Evaluation of various antimicrobial agents to reduce the load of *Salmonella* and *Campylobacter* in poultry processing

by

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ABSTRACT

Poultry meat and products have been highly associated with outbreaks related to *Salmonella* and *Campylobacter*, and different interventions have been used for the reduction of these pathogens, with more emphasis on the processing plant facility with antimicrobial evaluation. In recent years, the consumer has increased the pressure on chemical-free food products. But novel interventions have not been adopted due to the high costs they represent. We conducted two studies with the objective of exploring new potential antimicrobial alternatives. Briefly, to accomplish these objectives, an *in vitro* experiment was conducted using a 96 wells plate inoculated with 10^4 CFU of *Salmonella* Typhimurium or *Campylobacter*. Plates were exposed to LED light (430 nm) for 0 or 5 mins to evaluate photodynamic therapy as an alternative using two different photoactive compounds, curcumin (CUR) and chlorophyllin (CH) at 100, 500 and 1000 ppm in comparison with a common antimicrobial used in the industry peracetic acid (PAA) at 100, 200, 300 ppm. These results indicate that CUR and CH were ineffective as antimicrobials under evaluated conditions, particularly compared to the commonly used antimicrobial, PAA. In the second study, pelargonic acid (PA) and lactic acid (LA) were evaluated against *Salmonella* Infantis (10^5 CFU/ml) alone and in combination with surfactants Saponin and Tween 80 at different concentrations *in vitro* and based on the results on chicken wing flats. PA was effective in the reduction of *Salmonella* Infantis in vitro but was ineffective on the chicken wing flats compared with a low concentration of 75 ppm PAA. These new alternatives needs more evaluation and standardization for be considered as potential antimicrobial for the poultry industry in the future.

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LIST OF ABBREVIATIONS

PAA	Peracetic acid
CUR	Curcumin
CH	Cholophyllin
MHB	Mueller Hinton Broth
BF-BEB	Blood Free Bolton Enrichment Broth
PA	Pelargonic acid
LA	Lactic Acid
SAP	Saponin
TW80	Tween 80

CHAPTER 1: LITERATURE REVIEW

1.1 INTRODUCTION

Chicken meat has consistently been recognized as a source of foodborne pathogens. This concern starts from the farm production of live birds until the final product is available to consumers. *Salmonella* and *Campylobacter* are two of the main pathogens related to poultry meat and products. A specific serovar *Salmonella* Infantis, is one of the main focuses of this thesis. Food safety covers all aspects of food, from chemical to biological contamination. Food antimicrobials are compounds that are generally used to extended the lag phase or kill microorganisms. Poultry processing has moved from chlorine as an antimicrobial intervention during poultry processing to peracetic acid (PAA), which is the most common chemical intervention.

The main concern in using PAA as a chemical antimicrobial in the poultry processing industry is the occupational health concern. PAA is corrosive and irritating to eyes, mucous membranes of the respiratory tract, and skin. Poultry processing plants can even spend more than a million dollars per year to minimize the presence of these pathogens in the final raw product. Taking this in consideration, the increase in regulatory restrictions and negative consumer perception of chemical compounds in foods have contributed to the pressure for the development of alternative antimicrobial agents.

Photo-active compounds can be an alternative, such as curcumin and chlorophyllin, which have demonstrated an antimicrobial activity followed by light activation (Castano et al., 2004; Gao and Matthews, 2020).Photo-inactivation of foodborne pathogens applies to the disinfection of food products because it is a simple two-step procedure using photo-active substances; 1) the photoactive compound and 2) harmless visible light. In recent years the

implementation of organic acids as an alternative for the reduction of different microorganisms has increased.

Some of these organic acids are pelargonic acid and lactic acid, which have been evaluated for their antimicrobial activity. But, the poor solubility in water phase is one of the main concerns. Surfactant type and concentration can either synergistically strengthen or impede the antimicrobial activity of an emulsion. Saponin and Tween 80 are surfactants that are approved to use in the food industry.

1.2 FOOD SAFETY CONCERNS AND PATHOGENS RELATED TO POULTRY PROCESSING INDUSTRY

1.2.1 *Salmonella* and *Campylobacter* are foodborne pathogens

Chicken meat has consistently been recognized as a possible source of foodborne pathogens. This concern starts from the farm production of live birds until the final product available to consumers. *Salmonella* and *Campylobacter* are two of the main pathogens related to poultry meat and products. Any disease or toxic nature caused by or possibly caused by the consumption of water or foods is called foodborne disease or illness (Adams and Moss, 2008). *Campylobacter* and *Salmonella* were reported as the most frequent bacterial cause of foodborne illnesses in 2016 according with the numbers reported by FoodNet. For *Campylobacter* was reported 8,547 and *Salmonella* 8,172 illnesses, respectively (CDC, 2017).

Food control is necessary for consumers as well for the industry to gain the confidence of consumers and strengthen the market. The foodborne diseases are important to governments, food industries and the common public (Adak et al., 2002). Food safety covers all aspects of food from chemical to biological contamination. Different tools and programs have been implemented to promote food safety from farm to fork such as Microbiological Risk Assessment

(MRA) and Hazard Analysis Critical Control Point (HACCP) systems (Perni et al., 2009; WHO, 2010).

1.2.2 *Salmonella* in poultry

Salmonella spp. has been identified as one of the primary etiological agents attributed to food-borne illnesses of poultry origin (Painter et al., 2013). Consumption of food contaminated with *Salmonella* can result in non-typhoidal salmonellosis. According to the Center for Disease Control (CDC), *Salmonella* causes about 1.35 million infections, 26,500 hospitalizations, and 420 deaths in the United States every year (CDC, 2021a). More than 2,500 described serovars of *Salmonella,* and many of those related to human infections are highly present in broiler meat (Foley et al., 2008). This is because the primary reservoir is the intestinal tract of animals such as chickens, and colonization is favored by intensive animal production to fill the consumers demand (Bryan and Doyle, 1995).

Contaminated poultry, meat, and eggs are important vehicles of *Salmonella* infections, there are different factors involved in the colonization of *Salmonella* in poultry, including the age and genetic susceptibility of the birds, bird stress due to overcrowding or underlying illness, the level of pathogen exposure, competition with gut microflora for colonization sites, and the strain of *Salmonella* present (Bailey, 1988; Foley et al., 2008).

Bacteria can easily attach to many surfaces (Berkeley et al., 1980). One suggestion made in 1974 explain that bacteria can be easily attached to the skin of broiler chickens during, in this experiment they evaluated different parameters and concluded that flagellated bacteria are attached more easily than non-flagellated bacteria (Notermans and Kamplemacher, 1974). It has been demonstrated that bacteria are firmly attached to carcasses before processing is initiated, and *Salmonella* is a clear example (Lillard et al., 1989). To obtain better results in the processing

plant, it is important to obtain *Salmonella*-free carcasses, and consequently, it is essential to look for alternatives to reduce *Salmonella* load on birds (Lillard, 1989). Once *Salmonella* is attached to the skin it is extremely difficult to remove or control in the plant (Lillard, 1989). Other sources are the leakage of intestinal contents/feces during processing, contaminated processing equipment, water, and the hands of processing workers (Thames and Sukumaran, 2020).

The Food Safety and Inspection Service (FSIS) is worried about the incidence of Salmonellosis related to poultry and for that reason has a complete guideline for controlling *Salmonella* in raw poultry. Guidelines include the implementation of HACCP programs, sanitation, and the use of antimicrobial interventions. The performance standards given by the USDA are not specifically to determine the prevalence of *Salmonella* on different products but to monitor the effectiveness of the processing procedures in limiting the contamination (FSIS, 2019). The maximum acceptable percentages of those positive for Salmonella in a 52-week period are 9.8%, 25% and 15.4% for broiler carcasses, comminuted chicken and chicken parts, respectively (USDA-FSIS, 2019).

1.2.3 *Salmonella* Infantis

The genus *Salmonella* contains two species: *S. enterica* and *S. bongori*. There are many subspecies and serotypes within each species. There are approximately 2500 different serotypes identified in the *S. enterica* species described by the World Health Organization. Even with these high numbers only a fraction are responsible for the majority of foodborne outbreaks. Based on a surveillance from the CDC, in 2018 the three most common serotypes related to human infections were *S*. Enteritidis, *S*. Newport and *S*. Typhimurium (CDC, 2018).

Three of the predominant serotypes found in broiler meat were *S*. Typhimurium, *S*. Enteritidis, and *S*. Heidelberg (Foley et al., 2008; Antunes et al., 2016; Heredia and Garcia,

2018). From 1990 to 2010 at least 28 outbreaks of human *Salmonella* infections have been linked to live poultry exposure, with an occurrence of 1 to 4 outbreaks per year (CDC, 2012a).

In recent years, the attention has focused on *Salmonella* Infantis, which is also commonly isolated in broilers (Ferrari et al., 2019). Poultry is considered a significant sources of *S*. Infantis (Gymoese et al., 2019). Around 2 to 4% of the serotypes found in poultry meat and products are Infantis (USHHS-NARMS, 2019). Another essential factor of *S*. Infantis is the high antimicrobial resistance (USHHS-NARMS, 2019). Since 2010, just in the United States, *S.* Infantis has been among the top 10 serotypes causing human illnesses yearly (CDC, 2018).

A report from the CDC in 2018 had shown a *S.* Infantis strain, resistant to multiple antibiotics resulted in 129 sick people, 25 hospitalizations and one death (CDC, 2018).

1.2.4 *Campylobacter* in poultry

The genus of *Campylobacter* is composed of 16 species. The species that are highly associated with human infections are *C. jejuni* and *C. coli* (Fouts et al., 2005). These two species are most often detected in cases related to poultry. *C. jejuni* is typically found in the gastrointestinal tract of chickens where the conditions are favorable for colonizing the mucus on the epithelial cells (Newell and Fearnly, 2003).

Campylobacter on poultry farms are most commonly ingested by birds during the growing phase and once ingested they rapidly colonize the gastrointestinal tract, frequently affecting the ceca, large intestine, jejunum and cloaca, and can easily spread throughout the flock (Beery et al., 1988). The most commonly associated cause of human infections is cross-contamination which can happen in the consumer's house (Thames and Sukumaran, 2020). In the processing plant this can happen where feces and digesta can contact broiler meat products (Oyarzabal, 2005).

Poultry is the second principal product associated with *Campylobacter* infections, and every year there are around 1.5 million illnesses reported in the United States (CDC, 2021b). It is estimated that around 20-30% of these cases are related to poultry meat consumption (Skarp et al., 2016).

Campylobacter infections represent around a $6.9 billion loss to the poultry industry. Based on CDC data, between 2004 and 2012 the number of confirmed outbreaks reached 347 and represents around 1.9% of all foodborne outbreaks in United States (Geissler et. al., 2012). Between October 2018 and September 2019, the presence of *Salmonella* and *Campylobacter* in chicken parts was 8.77 and 17.60%, respectively, and in the case of the whole carcasses was 3.62 and 21.15% respectively (USDA-FSIS, 2020a)

1.2.5 Interventions for pathogenic reduction on poultry

On-farm, the most common contamination points are surfaces, air (aerosols), and even liquid that can encompass bacteria (Vihavainen et al., 2007). Another critical concern is the processing plants, which focus on bacterial cross-contamination. During the processing steps to obtain the final meat product, contamination can occur from equipment surfaces, water, and animal microbiota. Different bacteria are present in the environment and air and can easily contaminate the broiler meat (Demirok et al., 2013). In the past years, different intervention points have been created to reduce this contamination and try to obtain safer chicken meat at production and specifically at processing.

Interventions during poultry processing have increased for reducing the bacterial contamination in the final product; some of these practices are cold water and air chilling procedures and have shown effects in diminishing *Salmonella* and *Campylobacter* counts, but there are still some gaps to fill and look for alternatives to reduce this bacteria count, especially

in the shelf life of cuts (CDC, 2012b). The Centers for Disease Control and Prevention (CDC) have reported that around 48 million people (1/6) are affected by a foodborne illness every year (CDC, 2012b) Poultry is one of the main sources of *Salmonella*, and 1 in every 25 packages of chicken at the grocery stores is contaminated with this pathogen (CDC, 2021a). In the case of *Campylobacter*, a single drop of contaminated raw chicken can be enough to infect a person (CDC, 2021b). The USDA's Economic Research Service (ERS) estimated that *Salmonella* infections cost around $2.83 to $11.3 billion annually for the poultry industry. Poultry is estimated to cause about 19% of salmonellosis, higher than any other food.

1.2.6 Antimicrobials

Food antimicrobials are compounds that are generally used to extend the lag phase or kill microorganisms. Antimicrobials are classified as "traditional" or "naturally occurring" (Davidson, 2001). Many traditional antimicrobials are approved for use in the United States and other international regulatory agencies. In the case of naturally occurring antimicrobials, these are the ones that originate from microbial, plant, and animal sources (Davidson and Harrison, 2002). Some antimicrobials can specifically work just to control the growth of specific foodborne pathogens (Davidson and Harrison, 2002).

Chemical antimicrobials are the most common type used in the poultry industry (Kim et al., 2017). Until 1997, the most common antimicrobial intervention was chlorine, but in recent years has changed to peracetic acid as the primary one used, in addition to cetylpyridinium chloride and acidified sodium chlorite (Capita et al., 2000; Zhang et al., 2019).

1.2.7 Antimicrobial interventions in poultry

The most commonly used antimicrobials are peracetic acid (PAA), cetylpyridinium chloride (CPC), chlorine, and others. Each of these antimicrobials has advantages and disadvantages (Nagel et al., 2013). These interventions in poultry are primarily used to reduce the microbial load of pathogenic bacteria such as *Salmonella* and *Campylobacter* and are applied during spray washing, chilling, or post chilling (Anang et al., 2010). CPC is a chemical that has been safely used for over 30 years and has been able to reduce *Salmonella* counts on poultry tissues, preventing bacterial attachments and subsequently reducing the risk of cross-contamination (Breen et al., 1997).

Chlorine has been used due to the efficacy, availability, and relatively low cost for poultry processors. Chlorine not only reacts with bacteria but also with organic and inorganic material that can be present (Tsai et al., 1992). However, since 1997 the European Union has banned the import of chicken carcasses treated with chlorine (Schraer and Edgington, 2019). With this information in mind, poultry processing has moved to use another antimicrobial intervention during poultry processing and PAA as the new industry standard for the poultry industry.

1.2.8 Peracetic acid (PAA) in poultry processing

PAA is used in raw poultry products and is approved by the U.S. Food and Drug Administration (FDA) (21 CFR 173.370). The maximum allowed concentration is 2,000 ppm of peroxyacids and 1,435 ppm of hydrogen peroxide, and its use depends on the application (USDA-FSIS, 2020b). In the poultry industry, PAA generally is used in different concentrations varying from 50 to 2000 ppm (USDA, 2016). PAA decomposition rate is positively correlated with pH, temperature, and organic matter content and negatively with initial PAA concentration

(Chen and Pavlostathis, 2019). The presence of hydrogen peroxide makes PAA a strong oxidizing agent, in that way affecting the bacteria cell wall permeability, denaturing proteins and enzymes, and inhibiting other cellular activities (Humayoun et al., 2018).

Several studies have demonstrated the efficacy of PAA for the reduction of different microorganisms. *In vitro*, 7 to 11 ppm of PAA was enough to obtain a 5 log reduction in counts of gram-negative bacteria (*Escherichia coli, Pseudomonas aeruginosa*, and *Salmonella*) and gram-positive bacteria (*Bacillus subtilis, Enterococcus faecalis, Listeria monocytogenes,* and *Staphylococcus aureus*) (Bridier et al., 2011).

Generally, during the poultry processing steps, the antimicrobial interventions of PAA are in the chiller tanks and in 2011, PAA was the most commonly used antimicrobial (Chen et al., 2014). In a survey conducted in 2016 with 167 U.S. poultry processing plants, the majority used PAA as an antimicrobial intervention for chicken parts (146/167) and carcasses (124/167) (Ebel et al., 2019). In Table 1.1, are different examples of PAA as a principal chemical intervention on poultry products.

The main concern in using PAA as a chemical antimicrobial in the poultry processing industry is the occupational health concern. PAA is corrosive and irritating to eyes, mucous membranes of the respiratory tract, and skin (National Academy of Sciences, 2010). High exposure to airborne chemicals can quickly affect workers and as result, produce severe irreversible effects and even death (CDC-NIOSH, 2020)

The use of these chemical antimicrobial interventions represents a high cost for the poultry processing plants. Processing plants can even spend more than a million dollars per year to minimize the presence of pathogens in the final raw product. These chemicals, such as PAA, are expensive and can contribute to unintended consequences such as producing undesirable

secondary compounds, diminished meat quality, and equipment corrosion. Taking this in consideration and now with the increase in regulatory restrictions and negative consumer perception of chemical compounds in foods have contributed to the pressure for the development of alternative antimicrobial agents.

The large antimicrobial volume required and the cost associated with the implementation and use of novel antimicrobial treatments are often prohibitive compared to standard chemical sanitizing treatments (Cano et al., 2021).

Photo-active compounds can be an alternative, such as curcumin and chlorophyllin, which in previous research have demonstrated an antimicrobial activity when activated by light. There is a critical need to fill significant gaps in knowledge about the ability of these natural compounds as antimicrobial agents for food, particularly to develop a suitable decontamination strategy for raw poultry.

1.2.9 Photodynamic therapy as alternative

For many years light has been used for therapeutic purposes, but in the last few years has been developed as a new alternative photodynamic therapy. Photodynamic therapy needs a photosensitizer with the presence of light and oxygen to treat specific diseases (Felsher, 2003). Photodynamic therapy has also been evaluated as an alternative to use in food safety related to oysters, cucumbers, peppers, and chicken meat (Tortik et al., 2014; Wu et al., 2015; Wu et al., 2016).

The compounds that can improve or impede photodynamic therapy are called photosensitizers. The ability of this compound is based on phototoxicity. In Figure 1.1 , how the photophysical and photochemical process of photodynamic therapy inactivation works is explained (Castano et al., 2004; Li et al., 2020).

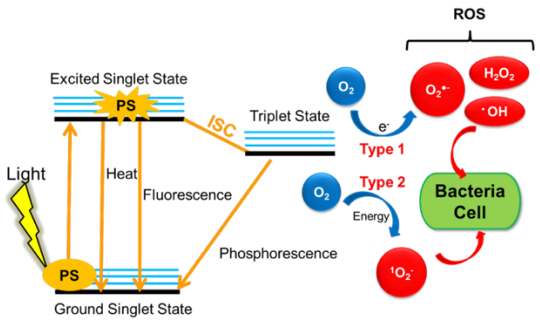

Figure 1.1 Photodynamic mechanism system

The mechanism is based on the ground state of the photosensitizer; this has two electrons with opposite spins (singlet state), all of these in the low-energy molecular orbital. When light absorption occurs (photons), one of these electrons is boosted into a high-energy orbital but keeps its spin that was the first excited singlet state. All this process happens in a short-lived (nanoseconds) period, and the species can lose its energy by emitting light (fluorescence) or by internal conversion into heat (Castano et al., 2004).

The photosensitizer excited triplet can undergo two kinds of reactions; In type I reactions, it can react directly with a substrate, such as a cell membrane or a molecule, and transfer a proton or electron to form a radical anion or cation. These radicals can further react with oxygen to produce reactive oxygen species (ROS). Or in type II, the triplet photosensitizer can transfer its energy directly to molecular oxygen to form the excited state of singlet oxygen (Castano et al., 2004; Li et al., 2020).

The excited singlet oxygen ($^1O_2$) can react indiscriminately with proteins, nucleic acids, and lipids and cause membrane disruption and DNA damage (Li et al., 2020). In the case of the phototoxicity present in the photosensitizer, it is strictly related to the photoproducts generated in the excited triplet state (Gao and Matthews, 2020). It can oxidize a variety of cellular components, leading to the destruction of the cell membrane, proteins, DNA, and RNA resulting

in cell death (Michaeli and Feitelson, 1994; Böcking et al., 2001; Haeubl et al., 2009; Gao and Matthews, 2020).

Photo-inactivation of foodborne pathogens applies to the disinfection of food products because it is a simple two step procedure using photo-active substances; 1) the photoactive compound and 2) harmless visible light. In the case of poultry processing, the surface contamination is a critical point and would represent an ideal case study. The raw poultry products can be drenched with a solution of the photosensitizer and then exposed to light for a predetermined period.

An appropriate light wavelength that facilitates photo-inactivation and represents no worker safety issues is required. After investigating the absorption spectrum of photosensitizer curcumin (Figure 1.2). A wavelength near 410 nm was appropriate for use with the curcumin.

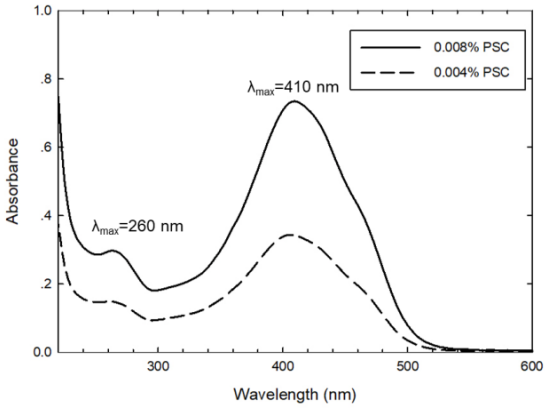

Figure 1.2 Absorption spectrum of curcumin (Gao and Matthews, 2020)

1.2.10 Curcumin as potential photosensitizer

Curcumin (CUR) is a hydrophobic polyphenol derived from the rhizome of the herb *Curcuma longa* and has different biological properties related to pharmacological activities. In the chemical composition, curcumin is a bis-α, β-unsaturated, β-diketone (diferuloylmethane, Figure 1.3) (Anand et al., 2007).

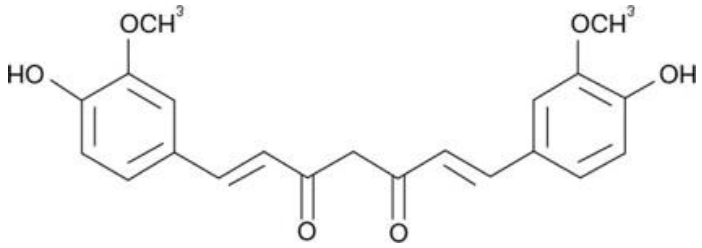

Figure 1.3 Curcumin chemical structure

The use of curcumin in foods has been approved as a colorant or flavor by both European Union and the United States. In the United States, curcumin has been categorized as Generally Recognized as Safe (GRAS) and can be used in different ways such as flavor, flavor enhancer, or as an ingredient in soups, snacks foods, baked goods, imitation dairy products, and seasoning. The maximum permitted use in foods is 20 milligrams per serving (Gao and Matthews, 2020).

Curcumin has a relatively high LD_{50} value, up to 2.0 $g.kg^{-1}$ (Srimal and Dhawan,1973). In a three-month human clinical trial, it was found that humans present a high tolerance towards curcumin, and the dose can be up to 8 g/day (Cheng et al.,2001).

Curcumin extraction

Based on the origin and soil conditions, where the curcumin grows influences the curcuminoids content, and generally is between 2 to 9%. Curcumin is the major component, and cyclic curcumin is the minor (Priyadarsini, 2014). Curcumin as a food additive is extracted from turmeric using solvents. Some solvents that can be used are acetone, carbon dioxide, ethyl acetate, methanol, ethanol, and hexane (Stankovic, 2004). Even when chlorinated solvents are very efficient for curcumin extraction, they are not usually employed due to their non-acceptability in the food industry (Priyadarsini, 2014). Methods using heat have been tried and have demonstrated better results than the chemical methods; a temperature increase between 60

to 80° C improves the curcumin extraction (Kim et al., 2013). Currently, researchers are still looking for alternative methods for employing food grades solvents like triacylglycerols, due to the high demand for the product (Takenaka et al., 2013).

Curcumin in water is poor at acidic and neutral pH. In the case of neutral conditions, only 1.34 µg/mL can be dissolved in 1 L of distilled water; it has a higher solubility in 99.5% EtOH solvent (Carvalho et al., 2015). Solubility is an essential factor that can affect the photodynamic inactivation of a photosensitizer because if precipitated it cannot interact with the cells. Curcumin has higher solubility in alkaline solutions (Wang et al., 1997).

Curcumin as potential antimicrobial

Some studies have reported using curcumin and light as an antimicrobial for foods (Table 1.2). In these studies (de Oliveira et al., 2018), curcumin in produce showed a reduction of 3 log of inoculated *E. coli* O157:H7 and *Listeria innocua* levels when low levels of curcumin (1 to 10 ppm) were sprayed on spinach, lettuce, or tomato followed by 5 to10 minutes of exposure to UV-A light. Curcumin has been used to extend oyster shelf life, reduce norovirus in oysters (Chen et al., 2021), extend Hami melon shelf life (Lin et al., 2019), reduce *Aspergillus flavus* spores on maize (Temba et al., 2019), and *Staphylococcus aureus* on vegetables (Tortik et al., 2014) and all have been shown to be positively impacted by the use of curcumin as a photo-active antimicrobial.

While most food safety research using curcumin has been conducted on produce or seafood, some studies have evaluated the use of curcumin as a potential antimicrobial in poultry meat and products. In one of these, *Staphylococcus aureus* was reduced by 1.7 log on cooked chicken meat, using a treatment of curcumin polyvinylpyrrolidone compounds, followed by light activation (Tortik et al., 2014).

In other research conducted in 2018 by Gao, water-soluble curcumin (95%) concentrations from 0 to 300 ppm were evaluated against *Listeria monocytogenes*, *E. coli*, O157:H7, and *Staphylococcus aureus* on media and chicken skin, with exposure light of 1, 2.5, 5 and 10 min and *Salmonella enterica* using curcumin concentrations in a range from 0 to 2,000 ppm. When they applied the 10 min of exposure light at CUR concentrations of 10, 30, and 300 ppm, *L. monocytogenes* was reduced by approximately 3 log. For *Salmonella* the highest reduction of 1.8 to 3.6 log was reached at 200 ppm CUR and 10 min of exposure to light (Gao, 2018).

1.2.11 Chlorophyllin as potential photosensitizer

Chlorophyllin are semi-synthetic porphyrins obtained from chlorophyll (Figure 1.4). Porphyrin molecules are of interest for their antimicrobial activity (Lopez-Carballo et al., 2008). These molecules are used in dietary supplements, as food colorants, in cosmetics, as an internal deodorant, and as an accelerant in wound healing (Kephart, 1955). If these molecules are activated by visible light and air, they generate a singlet oxygen and cytotoxic free radicals in the majority of live cells (Romanova et al., 2003).

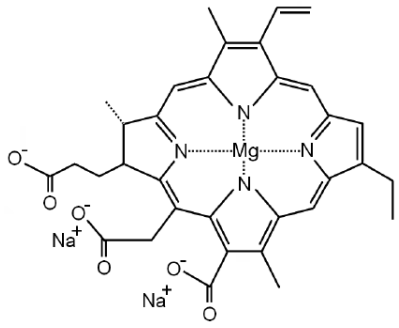

Figure 1.4 Chlorophyll chemical structure

There are two main types of chlorophyll and they are differentiated as a and b. When any of these molecules are exposed to weak acids, oxygen or light (Figure 1.5). This accelerates their oxidation there is a formation of numerous degradation products (Jeffrey et al., 1997; Hosikian et al., 2010).

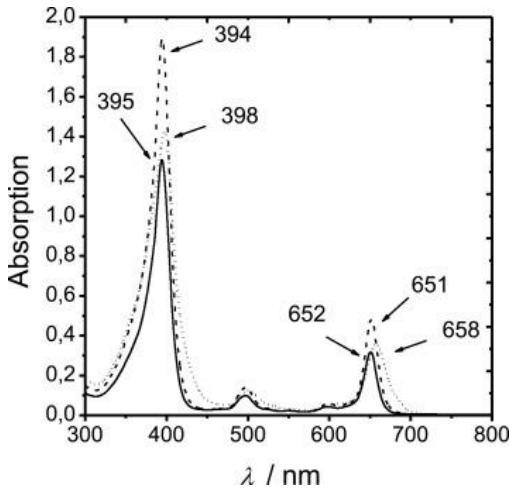

Figure 1.5 Absorption spectrum of chlorophyll (Kustov et. al., 2019)

Chlorophyllin extraction

To quantify the amount of chlorophyll that may contain a particular species, the intracellular chlorophyll must first be extracted and the most common method involves organic solvent extraction (Jeffrey et al., 1997; Simon and Helliwell, 1998). In this process, the organic solvent penetrates through the cell membrane and dissolves the lipids as well as the lipoproteins of chloroplast membranes (Jeffrey et al., 1997). It has been found that cell disruption, grinding, homogenization, ultrasound or sonication significantly improve the effectiveness of chlorophyllin extraction (Jeffrey et al., 1997; Simon and Helliwell, 1998).

Since chlorophyllin is highly reactive, the yield of a particular extraction procedure is also affected by the degradation products that can be obtained. These degradation products are

produced when their molecules are exposed to excess light, oxygen/air, high temperature, and acidic or basic conditions (Jeffrey et al., 1997; Cubas et al., 2008).

Chlorophyll as potential antimicrobial

Microbiological studies have shown that even when the pigments are highly diluted, they can effectively kill gram-positive bacteria and fungi but the efficacy against gram-negative bacteria is still questionable (Suvorov et al., 2021). Some of the studies conducted using chlorophyllin as potential antimicrobial are presented in Table 1.3.

However, the use of chlorophyllin as an antimicrobial for foods has had minimal attention. Reductions in *Listeria monocytogenes* and *Salmonella enterica* have been demonstrated *in vitro* and on produce (Luksiene and Paskeviciute 2011). When chlorophyllins were incorporated into a gelatin polymer matrix, *Staphylococcus aureus* and *Listeria monocytogenes* were significantly reduced on inoculated cooked frankfurters (López-Carballo et al., 2008).

Even when photodynamic therapy is a potential alternative to consider as a novel intervention in poultry processing facilities, there are still some concerns, especially regarding how the photosensitizers can affect some sensory characteristics. It is essential to start testing this new alternative by conducting more laboratory-level and practical pilot plant research using different photosensitizers.

1.2.12 Organic acids as an alternative

In recent years the implementation of organic acids as an alternative for the reduction of different microorganisms has increased. Different factors can affect the antimicrobial effect expected such as the chemical formula, physical form, pKa value, nature of the microoganisms and others (Davidson et al, 2005; Coban, 2020). Another important consideration is that their

efficacy is often diminished by their poor solubility in water and phase separation in aqueous medium (Dev Kumar and Micallef, 2017).

Generally, organic acid treatments are cheap, simple, and fast (Hinton and Corry, 1999). Organic acids are defined as carbon containing compounds with weak acidic properties and sometimes are synthesized by plants (Theron et. al., 2010; Anyasi et al., 2015). An important characteristics of organic acids is that they are Generally Recognized As Safe (GRAS) by the FDA and can be used as food additives (21 CFR 170.30) (USDA-FDA, 2019). Two examples of these organic acids are Pelargonic acid (PA) and Lactic acid (LA) which can be used as potential alternatives in the poultry processing industry.

Pelargonic acid

Pelargonic acid (PA), which was originally obtained from *Pelargonium* leaves, but is now usually prepared synthetically (Figure 1.6) is a clear to yellowish oily liquid, and insoluble in water, but soluble in alcohol and organic solvents (Sahin et al., 2006).

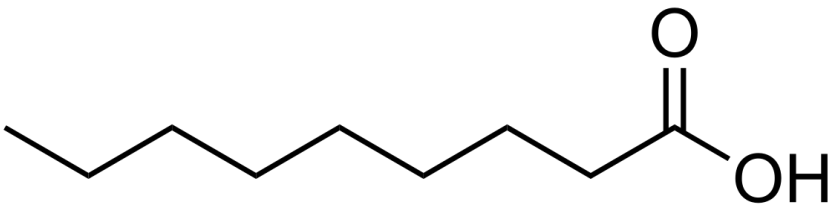

Figure 1.6 Pelargonic acid chemical structure

The chemical structure is a fatty acid and has an antimicrobial effect due to its ability to insert and subsequently disrupt the lipid bi-layer of cellular membranes (Ciriminna et al., 2019). Following this insertion, the membrane fluidity increases, and induces disorganization and conformational changes within the membrane. Following this, the membrane leaks, the

intracellular components collapse and eventually the cell will lysic (Pohl et al., 2011; Dev Kumar and Micallef, 2017; Ciriminna et al., 2019; Dev Kumar et al., 2019).

PA has been also used as an antifungal. Another advantage is that it is GRAS and some of the components are obtained from the tomato exometabolome. PA has been shown to have better antimicrobial results when it is in combination with surfactants such as Saponin (Dev Kumar et al., 2020). *In vitro* antimicrobial studies to obtain the antimicrobial activity of PA have been carried out by the agar-disk diffusion method against different organisms (*Bacillus cereus*, *Salmonella typhimurium*, and *Escherichia coli*). The initial inoculum for each bacteria was 10^6 CFU/mL and they used 100 % of PA. In the results, it was demonstrated that PA was efficient in the reduction against Gram-positive and Gram-negative bacteria (Sahin et al., 2006).

PA antimicrobial activity had been tested against different *Salmonella* serotypes Angona, Saintpaul, Newport, Montevideo, and Kentucky on organic grape tomatoes inoculated with 200 CFU/µl sample and PA at a concentration of 1 M and 0.1% (w/v) saponin. PAA, chlorine, and water were evaluated as controls. The results demonstrated that on tomatoes treated with PA, *Salmonella* counts decreased significantly compared to tomatoes treated with chlorine, PAA, or water. This experiment also evaluated the effect of time: On day 0, PA resulted in a significant reduction over chlorine and PAA; after 1-day storage, the results were the same for the three treatments and a similar trend was observed after 7-day storage (White et al., 2021). PA can be used in a solution up to 1% as antimicrobial compounds in foods (Flavor and extract manufacturers association, 2018).

Lactic acid (LA)

Lactic acid (LA), which formula is $C_3H_6O_3$ (Figure 1.7) with a molecular weight of 90 g/mol, has been widely used not only in food industry but also in other industries such as

pharmaceutical, textile and biodegradable production (Hofvendahl and Hahn-Hagerdal, 2000). In 2013, the lactic acid market was around 714,000 tons and for 2020, was expected to be 1,960,000 tons (Cubas-Cano et al., 2018).

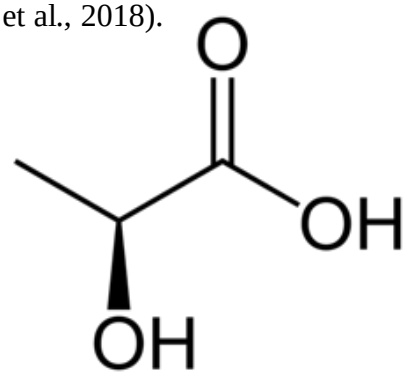

Figure 1.7 Lactic acid chemical structure.

Lactic acid application in foods is not only direct but has been also used as spray on food materials to decrease the spoilage microorganism growth on the product. Different studies have been conducted to demonstrate the efficacy of LA in the reduction of different microorganisms. In one study, where LA was evaluated in a concentration of 2% against *Listeria monocytogenes* on beef cubes, it was shown that LA reduced pathogen population by 1.7 log/6 cm^2 (Khateib et al., 1993).

In the case of *Salmonella,* which is the most common foodborne pathogen associated with poultry, there was observed a reduction in broiler carcasses when LA was applied as spraying or dipping at 1.3 and 2.3 log CFU/mL, respectively (Laury et al., 2009).

Because organic acid properties can be affected by their solubility, there have been some studies using a surfactant as a possible way to modify this property. This result may allow the organic acid to affect bacteria.

1.2.13 Surfactants

Surfactants have been used in the food industry for many centuries. There are different surfactants in the industry; some are natural, and others are synthetically obtained. Synthetic

surfactants, such as sorbitan, esters, and their ethoxylates, have increased in popularity to use in food emulsion (Larsson and Friderg, 1990).

When surfactants are in conjugation with other molecules they can form emulsions, and these emulsions are important for the food system. There are three types of emulsions in food: 1) oil-in-water emulsion where droplets of oil are suspended in an aqueous continuous phase and are the most versatile, 2) water-in-oil emulsion where the stability depends more on the properties of the oil, and 3) water-in-oil-in-water where the droplets themselves contain water droplets (Kralova and Sjoblom, 2008).

The surfactant type and concentration can either synergistically strengthen or impede the antimicrobial activity of an emulsion (Ziani et al., 2011). In this body of work, the selected surfactants were Tween 80 and Saponin to be evaluated in conjugation with PA and LA.

Saponin

Saponin naturally occurs in different plants and has been obtained from the bark of the Chilean tree *Quillaja saponaria*. Additionally, some have been created synthetically for food use (Fenwick and Oakenfull, 1983). One of the characteristics of the surfactant, such as saponins, is their property to form a stable soap-like foam upon shaking in an aqueous solution (Faizal and Geelen, 2013). Thanks to the amphiphilic nature of this surfactant, the saponin molecules can form micelles in aqueous solutions. The structure, size and shape of the molecules depend on the plant origin, temperature, pH, and the presence of electrolytes in the solution (Stanimirova et al., 2011).

The antimicrobial activity of saponin extracted from Sorghum Bicolor was tested against three pathogens, *Staphylococcus aureus, Escherichia coli,* and *Candida albicans*. The results showed than saponins inhibited the growth of *S. aureus*, but did not demonstrate any significant

reduction on *E. coli* and *C. albicans*, potentially due to the gram-negative bacteria and fungus characteristic of the protective effect linked to microbial membranes (Soetan et al., 2011). This antimicrobial property of a micelle depends on its availability in the aqueous phase (Hilbig et al., 2016).

When Quillaja-Saponin was used in combination with PA to create an emulsion, it was effective in the reduction of *S.* Newport that was more sensitive than *S.* Typhimurium and Oranienburg. A positive correlation was observed between higher micellar size and inhibitory activity (Dev Kumar et al., 2020). It is important to consider the concentration used because this can affect the antimicrobial property of an emulsion (Ziani et al., 2011).

Tween 80

Tween 80 is non-ionic surfactant with popular use as emulsifier in different industries like cosmetics, pharmaceuticals, and food. It is approved by the FDA for use up to 1% in selected foods (Chassaing et al., 2015).

The antimicrobial activity of Tween 80 has been demonstrated to positively or negatively affect bacterial growth. In an experiment evaluating the effect of commercial Tween 80 when tested against *S. aureus* in this scenario, Tween 80 stimulated the growth of the pathogen on batch culture and biofilms (Nielsen et al., 2016). In the case of *Listeria monocytogenes*, there was no effect in the batch culture and in *Pseudomona fluorescens* Tween 80 reduced biofilm formation (Nielsen et al., 2016).

This emulsifier has a hydrophilic-lipophilic balance of 15.0, which allows it to be stable in oil-water emulsions. Another characteristic is its low toxicity when compared with other synthetic surfactants with a low-cost associated (McClements, 2015). According to the FDA, the consumption should be limited to a daily intake of 25 mg/kg (McClements, 2015).

Dev Kumar et. al. (2020) tested the antimicrobial properties of Tween 80 as an emulsifier against different *Salmonella* serotypes, but this was not as effective as Quillaja saponin. Other experiments had also demonstrated that Tween 80 has not been effective against *Escherichia coli* O157:H7 and *Listeria monocytogenes* (Hilbig et al., 2016; Ma et al., 2016). It is essential to use these emulsifiers as a natural synthetic derived for antimicrobials intervention in the poultry industry.

1.3 SUMMARY

- *Salmonella* and *Campylobacter* are highly associated with poultry meat and products and represent a high cost for the poultry industry interventions.
- Different interventions have been used in poultry live and processing systems for the reduction of *Salmonella* and *Campylobacter*.
- Peroxyacetic acid is the most common antimicrobial intervention used in the poultry industry, but consumer concerns about chemicals in their food has put pressure to move to natural alternatives.
- Photodynamic therapy is a new alternative to apply for the disinfection of food products because it is a simple two-step procedure using photo-active substances; 1) the photoactive compound and 2) harmless visible light.
- Curcumin and chlorophyllin are potential photo-active compounds that have been demonstrated to have antimicrobial activity against different pathogens and are considered natural interventions.
- Organic acids have been approved for their use as antimicrobials in food, representing a new alternative to the common chemical interventions in the poultry industry.

- Emulsions formed with surfactants like Saponin and Tween 80, can either synergistically strength or impede the antimicrobial activity of some organic acids.

1.4 KNOWLEDGE GAP IN LITERATURE SPECIFICALLY FOR NEW ANTIMICROBIAL ALTERNATIVES TO USE IN POULTRY PROCESSING

The desire for alternative approaches to microbial control has resulted in considerable interest concerning novel methods of disinfection and decontamination. Increasing regulatory restrictions and negative consumer responses to chemical compounds in foods and the use of antibiotics in agriculture have also contributed to the pressure to develop alternative compounds for use as antimicrobial agents. Novel methods should be safe, effective, practical, and cost-appropriate. Alternatives to antimicrobial treatments are needed that are more efficacious and acceptable to consumers in the United States and globally. Therefore, it is necessary to investigate these alternatives in poultry processing. With this in mind, we conducted two different studies entitled:

1. *In vitro* effect of photo-active compounds curcumin and chlorophyllin against *Salmonella* and *Campylobacter*. (Chapter 2)
2. Effect of organic acids alone and in combination with surfactants Tween 80 and Saponin in the reduction of *Salmonella* Infantis in vitro and on chicken wings. (Chapter 3)

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Table 1.1 PAA treatment in different chicken products as antimicrobial

Step/Product	Treatment	Quantitative reduction	Reference
Prechill tank interventions: immersion			
Chicken carcass	PAA, 50 ppm, 25 min	APC: 0.5	(Guastalli et al., 2016)
	PAA, 200 ppm, 15 min	APC: 0.9	(Steininger et al., 2018)
	PAA, 200 ppm, 20 min	S: 0.3, C: 2.1[b], TVC: 0.1	(Chousalkar et al., 2019)
Prechill tank interventions: spray			
Chicken carcass	PAA, 400 ppm, 30 s	C:1.2	(Purnell et al., 2014)
	PAA, 500 ppm, 20 s	S: 0.1, EC: 0.6[b], C:0.4[b]	(Dittoe et al., 2019)
Chill tank interventions			
Chicken carcass	PAA, 5,000 ppm, 45 min	S(%): 5.0[b]	(Vadhanasin et al., 2004)
Post chill tank interventions: immersion			
Chicken carcass	PAA, 750 ppm, 15 s	S(%): 0.0, C: 2.2[b], APC: 4.1[b]	(Kim et al., 2017)
	PAA, 1,000 ppm, 30 s	S: 1.7[b], APC: 1.7[b]	(Lemonakis et al., 2017)
	PAA, 1,000 ppm, 20 s	S: 2.1[b], C: 2.0[b]	(Nagel et al., 2013)
Chicken wings	PAA, 1,000 ppm, 30 s	S: 1.3[b], ACP: 1.4[b]	(Kim et al., 2017)
	PAA, 700 ppm, 20 s	S: 1.5[b], APC:1.5[b]	(Scott et al., 2015)
	PAA, 1,000 ppm, 30 s	C: 2.3[b]	(Shen et al., 2019)
Chicken breast	PAA, 400 ppm, 10 min	APC:1.1[b]	(Moghassem Hamidi et al., 2021)
Chicken skin	PAA, 400 ppm, 20 s	S: 1.7[b]	(Sukumaran et al., 2015)
	PAA, 220 ppm, 15 min	APC: 0.3	(Del Rio et al., 2007)

[a] S, Salmonella counts; S (%), Salmonella prevalence; C, Campylobacter counts; EC, E. coli counts; TVC, total viable counts; APC, aerobic plate counts; PAA, peracetic acid

[b] Reduction was significantly different from the control, $P < 0.05$

Table 1.2 Summary of photoinactivation on bacteria using curcumin

Microorganisms	CUR concentration (μM)	Wavelength (nm)	Ilumination time (min)	Log reduction	Reference
Staphylococcus aureus	5-50	435	60	>6	(Winter et al., 2013)
	50 and 100	435	60	2.6 (cucumber) 2.5 (pepper) 1.7 (chicken)	(Tortik et al., 2014)
Enterococcus faecalis	5	450-500	4	5.5	(Pileggi et al., 2013)
Escherichia coli	50	435	60	3	(Winter et al., 2013)
	10	400-500	30	4.16	(Hegge et al., 2012)
	25	300-500	30	2.8	(Haukvik et al., 2010)
Vibrio parahaemolyticus	10	470	60	5 (Oyster)	(Wu et al., 2016)
Listeria monocytogenes	40	300-600	1 and 10	2.9	(Gao, 2018)
Salmonella enterica	0-2,000	300-600	1, 2.5, and 5	1.5 (Chicken skin)	(Gao and Matthews, 2020)

Table 1.2 Summary of photoinactivation on bacteria using chlorophyllin.

Microorganisms	CH concentration	Wavelength (nm)	Ilumination time	Log reduction	Reference
Listeria monocytogenes	1 mmol l^{-1}	400	20min	1.8 $\log_{10}$ CFU/g	(Luksiene and Paskeviviute, 2011)
Total aerobic mesophiles	1 mmol l^{-1}	400	20min	1.7 $\log_{10}$ CFU/g	(Luksiene and Paskeviviute, 2011)
Fungi and yeast	1 mmol l^{-1}	400	20min	0.86 $\log_{10}$ CFU/g	(Luksiene and Paskeviviute, 2011)
Staphylococcus aureus	0.1ml	1270	2ns*	0.1 $\log_{10}$ CFU/mL	(Kustov et. al., 2019)
Escherichia coli	0.1ml	1270	2ns*	10^3 CFU/mL	(Kustov et. al., 2019)
Salmonella spp.	10g/100mL	30,000lux	5 min	4 $\log_{10}$ CFU/m	(Lopez-Carballo et. al., 2008)

*nanoseconds

CHAPTER 2: *IN VITRO* EFFECT OF PHOTO-ACTIVE COMPOUNDS CURCUMIN AND CHLOROPHYLLIN AGAINST *SALMONELLA* AND *CAMPYLOBACTER*

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2.1 ABSTRACT

Salmonella and *Campylobacter* are two of the most common foodborne pathogens associated with poultry meat. Regulatory restrictions and consumer concerns have increased the need for natural antimicrobials and emerging novel technologies. The objective of this study was to determine the antimicrobial activity of two natural photo-active compounds curcumin (CUR) and chlorophyllin (CH) followed by LED light exposure for the reduction of *Salmonella* and *Campylobacter*. Peroxyacetic acid (PAA), was also evaluated as a control. In 96 well plates, CUR and CH at concentrations of 100, 500, and 1,000 ppm, PAA at 100, 200, and 300 ppm, and distilled water (DW) were evaluated. Each well was inoculated with 10^4 CFU of *Salmonella* Typhimurium or *Campylobacter*. Plates were exposed to LED light (430 nm) for 0 or 5 mins Data were analyzed for each antimicrobial by concentration and light exposure using the GLM procedure with means separated by Tukeys HSD with significance at a P value of ≤ 0.05. For *Salmonella*, there was a significant main effect of treatment ($P<0.0001$), but time ($P=0.3681$) and treatment*time interaction ($P=0.9999$) were not significant. No detectable reductions were observed for *Salmonella* or *Campylobacter* when treated with CUR, CH, or 100 ppm PAA.

However, when *Salmonella* was treated with 200 ppm PAA, counts were reduced to 2.5 $\log_{10}$ CFU/mL. When *Salmonella* was treated with 300 ppm PAA, counts were below detectable levels. For *Campylobacter*, there was a significant main effect of treatment (P<0.0001) and a treatment*LED light interaction (P=0.0354), but LED light (P=0.3325) was not significant. *Campylobacter* was reduced when treated with 200 ppm PAA. However, no further reductions were observed when *Campylobacter* was treated with 300 ppm PAA (2.2-2.7 $\log_{10}$ CFU/mL). These results indicate that CUR and CH were not effective as antimicrobials under this evaluated conditions, particularly in comparison to the commonly used antimicrobial, PAA.

Keywords: *Salmonella*, *Campylobacter*, curcumin, chlorophyllin, Peroxyacetic acid, photosensitizer

2.2 INTRODUCTION

Poultry meat has been recognized as one of the major sources of the foodborne bacterial pathogens *Salmonella* and *Campylobacter.* According to the Center for Disease Control (CDC) every year *Salmonella* causes about 1.35 million infections, 26,500 hospitalizations, and 420 deaths in the United States (CDC, 2022). Poultry is the second principal product associated with *Campylobacter* infections and every year there are around 1.5 million illnesses estimated in the United States (CDC, 2022)

There are more than 2,500 described serovars of *Salmonella* and many of those related to human infections are highly present in broiler meat (Foley et al., 2011). Poultry is one of the main reservoirs for *Campylobacter* and the species most commonly associated with poultry are *jejuni* and *coli* (Sheppard and Maiden, 2015). Different interventions have been implemented on-farm and in processing plants with the goal of reducing the microbial load of poultry meat. Poultry meat is processed in a highly automated industry with high volume and as a

consequence, there are many cross-contamination points to consider (Bauermeister et al., 2008). Several antimicrobial interventions have been tested in poultry during processing, but currently the most extensively used is peracetic acid (PAA).

PAA has been widely distributed in different applications as a sanitizer or disinfectant (Luukkonen and Pehkonen 2017). The use of PAA for microbial inactivation demonstrates high sterilization stability and this characteristic has enabled PAA to become a substitute for chlorine, especially in food industry interventions (Domínguez Henao et al., 2018). Poultry carcass interventions using PAA are applied in different ways and locations in the processing plant, including short-term spray or dip applications and longer term in the immersion chilling tanks. However, PAA represents a high cost, has somewhat limited effectiveness, is corrosive to metal equipment, and presents personnel safety concerns.

Novel interventions for the reduction of pathogens are continuously needed in the poultry industry. Natural compounds with antimicrobial activity are a promising alternative to traditionally used chemical antimicrobials for food safety. Some of these natural alternatives are known as photosensitizers. Photosensitizers have molecules that are activated by light exposure (Wan and Lin, 2014). Different photosensitizers have been evaluated against microorganisms on food and on food contact surfaces (D'Souza et al., 2015). Photodynamic therapy consists of a non-thermal technology that involves a simultaneous interaction between non-toxic photosensitizers, light of an appropriate wavelength, and molecular oxygen to produce a microbial reduction (Corrêa et al., 2020).

Following application, this mechanism starts with a photochemical activation of the compound that is used as a photosensitizer and results in the production of Reactive Oxygen Species (ROS) (Buytaert et al., 2007). LED light produces excitation of the photosensitizer and

induces an excited singlet state, which can undergo two kinds of reactions: decay into the ground state by fluorescence or electron spin conversion into a triplet state. Molecules in the triplet state can react with different substrates and as consequence form free radicals to interact with oxygen, and this molecule can transfer energy directly to molecular oxygen, forming ROS (Kushibiki et al., 2015).

Curcumin is a bright yellow chemical derived and extracted from *Curcuma longa* and is one of the most studied photosensitizers (Gao and Matthews, 2020). Curcumin has been used to extend oyster shelf life, reduce norovirus in oysters (Chen et al., 2021), extend Hami melon shelf life (Lin et al., 2019), reduce *Aspergillus flavus* spores on maize (Temba et al., 2019), and *Staphylococcus aureus* on vegetables (Tortik et al., 2014) and all have been shown to be positively impacted by the use of curcumin as a photo-active antimicrobial.

Chlorophyllin is a brilliant green and semi-synthetic porphyrin obtained from natural chlorophyll (López-Carballo et al., 2008). Chlorophyllin has been demonstrated to inactivate several food-related microorganisms, such as *Bacillus cereus* (Luksiene et al., 2010) and *Listeria monocytogenes* (Luksiene and Paskeviciute, 2011) when exposed to light at ~400 nm wavelength. However, chlorophyllin as an antimicrobial for application to foods has had minimal attention.

Photodynamic therapy interventions have been shown to be effective for the reduction of *Salmonella* using the photosensitizer curcumin (Gao and Matthews, 2020). However, efficacy against *Campylobacter* is unknown. Consumer demand is increasing for natural antimicrobials in food (Fernández-López et al., 2005). The objective of this study was to determine the antimicrobial activity of two natural photo-active compounds curcumin (CUR) and chlorophyllin (CH) followed by LED light activation for the reduction of *Salmonella* and *Campylobacter.*

2.3 MATERIALS AND METHODS

Experimental design

Photo-active antimicrobials curcumin (**CUR**) and chlorophyllin (**CH**) at concentrations of 100, 500, and 1,000 ppm were evaluated for the reduction of *Salmonella* Typhimurium or *Campylobacter jejuni*. In addition, peracetic acid at 100, 200, and 300 ppm was evaluated for the reduction of *Salmonella* Typhimurium and *Campylobacter jejuni*. For both *Salmonella* and *Campylobacter* trials, a positive control with pathogen and growth media, and a negative control with only growth media were included. Antimicrobial and pathogen concentrations were either held for 5 min under laboratory ceiling fluorescent light or exposed to activating light within an LED light box for 5 minutes. Following LED light, no LED light exposure, each of the treatments were plated for enumeration.

***Salmonella* and *Campylobacter* inoculum preparation.** *Salmonella enterica* serotype Typhimurium was prepared for inoculation by plating from a glycerol stock stored at -80 °C onto plate count agar (Hardy Diagnostics, Santa Maria, CA, USA). The colonies were collected from plate count agar plates after the incubation period of 24 h at 37 °C and then suspended in sterile saline to achieve an optical density of approximately 0.12 which yields about 10^8 CFU/mL. The initial inoculum was further serially diluted in 2X Mueller Hinton Broth supplier (**MHB**) to obtain a final inoculum of 10^5 CFU/mL. A poultry-associated field strain of *Campylobacter jejuni* was used for inoculum preparation by plating a stored glycerol stock at -80 °C onto Campy Cefex agar (Neogen Corporation, MI, USA). After incubation for 48 h at 42 °C under microaerobic conditions, colonies were collected and then suspended in sterile saline to achieve an optical density of approximately 0.12 which yields about 10^8 CFU/mL. The initial inoculum

was further serially diluted in 2X blood-free Boltons Enrichment Broth (**BF-BEB**) to obtain a final inoculum of 10^5 CFU/mL.

Antimicrobial stock solution preparation, A stock solution of curcumin (97% curcuminoid content, TCI America, Portland, OR) was prepared using 200 mg of curcumin dissolved in 10 mL of 95% ethanol followed by dilutions with sterile distilled water (**SDW**) to obtain concentrations of 200, 1,000 and 2,000 ppm. For chlorophyllin (Spectrum Chemical Mfg. Corp, California, USA) a stock solution using 100 mg was diluted with 10 mL SDW followed by dilutions with SDW to achieve 200, 1,000, and 2,000 ppm. Peracetic acid (35% concentrated, Pfaltz & Bauer, Waterbury, CA) was diluted with SDW to obtain the desired concentrations of 200, 400, and 600 ppm for *Salmonella* and *Campylobacter* trials. PAA concentration was confirmed using the N-N-diethyl-p-phenylenediamine method with K-7913 Peracetic Acid Vacu-Vials (Chemetrics, Midland, VA). For each well assay, antimicrobial concentrations were further diluted by half to yield 100, 500, and 1,000 ppm final concentrations for CUR and CH or 100, 200, and 300 ppm final concentrations for PAA.

96 wells plate assay. In a series of 96 well plates, 125 µL of each concentration of the antimicrobial stock solutions of CUR, CH, or PAA was added to wells. Then 125 µL of the 10^5 CFU/mL inoculum prepared in 2X MHB for *Salmonella* or 2X BF-BEB for *Campylobacter* were added. For the negative controls, 250 µL of MHB or BF-BEB was used without *Salmonella* or *Campylobacter*. For the positive control, 125 µL of SDW and 125 µL of the bacterial inoculums were used. Because the CUR stock solution was initially prepared with ethanol, an additional control was prepared combining 125 µL of 9% ethanol with 125 µL of the 10^5 CFU/mL inoculum of *Salmonella* or *Campylobacter* in the well, mimicking the procedure used for the CUR treatment. The arrays of treatments were prepared in two sets of 96 well plates. One set of

plates remained on the laboratory bench uncovered and the other set was exposed for 5 minutes in the LED lightbox. After the completion of the exposure time, serial dilutions were prepared and plated for *Salmonella* on XLT4 agar plates in duplicate and aerobically incubated for 24 h at 37°C. *Campylobacter* dilutions were plated in duplicate on Campy Cefex agar and incubated under microaerobic conditions for 48 h at 42°C. Each combination of antimicrobial concentration, pathogen type, and light exposure time was completed in triplicate. The minimum level of detection for either *Salmonella* or *Campylobacter* was 10 cells or 1.00 $\log_{10}$ CFU/mL.

Light apparatus. A lightbox was composed of eight solderless light strips with eight LEDs (7.2 W; LED Group Buy) evenly distributed with a distance between each string of lights of 3.18 cm across an interior stainless-steel top. Each LED emits a peak wavelength of 430 nm. The total height of the LED box was 22.1 cm and a width of 22.6 cm. This LED box was constructed similarly to a previously assembled lightbox by Gao and Matthews (2020). The distance between the 96 wells plate and the LED lights was 21.6 cm.

Statistical analyses. *Salmonella* and *Campylobacter* counts were transformed into $\log_{10}$ CFU/mL before data analysis. Data for each pathogen were analyzed for the main effects and interactions of antimicrobial concentration and light exposure time using the GLM procedure with means separated by Tukeys HSD with significance at a P-value of ≤ 0.05. All data were analyzed using SAS Studio, release 3.8 Enterprise Edition. The sample size n = 3 for each treatment and LED light (0 or 5 min) exposure.

2.4 RESULTS AND DISCUSSION

For *Salmonella*, there was a significant main effect of antimicrobial treatments ($P<0.0001$), but light time ($P=0.3681$) and treatment*light time interaction ($P=0.9998$) were not significant. When *Salmonella* was treated with CUR at 100, 500, or 1000 ppm, final counts

ranged from 4.7 to 4.9 $\log_{10}$ CFU/ml, did not differ by concentration or light exposure, and did not differ from the positive or ethanol controls (Table 2.1). For CH, final counts ranged from 4.5 to 4.9 $\log_{10}$ CFU/mL, did not differ by concentration or LED light exposure, and did not differ from the positive or ethanol controls. When *Salmonella* was treated with 100 ppm PAA, counts ranged from 4.5 to 4.8 $\log_{10}$ CFU/mL and did not differ from the positive control. However, counts were reduced following treatment with 200 ppm PAA (2.5 $\log_{10}$ CFU/mL). When *Salmonella* was treated with 300 ppm PAA, counts were below detectable levels.

For *Campylobacter*, there was a significant main effect of antimicrobial treatments (P<0.0001) and a treatment*light time interaction (P=0.0354), but light time (P=0.3325) was not significant. When *Campylobacter* was treated with CUR at 100, 500, or 1000 ppm final counts ranged from 4.5 to 4.7 $\log_{10}$ CFU/mL and did not differ by concentration or light exposure (Table 1.2). For CH, final counts ranged from 4.5 to 4.6 $\log_{10}$ CFU/mL and did not differ by concentration or light exposure. When *Campylobacter* was treated with 100 ppm PAA the counts ranged from 4.3 to 4.4 $\log_{10}$ CFU/mL and did not differ from the positive control. *Campylobacter* was reduced to 2.7 to 2.9 $\log_{10}$ CFU/mL when treated with 200 ppm PAA. However, no further reductions were observed when *Campylobacter* was treated with 300 ppm PAA (2.3 to 2.7 $\log_{10}$ CFU/mL).

Neither CUR nor CH at 100 to 1,000 ppm were effective at reducing the levels of *Salmonella* or *Campylobacter* with or without exposure to 5 min of activating LED light. The antimicrobial and light exposure time combination used in this study was selected based on a reasonable timeframe for which poultry products could be treated on a production line. However, only when the commonly used antimicrobial PAA was evaluated, were significant microbial reductions observed.

The use of CUR as an antimicrobial has been previously evaluated and efficacy has been demonstrated in several food matrices. In the experiment conducted by (Gao and Matthews, 2020) they evaluated water soluble curcumin (95%) concentrations from 0 to 300 ppm against *Listeria monocytogenes* in media and on chicken skin, with exposure time to LED light of 1, 2.5, 5, and 10 min. They also evaluated CUR concentration in a range from 0 to 2,000 ppm against eight *Salmonella* strains. When they applied the 10 min of exposure to light at CUR concentrations of 10, 30, and 300 ppm, *L. monocytogenes* was reduced by approximately 3 log CFU/mL on media. *Salmonella* was reduced by 1.8 to 3.6 log CFU/mL when treated with 200 ppm CUR with 10 min of exposure to light on media. On chicken skin, the results were similar between the reduction of *Listeria monocytogenes* and *Salmonella* with 3 $\log_{10}$ CFU/cm^2 using CUR. Using a 10 min exposure time was not considered for this experiment because our principal aim was to evaluate the potential for use in a poultry processing plant using these treatments as a new alternative for antimicrobial intervention.

When CUR between 1 and 10 ppm and UV-A light were used for the sanitation of wash water for fresh produce applications, *E. coli* O157:H7 was reduced by 3 $\log_{10}$ CFU/mL and *Listeria innocua* was reduced by more than 5 $\log_{10}$ CFU/mL (de Oliveira et al., 2018). However, UV-A light is considered an ultraviolet longwave emission (Harm, 1980) and is in the wavelength range from 320 to 400 nm. UV irradiation with a range from 250-260 nm is lethal to different microorganisms such as bacteria, viruses, protozoa, fungi, yeast, and algae, but the maximum effect is at 254 nm with higher wavelengths decreasing in lethality (Bintsis et al., 2000). The wavelength of 430 nm used in the current study was intended to activate CUR and was not expected to exhibit any antimicrobial activity alone.

Other studies evaluating CUR against *Salmonella* did not demonstrate antimicrobial efficacy. The lack of *Salmonella* reductions observed in the current study may have also been due to differences in concentration or CUR solubility. In the current study, we evaluated non-water-soluble CUR with higher concentrations of 500 and 1,000 ppm. In previous work it has been shown that at higher CUR concentrations, a self-shielding effect can occur (Barr et al., 1990). At low concentrations CUR is able to absorb light energy and be excited, which is required for the ROS molecule production and subsequently the cell damage to obtain an antimicrobial effect. However, when CUR is at a high concentration >100 ppm, the large number of CUR molecules can block the light source and interfere with the photodynamic therapy. This self-shielding effect could explain why increasing CUR concentrations did not detectably impact antimicrobial activity.

In this study non-water-soluble CUR and 95% ethanol was used as the first diluent agent. In a similar study where ethanol was used as a diluent agent against twenty four pathogenic bacteria isolated from chicken and shrimp, the concentrations of CUR with antimicrobial activity ranged from 125 to 1,000 ppm (Lawhavinit et al., 2010). CUR diluted with ethanol demonstrated antimicrobial activity against *Vibrio*, *Staphylococcus*, and *Bacillus*, (gram negative and gram positive) but did not inhibit *Salmonella* in a disk diffusion assay.

Based on these previous studies, it appears that the use of CUR in conjunction with activating light has a stronger antimicrobial activity against Gram-positive than Gram-negative bacteria (Adamczak et al., 2020). *Salmonella* and *Campylobacter,* the two evaluated pathogens in the present study, are Gram-negative bacteria (Schleifer and Kandler, 1972). The cell membrane structure of the Gram-negative bacteria may have reduced the penetration of the singlet oxygen derived from the photo-activation mechanism. This reduced effectiveness against Gram-negative

bacteria for CUR as a photo-active antimicrobial can partially explain why *Salmonella* or *Campylobacter* were not reduced in this study. The gram-negative bacteria cell wall outer membrane represents an obstacle to the antimicrobial effect that has been observed in Gram-positive bacteria (Romanova et al., 2003). This outer membrane plays an important role in antibiotic resistance for gram negative bacteria and also can act as a shield of the cytoplasmatic membrane and prevent porphyrins from entering the cell and reacting as photo-sensitizers, subsequently producing a protective effect (Ehrenberg et al., 1985). To obtain reductions in gram-negative bacteria, application of the photo-sensitizer compound mixed with ethylene diamine tetraacetic acid (EDTA) or increasing the positive charge may be more effective (Malik et al., 1992).

Chlorophyllin (CH) has also previously been demonstrated to be effective as a photoactive antimicrobial. Strawberries inoculated with *Listeria monocytogenes* soaked in CH for 5 minutes, then exposed to 400 nm light for 20 min reduced *Listeria* by 1.8 $\log_{10}$ CFU/g, total aerobic mesophiles by 1.7 $\log_{10}$ CFU/g, and yeast and fungi by 0.86 $\log_{10}$ CFU/g (Luksiene and Paskeviciute, 2011). When water-soluble sodium magnesium chlorophyllin E-140 and water-soluble sodium copper chlorophyllin E-141 were evaluated against *S. aureus*, *Listeria monocytogenes*, *E. coli*, and *Salmonella* spp. as an edible and coating film for cooked frankfurters, 4 to 5 log CFU/mL reductions were observed for *S. aureus* and *Listeria* following exposure to high intensity white light (10,000 to 50,000 lux) for 5 or 15 minutes (López-Carballo et al., 2008). However, in the case of *E. coli* and *Salmonella* none of the treatments were effective for pathogen reduction. Although some antimicrobial efficacy for CH had been previously demonstrated, neither *Salmonella* nor *Campylobacter* evaluated in this study were reduced, potentially due to the short light activation time (5 min).

Distance and angle between the light source and photo-active compound could also influence the antimicrobial activity. In contrast with the present study where the light strips were 21.6 cm from the plates, the previous work demonstrating antimicrobial efficacy against *Salmonella* had only a 2 cm distance between the light source and well plate (Gao, 2018). Although successful pathogen reductions were observed at a 2 cm distance, this is not likely to be practical for use in a commercial poultry processing facility where large variability exists in the size of products. LED shape can also influence light distribution. When different shapes of LEDs were evaluated, square-shaped LEDs had an average of 79.4° angle distribution compared with round-shaped LEDs with a 87.5° angle (Kee Xiao Ying and Lim, 2022). The angle of application could affect the distribution of light across the 96 well plate depending on the position of each LED in relation to the position of the wells.

PAA is currently the most common antimicrobial used in the poultry industry in the U.S. for the reduction of *Salmonella* and *Campylobacter* on raw poultry products. There have been multiple previous studies demonstrating the efficacy of PAA against both *Salmonella* and *Campylobacter* following short term treatment, which is applicable for the poultry processing environment (Humayoun et. al., 2018; Chen and Pavlostathis 2019; Cano et.al., 2021; Vaddu et al., 2021). The approved concentration for use is up to 2,000 ppm but typical use concentrations range from 50 to 500 ppm (USDA, 2016). The results of the current study agree with the previous literature and demonstrated that PAA at 200 and 300 ppm reduced both *Salmonella* and *Campylobacter* by 2 $\log_{10}$ CFU/mL or to below detectable levels. No synergistic effect was observed between the LED light and PAA.

Although CUR and CH were not effective at the parameters applied, PAA reduced both *Salmonella* and *Campylobacter*. Overall, the use of CUR and CH as photo-active antimicrobials

with activating light was not effective for the reduction of *Salmonella* or *Campylobacter* when a treatment time of 5 min was applied. Application parameter changes such as increases in exposure time or light intensity will be necessary if CUR or CH are to be effective as potential antimicrobials during poultry processing.

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Table 2.3 *Salmonella* recovery counts (Log_{10} CFU/mL) following treatment with curcumin, chlorophyllin or peracetic acid and LED light exposure time.

		LED exposure time	
Treatment	Concentration	0 min	5 min
Curcumin	100	4.90 ± 0.13	4.84 ± 0.18
	500	4.74 ± 0.14	4.72 ± 0.09
	1,000	4.73 ± 0.20	4.79 ± 0.16
Chlorophyllin	100	4.74 ± 0.17	4.90 ± 0.16
	500	4.59 ± 0.18	4.68 ± 0.09
	1,000	4.59 ± 0.17	4.88 ± 0.17
Peracetic acid	100	4.56 ± 0.34^{A}	4.85 ± 0.17^{A}
	200	2.51 ± 0.20^{B}	2.53 ± 0.94^{B}
	300	0^{C}	0^{C}
Positive Control	-	4.51 ± 0.15	4.63 ± 0.17
Ethanol Control	-	4.62 ± 0.16	4.76 ± 0.16

[A-C] Values within a column within a treatment with different superscripts are significantly different ($P \leq 0.05$).

Sample size n = 3.

Table 4.2 *Campylobacter* recovery counts (Log_{10} CFU/mL) following treatment with curcumin, chlorophyllin or peracetic acid and LED light exposure time.

		LED exposure time	
Treatment	Concentration	0 min	5 min
Curcumin	100	4.55 ± 0.06	4.65 ± 0.01
	500	4.59 ± 0.02	4.72 ± 0.03
	1,000	4.60 ± 0.02	4.56 ± 0.02
Chlorophyllin	100	4.59 ± 0.02	4.55 ± 0.02
	500	4.49 ± 0.02	4.52 ± 0.04
	1,000	4.50 ± 0.08	4.50 ± 0.08
Peracetic acid	100	4.31 ± 0.04^{A}	4.36 ± 0.08^{A}
	200	2.93 ± 0.15^{B}	2.71 ± 0.04^{B}
	300	2.71 ± 0.02^{B}	2.28 ± 0.27^{B}
Positive Control	-	4.43 ± 0.02	4.46 ± 0.05
Ethanol Control	-	4.47 ± 0.03	4.52 ± 0.02

$^{A-B}$ Values within a column within a treatment with different superscripts are significantly different ($P \leq 0.05$).

Sample size n = 3.

CHAPTER 3: EFFECT OF PELARGONIC ACID ALONE AND IN COMBINATION WITH SURFACTANTS SAPONIN AND TWEEN 80 IN THE REDUCTION OF *SALMONELLA* INFANTIS *IN VITRO* AND ON CHICKEN WING FLATS

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3.1 ABSTRACT

Salmonella Infantis has been highly associated with chicken products. Pelargonic acid (PA) has been previously shown to reduce *Salmonella*. Lactic acid (LA) and peracetic acid (PAA) are organic acids currently in use by the beef and poultry processing industries. Surfactants can be used to strengthen antimicrobial activity. The objective of this study was to determine the antimicrobial activity of PA, LA, and PAA alone and in combination with two different surfactants, Tween 80 (TW80) or Saponin (SAP), for the reduction of *Salmonella* Infantis *in vitro* and on chicken wing flats. In 24 well plates, PA concentrations of 300, 500, or 700 ppm, LA concentrations of 1,000, 2,000, or 5,000 ppm, and PAA at 75 or 135 ppm were evaluated alone and in combination with 0.50% of either TW 80 or SAP. Each well was inoculated with 10 µl of a 10^7 CFU/ml *Salmonella* Infantis inoculum and 250 µl of the treatments, with a contact time of 5 minutes. For the wing flats, each one was inoculated with 10^6 CFU/mL of *Salmonella* Infantis followed by dipping for 30 s in PA at 300 or 1000 ppm with SAP at 0.05% w/v or 0.50% w/v or PAA at 75 ppm with TW80 or SAP at 0.05%. Sterile Distilled Water (SDW) was used as

the control. Treatments were serially diluted and plated onto XLT4 agar plates for evaluation of *Salmonella* reductions. Count data were transformed to $\log_{10}$ CFU/mL and reductions analyzed using the GLM procedure with means separated by Tukeys HSD with significance at $P \leq 0.05$. *In vitro* results demonstrated that PA did not reduce *Salmonella* Infantis alone or in combination with TW 80, but with SAP reduced *Salmonella* Infantis below detectable levels (0.70 $\log_{10}$ CFU/mL, $P<0.0001$). LA was effective at levels > 1000 ppm alone or in combination with TW 80. *Salmonella* was reduced below detectable levels (0.70 $\log_{10}$ CFU/mL, $P<0.0001$). On chicken wing flats PA did not reduce *Salmonella* Infantis when applied in combination with 0.05% or 0.50% SAP ($P>0.0845$). Overall, PAA at 75 ppm was effective in combination with 0.05% SAP or 0.05% TW 80 for the reduction of *Salmonella* Infantis ($P<0.0001$)

KEYWORDS: Pelargonic acid, Lactic acid, Peroxyacetic acid, Tween 80, Saponin

3.2 INTRODUCTION

The Centers for Disease Control and Prevention (CDC) estimates that *Salmonella* is one of the principal pathogens causing human foodborne illnesses (CDC, 2012). There are about 2,650 serotypes of *Salmonella* identified, and *Salmonella* Infantis is one of these (Ferrari et al., 2019). Since 2010, just in the United States, *S.* Infantis has been among the top 10 serotypes causing human illnesses yearly (CDC, 2018). Poultry is considered one of the significant sources of *S.* Infantis and around 2% to 4% of the serotypes found in poultry meat and products are Infantis (USDA-NARMS, 2016; Gymoese et al., 2019). *S.* Infantis causes human gastroenteritis and is transmitted through ingesting contaminated food or water (Boyle et al., 2007; Shahada et al., 2006). Another important characteristic is the high antimicrobial and multidrug resistance (Fonseca et al., 2006).

Salmonella Infantis is highly associated with poultry meat, and for that reason, several antimicrobial interventions have been considered on the farm and in the processing plant. Addressing the presence of *Salmonella* is important not only in the harvest process but also in poultry parts. The consumption of chicken meat in the United States ranks as the highest consumed among meat species (USDA, 2016). According to a 2016 report from USDA-FSIS, the prevalence of *Salmonella* on chicken parts including wings is around 25% (USDA, 2016; Vaddu et al., 2021).

It is important to identify new alternatives for the reduction of *Salmonella* on chicken carcasses and parts. Peroxyacetic acid (PAA) is most often used in poultry processing plants in the U.S. It has been approved by the U.S. Food and Drug Administration (FDA) (21 CFR 173.370), with a maximum concentration of 2000 ppm of peroxyacids and 1435 ppm of hydrogen peroxide, depending on the application (USDA-FSIS, 2020). The main concern with PAA is the worker's health because exposure can have an irritating effect on the eyes, mucous membrane of the respiratory tract, and skin (National Research Council (US), 2010).

Considering the health concerns related to PAA, it is essential to look for new antimicrobial options for poultry processing. Organic acids are found naturally in various fruits and fermented products and have antimicrobial activity against some foodborne pathogens (Beuchat, 1996). The antimicrobial effect of specific fatty acids is due to the ability to disrupt bacterial membrane lipids, alter membrane fluidity, or by hydroperoxide formation, creating oxidative damage (Dev Kumar and Micallef, 2017). These treatments are cheap, simple, and fast (Hinton and Corry, 1999), and are generally recognized as safe (GRAS) by the FDA for meat products. However, organic acid antimicrobial efficacy is often affected by poor solubility in water and phase separation in an aqueous medium (Dev Kumar and Micallef, 2017). A possible

alternative to obtain the desired antimicrobial effect is through combination with surfactants and subsequent emulsion formation.

Under this organic acid category is pelargonic acid, also known as nonanoic acid, which naturally occurs in plants, including tomatoes, and can be present in animals (Dev Kumar and Micallef, 2017). Pelargonic acid has previously been demonstrated to have an antimicrobial effect against *Salmonella*. Still, this effect is conditioned by the emulsion formed with surfactants, and better results were obtained in combination with the surfactant Quillaja Saponin (Dev Kumar and Micallef, 2017). Lactic acid is another organic acid, naturally found in different fruits and fermented products (Stanojevic-Nikolic et al., 2015). Lactic acid was effective in the reduction of *E. coli* O157:H7 and *Salmonella* Typhimurium on round beef carcass by 5.2 log CFU/cm^2 when sprayed at 2% lactic acid concentration (Castillo et al., 2000).

There are natural and synthetic surfactants used in the food industry including Saponin which is a surfactant naturally occurring in plants. When Saponin is agitated with water it forms a soapy lather (Rai et al., 2021). Saponin is eco-friendly, biodegradable, and non-toxic and can be considered an option for poultry processing plant interventions (Olezek and Bialy, 2006).

Surfactant type and concentration can either synergistically strengthen or impede the antimicrobial activity of an emulsion (Ziani et al., 2011). As another option, Tween 80 is a surfactant widely used in the pharmacy and food industry due to its low toxicity characteristics. It is also approved by the FDA to be used in certain foods up to 1% (Chassaing et al., 2015).

Surfactants affect the antimicrobial activity of an emulsion due to their placement on the colloidal particle and their charges, affecting the total particle size in contact with the target bacteria cell (Oakenfull, 1981). Saponin or Tween 80 in combination with the organic acids, will

provide a nonionic emulsion where hydrophilic and hydrophobic tails are in contact to provide a stable emulsion (Akbari and Nour, 2018).

The objective of this study was to determine the antimicrobial activity of three different organic acids; pelargonic acid (PA), lactic acid (LA), and peracetic acid (PAA) alone and in combination with two different surfactants, Saponin or Tween 80, for the reduction of *Salmonella* Infantis *in vitro* and on chicken wing flats.

3.3 MATERIALS AND METHODS

***Salmonella* inoculum preparation.** *Salmonella* Infantis previously isolated from a poultry source was prepared for inoculation by plating from a glycerol stock stored at -80 °C onto an XLT4 agar plate (Fisher Scientific, Waltham, MA). A colony was collected from XLT4 agar plates after the incubation period of 24 h at 37 °C and struck onto plate count agar (Hardy Diagnostics, Santa Maria, CA, USA). The colonies were collected from plate count agar plates after the incubation period of 24 h at 37°C and then suspended in Phosphate-Buffered Saline (**PBS**) to achieve an optical density of approximately 0.12 which yields approximately 10^8 CFU/ml. The initial inoculum was further serially diluted in PBS to obtain a final inoculum of 10^7 CFU/ml.

Experiment 1. Experiment 1 was conducted in triplicate to evaluate three different organic acids to reduce *Salmonella* Infantis. Pelargonic acid (**PA**) at 300, 500, or 700 ppm, lactic acid (**LA**) at 1000, 2000, or 5000 ppm, and peracetic acid (**PAA**) at 75 or 135 ppm were evaluated alone and in combination with surfactants Saponin or Tween 80 at 0.50%. PBS without *Salmonella* was used as the negative control. Organic acid concentrations and *Salmonella* were held for a 5 min contact time prior to plating each treatment in duplicate for enumeration.

Three different working solutions at 300, 500, and 700 ppm of PA (97%, Acros Organic, New Jersey, USA) were prepared in Sterile Distilled Water **(SDW)** and vortexed for 1 min. Then surfactants of either Saponin (VWR, Pennsylvania, USA) or Tween 80 (VWR, Pennsylvania, USA) was added for a concentration of 0.05% and vortexed for 1 min. For LA (85%, Acros Organic, New Jersey, USA) three different working solutions at 1000, 2000, and 5000 ppm were prepared in SDW and vortexed for 1 min, then Saponin or Tween80 were added at a concentration of 0.05% and vortexed for 1 min.

Using 3 wells for each treatment in a sterile 24 well plate, 250 µL of each concentration of the organic acids of PA, LA, or PAA alone and in combination with the surfactants Saponin or Tween 80 were inoculated with 10 µl of 10^7 CFU/ml of *Salmonella.* For the positive control 250 µl of PBS was inoculated with 10 µl of *Salmonella*. The negative control was 260 µl of PBS alone. The contact time between the treatments and *Salmonella* was 5 minutes. Serial dilutions were prepared and plated onto XLT4 agar plates in duplicate and incubated for 24 h at 37°C for enumeration. Following Experiment 1 the concentrations and surfactant combinations that demonstrated the most *Salmonella* reduction were selected for use in Experiment 2.

Experiment 2. For Experiment 2, in Trial 1, five chicken wing flats per treatment were inoculated with 100 µL of 10^6 CFU/mL *Salmonella* Infantis and dip treated with PA at 300 or 1000 ppm in combination with 0.05% or 0.50% saponin. SDW was used as the control. Following treatment, each wing flat was rinsed and rinsates plated in duplicate for enumeration. Based on the results of Trial 1, treatments were adjusted to include PAA in Trials 2 and 3. LA was not evaluated in Experiment 2 due to a greater interest in evaluating PA.

For Experiment 2, Trial 2 and 3, eight chicken wing flats were inoculated with 100 µL of 10^6 CFU/mL *Salmonella* Infantis and dip treated with PA at 300 or 1000 ppm in combination

with Saponin at 0.05% or 0.50%. PAA was added as treatment at 75 ppm in combination with Saponin or Tween 80 at 0.05%. SDW was used as the control. Following treatment, each wing flat was rinsed and rinsates plated in duplicate for enumeration.

PA was prepared with SDW at 300 or 1000 ppm alone and in combination with 0.05% or 0.50% Saponin. PA and Saponin concentrations were calculated based on a final volume of 1000 mL. The emulsion was initially prepared using 25 ml of SDW in combination with the organic acid and the surfactant and vortexed for 1 minute, this emulsion was then added to 975 ml of SDW to yield the final concentration combinations for treatment application.

Peracetic acid (35% concentrated, Pfaltz & Bauer, Waterbury, CA) was diluted with SDW to obtain the desired target concentration of 75 ppm in 1000 mL. This emulsion was initially prepared using 25 mL PAA and Tween 80 and vortexed for 1 minute, this emulsion was then added to 975 ml of SDW for the final treatment application. Final PAA concentration was confirmed using the N-N-diethyl-p-phenylenediamine method with K-7913 Peracetic Acid Vacu-Vials (Chemetrics, Midland, VA).

Chicken wing flats were arranged on a clean wire rack and drop inoculated with 100 µl of 10^6 CFU/ml of *Salmonella* Infantis. The inoculum was then spread across the visible skin surface with a sterile plastic spreader and was allowed a contact time of 1 h at room temperature. For each treatment, wing flats were individually immersed in a sterile cup with 100 ml of the designated treatment, capped, and gently inverted for 30 seconds. Each flat was removed with a sterile hemostat, allowed to drip for 10 s, and placed in a sample bag with 100 mL of buffered peptone water (BPW). Each wing was gently massaged for 30 s, and 100 µl was plated in duplicate onto XLT4 agar plates and incubated at 37°C for 24 h.

Statistical analyses. *Salmonella* count data were transformed to $\log_{10}$ CFU/mL and analyzed using the GLM procedure with means separated by Tukeys HSD with significance at $P \leq 0.05$. All data were analyzed using SAS Studio, release 3.8 Enterprise Edition.

3.4 RESULTS AND DISCUSSION

In Experiment 1 on Table 3.1, when PA was evaluated in well plate assay, PA did not reduce *Salmonella* Infantis when applied alone (3.27 to 3.46 $\log_{10}$ CFU/mL) or combined with Tween 80 (3.22 to 3.46 $\log_{10}$ CFU/mL) when compared to the control (3.62 $\log_{10}$ CFU/mL). However, when combined with Saponin, PA at 300, 500, and 700 ppm reduced *Salmonella* below the level of detection (7.0 $\log_{10}$ CFU/mL, $P<0.0001$). When LA was applied at 1000 ppm, *Salmonella* was not reduced when applied alone (2.61 $\log_{10}$ CFU/mL) or in combination with Saponin (3.13 $\log_{10}$ CFU/mL) or Tween80 (1.73 $\log_{10}$ CFU/mL). However, *Salmonella* Infantis was reduced below detectable levels when used at a concentration of 2000 ppm in combination with Tween80 or alone ($P<0.0001$). When LA at 2000 ppm was in combination with Saponin no reduction of *Salmonella* was observed compared to the control. When LA was used at 5000 ppm alone or in combination with either Saponin or Tween 80 *Salmonella* was reduced below detectable levels ($P<0.0001$).

For Experiment 2 on Table 3.2, Trial 1, PA at 300 or 1000 ppm did not reduce *Salmonella* Infantis when applied in combination with 0.05% or 0.50% Saponin ($P=0.0845$) when compared with SDW control. In Trial 2, PA at 300 or 1000 ppm did not reduce *Salmonella* Infantis when applied in combination with 0.05% or 0.50% Saponin, however PAA at 75 ppm in combination with either 0.05% Saponin or 0.05% Tween80 was effective in for the reduction of *Salmonella* Infantis to 0.60 $\log_{10}$ CFU/mL or 0.44 $\log_{10}$ CFU/mL, respectively ($P<0.0001$). In Trial 3, PA at 300 or 1000 ppm in combination with Saponin did not reduce *Salmonella* Infantis

in comparison to the control. However, PAA at 75 ppm in combination with Saponin did reduce *Salmonella*, combination with Tween80 reduced *Salmonella* levels by 0.76 $\log_{10}$ CFU/mL. When all trials were combined, PA combined with Saponin did not differ from the control, but PAA combined with either Saponin or Tween80 decreased *Salmonella* recovery by 0.78 $\log_{10}$ CFU/mL or 1.06 $\log_{10}$ CFU/mL, respectively.

Overall, PA demonstrated efficacy in *in vitro* tests in Experiment 1, but did not reduce *Salmonella* when applied to chicken wing flats in Experiment 2. These results are similar to Dev Kumar et al. (2020) where PA was evaluated at 15 to 31 mM (2400 to 4900 ppm) in combination with surfactants Tween 80, Triton X100, Sodium Dodecyl Sulfate (SDS) and Quillaja Saponin at 1%, 0.1% and 0.01% (w/v) against *Salmonella* Newport, Oranienburg, and Typhimurium. Micelle/droplet size and minimal inhibitory concentration (MIC) were evaluated using a modified 96 well plate Resazurin assay using an initial concentration of 10^9 CFU/ml and two different exposures times of 30 s and 5 min. When PA formed an emulsion with Quillaja Saponin, the antimicrobial activity was improved by a reduction of greater than 6 $\log_{10}$ CFU/mL for the three *Salmonella* serotypes and in this case against *Salmonella* Infantis.

In another experiment evaluating Saponin as potential emulsifier, Saponin was purified from Quillaja Saponaria Molina bark by silica and reverse phase chromatography (Kensil et al., 1991). One of the main differences that makes Quillaja Saponin not a potential emulsifier to use in foods is the undesirable side effects (Kensil et al., 1991) due to the high concentration of tannins can be associated with some antinutritional effects (Sharma et al., 2019). In the current study, Saponin which is derived from Quillaja Saponin was evaluated alone at a concentration of 0.05% with no antimicrobial activity observed against *Salmonella* Infantis. However, Saponin when was evaluated in combination with PA at 300, 500, and 700 ppm, *Salmonella* Infantis was

reduced below detectable levels. However, this antimicrobial effect was not observed when evaluated on the chicken wings flats.

Quillaja Saponin has also been demonstrated to have an impact on the growth of *E. coli*, improving the growth rate at lower concentrations and slowing the growth rate at higher concentrations when was applied in *Yucca schidigera*, which indicates in some cases a probably concentration-dependent response (Sen et al., 1998). The antimicrobial ability of a micelle is related to the availability in the aqueous phase when an emulsion is formed (Hilbig et al., 2016). Emulsions often incorporate essential oils in aqueous food matrices (Hilbig et al., 2016). Multiple emulsions are very complicated dispersion systems and the principal characteristic is a low thermodynamic stability (Muschiolik, 2007).

PA antimicrobial activity has been tested against different *Salmonella* serotypes Angona, Saintpaul, Newport, Montevideo, and Kentucky on organic grape tomatoes inoculated with 10^9 $\log_{10}$ CFU/sample in a concentration of 0.1% (w/v) saponin. PAA, chlorine, and water were evaluated as controls. The results demonstrated that on tomatoes treated with PA, *Salmonella* counts significantly decreased in comparison to tomatoes treated with chlorine, PAA, or water (White et al., 2021).

There are differences between emulsions, in this case a nanoemulsion formed between Pelargonic acid and Tween 80 which is thermodynamically unstable and a microemulsion between Pelargonic acid and Saponin which is thermodynamically stable under specific conditions and can be easily formed by mixing water, oil, and surfactants (McClements, 2012). In this case, where PA is combined with saponin to create a microemulsion, the surfactant molecules are arranged in an oil-in-water manner so that their non-polar tails are associated with each other and create a hydrophobic core (McClements, 2012).

PA combined with Tween 80 forms a nanoemulsion. Tween 80 is non-ionic in nature and some have reported that it can enhance the antimicrobial activity in some products (Donsi et al., 2011) or can reduce the antimicrobial activity due to interaction with the emulsion active layer (Jumaa et.al., 2002) or induction of emulsion instability phenomena (Sznitowska et. al., 2002).

In another experiment evaluating the effect of Tween 80 alone or as an emulsion with different essential oils, the results indicated that it was not effective in the reduction of a *Listeria monocytogenes* cocktail. When the concentration of Tween 80 increased, the antimicrobial activity decreased (Ma et al., 2016). In the current study, Tween 80 was effective in reducing *Salmonella* Infantis only when used in combination with higher concentrations of LA (2000 and 5000 ppm).

In case of surfactants like Tween 80, the micelles can reduce the ability of the compounds to interact with the bacteria cell membranes and act as a possible source of carbon for the bacteria (Ma et al., 2016; Inouye et. al., 2001). Similar results were obtained by Dev Kumar et al., (2020) where the emulsion obtained with PA and Tween80 had a lower efficacy against *Salmonella* Newport, Oranienburg and Typhimurium in comparison with Quillaja Saponin. Another important observation was that the surfactant type and concentration affect the inhibitory and bactericidal activities of PA.

Combinations of LA with surfactants did not improve antimicrobial activity. These results are similar to previous research evaluating the antimicrobial activity of LA. In another experiment that was conducted to evaluate the effect of LA on the growth of *Salmonella, E. coli,* and *Listeria* using *in vivo* antimicrobial susceptibility, the initial bacteria inoculum was ~10^7 CFU/mL and was incubated in lactic acid for 6 h prior to plating to determine viability. *Salmonella* was exposed to 0.25% LA was completely killed, and after 2 h exposure the same

result was observed for *E.* coli. When 0.5% LA was evaluated, 1 h exposure killed *Salmonella* and *E.* coli and 2 h of exposure killed *Listeria*. Viability of *Salmonella, E. coli* and *Listeria* was eliminated following 6 h exposure to LA (Wang et al., 2015). Although previous studies demonstrated high antimicrobial activity present in LA, in the current study of LA effect was not evaluated over long time treatment against *Salmonella* Infantis but only for a short contact time of 5 min which was effective in reducing counts below the level of detection when applied at 2000 ppm.

Other research, where they evaluated the effect of LA against *E. coli, Salmonella* Enteritidis, *Pseudomonas aeruginosa*, *Staphylococcus aureus,* and *Listeria monocytogenes* using 102, 209, and 321 mg/µL of isolated LA. The initial bacteria inoculum was 10^6 CFU/mL, and the antimicrobial effect was evaluated using the disc diffusion method. The results showed that LA inhibits the growth of all tested bacteria and when the concentration of LA increases, the inhibition area increases. LA was more effective against gram-positive bacteria than gram-negative (Stanojevic-Nikolic et al., 2015).

PAA at 75 and 135 ppm was effective in the reduction of *Salmonella* Infantis alone or in combination with saponin or Tween80. Concentrations of PAA between 50 and 500 ppm are typically used for antimicrobial interventions in commercial poultry processing facilities in the US (USDA, 2016) and are known to reduce *Salmonella* levels (Vaddu et al., 2021).

After conducting the *in vitro* experiments, chicken wings were evaluated to represent a worst-case scenario poultry part because the skin can act as a protective niche for microorganisms and prevent exposure to antimicrobial interventions in poultry processing plants. As expected, in this experiment with chicken wings flats, when PAA at 75 ppm was added as

treatement, efficacy was demonstrated in the reduction of *Salmonella* Infantis used in combination with 0.05% saponin or Tween 80.

PA has potential as an antimicrobial when combined as an emulsion with saponin but application parameters need to be further adapted to provide efficacy on poultry products. LA did not appear to be improved by emulsion with the selected surfactants, and PAA, which is the commonly used antimicrobial in the poultry industry, was demonstrated to be effective reducing *Salmonella* Infantis.

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Table 3.5 Experiment 1, *Salmonella* Infantis levels following treatment with pelargonic acid, lactic acid, or peroxyacetic acid alone or in combination with saponin or Tween 80 surfactants.

		Salmonella Infantis Log_{10} CFU/mL		
Organic acid	Concentration (ppm)	Surfactant concentration		
		0.05% Saponin	0.05% Tween 80	None
Pelargonic Acid	300	ND[1]	3.37 ± 0.10	3.46 ± 0.04
	500	ND	3.22 ± 0.11	3.36 ± 0.03
	700	ND	3.46 ± 0.07	3.27 ± 0.08
Lactic Acid	1000	3.13 ± 0.10	1.73 ± 0.86	2.61 ± 0.59
	2000	2.90 ± 0.18	ND	ND
	5000	ND	ND	ND
Peroxyacetic Acid	75	ND	ND	ND
	135	ND	ND	ND
Positive Control	-	3.55 ± 0.06	3.52 ± 0.04	3.62 ± 0.03
Negative Control	-	ND	ND	ND

[1] ND = not detected at a detection level of 7 log_{10} CFU/mL

Table 3.6. Experiment 2, *Salmonella* Infantis levels following treatment with pelargonic acid or peroxyacetic acid in combination with saponin or Tween 80 surfactants on inoculated chicken wing flats.

	Organic acid	Concentration ppm	Surfactant concentration	Log_{10} CFU/mL
Trial 1				
	Pelargonic Acid	300	0.05% Saponin	2.32 ± 0.09
			0.50% Saponin	2.31 ± 0.05
		1000	0.05% Saponin	2.14 ± 0.10
			0.50% Saponin	2.25 ± 0.06
	Positive Control	-	-	2.44 ± 0.03
Trial 2				
	Pelargonic Acid	300	0.05% Saponin	1.54 ± 0.08^{AB}
			0.50% Saponin	1.78 ± 0.07^{A}
		1000	0.05% Saponin	1.00 ± 0.09^{BC}
			0.50% Saponin	1.01 ± 0.22^{BC}
	Peroxyacetic Acid	75	0.05% Saponin	0.60 ± 0.19^{C}
			0.05% Tween 80	0.44 ± 0.17^{C}
	Positive Control	-	-	1.41 ± 0.08^{AB}
Trial 3				
	Pelargonic Acid	300	0.05% Saponin	2.13 ± 0.04^{A}
			0.50% Saponin	2.01 ± 0.13^{AB}
		1000	0.05% Saponin	1.76 ± 0.12^{AB}
			0.50% Saponin	2.06 ± 0.12^{A}
	Peroxyacetic Acid	75	0.05% Saponin	1.48 ± 0.15^{BC}
			0.05% Tween 80	1.07 ± 0.19^{C}
	Positive Control	-	-	1.83 ± 0.06^{AB}
Overall				
	Pelargonic Acid	300	0.05% Saponin	1.93 ± 0.08^{A}
			0.50% Saponin	1.99 ± 0.07^{A}
		1000	0.05% Saponin	1.56 ± 0.12^{B}
			0.50% Saponin	1.70 ± 0.15^{AB}
	Peroxyacetic Acid	75	0.05% Saponin	1.04 ± 0.16^{C}
			0.05% Tween 80	0.76 ± 0.15^{C}
	Positive Control	-	-	1.82 ± 0.09^{AB}

$^{A-C}$ Values within a column within a trial with different superscripts are significantly different ($P \leq 0.05$).

CHAPTER 4: CONCLUSIONS AND FUTURE IMPLICATIONS

Antimicrobials interventions are important in the poultry processing industry. The need of start looking for new alternatives is a concern in the last years due to the high demand of chemical-free products. Based on the findings on Chapter 2, photodynamic therapy using photo-active compounds still needs refinement, for it can be used under these conditions as an alternative in the poultry processing industries without causing no kind of delay in the production line, because in this thesis 5 minutes contact time was not as effective as peracetic acid for reduce *Salmonella* and *Campylobacter*. On Chapter 3, pelargonic acid in combination with Saponin was effective in the reduction of *Salmonella* Infantis *in vitro*, but when evaluated on chicken wing flats was not as effective as peracetic acid at 75 ppm in combination with Saponin or Tween 80 that reduce below detectable levels. There are different factors that can influence the effectiveness of this organic acids, natural compounds, and surfactants that still needs to be addressed and evaluate on laboratory scale and most important on poultry processing real scenarios. These findings are important to start considering new options to evaluate with different concentrations, time and implications in poultry meat and products, and in the future start having new natural alternatives for the consumer demand and regulatory restrictions that affect the actual processing procedures.