

ABSTRACT

THANISSERY RAVINDRANATH, RAJANI. Application of Thyme-Orange Essential Oils to Reduce *Salmonella* and *Campylobacter* on Processed Broiler Chicken. (Under the direction of Drs. Douglas P. Smith and Sophia Kathariou).

Poultry meat is often linked to human illness outbreaks caused by *Salmonella* and *Campylobacter*. Natural antimicrobial interventions are gaining interest among consumers. Essential oils are plant-derived compounds that have a potential to be used as natural antimicrobials in food. Preliminary studies were conducted to screen the oils of thyme, orange, rosemary, clove, and thyme-orange oil combination (TOC) against *Salmonella* and *Campylobacter* using disc diffusion and macro broth dilution techniques. A concentration of 0.14% v/v of TOC was found to be effective against both pathogens. Two separate experiments were conducted to evaluate the effect of TOC on reducing *S. Enteritidis* (SE) and *C. coli* numbers on inoculated broiler breast fillets and on whole wings dipped or marinated with TOC. Three independent trials were conducted for the dip study and two independent trials for the marination study. In the dip study, each part was dipped in a mixture of inoculum containing SE and *C. coli* and then subjected to a dip treatment in one of four solutions: 0.25% TOC; 0.5% TOC; DMSO (Dimethyl sulfoxide used as emulsifier for the essential oils) or control (only water) for one minute. In the marination study, 12 breast fillets or wings were vacuum tumbled for 20 min with the marination solution either with or without 0.5% TOC. Results from the three replicate trials of the dip study showed that 0.5% TOC significantly reduced ($P < 0.05$) SE by 2.4 and 4.1 log cfu/mL on breast fillets and whole wings, respectively, and *C. coli* by 4.5 log cfu/mL on breast fillets. Although 0.5%

TOC resulted in 4.80 log cfu/mL reduction of *C. coli* on the wings, compared to 2.2 logs for control, results were not significant due to variation between different trials. TOC at 0.25% level performed similar to the DMSO and control treatments. Results from the two replicate trials of the marination study indicated that TOC at 0.5% in the marinade solution applied by vacuum tumbling significantly reduced ($P < 0.05$) numbers of viable SE on broiler breast fillets by 2.6 and 2.3 log₁₀ cfu/ml, respectively, and *C. coli* on whole wings by 3.6 and 3.1 log₁₀ cfu/ml. The un-inoculated chicken parts were positive after marination with inoculated parts, indicating cross-contamination. However, the numbers of bacterial cells recovered from the TOC- treated samples were significantly lower ($P < 0.05$) than the numbers recovered from the untreated samples. A third experiment was conducted to evaluate the effect of 0.5% TOC- containing marinade on the shelf life of broiler breast fillets and whole wings. The total aerobic and facultative mesophiles (TAM) occurring naturally on these products during refrigerated storage for 14 d (at 1, 7, 10, and 14 d) were enumerated. One set of duplicate plates were incubated at 35°C and another set at room temperature (20-25°C) for 48h. TOC marinade was able to significantly ($P < 0.05$) reduce TAM numbers on the breast fillets on days 1, 7 and 10 compared to the controls, with a log reduction of 0.3, 0.9-1.4, and 0.7-1.1 on d 1, 7, and 10, respectively, at both incubation temperatures. The difference in TAM between the treated and untreated whole wings was not significant.

In conclusion, 0.5% TOC applied on broiler breast fillets and whole wings by dip or marination can be successfully used as natural antimicrobial to reduce *Salmonella* and *Campylobacter* numbers on the products. Marination with 0.5% TOC can also be used to

reduce TAM on skinless breast fillets; however, higher concentration may be required for skin-on poultry products.

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Application of Thyme-Orange Essential Oils to Reduce *Salmonella* and *Campylobacter* on
Processed Broiler Chicken

by
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DEDICATION

To my beloved family members Kalyan Divakala, Ravindranath Thanissery, Kairely
Thanissery, and Rajeesh Thanissery

BIOGRAPHY

Rajani Thanissery is the first of the two siblings of Thanissery Ravindranath and Thanissery Kairely, and was raised in Pondicherry, India. She obtained her degree in Veterinary medicine from Pondicherry University, India in 2003. As a veterinarian she worked for 4 years at the Animal Husbandry Department, Government of Kerala, India. In 2008, Rajani decided to move to USA to pursue her Master Degree from Auburn University, where her research primarily focused on developing strategies to control *Clostridium perfringens* in broilers. Upon graduation, she joined Dr. Smith's research lab at NCSU. Her current area of research is to study the effect of essential oil blends to control *Salmonella* and *Campylobacter* on processed broiler meat. She hopes to use her expertise to improve the safety and wholesomeness of poultry products.

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CHAPTER 1

REVIEW OF LITERATURE

1.10 PROCESSED BROILER CHICKEN

1.11. General Overview:

Poultry has been an important part of agriculture in the US since the 1800s with backyard flocks of dual purpose birds on family farms. By the beginning of the 20th century, meat which was a byproduct of the egg enterprise became available during summer months. Gradually there was a shift from seasonal to year round production. In 1930s began the development of the broiler – a chicken raised exclusively for its meat. By 1940s vertical integration brought about a major change in the structure of the broiler industry [1]. In such integrated production, a single company controls ownership of each stage starting from the hatchery and the production (which includes the feed mill) to the processing plant. The tremendous growth that formed the poultry industry of today was marked by three interacting trends: a) Increase in per capita consumption of poultry when compared to red meat. An average American consumed about a half a pound of chicken per year in 1928, and by 2012 the figure has increased to almost 82 pounds [2]. b) A shift from ice packed to pre packed branded poultry. What started as ‘New York dressed chicken’ with only blood and feathers removed, was gradually moved to evisceration and packing in ice and wooden crates, and then to pre packed branded poultry in ready to cook form. c) Shift from whole birds to cut – up/parts and further processed products for added value in response to the consumers demand for reduced preparation time. In 1962, 83% of broiler meat was sold as whole carcass, 15%

as cut-up/parts and 2% as further processed. By 2012, there was a complete shift with 12% sold as whole carcass, 41% as cut-up/parts and 47% as further processed. [3]. This extraordinary growth of the industry is due to the rapid adoption of improved technology in breeding, nutrition, disease control, housing, processing, packaging, and distribution. The use of technology by the vertical integrators has resulted in one of the lowest production costs in the world for animal protein. The retail price in 2012 for broilers was 133.5 cents per pound, when compared to 525 and 360 for beef and pork respectively [4]. With the growing concern for reducing cholesterol and fat from the diet, chicken (which is considered a healthier alternative) consumption surpassed both pork and beef by 1992 making the poultry industry one of the largest meat trade industries in the US. [1].

The U.S. Department of Agriculture has classified major poultry and poultry products. The types of poultry are chicken, turkey, duck, geese, guineas, and pigeons. The various classes of ready-to-cook chicken includes Rock Cornish game hen or Cornish game hen, Rock Cornish fryer or hen, Broiler or fryer, Roaster or roasting chicken, Capon, Hen, fowl, or baking or stewing chicken, cock or rooster. The most popular chicken is a broiler or fryer which is a young chicken (usually 6-7 weeks of age) of either sex with tender-meat with soft, pliable, smooth-textured skin and flexible breastbone cartilage. The carcass is sold whole, or components of carcass may be cut-up for individual sale. Carcass cut-up often includes removal of breast, thigh, drumstick, and wings. Breast and thigh are commonly deboned before sale. Further processing of whole carcass or deboned meat may be done to obtain added value products such as hot dogs, chicken nuggets, or sausages. Some products

are cured or smoked for added flavor. A few products may not require additional preparation on purchase and are sold as 'ready-to-eat' [5].

1.12. Broiler Rearing and Processing

Different segments involved in the production and processing of poultry are the broiler breeder housing operation, hatchery, grow-out farms, feed mill and processing plant.

Broiler Breeder Housing Operations:

The ideal characteristics of a meat type bird are a large frame body, with long, broad, and heavily muscled breast. Cornish are a breed of chicken that carry the desired traits of a meat type bird. Hence, most of the commercially developed meat type chickens have Cornish blood lines. The modern day commercial broiler lines had undergone years of genetic selection for traits such as feed conversion, carcass yield, breast meat yield, mortality, and leg quality. The breeder flock lay fertile eggs that develop to produce broilers [6].

Hatchery:

The hatchery is responsible for the incubation and hatching of the eggs obtained from the broiler breeders. The eggs after lay can be held for a maximum of 7-10 days under proper storage conditions without affecting the hatchability. A typical hatchery has two major types of equipments an incubator (setter) and a hatcher. The incubator provides appropriate temperature, humidity, ventilation, and turning for optimum growth of the embryos. The fertile eggs are incubated in the setter for 18 days. After 18 days the eggs are transferred from the setter to the hatcher. The hatcher provides the ideal temperature and

humidity for the hatching of eggs. The chicken eggs generally hatch within three days, resulting in a total incubation period of 21 days. [7]. In-ovo vaccines might be administered in the hatchery. The first sorting is made to cull the weak and crippled chicks immediately after removing the trays from the hatcher. The second sorting may be done to sex the chicks and separate the male and females. Sexing is practiced by few growers and all primary breeders to allow optimum management and feeding to meet the needs of each sex. The chicks are vaccinated, loaded into boxes and then delivered to grow-out farms [8].

Grow out Farms:

In the grow-out farms, the broiler is grown to specified carcass weight. The target weight is determined by the consumers based on the purpose for which the birds are grown. Smaller weight for fast food products and large weights are preferred for deboning operations. Typically, a broiler weight of 5 lb can be achieved in about 5 weeks. The grow-out houses are either curtain sided or tunnel ventilated [9]. The house contains automated systems to supply feed and water *ad libitum*. Before the day old chicks arrive at the environmentally controlled growing unit the whole house is heated and brooder rings are placed around each unit. Extra feed pans and waters are kept in the brooding area to ensure easy access of feed and water. For the first few days the temperature of the house is about 33°C after which it is gradually lowered as per the requirement of the growing chicks. The broiler nutrition is designed to produce the lowest feed cost per pound of product produced. Typically a 3 -5 phase feeding program is employed with fixed feed intake per bird for each diet. The different phases of feeding are pre-starter, starter, grower, finisher, and withdrawal

feed which depend on the target body weight. The market weight broilers are caught, loaded into coops, and transported to the processing unit. [10].

Feed Mill:

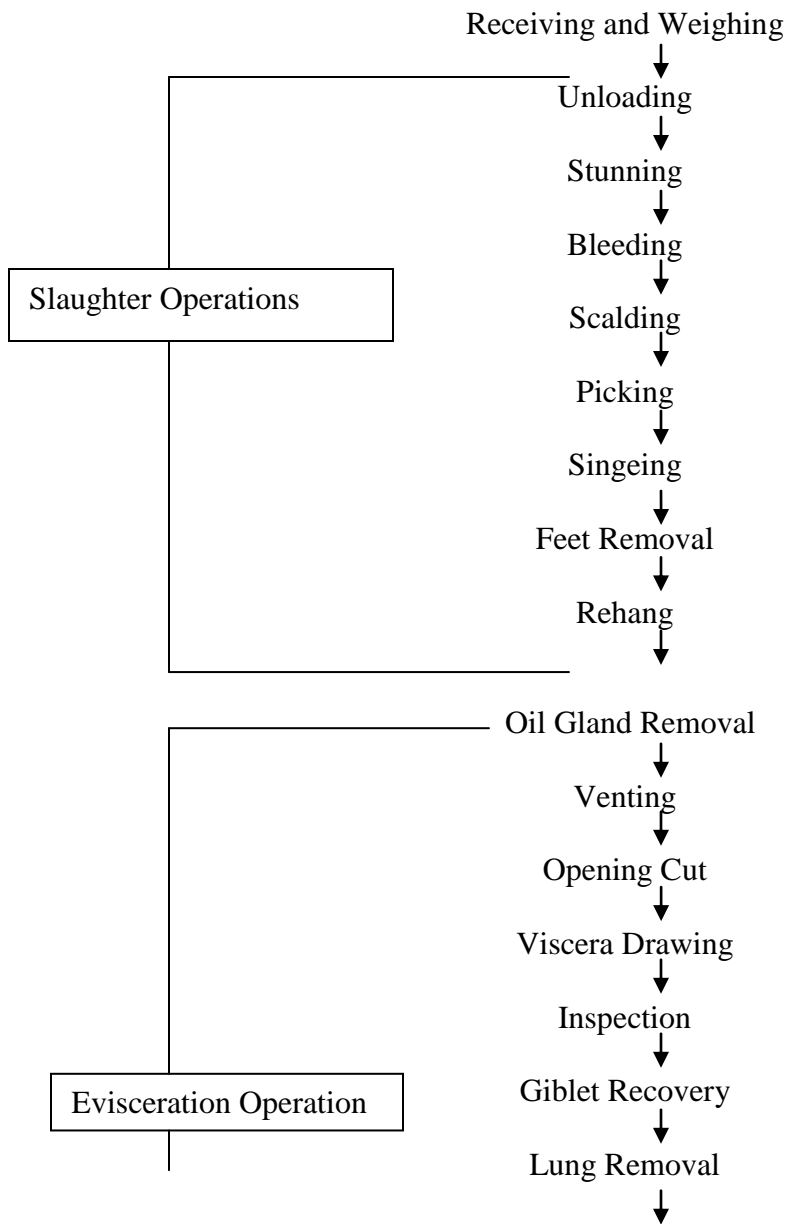
Feed accounts to about 65-70% of the production cost. Hence it is necessary to ensure effective utilization of the feed. Feed is given in different physical forms (mash, pellet, and crumbles) to different age groups of broilers. The key to feed milling is lowering the feed cost while maintaining the standard operating procedures, quality assurance and other preventive and maintenance programs [11].

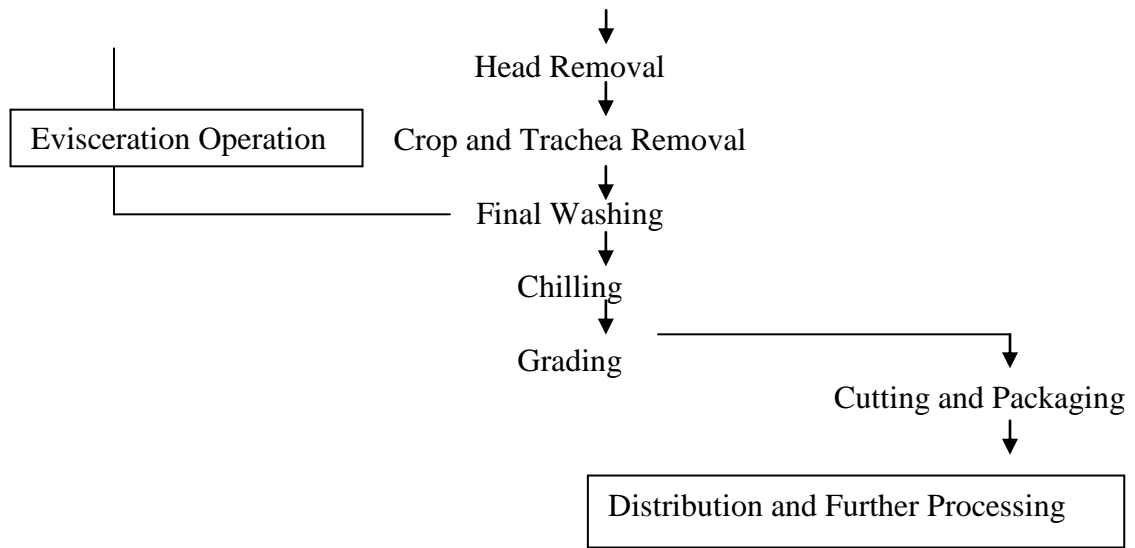
Processing Plant:

The basic steps involved in a typical poultry operation are listed in Fig 1 The step may vary slightly and have different levels of automation depending on the needs of the processor [12].

Figure 1: Steps involved in a typical poultry processing operation¹

¹Adapted from Barbut, S. 2010. Primary processing of poultry. Page 82 in Poultry products processing: an industry guide. CRC Press





1.13. Nutritional facts and Comparison to Red Meat

Muscle food from poultry and other farm animals contribute significantly to intake of energy and other essential nutrients such as protein, poly unsaturated fatty acids (PUFA), several trace minerals, and most B vitamins. Nutrient composition of raw poultry, pork, and beef are listed in Table 1. Chicken meat has a slightly higher protein than other red meat. A food is considered complete if it supplies all the indispensable amino acids in the proportion required by the body. The essential amino acid content in broiler breast meat is on an average 19 -110mg/g, and the corresponding value of thigh muscles ranges from 14 -93 mg/g [13]. The fatty acid profile of chicken meat shows greater amount of unsaturated fatty acid than saturated fatty acid. However the fat content in the skin could be a major contributor of fat to the edible portion. Cholesterol level is also slightly lower in chicken meat when compared to beef and pork. Dietary cholesterol is a potent regulator of serum cholesterol levels which has been correlated with the development of atherosclerosis in a percentage of human population. Therefore chicken meat which shows high nutritive value, lower levels saturated fatty acids, higher proportion of PUFA, and lower cholesterol content can be considered a healthy food; due to economical production it is also an affordable food for low income groups [14].

Table 1: Nutrient composition of raw broiler, pork, and beef (meat only). Value per 100g

Components	Units	Raw Broiler meat	Raw Pork	Raw Beef
Water	g	75.46	49.83	57.26
Energy	kcal	119	376	291
Protein	g	21.39	13.91	17.32
Total Lipid	g	3.08	35.07	24.05
Fatty acids Total	g	0.790	12.44	9.75
Saturated				
Fatty acids Total monounsaturated	g	0.900	15.93	10.47
Fatty acid Total Polyunsaturated	g	0.750	3.8	0.920
Cholesterol	mg	70	74	74

Source: USDA, 2012. National Database for Standard Reference. Release 25.

<http://ndb.nal.usda.gov/ndb/search/list>. Accessed June, 2013

1.14. Quality and Safety Concerns

The term quality includes both subjective and objective components. The scientifically measurable characteristics such as color, texture, drip loss, and flavor are described as ‘quality attributes’. There are differences in preferences to skin color among consumers. Some prefer a corn-fed yellow color, whereas others prefer a whiter appearance. Color is particularly relevant in skinless cooked products where a red or pink color gives the impression of undercooking, and black or dark bones are recognized as defects. [15]. The color of meat depends on the quantity and the chemical state of the pigment myoglobin which can be substantially affected by various factors such as breed, sex, muscle type, feed,

chilling methods, light, storage temperature, oxygen content, and duration of storage. Another concern of refrigerated poultry meat is the pale chicken muscle syndrome which is used to describe pale muscle that exhibits lower pH and decreased water-holding capacity. [16]. Chicken meat has a higher tenderization rate with more than 80% of tenderization occurring within 10h of slaughter. Factors such as *ante mortem* stress and induction of muscular spasms during stunning can negatively affect the ripening of meat. The problems of drip accumulates in the container during refrigeration occurs to a lesser extent in poultry meat. The drip loss depends on the cut surface area: volume ratios. Excessive drip losses can result in dryness, and loss of flavor. Raw poultry has little flavor, however cooked products develop a typical poultry flavor [17]. The relatively high PUFA makes poultry meat more susceptible to lipid oxidation and off flavors during refrigerated storage. Spoilage is generally not recognized when the microbial load is below 10^6 . Above this limit obvious signs of spoilage ranging from off odors, sliminess, to structural changes of the product may be noticed. The organisms that predominate at spoilage are *Pseudomonas* spp (*P. fragi*, *P. lundensis*, *P. fluorescens* biovars), *Acinetobacter*, *Moraxella*, and *Psychrobacter*. Other spoilage bacteria include *Shewanella putrefaciens*, and some psychrotrophic strains of Enterobacteriaceae. Yeast such as *Candida* and *Yarrowia* are also associated with spoilage. The chicken part, initial microbial load, pH of meat, storage conditions, and period of storage all determine the type of microorganisms present [18].

Consumption of poultry meat is becoming popular since high quality protein is provided at affordable prices. Public awareness and concerns regarding food safety issues

have also increased. The possible food safety hazards associated with poultry can be classified into microbial, chemical and physical.

The most common microbial agents are *Salmonella*, *Campylobacter*, *E.coli*, *Listeria* and *Clostridium perfringens* [19]. *Salmonella* and *Campylobacter* are diagnosed as frequent causes of food related gastroenteritis in the United States. An estimated one million cases of *Salmonella* and 800,000 cases of *Campylobacter* are reported annually in the US [20]. Both pathogens are common inhabitants of the intestinal tract of poultry leading to the contamination of meat during evisceration and processing [21]. Prevalence of *Campylobacter* on poultry products tends to be relatively higher than *Salmonella*. However, *Salmonella* appears to survive better in the environment. *Campylobacter* is particularly sensitive to drying, and growth occurs only under conditions of reduced oxygen, high moisture, and temperature above 30°C. However, they are found to survive different processing conditions and remain detectable by culture methods. Both *Salmonella* and *Campylobacter* cause a self limiting acute gastroenteritis which generally lasts for a week, although other symptoms are different. Complications and death might occur in immuno-compromised and elderly individuals [22, 23]. Verotoxin producing *E. coli* is capable of colonizing poultry without showing any external symptoms; however human outbreak from such strains has rarely been related to poultry [24]. *Listeria monocytogenes* is a pathogen that is capable of causing illness in both animals and humans. The ubiquitous distribution of the organism in the environment, and its ability to grow at cold temperatures makes *L. monocytogenes* a risk for post process contamination of ready-to-eat poultry meat. In the United States there is zero

tolerance policy for *L. monocytogenes* in RTE products. [25]. *Clostridium perfringens* causes a clinical condition called necrotic enteritis in chicken. Although its importance as a food borne pathogen is not extensively researched, *C.perfringens* is reported as the third leading cause (following Norovirus and nontyphoidal *Salmonella*) of foodborne illness in the United States [20]. *C. perfringens* spores are known to have exceptional resistance to heat, which favor their germination and growth under conditions of inadequate cooking and improper storage [26]. In addition to the direct implication on human health, it is hypothesized that antibiotic resistant strains of bacteria are being transferred from food animals to humans. Some of the resistant strains of bacteria are multi drug resistant *Salmonella*, macrolide and fluoro-quinolone resistant *Campylobacter*, glycopeptide, or streptogramin resistant enterococci, and multi drug resistant *E.coli*. The speculated risk of generating antibiotic resistant human pathogens has lead to the ban of antibiotic feed additives [27].

Perceived chemical hazard concerns associated with poultry meat are presence of veterinary drug residues, heavy metals, hormone like substances, environmental contaminants and mycotoxins. The federal regulation sets limits for the presence of drug residues in meat, and the US. Department of Agriculture Food Safety Inspection services (USDA FSIS) through its National Residue Program tests meat products for approved and unapproved veterinary drugs, pesticides, hormones, and environmental contaminants [28].

Bone, metal, foreign material, glass, insect, wood, rock, plastic, gristle are some of the physical hazards that can be associated with poultry products. The plants Hazard Analysis Critical Control Point (HACCP) plan or prerequisite program generally includes the

procedures and safeguards to ensure that such materials are not introduced into the product [29].

1.20. SALMONELLA

1.21. Nomenclature and Taxonomy

Salmonella spp. are members of the family Enterobacteriaceae and are related to *E.coli*. They are facultatively anaerobic, non spore forming gram negative rods. Most serotypes are motile (with peritrichous flagella) with the exception of serovars Pullorum and Gallinarum which are non motile. *Salmonella* nomenclature is quite complex and has evolved over time. [30]. The current system used by the CDC is based on the immune reactions of two surface antigens designated as O and H antigens. The O antigen (somatic antigen) is a carbohydrate antigen and is the lipopolysaccharide of the outer membrane. H antigen is a protein antigen and is present in the flagella. The genus *Salmonella* is divided into two species. *Salmonella enterica* and *Salmonella bongori*. *Salmonella enterica* is further subdivided into 6 subspecies which are differentiated based on biochemical reactions and genomic relatedness. The sub species are I-*enterica*, II-*salamae*, IIIa -*arizonae*, IIIb-*diarizonae*, IV- *houtenae*, VI- *indica*. The serotypes usually refer to the geographic location from where it was first isolated. When written, the serotype follows the genus, is not italicized, and the first letter is capitalized. Genus *Salmonella*, species *enterica*, subspecies *enterica*, and serotype Enteritidis is often shortened as *S. Enteritidis*. There are over 2500 *Salmonella* serotypes of which approximately 60% belongs to the *subspecies* I, and within *S. enterica* subspecies I the most common O-antigen serogroups are A, B, C1, C2, D and E.

Ninety nine percent of strains in this serogroups cause illness in humans and warm blooded animals. The other 5 subspecies and the species *bongori* are rarely isolated from human, and are commonly associated with cold blooded animals and the environment [31]. The most common *S. enterica* serovars associated with human infection are Enteritidis, Newport, Typhimurium, Javiana, Heidelberg, Saintpaul, Muenchen, Montevideo, and Infantis. The predominant serotypes identified in 2010 in meat and poultry products are Kentucky, Hadar, and Derby [32]

1.22. Biochemical and Molecular Characteristics

The genus *Salmonella* catabolizes D-glucose and other carbohydrates to produce acid and gas. This characteristic forms the basic for biochemical identification of *Salmonella* in triple sugar iron agar (TSI), brilliant green, and xylose-lysine-deoxycholate agar medium. Typical *Salmonella* isolates decarboxylates lysine to cadaverine in lysine iron agar. It fails to hydrolyse urea, but generates H₂S which is shown as a black color in TSI [33]. Traditionally serotyping involving the agglutination of the surface antigens (O and H) have been used for the biochemical confirmation of *Salmonella*. Various genotyping methods are practiced for molecular characterization of *Salmonella* and differentiation to serotype levels. Molecular methods include pulsed field gel electrophoresis, restriction fragment length polymorphism, ribotyping, and multilocus sequence typing [34]

1.23. Growth and Survival of Salmonella in Raw Broiler Meat

The optimum temperature requirement for the growth of *Salmonella* is 35-37°C. However the organism is quiet resilient and is found to grow at temperatures as low as 2-4°C,

and to elevated temperatures as high as 54°C. *Salmonella* is also found to survive extended period of storage at freezing temperatures. Other factors that affect the growth and survival of *Salmonella* are water activity (a_w), pH, osmolarity, nutrient content of the food, phase of the growth curve of the cells, and pre exposure of the cells to certain stress factors [35]. The appropriate water activity for growth of *Salmonella* is 0.99. The water activity in fresh poultry meat is 0.99 – 1.00 which is optimum for the growth of *Salmonella*. During the recent years the presence of *Salmonella* in low moisture processed food has become a particular concern, because outbreaks from low moisture processed food often impact a large number of people. [36]. The approximate pH that permits the growth of *Salmonella* in food is 7-7.5, however it is found to grow in a pH ranging from 4.2 to 9.5. The pH of poultry meat is between 6.2-6.4, which is favorable for the growth of *Salmonella*. The fermentation procedure provides an environment for *Salmonella* to develop acid tolerance. This has been of specific concern for its survival in low acid fermented meat products such as sausages. Acid tolerance is also found to confer cross tolerance to high temperatures, osmotic stress, and certain antimicrobials. [37]. Salt concentration of 3-4 % is found to inhibit the growth of *Salmonella*. However *Salmonella* develops tolerances to high salt concentration in acidic conditions with an increasing temperature [33].

1.24. Transmission to Humans and Pathogenesis

In the United States, nontyphoidal *Salmonella* is estimated to cause more than one million illnesses, 19,336 hospitalizations, and 378 deaths every year. [20]. *Salmonella* is a

normal inhabitant in the gastrointestinal tract of food animals, and during the evisceration process the carcass can be easily contaminated with *Salmonella*. [21]. Fresh and processed poultry products account for 29% of *Salmonella* infection in humans. The trans-ovarian transmission of *S. Enteritidis* in chickens had made this serovar a particular concern in the consumption of raw or under cooked eggs. Apart from this a number of non poultry food sources (jalapeno peppers, tomatoes, peanut butter, ground beef) and non food items (pets such as turtles and African dwarf frogs) have been implicated as potential sources [39]. Infants and the immunocompromised are more susceptible to illness when compared to healthy individuals. The infectious dose ranges from 10 cells to 10^7 depending on the kind of food and the serovar. Food containing high fat protects the bacteria from gastric juices, thereby requiring a very low infectious dose. [39]. As the bacteria are consumed by individual there are several no specific host defense mechanisms that synergistically work to prevent its colonization in the intestine. Failure of such mechanisms will result in the attachment of bacteria to the intestinal cells. Most serovars have flagellar filaments and fimbriae that help in the process of attachment. Several genes in the *inv* pathogenicity island encode for enzymes and activators that help in the invasion of *Salmonella* into the bacterial cells. Once it internalizes the host cell the bacteria starts multiplying within the endocytic vacuoles. Some serotypes possess virulence plasmid that encodes for attachment and multiplication within the host cells. Other *Salmonella* virulence factors are siderophores, enterotoxin and a cytotoxin. Nontyphoidal salmonellosis results in a self limiting gastroenteritis which usually last for 8 to 72 hours or sometimes up to 5 days.

Complications and even rare cases of death can result from systemic cases. Asymptomatic carriers and chronic conditions such as reactive arthritis, Reiter's syndrome, and ankylosing spondylitis are also reported [33].

1.30. CAMPYLOBACTER

1.31. Nomenclature and Taxonomy

Campylobacter was first identified in 1906 as 'peculiar organisms' in the uterine mucus of pregnant sheep. Later in 1927, Smith and Orcutt named the group of organisms as *Vibrio jejuni*. The genus *Campylobacter* was first proposed in 1963 by Sebald and Veron after distinguishing this group of organisms from the true *Vibrio spp* due to their non-fermentative metabolism, low DNA base composition, and microaerophilic requirement (5% oxygen and 10% carbon dioxide). The family Campylobacteraceae consists of two genera *Campylobacter* and *Arachobacter*. [40]. The genus *Campylobacter* comprises 16 species, which are distinguished based on 16S rRNA gene sequence comparison. The members of this genus are associated with a wide variety of diseases in animals and humans, but some are considered commensal organisms. Within the genus three species *C. jejuni*, *C. coli*, *C. lari* account for majority of the human infections and are commonly referred as thermophilic species. *C. coli* is frequently encountered in pigs, and is considered as the second most common *Campylobacter* species associated with human illness. [23]. *Campylobacter jejuni* has two subspecies, *C. jejuni* subspecies *jejuni* (which is often referred as *C. jejuni*), and *C. jejuni* subspecies *doylei*. *C. jejuni* occurs as a commensal in chicken, and is considered as an important food borne pathogen. *C. jejuni* subspecies *doylei* is considerably distinct from

C. jejuni and is not found to have any animal host [41].

1.32. Biochemical and Molecular Characteristics

The genus *Campylobacter* does not ferment or oxidize carbohydrate substrates but instead derives energy from amino acids or tricarboxylic acid cycle intermediates. They reduce nitrates and are oxidase and catalase positive. *C. jejuni* hydrolyzes both hippurate and indoxyl acetate, therefore these tests can be used for the differentiation of *C. jejuni* from *C. coli*. The organism appears as slender spiral rod under a phase contrast microscope. Sometimes two or more rods unite to form a seagull or V shape. The darting motility is one of the characteristic features of the genus *Campylobacter* [40]. When the cells age they form a coccoid shape which is described as viable but non culturable state (VBNC). However there is mixed reports about the existence and survival of the VBNC [42, 43]. Both *C. jejuni* and *C. coli* can undergo transformation and conjugation naturally, and strains containing plasmids and phages have been described [44]. Several selective media containing oxygen scavengers such as blood, ferrous iron, and pyruvate are used to culture *Campylobacter*. Selective broth such as Bolton broth, *Campylobacter* enrichment broth, and Preston broth, and selective agar such as charcoal cefoperazone deoxycholate (CCDA), Butzlers agar, Campy Cefex and modified CCDA are formulated for the routine laboratory isolation of *Campylobacter spp.* The most effective confirmation method has been some rapid techniques such as polymerase chain reaction (PCR), real time PCR, and molecular typing techniques such as random amplification of polymorphic DNA, and PFGE [45].

1.33. Growth and Survival of *Campylobacter* in Raw Broiler Meat

C. coli and *C. jejuni* are normal inhabitants of the gastrointestinal tract of poultry. During the evisceration process the broiler meat can easily be contaminated with *Campylobacter* [46, 47]. *Campylobacter* is relatively sensitive to the environment, which makes it less likely to survive outside the body of the host for long periods. The cells are sensitive to freezing, drying, standard concentrations of common disinfectants, salt concentrations above 1%, and other treatments such as ultraviolet and gamma irradiation [48]. The environmental susceptibility is mainly due to its inability to grow at temperatures below 30°C, microaerobic requirements, and its sensitivity to drying, oxygen, and low pH. Their optimum growth environment is 42°C, with pH 6.5 – 7.5. *Campylobacter* is susceptible to low pH, and is killed at pH 2.3. The D-value is less than a min at 60°C, therefore the organism does not survive if food is cooked to the appropriate temperature. Despite reported sensitivity, they are found to survive different processing conditions, and remain detectable by culture methods possibly due to their high initial numbers or due to their ability to adapt. Studies show that the organism can persist on chicken carcass even after storage at refrigeration (4°C) or freezing (-20°C) temperatures [49, 50]. Data shows that under refrigerated conditions, *Campylobacter* numbers can remain unchanged for over 7 days [51].

1.34. Transmission to Humans and Pathogenesis

In the United States, *Campylobacter spp* are estimated to cause more than 800, 000 illnesses, 8463 hospitalizations, and 76 deaths every year. [20]. Poultry is the major vehicle, and is attributed for the transmission of 72% of the estimated illnesses to humans [19]. Most

of the human illnesses occur as sporadic cases. Outbreaks are reported from the consumption of untreated water, raw milk, and contaminated chicken. The infectious dose can vary from < 1000 cells to 10^8 cells depending on the strains [52]. The polar unsheathed flagella and the corkscrew motility enhance invasion of the mucus layer and adherence to the intestinal epithelium. The motility along with the chemotaxis provides *C. jejuni* the ability to colonize the enteric cells [53]. The epithelial damage is further aggravated by the production of cytotoxins and enterotoxins. However, the strain and immune status of the individuals determines the range of toxic activity [54]. *Campylobacter* does not produce siderophores unlike *Salmonella*, but has several ferric iron acquisition systems for iron uptake [55]. They also have an oxidative stress defense and a heat shock response mechanism to deal with toxic oxygen and changes in temperature respectively. In developing countries there is high rate of asymptomatic carriers, while in developed countries the disease is manifested as acute inflammatory gastroenteritis. Symptoms often start as abdominal cramps, followed by fever, malaise, profuse diarrhea with or without mucus and blood which is self limiting lasting for approximately 5-8 days. In rare cases, *C. jejuni* has been implicated with a long term sequelae affecting the peripheral nervous system called Guillain Barre' syndrome. It is estimated that 1 in 1000 infected are at the risk of developing this autoimmune nuerological disorder [56].

1.40. CONTAMINATION OF RAW BROILER CHICKEN AT PROCESSING

Contamination by *Salmonella* and *Campylobacter* occur at three levels: i). Primary production, ii) Processing, and iii) Distribution, handling, and preparation.

At primary production, there are numerous sources of *Salmonella* and *Campylobacter* entry the commercial poultry flocks. For *Salmonella*, vertical transmission is one of the major sources of contamination. Other risk factors include contamination from the hatchery, bird to bird transmission, and contamination from the production facility environment (feed, water, insects, rodents, litters, wild birds and air) [57, 58]. For *Campylobacter*, the transovarian transmission is recently being accepted as a route of transmission by some researchers [38]. Unlike *Salmonella*, the flock is less likely to acquire *Campylobacter* from the hatchery or feed. However insects, rodents, and untreated water are reported to be more common sources. There are some basic differences in the features of intestinal carriage of *Salmonella* and *Campylobacter* by poultry. Although the preferred site for colonization of both pathogens is ceca, *Campylobacter* prefers to swim freely within the mucus in the crypts. *Salmonella* infection generally occurs at 2nd or 3rd week of grow-out. The host susceptibility to *Campylobacter* is not age related, but once infected the carriage level is relatively high, and remains colonized until processing [59].

The *Salmonella* and *Campylobacter* level of the flock upon arrival influences subsequent levels of the fully processed carcass. Birds harbor pathogens on their feather, feet, skin, and intestinal tract [11]. Contamination can occur directly from birds or indirectly via equipments and workers. For *Salmonella*, even if only 3-4% of the entering flock is found

positive it tends to survive and even reproduce in some processing areas, resulting in cross contamination from infected to uninfected carcasses. In spite of the low initial levels, the prevalence in ready to cook broiler meat at retail is reported to range from 4-61% for *Salmonella*. [60]. This emphasizes the importance of cross contamination at slaughter. *Campylobacter* status of the entering flock is generally very high (up to 100%). Although *Campylobacter* is incapable of growth outside the host, it is found to remain viable at low temperatures for a considerable time. Prevalence in ready to cook broiler meat at retail in the United States is up to 76% for *Campylobacter* [61, 62].

The processing operation can be divided into four stages based on their microbiological impacts. 1). Unloading and shackling of birds. 2). Stunning, killing, scalding and defeathering. 3). Evisceration. 4) Chilling and packaging. Unloading and shackling even if done in a least stressful manner can result in wing flapping and subsequent dispersion of dust and microbes in the air. In a study conducted by Zotolla et al, *Salmonella* was regularly isolated from air samples in this area [63]. The stunning water and the killing knife could act as a source of contact contamination from infected to uninfected carcasses [64, 65]. Microbes on the skin, feather, and fecal matter are washed into the scald water. The possibility of transferring *Salmonella* and *Campylobacter* from one batch to another in a scald tank depends on the survival time of pathogens. Survival time is influenced by the temperature of scald water, pH, and the presence of organic matter. Slavik et al [66] reported that hard scald temperatures (60°C) have greater effect in reducing both *Salmonella* and *Campylobacter* when compared to soft scald (51-52°C). The pH of the scald tank is usually 6, due to

dissociation of ammonium urate present in the feces. However lowering or raising the pH has been beneficial in reducing levels of both *Salmonella* and *Campylobacter* [67, 68]. Numbers of both pathogens increases significantly during the defeathering process. The increase is mainly attributed to the passage of gut contents through the vent that contaminates the rubber fingers of the automated feather picking machine [69]. Evisceration involves opening the abdominal cavity, and removing the viscera. It is a very critical point because breaking of the intestinal tract can lead to contamination of the internal cavity of carcass, contamination of machines, and all the subsequent carcasses. Rupture of both ceca and crop are considered as primary sources for the contamination of both *Salmonella* and *Campylobacter* [46, 70, 71]. Before the carcass is transferred to the chill tank, washing of the eviscerated carcass minimizes the introduction of organic matter and microbes in to the chill tank. Overall, bird washers are found to reduce pathogens [72, 73]. Immersion chilling is accomplished by carcass immersion in cold water or ice water mix. The benefits of immersion chilling include efficient heat transfer, maintenance of the appearance of product, and reduction in bacterial numbers. Several studies report a decrease in microbial load [72-76]. However the problem with immersion chilling is to prevent the build-up of microbes which could be a source of cross contamination [76]. Alternatives to immersion chilling are dry air or evaporative spray chilling, cryogenic chilling, or controlled continuous immersion chilling systems. According to a study conducted by Huezo et al, chilling methods did not have an effect on the prevalence of *Salmonella* and *Campylobacter* recovered from carcasses [77]. Cross contamination can also occur during product handling and contact with

contaminated surfaces [78]. Dookeran et al reported a *Salmonella* contamination of 2.49% at broiler production, an increase to 3.95% during transportation to the processing plant, further increasing to 51.32% during processing and 77.14% at retail. Thus cross contamination during processing and handling is an important issue in poultry processing [79]

1.50. POST-HARVEST CONTROL OF *SALMONELLA* AND *CAMPYLOBACTER* ON RAW BROILER CHICKEN

1.51. Introduction

Many stages are involved in the production and processing of fresh poultry products. Hence assurance of microbiological safety is complex and requires interventions throughout the farm-to-table continuum [80]. Interventions at processing should reduce the existing contamination, prevent cross contamination, and minimize introduction of additional contaminants by sanitation interventions, processing treatments, or antimicrobial procedures [81].

Good sanitation is not only necessary to improve the shelf life of the product, but also to maintain the company's reputation and meet legal requirements. The type of equipment used for processing should have some basic hygienic design principals. The material used for equipment making should have inert and easily cleanable surfaces, and should be readily accessible for cleaning. The method used for cleaning is based on the type and characteristic of the soil found in the plant. Since meat contains protein, fat, and moisture alkaline solutions such as 1.5% sodium hydroxide, alkaline phosphates, enzymes and synthetic detergents are generally used. [82]. The steps involved in sanitation includes manual removal of heavy soil,

rinsing with water (temperature below 55°C), washing with an alkaline solution, rinsing with clean water, acid washing to neutralize the alkali, sanitizing with a chlorine solution, iodine, or quaternary ammonium compounds, and the final rinse step. Another system of cleaning is Clean-in-place (CIP) which is a closed system of cleaning using heavy duty detergents [83]

Antimicrobials are applied at various stages of processing to control foodborne pathogens and spoilage microorganisms. Generally, antimicrobial application at pre-chill inside-outside bird washers (IOBW), immersion chill step, and post-chill spray or dip applications result in microbial reduction. However the results may or may not be consistent for reduced *Salmonella* and *Campylobacter* numbers [47, 84, 85].

1.52. Mechanism of Microbial Attachment to Poultry Carcasses

Understanding bacterial attachment is very important to develop interventions to control pathogens. Attachment of bacteria to the carcass takes place in two steps. An initial reversible phase when the bacteria is trapped within the water in contact with the meat, and the second irreversible phase when the bacteria forms a physical attachment involving extracellular polysaccharides. [86]. Generally, the bacterial attachment occurs from specific interactions between cell surface receptors and ligands on carcasses. The interaction is dependent on various intrinsic (type of bacteria, presence and activity of flagella) and extrinsic (temperature, pH, contact time, presence of organic matter, type of environment or liquid medium) factors. The optimum conditions for bacterial attachments were found to be 21°C and pH 8.3 to 8.4. Contact time and bacterial attachment are directly proportional to the concentration of bacteria [87].

1.53. Regulations and Prevention Programs

According to a recent estimate by Scallan et al [20], one in six Americans become sick every year from foodborne illnesses. These illnesses occur as a result of unintentional contamination of food by biological hazards. Keeping food safe is very challenging as the disease causing agent can enter the food chain at any point in the farm-to-fork continuum. Several government agencies share the responsibilities to minimize the associated risk, and ensure the quality and safety of food [81].

Monitoring Agencies. Two agencies that work together to prevent the occurrence of foodborne illness in the United States are the United States Department of Agriculture and the Food and Drug Administration (FDA).

United States Department of Agriculture

USDA is composed of 17 agencies, 15 offices, and 7 mission areas. The agencies associated with monitoring poultry products safety and qualities are Agricultural Marketing Service (AMS), Animal and Plant Health Inspection Service (APHIS), and Food Safety and Inspection Service (FSIS) [33]. Agricultural Marketing Service facilitates efficient and fair marketing of agricultural products. It ensures the quality and availability of wholesome food across the country. AMS also provides voluntary tools such as grading, certification, auditing, inspection, and laboratory analysis at a cost [88]. Animal and Plant Health Inspection Service works to ensure the health of plants and animals, regulates genetically engineered organisms, administers the Animal Welfare Act and carries out wildlife damage management activities [89].

Food Safety Inspection Service inspects meat, poultry, and egg products for safety and proper packaging. The services by the agency include ante mortem inspection (from arrival to live hang), post mortem inspection, and post-chill microbiological testing for *E.coli* numbers, and incidence of *Salmonella* and *Campylobacter* [81, 90].

Food and Drug Administration (FDA)

FDA is responsible for regulating labeling and safety of all food products (except meat and poultry). In December 2010, the Food Safety Modernisation (FSMA) act was signed into a law. FSMA will give FDA more enforcement tools, and one new aspect is the risk based inspection of food processing facilities [91, 33].

Policies and Microbial Performance Standards

Effective in May 1997 a zero tolerance policy for visible fecal contamination was implemented [93]. Post chill carcasses are tested for numbers of generic *E. coli*, and incidence of *Salmonella* and *Campylobacter*. For young chickens, 1 carcass in 22,000 are sampled for *E.coli* by whole carcass rinse procedure. For turkeys, 1 carcass in 3000 is sampled for *E. coli* by whole carcass rinse or sponge procedure. No single result with more than 1000 cfu/ml, and no more than 3 results having >100 cfu/ml out of 13 consecutive test will be considered a pass [94].

Effective in July 2011, *Salmonella* prevalence should be no more than five positive samples in the 51 sample set for young chickens, and no more than four positives in the 56 sample set for turkeys for an establishment to pass. For *Campylobacter*, prevalence should be

no more than 8 positive samples in the 51 sample set for young chickens, and no more than four positive samples in a 56 sample set for turkeys [95].

Healthy People 2020

The US Department of Health and Human Services launched Healthy People 2020 in December 2010. This sets the nations health objectives for health promotion and disease prevention.

Food Safety Objectives of Healthy People 2020 for *Salmonella* and *Campylobacter*

Salmonella: Baseline is 15 cases per 100,000 populations per year, and the target is 11.4 cases per 100,000.

Campylobacter: Baseline is 12.7 cases per 100,000 populations per year, and the target is 8.5 cases per 100,000.

For number of outbreaks associated infections per year due to pathogens (Shiga toxin producing *E. coli*, or *Campylobacter*, *Listeria*, or *Salmonella*) associated with poultry, baseline is 258 cases per year, and the target is 232 cases per year [96].

FoodNet

FoodNet is an active surveillance network that tracks trends for infections transmitted through food, and attributes illness to specific food and settings. It is a collaborative program among Center for Disease Control (CDC), 10 state health departments, USDA-FSIS, and FDA. The pathogens that are currently tested are *Campylobacter*, *Cylospora*, *Listeria*, *Salmonella*, *Shiga toxin producing E. coli* (STEC) O157H7, *Shigella*, *Vibrio*, and *Yersinia* confirmed by laboratory testing of samples from patients. [97].

According to the FoodNet trends monitored during 1996 to 2012, *Campylobacter* and *Vibrio* incidence has increased in 2012 when compared to the 2006-2008 data, whereas incidence of all other pathogens remained unchanged [98].

1.54. Chemical Antimicrobials

Chlorine and Chlorine dioxide

The use of chlorine (Cl_2) in poultry processing was first suggested by Gorgeline et al in 1951 [99]. It is considered as one of the cheapest and most widely used antimicrobial compounds to reduce contamination in poultry processing [100]. Several researchers have proven the bactericidal ability of Cl_2 and chlorine dioxide (ClO_2) at a concentration less than 50 ppm. [100, 101, 102, 103]. In a study conducted by Lillard [101], when water was treated with 20 and 34 ppm Cl_2 , fecal coliforms and total aerobic plate counts were significantly reduced. Prevalence of *Salmonella* was also significantly reduced from 14.3% in untreated to 1.9% in treated broiler carcasses. However 17% of the water sample itself tested positive for *Salmonella*. Others researchers indicate that very high concentration ($>100\text{ppm}$) of Cl_2 would be required to have a significant effect on microbial reduction and improving shelf life [104, 105]. In a study conducted by Northcutt et al it was shown that spray washing broiler carcasses with chlorinated water (0 or 50 ppm) at different temperatures (21.1, 43.3, 54.4C), adding Cl_2 or/ and elevating water temperatures did not have an effect on reducing *Campylobacter* and nalidixic acid resistant *Salmonella*. [106]. Free Cl_2 forms hypochlorous acid which is the antimicrobial agent. The main drawback with the use of Cl_2 is that presence of organic compounds or a high pH which limits the availability of free Cl_2 , thereby reducing

its bactericidal effect. The advantage of using ClO_2 is that it is odorless, does not react with nitrogenous compounds, and is more soluble in water than chlorine [101]. There are mixed results on the antimicrobial activity of chlorinated water when used in various steps in processing, and many researchers believe that chlorine is relatively ineffective against *Salmonella* [106, 107].

Trisodium phosphate

In October, 1992, trisodium phosphate (TSP) was approved by the USDA for use in broiler slaughter processing operations [108]. TSP (8-12%) solution is used for dipping or spraying carcasses with a contact time of 15s and temperature of application of 20-30°C. The bactericidal effect of TSP is primarily attributed to its high pH (12), ionic strength (causes autolysis), and ability to remove thin layers of lipids (detergent effect) from the carcass surfaces [109]. In a study conducted by Kim et al [110] the incidence of *Salmonella* was lower (52 and 64% in 15s and one-day) for 10% TSP treated carcass when compared to (96 and 88%) untreated carcasses. *Campylobacter* numbers on carcasses were decreased by 1.5 log and 1.2 log in 1 and 6 day stored 10% TSP treated (as post chill dip) carcasses stored at 4°C, however storage at 10°C only reduced *Campylobacter* levels by 0.16 log [111]. The advantage in the use of TSP is that, the residual hydroxyl radicals in the treated meat suppresses further growth of microbes, when most other chemical decontaminants dissociate and do not have any effect in preventing further contamination. However the disadvantage is the release of large amount of phosphates that have disposal and water treatment issues [112].

Cetylpyridinium Chloride

In April 2004, FDA approved the use of cetylpyridinium chloride (CPC) as an antimicrobial agent to treat the surface of raw meat. The concentration of CPC use should not exceed 0.3g/lb of raw poultry carcass. CPC is a cationic surfactant, which forms weakly ionized compounds that inhibits bacterial metabolism. In a study conducted by Kim et al [113], 0.1% CPC solution sprayed on chicken skin surface for 1 min reduced the number of *Salmonella* by 0.9 -1.7 log units. Arritt et al noticed a *Campylobacter* reduction of 2.89 and 1.42 log cfu/ skin using 0.5% and 0.1% CPC. Although CPC is required in smaller quantity, 0.1% CPC was found to be less effective than 10% TSP [114].

Acidified Sodium Chlorite

In January 1999, USDA approved the use of acidified sodium chlorite (ASC) for antimicrobial treatment in poultry. ASC is used at a concentration of 50-150 ppm in the wash solution [115]. Kere et al [116] compared continuous online spray system using ASC versus standard offline reprocessing and determined that continuous system reduced the incidence of *Salmonella* to 10% versus 31.6% for offline reprocessing, and *Campylobacter* to 49.1% compared to 73.2% for offline reprocessing.

Hydrogen Peroxide

The bactericidal action of hydrogen peroxide (HP) is due to the formation of free radicals that damage nucleic acid, proteins and lipids. Mulder et al [117] compared the efficacy of lactic acid (1%), HP (0.5%), and L-cysteine in inhibiting *S. Typhimurium* and identified that lactic acid and HP treatments resulted in a reduction of 4 logs, whereas L-cysteine did

not have any bactericidal effect. Zhao et al [118] reported a 2 and 4 log reduction in *Campylobacter* on chicken wings when treated with 0.1% and 0.2% HP for 2 min. However the disadvantage of HP is that it is reported to shown temporary bleaching of carcasses due its strong oxidizing capacity [117, 119].

Organic Acids

Organic acids such as acetic, lactic, and citric acids are used for the decontamination of poultry carcasses and parts. Acetic acid yields 0.8 -2.0 log a reduction of *Salmonella* [120]. Jimenez et al [121] observed a 99% reduction in the initial load of *S. Hadar* on poultry skin using a double sequential decontamination with acetic acid for 30s. Zhao and Doyle [118] reported that 2% acetic acid showed a 5 log reduction in *C. jejuni* within 2 min, however 1% lactic acid did not substantially reduce *Campylobacter* population.

1.55. Natural Antimicrobials.

Currently, the chlorine based substances, TSP, ASC, and organic acids are widely used for decontamination of poultry carcasses and parts. Based on the studies evaluated in this review chemical antimicrobials have yielded results varying from 0.9 to 4 log reductions of *Salmonella* and *Campylobacter* depending on the method of application, contact time, and temperature. The negative aspect of chlorine based substances is the presence of organic matter that hinders its functionality. The banning of meat rinsed with chlorine by the European Union due to health concerns also limits its use. Several of the chemicals that are being used have corrosive properties, have shown sensory changes, or have a bleaching effect on meat [119]. The use of other chemical decontaminants such as TSP is limited due to

environmental concerns. However, one of the important concerns is that chemical antimicrobials generally have shown a lower log reduction in bacterial cells rendering the surviving cells resistant to various stressors. Therefore scientists are looking for natural means to reduce microbial load, prevent further cross contamination, and lessen the percentage of resistance strains. The use of natural antimicrobials also has a positive image among general public [112].

Several plant derived compounds such as spices, herbs, fruit extracts, vegetable extracts, and essential oils have been evaluated for their potential effect on reducing pathogens, spoilage organisms, and improving shelf life [122-126]. In general, the antibacterial, antifungal, and antioxidant property is due to presence of phenolic compounds containing active hydroxyl groups [127]. An in depth *in-vitro* investigation on the antimicrobial effect of plant derived compounds such as basil, bay leaves, caraway, cardamom, celery seeds, cinnamon, cloves, coriander, cumin, dill, fennel, garlic, ginger, lemon, lemon grass, lime, marjoram, mint, nutmeg, onion, parsley, pepper, rosemary, sage, thyme, and oregano on *Salmonella* and *Campylobacter* has shown very promising results [127-129].

1.60. ESSENTIAL OILS

1.61. Introduction

The term essential oils (EO), derived from ‘Quinta essential’, was first used in the 16th century to indicate the active component of a drug by Paracelsus von Hohenheim [130].

EO are volatile, complex mixtures produced by aromatic plants as secondary metabolites. They are generally colorless, have high refractive index, soluble in organic solvents, insoluble or have limited solubility in water, and are steam distillable. The volatile nature of EO at temperatures between 50 and 320°C gives them a characteristic odor, and makes them distinct from mineral or fatty oils [131]. They are generally obtained by distillation or pressing whole plants or specific parts such as flowers, buds, seeds, leaves, twigs, bark, herbs, wood, fruits, and roots [132]. EO function as allelopathic agents, wound healing agents, thermo tolerant agents and repellants or attractants of insects, pathogens, other plants and herbivores [133].

The human use of the harvested EO primarily consisted of perfumes, flavoring, and cosmetics. The first known use as antibacterial agents was by the ancient Egyptians who used aromatic plants in embalming to stop bacterial growth and prevent decay. EO are now known to possess antibacterial, antiviral, antifungal, antioxidant, insecticidal, and anticancer properties. [130, 131].

Most oils are made up of 20-60 compounds from a variety of chemical classes, predominantly terpenoids, phenylpropanoids, and other less important ingredients. The monoterpenes which could be an alcohol, aldehyde, ketone, or ether constitutes 90% of the oil, and is described as major component. The aromatic phenylpropane groups and products of terpene degradation form the minor components of the oil [131].

When consumed by humans most terpenoids are metabolized in the liver by hydroxylation or glucuronidation, and later eliminated by kidneys. Some terpenoids such as menthol and cineole are found to remain unchanged and they are eliminated through the respiratory tract or skin. Since essential oils are very lipophilic they tend to concentrate in the adipose tissue. A few studies have looked at the plasma concentrations and reported a value of 5-20 μ M. The toxicity by oral intake is very low with an LD₅₀ of 2-5g/kg [132, 133]. During storage of EO several reactions such as photoisomerization, photocyclization, generation of peroxides and free acids, thermoisomerization, transition-metal catalyzed decay initiates the decomposition of EO. Therefore the properties of EO are gradually lost during storage. Hence EO must be stored away from light and heat. The age of the EO are determined by iodine titer, peroxide determination, and chemiluminescence methods [131].

1.62. Antimicrobial Properties of Essential Oils

One of the first known studies on the antibacterial activity of essential oils was reported by Chamberland in 1887 in which the antibacterial potential of cinnamon oil to inhibit anthrax spores was demonstrated [122]. Later the antibacterial activity of various essential oils against several genera of pathogenic and spoilage bacteria were intensively

investigated [126, 127, 129, 134-136]. Deans and Richie [127] investigated the antimicrobial activity of 50 plant essential oils against 25 genera of bacteria and found varying levels of inhibition among oils and between strains of bacteria. Some EO exhibited antibacterial activity against foodborne pathogens [135, 137, 138]. Kim et al studied the antibacterial effect of 11 essential oils against 5 foodborne pathogens and identified that

Carvacrol was highly bactericidal against *S. Typhimurium*, and 500 µg of citral, geraniol, and perillaldehyde completely killed *S. Typhimurium* in laboratory media [135]. Smith-Palmer [129] reported that of the 21 essential oils tested against 5 foodborne pathogens the oils of bay, cinnamon, clove, and thyme had the highest activity against *C. jejuni*, *S. Enteritidis*, *E. coli*, *Staphylococcus aureus* and *Listeria monocytogenes*. Generally foodborne pathogens are found to be more sensitive to a group of essential oils that have higher percentage of phenolic compounds such as thymol, carvacrol, and eugenol [139, 140]. Often the antimicrobial activity of the oil is due to the complex interaction between different compounds present in the oil, although its bioactivity would be closely related to the main component. The large variety of active compounds prevents microbes from developing resistance [127].

Some studies investigating the antimicrobial activity of gram positive and gram negative bacteria suggest that EO are more effective on gram positives than gram negatives [129, 139, 141]. The reason for the resistance is suggested that gram negatives possess an outer membrane made of lipopolysaccharide that restricts the diffusion of hydrophobic oils. However, other researchers have found gram negatives to be more sensitive. In a study conducted by Tassou et al [142], *Salmonella* (gram negative) was found to be more sensitive to mint essential oil than *Listeria* (gram positive). Yet another group of scientists does not believe in any difference in sensitivity due to gram reactions [127].

In-vitro work with essential oils and their components showed positive results, however higher concentration was required in food systems. Antimicrobial activity of rosemary extract in chicken meat juice was 4 times the level required in laboratory medium

[143]. The essential oil levels tested on food models in various studies have been between 1-3% in order to retain the organoleptic properties and sensory attributes of the food tested. Very few studies have looked at the effectiveness of essential oils to inhibit *Salmonella* and *Campylobacter* on raw poultry meat. Dickens and Ingram [144] evaluated the effectiveness of an herbal extract (0.5%Protecta II) on a NaCl carrier to reduce total aerobes, coliforms, *Campylobacter*, and generic *E. coli* on broiler carcasses subjected to a simulated chill for 30m at 1°C. All pathogens tested were reduced to a level below detection limit. In a study conducted to evaluate the antimicrobial effect of 3 fruit extracts lime, plum, and sour orange peel against *C. jejuni* and *C. coli* on poultry skin incubated for 48 hours at 4°C resulted in > 4 log reduction of *Campylobacter*. The flavor of a mixture of lime and plum treated chicken wings was ranked best by the sensory panelist. [125]. Another study tested the antimicrobial activity of coriander oil against *C. jejuni* on ground chicken meat incubated at 4°C and 32°C for 3 h. At 4°C, the control chicken meat which was not treated with any oil had a 5 log cfu of *C. jejuni*. A treatment with 0.5% oil v/w completely inhibited *C. jejuni*, on chicken meat whereas 0.1% and 0.25% v/w of oil reduced *C. jejuni* by 3 and 1 log respectively [144]. A *Campylobacter* reduction of 3 logs with a synergistic effect of pre-freezing with rosemary extract treatment of chicken meat model was reported by Piskernik et al [143].

1.63. Chemistry and Bioactivity Thyme, Rosemary, Clove, and Orange Essential Oils

Thyme Oil

Thyme oil is obtained from a perennial herb '*Thymus vulgaris*' which is native to southern Europe. The dried plant material contains at least 1 to 2.5% of EO which is typically

extracted from the leaves of thyme. The major component of thyme oil is thymol, which constitutes 30-55% of the oil. It could also contain other monoterpenes such as carvacrol (1-5%), and precursors of thyme such as *p*-cymene (15-20%) and γ -terpinene (5-10%). All the major and minor terpenes in thyme oil are listed in Figure 2 [146].

The antibacterial activity of thyme oil is mainly attributed to thymol and carvacrol. Both these compounds have similar structure, differing only in regards to the position of the hydroxyl groups on the phenolic ring. These compounds appear to interact with the bacterial cell membrane by hydrogen bonding, dissolve the phospholipids bilayer, altering membrane permeability and resulting in the leakage of cellular contents and cell death. [139]. Thymol is found to be more undissociated at a pH of 5.5 than 6.5, which makes them more hydrophobic, hence attach better to the hydrophobic areas of bacterial cell membrane [147]. Dean and Ritche [127] and Smith-Palmer reported thyme oil as one of the most inhibitory oils tested. Thyme exhibited a strong antibacterial activity against *Salmonella* showing a zone of inhibition of 11-26 mm and minimum inhibitory concentration of 0.04%. [127, 129]. Burt et al [148] reported that 20% carvacrol vapors significantly reduced *S. Enteritidis* numbers on chicken meat, and 40% carvacrol vapors completely eliminated all cells within 3h at 37°C. In a study conducted by Yu et al [149], thymol when applied as a wash on grape tomatoes resulted in a >4.6 log reduction of *S. enterica* population. *Campylobacter* is also found to be sensitive to thyme oil. Friedman [150] reported a bactericidal activity of 0.02% of thyme oil against *Campylobacter* under *in-vitro* conditions. Goswami [151] reported that chicken meat coated with thyme oil completely eliminated *Campylobacter* and reduced

Salmonella by 2 logs during storage at 4°C for 12 days.

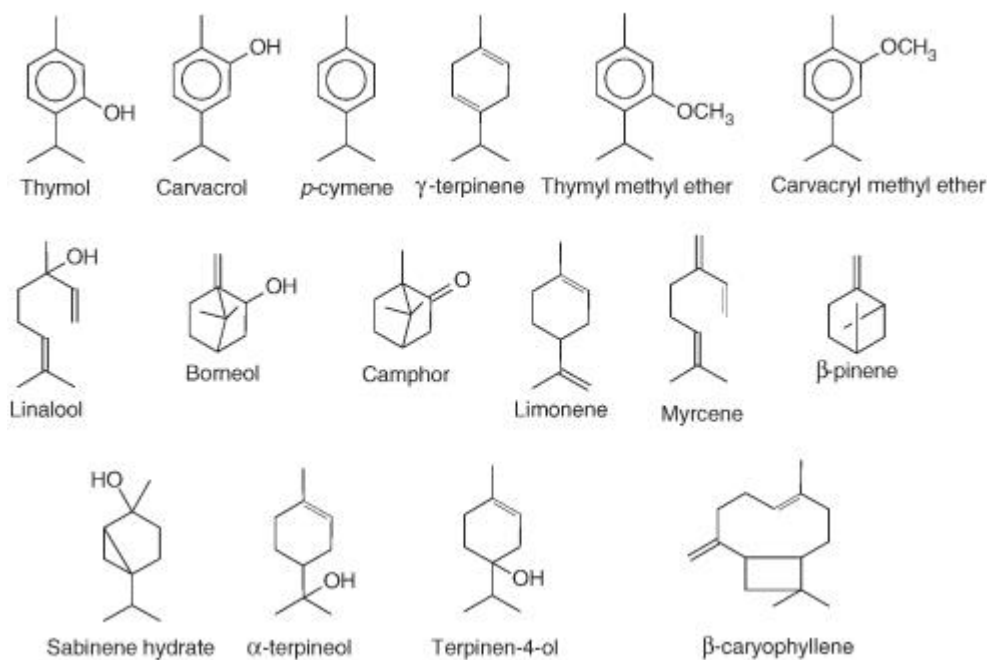


Figure2: Terpenes in the essential oil of thyme¹

¹Adapted from Peter, K. 2004. Handbook of Herbs and Spices: Volume 2. Woodhead publishing.

Rosemary Oil

Rosemary oil is obtained from an herb '*Rosmarinus officinalis*' which is native of Mediterranean regions. The oil may be extracted from the whole plant or only from the leaves and flowering tops by steam or water distillation, and it constitutes 1-3% of the dried plant. The oil consists of 1,8-cineole (30-40%), α -pinene (25%), camphor (15-25%), borneol

(16-2%), bornyl acetate (7%) [146]. Friedman [150] reported that rosemary oil had a significant antibacterial activity against both *Salmonella* and *Campylobacter* at a concentration of 0.45 and 0.06% respectively.

Clove Oil

Clove oil is obtained from the buds of '*Syzygium aromaticum*'. It is traditionally used in dental care as an antiseptic and analgesic agent. The major components of clove oil are eugenol (75%-85%), eugenol acetate (8-15%) and small amounts of β -caryophyllene. Eugenol is the antibacterial component of clove oil [152]. Devi [153] reported that eugenol acts on *S. Typhi* by disrupting its cell membrane. Clove oil was found to be effective against both *Salmonella* [150, 154] and *Campylobacter* [154, 155].

Orange Oil

Orange oil is extracted from the peel of oranges from '*Citrus sinensis*', and is one of the important byproducts of orange processing. They are used as flavoring ingredients in citrus products. Orange oil is composed mostly of d- limonene (> 90%), which is the antibacterial component of the oil. The typical volatile compounds present in sweet orange oil are listed in Table 2. Mechanism of actions of orange oil has not been thoroughly researched, but may be similar to other EO. Morphological changes to the *Enterococcus faecalis* was noticed when exposed to citrus oil vapors [156]

Dabbah et al [157] reported that *S. Senftenberg* was reduced to by 93% when 1000 μ l of orange essential oil was added to one liter of agar medium. Nazer et al [158], reported a 10% reduction of *Salmonella* with 0.5% citrus oil, and 100% reduction with 3.5% oil. Seven

citrus oils tested against 11 serotypes of *Salmonella* produced an MIC ranging from 0.125 to 0.5%. [159]. *Campylobacter* was significantly reduced by orange oil fractions both on agar media and chicken skin samples [160, 161].

Table 2: The typical volatile compounds present in sweet orange oil¹.

Volatile compounds	Sweet orange (%)
Methanol	0.37
Isopropanol	0.91
α -Pinene	6.37
Butylacetate	0.00
3-Heptanone	0.00
Limonene	88.21
Ocimene	0.00
Nonanol	0.00
Linalool	0.02
α -Terpineol	0.71
Valencene	0.00
α -Terpinene	0.00
β -Ionone	0.00
β -Pinene	0.00
Terpinen-4-ol	0.00
p-Cymene	2.37
Citral	3.00

¹Fisher, K., and C. Phillips. 2008. Potential antimicrobial uses of essential oils in food: is citrus the answer? Trends Food Sci. Technol. 19:156-164.

1.64. Synergism between Essential oil Components

Essential oils are complex mixtures of numerous molecules. Therefore, the antibacterial activity of the essential oil is the net result of the activity of the major component, and its interaction with the minor components [132]. These interactions could

lead to modulations in the activity of the major component which could be additive [139], synergistic [162], or even antagonistic [163]. The synergistic effect of the major components of thyme oil, thymol and carvacrol with the other EO components such as eugenol, cymene, linalool, and cinnamaldehyde has been reported [164-167]. The minor components favors the transport of the major components into the cell by altering the hydrophobicity, assisting attachment on cell walls and membranes, or assisting in cellular distribution thereby creating a synergistic effect. Since the minor components also play a critical role in the antibacterial activity of the oil, it is more meaningful to use the whole oil instead of the individual components [133].

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CHAPTER 2

A mix of thyme orange oils inhibits *Salmonella* and *Campylobacter* invitro

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2.10. SUMMARY

The demand for foods that are free of pathogens and chemical residues has increased interest in the use of plant-based products as natural antimicrobials. Essential oils (EOs) from plants are natural compounds that have been shown to have antimicrobial properties against food borne pathogens. The objective of this study was to determine the effect and concentration of four selected EOs to inhibit *Salmonella enterica* (three nalidixic acid-resistant strains, each of a different serovar, and a mix of all three strains) and *Campylobacter* (two strains of *Campylobacter jejuni*, one strain of *C. coli* and a mix of all three). The disc diffusion method (DDM) was used to screen the oils of thyme, orange, rosemary, and clove oil. The MIC (minimum inhibitory concentration) or MBC (minimum bactericidal concentration) of the EOs were tested using a two fold broth dilution method at concentrations ranging from 1.000 to 0.008% (v/v). Two independent experiments were performed. A zone of inhibition (ZI) was expressed in mm, and concentrations expressed as percentage. All the oils demonstrated antibacterial activity against the strains tested. However, thyme oil demonstrated the strongest inhibitory activity against *Salmonella* (ZI of 18.5 mm). In general *Campylobacter* was more susceptible, with the plates containing thyme or clove oil showing no growth. Orange oil was also highly effective on *Campylobacter*, with a mean ZI of 17.5mm. The least costly treatment effective against both *Salmonella* and *Campylobacter* involved thyme and orange oil combination (TOC). These two EOs were combined on a 50:50 proportion and their effects were tested on the same strains of bacteria. TOC had a mean ZI of 20.5mm for *Salmonella* and 21.3mm for *Campylobacter*. TOC

demonstrated a synergetic effect against *Salmonella*, but no such effect was noticed for *Campylobacter*. On an average 0.14% TOC was required to inhibit both pathogens. Hence, TOC can be considered as a potential antimicrobial for future studies on food systems.

2.20. DESCRIPTION OF PROBLEM

Salmonella and *Campylobacter* continue to be leading causes of human foodborne illness worldwide. In the US alone, an estimated one million cases of *Salmonella* and 800,000 cases of *Campylobacter* are reported annually [1]. Contaminated poultry and poultry products are major vehicles for the transmission of both pathogens to humans [2, 3]. Prevalence in broiler meat at retail in the United States ranges from 4-61% for *Salmonella* and up to 76% for *Campylobacter* [4-6]. Post-harvest pathogen reduction is achieved primarily using chlorinated water or spraying with broad spectrum antimicrobial agents such as peroxyacetic acid, trisodium phosphate, acidified calcium sulfate, organic acids and cetyl pyridinium chloride [7]. To comply with recent food safety enhancement efforts and stricter performance standards, processors have increased the rinse water volume or the use of chemicals such as those listed above. However, organic producers have restrictions in the use of chemical interventions [8]. With the increasing demands for foods that are free of pathogens and chemical residues, plant-based natural antimicrobials are becoming popular. Plant essential oils (EOs) are naturally-derived compounds known historically for their antiseptic properties. However, their use in foods is primarily as flavor additives in soft drinks and sweets, and more recently as preservatives [9-12]. These oils are classified as

Generally Recognized As Safe (GRAS) food additives in the US [9]. The acute toxicity by oral intake is generally very low. The major components and LD₅₀'s of thyme and orange oils have been listed in Table 3. Generally the concentration in food is kept low in order to minimize any sensory or flavor changes [10].

EOs are volatile, complex mixtures produced by aromatic plants as secondary metabolites. Most oils are made up of 20 – 60 compounds from a variety of chemical classes, predominantly terpenes and their derivatives. Often the antimicrobial activity of the oil is due to the complex interactions among these compounds, although the bioactivity of the oil would be closely related to the main component. The large variety of active compounds prevents microbes from developing resistance [11]. Previous reports of *in vitro* studies show that thyme, orange, rosemary, and clove oils were effective either against *Salmonella* or *Campylobacter* [12, 13, 14, 15]. Therefore, these four oils were selected to study the effect of various concentrations on inhibition of *Salmonella* and *Campylobacter*. The purpose of the study was to identify an inexpensive combination of EOs which can be safely used in poultry food systems to reduce pathogens.

2.30. MATERIALS AND METHODS

Test Compounds

Thyme oil, clove oil, rosemary oil [16] and orange oil [17] were obtained in certified food grade form. TOC was a blend of thyme and orange oil at 50:50 ratios. Dimethyl sulfoxide [18] was added to increase the solubility of the EOs in aqueous media.

Test Bacteria

Salmonella. *S. Enteritidis*, *S. Montevideo*, *S. Heidelberg* stock cultures were grown on nutrient agar slants [19]. Test strains were transferred to Brain Heart Infusion medium (BHI) [19] before use. Each working culture was individually inoculated to fresh BHI and incubated at 37°C for 12 hrs to obtain 10⁸ cfu/ml. Equal volumes of individual cultures were mixed to obtain mixtures of the three strains.

Campylobacter. Field isolates *C. jejuni* (11601MD, from the intestine of a turkey), *C. jejuni* (RM1221, from chicken skin) and *C. coli* (RM2228, a multi-drug resistant isolate from chicken carcass) was used. The cultures were preserved at -80C in brain heart infusion medium with 20 % glycerol. Test strains were transferred to Muller Hinton agar (MHA) [19] plates before use. The test organisms were further subcultured in Muller Hinton broth (MHB) and incubated at 42°C for 24 hrs under microaerobic conditions to obtain 10⁷ cfu/ml. The individual strains were combined in equal volumes to obtain strain mixture.

Disc diffusion method

Disc diffusion is a method to initially screen chemicals to determine antimicrobial activity. The zone of inhibition indicates the relative antibacterial activity [9]. The agar media plates (Brilliant Green Agar with sulfadiazine [20] for *Salmonella* and CCDA [19] for *Campylobacter*) were swabbed with the bacterial suspensions (10⁷-10⁸ cfu/mL). Sterile filter paper discs (6.0 mm in diameter) were soaked with 10 µL individual EO or TOC (5µl thyme oil and 5µl orange oil) and placed on the surface of inoculated agar plates. All plates were left at room temperature for 30 minutes to allow diffusion of oil before inverting the plates

for incubation. *Salmonella* plates were incubated at 37°C for 24 h, and *Campylobacter* plates under microaerobic conditions at 42°C for 48 hours. Control plates lacked exposure to any EOs. Two independent experiments were performed and zones of inhibition (ZI, mm including the 6mm of the disk) were expressed as mean values.

Macro broth dilution technique

Minimum inhibitory concentration (MIC) or minimum bactericidal concentration (MBC) of thyme, orange, clove, rosemary and TOC was determined using a macro broth dilution technique. Serial 2-fold dilutions of the EOs were made in tubes using BHI broth for *Salmonella*, and MHB for *Campylobacter*, in a total volume of 10 ml DMSO was added to increase the solubility of the EOs. The concentration of the oils in the test tubes ranged from 1.000 to 0.008% (v/v). Overnight broth cultures (0.5 ml of 10^7 - 10^8 cfu/ml) of *Salmonella* or *Campylobacter* were then added to the tubes. Positive controls contained no EO or DMSO. Negative controls were tubes containing DMSO but no EO. The *Salmonella* tubes were incubated at 37°C for 24h, and *Campylobacter* tubes at 42°C under microaerobic conditions for 48h. Turbidity was visually determined for tubes containing *Salmonella*, and the least concentration of the oil at which there was no visible growth was recorded as the MIC. The turbidity of orange oil impaired visual determination of MIC and therefore the MBC was determined by plating 100 µL from each dilution on to BGA plates. MBC was defined as the lowest concentration at which there was no growth in the plates. MBC was determined for all oils tested for inhibition of *Campylobacter*. This is because the turbidity that develops from *Campylobacter* growth in broth is not sufficient for determination of growth. Results are

reported as mean values from two replicate trials, with each dilution tested

Statistical Analysis

ZI are expressed in mm. Differences in ZI between different treatments were tested by one-way ANOVA using the General Linear Models procedure of SAS [21]. Statements of statistical significance were based upon $P < 0.05$. Tukeys test was applied for means separation Data are presented as means \pm SEM.

2.40. RESULTS AND DISCUSSION

The results from the present study showed that the essential oils had varying degrees of growth inhibition against the microorganisms tested. The disc diffusion results for *Salmonella* are summarized in Table 4. The oils of thyme, clove, and rosemary had zones of inhibition against all strains of *Salmonella* at a concentration of 10 μ l/disc. Thyme oil had the widest ZI ($p < 0.05$) at an average of 18.5 mm, followed by clove (13.8) and rosemary (7.8). Similar properties have been observed by other researchers. Palmer-Smith [16] recorded 11.1mm for thyme, 11.1 for clove, and 9.3 for rosemary oil against *S. Enteritidis*, while Dean and Ritchie [11] recorded a ZI of 26 mm for thyme, 16 for clove, and 5 for rosemary oil against *S. Pullorum*. Orange oil did not have any effect on *S. Heidelberg* and the mixture, and exhibited the lowest antibacterial effect on *S. Montevideo* and *S. Enteritidis* with a mean ZI of 7 mm. Low activity of orange oil on *Salmonella* has also been reported by others [11, 22].

The cost of thyme oil is 60 cents/ml whereas orange oil is available for only 12 cents/

ml. Therefore, these EOs were combined to arrive at a least cost treatment. In the present study, an interesting observation was made when the combined activity of thyme and orange oils were tested against *Salmonella*. TOC produced an average ZI of 20.5 mm which was greater than the effects of individual thyme and orange oils, indicating a synergistic effect.

Usually combinations of essential oils or their purified major components targets multiple biochemical processes in the bacteria leading to a synergistic, additive or sometimes even antagonistic effects. The possible mechanism of action of this oil blend is due to interactions between the phenolic compounds from thyme oil and the alkyl groups from the orange oil. Phenolic compounds are hydrophobic and antimicrobial. The major phenolic compounds of thyme oil are carvacrol [2-methyl- 5-(1-methylethyl) phenol] and thymol (2-isopropyl-5-methylphenol). The hydrophobicity of the phenolics enables them to attach to the lipid bi-layer of the cytoplasmic membrane leading to the leakage of ions and other critical molecules, resulting in death of the cell [23, 24, 25]. The major component of orange oil is limonene (1-methyl-4-(1-methylethenyl)-cyclohexene) a type of alkyl group which is more active than other forms such as p-cymene. Alkyl substitution of phenolic compounds renders an increased antimicrobial activity [26].

Although the disc diffusion study was done for the initial screening of EOs, one limitation concerned the difference in solubility of the different oils in the agar media. Hence the macro broth dilution method was used to confirm the findings of the disc diffusion assay and to determine the inhibitory concentrations. EOs are not soluble in water and it was therefore necessary to add DMSO as an emulsifier.

The negative controls with DMSO only showed no antibacterial activity. The MIC and MBC values for *Salmonella* are listed in Tables 6 and 7. The average MIC values for the oils of thyme, clove, and rosemary were 0.06%, 0.11%, and 0.88%, respectively. A higher concentration of orange oil (MBC, >1%,) was required to inhibit *Salmonella*. The average concentration of TOC required for a similar bactericidal effect was 0.14%. The inhibitory concentrations reported in other essential oil studies varied from 0.04 - 2% (thyme) and 0.04-2% (clove) to >1-2 (rosemary) and >2% (orange) [12, 22]. The synergistic effect of the major components of thyme oil, thymol and carvacrol with the other essential oil components such as eugenol, cymene, linalool, and cinnamaldehyde has been reported [27-30]. Certain studies suggest that the whole EO has higher antibacterial effect than the individual component, suggesting that the minor components in the oil are also crucial for the observed activity [31, 32]. Hence in this study we used a combination of whole oils. To our knowledge, this is the first report on anti-*Salmonella* effects from the blend of thyme and orange oils.

Campylobacter was found to be relatively sensitive to all the oils tested. The results of disc diffusion assay for *Campylobacter* are presented in Table 5. Oils of thyme and clove showed the strongest antibacterial activity, followed by rosemary and orange oils (ZI of 13.5 and 17.5, respectively). TOC produced a ZI of 22 for *C. jejuni* and 21 for *C. coli*. No growth was noticed even at the highest dilution of oil tested in the present study. However there was growth in the control and DMSO tubes. Therefore, the exact MIC or MBC could not be determined. In other studies, *C. jejuni* was found to be 5 to 10 fold more sensitive to many EOs than other species of bacteria [33, 14], possibly reflecting the fastidious growth

requirements of the organism. On the other hand, Smith-Palmer et al (1998) reported that *Campylobacter* to be the most resistant organism among those they investigated. Differences in activity between studies could be due to the differences in the composition of the oils. In general, the antimicrobial activity of the EOs depends on their chemical composition which is determined by the genotype of the source plants, region and environmental conditions in which they are grown [34, 35].

2.50. CONCLUSIONS AND APPLICATIONS

1. The antibacterial activity of thyme, orange, clove, and rosemary against *Salmonella* and *Campylobacter* has been reported in various publications. These findings were confirmed in the present investigation.
2. *Campylobacter* was found to be more sensitive to the essential oils tested than *Salmonella*
3. Thyme orange oil combination when used at 0.14% is found to be effective against both *Salmonella* and *Campylobacter* grown in laboratory media. Hence TOC can be considered as a potential antimicrobial for future studies on poultry food systems

Table 3. List of selected essential oils, their major active ingredients and LD₅₀'s (Oral)

Common name	Plant source	Major components	^a LD ₅₀ (g/kg)
Thyme oil	<i>Thymus vulgaris</i>	Thymol and carvacrol	2-5
Sweet Orange oil	<i>Citrus sinensis</i>	Limonene	>5

^a Lethal dose 50 of essential oils determined in rats. LD₅₀ values represent the lethal dose of oil per unit weight for killing of 50% population of test animals

Table 4. Effect of essential oils on *Salmonella*

Oil	<i>S. Heidelberg</i>	<i>S. Montevideo</i>	<i>S. Enteritidis</i>	Strain cocktail
Thyme	17 ^b	19 ^b	19 ^b	19 ^a
Orange	0 ^e	7 ^d	7 ^d	0 ^d
Rosemary	9 ^d	7 ^d	8 ^d	7 ^c
Clove	13 ^c	15 ^c	14 ^c	13 ^b
TOC	19 ^a	23 ^a	21 ^a	19 ^a
SEM	0.04	0.03	0.05	0.03

Zone of inhibition is expressed as diameter in millimeters

Values represent means of inhibition zones of two independent experiments; $n=8$

^{a,b,c,d,e} Within columns, means without common subscripts are significantly different ($p < 0.05$)

Table 5. Effect of essential oils on *Campylobacter*

Oil	<i>C. jejuni</i> 11601MD	<i>C. jejuni</i> RM1221	<i>C. coli</i> RM2228	Strain cocktail
Thyme	NG	NG	NG	NG
Orange	18 ^b	16 ^b	19 ^b	17 ^b
Rosemary	17 ^b	15 ^b	11 ^c	11 ^c
Clove	NG	NG	NG	NG
TOC	22 ^a	22 ^a	21 ^a	20 ^a
SEM ¹	0.80	0.79	0.38	0.55
SEM ²	0.92	0.91		

Zone of inhibition is expressed as diameter in millimeters

NG – No growth

Values represent means of inhibition zones of two independent experiments; $n=8$; $n=6$ for Orange treatment

^{a,b,c}Within columns, means without common subscripts are significantly different ($p < 0.05$)

¹SEM when $n=8$

²SEM when $n=6$

Table: 6 Minimum inhibitory concentration of essential oils on selected strains of *Salmonella*

Oil	<i>S. Heidelberg</i>	<i>S. Montevideo</i>	<i>S. Enteritidis</i>	Cocktail
Thyme	0.06	0.06	0.06	0.06
Rosemary	1	0.75	1	0.75
Clove	0.06	0.15	0.15	0.06

Inhibition concentrations are average values of two replicate trials

Table: 7 Minimum bactericidal concentration of essential oils on selected strains of *Salmonella*

Oil	<i>S. Heidelberg</i>	<i>S. Montevideo</i>	<i>S. Enteritidis</i>	Cocktail
Orange	>1	>1	>1	>1
TOC	0.06	0.25	0.125	0.125

Inhibition concentrations are average values of two replicate trials

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CHAPTER 3

Reduction of *Salmonella* Enteritidis and *Campylobacter coli* inoculated on broiler breast

fillets and whole wings by a mix of thyme and orange oils.

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3.10. SUMMARY

Poultry products are important vehicles for the transmission of both *Salmonella* and *Campylobacter* to humans. Recent research has demonstrated that low concentrations (0.14% v/v) of thyme and orange oil combination (TOC) were effective against both *Salmonella* and *Campylobacter* species *in vitro*. Three separate trials were conducted to determine if TOC applied to broiler breast meat and wing would reduce pathogens. A total of 36 breast fillets and 36 wings were used for the three trials. TOC was mixed with Dimethyl sulfoxide (DMSO) and added to sterile water to achieve a final concentration of 0.25% and 0.5% v/v of TOC. Each breast fillet or wing was dipped in a mixture of inoculum containing both *S. Enteritidis* and *C. coli*, and then dipped in one of the four test solutions: 0.25% TOC, 0.5% TOC, DMSO (only) or control (water with no TOC or DMSO) for one minute. Parts were placed individually in sterile bags, shaken with buffered peptone water, serially diluted and then plated. Results from the three replicate trials showed that 0.5% TOC treatment significantly reduced ($P < 0.05$) *S. Enteritidis* by 2.4, and 4.1 log cfu/mL on breast fillets and wings, and *C. coli* by 4.5 log cfu/mL on breast fillets. Although 0.5% TOC resulted in 4.80 log cfu/mL reduction of *C. coli* on the wings, compared to 2.2 logs for control, results were not significant due to variation. 0.25% TOC performed similar to the DMSO and control treatments. In conclusion the 0.5% TOC reduced both *Salmonella* and *Campylobacter* on broiler parts, and could be used to enhance food safety of raw chicken products.

3.20. DESCRIPTION OF PROBLEM

Consumption of food contaminated with bacteria has been estimated to cause 48 million illness, 128,000 hospitalization, and 3000 deaths every year in the United States. Of those illnesses 1,027,561 and 800,000 cases are attributed to *Salmonella* and *Campylobacter* respectively [1]. Poultry products are often implicated as one of the major vehicles for the transmission of *Salmonella* and *Campylobacter* infections to humans [2-5]. Prevalence of both pathogens within or on broilers on farms [6, 7], at the processing facility [7, 8], and at retail [9, 10] has been established. Contaminated poultry meat is responsible for an estimated \$2.5 billion in disease burden losses annually [11]. Furthermore, recalled contaminated meat products directly contribute to industry costs, and may adversely affect the demand for such products [12]. Consequently, methods have been developed to substantially reduce microbes at different stages of processing. Various systems include: water-rinse/spray/steam, ultrahigh pressure, irradiation, ultrasonic energy, UV light, and chemicals (chlorine, chlorine dioxide, trisodium phosphate, cetylpyridinium chloride, acidified sodium chlorite, hydrogen peroxide and organic acids) [13-24]. Currently, USDA approved antimicrobials are used as prechill and postchill dips or spray applications [25]. Chlorine is one of the cheapest and most widely used decontaminant chemicals in poultry processing. However there are mixed results on the antimicrobial activity of chlorine, and many researchers believe that chlorine is relatively ineffective against *Salmonella* [26, 27]). The functionality of chlorine is further reduced by the presence of organic matter [19]. Other chemical antimicrobials have yielded results

between 0.9 and 4 log reductions of *Salmonella* and *Campylobacter* depending on the method of application, contact time, and temperature [20-24]. However, the application or functionality of these chemicals is limited due to their corrosive nature, bleaching effect, sensory changes, and disposal issues [28]. The ban of chemical decontaminants in the European Union further limits their use [29]. Another concern is the very low log reduction in bacterial cells which renders the survivors resistant and more infective [30]. Therefore scientists are looking for natural means to reduce microbial load, prevent further cross contamination, and lessen the percentage of resistant strains. Consumers also show increasing preference for natural products, which would include antimicrobials.

Essential oils are known to possess antibacterial properties against foodborne pathogens and have the potential to be used as natural antimicrobials in food [31, 32]. In a previous *in-vitro* study by disc diffusion and broth dilution techniques, *Salmonella* and *Campylobacter* was inhibited by a mix of thyme and orange oils [Unpublished data]. Thyme oil or its major component thymol has shown great efficacy in inhibiting *Salmonella* and *Campylobacter* on chicken breast in other studies [33, 34]. However its use for commercial scale applications has been limited due to its high cost. Therefore, to arrive at a cost effective treatment, 50% of thyme oil was replaced with 50% sweet orange oil which is available at relatively low cost. The mixture exhibited a synergistic effect against *Salmonella*. The antibacterial activity of thyme and orange oil is attributed to the presence of high concentration of phenolic compounds thymol and carvacrol from thyme oil, and limonene (alkyl group) from orange oil. The possible synergistic effect of the combination could be

due to the alkyl substitution of phenolic compounds, which increased the hydrophobicity and thereby antimicrobial activity [35, 36].

Salmonella Enteritidis (SE) is a serotype that is frequently isolated from table eggs, and also found in poultry meat [37]. *C.jejuni* and *C.coli* are the two important thermophilic species of *Campylobacter* that are often linked with foodborne illness in the United States [38]. Therefore, in this study the antimicrobial effect of TOC on SE and *C. coli* was examined on boneless skinless broiler breast fillets and whole wings. These cut up parts were chosen in order to determine the antimicrobial effect on both skin on and skinless fresh poultry products. A level of 0.14% TOC was required to inhibit *Salmonella* and *Campylobacter* in the vitro antibacterial assay. However a higher concentration would likely be required to achieve similar effects in food [39]. Two fold and four fold ratios were used to make 0.25% and 0.5% TOC respectively. In this study, 0.25% and 0.5% TOC was used in a dip application on inoculated broiler breast fillets and wings to evaluate antimicrobial activity against SE and *C. coli*.

3.30. MATERIALS AND METHODS

Test Compounds and preparation of dip solutions

Thyme oil [40] and orange oil [41] were obtained in certified food grade form. TOC was a blend of thyme and orange oil used at 50:50 ratios. The stock solution of oils were prepared by diluting the essential oils with 50% Dimethyl sulfoxide [42] which was used as an oil solubilizer. The dip solutions to be tested were prepared by mixing the stock solution with sterile non chlorinated water to achieve a final concentration of 0.25% and 0.5% TOC.

The control dip solution contained only water with no TOC or DMSO. In order to isolate any antibacterial effect of DMSO a dip solution containing water with DMSO but no TOC was investigated.

Bacterial cultures and preparation of inoculum

Bacterial marker strains were a 200 ppm nalidixic acid resistant SE and a 100 ppm nalidixic acid resistant *C. coli*. The stock cultures were stored at -80°C. SE was sub-cultured into Brain heart infusion medium (BHI) [43] before each use at 37°C for 24 h. The culture was streaked on Brilliant Green Agar with sulfadiazine [44] (BGA) containing 200 ppm nalidixic acid. Isolated colonies were inoculated into fresh BHI and incubated at 37°C for 12 hrs. The frozen stock culture of *C. coli* was sub-cultured into Muller Hinton (MH) agar medium [44] before each use. The culture was further streaked on *Campylobacter* blood-free agar base with cefaperazone (CCDA) plates [43] containing 100 ppm nalidixic acid. Isolated colonies were inoculated into fresh MH broth and incubated at 42°C under microaerobic conditions for 24 h. Equal volumes of both SE and *C.coli* strains were mixed which resulted in a composite inoculum for the microbial challenge of breast fillets and wings.

Dip Application and microbiological procedures

Chicken carcasses were obtained from a local processing plant and were removed from the evisceration line after the final wash step. Carcasses were transported to the research facility and immediately upon arrival the whole carcass was cut-up to separate the breast fillets and wings. A total of 36 breast fillets and 36 wings were used for the three trials. For each trial samples (which were maintained at temperatures below approximately 10°C)

were divided into 4 groups: 0.5% TOC, 0.25% TOC, DMSO, and control. Four breast fillets and 4 wings were used for each TOC treatments, and 2 breast fillets and 2 wings for the DMSO and control treatments. Each breast fillet or wing was dipped in a mixture of inoculum containing both SE and *C. coli* for approximately 30 seconds. Short duration was used not to kill or injure any *Campylobacter* cells. All the test and the control samples received equal time. The chicken parts were inoculated with 9.99, 9.05, 9.58 log cfu/ml of *Salmonella* and 6.99, 7.66, 7.13 log cfu/ml of *Campylobacter* for the three replicate trials respectively. The inoculated samples were then dipped in one of the three test solutions or the control for one minute. The treated samples were placed individually in sterile bags, shaken with 25 mL 1% buffered peptone water [43]. The rinsate was serially diluted (10 dilution), and plated in duplicates using a spiral plater [45]. BGA plates containing 200ppm nalidixic acid used for *Salmonella* enumeration was incubated at 37°C for 24 hours. CCDA plates containing 100 ppm nalidixic acid used for *Campylobacter* enumeration was incubated at 42°C for 48 hours. The number of colonies were counted and expressed as log₁₀ cfu/mL. One breast fillet and one wing was sampled before each experiment to determine the presence of naturally occurring strains with high level of resistance to nalidixic acid.

Statistical Analysis

Salmonella and *Campylobacter* numbers were converted to log₁₀ cfu/mL. Log reductions between the levels inoculated on the chicken parts and control or treated samples were calculated. Data were analyzed by General Linear Models procedure of SAS using treatment (control, DMSO, 0.25% TOC, and 0.5% TOC) and trial as main effects. [45]. All

first order interactions were tested for statistical significance ($P < 0.05$) by residual error Mean Square. Data was pooled across trials and any significant trial treatment interaction was included as an error in the model statement. Statements of statistical significance were based upon $P < 0.05$. Tukeys test was applied for means separation. Data are presented as means \pm SEM.

3.40. RESULTS AND DISCUSSION

The log reduction (\log_{10} cfu/ml) of SE recovered from inoculated broiler breast fillets and wings before or after a dip application for 1 minute in untreated water or treatment levels is shown in Table 8. The SE count on the control chicken parts was reduced by 0.9 log due to rinse effect. A dip application in 0.5% TOC solution for 1 min reduced SE by 2.4 and 4.5 logs on inoculated breast fillets and wings respectively. This reduction was significantly higher than the other treatments ($P < 0.05$). TOC at 0.25% also significantly lowered (1.6 and 2.5 log reduction on breast fillets and wings) counts when compared to control but was not different from the DMSO treatment. The antimicrobial activity of thyme and orange oils has long been recognized [31, 32, 46] and is attributed to its major components of thymol, carvacrol, and limonene. [47, 48]. These components or combinations with other oils could produce a synergistic, additive, or even antagonistic effects [47, 49, 50]. Dean and Ritche [46] and Smith-Palmer [32] reported thyme oil as one of the most inhibitory oils tested against several genera of bacteria. In previous research, TOC exhibited a strong antibacterial activity against both *Salmonella* and *Campylobacter* with a minimum bactericidal concentration of 0.14% [Unpublished data]. In-vitro work with essential oils and their

components showed substantial results, however higher concentration was required in food systems. Antimicrobial activity of rosemary extract in chicken meat juice was 4 times the level required in laboratory medium [39]. The essential oil levels tested on food models in various studies have been between 1-3% in order to retain the organoleptic properties and sensory attributes of the food tested [51]. Previous research on the antibacterial effects from the blend of thyme and orange has not been found. However, a few studies determined the effect of thyme or orange oil or their major components on poultry products and found significant results. Goswami et al [34] found a 2 and 3 log reductions of *Salmonella* on storage day 4 and 8 when chicken breast fillets were coated with 0.5% thyme oil. Burt et al [53] reported that 20% carvacrol vapors significantly reduced *S. Enteritidis* numbers on chicken meat, and 40% carvacrol vapors completely eliminated all cells within 3h at 37°C. Lu and Wu [52] used thymol in combination with organic acids or surfactants and achieved a log reduction of 2.20 and 2.23 cfu/g on breast fillets treated for 2 mins. Fratianni [33] reported a reduction in *Salmonella* on day 4, 14, and 21 of storage of thyme oil treated wings. Citrus oils were found to be effective against different strains of *Salmonella in-vitro* [48]; however the activity in food varied depending on the nutrient composition of the food [54].

Mean log reduction of 100 ppm nalidixic acid-resistant *C. coli* on broiler breast fillets and whole wings is presented in Table 9. TOC at 0.5% level significantly ($P<0.05$) reduced *C.coli* on breast fillets by 4.1 logs. A 1.8 log reduction was achieved on the breast fillet controls by rinse effect, and was comparable to the reduction achieved by DMSO and 0.25% TOC treatments. Wings treated with 0.5% TOC showed a difference of 4.80 logs compared

to 2.18 logs for control; however the results were not significant due to variation. *Campylobacter* has been found to be very sensitive to most essential oils due to their fastidious nature [39, 55]. Goswami [52] reported that chicken breast meat coated with thyme oil completely eliminated *Campylobacter* during storage at 4°C for 12 d. In a study, sour orange peel oil was found to reduce *C. jejuni* and *C. coli* by greater than 4 logs on poultry skin incubated for 48 hours at 4°C. In the present study a difference in reduction on the skinless breast fillets versus skin on wing was observed. Possibly *Salmonella* and *Campylobacter* were entrapped in deeper layers of skin which provided a microenvironment for the attached bacteria providing protection from antimicrobial agents. [56-58]. This scenario also explains why fewer cells and more variable results were observed with wings versus breast fillets. The difference in composition of protein, carbohydrate, fat, and pH of different food is also found to affect bacterial sensitivity [60]. Since the contact time was only 1 min for antimicrobial treatments, the skin may have affected the results and increased variation with wings. Tests on parts prior to inoculation and treatment showed that no nalidixic acid resistant strains of *Salmonella* and *Campylobacter* were present.

3.50. CONCLUSIONS AND APPLICATIONS

1. Findings are in agreement with previous studies on the antimicrobial effect of essential oils on raw chicken products. In particular the combination of thyme and orange oil was effective against both *Salmonella* and *Campylobacter* on broiler breast fillets and whole wings.
2. TOC at 0.5% level significantly reduced *Salmonella* Enteritidis on fresh broiler breast

fillets and wings by 2.44 and 4.53 log cfu/mL

3. TOC at 0.5% level significantly reduced *Campylobacter coli* on fresh broiler breast fillets by 4.1 logs

4. TOC at 0.5% level has a potential to be used to control *Salmonella* and *Campylobacter* on poultry products, however future studies are needed to evaluate sensory attributes.

Table 8. Log reduction of *Salmonella* Enteritidis on broiler breast fillets and whole wings by dip application for 1 min with DMSO or TOC at 0.25 and 0.50%

Treatment	Breast fillets	Wings
^A Control	0.9 ^c	0.9 ^c
^B DMSO	1.4 ^b	1.4 ^{bc}
^C 0.25% TOC	1.6 ^b	2.5 ^b
^D 0.5% TOC	2.4 ^a	4.5 ^a
^E SEM	0.07	0.25
^F SEM	0.10	0.35
<i>P</i> -value	<0.0001	0.02

Number expressed at log₁₀ cfu/ml

Values represent means ± SEM of log reduction of three replicate trials

^A Samples dipped in water not treated with either TOC or DMSO; *n*=6

^B Samples dipped in water containing DMSO, but no TOC; *n*=6

^C Samples dipped in water containing 0.25% TOC and DMSO; *n*=12

^D Samples dipped in water containing 0.5% TOC and DMSO; *n*=12

^E SEM when *n*=12

^F SEM when *n*=6

^{a,b,c} Within columns, means without common subscripts are significantly different (*p* < 0.05)

Table 9. Log reduction of *Campylobacter coli* on broiler breast fillets and whole wings by dip application for 1 min with or without DMSO or TOC at 0.25 and 0.50%

Treatment	Breast fillets	Wings
^A Control	1.8 ^b	2.2
^B DMSO	2.1 ^b	2.2
^C 0.25% TOC	2.2 ^b	3.4
^D 0.5% TOC	4.1 ^a	4.8
^E SEM	0.13	0.10
^F SEM	0.19	0.14
<i>P</i> -value	0.04	0.07

Number expressed at log₁₀ cfu/ml

Values represent means ± SEM of log reduction of three replicate trials

^A Samples dipped in water containing neither TOC or DMSO; *n*=6

^B Samples dipped in water containing DMSO, but no TOC; *n*=6

^C Samples dipped in water containing 0.25% TOC and DMSO; *n*=12

^D Samples dipped in water containing 0.5% TOC and DMSO; *n*=12

^E SEM when *n*=12

^F SEM when *n*=6

^{a,b} Within columns, means without common subscripts are significantly different (*p* < 0.05)

3.60. REFERENCES AND NOTES

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CHAPTER 4

Marinade with thyme and orange oils reduces *Salmonella* Enteritidis and *Campylobacter coli* on inoculated broiler breast fillets and whole wings

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4.10. ABSTRACT

Essential oils are known to possess antimicrobial properties, and have a potential to be used as a natural antimicrobial in food. In a previous study thyme orange essential oil combination (TOC) used at 0.5% level as a dip application on chicken cut-up parts had a significant antibacterial effect against *Salmonella* and *Campylobacter*. A study was designed to evaluate the effect of salt-phosphate marinade solution containing 0.5% TOC to i). reduce *Salmonella* Enteritidis (SE) and *Campylobacter coli* numbers on broiler breast fillets and whole wings marinated by vacuum tumbling ii). reduce cross contamination of both pathogens between inoculated and un-inoculated parts during marination. A total of 52 skinless breast fillets and 52 whole wings were used for the two replicate trials. For each trial, each cut-up part was randomly assigned to one of the 5 groups: Treatment 1: Un-inoculated parts marinated without TOC, Treatment 2: Inoculated parts marinated without TOC, Treatment 3: Un-inoculated parts marinated with TOC, Treatment 4: Inoculated parts marinated with TOC, and Control: non-marinated inoculated parts. Inoculation was done by dipping the samples in an inoculum containing a mixture of SE and *C. coli*. The treatment samples were marinated by vacuum tumbling. All control and treated samples were immediately evaluated to determine SE and *C. coli* numbers. Results indicated that TOC at 0.5% level in the marinade solution applied by vacuum tumbling significantly reduced ($P < 0.05$) numbers of viable SE by 2.6 and 2.3 log₁₀ cfu/ml on broiler breast fillets and *C. coli* by 3.6 and 3.1 log₁₀ cfu/ml on whole wings. The un-inoculated chicken parts after marination with inoculated parts were positive indicating cross contamination. However the number of

bacterial cells recovered from the TOC treated samples were significantly lower ($P < 0.05$) than the numbers recovered from the untreated samples. Marination with a salt phosphate formulation containing 0.5% TOC can successfully be used to reduce *Salmonella* and *Campylobacter* numbers on poultry products.

4.20. INTRODUCTION

In the United States, processed meat (whole carcasses, parts, or further processed products) is often marinated before final sale, and is estimated that more than 50% of the raw poultry may be marinated prior to consumption (Smith and Acton, 2001). Water, salt, and phosphate is typically applied as marinades to raw meat by various methods such as soaking, injection, or vacuum tumbling. Marination is applied to improve poultry meat quality. Enhanced flavor and tenderness, extended shelf life, and improved yield are some of the attributes of marinated meat products (Smith and Young, 2007). More recently, marination has been used to improve functionality and safety of meat (Perko-Makela et al., 2000; Friedman et al., 2006; Isohanni et al., 2010).

The major microbial safety concern with the consumption of contaminated poultry products is gastroenteritis caused by *Salmonella* and *Campylobacter* (Humphrey et al., 1988; Pearson et al., 2000; Effler et al., 2001; Kimura et al., 2004). Both these pathogens are leading causes of foodborne illness responsible for hospitalizations and deaths in the United States. An estimated one million cases of *Salmonella* and 800,000 cases of *Campylobacter* are reported annually in the US (Scallan et al., 2011). Both pathogens are common

inhabitants of the intestinal tract of poultry. During the evisceration process the broiler meat can easily be contaminated with *Salmonella* and *Campylobacter* (Smith et al., 2007). Prevalence in broiler meat at retail in the United States ranges from 4-61% for *Salmonella* and up to 76% for *Campylobacter* (Bokanyi et al., 1990; Cui et al., 2005; Zhou et al., 2007). Higher prevalence of *Campylobacter* over *Salmonella* has been reported (Cason et al., 1997). However *Salmonella* appears to survive better, and even reproduce in some processing areas, resulting in cross contamination from infected to uninfected carcasses (Carraminana et al., 1997). *Campylobacter* is particularly sensitive to drying, and growth occurs only under conditions of reduced oxygen, high moisture, and temperature above 30°C. Nevertheless, they survive different processing conditions and remain detectable by culture methods. The organism's high initial numbers, survival under different conditions, and low human infectious dose highlights the need for successful intervention strategies (Keener et al., 2004).

Many stages are involved in the production and processing of fresh poultry products. Hence assurance of microbiological safety is complex and requires interventions throughout the farm-to-table continuum (White et al., 1997). Interventions at processing should be designed to reduce the existing contamination, prevent cross contamination, and minimize introduction of additional contaminants by sanitation interventions, processing treatments, or antimicrobial procedures (Sofos, 2008). Antimicrobial marinade formulations could be used as one of the intervention strategies to reduce *Salmonella* and *Campylobacter* numbers on the final product.

The demand for organic products and the interest in natural alternatives to antimicrobials has been expressed by consumers. Such compounds also benefit producers in regard to labeling for those groups of consumers. In a previous study thyme and orange essential oil combination was found to be effective in reducing *Salmonella* and *Campylobacter* both in-vitro (at 0.14% level) and by a dip application (0.5% level) on chicken breast meat and wings (Unpublished data). Therefore TOC could be an ideal candidate for use in marinade formulations as an antimicrobial to control pathogens in poultry products. The objective of this study was to evaluate the effect of salt-phosphate marinade solution containing 0.5% TOC to: i). Reduce *Salmonella* Enteritidis and *Campylobacter coli* numbers on broiler breast fillets and whole wings marinated by vacuum tumbling; and ii). reduce cross contamination of both pathogens between inoculated and uninoculated parts during marination.

4.30. MATERIALS AND METHODS

Preparation of Marination Solutions

The marination solution was composed of 91% tap water, 6% (wt/vol) NaCl, and 3% (wt/vol) of commercial food-grade sodium tripolyphosphate (Brifisol STPNEW, B.K. Giuline Corporation, Simi Valley, CA). The solution was prepared and stored at 4°C. Thyme oil (Spectrum Chemicals Manufacturing Corporation, Gardena, CA) and orange oil (Sigma-Aldrich Co. LLC, St. Louis, MO) were both certified food grade form. TOC was a blend of thyme and orange oil used at 50:50 ratios. The test marinade solution was made just before

each trial by adding 0.5% TOC to the marination solution.

Bacterial Cultures and Preparation of Inoculum

Bacterial marker strains were a 200 ppm nalidixic acid resistant SE and a 100 ppm nalidixic acid resistant *C. coli*. The stock cultures were stored at -80°C. SE was sub-cultured into Brain heart infusion medium (BHI, Oxoid ltd, Basingstoke, Hampshire, England) before each use by incubation at 37°C for 24 h. The culture was streaked on Brilliant Green Agar with sulfadiazine (BGA, Neogen, Lansing, Michigan) containing 200 ppm nalidixic acid. Isolated colonies were inoculated into fresh BHI and incubated at 37°C for 12 hrs. The frozen stock culture of *C. coli* was sub-cultured into Muller Hinton (MH) agar medium (Neogen, Lansing, Michigan) before each use by incubation at 42°C for 48 h. The culture was streaked on *Campylobacter* blood-free agar base with cefaperazone plates (CCDA, Oxoid ltd, Basingstoke, Hampshire, England) containing 100 ppm nalidixic acid. Isolated colonies were inoculated into fresh MH broth and incubated at 42°C under microaerobic conditions for 24 h. Equal volumes of both SE and *C.coli* strains were mixed which resulted in a composite inoculum for the microbial challenge of breast fillets and wings.

Marination Procedure

A total of 52 skinless breast fillets and 52 whole wings were purchased from a local grocery store for the two replicate trials. It was ensured that the chicken cut-up parts were not previously marinated. For each trial, each cut-up part (which was maintained at temperatures below approximately 10°C) was randomly assigned to one of the 5 groups: Treatment 1: Uninoculated parts marinated without TOC (6 samples), Treatment 2: Inoculated parts

marinated without TOC (6 samples), Treatment 3: Uninoculated parts marinated with TOC (6 samples), Treatment 4: Inoculated parts marinated with TOC (6 samples), and Control: non-marinated inoculated parts (2 samples). Inoculation was done by dipping the samples in a mixture of inoculum containing both *S. Enteritidis* and *C. coli*. The inoculum contained approximately 10^9 cfu/mL *Salmonella* and 10^7 cfu/mL *Campylobacter* cells. The chicken cut-up parts were then allowed to attach for 5 min at room temperature. Following inoculation and attachment the parts were vacuum tumbled with a dual Injectstar MC 40 tumble-marinator (Inject Star, Brookfield, CT). A vacuum of approximately 23 mm Hg was drawn using a vacuum pump, and the container remained sealed during the marination process. The treatment samples were marinated for 20 min en vacuo with 10% (vol/wt) of a prechilled (4°C) marination solution.

Micro Analysis

The control and the treated samples were evaluated immediately for bacterial numbers. For the enumeration the samples were placed individually in sterile bags, shaken with 25 mL 1% buffered peptone water (Oxoid Ltd, Basingstoke, Hampshire, England) for 1 min. The rinsate was serially diluted (10 dilution), and plated in duplicates. BGA plates containing 200 ppm nalidixic acid used for *Salmonella* enumeration was incubated at 37°C for 24 hours. CCDA plates containing 100 ppm nalidixic acid used for *Campylobacter* enumeration was incubated at 42°C for 48 hours. The number of colonies were counted and expressed as \log_{10} cfu/mL. One cut-up part was sampled before each trial to determine the presence of any naturally occurring bacterial strains with high levels of resistance to nalidixic acid.

Statistical Analysis

Salmonella and *Campylobacter* numbers were converted to log₁₀ cfu/mL. Data were analyzed by General Linear Models procedure of SAS using treatment and trial as main effects. (SAS Institute, 2004). All first order interactions were tested for statistical significance ($P < 0.05$) by residual error Mean Square. Data was pooled across trials and any significant trial treatment interaction was included as an error in the model statement. Statements of statistical significance were based upon $P < 0.05$. Tukeys test was applied for means separation. Data are presented as mean \pm SEM.

4.40. RESULTS AND DISCUSSION

SE counts (log₁₀ cfu/ml) on broiler breast fillets and wings marinated with or without 0.5% TOC is presented in Table 10. Marination with 0.5% TOC reduced SE by 2.6 and 2.3 logs on inoculated breast fillets and wings when compared to the controls. This reduction was significantly higher than the reductions achieved by using marinade without TOC (1.2 and 1.0 logs; $P < 0.05$). The un-inoculated breast fillets contained as many bacterial cells as the inoculated samples both in the TOC treated and non treated samples, indicating that cross contamination occurred during marination. However the TOC treated un-inoculated breast fillets (5.8 log₁₀ cfu/ml) showed a lower count than non treated fillets (7.4 log₁₀ cfu/ml) indicating a preventive effect. The SE count on whole wings was similar for the inoculated and un-inoculated non treated samples. However for the TOC treated samples, the un-inoculated wings showed significantly lower counts (6.1 log₁₀ cfu/ml, $P < 0.05$) than the

inoculated samples (6.4). Decontamination of meat by vacuum tumbling using antimicrobial solutions such as lactic acid, trisodium phosphate, cetylpyridinium chloride and plant extracts has been studied, and vacuum tumbling is reported as an effective method for antimicrobial application on chicken parts (Pohlman et al., 2002; Deumier, 2006; Over et al., 2009). In a previous study TOC was found to be effective in reducing *Salmonella* and *Campylobacter* both in-vitro and by dip application on broiler breast fillets and wings (Unpublished data). Treatments with natural antimicrobials by dip or spray application showed slightly lower or similar reductions in *Salmonella* spp (Goswami et al., 2009, Lu and Wu 2011). A 2.2 log reduction of *Salmonella* was achieved by a dip application of chicken breast fillets in a solution containing thymol, sodium dodecyl sulfate, and acetic acid, whereas a higher concentration of chlorine (200ppm) was required to yield similar results (Lu and Wu 2011). Also chicken breast coated with 0.5% thyme oil decreased *Salmonella* counts by 2 and 3 logs on storage day 4 and 8 (Goswami et al., 2009).

Table 11 summarizes the antimicrobial effect of the marinade with or without 0.5%TOC on *C. coli* on the surface of breast fillets and whole wings. The marinade containing TOC significantly reduced *C. coli* counts by 3.6 and 3.1 log₁₀ cfu/ml on the breast fillets and wings when compared to the non marinated controls ($P<0.05$). A 5.0 and 3.8 log₁₀ cfu/ml cells were recovered from the un-inoculated non treated breast fillets and wings indicating cross contamination. However fewer *Campylobacter* cells were recovered (2.3 and 2.6 log₁₀ cfu/ml) from the TOC treated un-inoculated breast fillets and wings when compared to the non treated samples (5.0 and 3.8. log₁₀ cfu/ml). Additionally, while both the inoculated

and un-inoculated breast fillets of the untreated group showed similar counts, in the treated group the un-inoculated samples ($2.3 \log_{10}$ cfu/ml) showed a significantly lower number of cells than the inoculated samples ($3.1 \log_{10}$ cfu/ml) indicating a small level of protection to cross contamination conferred by TOC. Similar or slightly lower reduction has been reported using various antimicrobials. *C. jejuni* was decreased by 0.5 log to 2 log on chicken meat medallions treated with marinades containing organic acids, and a 1.2 log reduction after three day storage of chicken breast fillets marinated with pomegranate syrup, lemon juice, and white wine vinegar (Birk et al., 2010). Goswami et al (2009) reported a complete elimination of *Campylobacter* on chicken breast fillets treated with 0.5% thyme oil during 12 d storage at 4°C.

The antibacterial activity of thyme oil is mainly attributed to the phenolic compounds namely thymol and carvacrol. These compounds appear to interact with the bacterial cell membrane, dissolving the phospholipids bilayer and resulting in leakage of cellular contents and cell death (Juven et al., 1994). Orange oil is composed mostly of d- limonene (> 90%) which is the antibacterial component of the oil. Mechanism of action of orange oil has not been thoroughly researched, but may be similar to other essential oils (Fisher and Phillips, 2008). The antimicrobial activity of the essential oil is the net result of the activity of the major and minor components. Interactions between various components of the essential oil or other oils could result in a synergistic, additive or antagonistic effect. Therefore it is more meaningful to use the whole oil instead of the individual components (Utee, 2000; Lambert et al., 2001; Delaquis., et al 2002).

TOC added at 0.5% level in the marinade solution and applied by vacuum tumbling significantly reduced numbers of viable SE and *C. coli* on broiler breast fillets and whole wings. Marination without TOC reduced inoculated part numbers, but the reduction seems to be due to spread to uninoculated parts. The un-inoculated chicken parts when marinated with inoculated parts had marker strain bacteria present indicating cross contamination. However the number of cells recovered from the TOC treated samples were significantly lower than the numbers recovered from the untreated samples. Therefore marination using a salt phosphate formulation containing 0.5% TOC can successfully be used to reduce *Salmonella* and *Campylobacter* numbers on contaminated poultry products and may provide some protection against low levels of cross contamination.

Table 10. Numbers of *Salmonella* Enteritidis (\pm SEM) on broiler breast fillets and whole wings marinated with or without 0.5% TOC, either with or without bacteria inoculated prior to marination

Treatment	Bacteria	Breast fillets	Whole wings
^A Control	Yes	8.8 \pm 0.5 ^a	8.6 \pm 0.4 ^a
1-Without TOC	No	7.4 \pm 0.2 ^b	7.5 \pm 0.2 ^b
2-Without TOC	Yes	7.6 \pm 0.3 ^b	7.6 \pm 0.2 ^b
3-With TOC	No	5.9 \pm 0.2 ^c	6.1 \pm 0.2 ^d
4-With TOC	Yes	6.2 \pm 0.2 ^c	6.4 \pm 0.3 ^c
^B SEM		0.07	0.04
^C SEM		0.10	0.06
<i>P</i> -value		<0.0001	0.0006

^A Samples that were not subjected to marination

Number expressed at log₁₀ cfu/mL; *N*=52

Values represent mean of two replicate trials

^BSEM when *n*=12

^CSEM when *n*=4 (Control treatment)

^{a,b,c,d} Within columns, means without common subscripts are significantly different (*P* < 0.05)

Table 11. Numbers of *Campylobacter coli* (\pm SEM) on broiler breast fillets and whole wings marinated with or without 0.5% TOC, either with or without bacteria inoculated prior to marination

Treatment	Bacteria	Breast fillets	Whole wings
^A Control	Yes	6.7 \pm 0.2 ^a	5.7 \pm 0.6 ^a
1-Without TOC	No	5.0 \pm 0.1 ^b	3.8 \pm 0.3 ^b
2-Without TOC	Yes	4.9 \pm 0.2 ^b	3.8 \pm 0.3 ^b
3-With TOC	No	2.3 \pm 0.1 ^d	2.6 \pm 0.4 ^c
4-With TOC	Yes	3.1 \pm 0.2 ^c	2.6 \pm 0.3 ^c
^B SEM		0.13	0.10
^C SEM		0.19	0.14
<i>P</i> -value		<0.0001	0.0016

^A Samples that were not subjected to marination

Number expressed at log₁₀ cfu/mL; *N*=52

Values represent mean \pm SEM of log reduction of two replicate trials

^BSEM when n=12

^CSEM when n=4 (Control treatment)

^{a,b,c,d} Within columns, means without common subscripts are significantly different (*P* < 0.05)

4.50. REFERENCES

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CHAPTER 5

Effect of marinade containing thyme and orange oils on broiler breast fillet and whole wing aerobic bacteria during refrigerated storage

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5.10. SUMMARY

Raw meat is naturally contaminated with both pathogenic and spoilage organisms. Natural antimicrobial interventions are gaining interest among consumers. In a previous study a marinade containing thyme orange essential oils (TOC) at the 0.5% level was found to inhibit *Salmonella* and *Campylobacter*. The objective of this study was to evaluate the effect of salt-phosphate marinade solution containing 0.5% TOC applied by vacuum tumbling on the shelf life of broiler breast fillets and whole wings. The total aerobic and facultative mesophiles (TAM) occurring naturally on these products during refrigerated storage for 14 d (at 1, 7, 10, and 14 d) were enumerated. A total of 48 skinless breast fillets and 48 whole wings were used for each of the two trials. For each trial, twelve of the 24 breast fillets or wings were marinated using the control marinade solution and the remaining twelve were marinated in the test marinade solution. On d 1, 7, 10, and 14, three treated and three control breast fillets and wings were randomly selected for TAM enumeration. Duplicate plates were plated and incubated at 35°C for 48h. TOC marinade was able to significantly ($P<0.05$) reduce TAM numbers on days 1, 7 and 10 compared to the controls. A log reduction of 0.3, 0.9, and 1.1 was recorded on d 1, 7, and 10, respectively. The difference in TAM between the treated and untreated whole wings was not significant. Therefore 0.5% TOC in marinade can be used as a natural antimicrobial to reduce TAM on skinless breast fillets; however, higher concentration may be required for skin-on products.

5.20. DESCRIPTION OF PROBLEM

Poultry meat has high nutritive value and is also an affordable source of protein for low income groups [1, 2]. A large proportion of poultry meat is sold fresh as ‘ready-to-cook’ [3]. Raw meat is naturally contaminated with both spoilage and pathogenic bacteria. The bacteria predominantly found on fresh meat are mesophiles. They have an optimum growth temperature of 35°C, and their growth is generally minimal below 10°C. This group includes pathogenic organisms such as *Salmonella*, *Campylobacter*, *Clostridium perfringens*, *Staphylococcus*, and *E.coli* [4, 5]. The bacteria that predominate in spoiled poultry meat are generally psychrotrophs. This group includes *Pseudomonas*, *Acinetobacter*, and Enterobacteriaceae which multiplies rapidly between temperatures 20 and 30 C, and grows well at refrigeration temperatures [6]. In recent years several studies have been conducted to improve quality and safety of poultry meat [7-11]. Much attention is focused on natural preservatives such as plant extracts that were traditionally used to enhance sensory attributes and improve shelf life [7, 11]

Essential oils are volatile, complex mixtures produced by aromatic plants as secondary metabolites. They are generally obtained by distillation or pressing whole plants or specific parts such as flowers, buds, seeds, leaves, twigs, bark, herbs, wood, fruits, and roots [12]. One of the first known studies on the antibacterial activity of essential oils was reported by Chamberland in 1887, in which the antibacterial potential of cinnamon oil to inhibit anthrax spores was demonstrated [13]. Later the antibacterial activity of various essential oils against several genera of pathogenic and spoilage bacteria were intensively investigated [14-

19]. The results showed varying levels of inhibition among oils and between strains of bacteria. Therefore it is necessary to identify the oils with specific inhibition against the microbes of interest.

In the United States more than 50% of the processed meat may be marinated, which includes products that are marinated in commercial settings, at retail outlets, and at home [20]. Typically, a commercial marinade contains water, salt, and phosphate which are applied to meat by soaking, injection or vacuum tumbling [21]. The effect of marinades to improve flavor, tenderness, increase water holding capacity and yield, and reduce off flavors has been studied [21-23]. Recently, the study on the effect of marinades on improving the microbial quality of meat is gaining interest. In a previous study thyme and orange essential oil combination (TOC) was found to be effective in reducing *Salmonella* and *Campylobacter* both in-vitro (at 0.14% level) and on chicken breast meat and whole wings (at 0.5% level) by a dip application or marination (Unpublished data). Therefore, studies were conducted to evaluate the effect of the same combination on naturally occurring general mesophile populations on poultry products. The objective of the study was to evaluate the effect of salt-phosphate marinade solution containing 0.5% TOC applied by vacuum tumbling on the shelf life of broiler breast fillets and whole wings by enumerating the total aerobic and facultative mesophiles occurring naturally during refrigerated storage for 14 d (at d 1, 7, 10, and 14).

5.30. MATERIALS AND METHODS

Thyme and Orange Essential Oil

Thyme oil [24] and orange oil [25] were in certified food grade form. TOC was prepared by combining thyme and orange oil at 50:50 ratios.

Marination Solutions

The two types of marination solutions prepared for the trials were the test and the control solutions. The control marination solution consisted of 91% tap water, 6% (wt/vol) NaCl, and 3% (wt/vol) commercial food-grade sodium tripolyphosphate [26] which was prepared and stored at 4°C. The test marinade solution was made just before each trial by adding 0.5% TOC to the control marination solution.

Marination Procedure and Storage

A total of 48 skinless breast fillets and 48 whole wings were used for the two trials. Breast fillets or whole wings were either obtained from a local grocery store or from birds slaughtered at North Carolina State University research facility. For each trial, twelve of the 24 breast fillets or wings were marinated using the control marinade solution and the remaining twelve were marinated in the test marinade solution. Marination was applied by vacuum tumbling using an Injectstar MC 40 tumble marinator [27]. A vacuum of approximately 23 mm Hg was drawn using a vacuum pump, and the chicken parts were marinated for 20 m en vacuo with 10% (vol/wt) of prechilled (4°C) marination solution. The pH of the marination solution was approximately 7.2 for both trials. The marinade pick up weight % was on an average 10% for breast meat and 6% for wings. The chicken parts were

removed from the tumbler, packed individually in whirl pack bags and stored at 4°C for 1, 7, 10, or 14 d. On each of these days three treated and three control breast fillets and wings were randomly selected for microbial analysis.

Microbial Analysis

For the enumeration of total aerobic mesophiles, the chicken parts were placed individually in sterile bags and shaken with 25 mL 1% buffered peptone water [28] for 1 min. In both trials the rinsates were serially diluted (10 dilution), plated in duplicates on standard plate count agar [28] and incubated at 35°C for 48 h. The number of colonies on duplicate plates were counted and averaged to determine \log_{10} cfu/mL values for each chicken part.

Statistical Analysis

Mesophile numbers were converted to \log_{10} cfu/mL. Data were analyzed by General Linear Models procedure of SAS using treatment and trial as main effects. [29]. All first order interactions were tested for statistical significance ($P < 0.05$) by residual error Mean Square. Means for each day and treatment was pooled across trials for the breast meat data since there was not significant trial treatment interaction. For the wing data any interaction was included as an error in the model statement and the data was pooled across trials. Statements of statistical significance were based upon $P < 0.05$. Data are presented as mean \pm SEM.

5.40. RESULTS AND DISCUSSION

The effect of TOC marinade on TAM on skinless breast fillets is presented in Table 12. The initial levels were 4.5 (treated) and 4.8 (control) log cfu/mL, and during the storage for 14 days the counts significantly increased in both control and treated groups. The initial level observed on day 1 is considered normal and comparable to the levels reported in other studies [10, 30]. The essential oils in the marinade was able to significantly ($P<0.05$) reduce TAM numbers on days 1, 7 and 10 compared to the controls. A log reduction of 0.3, 0.9, and 1.1 was recorded on days 1, 7, and 10 respectively. However, by day 14 the differences in counts were not significant.

The results on the effect of TOC marinade on the TAM naturally occurring on whole wings are presented in Table 13. There were no significant differences due to treatment. At day 7, there was a 2 log difference between the treated and untreated group. However the results were not significant due to a trial treatment interaction. The level of antimicrobial activity obtained by 0.5% TOC in this study is similar or slightly lower when compared to other chemical decontaminants. Dip treatment of chicken legs with 220 ppm peroxyacids (0.33 log), and 2% citric acid (1.21 log) has shown similar log reduction in total aerobic mesophiles. However decontaminants such as 12% trisodium phosphate (1.74 log), and 1200 ppm acidified sodium chlorite (1.97) has shown slightly higher reductions [9]. Testing the effect of essential oils on total mesophile counts have had mixed results. Essential oil of mustard significantly reduced total aerobic mesophiles on acidified chicken meat models after two days of storage when compared to the untreated controls [7]. Barbosa et al [31] tested the

effect of essential oils of oregano, thyme, marjoram, ginger, and clove against naturally occurring microbiota (mesophiles and psychotrophic organisms) on minced meat recorded at 0, 6, and 24 h. They did not find any significant reduction due to treatment with essential oils, and the highest log reductions of 1.3 log and 1.0 log was achieved by ginger and thyme oil. Turgis et al [32] found that essential oils used alone did not have an effect in reducing total aerobic mesophile number on ground beef. However reductions (more than 4 logs) were achieved when essential oils were combined with irradiation treatment.

In the present study a significant difference TAM counts due to TOC treatment was noticed on the skinless breast fillets but not on the skin-on wings. This could be due to the complex nature of the skin which entraps microbes in its deeper layers and provides protection from the antimicrobial agents [33, 34]. Also the wings picked up 40% less marinade than the breast fillets, so the concentration of TOC was diminished in the wings. Hence higher concentration of TOC or a different marinade application such as injection may be required to increase uptake and reduce mesophiles on skin-on products. TOC marinade may not result in significant kill of mesophiles on breast meat (as shown by small or no reduction on d 1), but does appear to affect growth of bacteria at d 7 and 10 as compared to control. The effect disappeared by d 14 however.

5.50. CONCLUSIONS AND APPLICATIONS

1. TOC at 0.5% level significantly reduced TAM on marinated broiler breast fillets at up to 10 d of refrigerated storage.

2. The difference in TAM noticed between the treated and untreated whole wings was not significant. Increased marinade uptake (and higher TOC concentration in finished product) might be required for skin-on poultry products.

Table 12: Numbers of Total Aerobic Mesophiles (\log_{10}) of broiler breast fillets marinated without essential oils (control) or with thyme orange oil (treated) and stored at 4°C for up to 14 d.

Days	^A Control	^B Treated	Log reduction	SEM	<i>P</i> -value
1	4.8	4.5	0.3	0.08	0.0327
7	7.6	6.8	0.9	0.13	0.0023
10	9.3	8.2	1.1	0.33	0.04
14	10.4	9.6	0.9	0.38	0.18

n=6

^AControl parts that were marinated in salt-phosphate marinade

^BTreated parts that were marinated in salt-phosphate marinade containing 0.5% TOC

Table 13: Numbers of Total Aerobic Mesophiles (\log_{10}) of whole wings marinated without essential oils (control) or with thyme orange oil (treated) and stored at 4°C for up to 14 d.

Days	^A Control	^B Treated	Log reduction	SEM	<i>P</i> -value
1	5.1	4.7	0.4	0.20	0.42
7	7.9	5.9	2.0	0.52	0.26
10	9.2	8.4	0.8	0.10	0.45
14	9.9	9.5	0.4	0.16	0.19

n=6

^AControl parts that were marinated in salt-phosphate marinade

^BTreated parts that were marinated in salt-phosphate marinade containing 0.5% TOC

5.60. REFERENCES AND NOTES

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