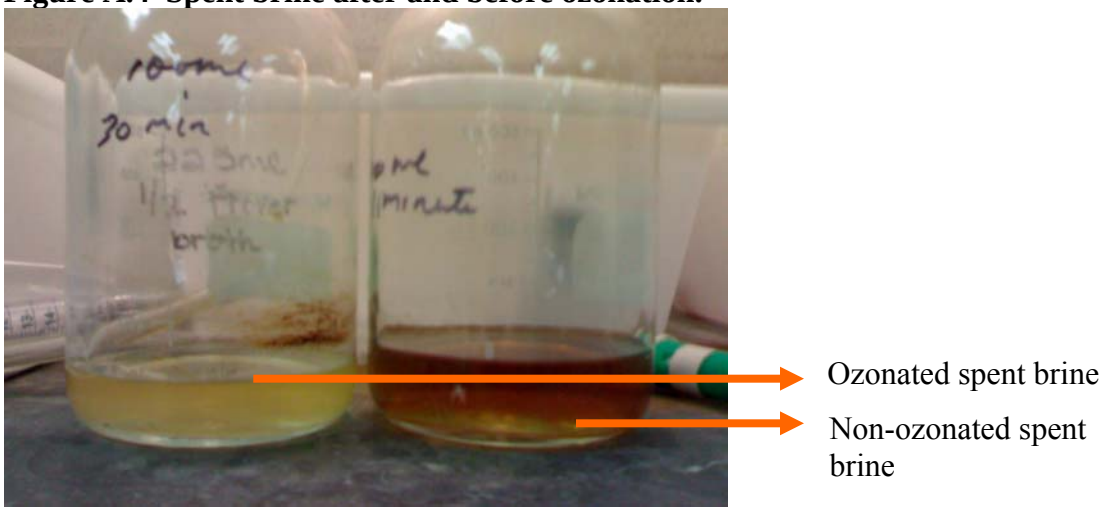

Image by Govindaraj Dev Kumar