IN THE PURSUIT OF POULTRY: β-PHENYLETHYLAMINE AND ETHYL ACETOACETATE AS ANTIMICROBIALS ON CHICKEN

A Thesis
Submitted to the Graduate Faculty
of the
North Dakota State University
of Agriculture and Applied Science

By

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In Partial Fulfillment of the Requirements
for the Degree of
MASTER OF SCIENCE

Major Department:
Microbiological Sciences

April 2022

Fargo, North Dakota

North Dakota State University
Graduate School

Title

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MASTER OF SCIENCE

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ABSTRACT

This research examines the effect of β-phenylethylamine (PEA), a natural trace amine commonly found in food, and ethyl acetoacetate (EAA), an FDA approved flavoring agent and food additive, as novel antimicrobials on store-bought chicken thighs in a 5-minute immersion. In the first part of this experiment, 5%, 7.5%, and 10% treatments of β-phenylethylamine and ethyl acetoacetate were compared to control H_2O treatments utilized on chicken thighs. 10% treatments of PEA and EAA had significant reductions in counts of total aerobic bacteria and *Pseudomonas* spp. grown at 20°C by >1 log_{10} CFU/g of chicken meat. In the next experiments regarded 10% EAA as an antimicrobial on potential pathogens on chicken meat. The treatments of 10% EAA only succeeded in partial efficacy in the reduction of inoculated *Salmonella spp.* and *Campylobacter* spp. on chicken thighs.

ACKNOWLEDGMENTS

My immense gratitude goes out to my committee members and mentors: Dr. John McEvoy, Dr. Samiran Banerjee, Dr. Teresa Bergholz, Dr. Birgit Prüβ, and Dr. Shelley Horne for their counsel and esteemed guidance. To my friends and family that supported me throughout my research, I am immensely grateful for your continued upholding of me and my ambitions. And finally, to my mother, who instilled perseverance and the strength to succeed within me.

I would like to give a sincere thank you to Dr. Teresa Bergholz for the *S. enterica* R6-0020 strain and to Dr. Catherine Logue for the invaluable knowledge on the growth and culturing of *Campylobacter* spp.

My research was funded by grant 19-458A from the North Dakota Agricultural Products and Utilization Commission.

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LIST OF ABBREVIATIONS

ADHD .... Attention Deficit Hyperactivity Disorder

ANOVA .... Analysis of Variance

APT .... All-purpose Tween Agar

ASC .... Acidified sodium chlorite

BHI .... Brain and Hearth Infusion broth

CDC .... Center for Disease Control and Prevention

CFU .... Colony Forming Units

CPC .... Cetylpyridinium chloride

EAA .... Ethyl Acetoacetate

EU .... European Union

FDA .... Federal Drug Administration

FSIS .... Food Safety and Inspection Service

GRAS .... Generally Regarded As Safe

LAB .... Lactic-Acid producing Bacteria

LB .... Luria Bertani

LD_{50} .... Lethal Dose for 50% of the population

MHA .... Mueller-Hinton Agar

MRD .... Maximum Recovery Diluent

NIFA .... National Institute of Food and Agriculture

OD_{600} .... Optical Density at 600 nm wavelength

PBS .... Phosphate Buffered Saline

PAA .... Peracetic acid

PCA .... Plate Count Agar

PEA .... β-phenylethylamine

PSA .... *Pseudomonas* Selective Agar

SCCS .... Scientific Committee on Consumer Safety

SHY .... Sodium Hypochlorite

SSA .... *Salmonella-Shigella* Agar

TAB .... Total Aerobic Bacteria

TSB .... Tryptic Soy Broth

TSP .... Trisodium Phosphate

USDA .... United States Department of Agriculture

1. LITERATURE REVIEW

1.1. Introduction

It is in human nature to find out ways to work smarter, cleaner, and more efficient. The world's overall population of humans reached that of 7.9 billion in 2021 and with it has increased pressure on the ceaseless struggle to feed people. Having an extended shelf life and safer controls on pathogens is a functional and required step in the process of stifling hunger, reducing the impact of foodborne pathogens, and increasing security and reliability of food products.

When regarding foodborne pathogens, there is a heavy need for control as the impact of disease cripples both the individual and the community, causing loss of income and incurring food-scares amongst the population. Spoilage organisms result in food waste as the color, texture, taste, and odor of the product would result in decreased edibility. There is potential overlap between organisms that can cause spoilage and organisms utilized in for fermentation in food processing. The distinction between these is both in the purpose the organism is used for and in the outcome of the food product, although the fermentation of chicken meat is uncommon. Since 2016, chicken has been the most produced meat worldwide (Shahbandeh et al. Statista. 2021(1)), and therefore it is a topic of concern as it is a predominant source of bacterial contamination by *Salmonella* and *Campylobacter* (Chai et al. Epidemiol. Infect. 2016 (2)). With current pre-market washes both coming into question for safety and there simply being a requirement to find a more successful route of washing, β-phenylethylamine and ethyl acetoacetate are possible candidates of efficient and safe reduction of spoilage contamination of chicken. To consider pathogenic organisms on chicken, EAA is further explored and further exploration into EAA as a safe inhibitor of *Salmonella* spp. and *Campylobacter* spp.

1.2. Foodborne Pathogens

Food safety and hygiene are consistently important and of a high maintenance priority in society and is an ongoing science to provide improvements. It is a human right to have food, and in relation, that food should be safe for consumption. The Center for Disease Control (3) estimates that there are 48 million foodborne disease cases every year, a hospitalization rate of 128,000. 3,000 people die from foodborne diseases each year in the United States. This rate of illness heavily results in; the individual incurring the loss of income, a decrease in both individual and community productivity, increase the burden of the healthcare system, and decrease in the confidence of the food supply chain (FSIS Guidelines (4, 5)). For a distinct definition: a foodborne disease outbreak is defined as two or more illnesses caused by the same source which are linked to eating the same food. The total number of people effected by foodborne illness relating to an outbreak may be more than the total number recorded. Within the reported cases of foodborne illness, 20-22% of cases require hospitalization (Chen et al. Pediatr Neonatol. 2013 (6)). As hospitalizations occur and the recalls are published, this can result in a fear response within consumer crowds and therefore cause 'food scares' (Henson et al. Food Policy. 1999 (7)). Food scares often result in the spread of misinformation of actual danger, which can lead to a lack of money flow from purchasing groceries, food waste from non-purchased products, and, possibly, malnutrition (Henson et al. Food Policy. 1999(7)). As foodborne illness outbreaks occur, there will also be political pressures to instill higher-grade mechanisms for the assurance of food safety regarding popular, easily produced, and relatively affordable food options. As of the year 2021, production of children hit 135 million metric tons of meat and has been the top grossing meat product since 2016 (Antunes et al. Clin. Microbiol. Infect. 2016 (8)). Poultry products, including chicken products, are a long-standing source for

the spread of bacteria. Raw, rare, or any form of partially cooked chicken is hardly eaten on purpose in the US, whereas rare steaks and burgers are commonly served in restaurants and in-home kitchens. Despite cooking chicken thoroughly, it is a predominant source of foodborne illness (Antunes et al. Clin. Microbiol. Infect. 2015 (9)). The most commonplace pathogens associated with chicken foodborne outbreaks are non-typhoidal *Salmonella* spp. and *Campylobacter* spp. (Antunes et al. Clin. Microbiol. Infect. 2015, Chai et al. Epidemiol. Infect. 2016(9, 10)). There are many modes in which bacteria can be introduced to the animal and the edible parts of an animal, usually sourcing from the gastrointestinal tract.

Before slaughter in transportation and/or holding-pens, close quarters and defecation of animals can spread contaminated feces to uninfected animals (Marin et al. Poult Sci. 2009(11)).

After slaughter, contamination of the carcass can occur in the scalding step where the bird is initially immersed in water at either 50-52°C (soft-scald) or 56-58 C (hard-scald) (Rouger et al. Microorganisms. 2017(12)). A large source of contamination occurs in the plucking process where microorganisms on the outside of the bird become aerosolized or spread with the rubber fingers used to remove feathers (Arnold et al. Poult Sci. 2007(13)). The evisceration step is where the intestines are removed, which poses a threat of fecal microbiota spread is the intestines are punctured. The washing step, which is used as a bacterial intervention point that applies antimicrobials to reduce the pathogenic load, can also spread the contamination between carcasses (Russel et al. Poult Sci. 2007(14)). Observationally, the portioning of poultry part could also lead to contamination if individuals or machinery are exposed to microbes.

1.2.1. *Salmonella* spp.

Salmonella is a gram-negative bacterium that includes two species; *S. enterica* and *S. bongori*, with over 2500 serotypes identified by WHO (Thames et al. Foods. 2020(15)). While there are so many serotypes, only a few results in the proliferation of foodborne diseases. The infectious dose of the pathogenic *Salmonella* is between 10^6 and 10^8 bacteria for a healthy human adult. However, lower bacterial counts can cause diseases in those who are immunosuppressed, infants, and/or the elderly (Chen et al. Pediatr Neonatol. 2013(6)). When *Salmonella* presents itself in an infected human, the most common disease is acute gastroenteritis, which gives a plethora of symptoms; fever and chills, nausea and vomiting, abdominal cramping, and diarrhea, which may be bloody (Chen et al. Pediatr Neonatol. 2013(6)). Reactive arthritis, called Reiter's syndrome, is a sequelae illness caused by *Salmonella* infections (Dworkin et al. Arch. Clin. Infect. Dis. 2001(16)). There is an increasing rate of resistance to traditional agents (*i.e.*, ampicillin, chloramphenicol, and trimethoprim–sulfamethoxazole) have turned the treatment of invasive salmonellosis into a clinical dilemma (Chen et al. Pediatr Neonatol. 2013(6). Salmonella is a zoonotic-capable organism, and poultry populations are frequently colonized with *Salmonella,* becoming unaffected carriers, and transmitting it between one another by vertical and horizontal transmission (Barrow et al. Avian Pathol. 2012, Cosby et al. J. Appl. Poult. Res. 2015 (17, 18)). Improper storage, handling, and cooking can lead to pathogens on the poultry can lead to the cross-contamination of foods and household objects, thus leading to potential infections of humans and animals (Manios et al. 2014, Ravishanker et al. Food Micro. 2010, Sarjit et al. Journ of Food Protec. 2017(19–21)). The CDC estimates that 1 in every 25 packages of chicken at the grocery store are contaminated with *Salmonella.* This provides a

substantial need for better control of chicken-borne *Salmonellosis* before they can get to the consumer.

Table 1: *Salmonella spp.* outbreaks on chicken and poultry products in the United States between 2015 and 2021 (CDC(22)).

Date	Pathogen	Total Cases	Hospitalizations	Mortality	Products Linked
Oct 13, 2021	*Salmonella* Enteritidis	36	12	-	Raw Frozen Breaded Stuffed Chicken Products
May 18, 2021	*Salmonella* Hadar	33	4	-	Ground Turkey
May 7, 2019	*Salmonella* Schwarzengrund	7	1	-	Butterball Brand Ground Turkey
Feb 21, 2019	*Salmonella* Infantis	129	25	1	Raw Chicken Products
Dec 7, 2018	*Salmonella* I 4,[5],12: i:-	25	11	1	Chicken
April 16, 2018	*Salmonella* Typhimurium	265	94	1	Chicken Salad
Oct 16, 2015	*Salmonella* Enteritidis	15	4	-	Raw, Frozen, Stuffed Chicken Entrees

1.2.2. *Campylobacter* spp.

Campylobacter spp. are Gram-negative, motile, and non–spore-forming microaerophilic bacteria with a helical shape that changes to filamentous or coccoid as an adaptive response to environmental stresses (Hakeem et al. Front. Cell. Infect. Microbiol. 2021(23)). *Campylobacter* infections, or campylobacteriosis, is a major cause of diarrheal gastroenteritis worldwide (Kaakoush et al. Clin Microbiol Rev. 2015 (24)). Sequelae illnesses in relation to

Campylobacteriosis can include Guillain-Barré Syndrome and reactive arthritis, adding more to the clinical cost of the infection (Altekruse et al. Emerg. Infect. Dis. 1999(25)). In food sources, *Campylobacter* spp. can be carried in the gut or liver of slaughtered animals and be transferred to edible parts during processing, much like that described of *Salmonella* spp. Of the species pathogenic to humans, 90% of the disease is caused by *C. jejuni* and most of the rest by *C. coli* (Gillespie et al. Emerg. Infect. Dis. 2002 (26)). *Campylobacter* spp. infections have been heavily correlated to contaminated chicken, but do occur in water, raw milk, and other meats or seafoods. Relative to *Salmonella, Campylobacter* spp. have a very low infectious dose of 500 ≤ organisms for a healthy human (Robinson et al. BJM. 1981(27)). In 2015, National Antimicrobial Resistance Monitoring System (NARMS) testing found *Campylobacter* on 24% of raw chicken bought from retailers (CDC (28)). Within poultry houses; the source of transmission is wide and the horizontal route can be caused by farm visitors, wild birds, insects, amoebae, yeasts, and molds, which provides an almost inescapable reach of *Campylobacter* (Hiett et at. Appl. Environ. Microbiol. 2002, Axelsson-Olsson et al. Appl. Environ. Microbiol. 2005, Newell et al. Appl. Environ. Microbiol. 2011(29–31)).

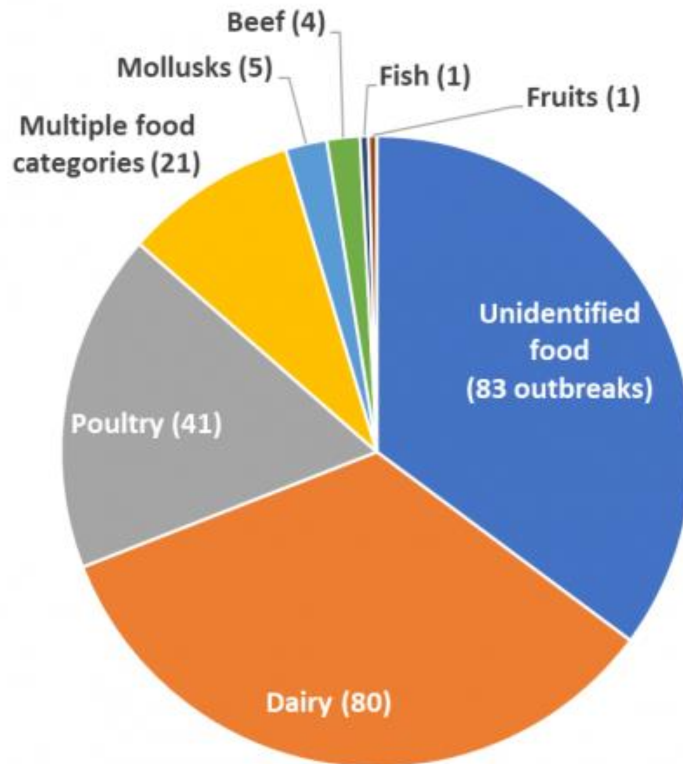

Figure 1: Outbreaks caused by *Campylobacter* in the United States, by food category, 2010-2017. CDC. (CDC (32)).

1.3. Spoilage

Food spoilage is different from foodborne pathogen cases and outbreaks, as the effect of the microorganism is witnessed before the consumption; an off-smelling odor, slime coating/biofilm, and/or discoloration (Petruzzi et al. 2017 (33)). Food spoilage is the main source of excessive food loss even with the modern techniques of spoilage inhibition (Gram et al. Int. J. Food Microbiol. 2002 (34)). The bacterial load would differ across chicken as the contamination of spoilage and pathogenic organisms are often traced back to the animal gut and the current microbiota consisting there (Marmion et al. Food Microbiol. 2021 (35)). It is not just constrained to bacterial contamination, but to that of molds and yeasts as well (Sohaib et al. J. Food Sci. Technol. 2015, Review (36)). Speculatively, differences in the microbial load and species will change the observed effect and speed of spoilage of the chicken, impacting shelf-life. Food

antimicrobials are often utilized to reduce the pathogenic bacterial load on food but may also reduce any spoilage organisms to the same effect. Within the processing of chicken, the washing step(s) is the procedure which is used to lower bacterial loads by applying the food antimicrobials. Antimicrobials, such as chlorine, are also added in the chilling tanks. The chilling step is utilized to keep the bacterial loads from increasing, but not directly for the reduction of bacteria. The effect of the washing step is observed in diminishing *Salmonella* spp. and *Campylobacter* spp. but has little to no difference in its impact on shelf-life (Demirok et al. Poult Sci. 2013 (37)). One of the main perpetrators of spoilage on chicken is *Pseudomonas* spp., which is a psychrophilic organism and capable of proliferation in temperatures between -5°C and 30°C. Other common spoilage associated organisms that are isolated from fresh chicken meat in aerobic conditions were *Enterococcus* spp. and *Shewanella* spp. (Russel et al. Poult Sci. 1995 (38)). Under modified atmospheric packaging, or vacuum sealed packages, the common spoilage associated microorganisms on chicken meat were *Lactobacilli* spp*.,* *Enterobacteria* spp., and *Brochothrix thermosphacta* (Jiménez et al. J Appl Micro. 1997 (39)). Spoilage can be caused by more than one microorganism at a time, including those that cause foodborne illness if consumed.

1.4. Current Prevention

There are both pre-slaughter and post-slaughter methods of foodborne pathogen reduction. From USDA-FSIS 2021 Guideline for Controlling *Salmonella* and *Campylobacter* in Raw Poultry, recommendations for pre-slaughter includes that of immunization, pre- and probiotics, general cleanliness in hatcheries/grow-out farms, and keeping transportation cages clean (4, 5). Establishments that process poultry are required to document the procedures they use to reduce or prevent the contamination throughout the slaughter and carcass processing steps.

The management systems in place to mitigate the contamination are: Hazard Analysis and Critical Control Points (HACCP), Sanitation Standard Order of Procedure (SOP), or other programs or reduce hazards that are in accordance with 9 CFR 417.5. The HACCP plan dictates a critical control point (CCP) that is defined as a step in the food process that controls, such as antimicrobials, are applied to result in food hazards, such as pathogens, being reduced, removed, or prevented. Antimicrobial interventions, which are part of the HACCP system and CPP, are followed up with routine sampling that registers whether the interventions were effective. The target organisms for antimicrobial procedures are commonly *Salmonella* and *Campylobacter*. The reduction of these organisms is not monitored by $\log_{10}$ CFU/g of meat, but by the amount of sampling that comes back positive for the pathogen. However, the implication of a better antimicrobial would directly correlate with the reduction of samples coming back positive, which allows for research into antimicrobials to be applied in a $\log_{10}$ CFU/g reduction scale.

Water baths and antimicrobial washes have been used to decrease spoilage and pathogenic bacteria loads. Poultry scalding baths are utilized to help loosen the feather follicles prior to the plucking step, but also aids in the removal of fecal matter and bacteria from the outside of the bird. However, this can often promote the transfer of bacteria from one carcass to another (Göksoy et al. Poult. Sci. 2004 (40)).

Antimicrobials are used in multiple steps of the processing of chicken, attempting to broaden the ability of those antimicrobials to cut down the contamination and increase food safety: these usually occur with equipment management/cleaning, carcass washing, reprocessing, immersion treatment, and post-chill treatment (USDA-FSIS (4, 5)). Peracetic acid (PAA) is a compound of acetic acid and hydrogen peroxide and is a commonly used antimicrobial to reduce the pathogenic load on poultry, effective due to combined acidic and oxidizing properties

(Kataria et al. Poult Sci. 2020, Fatemi et al. J. Food Prot. 1999 (41, 42)). When tested as an immersion dip at 500 ppm of PAA for 30s, *Salmonella* and *Campylobacter* were reduced by 1.76 and 1.78 log_{10} CFU/ml, respectively (Kumar et al. Poult Sci. 2020 (43)). In another test, *Salmonella* and *Campylobacter* were reduced in a post chill immersion in 400 ppm of PAA for 20s which resulted in the reduction of 2.02 and 1.93 log_{10} CFU/ml, respectively (Nagel et al. Int. J. Food Microbiol. 2013 (44)). PAA is disadvantageous due to high cost, yield loss, fat loss on meat pieces, discoloration of meat, and weak carcinogenicity in higher concentrations or after prolonged/repeated exposure; it also causes serious eye and skin damage (USDA-FSIS, Auer et al. Equine Surgery. 2012 (4, 5, 45)). PAA is approved for exported products while also being the most used antimicrobial in 2010 in on-line reprocessing, inside-outside bird washers, carcass chilling and post-chill treatment (Wideman et al. Poult. Sci. 2016, USDA-FSIS (4, 5, 46)). Cetylpyridinium chloride (CPC) is another antimicrobial agent used on raw poultry. CPC is effective at lowering the counts of *Salmonella* and *Campylobacter* but requires extra washes with potable water afterward and the wastewater is estimated to have a detrimental effect on the microbial kill-off during future wastewater treatment (Beers et al. Int. J. Poult. Sci. 2006 (47)). In a study by Kim and Slavik, chicken skin samples were inoculated with *Salmonella typhimurium* treated with an immersion of 2.5 mL 0.1% CPC solution. Chicken skin samples were either incubated at 1 or 3 minutes followed by an immediate rinse with 5 ml of H_2O. As an alternative, the CPC was removed after 1 minute and the skin was left for 2 minutes before rinsing with H_2O. These tests resulted in reductions ranging from log_{10} 1.0 to log_{10} 1.6 with longer immersion times resulting in higher reductions (Kim and Slavik et al. J. Food Prot. 1996 (48)). Zhang et al. tested CPC on chicken with concentrations of 0.35% and 0.6%, used in contact times of 10, 20, and 30 seconds in an immersion wash on inoculated drumsticks; CPC had no differences in

effectiveness on *S. typhimurium* at the three times under 0.35% CPC. 0.60% CPC treatments on *S. typhimurium* had a significant ($p>0.05$) increased effectiveness for the 30 second contact(49). The treatment of 0.6% CPC significantly reduced the $\log_{10}$ CFU/mL of *S. typhimurium. C. jejuni* in Zhang et al's study was reduced by 0.8 $\log_{10}$ with no effect correlating with the time or concentrations used (49). CPC in higher concentrations is known to be a toxic agent that can cause severe damage and even fatal if inhaled, serious damage to eyes, skin irritation, and can be harmful if swallowed (50). Acidified sodium chlorite (ASC) yielded conflicting results with one study showing an effective reduction in *Campylobacter* spp. reduced by 1.7 $\log_{10}$, but another reduced counts by 0.2 log10 (Kemp et al. J. Food Prot. 2000, Oyarzabal et al. J. Food Prot. 2005 (51, 52)). ASC treated full broiler carcasses exhibited an averaged reduction of $\log_{10}$ CFU/ml from 2.78 to 1.23 (Sexton et al. Int. J. Food Microbiol. 2007 (53)). In a study by Hwang et al. using a solution of 0.5% lactic acid/0.05% sodium benzoate, raw chicken wings inoculated with *Salmonella, Campylobacter jejuni, Listeria monocytogenes, Staphylococcus aureus*, or *Escherichia coli* O157:H7 had a reduced load of *Salmonella, C. jejuni*, and *E. coli* O157:H7. This effect was less severe in *L. monocytogenes* and *S. aureus* (Hwang et al. Int. J. Food Microbiol. 1995 (54)). Today's carcasses are commonly sanitized in processing plants through a series of washes using chlorinated water to reduce surface contamination. However, due to customer perception, occupational health, and safety concerns regarding the use of chlorinated water, there is a need to find alternative ways of sanitization (Chousalkar et al. Int. J. Environ. Res. 2019 (55)). Chlorine is a widely used antimicrobial for both water and food as a municipal regulation as its oxidizing capabilities are responsible for killing a wide array of pathogens and viruses. Chlorine disrupts several aspects of the cell's biology, including the cell membrane (Virto et al. Appl. Environ. Microbiol. 2005)(56). Cold water used for chilling carcasses after

evisceration can act as a source of cross-contamination between carcasses, but also has a decontaminating effect when 5 mg/kg chlorine is added to the water (Demirok et al. Poult Sci. 2013 (37)). However, chlorine was banned from being used by the European Union (EU) on food in 1997 due to safety concerns. Table 1 and Figure 1 reflect recent outbreaks due to *Salmonella* spp. and *Campylobacter* spp. This provides relevant reason to research new, novel and food-safe antimicrobials for the reduction of pathogenic organisms.

As the outlook on antimicrobials change and the socioeconomic desire for higher standards come forward, novel options are being appraised for their capabilities against spoilage and pathogenic bacteria. β-phenylethylamine and ethyl acetoacetate are two promising novel antimicrobials but require the proper investigative experimentation to analyze the true effect they may have on the spoilage and pathogenic bacterial loads in chicken products.

1.5. β-phenylethylamine

β-phenylethylamine is a molecule that weighs 121.18 g/mol with a formula of $C_8H_{11}N$. It has been found in several parts of the mammalian brain (Philips et al. Biol Psychol. 1978, Boulton et al. J. Neurochem. 1975 (57, 58)). Changes in the PEA metabolism have been demonstrated in various human disorders including phenylketonuria, migraine, schizophrenia, attention deficit hyperactivity disorder (ADHD) and deficiencies in PEA can lead to depression (Sotnikova et al. J. Neurochem. 2004, Sabelli et al. J. Neuropsychiatry Clin. Neurosci. 1996 (59, 60)). Treatments utilizing PEA against depression showed a 60% relief rate in patients with no apparent side effects (Sabelli et al. J. Neuropsychiatry Clin. Neurosci. 1996 (60)). While being present naturally in the mammalian brain, it also occurs in several types of food. In chocolate, it is not produced as a biological product. Instead, it is formed, or increased, as a result of the thermal processing of cocoa (Granvogl et al. J. Agric. Food Chem. 2006 (61)). In eggs, which are

a staple food choice, 38.0 mg/kg of PEA have been detected in the albumens (Figueiredo et al. Poult Sci. 2013 (62)).

While also occurring in food naturally, PEA can be found as a byproduct of bacteria in food spoilage and fermentation cases, resulting due to a tyrosine-decarboxylase *(TyrDC*) encoding gene that allows for both decarboxylase activity against tyrosine and phenylalanine, the latter leading to the production of PEA (Marcobal et al. Syst. Appl. Microbiol. 2004, Landete et al. Int. J. Food Microbiol. 2007 (63, 64)). *Enterococci* is a lactic acid producing bacteria (LAB) and in cases of meat spoilage and the production of traditional cheese, it fulfills both roles as spoilage organism and a fermenter, respectively. In a study to identify the different *Enterococcus faecium* strains, Marcobal et al. found that some contained the putative *tyrDC* that allowed for the encoded decarboxylase activity to produce PEA (Marcobal et al. Syst. Appl. Microbiol. 2004 (63)). Ten commercial red wines from Utiel-Requena with accomplished malolactic fermentation were analyzed for amines and the amine-producing LAB. In a study on colonies of LAB in red wines, amine-producing bacteria were screened to produce tyramine and PEA, in which certain strains of *Oenococcus oeni, Lactobacillus hilgardii, Lactobacillus brevis,* and *Pediococcus parvulus* all produced PEA within the wine samples (Landete et al. Int. J. Food Microbiol. 2007(64)).

While being natural in the body and in the food we consume, there have also been several treatments using PEA against microbes. PEA has been proven to be heat-safe, as using it as an antimicrobial in products that will/could be cooked requires it to not change its chemical formation into possibly dangerous compounds. In tests performed by Horne et al., PEA was heated to 73.9 °C or 93.3 °C and with the use of gas chromatography and mass spectrometry was shown to be unaltered by the heating process (Horne et al. Antibiotics 2021 (65)). Against *E. coli*

O157:H7, also a documented food pathogen, treatments of PEA reduced bacterial counts by 90% at a concentration of 150 mg/ml and 85% at 70 mg/ml (Lynnes et al. Meat Sci. 2014 (66)). In a study by Muchaamba et al, the effect of PEA was tested as a treatment against *L. monocytogenes*. Results that showed that PEA not only inhibited the growth of *L. monocytogenes* completely at 8mg/mL in Brain-Heart Infusion broth but also discouraged biofilm activity at lower concentrations (Muchaamba et al. Foods. 2020 (67)). In a medical setting, PEA has also been shown to have efficacy as a liquid media in catheter flushes for the inhibition of biofilm formation, bacterial cell counts, and growth (Schroeder et al. J. Med. Microbiol. 2018 (68)). PEA has been tested as an antimicrobial on beef broth and beef muscle. In beef broth, it was determined that the minimal bactericidal concentration against *E. coli* and *Salmonella* spp. was 25.15% for PEA and 20.80% for EAA (Horne et al. Antibiot. 2021 (65)). In Lynnes et al's study, PEA treatments significantly reduced inoculated *E. coli* 0157:H7 on beef muscle, reducing counts by 75% overall. Further research into PEA as a novel antimicrobial is a speculatively worthwhile process (66).

1.6. Ethyl Acetoacetate

Ethyl Acetoacetate (EAA) is a chemical intermediate with a molecular weight of 130.14 and a formula of $CH_3COCH_2COOC_2H_5$ and is commonly used for synthetic dyes and drugs. It is approved as a food additive by the FDA under 21CFR172.515 and used for flavoring under Flavis No. 9.402.(FDA, Code of Federal Regulations). It is a sweet or fruity-smelling substance that, under observation, goes into solution slowly with water. In a panel discussing the safety of EAA, for usage as a fragrance ingredient, risk-assessment tests were performed including genotoxicity, repeated dose toxicity, developmental and reproductive toxicity, local respiratory toxicity, phototoxicity/photo-allergenicity, skin sensitization, and aquatic environmental safety.

The results gave way to EAA presenting no concern in all assessments (Api et al. Food Chem. Toxicol. 2019 (69)). In the same risk assessment study, EAA proved to not increase the bacterial cell counts of any of *Salmonella typhimurium* TA98, TA100, TA1535, TA1537, TA1538 or *Escherichia coli* WP2uvrA nor did it prove to be mutagenic within the results of an Ames test (Api et al. Food Chem. Toxicol. 2019 (69)). For claims of edible safety, in a 28-day treatment bracket, EAA was dosed to 32 rats separated by sex based on weight at intervals of 100 mg/kg, 300 mg/kg, and 1,000 mg/kg. This resulted in post-mortem necropsies that showed no damage of the tissues. The living animals showed no noted behavioral changes, mean body weight changes based on male and female differentiation, or intake of foods (Cook et al. Food Chem. Toxicol. 1992 (70)). Despite being a safe edible material and lacking in risk concerns, the use of EAA in food also brings up the topic of heating and the possibility of denaturing into potentially harmful serotypes. In the same study as PEA, the same test was done to samples of EAA that were heated to 73.9 °C or 93.3 °C. With the use of gas chromatography and mass spectrometry also indicated that EAA was unaltered by the heating process. This proved that EAA a safe-to-use option prior to cooking (Horne et al. Antibiotics 2021 (65)). EAA has been used in several antimicrobial experiments to provide a reasonable, plausible use as a new wash on food products to prevent cases of foodborne illnesses and lengthen the shelf-life by decreasing spoilage organisms. Against *Cronobacter sakazakii*, *Serratia marcescens,* and *Yersinia enterocolitica*, EAA was used as a treatment to inhibit biofilm production and planktonic cell growth (Horne et al. Appl Micr. 2018 (68)). In this study, biofilm production and planktonic bacterial growth of *Y. enterocolitica* were observed at incubation temperatures of 25°C and 37°C. With treatments EAA at 5 mg/ml, 10 mg/ml, 15 mg/ml, and 20 mg/ml, the inhibition of colony forming units of *Y. enterocolitica* were reduced compared to an H_2O control (Horne et al. Appl Micr. 2018 (68)). In the same

study, *C. sakazakii* and *S. marcescens* were treated with EAA at respective incubation temperatures of 37°C and 30°C. *C. sakazakii* and *S. marcescens* exhibited a significant overall decrease in biofilm amounts at both temperatures with a EAA treatments (Horne et al. Appl Micr. 2018 (68)). In unpublished research from our own laboratory, EAA was bactericidal at 8% against planktonic cells of *Salmonella enterica* (Dr. Horne and Dr. Pruess personal communication).

To further a strengthening relationship between EAA and its use as an antimicrobial, research into its effect on both spoilage and pathogenic organisms in relation to certain products must be tested and analyzed for areas of significance. Another invaluable step in this direction is the use of EAA on pieces of meat, one of which has been done on ground beef (Horne et al. Antibiotics 2021). The capabilities of EAA as an antimicrobial and as a food-processing aid are still mostly unexplored. This, currently, leaves a wide-open place to test it against spoilage and pathogenic organisms on different categories and types of produce.

1.7. Objectives

Within this thesis, there are two interconnected experiments.

- The first experiment was to test 5%, 7.5%, and 10% treatments of PEA and EAA on spoilage organisms on store-bought chicken. We tested the total aerobic bacteria on PCA, *Pseudomonas* spp., and *Lactobacilli* spp. to test the efficacy of each treatment against the collective aerobic bacteria and two spoilage associated bacteria by comparison to an H_2O control. *Pseudomonas* spp. and *Lactobacilli* spp., respectively, were utilized as representative species of spoilage involving one organism under aerobic-growth or anaerobic-growth-requirements.

- The objective of the second experiment was to introduce common poultry-associated pathogenic bacteria, apply a 10% EAA treatment, and analyze the effect of the 10% EAA treatment on the added pathogens. 10% EAA was chosen in part due to the results of the first experiment and to fulfill background research for a patent. Two strains of *Salmonella* spp. and *Campylobacter* spp., respectively, were utilized to postulate the efficacy of 10% EAA.

Both sets of experiments aid in the observation of PEA and EAA as novel antimicrobials on poultry.

2. MATERIALS AND METHODS

2.1. Chicken Preparation

Skinless chicken thighs were bought from a grocery store the day of the experiment and moderately (between 75g and 95g) sized thigh pieces were selected for the experiment. These chicken thighs were transferred to zip-lock bags immediately with gloves.

2.1.1. Preparation of PEA, EAA, and H_2O Treatments

200 mL aliquots of 5%, 7.5%, and 10% PEA-HCl (TCI America, Portland, OR) and EAA (Alfa Aesar, Ward Hill, MA) in sterile H_2O were made the day of the experiment. After being brought into solution by vortexing, the solutions were passed through a 0.2 µm filter for sterilization. The control H_2O treatment was heat sterilized.

2.2. Experiment 1: Spoilage Flora of Chicken

Single chicken thighs were initially washed with phosphate buffered saline (PBS) by adding 200 ml of PBS to the chicken in the zip-lock bags. The chicken thighs were washed by immersing them in the PBS and agitating them by hand initially before allowing them to sit immersed for 5 minutes before being aseptically transferred to sterile mesh racks to drip for 10 minutes. The PBS rinse was used to keep the process as uniform as possible between the spoilage experiments and the pathogenic experiments. After being allowed to drip for the full 10 minutes, the chicken was placed into new zip-lock bags. The 200 ml solutions of PEA, EAA or the H_2O were added to the bags as treatments. The chicken thighs were treated by immersing them in the treatments and agitating them initially before allowing them to sit immersed for 5 minutes before being transferred to drip dry on a sterile mesh rack for 10 minutes. The chicken thighs were placed in stomacher bags, reweighed, and placed in a 4°C incubator for 30 minutes. The final weight was used to calculate the amount of maximum recovery diluent (MRD) to be

added. One ml of MRD was added for every 5 g of chicken. After the 4°C incubation, the calculated MRD was added to the stomacher bags. The chicken was homogenized by use of the Seward Stomacher 400 Circulator (Cole Parmer, Vernon Hills, IL) for 30 s at 230 rpm. Homogenate was withdrawn from the stomacher bags and placed within 15 ml centrifuge tubes. For the dilution series, the homogenate was diluted in four 1:10 steps to a factor of 10^{-4}. 100 µl of each dilution was spread onto agar plates. For the full workflow breakdown, reference Figure 2.

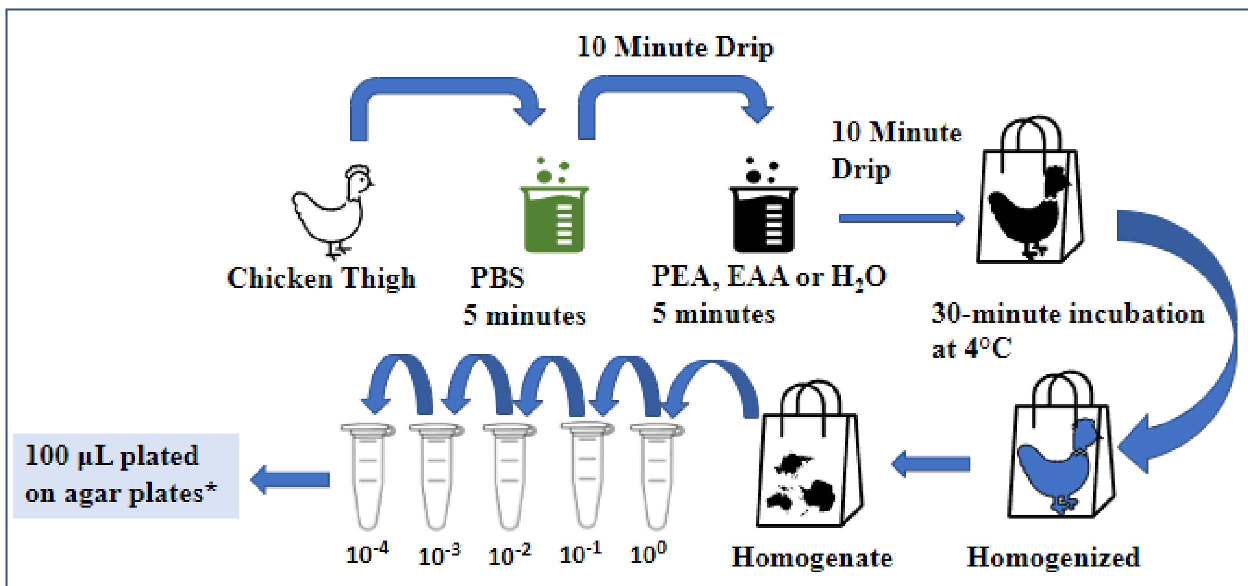

Figure 2: Workflow for the plating of spoilage organisms.

2.2.1. Enumeration of Spoilage Microbes

100 µl of the dilutions were plated on two plates each of Plate Count Agar (PCA), *Pseudomonas* Selective Agar (PSA), and All-Purpose Tween agar (APT), using a lazy-L spreader. PCA and PSA plates were incubated at ~ 20°C. APT plates were incubated anaerobically at 30°C for 48 hours. Colonies were counted on all dilutions possible. Recipes for all media are shown in Table 2.

Table 2: Diluent and selective agar plates.

Name	Abbrev.	Purpose	Composition	Brand
Maximum recovery diluent	MRD	Diluent	1 g/l peptone, 8.5 g/l NaCl, pH 7.0	Becton Dickinson
Plate count agar	PCA	Total aerobic bacterial counts at ~20°C	5 g/l tryptone, 2.5 g/l yeast extract, 1 g/l glucose, 15 g/l agar, pH 7.0	Difco BD
Pseudomonas agar	PSA	Detection of pseudomonads at ~20°C	16 g/l gelatin peptone, 10 g/l casein hydrolysate, 10 g/l K_2SO_4, 1.4 g/l $MgCl_2$, 0.5 mg/ml cetrimide, 0.5 mg/ml fucidin, 2.5 mg/ml cephalosporin, 11 g/l agar, pH 7.1	Oxoid
All purpose tween agar	APT	Detection of lactobacilli at 30°C	7.5 g/l yeast extract, 12.5 g/l pancreatic digest of casein, 10 g/l dextrose, 5 g/l sodium citrate, 0.001 g/l thiamine HCl, 5 g/l NaCl, 5 g/l K_2HPO_4, 0.14 g/l $MnSO_4 \cdot H_2O$, 0.8 g/l $MgSO_4 \cdot 7H_2O$, 0.04 g/l $FeSO_4$, 0.2 g/l polysorbate, 15 g/l agar, pH 6.7	Difco BD
Luria Bertani agar	LB	*Salmonella* growth	10 g/l tryptone, 5 g/l yeast extract, 5 g/l NaCl, 15 g/l agar	Difco BD
Tryptic soy broth agar	TSB	*Campylobacter* growth	17 g/l pancreatic digest casein, 3 g/l papaic digest of soybean, 2.5 g/l dextrose, 5 g/l NaCl, 2.5 g/l K_2PO_4, 15 g/l agar	Difco BD

Table 2: Diluent and selective agar plates (continued).

Name	Abbrev.	Purpose	Composition	Brand
Brain heart infusion	BHI	*Salmonella* and *Campylobacter* growth	7.7 g/l calf brain infusion solids, 9.8 g/l beef heart infusion solids, 10 g/l protease peptone, 5 g/l NaCl, 2 g/l glucose, 2.5 g/l Na_2HPO_4	Difco BD
Shigella Salmonella agar	SSA	Detection of *Salmonella*	5 g/l beef extract, 2.5 g/l pancreatic digest of casein, 2.5 g/l peptic digest of animal tissue, 10 g/l lactose, 8.5 g/l bile salts mixture, 8.5 g/l sodium citrate, 8.5 g/l sodium thiosulphate, 1 g/l ferric citrate, 0.025 g/l neutral red, 15 g/l agar, 0.33 mg/l brilliant green, pH 7.0	Difco BD
Müeller Hinton agar[1]	MHA	Detection of *Campylobacter*	2 g/l beef extract, 17.5 g/l acid hydrolysate of casein, 1.5 g/l starch, 12.5 mg/l sodium pyruvate, 12.5 mg/l ferrous sulfate, 12.5 mg/l sodium metabisulphite, 5,000 IU/l polymyxin B, 10 mg/l rifampicin, 10 mg/l trimethoprim lactate, 10 mg/l amphotericin B, 17 g/l agar, pH 7.3	DifcoBD/ Oxoid/ HiMedia Laboratories

[1]Note that MHA plates were supplemented with sodium pyruvate, ferrous sulfate, and sodium metabisulphite as *Campylobacter* growth supplement (liquid) SR0232E from Oxoid. Polymyxin, rifampicin, trimethoprim lactate, and amphotericin were added as *Campylobacter* selective supplement IV, modified (Preston Selective Supplement) from HiMedia Laboratories Pvt. Ltd. (Mumbai, India).

2.2.2. Analysis of Spoilage Microbes

Each experiment was performed in four biological replicates (different thighs from different packaging) with two technical replicates per biological replicate (homogenates plated on two identical plates). Counts obtained from the dilution series were converted to CFU/mL in the 10^0 (undiluted) samples. This was done by multiplying the counts with their dilution factor. CFU/g of chicken was then calculated by multiplying the CFU/mL by a factor of 2. The factor of 2 was computed from the 5-fold concentration of the chicken in MRD and the 1:10 dilution from plating 100 µl of the homogenate on each plate (10/5=2).

Averages were first calculated from the two plate replicates. Log_{10} CFU/ g of chicken was calculated for the four biological replicates from the average of the two plate replicates. Averages and standard deviations were calculated across the four biological replicates. For each concentration data set (5%, 7.5%, and 10% treatments of PEA and EAA separately), a comparison between the $\log_{10}$ CFU/g PEA and EAA treatment data and the replicate control H_2O were calculated as $\log_{10}$ reduction. This was calculated as $\log_{10}(a/b)$, where 'a' is bacterial count of the control H_2O and the 'b' are bacterial counts of PEA or EAA at each concentration. To analyze the data, a one-way ANOVA was performed to compare the log reductions of the combination of treatment and concentrations. A second statistical analysis was performed with a paired *t*-test to compare the treatments of PEA or EAA $\log_{10}$ CFU/g of chicken values against the H_2O $\log_{10}$ CFU/g of chicken. For data analysis, statistically significant p-values are > 0.05. This was done for each concentration of PEA and EAA.

2.3. Inoculated with Pathogens

All bacteria were stored at -80°C prior to the experiments. *Salmonella* spp. were incubated at temperatures of 34°C and *Campylobacter* spp. was incubated at temperatures of 42°C in a microaerophilic environment. All four bacterial strains were made resistant to 50 µg/ml of nalidixic acid by use of Taormina et al.'s method (71). All bacteria strains are detailed in Table 3.

Table 3: Pathogenic strains

Bacterial strain	**Alternative designation**	**ATCC #**	**Characteristic**	**Reference**
S. enterica serovar Typhimurium FSL R6-0020	TB0041	Not deposited	Source: Bovine feces Genome sequenced: no	www.foodmicrobe-tracker.com Vangay et al. J. Food Prot. 2013(72)
S. enterica subsp. *enterica* (ex Kauffmann and Edwards) Le Minor and Popoff serovar Typhimurium	LT2	ATCC 19585	Source: Lab modified Genome sequenced: yes	Laure et al. Food Sci. Biotechnol. 2021(73) Nguyen et al. Sci. Rep. 2020(74)
C. jejuni subsp. *jejuni* (Jones *et al*.,) Veron and Chatelain	NCTC 11168	ATCC 700819	Source: Human feces Genome sequenced: yes	Sher et al. Front. Microbiol. 2020(75)
C. coli (Doyle) Veron Chatelain	CIP 7080	ATCC 33559	Source: Swine feces Genome sequenced: yes	Sithole et al. Pathogens. 2021(76)

2.3.1. *Salmonella* spp. Inoculum

For our working stock, *Salmonella* spp. was stored on Luria Bertani broth (LB) agar plates, supplemented with 50 µg/ml of nalidixic acid, and placed in a 4°C cold storage. For inoculum preparation, *Salmonella* spp. were grown overnight in Brain Heart Infusion (BHI), supplemented with 50 µg/ml nalidixic acid. Cultures were incubated at 34°C. These overnight cultures of *Salmonella* were then diluted 1:10 into 20 ml of BHI, incubated at 34°C for 2 hours, and diluted to an OD_{600} of 1.0 with BHI. Bacteria in the 1.0 OD_{600} BHI culture were enumerated by plating onto LB plates. CFU/ml were determined to be between 3.01 x 10^8 and 3.37 x 10^9. This culture was further diluted 1:100 into 200 ml of PBS to form the inoculum. Each chicken was inoculated with a quantity of bacteria that range from 6 x 10^8 to 6.8 x 10^9 CFU.

2.3.2. *Campylobacter* spp. Inoculum

Campylobacter spp. followed a similar process with a change in incubation times and was consistently incubated at 42°C under microaerophilic conditions. *Campylobacter* spp. was plated on a weekly basis on Tryptic Soy Broth (TSB) agar plates, supplemented with 50 µg/ml nalidixic acid. To prepare the inoculum, *Campylobacter* spp. was grown for 3 days in 200 ml BHI. Due to the microaerophilic and fastidious nature of *Campylobacter* spp., this inoculum did not undergo any further modifications and was directly used. A sample of the inoculum was plated and the bacteria was enumerated. CFU were determined to be between 1.5 x 10^9 and 5.3 x 10^9 CFU of *C. jejuni* and between 3 x 10^6 and 1 x 10^7 CFU for *C. coli.*

2.3.3. Experiment 2: Inoculation with Pathogens

Chicken thighs were placed within zip-lock bags. Two chicken thighs were inoculated with a pathogenic strain (Table 2). Two additional chicken thighs were treated with a sterile control of either PBS for *Salmonella* spp. or BHI for *Campylobacter* spp. experiments. The

experiments using the pathogens are comparable to the spoilage microbes experiments and were performed in an identical way with one modification: chicken was incubated for five minutes with 200 ml of the pathogenic inoculum or the sterile control. After the 5-minute incubation, the chicken was drip dried for ten minutes on sterile mesh racks. The chicken was moved to new zip lock bags for treatment, which are as follows:

- chicken inoculated with bacteria: one treated with 10% EAA and one with H_2O.
- control chicken with no added bacteria: one treated with 10% EAA and one with H_2O

After the 5-minute treatment, the chicken was transferred to sterile racks and allowed to drip dry for 10 minutes. Chicken thighs were then transferred to stomacher bags, reweighed, and placed in a 4°C incubator for 30 minutes. To enumerate bacteria, 1 ml of MRD was added per 5 g of chicken. The chicken was homogenized by use of the Seward Stomacher 400 Circulator (Cole Parmer, Vernon Hills, IL) for 30 s at 230 rpm. Homogenate was withdrawn from the stomacher bags and placed within 15 ml centrifuge tubes. For the dilution series, the homogenate was diluted in four 1:10 steps to a factor of 10^{-4}. 100 µl of each dilution was spread onto agar plates. For the full workflow breakdown see Figure 3.

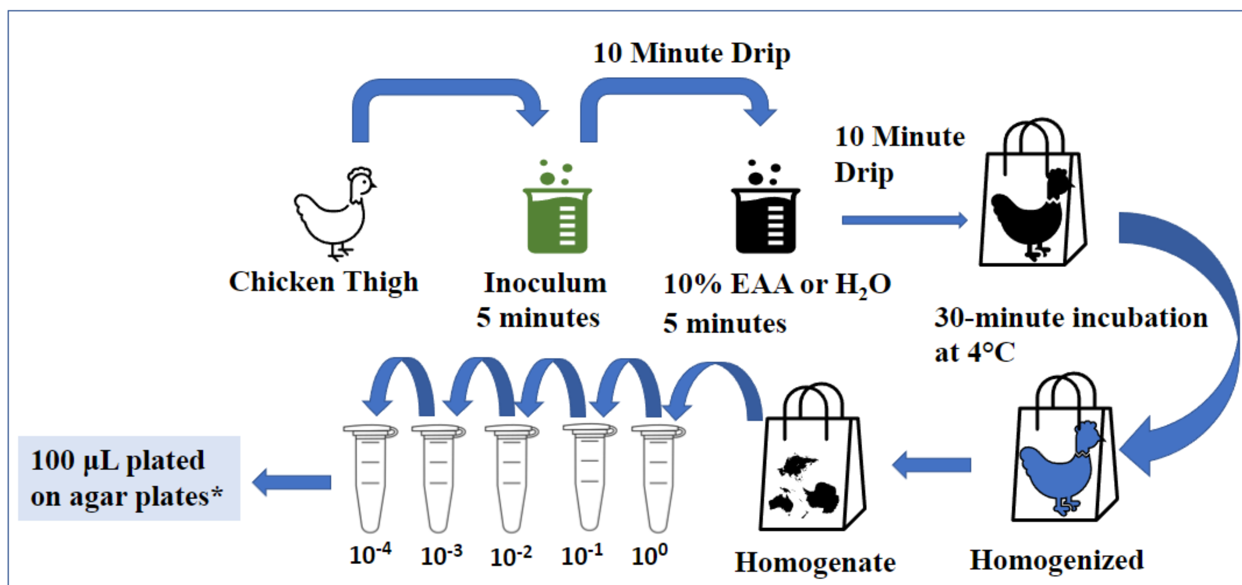

Figure 3: Workflow of pathogen-inoculated chicken treated with 10% EAA.

2.3.4. Enumeration of Pathogens

Each dilution was plated on two PCA plates for all homogenized chicken thighs.

Salmonella-Shigella Agar (SSA, Table 2) was used for the enumeration of *Salmonella* spp. inoculated chicken thighs. The *Salmonella* spp., due to being Typhimurium, utilized in this experiment appeared as black colonies after incubation due to the production of hydrogen sulfide. Two types of SSA were utilized in this experiment: a standard version and a version supplemented with 50 µg/ml nalidixic acid. Each replicate homogenate for the thighs inoculated with the *Salmonella* spp. and those uninoculated were plated on these SSA plates. Both types of SSA plates were incubated at 34°C for 48 hours. Colonies were counted on all dilutions possible.

Mueller-Hinton Agar (MHA, Table 2) with additional *Campylobacter* selective supplement and *Campylobacter* growth supplement was used for the enumeration of *Campylobacter* spp. Two types of MHA were utilized in this experiment: a standard version and a version supplemented with 50 µg/ml nalidixic acid. Each replicate homogenate for the thighs inoculated with the *Campylobacter* spp. and those uninoculated were plated on these MHA

plates. MHA plates were incubated under microaerophilic conditions at 42°C for 48 hours. Colonies were counted on all dilutions possible.

2.3.5. Analysis

Each experiment was performed in four biological replicates (different chicken thighs) with two technical replicates (homogenates plated on two identical plate sets). The analysis of the data was similar to that of the spoilage bacteria until the point where the $\log_{10}$ CFU/g of chicken data were calculated. A statistical analysis was performed on the $\log_{10}$ CFU/g of chicken data with a paired *t*-test (*p*-value <0.05) to compare the data between the EAA and H_2O treated chicken thighs. Log_{10} reductions were calculated with $\log_{10}(a/b)$, where ‘a’ is the bacterial counts from the control H_2O treatment and ‘b’ is the bacterial counts from the 10% EAA treatments.

3. RESULTS

3.1. PEA and EAA at a Concentration of 10% Decrease Spoilage Organisms on Chicken Thighs by More Than a Log

Spoilage organisms on chicken thighs were determined after treatments with PEA or EAA at the range of concentrations. The log_{10} reduction is the difference between the treatment and the H_2O control. The one-way ANOVA was performed on the log_{10} reduction data. Table 4 portrays the significant differences between log_{10} reductions of CFU/g of chicken data obtained at different concentrations of either PEA or EAA. For treatments of PEA, 10% is effectively better than 5% at the reduction of total spoilage bacteria from PCA plates (*p*-value of 0.03). For EAA the 10% treatment is also significantly more effective than 5% at reducing bacteria on PCA plates (*p*-value of 0.002) and *Pseudomonas* spp. on PSA plates (*p*-value of 0.04). All other comparisons did not yield statistically significant differences (Table 4).

Table 4: One-way ANOVA conducted on the log_{10} reduction of total aerobic bacteria, *Pseudomonas* spp., and *Lactobacilli* spp. by treatments of PEA and EAA

PEA		PCA			**EAA**		PCA		
PCA		5%	7.5%	10%	PCA		5%	7.5%	10%
	5%	-	No	0.0308		5%	-	No	0.0021
	7.5%	No	-	No		7.5%	No	-	No
	10%	0.0308	No	-		10%	0.0021	No	-
PEA		*Pseudomonas* spp., PSA			**EAA**		*Pseudomonas* spp., PSA		
PSA		5%	7.5%	10%	PSA		5%	7.5%	10%
	5%	-	No	No		5%	-	No	0.0416
	7.5%	No	-	No		7.5%	No	-	No
	10%	No	No	-		10%	0.0416	No	-
PEA		*Lactobacilli* spp., APT			**EAA**		*Lactobacilli* spp., APT		
APT		5%	7.5%	10%	APT		5%	7.5%	10%
	5%	-	No	No		5%	-	No	No
	7.5%	No	-	No		7.5%	No	-	No
	10%	No	No	-		10%	No	No	-

One-way ANOVA results mirrored on the dashed vertical. Entered and highlighted numerical values are expressions of significance based on p-value <0.05.

Log_{10} CFU/g of chicken data are presented in the Figures 4 - 6 for total bacterial counts from the PCA plates (Figure 4), *Pseudomonas* spp. counts from the PSA plates (Figure 5), and *Lactobacillus* spp. from the APT plates (Figure 6).

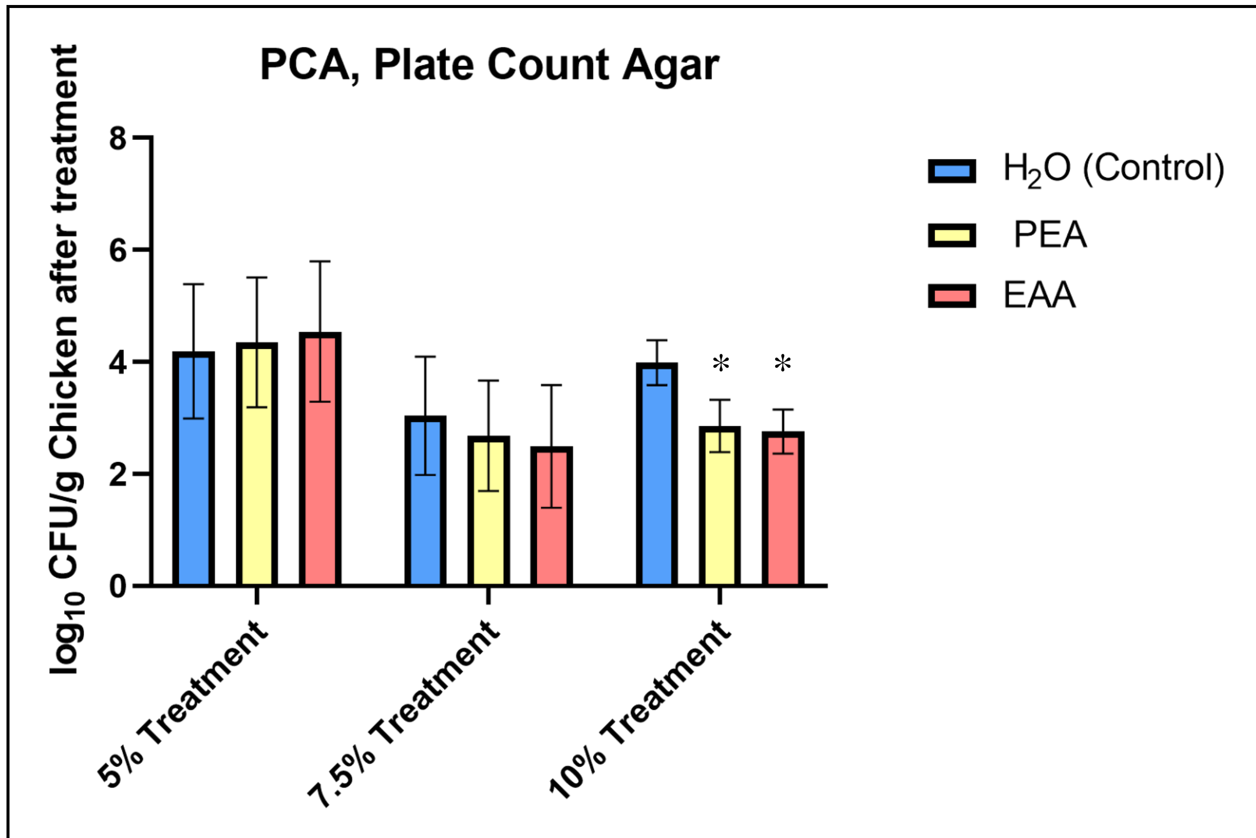

Figure 4: Spoilage bacteria counts on PCA, plate count agar. Log_{10} CFU/g of chicken of total aerobic bacteria at ~20 °C as compared between the H_2O control (blue), the PEA treatment (yellow), and the EAA treatment (red). The experiment was done at concentrations of 5%, 7.5%, and 10% of the antimicrobials. Note that a separate H_2O control experiment was performed with each of the treatments. A * describes a significant difference ($p>0.05$).

10% PEA and 10% EAA had the greatest effect on the log_{10} CFU/g of chicken that were obtained from the PCA agar. Treatments of chicken with 10% PEA and 10% EAA reduced total bacterial counts by 1.18 log_{10} (p-value 0.002) and 1.24 log_{10} (p-value 0.0005), respectively. Log_{10} reductions were calculated by the comparison of the treatment data with that of the H_2O control (Figure 4).

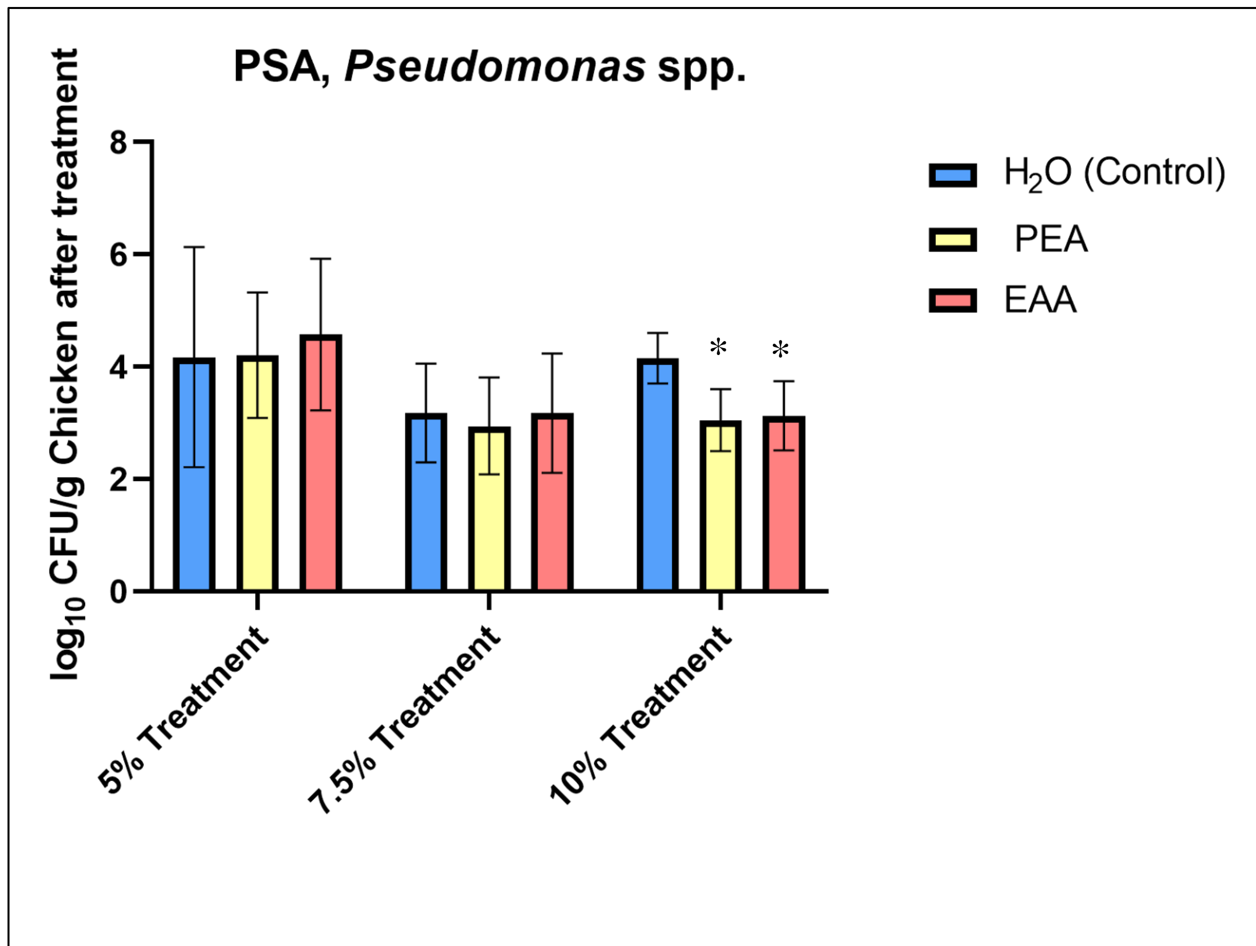

Figure 5: Spoilage bacterial counts on PSA, *Pseudomonas* spp. The $\log_{10}$ CFU/g of chicken of *Pseudomonas* spp. enumerated from the H_2O control (blue), the PEA treatment (yellow) and the EAA treatment (red). The experiment was done at concentrations of 5%, 7.5%, and 10% of the antimicrobials. Note that a separate H_2O control experiment was performed with each of the treatments. A * describes a significant difference.

10% PEA and EAA also had the greatest effect on the *Pseudomonas* spp. $\log_{10}$ CFU/g of chicken that were obtained from the PSA agar. Treatments of chicken with 10% PEA and 10% EAA reduced the *Pseudomonas* spp. counts by 1.14 $\log_{10}$ (*p*-value 0.004) and 1.03 $\log_{10}$ (*p*-value 0.008), respectively. Log_{10} reductions were calculated by the comparison of the treatment data with that of the H_2O control (Figure 5).

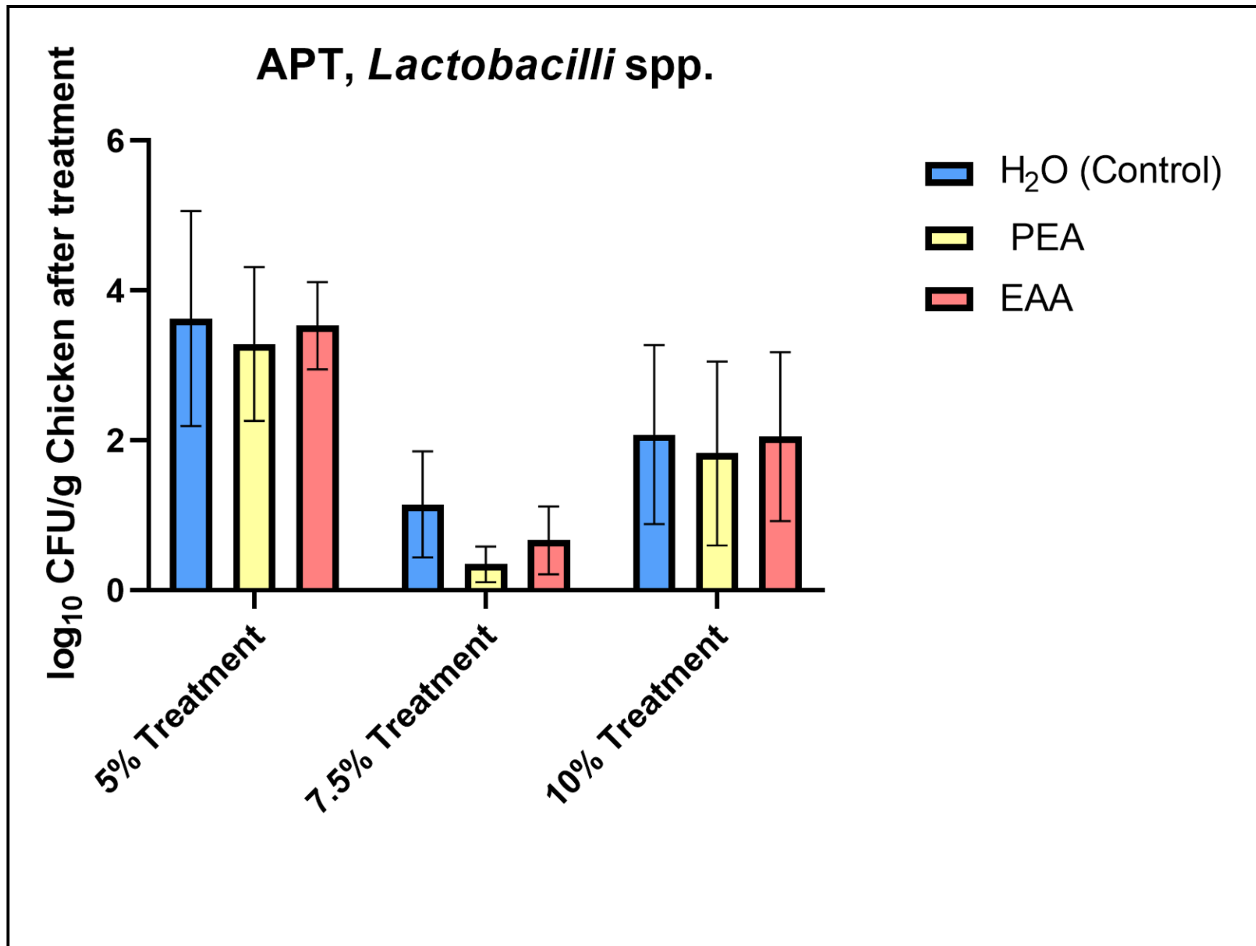

Figure 6: Spoilage bacterial counts on APT, *Lactobacilli* spp. The $\log_{10}$ CFU/g of chicken of *Lactobacilli* spp. enumerated from the H_2O control (blue) and 5%, 7.5%, and 10% PEA (yellow) and EAA (red). Note that a separate H_2O control experiment was performed with each of the treatments.

Lactobacilli spp. (Figure 6) yielded no significance of differences across PEA and EAA against the H_2O control.

A one-tailed paired *t*-test was used to test for significance of the differences between the H_2O control and the treatments. The *p*-values from the *t*-tests that compared the $\log_{10}$ CFU/g of chicken from the 10% PEA and 10% EAA treatments to the H_2O control on PCA plates were 0.002 and 0.001, respectively. On PSA plates, the *p*-values from the *t*-tests of the compared $\log_{10}$ CFU/g of chicken from the 10% PEA and 10% EAA treatments to the H_2O control was 0.004 and 0.008, respectively. Comparisons of the treatment data and the H_2O control on APT plates

yielded no significant *p*-values from the one-tailed paired *t*-test. A short comparison implies a close relationship between the ANOVA and the *t*-tests; all three significant *p*-values from the ANOVA were found significant with the *t*-tests. The *t*-tests included one more significant *p*-value from the comparison of 10% PEA and H_2O on PSA plates.

3.2. 10% EAA Reduces *Salmonella* spp. and *Campylobacter* spp. by Less Than a Log

Pathogenic organisms inoculated on chicken thighs were enumerated on selective agar plates for *Salmonella* spp. and *Campylobacter* spp. The EAA treatments were done at a concentration of 10% and compared to the H_2O control. Data in Figures 7-10 is expressed as $\log_{10}$ CFU/g of chicken obtained from the H_2O control and the 10% EAA treatments. A one-tailed paired *t*-test was used to evaluate for significance of the difference between the $\log_{10}$ data from the two treatments.

3.2.1. Results From the SSA and MHA Plates That Were Supplemented with Nalidixic Acid

10% EAA treatments reduced counts of inoculated nalidixic acid resistant *S. enterica* FSL R6-0020 by a statistically significant 0.36 $\log_{10}$ (*p*-value of 0.011). Counts of *S. enterica* ATCC19585 were reduced by 0.38 $\log_{10}$, but the corresponding *t*-test favored the null hypothesis (Figure 7).

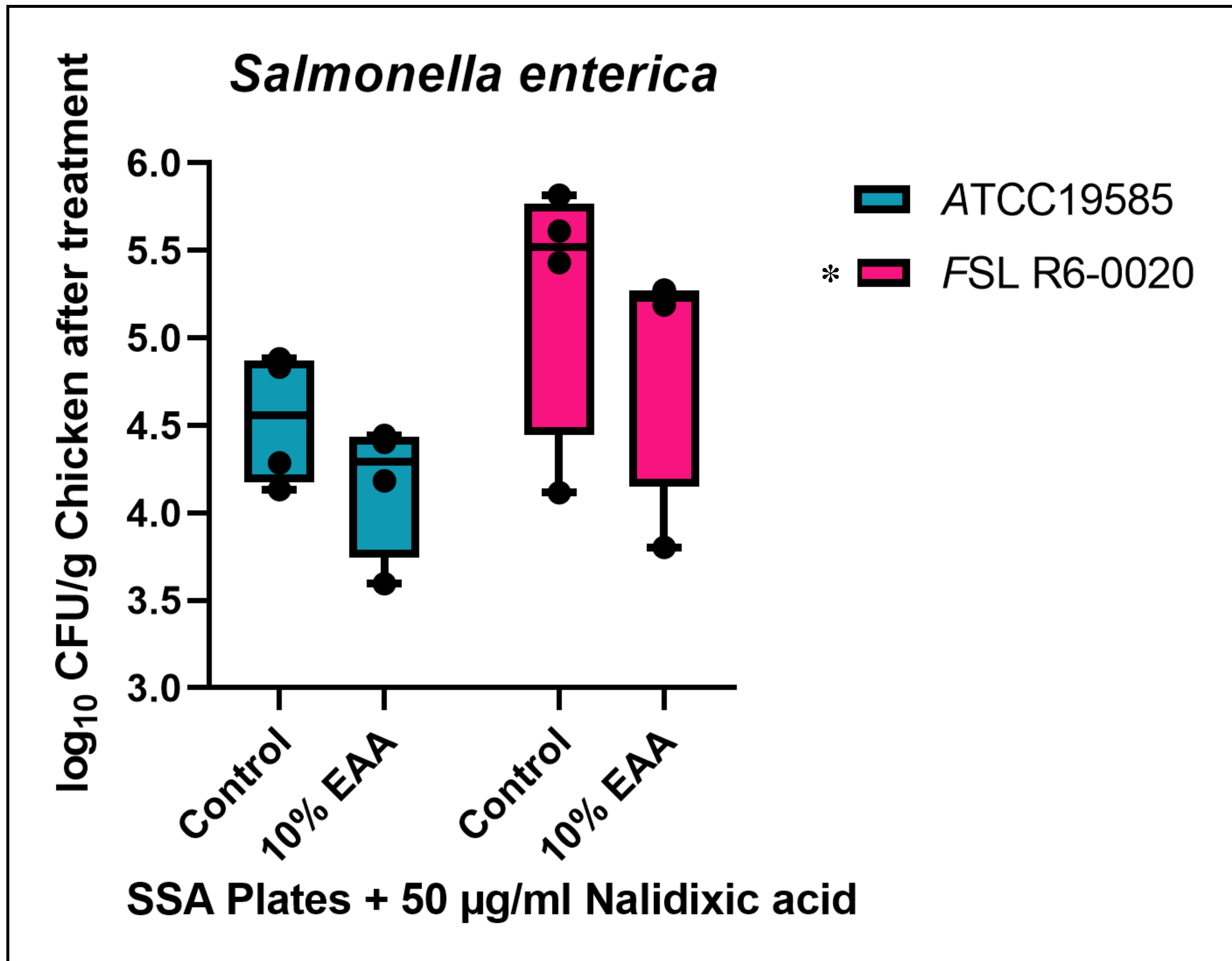

Figure 7: Salmonella enterica counts on SSA plates + 50 µg/ml Nalidixic acid. Box-and-whisker plots comparing the control H_2O to EAA washes on chicken inoculated with nalidixic acid resistant ***S. enterica*** **serovar Typhimurium FSL R6-0020** and ***S. enterica*** **subsp.** ***enterica*** **(ex Kauffmann and Edwards) Le Minor and Popoff serovar Typhimurium ATCC19585.** Data was retrieved from Salmonella Shigella Agar (SSA), supplemented with 50 µg/ml Nalidixic acid. A * describes a significant difference.

10% EAA treatments reduced counts of inoculated nalidixic acid resistant *C. jejuni* by 0.44 $\log_{10}$ CFU/g of chicken (*p*-value of 0.027). Counts of inoculated *C. coli* were reduced by 0.24 $\log_{10}$ CFU/g of chicken but the corresponding *t*-test favored the null hypothesis (Figure 8).

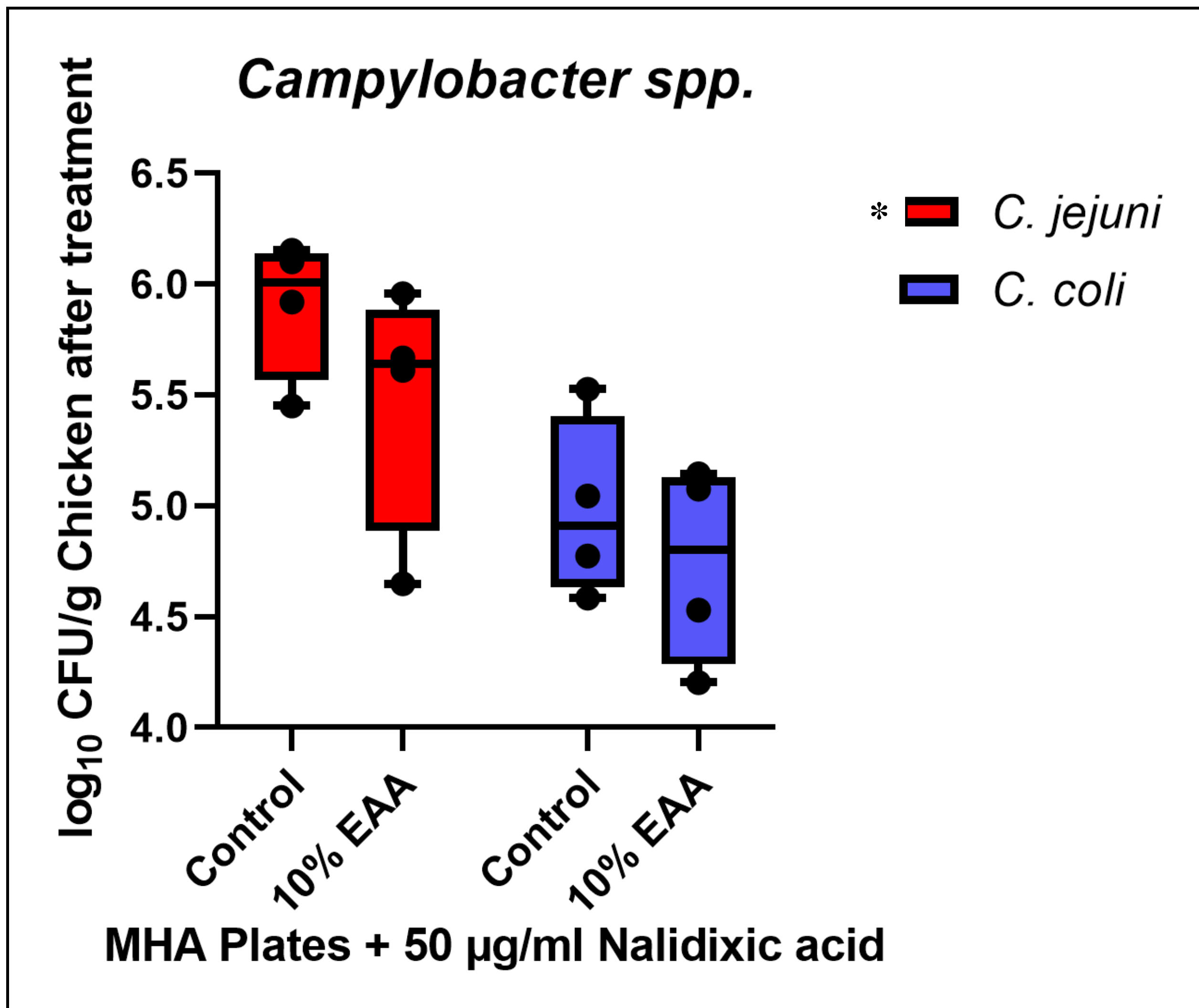

Figure 8: *Campylobacter spp.* counts on MHA plates + 50 µg/ml Nalidixic acid. Box-and-whisker plots comparing H_2O to EAA washes on chicken inoculated with nalidixic acid resistant ***C. jejuni* subsp. *jejuni* (Jones *et al*.,) Veron and Chatelain** and ***C. coli* (Doyle) Veron Chatelain.** Data were retrieved from Meuller Hinton Agar (MHA), 50 µg/ml nalidixic acid. A * describes a significant difference.

3.2.2. Results from the SSA and MHA plates without nalidixic acid

The 10% EAA treatment reduced overall counts of *Salmonella* spp. on chicken inoculated with *S. enterica* ATCC19585 and FSL R6-0020 by 0.62 $\log_{10}$ (p-value 0.011) and 0.22 $\log_{10}$ (p-value 0.024), respectively (Figure 9).

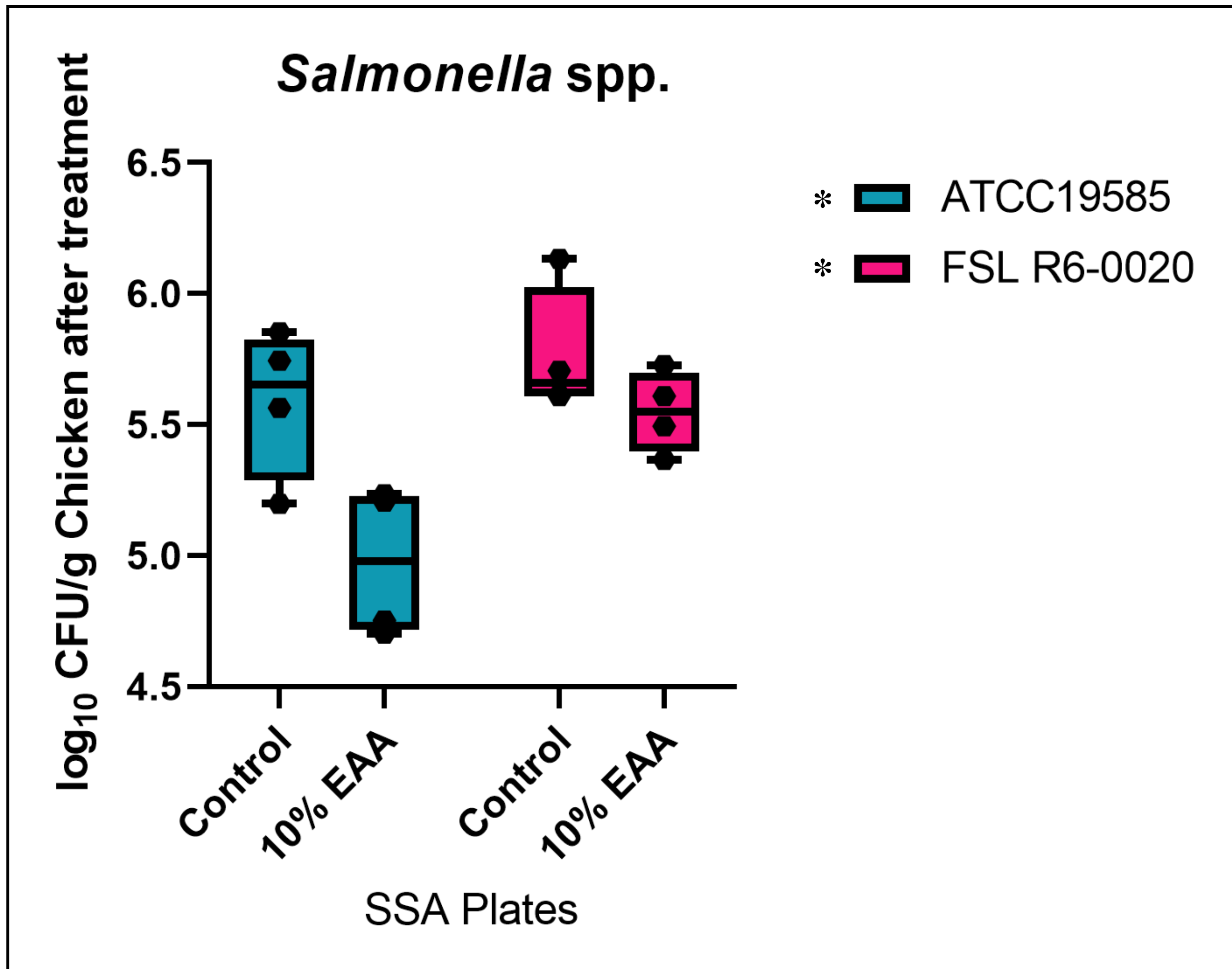

Figure 9: *Salmonella spp.* counts on SSA plates. Box-and-whisker plots comparing H_2O to EAA washes on chicken inoculated with nalidixic acid resistant ***S. enterica* serovar Typhimurium FSL R6-0020 and *S. enterica* subsp. *enterica* (ex Kauffmann and Edwards) Le Minor** and **Popoff serovar Typhimurium ATCC19585.** Data was retrieved from Salmonella Shigella Agar (SSA). A * describes a significant difference.

The 10% EAA treatment reduced overall counts of *Campylobacter* spp. on chicken inoculated with *C. jejuni* resulted in a statistically significant reduction in overall *Campylobacter* spp. of 0.41 log_{10} (p-value 0.009). Counts of *Campylobacter* spp. on chicken inoculated with *C. coli* were reduced by 0.62 log_{10}, but the corresponding *t*-test favored the null hypothesis (Figure 10).

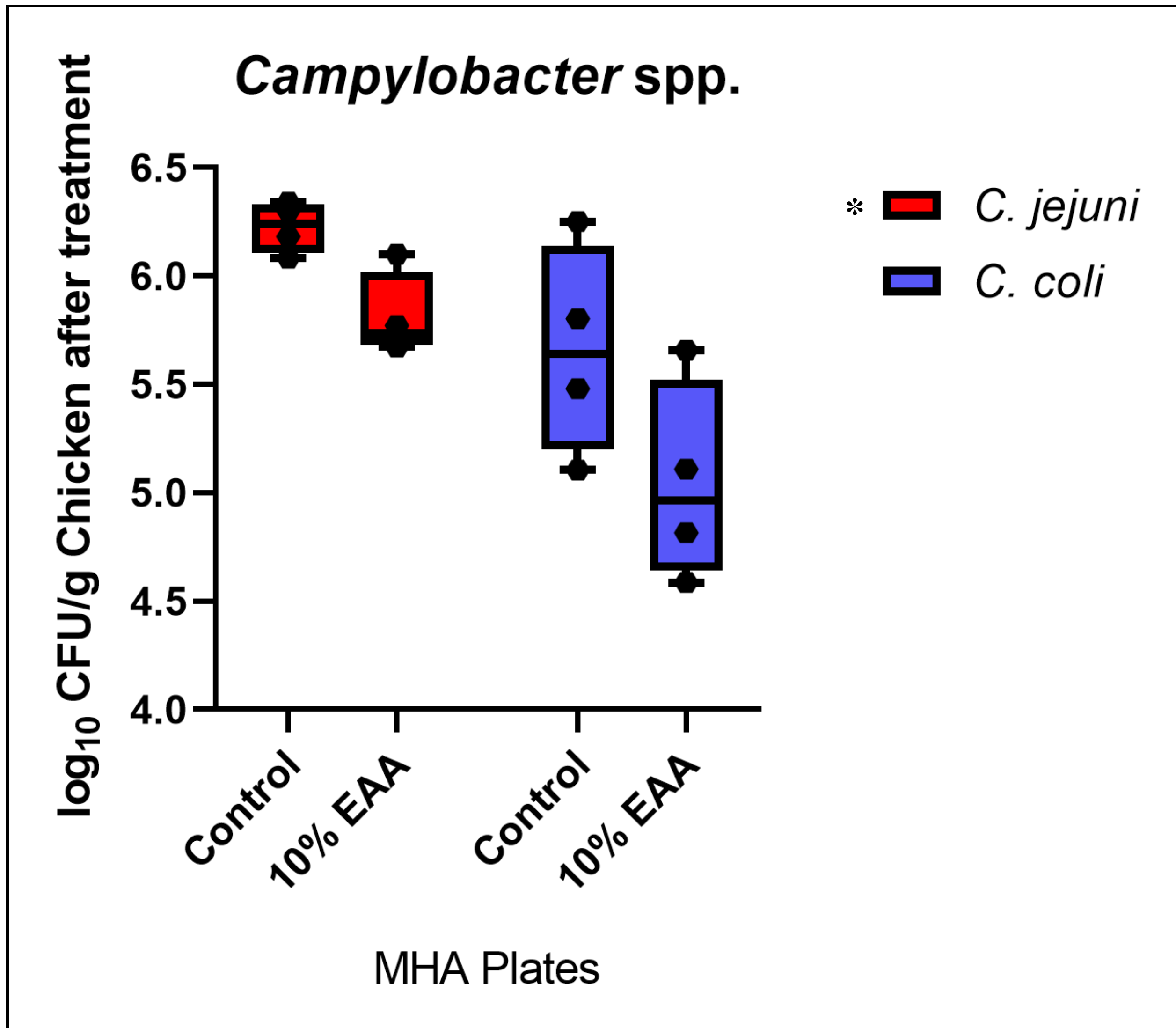

Figure 10: *Campylobacter spp.* counts on MHA plates. Box-and-whisker plots comparing H_2O to EAA washes on chicken inoculated with nalidixic acid resistant ***C. jejuni* subsp. *jejuni* (Jones *et al*.,) Veron and Chatelain** and ***C. coli* (Doyle) Veron Chatelain.** Data was retrieved from Meuller Hinton Agar (MHA). A * describes a significant difference.

3.3. Log Reductions

PEA was effective at significantly reducing $\log_{10}$ of total bacterial counts on PCA and *Pseudomonas* spp. counts on PSA by 1.18 (*p*-value 0.002) and 1.14 (*p*-value 0.004), respectively. EAA was effective at significantly reducing $\log_{10}$ of total bacterial counts on PCA and

Pseudomonas spp. counts on PSA by 1.24 (*p*-value 0.0005) and 1.03 (*p*-value 0.008), respectively.

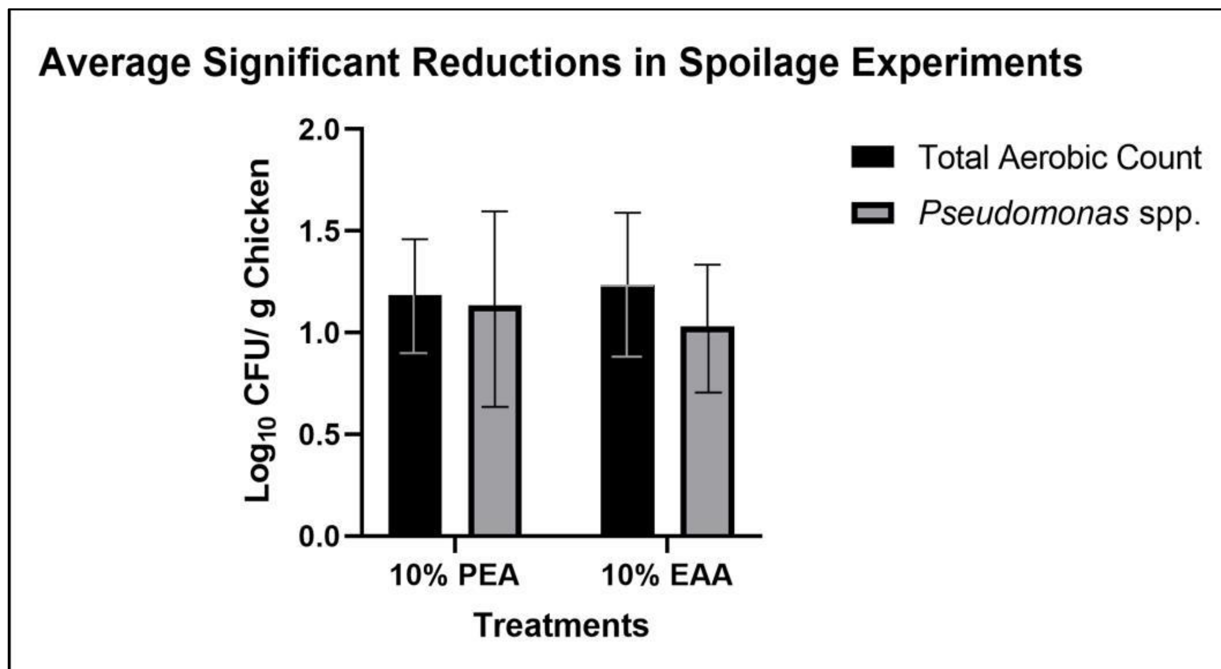

Figure 11: Average significant reductions from spoilage experiments. Log_{10} CFU/g microbial reductions in accordance with the total aerobic plate counts enumerated from PCA plates and enumerated *Pseudomonas* spp. that had significance of the difference proven by the one-tailed paired *t*-test (p-values <0.05). Log_{10} reductions were calculated by the comparison of the treatment data with that of the H_2O control.

In the pathogen experiment, 10% EAA on chicken thighs externally inoculated with pathogens *Salmonella* spp. and *Campylobacter* spp. provided partial efficacy in reducing the added pathogens. EAA was only effective at reducing the inoculated *S. enterica* FSL R6-0020 counts and *C. jejuni* counts by 0.36 log_{10} (*p*-value 0.011)) and 0.44 log_{10} (*p*-value 0.027), respectively (Figure 12A), when bacteria were enumerated on SSA and MHA plates, supplemented with nalidixic acid. On unsupplemented MHA plates, 10% EAA treatments reduced the counts on chicken thighs inoculated with *S. enterica* ATCC19585 and FSL R6-0020 by 0.62 log_{10} (*p*-value 0.011) and 0.22 log_{10} (*p*-value 0.024), respectively (Figure 12B). On the

unsupplemented MHA plates, 10% EAA also reduced the $\log_{10}$ *C. jejuni* by 0.41 (*p*-value 0.009) (Figure 12B).

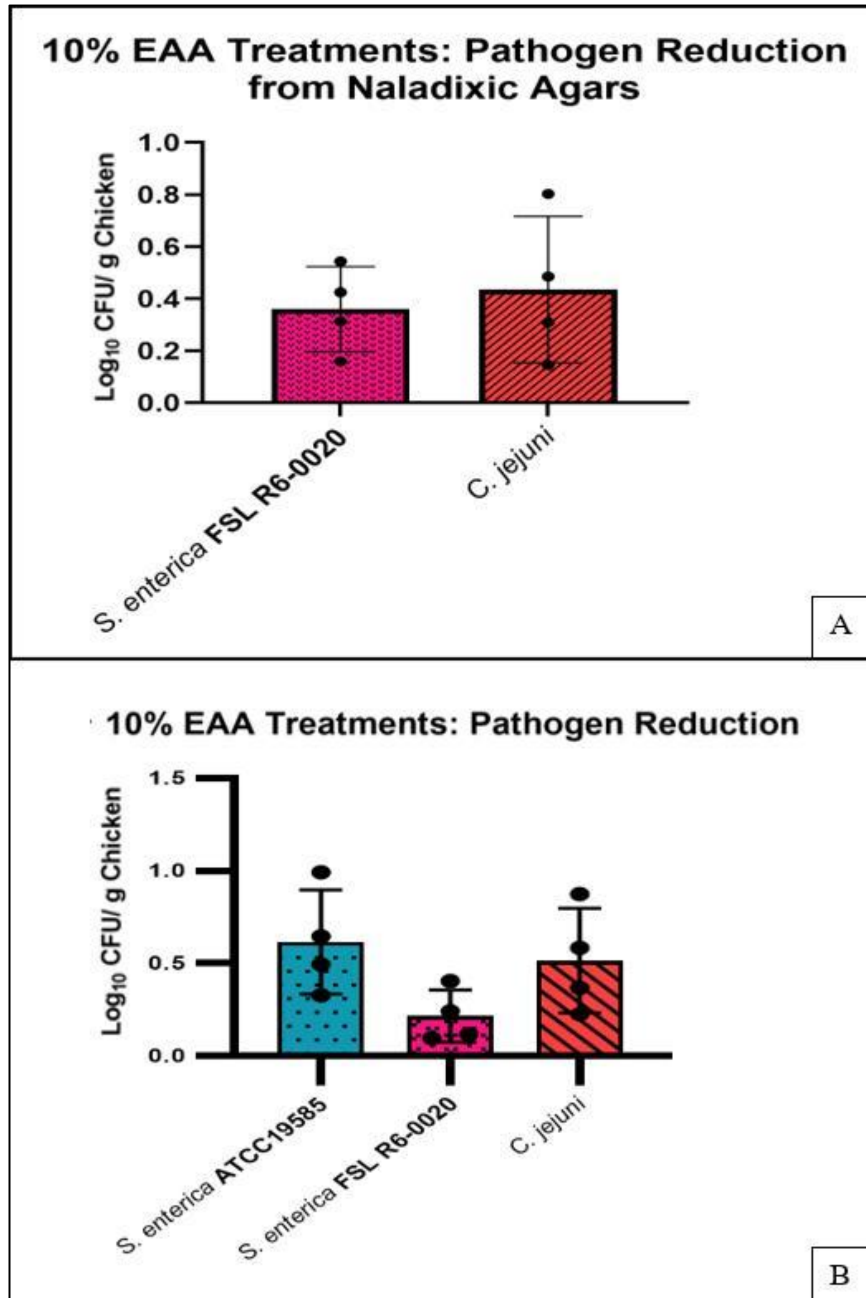

Figure 12: Average significant reductions from pathogen experiments. A and B are the $\log_{10}$ reductions of associated microbes that had significance of the difference proven by the one-tailed paired *t*-test (*p*-values <0.05). Log_{10} reductions were calculated by the comparison of the treatment data with that of the H_2O control. **A**. Log_{10} CFU/g reductions of *S. enterica* FSL R6-0020 and *C. jejuni* from agars supplemented with 50 µg/ml nalidixic acid. **B**. Log_{10} CFU/g reductions of *S. enterica* ATCC19585 and *S. enterica* FSL R6-0020 from SSA plates and *C. jejuni* from MHA plates.

4. DISCUSSION

We conclude that 10% treatments of β-Phenylethylamine-HCl (PEA) and Ethyl Acetoacetate (EAA) were effective at reducing total aerobic and *Pseudomonas* spp. spoilage bacteria contaminating chicken thighs. 10% treatments of EAA were partially effective at reducing the poultry pathogens *Salmonella* spp. and *Campylobacter* spp.

Relevant to the research currently being done and the research that has taken place within this lab, a patent on EAA as a novel antimicrobial has been submitted and is pending (Pruess et al. 2019). The pending patent is listed as US 2019/0082688, published on 21 March 2019. As of the completion of these experiments on poultry and the treatment of beef, there is a current exploration of EAA used as a treatment on tomatoes. This research was performed after the publication of the patent. The pending patent on EAA was the reason why the pathogen experiments were done with EAA, and not PEA. During the discussions on the patent, it was brought to our attention that processing aids are easier to commercialize than actual treatments. PEA and EAA are used as antimicrobials on chicken as processing aids, where the antimicrobial gets washed off later in the processing. For our experiment, we still use the word treatment because the chicken was treated with PEA or EAA.

When treating spoilage microbes, the desired outcome is an extended shelf-life. Treatments of chicken thighs with PEA or EAA were carried out within a day and therefore do not explore how much time would be required to reach above the spoilage level of 1×10^7 CFU/g for meat (EFSA BIOHAZ Panel et al. EFSA Journ. 2016 (77)). If PEA and EAA reduced the spoilage organisms by 1 $\log_{10}$, then this spoilage level 1×10^7 would be reached later, assuming growth of the spoilage bacteria on water or PEA/EAA treated chicken is the same. If PEA and EAA were residual on the chicken, it could be possible that there would be continued reduction

in growth and a further extension of the shelf-life. This would be required to be tested experimentally.

For the experiments regarding the 10% EAA treatment on chicken pathogens, the two strains of *Salmonella enterica* and the two strains of *Campylobacter* spp. (Table 3) were used to postulate the efficacy of the 10% EAA treatment. For the research into the efficacy of 10% EAA treatments on pathogens on chicken thighs, there are two separate sets of data (Figures 7 and 8, Figures 9 and 10). The first data set on figures 7 and 8 were counts enumerated from SSA/MHA plates, supplemented with nalidixic acid. The second set of data on Figures 9 and 10 were enumerated from unsupplemented SSA/MHA plates. The analysis of the first set of plates allowed for the enumeration of just the inoculated nalidixic acid resistant *Salmonella* spp. and *Campylobacter* spp. (Figures 7 and 8). The analysis of the second set allowed for the enumeration of native *Salmonella* spp. and *Campylobacter* spp., nalidixic acid resistant inoculated *Salmonella* spp. and *Campylobacter* spp., and inoculated *Salmonella* spp. and *Campylobacter* spp. that lost their resistance to nalidixic acid (Figures 9 and 10). The inclusion of this data is supplementary. Chicken with no added pathogens and a treatment of H_2O resulted in an average of 0.88 and 1.26 $\log_{10}$ CFU/g of meat for the replicate controls conducted in the experiments utilizing ATCC19585 and FSL R6-0020, respectively.

As a note on the *Salmonella* spp., there was utilization of the *S. enterica* ATCC19585 strain, which falls under the LT2 strain. *Salmonella enterica* serovar Typhimurium LT2 is a strain of *Salmonella* spp. first isolated in 1948 (Lilleengen, K. Acta Pathol. 1948 (78)). Within the LT2 genome, there is a defect within the gene *rpoS* (RNA polymerase, sigma S, also called *katF*), a global stress regulator, that leads to the avirulence of the LT2 clade (Swords et al. Infect. Immun. 1997 (79)). Partial to this, *rpoS* positively regulates the curli-operons clustered on the

agfBA and *agfDEFG* (Prigent-Combarat et al. J. Bacteriol. Res. 2001, Ibanez-Ruiz et al. J. Bacteriol. Res. 2000 (80, 81)). In a study on the bacterial attachment of *S. enterica* on alfalfa plant tissue, the *rpoS* mutant's ability to attach was reduced by 1 $\log_{10}$ compared to the wildtype (Barak et al. Appl. Environ. Microbiol. 2005 (82)). Within the experiments presented in this thesis, the *S. enterica* ATCC19585 LT2 strain enumerated from SSA plates, supplemented with nalidixic acid, was not significantly reduced on inoculated chicken thighs treated with 10% EAA in comparison to the control H_2O treatments. Comparing the recovered *S. enterica* ATCC19585 and *S. enterica* R6-0020 washed with the control H_2O the average counts enumerated from SSA, supplemented with nalidixic acid, were 5.59 and 5.77 $\log_{10}$ CFU/g. This means that the deficit attachment due to the *rpoS*^{LT2} is not the cause for the lack of reduction by EAA. In the parallel, ongoing, experiment utilizing EAA treatments on tomatoes, ATCC19585 was completely washed off by the control H_2O in some but not all of the experiments (Dr. Horne and Dr. Pruess personal communication). Chicken meat and the outside side of tomatoes are distinctly different surfaces. While these results are conflicting, chicken thighs are a striated muscle and are nutrient rich in comparison to the outside of a tomato. Speculatively, the *rpoS*^{LT2} may impact the attachment differently on different surfaces.

Comparisons of food antimicrobials require a deeper look beyond just the $\log_{10}$ reductions of certain organisms. These food processing aids must be acknowledged for what they are used for: edible products. This implies a greater need for safer antimicrobial applications and that the reduction of the microbiota must outweigh the implicit hazards that these treatments pose. The PEA utilized within this experiment is in a hydrochloride form, which was awarded with GRAS (Generally Recognized As Safe) status by the FDA. EAA is FDA approved as a flavoring agent for food (Flavis No 9.402) and the FDA approved it as a food additive under 21CFR172.515.

With reference to EAA, which has been assessed for both its capability in spoilage and pathogen reduction, it is also relatively cheap and is already utilized as an edible food additive. Unlike the majority, excluding the PoultrypHresh™ (a brand of food-processing antimicrobial manufactured by CMS TECHNOLOGY, INC. Table 5), EAA is not toxic if swallowed and could be utilized with European Union (EU) trade, as chlorine-treated poultry has been banned since 1997 under Article 3 Regulation (EC) No 852/2004.

Currently utilized antimicrobials used on chicken are included in Table 5 and 6 for the purpose of comparison. Concentrations of these antimicrobials are well below the toxic dose and are described in the U.S. Department of Agriculture Food Safety and Inspection Service (USDA-FSIS) Directive 7120.1 Rev. 43 (October 5, 2017). The approved concentrations for treatments of Peracetic acid (PAA) vary from 0.005% to 0.2% ppm and have been noted to be corrosive to skin at a 10% concentration within 3 minutes (National Research Council US Committee, 2010 (83)). Cetylpyridinium chloride (CPC) treatments (Table 6) is approved at 0.9% and have a 50% lethal dose (LD_{50}) of 560.3 mg/kg of bodyweight in a rat model (Scientific Committee on Consumer Safety, SCCS. 2015). Acidified sodium chlorite (ASC) goes up to 0.12%, chlorine is allowed between 0.0020% – 0.0050% and a dose of 10-15 g is lethal and can cause methemoglobinemia at lower doses (Lin et al. Renal Failure. 1993 (84)). Trisodium phosphate (TSP) is allowed between 8-12% and is identified as non-toxic.

In Table 5, comparisons are broken down between the experimental PEA and EAA treatments and the commonly utilized poultry processing aids used for the reduction of spoilage bacteria. This is, by far, not a complete summary of every concentration, application time, or step used in poultry processing.

Table 5: 10% PEA and EAA processing aids compared to the efficacy of applied antimicrobials on the total viable count and *Pseudomonas* spp. on chicken.

Abbrv.	Antimicro-bial Type	Hazards	Treatment	Time	Conc.	Results	Ref.
PEA	--	‣Eye irritation ‣Skin irritation ‣ Digestive and respiratory tract burns	Tested as an Immersion Wash	5 min	10%	1.18 log_{10} reduction PCA counts	This study.
						1.14 log_{10} reduction *Pseudomonas* spp.	
EAA	--	‣Eye irritation ‣Skin irritation ‣Digestive and respiratory tract irritation	Tested as an Immersion Wash	5 min	10%	1.24 log_{10} reduction PCA Counts	
						1.03 log_{10} reduction *Pseudomonas* spp.	
PAA	Organic acid and oxidant	‣ Weakly carcinogenic ‣Skin damage ‣Eye damage ‣ Harmful if swallowed ‣Respiratory irritation	Immersion Wash 4°C	20 min	0.02%	0.1 log_{10} reduction Total viable count	Chousalkar et al. Int. J. Environ. Res. 2019 (55)
ASC	Oxidative effect of chlorous acid	‣May cause fire or explosion; strong oxidizer ‣Toxic if swallowed ‣Fatal in contact with skin ‣Severe burns and eye damage ‣May cause damage to organs through prolonged or repeated exposure	Immersion Wash 4°C	20 s	0.09%	1.5 log_{10} reduction Total viable count	Chousalkar et al. Int. J. Environ. Res. 2019 (55)

Table 5: 10% PEA and EAA processing aids compared to the efficacy of applied antimicrobials on the total viable count and *Pseudomonas* spp. on chicken (continued).

Abbrv.	Antimicrobial Type	Hazards	Treatment	Time	Conc.	Results	Ref.
SHY	Chlorine	‣Irreversible skin and eye damage ‣ Corrosive ‣Fatal if swallowed ‣ Harmful if inhaled **Targeted Organs:** Blood	Immersion Wash 4°C	20 min	0.005%	0.1 $\log_{10}$ reduction Total viable count	Chousalkar et al. Int. J. Environ. Res. 2019 (55)
Poultry pHresh		**GRAS** ingredients	Immersion Wash 4°C	12 s	*Added until desired pH	Non-significant reduction.	Chousalkar et al. Int. J. Environ. Res. 2019 (55)
TSP	Alkaline Detergent	‣Eye irritation ‣Skin irritation ‣Respiratory irritation	Immersion	15 s	10%	>1.8 $\log_{10}$ reduction *Pseudomonas* spp.	Colin et al. Bristol University Press. 1996 (85)
			Spray	17 s	10%	0.74 $\log_{10}$ reduction Aerobic plate counts	Yang et al. 1998 (86)
			Immersion	15 min	10%	0.51 $\log_{10}$ reduction Aerobic plate counts	Lillard et al. J. Food Prot. 1994 (87)

β-Phenylethylamine-HCl (PEA), Ethyl acetoacetate (EAA), Peracetic acid (PAA), Acidified sodium chlorite (ASC), Sodium hypochlorite (SHY), and Trisodium phosphate (TSP). Hazards were assessed through FischerSci.com. Concentrations of the antimicrobials were translated into percentages for this table. Reductions displayed are aerobic plate counts, displayed as either PCA counts or total viable counts, and *Pseudomonas* spp.

In the comparison (Table 5) $\log_{10}$ reductions of total counts on PCA and *Pseudomonas* spp. 10% of PEA and EAA were only surpassed by the treatments of 10% trisodium phosphate (TSP) and 0.09% acidified sodium chloride (ASC).

Comparisons of 10% treatments of PEA and EAA to Table 5.

- PAA applications are less effective at 0.02% for 20 minutes than the 10% PEA and EAA treatments at 10-minute immersions. The PAA was able to reduce the total count by 0.1 log_{10} while the PEA and EAA treatments reduced the total counts by >1 log_{10}. The treatments of 10% PEA and EAA were more effective at a shorter time. PAA is carcinogenic.
- ASC is more effective at reducing the total aerobic bacteria than treatments of 10% PEA and EAA in a much shorter time frame. However, the hazards of ASC treatments include causing possible fires and explosions, it is toxic if swallowed, possibly fatal if it encounters the skin, can cause severe burns, and can cause damage to the organs when repeatedly exposed. The severity of these risks is incomparable to the edible quality of PEA and EAA.
- SHY is made from a solution of reacting chlorine and sodium hydroxide. It goes by the alias ‘bleach’, which is corrosive, fatal if swallowed, can cause irreversible burns, and is harmful if inhaled. It is both ineffective at a longer time compared to that of the 10% PEA and EAA.
- PoultrypHresh™ is made up of GRAS ingredients, which are non-harmful and was used at a shorter application time. However, it had no effect on the total plate counts.
- TSP is comparatively more efficient at the reduction of *Pseudomonas spp.* than both 10% PEA and EAA. TSP was also used in the same concentration for a shorter application time. However, reductions of total plate count are more effective by PEA and EAA.

10% PEA and EAA are effective in comparison to the other antimicrobials at reducing the $\log_{10}$ of total viable counts and *Pseudomonas* spp. on chicken. With added benefit, compared to most commercialized food processing aids, they are less toxic.

Table 6 compares the 10% EAA treatment against commonly utilized poultry processing aids specifically for *Salmonella* spp. and *Campylobacter* spp. $\log_{10}$ reductions from counts on SSA or MHA supplemented with 50 µg/ml of nalidixic acid. Coliforms were added to this table, although this would include other microorganisms in the *Enterobacteriaceae* family.

Table 6: 10% EAA treatments compared to the efficacy of applied antimicrobials on *Salmonella* spp., *Campylobacter* spp., and total coliforms on chicken.

Abbrv.	Antimicro-bial Type	Hazards	Treatment	Time	Conc.	Results	Ref.
EAA	--	‣Eye irritation ‣Skin irritation ‣Digestive and respiratory tract irritation	Tested as an Immersion Wash	5 min	10%	0.36 $\log_{10}$ reduction *S. enterica* FSL R6-0020	This study.
						0.44 $\log_{10}$ reduction *C. jejuni*	
PAA	Organic acid and oxidant	‣ Weakly carcinogenic ‣Skin damage ‣Eye damage ‣ Harmful if swallowed ‣Respiratory irritation	Immersion	30 s	0.05%	1.76 $\log_{10}$ reduction *Salmonella* spp.	Kumar et al. Poult Sci. 2020 (43)
						1.78 $\log_{10}$ reduction *Campylobacter* spp.	
			Post chill immersion	20 s	0.04%	2.02 $\log_{10}$ reduction *Salmonella* spp.	Nagel et al. Int. J. Food Microbiol. 2013 (44)
						1.93 $\log_{10}$ reduction *Campylobacter* spp.	

Table 6: 10% EAA treatments compared to the efficacy of applied antimicrobials on *Salmonella* spp., *Campylobacter* spp., and total coliforms on chicken (continued).

Abbrv.	Antimicro-bial Type	Hazards	Treatment	Time	Conc.	Results	Ref.
CPC	Quaternary ammonium	‣Fatal if inhaled ‣Skin irritation ‣Eye damage ‣Harmful if swallowed	Immersion *Skin	1 min	0.1%	~1 $\log_{10}$ reduction *S. typhimurium*	Kim and Slavik et al. J. Food Prot. 1995 (48)
				3 min		1.6 $\log_{10}$ reduction *S. typhimurium*	
		Target Organs: Respiratory system, eyes, skin.	Spray *Skin	Dwell: 1 min	0.1 %	0.9 $\log_{10}$ reduction *S. typhimurium*	
						1.7 log10 reduction *S. typhimurium*	
			Immersion	10, 20, 30 s	0.35% and 0.60%	0.8 $\log_{10}$ reduction *C. jejuni* Non-significant between time and concentration	Zhang et al. J. Appl. Poult. Res. 2019 (49)
			Immersion	10 min	0.8%	4.9 $\log_{10}$ reduction *S. typhimurium*	Breen et al. J. Food Prot. 1997 (88)
ASC	Oxidative effect of chlorous acid	‣May cause fire or explosion; strong oxidizer ‣Toxic if swallowed ‣Fatal in contact with skin ‣Severe burns and eye damage ‣May cause damage to organs through prolonged or repeated exposure	Immersion *Citric acid activated	5 s	0.12%	0.93 $\log_{10}$ reduction Total coliforms	Kemp et al. J. Food Prot. 2000 (51)
			Spray *Citric acid activated	Spray: 15 s Dwell: 30 s		0.52 $\log_{10}$ reduction Total coliforms	
			Immersion		0.12%	0.9 log10 reduction of *Salmonella* spp.	İlhak et al. J. Food Sci. Technol. 2018 (89)
			Spray	15 s	0.1%	1.6 log10 reduction of *Campylobacter* spp.	Purnell et al. Food. Bioproc. Tech. 2013 (90)

Table 6: 10% EAA treatments compared to the efficacy of applied antimicrobials on *Salmonella* spp., *Campylobacter* spp., and total coliforms on chicken (continued).

Abbrv.	Antimicrobial Type	Hazards	Treatment	Time	Conc.	Results	Ref.
SHY	Chlorine	‣Irreversible skin and eye damage ‣ Corrosive ‣Fatal if swallowed ‣ Harmful if inhaled **Targeted Organs:** Blood	Immersion	1 min	0.05% * 2 pH	0.90 $\log_{10}$ reduction Total coliforms	Bartenfeld et al. 2014 (91)
			Immersion	23 s	0.003%	No difference in *Salmonella* spp.	Chen et al. J. Food Prot. 2014 (92)
					0.003%	No difference in *Campylobacter* spp.	
TSP	Alkaline Detergent	‣Eye irritation ‣Skin irritation ‣Respiratory irritation	Immersion	15 min	10%	2 $\log_{10}$ reduction *Salmonella* spp.	Lillard et al. J. Food Prot. 1994 (87)
			Spray	30 s	10%	2.2 $\log_{10}$ reduction *Salmonella* spp.	Xiong et al. J. Food Prot. 1998 (93)
			Immersion	15 s	10%	Complete reduction of *Campylobacter* spp.	Colin et al. Bristol University Press. 1996 (85)

Ethyl acetoacetate (EAA), Peracetic acid (PAA), Cetylpyridinium chloride (CPC), Acidified sodium chlorite (ASC), Sodium hypochlorite (SHY), and Trisodium phosphate (TSP). Hazards were assessed through FischerSci.com. Concentrations of the antimicrobials were translated into percentages for this table.

The 10% EAA treatment compared to the other listed antimicrobials at reducing *Salmonella* spp. and *Campylobacter* spp. (Table 6).

- Both *Salmonella* spp. and *Campylobacter* spp. were reduced by PAA applications much more efficiently and effectively than the 10% EAA treatment.
- A CPC immersion is effective at removing > 1 $\log_{10}$ of *Salmonella* spp. from chicken skin at 0.1% concentration at 1 to 3 minutes. For 10 minutes at a concentration of 0.8% of CPC, there was a 4.9 $\log_{10}$ reduction of *S. typhimurium*. *C. jejuni* had just below 1 $\log_{10}$

reduction at the different times and different concentrations. 10% EAA reduced *Salmonella* spp. (R6-0020) and *C. jejuni* less effectively at 10 minutes by 0.36 and 0.44, respectively.

- ASC has > 1 $\log_{10}$ reduction for both spray and immersion treatments on coliforms. On the treatment of *Salmonella* spp. and *Campylobacter* spp. they were reduced by 0.9 $\log_{10}$ and 1.6 $\log_{10}$, respectively, which is much greater than that of the 10% EAA treatments.
- SHY at an immersion for 1 minute at 0.05%, the total coliforms were reduced by 0.90 $\log_{10}$. This was from Bartenfeld et al.'s study on high content chlorine washes on broiler chickens and the concentration would not be acceptable in actual food processing. In Chen et al.'s experiment, which used 0.003%, there was a nonsignificant difference between the inoculated positive controls, the H_2O treatment, and that of the chlorine treatment (91, 92).
- TSP is comparatively more efficient at the reduction of *Salmonella* spp. and *Campylobacter* spp. than 10% EAA treatments.

10-minute treatments of 10% EAA are not as effective at reducing *Salmonella* spp. and *Campylobacter* spp. as other treatments listed in Table 6.

In order to ensure that PEA and EAA are safe, they also must be heat stable. A study done by Horne et al. provided evidence that the compounds of PEA and EAA did not change when heated to 73.9 °C or 93.3 °C (Horne et al. Antibiot. 2021 (65)). This is invaluable information as a trace amount of either PEA or EAA could remain on not only chicken, but any other food product it is used on. Therefore, when the consumer cooks the product there is no danger of PEA and EAA undergoing a conformational change or breaking down into separate molecules that could potentially cause higher toxicity. In the same study, it was determined that

the minimal bactericidal concentration against *E. coli* and *Salmonella* was 25.15% for PEA and 20.80% for EAA in beef broth (Horne et al. Antibiot. 2021 (65)).

Within processing plants, the application of antimicrobials occurs mostly within equipment spraying, carcass washings, immersion chilling, and post-chill treatment (Bourassa et al. Food Safety. 2017 (94)). In our experiment, PEA and EAA treatments were applied to store-bought chicken thighs before the treated thighs were incubated at 4°C for 30 minutes. We recommend PEA and EAA to be used as a processing aid at the pre-chill stage. This is a popular step to apply aids and antimicrobials to products. Certain treatments may have different time constraints for the addition of antimicrobials, and they may be allocated to a full immersion or a spray application that is washed away. The functionality of PEA and EAA as an antimicrobial on chicken was tested as an immersion/dip treatment for a time span of 5 minutes. Our treatments also lacked a rinsing step to remove excess solution and instead were allowed to drip off excess before being incubated at 4°C for 30 minutes. Although untested in this experiment, there is a reasonable suggestion that there would be traceable PEA and EAA left on the chicken thighs. The amount of time the antimicrobials are applied for could range from a >1 minute dip at a higher concentration or go up to 60–120-minute applied treatment with a lower concentration of the antimicrobials (Bourassa et al. Food Safety. 2017 (94)). EAA, having only been tested for 5 minutes, could interact with issues based on the company utilizing it.

This study has provided a reasonable claim that the use of 10% PEA and EAA are effective at the reduction of the spoilage organisms and 10% EAA is not very effective at reducing poultry pathogens. The future for the antimicrobial food processing aid EAA depends on our commercialization efforts of the pending patent. Since EAA has been utilized as a food processing aid on beef, chicken, and currently on tomatoes (Horne et al. Antibiot. 2021, Lynnes

et al. 2014 (65, 66)), it may be nearing the end of its collective-stage research and prospects may lie with how interested companies would like to commercialize it on a factory scale. This would include possibly retesting with different washing times dependent on the company's own time-scales and which part of the food processing chain EAA processing aids would be instituted. However, before EAA can be added to the food processing chain, it is speculated that there will be a requirement for a sensory study to analyze the effect of what the wash could have on odor, taste, optical appearance, and consumer acceptance of a new antimicrobial.

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