Impact of Sanitizers on Salmonella enterica Contamination and Microbial Populations of Poultry Processing Reuse Water

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Impact of Sanitizers on *Salmonella enterica* Contamination and Microbial Populations of Poultry Processing Reuse Water

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science in Food Science

by

Andrew Micciche
University of South Florida
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Abstract

As human populations increase in numbers, access to clean, fresh water is becoming increasingly difficult to balance between agricultural and municipal demands. Water scarcity is a limiting factor of food production in many countries, whether they are emerging or established economies. In conventional poultry processing systems, access to water is particularly critical for the maintenance and disinfection of processing areas, as well as in processing operations such as scalding, chilling, and carcass washing. The need for sustainable alternatives to single-use water supplies is becoming increasingly more urgent, and as a result, the implementation of water reuse in poultry processing plants has emerged as an attractive alternative means to meet water requirements during processing. To effectively reuse water, it is essential to decontaminate the water with chemical sanitation.

Currently, peracetic acid (PAA) is widely utilized in poultry processing water to disinfect the final product and reduce bacterial loads in the water. However, PAA can be corrosive and potentially dangerous to processing workers. As such, sodium bisulfate (SBS) may be utilized in poultry processing facilities due to its antimicrobial potential. This thesis investigates the impact these acid-based sanitizers have on bacterial loads and potential Salmonella Typhimurium contamination within poultry processing reuse water. A literature review is provided, detailing the reuse of water within conventional poultry processing systems, the efficacy of acid sanitizers, and an evaluation of potential mitigation strategies (Chapter 1). It also details the rationale for decreasing the environmental footprint of poultry processing and how reusing water plays a potential role in this aspect. The first research chapter evaluates the ability of acid sanitizers to inactivation Salmonella Typhimurium in reuse poultry processing water (Chapter 2). The second research chapter evaluates the ability of acid...
sanitizers to impact bacterial loads and the microbiome in reuse poultry processing water (Chapter 3). Data presented herein will provide novel insight into reuse water, remediation of *Salmonella*, and impacts on the microbiome caused by application of acid sanitizers.
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Dedication

Dedicated to Laura Gardner and my family.
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I. Introduction

Bacterial contamination, particularly of pathogens such as *Salmonella*, has been a concern for poultry processing due to potential adulteration of the finished product leading to spoilage or even a risk to public health. To combat product adulteration, various regulatory processes have been implemented such as Hazard Analysis Critical Control Point (HAACP) programs. Within these HAACP programs, water-soluble sanitizers such as chlorine and peracetic acid (PAA) have been utilized. In an effort to meet product safety standards, water usage has increased to over 30 L/bird (Walsh *et al.*, 2018). Environmental and water scarcity concerns indicate that to sustainably utilize such high volumes of water, it may need to be reused.

The reuse of water in poultry processing facilities is regulated by the United States Department of Agriculture (USDA). The water cannot be utilized for downstream processing steps and should be equivalent to potable drinking water (Sanitation Performance Standards Compliance Guide, 2016). To reduce chemical contamination, filtration is utilized. To reduce microbial loads, sanitizers can be employed. Chlorine and peracetic acid (PAA) have been considered the sanitizers of choice for the U.S. poultry industry. Chlorine can form dangerous byproducts, bacterial resistance has also been documented, and free chlorine cannot exceed 5 ppm in reuse water (Fawell, 2000). Peracetic acid, while recently approved for use in washes up to 2000 ppm, can damage equipment and decay into acetic acid rendering it potentially unsuitable for water reuse (Kim *et al.*, 2017). As such, alternative sanitizers must be investigated. This thesis discusses several alternative sanitizers and explores the use of the dry acid sanitizer sodium bisulfate (SBS).

The central hypotheses of this thesis are that 1) Sodium Bisulfate (SBS) lowers
Salmonella concentrations within untreated reuse water compared to PAA, 2) SBS reduces indicator organism concentrations and the total microbial population compared to PAA, and 3) SBS and PAA will statistically significantly alter the microbial populations of reuse water (q < 0.05).

The thesis organization is as follows:

- **Chapter 1**: A literature review outlining the reuse of water in poultry processing facilities, the application of conventional sanitizers (PAA and chlorine), and the use of an alternative acid sanitizer (SBS). This chapter will also briefly discuss microbiome technology.

- **Chapter 2** is a peer-reviewed publication demonstrating the effect PAA and various concentrations of SBS have on Salmonella inoculated commercial poultry processing reuse water.

- **Chapter 3** demonstrates the effect PAA and various concentrations of SBS have on the microbiome of commercial poultry processing reuse water

  Ultimately, this thesis delivers a new understanding of the potential use of acid sanitizers in reuse water for poultry processing. By investigating these acid sanitizers, SBS was determined to be an effective alternative to PAA in reuse water. It is also recognized that SBS modulates the reuse water microbiome in unique ways compared to PAA. This thesis serves as a stepping stone to developing effective sanitation methods within reuse water for poultry processing plants. That knowledge may be used to improve product safety while implementing water reuse for industrial purposes.
II. Chapter 1. The implementation and food safety issues associated with poultry processing reuse water for conventional poultry production systems in the United States

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Abstract

As human populations increase in numbers, access to clean, fresh water is becoming increasingly difficult to balance between agricultural and municipal demands. Water scarcity is a limiting factor of food production in many countries, whether they are emerging or established economies. In conventional poultry processing systems, access to water is particularly critical for the maintenance and disinfection of processing areas, as well as in processing operations such as scalding, chilling, and carcass washing. Therefore, poultry processing plants use an excessive amount of water, limiting where facilities can operate, increasing overhead costs, and ultimately resulting in potential environmental concerns. The need for sustainable alternatives to single-use water supplies is becoming increasingly more urgent. As a result, the implementation of water reuse in poultry-processing plants has emerged as an attractive alternative means to meet water requirements during processing. Because the water is reused, it is essential to decontaminate the water with chemicals, such as peracetic acid and chlorine, and improve water filtration strategies to kill and remove potential pathogens and contaminants. However, questions remain as to the efficacy of commonly used disinfectants to achieve that goal. Thus, novel strategies must be developed to improve the capabilities of poultry processing plants to counter water insecurity worldwide. These new stratagems must be economical and must enable poultry processing plants to reduce their environmental footprint while meeting new food safety challenges. The current review will focus exclusively on water reuse in conventional poultry processing in the United States. The specific objectives of this review are to discuss the approaches for treating processing water in poultry processing systems, including reuse water systems, as well as investigate possible substitutes for maintaining food safety.
Introduction

Water scarcity, while a constant concern for developing countries, is rapidly emerging as a global concern (Beekman, 1998; Casani et al., 2005). Alcamo (1997; 2000) estimates that by 2025, half the world’s population will be living in countries facing considerable water stress or scarcity issues (Rijsberman, 2006). Several factors, including climate change, population growth, dietary shifts towards animal protein, irrigated agriculture, seawater intrusion, and increasing demands for domestic and industrial water, all contribute to this emergent problem (Meneses et al., 2017). Globally, the average water footprint is 7.45 x10^{15} \text{ L per year} (Hoekstra and Chapagain, 2005; 2006), while the estimated minimum basic water need is 50 \text{ L per capita per day} (Gleick, 1996; 1998). However, in 2010, the United States alone used 1.1 trillion liters of potable fresh water per day, or over 3000 \text{ L per capita per day} (Maupin et al., 2014). This substantial demand for fresh water comes primarily from horticulture, livestock, and energy needs, which accounts for 80% of water usage (Shannon et al., 2008). With expected growth in the global human population, and only 0.007\% of the world’s water supply clean and accessible, there is a critical and current need for water conservation practices and technologies, especially in the food industry (United States Census Bureau, 2011; United Nations Department of Economics and Social Affairs, 2017).

From 1998 to 2008, water use in the food industry increased by approximately 40\% and has continued to grow (Klemes et al., 2008; Meneses et al., 2017). These water requirements have become limiting factors for economic growth in China and India (Klemes et al., 2008). As an example of this extensive water consumption, the Australian food processing industry utilizes 30\% of the water used in all industrial facilities in the nation (Department of Agriculture and Water Resources, 2007). In 2005, the Netherlands food industry was the third largest user of
water at 247 billion L in the country behind only the chemical and refinery industry (Casani et al., 2005). In poultry processing, water usage is particularly critical as it facilitates the maintenance and disinfection of processing areas and aids in many basic operations such as scalding, chilling, and carcass washing (Luján-Rhenals et al., 2017). Water also helps with meeting regulations for pathogen reduction, Hazard Analysis and Critical Control Point (HACCP), and from other regulatory bodies such as the United States Department of Agriculture-Food Safety and Inspection Service (USDA-FSIS) (Northcutt and Jones, 2004; Sanitation Performance Standards Compliance Guide, 2016). However, the environmental and financial costs of this water use are notable. For example, in 1977, Wesley estimated that the poultry industry in Virginia would save $150,000 ($600,000+ adjusted for inflation) annually if they cut the water usage in one poultry processing facility in half from 1.9 to 0.95 L per bird (Lillard, 1979). However, current practices and regulations put water use per bird to range from 21 to 30 L, revealing a dramatic increase in consumption for fresh water in processing operations (Walsh et al., 2018; Northcutt and Jones, 2004; Kiepper, 2003). Therefore, to continue to meet these food safety regulations, while reducing costs and environmental pressures, alternative production systems involving water reuse have been implemented (Andelman and Clise, 1977; Meneses et al., 2017). Water reuse in the overall food industry has recently been reviewed by Meneses et al. (2017). The current review will focus exclusively on water reuse in conventional poultry processing in the United States. The specific objectives of this review are to discuss the approaches for treating processing water in poultry processing systems, including reuse water systems, as well as investigate possible substitutes for maintaining food safety.
Water Use in Poultry Processing Systems

In 2017, over 41.6 billion pounds of chicken was processed and produced in the U.S. with projections of 42.5 billion pounds of chicken to be produced in 2018 (USDA, 2018). To generate this quantity and meet demand, streamlined plants can process up to 140 birds/minute per line (9 CFR 381.69) (Owens et al., 2000). Due to the rate of processing, numerous sanitization steps are needed which requires extensive water usage (Menesses et al., 2017). The conventional poultry processing system is outlined in Figure 1.1. To reduce cross-contamination water is used at nearly all points including killing, bleeding, scalding, defeathering, evisceration, washing, and chilling (Keener et al., 2004; Guerin et al., 2010; Park et al., 2015).

As defined by Avula et al., (2009), the average water consumption of a poultry processing plant is 26.5L/2.3kg bird. In comparing processing operational steps, they described the steps that utilized the most amount of water per bird as evisceration (7.57 L/Bird), the wash steps (4.25 L/Bird), the deboning and cut-up steps (3.03 L/Bird), and the chilling step (2.12 L/Bird). Water is also utilized in the defeathering and scalding steps, and variations in water usage are present depending on the plant and the finished products. For instance, plants that process whole chicken carcasses may not utilize substantial quantities of water during the deboning step if one is included at all. Processing waters can be used to lower the temperature of a bird, such as chiller water, as well as a lubricant for the machinery, but they are also utilized to disinfect the bird and remove unwanted components, such as the gastrointestinal tract (GIT) (Avula et al., 2009). These processing waters are often treated as wastewater after use due to their high concentration of proteins, fats, carbohydrates, as well as grit and other inorganic materials (Fonkwe et al., 2001; Avula et al., 2009).

Furthermore, wastewater can not only accumulate microbiological contaminants but be
an environment that promotes the growth of bacteria including pathogens (Meneses et al., 2017). From 2009 to 2015, foodborne disease was caused by Salmonella, Campylobacter, and Shiga toxin producing Escherichia coli (STEC) in 23,662, 2,395, and 2,378 confirmed U.S. cases (Dewey-Mattia et al., 2018). Respectively, of these cases, 3,168, 134, and 672 hospitalizations occurred due to these foodborne diseases (Dewey-Mattia et al., 2018). In 2013, the costs of these foodborne illnesses have been estimated to be $275 million (USDA, 2017). These pathogens can contaminate poultry products and a CDC study has found that from 2009 to 2015, of 1,281 outbreaks traced back to a single source, poultry was responsible for 10% of the outbreaks ranking it the highest single source contributor to these outbreaks (Gremillion et al., 2018). These contaminants can be introduced to the processing waters at many points in processing. During evisceration, this water may come in contact with the ceca, crop, and the remainder of the GIT, which harbor significant levels of bacterial populations. Cecal content, in particular, can contain up to $10^{11}$ cells/g of digesta and this digesta can spill out during evisceration as organs may be ruptured (Gong et al., 2002; Stanley et al., 2014; Hargis et al., 1995). To prevent contamination on the carcass, multiple wash steps are often employed (Keener et al., 2004). Sprays of low levels of chlorine and peracetic acid are utilized at various locations to help ensure a pathogen-free product (Owens et al., 2000). Often washers, such as the inside-outside bird washer, use sanitizers, that include chlorine at 20 to 50 ppm, to reduce contamination from blood, tissue, or fecal matter, with varied success (Keener et al., 2004). For example, one poultry processing plant, despite using 9 L of water per bird for carcass washing, more than double the average, only achieved 0.5 log CFU reductions in Campylobacter (Bashor et al., 2004).

Furthermore, chiller water is also used to sanitize the poultry product. Often the last line of poultry processing before cut-up, packaging, and distribution, chillers are filled with cold
water, ice, and typically a sanitizer such as peracetic acid or chlorine (Keener et al., 2004). While their purpose is to rapidly cool the bird and prevent bacterial growth (USDA 2003a), they also serve as a final step to sanitize the carcass, and up to 50 ppm of chlorine can be added to these waters (Keener et al., 2004). Chiller water often contains 600 to 800 mg/L of total solids, and 30% of those are grease and fat (Avula et al., 2009). Because of the content of these waters and despite the previous wash steps, Northcutt et al., 2008 recovered 2.6, 2.9, and 2.6 log CFU/mL of *E. coli*, coliforms, and *Campylobacter* respectively. Furthermore, 9 of 40 post-chill carcasses were found to be positive for *Salmonella*, and this was not affected by the reuse or reconditioning of the chiller water (Northcutt et al., 2008).

In 1999, a survey estimated a typical poultry plant spends $500,000 to $1 million on water for carcass washers alone despite their low reduction potential on pathogen concentrations such as *Campylobacter* (Jackson et al., 1999; Keener et al., 2004). This survey found water costs per year for a typical plant to be $1.2 million and for water and sewer costs to be an average $0.4 per L and $0.58 per L (Jackson et al., 1999). Almost equally important is the environmental costs that can occur should water be improperly disposed of. In 1995, a large poultry waste lagoon ruptured and spilled 32.6 million L of waste into a nearby creek which diluted to 1 mg/L over 90 km downstream. This spill introduced high nitrogen and phosphorous loads of 92.1 mg/L and 6 mg/L as well as caused dense phytoplankton blooms, and *Clostridium perfringens* was detected in the range of 40,000 CFU/mL (Mallin et al., 1997). This spill consisted of conventional poultry processing waste and wastewater (Pellow et al., 2004).

With growing environmental and economic pressures, conventional poultry processing facilities will need to seek alternatives to their water consumption such as water systems that incorporate reuse water. By reusing water, economic concerns may be abated, while cleaning the
water on-site should mitigate environmental damage.

**Reuse of Processing Water**

Reuse of water is defined as recovery from a processing step, including from the food matrices, its reconditioning treatment, if applicable; and its subsequent use in a food manufacturing operation (Meneses *et al.*, 2017). The reuse of water has been approved in poultry production systems provided that critical control points are identified, and the water be equivalent to potable, or drinkable, water from a safety standpoint (Sanitation Performance Standards Compliance Guide, 2016; U.S. EPA, 2012; Codex Alimentarius, 2007). Typically including sensory, chemical, and microbiological characteristics, water reuse regulations are determined at the state level in the U.S. with guidelines provided by the USDA and the Environmental Protection Agency (EPA) (Meneses *et al.*, 2017; AWWA, 1996; U.S. EPA, 2012; Sanitation Performance Standards Compliance Guide, 2016). One specific guideline, 9 CFR 416.2 (g) (3), states that to use water more than once for the same purpose, measures must be taken to reduce physical, chemical, and microbiological contamination to prevent adulteration of the finished product (Sanitation Performance Standards Compliance Guide, 2016).

These contaminants vary depending on the source and use of the water and can be found in the Sanitation Performance Standards Compliance Guide. Typically, these adulterants include oils, proteins, macroparticles, organic compounds, and bacteria (Meneses *et al.*, 2017). Sanitation standards of poultry reuse water and other regulated waters in the U.S. are detailed in Table 1.1. In poultry reuse waters, total bacterial counts are limited to less than 500 colony forming units (CFU) /mL (Sanitation Performance Standards Compliance Guide, 2016; Casani *et al.*, 2005). There is also a zero tolerance for fecal coliforms, *Salmonella*, and *Staphylococcus aureus*.
Furthermore, turbidity is a physical parameter that is considered when contamination is present and measured with a nephelometer that observes light scattering. In the reuse of processing water, it must not exceed 5 Nephelometric Turbidity Units (NTU’s) which is the maximum turbidity suggested for drinking water as set by the World Health Organization (WHO, 2011). Closely related to this physical parameter is the total solid load which impacts how much light scattering occurs. Chemical parameters that must be met include nitrogen and biological and chemical oxygen demand (BOD and COD) which must comply with state requirements (Sanitation Performance Standards Compliance Guide, 2016; Casani et al., 2005). An additional guideline provided in the Code of Federal Regulations, states that reconditioning equipment must be approved and a 60% reduction in total bacterial load must be observed. Furthermore, light transmission, which is indicative of physical contamination, must be at least 60% of potable water used in the same process (Saravia et al., 2005; Sheldon and Brown, 1986).

To follow these guidelines while maintaining quality and safety throughout processing, several techniques have been employed to reduce upstream contamination including physical and chemical treatments (Menesses et al., 2017). The use of these treatments within the food industry has been documented in Menesses et al., (2017) and Casani et al., (2005). Physical methods, such as filtration systems, are often utilized to reduce chemical and physical adulterants, while chemical treatments such as chlorine and peracetic acid are added primarily to reduce microbial and pathogen loads (Casani et al., 2005). Each treatment, however, possesses deleterious properties (Casani et al., 2005; Stampi et al., 2001). As a consequence, these conventional treatments will be discussed for potential use in reuse water systems.
Chemical Sanitation Methods

Chlorine

Even before it was discovered as an element in 1809, chlorine was utilized in the textile industry as a bleaching agent. Once its disinfection properties were identified in the 19th century, chlorine was utilized for the treatment of water, and in 1881 hypochlorite was shown to be deleterious to bacteria (Wei et al., 1985). Hypochlorite, a liquid, was soon utilized to reduce exposure to chlorine gas but deaths and health problems persisted (Winder, 2001; Cameron, 1870). Globally, chlorine is the most commonly used chemical oxidant for municipal water disinfection (Deborde and Von Gunten, 2000). This is due to its capability to kill microorganisms as well as serve as a taste and odor control (Hoff and Geldreich, 1981; Wolfe et al., 1984). Despite U.S. EPA restrictions of only 4 ppm of chlorine in drinking water, its use as a sanitization method has been shown to be nearly 100% effective against planktonic bacteria when paired with a sand filtration system (Dunlop et al., 2002; U.S. EPA, 1992). In municipal wastewater systems chlorine is also utilized as a sanitizer for the disinfection and removal of pathogenic and nonpathogenic bacteria. While this practice has been commonplace in many developed countries, the long-term effects of on the environment of discharging chlorinated waste have not been largely elucidated (NSW Environmental Protection Agency, 2002). It is known that chlorine residues are toxic to aquatic organisms and they tend to persist and bioaccumulate within marine life (NSW Environmental Protection Agency, 2002). Furthermore, all forms of chlorine-based sanitizers are highly corrosive and toxic, and therefore it is encouraged to use the minimum amount of chlorine sanitizer necessary to reduce bacterial loads for each application. One treatment plant in Geneva, New York was able to meet fecal coliform limits, 200 CFU/100 ml, without exceeding 0.25ppm/L of chlorine in their effluent (NSW
Environmental Protection Agency, 2002; Sanders et al., 2013). Other studies have shown 100-fold reductions of enterococci and fecal coliforms in sewage effluents (Tyrell et al., 1995). The bactericidal effects of chlorine use in municipal water and wastewater are also noted in the food industry.

With the use of chlorine-based products in the food industry not only were pathogenic bacterial levels significantly reduced, but a decrease of microbial slime and spore counts were also observed throughout the processing facilities (Wei et al., 1985; Mercer and Somers, 1957). By neutralizing essential amino acids, and exhibiting other bactericidal mechanisms such as lowering pH, chlorine-based products were considered essential for mid-20th-century food safety (Wei et al., 1985; Frigeburg, 1957; Campers and McFeters, 1979; Foegeding, 1983). In the fresh produce industry chlorine, ozone, peracetic acid (PAA), and chlorine dioxide (ClO₂) have all been utilized to reduce pathogenic bacteria (Banach et al., 2015). Chlorine dioxide (ClO₂) in particular has been reported to be sevenfold more effective than chlorine or hypochlorite in poultry chiller water (Lillard, 1980; Tsai et al., 1995). It has also been shown to reduce Salmonella incidence in chicken with a concentration as low as 5 ppm, compared to hypochlorite recommended use at 50 to 100 ppm (Lillard, 1980; Tsai et al., 1995; Casani and Knøchel, 2002).

Sodium hypochlorite and other chlorine-based sanitizers function by saponifying fatty acids and glycerol creating byproducts such as chloramines which in turn convert amino acids into forms that antagonize cellular metabolism (Estrela et al., 2002). Acidified sodium chlorite has also been shown to be an effective antimicrobial, but must be in an acidic environment (pH 2.5 to 3.2), which can be generated by using citric acid (Warf, 2001; Allende et al., 2009).

Despite the efficacy of chlorine-based products as sanitizers, they are being phased out from many food production systems and are banned in poultry in the European Union since 1997.
Several studies have identified chlorine resistance in Gram-positive bacteria and microorganisms in biofilms (Patterson, 1968; Bolton et al., 1988; Ryu and Beuchat, 2005). Biofilms are bacterial communities that live commensally and attach to surfaces and each other (Costerton, 1995). These biofilms increase the resistance of their residential bacterial cells to antibiotics and environmental stressors such as sanitizers (Frank et al., 2003; Kumar and Anand, 1998; Ryu and Beuchat, 2005). Furthermore, while microbial slimes can be removed in the presence of high concentrations of chlorine, it has relatively low activity on microorganisms harbored within the biofilms. (Deborde and Von Gunten, 2008; Scher et al., 2005). *Salmonella enterica* serovar Typhimurium, when subjected to 50 ppm of sodium hypochlorite for 15 minutes, was completely reduced below the limits of detection (<10 CFU/mL) from an initial concentration of 1 x 10^8 CFU/mL (Scher et al., 2005). However, when sodium hypochlorite was applied to a pellicle of *Salmonella*, an air-liquid biofilm, less than a 1 log CFU reduction was observed (Scher et al., 2005). At 250 ppm of sodium hypochlorite, only a 4 log CFU reduction was observed (Scher et al., 2005). This clear increase in resistance encourages higher chlorine use, but this can become dangerous to workers.

The threshold for chlorine gas that should never be exceeded is 1 ppm and is set by the Occupational Safety and Health Administrations (OSHA) Permissible Exposure Limit (PEL) (OSHA, 2017a). If exceeded, chlorine gas can damage the lungs causing difficulty breathing, coughing, throat irritation, impaired sense of smell, and in extreme cases chronic issues such as bronchitis, emphysema, and permanent pulmonary damage (Winder, 2001). Chlorine can also cause chemical burns and be an irritant in the case of skin or eye contact. An additional concern with chlorine use is that in the presence of high organic matter free chlorine forms trihalomethanes, such as chloroform, which pose a significant danger to plant workers and
equipment (Tsai, 1992; Casani et al., 2005; Fawell, 2000). The corrosion rate of chlorine depends on a number of factors, mainly chlorine concentration and material (Avery et al., 1998; Nielsen et al., 2000). Cast iron, even at low concentrations of chlorine (<2mg/L) degrades by 0.1 mm/year, whereas stainless steel (SS) corrosion is insignificant at low concentrations except for localized buildup areas (Avery et al., 1998). However, at higher concentrations (5 mg/L to 20 mg/L) many types of SS alloys were not resistant to corrosion, and specific types of SS such as Types 304 and 316 are needed to withstand long exposures to high concentrations of chlorine (Avery et al., 1998). This can factor into the cost and be a detriment to poultry processing.

Furthermore, the by-product chloroform is considered carcinogenic, and its inhalation can impair the kidneys and cause liver necrosis (OSHA, 2017a). Poultry processing water, in general, contains a high level of organic material that reacts with chlorine to produce these by-products and this is a concern in the use and reuse of processing water when sanitizing with chlorine or chlorine dioxide (Northcutt et al., 2008; Casani et al., 2005; Meneses et al., 2017; White, 2010). These health concerns have led to regulations limiting reused chiller water to have no more than 5 ppm of free available chlorine, which can be measured with a test kit or amperometric titration system (Tsai et al., 1992; USDA, 2003b; Northcutt et al., 2008).

Chlorine-based sanitizers are also sensitive to temperature, pH, and residence time within the chiller water. As pH increased from pH 7.0 to 8.5 to 9.8 to 10.7, the killing power of chlorine against E. coli decreases, while raising temperature generally increases lethality (Butterfield et al., 1943; Nagel et al., 2013). However, this may result from an increase in temperature of the wash also reducing aerobic microbial populations independent of chlorine presence (Kelly et al., 1981). Due to the organic load within chillers, the bactericidal effects of chlorine sanitizers were found to be extremely low after 5 minutes of treatment (Tsai et al., 1992; Oyarzabal, 2005). It
was determined that for a chiller tank with total dissolved solids of 3,500 ppm over 400 ppm of chlorine would be needed to saturate the demand from organic compounds which react with chlorine and produce by-products that are hazardous and ineffective as bactericidal agents (Tsai et al., 1992). Within this chiller tank, there was no available free chlorine, the agent of sanitation, after 30 minutes of chlorination with concentrations up to 300 ppm (Tsai et al., 1992). This was determined through a colorimetric assay. As a consequence, to utilize chlorine effectively as a sanitizer under these conditions, especially in the presence of high organic loads, it must be regulated and these physical and chemical characteristics can fluctuate in reuse water systems on a day to day basis (Mead and Thomas, 1973; White, 2010; Northcutt et al., 2008). Due to these issues, poultry processing water, especially those in reuse systems, are increasingly utilizing peracetic acid as a sanitizer instead of chlorine.

**Peracetic Acid and Hydrogen Peroxide**

By breaking down into acetic acid, water, and oxygen, PAA has been considered a safer alternative to chlorine and is currently approved for poultry processing water use by the U.S. Food and Drug Administration (FDA) at up to 2000 ppm (21 CFR 173.370) (FCN No. 1465) (Kim et al., 2017; Warburton, 2014). Studies show PAA is effective against *Salmonella* and *Campylobacter* at a concentration of 20 and 200 ppm respectively, and this is likely achieved through membrane oxidation and acidifying the water (Bauermesiter et al., 2008; Oyarzabal, 2005). While the mechanism of disinfection is debated, PAA is believed to function by disrupting enzymatic activity through the binding and reacting with sulfur and double bonds, which denatures proteins (Block, 1991; Kitis, 2004; Lefevre et al., 1992; Liberti et al., 1999). It has also been suggested PAA may disrupt intracellular solute concentrations and impair
bacterial cell replication as well as inactivate catalase, which breaks down toxic hydroxyl radicals (Block, 1991; Lubello et al., 2002). Unlike chlorine, PAA is not significantly inhibited by a high organic load, and this has made it a popular alternative in poultry processing (Casani et al., 2005; Lillard, 1979; McKee, 2011). However, in the presence of highly concentrated organic matter the rate of disinfection may be impacted (Gehr et al., 2003; Gehr and Cochrane, 2002). At 200 ppm PAA in chiller water did not influence the sensory characteristics of poultry products including flavor appearance or product acceptability (Bauermesiter et al., 2008).

Hydrogen peroxide used in combination with peracetic acid has been shown to be beneficial as a synergistic sanitizer improving peracetic acid reductions of Escherichia coli, Staphylococcus aureus, and Pseudomonas aeruginosa. (Alasri et al., 1992; McKee, 2011). Hydrogen peroxide (H$_2$O$_2$) is an intermediary in the degradation of peracetic acid into acetic acid, but H$_2$O$_2$ also has some antimicrobial capacity (Bauermeister et al., 2008; Wagner et al., 2002). However, in the municipal effluent, concentrations of 106 to 285 mg/L of H$_2$O$_2$ were required to sufficiently reduce fecal coliforms compared to 0.6 to 1.6 mg/L of PAA (Wagner et al., 2002). Peracetic acid functions similarly to other peroxides in that it releases active oxygen that oxidizes double bonds, sulfur bonds, and sulfhydryl groups of proteins and enzymes that are located intra- and intercellularly (Block, 1991; Kitis, 2004). The sanitation effect of H$_2$O$_2$ can be improved by the addition of PAA as the inactivation of catalase improves the persistence of H$_2$O$_2$ (Block, 1991; Kitis, 2004). This effect, along with the equilibrium reached between H$_2$O$_2$ and PAA in solution means that these chemicals are often paired to achieve synergistic effects.

The PAA-based sanitizers are used in poultry processing systems reuse water and can also be combined with diatomaceous earth filtration to improve safety (Casani et al., 2005; Lo et al., 2005). However, peracetic acid is corrosive to equipment and can be caustic to exposed skin,
eyes and respiratory systems of personnel handling these materials (Casani et al., 2005; Peracetic acid-MSDS, 2013). Furthermore, the potential for microorganisms to recover from the sanitization treatment and grow may exist due to the decomposition of PAA into acetic acid (Kitis, 2004; Stampi et al., 2001). This rapid decomposition can result in a short shelf life, of a few days to weeks, if not refrigerated or placed away from light (Deshpande et al., 2013; 2014; Walsh et al., 2018).

Ozone

While some chemical sanitizers are considered corrosive ozone can be utilized as a sanitizer without corrosive damage to equipment (Guzel-Seydim et al., 2004). Ozone (O₃) is generated when oxygen molecules are subjected to high voltage (Horvath et al., 1985). It has been utilized in water disinfection of swimming pools, wastewater treatment plants, and in bottled water production (Guzel-Seydim et al., 2004; Rice et al., 1981; Legeron, 1982). Ozone solubility depends on pH, temperature, ozone bubble size, and water purity, which are difficult to regulate in reuse water systems (Rice et al., 1981). Typically, at 27°C, 270 mL of ozone can be dissolved into a liter of pure water (pH 7.00) (Rice et al., 1981). Water treated with ozone has also been used to chill poultry carcasses with no changes in sensory characteristics (Sheldon and Brown, 1986; Casani et al., 2005). The FDA has affirmed the GRAS status of ozone as a sanitizer but only if the provided applications and concentrations utilized are in agreement with good manufacturing practices (Oyarzabal, 2005; Graham, 1997; FDA, 1999).

Ozone has been approved as a sanitizer to reuse poultry chiller water (Kim et al., 1999). Like chlorine, ozone oxidizes critical bacterial membrane proteins, but ozone possesses 1.5 times higher oxidizing effect compared to chlorine (Oyarzabal, 2005; Xu, 1999). This oxidative
potential allows for the attack of bacterial membrane glycoproteins and glycolipids, which causes membrane permeability and lysis (Guzel-Seydim et al., 2004; Khadre and Yousef, 2001). Ozone can also decrease pH. For example, after 50 minutes of ozonation Sheldon and Chang (1987) observed a decreased chiller water pH from 6.9 to 5.6. To generate ozone on a commercial level the corona discharge method is utilized, which splits diametric oxygen into free radicals that, in turn, react with available diametric oxygen to form ozone (Rice et al., 1981). Depending on the system 8 to 16 kWh is required to generate 1 kg of ozone, and this is achieved by utilizing high and low-tension electrodes separated by a dielectric medium with a narrow discharge gap (Rice et al., 1981). When sufficient kinetic energy exists within the excited electrodes collision occurs through the narrow gap interacting with oxygen to form ozone and produces a 1 to 3% ozone mixture (Rice et al., 1981). Unlike chlorine, ozone’s killing potential is not as drastically impacted by the presence of organic material, and ozone decomposes into nontoxic residues (Graham, 1997; Xu, 1999; Restaino et al., 1995). This effect has been utilized to reduce pathogenic Escherichia coli, Listeria monocytogenes, Pseudomonas spp., Bacillus spp., Salmonella spp., and yeasts (Restaino et al., 1995; Guzel-Seydim et al., 2004; Almeida and Gibson, 2016). By using a recirculating ozone reactor, Restaino et al., (1995) tested the efficacy of 0.188 mg/L of ozone (concentration determined at the outlet) against 10^6 CFU or spores/mL of several pathogens. The authors reported, at time zero, 5 log reductions of S. Typhimurium and E. coli along with more than a 4 log reduction of Listeria, and 3 log reductions of B. cereus and P. aeruginosa, but only a 1 log reduction in Aspergillus niger (CFU/mL) (Restaino et al., 1995). Using a dipper well ozone sanitation system, a 5 log reduction of E. coli and Listeria innocua was observed at 30 seconds after exposure to 0.45 to 0.55 ppm of residual ozone in Almeida and Gibson (2016). In Fabrizio et al. (2002), carcass surfaces were inoculated with Salmonella and
allowed to attach, allowing for 3 log CFU/mL of rinsate (Fabrizio et al., 2002). These pathogen-
inoculated carcasses were added to an immersion chiller at 4°C with ozone (10 mg/L) (Fabrizio et al., 2002). This led to a 0.75 log CFU/mL reduction before storage and a 1 log CFU/mL reduction after storage but required pre-enrichment with tetrathionate to be detectable (Fabrizio et al., 2002). Ozone also reduced aerobic plate counts by 0.5 log CFU/mL which was statistically significant compared to all other treatments tested by Fabrizio et al. (2002). This was compared to 20 ppm of chlorine which was reported to be less effective with no immediate reduction and a 0.25 log CFU/mL reduction of Salmonella before and after storage in at 4°C respectively (Fabrizio et al., 2002). These low reductions were likely due to the low level of Salmonella inoculation. Comparatively, a one-hour ozone treatment has been shown by Selma et al., (2008) to reduce the microbiota populations of vegetable wash water by 5.9 log CFU/mL.

Regardless of these bacterial reductions, there are several drawbacks to utilizing ozone as a sanitizer such as human health concerns, instability, and degradation (Kim et al., 1999; Fabrizio et al., 2002; Hoof, 1982; Casani et al., 2005). Ozone primarily affects the respiratory tract in humans and generates headaches, dizziness, and a burning sensation in the eyes and throat (Hoof, 1982; Guzel-Seydim et al., 2004). When exposed to chronic toxicity, memory can be affected, and an increased prevalence of bronchitis and muscular excitability can be observed (Hoof, 1982). Due to these effects, OSHA guidelines state that levels are not to exceed 0.1 ppm of air for 8 hours of light work and no more than 0.05 ppm for heavy work, where the definitions of light and heavy workloads are defined by OSHA, (2017ab). Additionally, while from a health perspective rapid degradation of ozone is beneficial, utilizing the product as a sanitizer can be difficult when considering its short half-life of 2 to 165 minutes (Khadre et al., 2001; Wynn et al., 1973; Wickramanayake, 1984). The degradation rate of aqueous ozone is dependent on
alkalinity, mechanical stirring, water impurities, and temperature (Khadre et al., 2001; Hill and Rice, 1982). For instance, while ozone’s half-life at 20°C is 20 to 30 minutes a shorter half-life of 2 to 4 minutes was observed at pH 7.0 and 25°C while stirring occurred (Wickramanayake, 1984; Wynn et al., 1973). At pH 9.0, no ozone was detected by Kim (1998) while lower pH levels appeared to be more favorable to ozone stability.

Independent of these factors, this degradation forces on-site generation of ozone, which may be a potential practical obstacle for some poultry processing operations (Fabrizio et al., 2002; Pryor and Rice, 1999). Furthermore, while organic loads found in chiller tanks have been shown not to impact ozone initial killing potential, 20 ppm of bovine serum albumin, a protein concentrate, significantly impacts the stability of ozone which reduces its killing potential over time (Restaino et al., 1995; Horvath et al., 1985). As a consequence of these negative effects, ozone has been investigated more as a synergistic sanitizer rather than a standalone application (Khadre et al., 2001). Since ozone disrupts membrane permeability, combining it with chlorine allows for deleterious effects on parasites such as Cryptosporidium parvum (Khadre et al., 2001; Gyurek et al., 1996). This occurs by denaturation of membrane proteins due to the oxidation potential of ozone, which eventually leads to leakage and cell death (Zhang et al., 2011). Ozone has been utilized along with filtration in poultry chiller water and reduced microbial counts by 99% and have been shown by Unal et al. (2001) to inactivate E. coli and L. monocytogenes when utilized with a pulse electrified field (Sheldon and Brown, 1986).

The potential for ozone as an independent sanitizer or synergistic sanitizer is promising. However, while significant log reductions of pathogens in poultry chiller water have been indicated, there are concerns with worker safety and the need for an on-site generation due to ozone’s short half-life.
As such, alternative sanitizers still need to be investigated to achieve further food safety improvements.

**Sodium Bisulfate**

The advantages of chlorine, peracetic acid, and ozone, are clear as they can greatly reduce microbial loads and do not alter product quality if used in low-dose quantities resulting in their widespread use in poultry processing (Casani *et al.*, 2005; Northcutt and Jones, 2004; Wei *et al.*, 1985; Gehr *et al.*, 2003). However, these sanitizers can be corrosive, dangerous to workers, difficult to transfer in large quantities, and ineffective against biofilms in low doses. Sodium bisulfate may be an alternative solution as it is a dry acid which is soluble in water and is generally recognized as safe (GRAS, 21 CFR 582.1095) (Jones-Hamilton, 2018; USDA, 2015). Recently, SBS has been listed as a safer choice as an antimicrobial and processing aid by the environmental protection agency (EPA, 2018). Sodium bisulfate can be transported as a granular solid. It dissociates into nontoxic sodium, hydrogen and sulfate ions and has a pKa of 1.99 (Sun *et al.*, 2008). It also possesses the ability to lower the pH of water without producing off flavors in finished products (Sun *et al.*, 2008). Based on the U.S. National Fire Protection Agency (NFPA) code 704, sodium bisulfate is a class 2 health hazard where intense or continued exposure could cause injury, compared to PAA and hypochlorite, which are class 3 health hazards that could cause serious temporary or moderate residual injury on brief exposure (USDA, 2015; Sodium bisulfate-MSDS, 2013). Approved as a food ingredient and general purpose animal feed additive, SBS has wide range of applications including use in beverages, soups, dressings, ready-to-eat meals, vegetables, fruits, pet food, poultry feed, and in drinking water (Calvo *et al.*, 2010; Kassem *et al.*, 2012; Sun *et al.*, 2008; Kim *et al.*, 2018). Sodium
bisulfate is especially attractive to alternative processing facilities as it is considered ‘natural’ according to the FDA and the International Association of Natural Product Producers (IANPP) (Kim et al., 2018).

Sodium bisulfate has several potential properties that would make it an ideal alternative sanitizer, and while limited, there is information regarding its use as a sanitizer in the food industry. *Campylobacter* levels were reduced by 2 to 3 log over a six week period when 1.13 kg or 1.81 kg SBS was applied to 4.6m² poultry litter (Line, 2002). In Micciche et al., (2018) SBS was effective in reused poultry processing water by reducing 1.5e8 CFU/100mL of *Salmonella* to below the limit of detection, which was not accomplished by 200 ppm PAA. Dittoe et al. (2018) also demonstrated a 2 log reduction of *Salmonella* on poultry carcass rinses. To reduce *S. Typhimurium* on chicken carcasses 10% SBS solution was sprayed reducing concentrations by 2.4 logs (Yabin et al., 1997). Total aerobic microbial populations were decreased by 1.61 logs, and *Salmonella* counts were reduced by 2 logs when 5% SBS was utilized in the inside-outside bird washer (Yang et al., 1998). However, at this concentration slight discoloration was observed on the chicken carcass (Yang et al., 1998). The antimicrobial efficacy of SBS has also been tested on apples. When washed with a 1% solution of SBS and 60 ppm PAA reduced *Listeria innocua* by 5 logs after seven days storage compared to 150 ppm of chlorine which had only a 3.5 log reduction (Kim et al., 2018). When the treatment was increased to a 3% SBS solution with 60 ppm PAA, the 5 log reduction was also observed at day 14 (Kim et al., 2018). This bactericidal activity on apples corroborates findings in Fan et al. (2009) which demonstrated reduced total aerobic plate counts by SBS on apple slices. Further investigations into SBS efficacy as sanitizers must be performed, but due to its ability to be transported as a solid, and its relative safe use, there is noted interest on its use in alternative poultry processing.
Physical Treatment - Filtration

Despite the efficacy in reducing microbial populations chemical sanitizers alone are not sufficient in treating processing water. This is because there is a need to remove debris, dissolved solids, blood, fat, tissue, fecal matter and other particulates that collect in the water (Sanitation Performance Standards Compliance Guide, 2016). In order to remove these dissolved solids, filtration systems may be utilized, and these could also assist in reducing microbial loads.

Filtration systems can be broken into three types, macro-, micro-, and ultrafiltration (Saravia et al., 2005). Unlike chemical treatments, filtration systems can concentrate and remove oils, macrosolutes, and organic compounds (Meneses et al., 2017). Macrofiltration systems collect particles 5 µm in size and are not suitable for bacterial concentrates, whereas microfiltration typically picks up particles up to a tenth of a micron in size (Saravia et al., 2005). Ultrafiltration can concentrate particles with a molecular weight greater than 1,000 leaving only low molecular weight organic solutes and salts (Sarvia et al., 2005; Lo et al., 2005). This is accomplished by utilizing pressures up to 145 psi and selective fractionation (Sarvia et al., 2005; Lo et al., 2005). Ultrafiltration has been found to reduce 85% of total solids and 95% of chemical oxygen demand of poultry processing wastewater (Shih and Kozink, 1980; Avula et al., 2009). This conditioning has been utilized on chiller overflow and produced chemical compositions similar to tap or potable water (Mannapperuma and Santos, 2004; Avula et al., 2009). Mannapperuma and Santos (2004) designed a spiral membrane ultrafiltration system to recondition poultry overflow water. It was found that 480 L/min could be processed with an 80% reuse of water in this system. This would allow for 346 L/min of freshwater to be replaced and it was determined with an initial capital cost of $300,000 a full-scale processing
facility would see a 2.4 year simple payback period for their investment. In a pilot processing plant, it was estimated $60,000 of savings for a typical processing plant per year could be obtained if chiller reuse water system with ultrafiltration would be implemented. Savaria et al., (2005) detailed the economic feasibility of ultrafiltration reuse water systems and found them feasible for large-scale poultry production plants even when factoring in the 5-year and 20-year life expectancy for the membrane and the unit, respectively (Saravia et al., 2005). They also estimated over 600,000 L of water per day could be recycled through this process when only considering reusing chiller water (Savaria et al., 2005; Avula et al., 2009).

Despite these potential advantages several hurdles remain before filtration and reuse water systems become commonplace in poultry processing plants. For example, due to the high startup cost, filtration units may not be applicable to smaller processing facilities or pilot plants. An additional concern lies with the potential for organisms to survive on filtration concentrates and form biofilms which may be a worker safety concern when removing the filtrate buildup (Casani et al., 2005; Drozd and Schwartzbrod, 1997). To remedy this concern, some of the previously discussed chemical sanitizers are often used with filtration to ensure reduction of microorganisms and chemical contamination (Chang et al., 1989; Casani et al., 2005; Northcutt and Jones, 2004). For instance, Lo et al., (2005) utilized chlorine resistant (up to 50 ppm) filters and were able to reduce COD below 200 mg/L while recovering crude protein by-products. Lillard (1979) investigated broiler necks submerged in chlorinated chiller water which had been passed through a diatomaceous earth filter. The conclusion was drawn that, by using these two sanitization methods, water did not have to be potable to maintain quality (Lillard, 1979). Regarding microbial load, neither total aerobic counts, fecal coliforms, nor Salmonella populations were significantly different between reused chlorinated, filtered chiller water and
potable water (Lillard, 1979). However, with the aforementioned issues with traditional chemical sanitizers, alternative treatments should also be investigated for independent use or in combination with conventional sanitization methods in reuse water.

**Conclusions**

With environmental and financial pressures growing, the commercial poultry industry like the rest of the food industry must consider alternative measures in the near future to conserve one of the earth’s most precious resources (Meneses *et al.*, 2017). Reuse water systems have been demonstrated to be effective in traditional processing plants (Andelman and Clise, 1977; Casani *et al.*, 2005). With food safety in mind, proper sanitation is required to avoid cross-contamination. These sanitizers may result in the necessity of using caustic, corrosive, and potentially dangerous chemicals which may not be cost-effective or viable for alternative production systems (Casani *et al.*, 2005). As such, additional sanitizer sources must be developed and further investigated to improve food and operator safety. The organic poultry processing industry may have insights into these sources. Bacteriocins, essential oils, and bacteriophages have been investigated in this alternative poultry processing market and with further experimentation may be found useful for conventional poultry processing (Sirsat *et al.*, 2009; Calo *et al.*, 2015). Sanitizer stability and interaction with other components in the poultry processing water would be of particular interest in reuse systems to determine their long-term viability.

Sodium bisulfate in particular should be explored for its use within poultry processing waters as it is regarded as a safer choice and operates as an acidifier like the commonly used peracetic acid. To investigate how this alternative sanitizer performs in reuse water its impact on
Salmonella, spoilage organisms, and general bacteria loads must be investigated. The use of 16S rRNA gene amplicon sequencing may also yield valuable insights into how the acidifier impact the microbiome, including the presence of Pseudomonas and other spoilage organism taxa. Once viable, alternative sanitizers implemented with reuse water systems in conventional poultry processing may be a part of the solution to water scarcity.
<table>
<thead>
<tr>
<th></th>
<th>Poultry Processing(^1)</th>
<th>Drinking Water(^1)</th>
<th>Treated Municipal Wastewater (Sewage)(^1)</th>
</tr>
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<tbody>
<tr>
<td>U.S. Regulations</td>
<td>9 CFR 416.2(g)</td>
<td>40 CFR 141</td>
<td>40 CFR 503</td>
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<tr>
<td>Total Plate Count</td>
<td>500 CFU/mL(^*)</td>
<td>Not Tested</td>
<td>Not Tested</td>
</tr>
<tr>
<td>Total Coliform</td>
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<td>0 ppm</td>
<td>Not Tested</td>
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<tr>
<td>Fecal Coliform</td>
<td>0 CFU/mL(^*)</td>
<td>0 ppm</td>
<td>1000 MPN/g(^*)</td>
</tr>
<tr>
<td><em>Escherichia coli</em></td>
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<td>0 ppm</td>
<td>235 CFU/100ml(^3)</td>
</tr>
<tr>
<td><em>Salmonella</em></td>
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<td>3 MPN/4g(^*)</td>
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<tr>
<td><em>Staphylococcus</em></td>
<td>0 CFU/mL(^*)</td>
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<td>Not Tested</td>
</tr>
<tr>
<td>Turbidity</td>
<td>5 NTU(^*)</td>
<td>1 NTU(^*)(^2)</td>
<td>Not Tested</td>
</tr>
</tbody>
</table>

\(^*\)CFU: Colony Forming Units NTU: Nephelometric Turbidity Unit MPN: Most Probable Number
\(^1\)Additional state regulations may be imposed
\(^2\)Currently U.S. Drinking plants strive for under 0.1 NTU (Edzwald and Tobiason, 1999)
\(^3\)Arizona based regulation (Sanders et al., 2013)
Figure 1.1: Depicts the layout of a typical poultry slaughter and processing facility. The outflow from each area is also detailed. The figure demonstrates that at almost every step water is a component of the waste (Avula et al., 2009; Keener et al., 2004). Steps in green highlight regions where physical and chemical treatment may be most practical for water reuse (Avula et al., 2009).
References


III. Chapter 2. Comparison of Acid Sanitizers on *Salmonella* Typhimurium Inoculated Commercial Poultry Processing Reuse Water

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Abstract

When considering the cost associated with producing potable water and the environmental concerns associated with water scarcity, the reuse of processing water has received increasing interest. While water reuse during poultry processing can be environmentally friendly, it also brings potential food safety and cross-contamination concerns, through the reuse of water which may harbor human-host pathogens. Therefore, in order to effectively utilize reuse water systems, to mitigate environmental and cost concerns with water use, antimicrobials must be investigated to reduce bacterial and pathogen load. Currently, peracetic acid (PAA) is commonly used in processing water up to 2,000 ppm but can be corrosive with documented public health concerns. As such, sodium bisulfate (SBS) may be utilized as it has the potential to be an important anti-microbial in poultry processing facilities as seen in its application in the produce industry. In this study, SBS, PAA, and industrial grade PAA (IG-PAA) were evaluated against microbial populations in water reuse systems inoculated with Salmonella Typhimurium. Fresh, untreated processing plant reuse water was collected at the end of a poultry processing shift. The water was utilized within one hour of collection, minimum inhibitory concentrations were established, change in pH was investigated, and plate counts after Salmonella addition were performed. When 3x10^7 CFU of Salmonella were added to each microcosm, a 4 to 5 log reduction with 200 ppm PAA was observed compared to a reduction below the limit of detection observed by 5 minutes of treatment with 1%, 2%, or 3% SBS (P < 0.05). The results of this study indicate that SBS is an effective alternative to PAA for decreasing foodborne pathogen contamination in poultry reuse water.
Introduction

Water scarcity continues to be a global concern (Beekman, 1998; Casani et al., 2005; Meneses et al., 2017; Faour-Klingbeil and Todd, 2018). By 2025 almost half of the world’s population will face severe clean water scarcity issues (Alcamo 1997; 2000; Micciche et al., 2018). Factors impacting water scarcity include climate change, shifts in the human diet, and an increase in water use for industrial processing (Meneses et al., 2017). In the food industry, water use increased by 40% throughout a decade from 1998 to 2008 (Klemes et al., 2008; Meneses et al., 2017). To face these environmental, and eventually economic pressures, food processing industries need to develop novel water conservation technologies.

In poultry processing, 21 to 30 liters of potable water are used per bird (Walsh et al., 2018; Northcutt and Jones, 2004; Kiepper, 2008). There have been studies suggesting water usage could be reduced in poultry processing (Meneses et al., 2017; Lillard, 1979). However, the use of contaminant-free water is pivotal for food safety and the consideration of Hazard Analysis and Critical Control Point (HACCP) plans (Northcutt and Jones, 2004; USDA, 2016). As a consequence, to reduce the amount of water utilized in poultry processing without compromising on efficiency or product safety, reuse water systems can be implemented (Micciche et al., 2018).

Reuse of water or “the recovery and reconditioning of water from a processing step followed by its subsequent use” is covered in 9 CFR 416.2 and is enforced by the United States Department of Agriculture (USDA) (Meneses et al., 2017; USDA, 2016). According to the law, the reuse of processing water from upstream steps should not be utilized for downstream processing steps (USDA, 2016). Also, reuse processing water should be equivalent to potable, or drinkable, water (Meneses et al., 2017; USDA, 2016). To ensure chemical equivalency, filtration systems are often utilized. For microbial and pathogen elimination, sanitizers are employed
(Northcutt and Jones, 2004). Chlorine has long been the sanitizer of choice for the U.S. poultry industry, but it can be hazardous to workers due to its potential to form dangerous by-products, and bacterial resistance has been documented (Dunlop et al., 2002; Casani and Knøchel, 2002; Casani et al., 2005; Ryu and Beuchat, 2005). Furthermore, the European Union has banned the use of chlorine since 1997 and USDA regulations prevent the amount of free chlorine in reuse water to exceed 5 ppm (USDA, 2003; Johnson, 2015). As a consequence, alternative acid sanitizers such as peracetic acid (PAA) and sodium bisulfate (SBS) are of interest.

Peracetic acid has been utilized as a conventional sanitizer for poultry processing due to its effectiveness against Campylobacter and Salmonella, which are common poultry processing pathogens (Kim et al., 2017; Bauermeister et al., 2008a; Oyarzabal, 2005). However, while its legally allowed maximum concentration has recently increased to 2000 ppm (21 CFR 173.370), PAA is corrosive to equipment and hazardous to workers (Kim et al., 2018; Casani et al., 2005). Furthermore, there is evidence that peracetic acid can decay into acetic acid within 30 minutes on poultry carcasses (Walsh et al., 2018). Sodium bisulfate may be an alternative to peracetic acid as it has shown to be an effective antimicrobial against Salmonella on chicken parts (Dittoe et al., 2018a). SBS is an inorganic acidifier with a pKa of 1.96 (Knueven, 1999). This low dissociation constant allows for highly acidic conditions, which Salmonella’s acid tolerance mechanism and acid-shock proteins may not be able to compensate for, which would result in cell death (Knueven, 1999; Foster and Hall, 1991; Ryan et al., 2016). It has also been shown to be effective against Campylobacter when utilized pre-harvest (Line, 2002), and a 10% SBS spray decreased Salmonella counts by 2 logs on poultry carcasses (Li et al., 1997; Micciche et al., 2018). As such, SBS may be an ideal candidate for poultry processing reuse water systems. In the current study, we report on the antibacterial effectiveness of three acid sanitizers (peracetic
acid, sodium bisulfate, and industrial-grade peracetic acid) added to reuse poultry processing water. More specifically, we hypothesized that SBS, compared to PAA based acidifiers, would achieve a higher rate of reduction of Salmonella Typhimurium populations over time when reuse water microcosms were inoculated with the bacteria. In the current study, we utilized concentrations of SBS and PAA that have been reported not to affect the sensory characteristics of poultry carcasses (Ditoe et al., 2018a).

**Materials and Methods**

*Bacterial Strain*

The Salmonella Typhimurium UK-1 (ATCC 68169) used in this study was originally isolated from a chicken spleen inoculated 3 days prior with a virulent S. Typhimurium strain from an infected horse and the complete genome was sequenced by Luo et al. (2011) (Curtiss et al., 1991). It was maintained in Luria Bertani (LB) broth (Difco Laboratories, Detroit, MI, USA) at 37 °C and at a concentration of 3 x 10⁹ CFU (Colony Forming Units) /mL. Concentrations were verified through plating onto Xylose Lysine Desoxycholate (XLD) agar Difco Laboratories, Detroit, MI, USA).

*Water Collection*

Processing water samples were collected from a commercial poultry processing facility at the intake of the reuse water sanitization system. Intake waters were chlorinated (20 to 50 ppm total chlorine) and used in an inside-outside bird washer before water reuse collection. Water was transported to the laboratory at room temperature and utilized within one hour of sample collection.
Salmonella Killing Assay of Reuse Water Microcosms

Upon arrival, 20 mL of reuse water was individually added to sterile Erlenmeyer flask alone or with acid sanitizers to generate the following concentrations: 3% SBS (w/v) (Jones-Hamilton Co, Walbridge, Ohio), 2% SBS (w/v), 1% SBS (w/v), 0.02% PAA (v/v) (Sigma-Aldrich, St. Louis, MO, USA), or 0.02% Industrial Grade PAA (v/v) (IG-PAA). The composition of the PAA stock solution was acetic acid at approximately 45%, peracetic acid at approximately 39%, hydrogen peroxide at approximately 6%, and water at approximately 10% (Sigma-Aldrich, St. Louis, MO, USA). The composition of the IG-PAA stock solution was acetic acid 33 to 39%, hydrogen peroxide 8.5 to 10.5%, peracetic acid 20 to 23%, water 28 to 35%.

Three technical replicates were performed per trial, with a total of 6 independent trials in all. Each trial was repeated on a separate day using freshly collected poultry processing water from the same source. At every time point, the pH was also measured using a Symphony B10P (VWR, Radnor, PA, USA).

The Salmonella killing assay was performed by adding 100 µL 3 x 10⁹ CFU/mL Salmonella Typhimurium UK-1 to the reuse water microcosms with the acid sanitizers (1%, 2%, and 3% SBS, PAA, or IG-PAA). A no treatment (NT) control was also included. A 100 µL aliquot of the microcosms were plated onto XLD immediately and samples were collected every 5 minutes for 30 minutes. Incubation of plates occurred at 37 °C for 24 hours. The limit of detection (LOD) was determined to be 10 CFU/mL (Sutton, 2011).

Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC)

One ml of a 1% triphenyl tetrazolium chloride solution (TTC) was added to 100 mL of reuse water filtered with a sterile cheesecloth. To each well of a 96-well plate, 50 µL of reuse
water with TTC was added. Stock solutions of each acid sanitizer were prepared in deionized water in the following concentrations: 12% SBS, 0.8% PAA, 0.8% IG-PAA. Further serial dilutions (1:1) were made ranging from 6% to 0.09% for the SBS treatment and 0.4 % to 0.00625% for the PAA and IG-PAA treatments. This was performed by thoroughly mixing 50 µL of stock acidifier to the first well with 50 µL of reuse water and transferring 50 µL of acidifiers water to the next well for subsequent mixing and transfer. For positive control wells, 50 µL of phosphate buffered saline (PBS) was added in place of acid sanitizers. To each well, 50 µL of LB inoculated with $3 \times 10^8$ CFU/mL of *Salmonella* UK-1 was added and mixed. For negative control wells, 50 µL of sterile LB was added. This further diluted each sanitizer concentration in the well by a factor of 2. The plate was placed in an Infinite M200 microtiter plate reader (Tecan Männedorf, Switzerland), set to 37 °C, shaken every 15 minutes, and reads were taken at 24 hours at 480 nm. Changes in absorbance were indicative of bacterial growth and analyzed. For each well that had no change in absorbance an MBC test was performed by plating 50 µL onto XLD which was incubated at 37 °C for 24 hours. The concentration of sanitizer was considered to be bactericidal if no growth was detected.

**Statistics**

A one-way analysis of variance (ANOVA) was performed using nonparametric test (Kruskal-Wallis, Siegel and Castellan, 1988) for the *Salmonella* killing assay and a parametric test for the change in pH. When the ANOVA indicated a significant result, differences between the mean values were determined using pairwise comparisons test. Statistical significance was determined at 0.05 level. All data analyses were conducted using R (Version 3.5.1; R Core Team., Vienna, Austria).
Results

Reuse Water Microcosms-Change in Salmonella Concentrations

Because time point 0 was measured immediately after the addition of Salmonella and acid sanitizers, there were initial killings observed in all treatment groups when compared to the no treatment control. The average detected log CFU/mL at time point zero was 6.84, 4.66, 4.38, 4.34, 5.47, 6.73 for the negative control, PAA, Ig-PAA, and 1%, 2%, and 3% SBS groups respectively. The standard deviations were 0.03, 0.218, 0.33, 0.59, 0.15, and 0.13 respectively. Both the 2% and 3% SBS treatments were statistically not different compared to the control at time 0 (P > 0.05), whereas the PAA, IG-PAA, and 1% SBS were statistically significant (P < 0.05). By the 5-minute time point, no Salmonella counts were detected for the SBS treatments (Figure 2.1). Replicates within the PAA treatment varied with the time required to reduce Salmonella below the limit of detection (Figure 2.1). It also took 25 minutes for one replicate of IG-PAA to be reduced below the limit of detection. One replicate for standard PAA persisted after 30 minutes. No significant change over time was detected for the no treatment control.

Reuse Water Microcosms-Change in pH

There were no significant changes in pH over time across treatments (Figure 2.2). There were similarities between the pH of reuse with or without the addition of S. Typhimurium for each treatment. There was a significant treatment effect on pH. At time point 0 and 30 minutes, all treatments were significantly different from each other except for the 3% and 2% SBS treatment and the 2% and 1% SBS treatment (P < 0.05) (Table 2.1). At 30 minutes the average pH for 1%, 2% and 3% SBS were 1.58, 1.36 and 1.23. For PAA, the average was 4.02, and IG-PAA average pH was at 5.13. The average for the negative control was 7.52.
When pH was compared to *S*. Typhimurium population levels for one of the blocks (n=3), the lower the pH, the greater the population of viable *S*. Typhimurium at time point zero, except the negative control (Figure 2.3A). However, at 5 and 10-minute time points, this relationship was not present as all replicates exhibited no growth in this block except for PAA (Figure 2.3B, C).

*Minimum Inhibitory and Bactericidal Concentration Assays*

The MIC for SBS, PAA, and IG-PAA were 0.375%, 0.05%, and 0.10%. The MBC for SBS, PAA, and IG-PAA were 0.75%, 0.10%, and 0.10%, respectively. These concentrations were validated using the microtiter plate reader and are illustrated in Figure 2.4 A, B, and C. An increase in OD at 480 nm was indicative of dehydrogenation of TTC, and therefore metabolic activity and bacterial presence (O’Bryan et al., 2008). The MIC was the lowest concentration that did not exhibit a change in absorbance compared to the negative control. The MIC values were higher for SBS and PAA compared to their MIC responses.

*Discussion*

Acidifiers are often used to control biological contamination within poultry processing facilities and have been used in poultry feed, in the form of organic acids, to control bacteria (Paster et al., 1987; Smyser and Snoeyenbos, 1979; Dittoe et al., 2018b; Kurschner and Diken, 1997). The acidifying properties of compounds such as PAA and SBS allow for the decrease of pH which can interfere with cellular metabolism, particularly through disruption of the proton gradient (Kim et al., 2005). Treatment of poultry processing chiller water with peracetic acid has been shown to reduce bacterial loads, as well as *Salmonella* and *Campylobacter* concentrations
Peracetic acid at 200 ppm the chiller tank did not significantly impact sensory characteristics of the finished product (Bauermeister et al., 2008a,b). Other foodborne pathogens have been shown to be significantly reduced within 10 minutes by the application of 0.1% PAA (Briñez et al., 2006). In Bauermeister et al., (2008a), PAA reduced S. Typhimurium concentrations between 1.5 and 2 log CFU/carcass when inoculated with $10^6$ CFU/carcass compared to 0.03% sodium hypochlorite which only produced a 3 log reduction.

Our data are in congruence with the results reported in Bauermeister et al., (2008a), a 3 to 4 log CFU/mL reduction with PAA (Bauermeister et al., 2008b). However, our results indicate that within the microcosms containing reuse water, SBS appeared to be more consistently effective at producing reductions in Salmonella populations than PAA or IG-PAA after 5 minutes. At timepoint zero there were apparent initial reduction in S. Typhimurium populations. Other studies which have investigated SBS or PAA in different matrices such as apples have not observed initial reductions of pathogens (Kim et al., 2018). The 3% SBS treatment showed no significant change at timepoint zero compared to the negative control despite containing the most acidifier and lowest pH. This may be due to experimental error, but the reproducibility between replicates suggests there may be other possibilities that will require future studies to determine the specific mechanism(s) associated with the response of S. Typhimurium to SBS.

Nonparametric tests were used for timepoint zero comparisons of the Salmonella concentrations because they do not require any distributional assumption, which is advantageous for data acquired from the small sample size. On the other hand, ANOVA F- or t-tests require the assumption of normality and equal variance, which is not always possible to assume for small sample sizes (<30), especially when it is impossible to accurately test the assumption with our sample size (n=6).
Furthermore, the concentration of residual chlorine was not determined in this experiment. However, based on the negative control data, the impact of any previously added antimicrobials was not a significant source of pathogen reduction. Additionally, tetrathionate enrichment after acid neutralization with neutralizing buffered peptone water was performed and indicated no recovery of *S. Typhimurium* in the SBS treated water (data not shown). The decay of peracetic acid into acetic acid, was not tested in this experiment. However, this may help explain the survival and recovery of *S. Typhimurium* populations in peracetic acid compared to the total reduction observed with SBS. Walsh *et al.*, (2018) demonstrated that within 15 minutes 15.8 mg PAA/kg chicken decreased to 0.4 mg PAA/kg chicken resulting in a near equivalent increase in acetic acid concentrations. There is potential for microorganisms, such as *Salmonella*, to utilize acetic acid as a food source and recover from sanitization (Kitis, 2004; Stampi *et al.*, 2001).

The MIC and MBC data in this experiment indicate that over a 24-hour period, lower concentrations of PAA and IG-PAA are needed for inhibitory and bactericidal activity. To ensure chemical consistency, filtration was applied for the MIC assay, which impacts the organic loads present within the water. The presence of highly concentrated organic matter can slow the rate of disinfection with PAA (Casani *et al.*, 2005; Lillard, 1979; McKee, 2011; Gehr *et al.*, 2003; Gehr and Cochrane, 2002). This effect may influence the MIC and MBC results.

The main objective of this study was to evaluate the use of acidifiers for biological sanitation of poultry processing water in an effort to reuse and conserve water within the processing facility. Our results indicate that, in microcosms, total reduction can be observed with SBS, while no recovery of *S. Typhimurium* was found for SBS treatments via tetrathionate enrichment or direct plating after 5 minutes. This may be due to the difference in concentration
and the pH of these treatments. However, the concentrations of SBS were utilized due to previous results indicating no impact on sensory characteristics of skin on chicken carcasses (Dittoe et al., 2018a). The limit of peracetic acid concentration has recently been increased from 200 to 2000 ppm in poultry processing, and studies have detailed how PAA can be corrosive to equipment and caustic to the skin of personnel (Walsh et al., 2018; Peracetic acid, 2013; Casani et al., 2005). While SBS is generally recognized as safe (GRAS) (FDA, 2018), it should be noted that low pH can also be dangerous to personnel and further studies may be needed to validate the efficacy of SBS at a pH that is more typically seen within the poultry processing facility (Bauermeister et al., 2008a). The concentrations of SBS tested in this study were shown to be more effective in reducing S. Typhimurium in poultry processing reuse water microcosms than PAA or IG-PAA.

Conclusions

Environmental pressures will soon be felt throughout all food industries as fresh water becomes increasingly scarce. To combat this, the poultry industry may utilize reuse water systems but will face the risk of incomplete sanitation or an inability to reduce organic load. Our results showed a significant reduction of S. Typhimurium in microcosms containing acid sanitizers. In this study it was demonstrated that sodium bisulfate could be utilized in preventing S. Typhimurium contamination of processing reuse water. These results provide initial insights into the use of alternative antimicrobial treatments in alternative poultry production systems. A next logical step will be to determine if treated reuse water will reduce microbial cross contamination on chicken carcasses and not impact shelf life when substituted for fresh water. Lianou and Koutsoumanis (2013) concluded that phenotypic responses and genetic variation
among strains of the same foodborne pathogen are important to understand inactivation and growth characteristics. In *Salmonella* specifically, Andino and Hanning (2014) detail the importance of genetic variation within serovars in regard to virulence and control at the pre- and post-harvest level. As such, a multiple strain (cocktail) of *Salmonella* should be considered to determine if genetic variation plays a factor. In addition, testing at a pilot scale level will determine if SBS can practically be utilized on a much greater scale to reduce microbial contamination in poultry processing reuse systems. Finally, elucidating the mechanism(s) of the response by *Salmonella* and other microorganism to SBS should provide additional information for optimizing concentrations and more efficient means for application to reduce bacterial loads in reuse water.
## Tables

### Table 2.1: Impact of acidifiers on pH of *S. Typhimurium* inoculated reuse water at time point 0 and 30

<table>
<thead>
<tr>
<th>Treatment- Timepoint</th>
<th>Average pH(^1,2)</th>
<th>Lower CI(^3)</th>
<th>Upper CI(^3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Negative Control-0</td>
<td>7.47(^a)</td>
<td>7.35</td>
<td>7.59</td>
</tr>
<tr>
<td>PAA-0</td>
<td>4.06(^b)</td>
<td>3.94</td>
<td>4.17</td>
</tr>
<tr>
<td>IG-PAA-0</td>
<td>5.08(^c)</td>
<td>4.96</td>
<td>5.19</td>
</tr>
<tr>
<td>1% SBS-0</td>
<td>1.59(^d)</td>
<td>1.47</td>
<td>1.70</td>
</tr>
<tr>
<td>2% SBS-0</td>
<td>1.34(^d,e)</td>
<td>1.23</td>
<td>1.46</td>
</tr>
<tr>
<td>3% SBS-0</td>
<td>1.21(^e)</td>
<td>1.09</td>
<td>1.33</td>
</tr>
<tr>
<td>Negative Control-30</td>
<td>7.52(^a)</td>
<td>7.39</td>
<td>7.66</td>
</tr>
<tr>
<td>PAA-30</td>
<td>4.02(^b)</td>
<td>3.89</td>
<td>4.15</td>
</tr>
<tr>
<td>IG-PAA-30</td>
<td>5.13(^c)</td>
<td>5.00</td>
<td>5.26</td>
</tr>
<tr>
<td>1% SBS-30</td>
<td>1.58(^d)</td>
<td>1.45</td>
<td>1.71</td>
</tr>
<tr>
<td>2% SBS-30</td>
<td>1.36(^d,e)</td>
<td>1.23</td>
<td>1.49</td>
</tr>
<tr>
<td>3% SBS-30</td>
<td>1.23(^e)</td>
<td>1.10</td>
<td>1.36</td>
</tr>
</tbody>
</table>

\(^1\) Based on least square means  
\(^2\) Values with different superscripts indicate significantly different (P < 0.05)  
\(^3\) Based on 95% Tukey’s adjusted confidence interval
Figure 2.1: *Salmonella* Typhimurium Killing Assay over Time in Each Treatment

*S. Typhimurium* inoculated microcosms with five acidifier treatments were immediately plated onto XLD (n=6). Every 5 minutes after that for 30 minutes, plating occurred, and log-transformed counts were graphed. A value of 0.1 log CFU/mL was applied to visualize overlapping data points. Data points at zero were below the limit of detection (10 CFU/mL). The solid blue, cyan, and magenta line represents 1%, 2%, and 3% SBS treatments. The dashed red and green lines represent 200 ppm PAA and 200 ppm IG-PAA respectively. The black dashed line represents the no treatment negative control.
Figure 2.2: Impact on reuse water pH over time. The pH of reuse water with or without *Salmonella Typhimurium* inoculation after treatment with acidifiers. The average pH over three replicates at each time point is reported. *S. Typhimurium* was inoculated at time point zero at a concentration of $3 \times 10^7$ CFU/mL. The green diamonds represent pH of reuse water with *S. Typhimurium*. The red circles represent pH of reuse water without *Salmonella* addition. These data points are similar enough that overlaps occur.
**Figure 2.3:** Comparison of pH and *Salmonella* Typhimurium concentration at 0, 5, and 10 minutes. A) Represents comparisons of pH and *S.* Typhimurium concentration at time point 0 minutes. The pH was adjusted using the acidifier treatments. The inoculum added initially was $3 \times 10^7$ CFU/mL *S.* Typhimurium UK-1 to each microcosm (n=3). Plots (B) and (C) represent the comparison of pH and *Salmonella* concentration at 5 and 10 minutes and were generated in the same manner as (A).
Salmonella concentration (Log CFU/mL) vs pH at 5 minutes

- Neg. Con.
- PAA
- IG–PAA
- 1% SBS
- 2% SBS
- 3% SBS
Figure 2.4: (A) MIC of SBS against S. Typhimurium in reuse water with 0.01% TTC. One mL of 1% TTC was added to filtered reuse water which was inoculated with $5 \times 10^7$ CFU/mL S. Typhimurium UK-1. After a 24 hour incubation at 37 °C with shaking, the absorbance at 480 nm was taken and plotted against concentration (n=6). The MIC was determined to be 0.375% (3750 ppm). Plots (B) and (C) represent MIC against PAA and IG-PAA, respectively, and were generated in the same manner as (A), but using their respective acid sanitizers. The MIC was 0.05% (500 ppm) and 0.10% (1000 ppm), respectively.
MIC of PAA against S. Typhimurium

OD (480nm) vs PAA concentration
MIC of IG-PAA against S. Typhimurium

- OD (480nm)
- IG-PAA concentration
References


IV. Chapter 3. Comparison of acid sanitizers on the microbial composition of commercial poultry processing reuse water

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Abstract

Poultry plants have been evaluating the reuse of processing water. This is due to the environmental and financial concerns associated with water scarcity. There is a major concern with cross-contamination, as reused water may harbor human pathogens, spoilage microorganisms, or bacteria that readily form biofilms. Cost-effective strategies must be investigated to utilize reuse water and decrease the bacterial load before reuse. The most commonly used sanitizer in processing waters is peracetic acid (PAA), which has been approved for use in concentrations up to 2000 ppm. However, at high concentrations, PAA can be corrosive and hazardous to workers. Dry acid sanitizers, such as sodium bisulfate (SBS), may be utilized instead to mitigate these concerns. Sodium bisulfate has been demonstrated to have antimicrobial properties when used in the produce industry and in poultry litter. In this study, SBS and PAA were evaluated against microbial populations in reuse microcosms. Fresh, commercial poultry processing plant reuse water was collected at the end of a processing shift. The water was utilized within one hour of collection in microcosms, and plated onto TSA and XLD for the determination of aerobic bacteria counts and Enterobacteriaceae counts, respectively. Total genomic DNA was also extracted from microcosms for downstream microbiome evaluation. Treatments of 1%, 2%, and 3% SBS, and 200 ppm PAA reduced aerobic bacteria populations below the limit of detection, with the exception of 1% SBS at timepoint 30 minutes and 1% and 2% SBS at the 60-minute time point. All treatments were significantly different from the control (P < 0.05). Enterobacteriaceae counts were also significantly reduced below the limit of detection with the exception of 2% SBS at timepoint 30 minutes (P < 0.05). β-diversity metrics indicated that PAA did not significantly alter the microbiome of the reuse water compared to the control, while the SBS treatments did (q < 0.05).
Compositional analysis indicated that SBS significantly increased the operational taxonomic unit (OTU) abundance of some Enterobacteriaceae and Pseudomonas species but decreased others. The results of this study indicate that SBS is an equally effective sanitizer to PAA for reduction of aerobic bacteria in poultry reuse water; however, SBS impacts the microbiome in a unique way requiring further investigation.

**Introduction**

When factoring in seasonal variation, two-thirds of the world’s population (4 billion people) face severe water scarcity at least one month in any given year (Mekonnen and Hoekstra, 2016). With an increase in global population and the concomitant economic demands, these problems will likely become more pronounced. (Alcamo et al., 2007; Arnell, 2004; Schewe et al., 2014). In the U.S. there are large portions of the southwest, including the Great Plains and southern California, that already face considerable freshwater restrictions (Averyt et al., 2011). Water restrictions can be largely attributed to agricultural use, as 80% of water usage is relegated to meet primarily horticulture, livestock, and energy demands (Shannon et al., 2008). These environmental concerns, in turn, will generate economic pressure and food processing industries may need to respond through water conservation technologies.

In poultry processing, relatively contaminant-free water is used to deliver sanitation methods (Saravia et al., 2005; Micciche et al., 2018). Currently, on average, 26.5 liters of water are used per bird (Avula et al., 2009). Although studies have suggested that this usage could be reduced, doing so may pose an issue with effective implementation food safety measures (Lillard, 1979; Walsh et al., 2018). This is because water use is a primary component of Hazard Analysis and Critical Control Point (HACCP) plans, and even without sanitizers, water can be
effective as a wash in reducing biofilm-forming bacteria (USDA, 2016; Srey et al., 2013). For instance, the wash step uses 4.25L of water per carcass, and it is this water that has been proposed for reuse (Avula et al., 2009).

Water reuse is covered by 9 CFR 416.2, enforced by the United States Department of Agriculture (USDA), and is allowed if the water is equivalent to potable standards (USDA, 2016). Furthermore, the water should only be used in the same or upstream steps (USDA, 2016). Sanitizers such as chlorine or peracetic acid (PAA) are often employed during the wash steps in poultry processing (Tsai et al., 1992; Bauermeister et al., 2008; Kitis, 2004). These sanitizers are applied to the carcass to promote product safety, but they are also applied to reduce the bacterial load in the water during subsequent reuse (Micciche et al., 2018; 2019; Casani et al., 2005). However, free chlorine is regulated in reuse water to no more than 5 ppm (USDA, 2003). Studies have shown that low levels of chlorine (5 ppm) impact microbial populations in drinking water but allow for subsequent regrowth of biofilm producing bacteria such as Pseudomonas (Bertelli et al., 2018; Fish and Boxall, 2018). Peracetic acid may be utilized in higher concentrations and has been demonstrated to have bactericidal properties on pathogenic biofilms (Fatemi and Frank, 1999). As a consequence, acid sanitizers such as PAA have been utilized in poultry processing reuse water systems.

Peracetic acid has been shown to be effective against microbial populations on processed carcasses, including pathogens such as Campylobacter and Salmonella (Nagel et al., 2013). Currently, the maximum concentration allowed is 2000 ppm, which is a 10-fold increase from previous standards (21 CFR 173.370) (Casani et al., 2005; Walsh et al., 2018). Peracetic acid has been demonstrated to be corrosive towards equipment, hazardous towards workers, and can rapidly decompose into acetic acid which has minimal antimicrobial activity (Walsh et al., 2018;
Sodium bisulfate may be a suitable alternative as studies have shown its effectiveness against *Campylobacter* in poultry litter and against *Salmonella* on chicken drumsticks (Line, 2002; Dittoe *et al.*, 2019). *Salmonella* concentrations were also reduced on poultry carcasses using a 10% SBS wash (Yabin *et al.*, 1997; Line, 2002). Our previous findings indicated that 1%, 2%, and 3% SBS was effective against *Salmonella Typhimurium* in reuse water as concentrations were reduced below the limit of detection and no sublethal recovery was detected (Micciche *et al.*, 2019).

Microbial populations are known to persist in reuse water treatment systems used to process the drinking water supply (Stamps *et al.*, 2018). This occurs despite filtration and sanitation mechanisms including ultraviolet (UV) radiation (Stamps *et al.*, 2018). The goal of sanitation within water reuse should be to reduce bacterial loads with noted attention towards reducing concentrations of particular genera. Specifically, sanitation programs should be directed towards eliminating pathogens, biofilm forming bacteria, and, in the case of the food industry, spoilage organisms. This will help ensure food safety for subsequent uses of this water. The objective of this study was to investigate the microbial populations within reuse water using traditional microbiological and 16S rRNA gene amplicon sequencing techniques before and after the inclusion of acid sanitizers.

**Materials and Methods**

**Water Collection**

Processing waters were collected from an Arkansas commercial poultry processing facility at the intake of the reuse water sanitization system as described in Micciche *et al.*, (2019).
(n=5). Intake waters were chlorinated with 20 to 50 ppm of total chlorine at the facility. These were used in an inside-outside bird washer before water reuse. Water was transported to the laboratory at room temperature and utilized within one hour of sample collection.

**Microcosms**

Upon arrival, an aliquot (100µL) of each water sample was plated onto Tryptic Soy Agar (TSA) and Xylose Lysine Deoxycholate (XLD) (Difco Laboratories, Detroit, MI). Plates were incubated at 37°C for 24 hours. Furthermore, one mL was aliquoted, centrifuged at 6,700g for 10 minutes, and the pellet was frozen at -20°C for downstream DNA extractions. Reuse water was added to an Erlenmeyer flask with acid sanitizers to a volume of 20mL to generate the following concentrations: 0.02% PAA (v/v) (Sigma-Aldrich, St. Louis, MO, USA), and 1%, 2%, and 3% SBS (w/v) (Jones-Hamilton Co, Walbridge, Ohio). There were also no treatment controls included which did not have any sanitizer. Microcosms were placed in a shaking incubator at 25°C and covered to simulate turbulent flow through piping. After 30 and 60 minutes, aliquots were plated onto TSA and XLD, and for DNA extractions. A time point of 60 minutes was chosen as the endpoint of sample collection as waters are reused at this particular processing facility within one hour of sanitation. Plates were incubated for 24 degrees at 37°C. This was performed for each biologically independent replicate (n=5).

**DNA Extractions**

The DNA was extracted from frozen pelleted reuse water using the Gram-positive protocol for the QIAGEN DNeasy Blood & Tissue Kit (Qiagen, Valencia, CA), with the final elution from the spin column utilizing 50 µL nuclease free deionized water instead of the elution
buffer ATE. The DNA concentration was measured with a NanoDrop 1000 Spectrophotometer (Thermo Fisher Scientific, Waltham, MA) and diluted to 10 ng/µL. The extracted, diluted DNA was stored at -20°C until use in sequencing analysis.

Library Preparation and Sequencing

A library based on the V3-V4 region of 16S rRNA gene was created as detailed in Kozich et al. (2013) and Park et al. (2017). Briefly, dual-index primers, which included unique eight nucleotide barcode sequences were utilized to amplify each DNA sample. Sample amplification was verified using gel electrophoresis. Normalization was performed on PCR products using a SequalPrep™ Normalization kit (Life Technologies). Five µL of each sample was combined to generate a pooled plate library and concentrations were determined using a KAPA Library Quantification Kit (Kapa Biosystems, Woburn, MA), while product size was assessed using an Agilent 2100 Bioanalyzer System (Agilent, Santa Clara, CA). Each library was diluted to 20 nM and was mixed with HT1 Buffer along with a PhiX control v3 (20 nM) and 0.2 N fresh NaOH, to generate a final concentration of 12 pM. The resulting sample was combined with PhiX control v3 (5%, v/v) and 600 µL were loaded onto a MiSeq v2 (500 cycles) reagent cartridge.

Sequencing Analysis

Both demultiplexed R1 and R2 sequencing reads files were downloaded from the Illumina BaseSpace website and the QIIME2 pipeline (version 2018.11) was utilized for sequencing data processing (Bolyen et al., 2018). Paired end sequences were demultiplexed and then denoised through DADA2 (Callahan et al., 2016). The assembled sequences were utilized to
construct operational taxonomic units (OTUs) with 97% identity. Representative sequences were classified based on the Greengenes 16S rRNA gene database from phylum to genus levels (February 4th, 2012) (DeSantis et al., 2006). Subsequently, α- diversity (Observed OTUs, Shannon Diversity, Pielous Evenness, Faith PD) and β-diversity (Bray-Curtis, Jaccard Distance, Weighted and Unweighted UniFrac) were generated with the script -Qiime diversity core-metrics-phylogenetic within the QIIME2 2018.11 package. Principal coordinate analysis (PCoA) of β-diversity was also performed and plotted with QIIME2.

Statistical Analysis
A one-way analysis of variance (ANOVA) was performed using nonparametric test (Kruskal-Wallis, Siegel and Castellan, 1988) for aerobic and Enterobacteriaceae plate counts at time zero. When significant results were indicated by ANOVA, a pairwise comparisons test was performed on the differences between the mean values. Significance was determined at an α-level of 0.05. Non-microbiome data analyses were conducted using R (Version 3.5.1; R Core Team., Vienna, Austria). Pairwise comparisons of α-diversity of microbiome samples was also performed using nonparametric ANOVA (Kruskal-Wallis, Siegel and Castellan, 1988). Significance was determined at a false discovery rate (q-value) of 0.05. Pairwise comparisons of β-diversity were performed using permutational multivariate analysis of variance (PERMANOVA) with a significance level of (q < 0.05) (Anderson, 2001). Comparisons of significantly different OTUs across sample groups were performed by ANCOM through the QIIME2 q2-composition plugin (Mandal et al., 2015).
Results

Impact of Acid Sanitizers on Microbial Load

Data presented in Figure 1 and 2 show reductions of recoverable aerobic bacteria and Enterobacteriaceae at time point 30 and 60. As expected, no significant differences between the plate counts were observed before the addition of treatments (time point 0) (P > 0.05). At time point 30, when compared to the control, all treatments significantly reduced aerobic plate counts (greater than 5 log CFU) below the limit of detection (less than 5 CFU/mL) except 1% SBS, which retained a 1 log CFU/mL. At time point 60, detectable aerobic plate counts were observed for the 1% SBS and 2% SBS treatments. Compared to the control, all treatments at time points 30 and 60, except 2% SBS at time point 30, showed reductions of Enterobacteriaceae below the limit of detection (less than 30 CFU/mL).

Alpha and Beta-diversity of the Acid Sanitized Microcosms

Pairwise comparisons of α-diversity of treatments and time points were performed for each diversity metric (Observed OTUs, Shannon Diversity, Pielou’s Evenness, Faith PD) (Table 1). Kruskal-Wallis one-way ANOVA indicated no significant differences in α-diversity for any treatment across the diversity metrics at a significance level of q=0.05. Pairwise comparisons of β-diversity of treatments and time points were performed for each diversity metric (Bray-Curtis, Jaccard Distance, Weighted and Unweighted UniFrac) (Table 2). A PERMANOVA pairwise comparison test with 999 permutations was run, and significant differences were detected when evaluating the Bray-Curtis, Jaccard, and Unweighted UniFrac Distance Metrics. Sodium bisulfate treatments at time point 30 and 60 were significantly different from all treatments at timepoint 0, all PAA treatments, and all no treatments (NT) (q < 0.05). Peracetic acid treatments
at all timepoints were statistically similar to all NT samples at all timepoints (q > 0.05). No significant differences were detected between comparisons of Weighted UniFrac Distances. B-diversity metrics were visualized graphically through principal coordinate analysis (PCoA) plots with time fixed on the x-axis (Figure 3).

**Taxonomic Variation of the Acid Sanitized Microcosms**

Phylum level taxonomic profiles are detailed in Figure 4. The eight most abundant phyla represent 97% of the OTUs across all treatments and timepoints. At the phylum level, **Firmicutes** and **Proteobacteria** appeared to be the most abundant across all samples except one sample (2% SBS-30 minute) which had a high abundance of Cyanobacteria. Significant differences within taxonomic composition were determined via ANCOM. Table 3 details 45 OTUs that were determined to be significantly different across treatments (q < 0.05). The assumption that less than 25% of the OTUs do not significantly change is satisfied. The OTU (**Pseudomonas**) with the highest W value increased in relative abundance in the SBS treatments over time and has a low abundance in the PAA and NT samples. Other OTUs that were significantly different across treatments that represented **Pseudomonas** did not follow this observance and were higher in abundance in PAA and the NT. OTUs that correspond to **Lactobacillus** were identified more in the NT and PAA treatment. OTUs that correspond to **Enterobacteriaceae** were identified in all treatments. The abundance of the OTU that corresponds to **Campylobacter** was highest in PAA treatments while the abundance of OTU that corresponds to **Listeria** and **Escherichia coli** was higher within the SBS treatments. Of the ten most abundant OTUs that are significantly different across treatments, three corresponded to **Enterobacteriaceae** while three corresponded to
Pseudomonas (Figure 5). Aeromonas, Acinetobacter, and Shewanella were also represented in the volcano plot.

Discussion

Acidifiers may be used to control biological contamination within poultry processing facilities (Paster et al., 1987; Dittoe et al., 2018). Peracetic acid has been widely used due to its sanitation capabilities including against pathogens such as Salmonella and Campylobacter while not significantly impacting sensory characteristics of the finished product (Bauermeister et al., 2008; Kitis, 2004; King et al., 2005; Nagel et al., 2013). With this study, we aimed at evaluating the impact of SBS and PAA on bacterial loads in reuse water for poultry processing. The mechanism of disinfection for PAA is through the disruption of enzymatic activity by interacting with cysteine and double bonds (Block, 1991; Kitis, 2004; Lefevre et al., 1992; Liberti et al., 1999). The response mechanisms of bacteria to SBS, however, is not well defined although it has been regarded as a safer alternative as a sanitation and processing aide by the United States Environmental Protection Agency (EPA, 2018; Micciche et al., 2018). A prior study found that SBS could be used at concentrations of 1, 2, and 3% SBS with no discoloration of skin on poultry products (Dittoe et al., 2019). While 1% SBS did reduce aerobic bacterial loads by approximately 4 log CFU/mL, only 3% SBS and 200ppm PAA reduced the bacterial loads below the limit of detection.

Furthermore, there appeared to be regrowth of aerobic bacteria in the 1% SBS treatment. However, a longer time trial would be necessary to determine this. In our previous study, the same concentrations of SBS utilized here were able to completely reduce 3 x 10^7 CFU/mL concentrations of Salmonella below the limit of detection on plate counts, while 5 log CFU/mL reduction was observed using 200ppm PAA (Micciche et al., 2019). In Micciche et al., (2019)
the pH of SBS within microcosms was below 2. This indicates that the detected aerobic bacteria may be acidophiles.

Sustained alpha diversity for each treatment at each time point indicates that there was no significant variation within our biological replicates in terms of OTU richness, evenness, and taxonomy (Lozupone and Knight, 2008; Kim et al., 2017). Significant variation between the SBS treatments at time point 30 and 60 compared to time point 0 and the NT and PAA treatments suggest a compositional shift caused by the addition of the acidifier that did not occur when 200 ppm PAA was applied. While Bray-Curtis, Jaccard, and Unweighted UniFrac produced indicated these significant variations, when abundance was factored into the phylogenetic distance (Weighted UniFrac), there was no significant effect of treatment. This indicates that distinct phylogenetic groups are apparent, but the predominant abundance of taxonomic groups remain which are likely composed of the bacteria that were impacted by the sanitation and unable to grow (Lozupone and Knight et al., 2005). These potential shifts in the microbiome, further validated by the ANCOM results, suggest that some bacteria persisted and survived throughout all treatments despite low and no growth on the APC and XLD plates. This indicates that these bacteria remain viable but have entered a non-culturabl (VBNC) physiological state and that those microbiological techniques alone are not sufficient for detecting microorganisms within processing water. Several studies have indicated that pathogens, such as *Campylobacter* and *Salmonella*, as well as spoilage organisms, such as *Pseudomonas*, may persist in a VBNC state (Byrd et al., 1991; Keener et al., 2004; Mascher et al., 2000; Gupte et al., 2003).

The use of chlorine (> 0.1 mg/L free chlorine) within drinking water distribution systems has been shown to decrease bacterial diversity and created homogenous populations dominated by *Pseudomonas* (Bertelli et al., 2018). Within our samples, while the acidifiers modulated
which *Pseudomonas* OTUs are present, the genus was found to persist within all treatments and the control. This was also true with *Enterobacteriaceae*, which like *Pseudomonas*, contain species identified as spoilage organisms, potential pathogens, and biofilm formers (Shrivastava et al., 2004; Bertelli et al., 2018; Bush, 2010). Operation taxonomic units that correspond to potential pathogens such as *Campylobacter, Listeria, Aeromonas*, and *E. coli*, are also significantly impacted by the acid treatments but vary in relative abundance across the acidifier treatments. This suggests that a combination of acid sanitizers may be required to reduce VBNC bacteria of concern.

**Conclusions**

The scarcity of fresh potable water, driven through environmental factors, will be felt throughout the food industry. The reuse of water is a potential mitigation strategy for the poultry industry, but incomplete sanitation and persistence of pathogens and spoilage organisms is a concern. Acid sanitizers, such as SBS or PAA, may be used to alleviate this concern and our traditional microbiological techniques demonstrated this. However, through 16S sequencing, significant shifts in the microbiome were observed after the addition of sanitizers, which indicates the presence of VBNC cells. Furthermore, increases of OTUs corresponding to spoilage organisms and potential pathogens were identified over time within both the SBS and PAA treatments. As a consequence, while acid sanitizers may be utilized in poultry production reuse water systems, careful attention must be paid to the presence of VBNC spoilage organisms and pathogens.
### Table 3.1. Pairwise comparison of α-diversity

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<th>NT-60</th>
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**NOTES:**
- The table shows the pairwise comparison of α-diversity using SBS (Sample Based Similarity) and SBS-30 (Sample Based Similarity with a threshold of 30%).
- The values are calculated based on OTUs (Operational Taxonomic Units) observed.
- The Shannon Diversity values are also provided for each treatment.
<p>| Treatment | NT-0 | NT-30 | NT-60 | 1% SBS-0 | 1% SBS-30 | 1% SBS-60 | 2% SBS-0 | 2% SBS-30 | 2% SBS-60 | 3% SBS-0 | 3% SBS-30 | 3% SBS-60 | 200 ppm | 200 ppm |
|-----------|------|-------|-------|----------|----------|----------|----------|----------|----------|----------|----------|----------|----------|----------|----------|
| 3% SBS-0  | 0.64 | 0.72  | 0.72  | 0.56     | 0.12     | 0.31     | 0.92     | 0.12     | 0.12     | ---      | ---      | ---      | ---      | ---      | ---      |
| 3% SBS-30 | 0.12 | 0.21  | 0.28  | 0.21     | 0.72     | 0.23     | 0.12     | 0.56     | 0.20     | 0.34     | ---      | ---      | ---      | ---      | ---      |
| 3% SBS-60 | 0.21 | 0.82  | 0.72  | 0.56     | 0.28     | 0.85     | 0.21     | 0.72     | 0.21     | 0.21     | 0.21     | ---      | ---      | ---      | ---      |
| 200 ppm PAA-0 | 0.92 | 0.45  | 0.21  | 0.72     | 0.12     | 0.31     | 0.28     | 0.64     | 0.21     | 0.64     | 0.82     | 0.21     | ---      | ---      | ---      |
| 200 ppm PAA-30 | 0.72 | 0.82  | 0.12  | 0.64     | 0.12     | 0.21     | 0.72     | 0.21     | 0.64     | 0.28     | 0.82     | ---      | ---      | ---      | ---      |
| 200 ppm PAA-60 | 0.92 | 0.21  | 0.45  | 0.92     | 0.12     | 0.56     | 0.72     | 0.45     | 0.12     | 0.64     | 0.12     | 0.12     | 0.82     | 0.21     | ---      |
| NT-30     | 0.14 | ---   | ---   | ---      | ---      | ---      | ---      | ---      | ---      | ---      | ---      | ---      | ---      | ---      | ---      |
| NT-60     | 0.19 | 0.92  | ---   | ---      | ---      | ---      | ---      | ---      | ---      | ---      | ---      | ---      | ---      | ---      | ---      |
| 1% SBS-0  | 0.59 | 0.14  | 0.19  | ---      | ---      | ---      | ---      | ---      | ---      | ---      | ---      | ---      | ---      | ---      | ---      |
| 1% SBS-30 | 0.06 | 0.38  | 0.23  | 0.06     | ---      | ---      | ---      | ---      | ---      | ---      | ---      | ---      | ---      | ---      | ---      |
| 1% SBS-60 | 0.35 | 0.72  | 0.88  | 0.21     | 0.26     | ---      | ---      | ---      | ---      | ---      | ---      | ---      | ---      | ---      | ---      |
| 2% SBS-0  | 0.92 | 0.38  | 0.72  | 0.83     | 0.06     | 0.26     | ---      | ---      | ---      | ---      | ---      | ---      | ---      | ---      | ---      |
| 2% SBS-30 | 0.14 | 0.11  | 0.14  | 0.11     | 0.28     | 0.72     | 0.28     | ---      | ---      | ---      | ---      | ---      | ---      | ---      | ---      |
| 2% SBS-60 | 0.28 | 0.83  | 0.06  | 0.14     | 0.92     | 0.46     | 0.28     | 0.28     | ---      | ---      | ---      | ---      | ---      | ---      | ---      |
| Pielou's Evenness |
| 3% SBS-0  | 0.72 | 0.92  | 0.92  | 0.06     | 0.26     | 0.06     | 0.09     | 0.11     | 0.11     | 0.72     | ---      | ---      | ---      | ---      | ---      |
| 3% SBS-30 | 0.06 | 0.06  | 0.06  | 0.83     | 0.26     | 0.09     | 0.14     | 0.11     | 0.72     | ---      | ---      | ---      | ---      | ---      | ---      |
| 3% SBS-60 | 0.14 | 0.92  | 0.59  | 0.11     | 0.46     | 0.72     | 0.23     | 0.19     | 0.46     | 0.23     | 0.06     | ---      | ---      | ---      | ---      |
| 200 ppm PAA-0 | 0.83 | 0.23  | 0.23  | 0.59     | 0.06     | 0.46     | 0.19     | 0.46     | 0.23     | 0.46     | 0.72     | 0.46     | ---      | ---      | ---      |
| 200 ppm PAA-30 | 0.28 | 0.14  | 0.06  | 0.19     | 0.09     | 0.46     | 0.23     | 0.14     | 0.23     | 0.92     | 0.46     | 0.38     | 0.23     | ---      | ---      |
| 200 ppm PAA-60 | 0.38 | 0.11  | 0.11  | 0.92     | 0.09     | 0.21     | 0.19     | 0.23     | 0.06     | 0.92     | 0.06     | 0.23     | 0.23     | 0.28     | ---      |</p>
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1Pairwise comparisons were performed through Kruskal Wallis one-way ANOVA with a significance level of \( q = 0.05 \)
### Table 3.2. Pairwise comparison of β-diversity

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<td>NT-0 0.03 NT-30 0.03 NT-60 0.03</td>
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**Pairwise Comparison (q-values)**

<p>| Treatment | NT-0 | NT-30 | NT-60 | 1% SBS-0 | 1% SBS-30 | 1% SBS-60 | 2% SBS-0 | 2% SBS-30 | 2% SBS-60 | 3% SBS-0 | 3% SBS-30 | 3% SBS-60 | 200 ppm PAA-0 | 200 ppm PAA-30 | 200 ppm PAA-60 |
|-----------|------|-------|-------|----------|----------|----------|----------|----------|----------|----------|----------|----------|-----------|-------------|-------------|-------------|
| NT-30     | 0.74 | 0.74  | 0.74  | 0.74     | 0.74     | 0.74     | 0.74     | 0.74     | 0.74     | 0.74     | 0.74     | 0.74     | 0.74      | 0.74         | 0.74         |
| NT-60     | 0.36 | 0.36  | 0.36  | 0.36     | 0.36     | 0.36     | 0.36     | 0.36     | 0.36     | 0.36     | 0.36     | 0.36     | 0.36      | 0.36         | 0.36         |
| 1% SBS-0   | 0.03 | 0.03  | 0.03  | 0.03     | 0.03     | 0.03     | 0.03     | 0.03     | 0.03     | 0.03     | 0.03     | 0.03     | 0.03      | 0.03         | 0.03         |
| 1% SBS-30  | 0.03 | 0.03  | 0.03  | 0.03     | 0.03     | 0.03     | 0.03     | 0.03     | 0.03     | 0.03     | 0.03     | 0.03     | 0.03      | 0.03         | 0.03         |
| 1% SBS-60  | 0.03 | 0.03  | 0.03  | 0.03     | 0.03     | 0.03     | 0.03     | 0.03     | 0.03     | 0.03     | 0.03     | 0.03     | 0.03      | 0.03         | 0.03         |
| 2% SBS-0   | 0.03 | 0.03  | 0.03  | 0.03     | 0.03     | 0.03     | 0.03     | 0.03     | 0.03     | 0.03     | 0.03     | 0.03     | 0.03      | 0.03         | 0.03         |
| 2% SBS-30  | 0.03 | 0.03  | 0.03  | 0.03     | 0.03     | 0.03     | 0.03     | 0.03     | 0.03     | 0.03     | 0.03     | 0.03     | 0.03      | 0.03         | 0.03         |
| 2% SBS-60  | 0.03 | 0.03  | 0.03  | 0.03     | 0.03     | 0.03     | 0.03     | 0.03     | 0.03     | 0.03     | 0.03     | 0.03     | 0.03      | 0.03         | 0.03         |</p>
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**Jaccard Distance Metric**

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1Pairwise comparisons were performed through PERMANOVA with 999 permutations.
2Significant values are bold and underlined (q < 0.05)
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1. Taxonomic Grouping: The classification of bacterial taxa at different taxonomic levels.
2. Operational Taxonomic Unit Abundance: The number of reads for each taxonomic unit in the treatment samples.

5. The values in the table represent the number of reads for each taxonomic unit in the treatment samples.
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<sup>1</sup>The 45 OTUs detailed here were determined to be significant (W > 2090)

<sup>2</sup>Unable to be definitively identified at the genus level

<sup>3</sup>Unable to be definitively identified at the family level

<sup>4</sup>Identified as a previously uncultured and undetailed bacterium

<sup>5</sup>A pseudocount of 1 was applied
**Figures**

**Figure 3.1.** Aerobic bacterial concentrations of reuse water subjected to acid treatments Microcosms were plated onto TSA prior to acidifier treatment (n=5). Thirty and sixty minutes after the addition of the sanitizer treatment, plating occurred, and log-transformed counts were graphed. A value of 0.1 log CFU was applied to visualize overlapping data points. Data points at zero were below the limit of detection. The blue, orange, and green line represents 1%, 2%, and 3% SBS treatments. The purple line represents 200 ppm PAA and the red line represents the no treatment control.
Figure 3.2. *Enterobacteriaceae* concentrations of reuse water subjected to acid treatments. Microcosms were plated onto XLD prior to acidifier treatment (n=5). Thirty and sixty minutes after the addition of the sanitizer treatment, plating occurred, and log-transformed counts were graphed. A value of 0.1 log CFU was applied to visualize overlapping data points. Data points at zero were below the limit of detection. The blue, orange, and green line represents 1%, 2%, and 3% SBS treatments. The purple line represents 200 ppm PAA and the red line represents the no treatment control.
Figure 3.3. β-Diversity Principal Coordinate Analysis (PCoA) Plots. A) Bray-Curtis PCoA plot with Time as a fixed to the X-axis. Timepoint 0 is represented by circles, timepoint 30 minutes is represented by cylinders, and timepoint 60 is represented by diamonds. No treatment samples are red, 1% SBS samples are blue, 2% SBS samples are orange, 3% SBS samples are green, and 200ppm PAA samples are purple. Plots (B), (C), and (D) represent the Jaccard Distance, Unweighted UniFrac, and Weighted UniFrac plots respectively with Time as fixed to the X-axis.
Figure 3.4. Phylum Level Taxonomy Bar Plots. Relative abundance of major phyla among different groups. The eight most abundant phyla are detailed in the figure legend.
Figure 3.5. Volcano plot produced by the ANCOM method. The 10 most significant bacterial taxa are labelled. The absolute difference of the OTUs is represented on the x-axis (centered log ratio). The statistical significance of differential abundance is represented on the y-axis (W value). A W value above 2090 was determined to be significant by the ANCOM method.
References


V. Conclusion

With environmental concerns on the scarcity of fresh potable water, there is a need for industries to conserve and reuse water. However, prevalence and persistence of spoilage organisms and foodborne pathogens, such as *Salmonella*, within poultry processing waters indicates that appropriate sanitization is necessary prior to reuse. Economic loss can be accrued by the presence of foodborne pathogens, spoilage organism cross contamination, or re-contamination of food items due to reservoirs of microorganisms being established in the reuse water. Research continues on understanding the underlying mechanisms of acidifiers and sanitizers and how they impact bacterial loads and *Salmonella* within the processing environment. Future studies may investigate the neutralized acidifiers to determine if there is an underlying mechanism.

This thesis was focused on the impact of PAA and SBS on bacterial loads and *Salmonella* in processing waters in an effort to sanitize the water for its subsequent reuse. The objective of this research was to observe any differences between sanitizer efficacy and investigate how the microbial composition shifts in these waters. The results from Chapter Two (the *Salmonella* killing assay) provided evidence that *Salmonella Typhimurium* was more susceptible to sanitization from SBS at 1, 2, and 3% concentrations than 200 ppm (0.02%) PAA. Sodium bisulfate was able to reduce concentrations below the limit of detection after 5 minutes of treatment. However, the minimum inhibitory concentration was lower for PAA. This suggests further research including trials on SBS microcosms that use the MIC. In Chapter Three, while both treatments significantly reduced aerobic and *Enterobacteriaceae* plate counts, shifts in the microbiome were observed with SBS treatments having a significantly different beta-
diversity compared to the no treatment and PAA treatment. Analysis of composition testing also indicated that 45 OTUs were significantly different in composition across groupings suggesting an impact on pathogen and spoilage organism loads. It may be necessary to use a combination of sanitizers to reduce bacteria and prevent any regrowth of spoilage organisms. Future studies will help determine the use of these acidifiers to sanitize water for reuse in poultry processing. One particular avenue of interest is testing how multiple reuses of the sanitizers impacts their efficacy.
Appendix

Journal Permission

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I hope I have addressed your question. Please let me know if you have any other questions or concerns.

Best wishes,
Isobel

--
Isobel Martin
Review Operations Team Lead

--

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Review Operations Manager: Judyta Sorokowska-Yamin

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IBC Approval Letter: 18030

March 9, 2018

MEMORANDUM

TO: Dr. Steven Ricke

FROM: Bob Beitle, Acting Biosafety Committee Chair

RE: New Protocol

PROTOCOL #: 18030

PROTOCOL TITLE: The Use of SBS in Poultry Re-Use Water to Reduce Salmonella

APPROVED PROJECT PERIOD: Start Date March 8, 2018 Expiration Date March 7, 2021

The Institutional Biosafety Committee (IBC) has approved Protocol 18030, “The Use of SBS in Poultry Re-Use Water to Reduce Salmonella”. You may begin your study.

If modifications are made to the protocol during the study, please submit a written request to the IBC for review and approval before initiating any changes.

The IBC appreciates your assistance and cooperation in complying with University and Federal guidelines for research involving hazardous biological materials.
University of Arkansas
Institutional Biosafety Committee
Registration for Research Projects
Form 1: GENERAL INFORMATION

Principal Investigator Name: First: Steven M.I. C Last: Ricke

Please check the boxes for any of the forms below that are applicable to the research project you are registering. The General Information Form (Form 1) MUST be completed on all submitted project registrations, regardless of the type of research.

- [x] General information (must be completed) (FORM 1)
- [ ] Recombinant DNA (Even if it is exempt from the NIH Guidelines) (FORM 2)
- [x] Pathogens (human/animal/plant) (FORM 3)
- [ ] Biotoxins (FORM 4)
- [ ] Human materials/nonthuman primate materials (FORM 5)
- [ ] Animals or animal tissues and any of the above categories; transgenic animals, plant tissues; wild vertebrates or tissues. (FORM 6)
- [ ] Plants, plant tissues, or seed and any of the above categories; transgenic plants, plant tissues, or seeds (FORM 7)
- [x] CDC regulated select agents (FORM 8)
- [x] Notice to Pat Walker Health Center (FORM 9)

1. To initiate the review process, you must attach and send all completed registration forms via email to: ibc@uark.edu. ALL REGISTRATION FORMS MUST BE SUBMITTED ELECTRONICALLY.

2. To complete your registration, you must print out page 1 of this form, sign, date (by the Principal Investigator), and mail to: Compliance Coordinator-IBC, 1 Admin. Building, Fayetteville, AR 72701. You may also FAX it to 479-575-3848.

As Principal Investigator:
- [x] I attest that the information in the registration is accurate and complete and I will submit changes to the Institutional Biosafety Committee in a timely manner.
- [x] I am familiar with and agree to abide by the current, applicable guidelines and regulations governing my research, including, but not limited to, the NIH Guidelines for Research Involving Recombinant DNA Molecules and the Biosafety in Microbiological and Biomedical Laboratories.
- [x] I agree to accept responsibility for training all laboratory and animal care personnel involved in this research on potential biohazards, relevant biosafety practices, techniques, and emergency procedures.
- [x] If applicable, I have carefully reviewed the NIH Guidelines and accept the responsibilities described therein for principal investigators (Section IV-B-7).
- [x] I will submit a written report to the Institutional Biosafety Committee and the Office of Recombinant DNA Activities at NIH (if applicable) concerning: any research related accident, exposure incident, or release of rDNA materials to the environment; problems pertaining to the implementation of biological and physical containment procedures; or violations of the NIH Guidelines.
- [x] I agree that no work will be initiated prior to project approval by the Institutional Biosafety Committee.
- [x] I will submit in a timely fashion my annual progress report to the IBC.

Signature (PI): ________________________________ Date: __________________________
CONTACT INFORMATION

Principal Investigator:
Name: Dr. Steven Rieke
Title: Professor
Department: Food Science
Campus Address: FDSC E3
Phone#: A/C 575-4678 E-Mail: sricke@uark.edu
Fax#: A/C 575-6936

Co-Principal Investigator:
Name: Dr. Kristina Feye
Title: Postdoctoral Associate
Department: FDSC
Campus Address: BIOR 134
Phone#: A/C 281-705-2948 E-Mail: feye@uark.edu
Fax#: A/C 575-6936

PROJECT INFORMATION

Have you registered ANY project previously with the Institutional Bio Safety Committee? (Check one) Yes No
Are you registering a new project or renewing a previous project registration?

Yes

Project Title: The effect of various antimicrobial substances as poultry processing interventions on the
Project Duration: Start Date 5/25/2018 End Date 5/25/2020

Indicate what containment conditions you propose to use (check all that apply):
- Biosafety Level 1 (2,3)
- Animal Biosafety Level 1 (2,3a,3b)
- Plant Biosafety Level 1 (3)
- Biosafety Level 2 (2,3)
- Animal Biosafety Level 2 (2,3a,3b)
- Plant Biosafety Level 2 (3)
- Biosafety Level 3 (2,3)
- Animal Biosafety Level 3 (2,3a,3b)
- Plant Biosafety Level 3 (3)

NOTE: Hyperlinks for references throughout this form are available on the last page of this form.

References for Biosafety criteria:
(2) Biosafety in Microbiological and Biomedical Laboratories (BMBL) - 4th edition. CDC - Dept. of Health & Human Services - http://www.cdc.gov/niosh/biosafety/bmbl4/bmbl4toc.htm
(3) NIH Guidelines for work involving recombinant DNA molecules; "http://www4.od.nih.gov/oba/IBC/IBCindexexp.htm"

If working at BL-2, has your laboratory received an onsite inspection by the Biosafety Officer or a member of the IBC?
Yes  Date (if known) 05/30/2013 No (If No - schedule the inspection with the BSO)
Please provide the following information on the research project (Please DO NOT attach or insert entire grant proposals unless it is a Research Support & Sponsored Programs proposal):

**Project Abstract:**

Salmonella and Campylobacter are major concerns to the poultry industry as they are two of the leading causes of foodborne illness in the United States. Currently, poultry processing facilities employ various chemical and antimicrobial interventions to reduce pathogen loads on poultry products. However, these interventions are limited in their reduction of foodborne pathogens. Thus, various novel antimicrobial compounds are being explored as either alternatives or additives to current antimicrobials in processing facilities. (Sodium bisulfide) has been shown to inhibit Salmonella in poultry rinsates. The re-use of water by poultry plants is becoming an increasingly important and cost effective strategy for the poultry processing industry. Thus, it is essential to develop a strategy to utilize re-use water that is cost-effectively and promotes food safety. SBS is a strong antimicrobial candidate due to its documented anti-Salmonella effects. It is not known if those anti-Salmonella effects occur in re-use water. Further, it is also unknown as to the impact the use of SBS has on the re-use water microbiota and Salmonella biology. This project will ascertain the answers to those questions, as well as determine an optimal concentration for SBS in re-use water.
Specific Aims:

Objective 1: To identify the optimal applications (chiller, post-chiller, spray, dip) and concentrations of various antimicrobial compounds (surfactant, acidifier, etc.) in processing environments.

Objective 2: To determine the efficacy of various antimicrobial compounds (surfactant, acidifier, etc.) on mitigating the presence of inoculated pathogens such as Salmonella, Campylobacter, and etc.

Objective 3: To determine the impact various antimicrobial compounds (surfactant, acidifier, etc.) have on the microbiome of poultry products.
Relevant Materials and Methods: (this information should be specific to the research project)

Objective 1: Collaborating companies will supply fresh and un-treated re-use water from beginning and end of three shifts. Treated water is the generally regarded as safe water from the collaborating plant as it is actively monitored for pathogen adulteration and actively re-used in their facility. Un-treated water will be the water used in the plant that has not been treated by Tyson for re-use. On the day of the experiment, samples will be collected by a third party and dropped off to our lab. Salmonella enterica DT104 will be spiked into the different water and allowed to incubate stagnantly at 25°C for up to 1 hour, with subsampling at each time point (T=0, 15, 30, 60 minutes). Sub-sampling will require vortexing, which will occur in the biosafety hood. Centrifugation will occur outside of the biosafety hood. The Salmonella from each sub-sample will be quantified on nalidixic acid containing (20 g/mL) Xylose Lysine Deoxycholate (XLD) plates, XLD, and Tryptic Soy Agar. The serial dilutions from each time-point will then be subject to the standard USDA BAM enrichment protocol, and validated via qPCR analysis. The modified BAM protocol is as follows, dilutions will be grown overnight for pre-enrichment in buffered peptone water with sodium tetraphionate. The cultures will be diluted the next day (1:10) in Rappaport-Vassiliadis medium and grown overnight. The incubation periods will be in enclosed oscillating incubators. Care will be taken to ensure that samples are handled to minimize aerosolizing the cultures. As a result, throughout the entirety of the Masks, goggles, lab coats, and gloves will be donned for the whole of experiment and at all stages to reduce personal exposure risks.

Objective 2: The non-enriched neat dilution (or undiluted) of the rinsates will be subject to DNA extraction and microbiome analysis.

Objective 3: Non-enriched isolates of Salmonella will be subjected to a 96-well micro-well antibiogram and virulence assays. Briefly, 200 µL of fresh Luria broth will be aliquoted into 96-well plates. Salmonella will be individually picked and inoculate each well of the plate. The Salmonella will be grown overnight at physiological temperature, then pin replicated into 96 well-plates containing doubling dilutions of SBS or breakpoint concentrations of enrofloxacin, cefurox, chloramphenicol, cefepime, azithromycin, minocycline, ceftriaxone, augmentin, ampicillin, sulfisoxazole, trimethoprim-sulfamethoxazole, tetracycline, gentamicin, or streptomycin. Resistant isolates will be tested for the presence of the Salmonella genomic island 1 (SGI1) integron and other antibiotic resistance genes using standard endpoint PCR. Further, isolates recovered from the non-enriched cultures will be evaluated for changes in virulence using an established invasion assay and qPCR.

Biohazards: Possible exposure to pathogens via aerosols. To protect researchers from this risk, scientists will wear nitrile gloves, a face shield, goggles, a lab coat, and observe standard aseptic procedures. All activities that produce aerosolization, such as vortexing, will be done in a biosafety cabinet. Researchers will follow those above protective personal equipment and University of Arkansas biosafety procedures at all times.

* This information can be attached as an electronic document (Word or Word Perfect formats) or entered in the space provided (If you add an attachment, to your e-mail or FAX. Please indicate "see attached" and list the file name in the space provided):
PERSONNEL QUALIFICATIONS & FACILITY INFORMATION:
List all personnel (including PI and Co-PI) to be involved in this project:

<table>
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<tr>
<th>Name</th>
<th>POSITION</th>
<th>QUALIFICATIONS/TRAINING/RELEVANT EXPERIENCE</th>
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<tbody>
<tr>
<td>Steven C. Ricke, PhD</td>
<td>Bacteriology/Anir</td>
<td>20 years experience with anaerobic bacteria and foodborne pathogens</td>
</tr>
<tr>
<td>Peter Rubinelli, PhD</td>
<td>Molecular Cell Biol</td>
<td>20 years experience with pathogens</td>
</tr>
<tr>
<td>Kristina Feye, PhD</td>
<td>Biomedical Sciences</td>
<td>5 years experience with pathogens, 3 years experience</td>
</tr>
<tr>
<td>Laura Meyer, MS</td>
<td>Animal Science, Prog</td>
<td>2 years experience with BSL2 pathogens</td>
</tr>
<tr>
<td>Jennifer Wages, MS</td>
<td>In Operational Mar</td>
<td>15 years experience with pathogens</td>
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<tr>
<td>Andrew Miccichi, grad student</td>
<td></td>
<td>5 years experience with pathogens</td>
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<tr>
<td>Dana Ditoe, MS</td>
<td>Poultry Science</td>
<td>4 years experience working with pathogens</td>
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<tr>
<td>Zhaohao Shi, grad student</td>
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<td>3 years experience working with pathogens</td>
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<tr>
<td>Aaron Bodie, grad student</td>
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<td>3 years experience working with pathogens</td>
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<tr>
<td>Julie Atchley</td>
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<td>4 years experience working with pathogens</td>
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Additional Personnel Information (if needed):

Conner Sherman, undergraduate, 0 years of experience with pathogens
Madison Looper, undergraduate, 0 years experience with pathogens
List all the laboratories/facilities where research is to be conducted (specify building, room number and category for each (e.g. laboratories, cold/warm rooms, animal care facilities or farms, growth chambers and greenhouses, biological material storage areas, tissue culture rooms.))

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Additional Laboratory/Facility Information (if needed):

* Biohazard signs are required for entrances to Biosafety Level 2 areas (including Animal Biosafety Level 2 areas). The Office of Environmental Health & Safety will supply these signs.

If an updated biohazard sign is required, please indicate the location and what agents/organisms/hazards should be listed on the sign in addition to what is being registered. Describe below:
SAFETY PROCEDURES:

Please indicate which of the following personal protective equipment (PPE) will be used to minimize the exposure of laboratory personnel during all procedures requiring handling or manipulation of the registered biological materials.

GLOVES:

[ ] Latex  [ ] Vinyl  [x] Nitrile  [ ] Leather  [ ] Other (specify)

FACE & EYE PROTECTION:

[ ] Face Shield  [x] Safety Goggles  [ ] Safety Glasses  [ ] Other (specify)

CLOTHING PROTECTION:

[ ] Disposable clothing protection  [ ] Re-usable Coverall

[ ] Re-usable Lab Coat  [ ] Other (specify)

How will protective clothing be cleaned once dirty or contaminated? (Check all that apply)

[ ] Autoclaved prior to laundering or disposal  [ ] Laundered in on-site facilities with bleach

[ ] Laundered with qualified commercial service  [ ] Other (specify)

Outline procedures for routine decontamination of work surfaces, instruments, equipment, glassware and liquid containing infectious materials (Autoclaving or fresh 10% bleach as a chemical disinfectant are preferred treatments; please specify and justify exceptions):

Work surfaces will be decontaminated with a freshly prepared 10% bleach solution before and after working. Exception is biosafety cabinets which will be disinfected before and after use with Lysol® No Rinse Sanitizer in order to avoid the corrosiveness of the bleach on the metal of the biosafety cabinets. Instruments and equipment will be decontaminated by wiping down with 10% bleach. Paper towels used for these purposes will be discarded in biohazard bags. Glassware, waste, and disposable tubes will be autoclaved under standard conditions (15 psi, 121 C, 20 min). Disposable items (pipette tips, pipets, etc) will be discarded into a biohazard bag for autoclaving before disposal.
Describe waste disposal methods employed for all biological and recombinant materials used. (Please include methods involving the following types of waste)**

**Sharps:** Placed into 10% bleach solution for decontamination followed by discarding into sharps waste container

**Cultures, stocks, and disposable labware:**
- Liquid cultures in tubes will be autoclaved prior to disposal down a drain.
- Cultures in petri dishes are disposed of in biohazard bags which are autoclaved.

**Pathological Waste:** Not Applicable

**Other:** Rinsates will be disposed of in biohazard bags which are autoclaved and disposed of in the trash.

**For more information, please reference the Biological Safety Manual at http://ehs.uark.edu/DocumentPages/BiosafetyManual04.pdf**

Indicate autoclave location(s) used for waste disposal and describe autoclave validation procedures:

Autoclaves are located in Biomass 101. The autoclaves are checked monthly using SteriGage test strips (3M) and SporAmpule vials to ensure autoclaves completely sterilize all bacterial life forms including spores.

Will you be using a biological safety cabinet? (choose one)  
- Yes  
- No

If yes, please provide the following information:

<table>
<thead>
<tr>
<th>SERIAL NUMBER</th>
<th>CERTIFICATION EXPIRATION DATE</th>
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<td>Biosafety Cabine</td>
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Form 1: GENERAL INFORMATION, contd.

Indicate if any of the following aerosol-producing procedures will occur:

- Centrifuging
- Grinding
- Blending
- Vigorous shaking or mixing
- Sonic disruption
- Pipetting
- Dissection
- Stomacher
- Inoculating animals intranasally

Other (please describe):

Describe the procedures/equipment that will be used to prevent personnel exposure during aerosol-producing procedures:

Centrifugation and Stomacher: Centrifugation and Stomacher use will occur outside of a biosafety hood. Researchers will observe proper PPE and procedures which will include: a laboratory coat buttoned over street clothes, two pairs of gloves, goggles and a face shield.

Pipetting, Vortexing (Vigorous Shaking): Mechanical pipettors will always be used. This will occur in the biosafety cabinate at all times. Researchers will observe proper PPE and procedures which will include: a laboratory coat buttoned over street clothes, two pairs of gloves, goggles and a face shield.

EMERGENCY PROCEDURES

In the event of personnel exposure (e.g. mucous membrane exposure or parenteral inoculation), describe what steps will be taken including treatment, notification of proper supervisory and administrative officials, and medical follow up evaluation or treatment.

In the event of accidental exposure of personnel the person exposed should notify the laboratory supervisor immediately. Treatable exposures will be treated by use of the first aid kit. Mucous membrane exposure or puncture with contaminated material will result in the person being taken to the Health Center for evaluation.
Form 1: GENERAL INFORMATION, contd.

In the event of environmental contamination, describe what steps will be taken including a spill response plan incorporating necessary personal protective equipment (PPE) and decontamination procedures.

In case of a large environmental spill, persons interacting with spilled area should protect themselves with personal protective equipment such as gloves, eye mask, coat, etc. The area will be secured to prevent spreading. Appropriate disinfectant dependent upon spill will be selected. Freshly prepared 10% bleach as a chemical disinfectant is a preferred treatment. All spill-related material will be autoclaved. All individuals exposed to the environmental contamination will be sent to receive medical attention. The incident will be reported to biological safety officer and principal investigator.

TRANSPORTATION/SHIPMENT OF BIOLOGICAL MATERIALS

As per the Department of Transportation 49 CFR Parts 171-173 (5), some biological materials are regulated as hazardous materials and require special training of all personnel involved in shipping. Will you be transporting or shipping any of the following off campus? (Yes or No)

☐ Yes  ☑ No

If yes, check all that apply.

☐ Cultures of human or animal pathogens

☐ Environmental samples known or suspected to contain a human or animal pathogen

☐ Human or animal material (including excreta, secretions, blood and its components, tissue or tissue fluids, and cell lines) containing or suspected or containing a human or animal pathogen
Form 1: GENERAL INFORMATION, contd.

Have you or anyone in your lab involved in packaging, labeling, or completing/signing paper work received training to ship infectious substances or diagnostic specimens within the past 3 years? (Choose Yes or No.)

☐ Yes
☒ No

If yes, please provide the following information.

<table>
<thead>
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REFERENCES

1. Research and Sponsored programs Research Compliance: http://avcf.uark.edu/


4. University of Arkansas Office of Environmental Health & Safety: http://ehs.uark.edu/

Andrew Micciche

Graduate Student at the University of Arkansas

- Interests in food safety in alternative poultry processing systems, including water reuse systems.
- Future career path in the food industry with focus towards a managerial role in food safety and quality assurance.

Education

UNIVERSITY OF ARKANSAS, Fayetteville, AR
Masters in Science, 3.89 GPA
Major Adviser: Dr. Steven Ricke
- Major: Food Science
- Concentration: Food Safety
- Food Science Graduate Assistantship
- Expected Graduation year: August, 2019

UNIVERSITY OF SOUTH FLORIDA, Tampa, FL
Bachelor of Science, 3.99 GPA
- Major: Microbiology
- Minor(s): Psychology, General Business
- University of South Florida’s Deans List of Scholars (awarded every semester), 2013-2017
- University of South Florida’s Honors College Student, 2013-2017
- GRE: 161V/163Q/5.0 Writing
- Graduation Date 5/2017

Recent Awards and Grants

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<td>Kosove Society Finalist, 2013</td>
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<td>Florida Academic Scholars, 2013-2017</td>
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Publications and Presentations

Publications

Manuscripts in Submission
Manuscripts in preparation


Presentations

1) Olson, E., Micciche A., Ricke S., and Ghosh A. 2019. Understanding the characteristics of bacterial isolates obtained from commercial poultry feed using whole genome sequencing approach. 16th annual Capitol Graduate Research Summit, Capitol Graduate Research, Topeka, KS February 26, 2019

2) Micciche A., Feye K., Wages J., Knueven C., Ricke S., (September, 2018). Impact of acid treatments on Salmonella in reuse poultry processing waters. Poster presented by Andrew Micciche at the Arkansas Association for Food Protection, Fayetteville, AR.

3) Micciche A., Feye K., Wages J., Knueven C., Ricke S., (September, 2018). Impact of acid treatments on the microbial populations of reuse poultry processing waters. Poster presented by Andrew Micciche at the Arkansas Association for Food Protection, Fayetteville, AR.

4) Olson E., Micciche A., Ricke S., Ghosh A., (September, 2018). Understanding the characteristics of bacterial isolates obtained from commercial poultry feed using whole genome sequencing approach. Poster presented by Elena Olson at the Arkansas Association for Food Protection, Fayetteville, AR.


12) Micciche A., (2016, April). Innovation through gunpowder: The advancement of incendiary weapons during the Five Dynasties and Ten Kingdoms period and the Song dynasty. Poster presented at the Undergraduate Research Colloquium at the University of South Florida, Tampa, FL.
Research Experience

**DR. RICKE’S RESEARCH LABORATORY, Fayetteville AR-**
Center for Food Safety Laboratory focusing on pathogen reduction through vaccinations, prebiotics, probiotics, impacting the GIT microbiome, and acidifiers post-harvest.

**Graduate Student, 08/2017 to Present**
Current works focuses on the identification of the microbiome within feed, including DNA extraction optimization, as well as the remediation of *Salmonella* within the processing environment. Specific processing environment focuses include the chiller tank and reuse water systems. Assisted with writing a Grant on Reducing *Salmonella* and *Campylobacter* on Chicken Carcasses by Sodium Bisulfate (SBS) and Investigation into its Reuse to Reduce Water Consumption through the National Association for Meat Institute.

**Selected Accomplishments:**
- Awarded two first place and one second place award for my poster presentations at the Arkansas Associate for Food Protection
- Was awarded the Distinguished Doctoral Fellowship to complete my research within this laboratory
- Was able to present my work through three presentations at the 2018 Poultry Science Association conference in San Antonio, TX. Awarded a Travel Grant through the Department of Food Science

**DR. HARWOOD’S RESEARCH LABORATORY, Tampa FL-**
Water Quality and Microbial Ecology Laboratory focusing on Microbial Source Tracking (MST), understanding the ecological role of *Vibrio vulnificus*, evaluation of fungal capture of heavy metals in battery degradation, and understanding the ecological of vancomycin resistant enterococci.

**Undergraduate Researcher, 06/2016 to 06/2017**
Laboratory Technician, 06/2017 to 08/2017
Worked on a series of trials related to measuring the effectiveness of hollow fiber filtration and foam elution by identifying the capture rate of Human Polyomavirus (HPyVs) and fecal indicator bacteria from sewage spiked waters. Performed DNA extraction and PCR techniques in order to ascertain the effectiveness of several MST markers. Performed qPCR on cyanide resistant bacteria isolates.

**Selected Accomplishments:**
- Completed an honors thesis approved to test antimicrobial efficacy of chitosan microparticles on multiple strains of *Vibrio vulnificus*.

**DR. LIMAYEM’S RESEARCH LABORATORY, Tampa FL-**
Microbiology lab focusing on the effectiveness of alternative large scale antimicrobials for usage in biofuels and biomedical industries.

**Undergraduate Researcher, 03/2015 to 05/2016**
Laboratory Technician, 09/2015 to 07/2016
Worked on a series of experiments and graduate level analysis of data and literature. Performed DNA extraction and PCR techniques in order to ascertain the identity of isolated contaminants within algae samples. Prepared and investigated minimum inhibitory concentration experiments to serve as the quantitative data for a patent pending promising antimicrobial. Contributed and assisted in the writing, data generation, and revision of two published manuscripts and three poster presentations.

**Selected Accomplishments:**
• Received praise for the ability to assess literature and write graduate-level reports pertaining to the requested information and effectively represented laboratories work through my Goldwater nomination.
• Offered position of part-time Laboratory Technician on basis of dedication to the lab as well as leadership, organization, and time management skills.
• Lead undergraduate contributor on both manuscripts pending publication, 2nd author of latest publication, lead poster presenter at ASM 2016.

**DR. MALMBURG’S & DR. DUBÉ’S RESEARCH LABORATORY, Tampa FL**
Cognition modeling lab specializing in electroencephalograph analysis and memory modeling.

**Undergraduate Researcher,** 11/2013 to 12/2014
Worked on a series of experiments with increasing independence. Ran participants in EEG and memory modeling experiments and assisted in EEG data analysis. Participated greatly in lab discussion and proposed many ideas and confounds for future experiments. Working with a graduate student, we designed and helped code (using Python) for an experiment that analyzed the effect of contrast when participants are given multiple variables to remember.

**Selected Accomplishments:**
• Received a positive letter of recommendation from Dr. Dubé for my work within the lab.
• Was able to accurately ascertain the effectiveness of OpenSesame over PsychoPy and Authorware.

**Professional Experience**
**USF HONORS COLLEGE** and **USF INTO, Tampa FL,**
**Paid Tutor,** 10/2014 to 07/2017
Developed innovative explanations for a variety of difficult concepts in chemistry, organic chemistry, statistics, biology, and psychology. Used an all-encompassing approach to clarify concepts for tutees struggling with majority of the coursework.

**Selected Accomplishments:**
• Increased tutees average test grade by ~18%.
• Worked extensively with one tutee to improve their grade from a C to an A.
• Earned recommendations from tutees for excellence in explaining concepts in unique ways that made them more simplistic and drastically improved understanding.

**Skills**
- MIC/MBC analysis
- PCR and qPCR sequencing
- Poultry Necropsies
- Pre-harvest bird management
- Post-harvest carcass management
- 16S rRNA microbiome sequencing
- DNA extraction
- Membrane filtration
- Creative Team Leadership
- Teaching/Tutoring
- R and statistical programming
Technological Experience

**Very Good Knowledge:** MS Office-Certified, ChembioDraw, Google Drive (Docs, Sheets, Slides), Dropbox

**Moderate Knowledge:** Python programming language, Endnote, BLAST, QIIME 2

**Basic Knowledge:** R (data analysis), PsychoPy, Authorware, JMP

Extracurricular Activities

**Clubs & Organizations**

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<th>Club</th>
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<tr>
<td>Food Science Club</td>
<td>2017-</td>
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<tr>
<td>Community Organizer</td>
<td>2017-</td>
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**Community Service**

<table>
<thead>
<tr>
<th>Service</th>
<th>Year</th>
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<tbody>
<tr>
<td>Soup Kitchen-Pantry Stacker, Cook, Cleaner, and Server</td>
<td>2009-</td>
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<tr>
<td>Dominican Republic Service Trip</td>
<td>2013</td>
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References Provided on Request