Methods of Inactivation for Foodborne Pathogens: an investigation into ozone gas and UV irradiation

A Thesis Presented for the
Master of Science
Degree
The University of Tennessee, Knoxville

Addison Kay Myers
December 2023

Copyright © 2023 by Addison Kay Myers
All rights reserved.

ACKNOWLEDGEMENTS

I would like to first thank my advisor and mentor, Dr. Mark Morgan, for trusting me to work with him beyond my undergraduate years and giving me the opportunity to pursue further education under his understanding, knowledgeable, and ever entertaining direction.

Thank you to my committee members, Dr. Scott Leneghan, Dr. David Golden, and Dr. Doris D'Souza, for your guidance, time, and support over the time of my project.

Thank you to Dr. Xiaocun Sun for assistance with statistical analysis for both projects.

I would like to thank my parents, for their unwavering support of my academic endeavors and for instilling in me a love of learning that never fades.

To Aaron and Niloo, your attitudes towards frustrating challenges gave me the confidence to tackle many difficulties. You are both lights in my life and made the lab a place I looked forward to walking into day after day.

To Nikki, Sydney, Claire, and all of my close friends, thank you for listening to me through my tough times and celebrating me in my good ones. Thank you for believing in me.

ABSTRACT

These studies were conducted to investigate the efficacy of two disinfectant methods, ozone and UV irradiation, against *Escherichia coli* and *Eimeria acervulina* under specified conditions. Ozone gas is a widely studied disinfectant, however, inconsistencies in reporting ozone mass used in treatment as well as differences in log reduction for similar methods has led to complexity in the direct comparison of studies. The objective of this study was to elucidate the sanitizing potential of ozone gas and UV light. 10^6 CFU/mL *E. coli* dried on a stainless-steel surface was exposed to 10 parts per million by volume ozone gas in air across three time points. Log reduction for *E. coli* after treatment with 10 ppmmass ozone gas for ninety minutes was found to be 1.65 log; reduction decreased as time decreased. *Eimeria acervulina* was used as a surrogate for *Cyclospora cayetanensis*, a foodborne pathogen that infects humans. In recent years, incidences of *Cyclospora* contamination on crops has been increasing. This recent increase has prompted a search for novel methods of inactivation of the parasite. *Eimeria* oocyts were treated under 254 nm UV-C light for 1, 3, and 5 minutes. In a separate experiment, oocysts were treated with up to 4.93 ppm by mass ozone bubbled in water. Oocysts treated with UV and aqueous ozone were found to sporulate significantly less than those used as a control.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER I:
LITERATURE REVIEW

Ozone

Well before it was accepted by the Food and Drug Administration (FDA) as an "Antimicrobial Agent for the Treatment, Storage and Processing of Foods in Gas and Aqueous Phases", ozone had been shown to be a practical and effective disinfectant of surfaces and foods (R. Rice & Graham, 2001). Though extensive research has been conducted on ozone, the literature is confusing across the board regarding the mass of ozone used for treatment, methods for recovery of bacteria, and inconsistencies in reported log reduction. The goal of this project was to elucidate the effect of ozone gas on log reduction for a common foodborne bacteria, *Escherichia coli*, commonly found in the gastrointestinal tracts of humans and animals, under specified conditions.

Background

Ozone is an unstable triatomic allotrope of oxygen with a distinctive aroma, named after the Greek word "ozein", meaning smell (Pekárek, 2003). Due to its unstable nature, advantages and disadvantages arise when looking at it as a disinfectant. Instability requires near-constant monitoring of concentrations to ensure available ozone is present when applied as a disinfectant. The half-life of ozone ranges from seconds to days, depending on atmospheric conditions; these conditions will be discussed in detail in a later section. However, the unstable nature of ozone can be advantageous; when sterilizing open areas or applying it in solution, the disinfectant can dissipate to safe levels on its own. This can be sped up by a catalytic converter when used as a gas or by agitation when used in aqueous solution (Hudson et al., 2009).

Ozone gas is used in the medical field on equipment that cannot be sterilized using thermal methods and has proven to be a successful disinfectant for use on N95 masks and intubation equipment (Lopes et al., 2015; Manning et al., 2021).

Ozone was granted GRAS approval for use by the FDA in 1982 with a maximum residual concentration in bottled water set at 0.4 mg/L. However, ozone was not approved for use outside of bottled water, thus forcing those wishing to use it to submit Food Additive Petitions (FAP) to the FDA (R. Rice & Graham, 2001). In 2000, a FAP was filed by Electric Power Research Institute in the hopes of providing a single, comprehensive document that includes data on most applications for ozone in gaseous and aqueous phases on food. The FDA approved this

in 2001 (R. Rice & Graham, 2001). Current EPA guidelines limit worker ozone exposure to 0.08 ppm for 8 hours ("40 CFR § 50.10," 2015). OSHA's guidelines are similar, with an Immediately Dangerous to Life and Health limit at 5 ppm and Permissible Exposure Limit (8-hour Time-Weighted workday exposure) at 0.1 ppm. The Threshold Limit Value (TLV) for exposure for light, moderate, and heavy work is 0.1 ppm, 0.08 ppm, and 0.05 ppm, respectively. However, the TLV is a recommendation and not enforceable by OSHA (OSHA, 2022).

Methods of Generation

Ozone can be generated in a variety of ways, some more efficient than others. Photochemical generation of ozone can be conducted by exposing oxygen gas to short wavelengths of light. Vacuum-UV light below 240 nm (typically 172 or 185 nm) can excite oxygen molecules to the point of separation, where they will then form triatomic bonds (Hashem et al., 1997), (Claus, 2021). This is similar to how the stratospheric ozone layer is formed. The ozone layer in the atmosphere is created by UV rays close to 185 nm. Ozone then reacts with UV-C at 254 nm, which is highly hazardous to human health, absorbing it and preventing it from passing through the stratosphere. This method of generation has a relatively lower yield than other methods, so alternative methods are more commonly employed.

Most processes in research and industry generate ozone using non-thermal plasma in a process called corona discharge. Corona discharge is a process by which high voltage is passed through an oxygen containing gas or pure oxygen. This creates an electric field which ionizes the gas molecules, generating ozone. This process requires a high voltage power supply and can produce significant amounts of heat, so power demands and cooling measures must be considered. Several ozone manufacturers warn about the presence of water vapor in such generators, as well as using air as opposed to dry oxygen (P. Anderson & Aubermann, 2023). Corona discharge will ionize all species of gas present, therefore, if air (78% N_2) is used as the feed gas, the formation of nitrous oxides will occur. If water vapor is present on top of this, the nitrous oxides will be converted to nitric acid, which forms a film on the equipment and can clog the discharge gap (Bruggemann et al., 2018). Nitric acid can not only corrode equipment but is also harmful to human health if inhaled. Precautions should be taken to prevent nitric acid

formation when utilizing corona discharge. Ozone generated from this type of generator can be pumped into the air or into solution using a diffuser stone.

Units of Measurement

Throughout studies, the efficacy of ozone has been shown to be influenced by pH, relative humidity (RH), and Ct. Ct is calculated by multiplying the concentration of ozone used by the time (in minutes) applied; concentrations are usually in mg/L or ppm.

A frequent challenge encountered when reviewing papers discussing ozone as a disinfectant is the lack of consistency with regard to units of concentration. There is no one standard unit for which to report the concentration of ozone either in gas or water, which makes the direct comparison of methods difficult. The most commonly used units are mg/L and ppm.

It is crucial to note the importance of specificity when reporting concentration. Parts per million, or ppm, should be reported on a mass, volume, or molar basis and the medium it is in (water, air, O_2) should be noted as well. As (Chawla et al., 2012) described, the difference between these various units of measurement can be drastic. Assuming the vessel size is 100 L and pressure is 14.7 psi, they compare the mass of ozone in five different types of ppm measurements: ppm_{mass} (O_3 in O_2); ppm_{mass} (O_3 in air); ppm_{vol} (O_3 in O_2); ppm_{vol} (O_3 in air); and ppm_{mass} (O_3 in H_2O). Each respective measurement reads 10 ppm however, the *total mass* of ozone present ranges from approximately 1.4 mg (in 10 ppm_{mass} O_3 in air) to over 1000 mg (in 10 ppm_{mass} O_3 in H_2O) . See **Figure 1** for details from this review. A lack of specificity in reporting the basis and medium for ozone concentration can result in confusion and difficulty when comparing findings.

Another common mistake that is made when reporting concentration is using ppm and mg/L interchangeably. The units are interchangeable for O_3 in H_2O, but not for O_3 in O_2 or O_3 air. For aqueous ozone, 1 ppm_{mass} is equal to 1 mg/L (this is because ppm is calculated using molarity and molar mass), however, in gaseous ozone, 1 ppm_{vol} is equal to 0.00214 mg/L. Conversely, 1 mg/L is equal to 467 ppm_{vol} in air (OzoneSolutions, 2022).

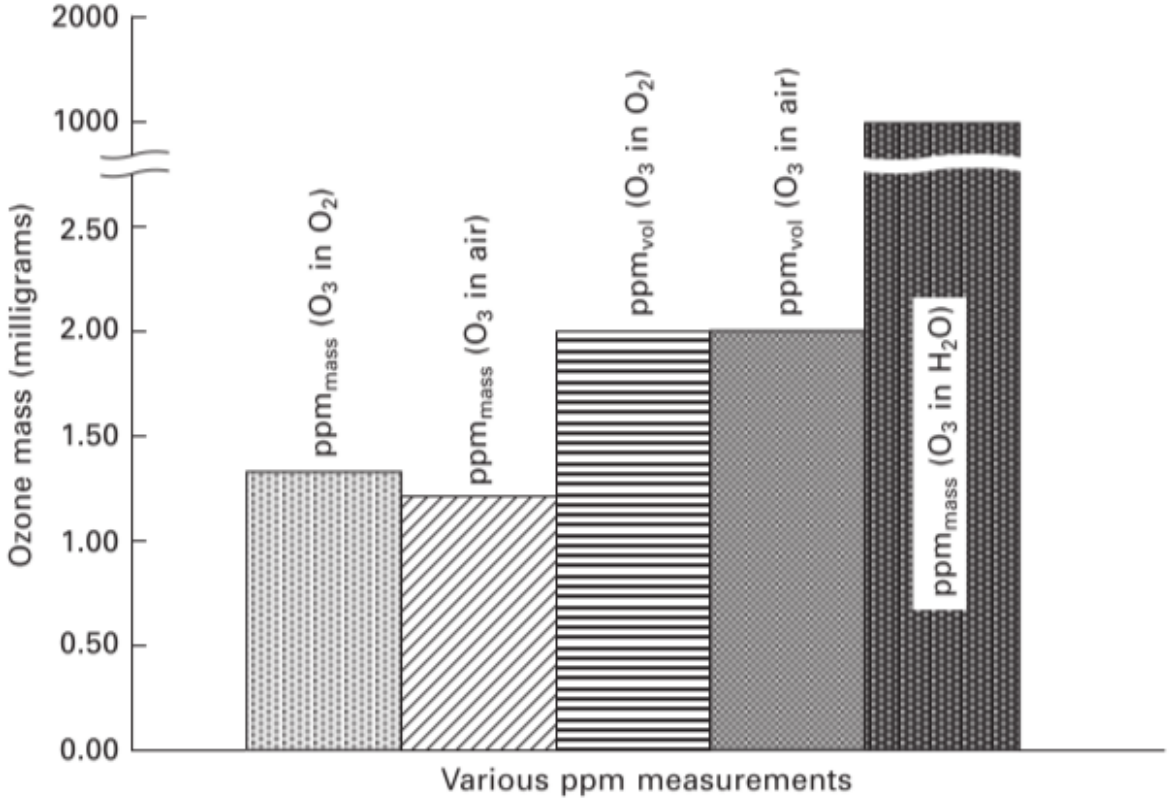

Figure 1: Mass of ozone calculated from 10 "ppm" measurements indicated by five hypothetical detectors. Each respective measurement reads "10 ppm", however, the total mass of ozone present ranges from approximately 1.4 mg (in 10 ppmmass O_3 in air) to over 1000 mg (in 10 ppmmass O_3 in H_2O).

Calculations are shown below for gaseous ozone at STP.

$$1\ \text{mg/L}\ O_3 = 467\ \text{ppm}\ O_3\text{: } \left(\frac{1000\ \text{mg}}{\text{m}^3}\right)\left(\frac{22.4\ \text{L/mol}}{48\ \text{g/mol}}\right)\left(\frac{(273+0)\text{K}}{273\ \text{K}}\right)\left(\frac{1\ \text{atm}}{1\ \text{atm}}\right) = 466.667\ \text{ppm}$$

$$1\ \text{ppm}\ O_3 = 2.14\ \text{mg}/\text{m}^3\text{: } (1\ \text{ppm})\left(\frac{48\ \text{g/mol}}{2.4\ \text{L/mol}}\right)\left(\frac{273\ \text{K}}{(273+0)\text{K}}\right)\left(\frac{1\ \text{atm}}{1\ \text{atm}}\right) = 2.14\ \text{mg/m}^3$$

Methods of Measurement

While several methods exist to quantitatively analyze the concentration of ozone present in water, the two most common methods are volumetric titration and spectrophotometry. The concentration of ozone in water can be determined by iodometric titration, following the methods as described by Birdsall et al. (Birdsall et al., 1952). However, this is not selective for ozone, and results can be influenced by other oxidizers present. AccuVac (Hach, USA) ozone ampules contain a small amount of malonic acid to prevent interference from chlorine, as well as indigo trisulfonate on the edges of the glass ampules. This indigo dye is bleached by ozone as the concentration increases. The resulting solution is analyzed spectrophotometrically at 600nm (Hach, USA). This method is accurate and not time consuming, however, it is costly and produces large amounts of glass waste.

Common forms of gaseous measurement include electrochemical sensors and gas detector tubes. Electrochemical sensors utilize electrodes and a sample pump to provide a continuous reading of a sample of gas pulled through the monitor. The gas is oxidized between the electrodes and the resulting current produced is used to report concentration. Gas detector tubes are thin, glass tubes that are typically filled with silica gel and a chemical reagent that undergoes a colorimetric change upon reaction with the gas of interest (RSComponents, 2002). Air is drawn into the tube using a piston pump. Higher concentrations of gas will result in more a pronounced color change further along the tube. Graduations along the tube correspond to the concentration of the gas, enabling quick, accurate readings. Detector tubes can provide accurate readings that require no further analysis for a relatively low cost.

Factors Influencing Efficacy

Multiple variables can be manipulated to create ideal treatment conditions for using ozone as an antimicrobial agent. The following factors have the most influence on the efficacy of the ozone used to kill microorganisms either in a gas phase or dissolved in water.

Relative Humidity

Numerous authors have found relative humidity (RH) significantly affects log reduction and microbial inactivation when using gaseous ozone. Foarde et al.; Ishizaki et al.; and Hudson et al. examined the role RH has in treatments with gaseous ozone. These studies compare multiple RH values, demonstrating the differences in results between them, and stressing the importance of creating a high RH environment when using ozone gas as a disinfectant. These three authors' teams, therefore, provide evidence supporting the hypothesis that high RH values aid in significant reduction of various microorganisms. Foarde et al. observed a 3 log reduction of *Penicillium glabrum* at 90% RH and 6.2 ppm_{vol} gaseous ozone in air. However at 30% RH, 9.8 ppm_{vol} was required for the same effect. Similar results were seen for *P. chrysogenum. Rhodotorula glutinis* exposed to 5.5 ppm_{vol} and 90% RH experienced a 3 log reduction; no concentration of ozone (in the range of 3 – 10 ppm_{vol} at 30% RH resulted in even a 1 log decrease (Foarde et al., 1997). Six strains of *Bacillus* were exposed to 3.0 ppm_{vol} ozone gas in air for 0-6 hours. An RH ³ 80% was needed to obtain an appreciable (3 log) decrease in the number of survivors within 6 hours, which was not attainable at relative humidity values below 50%. The lag phase of disinfection was also decreased as RH increased, shortening the time before exponential decrease occurs during exposure (Ishizaki et al., 1986).

Sharma & Hudson, 2008 focus on ozone as an antiviral agent. In their 2008 study, ozone was used to decontaminate viruses (10^5 - 10^8 PFU/mL) in various-sized rooms. Field tests conducted at ambient humidity (40%) yielded a peak average log reduction of 2.24 PFU/mL after Herpes Simplex Virus was dried onto a glass slide and left in a 65 m^3 room where it was exposed to rising ozone levels. Ozone levels rose over a period of 15 minutes to a maximum concentration of 28 ppm_{vol} ozone gas in air; this concentration was then maintained for a total exposure time of 60 minutes. Influenza H3N2, human isolate, was treated in a similar manner. At

38% RH and exposure to up to 20 ppm_{vol} for 20 minutes, log decrease was 0.097 PFU/mL. At 70% RH, this decrease was 2.57 PFU/mL log for the same ozone concentration of 20 ppm_{vol} for 20 min. These results show a direct comparison of two humidities, with 70% RH resulting in a 2.473 log increase in viral particle inactivation (Hudson et al., 2009). Relative humidity has been proven throughout studies to play a vital role in the efficacy of gaseous ozone on dry microorganisms. As RH increases, so does inactivation of an array of microorganisms. While RH is specific to gaseous ozone, there is another factor most relevant to ozone in aqueous forms, pH.

pH and Temperature

As pH trends towards basic, ozone decomposition is accelerated. This may be due to the catalytic activity of the hydroxyl ion (OH^-) (Alder & Hill, 1950). The reactivity of ozone with amino acids and peptides was reported to be higher at neutral and basic pH. Ozone was found to be most stable in water at a pH of 5.0 (Khadre et al., 2001). Venosa and Opatken (1972) have used Henry's law and its relationship to the solubility of oxygen and ozone in water as well as their concentrations in air to show the solubility of ozone in water is roughly thirteen times that of oxygen at 0-30 ˚C (R. G. Rice et al., 1981). Ozone solubility in water decreases with temperature: at zero degrees Celsius, 0.640 liters of ozone will dissolve per liter of water, resulting in a solubility ratio of 0.640 L_{Oz}/L_W. At 27 ˚C, this value drops to 0.270 L_{Oz}/L_W, and at 60˚C, ozone is no longer soluble in water (Guzel-Seydim et al., 2004). For concentrations at multiple temperatures, see **Table 1**.

Ozone as a Disinfectant

Microorganisms exhibit diverse responses to ozone treatment, and Ct values could offer a valuable tool in comparing resistance on a direct scale. Using Ct values, Korich et al found *Cryptosporidium parvum* to be 30 times more resistant than *Giardia lamblia* when exposed to aqueous ozone at 25˚C and pH 7. *C. parvum* required a Ct value of 5-10 (1 mg/L for 5-10 minutes) to achieve 99% inactivation, while *G. lamblia* required a Ct value of 0.16 (0.082 mg/L for 1.9 minutes) for the same (Korich et al., 1990; Wickramanayake et al., 1985).

Table 1: Mass of ozone able to be dissolved in water at various temperatures. Assuming Cg is equal (CG= 100 g/m^3) and 1 atm at each temperature for direct comparison.

Temperature, C	SR	mg/L Dissolved
0	0.64	64
5	0.5	50
10	0.39	39
20	0.24	24
30	0.15	15
40	0.1	10

$C_L = C_G \times S \times P$, C_L = dissolved concentration in liquid (mg/l); C_G = gas concentration (g/m^3); SR = solubility ratio; P = gas pressure (atm).

Based on the Ct values required for inactivation, the authors recommend using chlorine dioxide, chlorine, or monochloride in conjunction with additional disinfectants to ensure inactivation of *C. parvum*, however, ozone is suitable to use alone.

Protozoal Parasite Inactivation

Peeters (1989) and Liou (2002) used bioassays on mice and pheasants to evaluate the efficacy of ozone gas on the inactivation of *Cryptosporidium parvum* and *Eimeria colchici*. Neonatal mice were inoculated with a range of concentrations of treated *C. parvum* oocysts. Upon ozone generation, 14.1 mL of ozonated water was mixed with 1.6 mL of oocyst suspension. Treatment of 10^4 oocysts/mL with an initial value of 1.1 mg/L ozone for 6 minutes rendered water noninfectious to mice. To disinfect water containing 5×10^5 oocysts/mL, 2.25 mg/L of ozone for 8 minutes was required (Peeters et al., 1989). Oocyst shedding by 14-day-old pheasants inoculated with 5,000 *Eimeria colchici* oocysts treated with aqueous ozone were studied by Liou et al (2002). Sixty minutes of exposure to aqueous ozone with a continual concentration of 3.82 mg/l inhibited oocyst sporulation by 28.3% and reduced oocyst shedding by 58.2% (Liou et al., 2002). More recently, a 23.7% reduction in sporulation of *Eimeria* spp. was found following treatment with 2 mg/L ozone for five minutes (Morgoglione et al., 2021).

Bacterial Inactivation

An over 2 log reduction in *Bacillus cereus* was found following exposure to 0.12 mg/L ozone in water for 5 minutes. However, to achieve the same level of inactivation in the spores of *B. cereus* at the same timepoint, 2.29 mg/L of ozone in water was required (Broadwater et al., 1973). *E. coli* treated in water in a continuous flow reactor for 1.67 minutes at 0.23-0.26 mg/L ozone resulted in a 4.0 log reduction (Farooq & Akhlaque, 1983). *E. coli* in raw wastewater (having a higher organic load) in the same reactor type was treated with an initial concentration of 2.2 mg/L ozone (residual 0.06 mg/L) and achieved a 3.0 log reduction (Kaess & Weidemann, 1968).

Ozone has shown promising results in bioassays, reducing mortality and shedding in pheasants fed ozone-treated *Eimeria* when compared to non-treated oocysts. However, the

presence of organic content will increase the initial ozone demand, resulting in a precipitous drop immediately following application of ozone As well as decreased efficacy if the organic matter is not accounted for when determining strength of ozone to be applied. Thus, organic matter must be accounted for when determining Ct to optimize the disinfection potential of ozone. These studies highlight the significance of understanding varied responses of microorganisms to ozone treatment, the ease of use with Ct values, and the impact of organic demand on ozone efficacy.

Oxidizers other than Ozone

Ozone is a powerful oxidizer with an oxidation potential of 2.07 V. Chlorine and chlorine dioxide (ClO_2) have oxidation potential values of 1.36 and 0.95 V, respectively. HO^-, a radical produced as ozone decomposes in water, has an oxidation potential of 2.80 V. Chlorine dioxide is a highly reactive gas that is typically generated on-site, similar to ozone, due to the speed of its decomposition. Chlorine dioxide can safely disinfect water without leaving behind potentially harmful byproducts, however, chlorination of water can result in the presence of Halogenated trihalomethanes (THMs) and haloacetic acids (HAAs). Unlike ozone, the efficacy of chlorine dioxide is minimally changed as pH (6-11) changes (Ruffell et al., 2000).

Exposure to an initial concentration of 2.25 mg/L ozone in demineralized water) that sat for eight minutes resulted in a 96% reduction of oocyst production in mice in for *C. parvum* oocysts, while exposure of the same amount of oocysts to an initial concentration of 0.43 mg/L chlorine dioxide for 30 minutes resulted in 94.3% inactivation (Peeters et al., 1989).

Chlorine gas is also highly soluble and can be used in gaseous state, dissolved in water as hypochlorous acid (HOCl), and as a salt of hypochlorite (OCl^-). HOCl and OCl^- constitute free chlorine; the chlorine demand must first be met before residual chlorine can disinfect water or surfaces. Korich (1990) compared the resistance of *Cryptosporidium parvum* and *Giardia lamblia* to ozone, chlorine dioxide, and chlorine in water. Treatment of 10^8 oocysts with 80 mg/L chlorine decreased excystation from 80% to 20% after one hour, and to 0% after two hours. Exposure to a constant aqueous ozone concentration of 1 mg/L for five minutes resulted in a decrease from 84% excystation to 0%. Oocysts exposed to chlorine dioxide required 1.13 mg/L for one hour to decrease excystation from 87% to 0% (Korich et al., 1990).

When generated appropriately, ozone will not leave behind harmful by products, like chlorinated organic compounds. One reaction to take note of, particularly in wastewater treatment facilities, is the formation of bromate upon reaction of ozone with bromide-containing water. Chlorine dioxide and ozone must both be generated on-site, as the concentration will decrease rapidly over time. Chlorine-based disinfectants can be kept in liquid or gaseous states until ready to use. While chlorine is initially a cheaper alternative to chlorine dioxide and ozone generation, the Ct values required for inactivation of microorganisms is much higher for chlorine than both of the other disinfectants. For this reason, chlorine is commonly used to sanitize food contact surfaces with a ppm below 50-200 ppm for two minutes ("21 CFR § 178.1010," 2023).

Ultraviolet Irradiation

Background

Ultraviolet light has a wavelength of 100-400 nm, in which UV-C and far UV-C can be found. UV-C occupies the range of 200-280 nm, and far UV-C the range of 200-230 nm. For the subdivisions of the entire spectrum of UV light, see **Figure 2**. Wavelengths that fall under the UV spectrum are emitted by the sun, however, UV light can be transmitted artificially by plasma discharge lamps and light-emitting-diodes (LEDs). Plasma discharge lamps contain a gas mixture that is excited when a voltage is applied across the lamp filament. As the electrons in the gas fall to ground state, photons are emitted. Unlike light sources that use gas discharge or filaments, UV LEDs are semiconductors that emit light when an electric current is passed through it. The mechanism of UV disinfection is through damage of genetic material. Photons absorbed by RNA and DNA can damage the structures by creating thiamine dimers, free radicals, and other issues that render microorganisms unable to replicate and repair themselves.

The “dose” of UV is determined by the integrated value of irradiance, expressed as wattage over surface area, and time in minutes. For example, if a surface is exposed to 10 W/cm^2 for 3 minutes, the energy fluence would be 30 J/cm^2. This is not an exact value, as the angle of light, the surface area of the cell exposed, and other factors influencing the delivery of UV light all will affect the energy fluence an individual cell receives. In laboratory settings, these factors can be controlled, however, in real-world applications, the energy fluence for an item (whether it be food, liquid, or another material) may vary across its surface.

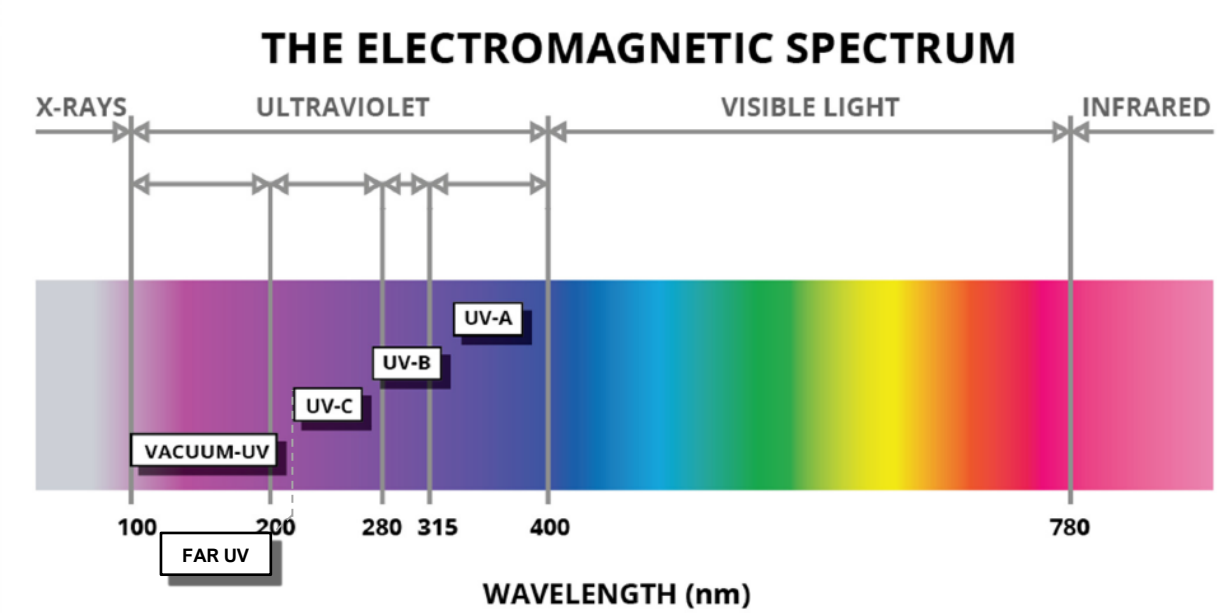

Figure 2: The electromagnetic spectrum

Careful consideration must be taken when using UV light as a disinfectant. Far UV, in the wavelengths of 282 and 222 nm are safe to use around humans without additional eye or skin protection. PPE is recommended when using UV light emitted at 254 nm, as it is harmful to humans and can cause photokeratitis and a sunburn like burn on the skin (Sholtes et al., 2021).

Use in the Food Industry

UV light is used as a non-thermal disinfectant that does not require the use of harmful chemicals and leaves no residual disinfectant in food or beverages. UV light is used in wastewater treatment plants to rid contaminated water of harmful microorganisms and in recent years has been studied as a disinfectant for food products. Survival of pathogens relevant to the food industry has been measured following exposure to UV radiation, also referred to as Ultraviolet Germicidal Irradiation (UVGI) (Reed, 2010). One study conducted on several pathogenic and indicator microorganisms found bacteria, coliforms, and standard plate count microorganisms that received comparable fluences of UV light were inactivated 99%. Bacterial spores and viruses were shown to be 9 and 3-4 times more resistant to inactivation than *E. coli* (Chang et al., 1985). A fluence of 10 mJ/cm^2 was sufficient to reduce several bacteria, including *Listeria monocytogenes*, *Staphylococcus aureus* (Methicillin-resistant *Staphylococcus aureus*), and *E. coli* by 4 log CFU/mL and inactivate three respiratory viruses, including SARS-CoV-2 and H1N1 (Ma et al., 2023). Ma et al. found no significant difference in the UV dose required to inactivate Gram-negative and Gram-positive bacteria on sterile petri dishes.

CHAPTER II:
EFFICACY OF OZONE GAS AGAINST *ESCHERICHIA COLI* DRIED ON STAINLESS STEEL SURFACES

Abstract

This study was conducted to test the efficacy of ozone gas on *Escherichia coli* ATCC 11229 on a stainless-steel surface. Gaseous ozone has been explored for decades as an alternative disinfectant to thermal processes. Ozone gas was generated onsite and pumped into a glovebox containing 5 x 2.5 centimeter stainless steel coupons inoculated with 100 uL of 10^7 CFU/mL *E. coli* (for a final population of 10^6 CFU/mL per coupon). The coupons sat until dry and were subsequently treated in duplicate with 10 ppm_{volume} ozone gas in air for 30, 60, or 90 minutes. Recovery took place using 32 mL phosphate buffered saline (PBS) and Tween80 mixture in a 50 mL centrifuge tube which contained one treated coupon and was vortexed for one minute before being ten-fold serially diluted and surface-spread plated. The average log reduction for 30, 60, and 90 minute treatments were 1.20 ± 0.16, 1.61 ± 0.16, and 1.65 ± 0.16 log, respectively. Log reduction seemed to plateau around 90 minutes. This research aims to explore the ability and limitations of gaseous ozone as a disinfectant against *E. coli*.

Introduction

Escherichia coli

Escherichia coli is a gram negative, facultative anaerobe commonly found in the gastrointestinal tracts of humans and many farm animals. These bacteria are serotyped based on specific combinations of surface antigens O and H; there are over 180 O-groups and over 50 H-groups, which leaves the possibility for nearly ten thousand serotypes (Fratamico et al., 2016). While most serotypes of *E. coli* are commensal, it is an opportunistic organism, and there are a few strains in particular of the utmost public health concern.

Pathology

Pathogenic *E. coli* are organized via the mechanism by which they cause infection. EHEC (also referred to as Shiga toxin-producing *E. coli* (STEC)) are enterohemorrhagic *E. coli*. A strain of EHEC that is prominent in EHC-associated foodborne outbreaks is *E. coli* O157:H7. *E. coli* O157:H7 is a Shiga toxin-producing strain that damages the intestinal mucosa, which can lead to bloody diarrhea and severe infection. Infection with *E. coli* O157:H7 can also cause

hemolytic uremic syndrome (HUS), a potentially fatal condition affecting the kidneys. Raw or undercooked hamburger meat was originally connected to this dangerous bacterium. *E. coli* O157:H7 has since been associated in outbreaks with foods such as apple juice, unpasteurized milk, ready to eat meats, and water sources near animals (JohnsHopkins, 2023). Other forms of pathogenic *E. coli* include enterotoxigenic *E. coli* (ETEC) and enteropathogenic *E. coli* (EPEC). ETEC is the most common cause of traveler's diarrhea and both ETEC and EPEC are responsible for the majority of diarrheal infections in children in developing countries (Ochoa & Contreras, 2011; Qadri et al., 2005). The World Health Organization estimates approximately 111 million illnesses and 63,000 deaths attributed to diarrheagenic *E. coli* globally each year (Collins et al., 2023).

Relevance to food industry

E. coli infections can be acquired by coming into contact with food or water contaminated with feces. Food that are commonly associated with *E. coli* contamination are: raw ground beef, raw sprouts, raw milk and raw milk products, and unpasteurized juice. Controlling *E. coli* in raw milk and juice is fairly straightforward, pasteurization of these products destroys harmful bacteria present, providing a strong line of defense against infection. The prevalence in raw ground beef is due to the mixing of meat from several cows when grinding. If meat from a few cattle contain *E. coli*, the mixing surely increases the likelihood of contaminating much more. To combat this, the FDA recommends cooking food to at least 155˚F for 15 seconds.

As people become inclined to eat healthier and more environmentally friendly, plant-based diets and consumption of fresh produce continues to rise (Bourassa, 2023). The US has seen substantial increases in consumption of fresh produce, unfortunately, this increase has contributed to a higher prevalence of foodborne outbreaks in these products. The USDA estimates a total of approximately $343 million were lost to medical expenses and loss of ability to work from *E. coli* STEC O157:H7 and non-O157:H7 alone from 2013-2018 (adjusted for inflation for 2018) (USDAERS, 2018). This does not account for the cost incurred to the industry. A computational simulation estimated that outbreak at a single fast-food restaurant would cost the establishment $3968 – $1.9 million. This estimate increased to $2.6 million for a

fine-dining restaurant, assuming a 250 person outbreak with high lost revenue, lawsuits, and legal fees (Bartsch et al., 2018).

A common source of transmission is cross contamination. This can occur when a contaminated food spreads a pathogen to another food article that was previously not contaminated. Equipment used to transport food throughout the facility may become contaminated and spread to other produce. Those who work in processing may unknowingly cross contaminate foods via their gloves and clothing. To prevent cross contamination, employees must adhere to Good Manufacturing Practices (GMPs). Proper storage, handwashing procedures, and preparation methods must be conducted from the facility to the establishment preparing the food for a meal, as the final cook temperature of plant foods is not required to be as high as for raw meat products, and vegetables are commonly consumed raw. Additionally, in establishments where foods are cooked or reheated, operators must ensure the cook times outlined in the US Food Code are followed.

Cross contamination of produce can occur along any point in the production chain; accordingly, hazard analysis and proper sanitization practices must be in place. In 2022, an *Escherichia coli* O157:H7 outbreak was linked to packaged salads and baby spinach, resulting in eight hospitalizations and one death (CDC, 2023). The CDC reminds consumers that the "safest produce is cooked, the next safest is washed", however, in a 2021 poll conducted by Gurney's Seeds and Nursery Co., when asked to rate the frequency at which they wash their vegetables (where 1 is least often and 5 is most often), the average response out of >3200 was 4.15. This frequency is 6.99% less than the national average for Tennessee inhabitants (Gurney's, 2021). Consumers cannot be relied upon to ensure their food is safe, this responsibility partly falls on the producer, processor, and distributors to ensure effective sanitization and cross-contamination prevention procedures are in place. Whole Genome Sequencing showed that *E. coli* from those infected in the 2022 leafy greens outbreak was closely related to a strain that caused an outbreak following contact with recreational waters (CDC, 2021). *E. coli* can survive in water, soil, manure, seeds, and plethora of other environments that may contact food. Effective sanitization methods post-harvest is an essential line of defense against foodborne outbreaks.

Materials and Methods

Growth of Culture

A vial of approximately 1 mL of *Escherichia coli* ATCC 11229 (Manassas, VA) was added to 9 mL nutrient broth (NB) and grown aerobically at 37 ˚C overnight. This culture was transferred every 24 ± 2 hours for two consecutive days and then used for the following experiment. The population of the overnight culture was between 4.4×10^7 and 5.8×10^8 CFU/mL, which was checked every day of the trials by serially diluting and plating.

Inoculation of Coupons

Sterile stainless-steel coupons with #2B finish measuring approximately 5 x 2.5 centimeters were used as a test surface. To minimize organic load on the ozone and facilitate spreading on the stainless-steel coupon, 1 mL of the culture was transferred from NB to 9 mL of PBS containing 1% Tween80 (v/v). Coupons were set in empty petri dishes for ease of handling. One hundred microliters of the culture in PBS + Tween80 mixture was aseptically added to each coupon and spread evenly across the surface using a pipette tip. These coupons dried in a biosafety cabinet (RH: 25-40 %) with the petri dish lids off until visibly dry, approximately 1.5 hours. Upon drying, the coupons were removed and separated according to treatment time.

Ozone Generation and Treatment

Ozone was generated via corona discharge from air and was pumped into a sealed glovebox. The glove box used had one opening covered by heavy plastic sheets that were lifted only to measure the concentration or transfer coupons in and out for treatment. A small fan inside the box circulated ozone in air for even distribution. Based on the readings from two sensors placed inside the treatment chamber, a control box switched the ozone generator on or off to achieve a concentration of 10 ± 1 ppm_{volume} O_3 in air. The glovebox had a relative humidity between 25 and 40%, normal atmospheric pressure, and no significant variation from room temperature (20-23 ˚C). Ozone concentration was verified during each treatment using a No. 18EL Gastec detection tube. The coupons were treated in duplicate or triplicate for 0, 30, 60, or 90 minutes. The experiment was repeated on six different days.

Bacteria Recovery and Enumeration

Following ozone treatment, all coupons were transferred to a 50 mL centrifuge tube, fully immersing them in 32 mL of a neutralizing solution made of PBS with 6% Sodium Thiosulfate (w/v) and 1% Tween80 (v/v). The centrifuge tube containing the coupon was vortexed for 15 seconds, lifted, and started again until the total vortex time was one minute. The coupon was left in the tube, from which a serial dilution was performed. One hundred microliter aliquots were spread-plated in duplicate on Nutrient Agar (NA). The plates were incubated at 37 ˚C and colonies were counted the following day (18-20 hours later).

Analysis

The effects of time on log recovery and log reduction were examined using mixed model analysis for randomized block design with date as a random block effect. Ranked transformation was applied when diagnostic analysis on residuals exhibited violation of normality and equal variance assumptions using Shapiro–Wilk test and Levene's test. Post hoc multiple comparisons were performed with Tukey's adjustment. Statistical significance was identified at $p<0.05$. Analyses were conducted in SAS 9.4 TS1M8 (SAS institute Inc., Cary, NC).

Results

Exposure to gaseous ozone in air was shown to be detrimental to *E. coli* growth and survival. As shown in **Figure 3**, log reduction of *E. coli* reached 1.67 log CFU after 90 minutes of exposure to a constant concentration of 10 ppm_{volume} ozone gas. There was no significant difference between 30 and 60 minutes or 60 and 90 minutes, however, the increase in bacterial log reduction between 30 and 90 minutes is statistically significant. Similarly, **Figure 4** displays a decrease in log recovery as time of exposure is lengthened. Fewer viable bacteria were recovered as treatment time increased. As with log reduction, there is no difference between the groups 30 & 60 or 60 & 90. The control group (time zero) is significantly different from all other groups. The limit of detection for *E. coli* was 320 CFU/cpn. Data were included for all counts that were above this limit. On average, 5.31 ± 0.42 log CFU *E. coli* were recovered from the untreated coupons, which were inoculated with 10^6 CFU/cpn.

Discussion

Exposure to ozone gas negatively impacts the survival of *Escherichia coli*, however, this effect is not as significant as previously reported. The present study employed 10 ppm_{volume} ozone in air, resulting in an average log reduction of 1.62 after 90 minutes of exposure. A dose of 0.02 mg/L ozone in a gaseous O_3/O_2 mixture (1%/99%) for five minutes completely inhibited the growth of *E. coli* (Fontes et al., 2012).

Differences in log reduction may be attributed to numerous factors. Bacteria were treated during the stationary phase of their growth. Treatment with ozone gas during the growth or exponential phase may result in a higher log reduction, however, this research sought to simulate a scenario representing conditions in manufacturing facilities, where bacteria may not be treated until 18-24 hours after they have contaminated a surface. As discussed previously, reporting of concentration in current literature may not accurately describe the mass of ozone used while treating microorganisms, which could account for large differences in expected versus actual log reduction when conducting similar methods. The relative humidity of the treatment chamber was not intentionally altered throughout this study, which was conducted over several days. It would not be unreasonable to assume the RH changed in the laboratory the coupons were prepared in or in the glovebox they were treated in. This could have an effect on the log reduction of *E. coli* on the surface of the coupons. Hudson (2009) and Dubuis (2020) showed a positive effect of increasing relative humidity on the efficacy of ozone gas against viruses (Dubuis et al., 2020)**.** Both studies saw improved efficacy as an increase in log reduction as RH was raised. Indeed, the fluctuations in log reduction in the present study from experimental day to day may in part be due to changes in relative humidity.

Conclusion

Ozone gas is a powerful, cost-effective alternative to other oxidizers commonly used in the food industry. It has been shown to be an effective disinfecting agent against an array of pathogens, including *Escherichia coli*. In high concentrations, ozone may negatively affect the sensory qualities of produce, particularly leafy greens, however, ozone has also been found to inhibit browning-related enzymes, prolonging shelf life (Ölmez & Akbas, 2009) (Rico et al., 2006).

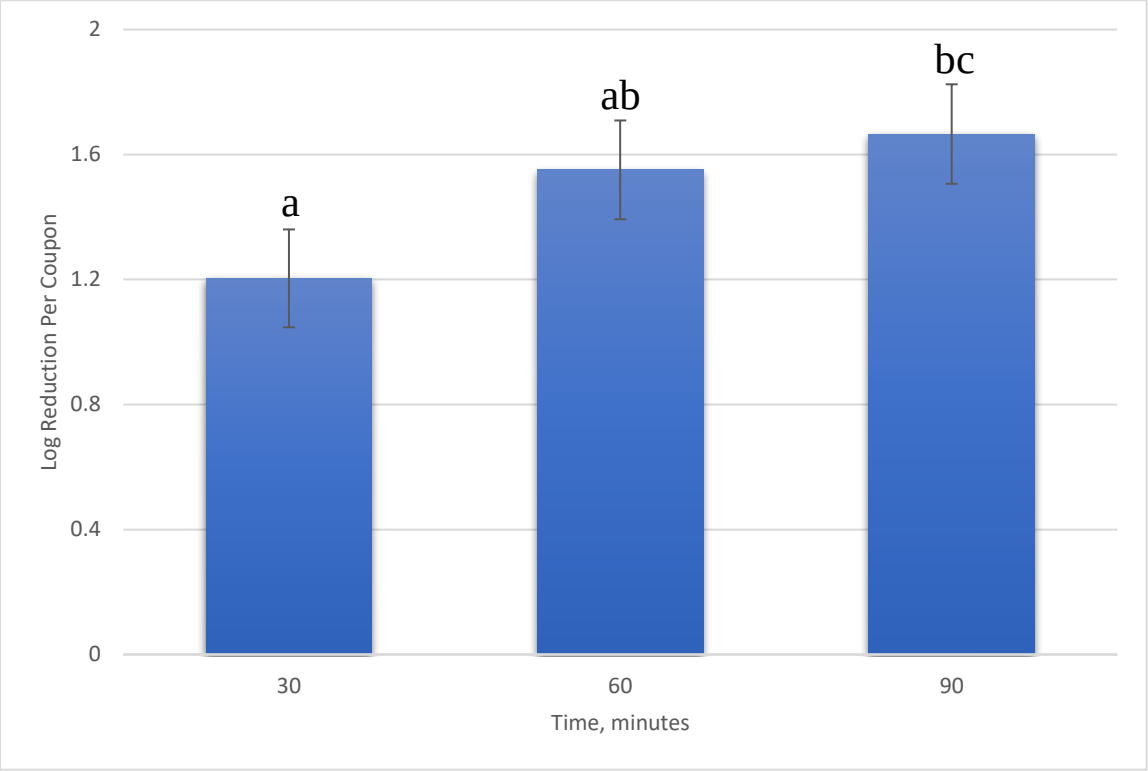

Figure 3: Effect of exposure to 10 ppm_{volume} O_3 in air at three timepoints on *E. coli* dried on stainless steel. Standard Error (0.16) shown. Analysis conducted using mixed model analysis for randomized block design with experiment date as a random block effect. Ranked transformation was applied when diagnostic analysis on residuals exhibited violation of normality and equal variance assumptions using Shapiro–Wilk test and Levene's test. Post hoc multiple comparisons were performed with Tukey's adjustment. Statistical significance was identified at $p<0.05$. Analyses were conducted in SAS 9.4 TS1M8 (SAS institute Inc., Cary, NC).

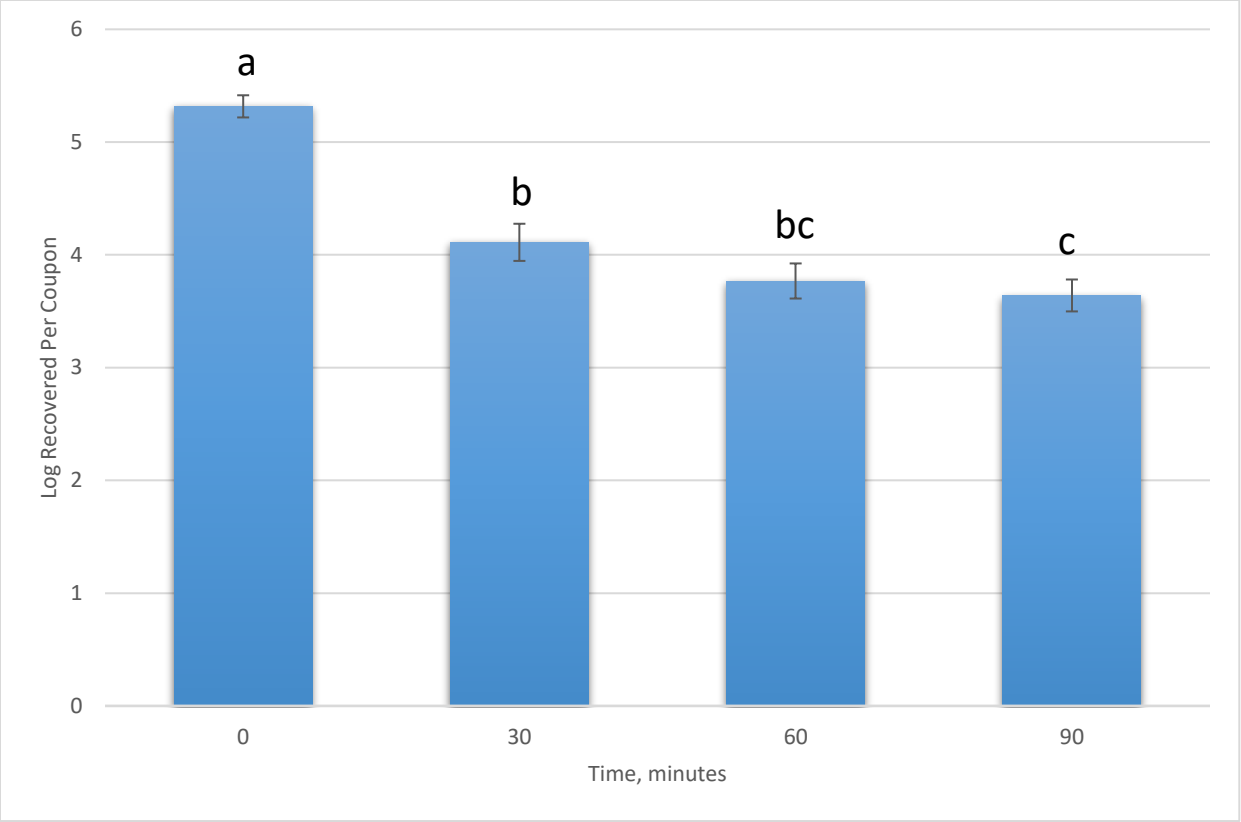

Figure 4: Log recovery of *E. coli* after 0, 30, 60, and 90 minutes of exposure to 10 ppm_{volume} O_3 in air. Standard error (0.16) shown. Analysis conducted using mixed model analysis for randomized block design with experiment date as a random block effect. Ranked transformation was applied when diagnostic analysis on residuals exhibited violation of normality and equal variance assumptions using Shapiro–Wilk test and Levene's test. Post hoc multiple comparisons were performed with Tukey's adjustment. Statistical significance was identified at $p<0.05$. Analyses were conducted in SAS 9.4 TS1M8 (SAS institute Inc., Cary, NC).

The purpose of this study was to elucidate the effect of gaseous ozone over time on *E. coli* dried on stainless steel, with an emphasis on clear reporting of the concentration of ozone used during treatment. A peak log inactivation of 1.62 ± .16 was achieved following 90 minutes of exposure to 10 ±1 ppm_{volume} O_3 in air. Subsequent studies seek to elucidate the effects of RH on effectiveness of ozone gas against E. coli and ozone gas on droplets containing *E. coli* (on stainless steel surfaces) under similar conditions as described above. Initial results show a greater log reduction (> 3 log CFU/mL) on average than found in the present study.

CHAPTER III:
INACTIVATION OF *EIMERIA ACERVULINA* OOCYSTS

A version of this chapter was originally submitted for publication by Aaron A. Baumann[1,*], Addison K. Myers[2,*], Niloofar Khajeh-Kazerooni[1], Benjamin Rosenthal[3], Mark Jenkins[3], Celia O'Brien[3], Lorraine Fuller[4], Qixing Zhong[2], Mark Morgan[2], and Scott C. Lenaghan[1,2,+]

* *Authors contributed equally.* AAB, AKM, QZ, MM, and SCL conceived and designed the experiments. AAB, AKM, and NKK performed experiments and analyses. BR, MJ, CO, and LF prepared critical samples for all experiments. AAB, AKM, and SCL wrote the manuscript. In the following chapter, I included additional information on our work with UV irradiation on *Eimeria acervulina*. Additional differences include the structure of my chapter. The following chapter presents figures immediately after their mention, includes additional sections for the sake of clarity and portions that were rewritten completely by myself.

[1] Center for Agricultural Synthetic Biology (CASB), University of Tennessee, Knoxville, TN, USA
[2] Department of Food Science, University of Tennessee, Knoxville, TN, USA
[3] USDA
[4] University of Georgia

Bauman et al. (2023). "Aqueous ozone exposure inhibits sporulation in the *Cyclospora cayetanensis* surrogate *Eimeria acervulina*" Journal of Food Protection, *VOLUME(issue),* pg, DOI

Abstract

Ozonation is a potent disinfecting agent used to treat potable water and wastewater, effectively clearing protozoa such as *Giardia* and *Cryptosporidium spp*. It is unclear whether ozone treatment of water or fresh produce can reduce the spread of the emerging parasite *Cyclospora cayetanensis*, which causes cyclosporiasis in humans. Obtaining viable *C. cayetanensis* oocysts is a challenging task due to delays in case reporting and standard practices in health departments, which add fixatives to collected specimens, thus eliminating their utility for inactivation assays. Recently, researchers have sought to bolster understanding of the biology of *C. cayetanensis* by studying the surrogate *Eimeria acervulina*, a closely related and easily cultured poultry parasite of economic significance. The consequences of ozone treatment on *E. acervulina* oocysts were examined using sporulation state as an indicator of infectious potential. When treated with ozonated water acidified with citric acid, the sporulation capacity was reduced in a dose-dependent manner; treatment with up to 4.93 mg/L initial concentration of ozone resulted in a

93% inactivation of sporulation by 7 days post treatment. This developmental arrest was accompanied by transcriptional changes in genes involved in regulating the response to reactive oxygen species (ROS) in a time course that is consistent with the production of oxygen free radicals. Our study shows that ozone is highly effective in preventing sporulation in *E. acervulina*, a model coccidian used as a surrogate for *Cyclospora*. Furthermore, we provide evidence for a molecular response to general oxidative stress, with several well-characterized antioxidant enzymes showing ozone inducible expression.

Introduction

Cyclospora cayetanensis is a human parasite that causes the intestinal illness Cyclosporiasis. This parasite is ingested through contaminated food, water, or surfaces that have been in contact with it. Gastrointestinal illness symptoms begin approximately seven days post-ingestion and include dehydration, fatigue, and diarrhea. Oocysts are shed from their host and sporulate one to two weeks later. Immunocompromised populations are particularly at risk for severe illness caused by *Cyclospora* (Sarfo et al., 2022*)*. Cyclosporiasis is most common in tropical and subtropical regions, however, since the first the first domestic outbreak in the United States in 1990, almost every year has seen an outbreak of cyclosporiasis, with the first instance of domestic transmission reported in 2018 (Almeria et al., 2019). There are several operational hurdles to studying *Cyclospora* biology. The scarcity of viable oocysts for evaluating disinfection measures is a major obstacle since there is currently no *in vitro* method to culture *C. cayetanensis*. A previous attempt to propagate the parasite in willing human volunteers was unsuccessful (Alfano-Sobsey et al., 2004). Furthermore, the submissions through public health departments that serve as the primary source for *C. cayetanensis* samples are invariably subject to chemical fixation and preservation, rendering them unsuitable for inactivation assays or transcriptional studies.

Recent efforts to hasten progress in studying *Cyclospora* biology have focused on *Eimeria acervulina*, a close relative of *C. cayetanensis* that causes coccidiosis in poultry (Tucker et al., 2022). Several aspects of *E. acervulina* biology support this approach. Importantly, *E. acervulina* infects regions of the chicken digestive tract that are comparable to infection observed by *C. cayetanensis* in the human digestive tract. Like *C. cayetanensis*, the spread of

avian coccidiosis occurs via fecal-oral transmission, with shedding of *E. acervulina* oocysts in the feces of infected birds reaching the infective, sporulated stage in the environment. Of practical importance, *E. acervulina* is harmless to human researchers and can be propagated in great quantities in poultry. Ingestion of a single oocyst leads to the excretion of 10^3-10^4 oocysts when passed through an avian host (Tucker et al., 2022). *E. acervulina* therefore represents an exceptional experimental platform to study the effects of decontamination procedures for fresh produce, such as the use of ozone.

Ozone is a powerful oxidizing agent that is commonly used for water disinfection and treatment. The ozone molecule is unstable and decomposes rapidly in water. The rapid release of free radicals oxidizes organic and inorganic compounds, making ozone an effective antimicrobial for a wide range of microorganisms, including viruses, bacteria, and protozoan parasites such as *Giardia* (Morrison et al., 2022)and *Cryptosporidium* (Pereira et al., 2008). Ozonated water was previously shown to reduce the incidence of Giardia contamination in drinking water (Kondo Nakada et al., 2020) and has proven effective to inactivate *Cryptosporidium parvum* (Morrison et al., 2022). Several studies have achieved ≥ 3 log reduction of pathogenic bacteria on berries, peppers, and leafy greens washed with ozonated water (Achen & Yousef, 2001; Beltrán et al., 2005; Bialka & Demirci, 2007). In addition to its disinfecting properties, ozone treatment has demonstrated benefits produce, including, but not limited to, the reduction of several types of pesticide residues on produce (Glowacz et al., 2015). Strawberries treated with ozone exhibited enhanced textural qualities through storage (Piechowiak et al., 2022). However, as exposure time increases, consequences begin to arise, such as a reduction in vitamin content or the development of unwanted cosmetic traits (reviewed in (Sachadyn-Krol & Agriopoulou, 2020)).

Here, we evaluate the effects of ozone on *E. acervulina* sporulation. Since the distinct morphological incongruence between unsporulated and sporulated oocysts is readily apparent via light microscopy, the proportion of sporulated to total oocysts was used as a metric to evaluate the disinfection capacity of ozone across a range of doses. At an initial dose of 4.93 mg/L ozone, the highest initial dose achieved, a sporulation rate of only 4% was observed in treated samples. The sporulation alone was not affected, bacterial aggregation to oocyst walls via light microscopy was confirmed. Previous studies have speculated that changes in surface glycoproteins rendered oocysts susceptible to bacterial invasion (Liou et al., 2002). The present

study presents evidence for a molecular response to oxidative stress, leading to developmental arrest.

Eimeria as a Surrogate Organism

Recent efforts to progress understanding have employed *Eimeria acervulina,* a closely related apicomplexan that causes coccidiosis in poultry, as a surrogate organism for *Cyclospora cayetanensis* (Tucker et al., 2022).

Obtaining sufficient numbers of *C. cayetanensis* oocysts is challenging, and finding viable oocysts is likewise difficult. Non-human primates have been found to be infected with *C. macacae* and possibly *C. cayetanensis* (Marangi et al., 2015), raising questions of host specificity of *C. cayetanensis*, however, every attempt to propagate them either in cell culture or other animals, including humans (Alfano-Sobsey et al., 2004), has failed. Successful reproduction of these parasites would likely require a human host, presenting a plethora of ethical and logistic issues. The primary source of oocysts is from outbreaks, however, laboratory handling conditions at public health institutions often render the parasites unsuitable for subsequent research. These roadblocks hinder progress in research on *Cyclospora*. A solution would be to use a surrogate organism.

Phylogenetic analysis reveals that *Eimeria*, particularly the species that infect poultry, is closely related to C*yclospora. Eimeria* and C*yclospora* both contain sporocysts that enclose a pair of sporozoites. There are four such sporocysts in Eimeria and two in C*yclospora*. One thousand to ten thousand oocysts can be produced as a result of the ingestion of a single oocyst, rendering inoculation of chickens a substantially efficient process for sourcing oocysts. Oocysts can be stored at refrigeration temperatures for several months and remain viable for testing. They pose no threat to humans, and work can be conducted on them in a BSL2 laboratory.

The benefit of using *Eimeria* as a surrogate organism is two-fold. *Eimeria* is the cause of poultry illness that has an estimated annual impact of $14B on the industry (Blake et al., 2020). Contributions to the study of methods to control *Eimeria* will directly impact the poultry industry in a positive manner. *Eimeria* populations can be multiplied through chickens with more ease and less ethical constrictions than *Cyclospora* in humans. *Eimeria* is more abundant, poses much less of a risk to researchers, and is an ethical alternative to methods that would have to be used to

propagate equivalent numbers of *Cyclospora*. Owing to their biological similarities, the time course for *Eimeria* sporulation under different conditions could be studied and inferences could be made on the maturation cycles of *Cyclospora*. The phylogenetic relationship of these two apicomplexans enables studies on *Eimeria* to be translatable to *Cyclospora*. Methods optimized using *Eimeria* can be verified once a sufficient amount of *Cyclospora* becomes available. *Eimeria* can also be used to assess specificity of assays. If PCR detection of *Cyclospora* is being employed, the presence of *Eimeria* in the matrix with *Cyclospora* will provide an excellent opportunity to test the specificity of the assay. Amplification of *Cyclospora* and not the closely related *Eimeria* will ensure the primer sets were appropriately chosen.

Methods

Isolation of Eimeria

Eimeria acervulina was acquired via feces from infected chicken. Samples were prepared and sent by Mark Jenkins with the USDA Animal Parasitic Diseases Laboratory. Upon receipt, the feces were transferred to a 2000 mL bottle. Deionized water was added until the total volume was ~900 mL, thinning the consistency enough to be mixed. Following the addition of a large stir bar, the bottle was capped and left to mix for 15 minutes. The slurry was carefully massaged through a funnel lined with cheesecloth before being poured into 50 mL centrifuge tubes. These tubes were centrifuged at 939 ×g for 10 minutes (Thermo 1000 Rotor in Thermo Scientific X Pro Series). The supernatant was poured off and pellets were resuspended in a 6.14 M NaCl solution at a 1:5 ratio. These were centrifuged for 10 minutes at 939 ×g and sat for 10 minutes, after which *Eimeria* were collected from the top fraction using a pipette. The collected *Eimeria* were washed and centrifuged at 1,467 ×g for 15 minutes. The supernatant was removed, and water was added back in the same ratio as before. This wash was completed twice more to remove salt. After resuspending the pellets in water, the isolated oocysts were consolidated into a single tube with an equal volume of 5% potassium dichromate and another equal volume of millipure water. Oocysts were stored at 4˚C until ready to be used.

Treatment with UV Irradiation

Oocysts were washed of potassium dichromate by centrifuging down to a pellet and adding water. This process was completed until the supernatant had very little orange color. The washed oocysts were pipetted into a 96 well plate 25 uL at a time for treatment. There were approximately 2.6×10^8 oocysts/mL. Oocysts were treated for 1, 3, and 5 minutes at both wavelengths: 222 and 282 nm.

UV irradiance was determined by holding a detector at various distances from the bulb and recording intensity according to the IL1700 research radiometer (International Light Technologies). The UV lamps were positioned above the oocyst samples to generate the following irradiance levels: 8.5×10^{-5}, 6×10^{-5}, 5×10^{-4}, 3×10^{-4},1×10^{-4}, 2.5×10^{-5}, and 7.5×10^{-4}, watts/cm^2. These irradiance levels were a measure of the intensity of the UV light at each distance. Each one corresponds to an individual distance the oocysts were away from the bulb. Integrated irradiances (Radiant Energy Density, fluence) based on the exposure times can be found in **Table 2**. Seven fluences, along with a positive and negative control, were tested for each wavelength. Oocysts exposed to the highest irradiance (shortest distance) for 10 minutes were used as a positive control.

Exposure of *E. acervulina* to UV

For wavelengths of 222 and 282, a wire shelving unit with mobile shelves was used to adjust distances of the oocysts from the Sterilray™ Microbe Buster™ bulb, thus adjusting irradiance. The bulb sat in a ballast on the top shelf, and a shelf with a 96-well plate was placed below it at predetermined distances. Oocysts were treated at each fluence (**Table 2**) in the presence or absence of potassium dichromate for 1, 3, and 5 minutes. After treatment, plates were wrapped in parafilm and incubated in the dark in 2.5% potassium dichromate or distilled water at 25˚C for 7 days before scoring.

Aqueous Ozone Generation, Treatment, and Residual Measurements

Ozone was generated from pure, dry O_2 with an Aqua-8 portable ozone generator (A2Z Ozone Inc.). The ozone was bubbled through a 1” diffuser stone into the bottom of a graduated cylinder with 500 mL of sterile Milli-Q Deionized water. Ozone concentration was measured

using Ozone AccuVac® Ampules (Hach), which contain an indigo blue reagent that reacts quantitatively with ozone and are measured with a spectrophotometer (Hach DR10900). Multiple ozone decay vs time curves were generated to determine an appropriate treatment concentration and time. First, a curve for ozone decomposition in sterile Milli-Q DI water with a pH of 6.98 was constructed by taking a reading every 10 minutes for 40 minutes. This curve is plotted in **Figure 7**. An ozone decomposition curve was also plotted for aqueous ozone concentration in tap water (pH of 7.0), which is seen in **Figure 7**. Next, 500 mL of Milli-Q DI water pH was adjusted to 1.925 by adding 12.1 g citric acid monohydrate to bring the molar concentration of citric acid to 0.2M. Following a six-minute sparge with ozone, the concentration of ozone in the 0.2M citric acid solution was measured to be 4.93 mg/L. The rate of decomposition for ozone in a 0.2M citric acid solution more closely follows a linear curve rather than a logarithmic one. The citric acid is stabilizing the ozone concentration, preventing the sudden initial drop that is seen in unbuffered water.

Fourteen milliliter aliquots of ozonated water were added to three 15 mL conical tubes for oocyst treatment. One milliliter of suspended *Eimeria* oocysts (2.6×10^8 oocysts/mL) were added to each biological replicate tube. As the ozone concentration in the original batch dropped over time, additional mixtures of *Eimeria* and ozonated water were created. This resulted in a total of seven initial ozone concentrations tested on *Eimeria* oocysts. The concentration values of ozone that were used included: 4.93, 4.44, 3.68, 2.48, 1.26, 0.78, and 0 mg/L. These were the initial concentrations at which *Eimeria* were treated – the oocysts were held in the treatment tubes while the ozone concentration dropped and eventually had completely dissipated. Treatment times lasted for approximately 60 minutes. Following treatment, oocysts were isolated by centrifugation and resuspended in 5% potassium dichromate and kept in the dark at 25°C for 7 days.

RNA Extraction

Samples for molecular analysis were collected at t=0, 6 and 18 hours following exposure. Oocysts were concentrated through centrifugation and resuspended 1:1 in Trizol reagent. Samples were stored at -80 ˚C unless RNA was isolated immediately.

Table 2: Integrated values for UV irradiation. Radiant energy density, or fluence, is the integrated value of irradiance over time. Equal fluences are marked with a letter subscript for clear delineation in Table 3.

	Radiant Energy Density, J/cm^2		
	Time, seconds		
Irradiance, W/cm^2	60	180	300
8.50×10^{-5}	0.005	0.015_a	0.026
6.00×10^{-5}	0.004	0.011	0.018_a
5.00×10^{-4}	0.030_a	0.090_a	0.015
3.00×10^{-4}	0.018_b	0.005	0.090_b
1.00×10^{-4}	0.006	0.018_c	0.030_b
2.50×10^{-4}	0.015	0.045_a	0.075
7.50×10^{-4}	0.045_b	0.135	0.225

Table 3: Average inhibition of *Eimeria acervulina* oocysts at individual fluences for each wavelength. Equal fluences are marked with a letter subscript, which corresponds to Table 2 above. At several fluences, the bulb flickered or pulsed; these percent inhibition values have been removed in the interest of accuracy. Average inhibition was calculated using the following formula, where t = average fraction sporulated of treated oocysts at a given fluence, and ctl = average fraction sporulated of control, or not treated, oocysts: $\frac{t-ctl}{ctl}$ 100 Standard error of mean shown.

222		
Fluence, J/cm^2	Percent Inhibition	Standard Error
0.004	48.13	1.64
0.006	39.33	1.45
0.011	54.71	0.91
0.015	46.83	1.26
0.018_b	30.01	0.74
0.018_c	44.76	1.22
0.018_a	60.41	0.47
0.026	46.79	1.41
0.03_b	49.02	1.13
0.045_b	78.27	0.76
0.075	47.79	1.07
0.09_b	61.00	0.09
0.135	81.37	0.50
0.225	85.55	0.45

282		
Fluence, J/cm^2	Percent Inhibition	Standard Error
0.004	51.06	1.01
0.005	40.16	1.43
0.011	46.35	0.51
0.015	50.15	1.86
0.018_a	40.86	1.49
0.026	49.42	0.46
0.03_a	34.75	1.45
0.03_b	54.42	0.94
0.045_b	65.67	1.30
0.045_a	68.20	0.69
0.075	71.49	0.73
0.09_a	48.74	0.51
0.135	69.75	0.79
0.150	58.05	0.81
0.225	64.16	1.01

Oocysts were transferred to a bead beater tube with glass beads and run through a cycle of 6.0 m/s for 60 seconds in a MP Bio bead beater (FastPrep-24 5G, MP Biomedicals). RNA was then isolated using the Zymo RNA miniprep kit per the manufacturer's instructions, and isolates were purified using the RNA Clean and Concentrate kit from the same manufacturer (Zymo, USA). RNA concentration was determined using a QuBit fluorometer. Quantitative real-time PCR was performed using the PrimeScript One-step kit from Takara on an ABI QuantStudio3 for 40 cycles with a melting temperature of 56-60°C. Ct values were normalized to the expression of the EF2 reference gene. Relative quantification was preformed using the ΔΔCt method.

Analysis of Gene Expression

Candidate genes were identified using Gene Ontology (GO) searches of key words and phrases including "oxidative stress", "ozone response", and "DNA repair". BLASTx searches were used to identify cognate *E. acervulina* genes using mouse or human ortholog queries. Twenty-three gene targets were initially evaluated, and refined to those whose products have obvious roles in mediating response to oxidative stress. Conserved Domain Database (CDD) searches were preformed to identify conserved domains within the amino acid sequences of any unannotated or hypothetical proteins, elucidating the functional domains of these proteins and the possible roles they play in ozone response.

Reactive oxygen species assays

Production of reactive oxygen species was measured using three separate assays produced by Invitrogen. These included the Reactive Oxygen Species assay, the Amplex Red Hydrogen Peroxide/Peroxidase assay, and the MitoSOX Red mitochondrial superoxide indicator kits. All assays were performed according to the manufacturer's instructions, with the exception of the total reactive oxygen species kit, for which fluorescence detection on a Cytation 5 (Agilent Technologies) plate reader was substituted for flow cytometry. For this assay, autofluorescence of untreated *Eimeria*, as well as the dichromate substrate was measured, based on work demonstrating that aged *E. maxima* show enhanced autofluorescence in the GFP range during prolonged storage (Beer et al., 2018). For each assay, oocysts were spun down and washed to

remove residual ozone. For the MitoSOX assay specifically, the supernatant was collected and assayed to ensure extracellular superoxide was not detected.

Oocyst Imaging

At 7 days post-exposure, visual scoring for sporulation took place using confocal microscopy. Oocysts were imaged at 60x under oil immersion. Twenty images were taken of oocysts from each integrated value shown in **Table 2**. These images were analyzed by three trained individuals until at least 500 total oocysts from each treatment were scored. While the three individuals had consistent trends of inactivation within their own scoring, their totals for sporulated oocysts varied among them. For this reason, totals were averaged to account for variations in individual counts.

Statistical Analysis

Data are presented as the mean ± standard error of the mean (SEM). Statistical analysis was performed using SPSS version 29. One way ANOVA with Tukey HSD post-hoc test was used to measure differences among groups with $p<0.05$ used as a cutoff value for statistical significance.

Results

UV Irradiation

Radiant Energy Density (J/cm^2), the integrated dose of irradiance (W/cm^2) the oocysts were treated with, significantly affected sporulation of *Eimeria acervulina*, when treated for 5 minutes with 282 nm UV light, sporulation was inhibited by 85.55%. A list of relative inhibitions at every fluence can be found in **Table 3**. The three wavelengths tested inactivated *Eimeria* oocysts in a dose-dependent manner (**Table 3**). Potassium dichromate was found to have a protective effect for the oocysts. Oocysts suspended in a 2.5% potassium dichromate solution and exposed to UV irradiation had no statistical difference from untreated oocysts suspended in dichromate or water (**Figure 5).**

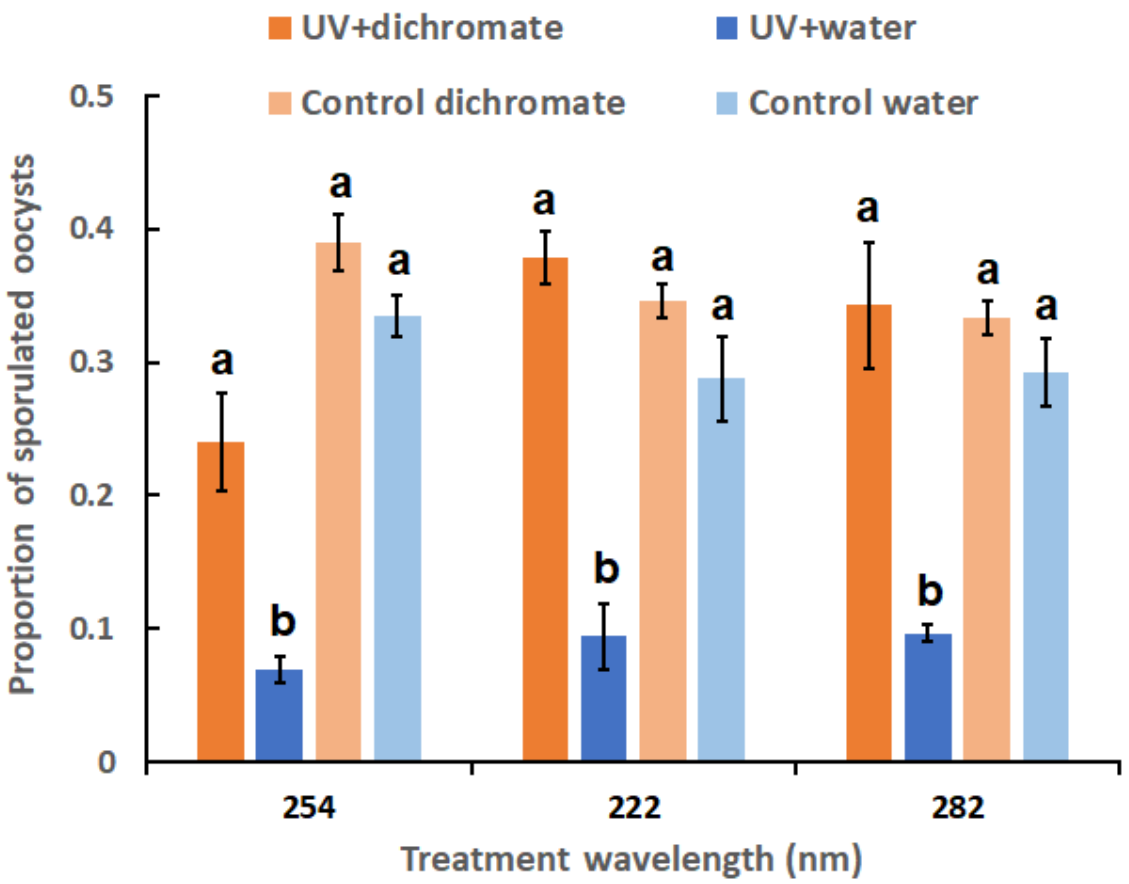

Figure 5: Exposure to UV light inhibits sporulation of E. acervuline oocysts; protective effect of potassium dichromate against UV irradiation at three wavelengths: 254, 222, and 282. Bars indicate mean standard error. Significant levels shown within each wavelength. $p < 0.05$.

Aqueous Ozone

Ozone spontaneously decomposes in water, in which its breakdown is influenced by various factors including pH, temperature, dissolved organic matter, and the presence of inorganic ions. This decomposition forms free radicals that oxidize inorganic and organic compounds. At low pH, ozone is more stable and reactive, since in acidic conditions the ozone molecule is protonated and exists in the form of ions such as $HOOO^-$ and H_2OO^{2-}, which can react more readily with organic compounds and pathogens, leading to increased disinfection efficiency. Ozone depletion in water is highly dependent on dissolved minerals and/or total organic content (TOC), as well as pH, as previously demonstrated (for reviews, see (Guzel-Seydim et al., 2004; Kim et al., 1999)). Indeed, in tap water a precipitous drop was initially seen between t=0 and t=10min, consistent with the phenomenon of the immediate ozone demand (IOD; (Xu et al., 2002)) zone (**Figure 6a**). In contrast, ozonated deionized water generated using a MilliQ system showed that this steep initial decrease was substantially attenuated but not completely eliminated (**Figure 6b**). The addition of citric acid monohydrate to a final concentration of 0.2M per Harihara et al., (2019) eliminated the IOD zone and provided a linear decomposition curve (Hirahara et al., 2019) (**Figure 6c, d**). Consistent with oocysts tolerating acidic conditions in the host alimentary canal, no effect on sporulation capacity was observed when oocysts were treated with 1M HCl in a previous assay (not shown). Therefore, the addition of citric acid was not expected to influence the sporulation rate. *Eimeria acervulina* oocysts were treated with a range of initial ozone concentrations from 0-4.93 mg/L to evaluate the subsequent effects on sporulation. Unsporulated *E. acervulina* oocysts are ovoid structures initially containing a round, disorganized cytoplasmic mass surrounded by a protective oocyst wall. Following sporulation the oocyst resembles a bag of marbles, harboring four distinct, round sporocysts and this morphology is readily distinguishable via light microscopy (**Figure 7a, b)**. Sporulation efficiency and thus infectivity was scored by imaging 96-well plates containing treated oocysts under 40x magnification on an Olympus Fv1000 confocal microscope. The number of fully sporulated oocysts with distinguishable sporocysts (**Figure 7c**) was counted and expressed as a percentage of the total number of oocysts in each field of view.

Liou and colleagues (2002) previously achieved partial inhibition of sporulation in *E. colchici* oocysts challenged with ozone; lower sporulation ratios and increased survival in birds

inoculated with treated oocysts were observed. Inhibition of sporulation was 18.4% in oocysts treated for 15 minutes with an ozone residual of 4.1 mg/L and 28.3% in oocysts treated for 60 minutes with an ozone residual of 3.8 mg/L (Liou et al., 2002) . A more recent study achieved a 27.3% reduction in sporulation capacity following treatment with 2mg/L aqueous ozone for 5 minutes (Morgoglione et al., 2021). In the present study, dose-dependent sporulation inhibition was observed. At the highest ozone concentration of 4.93 mg/L, 7% of oocysts sporulated by day 7, while the control group showed approximately 52% sporulated oocysts (**Figure 7c**).

Ozone is a more effective antimicrobial at low pH, since its stability is increased. Thus, the effect of buffering aqueous ozone with citric acid may play a substantial role in the relative increase in efficacy that is seen in the present studies. Nevertheless, our data indicate that aqueous ozone with a reduced pH is highly effective in preventing sporulation. No gross morphological changes were readily apparent via confocal microscopy following ozone exposure and incubation. While sporulation at 7 days post treatment was used as the assay, the possibility exists that sporulation was delayed rather than completely blocked. In the context of *E. acervulina* as a proxy for *C. cayetanensis*, which is distributed on fresh produce, a substantial heterochronic shift in time to sporulation remains an effective disinfection method since fresh produce must be consumed before spoilage.

Molecular Response to Ozone

The mechanism through which ozonation blocks sporulation in *Eimeria* has not been extensively studied. Bacterial aggregation along distinctly misshapen oocyst walls was reported for ozone-treated *E. colchici* (Liou et al., 2002). A similar phenomenon of fast-attachment of bacteria to the oocyst wall was observed in ozone-treated *Eimeria* spp. oocysts with no external morphological damage (Morgoglione et al., 2021). Subtle warping of the oocyst wall versus controls was observed, which showed entirely smooth oocysts with no surface anomalies (**Figure 7a, b**).

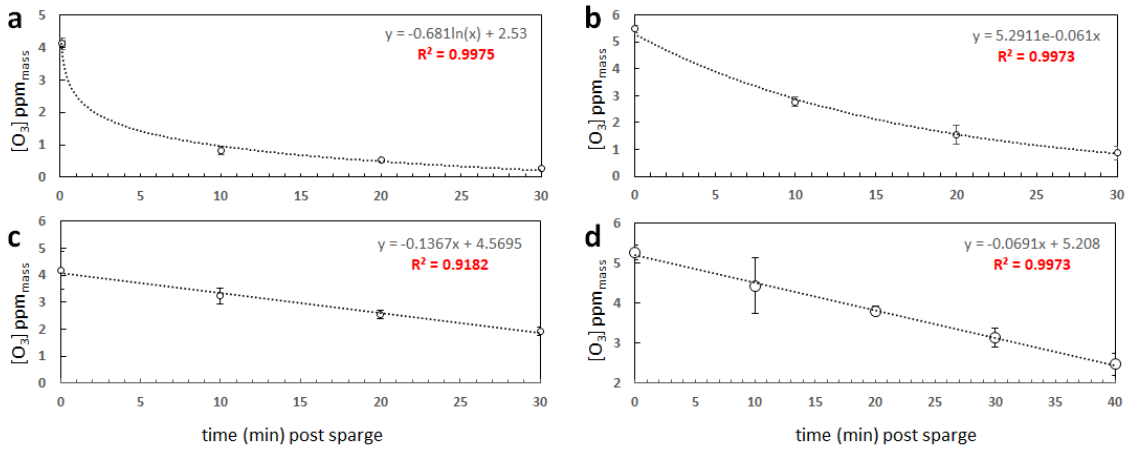

Figure 6: Effects of pH on ozone decomposition in water. Circles indicate average standard error. a) tap water; pH 7.0. b) MiliQ deionized water; pH 6.8. c) MiliQ deionized water with citric acid monohydrate added to a final concentration of 0.2 M and pH 1.9. d) MiliQ deionized water with citric acid monohydrate added to a final concentration of 0.2 M and pH 1.9. R^2 value shown to indicate change from logarithmic to linear decomposition upon lowering of pH via the addition of citric acid. The concentrations of ozone shown in graph d were used to inactivate *Eimeria acervulina.*

To study how ozone influences *E. acervulina* physiology at the molecular level, ROS assays were combined with qRT-PCR targeting transcripts involved in response to oxidative stress. Following a challenge with an initial dose of approximately 4 mg/L ozone that was allowed to sit until the concentration of ozone was below detection limits (one hour) RNA was collected from oocysts at t=0, 6, and 18 h post-exposure for targeted qRT-PCR transcriptional profiling.

According to a total ROS assay, the production of oxygen free radicals increased throughout the period of 0-24 h following ozone treatment (**Figure 8b**). A similar phenomenon of fast-attachment of bacteria to the oocyst wall was observed in ozone-treated *Eimeria* spp. oocysts with no external morphological damage.

Conversely, both H_2O_2 and mitochondrial superoxide accumulation decreased during the analysis window, consistent with the action of genes involved in scavenging free radicals and peroxides (**Figure 8a, b**).

Peroxiredoxin 1 and 2: EAH_00047270 and EAH_00058070

Peroxiredoxin (Prx) family members are widely represented in all living things as H2O2 scavengers, catalyzing the conversion of peroxides into water and alcohols. The human genome contains six isoforms, while E. coli harbors 3 and Arabidospsis thaliana expresses 9 unique Prx subtypes (Rhee, 2016). Two Prx family members were identified in *E. acervulina* using BLASTp searches against the species' genome. Prx1 (EAH_00047270) was upregulated most intensely immediately following exposure to ozone and elevated levels of expression relative to controls was sustained through the 18-hour timepoint (**Figure 8a)**. Prx1 family members are classified as typical 2-Cys Prxs, which function as homodimers that each contain one peroxidatic cysteine residue per subunit (Rhee, 2016). In mice, Prx1-/- mutants displayed significantly less pulmonary tissue inflammation following ozone exposure than their wild-type congeners, indicating a cellular role for Prx1 in mediating the innate immunological response to ozone (Yanagisawa et al., 2012). Prx2 (EAH_00058070) expression was significantly higher than controls extending to 18h, at which point the expression was elevated, but not significantly different from controls (**Figure 8a**).

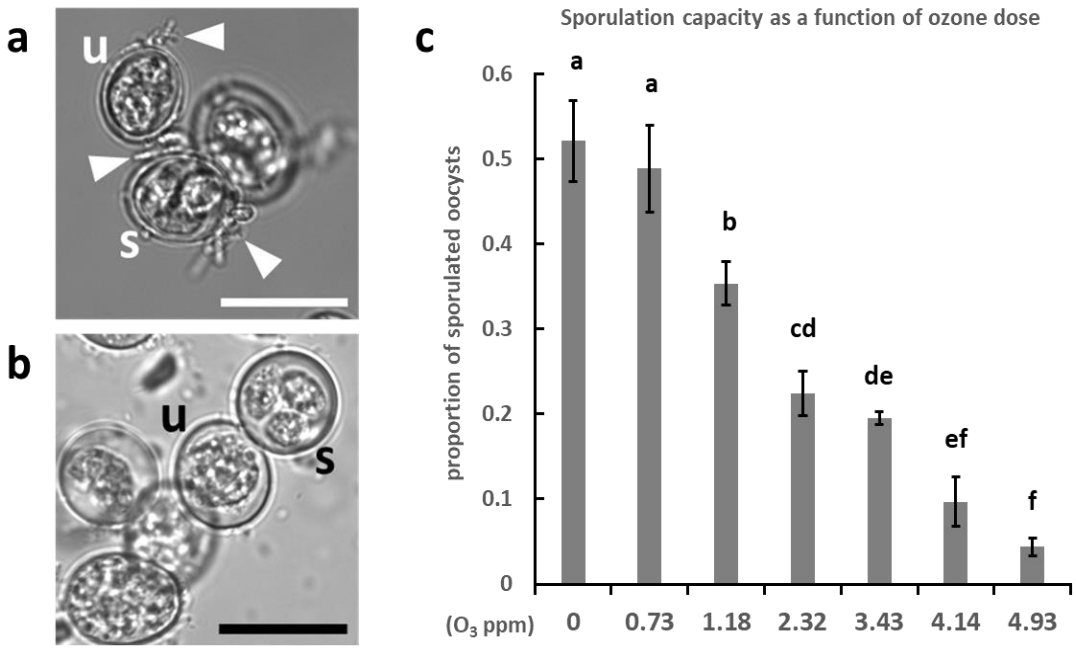

Figure 7: a) Arrows indicating bacterial aggregation along oocyst wall, and misshapen oocysts compared to controls below. Scale bars: 20 uM. u = unsporulated; s = sporulated b) Control oocysts received no aqueous ozone. Scale bars: 20 uM. u = unsporulated; s = sporulated. c) Oocyst sporulation is shown to be dose-dependent following exposure to ozone gas dissolved in water buffered to pH 1.9 with citric acid monohydrate. O_3 ppm by mass in water shown. Bars represent mean standard error for each dose. $p < 0.05$; repeated one-way ANOVA.

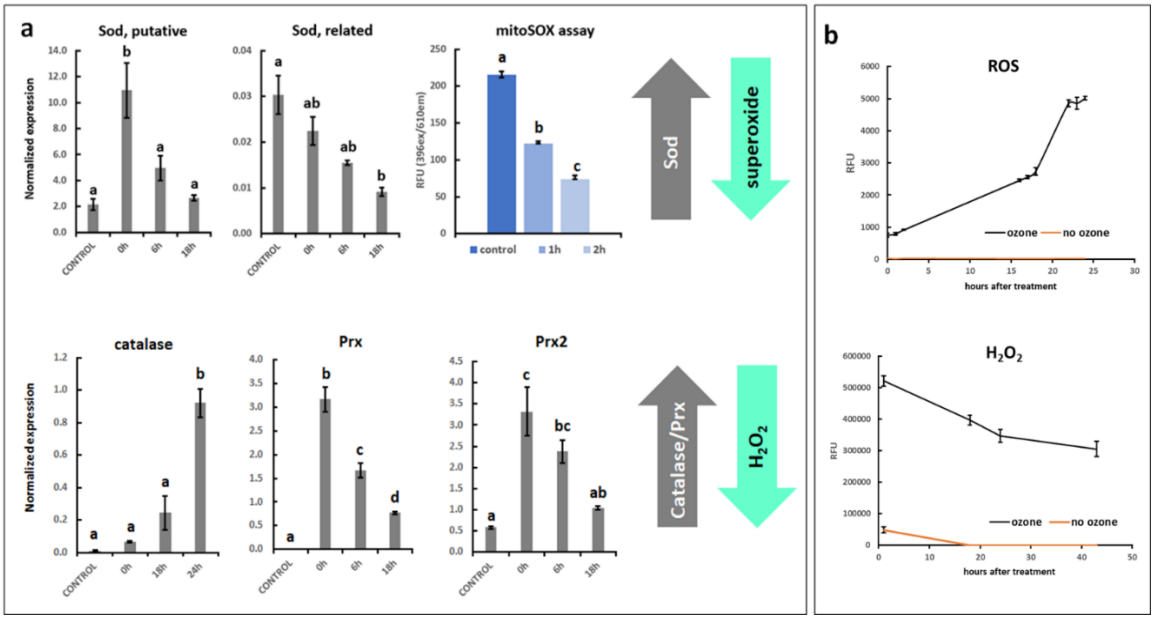

Figure 8: Several gene expression profiles in relation to the intracellular production of oxyradicals and peroxide a) Quantitative PCR results for superoxide dismutase (Sod), catalase, and peroxiredoxin (Prx) 1 and 2 transcripts and results of mitoSOX assay, a readout of mitochondrial superoxide formation. The mitoSOX readout was limited to 2 hours per manufacturer's recommendations. For all expression profiles shown, zero-hour time points represent the first RNA collection after a 1h exposure to ozone. $p<0.05$, one-way ANOVA. Bars indicate average ± standard error (n=3). b) Temporal profiles of total intracellular reactive oxygen species (top) and hydrogen peroxide (bottom) over the course of 24- and 48h, respectively. $p<0.05$, one-way ANOVA. Bars indicate average ± standard error (n=3).

Prx2 is also a typical 2-Cys Prx whose closest orthologs in related species include proteins annotated as both Prx2 and Prx6 with the latter annotation appearing more frequently (ex.: Cyclospora, Schistocerca, Periplaneta, Xenopus).

Superoxide dismutase: EAH_00053040 and EAH_00009710

Superoxide dismutase (SOD) catalyzes dismutation in a cell, metabolizing superoxide radicals to molecular oxygen and hydrogen peroxide.

There are three types of superoxide dismutases, each containing metal ions, and each localized to respective cellular locations (Nojima et al., 2019). The number of genes that encode sod vary across species; there are three copies in the silkworm Bombyx mori, (Nojima et al., 2019), seven in Arabidopsis (Kliebenstein et al., 1998), 18 copies in Gossypium (cotton), and five genes encode SODs in *C. elegans*. Each sod performs dismutation with different metal ions. SOD1 is a Cu/Zn containing sod, located in the cytosol. In response to the presence of H_2O_2, SOD1 localizes to the nucleus where it acts as a transcription factor (Tsang et al., 2014). Sod2 is located in the mitochondria and is a Mn or Fe dismutase, whereas sod3 is an extracellular dismutase. Two sod annotations were identified in the E. acervulina genome: EAH_00053040 ("superoxide dismutase, putative") and EAH_00009710 ("superoxide dismutase, related").

Quantitative RT-PCR analysis showed that EAH_00053040 transcripts were significantly upregulated upon ozone treatment (**Figure 8a**). According to the Amplex Red Hydrogen peroxide assay, peak H2O2 production was consistent with the peak sod expression, each of which decreased during the post-exposure period (**Figure 8a, b**). UniProtKB/TrEMBL conserved domains indicate that EAH_00053040 contains Fe or Mn superoxide dismutase domains, suggesting that EAH_005340 may be a mitochondrial SOD. Therefore, the MitoSOX kit was used to assess the mitochondrial superoxide levels, which decreased in ozone-treated oocysts in which EAH_0005340 levels were elevated in response to ozone. This supports the annotation that suggests this may be a mitochondrial SOD.

EAH_0009710 expression immediately following exposure to ozone was not statistically different from controls, additionally, expression waned at 6 and then 18 hours post-exposure (**Figure 9a, b)**. EAH_00053040 transcript abundance was statistically similar to control samples

by 6 hours post-exposure, however, EAH_0009710 levels were lower than controls at this timepoint.

In addition to genes whose products are directly involved in mediating the response to oxidative stress, we identified other target transcripts via GO searches using terms relative to ozone, response to oxygen radicals, and oxidative stress.

Eimeria acervulina *cation-transporting ATPase: EAH_00000740,*

EAH_00000740, annotated as *Eimeria acervuline* cation-transporting ATPase, harbors a HAD (haloacid dehalogenase) family domain. This superfamily includes ATPases, particularly EAH_00000740 shows close sequence identity with a Toxoplasma gondii P-type ATPase. P-type ATPases are cation-transporting, membrane-bound proteins with demonstrated roles in maintaining cation homeostasis under oxidative stress, as shown in human erythrocytes in response to ozone (Tukel et al., 1994). Expression of EAH_00000740 peaked immediately following ozone exposure and remained relatively elevated until transcription levels fell by 18 h (**Figure 10a)**.

Oxidation Resistance 1: EAH_00006130

EAH_00006130 is a TDLc domain containing protein whose expression levels were affected by exposure to ozone. TLDc [Tre2/Bub2/Cdc16 (TBC), lysin motif (LysM), domain catalytic] domain containing proteins function to protect against oxidation related stress in eukaryotes (Finelli & Oliver, 2017). Their mode of action remains unclear, but it was recently shown that TLDc proteins interact with V-ATPase (Eaton et al., 2021), a transmembrane proton pump with demonstrated activity in protecting against oxidative damage (Thorpe et al., 2004). The transcription of EAH_0006130 was most abundant at six hours following exposure to ozone (**Figure 9d**). The results of this study also show that both EAH_0006130 (OXR1) and Prx2 are upregulated relative to controls at 6 hours post ozone treatment, raising the possibility that this interaction may occur in *E. acervulina* in response to the intracellular accumulation of oxygen radicals.

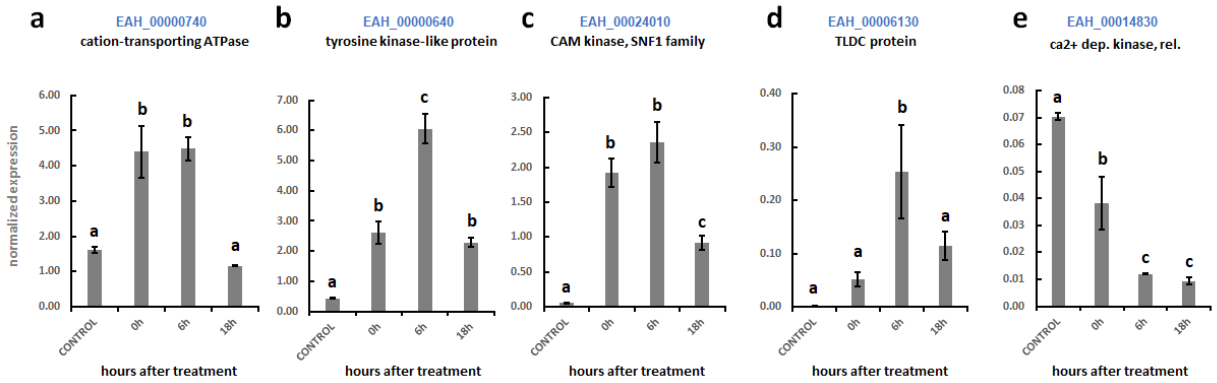

Figure 9: Time courses of the expression of genes identified using GO searches for "response to ozone" and "oxidative stress" are shown with their EAH annotation numbers. 0 h time points represent the first RNA collection after a 1 h exposure to ozone. $p<0.05$, one-way ANOVA. Bars indicate average ± standard error (n=3).

Five annotations consisting of three unique C. cayetanensis annotations were obtained via BLASTp searches; four of these annotations comprised two sets of duplicate sequences with different names. A multiple alignment of amino acid sequences supports C. cayetanensis LOC34621889 as the EAH_00006130 ortholog.

Peroxisomal catalase, putative: EAH_00041420

EAH_00041420, annotated as a putative peroxisomal catalase was identified in the *E. acervulina* genome, and the expression was monitored for 24 hours.
Catalase converts H_2O_2 to water and oxygen. Its induction in *E. coli* was reported to be more responsive than that of SOD in response to ozone (Whiteside & Hassan, 1987). Our data suggest that peak EAH_0006130 expression at 6 hours may likewise influence catalase levels in *E. acervulina* in response to oxidative insult (**Figure 9a**). Likewise, catalase plays a protective role in Listeria monocytogenes in protecting cells from ozone, but mutant strains showed that catalase plays a less significant role than SOD. However, a SOD overexpressing strain lacking catalase was hypersensitive to ozone, highlighting the role for catalase in protecting against oxidative stress (Fisher et al., 2000).

CAM kinase, SNF1 family, putative: EAH_00024010

The expression EAH_00024010 showed significant upregulation from 0 to 18 hours post ozone challenge relative to controls (**Figure 9c**). Conserved domain searches identified this gene product as harboring a CAM kinase domain, characteristic of stress-inducible kinases that monitor intracellular energy stores. CAM kinases serve as master regulators of energy homeostasis. SNF1 is a well characterized family member in yeast that responds to environmental stress. When yeast were treated with a selenium compound to induce oxidative stress and DNA damage, SNF1 was required to maintain the ratio of reduced to oxidized glutathione (Perez-Sampietro et al., 2013). Thus, EAH_00024010 upregulation in response to ozone challenge likely reflects the need for this protein in protecting against oxidative damage to DNA, or activity in other pathways that regulate intracellular energy stores under physiological stress.

Calcium-dependent protein kinase, related: EAH_00014830

EAH_00014830 produces a protein product that contains an STKc_CAMK and Ca2+ binding EF-hand superfamily domain. STKc_CAMK members are serine/threonine kinases and this family includes the well-characterized CamKII. This protein, which is activated by ROS (M. E. Anderson, 2015)is a critical sensor of oxidative stress in cardiac tissue(Swaminathan et al., 2012), where sustained CamKII expression is linked to apoptotic pathways. EAH_00014830 downregulation relative to controls from 0h – 18h post-treatment in treated oocysts indicates a cellular attempt to mitigate spurious or enhanced apoptosis in coordination with the upregulation of antioxidant enzymes under oxidative stress (**Figure 9e**).

Tyrosine kinase-like protein: EAH_0000640

EAH_0000640 encodes a protein that contains a PKc-like superfamily domain. Proteins containing this domain catalyze the transfer of a phosphoryl group from ATP to serine, threonine, or tyrosine residuals of other proteins. BLASTp searches against mammalian genomes identified mitogen activated protein kinase (MAPK) as the closest mammalian, zebrafish, and *Drosophila* ortholog. MAPK7 is activated in response to oxidative stress (Abe et al., 1996) and implicated in apoptotic signaling (Pi et al., 2004). EAH_0000640 transcription was upregulated following ozone exposure; levels were maintained through 6 hours until they attenuated at 18 hours post-exposure to levels no statistically different from controls (**Figure 9b**). Initial increased expression of EAH_0000640 in response to oxidative stress suggests a cellular attempt at mitigating apoptotic activity (Pi et al., 2004).

Conclusion

Initial studies conducted with UV have shown promising results in regard to its efficacy in inhibiting sporulation of *Eimeria acervulina*, relative sporulation was reduced by over 99% at every fluence and timepoint tested. The oxidative stress of UV irradiation likely induces a response similar to the one induced by ozone. This is supported by the dose-dependent curve and initial QRT-PCR analyses showing gene targets that were upregulated in response to ozone exposure have indeed been upregulated in response to UV exposure. Therefore, future studies will further elucidate the dose-dependent manner of inhibition with three wavelengths: 254, 222,

and 282 nm, as well as the effect of UV exposure in regards to the expression of several gene targets present in *Eimeria acervulina*.

In this study the efficacy of aqueous ozone to disrupt sporulation in a model coccidian was demonstrated. Consistent with previous reports (Liou et al., 2002), bacterial accumulation and mildly malformed oocyst walls, characterized by subtle warping of the near perfect ovoid structure were observed. A general cellular response of genes known to be related to oxidative stress and found with the search terms "response to ozone" and "oxidative stress" was studied. The cellular response of *Eimeria acervulina* to oxidative stress induced by aqueous ozone was supported by the upregulation of several enzymes and proteins involved in cellular protection from free radicals. Prx1, which was indicated in the immunological protection from ozone in mice, was significantly upregulated following exposure to ozone in this study (Yanagisawa et al., 2012). The steady attenuation of cellular hydrogen peroxide levels was concurrent with the expression levels of Prx1 and catalase, implicating these transcripts in the reduction of H_2O_2. The expression of antioxidant enzymes whose established roles deal specifically with detoxifying oxygen free radicals indicates a cellular attempt to mitigate the deleterious effects of excess reactive oxygen species. The current study supports the idea that OXR1 may induce catalase expression, as peak ORX1 expression preceded peak catalase production. Future work will be dedicated to conducting several assays to study the levels of free radicals in the cell, and identifying additional transcripts affected by the cellular response to ozone.

In contrast to methods that rely on continuous ozone exposure for disinfection, the method presented in this study treated oocysts with an initial concentration of ozone with was subsequently allowed to decompose to negligible levels by approximately one hour. Despite this reduction in ozone over time, the treatment showed significant effectiveness against oocysts. Treatment with a one-time application of 4.93 mg/L aqueous ozone resulted in 93.5% relative inhibition of oocyst sporulation (treated oocysts that sporulated make up 6.45% of control). This increased efficacy relative to previous studies is perhaps due to the improved stabilization of ozone in water provided by 0.2M citric acid, allowing oocysts to be exposed to a higher concentration of ozone for longer.

REFERENCES

21 CFR § 178.1010. (2023). *21 CFR*.

40 CFR § 50.10. (2015). *40 CFR*.

Abe, J., Kusuhara, M., Ulevitch, R. J., Berk, B. C., & Lee, J. D. (1996). Big mitogen-activated protein kinase 1 (BMK1) is a redox-sensitive kinase. *J Biol Chem, 271*(28), 16586-16590. https://doi.org/10.1074/jbc.271.28.16586.

Achen, M. A., & Yousef, A. E. (2001). Efficacy of Ozone Against Escherichia coli O157:H7 on Apples. *Journal of Food Science, 66*(9), 1380-1384. https://doi.org/https://doi.org/10.1111/j.1365-2621.2001.tb15218.x.

Alder, M. G., & Hill, G. R. (1950). The Kinetics and Mechanism of Hydroxide Ion Catalyzed Ozone Decomposition in Aqueous Solution1. *Journal of the American Chemical Society, 72*(5), 1884-1886. https://doi.org/10.1021/ja01161a007.

Alfano-Sobsey, E. M., Eberhard, M. L., Seed, J. R., Weber, D. J., Won, K. Y., Nace, E. K., & Moe, C. L. (2004). Human challenge pilot study with Cyclospora cayetanensis. *Emerg Infect Dis, 10*(4), 726-728. https://doi.org/10.3201/eid1004.030356.

Almeria, S., Cinar, H. N., & Dubey, J. P. (2019). Cyclospora cayetanensis and Cyclosporiasis: An Update. *Microorganisms, 7*(9). https://doi.org/10.3390/microorganisms7090317.

Anderson, M. E. (2015). Oxidant stress promotes disease by activating CaMKII. *J Mol Cell Cardiol, 89*(Pt B), 160-167. https://doi.org/10.1016/j.yjmcc.2015.10.014.

Anderson, P., & Aubermann, H. (2023). Technical Note No. 049: Corrosive Nitric Acid from Ozone Generators Harms Equipment. Retrieved from: https://twobtech.com/docs/tech_notes/TN049.pdf Accessed.

Bartsch, S. M., Asti, L., Nyathi, S., Spiker, M. L., & Lee, B. Y. (2018). Estimated Cost to a Restaurant of a Foodborne Illness Outbreak. *Public Health Rep, 133*(3), 274-286. https://doi.org/10.1177/0033354917751129.

Beer, L. C., Bielke, L. R., Barta, J. R., Faulkner, O. B., Latorre, J. D., Briggs, W. N., . . . Hargis, B. M. (2018). Evaluation of autofluorescent Eimeria maxima oocysts as a potential indicator of non-viability when enumerating oocysts. *Poult Sci, 97*(8), 2684-2689. https://doi.org/10.3382/ps/pey124.

Beltrán, D., Selma, M. V., Marín, A., & Gil, M. I. (2005). Ozonated Water Extends the Shelf Life of Fresh-Cut Lettuce. *Journal of Agricultural and Food Chemistry*, *53*(14), 5654-5663. https://doi.org/10.1021/jf050359c.

Bialka, K. L., & Demirci, A. (2007). Decontamination of Escherichia coli O157:H7 and Salmonella enterica on Blueberries Using Ozone and Pulsed UV-Light. *Journal of Food Science*, *72*(9), M391-M396. https://doi.org/https://doi.org/10.1111/j.1750-3841.2007.00517.x.

Birdsall, C. M., Jenkins, A. C., & Spadinger, E. (1952). Iodometric Determination of Ozone. *Analytical Chemistry*, *24*(4), 662-664. https://doi.org/10.1021/ac60064a013.

Blake, D. P., Knox, J., Dehaeck, B., Huntington, B., Rathinam, T., Ravipati, V., . . . Tomley, F. M. (2020). Re-calculating the cost of coccidiosis in chickens. *Vet Res*, *51*(1), 115. https://doi.org/10.1186/s13567-020-00837-2.

Bourassa, L. (2023). Vegan and Plant-Based Diet Statistics for 2023. Retrieved from: https://www.plantproteins.co/vegan-plant-based-diet-statistics/ Accessed.

Broadwater, W. T., Hoehn, R. C., & King, P. H. (1973). Sensitivity of three selected bacterial species to ozone. *Appl Microbiol*, *26*(3), 391-393. https://doi.org/10.1128/am.26.3.391-393.1973.

Bruggemann, N., Fiekens, R., Salvermoser, M., & Zhou, L. (2018). Importance of Feed Gas Quality for Ozone Generation. (Vol. 46). *Ozone News*.

CDC. (2021). Investigation Details. Retrieved from: https://www.cdc.gov/ecoli/2021/o157h7-12-21/details.html Accessed.

CDC. (2023). List of Outbreaks. Retrieved from: https://www.cdc.gov/foodsafety/outbreaks/lists/outbreaks-list.html Accessed.

Chang, J. C., Ossoff, S. F., Lobe, D. C., Dorfman, M. H., Dumais, C. M., Qualls, R. G., & Johnson, J. D. (1985). UV inactivation of pathogenic and indicator microorganisms. *Appl Environ Microbiol*, *49*(6), 1361-1365. https://doi.org/10.1128/aem.49.6.1361-1365.1985.

Chawla, A. S., Kasler, D. R., Sastry, S. K., & Youset, A. E. (2012). Microbial decontamination of food using ozone. In A. Demirci & M. Ngadi (Eds.), *Microbial Decontamination in the Food Industry* (pp. 495-532). Cambridge ; Philadelphia, Pa.: Woodhead Pub. Ltd.,.

Claus, H. (2021). Ozone Generation by Ultraviolet Lamps(dagger). *Photochem Photobiol, 97*(3), 471-476. https://doi.org/10.1111/php.13391.

Collins, J., Tack, D., Pindyck, T., & Griffen, P. (2023). Escherichia coli, Diarrheagenic. Retrieved from: https://wwwnc.cdc.gov/travel/yellowbook/2024/infections-diseases/escherichia-coli-diarrheagenic#:~:text=Epidemiology,certain%20types%20of%20diarrheagenic%20E Accessed.

Dubuis, M. E., Dumont-Leblond, N., Laliberte, C., Veillette, M., Turgeon, N., Jean, J., & Duchaine, C. (2020). Ozone efficacy for the control of airborne viruses: Bacteriophage and norovirus models. *PLoS One, 15*(4), e0231164. https://doi.org/10.1371/journal.pone.0231164.

Eaton, A. F., Brown, D., & Merkulova, M. (2021). The evolutionary conserved TLDc domain defines a new class of (H(+))V-ATPase interacting proteins. *Sci Rep, 11*(1), 22654. https://doi.org/10.1038/s41598-021-01809-y.

Farooq, S., & Akhlaque, S. (1983). Comparative response of mixed cultures of bacteria and virus to ozonation. *Water Research, 17*(7), 809-812. https://doi.org/https://doi.org/10.1016/0043-1354(83)90076-3.

Finelli, M. J., & Oliver, P. L. (2017). TLDc proteins: new players in the oxidative stress response and neurological disease. *Mamm Genome, 28*(9-10), 395-406. https://doi.org/10.1007/s00335-017-9706-7.

Fisher, C. W., Lee, D., Dodge, B. A., Hamman, K. M., Robbins, J. B., & Martin, S. E. (2000). Influence of catalase and superoxide dismutase on ozone inactivation of Listeria monocytogenes. *Appl Environ Microbiol, 66*(4), 1405-1409. https://doi.org/10.1128/AEM.66.4.1405-1409.2000.

Foarde, K. K., Vanosdell, D. W., & Steiber, R. S. (1997). Investigation of Gas-Phase Ozone as a Potential Biocide. *Applied Occupational and Environmental Hygiene, 12*(8), 535-542. https://doi.org/10.1080/1047322X.1997.10390043.

Fontes, B., Cattani Heimbecker, A. M., de Souza Brito, G., Costa, S. F., van der Heijden, I. M., Levin, A. S., & Rasslan, S. (2012). Effect of low-dose gaseous ozone on pathogenic

bacteria. *BMC Infectious Diseases, 12*(1), 358. https://doi.org/10.1186/1471-2334-12-358.

Fratamico, P. M., DebRoy, C., Liu, Y., Needleman, D. S., Baranzoni, G. M., & Feng, P. (2016). Advances in Molecular Serotyping and Subtyping of Escherichia coli. *Frontiers in Microbiology, 7*. https://doi.org/10.3389/fmicb.2016.00644.

Glowacz, M., Colgan, R., & Rees, D. (2015). The use of ozone to extend the shelf-life and maintain quality of fresh produce. *Journal of the Science of Food and Agriculture, 95*(4), 662-671. https://doi.org/https://doi.org/10.1002/jsfa.6776.

Gurney's. (2021). How Many People Wash Their Produce in Every State? In G. s. Team (Ed.).

Guzel-Seydim, Z. B., Greene, A. K., & Seydim, A. C. (2004). Use of ozone in the food industry. *LWT - Food Science and Technology, 37*(4), 453-460. https://doi.org/https://doi.org/10.1016/j.lwt.2003.10.014.

Hashem, T. M., Zirlewagen, M., & Braun, A. M. (1997). Simultaneous photochemical generation of ozone in the gas phase and photolysis of aqueous reaction systems using one VUV light source. *Water Science and Technology, 35*(4), 41-48. https://doi.org/https://doi.org/10.1016/S0273-1223(97)00007-3.

Hirahara, Y., Iwata, K., & Nakamuro, K. (2019). Effect of Citric Acid on Prolonging the Half-life of Dissolved Ozone in Water. *Food Saf (Tokyo), 7*(4), 90-94. https://doi.org/10.14252/foodsafetyfscj.D-19-00005.

Hudson, J. B., Sharma, M., & Vimalanathan, S. (2009). Development of a Practical Method for Using Ozone Gas as a Virus Decontaminating Agent. *Ozone: Science & Engineering, 31*(3), 216-223. https://doi.org/10.1080/01919510902747969.

Ishizaki, K., Shinriki, N., & Matsuyama, H. (1986). Inactivation of Bacillus spores by gaseous ozone. *Journal of Applied Bacteriology, 60*(1), 67-72. https://doi.org/10.1111/j.1365-2672.1986.tb01067.x.

JohnsHopkins. (2023). Escherichia coli O157:H7. Retrieved from: https://www.hopkinsmedicine.org/health/conditions-and-diseases/escherichia-coli-o157-h7 Accessed 2023.

Kaess, G., & Weidemann, J. F. (1968). Ozone treatment of chilled beef. *International Journal of Food Science & Technology, 3*(4), 325-334. https://doi.org/https://doi.org/10.1111/j.1365-2621.1968.tb01473.x.

Khadre, M. A., Yousef, A. E., & Kim, J.-G. (2001). Microbiological Aspects of Ozone Applications in Food: A Review. *Journal of Food Science, 66*(9), 1242-1252. https://doi.org/https://doi.org/10.1111/j.1365-2621.2001.tb15196.x.

Kim, J.-G., Yousef, A. E., & Dave, S. (1999). Application of Ozone for Enhancing the Microbiological Safety and Quality of Foods: A Review. *Journal of Food Protection, 62*(9), 1071-1087. https://doi.org/10.4315/0362-028x-62.9.1071.

Kliebenstein, D. J., Monde, R. A., & Last, R. L. (1998). Superoxide dismutase in Arabidopsis: an eclectic enzyme family with disparate regulation and protein localization. *Plant Physiol, 118*(2), 637-650. https://doi.org/10.1104/pp.118.2.637.

Kondo Nakada, L. Y., Urbano Dos Santos, L., & Guimaraes, J. R. (2020). Pre-ozonation of surface water: An effective water treatment process to reduce the risk of infection by Giardia in drinking water. *Environ Pollut, 266*(Pt 3), 115144. https://doi.org/10.1016/j.envpol.2020.115144.

Korich, D. G., Mead, J. R., Madore, M. S., Sinclair, N. A., & Sterling, C. R. (1990). Effects of ozone, chlorine dioxide, chlorine, and monochloramine on Cryptosporidium parvum oocyst viability. *Appl Environ Microbiol, 56*(5), 1423-1428. https://doi.org/10.1128/aem.56.5.1423-1428.1990.

Liou, C.-T., Wang, J.-S., & Ooi, H.-K. (2002). Effect of Ozone Treatment on Eimeria colchici Oocysts. *The Journal of Parasitology, 88*(1), 159-162. https://doi.org/10.2307/3285408.

Lopes, M. S., Ferreira, J. R. F., da Silva, K. B., de Oliveira Bacelar Simplício, I., de Lima, C. J., & Fernandes, A. B. (2015). Disinfection of corrugated tubing by ozone and ultrasound in mechanically ventilated tracheostomized patients. *Journal of Hospital Infection, 90*(4), 304-309. https://doi.org/https://doi.org/10.1016/j.jhin.2015.03.004.

Ma, B., Bright, K., Ikner, L., Ley, C., Seyedi, S., Gerba, C. P., . . . Linden, K. G. (2023). UV Inactivation of Common Pathogens and Surrogates Under 222 nm Irradiation from KrCl* Excimer Lamps. *Photochemistry and Photobiology, 99*(3), 975-982. https://doi.org/https://doi.org/10.1111/php.13724.

Manning, E. P., Stephens, M. D., Dufresne, S., Silver, B., Gerbarg, P., Gerbarg, Z., . . . Sharma, L. (2021). Disinfection of Pseudomonas aeruginosa from N95 respirators with ozone: a pilot study. *BMJ Open Respiratory Research, 8*(1), e000781. https://doi.org/10.1136/bmjresp-2020-000781.

Marangi, M., Koehler, A. V., Zanzani, S. A., Manfredi, M. T., Brianti, E., Giangaspero, A., & Gasser, R. B. (2015). Detection of Cyclospora in captive chimpanzees and macaques by a quantitative PCR-based mutation scanning approach. *Parasit Vectors, 8*, 274. https://doi.org/10.1186/s13071-015-0872-8.

Morgoglione, M. E., Bosco, A., Ciuca, L., Pepe, P., Coles, G. C., Cringoli, G., & Rinaldi, L. (2021). In Vitro Evaluation of Ozonated Water Treatment on the Viability of Eimeria Oocysts and Giardia Cysts from Water Buffaloes: A Proof-of-Concept Study. *Vet Sci, 8*(6). https://doi.org/10.3390/vetsci8060115.

Morrison, C. M., Hogard, S., Pearce, R., Gerrity, D., von Gunten, U., & Wert, E. C. (2022). Ozone disinfection of waterborne pathogens and their surrogates: A critical review. *Water Res, 214*, 118206. https://doi.org/10.1016/j.watres.2022.118206.

Nojima, Y., Bono, H., Yokoyama, T., Iwabuchi, K., Sato, R., Arai, K., & Tabunoki, H. (2019). Superoxide dismutase down-regulation and the oxidative stress is required to initiate pupation in Bombyx mori. *Sci Rep, 9*(1), 14693. https://doi.org/10.1038/s41598-019-51163-3.

Ochoa, T. J., & Contreras, C. A. (2011). Enteropathogenic escherichia coli infection in children. *Curr Opin Infect Dis, 24*(5), 478-483. https://doi.org/10.1097/QCO.0b013e32834a8b8b.

Ölmez, H., & Akbas, M. Y. (2009). Optimization of ozone treatment of fresh-cut green leaf lettuce. *Journal of Food Engineering, 90*(4), 487-494. https://doi.org/https://doi.org/10.1016/j.jfoodeng.2008.07.026.

OSHA. (2022). OZONE. Retrieved from: https://www.osha.gov/chemicaldata/9 Accessed.

OzoneSolutions. (2022). Ozone Equations: Concentration and Dosage. Retrieved from: https://ozonesolutions.com/pages/ozone-faq/o3-equations.html Accessed 2022.

Peeters, J. E., Mazas, E. A., Masschelein, W. J., Villacorta Martiez de Maturana, I., & Debacker, E. (1989). Effect of disinfection of drinking water with ozone or chlorine dioxide on

survival of Cryptosporidium parvum oocysts. *Appl Environ Microbiol, 55*(6), 1519-1522. https://doi.org/10.1128/aem.55.6.1519-1522.1989.

Pekárek, S. (2003). Non-Thermal Plasma Ozone Generation. *Acta Polytechnica, 43*(6). https://doi.org/10.14311/498.

Pereira, J. T., Costa, A. O., de Oliveira Silva, M. B., Schuchard, W., Osaki, S. C., de Castro, E. A., . . . Soccol, V. T. (2008). Comparing the efficacy of chlorine, chlorine dioxide, and ozone in the inactivation of Cryptosporidium parvum in water from Parana State, Southern Brazil. *Appl Biochem Biotechnol, 151*(2-3), 464-473. https://doi.org/10.1007/s12010-008-8214-3.

Perez-Sampietro, M., Casas, C., & Herrero, E. (2013). The AMPK family member Snf1 protects Saccharomyces cerevisiae cells upon glutathione oxidation. *PLoS One, 8*(3), e58283. https://doi.org/10.1371/journal.pone.0058283.

Pi, X., Yan, C., & Berk, B. C. (2004). Big mitogen-activated protein kinase (BMK1)/ERK5 protects endothelial cells from apoptosis. *Circ Res, 94*(3), 362-369. https://doi.org/10.1161/01.RES.0000112406.27800.6F.

Piechowiak, T., Skóra, B., & Balawejder, M. (2022). Ozonation process causes changes in PARP-1 expression and the metabolism of NADPH in strawberry fruit during storage. *Journal of Biotechnology, 357*, 84-91. https://doi.org/https://doi.org/10.1016/j.jbiotec.2022.08.012.

Qadri, F., Svennerholm, A. M., Faruque, A. S., & Sack, R. B. (2005). Enterotoxigenic Escherichia coli in developing countries: epidemiology, microbiology, clinical features, treatment, and prevention. *Clin Microbiol Rev, 18*(3), 465-483. https://doi.org/10.1128/CMR.18.3.465-483.2005.

Reed, N. G. (2010). The history of ultraviolet germicidal irradiation for air disinfection. *Public Health Rep, 125*(1), 15-27. https://doi.org/10.1177/003335491012500105.

Rhee, S. G. (2016). Overview on Peroxiredoxin. *Mol Cells, 39*(1), 1-5. https://doi.org/10.14348/molcells.2016.2368.

Rice, R., & Graham, D. (2001). US FDA regulatory approval of ozone as an antimicrobial agent–what is allowed and what needs to be understood. *Ozone News, 29*(5), 22-31.

Rice, R. G., Robson, C. M., Miller, G. W., & Hill, A. G. (1981). Uses of ozone in drinking water treatment. *Journal (American Water Works Association), 73*(1), 44-57. http://www.jstor.org/stable/41270552.

Rico, D., Martín-Diana, A. B., Frías, J. M., Henehan, G. T., & Barry-Ryan, C. (2006). Effect of ozone and calcium lactate treatments on browning and texture properties of fresh-cut lettuce. *Journal of the Science of Food and Agriculture, 86*(13), 2179-2188. https://doi.org/https://doi.org/10.1002/jsfa.2594.

RSComponents. (2002). Gas tube specifications. *Data Sheet* (p. 12).

Ruffell, K. M., Rennecker, J. L., & Mariñas, B. J. (2000). Inactivation of Cryptosporidium parvum oocysts with chlorine dioxide. *Water Research, 34*(3), 868-876. https://doi.org/https://doi.org/10.1016/S0043-1354(99)00187-6.

Sachadyn-Krol, M., & Agriopoulou, S. (2020). Ozonation as a Method of Abiotic Elicitation Improving the Health-Promoting Properties of Plant Products-A Review. *Molecules, 25*(10). https://doi.org/10.3390/molecules25102416.

Sarfo, F. S., Dompreh, A., Asibey, S. O., Boateng, R., Weinreich, F., Kuffour, E. O., . . . Eberhardt, K. A. (2022). The Clinical Features and Immunological Signature of Cyclospora cayetanensis Co-Infection among People Living with HIV in Ghana. *Microorganisms, 10*(7). https://doi.org/10.3390/microorganisms10071407.

Sholtes, K., Simons, R., Beck, S., Adeli, B., & Sun, Z. (2021). Ultraviolet Disinfection: Background and Basics. *UV Solutions* (Vol. 2021).

Swaminathan, P. D., Purohit, A., Hund, T. J., & Anderson, M. E. (2012). Calmodulin-dependent protein kinase II: linking heart failure and arrhythmias. *Circ Res, 110*(12), 1661-1677. https://doi.org/10.1161/CIRCRESAHA.111.243956.

Thorpe, G. W., Fong, C. S., Alic, N., Higgins, V. J., & Dawes, I. W. (2004). Cells have distinct mechanisms to maintain protection against different reactive oxygen species: oxidative-stress-response genes. *Proc Natl Acad Sci U S A, 101*(17), 6564-6569. https://doi.org/10.1073/pnas.0305888101.

Tsang, C. K., Liu, Y., Thomas, J., Zhang, Y., & Zheng, X. F. S. (2014). Superoxide dismutase 1 acts as a nuclear transcription factor to regulate oxidative stress resistance. *Nature Communications, 5*(1), 3446. https://doi.org/10.1038/ncomms4446.

Tucker, M. S., Khan, A., Jenkins, M. C., Dubey, J. P., & Rosenthal, B. M. (2022). Hastening Progress in Cyclospora Requires Studying Eimeria Surrogates. *Microorganisms, 10*(10), 1977. https://www.mdpi.com/2076-2607/10/10/1977.

Tukel, S. S., Bilgin, R., & Gul, S. (1994). Effects of ozone on the activity of erythrocyte membrane Na(+)-K+ ATPase. *Biochem Mol Biol Int, 33*(6), 1033-1040. https://www.ncbi.nlm.nih.gov/pubmed/7804127.

USDAERS. (2018). *Cost Estimates of Foodborne Illnesses*. https://www.ers.usda.gov/data-products/cost-estimates-of-foodborne-illnesses/.

Whiteside, C., & Hassan, H. M. (1987). Induction and inactivation of catalase and superoxide dismutase of Escherichia coli by ozone. *Archives of Biochemistry and Biophysics, 257*(2), 464-471. https://doi.org/https://doi.org/10.1016/0003-9861(87)90591-1.

Wickramanayake, G. B., Rubin, A. J., & Sproul, O. J. (1985). Effects of Ozone and Storage Temperature on Giardia Cysts. *Journal (American Water Works Association), 77*(8), 74-77. http://www.jstor.org/stable/41272515.

Xu, P., Janex, M.-L., Savoye, P., Cockx, A., & Lazarova, V. (2002). Wastewater disinfection by ozone: main parameters for process design. *Water Research, 36*(4), 1043-1055. https://doi.org/https://doi.org/10.1016/S0043-1354(01)00298-6.

Yanagisawa, R., Warabi, E., Inoue, K.-I., Yanagawa, T., Koike, E., Ichinose, T., . . . Ishii, T. (2012). Peroxiredoxin I null mice exhibits reduced acute lung inflammation following ozone exposure. *The Journal of Biochemistry, 152*(6), 595-601. https://doi.org/10.1093/jb/mvs113.

VITA

Addison Myers was born in Lafayette, Indiana in 1998. She spent most of her childhood in the Midwest before moving to Tennessee, where years later she received a Bachelor of Science degree in Food Science with a minor in Psychology at the University of Tennessee. She went on to pursue a Master of Science in Food Science, with a concentration in Food Safety and Microbiology, as well as a Graduate Certificate in Food Safety. She currently works in public service as an environmental health specialist.