DETECTION AND REDUCTION OF MICROBIAL LOAD IN POULTRY FACILITIES

by

JULIA SUSANNE MCELREATH

(Under the Direction of Brian James Jordan)

ABSTRACT

It is known that poultry hatchery cleanliness can impact chick quality and livability, so maintaining clean facilities and hatching eggs is essential for the commercial poultry industry. To assess hatchery disinfection procedures, accurate monitoring of microbial load on hatchery surfaces and in the air is critical. Traditional bacterial plating methods are the standard for cleanliness evaluation, but are relatively expensive and time consuming, taking days to provide data. ATP bioluminescence detection offers a more rapid and inexpensive alternative to traditional microbial detection techniques, but has not been validated for use in poultry facilities. It is also known that hatching eggs have an extensive microbial population that can impact chick health and hatchery cleanliness. Disinfection methods for reducing bacterial load could be beneficial, but washing hatching eggs can remove the protective cuticle layer and is not currently done in the US broiler industry. Traditional hatchery disinfectant procedures are problematic in terms of efficacy against bacterial pathogens and safety of humans and animals. A novel dry hydrogen peroxide (DHP) system can reduce bacterial loads on hard surfaces, but effects on the microbial load of the porous eggshell have not been investigated. The objectives of these studies were to compare ATP bioluminescence sampling to traditional bacteria enumeration methods to identify the most accurate tool for measuring microbial load, as well as to determine if a DHP

system can reduce the microbial load of the eggshell. Our results showed that ATP bioluminescence swabs produced results comparable to traditional bacterial enumeration methods in a hatchery on multiple surface types in a timely manner; thus we conclude that ATP bioluminescence assays could effectively be utilized as an alternative assessment method in a commercial hatchery. In regards to hatching egg disinfection using a DHP system, we concluded that treatment of hatching eggs with DHP resulted in an overall reduction of bacterial concentrations on the eggshell surface, and that a DHP system can be utilized in a commercial poultry hatchery. In conclusion, the need for rapid disinfection and detection of microbes in hatchery environment can be fulfilled with the use of ATP bioluminescence swabs and a DHP system.

INDEX WORDS: Poultry, hatchery, disinfection, DHP, hydrogen peroxide, microbial monitoring, ATP bioluminescence

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DEDICATION

This thesis is dedicated to my mentor, Dr. Brian Jordan for encouraging me to pursue the fields of research and poultry science. Throughout both my undergraduate and graduate experience, he continued to support my decisions and collaborate on studies while providing me with exceptional training and advice. This thesis is also dedicated to my parents, Chris and Mary Sue McElreath for continuing to reassure curiosity and passion. Thank you all for always lifting me up when I was down.

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CHAPTER 1

INTRODUCTION

Commercial poultry hatcheries in the United States hatched over 13 billion chicks in 2018, with eggs sourced from many different locations (USDA 2019). The central position of hatcheries in the poultry production chain results in transfer of bacteria from breeder to broiler, both on the eggs themselves and in hatchery contamination. Hatcheries receive eggs from breeder farms that can carry significant bacterial loads despite the appearance of cleanliness. Bacterial infection of broiler chicks during the incubation and hatching phase can cause increased incidences of omphalitis and salmonellosis as well as increased mortality within 7 days of age (Wilson 1949, Walker et al. 2002, Iqbal et al. 2006). Infected chicks can spread disease within the hatcher tray and later within the flock at the broiler farm, causing economic losses for both the farmer and the integrator (Bailey et al. 1994). Historically, antibiotics have been utilized in combination with vaccines to prevent bacterial contamination during *in ovo* vaccinations. However company-wide transitions to antibiotic-free and no antibiotics ever production has caused increases in *Escherichia coli* infections resulting in increased yolk sac infections and mortality (Cortés et al. 2004).

Reduced use of antibiotics in the industry emphasizes the need for comprehensive and routine disinfection procedures within the hatchery. Effective bacterial disinfection is traditionally achieved by using a combination of various chemicals, including formaldehyde, ozone, halogen solutions, aldehydes, quaternary ammonium, alcohols, phenols, and hydrogen peroxide (Brake and Sheldon 1990, Samberg and Meroz 1995, Rodgers et al. 2001, Gehan 2009,

Frame 2010). Formaldehyde gas is a known human carcinogen and can affect human neurological and respiratory function (World Health Organization 2006). Additionally, formaldehyde gas fumigation causes decreased motility of the tracheal cilia in chicks, increasing the already high risk of respiratory disease in the poultry house due to concentrated levels of ammonia and circulating dust (Pickrell 1991, Sander et al. 1995). Gaseous ozone applications can reduce hatchability and cause mutagenesis of select microorganisms (Whistler and Sheldon 1989a, Whistler and Sheldon 1989b). Chlorinated solutions are commonly used on machinery, floors, and countertops as per hatchery cleaning and disinfection protocol, but over time they can corrode surfaces and necessitate costly repairs (Schmidt 1997). Aerosolized hydrogen peroxide can significantly reduce concentrations of *E. coli* and *Salmonella* serovars and improve hatch, but a concentration over 75 parts per million (ppm) is known to be an immediate risk to human and animal respiratory and pulmonary systems (Sheldon and Brake 1991, Center for Disease Control 2018)

In response to the negative aspects of traditional sanitizers, a novel dry hydrogen peroxide (DHP) system was developed by Synexis Biodefense to reduce microbial load on hard surfaces. The system utilizes a black light with a membrane coated in a photocatalytic chemical (proprietary technology); as oxygen and water (in the form of humidity) from the environment flow through the membrane, the black light stimulates the chemical on the membrane to react with the oxygen and water to produce a gaseous form of hydrogen peroxide. The unique construction of the hydrogen peroxide molecule causes it to act as a true gas, disseminating to hard-to-contact areas and establishing a dilute equilibrium concentration. The effectiveness of DHP in poultry facilities, however, has not been tested.

To ensure effective cleaning and disinfection procedures are properly utilized in the hatchery, consistent and accurate microbial detection methods are necessary. Common traditional methods include agar contact plates, fluff sampling, and direct surface swabbing. These methods are shown to be accurate and require relatively little training, but they all require incubator space and a minimum of 24 hours to produce results. Furthermore, over time these traditional techniques can become costly when performing regular sampling of multiple surfaces and locations in a large-scale commercial hatchery. Adenosine triphosphate (ATP) bioluminescence assays have been implemented in the poultry industry to monitor bacterial load, specifically in the processing plant and on carcasses, since 1994 (D. Bautista et al. 1994, D. Bautista et al. 1995, G. Siragusa et al. 1996). Swabs utilizing ATP bioluminescence contain luciferin and luciferase solutions that react with ATP in the sample to produce an emission of light detectable by a luminometer. The light signal is directly proportional to the amount of ATP in the sample and is reported in relative light units (RLU).

The focus of this project is the detection and disinfection of microbial loads on hatching eggs and hatchery surfaces. The first study determined the effects of a DHP system on the surface of hatching eggs in a controlled laboratory setting and a research cooler facility. The second study compared traditional methods of microbial detection to general and organism-specific ATP bioluminescence swabs by sampling surfaces with a known bacterial concentration and surfaces in a commercial hatchery.

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CHAPTER 2

LITERATURE REVIEW

Introduction

Large-scale hatcheries are responsible for collection of hatching eggs from breeder farms and distribution of healthy, newly hatched chicks to the commercial broiler farm. In 2018, United States hatcheries incubated 13.9 billion hatching eggs, a 2% increase from 2017 (USDA 2019a). In 2018, 9.71 billion broiler-type chicks were hatched, equating to millions of chicks passing through commercial hatcheries on a daily basis (USDA 2019b). Commercial hatcheries require financial investments such as maintenance, cooling and heating, and employee wages. However, value is not generated for the integrator until carcasses are processed and sold, thus integrators aim to keep hatchery costs low. As a result, hatcheries are often not modern structures and do not have the most advanced equipment available, making cleaning and disinfection even more difficult. Cleaning and disinfection can be accomplished through a proper sanitation design created to make broiler hatcheries efficient and effective (Samberg and Meroz 1995). Many commercial hatcheries implement routine cleaning and disinfection with liquid solutions on solid surfaces, but hatching eggs are not traditionally washed and additional sanitizers can be required to improve hatch and reduce use of harmful chemicals.

Bacterial Contamination and Disease

The hatchery has been pinpointed as potentially the most significant point of contamination, regardless of grow-out conditions, due to chick vulnerability and exposure to high concentrations of pathogens in the immediate environment (Bailey et al. 2001). Insects,

rodents, and wild birds infected or carrying the organism act as reservoirs for bacterial and fungal contamination at the hatchery (USDA 2014). Contamination can also occur from management sources such as water supply, egg transport trucks, or clothing of hatchery personnel and visitors (USDA 2014). However, the primary source of contamination in the commercial hatchery is surfaces of soiled eggs incoming from the breeder farm (Bailey et al. 1994, Samberg and Meroz 1995). It is understood that newly hatched chicks are vulnerable to infection, and the high concentrations of bacteria and fungi in the hatchery can cause increases in mortality and disease in young chicks (Walker et al. 2002).

Microbial pathogens found in the hatchery, including Enterobacteriaceae spp., *Pseudomonas* spp., *Clostridium perfringens*, and fungal species, can cause contamination in eggs, chicks, and potentially entire flocks at the broiler farm (Samberg and Meroz 1995). *Salmonella* serotypes have been isolated from hatchery eggshells and equipment at concentrations upwards of 10^3, yet only a single *Salmonella*-contaminated eggshell is able to contaminate an entire hatching cabinet and potentially produce seeder birds that infect other birds during the grow-out process (Cox et al. 1990, Bailey et al. 1994, Cason et al. 1994, Bailey et al. 2001). Furthermore, similar *Salmonella* serotypes have been associated with the hatchery, multiple broiler farms, and the processing plant, though these serotypes were not often isolated at the breeder farm, indicating the hatchery as a source point of contamination for the chain of poultry production (Bailey et al. 2002). *Escherichia coli* is one of the most common bacteria isolated from diseased eggs and chicks in the hatchery environment, present in up to 8% of 21-day old eggs and designated as a potential cause of yolk sac infections (Cortés et al. 2004). This prevalence is attributed to the simple growth requirements of *E. coli* as well as the ability to grow in the presence of iron-chelating molecules found in the albumen, such as ovotransferrin

(Seviour et al. 1972). Despite declining antibiotic use in commercial hatcheries, multi-drug resistant strains of pathogenic *E. coli* have been detected in up to 11.2% of broiler samples during their first week of life (Baron et al. 2014). *Pseudomonas* species have been isolated from freshly hatched chicks in a commercial hatchery at concentrations of 10^2, but an isolate concentration of 10^1 is associated with up to 90% mortality in 14-day old broiler chicks (Walker et al. 2002). Broiler farm and hatchery isolates of *Pseudomonas* were identified as resistant to multiple drugs, including penicillin, bacitracin, erythromycin, naladixic acid, and tetracycline (Walker et al. 2002). Further, an infection of *Pseudomonas* within a 30-day old broiler flock can cause mortality losses of 18% in a single day (Fodor 2007). Eggshell exteriors can also carry disease-causing concentrations of *Clostridium perfringens*, with common ribotypes identified at the broiler farm and processing facility, to the extent of labeling the hatchery as a "critical control point" (Craven et al. 2001, Craven et al. 2003). Increased instances of *C. perfringens*-associated necrotic enteritis have been noted on broiler farms in association with reduced antibiotic use in the hatchery, accounting for up to 1% daily mortality and continuing for several days (Immerseel et al. 2004).

Additionally, fungal microbes including *Aspergillus* spp. are abundant in the air of the broiler hatchery, averaging a concentration near 70.4 colonies per hatchery room after a 30 second active air sample (Chute and Barden 1964). *A. fumigatus* spores range in size from 2-3µm in diameter, allowing for bypass of physical barriers and deep infection of the avian respiratory system (Fedde 1998). It has also been shown that certain species of *Aspergillus* produce toxins such as sterigmatocystin to induce embryo death in amounts as low as 5 micrograms (Schroeder and Kelton 1975). *Aspergillus* spp. and other fungal isolates in a hatchery environment can cause dermatitis as well as infections of the lungs and air sacs in young chicks (Eckman and Morgan-

Jones 1979). Retention of *Aspergillus* spores on the down of one-day-old chicks during transfer to the broiler farm can result in contamination of birds and litter at the farm (Arné et al. 2011). When spores are present at the farm, moisture, airflow, and animal movement can cause exponential growth of hyphae and increased release of airborne conidia that can cause aspergillosis in birds (Pinello et al. 1977, Debey et al. 1995).

The declining use of disinfectants and sanitizers harmful to human and bird health has exacerbated the persistence of microbial transmission from the commercial hatchery to the broiler farm and processing plant. Pathogens will continue to play a major role in the poultry industry, as it is impossible to maintain complete sterility in this field. It is the responsibility of the researcher to identify methods of disinfection that can be utilized in combination with routine cleaning to achieve the lowest level of contamination while meeting safety standards. It is the responsibility of the poultry industry to implement these methods in a cost-effective manner and produce quality birds.

Traditional Hatchery Disinfectants

Regular hatchery cleaning procedures are necessary to maintain cleanliness and reduced disease outbreaks. Treatment with a combination or rotation of disinfectants and sanitizers can be implemented to target multiple genera of bacteria and fungi on various surface types (Gehan 2009). It is critical that the proper disinfecting agent is utilized under the proper conditions to maximize efficacy and reduce complications such as health hazards or microbial resistance (Boothe 1998).

Common hatchery disinfectants are primarily based on quaternary ammonia, chlorine, or organic peroxides (i.e. peracetic acid), and each disinfecting agent has a different mechanism of action that results in microbial inactivation. Liquid surface disinfecting solutions containing

quaternary ammonium degrade protein and lipid structures, preventing most microbial attachment and invasion of the host cell (Suarez et al. 2003). Quaternary ammonia solutions tested in hatchery conditions were shown to be highly effective in reducing concentrations of coliforms, overall aerobic bacteria, *Staphylococcus aureus*, molds, and yeasts (Brake and Sheldon 1990, Rodgers et al. 2001). Organic peroxide and chlorine-based solutions are cellular oxidants, negatively affecting proteins, lipids, and nucleic acids (Suarez et al. 2003). Chlorine solutions can also significantly decrease aerobic, coliform, and fungal loads on the egg surface, but not as effectively as quaternary ammonia or peroxide solutions (Gehan 2009). Liquid peroxide disinfectants can completely kill eggshell microbial concentrations of aerobic bacteria, coliforms, and fungi (Gehan 2009).

Despite efficacy as antimicrobials, a 1995 study reported chemical resistance in 8% of bacteria isolated from three commercial hatcheries (Willinghan et al. 1996). Furthermore, Sander et al. (2002) reported varying efficacy of disinfectants against organisms of the same genus and species, implying rapid development of microbial chemical resistance. Reduced potency of disinfectants can also be reported when organic matter is present (Rodgers et al. 2001, Ruano et al. 2001). Additionally, occupational asthma has been associated with employees in close contact with concentrated chemical disinfectants (Purohit et al. 2000). However, long-term increases in bacterial resistance in addition to employee health concerns have increased research into alternative surface disinfectants in the hatchery, including combinations of formaldehyde, ozone, and hydrogen peroxide variants.

Formaldehyde Fumigation

Formaldehyde is a carcinogenic disinfectant, normally found in aqueous or gaseous forms and often referred to as ‘formalin’ (Tang et al. 2009). Its biocidal function derives from its

attachment to primary amide groups and amino acids to destabilize proteins; it also irreversibly damages DNA and RNA by alkylating purine and pyrimidine bases (Habeeb and Hiramoto 1968). An initial study by Pernot in 1909 investigated fumigation with formaldehyde gas for reduction of bacterial load on hatching eggs during incubation, and the method has been used extensively throughout the history of poultry since its proven efficacy. Lancaster and Crabb (1953) examined the effects of formaldehyde on eggs inoculated with *Salmonella* and reported that a 20 minute exposure to gas at a concentration of 6.5mg/ft3 were the minimal conditions for bactericidal effects. Williams later demonstrated in 1970 that formaldehyde gas is an effective disinfectant of eggshells, reducing bacterial concentrations on shells of eggs at the breeder farm by 99.66% when applied for 20 minutes. In regards to effects on *A. fumigatus*, a 2-minute exposure resulted in a >5 $\log_{10}$ reduction in spore counts (Whistler and Sheldon 1989). A field study reported a reduction of fungal conidia viability to <0.01% after a 24-hour application of gaseous formaldehyde at a concentrations of 2 parts per million (ppm) (Dennis and Gaunt 1974). However, negative effects of formaldehyde application caused researchers to investigate alternative application methods, including constant rate infusion (CRI), where a low concentration of formaldehyde is administered over several hours (Steinlage et al. 2002). Steinlage al. reported a significant decrease in aerobic bacterial CFU/m^3 in an egg hatcher when formaldehyde was administered via CRI or gas fumigation compared to a hatcher that received no disinfectant, yet no significant difference was observed between any of the hatchers that were treated with formaldehyde. Although ultimately, when administered during hatch, the CRI formaldehyde administration was overwhelmed by the bacterial load and no decrease was observed in aerobic bacterial counts (Steinlage et al. 2002).

Despite its effectiveness and low cost, the negative effects of formaldehyde on chick health are clear; tracheal cilia motility decreases and the respiratory tract becomes irritated (Sander et al. 1995). In regards to human health, repeated exposure to formaldehyde can cause respiratory disease, neurological effects, and birth defects (Tang et al. 2009). These extreme negative effects exposed by mounting research have caused the US Environmental Protection Agency and individual state legislatures to regulate or ban formaldehyde use, emphasizing the need for safer hatchery disinfectants while maintaining production and chick health (Kim and Kim 2010).

Ozone and Ultraviolet Light Disinfection

Ozone (O_3) is a polymerized oxygen molecule that, in gaseous form, has a blue hue and a distinct odor. It is known to have bactericidal, fungicidal, and sporicidal properties and has been implemented in a hatchery environment with successful reduction in microbial load (Whistler and Sheldon 1989, Kim et al. 1999). Normally applied in a dry gaseous form to reduce corrosion of metal equipment, ozone is produced by drawing air over high energy electrodes within an ozone generator (Whistler and Sheldon 1989, Kim et al. 1999). Its effectiveness as an antimicrobial agent derives from the strong oxidizing power of the free radicals formed as the ozone molecule decomposes, initially targeting lipid membranes and causing cell lysis (Rice et al. 1981, Kim et al. 1999). Moreover, ozone will spontaneously decompose to form oxygen, making it safe for human consumption (Rodriguez-Romo and Yousef 2005).

After a 3-minute exposure, results indicated a significant 3.0 log reduction of isolated *Salmonella* on inoculated eggshells (Rodriguez-Romo and Yousef 2005). However, Whistler and Sheldon noted continual “atypical growth” of *E. coli* cultures exposed to a 1.51% by weight ozone solution when compared to untreated colonies. Further testing revealed that surviving *E.*

coli colonies produced significantly greater concentrations of catalase, indicating possible mutagenesis in cultures exposed to ozone (Whistler and Sheldon 1989). Ozone application at 0.3-0.9 ppm for four hours was demonstrated by Dyas *et al.* to reduce *A. fumigatus* concentrations from 10^6 to zero, indicating that spores are relatively sensitive to exposure (Dyas et al. 1983). However, this experiment was conducted in a closed cupboard measuring $0.087m^3$ with prolonged exposure. In a study conducted in a prototype hatchery setter measuring $0.015m^3$, 1.51% ozone (by weight) reduced *A. fumigatus* spore concentrations more than 4 logs within 8 minutes of exposure (Whistler and Sheldon 1989).

Ultraviolet (UV) radiation affects cell health through a series of photochemical processes, forming free radicals that inhibit oxidative phosphorylation and destroy cell wall integrity (Sinha and Häder 2002, Rodriguez-Romo and Yousef 2005). It also causes cell death by inhibiting DNA transcription and replication by inducing cross-linking of pyrimidine nucleotides within cell DNA (Rodriguez-Romo and Yousef 2005). UV has been used to effectively reduce microbial loads on eggshell surfaces as well as in circulating air in the hatchery (Bailey et al. 1996, Kuo et al. 1997). Airborne Enterobacteriaceae and *Salmonella* concentrations were significantly reduced in hatchery conditions after continuous application of UV with no negative effects on hatchability (Bailey et al. 1996). On inoculated eggshell surfaces, application of UV for a brief 60 seconds resulted in a significant 4.3 log reduction of *Salmonella* (Rodriguez-Romo and Yousef 2005). When UV radiation is applied to *Aspergillus niger* spores at a low intensity of 1 W/m^2, 80% of spores were inactivated after a brief 15 minute application (Chen et al. 2009). Combined use of UV radiation and ozone for 1 minute can further reduce *Salmonella* concentration on eggshells by 4.1 and 2.1 logs compared to ozone-treated and UV-treated eggs, respectively (Rodriguez-Romo and Yousef 2005). Bacterial spores treated with both UV and

ozone were inactivated in less time than solo use of either treatment, resulting in a "synergistic sporicidal" effect (Kim et al. 1999).

Hydrogen Peroxide Disinfection

Hydrogen peroxide is commonly applied as an aqueous or aerosolized solution, appearing colorless and acting as a bacterial disinfectant by reacting with superoxide molecules to form a reactive hydroxyl radical that attacks cellular nucleic acids, lipids, and proteins (Linley et al. 2012). For industrial disinfection of hard surfaces, it is understood that a minimal concentration of 5% (v/v) is needed (Andersen et al. 2006). Aerosolized hydrogen peroxide has recently been used in the industry of food microbiology to decrease microbial load on surfaces by 6.0 logs after a brief 2 minute exposure (Wang and Toledo 1986). Furthermore, airborne microbial concentrations were significantly reduced in a commercial hatchery setting when treated with a 3% aerosolized hydrogen peroxide solution (Sander and Wilson 1999). Application of a 5% aqueous H_2O_2 solution by Sheldon and Brake (1991) to study the effects on eggshell microbial load resulted in a 5.3 log reduction of total bacterial load, as well as significant reductions in *Escherichia coli* and *Salmonella* spp. concentrations. Significant reductions in *S. typhimurium* concentrations was also observed after dipping hatching eggs in a 6% aqueous hydrogen peroxide solution (Padron 1995). Fungal concentrations are significantly impacted by hydrogen peroxide applications; a 99.5% reduction in *Aspergillus* spores was noted after disinfection using 6% aqueous hydrogen peroxide (Szymańska 2006).

Studies have indicated that overall, chick hatch is positively affected aqueous hydrogen peroxide application. A 2.0% increase was observed in hatchability of fertile eggs as well as a 2.2% decrease in early dead embryos when a 6% hydrogen peroxide solution was applied to eggs (Sheldon and Brake 1991). When compared to 3x formaldehyde fumigation, a 5% H_2O_2 (v/v)

solution can significantly reduce late dead embryo counts (Sheldon and Brake 1991). However, hydrogen peroxide is also known to be corrosive and dangerous to human respiratory systems when applied at high concentrations (Center for Disease Control 2018).

Using a combination of hydrogen peroxide and UV light can further reduce microbial load on eggshell surfaces (when compared to use of just one of these treatments) by generating hydroxyl radicals that rapidly kill bacteria in an 'advanced oxidative process' (AOP) (Bayliss and Waites 1979, Wells et al. 2010, Jones-Ibarra et al. 2019). Wells et al. reported a 2.8 $\log_{10}$CFU/egg reduction in concentrations of aerobic bacteria on the surface of treated eggs when compared to untreated eggs, with no significant effects on hatchability, embryo mortality, or chick weight (Wells et al. 2011). The combined treatment included a mist of 1.5% aqueous H_2O_2 and 8 minutes of exposure to UV light (Wells et al. 2011). This bacterial reduction on the eggshell surface was replicated by Gottselig, but it was noted that the process was not practical for a commercial setting due to time constraints (Gottselig 2011). To combat this, a system was developed by Coufal et al. (2015) that includes a conveyor belt to transport hatching eggs through a liquid hydrogen peroxide spray followed by a UV light chamber, thus effectively implementing an AOP to reduce microbial load on hatching eggs in a timely manner. By both reducing risks associated with high concentrations of hydrogen peroxide and increasing potential for use in a commercial environment, Coufal et al. have demonstrated the practicality of a commercial AOP system.

A system developed by Synexis Biosystems also addresses the issues of H_2O_2-associated health risks by producing a gaseous form of hydrogen peroxide at low concentrations for an extended period (Lee 2012, Lee and Stephens 2018). Dry hydrogen peroxide (DHP) is generated by applying low wavelength black light to activate a photocatalyst that then simultaneously

oxidizes water and reduces oxygen into H_2O_2 (Lee 2012). When hydrogen peroxide gas is produced, it diffuses into the environment at concentrations of 0.01-0.20 ppm, a value well below the workplace safety limit of 1.0 ppm established by the Occupational Safety and Health Administration (Lee 2012). The low concentration of H_2O_2 combined with the high air circulation commonly found in poultry environments requires extended application of the DHP system, ranging from one hour to four days, or continually (Lee and Stephens 2018). The system was shown to significantly reduce the eggshell microbial load after 72 hours; despite the time requirements, the DHP system is practical for a commercial poultry setting because it is a passive system that requires no unnecessary transfer of eggs (Lee and Stephens 2018). When a DHP system was applied in a field environment, results indicated positive effects on hatch; treatment of hatching eggs with DHP resulted in increased chick weight and hatchability in addition to decreased chick mortality (Lee and Stephens 2018).

Methods of Microbial Monitoring in Poultry Facilities

Accurate and consistent detection of bacterial loads on hatchery surfaces and air is essential to ensure that proper cleaning and disinfection methods are being applied. Health of both hatching chicks and hatchery personnel relies on accurate reporting of microbial load in the environment. Although visual evaluation can provide management with a general analysis of hatchery cleanliness, objective monitoring of surface and air microbial load is necessary. Many hatcheries lack expert personnel and microbiological facilities; thus, they must rely on simple yet comprehensive sampling techniques. Frequency and magnitude of microbial sampling varies between hatcheries based on size, integrator, and production type, and certain methodologies have proven consistent and efficient, and therefore are highly utilized amongst commercial hatcheries (Soucy et al. 1983).

Evaluation of fluff

In a 1958 study, Wright and Epps described testing of hatcher fluff as an accurate measurement of bacterial load in the environment. This sampling technique involves acquisition of fluff from a hatcher unit, suspension of fluff in a sterile enrichment broth or buffer solution, followed by plating of the liquid solution onto selective or general agar (Wright and Epps 1958). Liquid suspension can also be used as a template for polymerase chain reaction (PCR) to detect specific organisms (Hiett et al. 2002). Fluff samples have been identified as a potential source of *Salmonella* transmission after fluff acquired from poultry facilities was successfully utilized to serotype *Salmonella enterica* (Roy et al. 2002). In regards to *Campylobacter* detection, Hiett et al. (2002) determined that this method provides an accurate measurement of bacterial load in the hatchery.

Microbial fluff sampling can provide information on both hatchery sanitation effectiveness and bird health later in life. Wright and Epps provided a direct relationship between hatchery fumigation concentrations and fluff microbiological analysis. Formaldehyde fumigation of 1cc. per cubic foot reduced total bacterial counts from 3.2 to 1.7 million; higher concentrations of 1.5cc per cubic foot resulted in total bacterial counts of 68,200 (Wright and Epps 1958). One study in 1966 involving a turkey hatchery noted that increases in bacterial counts acquired from fluff samples were later associated with decreases in poult quality (Nichols et al. 1967). Fluff samples stored at room temperature are known to retain viable and high concentrations of certain *Salmonella* serotypes for up to 5 years after sampling, allowing for comprehensive knowledge on effects of *Salmonella* transmission via fluff (Miura et al. 1964). A significant increase in prevalence of *Salmonella* in commercial broiler-breeder hatcheries has been noted in Ontario using PCR analysis of fluff, and *Salmonella* serotypes have been identified

in both hatchery fluff and processed carcasses of the same flocks (Bhatia and McNabb 1980, Sivaramalingam et al. 2013). Microbial examination of fluff samples, especially when pooled from multiple hatches, can provide hatchery management with a broad understanding of contamination within the commercial facility.

Microbial plate culture methods

Agar plates are advantageous in that they can be customized to target specific bacterial types, such as Gram-staining positive and Gram-staining negative, and molecular techniques can be applied to isolated colonies for serotyping. Ease of use, convenience, and low cost of agar plates make them ideal for use in a hatchery where expertise and processing facilities can be limited. The main disadvantage of culture techniques is the difficulty in identifying ideal growth conditions of some bacterial and fungal species. When agar plate methods are compared to fluff and direct swab evaluation, they can provide a false impression of a clean environment; other conventional methods are able to detect lower levels of contamination compared to agar plates, providing more accurate results of hatchery cleanliness (Gehan 2009). Enumeration of colonies can also become complicated by swarming colonies, debris, and overlapping colonies on the agar (Soucy et al. 1983).

Use of Replicate Organism Detection and Counting (RODAC) agar plates that directly and consistently make contact with a flat surface are of much use in a hatchery setting for monitoring surface cleaning and disinfection regiments. They are convenient and effective in monitoring presumptive microbial load on hatchery equipment and surfaces, including belts, vaccine labs, and internal walls of setters and hatchers (Kim and Kim 2010). Plates are created so that the surface of the agar is slightly higher than the plate edge. The cover is removed and the agar is then gently pressed onto a flat surface, ensuring that the plate is not moved during contact

(Samberg and Meroz 1995). In terms of labor and cost, the design of contact plates is especially appealing for hatchery surveillance (Ernst 1987). Additionally these plate types are effective in detection and isolation of *Salmonella* species by flooding the surface with tetrathionate broth after a 16-20 hour incubation period (Samberg and Meroz 1995).

Microbial surface contamination can accurately be detected by contact plates, and a direct relationship has been identified between surface and aerobic microbial concentrations throughout the hatchery (Magwood 1964). Kim and Kim described effective quantitation of aerobic bacterial and fungal coliform concentrations using passive agar plates exposed for 10 minutes, observing comparable concentrations of microbes on RODAC and passive air plates (Kim and Kim 2010). Air quality within a hatchery is a reflection on hatchery cleanliness and contamination within the environment, and agar plates exposed to both passive and active air can provide comprehensive information on aerobic microbial concentrations. Instances of omphalitis in chicks occurred at higher rates in hatchers with poor air quality as determined by agar plate counts (Chute and Gershman 1961). Berrang et al. (1995) compared techniques of measuring airborne microbial contamination within hatching cabinets; an active air sampler pulling 180 liters of air detected a greater number of Enterobacteriaceae cells than a passive, open plate method, indicating that active sampling can provide advantages over passive sampling. Despite needing an incubator to culture plates and at least 24 hours to read results, microbial culturing methods are convenient and cost-effective, making them the microbial monitoring technique for many commercial hatcheries.

Swab techniques and evaluation

For direct swabbing methods, swabs are saturated in buffered solution before a standard area is swabbed in four directions; the swab is then streaked onto agar plates at the same angle at

which the surface was sampled (Magwood and Marr 1964). Alternatively, swabs can be suspended in liquid buffer before a brief vortex to release microbes, then plating the liquid onto various general and selective agar plates; liquid can also be utilized in molecular analysis, including PCR and sequencing (Gehan 2009). Direct surface swabbing provides an accurate determination of hatching cabinet bacterial, yeast, and mold concentrations as well as demonstrating high recovery and sensitivity (Soucy et al. 1983, Gehan 2009). In hatchery locations where fluff is not available for microbial testing or there is suspicion of microbial concentrations below the limit of detection of RODAC plates, direct swabs can provide accurate bacterial and fungal concentrations (Gehan 2009). This method is particularly useful in a hatchery setting for numeration of viable microbes within debris or other particulates, as it has been noted that direct swabs collect more residual debris than other microbial monitoring methods (Favero, McDade et al. 1968, Soucy, Randall et al. 1983). Magwood and Marr (1964) determined a proportional relationship between direct swabs taken from hatchery surfaces and embryo health, noting the ability of this method to detect potential pathogens at low concentrations.

Favero et al. described several limitations of direct swabbing in the field; residual chemicals on target surfaces can inhibit microbial growth and contaminate samples, providing false results, and enumeration of colonies can become complicated when overgrowth is observed in extremely contaminated locations (Favero et al. 1968). As with other traditional culturing techniques, a significant obstacle faced by direct swabbing is the difficulty in culturing certain bacteria and fungi.

Enumeration via the Most Probable Number (MPN) technique

The most probable number (MPN) technique provides an estimation of viable organism concentrations in a liquid sample (Cochran 1950). It was first described in 1915 by McCrady, and few modifications have been made since that time as it relies on the concept of probability and requires certain assumptions. Two assumptions are made when utilizing the MPN method, being 1) organisms are evenly distributed throughout the liquid solution and 2) when ideal growth conditions are implemented and an organism is present, growth will occur. If the sample is not well mixed or inhibitory factors are present, the MPN method can provide an underestimation of microbial concentrations (Cochran 1950). MPN technique includes acquisition of a liquid suspension containing a sample before serial diluting the liquid in sterile buffer and plating replicative dilutions on various agar types. The replicates are identified as positive or negative for growth, and mathematical analyses are calculated to estimate the probability of the organism existing in the original liquid sample (Cochran 1950). This method provides the benefit of calculating a selective bacterial and fungal titer which can be log transformed to produce a colony forming unit (CFU) equivalent. A study comparing MPN estimations to traditional plate counts of aerobic bacteria, coliforms, and *E. coli* reported >1 log difference between methods in 92.1% of all 277 samples processed (Line, Stern et al. 2011). Concerning fungal isolation, Koburger and Norden (1975) observed greater detection of fungi by MPN compared to traditional pour plate and plate inoculation methods.

MPN estimation is advantageous in poultry facilities where bacterial or fungal concentrations exceed those enumerable by RODAC plates or there is suspicion of swarming bacteria resulting in enumeration complications. It can be useful when detecting organisms whose concentrations are low or presence is masked by high contamination of competing

microbes (Capita and Alonso-Calleja 2003). Abundant research is available involving determination of MPN in poultry feed, litter, and gastrointestinal tracts, but little is reported on environmental MPN calculation in commercial hatcheries. Wallace *et al.* successfully determined MPN to monitor the seasonality of *Campylobacter* and its concentration flux throughout the chicken gastrointestinal tract (Wallace et al. 1997). MPN has been utilized to estimate *C. perfringens* cells and spores within the intestinal tract of two-day-old broiler chicks, but this study was not conducted in the hatchery environment (Stutz and Lawton 1984).

One major disadvantage of the MPN method is need for sterile facilities, laboratory-experienced workers, and the time needed to obtain results (Capita and Alonso-Calleja 2003). The tediousness of this technique is obvious in its need for multiple sets of vessels and multiple inoculations of several different dilutions. However, automated MPN instruments have been developed to reduce labor and temporal requirements of MPN estimates, potentially increasing application of MPN estimations in various poultry facilities, including hatcheries (Line et al. 2011).

ATP bioluminescence

Measuring ATP bioluminescence to estimate biomass concentrations is a technique developed by NASA that has since rapidly developed into a widely used assay for microbial quantification (Lindgren and Shaheen 1970, Karl and Larock 1975, Wang et al. 1979). It is often used in medical or food production environments to assess potential contamination points as well as cleaning and disinfection regiments (Bautista et al. 1992, Corbitt et al. 2000, Bellamy 2012). The luciferase enzyme and luciferin cofactor work to hydrolyze ATP and thus release a single photon; this light signal can be detected by a luminometer and reported in relative light units (RLUs), a unit that is directly proportional to the number of ATP molecules present (Larson et al.

2003). A strong positive correlation between RLU and traditional plate counts of *S. aureus* was reported by Omidbakhsh, Ahmadpour et al. (2014) across four ATP bioluminescence assays with a lower limit of detection of 2.40 x 10^2 CFU. The evidence of a strong association between microbial counts and RLU is supported by studies conducted in various environments and on different surface material (Ukuku et al. 2005, Leon and Albrecht 2007, Turner et al. 2010). In the poultry industry, ATP bioluminescence swabs are utilized mainly in carcass processing environments to determine microbial load on carcass and machinery surfaces and within water sources (Ellerbroek and Lox 2004, Bautista et al. 1995, Siragusa et al. 1996, Bautista, Vaillancourt et al. 1994). Bautista et al. (1995) reported good repeatability and a relatively strong positive correlation coefficient (*r*) of 0.85 between microbial counts and RLUs detected on poultry carcasses. Other researchers have reported similar findings, including Siragusa et al. (1996) who determined a correlation coefficient of 0.82 (Bautista et al. 1994, Cutter et al. 1996).

As with other microbial detection methods, some sanitizers and disinfectants can interfere with ATP bioluminescence assays, making it difficult to assess cleanliness of a surface that may contain chemical residue (Green et al. 1999, Omidbakhsh et al. 2014). It has been reported that ATP bioluminescence assays are not suitable for bacterial quantification because of the inaccuracy and inconsistency of RLU values compared to bacterial plate counts (Larson et al. 2003, Aiken et al. 2011, Vogel et al. 2014). Some ATP bioluminescence systems do not sufficiently lyse Gram positive bacterial cells, thus providing false negative values (Turner et al. 2010). Despite the disadvantages of ATP bioluminescence assays, they are generally very rapid and inexpensive tests that can provide an accurate presence/absence determination (Willis et al. 2007, Shama and Malik 2013). Poultry researchers have supported use of ATP bioluminescence

swabs for rapid determination of critical control points and overall microbial load on carcasses (Cutter et al. 1996, Bautista et al. 1997).

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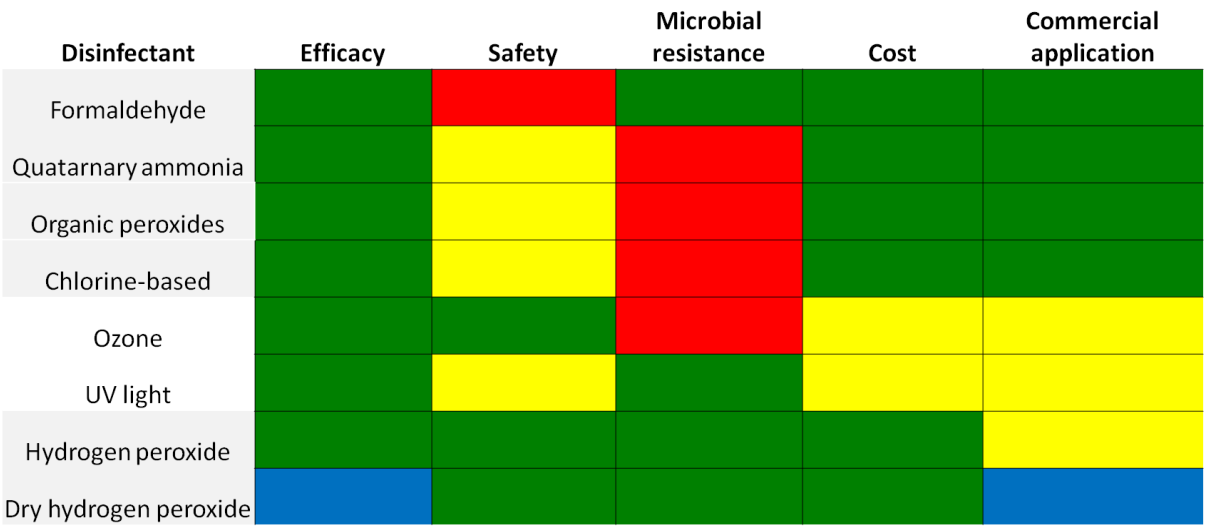

Figure 2.1: A colorimetric representation traditional and alternative disinfectants commonly used in the poultry industry (vertical) in regards to important characteristics (horizontal) for a "good" disinfectant. Relative fulfillment of each characteristic is displayed as positive (green), negative (red), neutral (yellow), and unknown (blue).

Detection method	Accurate	Rapid	Cost-effective	Commercially practical
Agar air plate				
RODAC plate				
Direct swab				
Fluff evaluation				
MPN enumeration				
ATP bioluminescence				

Figure 2.2: A colorimetric representation of important attributes (horizontal) for a successful microbial monitoring system, and the relative fulfillment of each traditional and alternative microbial monitoring technique (vertical). Positive (green), negative (red), neutral (yellow), and unknown (blue).

CHAPTER 3

REDUCING MICROBIAL LOAD ON HATCHING EGGS USING A DRY HYDROGEN PEROXIDE SYSTEM

Summary

An extensive microbial population exists on the surface of the eggshell of hatching eggs. Microbes present can potentially infect broiler chicks hatched from contaminated eggs, creating seeder birds that can infect entire flocks at the broiler farm (Bailey et al. 1994). Elimination of antibiotic use in the commercial hatchery environments generated a need for alternative disinfectants to ensure chick health and decrease instances of embryonic infection. Eggs are not traditionally washed in order to preserve the protective cuticle layer, but fumigation and vapor exposure has been previously utilized. The objective of this study was to determine if a novel dry hydrogen peroxide (DHP) gas could reduce the microbial load on hatching eggs, since it has previously been shown to reduce microbial loads on hard surfaces in hatcheries. The two trials described investigate effects of the DHP system on the presumptive total aerobic and gram-staining negative bacterial loads on the surfaces of hatching eggs. The first trial compares treatments in a controlled laboratory environment while the second trial examines effects of treatment in a research egg storage cooler environment. The overall microbial load of treated eggs in the laboratory decreased significantly over time. Treated eggs in the egg storage cooler trial experienced an increase in overall microbial load due to technical issues with the cooler facility and environmental cleanliness. However, the data indicates that the DHP system can prevent expansion or reduce microbial load on hatching eggs after prolonged exposure.

Introduction

The design and consistent implementation of an effective cleaning and disinfection protocol is fundamental for success of an operational commercial poultry hatchery to ensure bird health and minimize losses. Accumulation of bacteria due to poor disinfection can result in outbreaks of pathogenic organisms such as *Salmonella* spp., *Enterobacteriaceae* spp., and fungal species that can potentially result in economic losses (Kim and Kim 2010). Sources of bacterial and fungal contamination include but are not limited to accumulation of dust in hard to reach areas, transfer of diseases by personnel, and soiled eggshells of incoming eggs from breeder flocks (Samberg and Meroz 1995). Although some routes of contamination are preventable by increasing biosecurity and traditional cleaning methods, others are more easily controlled by application of proper disinfectants (Samberg and Meroz 1995). Various methods have been previously utilized to decrease microbial load on the eggshell, including formaldehyde fumigation, ozone, and hydrogen peroxide.

Despite its significant efficacy, formaldehyde gas produces detrimental health effects in both chicks and humans, emphasizing the need for safer hatchery disinfectants without compromising chick health and hatchery production (Scott et al. 1993). Ozone is a polymerized oxygen, known to have bactericidal properties with successful implementation for reducing microbial load in a hatchery environment (Whistler and Sheldon 1989). However, overexposure can cause increases in embryo mortality up to 37.5% compared to no disinfection, resulting in significant economic losses for the hatchery (Whistler and Sheldon 1989).

Hydrogen peroxide (H_2O_2) is a colorless liquid that functions as a disinfectant by reacting with superoxide ions to produce a reactive hydroxyl radical which then attacks cell DNA, lipid membranes, and proteins (Linley et al. 2012). Varying concentrations of hydrogen peroxide have

been used in the commercial industries for disinfection of surfaces and equipment with 5% v/v needed as the minimum concentration for effectiveness (Andersen et al. 2006). H_2O_2 can be applied in a liquid form as a surface decontaminate or microaerosolized to form vapor hydrogen peroxide (VHP). Sheldon and Brake (1991) applied aqueous 5% hydrogen peroxide solution to eggshell surfaces of hatching eggs and observed a 5.3 log reduction in total microbial load. Furthermore, a significant 2.0% increase was noted in hatchability of fertile eggs as well as a significant 2.2% decrease in early-dead after a 5% v/v H_2O_2 was sprayed.VHP has been utilized in food production settings and is able to reduce microbial concentrations on surfaces by 6 logs after 2 minutes of exposure (Wang and Toledo 1986). Exposure to 10% VHP is understood to be highly effective at against common viral and bacterial poultry pathogens (Neighbor et al. 1994). However, highly concentrated solutions and vapors can negatively affect human respiratory and pulmonary function as well as metal surfaces exposed over time (Neighbor et al. 1994, Center for Disease Control 2018).

Dry hydrogen peroxide (DHP) is a colorless, odorless substance emitted by the Synexis DHP system without the use of aqueous H_2O_2. The machinery consists of a membrane coated in titanium dioxide and a proprietary mix of other stabilizing chemicals, which acts as a catalyst that converts water and oxygen in the environment into the hydrogen peroxide molecule when activated by a non-germicidal black light (Lee 2012). Because of this unique method of generation, DHP behaves as a true gas and disseminates into the air to establish a dilute equilibrium concentration. Additionally, it is able to disinfect hard-to-contact areas that traditionally cannot be cleaned, such as behind or above large pieces of equipment present in a commercial hatchery setting.

Preliminary data has indicated that DHP is able to reduce concentrations of aerobic bacteria and fungi with prolonged exposure on solid surfaces (Lee 2012). A preliminary study by Melo et al. resulted in no negative effects on chick hatch, potentiating further research into the effectiveness of the DHP molecule on the microbial load of the porous surface of the eggshell. The primary target location for implementation of the system is the hatchery egg storage room. The 24-72 hour wait period in the cooler before transfer allows prolonged exposure to the DHP, which is fundamental for optimal effectiveness. Moreover, microbes tend to migrate inside of the egg in lower temperatures, thus targeting the microbes pre-existing in the environment could potentially reduce microbial infection in embryos (Cox et al. 2000, Fasenko 2007).

The objectives of the present trials were to evaluate the effects of a DHP system on the microbial load of porous eggshell surfaces in both a controlled laboratory setting and a research cooler setting.

Materials and Methods

Laboratory trial

All hatching eggs used for trials were acquired from an actively laying broiler breeder flock at the University of Georgia.

Three-hundred eggs were randomly assigned to either a treated or untreated group and sampled at 0, 24, 72, and 120 hours after assignment. The treated environment consisted of a sealed office at the Poultry Diagnostic and Research Center in Athens, Georgia; the area was treated with the Synexis DHP system for 7 days prior to egg placement. Untreated eggs were placed in a sealed biosecurity cabinet with the vent fan off. Sampling was performed using a most probable number (MPN) technique because of its wide usage in food microbiology communities.

Upon sampling, ten eggs were randomly selected from each group for washing; one egg was added per sterile Nasco 18 ounce Whirl-Pak® which contained 10mL of BD Difco™ tryptic soy broth (TSB) soybean-casein digest medium. Each egg was manually washed in separate Whirl-Paks for 60 seconds, removed from bags using sterile technique, and discarded. After all eggs were washed, 1mL of the sample broth was removed and added to the first column of a Fisherbrand™ 96-well DeepWell™ polypropylene microplate with 2mL wells; this was done in triplicate within the first column with two different egg samples added per plate (Figure 3.1). Ten-fold dilutions were made to the last column within the 96-well plate using 0.9mL aliquots of TSB. Six individual wells on the bottom row of one plate per sampling group were filled with un-inoculated TSB to act as negative controls. All plates were then covered with sterile foil and incubated at 37°C for 24 hours. Starting with the most dilute, 5µL of all sample dilutions was transferred onto lidded 96-well plates containing either MacConkey selective agar (MAC) or tryptic soy agar (TSA). MacConkey agar selects for negative gram staining bacteria while TSA generally selects for total aerobic bacteria. Agar plates were then covered with a sterile lid and incubated at 37°C for 24 hours.

Plates were analyzed by counting the number of samples with discernible growth out of the three replicates for each sample dilution. The sequence of numbers identified for each sample was used to calculate the MPN with the aid of an MPN calculator developed by Jarvis et al. in 2010. The MPN was log transformed to obtain a colony-forming unit (CFU) per mL equivalent. The MPN estimation method allows better sensitivity and recovery of lower concentration populations compared to traditional plate count methods (Oblinger and Koburger 1975). This method also tests a greater number of dilutions using fewer materials than traditional plating methods.

Research cooler trial

An egg storage cooler at the University of Georgia Poultry Science Research Farm was used for both the untreated and treated environments for this trial. One Synexis DHP unit was placed in the cooler with two units placed in the room outside of the cooler. Machines remained off for the untreated group and were turned on to pre-treat the environment for seven days before eggs were placed for the treated group. Beginning with the untreated group, 300 hatching eggs were placed in the cooler to be sampled at 0, 24, 72, and 120 hours after placement. Eggs were sampled using the same method as the laboratory trial. For sample analysis, the MPN was calculated using the same method as in Trial 1 before values were log transformed to obtain a CFU/mL equivalent.

Results

Laboratory trial

No significant reduction in presumptive microbial load was observed in the untreated group on either TSA or MAC media. A significant reduction was seen in bacterial load cultured on TSA media from treated eggshell surfaces at 72 hours and 120 hours post-treatment compared to time zero sampling ($P<0.05$) (Figure 3.2a). The concentration of microbes isolated from MAC media in both groups started low and remained low throughout the trial. Microbial load isolated from treated eggs on MAC media showed no statistically significant reduction, but a numerical reduction was observed compared to the baseline after treatment (Figure 3.2b).

Research cooler trial

The untreated group experienced a significant increase in total aerobic microbial load at 72 hours ($P<0.01$) and 120 hours ($P<0.0001$) compared to baseline levels. The treated group also displayed a statistically significant increase in bacterial load cultured on TSA at 120 hours

(*P<0.0001*), as seen in Figure 3.3a. Bacterial concentrations cultured on MAC media from untreated eggs increased at 24 hours post-treatment, but decreased at 72 hours post-treatment compared to 0 hours (*P<0.05*). On treated eggshell surfaces, microbial load isolated on MAC agar was reduced significantly at 72 hours (*P<0.001*) and 120 hours (*P<0.05*) compared to the 0 hour baseline; however a numerical increase in microbial load was observed from 72 hours to 120 hours post-treatment (Figure 3.3b).

Discussion

Laboratory trial

Statistically significant decreases in concentrations of aerobic bacteria indicate that the DHP system is able to reduce growth and possibly destroy microbes on the porous surface of hatching eggs when implemented in a controlled setting. Consistent detection of the DHP molecule was problematic due to the unique properties of the molecule itself. However, when utilizing a traditional hydrogen peroxide detection device, levels of H2O2 in the untreated biosecurity hood remained zero. Levels of presumptive hydrogen peroxide in the DHP-treated space varied greatly but remained greater than zero. This difference between the treated and untreated areas is promising, but more accurate detection methods should be developed.

Research cooler trial

When attempting to detect DHP using a device to measure traditional hydrogen peroxide (Dreager draw tube), levels of zero were obtained during both the treated and untreated portions of this trial. Fluctuations in isolated microbial load in both the treated and untreated groups were further investigated by inspection of the cooler environment after high levels of refrigerant inside of the cooler indicated a refrigerant leak. A thorough disassembly of the cooler showed that the conditioner coils were highly contaminated (visibly dirty). This led us to hypothesize that the

DHP molecules were targeting the incoming, airborne bacterial and fungal cells, reducing the ability to disinfect the eggshells. This would be supported by the inability to detect any significant concentrations of DHP in the cooler during the trial. It is also hypothesized that the refrigerant negatively impacted DHP effectiveness as well as detection, as peroxide molecules will also target volatile organic compounds (VOCs), like refrigerant coolant, for destruction. Dirty cooling fans and coils were identified and replaced, and the refrigerant leak was repaired. After maintenance, refrigerant levels in the cooler were zero and DHP was detected using the hydrogen peroxide-detecting device.

In summation, a DHP system can significantly reduce the microbial load on the surface of hatching eggs in 72 hours. This indicates that there is potential for system application in a poultry environment, possibly in the hatchery egg cooler where eggs are stored for up to 3 days. Further studies are needed to investigate the effects of a DHP system on hatch and chick health. Additionally, an accurate and consistent means of H_2O_2 detection are needed to truly attribute microbial reduction to the DHP system.

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Figures

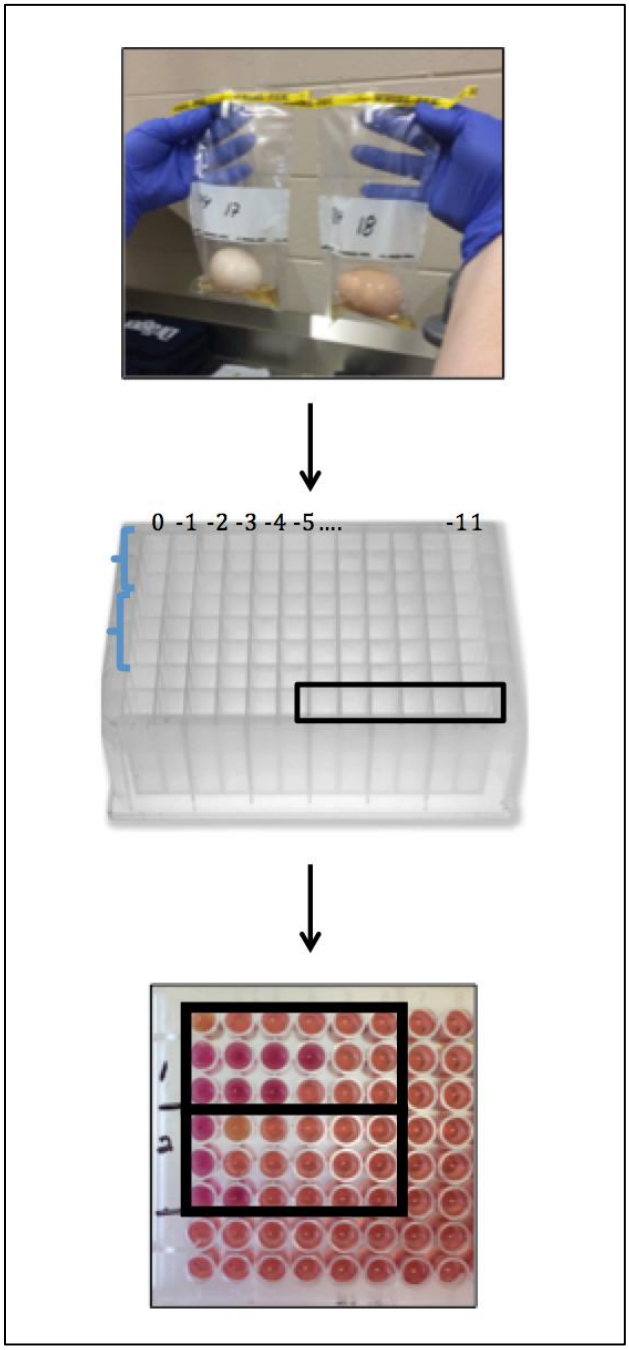

Figure 3.1: Methodology for calculation of the most probable number (MPN) of presumptive bacterial colonies on the eggshell surface. Beginning with manual washing of the egg, followed by serial dilution in TSB, then inoculation of agar media and reading of agar plates.

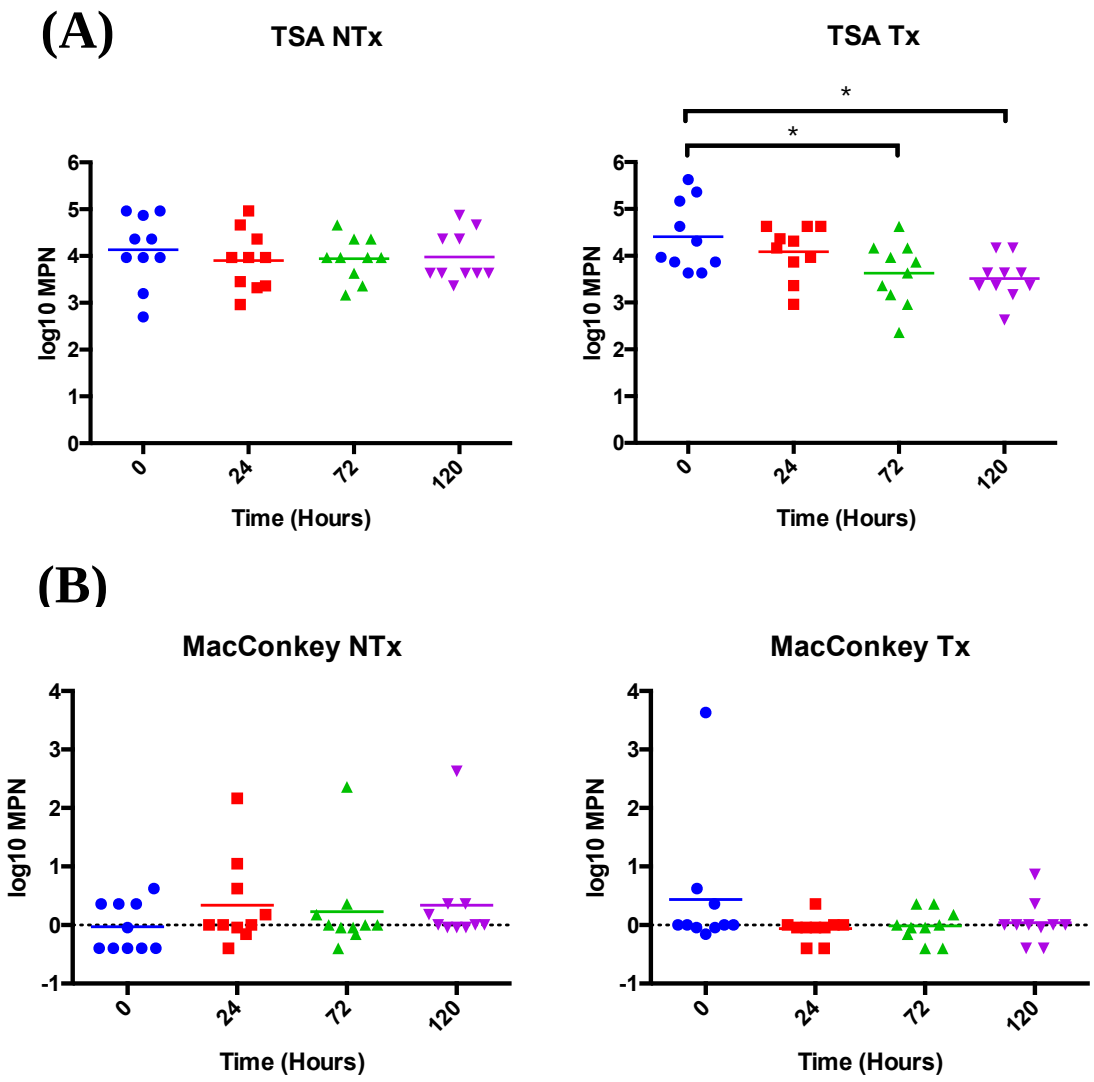

Figure 3.2: Comparison of $\log_{10}$ MPN values of bacterial loads on treated and untreated eggshells in a laboratory setting. Presumptive bacterial loads isolated on TSA (A) and MacConkey agar (B) from untreated (Ntx) and treated (Tx) eggs. Negative values indicate an MPN of less than one. Solid horizontal lines indicate statistical means for groups over time. Lines connecting time points indicate a statistical difference at *$P<0.05$*

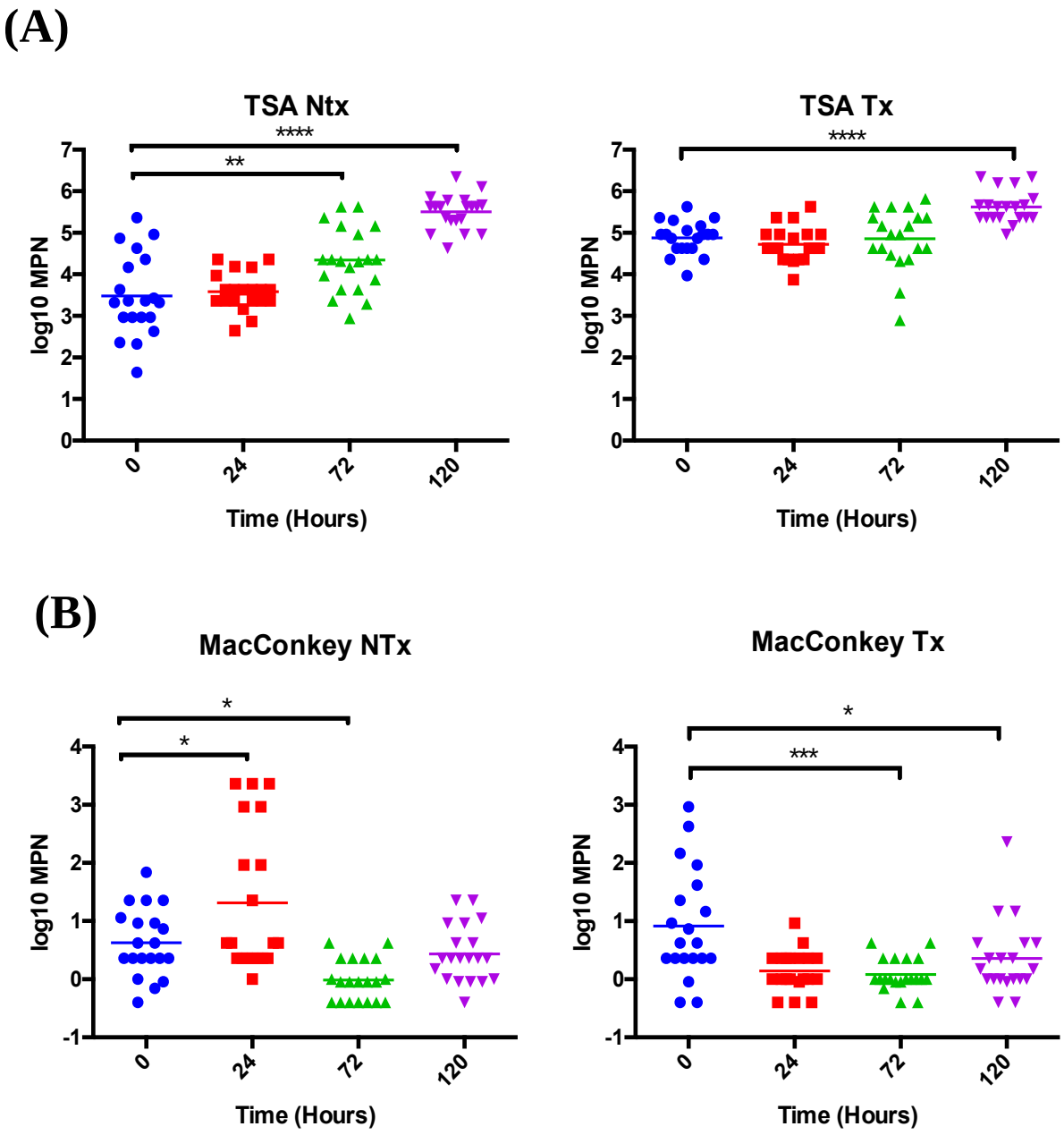

Figure 3.3: Comparison of $\log_{10}$ MPN values of bacterial loads on treated and untreated eggshells in a research cooler facility. Presumptive bacterial loads isolated on TSA (A) and MacConkey agar (B) from untreated (Ntx) and treated (Tx) eggs. Negative values indicate an

MPN of less than one. Solid horizontal lines indicate statistical means for groups over time. Lines connecting time points indicate a statistical difference at *$P<0.05$

CHAPTER 4

VALIDATION OF ATP BIOLUMIESCENCE SWABS TO DETERMINE MICROBIAL LOAD

Summary

Swabs utilizing ATP bioluminescence technology for measuring microbial load offer a fast and inexpensive alternative to traditional plating methods, reporting values as relative light units (RLU). These assays function through a chemical reaction involving the ATP present in living cells to generate luminescence detectable by a luminometer. However, swabs measuring different targets (total bacteria, specific class of bacteria, etc.) can produce inconsistencies in a field setting when compared to traditional agar plate inoculation. For this trial, three types of ATP bioluminescence swabs were obtained from a commercial company: Hygiena™ UltraSnap™, Hygiena™ MicroSnap™ Total Viable Count (TVC), and Hygiena™ MicroSnap™ Enterobacteriaceae (EB). UltraSnap™ swabs detect total surface ATP regardless of source; MicroSnap™ TVC swabs detect ATP from all bacterial cells and are Association of Agricultural Chemists (AOAC) International performance tested. MicroSnap™ EB swabs select for Enterobacteriaceae cells, and detect ATP from those sources. The objective of this study was to compare traditional and ATP bioluminescent sampling methods to identify the most accurate method for measuring microbial load in a controlled laboratory setting as well as a commercial hatchery setting. The laboratory trials utilized an *Escherichia coli* ATCC-25922 strain obtained from Microbiologics® and prepared in a tryptic soy broth (TSB) stock culture. Ten-fold serial dilutions were made in phosphate buffered saline (PBS) in triplicate and used to inoculate sterile

coupons and tryptic soy agar (TSA) plates. Coupons were swabbed with one of four swab types: Hygiena™ UltraSnap™, Hygiena™ MicroSnap™ TVC, Hygiena™ MicroSnap™ EB, or a cotton swab saturated with PBS. UltraSnap™ swabs were analyzed immediately using a Hygiena™ Ensure™ luminometer while both MicroSnap™ swab types were incubated for 7 hours. Cotton swabs were suspended in PBS and plated onto on TSA petri dishes to measure microbial load recovery using indirect plating methods. The hatchery trial consisted of swabbing adjacent surfaces in various critical locations to compare sensitivity of direct cotton swabs, UltraSnap™, MicroSnap™ TVC, and MicroSnap™ EB in a field setting. By comparing colony forming units (CFU) obtained from traditional plating methods to Hygiena™ swab RLUs and indirect plating CFUs, the most accurate and sensitive bacterial detection method available can be selected for field application in poultry settings.

Introduction

Proper cleaning and disinfection methods are essential in a commercial hatchery setting to protection health of hatching chicks and hatchery personnel, and accurate microbial sampling ensures that these measures are being implemented. Bacterial and fungal contamination in the hatchery can contribute to a variety of avian diseases upon hatch as well as on the broiler farm (Chute and Barden 1964, Cox et al. 1990, Samberg and Meroz 1995, Walker et al. 2002). Chick contamination of *Pseudomonas*, *Salmonella*, and fungal species is known to create seeder birds which can cause disease in entire flocks at the grow-out stage (Samberg and Meroz 1995).

Traditional microbial detection techniques, including most probable number (MPN) calculations, direct swabbing, air exposure plates, and fluff testing, can provide comprehensive results on the microbial population in the environment. Most of these methods, however, require at least 24 hours to provide results and can become costly when many locations are sampled in

duplicate or triplicate. MPN calculations provide useful data by reporting titers of bacterial contamination on hard surfaces or tissues, but the process requires a minimum of 48 hours and the amount of materials necessary can become costly (Wallace et al. 1997). Direct swabbing detects bacteria and fungi at low concentrations on surfaces, yet this method requires 24 hours and costs of materials can be a hindrance (Magwood and Marr 1964). Air exposure plates are convenient and consistent but can provide false positives, indicating lower levels of contamination than is actually present (Gehan 2009). Fluff testing for detection specific microbes is accurate and consistent, but because culturing can result in false positives, polymerase chain reaction (PCR) analysis is recommended; this process can be an obstacle for hatcheries based on expense and required training (Hiett et al. 2002).

Assays utilizing adenosine triphosphate (ATP) bioluminescence rely on a chemical reaction between luciferase and luciferin found in the swab sheath and ATP found in all cell types to produce light detectable by a luminometer. Rapid ATP bioluminescence assays have been implemented as a tool for microbial surveillance in poultry production plants, mainly on machinery and carcasses, but it is not greatly utilized in poultry hatchery environments (Bautista et al. 1994, Bautistat et al. 1995, Siragusa et al. 1996).

Hygiena™ produces ATP bioluminescence swabs and a luminometer that can provide results within seconds in the form of relative light units (RLU), directly proportional to the amount of ATP in a sample. Hygiena™ UltraSnap™ swabs detect total ATP within a surface area of 100cm^2. Protocols for more specific swab types, including MicroSnap™ Total Viable Count (TVC) and MicroSnap™ Enterobacteriaceae (EB), are equipped with bacterial colony forming unit (CFU) conversion charts and provide results in 6-8 hours. Additionally, both UltraSnap™ and MicroSnap™ TVC swabs are AOAC Performance Tested.

The purpose of these studies was to compare recovery and sensitivity of three types of Hygiena™ ATP bioluminescence swabs to traditional direct swabbing and plating techniques using known concentrations of bacteria within a laboratory setting. Additionally, we aimed to understand the possible application of these swab types within a commercial poultry hatchery by comparing detection and sensitivity to traditional direct swabbing.

Material and Methods

Laboratory trial 1

Both laboratory trials were conducted under a disinfected fume hood using sterile technique. For both trials a pure culture of *Escherichia coli* (ATCC®-25922™) was acquired from American Type Culture Collection® and cultured according to protocol on 100x15mm petri plates with BD Bacto™ tryptic soy agar (TSA). A single colony was picked using a sterile inoculation loop and grown in 10mL BD Bacto™ tryptic soy broth (TSB). To maintain working culture, 1mL of growing broth was passed every 24 hours into 9mL of fresh, sterile TSB. Both trials utilized 100cm^2 coupons cut from sheets of stainless steel and sterilized using an autoclave. Coupons were opened under the hood and handled only using sterile forceps.

Direct swabbing method Seven 10-fold serial dilutions were made from the working *E. coli* culture in sterile phosphate saline buffer. 0.1mL of the working culture and each dilution was used to inoculate 100x15mm petri plates with TSA in triplicate and spread using L-shaped spreaders to calculate the true concentration. We then inoculated 100cm^2 stainless steel coupons in triplicate with 0.1mL of the working culture and each dilution and used an L-shaped spreader to evenly distribute the inoculum. All inoculated coupons were allowed to dry fully for approximately 15 minutes. Three coupons were set aside and not inoculated to act as negative controls. Each coupon surface was thoroughly swabbed with a Puritan® sterile cotton-tipped

applicator with a wood handle that was saturated in sterile PBS. The swab was then streaked on a TSA plate while evenly rotating the wood handle (Figure 4.1a). All TSA plates were incubated at 37°C for 24 hours before colonies were counted.

UltraSnap™ swabbing method On day 2, seven10-fold serial dilutions were again made from the working culture of that day. We again inoculated TSA plates and stainless steel coupons in triplicate with 0.1mL of each dilution and spread the solutions using L-shaped spreaders (Figure 4.1b). Three coupons were set aside and not inoculated to act as negative controls. Coupon inoculum was allowed to dry for approximately 15 minutes before thoroughly swabbing with an UltraSnap™ swab according to protocol. UltraSnap™ swabs were immediately read using a calibrated Hygiena™ EnSURE™ luminometer to obtain an RLU value. TSA plates were incubated at 37°C for 24 hours before colonies were counted.

MicroSnap™ swabbing method The third sampling day, we diluted the working culture 10-fold seven times in PBS and inoculated TSA plates in triplicate with 0.1mL of each dilution and spread using an L-shaped spreader. Plates were incubated at 37°C for 24 hours before colonies were counted. Because both MicroSnap™ swabs types were tested with dilutions from this daily working culture, six stainless steel coupons were inoculated with 0.1 mL per dilution to allow sampling in triplicate for two swab types (Figure 4.1c). Six coupons were set aside and not inoculated to act as negative controls. Inoculum was spread using an L-spreader and left for approximately 15 minutes to fully dry. Each coupon surface was then fully swabbed according to protocol with either a MicroSnap™ TVC or MicroSnap™ EB detection swab. MicroSnap™ TVC swabs were incubated for 7 hours at 30°C and MicroSnap EB™ swabs were incubated for 7 hours at 37°C before all swabs were transferred to enrichment tubes - as per Hygiena™

protocol - and read using a calibrated Hygiena™ EnSURE™ luminometer to obtain an RLU value.

Laboratory trial 2

Working culture was diluted 10-fold seven times and used to inoculate TSA plates in triplicate. Plates were incubated at 37°C for 24 hours before colonies were counted. We then inoculated four stainless steel coupons with 0.1mL of each *E. coli* dilution and the working culture, one for each swab type: direct, UltraSnap™, MicroSnap TVC™, and MicroSnap EB™. The inoculum was spread using an L-shaped spreader and left to fully dry for approximately 15 minutes. Additionally, four coupons were left non-inoculated and swabbed with each swab type to act as negative controls. Coupons surfaces were swabbed according to protocol described in trial 1.

This process was repeated for two additional consecutive days with the daily working culture to generate three data points for each swab type (Figure 4.2).

Hatchery trial

The commercial hatchery that was sampled is divided into two equal wings each with an egg cooler, hatcher hall, and setter hall; in the center of the facility are shared areas including a chick processing room and a chick separator room. The hatchery was sampled on one day when hatch was not occurring, approximately 24 hours after routine cleaning and disinfection procedures had been performed according to hatchery standards. All four swabs types sampled a 100cm^2 area using a plastic template that was disinfected with 70% diluted ethanol between sampling locations. Nine samples of each swab type were taken in the chick processing area: four on plastic machinery belts, four on the concrete floor below belts, and one in the metal drain. Four samples of each swab type were taken in the chick separator area: one on a plastic

machinery belt, one on a steel machinery box, and two on the concrete floor near machinery. 12 samples of each swab type were taken in one of the egg coolers on the surface of hatching eggs from the same flock and lay date that had been in the cooler for approximately 48 hours.

Areas were sampled thoroughly as described in the laboratory trials and provided by Hygiena™ protocols. Direct swabs were streaked onto TSA plates immediately after sampling. Plates were transported back to the lab and incubated at 37°C for 24 hours before colonies were counted. UltraSnap™ swabs were read immediately using the EnSURE™ luminometer to obtain an RLU value. The liquid solution in the MicroSnap™ swabs was not released until they were transported back to the laboratory where they were then incubated for 7 hours as described in the laboratory trials. After the incubation period, MicroSnap™ swabs were read using the EnSURE™ luminometer to obtain an RLU value.

Results

Laboratory trials

Pictured in Figure 4.3, results of direct swabs showed approximately a 2.0-3.0 log loss in recovery when compared to CFU of the *E. coli* inoculum. Swabs resulted in a positive linear pattern with an upper limit of detection of approximately $10^{5.5}$ and a lower limit of $10^{2.75}$. Working culture plating resulted in a count that was Too Numerous To Count (TNTC). UltraSnap™ swab results also indicated an approximate 2.0-3.0 log loss in recovery based on CFU of *E. coli* inoculum. Again a positive linear pattern was observed with an upper limit of detection of approximately 10^7, at which point the reported RLU was at the maximum level, and a lower limit of detection of approximately 10^3. MicroSnap™ TVC swabs indicated a 4.0-5.0 log loss in recovery with a positive linear pattern but a narrow limit of detection; the upper limit was 10^7 with the lower limit of 10^5. MicroSnap™ EB swabs had similar results with a linear pattern

and a narrow limit of detection of 10^7 to 10^5 CFU. Results from the second laboratory trial indicated an improved recovery in both MicroSnap ™ swab types, although variability remained higher than UltraSnap™ and cotton swabs (Figure 4.4). Cotton swabs with colony growth TNTC are not reported in the figure. MicroSnap™, UltraSnap™, and cotton swabs resulting in an RLU or CFU of zero are also not reported because these data points were not representative of the rest of the data set. In calculating the replicate average, it was found that these values provided data unrepresentative of the data set. However, it was understood that field samples would provide us with data more representative of a hatchery environment.

Hatchery trial

Results from machinery in the chick separator room indicated similar variation and CFU and RLU values of direct and UltraSnap™ swabs, with a difference of less than 0.5 $\log_{10}$. MicroSnap™ TVC swabs resulted in much lower recovery compared to direct swabs (0.2 $\log_{10}$), while MicroSnap™ EB swabs resulted in RLU values of zero. Floor samples from the chick separator area resulted in similar levels of detection for all four swab types (2.0 $\log_{10}$ – 2.5 $\log_{10}$ CFU or RLU), but greater variation in both MicroSnap™ swab types (Figure 4.5).

Chick processing room machinery resulted in similar CFU and RLU values for direct and UltraSnap™ swabs, with respective replicate averages of 1.7 $\log_{10}$ and 1.5 $\log_{10}$. MicroSnap™ TVC swabs in this area resulted in lower RLU values than direct swab or UltraSnap detection ™, with an average of 0.3 $\log_{10}$ RLU. MicroSnap™ EB swabs resulted in RLU values of zero. Floor samples from the chick processing area resulted in similar CFU and RLU values and variation for direct (1.75 $\log_{10}$) and UltraSnap™ swabs (1.7 $\log_{10}$). Both MicroSnap™ swab types in this location resulted in lower recovery and greater variation than the other two swab types. The single drain sample in the chick processing area indicated greater recovery in the UltraSnap™

swab (3.0 $\log_{10}$ RLU) and MicroSnap™ EB (2.5 $\log_{10}$ RLU) with much lower recovery in the MicroSnap™ TVC swabs (1.9 $\log_{10}$ RLU).

Egg sampling resulted in similar variability and recovery for direct and UltraSnap™ swabs, with replicate averages of 3.2 $\log_{10}$ CFU and 3.0 $\log_{10}$ RLU respectively. MicroSnap™ TVC swabs resulted in lower levels of detection (1.9 $\log_{10}$ RLU) than the other two swab types, yet similar variability was noted. Results of MicroSnap™ EB swabs indicated slightly lower detection than direct and UltraSnap™ swabs, with a replicate average of 2.25 $\log_{10}$ RLU; however the variation of MicroSnap™ EB swab samples was much greater than the variation of the other swab types.

Discussion

Discrepancies in CFU calculations and RLU results acquired from AOAC performance-tested MicroSnap™ TVC swabs can be explained by protocol recommendations provided by Hygiena™, but not tested by AOAC. MicroSnap™ TVC protocol recommends one of three sampling methods: direct surface swabbing, addition of liquid directly to the enrichment device, or addition of suspension (e.g. 10% weight/volume food homogenate) directly to the enrichment device (Hygiena™, 2015). However the AOAC validation study tested the food homogenate protocol only, not taking into account the recovery of the cotton tip of the swabs (Meighan et al. 2016).

Differences in RLU values of UltraSnap™ and both MicroSnap™ swab types in the laboratory when a pure culture and sterile surfaces were used can be attributed to design of the MicroSnap™ swabs. The wands of these swabs are slightly longer than the UltraSnap™ swabs, resulting in less control and less pressure applied to the cotton tip by the user. The unstable applicator wand of the MicroSnap™ swab makes sampling with sufficient pressure more

difficult and awkward than sampling with the UltraSnap™ swabs. Overall, reported RLUs of the UltraSnap™ swabs were more repeatable with greater recovery than the MicroSnap™ swab. This comparison supports literature that reports difficulties in differentiating the source of ATP in ATP bioluminescence assays (Willis et al. 2007, Shama and Malik 2013).

In both laboratory and commercial hatchery settings, UltraSnap™ swabs displayed repeatability and recovery similar to direct swabs. UltraSnap™ swabs also produced RLU values comparable to direct swab CFU values on multiple surface types in the hatchery. These results, in combination with the ease of use, affordability, and convenience of Hygiena™ UltraSnap™ swabs, indicate a potential application in a field setting for microbial monitoring.

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Figures

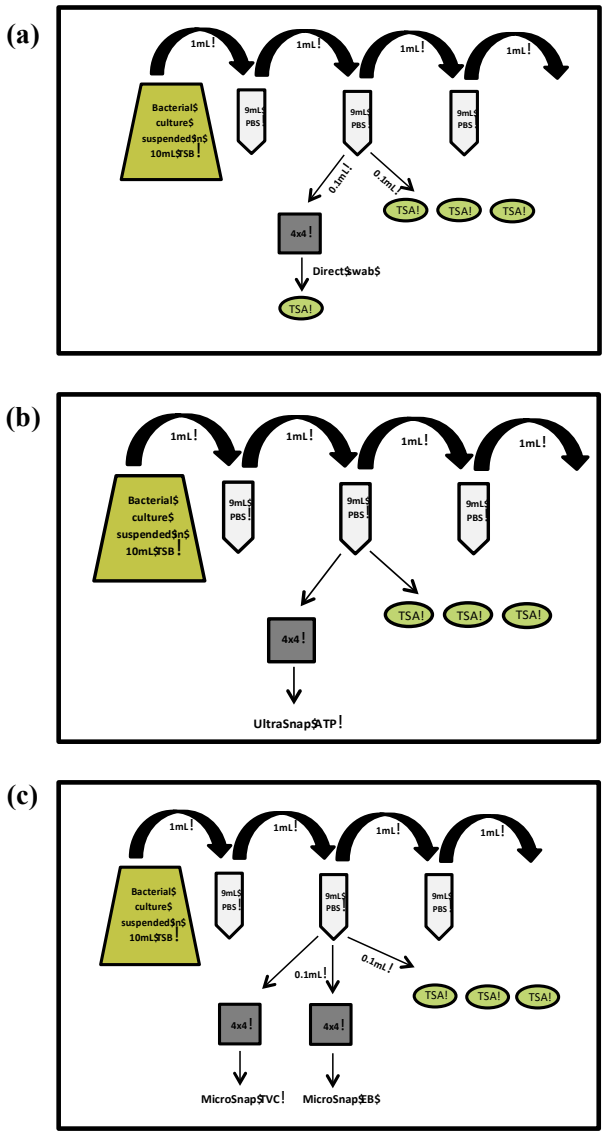

Figure 4.1: Experimental design of laboratory trial 1 comparing detection of microbial load between sampling methods with a known bacterial concentration. Day 1 with direct sampling only in panel (A), Day 2 sampling with UltraSnap™ swabs only in panel (B), and Day 3 sampling with both MicroSnap™ types in panel (C).

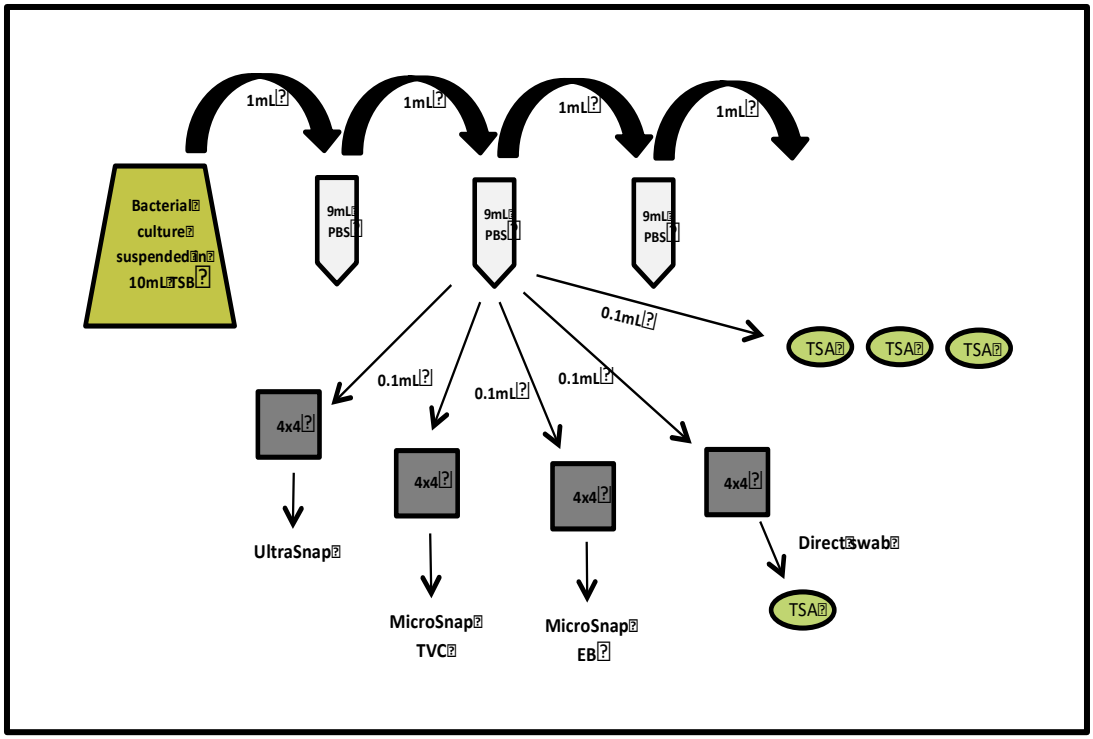

Figure 4.2: Experimental design of laboratory trial 2. All swab types were tested with one bacterial culture. This design was repeated over three days to produce three data points per swab type per culture dilution.

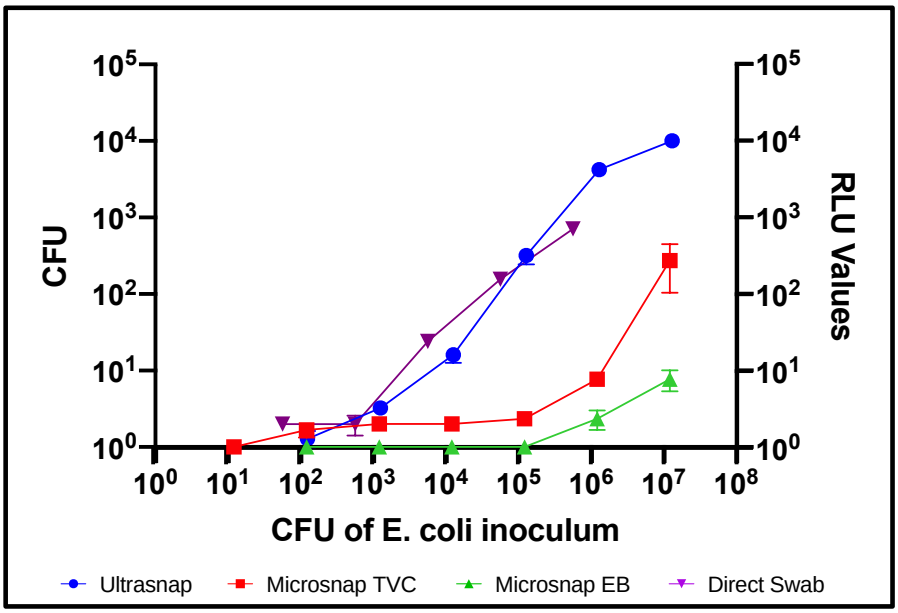

Figure 4.3: Results from the laboratory trial 1 comparing true concentration of bacterial inoculum to recovered CFU and RLU after swabbing.

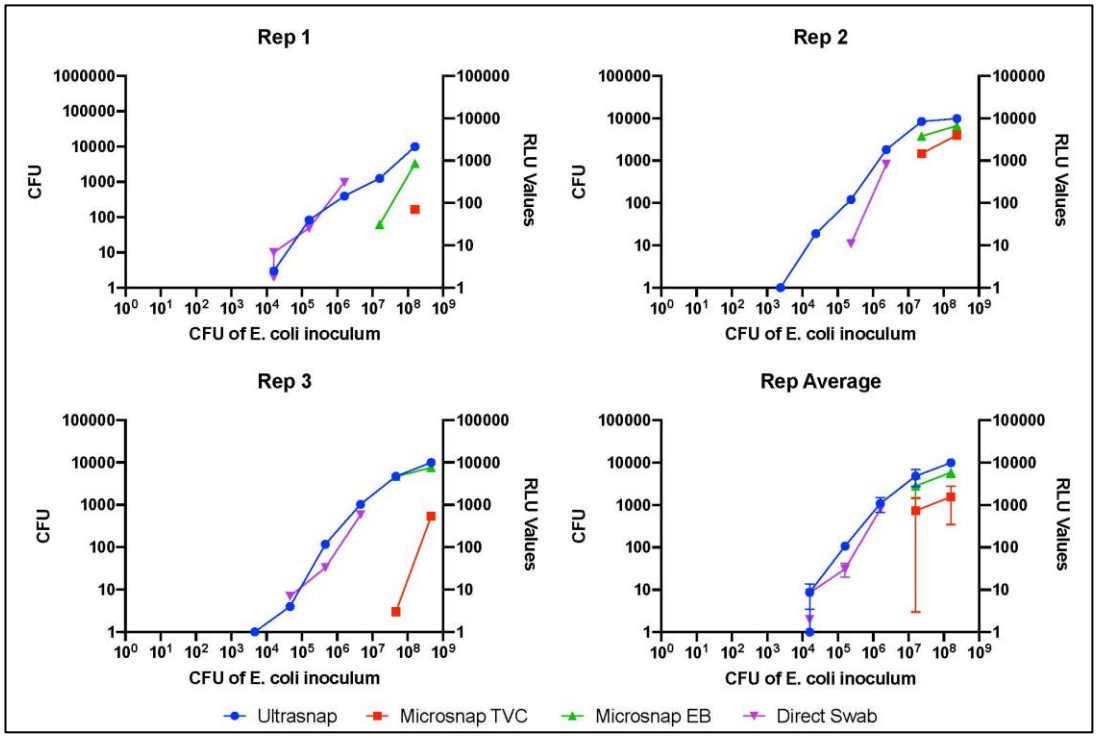

Figure 4.4: Results from the second laboratory trial comparing swab recovery to concentrations of three bacterial cultures over three consecutive days.

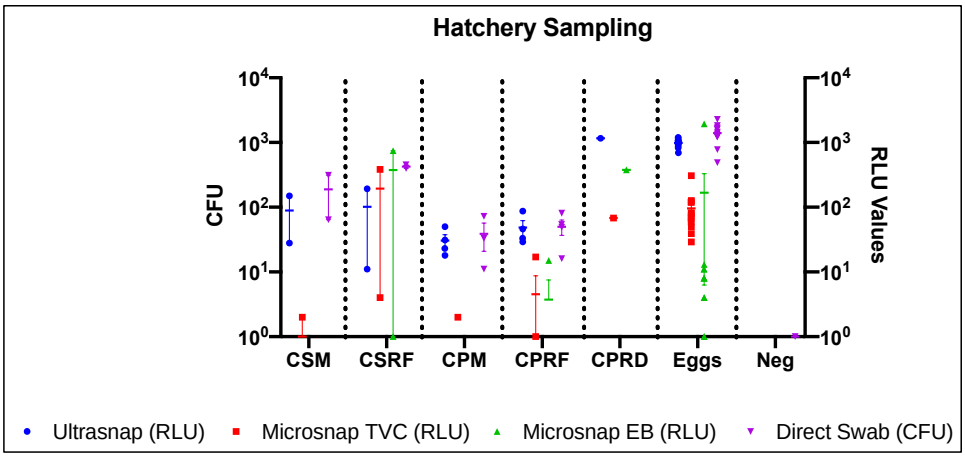

Figure 4.5: Results of hatchery sampling with direct swabs (CFU) and three Hygiena™ swab types (RLU).

CHAPTER 5

SUMMARY AND CONCLUSIONS

Commercial poultry hatcheries in the United States have been identified as significant sources of contamination in the integrated supply chain of poultry processing. Proper cleaning and disinfection methods must be implemented to control bacterial and fungal concentrations to maintain healthy birds and demanding increases in production. Furthermore, accurate and convenient methods of microbial detection must be implemented to obtain authentic results in a timely and cost-effective manner. The objective of these studies was to identify potential alternatives to microbial disinfection and detection within poultry facilities in the US.

The first study evaluated the effectiveness of a dry hydrogen peroxide system on disinfection of the hatching eggshell, a porous surface that traditionally is not washed in order to preserve the protective membrane. DHP acts as a true gas and has potential to decrease microbial loads in poultry facilities when combined with routine cleaning and disinfection procedures. Conducted in a controlled laboratory facility, this experiment included separate treated and non-treated groups that were sampled to determine presumptive total and Gram-negative eggshell bacterial concentrations over time. Enumeration was performed utilizing the MPN methodology, and significant decreases were observed in total cultured bacterial load after 72 and 120 hours of treatment with DHP. Despite promising results, a field trial was necessary to determine application of DHP in a hatchery setting.

The second trial, performed in a research egg cooler at a UGA poultry farm, included sampling techniques invariable to the laboratory trial. However, results were problematic in that

a significantly increased presumptive total and Gram-staining negative bacterial load was observed in both DHP treated and non-treated groups. Further investigation indicated contamination of the cooler fan in addition to a refrigerant leak, allowing us to assume a constant influx of bacteria and decreased effectiveness of the DHP molecules.

The final study of this project was determining and comparing the recovery and accuracy of swabs utilizing ATP bioluminescence to traditional cotton swabbing for microbial monitoring purposes. Hygiena™ UltraSnap™ and MicroSnap™ TVC and EB swabs were tested in addition to traditional cotton swabs in a controlled laboratory setting using known concentrations of *E. coli*. All swab types were also evaluated in a commercial hatchery setting on multiple surface types: porous eggshell, concrete wall, and plastic machinery. In terms of convenience and timeliness, Hygiena™ swabs provided a definite advantage over cotton swabs while providing comparable recovery; however recovery of MicroSnaps™ decreased 2-3 log units, most likely due to the wand design.

When evaluated together, this data shows that a DHP system has potential application in poultry facilities, and could be used as an alternative to antibiotics when proper C&D is implemented. ATP bioluminescence swabs could also be utilized in commercial poultry facilities as a method of microbial monitoring, providing rapid and accurate results with minimal variation from traditional swabbing techniques.