ADVANCED OXIDATION PROCESS IN EGG SANITIZATION

A Thesis

by

ANDREW CHRISTIAN REHKOPF

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Chair of Committee,	Tri Duong
Co-Chair of Committee,	Craig Coufal
Committee Members,	Allen Byrd
Head of Department,	David Caldwell

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ABSTRACT

The microbial quality of eggs entering the hatchery is an important critical control point for biosecurity, pathogen reduction, and food safety programs in poultry production. Developing interventions to reduce *Salmonella* contamination of eggs is important to improving the microbial food safety of poultry and poultry products. The hydrogen peroxide (H_2O_2) and ultraviolet light (UV) Advanced Oxidation Process (AOP) has been previously demonstrated to be effective in reducing *Salmonella* on the surface of experimentally contaminated eggs. Our objective was to evaluate the effect of treating eggs with an egg sanitizing apparatus using H_2O_2/UV AOP on *Salmonella* contamination during incubation, hatching, and in broiler chicks during grow-out. Experimentally contaminated eggs were treated using the automated H_2O_2/UV AOP egg sanitizer and incubated for 21 days. AOP sanitization reduced *Salmonella* greater than 7 $\log_{10}$ cfu egg^{-1} ($P < 0.05$) from the surface of experimentally contaminated eggs and reduced the number of *Salmonella* positive eggs by 65 % ($P < 0.05$) when treated 1 hour post-inoculation. AOP treatment also reduced the number of *Salmonella*-positive eggs during incubation. Additionally, *Salmonella* was recovered from more chicks hatched from untreated eggs than from eggs treated using the H_2O_2/UV AOP egg sanitizer ($P < 0.05$) through 14 days post-hatch. These data suggest reduction of *Salmonella* contamination on the surface of eggs using the H_2O_2/UV AOP egg sanitizer prior to incubation may reduce the gastrointestinal colonization of chicks by *Salmonella*.

DEDICATION

First and foremost I would like to thank the Lord Jesus for giving me the opportunity to study at Texas A&M and for the love, and strength He has given me.

This thesis is dedicated to my loving family who always believed in me. I would not be where I am today without their love and support which has allowed me to achieve more than I thought possible. My loving wife, Ashley, thank you for always being there when I needed you and lifting me up. To my parents, Henry and Kahlayah, thank you for your ever encouraging words and devotion to my education.

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TABLE OF CONTENTS

Page

LIST OF FIGURES

LIST OF TABLES

CHAPTER I

INTRODUCTION OF ULTRAVIOLET TECHNOLOGIES

1.1 INTRODUCTION

Salmonella is a leading cause of foodborne illness accounting for an estimated 1.2 million cases and an estimated total economic burden of $9.5 billion in the United States annually, the most for a foodborne pathogen (CDC, 2013; Hoffmann, et al., 2015). The Center for Disease Control recognizes poultry products as a primary route of *Salmonella* transmission leading to consumer illness (Painter, et al., 2013). Thirty four percent of salmonellosis in Europe is linked to contaminated poultry products, and in the U.S. poultry is responsible for twenty two percent of *Salmonella* outbreaks (Greig and Ravel, 2009). *Salmonella enterica* serovar Enteritidis (**SE**) is the most frequently isolated serotype from outbreaks (CDC, 2014) as it is capable of surviving in the antimicrobial environment of the albumin which allowed the serotype to find a niche reservoir in chicken eggs (Schroeder, et al., 2005; Braden, 2006; Kang, et al., 2006). This further demonstrates the importance of developing effective technologies capable of reducing SE in poultry products.

1.1.1 Salmonella *and Eggs*

Salmonella is readily capable of contaminating poultry eggs via vertical and horizontal transmission. Vertically transmitted *Salmonella* contaminates eggs during the laying process (Turnbull and Snoeyenbos, 1974; Gast and Beard, 1990), while horizontal transmission occurs when the eggshell is contaminated by external sources such as

incubators, the environment, or other infected birds (Smeltzer, et al., 1979; Cox, et al., 2000). The calcareous shell surrounding the egg is porous and penetrable by bacteria (Solomon, 2010) (**Figure 1**). The cuticle is a proteinaceous film covering the eggshell that provides a natural barrier to help prevent internal bacterial contamination (Peebles and Brake, 1986; Wang and Slavik, 1998), however, defects in the shell or thinning of the cuticle may lead to the invasion of the eggshell by bacteria on the surface (Mayes and Takeballi, 1983). *Salmonella* can readily penetrate across the cuticle of the egg and contaminate the internal contents (Williams, et al., 1968; Wang and Slavik, 1998). Additionally, standard incubation temperatures promote the penetration of *Salmonella* into the egg and developing embryo (Stokes, et al., 1956; Bailey, et al., 1996; Gole, et al., 2014).

Contamination of hatcheries represent a critical control point of *Salmonella* transmission in the poultry industry (Cox, et al., 1990; Bailey, et al., 1996). *Salmonella* does not cause significant deterioration of the egg allowing contaminated eggs to hatch (MacLaury and Moran, 1959). It has been demonstrated that as few as 5 contaminated eggs horizontally transmitted *Salmonella* to naïve chicks throughout the incubator during hatch (Bailey, et al., 1996) and that this transmission was independent of any physical contact with infected chicks or contaminated eggshell fragments (Cason, et al., 1994). Chicks infected with non-typhoid salmonellae do not always present clinical symptoms, allowing the presence of potential salmonellae reservoirs to go unnoticed (Cason, et al., 1994). Additionally, day-old chicks do not have an established gut microflora, and therefore, newly hatched chicks are readily colonized by *Salmonella* (Shaffer, et al.,

1957; Byrd, et al., 1998). Infected chicks leave the hatchery spreading the infection to naïve birds in poultry houses, ultimately reaching the consumer (Nakamura, et al., 1997). Therefore, within the integrated poultry industry, the hatchery has been suggested to be the most important source of *Salmonella* contamination (Cox, et al., 2007).

1.1.2 Current Control Strategies

There are no regulatory requirements for sanitizing table eggs, however, many U.S. egg producers voluntarily sanitize eggs using chlorous compounds or quaternary ammonium compounds (**QAC**s) (Howard, et al., 2012). Although the exact mechanism of chlorine-releasing agents in not fully understood, it is believed to be mostly due to the disruption of cellular function by the oxidation of cellular proteins resulting in cell death (McDonnell and Russell, 1999). At concentrations below 5 mM (260 ppm), chlorous compounds did not cause excessive disruption of the cell membrane (McKenna and Davies, 1988) and cell death was also accompanied by the disruption of ATP production (Barrette, et al., 1989). Chlorous compounds have been previously demonstrated to reduce *Salmonella* contamination on eggs (Knape, et al., 2001), however, *Salmonella* could still be detected on the surface of eggs after 5 min of complete submersion in chlorine dioxide (Choi, et al., 2015). Additionally, chlorine being a corrosive compound capable of causing severe damage to the skin and eyes of workers (White, 2010), the reactions of chlorous compounds being capable of producing carcinogenic byproducts (Navalon, et al., 2009; Burch, et al., 2015) and the evidence of increasing chlorine resistant bacterial populations are causes for concern (Ridgway and Olson, 1982; Hegstad, et al., 2010; Rakic-Martinez, et al., 2011). QACs have also been previously

demonstrated to be effective at reducing *Salmonella* on eggs (Cox, et al., 2007; Buhr, et al., 2013). However, QACs were unable to eliminate contamination from the surface of shell eggs (Musgrove, et al., 2006) and left a chemical residue on eggshells following treatment (Bierer, et al., 1961; Wang and Slavik, 1998), potentially affecting chicks during hatch. Furthermore, the concentrations of QACs (Bourassa, et al., 2002) or chlorous compounds (Patterson, et al., 1990) required to reduce *Salmonella* have been demonstrated to reduce the hatchability of eggs.

1.2 ULTRAVIOLET LIGHT SANITIZATION

1.2.1 Background

Ultraviolet (**UV**) light is electromagnetic radiation found on the electromagnetic spectrum between X-rays and visible light and includes wavelengths between 200 and 400 nm. UV light is subcategorized into UV-A (400-315 nm), UV-B (315-280 nm), and UV-C (280-100 nm) based on its respective wavelength range (ISO, 2007). In photochemistry, irradiance is defined as the radiation energy per unit area per unit time as measured at the surface (Melnikova and Vasilyev, 2005; Cohen, 2007). Although the SI units used to measure intensity are W m^{-2}, the intensity of UV when irradiating eggs is usually measured in mW cm^{-2}. The primary mechanism of microbial inactivation by UV irradiation is the dimerization of DNA bases (Jagger, 1967). The formation of these dimers within bacterial DNA prevent the duplication of DNA, ultimately leading to a reduction in the bacterial population. These bases have a peak UV absorption rate at a wavelength of 260 nm, which corresponds to the peak bactericidal effectiveness of UV irradiation which ranges between 260 and 270 nm (Gates, 1930). Pyrimidine bases are

10 times more reactive than purine bases to wavelengths of 254 nm, the predominant wavelength radiated from germicidal UV lamps (Jagger, 1967). The pyrimidine thymine requires the least amount of energy to form a dimer, consequently, the thymine-complex dimer is the predominant photoproduct of UV_{254} irradiation. UV-C includes the 254 nm wavelength within its 200-290 nm range and is therefore often referred to as germicidal UV.

1.2.2 Applications

The use of UV irradiation has been used across a variety of industries as a sterilizing agent (**Table 1**). Germicidal lamps are economical and readily available for producers to treat liquids, air systems, food products, and packaging materials (Bintsis, et al., 2000) and were effective in reducing contamination of contact lenses preventing microbial keratitis (Dobrogowski, 1989; Gritz, et al., 1990). Ultraviolet irradiation was previously demonstrated to degrade water pollutants and significantly reduce fecal indicator organisms (Shama, 1992; Legrini, et al., 1993). Ultraviolet light is an attractive method of sanitization for the food industry as it effectively reduced microbial contamination thereby extending the storage-life of meats without affecting consumer acceptability (Huang and Toledo, 1982; Stermer, et al., 1987). Additionally, the effectiveness of UV to reduce *Salmonella* makes it a reasonable method to decontaminate a wide range of food processing and packaging materials (Bank, et al., 1991; Gao, et al., 1997; Kuo, et al., 1997b; Unluturk, et al., 2008; Ge, et al., 2013).

1.3 APPLICATION OF ULTRAVIOLET LIGHT SANITIZATION IN EGGS

1.3.1 Benefits of UV Sanitization of Eggs

Excessive microbial contamination of hatching eggs can reduce hatchability (Quarles, et al., 1970; Williams, 1970), chick quality, and increase mortality (Reid, et al., 1961; Williams, 1970). However, using chemicals or surfactants to sanitize eggshells damages the cuticle on the egg surface, potentially allowing bacterial penetration of the egg (Kim and Slavik, 1996; Wang and Slavik, 1998; Favier, et al., 2000). Irradiation by UV does not expose eggs to chemicals or toxic byproducts and is environmentally safe (Scott and Swetnam, 1993b; Coufal, et al., 2003). The use of UV as a sanitizer does not adversely affect the egg or developing embryo like chemical sanitizers, such as chlorine dioxide and formaldehyde, have been demonstrated to potentially do (Patterson, et al., 1990; Sander, et al., 1995a; Sander, et al., 1995b). Furthermore, UV has been well documented to not affect the cuticle of eggs. An intact cuticle layer was seen after Coufal, et al. (2003) exposed eggs to 4 – 14 μW cm^{-2} of UV-C for 3 min (Coufal, et al., 2003) and continuous exposure of eggs to UV-C through 18 days of incubation did affect the cuticle layer or hatchability of fertile eggs (Scott, 1993; Berrang, et al., 1995; Bailey, et al., 1996).

Ultraviolet light can significantly reduce microbial contamination without excessively heating the egg. When compared to other irradiation treatments, such as microwave irradiation (Lakins, et al., 2008), UV irradiation is a low-heat treatment which avoids damaging the embryo or lead to unwanted embryonic development. In addition to being a low-heat process, UV cannot reach the developing embryo or cause

damage to its DNA (Gao, et al., 1997) as UV does not penetrate the eggshell (De Reu, et al., 2006).

Use of ultraviolet light as a sanitizer is easy to obtain, cost effective, and prototype sanitizing cabinets have been integrated into the production process for research purposes (Scott, 1993; Chavez, et al., 2002; Coufal, et al., 2003). Ultraviolet light is also used by the food industry because it does not affect consumer acceptance of food products. It is effective at reducing microbial contamination of poultry meats with no detrimental effects on the color, flavor, or appearance of the meat (Stermer, et al., 1987; Wallner-Pendleton, et al., 1994). Additionally as a low-heat process, UV treatment of shell eggs improves the consumer safety of shell eggs without denaturing proteins, allowing the retention of flavor (Dunn, 1996).

1.3.2 Factors Affecting UV Sanitization of Eggs

Early investigations into the use of ultraviolet light to sanitize chicken eggs demonstrated UV to be an attractive alternative to formaldehyde since UV is both safe for the workers and inexpensive. It has been demonstrated that irradiating hatching eggs with UV has similar bactericidal effects as treating eggs with formalin. Hatching eggs irradiated with UV for 1, 3, or 5 min at a distance ranging from 0.4 to 1.0 meters and eggs dipped in 1% formalin for 1, 5, or 10 min resulted in no Gram negative bacteria recovered from the egg surface (Scott, 1993). Subsequent research established that the bactericidal effect of UV radiation depends on exposure time and irradiance measured at the egg (Bank, et al., 1991; Berrang, et al., 1995; Gao, et al., 1997; Kuo, et al., 1997a; Chavez, et al., 2002).

It has been demonstrated that reduction of *Salmonella* increases as UV exposure duration increases (Berrang, et al., 1995). The percentage of *Salmonella* contaminated eggs was be reduced by 40% after 1 min and 63% after 5 min of exposure with an UV intensity of 600 μW cm^{-2}. Similarly, increasing results were seen when eggs were irradiated with 620 μW cm^{-2} UV (Kuo, et al., 1997a). Using an UV intensity of 620 μW cm^{-2}, counts of *Salmonella* were reduced by greater than 3 log_{10} cfu egg^{-1} after 1 min and approximately 4 log_{10} cfu egg^{-1} after 3 min of exposure. However, exposure durations of greater than 3 min did not result in a significant reduction.

The bactericidal effects of UV on *Salmonella* have also been demonstrated to increase with intensity. The inhibition of *Salmonella* plated onto tryptic soy agar (TSA) increased as UV irradiance increased (Bank, et al., 1991). Greater ranges in intensity are needed to detect a significant change in bactericidal effects while smaller differences in exposure time are needed to detect a similar change. Significant differences were not seen in bactericidal properties when irradiances were evaluated within a 600 μW cm^{-2} range (Scott, 1993; Berrang, et al., 1995), but significant differences were seen in greater ranges such as 2,600 μW cm^{-2} (Kuo, et al., 1997a). *Salmonella* was reduced on eggs treated with 100 μW cm^{-2} for 2 and 4 min by 2.6 and 2.0 log_{10} cfu egg^{-1} respectively. However, *Salmonella* was reduced on eggs treated with 1,500-2,500 μW cm^{-2} for 1 and 5 min by 3.4 and 4.3 log_{10} cfu egg^{-1} (Rodriguez-Romo and Yousef, 2005). Therefore, it seems that UV intensity, rather than exposure time, plays the more important role in eggshell sanitization.

Greater differences in microbial reduction are seen in shorter exposure times at high UV intensities. Total aerobic bacteria (**TAB**) was reduced by 2 $\log_{10}$ cfu egg^{-1} on eggs irradiated with 620 µW cm^{-2} UV for 15 min but TAB was only reduced an additional 0.4 $\log_{10}$ cfu egg^{-1} on eggs irradiated for 30 m (Kuo, et al., 1997a). However, similar reductions can be achieved in a shorter amount of time with a greater intensity. TAB was reduced by 2 $\log_{10}$ cfu egg^{-1} on eggs irradiated with 11 mW cm^{-2} for 8 min and a significantly greater reduction in TAB by 3 $\log_{10}$ cfu egg^{-1} was seen after 16 min of exposure (Wells, et al., 2010). This suggests that higher irradiances can be used to significantly reduce microbial contamination in short amounts of time. This shorter treatment time is critical to a commercial application as lengthy exposures risk excessively heating the eggs (Wells, et al., 2010).

Shorter exposure times leads to a practical method of eggshell sanitization for a commercial setting (Chavez, et al., 2002). Ultraviolet cabinets have been developed utilizing high intensities of UV light as potential commercial sanitizers. A cabinet producing 7.5 mW cm^{-2} UV was demonstrated to reduce total aerobic bacteria by 2 $\log_{10}$ cfu egg^{-1} after 48 s. Chavez, et al. (2002) and Coufal, et al. (2003) effectively reduced *Salmonella* by 4 $\log_{10}$ cfu egg^{-1} after 4 min of exposure to 14 mW cm^{-2} UV. A commercial UV-C disinfection system effectively reduces pathogen contamination on shell eggs using an intensity of 10 mW cm^{-2} (De Reu, et al., 2006). Eggshell contamination of *E. coli* and *Staphylococcus aureus* as well as TAB bacteria is significantly reduced after 4.7 s of exposure.

It has been demonstrated that egg rotation during irradiation enhances bactericidal effects of UV by providing more complete exposure of the eggshell surface to the UV light (Kuo, et al., 1997b). Microbial contamination of eggs irradiated with 4,350 μW cm^{-2} UV decreased as exposure time increased to 15 min, after which the reduction in microbial contamination was no longer significant. Rotation of the eggs during the 15 min UV treatment resulted in additional reduction of *Salmonella* bacteria (Kuo, et al., 1997b). *Salmonella* was reduced by 2.6 log_{10} cfu egg^{-1} on eggs irradiated with 100 μW cm^{-2} UV for 2 min, and *Salmonella* was reduced by 3.4 log_{10} cfu egg^{-1} on eggs irradiated with 2,000 μW cm^{-2} UV for 1 min of exposure with constant egg rotation (Rodriguez-Romo and Yousef, 2005). However, at higher intensities of 11 mW cm^{-2} egg rotation does not produce a significant difference. After repeated treatments of 2 min exposure, rotated and non-rotated eggs had an approximate 1.5 log_{10} cfu egg^{-1} TAB reduction (Wells, et al., 2011a). Therefore, egg rotation is not necessary when high intensities of UV are used.

Sanitization by pulsed UV light, a system that uses short bursts of high intensity UV light per s for a few hundred μs, has been demonstrated as a novel yet effective technology to significantly reduce *Salmonella* (Keklik, et al., 2010). Previous studies demonstrated that pulsed UV light reduced *Salmonella* on eggs 2 log_{10} cfu egg^{-1} within 1 s of pulsed treatments, and a reduction of up to 7 log_{10} cfu egg^{-1} after 1 min of UV pulses (Dunn, 1996; Keklik, et al., 2010). *Salmonella* was reduced on the eggshell surface by greater than 6 log_{10} cfu egg^{-1} when the maximum cumulative energy, by U.S. law, of 12

J cm^{-2} (USDA, 2010) was applied (Hierro, et al., 2009). Using pulsed UV technology, allows for rapid reduction in *Salmonella* contamination on eggshell surfaces.

Hatching eggs contaminated with *Salmonella* entering the incubators can contaminate other eggs in the incubator (Cason, et al., 1994), thereby serving as a critical source of horizontal contamination. Aerosolized *Salmonella* recovered within cabinets containing contaminated eggs was reduced in hatchers irradiated with 146 to 120 μW cm^{-2} UV (Bailey, et al., 1996). Continuous UV/air filtration throughout incubation and hatch has been demonstrated to increase hatchability by decreasing early and late embryo mortality (Scott, 1993). Treating incubator and hatcher air is not a means of direct egg sanitization but it could reduce the incidence of horizontal transmission (Avens, et al., 1975), and therefore, may play a strategic role in supporting egg sanitization.

Ultraviolet irradiation alone has been demonstrated to be effective at reducing *Salmonella* contamination on eggs. These antimicrobial properties are enhanced as the length and intensity of UV exposure increases. High intensities allow for shorter, more practical UV exposure times without sacrificing bactericidal effectiveness. Therefore, a short but high intensity treatment will potentially be the preferred method in commercial UV sanitization of eggs.

1.3.3 UV Advanced Oxidation Processes

Advanced Oxidation Processes (**AOP**s) are aqueous phase oxidation methods based on the *in situ* generation of highly reactive oxygen species (**ROS**) (Comninellis, et al., 2008). These ROS produced within an AOP system effectively oxidizes the outer

membrane of microorganisms, leading to cell death (Legrini, et al., 1993; Ikai, et al., 2010). The AOP methods which utilize UV light produce ROS as products of a photolytic reaction. The AOP represent one of the most promising disinfection technologies in the food industry (Selma, et al., 2008).

Ultraviolet light has also been demonstrated to improve the effectiveness of peracetic acid (**PAA**), which is an established eggshell disinfectant capable of reducing *Salmonella* (Hartman and Carlin, 1957; Cox, et al., 2007; Bauermeister, et al., 2008; Ge, et al., 2013). The PAA/UV AOP has been previously demonstrated to inactivate coliphages (Rajala-Mustonen, et al., 1997) and reduce *Salmonella* below countable levels on table eggs (Al-Ajeeli, et al., 2016). However, other pathogens such as *E. coli* exhibited some resistance to PAA (Baldry, et al., 1991; Gehr, et al., 2003; Jones, 2010).

Aqueous phase ozone (**O_3**) undergoing photolysis by UV light is another effective AOP (Peyton and Glaze, 1988). The O_3/UV AOP has been demonstrated to have a greater efficacy in reducing mesophilic bacteria and total coliforms in the wash water of fresh cut onions or escaroles than O_3 or UV alone (Selma, et al., 2008). A similar effect was also observed when applied to *Salmonella* contaminated eggs. Treatment of eggs with 12 to 14% (wt/wt) O_3 at 5 lb in^{-2} gauge, 4 to 8° C alone did not significantly reduce *Salmonella* contamination, but the additional application of 1,500 to 2,500 μW cm^{-2} UV resulted in a greater than 5 $\log_{10}$ cfu egg^{-1} reduction on the surface (Rodriguez-Romo and Yousef, 2005). Although effective, excessive treatment by O_3 does have negative effects and should be used with caution. Over exposing eggs to gaseous ozone significantly reduced hatchability and killed developing embryos

(Whistler and Sheldon, 1989). In addition to being a risk to hatching eggs, O_3 poses a severe inhalation, skin and eye hazard to those exposed to it (Scott and Swetnam, 1993a). Ozone is highly unstable in aqueous systems and is challenging to calculate the effects of organic matter will have on the agent (Legrini, et al., 1993; Min, et al., 2003). When O_3 is exposed to UV the net reaction results in the formation of hydrogen peroxide (**H_2O_2**) (Peyton and Glaze, 1988) and any hydroxyl radicals formed when O_3 reacts with UV are unable to escape this solvent cage (Glaze, et al., 1987). Although the O_3/UV AOP is an effective means of disinfection, the bactericidal properties are a result of hydrogen peroxide production instead of hydroxyl radicals formed from the initial O_3 molecule.

The net photolysis of H_2O_2 yields 2 hydroxyl radicals, per quantum of radiation absorbed, which can go on to form peroxyl radicals leading to secondary oxidation reactions (Legrini, et al., 1993). The H_2O_2/UV photolytic reaction is one of the most widely used AOPs (Bustillo-Lecompte and Mehrvar, 2015), and has been demonstrated to effectively inactivate vegetative bacteria, bacterial spores, and viruses (Bayliss and Waites, 1982; Mamane, et al., 2007; Ikai, et al., 2010). The use of H_2O_2 as a standalone sanitizer has been suggested to be equal to or better than formaldehyde in sanitizing hatching eggs (Sheldon and Brake, 1991). Hydrogen peroxide has been demonstrated previously to effectively reduce *Salmonella* contamination from experimentally contaminated eggs (Cox, et al., 2007; Buhr, et al., 2013). Following treatment of the eggs H_2O_2 readily evaporates leaving behind no chemical residue and presents minimal safety issues for workers or developing embryos (Sheldon and Brake, 1991; Scott and

Swetnam, 1993a; Padron, 1995; Keïta, et al., 2016). The bactericidal effects of H_2O_2 are enhanced following photolysis by UV (Ikai, et al., 2010; Wells, et al., 2010). The H_2O_2/UV AOP reduced more *Salmonella* and other pathogens on fresh produce compared to chlorous compounds (Hadjok, et al., 2008). The H_2O_2/UV AOP treatment of shell eggs resulted in significantly fewer bacterial counts for 2 weeks when compared to a PAA/UV AOP treatment and produced no negative effects on consumer quality (Al-Ajeeli, et al., 2016). Other benefits to using this system include the commercial availability of H_2O_2, it's infinite solubility in water, and poses less of a health risk than O_3 to workers (Legrini, et al., 1993; Scott and Swetnam, 1993a). These benefits coupled with the effectiveness as a sanitizer makes the H_2O_2/UV AOP system an attractive decontamination method for eggs.

Ultraviolet light can produce highly reactive hydroxyl radicals through photolytic reactions with various oxidative compounds. Rapid oxidation reduces microbial contamination more effectively than UV alone. These advanced oxidation processes are proving to be a novel approach that effectively and safely reduces *Salmonella* contamination on eggshells.

1.3.4 H_2O_2/UV Advanced Oxidation Process

The H_2O_2/UV AOP has been applied as a method of eggshell sanitization that is effective and practical for commercial use. When an 11 mW cm^{-2} UV intensity is used, it was determined that a 1.5% H_2O_2 solution followed by 8 min of UV exposure to be the optimum mixture to consistently treat eggs (Wells, et al., 2010). This H_2O_2/UV AOP method significantly reduced TAB on eggshells by 3.3 $\log_{10}$ cfu egg^{-1}. In addition to

effectively reducing microbial contamination, H_2O_2/UV AOP treatment of eggs will not adversely affect the flavor or texture of table eggs (Al-Ajeeli, et al., 2016). Moreover, no reduction in hatchability was observed when eggs were treated using the H_2O_2/UV AOP (Wells, et al., 2011b), even after 6 repeated application (Wells, et al., 2011a) suggesting the H_2O_2/UV AOP does not significantly alter the eggshell cuticle.

Higher H_2O_2 concentrations used for the H_2O_2/UV AOP does not necessarily lead to an increased bactericidal effect. More TAB bacteria were recovered from the surface of eggs treated with a 2.5% H_2O_2 concentration compared to a lesser concentration of 1.5% H_2O_2 used in an AOP (Wells, et al., 2010). These reports concur with earlier findings that suggest bacterial spores can be protected from the AOP by higher H_2O_2 concentrations. The inactivation of spores were more so due to production of free radicals within the spores from UV absorption than to the external radicals produced by H_2O_2 (Bayliss and Waites, 1979; Repine, et al., 1981). As the concentration of H_2O_2 increases, more UV radiation is absorbed by H_2O_2 molecules instead of spores or vegetative cells (Bayliss and Waites, 1982).

Gottselig, et al. (2016) demonstrated a practical AOP decontamination method for commercial production. Using a slightly modified UV chamber described by Coufal, et al. (2003) eggs were irradiated with 14mW cm^{-2} of UV after being sprayed with 3% H_2O_2. Repetitive applications of this H_2O_2/UV treatment results in significant microbial reductions using shorter UV exposure times. A significant and rapid reduction of *Salmonella* by almost 6 $\log_{10}$ cfu egg^{-1} from experimentally contaminated eggs was seen following 2 cycles of H_2O_2/UV treatment with 5 s of UV exposure. Aerobic plate counts

were reduced by 3 $\log_{10}$ cfu egg^{-1} after 2 15 s exposures cycles. An H_2O_2/UV AOP system, using the method first described by Gottselig, et al. (2016), was developed into a prototype egg sanitizer machine (**Figure 2**) (Al-Ajeeli, et al., 2016). The reduction of *Salmonella* was greater on eggs which had been sanitized with the H_2O_2/UV AOP than on eggs which had been treated with a PAA/UV AOP, PAA, QAC, or chlorine. Furthermore, the significant difference in *Salmonella* contamination continued 7 and 14 days after eggs are treated with the H_2O_2/UV AOP egg sanitizer (Al-Ajeeli, et al., 2016). Further development of this H_2O_2/UV AOP system could present an effective yet feasible means of eggshell sanitization in the poultry industry.

The UV/H_2O_2 AOP system is able to significantly reduce *Salmonella* contamination on eggshells. The use of H_2O_2 as a sanitizer is safe for the worker, consumer, and hatching embryo and in the presence of UV light, can rapidly reduce *Salmonella*. The development of the UV/H_2O_2 AOP egg sanitizer has the potential to significantly impact multiple sectors of the commercial poultry industry.

1.4 CONCLUSIONS

Currently, there are many available methods for eggshell disinfection that effectively reduce *Salmonella* populations on the egg surface with varying degrees of efficacy, yet none these methods are able to completely remove bacteria from the eggshell. After the discontinued use of formaldehyde, many chemical sanitizers have been screened as potential replacements but none have been chosen as a preferred method that is safe for both the workers and the environment. UV is environmentally safe and safe for workers but is not completely effective. The AOP process is safe for the

environment as well as for workers and is among the most trusted and effective sanitization methods. The H_2O_2/UV AOP provides a novel solution to egg sanitization without sacrificing hatchability or chick quality. An effective sanitization program in crucial to achieve high levels of hatchability and chick quality by reducing *Salmonella* as well as other microbial pathogens on eggs entering the hatchery (Sheldon and Brake, 1991; Bailey, et al., 1996). Pathogens such as *Salmonella* can invade the internal contents of eggs soon after lay therefore, sanitizing eggs soon after collection at the farm will be necessary (Padron, 1995; Coufal, et al., 2003). As sanitizers are further researched, practical methods for implementation into a commercial setting are needed. The H_2O_2/UV AOP system can easily be implemented at the farm level using the prototype egg sanitizer described in Al-Ajeeli, et al. (2016). Further development of advanced oxidation technologies is expected to significantly impact the commercial poultry industry by improving the overall health, productivity, and microbial food safety of poultry and poultry products (Gottselig, et al., 2016).

CHAPTER II

ADVANCED OXIDATION PROCESS SANITIZATION OF HATCHING EGGS REDUCES *SALMONELLA* IN BROILER CHICKS

2.1 INTRODUCTION

Salmonella is a leading cause of bacterial foodborne illness in the United States (Hoffmann, et al., 2015), with poultry and poultry products being recognized as a major reservoir for this important human pathogen (Wray and Wray, 2000). The isolation of identical *Salmonella* serotypes in the hatchery and from processed product suggests hatcheries could be an important initial source of *Salmonella* contamination in the poultry production chain (Bailey, et al., 2002; Kim, et al., 2007). Although additional *Salmonella* serovars including *Salmonella* Kentucky and *Salmonella* Enteritidis have increasingly been isolated from broilers, *Salmonella* Typhimurium has historically been the predominant cause of broiler-associated infections in humans (Foley, et al., 2011; Finstad, et al., 2012). *Salmonella* from infected broiler breeders can be transmitted pseudo-vertically via feces to newly laid eggs (Mine, et al., 2003), and contaminated eggs have been demonstrated to be an important source for the spread of *Salmonella* to uncontaminated eggs in incubators and during hatching (Cason, et al., 1994). Additionally, *Salmonella* Typhimurium contamination of newly hatched chicks has been demonstrated to transmit horizontally within growing broiler flocks (Byrd, et al., 1998). Thus, the reduction of *Salmonella* contamination of eggs prior to entering the hatchery is

a critical control point (Bailey, et al., 1996) for which the development of effective interventions could be useful to improve microbial food safety of poultry production.

Hydrogen peroxide (**H_2O_2**) (Sheldon and Brake, 1991; Padron, 1995; Sander and Wilson, 1999) and ultraviolet light (**UV**) irradiation (Kuo, et al., 1997b; Chavez, et al., 2002; Coufal, et al., 2003) have been demonstrated to be effective methods of sanitizing eggshell surfaces. The combination of H_2O_2 and UV irradiation as an Advanced Oxidation Process (**AOP**) represents an important alternative to conventional chemical sanitizers. AOPs are aqueous phase oxidation methods based on the generation of highly reactive oxygen species (Comninellis, et al., 2008). The photolysis of H_2O_2 by UV results in the generation of hydroxyl radicals (Legrini, et al., 1993). The H_2O_2/UV AOP has been demonstrated to be effective for the inactivation of vegetative bacteria, bacterial endospores, and viruses (Bayliss and Waites, 1982; Mamane, et al., 2007; Ikai, et al., 2010). H_2O_2/UV advanced oxidation has also been demonstrated to be effective in reducing total aerobic bacteria and both *Salmonella* Enteritidis and *Salmonella* Typhimurium, on eggshell surfaces (Wells, et al., 2010; Al-Ajeeli, et al., 2016; Gottselig, et al., 2016). Additionally, no reduction in hatchability was observed when eggs were treated using up to 6 repeated applications, suggesting the H_2O_2/ UV AOP does not significantly alter the eggshell cuticle (Wells, et al., 2011a). Although the effectiveness of the H_2O_2/UV AOP in reducing *Salmonella* contamination on eggshell surfaces has been demonstrated previously, the subsequent effect of this *Salmonella* reduction during incubation and in broiler chicks has not been evaluated. In this study we evaluated the effect of treating eggs using an automated H_2O_2/UV AOP egg sanitizer

on *Salmonella* contamination of experimentally contaminated eggs during incubation, hatching, and in broiler chicks raised to 14 days post-hatch.

2.2 MATERIALS AND METHODS

2.2.1 Eggs

Broiler hatching eggs (Cobb) were obtained from a commercial hatchery for use in studies in which eggs were hatched, whereas all other studies utilized eggs were obtained from the Texas A&M University Poultry Science Teaching, Research, and Extension Center (College Station, TX). Visibly clean, unwashed eggs were collected from White Leghorn hens within 24 h of lay immediately prior to use.

2.2.2 Experimental Salmonella Contamination of Eggs

Eggs were experimentally contaminated using a primary poultry isolate of *Salmonella* Typhimurium which had been selected for resistance to novobiocin and nalidixic acid and used previously in several studies in which broiler chickens were experimentally inoculated (Byrd, et al., 1998). *Salmonella* Typhimurium was cultured aerobically at 37 °C using tryptic soy broth (**TSB**, Difco, Detroit, MI), harvested by centrifugation, and re-suspended to 10^9 cfu mL^{-1} using either TSB, sterile phosphate buffered saline (**PBS**, Fisher Scientific, Pittsburgh, PA), buffered peptone water (**BPW**, Difco), or a sterile 10 % (wt/vol) suspension of feces in PBS (**FS**) as indicated. In order to evaluate inoculation methods, *Salmonella* Typhimurium suspended in PBS was transferred to the surface of eggs by pipetting 100 µL of inoculum onto the surface of eggs and spreading using a sterile disposable loop, spreading the inoculum using a gauze pad or cotton ball soaked in suspension, or rolling the egg on a sponge soaked in

suspension. In order to evaluate inoculation media, 100 µL of *Salmonella* Typhimurium suspended in TSB, PBS, BPW, or FS was transferred to the egg surface using a pipette as described previously. For the preparation of fecal suspension, chicken feces were oven dried at 100 °C for 24 h, ground into a powder, resuspended in PBS, and sterilized by autoclaving. All eggs were air dried for 1 h following experimental contamination. The inoculum was confirmed by enumeration using Xylose Lysine Tergitol-4 agar (**XLT-4**, Difco).

2.2.3 Incubation and Hatching of Eggs

Eggs were stored at 18 °C for 24 h with relative humidity maintained at ≥ 70% (Essick Air Inc., Little Rock, AR). Eggs were then transferred to incubators (GQF Manufacturing Company Inc., Savannah, GA.) and incubated under standard conditions.

2.2.4 Salmonella Recovery

Salmonella was enumerated from BPW egg surface rinsate as described by (Gottselig, et al., 2016). When indicated, *Salmonella* was selectively enriched from the surface rinsate, crushed eggshell with membranes (Webb, et al., 2014), and 25 g of homogenized egg contents consisting of the albumin and yolk (Jones, et al., 2012) using pre-enrichment in BPW, selective enrichment in Rappaport-Vassiliadis R-10 broth (**RV**, Difco), and selective isolation on XLT-4 agar. The gastrointestinal (**GI**) tract and yolk sac from developed embryos (18 d of incubation), the GI tract and retained yolk sac from newly hatched chicks (0 d post-hatch), and the cecum from 14 d old broiler chicks were aseptically collected for selective enrichment using RV and XLT-4. Eggs from which there were no colonies appearing on enumeration plates but were positive by

selective enrichment were assigned the lower limit of detection of the assay for statistical analysis (2.0 $\log_{10}$ cfu egg^{-1}).

2.2.5 Advanced Oxidation Process Treatment

Eggs were treated using an automated H_2O_2/UV AOP egg sanitizer (Al-Ajeeli, et al., 2016), composed of a wire chain conveyor belt on which eggs were placed in commercial incubator flats and carried through two treatment stages. Each stage consisted of a spray chamber in which eggs were sprayed with 3.2% (wt/vol) aqueous H_2O_2 and a UV chamber in which eggs were exposed to UV at an intensity of 10 mW cm^{-2} (G8T5 Germicidal lamps, General Electric Company, Cleveland Ohio). The speed of the conveyor was such that the complete run time for one flat of eggs was approximately 40 s.

2.2.6 Hatching and Grow-out

After 18 days of incubation, broiler hatching eggs were aseptically transferred to hatcher trays for the remaining incubation period. After 21 d of incubation, eggs were hatched and 200 chicks from each treatment group were placed for grow-out. Chicks were raised in floor pens under ABSL-2 biocontainment at the USDA-ARS Southern Plains Agriculture Research Center (College Station, TX) and provided potable water and an unmedicated broiler starter ration that met or exceeded NRC (1994) requirements *ad libitum*. At 0 and 14 d post-hatch, 20 chicks from each treatment were euthanized and the ceca were aseptically dissected for evaluation of gastrointestinal colonization by *Salmonella*. All procedures were performed as approved by the USDA animal care and use committee.

Tray liners were placed in hatchings trays immediately prior to the transfer of eggs to the hatchers for recovery of *Salmonella* after hatching. *Salmonella* was selectively enriched from tray liners by pre-enrichment using 300 mL BPW, selective enrichment using RV, and selective isolation using XLT-4 agar.

2.2.7 Statistical Analysis

Salmonella counts were $\log_{10}$ transformed and analyzed using the Kruskal-Wallis or Man-Whitney tests as appropriate for the number of treatment groups. Results from multiple independent assays were pooled for analysis and blocked by assay. Significantly different means were separated using the Steel-Dwass test *post hoc*. Prevalence of *Salmonella* from eggs and chicks was analyzed using Pearson's χ^2 test. Statistical significance was considered at $P \leq 0.05$.

2.3 RESULTS

2.3.1 Evaluation of Experimental Contamination Methods

The effect of the application method and medium used to experimentally contaminate eggs on the recovery of *Salmonella* was evaluated. *Salmonella* was enumerated from the surface of eggs upon which the inoculum was applied using a pipette, gauze pad, cotton ball, or sponge and uninoculated control eggs at 1 h post-inoculation and after 24 h of cooled storage (**Figure 3**). At 1 h post-inoculation, more *Salmonella* was recovered from eggs experimentally contaminated using a pipette, gauze pad, and cotton ball than from eggs experimentally contaminated using a sponge. However, at 24 h post-inoculation recovery of *Salmonella* was greater from eggs

experimentally contaminated using a pipette than from those inoculated using the other methods.

Salmonella was enumerated from the surface and selectively enriched from the surface rinsate and crushed shell and membranes of eggs that were experimentally contaminated with *Salmonella* using TSB, BPW, PBS, or FS, and uninoculated eggs up to 14 d of incubation (**DOI**). Over the time course of the experiment, the recovery of *Salmonella* and the number of eggs from which *Salmonella* was detected was greater when eggs were experimentally contaminated using TSB than from those inoculated using the other media. Counts of *Salmonella* recovered from the surface decreased over time for all treatments and there was no significant difference between inoculation media beginning at d 7 of incubation (**Figure 4A**). Overall, the number of eggs from which *Salmonella* was detected was greater when eggs were inoculated using TSB (**Figure 4B**). Significant differences were observed between inoculation media at d 7, with *Salmonella* being detected from more eggs inoculated using a TSB suspension than from eggs inoculated using the other media. However, no significant difference was observed in between any of the inoculation media by d 14.

Salmonella was enumerated from the surface of sanitized and unsanitized eggs that were experimentally contaminated with *Salmonella* suspended in TSB, PBS, FS, or BPW, and uninoculated eggs (**Figure 5**). *Salmonella* was not detected from any eggs which had been sanitized using the H_2O_2/UV AOP egg sanitizer. Treatment using the H_2O_2/UV AOP egg sanitizer reduced *Salmonella* on experimentally contaminated eggs up to 7 $\log_{10}$ cfu egg^{-1} when compared to unsanitized eggs. Based on these results, eggs

were experimentally contaminated by pipetting a *Salmonella* suspension in TSB onto the eggs for all subsequent assays.

2.3.2 Effect of Storage Time Prior to AOP Sanitization

The effect of the duration of storage prior to H_2O_2/UV AOP sanitization of eggs on the reduction of *Salmonella* was evaluated. Eggs were experimentally contaminated and stored for up to 7 d before being sanitized. Following sanitization, *Salmonella* was enumerated from the surface and selectively enriched from the surface rinsate, crushed shell and membranes, and contents of eggs. The reduction of *Salmonella* contamination was greater when eggs were stored for a shorter amount of time prior to the sanitization treatment, and *Salmonella* was not detected from the surface of any eggs treated with the egg sanitizer. Over the time course of the experiment, counts of *Salmonella* recovered from the surface of untreated eggs were significantly greater compared to treated eggs until 5 d post-inoculation (**Figure 6A**). Overall, the number of eggs from which *Salmonella* was detected was greater when eggs were left untreated (**Figure 6B**). However, no significant difference was observed beginning on 5 of cooled storage.

2.3.3 H_2O_2/UV AOP Sanitization Reduces Salmonella Contamination of Eggs during Incubation

The effect of H_2O_2/UV AOP sanitization on *Salmonella* contamination of eggs during 18 d of incubation was evaluated. *Salmonella* was enumerated from the surface and selectively enriched from the surface rinsate, crushed shell and membranes, and contents of experimentally contaminated eggs that were untreated or treated using the H_2O_2/UV AOP egg sanitizer. *Salmonella* was not detected from the surface of any eggs

that were treated using the egg sanitizer (**Figure 7A**), and *Salmonella* was detected from fewer treated eggs as compared to untreated eggs (**Figure 7B**). The counts of *Salmonella* recovered from the surface of untreated eggs decreased over time with no significant difference observed between treated and untreated eggs beginning on d 11 of incubation. Overall, the number of eggs from which *Salmonella* was detected was fewer when eggs were treated using the egg sanitizer. However, no significant difference was observed after d 4 of incubation.

2.3.4 H_2O_2/UV AOP Sanitization of Eggs Reduces Salmonella in Broiler Chicks

The effect of H_2O_2/UV AOP sanitization of eggs on the prevalence of *Salmonella* in chicks hatched from experimentally contaminated eggs was evaluated (**Table 2**). *Salmonella* was selectively enriched from the gastrointestinal tract of chicks at 0 and 14 d post-hatch. *Salmonella* was not detected in any chicks hatched from eggs treated using the egg sanitizer. Additionally, *Salmonella* was not detected on tray liners from hatchers containing eggs treated using the egg sanitizer but was detected on all tray liners from hatchers containing unsanitized eggs (data not shown). *Salmonella* was detected in the gastrointestinal tract of 5% and 10% of broiler chicks hatched from unsanitized eggs at 0 and 14 d post-hatch, respectively. Cumulatively through 14 d post-hatch, *Salmonella* was detected from 7.5 % of chicks hatched from unsanitized eggs, whereas *Salmonella* was not detected from any chicks from sanitized eggs ($P = 0.027$).

2.4 DISCUSSION

The development of interventions to reduce *Salmonella* present on eggs entering the hatchery is expected to contribute to reducing *Salmonella* contamination of eggs

during incubation and in newly hatched chicks (Cason et al., 1994). Once on the egg surface, invasion and survival of *Salmonella* in the egg results in a reservoir for potential infection of naïve chicks after hatch (Bailey, et al., 1996). Hydrogen peroxide (H_2O_2) (Sheldon and Brake, 1991; Padron, 1995; Sander and Wilson, 1999) and ultraviolet light (UV) irradiation (Kuo, et al., 1997b; Chavez, et al., 2002; Coufal, et al., 2003) have been demonstrated to be effective in reducing surface microbial contamination of eggs. When used together as an Advanced Oxidation Process (AOP), UV photolysis of H_2O_2 results in the rapid generation of highly reactive oxygen species able to effectively inactivate vegetative bacteria, bacterial spores, and viruses (Bayliss and Waites, 1982; Mamane, et al., 2007; Ikai, et al., 2010). The H_2O_2/UV Advanced Oxidation Process has been demonstrated previously to be an effective and safe method to reduce total aerobic bacteria and *Salmonella* on the surface of eggs by 3 and 5 $\log_{10}$ cfu, respectively (Wells, et al., 2010; Gottselig, et al., 2016). In this study, we evaluated the effect of H_2O_2/UV AOP sanitization of eggs on *Salmonella* contamination during incubation, hatching, and early grow-out.

Although the effectiveness of this and other egg sanitizing treatments (Cox, et al., 2007; Buhr, et al., 2013; Al-Ajeeli, et al., 2016) in reducing *Salmonella* contamination of eggs has been widely researched, few studies have followed the subsequent effect of the sanitizing treatments on *Salmonella* contamination of eggs through incubation and hatching and evaluated the colonization of chicks hatched from sanitized eggs. In this study, eggs were experimentally contaminated using a primary poultry isolate of *Salmonella* Typhimurium used widely in studies in which broiler chickens were

experimentally infected (Byrd, et al., 1998) in order to facilitate gastrointestinal colonization of broilers hatched from contaminated eggs. Although the isolate used in this study was marked with antibiotic resistance, antibiotics were not used in the recovery medium so that all *Salmonella* regardless of origin could be recovered and included in our analysis. Additionally, non-experimentally contaminated controls were included in the assays in order to further account for any naturally occurring *Salmonella*.

We evaluated several procedures to identify an appropriate method for the experimental contamination of eggs for use in this study. Although immersion in a microbial suspension is widely used for experimental contamination of eggs, it has been demonstrated to facilitate bacterial penetration through the eggshell pores (Berrang, et al., 1999), confounding evaluation of the effectiveness of surface-acting treatments on the reduction of microbial contamination (Musgrove, et al., 2010). Gottselig, et al, 2016 demonstrated that more *Salmonella* was recovered from crushed shells and membranes of H_2O_2/UV AOP sanitized eggs inoculated by immersion than from surface rinses, suggesting immersion of eggs promoted penetration of *Salmonella* through the pores limiting its exposure to the H_2O_2/UV surface treatment. In addition, counts of *Salmonella* have been reported to decline under normal conditions of egg incubation (Lancaster and Crabb, 1953; Cason, et al., 1993). Thus, an inoculation method that maximized surface contamination and minimized eggshell penetration was needed to evaluate treatment of eggs using H_2O_2/UV AOP sanitization. Of the non-immersive methods evaluated in this study, we determined using a pipette to inoculate eggs provided the most consistent application to the eggshell surface. Survival of *Salmonella*

on eggshells is reduced at higher temperatures with high relative humidity when compared to lower temperatures and low relative humidity (Simmons, et al., 1970; Radkowski, 2002). We evaluated the effect of the medium used to prepare the inoculum on the recovery of *Salmonella* from eggs during incubation. TSB, BPW, a fecal suspension, and PBS were evaluated for the persistence of *Salmonella* contamination of eggs during incubation. Overall, recovery of *Salmonella* during storage and incubation was greater from eggs experimentally contaminated with *Salmonella* Typhimurium suspended in TSB as compared to the other inoculation media. We speculate that nutrients contained in a growth medium such as TSB may promote the survival of *Salmonella* on eggshells during incubation. Additionally, we did not observe organic matter in the fecal suspension to reduce effectiveness of the sanitization treatment suggesting the H_2O_2/UV AOP may be less sensitive to reduced effectiveness by organic matter than other sanitizing treatments.

It has been demonstrated that enrichment from the pores and membranes of the eggshell may allow detection of microorganisms that may have been missed by a surface rinse alone (Musgrove, et al., 2005), suggesting that limiting enrichment to one component of the egg may result in failure to detect *Salmonella* present in another component. In this study, we enumerated *Salmonella* from the shell rinsate and selectively enriched for *Salmonella* from the eggshell rinsate, crushed shell and membranes, and the egg contents. There were several eggs for which *Salmonella* was not detected in the shell rinsate or crushed shell and membranes, but was detected in the egg

contents (data not shown). Thus, sensitivity of *Salmonella* detection from eggs was increased when all components of the egg were used.

Salmonella has been demonstrated to be able to penetrate eggshells almost immediately after contamination (Williams, et al., 1968), with greater penetration being observed with increased duration of exposure to *Salmonella* (Williams, et al., 1968; Berrang, et al., 1998). Additionally, eggs are routinely stored prior to incubation to delay the onset of embryonic development (Mauldin, 2002). In this study, we evaluated the effect of the duration of storage prior to sanitization on the reduction of *Salmonella*. *Salmonella* prevalence in eggs sanitized more than 3 d following initial contamination was similar to *Salmonella* prevalence in unsanitized eggs. However, no difference was observed in the percent of *Salmonella* positive eggs when sanitized after one d of storage. In a previous study, fewer *Salmonella* were recovered from the crushed shell and membranes of eggs sanitized 1 min following contamination than from those sanitized 24 h following contamination (Cox, et al., 1998). Thus, sanitizing eggs as soon after lay as possible is expected to minimize internalization of *Salmonella*.

We have demonstrated that treatment of eggs using H_2O_2/UV advanced oxidation can reduce *Salmonella* on the surface up to 7 $\log_{10}$ cfu egg^{-1} and reduce the number of *Salmonella* positive eggs up to 75% prior to being set for incubation. Additionally, we did not detect *Salmonella* on the surface of any eggs following treatment with the egg sanitizer. Quaternary ammonium compounds (**QAC**), chlorous compounds (Knape, et al., 2001), acidic electrolyzed oxidizing water (**EOW**) (Fasenko, et al., 2009), and peroxidase catalyzed compounds (**PCC**) (Kuo, et al., 1996) have also been demonstrated

previously to reduce *Salmonella* contamination of eggs. However, the concentrations of QAC required to reduce *Salmonella* on hatching eggs have been demonstrated to reduce hatchability (Bourassa, et al., 2002), and *Salmonella* was still detected on the surface of eggs following treatment with chlorous compounds (Choi, et al., 2015), EOW (Bialka, et al., 2004), or PCC (McKee, et al., 1998). However, repeated treatment with H_2O_2/UV was not observed to reduce hatchability or chick quality (Wells, et al., 2011a).

We have demonstrated the H_2O_2/UV Advanced Oxidation Process to be an effective surface-acting treatment for the reduction of *Salmonella* contamination of eggs. We have also demonstrated that H_2O_2/UV AOP sanitization of eggs prior to incubation reduced the incidence of *Salmonella* contamination in broiler chicks hatched from treated eggs through 2 weeks post-hatch. The horizontal transmission of *Salmonella* from contaminated eggs during hatching to chicks in the same hatcher (Cason, et al., 1994) and from infected to naïve chicks within the same flock (Byrd, et al., 1998) has been demonstrated previously. Although we did not evaluate *Salmonella* contamination from other sources after leaving the hatchery, our results suggest the H_2O_2/UV AOP egg sanitizer could be a potentially important intervention as part of a broader *Salmonella* reduction program.

In this study, we evaluated the effect of H_2O_2/UV Advanced Oxidation Process sanitization of experimentally contaminated eggs on *Salmonella* contamination during incubation, hatching, and early grow-out, and have demonstrated the effectiveness of this surface acting treatment in reducing *Salmonella* contamination of eggs and of the subsequent colonization by *Salmonella* of chicks hatched from treated eggs. The ability

of the automated H_2O_2/UV AOP egg sanitizer to dramatically reduce *Salmonella* contamination of eggs without reducing hatchability suggests significant advantages of H_2O_2/UV advanced oxidation over chemical sanitizers. We have demonstrated the application of this technology to be a potentially important intervention as part of a broader, overall *Salmonella* reduction program. The further development and application of this and other Advanced Oxidation Process technologies is expected to improve overall animal health, productivity, and microbial food safety of poultry.

CHAPTER III

CONCLUSIONS

We have demonstrated that treating eggs with the H_2O_2/UV Advanced Oxidation Process egg sanitizer reduces *Salmonella* contamination of eggs and may reduce the gastrointestinal colonization of chicks by *Salmonella*. Sanitizing eggs with H_2O_2/UV advanced oxidation prior to incubation, reduced *Salmonella* throughout incubation, hatch, and early grow-out. Although many eggshell sanitization methods are available that effectively reduce *Salmonella*, the ability of the automated H_2O_2/UV AOP egg sanitizer to rapidly reduce *Salmonella* contamination of eggs without reducing hatchability suggests significant advantages of H_2O_2/UV advanced oxidation over other methods. The H_2O_2/UV AOP provides a novel solution to egg sanitization that can easily be implemented as part of a broader, overall *Salmonella* reduction program. The further development and application of this and other Advanced Oxidation Process technologies is expected to significantly impact the food industry by improving the overall animal health, productivity, and microbial food safety of poultry and poultry products.

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APPENDIX

Figure 1. Diagram of chicken egg

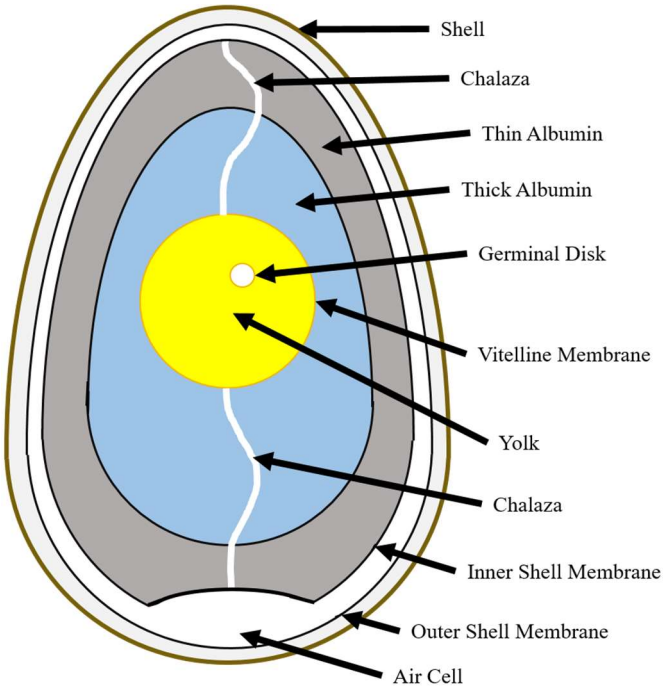

Figure 2. Schematic of egg sanitization device allowing for combined application of hydrogen peroxide and ultraviolet light via operator control.

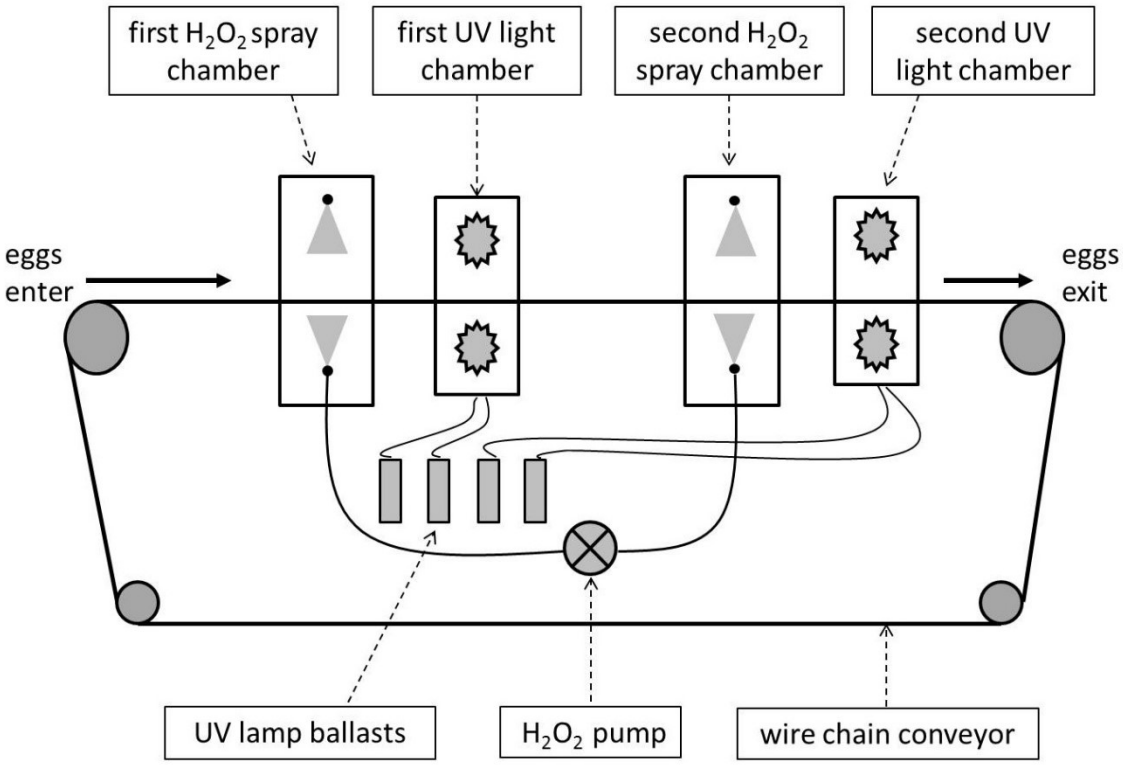

Figure 3. Evaluation of application methods.

Salmonella was recovered from the surface of eggs experimentally contaminated with *Salmonella* Typhimurium using a pipette, gauze pad, cotton ball, sponge, or uninoculated eggs (UNI) at 1 h (black) and 24 h (white) post inoculation. The mean ± SEM $\log_{10}$ cfu egg^{-1} *Salmonella* from 10 eggs per treatment from 2 independent assays are shown. Different letters indicate significantly different means ($P \leq 0.05$).

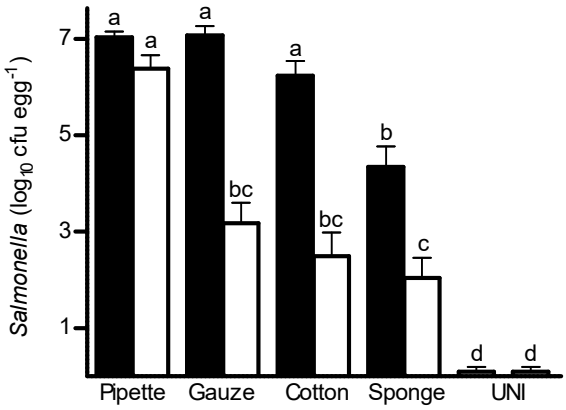

Figure 4. Evaluation of inoculation media.

Salmonella was recovered from eggs experimentally contaminated with *Salmonella* Typhimurium using TSB (filled circles), BPW (filled squares), PBS (open circles), FS (filled triangles), and uninoculated control eggs (open squares). Eggs were sampled -1, 0, 7 and 14 d of incubation. (**A**) Counts of *Salmonella* recovered from the surface are reported as the mean ± SEM $\log_{10}$ cfu egg^{-1} and (**B**) the prevalence of *Salmonella* is reported as the percent of eggs from which *Salmonella* was selectively enriched from the surface rinsate or shells and membranes crush. Means from 10 eggs per treatment from 2 independent assays are shown. Different letters indicate significantly different means ($P \leq 0.05$).

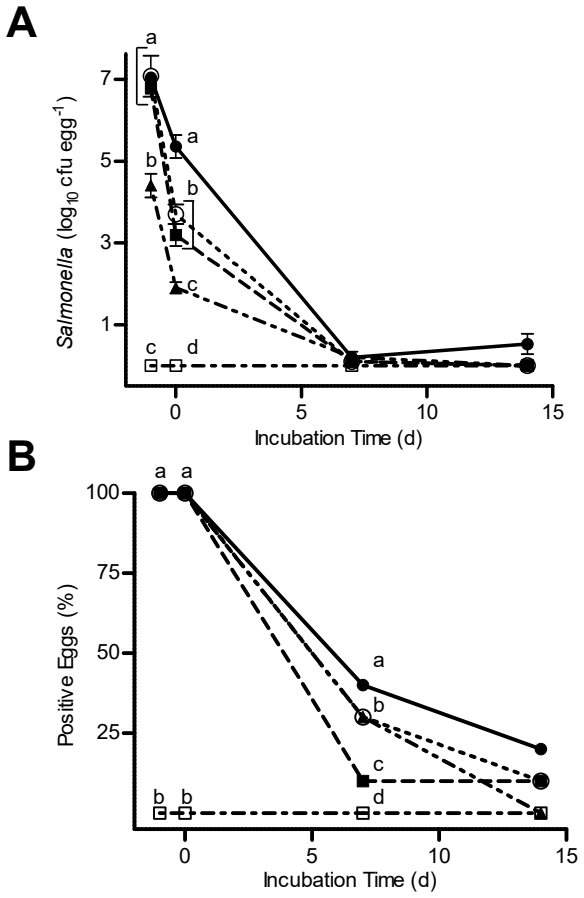

Figure 5. Effect of experimental contamination medium on H_2O_2/UV AOP reduction of *Salmonella* from the surface of experimentally contaminated eggs.

Salmonella was recovered from the surface of sanitized (white) or unsanitized (black) eggs experimentally contaminated with *Salmonella* Typhimurium TSB, BPW, PBS, FS, or uninoculated (UNI) eggs. The mean ± SEM $\log_{10}$ cfu egg^{-1} *Salmonella* from 10 eggs per treatment from 2 independent assays are shown. Different letters indicate significantly different means ($P \leq 0.05$).

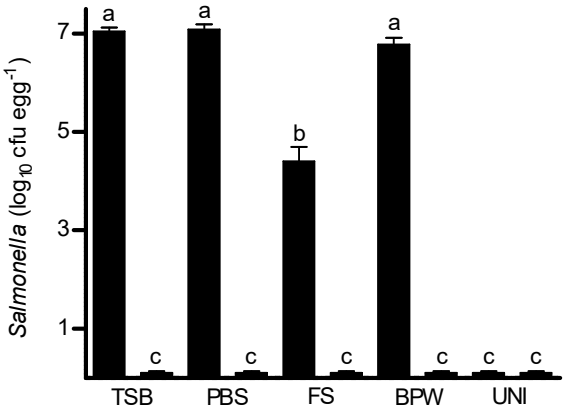

Figure 6. Effect of storage time prior to H_2O_2/UV AOP sanitization.

Eggs were experimentally contaminated with *Salmonella* Typhimurium and stored at 18 °C for 0, 1, 2, 3, 5, or 7 d prior to being sanitized. *Salmonella* was recovered from sanitized (squares) and unsanitized (circles) eggs. (**A**) Counts of *Salmonella* recovered from the surface are reported as the mean ± SEM $\log_{10}$ cfu egg^{-1} and (**B**) the prevalence of *Salmonella* is reported as the percent of eggs from which *Salmonella* was selectively enriched from the surface rinsate, shell and membrane crush, or egg contents. Results from 10 eggs per treatment at each time point from 2 independent assays are shown. * Indicates difference between groups at time point ($P \leq 0.05$).

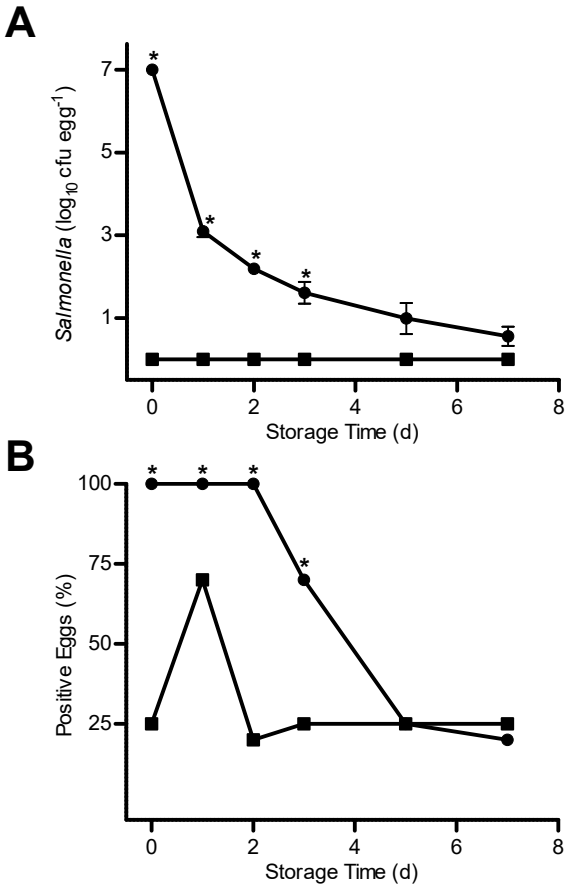

Figure 7. Treatment of eggs with H_2O_2/UV AOP reduces *Salmonella* contamination in eggs during incubation.

Salmonella was recovered from eggs experimentally contaminated with *Salmonella* Typhimurium. Eggs were sanitized (filled square) or unsanitized (filled circle) following experimental contamination and then sampled -1, 0, 4, 11, and 18 d of incubation. (**A**) Counts of *Salmonella* recovered from the surface are reported as the mean ± SEM $\log_{10}$ cfu egg^{-1} and (**B**) the prevalence of *Salmonella* is reported as the percent of eggs from which *Salmonella* was selectively enriched from the surface rinsate, shell and membrane crush, or egg contents. Means from 10 eggs per treatment from 2 independent assays are shown. * Indicates difference between groups at time point ($P \leq 0.05$).

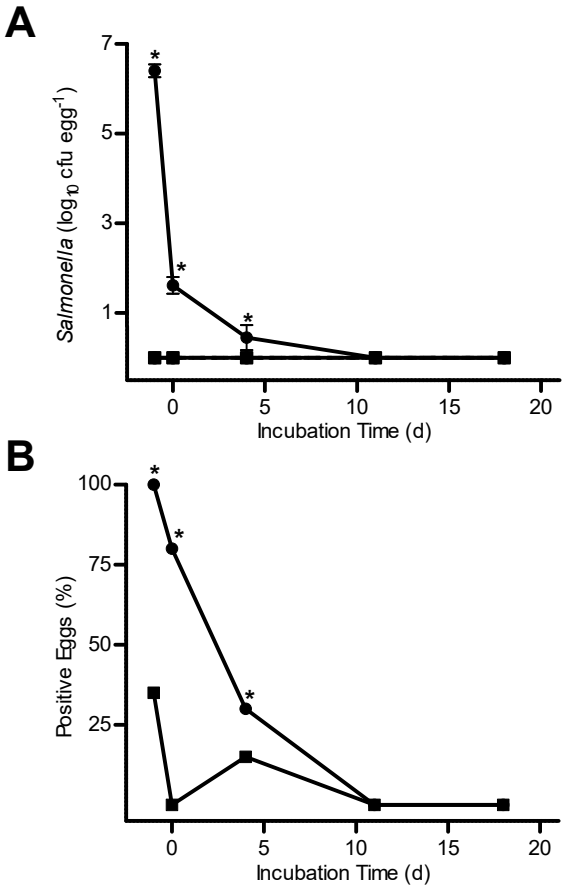

Table 1. Applications of ultraviolet light sanitization across various industries

Industry	Applied to	Effective against	Source
Food			
Carbohydrates			
	Bread loaves	Mold spores	Shama (1999)
	Sugar	*Bacillus stearothermophilus*	Weiser (1962).
Vegetables			
	Carrots	Preventative for fungal pathogens	Mercier, et al. (1994)
	Carrot juice	Total aerobic bacteria and total coliforms	Jo and Lee (2012)
	Packaged cucumbers	*Escherichia coli* and total coliforms	Tarek, et al. (2016)
	Tomatoes	*Escherichia coli* and *Salmonella enterica*	Mukhopadhyay, et al. (2014)
	Watercress	Psychotropic and mesophilic bacteria, molds, yeast, and Enterobacteriaceae	Hinojosa, et al. (2013)
Dairy			
	Yogurt packaging materials	Total aerobic bacteria and coliforms	Tamime and Robinson (1999)
Meat and poultry			
	Beef	*Pseudomonas*	Raymond A. Stermer (1987)
	Eggs	*Salmonella* Typhimurium	Kuo, et al. (1997a)
	Fish	*Pseudomonas*	Huang and Toledo (1982)
	Fish meal	*Salmonella, Escherichia coli,* and *Enterococcus*	Skowron, et al. (2014)
Ventilation			
	Laboratory air	*Escherichia coli, Micrococcus luteus, Bacillus subtilis*	Salie, et al. (1995)
	Hatcher air	*Enterobacteriaceae* and *Salmonella* Typhimurium	Bailey, et al. (1996)
Water			
	Drinking water	*Listeria monocytogenes*	Mikš-Krajnik, et al. (2017)
	Drinking water	*Enterococci* and total coliforms	Beck, et al. (2013)
	Waste water	*Escherichia coli, Bacillus subtilis,* vegetative cells and bacterial spores	Uslu, et al. (2015)

Table 2. Recovery of *Salmonella* from the GI tract of chicks hatched from sanitized and unsanitized eggs

	% *Salmonella* positive chicks		
Treatment group	0 d	14 d	Total
Inoculated eggs			
Sanitized	0.0	0.0	0.0[b]
Unsanitized	5.0	10.0	7.5[a]
Non-inoculated eggs			
Sanitized	0.0	0.0	0.0[b]
Unsanitized	0.0	0.0	0.0[b]
P	0.311	0.147	0.027
Cramer's V	0.160	0.229	0.239

[a-b] Different letters indicate groups are significantly ($P \leq 0.05$)