Efficacy of Essential Oils and Cold Plasma to Improve Food Safety of Produce, Raw Beef and Ready-to-eat Meat

by

Shijie Qin

A thesis submitted to the Graduate Faculty of
Auburn University
in partial fulfillment of the
requirements for the Degree of
Master of Science

Auburn, Alabama
August 7, 2021

Key Words: Antimicrobial, Foodborne, White Mustard Essential Oil, Cold Plasma, Lettuce, Illness

Approved by

Amit Morey, Chair, Associate Professor, Department of Poultry Science
Emefa Monu, Co-chair, Assistant Professor, Department of Poultry Science
Tung-shi Huang, Professor, Department of Poultry Science

Abstract

Foodborne diseases remain problematic in the U.S., resulting in illness, financial losses, and in some circumstances, death. Food processors have used traditional methods of chemical additives and thermal processes to reduce microbial loads, but current consumer trends are driving the food industry to develop natural, minimally processed antimicrobial technologies to enhance food safety. Essential oils derived from plants have shown to be effective against microorganisms. In addition, the development of novel non-thermal technologies have some potential applications in food safety.

In this study, we determined the minimal inhibitory concentration (MIC) of carvacrol and lauric arginate (LAE) against *E. coli* and compared it to the previously determined MIC of white mustard essential oil (WMEO). Using this data, we determined concentrations to use as a produce wash on lettuce challenged with *Escherichia coli*. Washes were made in various combinations, some with acetic acid, and compared to washes prepared with chlorine at 150 ppm as the industry standard. We also determined the effects of cold plasma at 5-, 10- and 15-minutes exposure against *E. coli* on raw whole-muscle beef and *Listeria innocua* on deli meat. To show if there were combined effects for treatments, rinses of tap water, chlorine, WMEO, carvacrol, WMEO + acetic acid, carvacrol + acetic acid and WMEO + acetic acid + carvacrol (WAC) were combined with cold plasma treatments of 5 and 10 minutes on lettuce challenged with *E. coli*.

The MICs for carvacrol and LAE against *E. coli* were determined to be 0.05% (w/v) [0.5 mg/mL] and 0.005% (w/v) [0.05 mg/mL], respectively. The previously determined MIC of WMEO was 0.84% (w/v) [8.4 mg/ mL].

The pH of the solutions were as follows: tap water 6.9 to 7.49; Chlorine 8.1 to 8.49; WMEO 3.4 to 3.99; WMEO + acetic acid 2.49 to 2.9; carvacrol 4.25 to 4.7; carvacrol + acetic

acid 3.75 to 4.51; WMEO + acetic acid + carvacrol (WAC) 3.1 to 3.76; LAE 4.6 to 5; LAE + acetic acid 2.7 to 3; and WMEO + acetic acid + LAE (WAL) 2.4 to 3.1.

In the experiment of combination of WMEO and LAE on lettuce against *E.coli* BAA-1296, the wash effect of tap water resulted in a 1.08 log reduction as compared to the control. The WMEO solution and the LAE solution caused 1.62 and 1.53 log reductions, respectively. Combining the antimicrobial compounds WMEO + acetic acid caused a 1.78 log reduction. Additional combinations, LAE + WMEO and WMEO + acetic acid + LAE (WAL), resulted in 1.82 log reductions for both washes, which is equivalent to the effect of chlorine solution (1.90 logs reduction, $p>0.05$).

In the experiment of combination of WMEO and carvacrol on lettuce against *E. coli* BAA-1296, the tap water caused a 0.77 log reduction. The WMEO solution and carvacrol alone caused 1.32 and 1.35 logs reduction respectively ($p>0.05$). The combination of the WMEO + acetic acid and carvacrol + WMEO caused 1.96 and 1.88 log reductions, respectively ($p>0.05$), which are greater than the WMEO and carvacrol alone. The WMEO+ acetic acid + carvacrol (WAC) caused a reduction of 2.32 logs, which did not have significant differences when compared to chlorine solution (2.28 logs reduction, $p>0.05$).

In the experiment of cold plasma on raw beef against *E. coli* k-12, the reductions of *E. coli* are averaged 1.75 logs with no significant differences ($P>0.05$) between treatment times. We chose the *E.coli* k-12 since it is an non-pathogenic strain that could be used in Physics Building.

In the experiment of cold plasma on ham against *Listeria innoca,* 5 minutes of cold plasma treatment resulted in a 1.02 log reduction of *Listeria*, while 10-minute caused less than 1.5 log reduction (1.35 log reduction) and 15-minute resulted in more than 1.5 logs (1.75 logs) reduction ($p<0.05$).

In the experiment of combination of cold plasma and WMEO-based produce wash on lettuce against *E. coli* k-12, wash effect (Tap water) caused a 0.48 log reduction. The produce wash (WAL) solution caused 2.71 logs reduction. The cold plasma treatment (5 and 10 minutes) alone caused 2.14 and 2.29 logs reduction respectively ($p>0.05$). Tap water combined with cold plasma (5 and 10 minutes) caused 2.31 and 2.39 logs reduction respectively ($p>0.05$). The WMEO and chlorine solutions without cold plasma treatment caused 2.71 and 2.55 logs reduction ($p>0.05$), respectively. Five minutes of cold plasma with chlorine could cause 2.81 log reductions, 10 minutes of cold plasma with chlorine could cause 3.13 logs reduction. A treatment of 5 minutes of cold plasma with produce wash solution could cause 3.41 logs reduction, 10 minutes of cold plasma with produce wash solution could cause 3.94 logs reduction, which is more effective than 150 ppm industry standard chlorine solution 2.56 logs reduction ($p<0.05$).

Our research showed that both WMEO-based produce wash and cold plasma have antimicrobial effect and the combination of them have a better effect.

Acknowledgments

I would like to express my deepest appreciation to my committee for the encouragement and guidance. I am deeply indebted to Dr. Monu for taking me as a graduate student and providing valuable advice and relentless support. You played a decisive role in every aspect of my research and study. I would like to extend my sincere thanks to Dr, Morey for your invaluable insight into my research and practical suggestions. I must also thank Dr. Huang for helpful advice and unwavering guidance.

I would also like to extend my deepest gratitude to Michelle, you made Auburn home to me and extended a great amount of assistance in my research, study, and life. Thank you for bringing Tina the sweet girl Dottie, who is bring me a lot of joys. I am extremely grateful to Tina for being my friend and providing helpful suggestions for my future career. I would like to thank Zoila, for your assistant with my research and your friendships.

Lastly, I cannot express my thanks to my parents, who give me unconditional love and support. Every step of my life would not have been possible without your support and nurturing.

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List of Abbreviations

BHI	Brain Heart Infusion
CDC	Centers for Disease Control and Prevention
CFU	Colony Forming Units
DBD	Dielectric Barrier Discharge
EAEC	Enteroaggregative *E. coli*
EHEC	Enterohemorrhagic *E. coli*
EIEC	Enteroinvasive *E. coli*
EPEC	Enteropathogenic *E. coli*
ETEC	Enterotoxigenic *E. coli*
FFRA	FDA-FSIS Risk Assessment
FSIS	Food Safety and Inspection Service
FSMA	Food Safety Modernization Act
GRAS	Generally Recognized As Safe
HDPE	High-Density Polyethylene
HUS	Hemolytic Uremic Syndrome
LAE	Lauric Arginate
LDPE	Low-Density Polyethylene
MIC	Minimal Inhibitory Concentration
MW	Microwave
PS	Polystyrene
RF	Radiofrequency

ROS	Reactive Oxygen Species
RTE	Ready-To-Eat
SLCM	Standard Liters Cubic per Minute
STEC	Shiga toxin-producing *E. coli*
TSA	Trypticase Soy Agar
TSB	Trypticase Soy Broth
USDA	US Department of Agriculture
VTEC	Verotoxigenic *E. coli*
WAC	WMEO + acetic acid + carvacrol
WAL	WMEO + acetic acid +LAE
WMEO	White Mustard Essential Oil

Chapter 1 Introduction

Food-borne diseases are a serious public health threat in the world today. According to estimates by the Centers for Disease Control and Prevention, 76 million foodborne illnesses occur in the United States each year, including 325,000 hospitalizations and 5,000 deaths (CDC 1999). According to a report from the Foodborne Disease Active Surveillance Network (FoodNet), *Salmonella*, *Campylobacter*, *Shigella*, *Cryptosporidium*, and pathogenic *Escherichia. coli* are the main causes of foodborne diseases. According to the US Department of Agriculture, food-borne diseases cost the United States $1 to 83 billion annually (USDA 2021) .The CDC reported that in all food-borne disease outbreaks, food-borne diseases caused by raw agricultural products increased from 8% of all foodborne outbraks in 1998-2001 to 16% of 2010-2013. The main causes of the outbreak of raw agricultural products are vegetables (38%), fruits (35%), and seed vegetables (11%)(Bennett and others 2018). The most common pathogens are Norovirus (54%), *Salmonella enterica* (21%) and *E. coli* that produces Shiga toxin (Bennett and others 2018).

Due to the potential health benefits and consumers' increasing demand for fresh foods, minimally processed fruits and vegetables are becoming more and more popular (Slavin and Lloyd 2012). Traditionally, chlorine is the most widely used disinfectant, usually used for cleaning and spraying fresh fruits and vegetables (Rosa M. Raybaudi-Massilla 2009). However, people are concerned about possible residual chlorine by-products such as chloroform in wastewater (Yousef 2001). Chloroform and other trihalomethanes (THM) compounds are known as carcinogens under high dose exposure (Rathbun 1996).Therefore, there is a need to explore

novel antimicrobial intervention strategies to reduce pathogens, improve food safety while catering to the consumer demands.

One such intervention is the use of plant-based essential oils and extracts as natural antimicrobials, several of them have received the Generally Recognized As Safe (GRAS) status (N. B. Ress 2003). Plant essential oils, extracts, and individual ingredients have shown antimicrobial properties against *E. coli, Mycoplasma pneumoniae, Staphylococcus aureus, Candia albicans, Candia glabrata, Rhodobacter sphaeroides, Listeria monocytogenes* and *Bacillus subtilis* (Reichling and others 2009). In addition, studies have shown that plant-derived antimicrobial agents will not induce drug resistance in Gram-positive and negative bacteria after prolonged exposure(Tomoyuki Ohno 2003). Plant-based essential oil may be lethal to microbial cells or can inhibit secondary metabolites and inhibit the growth of bacteria (Nazzaro and others 2013).

Another modern antimicrobial technology under investigation is atmospheric cold plasma which has been successfully applied to inactivate foodborne pathogens such as *E. coli, L. monocytogenes* and *S. aureus* (Niemira 2012) and fungi and other microorganisms (Misra and others 2019) and destroy the microorganisms on seeds (Adhikari and others 2020) or crops (Tamosiune and others 2020). Due to its non-thermal nature, cold plasma has no or little effect on the physical, chemical, nutritional and sensory properties of the product. The versatility, non-thermal, economical, and environmentally friendly nature of cold plasma make advantages over traditional sterilization techniques for food decontamination, enzyme inactivation, and wastewater treatment (Sarangapani and others 2016). However, cold plasma technology is still in its infancy and needs further research.

The purpose of this study is to investigate the antimicrobial effect of white mustard essential oil-based produce wash on lettuce and cold plasma on raw beef and ham and the combination of white mustard essential oil-based produce wash and cold plasma. To achieve this goal, several specific objectives are listed: determine the MIC for carvacrol and Lauric arginate and assess antimicrobial efficiency against foodborne pathogens in leafy greens, deli meat, and raw beef.

Chapter 2 Literature Review

2.1 Foodborne diseases

Foodborne diseases are major public health problem today. Globally, the most common causes of foodborne diseases are microbial pathogens, biotoxins and chemical pollutants, which seriously threaten the health of millions of people (Arendt and others 2013). In the United States, the most common causes of foodborne illness are viral or bacterial contamination. One USDA report estimated that the foodborne illness brought $15.5 billion economic burden each year (Sandra Hoffmann 2015). The long term disabilities, chronic conditions and latent impacts of foodborne disease are not included in the cost estimate of CDC and there are also underdiagnosed cases which were not included (Scallan and others 2011). There are many symptoms of foodborne diseases ranging from mild- diarrhea, vomiting, abdominal pain, fever, headache and muscle pain (Finn and others 2013), to severe- enterotoxin poisoning, autoimmune complications, meningitis, sepsis, hemorrhagic colitis, Hemolytic uremic syndrome (HUS) and miscarriages (Renaud and others 2013).

Several foods including raw poultry and eggs, meats, raw produce as well as fully cooked foods have been implicated in foodborne illnesses. Fresh produce has been found to be the source of *Salmonella,* pathogenic *Escherichia coli*, *Shigella*, *Yersinia*, *Listeria monocytogenes*, *Staphylococcus aureus* and *Clostridium* (Bintsis 2017). On the other hand, ready-to-eat meat products such as ham and deli have been shown to be contaminated with

Listeria monocytogenes (CDC 2021). In the period of 1980 – 2015, there are1966 cases related to meat and meat products, 476 hospitalized and 32 death (Omer and others 2018).

2.2 *Escherichia coli* and *Listeria* as foodborne pathogens

2.2.1 *Escherichia coli*

E. coli is a common facultative anaerobic bacterium with pathogenic and non-pathogenic serovars associated with humans and animals (Ji Youn Lim 2010). *E. coli* grows optimally at 30-40°C but could survive at 4°C for over 6 months (Whitman and others 2003).The pH resistance varies by strains, while O157:H7 is more tolerant at low pH than other non-O157 strains (Jyhshiun Lin 1996). *E. coli* can survive with versatile energy acquisition and ability to degrade various carbon substrates (Diaz and others 2001), which might allow it survived under various conditions. *E. coli* initially inhabits the intestinal tract of warm-blooded animals and could be discharged to environments through feces and contaminated water (Berthe and others 2013).Pathogenic *E. coli* are grouped into enteropathogenic *E. coli* (EPEC) such as O55, O86 and O111 (Levine 1987), enterohemorrhagic *E. coli* (EHEC) such as O157:H7, O26 and O45 (Delannoy and others 2013), enteroaggregative *E. coli* (EAEC) such as O147:H4 (Lothar H Wieler 2011), Shiga toxin-producing *E. coli* (STEC) such as O119, O7:H10 and O22:H16 (Aidar-Ugrinovich and others 2007), Enterotoxigenic *E. coli* (ETEC) such as O78:H11, O128:H12 and O78:H9 (Echeverria 1996) and Enteroinvasive *E. coli* (EIEC) such as O28ac, O29 and O112ac (Shigeru Matsushita 1993).

Enterotoxigenic *E. coli* (ETEC) can produce one or more toxins (Rodriguez 1997) that can be fatal in humans. These strains have specific adhesion fimbriae for intestinal adhesion and colonization. Enteropathogenic *E. coli* (EPEC) can produce one or more cytotoxins and can

adhere to and interfere with the electrolyte transport system of intestinal epithelial cells. Enteroinvasive *E. coli* (EIEC), like *Shigella*, EIEC only causes human diseases, and is a common pathogen of diarrhea in Chile, Thailand, India and Brazil (Kotloff and others 2018). Reported cases in other countries are mainly related to travel (Pasqua and others 2017).

Pathogenic *E. coli* infection is a serious disease that may cause hemorrhagic diarrhea, hemolytic uremic syndrome (HUS) and neurological symptoms(Scheiring and others 2008). Typically, symptoms start with mild non-bleeding diarrhea, which may be followed by a period of abdominal pain and brief fever (Tarr 1995). In some cases, HUS may develop into chronic kidney disease leading to kidney failure (Jokiranta 2017). Internationally, 5%-7% of cases infected with verotoxigenic (VTEC) *E. coli*, which belonged to EHEC, have HUS and the percentage can be as high as 30% (Majowicz and others 2014). Children under 5 years of age and the elderly are at the highest risk of the HUS(Akashi 1994). Approximately, 10% of children under 10 years of age are infected with *E. coli* O157:H7 and have obvious HUS symptoms and require hospitalization. Between 2003 and 2012, a total of 4928 people in the United States became ill due to *E. coli* infections, where 1272 were hospitalized, and 33 people died (Heiman and others 2015). An outbreak in 2020 involving 19 states caused by *E. coli* O157:H7 associated with leafy greens caused 20 people to be hospitalized and 4 developed HUS (CDC 2020).

2.2.2 Lettuce and *E. coli*

Fresh produce plays an important role in the diet. It provides consumers with a variety of vitamins, nutrients, and fiber. Fresh produce includes fruits, vegetables, herbs, seeds and nuts, divided into whole and ready-to-eat (RTE) types (Srivastava 2017). Since 1980, most high-income countries have increased their demand for healthy foods and have begun to produce and

consume agricultural products on a large scale (Ghose 2014). The World Health Organization recommends 400 grams of fruits and vegetables daily to prevent heart disease, cancer, and diabetes (WHO 2019). In the United States, the per capita consumption of fresh produce is also increasing (Clemens 2015). In the United States, the incidence of foodborne diseases related to fresh produce ranges from less than 1% in the 1970s and increased to 6% in the 1990s (Sivapalasingam 2004). Globalization and increasing international trade may increase risks of foodborne illness, especially when food may come from countries with lower food safety standards (K€aaferstein 2003). With the increase in cases of foodborne diseases and consumers' preference for minimally processed foods, foodborne diseases caused by fresh foods have attracted more and more attention.

Fresh agricultural products that have shells, wax-coverings and a low pH are less likely to be contaminated by microorganisms, while some fresh agricultural products such as lettuce with high moisture and nutrient contents, and with natural openings such as stomata and lenticels are easily contaminated by microorganisms (Choi 2019). Contamination of fresh agricultural products can occur at many points in farming, including contaminated seeds, water, soil, dust, insects before harvest, and during processing and transportation after harvest (Choi 2019). Since fresh produce such as lettuce is often eaten raw or undercooked, it is necessary to minimize pathogen contamination before eating (Yeni and others 2016). Although there is no way to completely eliminate foodborne pathogens from fresh produce, there are some methods to reduce pathogens in fresh food, such as using physical methods for scrubbing and rinsing, using chemical interventions, like chlorine, phosphates, quaternary ammonium compounds, acids and others, biological methods, irradiation methods and others (Parish 2003).

Most of the recent *E. coli* outbreaks are related to leafy vegetables (Probert and others 2017). Fresh lettuce is one of the RTE foods that has been implicated in foodborne disease outbreaks. Studies have shown that the *E. coli* detected in fresh vegetables is likely to come from the environment of food factories due to the contamination, where the equipment is difficult to clean. Between 1995 and 2005, there were more than 20 outbreaks of *E. coli* O157:H7 related to fresh-cut vegetables and spinach in the United States (Lynch and others 2009). An *E. coli* outbreak caused by fresh spinach in 2006 resulted in 199 cases, 102 hospitalizations and 3 deaths across the United States.

Table 2.2.2 Recent food recalls due to *E. coli* (FDA 2020)

Date	**Outbreak strain**	**Produce/product source (not company name)**
12/21/2020	O157:H7	Organic romaine heart
11/06/2020	O157:H7	Single head romaine lettuce
04/06/2020	O157:H7	Items prepared with romaine lettuce
03/17/2020	Not mentioned	Macadamia nuts
03/11/2020	O157:H7	Sandwiches
03/10/2020	O157:H7	Fresh cauliflower rice, veggie cauliflower rice blend and stir-fry mix with cauliflower
03/10/2020	O157:H7	Red leaf lettuce, green leaf lettuce, and cauliflower
01/24/2020	Shiga toxin producing *E. coli*	Cheese
12/27/2019	O123	Clover sprouts
11/27/2019	Not mentioned	Unbleached flour
11/27/2019	Not mentioned	Organic all-purpose flour

11/01/2019	O26	Unbleached all-purpose flour
10/04/2019	O26	All-purpose flour
10/03/2019	O26	Unbleached all-purpose flour
09/16/2019	O26	Unbleached all-purpose flour
09/13/2019	Not mentioned	Salads and wraps
07/17/2019	O121 and O103	Bison burgers & bison ground
06/21/2019	Not mentioned	Cookie and brownie mixes
06/14/2019	Not mentioned	Flour
06/13/2019	Not mentioned	Unbleached all-purpose flour
05/28/2019	Not mentioned	All-purpose flour
05/23/2019	Not mentioned	Flour
05/22/2019	Not mentioned	Flour
02/09/2018	O157:H7	Carb not beanit butter
02/09/2018	Not mentioned	Yogurt peanut crunch protein bar
02/09/2018	O157:H7	Original creamy soyNut butter
02/08/2018	Not mentioned	Ice cream
02/08/2018	O157:H7	Original creamy soyNut butter
02/06/2018	Not mentioned	flour
10/20/2017	Not mentioned	Salad and basil products

2.2.3 Raw beef and *E. coli*.

E. coli is a pathogen of concern in the meat processing industry. In the 1980s, it was discovered that eating undercooked ground beef infected with *E. coli* O157:H7 caused hemorrhagic colitis (Riley 1983). In the United States, between 1992 and 1993, an outbreak of *E. coli* O157:H7 related to the consumption of ground beef resulted in hundreds of hospitalizations and 4 deaths. This outbreak happened in a fast-food chain in Washington State and was related to the ground beef in hamburgers. This outbreak caused 501 cases identified and 45 cases of HUS. With further isolation, *E. coli* O157:H7 was found in frozen hamburger patties and this *E. coli* produced Shiga-like toxins I and II. The final report pointed out that the internal temperature of these patties were below 60°C, which caused this outbreak (Palul R. Cieslak 1997). These outbreaks have led the Food Safety and Inspection Service (FSIS) to require meat processing plants to establish a Hazard Analysis Critical Control Point (HACCP) plan for controlling *E. coli*. In the United States, there are 63,153 cases of domestic acquired foodborne diseases caused by *E. coli* each year (Scallan and others 2011). Some scientists think that beef is one of the most important factor (Gyles 2007).

2.2.4 *Listeria*

Listeria monocytogenes is a Gram-positive pathogen that is widely present in the environment and various foods and is the causative agent of listeriosis (NicAogain and O'Byrne 2016). It grows in a temperature range of about 0.4°C to 50.8°C (Gallagher and others 2007) and can tolerate a wide range of pH from 4.5-9.6. It can grow well in meat with a pH greater than 6.0 but does not grow well or at all when the pH is less than 5.0. It can tolerate salt (NaCl)of 115.0g/Land nitrite ($NaNO_2$) of 200 mg /L (McClure 1997). It is widely found in plants, soil, silage, sewage, slaughter plant waste, human and animal manure, food processing environments

and catering facilities (Beresford 2001). Adult animals such as chicks may be infected with *Listeria* from eating contaminated feed and water (Husu 1990). *Listeria* now includes 5 species: *Listeria monocytogenes*, *Listeria innocua*, *Listeria welshimeri*, *Listeria seeligeri* and *Listeria ivanovii*, with *L. monocytogenes* and *L. ivanovii* which are pathogenic in nature (S H Notermans 1991).

Foodborne related outbreaks of listeriosis in recent years have involved a variety of food types, including cabbage, lettuce, meat, cheese, and others (Garner and Kathariou 2016). Although listeriosis is rare, it is very important in public health resulting in serious disease with a high mortality rate (20%-30%).The Centers for Disease Control and Prevention (CDC) estimates that there are approximately 1600 cases of listeriosis in the United States each year, resulting in 260 deaths (CDC 2016). High-risk individuals, especially newborns, sicken pregnant women, adluts aged 65 or older and people with weakened immune systems are susceptible to *Listeria* infection (CDC 2016). In 1983, epidemiologists first confirmed in the laboratory that this pathogen can contaminate food (Schlech 1983).

Its ability to grow under refrigerated conditions and strong pathogenicity make *Listeria* a concern in refrigerated and ready-to-eat (RTE) foods that are not reheated or cooked. Several large-scale outbreaks of *Listeria* have been confirmed from contaminated RTE foods, including dairy products, vegetables and meat products (Schuchat 1991). An investigation between 1990 and 1999 showed that the following types of RTE foods are the most susceptible to *Listeria* contamination: uncooked dried meat, cooked sausage, cooked beef, roast beef, and cooked salty beef. The wide distribution of *L. monocytogenes* makes it possible to contaminate food at all stages of production. Although the food industry and regulatory agencies have strict, zero

tolerance policies to regarding *L. monocytogenes* in RTE foods, *Listeria* is still an important challenge to control due to the ability to reproduce during refrigeration (IR Foundation 2005).

2.2.5 Deli meat and *Listeria*

Deli meat is an RTE food commonly associated as the source of listeriosis outbreaks (Amezquita 2002). The prevalence of *L. monocytogenes* in RTE foods varies depending on the type of RTE food, the place of production and the year (Meloni and others 2009). According to a risk assessment conducted by the U.S. Food and Drug Administration (FDA) and the U.S. Agricultural Food Safety and Inspection Service (FSIS), 90% of cases of listeriosis infection in the United States are caused by food contamination. Several outbreaks related to the consumption of RTE deli meat have been reported. RTE deli turkey caused a multi-state listeriosis outbreak in the United States in 2000, involving 10 states, in which four people died and three miscarriages were attributed. In 2002, the multi-state outbreak linked to turkey deli caused 46 illnesses, 7 deaths and 3 stillbirths and miscarriages. In 2018, deli ham outbreak caused 4 hospitalized and 1 death. The FDA-FSIS Risk Assessment (FFRA) issued a report in 2003 that quantitatively estimated the risk of disease or death caused by *Listeria*-contaminated RTE food, investigating 23 different RTE food categories, including bologna sausage, cured, cooked and roast beef, ham, beef sirloin, thinly sliced chicken, chicken tenderloin, and thinly sliced turkey. Among these, cooked beef, bologna sausage and ham were associated with the most cases. The ranking was based on the per serving and per annum basis and included the growth of *Listeria* under refrigerated storage with extended periods and extensive consumption (FDA 2003).

2.3 Essential oils

Current antibacterial agents are mainly synthetic chemicals with many disadvantages, including carcinogenicity, acute toxicity, teratogenicity, etc., and may also pollute the environment (Guillen and others 2012). The public's distrust of synthetic antimicrobial agents has led scientists to turn to natural compounds to be incorporated in foods (Sofos 1998). Plant essential oils such as bergamot, cinnamon, citronella, geranium, ginger and jasmine can be extracted from leaves, bark, stems, roots, flowers and fruits (Ali and others 2015; Erasto and others 2004). Essential oils are not strictly oils but are aromatic compounds that are usually not water-soluble. Essential oils generally have a pleasant smell and sometimes have a very unique taste. Herbs and spices commonly used in food have provided us with a large number of essential oils with antimicrobial activity (Burt 2004). Essential oils are complex aromatic chemicals rich in aldehydes, phenols, fatty acids, esters and ketones (Moghaddam and Mehdizadeh 2017). Essential oils also have anti-inflammatory, antioxidant, anti-cancer and antiseptic agents, which can effectively kill bacteria and fungi. Essential oils inhibit cell growth as well as the production of toxic bacterial metabolites. The many characteristics of essential oils make them a versatile natural additive to replace antibiotics (Patra and Yu 2012). Generally speaking, essential oils are more effective in killing gram-positive bacteria than gram-negative bacteria. The hydrophobic molecules could easily penetrate the cell wall of gram-positive bacteria than the complex gram-negative bacteria (Nazzaro and others 2013). In one study, the citron essential oils were more effective on gram-positive *Listeria* than gram-negative *Salmonella* and *E. coli* (Belletti and others 2008).The antimicrobial activity of essential oils is determined by their chemical composition, involving a series of reactions throughout the cell (Burt 2004).

However, it is worth noting that the fat content, protein, water activity, pH and enzymes of the food may reduce the effect of essential oils (Burt 2004). Studies have shown that low pH at 6.55 can increase the solubility and stability of essential oils and enhance antibacterial activity against *Listeria*. In addition, increasing the salt content to 0.01% and lowering the temperature to room temperature can also enhance the activity of essential oils (Friedly and others 2009).

Essential oils are hydrophobic and can separate the lipids of bacterial cell membranes and mitochondria, and in the process make bacterial cells more permeable (Burt 2004). Essential oils such as *Mentha citrate,* basil and rosemary oil have been proven effective against *E. coli* O157: H7, *Salmonella* Typhimurium, *Staphylococcus aureus*, *Listeria monocytogenes*, *Campylobacter*, and other microorganisms (Chouhan and others 2017). Although essential oils could have considerable antimicrobial effects, the smell could become unacceptable at levels that are bactericidal (Winska and others 2019).

Essential oils can also be used in multiple hurdle techniques, which are combinations of several antimicrobial agents (Friedly and others 2009) to decrease microorganism loads. In addition to having a synergy with other essential oils, it can also work with organic acids and salts such as sodium(Ricle 2003). Organic acids can help destabilize the pH of cells and change the osmotic pressure to enhance the antibacterial properties of essential oils(Hirshified 2003)

2.4 White Mustard Essential Oil (WMEO)

Mustard is one of the earliest domesticated crops. It has been cultivated in northern Asia, Africa and Europe for thousands of years and can be traced back to the time of the Buddha Shakyamuni (600 BC) centuries ago. There is also records of its use by the Romans from the 4th century (Thomas and others 2012).

The three most common commercial mustards are yellow mustard, white mustard and black mustard. White mustard essential oil (WMEO) is a natural antibacterial essential oil obtained by defatting and moistening mustard powder and then performing solvent extraction (David and others 2013). WMEO is a substance rich in 4-hydroxybenzyl isothiocyanate (4-HBITC), which has been proven by multiple studies to have broad-spectrum antibacterial properties, making it an ideal antibacterial agent for food. WMEO has a low volatility but does have a strong mustard smell that has been associated with a burning of the tongue. Because WMEO is very unstable in the aqueous phase, solvent or supercritical carbon dioxide extraction is generally used (Agrawal and others 2020).

The application of this WMEO has broad prospects (Monu and others 2014) and studies have shown that mustard extract has antibacterial effects on *Salmonella* and *E. coli* (Turgis and others 2009). White mustard seeds are rich in sinalbin (4-hydroxybenzylglucosinolate) (Fahey 2001). After hydrolysis of the precursor sinalbin, p-HBITC, a non-volatile umami oily compound, can be obtained. This reaction can be catalyzed by endogenous myrosinase and further activated by low concentrations of ascorbic acid. WMEO formerly has Generally Recognized as Safe (GRAS) status as an additive in foods. WMEO is not approved as an additive in food, it has a potential to be used as a natural rinse agent. WMEO is a new natural antibacterial substance that has never been used in commercial food before, so it has great potential that can be used in food industry (Techathuvanan and others 2014).

2.5 Lauric arginate (LAE)

Lauric arginate (LAE), considered one of the most effective food antibacterial agents because of its broad spectrum, exhibits activity against microorganisms such as molds, yeasts,

Gram-negative bacteria and Gram-positive bacteria (Hassan and Cutter 2020). It has GRAS status as a preservative, with a maximum allowable concentration in food of 200 ppm(Soni and others 2010). LAE can be rapidly hydrolyzed and converted into naturally occurring amino acids after being absorbed by the human body (Ruckman and others 2004).

The antibacterial property of LAE comes from its effect on the cell membrane of microorganisms. LAE can destroy the bacterial cell membrane, thereby changing the membrane potential and membrane permeability, leading to the loss of materials in the cell, which leads to cell death (Rodriguez and others 2004).A study using LAE at concentrations ranging from 22-44 ppm with frankfurters challenged with *Listeria monocytogenes* reported reductions of about 5%(Porto-Fett and others 2010).

2.6 Carvacrol

Carvacrol is an essential oil extracted from oregano (*Oregano vulgare*). Carvacrol is a phenolic compound, with a very high concentration in some oils, potentially reaching 75% in oregano extracts (Fani and Kohanteb 2017). As an antimicrobial, carvacrol affects the integrity of the cell membrane, making the membrane permeable to potassium ions and protons, leading to a decrease in internal pH and changes in membrane potential, inhibiting the synthesis of ATP, and leading to cell death. It has a broad antibacterial activity against almost all Gram-positive and Gram-negative bacteria (Friedman 2002). Carvacrol also has anti-fungal (Chami 2005), insecticidal (Panella 2005) and anti-parasitic effects (Lindberg and others 2000). Existing studies have proven that carvacrol can inhibit the growth of *E. coli* O157:H7 in liquid foods, especially with the combination of stabilizers or ethanol solutions (Sara A. Burt 2005).

2.7 Chlorine

Chlorine is one of the most common disinfectants, and is moderately oxidative and could react with multiple components of bacteria cells. With the Reactive Chlorine Species (RCS) reactions, chlorine could have effects on pathogens in many ways (Fig 2.7). By chlorinating the lipid protein in the cell wall and attacking the amino group in nucleic acid, chlorine could cause the leakage of macromolecules of the cells, resulting in the death of bacteria (Denyer 1998). Chlorination of the lipids can lead to several consequences. First, the double bond of the unsaturated acid could be chlorinated to form chlorohydrin. Also, amines in the head group of lipids could be converted to chloramines, nitrogen radicals, and aldehydes. The radicals from the reaction with amine or peroxides could lead to lipid oxidation and degrade the polyunsaturated fatty acids to aldehydes via organic hydroperoxide intermediates (Niki 2009). The nitrogenous compounds in bacteria could react with HOCl and be inactivated due to the damage of DNA and RNA (Gray and others 2013). Most studies confirmed that RCS kill microbes by damaging multiple cellular components. However, the mechanisms of the RCS are still not fully known, with many possible explanations. Early studies noted that HOCl caused the rapid loss of glucose respiration and converted most cellular ATP pool to AMP (William C. Barrette 1987), and targeted the inner membrane and the cytoplastic enzymes of bacteria (Albrich and others 1986). A more recent study showed that the reduction might due to the oxidative unfolding and aggregation of essential bacterial proteins(Winter and others 2008). The industry is using NaOCl (50-200 mg/L) with 1-2 minute exposure to reduce the microbial populations in the water used in washing operation (Ryu 1997).

However, since chlorine could react with organic matter, it could also form some potentially hazardous by-products such as trihalomethane and chloramines, which are potentially

carcinogens. Also, the cracks, crevices and waxes in fruit and vegetables could hinder the effectiveness of chlorine. Although FAO stated that the use of a chlorine produce wash will not increase the health risk to the public, there is still a tendency to remove chlorine from the disinfection process(Ölmez and Kretzschmar 2009). Hypochlorous acid and hypochlorite is used in fresh-cut food industry and the concentrations are between 50-200 ppm for an maxium 5 min exposure time (Goodburn and Wallace 2013).

$$\boxed{R - NH_2 + HOCl \rightarrow R - NH - Cl + H_2O}$$

$$\boxed{R - CO - NH - R' + HOCl \rightarrow R - CO - NHCl - R' + H_2O}$$

Fig 2.7 Process of the oxidation by HOCl

2.8 Non-thermal technology and cold plasma

Heating may have adverse effects on foods, changing the original colors and textures, thus affecting the quality. Sterilization can damage the quality of foods, as well as increase productions costs with loss of energy by heat transmission (Niemira 2012). A method that can efficiently inhibit or kill microorganisms with low cost is desired by the food industry.

Plasma consists of charged ionized gases which can be generated using radiofrequency (RF), microwave (MW), dielectric barrier discharge (DBD) and biasing source. The chemical composition of cold plasma is very complex, and its composition and efficacy depend on the equipment design and system operating parameters, such as gas composition, flow rate, humidity, temperature, voltage, and frequency. The source will spill out positive and negative ions, quanta of electromagnetic radiation and neutral particles, etc. The vacuum ultraviolet release plasma is

effective in breaking most organic bonds such as C-H, C-C, C-O, and C-N, which are common bonds in high molecular weight hydrocarbon contaminants. Also, there are oxygen species created in plasma such as O_2^+, O_2^-, O_3, O, O^+, O^-, ionized ozone, metastably-excited oxygen, and free electrons that eliminate organic contaminants. After the cold plasma discharge, the active substance that can inactivate the microorganisms is produced and then returns to the original state (Ziuzina and others 2013). After these procedures, the resulting surface is ultra-cleaned and sterilized. The by-products of the plasma method are H_2O, CO, CO_2 and some lower molecular hydrocarbons (Pankaj and others 2014).

Studies have shown that most bacteria, especially anaerobic bacteria, are very sensitive to Reactive Oxygen Species (ROS) (Stoffels and others 2008). Oxygen and oxygen-containing free radicals can diffuse through the cell wall to the plasma membrane, causing the oxidation of protein and DNA chains and causing local damage (Gallagher and others 2007). In the plasma stream, active nitrogen and active oxygen are generated simultaneously, which directly affects microorganisms. Because reactive nitrogen species can accumulate on the surface of microorganisms, they can diffuse through cell membranes, resulting in a decrease in intracellular pH. The pH plays an important role in cell function, which can affect enzyme activity, biochemical reaction rates, protein stability and nucleic acid structures.

In recent years, cold plasma technology has gained some applications. Until recently, cold plasma technology was used in a small range under low-pressure conditions (Niemira 2012), but is now widely used in industries. It can be used for tiny particles to generate static electricity, which can produce ozone for cleaning and purification purposes. It also oxidizes, etches, and ashes the surface of objects to modify the hydrophobic surface to make it hydrophilic (Kim and others 2006).

Current applications for DBD technology include blood plasma sterilization, fresh produce (Klockow and Keener 2009), fish (3-5 log microbial reduction) (Chiper and others 2011) and meat (2 log microbial reduction) (Rod and others 2012). It is effective for a variety of product packaging, such as low-density polyethylene (LDPE), high-density polyethylene (HDPE), polystyrene (PS) and so on.

2.8 Colors measurement

Studies have shown that for consumers, color is an important basis for their judgment(Del-Valle and others 2005) of the freshness of food and plays an important role in their choices, affecting their taste and pleasure (Clydesdale 1993).

The L*a*b are parameters that measure for color and brightness. The brightness index (L) ranges from 0 (black) to 100 (white). The parameter a measures the chromaticity of red (+a) or green (-a), while the b parameter measures the chromaticity of yellow (+b) or blue (-b) (Misra and others 2014). In lettuce, L represents the luminosity of the lettuce where a lower value indicates a higher degree of browning; a is used to evaluate the change in the color of lettuce from green to red, and b is a measurement of the yellowness of the lettuce (Martin-Diana and others 2005). In the measurement of meat color, L is most related to the pinkness; a corresponds to the measure of redness, where a lower value indicates a decrease in redness, and b is related to the darkness (Brewer 2001)

Chapter 3 Material and Methods

3.1 Preparation of WMEO

Frozen stock solution of WMEO (Procter & Gamble, Mason, OH) containing 120 g/ liter 4-HBITC was thawed and dispersed by vortexing in 1:1 soy lecithin (Tokyo Chemistry, Portland, OR) and sucrose-palmitate (Alfa Aesar, Ward Hill, MA) solution to reach a final concentration of 0.84%. The 5% sucrose-palmitate solution was made by dissolving 2.5 g sucrose-palmitate in 50 mL water, and 2% soy lecithin solution was prepared using 2.5 g in 122.5 mL water.

3.2 Preparation of *E. coli* inoculum

Two different *E. coli* strains were used in this study. American Type Culture Collection (ATCC) *E. coli* BAA-2196 was used in the produce wash while *E. coli* K-12 was used in cold plasma and the combination of cold plasma and produce wash project. The *E. coli* k-12 strain was chosen since the physics building do not have biosafety lab. *E. coli* ATCC BAA-2196 strain was grown in trypticase soy broth (TSB, Hardy Diagnostics, Santa Maria, CA) with 100-ppm nalidixic acid (Sigma, St. Louis, MO) while *E. coli* K-12 strain was grown in TSB. Both strains were incubated at 37°C, 24h for two passages before being used. Cultures were centrifuged at 5,000g for 20 minutes at room temperature, the supernatant was discarded, the pallet was

resuspended in in an equal amount 0.1% peptone water (Oxoid Ltd., Hampshire, England), vortexed and centrifuged again. The process was repeated twice to obtain a pellet that was resuspended in equal volume of 0.1% peptone water and then serially diluted to obtain a population of 10^6 CFU (colony forming units)/mLfor *E. coli* ATCC BAA-2196 and 10^7 CFU/mL for *E. coli* K-12 49595.

3.3 Preparation of *Listeria* inoculum

Non-pathogenic *Listeria innoca* was used in this study. The *Listeria innoca* was grown in brain heart infusion broth (BHI, Hardy Diagnostics, Santa Maria, CA) at 37°C, 24 h for two passages before being used. After two successive passes, cultures were centrifuged at 5,000 *g* for 10 min at room temperature, washed, and resuspended in an equal amount of 0.1% peptone water to achieve the final population of 10^7 CFU/mL.

3.4 Minimum inhibitory concentration (MIC) assay

The MIC of carvacrol (Tokyo Chemistry, Portland, OR) and Lauric arginate (LAE; (Sigma, St. Louis, MO) were determined using microdilution assay. Various concentrations of the compounds, final concentration of carvacrol ranging from 0.0125% to 0.0625% (v/v), LAE ranging from 0.000625% to 0.005% (v/v), were tested against *E. coli* ATCC BAA-2196 at an initial population of approximately 10^5 CFU/mL. Samples were made by using carvacrol and LAE solutions to achieve a final working volume of 2 mL and incubated at 37°C for 24 h. Positive and negative controls were prepared using *E. coli* and TSB, respectively. The 96 wells plate with treatments were incubated at 37°C for 24 h, and OD was measured at 600nm. The MIC against *E. coli* was determined based on the concentration at which the microorganisms

exhibited visible growth. Our MIC test is using broth-based test as one of the well-recognized MIC testing method(Andrews 2001). The MIC is the lowest concentration that inhibit the cell growth and significant change of OD indicates the bacterial growth. The experiment was performed a total of three times using a OD measurement instrument and the MICs were averaged.

3.5 Preparation of produce wash solution

Nine different lettuce wash treatments were used in this study. The chlorine solution was prepared using household bleach at 7.25% sodium hypochlorite which was diluted to a final concentration 150-ppm chlorine by calculating the Chloride. The final concentration of WMEO was 0.84%. The concentration of the acetic acid stock solution was 4%, the stock LAE solution concentration was 0.02% and the carvacrol (Tokyo Chemistry, Portland, OR) stock solution concentration was 0.2%. All the combinations are showed in Table 1. below.

Table 3.5 Concentrations of compounds used to create the produce wash solutions

Treatment	Water	WMEO	Acetic acid	Carvacrol	LAE
Control	250 mL	-	-	-	-
150 ppm Chlorine	-	-	-	-	-
0.84% WMEO	229 mL	21 mL	-	-	-
0.84% WMEO + 2% Acetic acid	104 mL	21 mL	125 mL	-	-
0.1% Carvacrol	237.5 mL	-	-	12.5 mL	-
0.01% LAE	237.5 mL	-	-	12.5 mL	-
0.84%WMEO	216.5 mL	21 mL	-	12.5 mL	-

+ 0.1% Carvacrol					
0.84%WMEO + 2% Acetic acid + 0.1% Carvacrol (WAC)	91.5 mL	21 mL	125 mL	12.5 mL	-
0.84% WMEO + 2% Acetic acid+ 0.01% LAE (WAL)	91.5 mL	21 mL	125 mL	-	12.5 mL

3.6 Lettuce preparation and treatment

Romaine lettuce was stored at 4 °C for 24 hours before the experiments. Samples were cut into 25 g pieces and inoculated with 10^6 population of *E. coli* BAA 2196 for 1 minute immersion for WMEO-based produce wash and *E. coli* K-12 for combination of produce wash and cold plasma. The inoculum ssuspension was made by adding 1 mL 10^8 population of *E.* coli BAA 2196 and k-12 in 200 mL sterile deionized water. Samples were left in a bio-safety cabinet for 1 hour for bacterial attachment. After this process, samples were transferred out for produce wash.

The color of each piece of lettuce was measured by Hunter Lab colorimeter (Konica Minolta) before and after treatments. Sixteen samples of 25 g lettuce were then immersed and flipped continuously in the trays of 250 mL different solutions for 2 minutes with a rinse and placed into the stomacher bags, diluted with 0.1% peptone water for a 1/10 dilution and stomached by stomatcher. All the samples were serially diluted in 0.1% peptone water and

spread plated in duplicate on TSA with 100-ppm nalidixic acid. Plates were incubated for 24 h at 37°C, and log CFU per gram were ccalculated. The pH of each solution was measured using a pH meter. This experiment was performed in triplicate.

3.7 Preparation of beef and deli meat

The raw whole-muscle beef was partially frozen and cut using a sterile knife into 2x2 cm^2 pieces and circular ham coupons (area of 1.6 cm^2) were cut using a sterile punching tool. The beef pieces and lunch meat pieces were speared with 0.05 mL of *E. coli* K-12 and *Listeria innoca* inoculum, respectively to obtain a final population of 10^7 CFU/g. The inoculum was spread evenly, and samples were placed in the biosafety cabinet for 1 hour to allow for the attachment of bacteria. The thickness of beef and ham was 3mm and 1mm, respectively.

3.8 Effect of cold plasma on microbial survival

The cold plasma machine was developed by Ryan Gott, University of Alabama in Huntsville. The input gas used was helium at 3 SLCM (Standard Liters Cubic per Minute). The input voltage ranged from 6.8 kV to 7.04 kV and the samples were kept at a distance of 10 mm from the tip of the nozzle. The average current was 27 mA, and the pulse "gate" was at 6.5 kHz frequency with a width of 1.0μs.

Samples were transported on ice to the Auburn University Physics Department for cold plasma treatments. They were treated for 0, 5, 10 and 15 minutes under the plasma jet with a distance of 10mm. Samples were transported on ice back to the Poultry Science Building, Auburn University for microbial analysis. Samples of raw beef and ham were swabbed twice with a sterile cotton swabs dipped with 0.1% peptone water and vortexed in 10 mL 0.1% peptone

water. All the samples were 10 fold serially diluted in 0.1% peptone water and spread plated on TSA. Plates are inoculated for 24 hours at 37°C, and log colony forming units (CFU) per gram was determined. The color of all the samples were measured by Hunter Lab colorimeter (Konica Minolta). This experiment was performed in triplicate.

3.9 Effect of combination of produce wash and cold plasma on microbial growth survival

Romaine lettuce was stored at 4 °C for 24 hours before the experiments. Samples were 25g lettuce pieces and inoculated with 10^6 *E. coli* k-12 for 1 minute. The inoculum suspension was made by adding 1 mL 10^8 population of *E. coli* k-12 in 200 mL sterile deionized water. Inoculated samples were left in a bio-safety cabinet for 1 hour for bacterial attachment.

Color of each piece of lettuce was measured by Hunter Lab colorimeter (Konica Minolta) before and after treatments. Lettuce was immersed in the trays of different solutions for 2 minutes and cut into 1.6 cm^2 round pieces and transported to the Physics Department for cold plasma treatments on ice. They were treated for 0, 5 and 10 minutes under the plasma jet with a distance of 10 mm.After transported back ro Poultry Science Building, samples were vortexed in 10 mL 0.1% peptone water. All the samples were serially diluted in 0.1% peptone water and spread plated on TSA. Plates were incubated for 24 hours at 37°C, colonies were counted and reported as log colony forming units (CFU) per gram. The color of all the samples were taken by Hunter Lab colorimeter. This experiment was performed in triplicate.

Chapter 4 Results and Discussion

4.1 Minimum inhibitory concentration (MIC) assay

The MIC under optical growth conditions in 96 wells plate was determined for carvacrol and LAE against *E. coli* BAA-2196. The MIC was defined as the concentration of antimicrobial at which the microorganism had the growth under OD at 600 nm. Our results show that the MIC of carvacrol and LAE against *E. coli* was 0.05% (w/v) [0.5 mg/mL] and 0.005% (w/v) [0.05 mg/mL], respectively. Based on a previous study, the MIC of carvacrol could range from 25 mg/L (0.0025%) (Otero and others 2014) to 0.31% (Ye and others 2013) while the MIC of LAE could range from 11.8 ppm (0.0118%) (Ma and others 2013) to 0.025% (Becerril and others 2013) against *E .coli*, respectively. The differences of the results between the current study and those reported could be due to the differences in strains and inoculum levels used (Schuurmans and others 2009).

4.2 pH of solutions used in produce wash

The pH of the solutions was measured in each trial (Fig 4.2.1 and Fig 4.2.2). The pHs of the solutions were as follows: tap water 6.9 to 7.49; Chlorine 8.1 to 8.49; WMEO 3.4 to 3.99; WMEO + acetic acid 2.49 to 2.9; carvacrol 4.25 to 4.7; carvacrol + acetic acid 3.75 to 4.51;

WMEO + acetic acid + carvacrol (WAC) 3.1 to 3.76; LAE 4.6 to 5; LAE + acetic acid 2.7 to 3; WMEO + acetic acid + LAE (WAL) 2.4 to 3.1. The pH of WMEO + acetic acid, WAC and WAL were significantly lower than other treatments ($p<0.05$). With the combination of acetic acid, the pH was lower and might cause a synergistic or additive antimicrobial effect when used with other antimicrobial compounds (Eklund 1983; Hee Lee 1997).

When used in combination with essential oils such as WMEO, and carvacrol, the phenolic compounds in essential oil could change the structure and function of cellular membrane and cause disruption of proton motive force due to the loss of H^+-ATPase (R.J.W. Lambert 2001; Wahlqvist 2004). Moreover, the hydrophobicity of essential oil might increase and make them more easily dissolved in the lipids of bacteria membrane to get higher reduction (A.K. Karatzas 2001). In the current study, we observed that with the addition of the acetic acid, the microbial reductions *on Listeria innocua and E. coli* BAA-1296 and k-12 were significantly lower than the single compounds. The possible mechanism might be related to the increase of the 4-HBITC antimicrobial activity, caused by lowering pH (Ekanayake et al 2012). The compound, 4-HBITC is formed when sinalbin, a glucosinolate found in the seed, is wetted, causing myrosinase to hydrolyze it into 4-HBITC, sinapine and glucose (Ekanayake et al 2012). Further research has shown that an alkaline environment could cause instantaneous hydrolysis and lower the pH increasing the stability of 4-HBITC (Fig 4.2.3) (Ekanayake and others 2016). However, the pH of WMEO +acetic acid + carvacrol (WAC) was higher than WMEO + acetic acid, which this might due to the antimicrobial effect of carvacrol. Overall, in this study, the acetic acid could lower the pH of the solutions, resulting in a better antimicrobial effect.

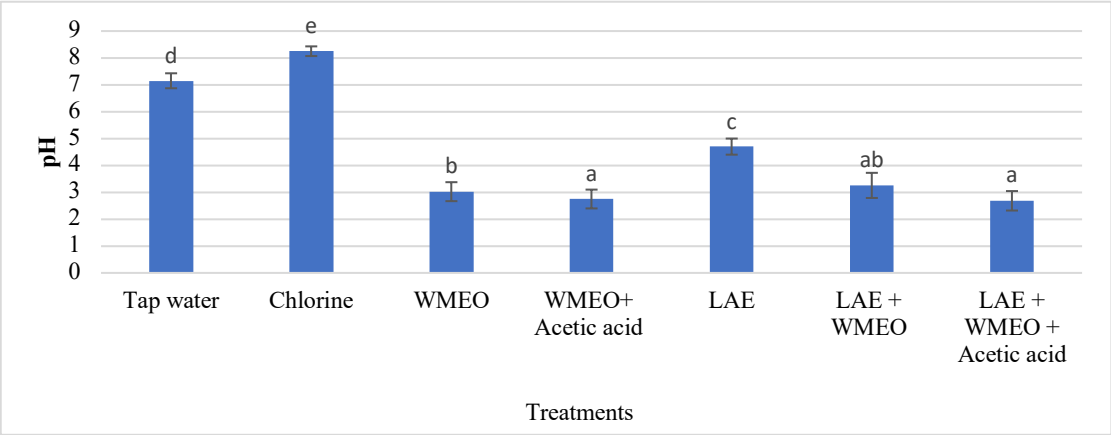

Fig 4.2.1 pH of produce washes containing combinations of WMEO, acetic acid and LAE

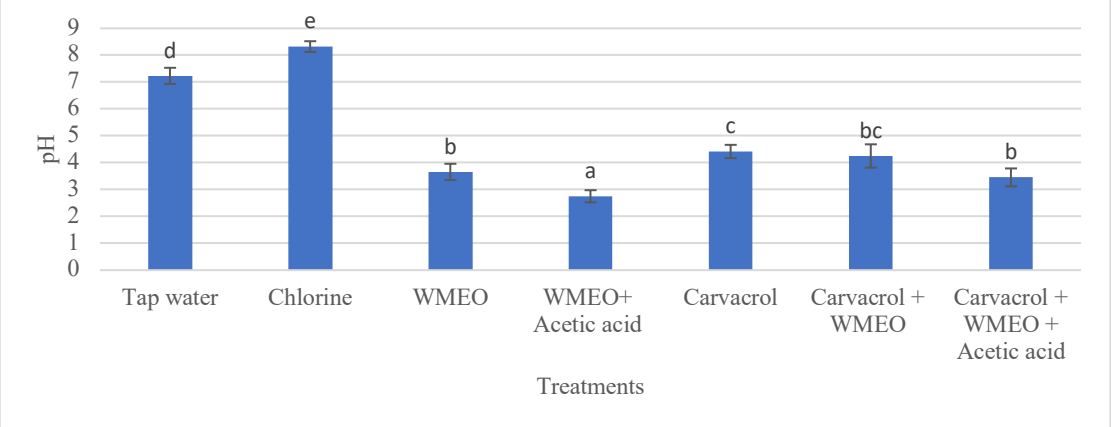

Fig 4.2.2 pH of produce washes containing combinations of WMEO, acetic acid and carvacrol

4.3 Effect of combination of LAE and WMEO-based produce wash on lettuce against *E. coli*

Based on the results of the previous MIC experiments, LAE was combined with WMEO and acetic acid. The results of the antimicrobial activities are found in Fig 4.3.1. The wash effect of tap water resulted in a 1.08 log reduction as compared to the control. The WMEO solution and the LAE solution caused 1.62 and 1.53 log reductions, respectively. Combining the antimicrobial compounds WMEO + acetic acid caused a 1.78 log reduction. Additional combinations, LAE + WMEO and WMEO + acetic acid + LAE (WAL), resulted in 1.82 log reductions for both washes, which is equivalent to the effect of industry standard chlorine solution (1.90 logs

reduction, $p>0.05$). All treatments differed significantly from the tap water wash, but there were not significant differences among antimicrobial treatments, showing no additive effects from combined washes as FICI is not larger than 1.

Higher bacterial reductions in the LAE treatment compared to tap water wash could be due to the action of LAE as a surfactant on proteinaceous cytoplasmatic membranes and outer membranes of Gram-negative as well as cell membranes and cytoplasm of Gram-positive bacteria (Luchansky and others 2005). The surfactant could absorb the cell membrane, cause the leakge of cell and lead to cell death.

WMEO contains the active compound 4-HBITC, which exhibits antimicrobial propertiesby inhibiting several metabolic pathways and damaging cellular structures. Based on the previous studies by Monu and Others (2014), we used 0.84% concentration of WMEO in the produce washes. Research has been conducted using WMEO in media and as a food additive. The commercialized product IsoGrad with similar concentration (0.85%) could cause 2 log reduction of *E.coli* in 0.5% peptone broth after 5 days at 6.5°C (A. Ekanayake 2006). Another study on the effect of WMEO against *E. coli* at 22°C in TSB at 0.83% and 0.84% resulted in about 4 log reductions after 24 h (Monu and others 2014). One study reported that WMEO at 0.84% in TSB for 24 h at 22 °C showed about 6-7 log reduction against *Salmonella* (Porter and Monu 2019). The higher reduction may be due to multiple reasons, including the continuous exposure to the WMEO for 24 h, and the fact that it was performed in media and not in a food matrix. In our study, the inoculated lettuce was only in contact with the WMEO for the time of the wash (2-minute) and was removed by rinsing, which could be a cause of lower microbial reductions compared to the studies discussed. Porter and Monu (2019) also investigated the antimicrobial effects of WMEO and WMEO + carvacrol in ground chicken challenged with

Salmonella and stored at 10 °C for 12 days. In their study, WMEO alone and in combination with carvacrol against *Salmonella* were found to have a 1.05 and 1.14 log reduction at 10 °C for 48h, respectively. These results are lower than our 1.32 and 1.88 log reduction of WMEO and WMEO +carvacrol produce wash. Our selection of higher WMEO concentration (0.84% compared to 0.75%), wash effect and different pathogens and target samples might be the reason of difference. Another study that involved 0.1% geranium essential oil in a Chinese cabbage produce wash solution for 5- minute caused 1.82 log reduction on *Salmonella* and 1.59 log reduction on *E. coli* (Park and others 2018).

Based on Techathuvanan and others (2014) study, LAE + WMEO was proven to have an additive effect at pH 6.0 and 27°C with fractional inhibitory concentration index (FICI) =1 as FICI between 0.5 - 4 was considered as additive effect (Meletiadis and others 2010). In addition, some triple combinations in the same study were tested against *E. coli,* and additive effects were found (Techathuvanan and others 2014). Our results did not show significant differences ($P>0.05$) between washes that included LAE and acetic acid possibly due to the different bacterial strains tested (*E. coli* O157:H7 ATCC BAA-1882). In a study from Muriel-Galet (2012), 0.25% of LAE was found to cause a 2.40 log reduction against *E. coli* on packaging films. The higher reduction might be due to the higher concentration used in their study (0.25% compared to 0.005%). The folds of lettuce leaves might provide an area of protection for the bacteria and limit the contact with the antimicrobial, one possible explanation for the difference in reductions. Also, the treated packaging film was in contract with pathogens 24 h, much more time than the 2 min wash in our study.

Researches have shown that Gram-negative bacteria are more sensitive to LAE than Gram-negative bacteria, which is also observed for other natural antimicrobials (Delaquis 2002).

This difference is mainly due to the outer membrane surrounding the cell wall in Gram-negative bacteria that restricts the diffusion of the hydrophobic compounds going through its lipopolysaccharide coverings (Russell 1995) which could explain a lower kill-rate of LAE in our study. In our study, combination of LAE with WMEO did not show an additional bacterial elimination probably due to the presence of two phases viz., the water soluble LAE and acetic acid and the WMEO in oil phase. Therefore, the LAE and carvacrol might not have enough interactions.

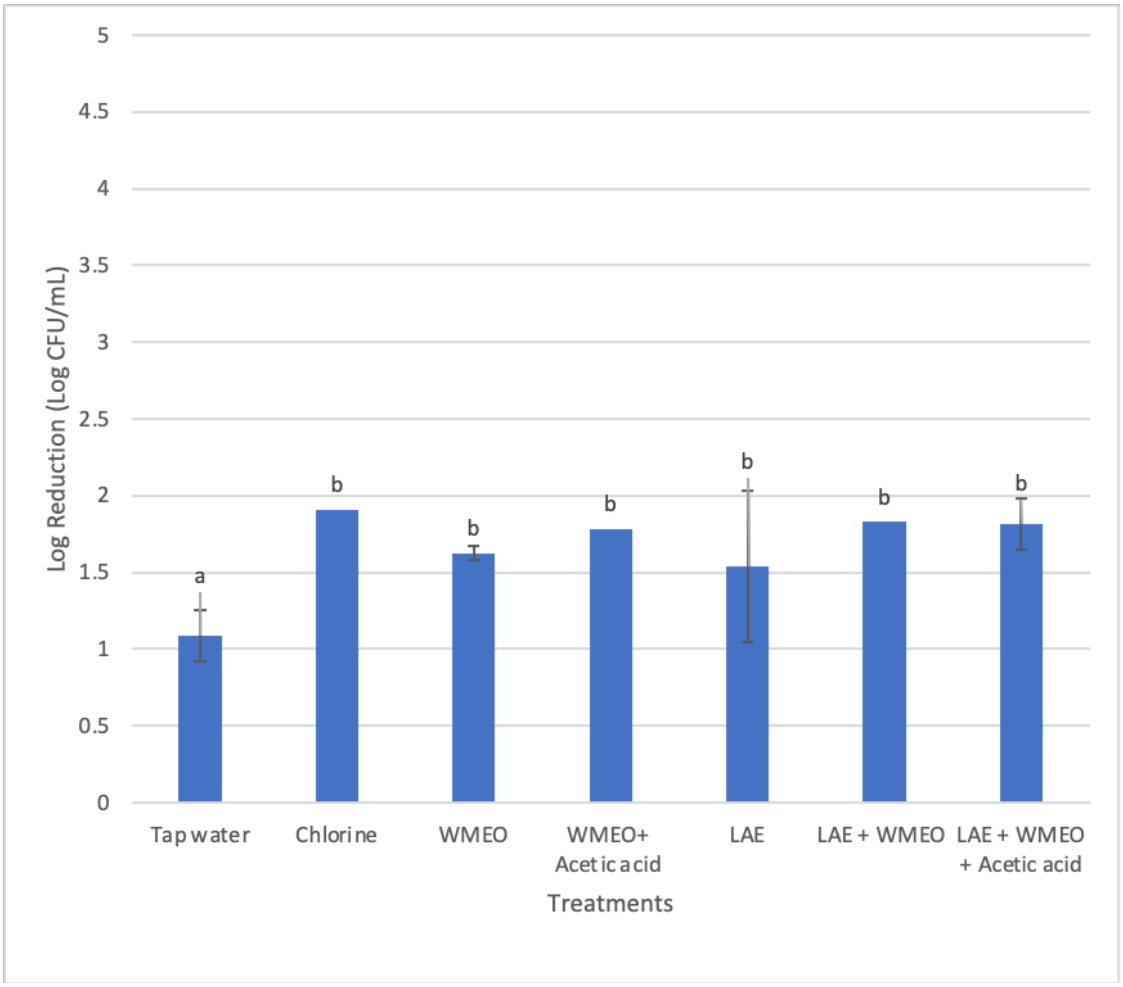

Fig 4.3.1 The reduction on *E. coli* on lettuce washes using a combination of white mustard essential oil (0.84%), lauric arginate (0.01%) and acetic acid (2%).

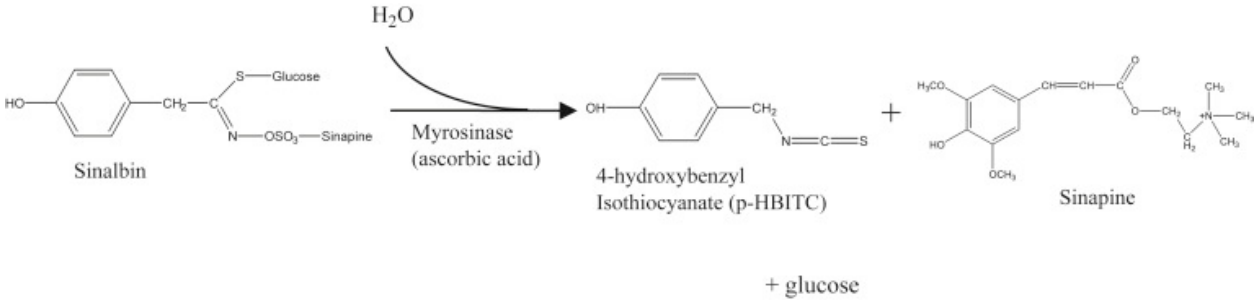

Fig 4.3.2 The reaction of the formation of 4-HBITC in white mustard plants (Ekanayake and others 2016)

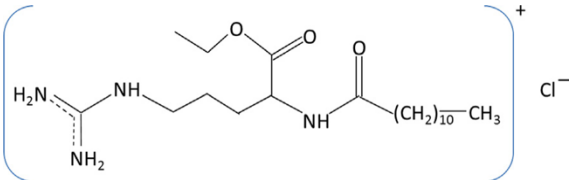

Fig 4.3.3 Chemical structure of lauric arginate

4.4 Effect of combination of carvacrol and WMEO-based produce wash on lettuce against *E. coli*

Based on the results of the previous MIC experiments, the carvacrol was combined with WMEO and acetic acid. The results (Fig 4.4.1), showed the tap water caused a 0.77 log reduction. The WMEO solution and carvacrol alone caused 1.32 and 1.35 log reductions in the population of *E. coli*, respectively ($p>0.05$). The combination of the WMEO + acetic acid and WMEO + carvacrol caused 1.96 and 1.88 log reductions, respectively ($p>0.05$), which is greater than the WMEO and carvacrol alone. The WMEO+ acetic acid + carvacrol (WAC) caused a reduction of 2.32 logs, which did not have significant differences when compared to chlorine solution (2.28 logs reduction, $p>0.05$) but was significant when compared to other treatments. All treatments were significantly different from the tap wash treatment.

The WMEO + acetic acid and WMEO + carvacrol caused more reduction than WMEO and carvacrol alone ($P<0.05$). These might be due to an additive effect of the combinations. Although WMEO alone could affect the metabolic pathway and increase the permeability of the bacterial cell (Ekanayake and others 2016), carvacrol could further decrease the membranes

integrity by increasing the unsaturated fatty acid, inhibits the metabolic pathway by depleting ATP pool to affect energy supply (Ultee and others 2002), and hindering the movement of *E. coli* by inhibiting the synthesis of flagellin (Rosanglea Di Pasqua 2006), resulting in more reduction. The addition of acetic acid could lower the pH, causing more damage to membranes, suppression of NADH and decrease of the rate of the transportation of substrates to hinder the metabolism of bacteria to cause more reduction (Mani-López and others 2012). The WMEO+ acetic acid + carvacrol (WAC) caused more reductions than either WMEO + acetic acid or WMEO + carvacrol ($P<0.05$), which showed the additive effect of the WAC solution. The antibacterial effect of carvacrol could be enhanced by a lower pH as well (Nostro and others 2017). According to the study by Nostro and others (2017), a lower pH of 6.0, carvacrol is less dissociated and more hydrophobic, making it more prone to bind to the hydrophobic regions of the membrane proteins and result in a better partition into the lipid phase of the bacterial membrane. Increasing the acidity might make carvacrol less dissociated and more hydrophobic, which might be another explanation for an increase in the efficacy of antimicrobials.

The antimicrobial activity of carvacrol is attributed to the presence of phenolic compounds (Lambert 2001) that interact with the cell membrane of microorganisms (Fig4.4.2). They are amphipathic and have a high affinity for cell membrane physio-chemical properties, which could affect both the lipid ordering and bilayer stability and result in the decreasing of membrane integrity and increasing proton passive flux across the membrane. Atomic force microscopy of the cell envelop after treatment has confirmed that it is a major target as the cell size, length and diameter decreased after contact (La Storia and others 2011). Ultee et al (2002) hypothesized that the hydroxyl group and delocalized electrons are important for phenolic compounds to act as a proton exchanger, reducing the gradient across the cytoplasmic membrane

between the lipid acyl chains, resulting in the collapse of the proton motive force, depleting the ATP pool and finally causing cell death. Due to the hydrophobicity, carvacrol can accumulate in the cell membrane, which could accelerate cell death from modifications of the membrane because of the hydrogen-bonding and proton-release ability of carvacrol. Another study showed that at sublethal concentrations, carvacrol could change the fatty acid compositions, increasing the unsaturated fatty acid content, as well as interfere with the protein synthesis and inhibit the synthesis of flagellin in *E. coli* (Rosanglea Di Pasqua 2006). With the combination of LAE, the permeability of the cell was increased, the metabolic process was further disrupted, and proteins were denatured, which resulted in more reduction (1.32 to 1.88 log reduction).

The current research demonstrated a higher kill with the WMEO + carvacrol in lettuce wash compared to 0.22 log reduction in *E. coli* in ground chicken as reported by Porter and others (2020) in ground chicken Our higher reduction may be due to the higher concentration of WMEO (0.84% compared to 0.75%), but a lower concentration of carvacrol was used in our study (0.05% compared to 0.1%). Another research studied the different ratios and combinations of natural essential oil antimicrobials that might lead to different antimicrobial activities (Vuuren 2008), which might be an explanation for the difference results in our study. In these studies, the temperature, time and research target were different. The result showed that WAC might be potential substituted for 150 ppm industry standard chlorine rinsing solution with many other parameters need to be investigated and the triple combinations were more effective than the combination of two compounds or WMEO and carvacrol alone. The WMEO + carvacrol + acetic acid might be potential alternative substitute for chlorine in produce wash.

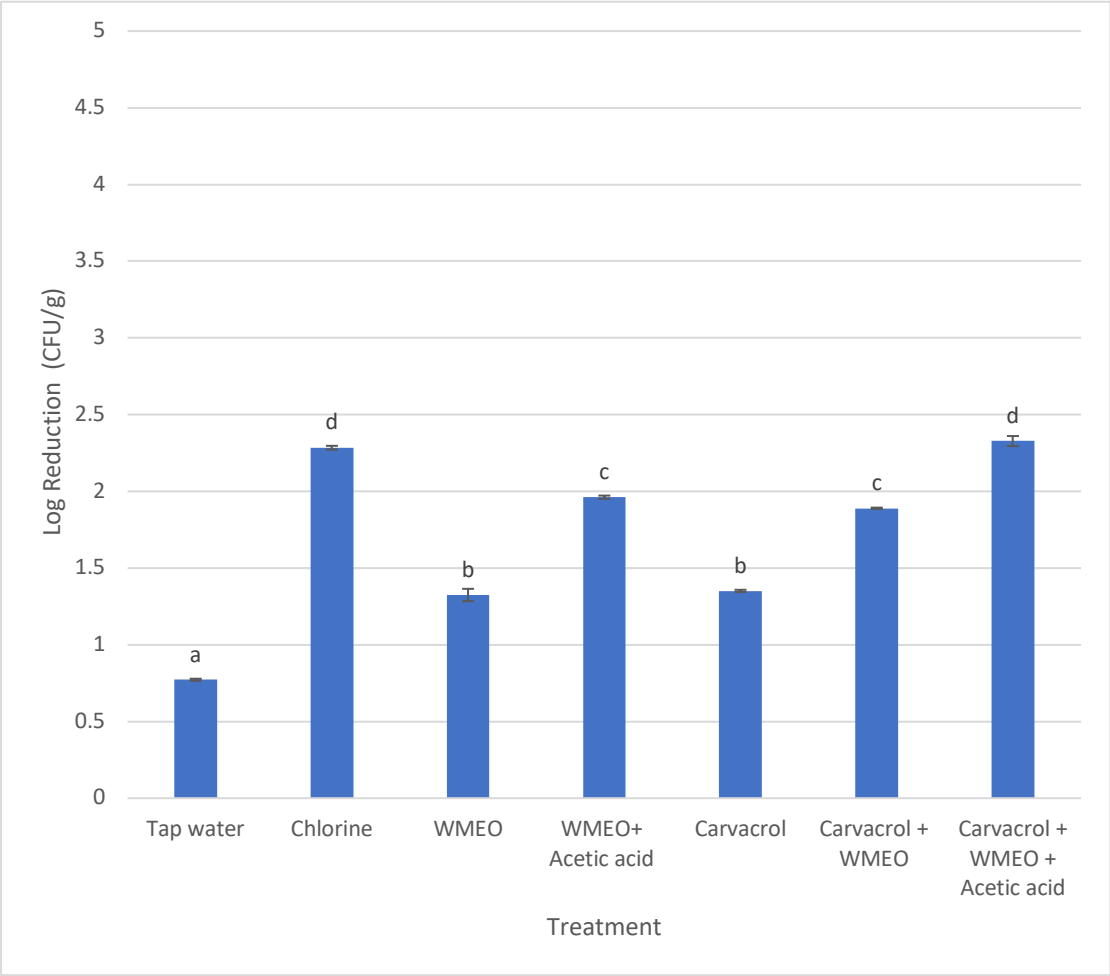

Fig 4.4.1 The reduction on *E. coli* on lettuce using produce washes composed of combination of white mustard essential oil (0.84%), carvacrol (0.1%) and acetic acid (2%).

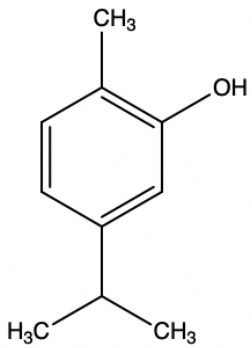

Fig 4.4.2 Molecular structure of carvacrol, 2-methyl-5-(1-methylethyl)-phenol

4.5 Effect of cold plasma against *E. coli* on raw beef

After the treatment of 5, 10 and 15 minutes of cold plasma on raw beef (Fig 4.5), the reductions of *E. coli* averaged 1.75 logs with no significant differences ($P>0.05$) between treatment times.

The free radicals from cold plasma could break the organic bonds in the high molecular weight hydrocarbon containments which includes pathogens. Also, the release of ROS could oxidize the proteins and DNA, resulting in the death of bacteria (Gallagher and others 2007). By diffusing through the cell membrane, the intercellular pH would decrease, and the stability of proteins and DNA chains would be affected, leading to the dysfunction of bacteria cells.

The mechanism of cold plasma technology has been thoroughly researched, but there are few applications on food (Thirumdas and others 2014). There is a report of cold plasma treatment on beef loin which resulted in 2.8 log microbial reduction (Bauer and others 2017), which might be due to our smaller diameter (0.4mm compared to 2mm), higher frequency (9KHz compared to 6.5kHz), higher input (8.16kV compared to 6.8 kV) and different gas composition. The higher input could create more ROS thus increase the bacterial reduction (Kyenam Lee 2006). Although it was longer distance (20 mm compared to 10 mm) and achieved a hogher bacterial reduction under shorter time (1 minute), this resulted in discoloration. The discoloration might be due to the oxidation of lipids caused by ROS, which was rare but was found by serval scientists (Jayasena and others 2015; Thirumdas and others 2016). Some scientists found the effect of cold plasma was initial but not time dependent, which is consistent with the finding of our study (Bauer and others 2017; Zhuang and others 2019). While in our study, no significant increase of microbial reduction was found with the increase of time, this might be due to the

unevenness of the surface of raw beef. Since the cold plasma could not penetrate and could only react to the surface of the food (Winter and others 2008), an uneven surface could have adverse effect of the treatment. In this beef study, the exposure time between 5 to 15 min showed no diffence statistically, which means that increasing exposure time during 5 to 15 min could not increase the microbial reduction of cold plasma.

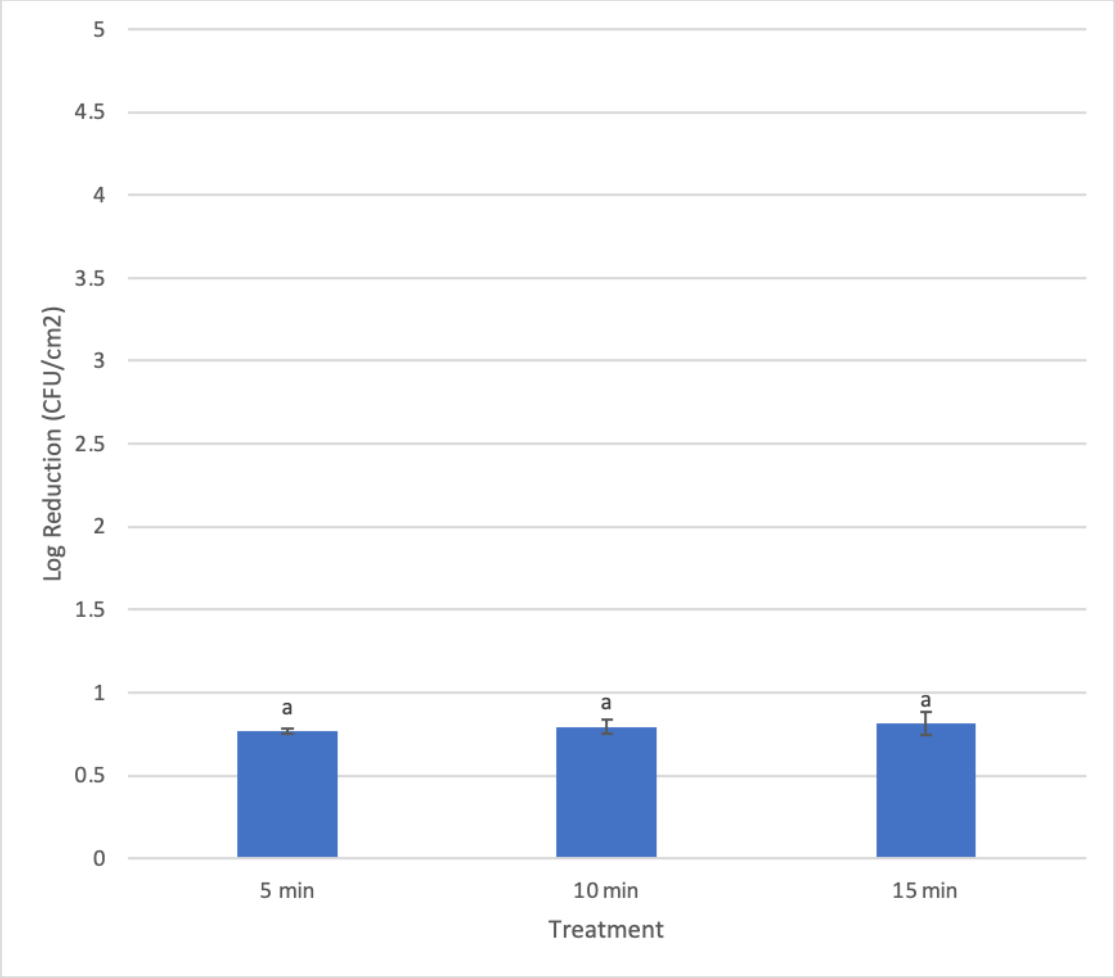

Fig 4.5 The reduction of *E. coli* inoculated on raw beef after cold plasma treatments.

4.6 Effect of cold plasma on ham against *Listeria*

After the treatment of 5-, 10- and 15-minute of cold plasma on ham (Fig 4.6), 5 minutes of cold plasma treatment resulted in a 1.02 log reduction of *Listeria*, while 10-minute caused

1.35 log reduction and 15-minute resulted in 1.75 log reduction ($p<0.05$). The reduction was increased with increasing the time of exposure.

In our study, the increase of 5 min treatment could approximately increase 0.35-0.4 log reduction. The surface of ham is smoother than the raw beef, which might allow samples a larger expose area than raw beef and caused the more reductions.

Previous research showed that 5- minute of cold plasma could cause 3.8 log reduction on strawberries against *Listeria* (Ziuzina and others 2014). They used a high-voltage (70kV) cold plasma in a container and exposed samples indirectly. Research showed that higher voltage could cause more reduction due to the higher energy dose and different ROS amount with more damage on the cell membrane integrity and DNA. The 15-minute exposure showed 1.75 log reduction, which was the best microbial reduction in this study.

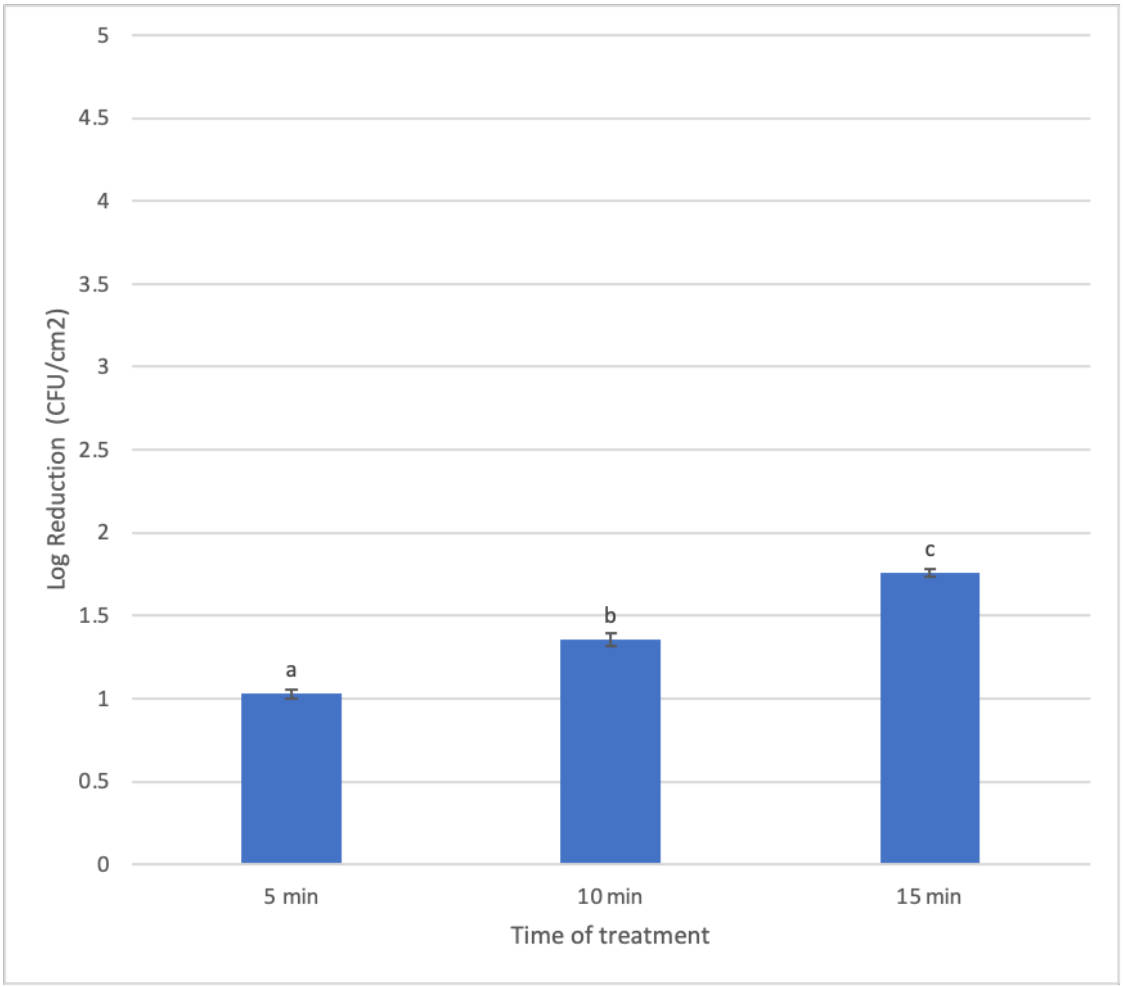

Fig 4.6 Reduction of *Listeria* spp. inoculated on ham after cold plasma treatments.

4.7 Effect of combination of cold plasma and WMEO-based produce wash on lettuce against *E. coli*

Based on the results of WMEO-based produce washes with LAE and carvacrol (Fig 4.7), we chose carvacrol since it had higher reduction (2.32 logs reduction compared to 1.82 logs reduction, respectively). For cold plasma treatment, we used 0-, 5- and 10-minute exposurebased on the prior experiments. Results are presented in Fig 4.7, the wash effect (Tap water) caused a 0.48 log reduction in the *E. coli* population. The produce wash (WAL) solution caused 2.71 logs reduction. The cold plasma treatment (5 and 10 minutes) alone caused 2.14 and 2.29 logs

reductions, respectively ($p>0.05$). Tap water combined with cold plasma (5 and 10 minutes) caused 2.31 and 2.39 logs reduction respectively ($p>0.05$). The WMEO and chlorine solutions without cold plasma treatment caused 2.71 and 2.55 logs reductions ($p>0.05$), respectively. Treatment of 5 minutes of cold plasma with chlorine caused 2.81 logs reduction, 10 minutes of cold plasma with chlorine caused 3.13 logs reduction. 5 minutes of cold plasma with produce wash solution caused 3.41 logs reduction and 10 minutes of cold plasma with produce wash solution caused 3.94 logs reduction, which was more effective than chlorine solution 2.56 logs reduction ($p<0.05$).

Our results showed that all treatments are significant different from the tap water alone ($P<0.05$), which showed WAC, chlorine and cold plasma treatment are more effective than simple tap wash treatment at reducing *E. coli* on lettuce($p<0.05$). The WAC treatment could increase the permeability of the cell, interfere with metabolic pathway and cause the disturbance of bacteria cell. The chlorine could attack the cell walls, release radicals to oxidize proteins and DNA and affect the enzyme system, while cold plasma could release ROS and oxidize the surface of cells and cause the death. These could have an addictive effect to the sole wash effect. However, increasing the exposure time of cold plasma in both tap water and no wash treatment could not cause more bacterial death, while in chlorine and WAC treatments, increasing the treatment time, higher microbial reductions were found. This might be due to the reactions between cold plasma and these chemicals. Researches showed that lower pH could be more suitable for cold plasma to eliminate microorganisms (Smet and others 2016), this might be an explanation for more reduction caused by WAC. Although there is no current research investigated how chlorine reacts with cold plasma, we speculate that some reactions happened with the combination of cold plasma and chlorine.

One study involved cold nitrogen plasma and clove oil against *E. coli* on lettuce and it caused 5.48 log reduction with the treatment of 3-minute (Cui and others 2016). The difference of the results might due to the treatment time. A longer dip time might result in better absorption of essential oil and cause higher reductions. However, the distance of plasma jet to samples was not reported in this research. As more hurdles were introduced, we could see higher microbial reduction was found, which was reported by serval publications(Leisner 2000; Singh and Shalini 2016). Based on our results, the WAC wash with 10- minute cold plasma could be an alternative to industry standard 150-ppm chlorine wash. In future study, we might introduce more hurdles to increase the microbial reduction.

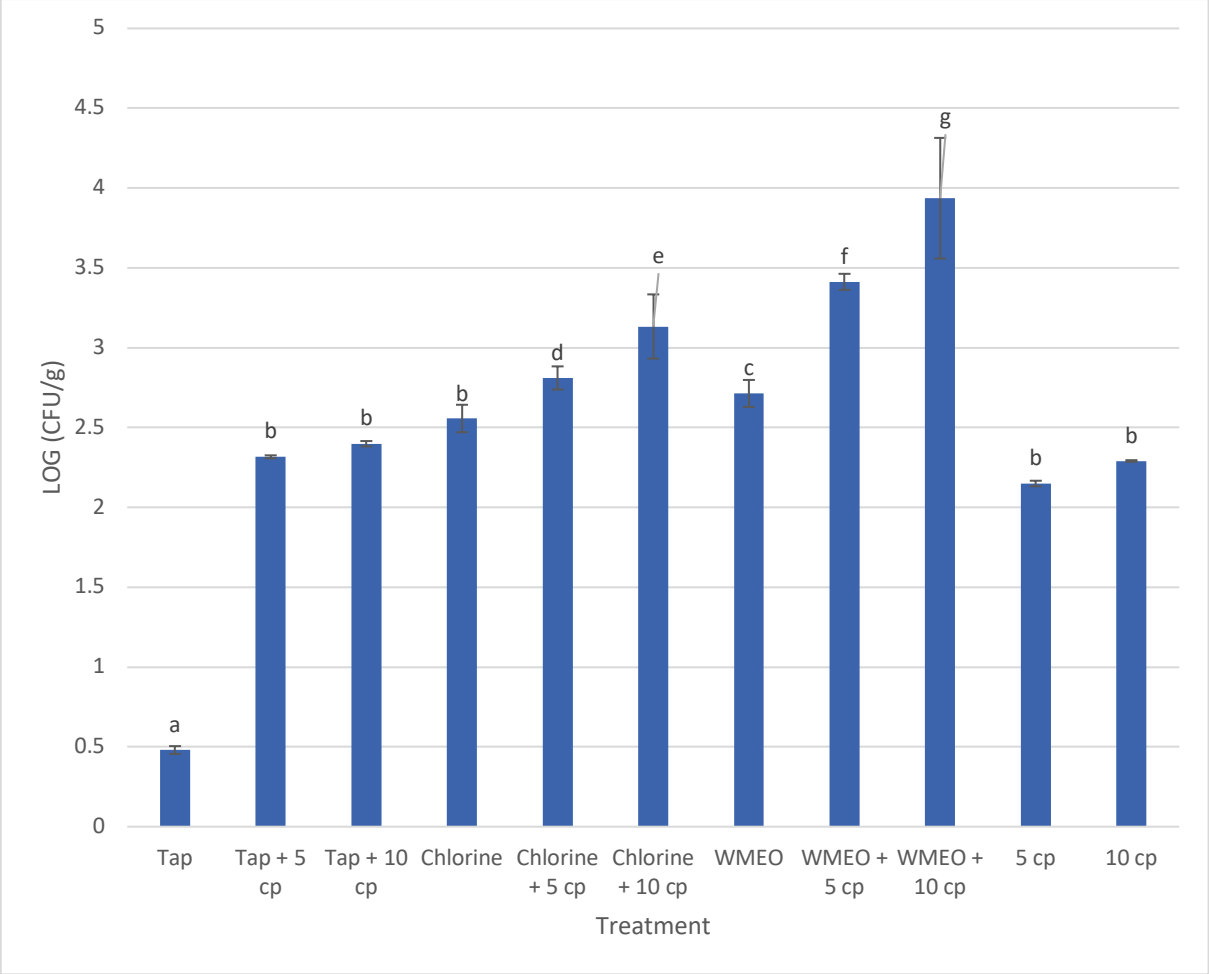

Fig 4.7 The reduction of bacteria level after treatments of WMEO-based wash and cold plasma against *E. coli* K-12 on lettuce (Tap = tap water; WAC = 0.84%WMEO + 2% acetic acid + 0.1% carvacrol; Chlorine = 150 ppm chlorine; 5 cp = 5 min cold plasma exposure; 10 cp = 10 min cold plasma exposure)

Chapter 5 Conclusion

The purpose of this study was to evaluate the white mustard essential oil-based produce wash on lettuce against *E. coli*, cold plasma on raw beef against *E. coli*, cold plasma on ham against *Listeria*, combination of white mustard essential oil-based produce wash and cold plasma on lettuce against *E. coli*.

After trials of cold plasma, we found that the length of time was not critical among 5–15-minute treatment and 5-minute exposure could bring about 1 log reduction. These showed the cold plasma could serve as an efficient potential invention against microorganisms. The next step was to combine cold plasma technology and WMEO-based produce wash. The result showed that the combination of cold plasma technology and WMEO-based produce wash had a better effect against *E. coli* on lettuce compared to the industry standard of 150-ppm chlorine.

Serval treatments involved in this study could have same or higher reduction when comparing to 150-ppm chlorine.

Lastly, without cold plasma technology, the WMEO + acetic acid, LAE + WMEO and LAE + WMEO + acetic acid, and carvacrol + WMEO + acetic acid did not have significant difference when compared to 150-ppm chlorine.

In conclusion, these studies show potential of cold plasma technology and WMEO-based produce wash and their combination to be used as antimicrobial methods to protect the food safety. However, further investigations should be conducted to involve more types of food and microorganisms, the efficacy with other hurdles, and under other conditions to increase the effectiveness and feasibility of application in the food industry.

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