ANTIOXIDANT, ANTIBACTERIAL, AND ANTIVIRAL EFFECTS OF TWO ESSENTIAL OILS, THEIR COMPONENTS, AND CAFFEIC ACID FOR USE AS FEED ADDITIVES IN POULTRY

by

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ABSTRACT

To search for a potential feed additive as an antibiotic alternative for poultry production, two essential oils, thyme and cinnamon bark oil were evaluated along with their respective components as well as the common phenolic acid, caffeic acid by *in vitro* and *in vivo* experiments. In an oxygen radical absorbance capacity (ORAC) assay, caffeic acid, thymol, and thyme oil proved to be better antioxidants compared to carvacrol, cinnamaldehyde and cinnamon bark oil. Using an antimicrobial assay with a growth indicator, minimum inhibitory concentrations (MICs) were determined to be 2.0, 0.4, 0.78, 0.84, 1.54, and 1.83 mg/mL for caffeic acid, thymol, carvacrol, cinnamaldehyde, cinnamon bark oil, and thyme oil, respectively against *Salmonella enterica* serovar Typhimurium. Lower MICs were recorded for *Salmonella enterica* serovar Kentucky, *Salmonella enterica* serovar Enteritidis Nal[r], and *Salmonella enterica* serovar Senftenberg. These compounds were not cytotoxic in chicken embryos and they were effective at inactivating Newcastle Disease Virus (NDV). Since thymol and thyme oil were effective antioxidant, antimicrobial, and antiviral agents based on the three assays so they were used as feed additives in three feeding trials. After a 28-day feeding trial, dietary thymol and thyme oil did not affect broiler growth performance and conversion efficiency compared to the control. Histopathology results indicated that supplementation did not cause significant lesion growth in kidney, liver, intestine and ceca samples. However, the supplementation increased the serum hydrophilic antioxidant status of the chickens. Although the addition of thymol showed no significant inhibition against cecal *Salmonella* for

chicks inoculated with 10^4 and 10^8 CFU/mL nalidixic acid-resistant *Salmonella*, the highest concentration of thymol (4g/kg) demonstrated some protective effects for SPF chicks against *S*. Enteritidis Nal^r preventing mortality.

CHAPTER 1
LITERATURE REVIEW

History of Essential Oils

       Essential oils (EOs) have a long and rooted history dating back to at least the 13th century when pharmacies were making EOs and their effects were included in pharmacopoeias (Burt, 2004). They are commonly obtained from plants by steam or hydro-distillation first developed in the Middle Ages by Arabs (Bakkali et al., 2008). EOs are extracted from plants generally located in countries with temperate to warm climates like the Mediterranean and tropical countries where they exist as an important and traditional part of the pharmacopoeia (Bakkali et al., 2008). Widespread use of EOs for medicinal purposes began in Europe around the 16th century, and by the 17th century pharmacies were stocking 15 – 20 different EOs (Burt, 2004). Tea tree oil has been a staple in Australian medicine as early as the 18th century. The bactericidal properties of EOs were first discovered by De la Croix from EO vapors in 1881 (Burt, 2004). Medicinal uses became secondary to using EOs as aromas in flavors during the 19th and 20th centuries, and currently the greatest use for EOs is in flavorings, perfumes, and pharmaceuticals (Burt, 2004). Essential oils have been widely used since the Middle Ages in bactericidal, virucidal, fungicidal, antiparasitical, insecticidal, medicinal, and cosmetic applications (Bakkali et al., 2008). They contain various volatile molecules e.g. terpenes and terpenoids, phenol-derived aromatic compounds, and aliphatic components (Bakkali et al., 2008). Proper storage of EOs is

important because they are volatile and therefore require airtight containers and little light to prevent compositional changes (Burt, 2004). Essential oils can have complex compositions with some including more than 60 individual compounds and major components constituting up to 85%. Phenolic compounds commonly found in EOs contribute greatly to the antibacterial and antioxidant activity. Synergistic effects have also been found in essential oils and are critical to their antibacterial activity. Presently, approximately 3000 essential oils are known with 300 commercially important in the pharmaceutical, agronomic, food, sanitary, cosmetic, and perfume industries (Bakkali et al., 2008).

Safety of Essential Oils

Because of the increased use of plant extracts in foods, their toxicity and stability are a major concern. The oils that come from herbs, spices, and fruits are hydrophobic in nature which is believed to contribute to their mode of action as antimicrobials. Being lipophilic, they can accumulate in the cell membrane of microorganisms, increasing the permeability of the membrane leading to leakage of enzymes and metabolites resulting in cell death (Stammati et al., 1999). Their toxicity to humans has been somewhat contradictory. The Food and Drug Administration as well as the Flavor and Extract Manufacturers' Association deem essential oils and their components as safe and thus they have GRAS status (Generally Recognized as Safe). The use of these compounds in foods is at extremely low levels that would not exhibit any toxic effects but the concern is what effects may be caused if higher doses are necessary. Recent work indicates that in eukaryotic cells, EOs can have prooxidant effects on inner cell membranes and organelles causing concentration and type

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dependent cytotoxic effects on living cells without genotoxicity (Bakkali et al., 2008). Stammati et al. (1999) performed *in vitro* assays using cinnamaldehyde, carvacrol, thymol, and carvone to evaluate cytotoxicity and genotoxicity. Results indicated mild to moderate cytotoxicity based on *in vitro* tests, but genotoxicity data raised no concerns (Stammati et al., 1999). *In vivo* these four compounds are considered to have no significant or marginal effects. The oral LD_{50} in rats for cinnamaldehyde is 2220 mg/kg body weight but no effect was found at doses of 2500 and 1000 ppm in a one year and 27 – 28 week rat study, respectively. For carvacrol and thymol, oral LD_{50} in rats is 810 mg/kg body weight and 980 mg/kg body weight, respectively but in a 19-week rat study, thymol was found to have no effect (Stammati et al., 1999). Essential oils can provoke depolarization of mitochondrial membranes in eukaryotic cells by decreasing membrane potential, affecting ion channels, and reducing the pH gradient (Bakkali et al., 2008). All of which results in increased membrane fluidity leading to cell death. While cytotoxic effects have been found, mutagenicity has not been associated with EOs suggesting that most are not carcinogenic (Bakkali et al., 2008). The specificity of EOs and the effects they cause have been questioned. While it appears that EOs are not specific in their biological effects like cytotoxicity, cytoplasmic mutant induction, gene induction, and antigenotoxic effects, EOs do exhibit specificity in their mode of action regarding production of reactive oxygen species (ROS) (Bakkali et al., 2008). It is clear that various studies offer different perspectives regarding the safety of essential oils and their pure compounds. Further research is necessary but overall their benefits greatly outweigh any negative reports.

Antioxidants: Health Benefits and Safety Assessment

Antioxidants are currently hot topics in the food industry with various companies advertising their products as good sources of antioxidants. Antioxidants have the ability to scavenge the body for ROS which can cause cellular damage. The most common forms of ROS are superoxide, the hydroperoxy radical, singlet oxygen, the hydroxyl radical, and hydrogen peroxide (Cerutti, 1985). Serious biological consequences can result from ROS damage like mutations, sister chromatid exchanges, chromosomal aberrations, cytotoxicity, carcinogenesis, and age-related cellular degeneration (Cerutti, 1985). Increasing evidence suggests that oxidative stress, an imbalance between oxidants and antioxidants, leads to serious biochemical reactions and is a contributing factor in several chronic diseases e.g. atherosclerosis, cancer, and neurodegeneration (Castro and Freeman, 2001). A strong correlation exists between a high intake of fruits and vegetables, the major source of antioxidants, and a 50% reduction in the risk of cancer (Castro and Freeman, 2001). Recently, a large scale study across Europe found a very small inverse relationship between intake of fruits and vegetables and reduced cancer risk (Boffetta et al., 2010). Fruits and vegetables contain polyphenols, a broad term encompassing a variety of antioxidant compounds, which are known to reduce oxidative stress, prevent chronic diseases, as well as possess anticancer, antiviral, and anti-inflammatory properties (Ninfali et al., 2005). Plant phenols are derived from a common intermediate, phenylalanine or its precursor shikimic acid (Arts and Hollman, 2005). These compounds can be divided into 10 different classes based on their chemical structure characteristically with at least one aromatic ring and one or more hydroxyl groups (Arts and Hollman, 2005). In

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addition to scavenging free radicals, polyphenols regulate nitric oxide, decrease leukocyte immobilization, induce apoptosis, inhibit cell proliferation and angiogenesis, and exhibit phytoestrogenic activity which contributes to their protective role in cancer and cardiovascular disease (Arts and Hollman, 2005). Polyphenols are the most abundant antioxidants with a total dietary intake possibly as high as 1 g/day (Scalbert et al., 2005). This is higher than all other classes of phytochemicals and known dietary antioxidants e.g. 10 times higher than vitamin C and 100 times higher than vitamin E and carotenoids (Scalbert et al., 2005). The main sources of polyphenols are of course fruits and vegetables, but they can also be found in fruit juices, tea, coffee, red wine, cereal, chocolate, and dry legumes (Scalbert et al., 2005). Antioxidants are beneficial to the human diet but their incorporation into the diet of animals like chickens could improve their overall health and help reduce serious poultry infections that cause mortality. Since heat stress can also cause increased oxidative stress in poultry leading to decreased body weight gain, feed consumption, and feed efficiency (Mujahid et al., 2005), poultry feed supplemented with antioxidants might minimize these negative effects.

While antioxidants are important for human and possibly animal health, it is important to note that different foods contain different amounts of antioxidants, and to know which are the best, is helpful in maintaining good health (Manach et al, 2005; Ninfali et al., 2005). The bioavailability of polyphenols differs from one to another with gallic acid and isoflavones being the most well-absorbed, followed by catechins, flavanones, and quercetin glucosides, and the least well-absorbed being procyanidans, galloylated tea catechins, and anthocyanidins (Manach et al., 2005; Williamson and

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Manach, 2005). Ninfali et al. (2005) conducted a study evaluating the antioxidant capacity of various fresh vegetables, herbs, and spices using the oxygen radical absorbance capacity (ORAC) assay in addition to the phenolic, flavanoid, and flavanol content to determine which are the most beneficial. Freshly harvested vegetables, herbs, and spices are able to offer maximum antioxidant effects because their phenolic and flavanol contents are intact unlike those stored for long periods or minimally processed (Ninfali et al., 2005).

An extensive study was performed with rats using dietary supplementation of thyme oil during their lifetime and investigating the antioxidant status of liver, kidney, and heart tissues as well as changes in activity of superoxide dismutase (SOD) and glutathione peroxidase (GSHPx), cellular antioxidant enzymes. Liver and heart SOD activities in old rats fed thyme oil were significantly higher than the control but activities in the kidney were not significantly different (Youdim and Deans, 1999). Liver GSHPx activity was significantly higher in thyme oil treated old rats than the old rat control. Kidney GSHPx activity declined with age but was significantly higher in old thyme oil rats than old control rats, and finally heart GSHPx activities were not significantly different in old rats (Youdim and Deans, 1999). The total antioxidant status in all three tissues declined with age; however in old rats, those receiving thyme oil showed significantly higher total antioxidant status in liver, kidney, and heart compared to old control rats (Youdim and Deans, 1999).

Phenolic components are believed to promote optimal health because of their antioxidant and free radical scavenging abilities (Undeger et al., 2009). Thymol and carvacrol, both of which are phenolics in thyme oil, were tested for their

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antioxidant capacity, ROS degeneration, and DNA damage. Undeger et al. (2009) reported that thymol and carvacrol had good antioxidant capacity; ROS degeneration was dose-dependent for thymol (50% at 50µM) with carvacrol less efficient using Trolox equivalent antioxidant capacity (TEAC). Both compounds did not induce DNA strand breakage at concentrations lower than $50 - 100$ µM (Undeger et al., 2009). This study confirmed other reports that thymol and carvacrol are definitely good antioxidants and at low concentrations can reduce ROS and have no effect on DNA.

The risk and safety associated with increased polyphenol intake has come into question as their obvious benefits have been elucidated. However, there are some that may have carcinogenic/genotoxic effects or that inhibit nonheme iron absorption which can lead to iron depletion (Mennen et al., 2005). The recommended intake of polyphenol-rich supplements varies with companies suggesting 50 mg/day isoflavones or $100 - 300$ mg/day grape seed extract which is rich in proanthocyanidins (Mennen et al., 2005). Tablets/capsules sold on the internet contain intakes almost 100 times higher than what is commonly found in Western diets (Mennen et al., 2005). Because of the varied intake levels of polyphenols, it is difficult to determine the exact doses that could possibly have toxic effects. However some studies done with rats have shown that caffeic acid at 2% induced forestomach and kidney tumors (Mennen et al., 2005).

The genotoxic effects observed *in vitro* may be due to the use of high concentrations at which point polyphenols can become prooxidants, and tissues rich in oxidative enzymes might be vulnerable to this prooxidant toxicity (Mennen et al., 2005). In the case of carvacrol, high concentrations (100 µM) showed a prooxidant

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effect and high concentrations of thymol (25 µM) increased DNA damage (Undeger et al., 2009). Phenolic compounds, which characteristically have shown great antioxidant capacity, are complex molecules with various biological activities (Halliwell et al., 2005). It is important to note that these effects have only been shown in *in vitro* or animal model studies and to date have not been proven to occur in humans (Mennen et al., 2005). The daily intake of polyphenols in everyday foods is at low concentrations compared to the concentrations used in research studies with the food matrix possibly influencing the effects of polyphenols (Mennen et al., 2005). Therefore, more research must be done to fully understand the mechanisms of polyphenols in humans and animals and the possible toxic effects as a result of abnormally high intake.

Antioxidant Assays

The antioxidant value of a particular food component is not just in the ability of the antioxidant to quench free radicals but also in the interactions within the food matrix especially its bioavailability and distribution (Collins, 2005). This makes *in vitro* assays difficult to interpret and therefore results may not be entirely accurate. There are several methods for determination of antioxidant capacity. Some common and widely used methods are TRAP (Total Radical-Trapping Antioxidant Parameter), TEAC (Trolox Equivalent Antioxidant Capacity), diphenylpicrylhydrazyl (DPPH), Folin-Ciocalteau Total Phenolic Assay, and oxygen radical absorbance capacity (ORAC) assay. ORAC measures the rate of decay of a fluorescent molecule using a microplate reader for fluorescence measurement. Trolox[®] (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), a water soluble vitamin E analog, is used as the calibration standard and the results are expressed as Trolox Equivalents (TE).

The TEAC assay is an inhibition assay like ORAC in that a sample is added to a radical producing compound and the results are measured by degree of inhibition of the free radical but the radical used is different (Wang et al., 2004). DPPH uses 1,1-Diphenyl-2-picrylhydrazyl as a stable free radical, which is red in color, to directly react with the test sample but DPPH is not a radical commonly found in biological systems (Mermelstein, 2008). Once the free radical has been scavenged the color changes to yellow. Results are measured using absorbance due to the color change rather than fluorescence like the ORAC assay. The preferred method of antioxidant capacity determination in this study was the ORAC assay due to its relative ease and efficiency in result output. ORAC uses a peroxyl radical, the most common free radical in the human body, making this assay more biologically relevant compared to other antioxidant assays (Mermelstein, 2008). ORAC has also been employed to determine total antioxidant capacity in human plasma (Prior et al., 2003). Many human trials have not been able to demonstrate antioxidant supplementation effects in plasma, and assays measuring DNA oxidation or plasma malondialdehyde concentrations may be more effective (Collins, 2005). Wang et al., (2004) performed antioxidant capacity determination on plasma from rats using the TEAC and ORAC assay to find correlations between the two methods. They determined that both methods were suitable for plasma antioxidant capacity but the inhibition time for the TEAC assay had to be considered. Results from the TEAC assay after 30 min correlated to ORAC results after 70 min, and since ORAC uses the peroxyl radical, it is more biologically relevant in comparison (Wang et al., 2004). Antioxidant determination in serum or plasma is difficult due to the inability to quantify specific

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antioxidant molecules as well as the interactions between molecules. Therefore, various methods can be used but may not be comparable.

Gomez-Ruiz et al. (2007) reported the antioxidant activity of caffeic acid as 4.5 µM TE and 1.3 µM TE using ORAC and TEAC, respectively. Caffeic acid is considered a good antioxidant found in coffee, tea, sweet potatoes, and sunflower seeds (Marinova et al., 2009). ORAC values for carvacrol and cinnamaldehyde found in literature were stated as 50 µmol TE/g and 45 µmol TE/g, respectively (Wang et al., 2008). Using the total equivalent antioxidant capacity (TEAC) assay, the antioxidant activity of cinnamon bark oil obtained from *Cinnamomum* species from China and Indonesia was reported as 61.75 and 108 mmol of Trolox/100 g of dry weight, respectively (Shan et al., 2005). Thyme oil from a *Thymus vulgaris* species from New Zealand had a TEAC value of 38 mmol of Trolox/100 g of dry weight and another study reported an ORAC value of 19.5 µmol TE/g of fresh weight (Shan et al., 2005; Zheng and Wang, 2001).

Antibacterial Assays

In vitro assays are a common way to determine the antibacterial efficacy of essential oils and like antioxidant assays, there are a wide variety from which to choose. Common methods include plate counts, turbidity, absorbance, bioluminescence, and many others (Gabrielson et al., 2002). Favored methods used by many of the studies discussed here tend to be zone inhibition methods or broth culture methods but they can be time consuming. Color change *in vitro* assays are an easy way to evaluate antibacterial effects of EOs. Resazurin salts are one of many compounds used for colorimetric microbial growth determination. Resazurin changes color from

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blue to pink upon reduction of the salt indicating growth. Tetrazolium salts are also a favored growth indicator and both resazurin and tetrazolium salts have been in use since the 1940s (Gabrielson et al., 2002). However, tetrazolium salts precipitate upon reduction and therefore resazurin became the indicator of choice for this study. It works by oxidizing the sample it is mixed with and changing color accordingly (Gabrielson et al., 2002). There are many *in vitro* studies documenting the antibacterial efficacy in coordination with the antioxidant activity of essential oils from plants like cinnamon, thyme, and oregano. The purified components of these oils also have antibacterial effects on common foodborne pathogens like *Escherichia coli*, *Campylobacter*, *Salmonella*, *Listeria monocytogenes*, and *Staphylococcus aureus* (Aeschbach et al., 1994; Chorianopoulos et al., 2004; Friedman et al., 2002; Kim et al., 1995).

Antibacterial Effects of Essential Oils and Plant Extracts

Chao et al. (2000) screened 45 essential oils to determine their effects on four gram-positive bacteria (including *Bacillus cereus* and *S. aureus*) and four gram-negative bacteria (including *E. coli* and *Pseudomonas aeruginosa*). The majority of the essential oils tested showed some inhibition but seven had high antimicrobial inhibition including cinnamon bark and savory [*Satureja* sp.] oils which were effective against both gram-positive and gram-negative bacteria (Chao et al., 2000). The major components of these oils include phenolic and terpinene derivatives both of which are considered to have bactericidal activity. The antibacterial activity of oils from black pepper, clove, geranium, nutmeg, oregano, and thyme were tested against 25 bacteria including animal and plant pathogens, food poisoning and spoilage bacteria (Dorman

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and Deans, 2000). Clove, oregano, and thyme oil inhibited all bacterial strains to some degree with thyme oil showing >90 mm of inhibition against 12 bacteria and the order of activity for each EO was thyme, oregano, clove, nutmeg, pepper, and geranium (Dorman and Deans, 2000). The individual components from each oil were also tested against each bacterial strain with the order of activity as thymol, carvacrol, α-terpineol, terpinen-4-ol, eugenol, etc. (Dorman and Deans, 2000). Thyme oil had the lowest minimum inhibitory concentration (MIC) of 0.03% (v/v) against *E. coli* using the broth microdilution method (Hammer et al., 1999). Many studies involving the antibacterial effects of essential oils have been from local herbs and spices often used in traditional medicinal and food preparations. For example, one study examined the antibacterial activity of oregano, bay laurel, Spanish lavender, and fennel species all native to Turkey against *E. coli* O157:H7, *L. monocytogenes*, *S.* Typhimurium, and *S. aureus*. Oregano and bay laurel EOs showed increasing inhibition of *E. coli* as the doses of both EOs increased with the highest dose (80μL/mL) showing complete inhibition (Dadalioglu and Evrendilek, 2004). Inhibitory effects on *L. monocytogenes* were in the order of Turkish oregano, bay laurel, fennel, and Spanish lavender with Turkish oregano more effective than fennel and Spanish lavender and bay laurel more effective than Spanish lavender (Dadalioglu and Evrendilek, 2004). The same results were also found against *S.* Typhimurium with no significant differences found among bay laurel, fennel, and Spanish lavender (Dadalioglu and Evrendilek, 2004). Finally, the order of EO inhibition against *S. aureus* was Turkish oregano, Spanish lavender, bay laurel, and fennel (Dadalioglu and Evrendilek, 2004). Turkish oregano was the most effective essential oil against all pathogens except *E. coli* O157:H7 and this was attributed to its

high carvacrol content whereas the weak antibacterial effects of Spanish lavender was attributed to its major component fenchone, a weak antibacterial agent (Dadalioglu and Evrendilek, 2004). Zhou et al. (2007) found cinnamaldehyde, thymol, and carvacrol to be affective growth inhibitors of *S. typhimurium* with MICs of 200, 400, and 400 mg/L, respectively. Synergistic effects were also found for combinations of cinnamaldehyde/thymol, cinnamaldehyde/carvacrol, and thymol/carvacrol meaning that combining essential oils and/or their components can offer a greater result than use individually. Synergism of EOs and their components is an important concept that contributes to their antibacterial effects. Lambert et al. (2001) found that carvacrol and thymol from oregano EO had synergistic effects against both *S. aureus* and *P. aeruginosa* and through mathematical equations, they determined that 96% of the inhibition caused by oregano oil could be attributed to the additive effects of thymol and carvacrol. In a study that screened 21 essential oils against *C. jejuni, S.* Enteritidis*, E. coli, S. aureus* and *L. monocytogenes*, bay, cinnamon, clove, and thyme oil were found to be the most inhibitory (bacteriostatic concentration 0.075% or less) against all five pathogens (Smith-Palmer et al., 1998). This study marked the first report of the antibacterial effect of EOs against *C. jejuni*. *C. jejuni* is a pathogen of major concern in the poultry industry in addition to *Salmonella* and antibiotic-resistant isolates of *Campylobacter* have begun to emerge worldwide in broiler chickens (Ravishankar et al., 2008). Cinnamaldehyde and carvacrol were used in this study against antibiotic-resistant *C. jejuni* isolates, and cinnamaldehyde performed better than carvacrol at inactivating the resistant isolates showing growth inhibition within 30 min of application (Ravishankar et al., 2008). These studies support the claim that EOs and

their components are effective against common foodborne pathogens which indicates the benefits for their use in the food industry as well as in animal feeds.

Essential Oils: Mode of Action as Antimicrobials

EOs have been proven to perform well *in vitro* as antimicrobials but their mode of action is still largely unknown. Their hydrophobicity enables them to traverse the bacterial cell wall causing increased permeability and leakage of ions and other essential molecules to bacteria (Burt, 2004). Membrane disruption can subsequently lead to lysis and cell death. EOs with a high phenolic content have been the most effective against bacteria especially foodborne pathogens. The mechanism of action for phenolic compounds involves disruption of the bacterial cell membrane, proton motive force, electron flow and active transport as well as coagulation of cell contents (Burt, 2004). Carvacrol and thymol, phenolic compounds that are structural isomers, can disintegrate the outer membrane of gram-negative bacteria causing release of lipopolysaccharides and increased membrane permeability. In addition to inhibiting the growth of bacteria, carvacrol has been shown to inhibit toxin production of *B. cereus* which is of vital importance to food safety (Burt, 2004). In general, EOs are more effective against gram-positive bacteria than gram-negative bacteria which is likely due to the outer membrane gram-negative bacteria possess. Lambert et al. (2001) used confocal microscopy and ethidium bromide to evaluate the effects of oregano EO on bacterial cells and found that a 0.1% addition of oregano oil, thymol, or carvacrol corresponded in a 90% increase in fluorescence compared to the control indicating dead or injured cells. In the same study, oregano oil caused an increase in potassium and phosphate leakage in *S. aureus* and *P. aeruginosa* as well as a marked decrease in

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the internal pH for both bacteria (Lambert et al., 2001). The results of this study suggest that membrane permeability and ion flow disruption are mechanisms of action used by EOs and their individual components. Griffin et al. (1999) studied the role of terpenoid structure and molecular properties in determining antibacterial activity, and they found that hydrogen bonding capacity and water solubility were the major discriminating factors. Specifically against gram-negative bacteria, small terpenoids able to form hydrogen bonds and interact with water may offer increased ability to pass through porins in the outer membrane of bacteria and therefore are more effective antibacterial (Griffin et al., 1999). This study concluded that terpene acetates and hydrocarbons had little antibacterial activity which was attributed to their limited hydrogen bond capacity and water solubility (Griffin et al., 1999). Ketones, aldehydes, and alcohols showed activity but with varying specificity and strength not associated with functional groups and instead with hydrogen bonding (Griffin et al., 1999).

Antiviral Effects of Essential Oils and Plant Extracts

Research on the antiviral effects of EOs has been conducted mostly outside of the U.S., specifically in Africa. Newcastle disease (ND) is an extremely contagious illness affecting commercial poultry flocks caused by Newcastle Disease Virus (NDV). Current measures taken to control the spread of NDV include vaccination, chemotherapy, and good husbandry. Studies with rural free-range poultry flocks in Tanzania used *Aloe* species as a method of NDV control (Waihenya et al., 2002). Results showed on day 2 post infection that chickens treated with *Aloe* had no clinical signs of infection unlike the untreated birds. *Aloe* did not just delay clinical signs but also mortality and appeared to reduce the severity of lesions found in the

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chickens post-mortem. Chinese herbs have also been used as an immune stimulator in chickens infected with NDV. The study found that at appropriate concentrations, the Chinese herbs were effective at increasing *in vitro* production of chicken embryos fibroblasts in response to the infection (Kong et al., 2006). *Aloe* and other plant extracts have the potential to protect chickens and other poultry from the spread of disease (Mtambo et al., 1999; Waihenya et al., 2002).

Antibiotic Use in the Poultry Industry

The use of antibiotics as growth promoters in the poultry industry has been under severe criticism. There are theories that pathogenic bacteria have the ability to become resistant to specific antibiotics so that they are more difficult to kill. In a recent study 58 *S. enterica* serovar Heidelberg isolates, a commonly isolated serovar in chickens, turkeys and swine, were screened and found 72% resistant to at least one antibiotic and 24% resistant to eight or more antimicrobial agents (Lynne et al., 2009). The American Veterinary Medical Association (AVMA) released a response in November 2009 to the Pew Commission's report on Industrial Farm Animal Production. The AVMA (2009) acknowledged the public health concern as a result of antimicrobial resistance but noted that a direct link between antimicrobials used in food animals and their subsequent resistance in humans has yet to be established. Instead of a focus on antimicrobial resistance issues, the AVMA said the major public health concern should be to reduce the spread of foodborne pathogens. They state that the use of antimicrobials is an essential tool for veterinarians to maintain animal health and strategic use should not be banned as routine use (AVMA, 2009). Published studies have reported antimicrobial resistance of pathogenic bacteria found in poultry

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especially *Salmonella* and *E. coli* and antibiotics like fluoroquinolones are of

particular concern since they are important in the treatment of invasive *Salmonella* and

Campylobacter infections in both humans and animals (Kilonzo-Nthenge et al., 2008).

In the same study, *Campylobacter* and *Salmonella* were isolated from chickens and

evaluated for antibiotic resistance. Multidrug-resistance (ampicillin, ciproflaxin,

erythromycin, kanamycin, and nalidixic acid) was found in *Campylobacter* spp. and

the resistance to ciproflaxin and erythromycin is of particular concern because both are

commonly used to treat campylobacteriosis in humans (Kilonzo-Nthenge et al., 2008).

Salmonella isolates from this study exhibited resistance to ampicillin, streptomycin,

and tetracycline which is of major concern to treating people with salmonellosis

(Kilonzo-Nthenge et al., 2008). Antibiotic resistance of pathogens will continue to be a

major public health concern and attention should be paid to the repeated use of

antibiotics with screenings to determine which ones are no longer effective.

Pathogen Contamination in Poultry

   Salmonella is one of the most common causes of foodborne illness with

multistate outbreaks occurring most recently in January 2009 as a result of

contaminated peanut butter, peppers, and alfalfa sprouts (CDC, 2009). Since 1989,

Salmonella enterica serovar Enteritidis has risen to be the leading cause of infection

among *Salmonella* spp. (Izumiya et al., 2003). Poultry products have the potential to

carry this pathogenic bacterium (USDA, 2009). *Salmonella* infection of poultry usually

begins with the breeder and works its way into commercial product (Horrox, 2007).

There are various ways for *Salmonella* to enter the breeding area but three routes

remain of utmost concern: day old chicks, the feed, and wild birds and vermin. Pests

are common vectors of enteric pathogens and it was reported that 90% of chickens

consuming a single infected adult or larval beetle became infected by *Campylobacter*

and 100% of birds were *Campylobacter*-positive after consuming 10 infected adult or

larval beetles (Doyle and Erickson, 2006). Besides the breeder, handling of poultry by

infected carriers can pass the infection onto the meat during processing thus causing a

contamination issue. The Food Safety and Inspection Service (FSIS) branch of the

USDA developed a HACCP plan specifically designed to reduce *Salmonella*

contamination in broiler production in 1996. In the data from 2002, 2003, and 2004,

one can observe an increase in positive *Salmonella* samples in broilers from 11.5% to

12.8% to 13.5%, respectively (USDA FSIS, 2009). While these results are below the

national baseline figures, *Salmonella* remains a constant health and safety concern. In

the most recent quarterly reports from FSIS regarding positive *Salmonella* samples in

2009 there was a decrease in the incidence of *Salmonella* in broilers and ground

chicken from the first (9% and 21.6%) to third quarter (7.1% and 18.3%) (USDA

FSIS, 2010). However looking at results from the past three years (2006 – 2009), it is

clear that the results fluctuate and a clear trend is not apparent. Raw poultry carcasses

are susceptible to contamination and therefore it is critical to reduce the possible routes

of contamination. One such route is the poultry farm itself. Essential oils can be

beneficial to promote the gut health of chickens and help reduce the risk of bacterial

infections as a feed additive. Becker and Galletti (2008) showed using *in vitro* tests

different plant extracts can be an alternative adhesion matrix for enteropathogenic

bacteria instead of the intestinal tract of the host itself. The antimicrobial properties of

essential oils may be mechanistically different from other natural antimicrobials like

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organic acids and salts, and these differences are important to identify since bacterial pathogens have the ability to develop resistance strategies (Ricke et al., 2005). Preharvest strategies are being employed to combat pathogen contamination and feed additives are a common solution. Studies have shown that adding chlorate to feed and/or water can selectively target bacteria that use nitrogen respiration but not beneficial anaerobes like those of the host's natural microbiota. Chlorate has successfully reduced *S.* Typhimurium colonization in crops and ceca of broilers and turkeys (Anderson et al., 2005). However studies continue on the safety of these products and they have yet to be used commercially. Since the consumer is becoming more health conscious desiring natural instead of artificial, essential oils can be that natural additive in poultry feed.

Antibiotic Alternatives to Promote Health and Control Pathogens in Poultry

In the United States, current organic farming regulations prohibit the use of antibiotics for any reason unless animals become infected with diseases for which there are no nonantibiotic treatments (Griggs and Jacob, 2005). Therefore animals are no longer considered organic once treated with an antibiotic which is why the search for alternatives has greatly increased. Current studies have focused on the use of probiotics, prebiotics, organic acids, and plant extracts as additives to feed and water to reduce poultry pathogen contamination and colonization. Essential oils have reduced pathogens like *Clostridium perfringens*, *E. coli* O157:H7, and *S.* Typhimurium in the gut of chickens without negatively affecting their natural gut biota and in some cases improving the gut microflora (Cross et al., 2007; Jang et al., 2007; Mitsch et al., 2004; Si et al., 2009). Baurhoo et al. (2007) showed that birds fed

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supplemented diets with purified lignin and mannanoligosaccharides and subsequently

challenged with *Escherichia coli* (O2 and O88 serotypes) had decreased cecal loads

containing *E. coli*. Guo et al. (2004) added polysaccharides from two mushrooms and

an herb to broiler feed and found no significant differences in body weight gain, feed

intake, and feed conversion ratio compared to broilers given antibiotics. However,

significant effects of extract type and concentration on growth performance were seen

in birds from 7 – 28 days of age, and it was determined that even though significant

differences were not seen, birds fed the extracts showed better growth performance

than non-supplemented broilers (Guo et al., 2004). In another study by Guo et al.

(2004), the same additives were analyzed for their effects on the cecal microbial

ecosystem of broiler chickens and found that these extracts stimulated the number of

potentially beneficial bacteria e.g. bifidobacteria and lactobacilli while reducing

potentially harmful bacteria like *Bacteroides* and *E. coli*. The addition of lactose to the

diets of chickens reduced cecal colonization of *S.* Typhimurium due to changes in

cecal pH and increases in propionic acid (Doyle and Erickson, 2006). Even

corporations are beginning to perform antibiotic alternative research. Novus

International, Inc. (St. Charles, MO, USA) studied the effect of organic acid blends

added to the drinking water against *Salmonella* colonization in commercial broilers

and found lower percentages of positive swabs taken from the pens receiving organic

acid water treatment as well as a 50% reduction of positive cecal swabs at 49 days for

treated birds (Quiroz et al., 2007). Butyric acid treatments were recently added to

broiler feed and the birds challenged with *S. enterica* serovar Enteritidis. Following

bacteriological analysis, the butyrate-based additives showed a significant reduction in

S. Enteritidis infection from day 27 until the end of the trial (day 42), and sodium butyrate protected with vegetable fats offered unique protection because active substances are slowly released during digestion so they are effective all along the gastrointestinal tract (Fernandez-Rubio et al., 2009). Competitive exclusion using probiotic cultures has also been studied as a method of preventing pathogen colonization in poultry. Establishing adult intestinal microbiota in day-old turkeys was very effective in reducing *Salmonella* colonization in these turkeys (Russell, 2010). The treatment of a lactic acid bacteria probiotic culture in day-old broilers showed a 60 – 70% and 89 – 95% reduction in *Salmonella* Enteritidis and *S.* Typhimurium, respectively (Russell, 2010). Oral inoculation of *B. subtilis* spores reduced intestinal colonization of *E. coli* O78:K80 in chicken within 24 hours of challenging (Griggs and Jacob, 2005). Bacteriophages have also been suggested as a method to control enteric pathogens in chickens because of their effectiveness and specificity of target, their natural occurrence in the environment, and their self-replicating and self-limiting abilities (Doyle and Erickson, 2006). Sklar and Joerger (2001) reported a decrease in positive cecal swabs for *S.* Enteritidis Nal[r] in birds that received phage treatment. However the results were not statistically significant compared to infected control birds receiving no treatment, but when phage was mixed with feed, these birds had the lowest average cecal counts.

Several studies have shown varying effects on the performance of chickens given feed supplemented with herbs, essential oils, or their components. In one study, thyme oil and yarrow herb improved the feed conversion efficiency and the growth performance of broilers (Cross et al., 2007). Another study by Cross et al.

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(2003) added thyme oil to feed in combination with the enzymes xylanase and glucanase to analyze bird performance. The addition of enzymes increased feed consumption and weight gain over the 42 days of the trial with lower feed conversion ratio during days 8 – 14. No overall effect of thyme oil in the diet was found on feed intake or weight gain throughout the 42 days. Jang et al. (2007) found no significant differences in all parameters related to growth performance in broilers fed a commercial feed with thymol throughout the entire study (3 – 35 days). This commercially supplemented feed is Crina Poultry Plus from DSM Nutritional Products Ltd. (Basel, Switzerland). The supplements include thymol, eugenol, piperine, and benzoic acid which have shown in trials to modulate gut microflora and improve digestive function in broilers as well as increasing weight gain (0.9% - 1.8%) and decreasing feed conversion ratio (1.2% - 1.7%) (Horrox, 2009). Crina Poultry and Crina Alternate, commercial EO blends, added to broiler feed were better able to modulate microbial ecology than antibiotics after being challenged with mixed *Eimeria* species (Hume et al., 2006). Dietary carvacrol was found to lower body weight gain but actually improve feed conversion efficiency in female broilers whereas thymol had no effect on general performance (Lee et al., 2003). Hernandez et al. (2004) did a study combining essential oil extracts from oregano, cinnamon, and pepper in one treatment and extracts from sage, thyme, and rosemary in another treatment and found no differences in feed intake or feed:gain ratio for male broiler chickens. Phytogenic feed additives (PFA) containing essential oil mixtures have also been studied to improve growth performance. A commercial PFA (*XTRACT*™, Pancosma S.A., Switzerland) containing 5% carvacrol, 3% cinnamaldehyde, and 2%

Capsicum oleoresin was found to have no significant effect on feed intake, growth, feed conversion ratio, dressing percentage or muscle breast percentage on male Ross 308 chickens (Muhl and Liebert, 2007). Garcia et al. (2007) used formic acid and plant extract treatments in broiler feed to evaluate the effects on performance and nutrient digestibility. Formic acid (5000 and 10000 ppm), 200 ppm of a plant extract blend from oregano, cinnamon, and pepper EO, and 5000 ppm of hydroalcoholic plant extracts from sage, thyme, and rosemary were added to broiler feed for 49 days. Feed conversion ratio was improved in birds receiving the formic acid and the 200 ppm extract blend treatments compared to the control and the ileal digestibility of all nutrients was significantly improved by the additives (Garcia et al., 2007). The formic acid also showed beneficial effects on the villus height of the intestinal mucosa of the chickens and the blend of oregano, cinnamon, and pepper EO significantly improved performance and nutrient digestibility compared to the other extract blend (Garcia et al., 2007). Essential oils and other plant extracts as feed additives have shown to promote intestinal health and improve digestion in pigs, turkeys, and broilers which help to prevent pathogen infection as well as increase nutrient absorption, respectively (Windisch et al., 2008).

Vitamin Content of Chicken Breast Meat using HPLC

Since chicken breasts are a good source of vitamins specifically B vitamins, it was important to determine if feed supplementation would affect the nutritional quality of breast meat. Vitamins are an important part of the diet and are available as supplements and through biological processes in the body. Muscle foods are considered to be a good source of vitamins especially vitamin B_{12} because it is

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normally found in animal products (Kinsman et al., 1994). However, the determination of vitamins in food products can be difficult because they can exist in various biologically active forms (Kinsman et al., 1994 and Esteve et al., 1998). The bioavailability of nutrients in muscle foods depends on various intrinsic factors of the meat itself (Kinsman et al., 1994). Some nutrients are more readily available than others including vitamin B_6 and niacin both of which were quantified in this study. Vitamins B_2 and B_6 may be present in food as free and phosphorylated forms or bound tightly to proteins in the food (Ndaw et al., 2000). B vitamins are water-soluble so their quantification is somewhat easier compared to fat-soluble vitamins.

Using high performance liquid chromatography for vitamin analysis in food allows for a more sensitive approach often chosen in many studies (Ndaw et al., 2000; Esteve et al., 1998; Tang et al., 2006; Zafra-Gomez et al., 2006). Simultaneous determination can be difficult due to interference between the vitamins. Usually vitamins are present in their free form in but their determination in meat tissue frequently requires a hot acid treatment to separate vitamins bound to proteins as well as an enzymatic treatment to dephosphorylate the vitamins (Ndaw et al., 2000; Tang et al., 2006). Tang et al. (2006) found that an acid hydrolysis step prior to enzymatic digestion does not seem to be justified when determining vitamins B_1, B_2, and B_6 in food. This study also found that a mixture of enzymes i.e. a protease (papain), a phosphatase (potato acid phosphatase), and an amylase (α-amylase) gave satisfactory chromatographic isolation of vitamins and were in good agreement with previous results (Tang et al., 2006). However, it is still not explicitly clear that one method over the other is better or that using both is not still useful. This study simultaneously

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determined the B vitamin content of chicken breast meat taken from birds fed supplemented feed using both an acid hydrolysis step and a mixture of enzymes for digestion. Using both methods would assure that vitamins were released for quantification by liquid chromatography.

REFERENCES

Aeschbach, R., J. Loliger, B.C. Scott, A. Murcia, J. Butlers, B. Halliwell, and O.I. Aruoma. 1994. Antioxidant actions of thymol, carvacrol, 6-gingerol, zingerone and hydroxytyrosol. Food Chem. Toxic. 32: 31–36.

American Veterinary Medical Association. 2009. The American Veterinary Medical Association response to the final report of the Pew Commission on industrial farm animal production.

Anderson, R.C., R.B. Harvey, J.A. Byrd, T.R. Callaway, K.J. Genovese, T.S. Edrington, Y.S. Jung, J.L. McReynolds, and D.J. Nisbet. 2005. Novel preharvest strategies involving the use of experimental chlorate preparations and nitro-based compounds to prevent colonization of food-producing animals by foodborne pathogens. Poult. Sci. 84: 649–654.

Arts, I.C.W. and P.C.H. Hollman. 2005. Polyphenols and disease risk in epidemiological studies. Am. J. Clin. Nutr. 81: 317S–325S.

Bakkali, F., S. Averbeck, D. Averbeck, and M. Idaomar. 2008. Biological effects of essential oils – a review. Food Chem. Toxic. 46: 446–475.

Baurhoo, B., A. Letellier, X. Zhao, and C.A. Ruiz-Feria. 2007. Cecal populations of lactobacilli and bifidobacteria and *E. coli* populations after in vivo *E. coli*

challenge in birds fed diets with purified lignin or mannanoligosaccharides. Poult. Sci. 86: 2509–2516.

Becker, P.M. and S. Galletti. 2008. Food and feed components for gut health-promoting adhesion of *E. coli* and *Salmonella enterica*. J. Sci. Food Agric. 88: 2026–2035.

Boffetta, P., E. Couto, J. Wichmann, P. Ferrari, D. Trichopoulos, H.B. Bueno-de-Mesquita, F.J.B. van Duijnhoven, F.L. Buchner, T. Key, H. Boeing, U. Nothlings, J. Linseisen, C.A. Gonzalez, K. Overvad, M.R.S. Nielsen, A. Tjonneland, A. Olsen, F. Clavel-Chapelon, M.-C. Boutron-Ruault, S. Morois, P. Lagiou, A. Naska, V. Benetou, R. Kaaks, S. Rohrmann, S. Panico, S. Sieri, P. Vineis, D. Palli, C.H. van Gils, P.H. Peeters, E. Lund, M. Brustad, D. Engeset, J.M. Huerta, L. Rodriguez, M.-J. Sanchez, M. Dorronsoro, A. Barricarte, G. Hallmans, I. Johansson, J. Manjer, E. Sonestedt, N.E. Allen, S. Bingham, K.-T. Khaw, N. Slimani, M. Jenab, T. Mouw, T. Norat, E. Riboli, and A. Trichopoulou. 2010. Fruit and vegetable intake and overall cancer risk in the European prospective investigation into cancer and nutrition (EPIC). J. Natl. Cancer Inst. 102: 529–537.

Burt, S. 2004. Essential oils: their antibacterial properties and potential applications in foods---a review. Int. J. Food Microbiol. 94: 223–253.

Castro, L. and B.A. Freeman. 2001. Reactive oxygen species in human health and disease. Nutrition. 17: 161–165.

Center for Disease Control. 2009. http://www.cdc.gov. Accessed October 2009.

Cerutti, P.A. 1985. Prooxidant states and tumor promotion. Science. 227: 375–381.

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Chao, S.C. and D.G. Young. 2000. Screening for inhibitory activity of essential oils on selected bacteria, fungi, and viruses. J. Essent. Oil Res. 12: 639–649.

Chorianopoulos, N., E. Kalpoutzakis, N. Aligiannis, S. Mitaku, G. Nychas, and S.A. Haroutounian. 2004. Essential oils of *Satureja*, *Origanum*, and *Thymus* species: Chemical composition and antibacterial activities against foodborne pathogens. J. Agric. Food Chem. 52: 8261–8267.

Collins, A.R. 2005. Assays for oxidative stress and antioxidant status: application to research into the biological effectiveness of polyphenols. Am. J. Clin. Nutr. 81: 261S–267S.

Cross, D.E., R.M. McDevitt, K. Hillman, and T. Acamovic. 2007. The effect of herbs and their associated essential oils on performance, dietary digestibility and gut microflora in chickens from 7 to 28 days of age. Brit. Poult. Sci. 48: 496–506.

Cross, D.E., K. Svoboda, R.M. McDevitt, and T. Acamovic. 2003. The performance of chickens fed diets with and without thyme oil and enzymes. Spring meeting of the WPSA UK branch. S18–S19.

Dadalioglu, I. and G.A. Evrendilek. 2004. Chemical compositions and antibacterial effects of essential oil of Turkish oregano (*Origanum minutiflorum*), bay laurel (*Laurus nobilis*), Spanish lavender (*Lavandula stoechas* L.), and fennel (*Foeniculum vulgare*) on common foodborne pathogens. J. Agric. Food Chem. 52: 8255–8260.

Dorman, H.J.D. and S.G. Deans. 2000. Antimicrobial agents from plants: antibacterial activity of plant volatile oils. J. Appl. Microbiol. 88: 308–316.

Doyle, M.P. and M.C. Erickson. 2006. Reducing carriage of foodborne pathogens in livestock and poultry. Poult. Sci. 85: 960–973.

Esteve, M.J., R. Farre, A. Frigola, and J.M. Garcia-Cantabella. 1998. Determination of vitamin B_6 (pyridoxamine, pyridoxal and pyridoxine) in pork meat and pork meat products by liquid chromatography. J. Chromatography A. 795: 383 – 387.

Fernandez-Rubio, C., C. Ordonez, J. Abad-Gonzalez, A. Garcia-Gallego, M. Pilar Honrubia, J. Jose Mallo, and R. Balana-Fouce. 2009. Butyric acid-based feed additives help protect broiler chickens from *Salmonella* Enteritidis infection. Poult. Sci. 88: 943–948.

Friedman, M., P.R. Henika, and R.E. Mandrell. 2002. Bactericidal activities of plant essential oils and some of their isolated constituents against *Campylobacter jejuni*, *Escherichia coli*, *Listeria monocytogenes*, and *Salmonella enterica*. J. Food Prot. 65: 1545–1560.

Gabrielson, J., M. Hart, A. Jarelov, I. Kuhn, D. MacKenzie, and R. Mollby. 2002. Evaluation of redox indicators and the use of digital scanners and spectrophotometer for quantification of microbial growth in microplates. J. Microbiol. Meth. 50: 63–73.

Garcia, V., P. Catala-Gregori, F. Hernandez, M.D. Megias, and J. Madrid. 2007. Effect of formic acid and plant extracts on growth, nutrient digestibility, intestine mucosa morphology, and meat yield of broilers. J. Appl. Poult. Res. 16: 555–562.

Gomez-Ruiz, J.A., D. Leake, and J. Ames. 2007. In vitro antioxidant activity of coffee compounds and their metabolites. J. Agric. Food Chem. 55: 6962–6969.

Griffin, S.G., S.G. Wyllie, J.L. Markham, and D.N. Leach. 1999. The role of structure and molecular properties of terpenoids in determining their antimicrobial activity. Flavour Fragr. J. 14: 322–332.

Griggs, J.P. and J.P. Jacob. 2005. Alternatives to antibiotics for organic poultry production. J. Appl. Poult. Res. 14: 750–756.

Guo, F.C., R.P. Kwakkel, B.A. Williams, W.K. Li, H.S. Li, J.Y. Luo, X.P. Li, Y.X. Wei, Z.T. Yan, and M.W.A. Verstegen. 2004. Effects of mushroom and herb polysaccharides, as alternatives for an antibiotic, on growth performance of broilers. Brit. Poult. Sci. 45: 684–694.

Guo, F.C., B.A. Williams, R.P. Kwakkel, H.S. Li, X.P. Li, J.Y. Luo, W.K. Li, and M.W.A. Verstegen. 2004. Effects of mushroom and herb polysaccharides, as alternatives for an antibiotic, on the cecal microbial ecosystem in broiler chickens. Poult. Sci. 83: 175–182.

Halliwell, B., J. Rafter, and A. Jenner. 2005. Health promotion by flavanoids, tocopherols, tocotrienols, and other phenols: direct or indirect effects? Antioxidant or not? Am. J. Clin. Nutr. 81: 268S–276S.

Hammer, K.A., C.F. Carson, and T.V. Riley. 1999. Antimicrobial activity of essential oils and other plant extracts. J. Appl. Microbiol. 86: 985–990.

Hernandez, F., J. Madrid, V. Garcia, J. Orengo, and M.D. Megias. 2004. Influence of two plant extracts on broiler performance, digestibility, and digestive organ size. Poult. Sci. 83: 169–174.

Horrox, N. 2007. Salmonella – an old adversary comes back! Int. Hatchery Practice. 19: 21.

Horrox, N. 2009. Nutrition maximizing the economic benefits. Int. Hatchery Practice. 23: *i –xi*.

Hume, M.E., S. Clemente-Hernandez, E.O. Oviedo-Rondon. 2006. Effects of feed additives and mixed *Eimeria* species infection on intestinal microbial ecology of broilers. Poult. Sci. 85: 2106–2111.

Izumiya, H., N. Nojiri, Y. Hashiwata, K. Tamura, J. Terajima, and H. Watanabe. 2003. *Salmonella enterica* serovar Enteritidis, Japan. Emerg. Infect. Dis. 9: 1650–1651.

Jang, I.S., Y.H. Ko, S.Y. Yang, and C.Y. Lee. 2007. Effect of a commercial essential oil on growth performance, digestive enzyme activity and intestinal microflora population in broiler chickens. Animal Feed Sci. Tech. 134: 304–315.

Kilonzo-Nthenge, A., S.N. Nahashon, F. Chen, and N. Adefope. 2008. Prevalence and antimicrobial resistance of pathogenic bacteria in chicken and guinea fowl. Poult. Sci. 87: 1841–1848.

Kim, J., M.R. Marshall, and C. Wei. 1995. Antibacterial activity of some essential oil components against five foodborne pathogens. J. Agric. Food Chem. 43: 2839–2845.

Kinsman, D.M., A.W. Kotula, and B.C. Breidenstein. 1994. Muscle Foods: Meat, Poultry and Seafood technology. Chapman & Hall, Inc., New York, NY.

Kong, X.-F., Y.-L. Hu, Y.-L. Yin, G.-Y. Wu, R. Rui, D.-Y. Wang, and C.-B. Yang. 2006. Chinese herbal ingredients are effective immune stimulators for chickens infected with the Newcastle Disease virus. Poult. Sci. 85: 2169–2175.

Lambert, J.D., J. Hong, G. Yang, J. Liao, and C.S. Yang. 2005. Inhibition of carcinogenesis by polyphenols: evidence from laboratory investigations. Am. J. Clin. Nutr. 81: 284S–291S.

Lambert, R.J.W., P.N. Skandamis, P.J. Coote, and G.-J.E. Nychas. 2001. A study of the minimum inhibitory concentration and mode of action of oregano essential oil, thymol and carvacrol. J. Appl. Microbiol. 91: 453–462.

Lee, K.-W., H. Everts, H.J. Kappert, K.-H. Yeom, and A.C. Beynen. 2003. Dietary carvacrol lowers body weight gain but improves feed conversion in female broiler chickens. J. Appl. Poult. Res. 12: 394–399.

Lynne, A.M., P. Kaldhone, D. David, D.G. White, and S.L. Foley. 2009. Characterization of antimicrobial resistance in *Salmonella enterica* serotype Heidelberg isolated from food animals. Foodborne Path. Dis. 6: 207–215.

Manach, C., G. Williamson, C. Morand, A. Scalbert, and C. Remesy. 2005. Bioavailability and bioefficacy of polyphenols in humans. I. Review of 97 bioavailability studies. Am. J. Clin. Nutr. 81: 230S–242S.

Marinova, E. M., A. Toneva, and N. Yanishlieva. 2009. Comparison of the antioxidative properties of caffeic and chlorogenic acids. Food Chem.. 114: 1498–1502.

Mennen, L.I., R. Walker, C. Bennetau-Pelissero, and A. Scalbert. 2005. Risks and safety of polyphenol consumption. Am. J. Clin. Nutr. 81: 326S–329S.

Mermelstein, N.H. 2008. Determining antioxidant activity. Food Tech. 62: 63–66.

Mitsch, P., K. Zitterl-Eglseer, B. Kohler, C. Gabler, R. Losa, and I. Zimpernik. 2004. The effect of two different blends of essential oil components on the proliferation

of *Clostridium perfringens* in the intestines of broiler chickens. Poult. Sci. 83: 669–675.

Mtambo, M.M.A., E.J. Mushi, L.D.B. Kinabo, A. Maeda-Machang'u, G.L.M. Mwamengele, M.G.S. Yongolo, R.P.C. Temu. 1999. Evaluation of the efficacy of the crude extracts of *Capsicum frutescens*, *Citrus limon*, and *Opuntia vulgaris* against Newcastle disease in domestic fowl in Tanzania. J. Ethnopharmacol. 68: 55–61.

Muhl, A. and F. Liebert. 2007. Growth, nutrient utilization and threonine requirement of growing chicken fed threonine limiting diets with commercial blends of phytogenic feed additives. J. Poult. Sci. 44: 297–304.

Mujahid, A., Y. Yoshiki, Y. Akiba, M. Toyomizu. 2005. Superoxide radical production in chicken skeletal muscle induces by acute heat stress. Poult. Sci. 84: 307–314.

Ndaw, S., M. Bergaentzle, D. Aoude-Werner, and C. Hasselman. 2000. Extraction procedures for the liquid chromatographic determination of thiamin, riboflavin and vitamin B_6 in foodstuffs. Food Chem. 71: 129 – 138.

Ninfali, P., G. Mea, S. Giorgini, M. Rocchi, and M. Bacchiocca. 2005. Antioxidant capacity of vegetables, spices and dressings relevant to nutrition. British J. Nutr. 93: 257–266.

Prior, R.L., H. Hoang, L. Gu, X. Wu, M. Bacchiocca, L. Howard, M. Hampsch-Woodill, D. Huang, B. Ou, and R. Jacob. 2003. Assays for hydrophilic and lipophilic antioxidant capacity (oxygen radical absorbance capacity ($ORAC_{FL}$)) of

plasma and other biological and food samples. J. Agric. Food Chem. 51: 3273–3279.

Quiroz, M.A., C. Hofacre, G.F. Mathis, C. Knight, and J. Dibner. 2007. Organic acid water treatment effective in decreasing *Salmonella* colonization and horizontal transmission in broiler chickens. Technical Brief Novus International, Inc. St. Louis, MO 63141.

Ravishankar, S., L. Zhu, B. Law, L. Joens, and M. Friedman. 2008. Plant-derived compounds inactivate antibiotic-resistant *Campylobacter jejuni* strains. J. Food Prot. 71: 1145–1149.

Ricke, S.C., M.M. Kundinger, D.R. Miller, and J.T. Keeton. 2005. Alternatives to antibiotics: chemical and physical antimicrobial interventions and foodborne pathogen response. Poult. Sci. 84: 667–675.

Scalbert, A., I.T. Johnson, and M. Saltmarsh. 2005. Polyphenols: antioxidants and beyond. Am. J. Clin. Nutr. 81: 215S–217S.

Shan, B., Y.Z. Cai, M. Sun, and H. Corke. 2005. Antioxidant capacity of 26 spice extracts and characterization of their phenolic constituents. J. Agric. Food Chem. 53: 7749–7759.

Si, W., X. Ni, J. Gong, H. Yu, R. Tsao, Y. Han, and J.R. Chambers. 2009. Antimicrobial activity of essential oils and structurally related synthetic food additives towards *Clostridium perfringens*. J. Appl. Microbiol. 106: 213–220.

Sklar, I.B. and R.D. Joerger. 2001. Attempts to utilize bacteriophage to combat *Salmonella enterica* serovar Enteritidis infection in chickens. J. Food Safety. 21: 15–29.

Smith-Palmer, A., J. Stewart, and L. Fyfe. 1998. Antimicrobial properties of plant essential oils and essences against five important food-borne pathogens. Letters Appl. Microbiol. 26: 118–122.

Stammati, A., P. Bonsi, F. Zucco, R. Moezelaar, H.-L. Alakomi, and A. von Wright. 1999. Toxicity of selected plant volatiles in microbial and mammalian short-term assays. Food Chem. Toxic. 37: 813–823.

Tang, X., D.A. Cronin, and N.P. Brunton. 2006. A simplified approach to the determination of thiamine and riboflavin in meats using reverse phase HPLC. J. Food Comp. Anal. 19: 831 – 837.

Ündeger, Ü., A. Basaran, G.H. Degen, and N. Basaran. 2009. Antioxidant activities of major thyme ingredients and lack of (oxidative) DNA damage in V79 Chinese hamster lung fibroblast cells at low levels of carvacrol and thymol. Food Chem. Toxic. 47: 2037–2043.

United States Department of Agriculture – Salmonella Initiative Program. http://www.fsis.usda.gov/Science/Salmonella_Initiative_Program_Criteria/index.asp. Accessed November 2009.

United States Department of Agriculture – Food Safety and Inspection Service. http://origin-www.fsis.usda.gov/PDF/FSRE-HACCP-Pathogen_Reduction-Salmonella.pdf. Accessed November 2009.

United States Department of Agriculture – Food Safety and Inspection Service. Quarterly progress reports on *Salmonella* testing of selected raw meat and poultry products.

http://www.fsis.usda.gov/Science/Quarterly_Salmonella_Results/index.asp.
Accessed February 2010.

Waihenya, R.K., M.M.A. Mtambo, and G. Nkwengulila. 2002. Evaluation of the efficacy of the crude extract of *Aloe secundiflora* in chickens experimentally infected with Newcastle disease virus. J. Ethnopharmacol. 79: 299–304.

Wang, C.C., C.Y. Chu, K.O. Chu, K.W. Choi, K.S. Khaw, M.S. Rogers, and C.P. Pang. 2004. Trolox-equivalent antioxidant capacity assay versus oxygen radical absorbance capacity assay in plasma. Clinical Chemistry. 50: 952–954.

Wang, C.Y., S.Y. Wang, and C. Chen. 2008. Increasing antioxidant activity and reducing decay of blueberries by essential oils. J. Agric. Food Chem. 56: 3587–3592.

Williamson, G. and C. Manach. 2005. Bioavailability and bioefficacy of polyphenols in humans. II. Review of 93 intervention studies. Am. J. Clin. Nutr. 81: 243S–255S.

Windisch, W., K. Schedle, C. Plitzner, and A. Kroismayr. 2008. Use of phytogenic products as feed additives for swine and poultry. J. Anim. Sci. 86: E140–E148.

Youdim, K.A. and S.G. Deans. 1999. Dietary supplementation of thyme (*Thymus vulgaris* L.) essential oil during the lifetime of the rat: its effects on the antioxidant status in liver, kidney and heart tissues. Mech. Ageing Dev. 109: 163–175.

Zafra-Gomez, A., A. Garballo, J. C. Morales, and L. E. Garcia-Ayuso. 2006. Simultaneous determination of eight water-soluble vitamins in supplemented foods by liquid chromatography. J. Agric Food Chem. 54: 4531 – 4536.

Zheng, W. and S. Y. Wang. 2001. Antioxidant activity of phenolic compounds in selected herbs. J. Agric. Food Chem. 49: 5165–5170.

Zhou, F., B. Ji, H. Zhang, H. Jiang, Z. Yang, J. Li, J. Li, and W. Yan. 2007. The antibacterial effect of cinnamaldehyde, thymol, carvacrol, and their combinations against the foodborne pathogen *Salmonella* Typhimurium. J. Food Safety. 27: 124–133.

CHAPTER 2

INTRODUCTION

Essential oils have been used in various fields for many years. Their antibacterial properties and low toxicity make them ideal as additives in food, cleaning products, and in animal feed. Research is increasing on the potential use of essential oils and their individual components as feed additives for poultry especially chickens. The increasing concern of antibiotic-resistant bacterial infections reaching the public as a consequence of antibiotic use in poultry and other meat production is influencing a search for antibiotic alternatives. Because of the commercial ramifications of a new additive in poultry feed, extensive testing and research must be done to evaluate the effects that essential oils could have on the growth performance and feed efficiency of the chickens. If either of these is negatively impacted, then it is not financially wise for producers to adopt a new feed regimen.

Consumers are increasingly concerned with the safety and quality of their food. An organic movement has emerged to become a mainstream topic, with grocery stores advertising organic and natural products. The current consumer is much more health conscious, and antioxidants are a major part of this recent health movement. Antioxidants are not just good for humans but for animals as well. The health and immune benefits they provide could promote healthier poultry flocks that are able to fight off infections better. Antioxidants like essential oils and their pure compounds are good feed additives to study the potential outcome of their effect.

OBJECTIVE

The purpose of this study was to analyze two essential oils, their respective components, and caffeic acid for their antioxidant, antibacterial, and antiviral properties and then apply the results to *in vitro* and *in vivo* experiments with chickens in order to find a potential feed additive that would not only improve growth performance but also improve the antioxidant status and pathogen resistance of chickens.

CHAPTER 3

ANTIOXIDANT, ANTIBACTERIAL, AND ANTIVIRAL ACTIVITY OF ESSENTIAL OILS AND PURE COMPOUNDS AND THEIR APPLICATION TO A *SALMONELLA* CHALLENGE IN SPF CHICKS

INTRODUCTION

Thyme oil and its major components, thymol and carvacrol, and cinnamon bark oil and its major component, cinnamaldehyde, have been shown to have good antioxidant and antibacterial properties in previous literature reports (Burt, 2004; Marinova et al., 2009; Shan et al., 2005; Zheng and Wang, 2001). Information regarding caffeic acid was limited and compared to the essential oils and pure compounds, well known to have good antioxidant and antibacterial activity. Caffeic acid is a phenolic acid and its antioxidant and antibacterial properties were thought to be similar to other phenolics like thymol and carvacrol. It was also necessary to determine the cytotoxicity of these compounds in the context of chickens. Specific pathogen free (SPF) chicken eggs provided a good environment for the evaluation of these compounds. The antibacterial effects of essential oils have been determined but little is known about their effects on viruses especially those that affect poultry. SPF chicken eggs again provided a model environment to test the antiviral effects of thyme and cinnamon bark oil, their major components and caffeic acid. Thymol and thyme oil performed well in each assay and therefore were chosen for an *in vivo* experiment with SPF chicks involving *Salmonella* challenge. The purpose of a *Salmonella*

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challenge was to determine what protective effects thymol and thyme oil could provide to either inhibit or prevent bacterial infection.

MATERIALS AND METHODS

Chemicals

Thyme and cinnamon bark oil, thymol, carvacrol, cinnamaldehyde, caffeic acid, sodium fluorescein, Trolox®(6-hydroxy-2,5,7,8 tetramethylchroman-2-carboxylic acid), AAPH (2,2′-Azobis(2-methylpropionamidine) dihydrochloride), resazurin sodium, were purchased from Sigma-Aldrich Chemical, Co. (St Louis, MO, USA). Culture media including Tryptic Soy Agar (TSA), Tryptic Soy Broth (TSB), and XLD were purchased from BD Biosciences (San Jose, CA, USA).

Essential oil and pure compound preparation

Liquid samples which included thyme and cinnamon bark oil, carvacrol, and cinnamaldehyde were prepared as 20% (v/v) solutions in 80% ethanol. Solid samples, thymol and caffeic acid, were prepared as 10% (w/v) solutions in 80% ethanol. These were considered to be stock solutions and were diluted accordingly depending on the assay used. Dilutions of at least ten minimized the effects of ethanol to be considered negligible.

Bacterial strains and culture conditions

The bacteria used for this study were obtained from the culture collection in the Department of Animal and Food Sciences at the University of Delaware (Newark, DE) and included: *Salmonella enterica* serovars Typhimurium, Kentucky, Enteritidis Nal[r], and Senftenberg. Stock cultures on TSA were stored at 4°C. Three mL

of TSB was added to a sterile test tube. A loop of bacteria culture from a tryptic soy agar TSA plate was added to the media. The test tube was then capped and placed in a 37°C incubator with shaking at 200 rpm for 18 – 24 hrs. Colonies of *Salmonella* on average grew to about 10^9 CFU/mL and were diluted as necessary.

Oxygen radical absorbance capacity (ORAC) assay

The ORAC assay followed the protocol of Wu et al. (2008). An aliquot (25 µL) of the diluted sample, blank (75 mM phosphate buffer, pH 7.4), or Trolox® calibration standards (6.25, 12.5, 25, 50, and 100 µM) was added to a well of a 96-well bottom reading microplate. Fluorescein solution, 150 µL at 0.004 µM, was added to each well of the plate, and then the plate was incubated at 37°C for 30 min before an aliquot of 25 µL AAPH solution (153 mM) was added to each well as a peroxyl generator to initiate the reaction. A Synergy™ 2 Multi-Detection Microplate Reader from BioTek Instruments, Inc. (Winooski, Vermont, USA) was programmed to record the fluorescence reading with an excitation wavelength of 485 nm and an emission wavelength of 528 nm at 1 min intervals for 1h using Gen5™ software. The final ORAC values were calculated using a linear regression model ($y = ax + b$) between the Trolox concentration (µM) and the net area under the fluorescence decay curve. Data are expressed as micromolar Trolox® equivalents (µM TE). A higher ORAC value indicated a higher antioxidant capacity.

Antimicrobial assay

The assay was modified based on previous studies (Friedman et al., 2002; Hansen et al., 2001). Stock solutions of samples were diluted accordingly directly with TSB media. Bacterial cultures were diluted to ~1000 CFU/50 µL. Using a sterile

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culture plate and working in a biosafety hood, 50 µL of bacterial cells were pipetted into each well. Then 100 µL of sample in TSB were added to each experimental well with controls as 100 µL TSB only with cells, 100 µL of 300 µg/mL kanamycin, and 150 µL of TSB only no bacteria. Fifteen µL of indicator (1 mg/mL resazurin sodium solution) were added to each well. The culture plate was then covered and placed in a 37°C incubator for approximately 24 hrs. After incubation MICs were determined as the lowest concentration of sample used that did not turn pink meaning had no growth. To be certain of MIC values, 5 – 10 µL of each MIC well and the concentration above and below was plated on TSA and placed in 37°C incubator for about 24 hrs. Minimum bactericidal concentrations (MBC) were determined if no new growth resulted following incubation. Results are based on at least three independent trials.

Cytotoxicity and Anti-NDV assays

It was important to test the cytotoxicity of the oils, their components, and caffeic acid. Testing was done using specific pathogen free (SPF) chicken embryos (Sunrise Farms Inc., Catskills, NY). The MIC (0.4, 0.78, 0.84, 1.54, and 1.83 mg/mL for thymol, carvacrol, cinnamaldehyde, cinnamon bark oil, and thyme oil, respectively) was used for each compound except caffeic acid (1 mg/mL) and prepared in sterile phosphate buffered saline (PBS). Eggs were candled to ensure fertilization and marked for hole punctures in the air sac and the chorioallantoic sac (CAS). Holes were then made in the eggs and 0.2 mL was injected by syringe into each egg via the CAS route. Four to five eggs were used per treatment with death recorded each day for one week. After a week, the eggs were opened and the embryos examined for any major differences between those that were injected and those that were not (control). Finding

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no cytotoxic effects in the embryos, the MIC for each compound was used in an anti-Newcastle Disease Virus (anti-NDV) assay. The virus strain used in each assay was NDV LaSota. The compounds were prepared the same way and viral dilutions of 10^{-4} to 10^{-9} were used with four SPF eggs per treatment and 8% ethanol (EtOH) was used as a control. One milliliter of each compound was combined with 1 mL of virus at each dilution, vortexed, and allowed to sit for 10 min. After the waiting period, 0.2mL of each virus-compound solution was injected into the CAS of SPF embryos. The eggs were candled every day for 1 week recording mortality. On the final day, the SPF eggs were placed in a refrigerator to kill the remaining live embryos for hemagglutination analysis. Fluid was removed from embryos that survived the week and those that died beyond the first $24-48$ hours post-infection. A few drops of fluid was mixed with chicken red blood cells and evaluated for the presence of hemagglutination indicating viral infection. The virus titer was then calculated to determine $\log_{10}$ reduction of virus within the embryos.

Another cytotoxicity assay was performed using only thymol (1, 2, 4 mg/mL) and thyme oil (4, 8, 16 mg/mL) at higher concentrations than their MIC because *in vitro* tests indicated that thymol and thyme oil were better antioxidant and antimicrobial agents than caffeic acid, carvacrol, cinnamaldehyde, and cinnamon bark oil. After determining these higher concentrations to be safe, two more anti-NDV assays were performed as detailed above using just thymol and thyme oil with virus dilutions 10^{-5} to 10^{-8} and 8% ethanol as the control. Hemagglutination was determined as well as the virus titer.

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Feeding Trials

All experiments were conducted with the approval of the Agriculture Animal Care and Use Committee (AACUC) of the College of Agriculture and Natural Resources, University of Delaware.

Feeding trials were conducted by supplementing antibiotic-free commercial starter feed (Powl's Feed, Peach Bottom, PA) with thymol.

Experimental Design Trial 1. The first trial involved 60 mixed sex specific pathogen free (SPF) chicks hatched from SPF eggs (Sunrise Farms Inc., Catskills, NY). Ten chicks were placed in each of 6 isolators. Thirty were fed with supplemented feed and challenged with 10^4 CFU/mL *S. enteritidis* Nal^r on day 3 of age, and the remaining 30 chicks served as control only receiving supplemented feed but not inoculation. Treatment groups were as follows: (1) Control -, no inoculation and 0.8 g/kg cellulose feed, (2) 0.8 g/kg thymol -, no inoculation and supplemented feed with 0.8 g/kg thymol, (3) 0.4 g/kg thymol -, no inoculation and supplemented feed with 0.4 g/kg thymol, (4) Control +, inoculation and 0.8 g/kg cellulose feed, (5) 0.8 g/kg thymol +, inoculation and supplemented feed with 0.8 g/kg thymol, and (6) 0.4 g/kg thymol +, inoculation and supplemented feed with 0.4 g/kg thymol. On day 7, chicks were swabbed and the swab plated on XLD with nalidixic acid and 0.1 mL peptone water. Plates were then incubated at 37°C for 24 – 48 hrs for *S.* Enteritidis Nal^r growth. On day 14 of the trial, chicks were euthanized and the ceca removed and placed in stomacher bags. To the bags 50 mL peptone water was added and stomached for one minute. A 10 mL aliquot was removed and placed in sterile 15 mL tubes. The 10 mL stock was diluted 10^2, 10^4, and 10^6. Each dilution and nondilution was plated

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on XLD with nalidixic acid and incubated at 37°C for 24 – 48 hrs for population count.

Experimental Design Trial 2. The second trial is based on results from Trial 1 with further modification again using mixed sex SPF chicks hatched from SPF eggs (Sunrise Farms Inc., Catskills, NY). Seventy SPF chicks were used with 10 chicks in each isolator for those inoculated with *S. enteritidis* Nal^r. Chicks were inoculated on the day they hatched with 10^4 and 10^8 CFU/mL. Treatment groups were (1) 10^8 CFU infection and 4 g/kg cellulose, (2) 10^8 CFU infection and 0.4 g/kg thymol, (3) 10^8 CFU infection and 4 g/kg thymol, (4) 10^4 CFU infection and 4 g/kg thymol, (5) 10^4 CFU infection and no feed additive, (6) no infection and 0.4 g/kg thymol, and (7) no infection and 4 g/kg thymol. Due to insufficient hatching, 8 chicks were placed in the group no CFU and 0.4 g/kg thymol and 9 chicks in the group no CFU and 4 g/kg thymol. Cecal swabs were taken on day 7 of the trial and plated with 0.1 mL of 0.1% peptone water onto XLD agar plates with nalidixic acid. The plates were incubated at 37°C for 24 hrs and examined for growth. On day 14, the final day of the trial the chicks were sacrificed and ceca removed. Ceca were weighed, placed individually in stomacher bags with 50 mL 0.1% peptone water and stomached for one minute. A 10 mL aliquot was removed and placed in sterile 15 mL centrifuge tubes. This stock was diluted with peptone water 10^{-2}, 10^{-4}, and 10^{-6}. 0.1 mL of each dilution and stock were plated on XLD with nalidixic acid and incubated at 37°C 24 – 48 hrs for population count.

Statistical Analysis

        Statistical analysis of *Salmonella* challenge data was done using fit model to evaluate block effect and oneway ANOVA and the Student's t-test for multiple comparison from the program JMP 8.0.1 distributed by SAS Institute, Inc (Cary, NC). Significance was determined using a p-value less than 0.05.

RESULTS AND DISCUSSION

Antioxidant Activity

        The purpose of the ORAC assay was to determine the antioxidant capacity of the essential oils and pure compounds. The ORAC value or antioxidant capacity of the tested materials was determined in relation to the Trolox standard curve and expressed as Trolox equivalents (TE) (Figure 1a and 1b). The ORAC values increased linearly for caffeic acid, thymol, carvacrol, cinnamaldehyde, cinnamon bark oil, and thyme oil with concentrations of $0.00025 - 0.002$, $0.001 - 0.008$, $0.005 - 0.04$, $0.2 - 1.7$, $0.05 - 0.7$, and $0.002 - 0.04$ mg/mL, respectively. The rank of antioxidant activities of the tested compounds was: caffeic acid > thymol > carvacrol > cinnamaldehyde. Thyme oil had higher antioxidant activities as compared to cinnamon bark oil. Synergistic effects were seen in the 1:1 binary combinations of thymol and caffeic acid (70 µM TE) and thymol and carvacrol (60 µM TE).
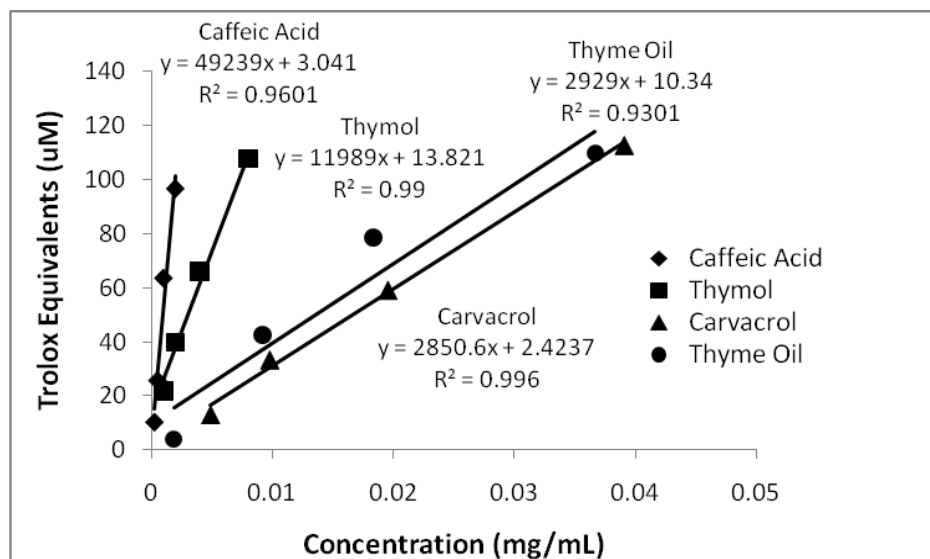
Figure 1a. Linear ORAC values for caffeic acid, thymol, carvacrol and thyme oil.
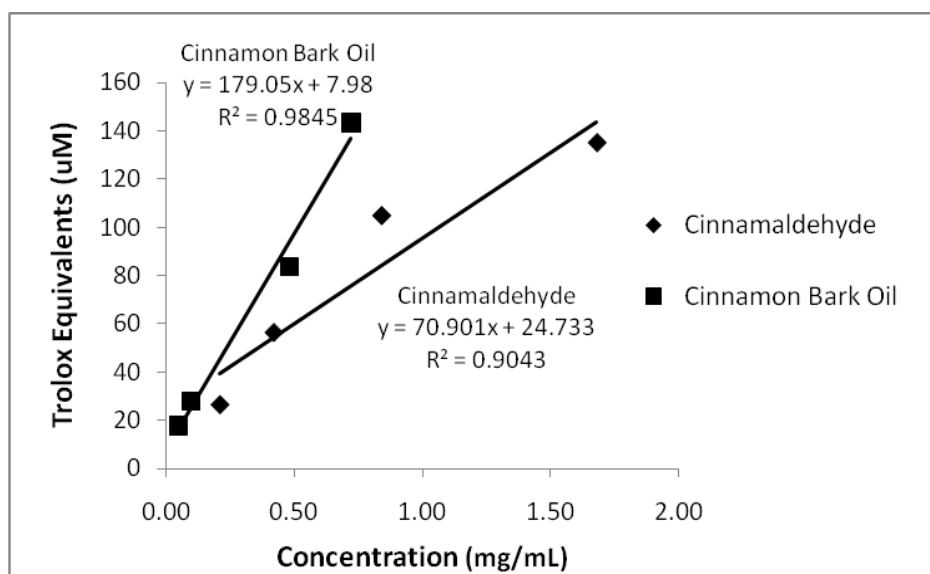

Figure 1b. Linear ORAC values for cinnamon bark oil and its component cinnamaldehyde.

The ORAC value for caffeic acid is comparable with that reported by

Gomez-Ruiz et al. (2007) which was 4.5 µM TE, which falls within the linear range of

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caffeic acid found in this study. Caffeic acid is considered a good antioxidant found in coffee, tea, sweet potatoes, and sunflower seeds (Marinova et al., 2009). ORAC values for carvacrol and cinnamaldehyde found in literature were stated as 50 µmol TE/g and 45 µmol TE/g, respectively (Wang et al., 2008). Using the total equivalent antioxidant capacity (TEAC) assay, the antioxidant activity of cinnamon bark oil obtained from *Cinnamomum* species from China and Indonesia was reported as 61.75 and 108 mmol of Trolox/100 g of dry weight, respectively (Shan et al., 2005). Thyme oil from a *Thymus vulgaris* species from New Zealand had a TEAC value of 38 mmol of Trolox/100 g of dry weight and another study reported an ORAC value of 19.5 µmol TE/g of fresh weight (Shan et al., 2005; Zheng and Wang, 2001). Caffeic acid and thymol in our study had the greatest oxygen radical scavenging ability. This may be due in part to the fact that both compounds are phenols. It has been reported that phenolic compounds exhibit antioxidant activity which is correlated with health benefits (Kähkönen et al., 1999). The high antioxidant capacity of cinnamon bark and thyme oil and their respective components make them a beneficial and natural alternative to currently used food and animal feed additives. These compounds are generally recognized as safe (GRAS) by the FDA and FEMA (Flavor and Extract Manufacturers' Association) as food additives. The findings of this study were in agreement with previous reports confirming the high antioxidant activity of thyme and cinnamon bark oil, their components, and caffeic acid.

Antimicrobial effects

These compounds all had antibacterial effects against the four strains of *Salmonella*. Results are shown in Table 1. *Salmonella* Typhimurium DT 104 was the

most resistant compared to the other three strains. Therefore, a higher concentration of each compound was necessary for inhibition. The use of caffeic acid resulted in a poorer performance as an antibacterial agent in contrast to its antioxidant effects. Against *S.* Typhimurium, thymol was superior and cinnamon bark oil stronger than thyme oil. Similar MICs were observed across the other three strains for each compound except carvacrol which required a higher concentration to inhibit *S.* Senftenberg compared to *S.* Kentucky and *S.* Enteritidis Nal[r].

Table 1. Minimum Inhibitoty Concentrations/Minimum Bactericidal Concentrations in mg/mL for six plant extracts against four strains of *Salmonella*.

Compound	*Se* Typhimurium	*Se* Kentucky	*Se* Enteritidis Nal[r]	*Se* Senftenberg
Caffeic Acid	2.0	1.0	1.0	1.0
Thymol	0.4	0.4	0.4	0.4
Carvacrol	0.78	0.195	0.195	0.78
Cinnamaldehyde	0.84	0.21	0.21	0.21
Cinnamon Bark Oil	1.54	0.77	0.77	0.77
Thyme Oil	1.8	1.5	1.5	1.5

Helander et. al. (1998) reported MIC values for thymol and cinnamaldehyde as 1 and 3 mM, respectively against *S.* Typhimurium. Thymol and cinnamaldehyde in this study had a 2.7 mM and 6.4 mM MIC, respectively, which is comparable although higher than the literature value. Carvacrol was reported to have a MIC of 250 μg/mL against *S.* Typhimurium, which is three times lower than the value in this study (Kim et al., 1995). Thyme oil's activity against *S.* Typhimurium was reported to range from 0.45 - > 20 μL/mL (Burt, 2004). In this study, we found that

thyme oil was effective as low as 0.002 µL/mL. The literature MIC value reported for caffeic acid against *S.* Typhimurium was 350 µg/mL which was much lower than 2 mg/mL reported here (Tuncel and Nergiz, 1993). Zhou et al. (2007) found cinnamaldehyde, thymol, and carvacrol to be affective growth inhibitors of *S.* Typhimurium with MICs of 200, 400, and 400 mg/L, respectively. Synergistic effects were also found for combinations of cinnamaldehyde/thymol, cinnamaldehyde/carvacrol, and thymol/carvacrol. Synergistic effects were also seen against *S.* Typhimurium in this study using 1:1 binary combinations of the same compounds at their MIC. The difference between the previously reported values and those shown here is the result of different methods and media used as well as the general preparation of samples and bacterial cultures. Also the differences between strains are most likely due to growth differences between strains and morphological differences within the bacteria.

The mode of action of these essential oils and purified compounds against bacteria has been greatly discussed. Generally essential oils have been more effective against Gram-positive bacteria e.g. *Listeria monocytogenes*, *Staphylococcus aureus*, and *Bacillus cereus* due to their lack of an outer membrane like Gram-negative bacteria (Chorianopoulos et al., 2004; Burt, 2004). Essential oils have been effective at penetrating the outer membrane of Gram-negative bacteria like *Salmonella* and *E. coli*, which is necessary for bactericidal activity (Helander et al., 1998). Also the composition of the oils and general structures of specific components have been reported as the reason why they are such effective antimicrobials. For example, synergistic effects were seen in this and other studies suggesting that more than one

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reactive component contribute to the antibacterial activity of thyme, sage, and oregano oils (Burt, 2004). Phenolic components, like thymol and carvacrol, are believed to be inherent to the antibacterial activity of their respective oils which is why individually both are effective (Burt, 2004).

Cytotoxic and antiviral effects

The results of the cytotoxicity tests using chicken embryos indicated that at the MICs there were no adverse effects on the embryos (Figure 2). Upon necropsy, all embryos appeared to be well developed with the same physical characteristics of the embryos that did not receive any test agent. Increasing the concentrations of thymol and thyme oil also did not have toxic effects on the embryos. Again their appearance was similar to that of the control embryos.
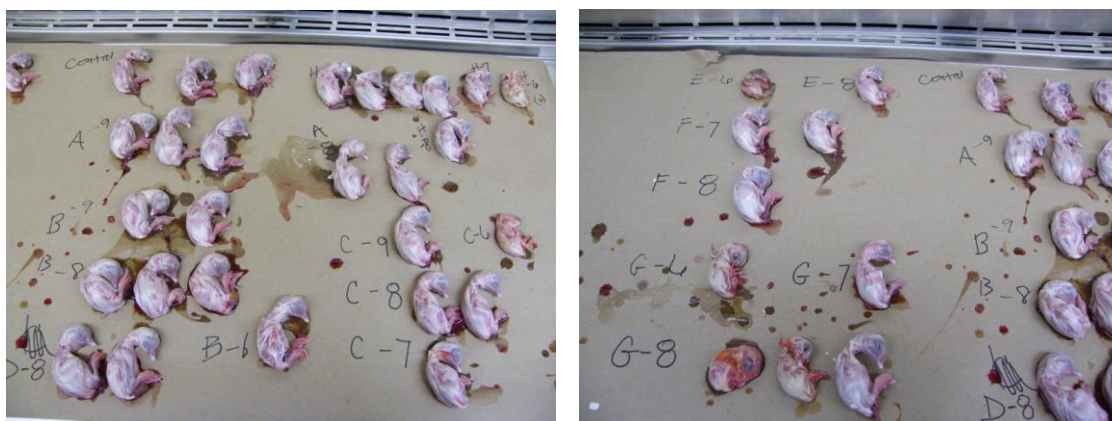

Figure 2. Photographs depicting embryo necropsy following cytotoxicity assay with both essential oils and each pure compound.

The results of the hemagglutination test after the first anti-NDV assay using all of the compounds at their MIC are as follows. The virus titer calculation can

be found in Table 2. At the lowest virus dilutions (10^{-4} and 10^{-5}) all of the fluids were positive for hemagglutination for each compound and control and most of the fluid came from dead embryos. Many compounds also had hemagglutination at 10^{-6} and 10^{-7} but the highest virus dilutions (10^{-8} and 10^{-9}) tended be negative for hemagglutination. Thymol had one negative result at the 10^{-6} dilution from an embryo that survived the whole week which was not seen for any other compound or the controls.

Table 2. Virus titer results for each compound and control from Anti-NDV #1

Sample	Virus Titer
Caffeic Acid, 1 mg/mL	$10^{8.2}$ / mL
Thymol, 0.4 mg/mL	$10^{7.5}$ / mL
Carvacrol, 0.78 mg/mL	$10^{8.5}$ / mL
Cinnamaldehyde, 0.84 mg/mL	$10^{8.2}$ / mL
Cinnamon Bark Oil, 1.54 mg/mL	$10^{7.5}$ / mL
Thyme Oil, 1.83 mg/mL	$10^{7.7}$ / mL
8% EtOH	$10^{7.7}$ / mL

Only the thymol and two essential oils showed either a reduction or maintenance of viral titer compared to the control. A greater than 2 $\log_{10}$ reduction is necessary for significance. Therefore thymol was considered to have performed best and since it is the major component of thyme oil, both were chosen for further anti-NDV assays.

The second anti-NDV assay used only thymol (1,2,4 mg/mL) and thyme oil (4,8,16 mg/mL) but the same control 8% ethanol. Fewer virus dilutions were used since the 10^{-4} dilution appeared to have too much virus for the compounds to

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inactivate and 10^{-9} was easily inactivated. Therefore virus dilutions of 10^{-5} to 10^{-8} were used in subsequent assays. Hemagglutination results for thymol at 2 and 4 mg/mL were all negative and most of the eggs from 4 mg/mL thymol survived throughout the week. The results for the thyme oil were more varied with some positive hemagglutination at high virus dilutions; however the mortality was low overall for each concentration of thyme oil. The virus titer was calculated and is presented in Table 3. While none of the compounds showed a greater than 2 $\log_{10}$ reduction, thyme oil (4 and 16 mg/mL) and thymol at 4 mg/mL showed 1.4 and 1.6 log reduction, respectively, which indicates that the virus may be inhibited by thymol and thyme oil.

Table 3. Virus titer results for thymol and thyme oil from Anti-NDV #2

Sample	Virus Titer
1 mg/mL Thymol	$10^{7.2}$ / mL
2 mg/mL Thymol	$10^{7.3}$ / mL
4 mg/mL Thymol	$10^{6.6}$ / mL
4 mg/mL Thyme Oil	$10^{6.8}$ / mL
8 mg/mL Thyme Oil	$10^{8.2}$ / mL
16 mg/mL Thyme Oil	$10^{6.8}$ / mL
8% EtOH	$10^{8.2}$ / mL

A third anti-NDV assay was performed to see if results from the second assay using only thymol and thyme oil were repeatable. Concentrations for thymol were 2, 4, and 6 mg/mL and for thyme oil were 4, 8, and 16 mg/mL with same ethanol control. Thymol at 1 mg/mL was not used because it did not show good inhibitory effects like 2 and 4 mg/mL in the previous assay. Both 2 and 4 mg/mL thymol had

total embryo survival after the one week. All eggs for all virus dilutions (10^{-5} to 10^{-8}) were negative for hemagglutination for 2 mg/mL thymol. Thymol at 4 mg/mL also had complete survival of embryos but hemagglutination testing revealed slight positives with delayed clumping in eggs from dilutions 10^{-6} to 10^{-8}. The 10^{-5} dilution had negative hemagglutination for all eggs. The highest concentration of thymol (6 mg/mL) had mortality which could be due to the high concentration of thymol and not solely the virus since mortality was greater in 10^{-7} and 10^{-8} dilutions of virus. All eggs from the 10^{-5} dilution survived and were all negative for hemagglutination. One egg from the 10^{-6} dilution died within the first 24 h which is considered non-specific death and is not considered due to infection. The embryos that survived were all negative for hemagglutination. The thyme oil concentrations again had varied mortality and hemagglutination results. The lowest thyme oil treatment (4 mg/ml) had little mortality and negative hemagglutination was seen in 10^{-5} to 10^{-7} dilutions but the 10^{-8} dilution was all positive. Thyme oil at 8 mg/mL had some non-specific death but no deaths were recorded for the rest of the week. All eggs tested for hemagglutination were negative. With the highest thyme oil treatment (16 mg/mL) more mortality was seen compared to the lower concentrations and positive hemagglutination was seen at the 10^{-7} dilution. Also the control (8% EtOH) had high mortality with positive hemagglutination at -5 and -8 dilutions and negative hemagglutination at 10^{-6}, 10^{-7}, and 10^{-8} dilutions.

The virus titer calculations (Table 4) showed 2.7 and 2.8 $\log_{10}$ reduction of virus for 6 mg/mL thymol and 8 mg/mL thyme oil, respectively. A 3.4 $\log_{10}$ reduction in virus was seen for 2 and 4 mg/mL thymol and 4 mg/mL thyme oil.

Table 4. Virus titer results for thymol and thyme oil from Anti-NDV #3

Sample	Virus Titer
2 mg/mL Thymol	10^6 / mL
4 mg/mL Thymol	10^6 / mL
6 mg/mL Thymol	$10^{6.7}$ / mL
4 mg/mL Thyme Oil	10^6 / mL
8 mg/mL Thyme Oil	$10^{6.6}$ / mL
16 mg/mL Thyme Oil	$10^{8.3}$ / mL
8% EtOH	$10^{9.4}$ / mL

These results were comparable to those seen in the second anti-NDV assay for 4 mg/mL thymol and 4 mg/mL thyme oil. Complete consistency between the two assays is not possible due to chicken embryo differences. Even though the concentrations of the compounds were kept the same, it is impossible to predict how each embryo will react to being injected with these samples and the virus. Essential oils used as antivirals have few literature citations and none against NDV; however, tea extracts have been studied as antivirals and showed inhibitory effects against adenovirus, Epstein-Barr virus, herpes simplex virus, HIV, and influenza virus (Friedman, 2007). Studies with rural free-range poultry flocks in Tanzania used *Aloe* species as a method of NDV control. Results showed on day 2 post-infection that chickens treated with *Aloe* had no clinical signs of infection unlike the untreated birds, and *Aloe* did not just delay clinical signs but also mortality and appeared to reduce the severity of lesions found in the chickens post-mortem (Waihenya et al., 2002). Chinese herbs have also been used as an immune stimulator in chickens infected with NDV, and the study found that the Chinese herbs were effective at increasing *in vitro*

production of chicken embryos fibroblasts in response to the infection (Kong et al., 2006).

S. enterica **serovar Enteritidis Nal^r challenge**

The results from the first feed trial that challenged chicks with *S.* Enteritidis Nal^r were not conclusive (Table 5). In the cecal swabs collected on day 7 there was no colony growth for birds not inoculated with *S.* Enteritidis Nal^r as expected. The *Salmonella* positive control plates and the *Salmonella* positive 0.4 and 0.8 g/kg thymol plates had black colonies indicating *S.* Enteritidis Nal^r growth. The cecal samples taken on day 14 were then plated on XLD were enumerated for colonies of *S.* Enteritidis Nal^r. Those plates for the uninoculated chicks had no growth as expected. For inoculated chicks, colony growth was similar.

Table 5. Average cecal counts and range of counts from *S. enterica* Enteritidis Nal^r challenge in SPF chicks (Trial 1)

Treatment	Average Cecal Counts (10^5cfu/g)	Range of Counts (cfu/g)
Control, 0.8 g/kg cellulose	$2.0 \pm .68^a$	$1.1 - 3.5 \times 10^5$
Thymol 0.4 g/kg	$1.3 \pm .54^b$	$7.4 \times 10^4 - 2.3 \times 10^5$
Thymol 0.8 g/kg	$1.5 \pm .39^{ab}$	$9.1 \times 10^4 - 2.2 \times 10^5$

[a,b] Means not connected by the same letter in a column are statistically different ($P < 0.05$).

There was a significant difference between the birds fed the control feed (0.8 g/kg cellulose) and those fed 0.4 g/kg thymol. However there was no log reduction in colonies. Also the control group had no deaths recorded whereas the 0.4 g/kg thymol treatment group had two chicks die throughout the course of the trial. It is possible that the inoculation amount, 10^4 CFU/mL at day 3 was not large enough to

colonize the chicks' ceca which was why higher cecal counts were not found in the inoculated control group. The amount of thymol added to feed may also have been an issue with a larger amount necessary to have pronounced effects against *Salmonella*.

Results from the second *Salmonella* trial (Trial 2) are presented in Table 6 where chicks were inoculated on day of hatch unlike day 3 in Trial 1. Inoculation of 10^8 CFU/mL was too high for the chicks and therefore no apparent affect from the thymol was observed. In treatment group 4 (10^4 CFU and 4 g/kg thymol) some inhibition or possibly protection by thymol was observed, but the results were not statistically significant from its control, treatment group 5 (10^4 CFU and No Additive). The lowest recorded cecal count was on a 10^5 scale compared to treatment group 5 (10^4 CFU and No Additive) which had counts as high as 10^9 cfu/g. Comparing average cecal counts for treatment groups 4 and 5 (10^4 CFU and 4 g/kg thymol and 10^4 CFU and No Additive), a 2-log reduction in *Salmonella* was observed.

Table 6. Average cecal counts and range of counts from *S. enterica* Enteritidis Nal[r] challenge in SPF chicks (Trial 2)

Treatment	Average Cecal Counts (10^8cfu/g)	Range of Counts (cfu/g)
(1) 10^8 cfu and 4 g/kg Cellulose	86.9 ± 140.4^{ab}	$4.2 \times 10^8 - 3.3 \times 10^{10}$
(2) 10^8 cfu and 0.4 g/kg Thymol	105.8 ± 136.5^{a}	$3.4 \times 10^8 - 3.4 \times 10^{10}$
(3) 10^8 cfu and 4 g/kg Thymol	1.8	$1.8 \times 10^8*$
(4) 10^4 cfu and 4 g/kg Thymol	0.3 ± 0.5^{b}	$3.2 \times 10^5 - 1.5 \times 10^8$
(5) 10^4 cfu and No Additives	10.3 ± 6.5^{ab}	$4.3 \times 10^8 - 1.8 \times 10^9$

[a,b] Means not connected by the same letter in column are statistically different ($P < 0.05$).
* Only one chick survived through day 14 of the trial and the treatment was not included in statistical analysis.

While the results were not conclusive, comparing these two treatment groups indicated a possible interaction between the thymol and the *Salmonella*. Nine of the ten chicks from the 4 g/kg thymol group survived the duration of the trial (14 days) which was the most for the groups that received bacterial inoculation 10^4 CFU/mL. The thymol appeared to have protected the remaining chicks from developing a severe *Salmonella* infection that caused mortality in the chicks with the higher inoculation amount. Further study is required to determine the actual effects of thymol on the *Salmonella* in the ceca of the chicks. A higher concentration of thymol and a range of inoculation amounts may be necessary for better and more definitive results.

CONCLUSION

The rank of antioxidant activities of the tested compounds was: caffeic acid > thymol > carvacrol > cinnamaldehyde. Thyme oil had higher antioxidant activities compared to cinnamon bark oil. Synergistic effects were seen in the 1:1 binary combinations of thymol and caffeic acid (70 µM TE) and thymol and carvacrol (60 µM TE). Caffeic acid had the greastest antioxidant activity but showed poor performance as an antibacterial agent. Thymol had the greatest antibacterial effects against *S*. Typhimurium having the lowest MIC. Carvacrol, the isomer of thymol, had a higher MIC than thymol against *S*. Typhimurium and *S*. Senftenberg but a lower MIC than thymol against *S*. Kentucky and *S*. Enteritidis Nal[r]. Cinnamon bark oil also performed better than thyme oil as an antibacterial against all four serovars. No

cytotoxic effects were found to be caused by these compounds on SPF chicken embryos.

Antiviral effects were inconclusive although greater than 2 $\log_{10}$ reduction in virus was seen for 2, 4, and 6 mg/mL thymol and 4 and 8 mg/mL thyme oil. Further studies are necessary to determine the antiviral activity of essential oils and their pure components. Results of the first *Salmonella* challenge trial with thymol supplemented feed did not show a $\log_{10}$ reduction in *S.* Enteritidis Nal[r] and the results for the second trial were not conclusive. However, thymol appeared to have protected the remaining chicks from (4) 10^4 cfu and 4 g/kg Thymol from developing a severe *Salmonella* infection that ultimately resulted in the death of chicks given the higher dose. Further *Salmonella* challenge trials are necessary to determine the interaction of the bacteria and the feed additives inside the gut of chickens and whether thymol can protect chickens from infection or reduce infection symptoms.

REFERENCES

Burt, S. 2004. Essential oils: their antibacterial properties and potential applications in foods---a review. Int. J. Food Microbiol. 94: 223–253.

Chorianopoulos, N., E. Kalpoutzakis, N. Aligiannis, S. Mitaku, G. Nychas, and S.A. Haroutounian. 2004. Essential oils of *Satureja*, *Origanum*, and *Thymus* species: Chemical composition and antibacterial activities against foodborne pathogens. J. Agric. Food Chem. 52: 8261–8267.

Friedman, M. 2007. Overview of antibacterial, antitoxin, antiviral, and antifungal activities of tea flavanoids and teas. Mol. Nutr. Food Res. 51: 116–134.

Friedman, M., P.R. Henika, and R.E. Mandrell. 2002. Bactericidal activities of plant
essential oils and some of their isolated constituents against *Campylobacter jejuni*,
Escherichia coli, *Listeria monocytogenes*, and *Salmonella enterica*. J. Food Prot.
65: 1545–1560.

Gomez-Ruiz, J.A., D. Leake, and J. Ames. 2007. In vitro antioxidant activity of
coffee compounds and their metabolites. J. Agric. Food Chem. 55: 6962–6969.

Hansen, L.T., J.W. Austin, and T.A. Gill. 2001. Antibacterial effect of protamine in
combination with EDTA and refrigeration. Int. J. Food Microbiol. 66: 149–161.

Helander, I.M., H. Alakomi, K. Latva-Kala, T. Mattila-Sandholm, I. Pol, E.J. Smid,
L.G.M. Gorris, and A. von Wright. 1998. Characterization of the action of
selected essential components on gram-negative bacteria. J. Agric. Food Chem.
46: 3590–3595.

Kähkönen, M.P., A. I. Hopia, H. J. Vuorela, J. Rauha, K. Pihlaja, T. S. Kujala, and M.
Heinonen. 1999. Antioxidant activity of plant extracts containing phenolic
compounds. J. Agric. Food Chem. 47: 3954–3962.

Marinova, E. M., A. Toneva, and N. Yanishlieva. 2009. Comparison of the
antioxidative properties of caffeic and chlorogenic acids. Food Chem.. 114:
1498–1502.

Shan, B., Y.Z. Cai, M. Sun, and H. Corke. 2005. Antioxidant capacity of 26 spice
extracts and characterization of their phenolic constituents. J. Agric. Food Chem.
53: 7749–7759.

Tuncel, G. and C. Nergiz. 1993. Antimicrobial effect of some olive phenols in a
laboratory medium. Letters Appl. Microbiol. 17: 300–302.

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Wang, C.Y., S.Y. Wang, and C. Chen. 2008. Increasing antioxidant activity and reducing decay of blueberries by essential oils. J. Agric. Food Chem. 56: 3587–3592.

Wu, C., S.K. Duckett, J.P.S. Neel, J.P. Fontenot, and W.M. Clapham. 2008. Influence of finishing systems on hydrophilic and lipophilic oxygen radical absorbance capacity (ORAC) in beef. Meat Sci.. 80: 662–667.

Zheng, W. and S. Y. Wang. 2001. Antioxidant activity of phenolic compounds in selected herbs. J. Agric. Food Chem. 49: 5165–5170.

Zhou, F., B. Ji, H. Zhang, H. Jiang, Z. Yang, J. Li, J. Li, and W. Yan. 2007. The antibacterial effect of cinnamaldehyde, thymol, carvacrol, and their combinations against the foodborne pathogen *Salmonella* Typhimurium. J. Food Safety. 27: 124–133.

CHAPTER 4

EFFECTS OF THYMOL AND THYME OIL SUPPLEMENTED FEED ON BROILER PERFORMANCE, SERUM ANTIOXIDANT LEVEL, AND VITAMIN CONTENT OF BREAST MEAT

INTRODUCTION

Thymol and thyme oil performed the best in previous *in vitro* and *in vivo* assays and therefore were chosen to be added to broiler feed in a 28 day feed trial. The purpose of this feed trial was to determine the effects both additives would have on the growth performance of broiler chicks. If negative effects were found, thymol and thyme oil would not be considered economically wise feed additives for the poultry industry. Histology samples were taken to analyze the possible effects thymol and thyme oil may have on the liver, kidney, intestine, and ceca of broilers. Damage to these organs by thymol and thyme oil could cause serious health complications and reduce the quality of the birds produced. Because thymol and thyme oil are antioxidants, it was important to determine if the antioxidant level of the broilers' serum changed as a result of feed supplementation. Finally the quality of the breast meat from the broilers was evaluated by determination of the B vitamin content to maintain that supplementation would not affect the nutritional quality of the breast meat. The main goal of these experiments was to evaluate how thymol and thyme oil added to feed would affect the general performance of broilers as well as their overall health and quality of their meat products.

MATERIALS AND METHODS

Chemicals

Thyme oil, thymol, trichloroacetic acid, riboflavin, nicotinamide, nicotinic acid, pyridoxamine dihydrochloride, pyridoxal hydrochloride, pyridoxine hydrochloride, taka-diastase, acid phosphatase, and papain were purchased from Sigma-Aldrich Chemical, Co. (St Louis, MO, USA). Trappsol®, randomly methylated BCD (RMCD) was purchased from CTD, Inc. (High Springs, FL, USA).

Feeding Trial

All experiments were conducted with the approval of the Agriculture Animal Care and Use Committee (AACUC) of the College of Agriculture and Natural Resources, University of Delaware.

Experimental Design. This feed trial examined the growth performance and feed conversion efficiency of mixed sex Ross broiler chickens with antibiotic-free feed (Powl's Feed, Peach Bottom, PA) supplementedwith thymol and thyme oil. Four hundred broiler chicks were obtained from Mountaire Farms (Milford, DE) and placed in 48 pens (24 pens/room) for seven days and received feed *ad libitum* throughout the trial. Two rooms (126A and 126B) were used in the experiment. On the seventh day, chicks were euthanizeded by cervical dislocation from each pen to yield five/pen. At random, two chicks from each room that were euthanized were bled for serum analysis. The 240 chicks were separated into 48 pens with 8 pens/treatment and 6 treatment groups. Treatments were based on the cytotoxicity results and two *in vitro* bioassays and were as follows: A (control 0.8g/kg cellulose), B (0.2g/kg thymol), C (0.4g/kg thymol), D (0.8g/kg thymol), E (2 mL/kg thyme oil), and F (4 mL/kg thyme

oil). The 240 chicks (five chicks/pen) were fed supplemented feed for 9 days. Cellulose was added to the control treatment because it is a common food additive and also fills the space left by adding thymol and thyme oil in the other treatment groups. On the ninth day, they were removed from the pens and two euthanized from each pen to yield three chicks/pen. Those two chicks euthanized from each pen were bled and breast meat removed. The trial proceeded for another 11 days, and then the remaining chicks were euthanized. Blood and breast meat was removed from two of the three chicks in each pen. Histological samples (kidney, liver, cecum, small intestine) were taken from four chicks (two/room) prior to receiving supplemented feed and subsequently taken from one chick/pen after each time period. Breast meat samples were used for a vitamin analysis. Conversion efficiency was calculated based on the gain of broilers and corrected for mortality. Histopathology was performed by Conrad Pope D.V.M., Ph.D., Professor of Veterinary Pathology and Histology, Dept of Animal and Food Sciences, University of Delaware (Newark, DE).

Hydrophilic and Lipophilic ORAC of Serum Samples

The protocol for this method was based on Wu et al. (2008) and Prior et al. (2003). The blood taken from the chicks was allowed to first sit for about 24 hrs in order to easily remove the serum. 100 µL of serum was mixed with 200 µL of ethanol and 100 µL of water in a 2mL microcentrifuge tube. 400 µL of hexane was added to this, mixed, and sat for $1 - 2$ min for separation. Then the samples were centrifuged for 5 min at 17,970 x g. The hexane layer was removed and placed in an alternate microcentrifuge tube. To the first tube, another 400 µL of hexane was added, allowed to settle, and centrifuged for 5 min at 17, 970 x g. The hexane layer was removed and

added to the previous hexane layer. The hexane extracts were dried under nitrogen atmosphere and then used for lipophilic ORAC. The resulting liquid after hexane extraction was used for hydrophilic ORAC. The procedure was performed as described above for antioxidant determination of plant extracts. Dilutions were made as necessary and results were reported as μmol TE/mL of serum sample.

Hydrophilic ORAC with protein removal required the resulting layer after hexane extractions to be mixed with 400 μL of 50% trichloroacetic acid (TCA). The mixture was centrifuged for 5 min at 17,970 x g followed by removal of 160 μL of supernatant which was added to 840 μL of 75 mM phosphate buffer pH 7.4. This was diluted as necessary with results reported as μmol TE/mL of serum sample.

For the lipophilic ORAC, the dried extracts were dissolved in 250 μL acetone and 750 μL of 7% RMCD (7g in 100 mL of DI water). The 75 mM phosphate buffer pH 7.4 was still used for flourescein and AAPH preparation. Trolox standards were dissolved in 7% RMCD which also served as the blank instead of the buffer. Results were reported as μmol TE/mL of serum sample.

Vitamin Analysis

Vitamin Standards. The vitamin standards were used to make standard curves for vitamin determination of chicken breast samples. Each standard was weighed to 10mg dissolved in either 0.1N HCl or 0.1M NaOH depending on the vitamin and diluted as necessary to create a standard curve. The standard curves were 0.05 – 1.0, 5.0 – 100, 1.0 – 25, 0.1 – 1.0, 0.5 – 10, and 0.5 – 5.0 μg/mL for riboflavin, nicotinamide, nicotinic acid, pyridoxamine, pyridoxal, and pyridoxine, respectively.

Sample Preparation. Breast meat samples were taken from euthanized birds after two time periods. The method for vitamin analysis of meat samples is based on that developed by Wu et al. (2008). Samples for vitamin analysis were pulverized using a small food processor and five grams were placed in an amber bottle with 44mL of 0.5M sodium acetate with pH adjusted to 4.5. Taka-diastase, acid phosphatase, and papain enzymes were added to each sample and placed in a 37°C water bath for 16 – 18 hours for enzymatic digestion to release vitamins. Following enzymatic digestion, 50% TCA was added and the temperature of the water bath raised to 55°C for 10 min to stop enzyme digestion. Samples were then removed from the water bath and the liquid transferred to 50mL centrifuge tubes. This liquid was centrifuged at 2616 x *g* for 10 min after which 1.4mL of the supernatant was removed and placed in a microcentrifuge tube. The supernatant was centrifuged at 15,403 x *g* for 10min and then 1mL was transferred to an amber HPLC vial for analysis.

HPLC Conditions. Analysis was performed using a Shimadzu HPLC with a C_{18} column kept at 35°C. The time program lasted 30min with mobile phase A (0.001M sulfuric acid) and 1% of mobile phase B (methanol) for 8 min increasing to 30% B at 25min. Fluorescence detection was used for vitamins B2 and B6 with excitation/emission wavelengths of 290/410 for vitamin B6 elution and changed to 400/520 at 20min for elution of vitamin B2. UV detection at 265nm was used for the detection of nicotinamide and nicotinic acid. Peak areas and retention times were compared to the vitamin standard curves to determine the level of each vitamin in breast meat samples.

Statistical Analysis

Statistical analysis of the conversion efficiency results, serum ORAC data, and vitamin analysis was done using a fit model to evaluate block effect and oneway ANOVA and Student's t-test for multiple comparison from the program JMP 8.0.1 distributed by SAS Institute, Inc (Cary, NC). Significance was determined using a p-value less than 0.05.

RESULTS AND DISCUSSION

Conversion Efficiency

Supplementation of poultry feed with thymol and thyme oil had no significant affect on the conversion efficiency of broiler chicks. Feed conversion efficiency for each treatment and the control is presented in Tables 7 and 8. On day 16, there was no significance difference between the treatments and the control. Following the second time period, day 28, a block effect from the two rooms was seen but there was no significant difference found between the treatments. The room difference however may be due to water leakage issues that resulted in wet feed in four pens in one room (126B) over a 24 h period.

Table 7. Growth performance and conversion efficiency of broilers day 16

Treatment	16 d BW	Gain	Feed Intake	Conversion Efficiency
	kg/bird with 8 pens/treatment			Day 16
A (Ctrl)	0.502 ± .015	0.364 ± .016	0.488 ± .119[*]	1.33 ± .28
B (0.2g/kg Thymol)	0.484 ± .034	0.344 ± .035	0.494 ± .037[*]	1.47 ± .21
C (0.4g/kg Thymol)	0.494 ± .007	0.352 ± .006	0.512 ± .056	1.45 ± .17
D (0.8g/kg Thymol)	0.488 ± .033	0.348 ± .032	0.472 ± .026	1.36 ± .13
E (2mL/kg Thyme Oil)	0.488 ± .044[**]	0.362 ± .017	0.468 ± .059	1.30 ± .17
F (4mL/kg Thyme Oil)	0.488 ± .017	0.348 ± .014	0.492 ± .057	1.42 ± .18

* One pen in treatment A and B contained wet feed and were not included in calculations.

** One pen contained a dead chick with weight included in conversion efficiency calculations only.

Table 8. Growth performance and conversion efficiency of broilers day 28

Treatment	28 d BW	Gain	Feed Intake	Conversion Efficiency
	kg/bird with 8 pens/treatment			Day 28
A (Ctrl)	1.22 ± .052	0.721 ± .040	1.10 ± .134[*]	1.48 ± .14
B (0.2g/kg Thymol)	1.23 ± .103	0.743 ± .132	1.12 ± .162[*]	1.46 ± .16
C (0.4g/kg Thymol)	1.16 ± .053[**]	0.673 ± .053	0.984 ± .065	1.46 ± .05
D (0.8g/kg Thymol)	1.21 ± .047	0.733 ± .030	1.22 ± .250[*]	1.59 ± .32
E (2mL/kg Thyme Oil)	1.18 ± .090	0.693 ± .091	1.04 ± .108	1.50 ± .10
F (4mL/kg Thyme Oil)	1.20 ± .086	0.710 ± .086	1.03 ± .149	1.45 ± .11

* Some pens contained wet feed which increased feed weight.

** One pen contained a dead chick with weight included in calculations.

The results were similar to a previous study using a commercial essential oil supplemented feed that found a feed conversion ratio of $1.21 - 1.23$ during day $0 - 13$ and $1.71 - 1.76$ during day $13 - 35$ for male broiler chickens (Oviedo-Rondon et al., 2005). Cross et al. (2007) used a combination of herbs and oils as supplements to

broiler feed including thyme oil (1 g/kg) and they concluded that compared to the control (1.72), the conversion efficiency of thyme oil (1.66) was not significant but birds given thyme oil performed the best since they had the greatest overall body mass, average gain, average feed consumption, and feed conversion efficiency compared to other treatments.

Several studies have resulted in varying effects on the performance of chickens given feed with herbs, oils, or specific compounds. Jang et al. (2007) found no significant differences in all parameters related to growth performance in broilers fed a commercial feed that had 290 g of active ingredients one of which was thymol throughout the entire study (3 – 35 days). Dietary carvacrol was found to lower body weight gain but actually improve feed conversion efficiency in female broilers. In the same study, thymol was also used and had no effect on general performance (Lee et al., 2003). Hernandez et al. (2004) did a study combining essential oil extracts from oregano, cinnamon, and pepper in one treatment and extracts from sage, thyme, and rosemary in another treatment finding no differences in feed intake or feed:gain ratio for male broiler chickens. While thymol and thyme oil did not negatively affect performance which is good, the conversion efficiency was not improved. Further studies using increased amounts of thymol and thyme oil might be necessary to conclude whether or not they can be effective feed additives.

Hydrophilic ORAC and Lipophilic ORAC of Serum

The antioxidant capacity of the serum taken from the chicks at three different times during the trial was determined by hydrophilic and lipophilic ORAC (Tables 9 and 10). Before supplemented feed was provided, the serum ORAC values

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were about 45 μmol TE/mL, 0.012 μmol TE/mL, and 18 μmol TE/mL for hydrophilic, lipophilic, and hydrophilic without protein ORAC, respectively. These values served as the baseline antioxidant level present in chicken serum. Treatment A (Control) had comparable hydrophilic ORAC values but much lower lipophilic ORAC when compared to that of the initial serum analyzed before supplementation. After nine days with supplemented feed (day 7 – 16), the hydrophilic ORAC values had an increase in serum antioxidant levels for treatment groups receiving thymol and thyme oil. Treatments C (0.4 g/kg thymol), E (2 mL/kg thyme oil), and F (4 mL/kg thyme oil) had a significant difference ($p < 0.05$) in the antioxidant level of serum compared to the control. From day 16 – 28, the antioxidant levels seemed to increase generally. Both thyme oil treatments (E and F) had a significant increase in serum antioxidant level compared to the control. Overall, both thyme oil treatments (E and F) seemed to show the best results across both time periods.

Table 9. Serum antioxidant level of broilers using ORAC day 16

Treatment	ORAC (µmol TE/mL)		
	Hydrophilic	Lipophilic	Hydrophilic w/o protein
A (Ctrl)	41.83 ± 1.49^c	$1.06 \pm .014^{ab}$	18.92 ± 0.39^a
B (0.2g/kg Thymol)	49.52 ± 6.03^b	$1.10 \pm .017^a$	16.60 ± 3.25^{ab}
C (0.4g/kg Thymol)	58.11 ± 1.30^a	$0.90 \pm .029^{bc}$	12.88 ± 4.30^b
D (0.8g/kg Thymol)	51.66 ± 7.31^b	$0.85 \pm .025^c$	14.79 ± 2.60^{ab}
E (2mL/kg Thyme Oil)	59.32 ± 0.60^a	$0.70 \pm .026^d$	16.53 ± 2.33^{ab}
F (4mL/kg Thyme Oil)	48.72 ± 3.74^b	$0.29 \pm .011^e$	18.44 ± 3.12^a

[a,b,c] Means not connected by the same letter in each column are statistically different ($P < 0.05$).

Following hydrophilic ORAC, the protein was removed using TCA from some serum samples at random and analyzed again for hydrophilic antioxidant

capacity. Thymol treatment at medium level (C) from day 16 had the lowest ORAC value. No significant differences were found day 28.

Lipophilic ORAC showed a decrease in antioxidant serum level day 16 for treatments A to F but day 28 the ORAC levels appeared relatively consistent across all treatment groups. The results after the first nine days of supplemented feed were that both thyme oil treatments (E and F) are statistically different from each treatment group and themselves having the lowest ORAC values. After the final 12 days of the trial, only treatment B (0.2 g/kg thymol) and F had significant difference from one another with B having the highest ORAC and F the lowest. Overall, the thyme oil treatments, specifically treatment F, seemed to have the lowest lipophilic ORAC across both time periods.

Table 10. Serum antioxidant level of broilers using ORAC day 28

Treatment	ORAC (µmol TE/mL)		
	Hydrophilic	**Lipophilic**	**Hydrophilic w/o protein**
A (Ctrl)	39.87 ± 0.52^c	$0.41 \pm .028^{ab}$	15.20 ± 2.83^a
B (0.2g/kg Thymol)	41.23 ± 2.13^c	$0.47 \pm .037^a$	16.36 ± 2.04^a
C (0.4g/kg Thymol)	39.52 ± 5.89^c	$0.38 \pm .032^{ab}$	16.67 ± 1.56^a
D (0.8g/kg Thymol)	41.23 ± 2.40^c	$0.32 \pm .027^{ab}$	13.86 ± 1.61^a
E (2mL/kg Thyme Oil)	50.41 ± 7.61^b	$0.42 \pm .022^{ab}$	14.86 ± 1.50^a
F (4mL/kg Thyme Oil)	57.50 ± 1.80^a	$0.28 \pm .018^b$	14.21 ± 2.36^a

[a,b,c] Means not connected by the same letter in each column are statistically different ($P < 0.05$).

Analysis of serum samples taken from the chicks indicates that the supplementation of feed with thymol or thyme oil has an effect on the antioxidant capacity of the serum. Hydrophilic antioxidants were more abundant than lipophilic antioxidants in the serum. Protein removal showed that much of the antioxidant

capacity of the serum is held in the proteins since ORAC values decreased dramatically after protein removal. In accordance with human serum ORAC analysis done by Prior et al. (2003), the protein portion greatly contributes to the hydrophilic ORAC values, which was seen in this study as well. Even with protein removal the ORAC values were greater than the lipophilic ORAC values for the serum.

Serum taken after the first time period, day 16, had the greatest increase in antioxidant level with treatment E (2 mL/kg thyme oil) for hydrophilic ORAC but serum taken after completion of the trial had the greatest increases due to treatment F (4mL/kg thyme oil) for hydrophilic ORAC. The thyme oil had the largest effect on the serum antioxidant levels of the chicks throughout the trial more than the thymol alone did. This is most likely due to the fact that the thyme oil has other components in addition to thymol which allow it to have a more noticeable effect on the antioxidant levels in the chicken's serum. The major differences in the hydrophilic and lipophilic ORAC values may be due to insufficient absorption of the lipophilic antioxidant in the serum. This increase in antioxidant levels of the hydrophilic portion of serum after supplementation with thymol and thyme oil is important to note because of the increased immune health that antioxidants promote. A higher antioxidant level allows for greater free-radical scavenging in the chickens which would help lower the risk of oxidative stress and its associated diseases that are of such great concern in the poultry industry.

Histopathology

No significant lesions were found on tissues taken from the four chicks prior to feed supplementation. Rare bile duct hyperplasia was found in one of the four

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but it is common in young chicks and minor inflammation of the gut was seen in two of the four chicks. Histology samples taken on day 16 showed lesions not associated with supplementation of thymol and thyme oil. Two chicks from treatment B (0.2 mg/kg thymol) showed minimal hepatocellular fatty infiltration but since it was not seen in the higher doses of thymol, a dose response was disregarded. One chick from treatment F (4 mL/kg thyme oil) had coccidiosis, and two from the same treatment had lymphomas but none were considered compound related. Following day 16 – 28, again lesions were not associated with the thymol or thyme oil supplementation. One of the control chicks (0.8 mg/kg cellulose) displayed a kidney lymphoma suspected to be due to Marek's Disease. Also minor bacterial infections were found in the ceca of two chicks from treatment E (2 mL/kg thyme oil) and one from treatment F but a dose response could not be established. Bacterial infections found in ceca of chickens that appear healthy are common. Even though coccidiosis and Marek's Disease was found, both of which are immunosuppressive, the overall results cannot be negated. The majority of chicks showed no significant lesions supporting the determination that the thymol and thyme oil did not impair the health of the birds.

Vitamin Content of Breast Meat

The amount of vitamin B_2 in the chicken breast meat appears to be rather consistent across all treatments and both time periods (Figure 3). It is important that the level remained consistent throughout the feed trial and that feed supplementation did not have any negative effects on the overall B_2 content of the breast meat. Statistical analysis revealed a block effect between the rooms for samples from the first time period. Vitamin B_2 levels for day 16 were 0.193, 0.131, 0.284, 0.181, 0.205,

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and 0.189 mg/100g of breast meat for treatments A – F, respectively from 126A.

Treatment C (0.4 g/kg thymol) had the highest level of B_2 in the breast meat and was

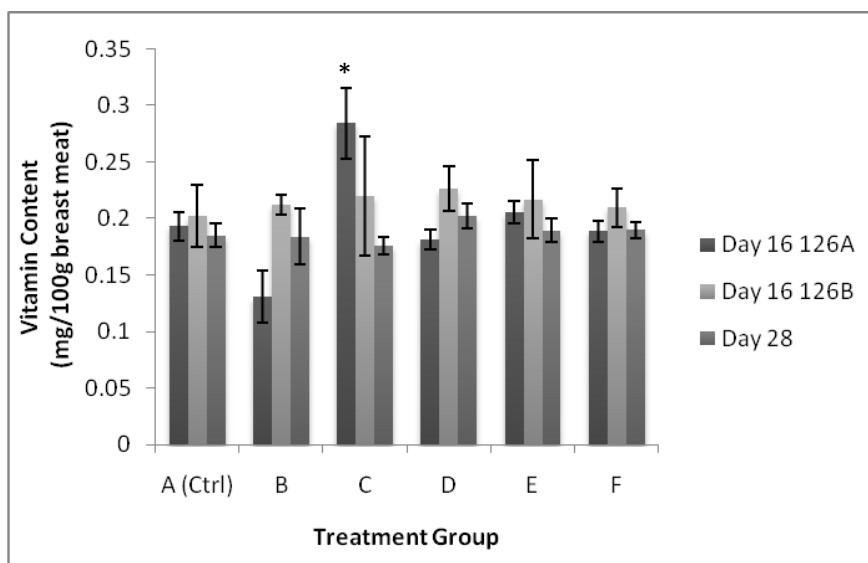significantly different ($p < 0.05$) from the control (Trt A, 0.8 g/kg cellulose).

Figure 3. The vitamin B_2 content of chicken breast meat from broilers. Treatment groups: A (0.8 g/kg cellulose), B (0.2 g/kg thymol), C (0.4 g/kg thymol), D (0.8 g/kg thymol), E (2 mL/kg thyme oil), and F (4 mL/kg thyme oil). * Designates significance ($p < 0.05$).

Vitamin B_2 levels for day 16 from 126B were 0.202, 0.212, 0.220, 0.226,

0.217, and 0.210 mg/100g of breast meat for treatments A – F, respectively. Treatment

groups in 126B were not found to be significantly different but treatment D had the

highest level of vitamin B_2 in the chicken breast meat and treatment A had the lowest

level. For samples analyzed from day 28, a block effect was not seen between the two

rooms. Vitamin B_2 levels were 0.185, 0.184, 0.176, 0.202, 0.189, and 0.190 mg/100g

of breast meat for treatments A – F, respectively. Treatment D contained the highest

level of vitamin B_2 in the breast meat but was not significantly different from the control.

Nicotinamide levels (Figure 4) in broiler breast meat were relatively consistent over the course of the feed trial. Statistical analysis revealed a block effect between the rooms for the first time period. Nicotinamide levels from day 16 for 126A were 2.06, 8.49, 3.33, 2.45, 2.71, and 2.44 mg/100g of breast meat for treatments A – F, respectively. Treatment B (0.2 g/kg thymol) had the largest level of nicotinamide and was significantly different ($p < 0.05$) from all other treatments. None of the other treatments were significantly different from the control which had the lowest nicotinamide level. Treatments in room 126B from day 16 were not significantly different from one another and vitamin levels were 2.23, 2.80, 2.70, 2.63, 2.43, and 2.14 mg/100g of breast meat for treatments A – F, respectively. As in 126A, treatment B had the highest level of nicotinamide in samples from 126B but treatment F (4 mL/kg thyme oil) had the lowest level.
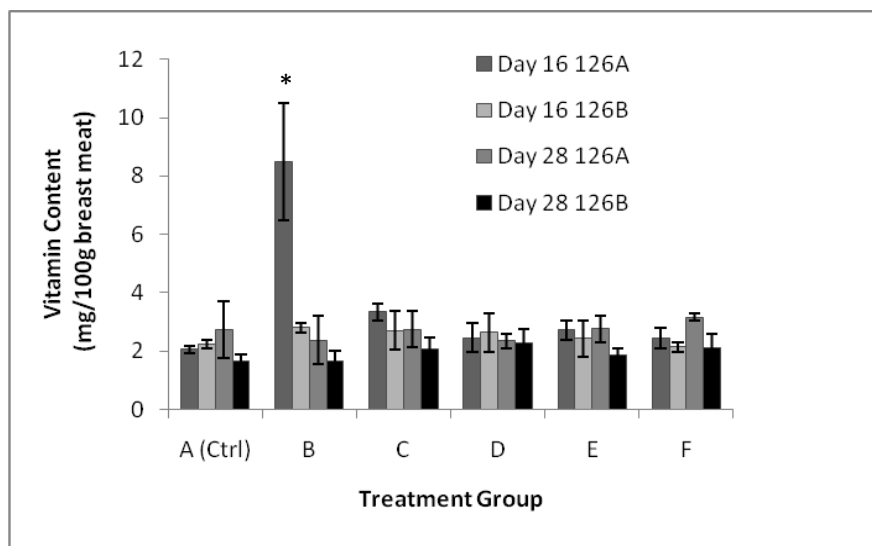
Figure 4. The vitamin B_3, nicotinamide content of chicken breast meat from broilers. Treatment groups: A (0.8 g/kg cellulose), B (0.2 g/kg thymol), C (0.4 g/kg thymol), D (0.8 g/kg thymol), E (2 mL/kg thyme oil), and F (4 mL/kg thyme oil). * Designates significance ($p < 0.05$).

Nicotinamide levels from day 28 also showed a block effect between the two rooms but neither room had treatment groups significantly different from the control. Vitamin levels in 126A were 2.73, 2.37, 2.75, 2.35, 2.76, and 3.15 mg/100g of breast meat for treatments A – F, respectively. Treatment F had the highest level of nicotinamide in broiler breast meat. Nicotinamide levels in 126B were 1.64, 1.66, 2.05, 2.28, 1.87, and 2.10 mg/100g of breast meat. Treatment D (0.8 g/kg thymol) had the highest vitamin level.

Nicotinic acid levels (Figure 5) in the broiler breast meat were generally less than the nicotinamide levels. Like nicotinamide, the levels of nicotinic acid remained rather consistent throughout the trial and a block effect was found between rooms for both time periods. The levels of nicotinic acid in samples from 126A for day

16 were 0.880, 1.84, 4.38, 1.28, 6.98, and 5.35 mg/100g of breast meat for treatments A – F, respectively. Treatment E (2 mL/kg thyme oil) showed the highest level of nicotinic acid with treatment F the second highest and both were significantly different ($p < 0.05$) from the control. Treatment C (0.4 g/kg thymol) was also significantly different from the control having the third highest nicotinic acid level. Samples from 126B did not show any significant difference with nicotinic acid levels 1.01, 0.949, 1.26, 0.908, 0.894, and 1.53 mg/100g of breast meat for treatments A – F, respectively. Treatment F had the highest level of nicotinic acid.
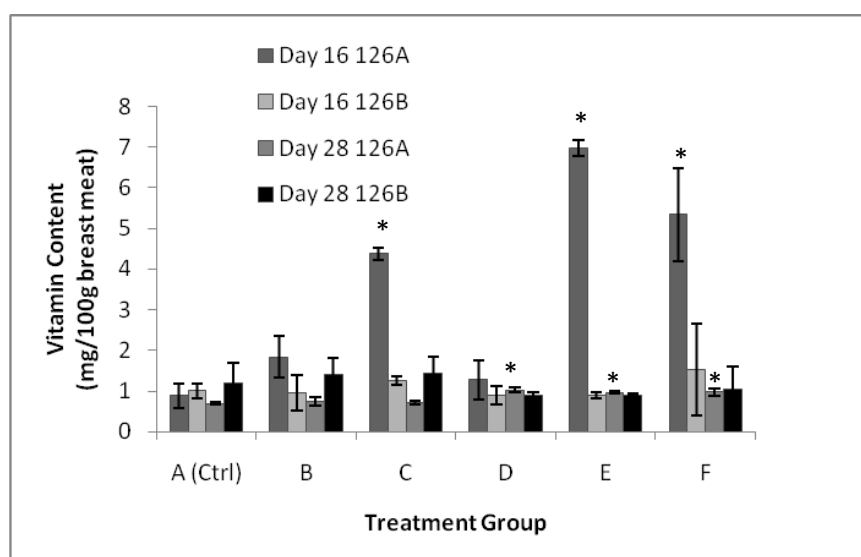

Figure 5. The vitamin B$_3$, nicotinic acid content of chicken breast meat from broilers. Treatment groups: A (0.8 g/kg cellulose), B (0.2 g/kg thymol), C (0.4 g/kg thymol), D (0.8 g/kg thymol), E (2 mL/kg thyme oil), and F (4 mL/kg thyme oil). * Designates significance ($p < 0.05$).

Treatments A – F from 126A and day 28 had nicotinic acid levels of 0.694, 0.748, 0.722, 1.03, 0.961, and 0.973 mg/100g of breast meat, respectively.

Treatment D had the highest level of nicotinic acid and was significantly different ($p < 0.05$) from the control. Treatment F and E had the next highest levels in that order and were significantly different from the control. Samples from 126B were not found to be significantly different from one another with levels for treatments A – F 1.20, 1.42, 1.43, 0.910, 0.902, and 1.05 mg/100g of breast meat, respectively. Treatment C had the highest nicotinic acid level and treatment E contained the lowest amount of the vitamin.

Analysis of breast meat from broilers contained levels of three vitamin B_6 derivatives, pyridoxamine, pyridoxal, and pyridoxine. Levels of pyridoxamine (Figure 6) in breast meat samples for day 16 were 0.184, 0.214, 0.165, 0.186, 0.186, and 0.212 mg/100g of breast meat for treatments A – F, respectively. No treatment was found to be significantly different from the control. Treatment B had the highest level and treatment C had the lowest level of pyridoxamine.
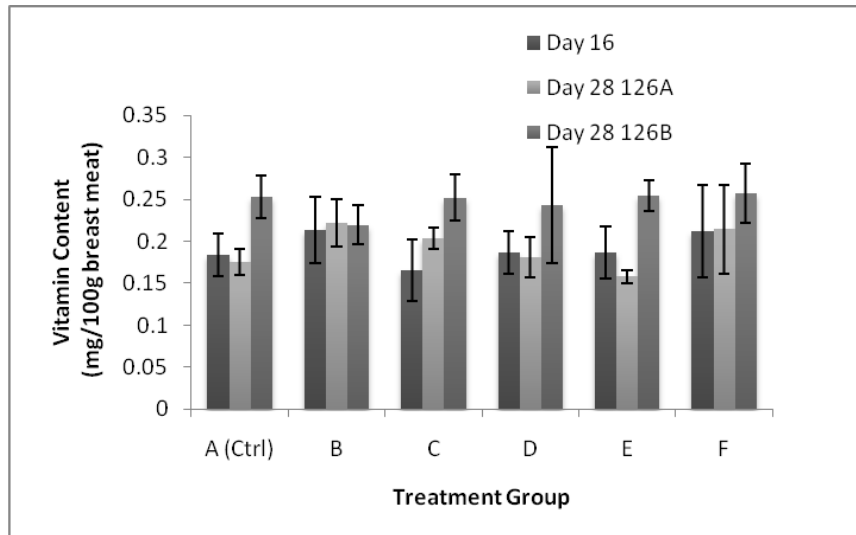
Figure 6. The vitamin B_6, pyridoxamine content of chicken breast meat from broilers. Treatment groups: A (0.8 g/kg cellulose), B (0.2 g/kg thymol), C (0.4 g/kg thymol), D (0.8 g/kg thymol), E (2 mL/kg thyme oil), and F (4 mL/kg thyme oil). * Designates significance ($p < 0.05$).

A block effect was seen between rooms for samples during the second time period. Levels of pyridoxamine for day 28 from 126A were 0.175, 0.222, 0.203, 0.181, 0.158, and 0.215 mg/100g of breast meat for treatments A – F, respectively. None of the treatments were significantly different from the control. Pyridoxamine levels for 126B were 0.253, 0.220, 0.252, 0.243, 0.255, and 0.257 mg/100g of breast meat. None of the treatment groups were significantly different from the control and treatment F had the highest level of pyridoxamine.

Pyridoxal results (Figure 7) from day 16 showed a block effect between rooms with pyridoxal levels of 0.176, 0.120, 0.271, 0.190, 0.100, and 0.276 mg/100g of breast meat for treatments A – F, respectively from 126A. Treatment F had the highest level of pyridoxal and was significantly different ($p < 0.05$) from the control. Treatment C had the second highest level and was also significantly different ($p <$

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0.05) from the control. Pyridoxal levels from 126B were 0.194, 0.256, 0.282, 0.276, 0.196, and 0.237 mg/100g of breast meat. Treatment C had the highest pyridoxal level and was found to be significantly different ($p < 0.05$) from the control. Treatment D had the second highest pyridoxal content and was also significantly different from the control. Samples from day 28 did not show a block effect. Pyridoxal levels were 0.267, 0.217, 0.248, 0.340, 0.291, 0.240 mg/100g of breast meat for treatments A – F, respectively. Treatment D had the highest level of pyridoxal and but was not significantly different from the control.
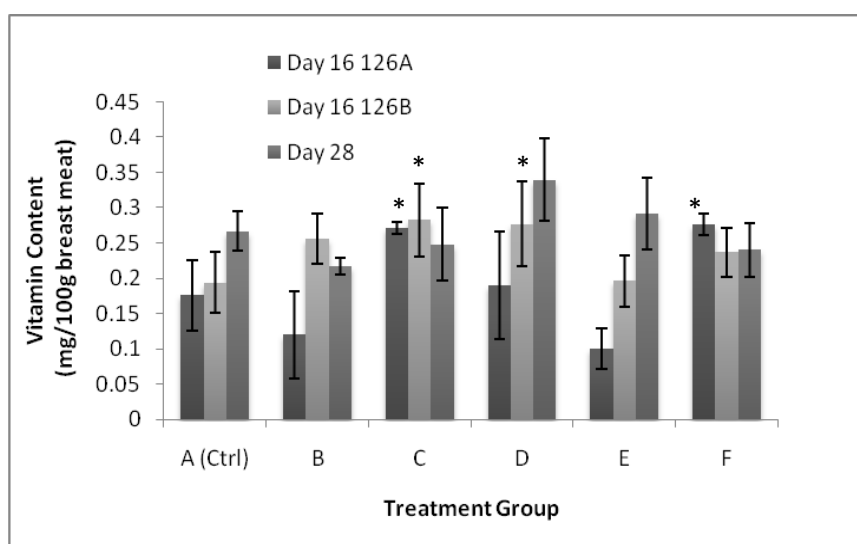

Figure 7. The vitamin B_6, pyridoxal content of chicken breast meat from broilers. Treatment groups: A (0.8 g/kg cellulose), B (0.2 g/kg thymol), C (0.4 g/kg thymol), D (0.8 g/kg thymol), E (2 mL/kg thyme oil), and F (4 mL/kg thyme oil). * Designates significance ($p < 0.05$).

The B_6 derivative, pyridoxine had the highest levels of the three B_6 compounds in the breast meat samples (Figure 8). Statistical analysis revealed a block

effect between the two rooms from day 16. Pyridoxine levels for samples from 126A during this time period were 0.781, 0.806, 1.06, 0.787, 1.02, 0.988 mg/100g of breast meat for treatments A – F, respectively. Treatment C was found to be significantly different ($p < 0.05$) from the control and had the highest pyridoxine level. Treatments E and F had the next highest pyridoxine levels in that order and were significantly different from the control. The pyridoxine levels from 126B were 0.789, 0.805, 0.808, 0.843, 0.806, and 0.802 mg/100g of breast meat for treatments A – F, respectively. Treatment D had the highest level of pyridoxine and was the only treatment found to be significantly different ($p < 0.05$) from the control.
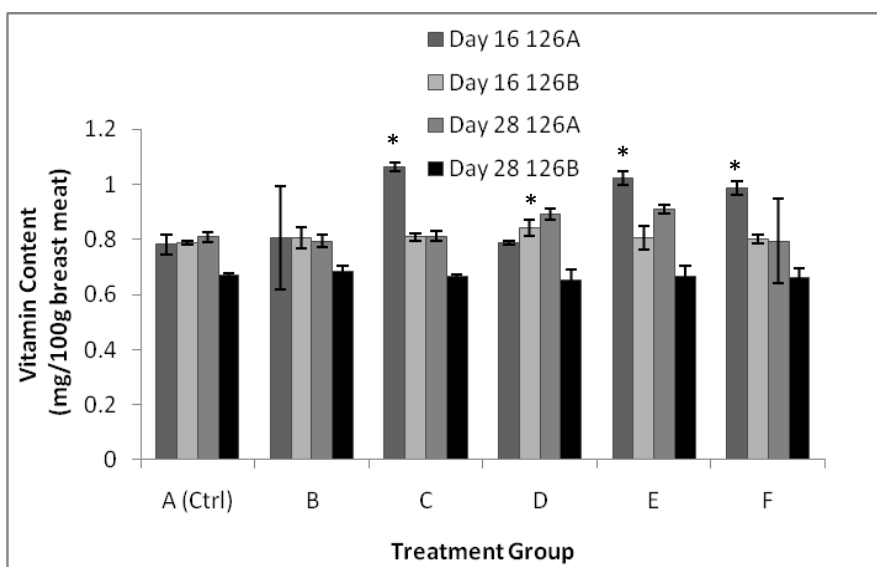

Figure 8. The vitamin B_6, pyridoxine content of chicken breast meat from broilers. Treatment groups: A (0.8 g/kg cellulose), B (0.2 g/kg thymol), C (0.4 g/kg thymol), D (0.8 g/kg thymol), E (2 mL/kg thyme oil), and F (4 mL/kg thyme oil). * Designates significance ($p < 0.05$).

Chicken breast samples from day 28 also showed a block effect between rooms. Pyridoxine levels for 126A were 0.808, 0.793, 0.812, 0.891, 0.910, and 0.794 mg/100g of breast meat. Treatment E had the highest pyridoxine level but was not significantly different from the control. The pyridoxine levels for 126B were 0.669, 0.682, 0.666, 0.654, 0.667, and 0.663 mg/100g of breast meat. None of the treatments were significantly different from one another or the control. Treatment B had the highest level of pyridoxine and treatment D had the lowest.

The vitamin analysis of breast meat from broiler chickens contained various levels of vitamins B_2, B_3, and B_6. Nicotinamide, a B_3 derivative, had higher levels than its counterpart nicotinic acid. The three B_6 derivatives also showed differences in their levels within breast meat samples. Pyridoxine seemed to have the most pronounced levels compared to pyridoxamine and pyridoxal. Feed supplementation did not have negative effects on the vitamin content since the levels remained rather consistent throughout the feed trial. Most of the treatment significance was observed during the first time period. According to the USDA raw chicken breast meat from broilers contains 0.100mg, 10.430mg, and 0.749mg of vitamins B_2, B_3, and B_6, respectively in 100g of breast meat. Vitamin B_2 results from this study are in agreement with the USDA data and are actually a little larger than the USDA data. Vitamin B_3 has the largest presence in chicken breast meat which is in accordance with the present study. Niacin exists largely as nicotinamide in meat due to postmortem hydrolysis of NAD (Ball, 2006). Adding the nicotinamide and nicotinic acid results together gives the relative niacin content but this value is still much lower in comparison to the USDA nutrient database. This could be due to the fact that the

USDA uses full-grown birds but this study only grew birds to 28 days. Most likely the discrepancy is due to the acid sensitivity of nicotinamide during the acid hydrolysis preparation of meat samples (Russell, 2000; Finglas and Faulks, 1987). Nicotinamide is pH sensitive and acidic conditions can degrade it however acid hydrolysis methods continue to be used. Figure 3 shows treatment B having a high level from day 16 in room 126A. This is because the sodium acetate used for acid hydrolysis was not adjusted to pH 4.5 and therefore the nicotinamide was not degraded as in subsequent samples. The three B_6 derivatives must also be added together in order to agree with the USDA data on vitamin B_6 content of broiler breast meat in which case they are greater than or equal to the USDA information.

Commercial chicken breast meat was also analyzed for comparison and the results were 0.150, 4.04, and 1.12 mg/100g of breast meat for vitamins B_2, B_3, and B_6, respectively. The results for B_2 and B_6 are in accordance with the current study and the USDA but the results for B_3 only agree with the current study and are lower than the USDA. Abdulrahman et al. (1993) reported riboflavin content of raw light muscle broiler meat as 0.14 mg/100g which is very similar to values reported here. Another study found much lower B_2 content in chicken breast meat, 0.03 mg/100g (Lombardi-Boccia et al., 2005). The latter study used a similar acid hydrolysis and enzyme digestion combination method for analysis but only one enzyme was used instead of a mixture which may account for the large difference. Also a UV detector was used for chromatography rather than a fluorescence detector which is commonly used for riboflavin detection. Lombardi-Boccia et al. (2005) also reported niacin levels of 8.0 mg/100g which are higher than this study.

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CONCLUSION

The results of the 28-day feed trial showed that thymol and thyme oil had no impact on bird performance. Using antioxidants as feed additives increased the hydrophilic antioxidant level in the serum of broilers which can help to control oxidative stress and imbalances and promote better immune health. Histopathology showed that thymol and thyme oil did not cause significant lesions to form in the liver, kidney, intestine, and ceca of broilers. This means that thymol and thyme oil would not cause organ damage to chickens and therefore would be safe for the chickens to consume. Feed supplementation with thymol and thyme oil did not affect the breast meat quality of broilers because all treatments showed similar levels of B_2, B_3, and B_6 in the breast meat samples. Further studies using increased amounts of thymol and thyme oil might be necessary to conclude whether or not they can be effective feed additives with corresponding dosage and cytotoxicity assessments to determine safety.

REFERENCES

Abdulrahman, A.S. and A.A. Dawood. 1993. Effects of cooking methods on thiamin and riboflavin contents of chicken meat. Food Chem. 48: 69 – 74.

Ball, G.F.M. 2006. Vitamins in foods: analysis, bioavailability, and stability. CRC Press, Taylor and Francis Group, Boca Raton, FL.

Cross, D.E., R.M. McDevitt, K. Hillman, and T. Acamovic. 2007. The effect of herbs and their associated essential oils on performance, dietary digestibility and gut microflora in chickens from 7 to 28 days of age. Brit. Poult. Sci. 48: 496–506.

Finglas, P.M. and R.M. Faulks. 1987. Critical review of HPLC methods for the determination of thiamin, riboflavin and niacin in food. J. Micronutr. Anal. 3: 251–283.

Hernandez, F., J. Madrid, V. Garcia, J. Orengo, and M.D. Megias. 2004. Influence of two plant extracts on broiler performance, digestibility, and digestive organ size. Poult. Sci. 83: 169–174.

Jang, I.S., Y.H. Ko, S.Y. Yang, and C.Y. Lee. 2007. Effect of a commercial essential oil on growth performance, digestive enzyme activity and intestinal microflora population in broiler chickens. Animal Feed Sci. Tech. 134: 304–315.

Lee, K.-W., H. Everts, H.J. Kappert, K.-H. Yeom, and A.C. Beynen. 2003. Dietary carvacrol lowers body weight gain but improves feed conversion in female broiler chickens. J. Appl. Poult. Res. 12: 394–399.

Lombardi-Boccia, G., S. Lanzi, and A. Aguzzi. 2005. Aspects of meat quality: trace elements and B vitamins in raw and cooked meats. J. Food Comp. Anal. 18: 39 – 46.

Oviedo-Rondon, E.O., S. Clemente-Hernandez, P. Williams, and R. Losa. 2005. Responses of coccidian-vaccinated broilers to essential oil blends supplementation up to forty-nine days of age. J. Appl. Poult. Res. 14: 657–664.

Prior, R.L., H. Hoang, L. Gu, X. Wu, M. Bacchiocca, L. Howard, M. Hampsch-Woodill, D. Huang, B. Ou, and R. Jacob. 2003. Assays for hydrophilic and lipophilic antioxidant capacity (oxygen radical absorbance capacity ($ORAC_{FL}$)) of plasma and other biological and food samples. J. Agric. Food Chem. 51: 3273–3279.

Russell, L.F. 2000. Quantitative determination of water-soluble vitamins. In L.M.L. Nollet (Ed.), Food Analysis by HPLC. Marcel Dekker, Inc., New York, NY. 403–492

United States Department of Agriculture. USDA National Nutrient Database for Standard Reference. http://www.ars.usda.gov/Services/docs.htm?docid=18877. Accessed January 25, 2010.

Wu, C., S.K. Duckett, J.P.S. Neel, J.P. Fontenot, and W.M. Clapham. 2008. Influence of finishing systems on hydrophilic and lipophilic oxygen radical absorbance capacity (ORAC) in beef. Meat Sci.. 80: 662–667.

CHAPTER 5

FUTURE WORK

Further critical studies are necessary to evaluate the effectiveness of thymol and thyme oil as poultry feed additives. Safety and risk assessment studies are essential to determine the best concentration of each compound to add to feed in order to have most benefits. While histology indicates that these compounds are safe, if higher amounts are used, another histological evaluation would be necessary. Another large scale feed trial would be performed with higher amounts of thymol and thyme oil with further analysis of serum and breast meat. Heat stress response analysis with these supplements would also be important to establish a commercial poultry production atmosphere and evaluate supplement effects. Mujahid et al. (2005) found an increase in heat stress of chickens resulted in enhanced superoxide production and significant body weight loss. Further *Salmonella* challenge trials are necessary to determine the interaction of the bacteria and the feed additives inside the gut of chickens and whether thymol can protect chickens from infection or reduce infection symptoms.

Antioxidant assays like ORAC have been suggested as inappropriate for the analysis of serum when evaluating supplementation effects in humans (Collins, 2005). Instead of measuring the radical scavenging abilities in the case of ORAC, evaluation of lymphocytes and lipids to oxidation is a better method to determine the effects of antioxidants in serum. The comet assay has shown to be successful in monitoring the antioxidant resistance of lymphocytes to DNA damage induced by hydrogen peroxide (H_2O_2) (Collins, 2005). Also looking at lipid oxidation using

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malondialdehyde as a marker is a good method for determining antioxidant protection in human serum studied (Collins, 2005). It would be beneficial to apply these methods to the serum analysis of thymol and thyme oil supplemented chickens to give a better indication of the mechanism of protection these compounds provide.

Lipid oxidation measurement could also be applied to the chicken breast meat samples taken from the birds. This method is very common in feed supplementation trials to determine the quality of the meat because oxidation is the frequent cause of deterioration in meat. By adding antioxidants to chicken feed, oxidative stress pre-slaughter can possibly be reduced to provide a better quality product. Post-slaughter changes occur in order for muscle to become meat and involve loss of cellular antioxidant defenses and increased lipid oxidation which can result in undesirable quality characteristics in the meat (O'Neill et al., 1998). The focus of research in this area has focused on producing meat with a greater monounsaturated fatty acid profile and the most common feed additive studied has been α-tocopherol (vitamin E). Lipid oxidation is measured in comparison to malondialdehyde using thiobarbituric acid-reducing substances (TBARS) assay.

Morrissey et al. (1997) found significant reduction in lipid peroxidation susceptibility of broilers after 4 weeks of α-tocopherol supplementation pre-slaughter and also found the presence of α-tocopherol in plasma and tissues of broilers following supplementation. Herbs and plant extracts have also been studied as feed additives to reduce the lipid oxidation of broiler meat. Oregano, as an herb and its essential oil have both shown to increase the antioxidant status of tissues while reducing TBARS and/or malondialdehyde in tissues which are indicators lipid oxidation (Botsoglou et

al., 2002; Young et al., 2003). Most recently thymol and carvacrol were added to broiler feed to evaluate lipid oxidation in meat and lower TBARS were detected in thigh meat from feed-supplemented birds compared to the control (Luna et al., 2010). Gao et al. (2010) found vitamin E supplementation of broiler feed suppressed lipid peroxidation in both the plasma and skeletal muscle tissue of the birds. Since vitamin E is often added to poultry feed, a feeding trial using feed without vitamin E would be beneficial to determine that the effects of the thymol and thyme oil on the serum of broilers seen in this study are not due to vitamin E.

Future analysis of the quality of meat produced from feed-supplemented broilers is necessary to determine the overall effects that supplementation may have. Sensory analysis should also be conducted to determine if the flavor profile of the meat changes as a result of supplementation. Sensory analysis on feed additives has focused on the addition of rapeseed meal and fishmeal as well as dietary additives like vitamin E and fats to broiler feed showing varying affects on odor and flavor of the meat (Poste, 1990; Ruiz et al., 2001). More study is necessary on how plant extracts can affect sensory attributes in chicken meat.

REFERENCES

Botsoglou, N.A., P. Florou-Paneri, E. Christaki, D.J. Fletouris, and A.B. Spais. 2002. Effect of dietary oregano essential oil on performance of chickens and on iron-induced lipid oxidation of breast, thigh and abdominal fat tissues. Brit. Poult. Sci. 43: 223–230.

Collins, A.R. 2005. Assays for oxidative stress and antioxidant status: application to research into the biological effectiveness of polyphenols. Am. J. Clin. Nutr. 81: 261S–267S.

Gao, J., H. Lin, X.J. Wang, Z.G. Song, and H.C. Jiao. 2010. Vitamin E supplementation alleviates the oxidative stress induced by dexamethasone treatment and improves meat quality in broiler chickens. Poult. Sci. 89: 318–327.

Luna, A., M.C. Labaque, J.A. Zygadlo, and R.H. Marin. 2010. Effects of thymol and carvacrol feed supplementation on lipid oxidation in broiler meat. Poult. Sci. 89: 366–370.

Mujahid, A., Y. Yoshiki, Y. Akiba, M. Toyomizu. 2005. Superoxide radical production in chicken skeletal muscle induces by acute heat stress. Poult. Sci. 84: 307–314.

Morrissey, P.A., S. Brandon, D.J. Buckley, P.J.A. Sheehy, and M. Frigg. 1997. Tissue content of α-tocopherol and oxidative stability of broilers receiving dietary α-tocopheryl acetate supplement for various periods pre-slaughter. Brit. Poult. Sci. 38: 84–88.

O'Neill, L.M., K. Galvin, P.A. Morrissey, and D.J. Buckley. 1998. Comparison of effects of dietary olive oil, tallow and vitamin E on the quality of broiler meat and meat products. Brit. Poult. Sci. 39: 365–371.

Poste, L.M. 1990. A sensory perspective of effect of feeds on flavor in meats: poultry meats. J. Anim. Sci. 68: 4414–4420.

Ruiz, J.A., L. Guerrero, J. Arnau, M.D. Guardia, and E. Esteve-Garcia. 2001. Descriptive sensory analysis of meat from broilers fed diets containing vitamin E

or β-carotene as antioxidants and different supplemental fats. Poult. Sci. 80: 976–982.

Young, J.F., J. Stagsted, S.K. Jensen, A.H. Karlsson, and P. Henckel. 2003. Ascorbic acid, α-tocopherol, and oregano supplements reduce stress-induced deterioration of chicken meat quality. Poult. Sci. 82: 1343–1351.