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Evaluation of the Effect of a Lippia organoides Essential Oil Extract on Clostridium perfringens Proliferation In Vitro and Necrotic Enteritis in Broiler Chickens

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Evaluation of the Effect of a *Lippia organoides* Essential Oil Extract on *Clostridium perfringens* Proliferation *In Vitro* and Necrotic Enteritis in Broiler Chickens

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Abstract

The purpose of the present research was to assess the effects of essential oils derived from the plant *Lippia organoides* on performance parameters, intestinal integrity, and necrotic enteritis (NE) in broiler chickens. To do this, a previously established challenge model for NE was utilized which included challenging with *Salmonella* Typhimurium on day 0, *Eimeria maxima* on day 18, and *Clostridium perfringens* on days 22 and 23. Treatment groups included a 1) non-challenged, negative control, 2) challenged control, and 3) challenged, *Lippia origanoides* (37ppm in the diet). Group 1 (negative control) had significantly ($P < 0.05$) higher body weight gain from d8-25 and d0-25 compared to both challenged groups. Feed intake was significantly different for all three groups ($P < 0.05$) at 8-25 days and 0-25 days with group 1 having the highest feed intake for both time periods. Total mortality was greater in the positive control when compared to both the negative and treatment groups. NE lesion scores were significantly different between all groups with the positive control having the highest mean lesion scores and the negative control having a mean lesion score of 0. The positive control group had the highest FITC-d amounts detected in the sera, being statistically higher than both the treatment group and negative group which were both statistically different from each other. At the present inclusion rate for the essential oil (37 ppm), there was an overall reduction in the negative impact from the NE infection. Further studies should be conducted to reach more significant conclusions.

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Dedication

For Colton, Josiah and Tucker, may you never doubt how capable you are.

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Chapter I. Thesis Introduction

For decades, antibiotic growth promoters (AGPs) have been used in poultry diets at subtherapeutic dosages to improve performance (1). Consumer concern for antibiotic use, the misuse of antibiotics, and multidrug resistance have caused a shift in the poultry industry. These AGPs are being implemented less and less, leading to an uptick in the occurrence of many bacterial diseases that were previously controlled by their use. Among the bacterial diseases on the rise, is necrotic enteritis (NE). NE is caused by an over-proliferation of *Clostridium perfringens* (CP), a Gram-positive, spore-forming bacterium in the gastrointestinal tract (GIT) of broiler chickens and turkeys (2). There is evidence that suggests predisposing factors contribute to the overgrowth of CP in the GIT (3). For instance, these predisposing factors directly change the landscape of the GIT by damaging the lining of the intestines, promoting inflammation and excessive mucin production. As a result, the composition and balance of the gut microbiota is disrupted. Common predisposing factors for NE in broiler chickens include co-infection with *Eimeria maxima* or dietary and environmental stressors (3).

Since antibiotic use is on the decline, there is a need for alternatives to mitigate NE in commercial poultry flocks. The incidence of NE may be reduced by directly targeting the CP or by indirectly controlling *E. maxima* cycling and limiting the use of dietary ingredients that promote CP proliferation in the GIT. An antibiotic alternative currently of interest for use in poultry diets is essential oils (EOs) and EOs derivatives. EOs are highly volatile compounds extracted from aromatic plants (4). The natural compounds have been used for the treatment of pain, inflammation, viral diseases and cancer in holistic medicine for centuries (5). As stated, EOs are highly volatile, very unstable, and subject to degradation in their natural form. One way to overcome this is to encapsulate the EOs in colloidal systems (6) or in polymer coatings and

alginates (7) as the outer shell of the EO microencapsulation. Microencapsulation of the EOs allows for easy and efficient mixing into different rations. Microencapsulation has also been shown to create a slower and more targeted release of the product in the GIT (2).

In this study, EOs were derived from *Lippa organoides* plants and microencapsulated through spray drying techniques. To replicate NE, broiler chicks were then exposed to *Salmonella* Typhimurium at day-of-hatch and *Eimeria maxima* at day 18 to promote enteritic inflammation and a damaged epithelial surface. These conditions provide an ideal environment for CP replication and previous research has shown how *Salmonella* challenge during the neonatal period followed by *Eimeria maxima* increases the NE-associated mortality (8). To ensure uniform enteric colonization by CP, chickens were orally challenged with CP at day 22 and 23 days of age. Compared to the PC, EOs improved performance parameters, including body weight at day 25 and body weight gain from day 0-25. However, there were no significant differences in feed conversion ratio between the PC and EOs. At day 25, NE-associated lesion scores in the group that received EOs in the diet at 37ppm were significantly ($P < 0.05$) lower than the PC group. Additionally, inclusion of EOs markedly ($P < 0.05$) reduced CP recovery in an *in vitro* digestion assay compared to the control. The impact of EOs on NE lesion scores and CP proliferation *in vitro* suggest that the EOs have a negative effect on CP replication. There were no differences in morbidity observed between the PC and EOs, but total mortality and NE-associated was significantly ($P < 0.05$) reduced in the EOs-treated group. The enzyme activity of superoxide dismutase (SOD) was also measured. SOD acts on superoxide anions and breaks them down into a hydrogen peroxide molecule and molecular oxygen (9). The PC had a significantly ($P < 0.05$) lower SOD level than the treated group indicating that more superoxide was being broken down, and not allowed to cause oxidative stress on the lining of the intestinal

tract. Fluorescein isothiocyanate dextran (FITC-d) is a fairly large particle that cannot pass through the GIT unless there is inflammation present. FITC-d is an established indicator for gut leakage in poultry (10) In this study, PC serum FITC-d levels were found to be significantly higher than the treatment group. This suggests that the PC had a higher rate of gut leakage and gut inflammation than the treatment group. Interferon-gamma is important in the induction of inflammation. They are produced by natural killer cells (11). Natural killer cells are part of the innate immune system activated by several things including intracellular pathogens (12) such as the predisposing factor for necrotic enteritis, *Eimeria maxima*. For this trial, the interferon-gamma levels found in serum were statistically higher in the PC group than in the treated group suggesting that there was more inflammation in the GIT of the PC group. The PC also had significantly higher levels of immunoglobulin-a (IgA) than the treatment group. IgA is an indicator of mucosal production in the GIT (13). Therefore it can be supposed that there was more mucus produced in the GIT of the PC group than in the treatment group.

From this trial, it can be concluded that *L. organoides* could be a viable alternative to reduce the severity of NE in broiler chickens. Further research is recommended to investigate the interaction of these EOs on *Eimeria* spp. and immunomodulatory effects on the host. Sustainable, non-antibiotic alternatives are needed to prevent NE in commercial poultry flocks.

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Chapter II. Literature Review

Essential oils as an alternative to antibiotics for necrotic enteritis. A review

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Abstract

Due to the removal of antibiotic growth promoters (AGPs) and consumer pressure for antibiotic-free (ABF) or no antibiotics ever (NAE) poultry production, there is a need for sustainable alternatives to prevent disease in commercial poultry operations. Without AGPs, there has been a rise in diseases that were traditionally controlled by subtherapeutic levels of antibiotics in the diet. This has impacted the health of commercial poultry and has been a significant cost to poultry producers. To mitigate this, the industry has started to investigate alternatives to antibiotics to treat these forthcoming health issues, such as necrotic enteritis (NE). NE is an enteric disease caused by an over proliferation of toxigenic *Clostridium perfringens* (CP) in the gastrointestinal tract. Although CP is a commensal in the avian intestinal tract, dysbiosis caused by inflammation and impaired intestinal integrity facilitates uncontrolled replication of CP. Infectious agents, such as *Eimeria maxima*, appear to be a predominant predisposing factor that promotes NE. However, non-infectious stressors, including dietary changes, have also been associated with NE to some degree. As a result of increased pressure to restrict the use of antibiotics, there is a need for research evaluating the efficacy of alternatives, such as plant-derived essential oils, as potential tools to mitigate NE in commercial poultry flocks.

Introduction

Over the last few hundred years, *Homo sapiens* have dominated all known animal species and altered every known ecosystem. This approach led to the genetic change of domestic animals and was undoubtedly driven by agriculture. The most important genetic changes in domestic animals occurred in the previous 60 years. Modern broiler chickens are likely the clearest example of these genetic improvements. Newborn chicks grow 31 % (55 g/bird) on day one, and 5,902%

(2,521 g/bird) on day 35 (1). Intensive genetic selection, diet, health, and management initiatives have led to these achievements. Nonetheless, maintaining the integrity of the gastrointestinal tract (GIT), the primary organ responsible for digestion and nutritional absorption, is critical for production. Because feed conversion accounts for over 70% of the cost of production in poultry and animal enterprises, subclinical coccidiosis or necrotic enteritis in chickens is more costly than acute infections (Figure 1). As the growing period of broilers shortens and feed efficiency improves, so do health and nutrition programs. Because the changes during intestinal development are microscopic, they are typically neglected—nevertheless, gut health influences overall health and productivity.

Importance of GIT health in poultry

The GIT is home to a varied microbial community known as gut microbiota (2), outnumbering somatic cells by tenfold, with 300,000 genes compared to 23,000 genes in chickens (3, 4). The enteric nervous system (ENS) has approximately one hundred million neurons and is referred to as the "second brain" of metazoans because of its importance in digestion (5). Approximately 80 percent of the immune cells in the body are found in the gut-associated lymphoid tissue (GALT). The Bursa of Fabricius, a lymphoid organ that is critical for B-lymphocyte growth and proliferation in avian species, is a component of the GALT (6). As an astonishment, the GALT comprises 80 percent of the plasma cells that are responsible for the production of secretory immunoglobulin A (IgA), the far more prevalent immunoglobulin (7).

A range of physiological processes, including secretion, absorption, digestion, and gut motility, are mediated by enteroendocrine cells (EECs), which also play a role in the etiology of intestinal mucosa atrophy and malignancies, both within and beyond the GIT (8). Gastrin, secretin, cholecystokinin, insulin, and glucagon were among the first GIT hormones to be discovered in

humans (9). The discovery of more than 50 gut hormones and bioactive peptides today confirms that the gut is the body's largest endocrine organ, performing an extensive spectrum of endocrinological, neuroendocrine, autocrine, and paracrine functions, as well as a variety of other roles (10). Enterochromaffin cells, a subset of several EECs, produce 90% of the neurotransmitter serotonin (5-hydroxytryptamine), which plays multiple biological roles in temperament, perception, reproduction, vasodilation, gut motility, wound healing, and vasoconstriction (11). Surprisingly, the gut microbiome modulates serotonin and other EEC-produced mood neurotransmitters like dopamine, oxytocin, and endorphins (12-14). Published research have shown that in humans, illnesses of the brain (such as schizophrenia, depression, Alzheimer's disease, Parkinson's disease, and autism) are associated with the kind of microbiota prevalent in the GIT (15, 16). The cliche "gut instincts" holds true in this case (17).

For more than a century, Eli Metchnikoff, the Nobel Prize-winning father of innate immunity, offered the breakthrough idea of ingesting live bacteria to boost health by modifying the intestinal microbiota (18, 19). Antibiotic resistance in bacteria (sometimes known as "superbugs") is a major problem in medicine and agriculture around the world. As the number of antibiotic-resistant bacteria grows, this concept is becoming increasingly relevant (20). According to recent research, nutritional approaches may be effective alternatives to antibiotics in some cases (21-23). In addition to increasing animal health, welfare, and production, boosting disease resistance in antibiotic-free animals is an important task in enhancing food safety. Chronic inflammation in the intestine is thought to be the root cause of 90% of all diseases (24). The gut microbiota influences the host's biology, metabolism, nutrition, immunity, and neuroendocrine system (25, 26). Short-chain fatty acids, gastrointestinal hormones, enteroendocrine and immune cells all play a role in these effects (27). The enteric nervous system and hormonal networks control GIT motility, which

is impaired in functional GIT diseases (28). The neuroendocrine network that connects the brain, the ENS, gut microbiota, and the GALT has a significant impact on the delicate intestinal epithelial barrier (29, 30). This barrier, which consists of a single layer of enterocytes with tight intercellular junctions, regulates the balance of tolerance and immunity to non-self antigens (31). Hence, gut integrity is critical in maintaining a healthy balance of health and disease (32). To keep the system in survival mode, chronic stress and chronic intestinal inflammation divert significant biological resources away from development and reproduction. Perhaps a more comprehensive definition of "gut health" should include the harmonious interaction of the microbiota-brain-gut axis (25, 33, 34).

All biological and physiological processes maintain the various microbiomes that live on mucosal surfaces in balance (35). Dysbiosis (loss of symmetry of the GIT microbiota) leads to loss of intestinal integrity (36). Dietary ingredients and the viscosity of gut contents influence microbes in the small intestine (37). Animal producers who have eliminated antibiotics from their production systems may use a combination of alternative products, improved management methods, stringent biosecurity, and successful immunization programs to achieve their health and productivity goals. However, chronic stress and persistent inflammation still harm modern animal production operations. Any source of chronic stress, whether biological, physical, chemical, toxic, or psychological, will cause oxidative stress and, if unabated, chronic intestinal and systemic inflammation (38-40). Chronic intestinal and systemic inflammation opens up the gut to opportunistic bacteria such as *C. perfringens*.

***Clostridium* spp. in the GIT**

To maintain gut homeostasis, a complicated mutualistic symbiosis maintains the host-microbiota connection (41). Commensal Clostridia (Class) in the Firmicutes (Phylum) make up a

large proportion of the gut microbiota (42). Clostridial spp. begin colonizing the intestine at hatch, live near intestinal cells, and play an important role in altering gut physiology and immunology (42). *Clostridium* (Genus) contains over one hundred beneficial species, and only a few are pathogenic (42). They represent the most significant butyric acid-producing organisms in the GIT (43). Commensal Clostridia play an active role in maintaining overall gut function (42). Hence, distinguishing beneficial Clostridial species from potentially virulent ones, like *Clostridium perfringens,* is critical (44). Clostridial cluster IV contributes to up to twenty percent of bacteria present in humans (45). *Clostridium* clusters XIVa and IV members consistently decreased in patients with gut inflammation (46). This implies that these organisms are vital to gastrointestinal homeostasis (44). Clostridiales (Order) also increased mucosal tolerance to commensal microbiota by boosting IL-10 and transforming growth factor-beta expression levels in the gut (47). Furthermore, Clostridiales such as *Ruminococcus* spp., *Faecalibacterium* spp., and *Lachnospiraceae* spp. are the remarkable bacteria that produce butyrate (48, 49), inducing profound physiological reactions in the gut (50). *Clostridium* cluster strains IV and XIVa are great inducers of T-regulatory cells and constitute a novel therapeutic alternative for intestinal inflammatory diseases (51). Interestingly, probiotics have been demonstrated to cause significant alterations in butyrate and other key SCFA, which have a considerable impact on gut physiology and immunology (52-57). Commensal *Clostridium* bacteria clearly are vital in gut homeostasis (42)**.** However, commensal Clostridial spp. such as *C. perfringens* rapidly proliferate when the broiler's epithelium is damaged (58). There are predisposing factors that increase *C. perfringens* overgrowth such as nutritional components (59) and coinfections with *Salmonella* spp. (60) or *Eimeria* spp. (61) (Figure 2). Mucin-2 is the most abundant mucin that is secreted by intestinal

epithelial cells (62). *Eimeria* spp. have been shown to have an effect on the relative mucin secretion in each area of the GIT (63).

Necrotic enteritis

Necrotic enteritis (NE) is caused by the ubiquitous bacterium *C. perfringens*. *C. perfringens* is an anaerobic, gram-positive, endospore-forming, nonmotile, bacterium that can survive and persist in harsh environmental conditions. As the chicken industry has reduced its usage of antibiotics, NE in both its clinical and subclinical forms have become a significant health, welfare, and performance problem (64). Because NE is a complex disease, that usually includes a co-infection of *Eimeria* spp. and *C. perfringens*, management without antibiotics requires sustainable alternative prophylactic or therapeutic strategies (65). The total global economic cost of NE was assessed to be more than $2 billion dollars in 2000 (66). The number of broiler chickens produced increased from 14.38 billion in 2000 to approximately 33 billion in 2020 worldwide (67). It was estimated that necrotic enteritis cost the United States poultry industry $6 billion annually (68).

C. perfringens is grouped into seven toxigenic categories (A−G) producing over twenty toxins (69). The poultry industry is particularly interested in *C. perfringens* types A, C, and G (70, 71). In this process, *C. perfringens* releases enzymes that break down host tissue, causing more tissue damage, inflammation, and disruption of the intestinal ecology, causing dysbiosis (72-74). These avian *C. perfringens* strains produce pathogenic toxins including NetB toxin, which has been identified as a major factor associated with NE in broilers (75)**.** However, some strains can cause NE without producing toxins (76), as proteolytic enzymes have also been identified to cause severe damage to the enterocytes (77) (Figure 3). Field outbreaks of NE had one *C. perfringens* clone prevalent in the intestines of all infected birds, rather than the variety of strains found in

healthy bird intestines. A single dominant *C. perfringens* strain associated with NE may be due to bacteriocin production (78). Intestinal *C. perfringens* overgrowth has been linked to intestinal mucosa injury, low pH, coccidiosis, nutritional factors, stress, and immunosuppression (75, 79, 80). Several investigators have evaluated different alternatives to reduce NE such as probiotics, prebiotics, synbiotics, and organic acids (81-84).

C. perfringens infection alone is not enough to cause necrotic enteritis in broiler chickens. Predisposing factors are a key player in creating the right environment for the proliferation of virulent *C. perfringens*, producing disease (85). These predisposing factors can include, but are not limited to, feed ingredients (86), coccidiosis caused by *Eimeria* spp. (87), environmental stressors (88), and exposure to *Salmonella* spp. (60).

Alternatives to antibiotics to control NE

Due to the removal of AGPs and the shift to antibiotic free production systems, research investigating antibiotic alternatives have been on the rise. The incidence of Clostridial-related diseases, including NE, increased with implementation of AGP bans (89). In an antibiotic-limited or antibiotic-free era, natural alternatives to optimize intestinal health and improve animal wellbeing and performance are desperately needed. Probiotics are live, beneficial microorganisms that have been shown reduce colonization by enteric pathogens (90-92). Direct-fed microbials (93), prebiotics (94-96), organic acids (97), plant extracts (98), essential oils (99, 100), and trace minerals (101) can help to improve intestinal microbial balance, metabolism, and gut integrity. Phytogenics have remarkable antioxidant, anti-inflammatory, antibacterial, and barrier integrity-enhancing assets. For example, supplementation with curcumin, a component in tumeric, reduced the severity of necrotic enteritis (102), salmonellosis (98, 102), and aflatoxicosis (103) in broiler

chickens as well as coccidiosis in Leghorn chickens (104). Additional investigations regarding phytogenics, specifically EOs and gut health are described below.

Essential oils

Essential oils (EOs) are a derivative of plants. Revered for their medicinal properties, EOs are natural, volatile compounds usually associated with a strong odor (105). EOs may consist of over 50 single components, but the major components constitute ~85% of the EO and are the primary components related to bacteriostatic or bactericidal activity (106). The mode of action(s) of EOs has been extensively reviewed (107). EOs inhibit *in vitro* proliferation of Gram-negative and Gram-positive bacteria by increasing membrane permeability of the cell wall and mitochondrial membranes (107). Antimicrobial efficiency of EOs is impacted by the compound's hydrophobicity (108). *In vitro* studies show evidence that when compared to standard antimicrobial agents, EOs have a similar effect on the growth inhibition of *C. perfringens* (109).

EOs have been increasingly popular as feed additives over the last two decades (110) due to their antibacterial, antiviral, antifungal, anti-inflammatory, digestive stimulant, immunomodulatory, and anti-hyperlipidemic properties (111-114**)**. Carvacrol and thymol are abundant in plants, such as *Lippia origanoides,* and stimulate enzyme secretion and improve digestion (115, 116) (Figure 4). Hence, EOs have been used as a feed additive in poultry diets without adverse effects (117). However, one of the most remarkable bioactive properties of EOs is their anti-oxidant effect, which prevents lipid peroxidation of the cell membrane and mitochondrial membrane phospholipids, as well as the denaturalization of proteins and DNA, thereby preventing multiple organ failure and other diseases **(**115, 116, 118,119).

At the moment of writing this paper, using the keywords *"Essential oils and necrotic enteritis"* in Google scholar, the results show an astonishing number of scientific manuscripts: 8,720. Most studies involve using EOs instead of antibiotics to reduce the severity of NE, demonstrating the bactericidal activity of EOs against *C. perfringens* (109**,** 120). Other recent studies have revealed a reduction in NE-induced intestinal damage (61, 121-124), reduction in mortality associated with NE (125, 126), regulation of the intestinal microbial communities (127-129)**,** modulation of short-chain fatty acids profiles (79, 130), improvement of the morphometric and barrier functions (126, 131), as well as, reduction of oocyst counts (132, 133) and dysbacteriosis (134, 135). The impact of EOs on performance parameters and intestinal lesion score in broiler chickens under different NE models has been summarized in Table 1.

EOs have been shown to alter the host's immune response. Dietary inclusion of carvacrol, cinnamaldehyde or oleoresin altered gene expression of intestinal intraepithelial lymphocytes, with dietary oleoresin having the greatest effect on transcriptional regulation (136). Furthermore, EOs limited pro-inflammatory cytokine production related to *C. perfringens* or *Eimeria* spp. challenge (137). Pathogenicity of *C. perfringens* was modulated by the inclusion of EOs (25% carvacrol, 25% thymol; 120mg/kg) in the diet which downregulated *in vivo* expression of *C. perfringens* virulence factors: VF 0073-ClpE, VF0124-LPS, and VF0350-BSH (129). The altered host ileal microbiome composition and *C. perfringens* virulence factor expression was likely reduced intestinal lesion scores and mortality in broiler chickens (129). The direct or indirect changes in the gut microbiome composition associated with EOs treatment is suggested to be a primary beneficial factor related to application of EOs as natural alternatives to antibiotics.

Combinations of EOs and organic acids have synergistic or additive effects that may improve poultry gut health and growth performance (138). Similar to dietary EOs fed alone, blends

of EOs and organic acids alter the composition of the gut microbiota, specifically increasing the abundance of *Lactobacillus* spp. (138) and SCFA concentration in the gut (139). As a result, the dietary blends can inhibit the overgrowth of *C. perfringens* in the gut perhaps lowering the incidence and severity of NE. For instance, encapsulated blends of EOs (thymol, vanillin, eugenol) and organic acids (fumaric, sorbic, malic, citric) have been shown to improve gut health and performance of NE-affected broiler chickens (140). Similarly, feeding microencapsulated blends of EOs and organic acids (BUTYTEC-PLUS or ACITEC-MC) to broiler chickens placed on used NE litter increased growth performance due to improved intestinal barrier function and integrity (135). Enteric inflammation associated with NE may have been reduced due to the anti-inflammatory effects of a dietary blend of encapsulated EOs and an organic acid (4% carvacrol, 4% thyme, 0.5% hexanoic, 3.5% benzoic, 0.5 % butyric acid) (131). Additionally, broiler chickens that received the EO and organic acid blend had improved intestinal integrity compared to the non-treated, challenged group (131).

Taken together, phytogenic compounds, such as EOs show promise as natural alternatives to mitigate the severity of NE-induced intestinal damage and performance losses. However, factors including the antimicrobial activity of the specific EO evaluated, EO dose, NE challenge model, and methods to determine efficacy of these naturally occurring AGP alternatives must be considered when designing experiments and comparing research findings.

Brief overview of methods to evaluate impact of antibiotic alternatives on intestinal integrity and enteric inflammation

Researchers could use enteric inflammation models to investigate alternative antibiotic growth promoters (AGP) and dietary supplements for livestock, including poultry. As a result, several intestinal inflammatory models including nutritional factors (37), management (141),

chemicals(142, 143), and environmental influences (100) have been used to evaluate the effect of AGP alternatives on enteric inflammation in a laboratory setting. A non-terminal approach, such as serum fluorescein isothiocyanate-dextran (FITC-d) concentration, can be used to assess intestinal permeability and tends to correlate with bacterial translocation in the liver (144). For the FITC-d assay, a 4-6kDa FITC-d molecule is utilized since it cannot translocate through an undamaged intestinal epithelium (145). Thus, an increase in FITC-d in the serum indicates that there has been damage to the intestinal epithelial barrier (145). Other reliable serum biomarkers, such as antioxidant biomarkers, isoprostane 8-iso-PGF2 and prostaglandin GF2, have been evaluated (104). Enterocyte biomarkers such as peptide YY, Fenterocellular signal-regulated kinase, citrulline, and mucin 2, as well as immune biomarkers peptide YY, enterocellular signal-regulated kinase, citrulline, and mucin 2, total or specific secretory IgA and interferon-gamma have been utilized (146, 147). IgA is closely associated with mucosal immunity in mammals and avian species (148, 149). It is the main immunoglobulin isotype in most mucosal secretions (149). Therefore, elevated IgA levels can be associated with elevated mucin production and an increased immune response. Interferon-gamma is a pro-inflammatory cytokine associated with intestinal inflammation and gut leakage (150). Thus, interferon-gamma levels in the serum can be used to assess inflammation. Reactive oxygen species, such as superoxide are free radicals that are created naturally through cellular respiration. Free radical accumulation is damaging (151). An enzyme, superoxide dismutase catalyzes superoxide into oxygen and hydrogen peroxide (152). Superoxide dismustase concentration in the sera has been used to assess oxidative stress in broiler chickens (146). Additionally, gene expression of other biomarkers, such as 1-acid glycoprotein, fatty acid-binding protein, and interleukins (IL-8, IL-1β,), mucin 2, transforming growth factor, and tumor necrosis factor have also yielded promising results (21, 153).

Inflammation alters expression of intestinal tight junction proteins followed by increased intestinal permeability (154). Futhermore, intestinal morphometric measurements, such as villus height, villus width, crypt depth, and crypt/villi ratio can be used to evaluate gut integrity. An increase in crypt depth and villus witdh was indicative of gut barrier failure in broiler chickens (153). The I See Inside (ISI) methodology, which employs both macroscopic and histological analyses, has been used to determine the impact of a treatment or challenge on an organs function. This method been used to assess effect of EOs and organic acids on ISI scores in NE-challenged broiler chickens (140).

Conclusion

The increased incidence of NE due to the removal of AGPs or shift to ABF or NAE production in commercial poultry systems has been associated with reduced performance and increased mortality (75, 155). *C. perfringens* is the causative agent for necrotic enteritis (156). Biological and non-biological factors may increase predilection for NE in a flock. Biological factors including *Salmonella* spp., *E. maxima*, or a diet change (60, 157). These predisposing factors promote *C. perfringens* replication by disrupting the microbiota composition and damaging the intestinal epithelial lining (85). Thus, NE can be consistently reproduced in a controlled laboratory setting by challenging with *Salmonella* Typhimurium at placement, *E. maxima* at 18 days-of-age, and *C. perfringens* challenge at 22 and 23 days-of-age (60, 158). This challenge model can be used to evaluate natural alternatives, such as plant-derived essential oils, to prevent NE in broiler chickens, as confirmed by a recent study published in our laboratory (99).

Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Author Contributions

MEC, BDG, and GT-I developed the conceptualization and wrote the first draft of the manuscript. GT-I drew and edited the figures. VP-G, XH-V, XS, JDL, and BMH participated in design, analysis, presentation, and writing of manuscript. All authors have read and agreed to the submitted version of the manuscript.

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Table 1. Impact of essential oils (EOs) on performance parameters and intestinal lesion score in broiler chickens under different necrotic enteritis (NE) models.

Essential oils / Source	Dietary inclusion	Performance parameter	NE lesion score	References
Citrus, oregano and annase EOs	1 g/kg of feed	Reduction in mortality (26% PC* vs 8% EOs).	1.33 vs 0.58 (PC vs EOs).	(123)
Ginger oil and carvacrol	1.5 g/kg of feed	EOs improve BW in 100 g compared to the PC.	3.0 vs 2.3 (PC vs EOs).	(159)
Capsicum oleoresin and turmeric oleoresin	4 mg of each oleoresin per kg of feed.	Improve BW ($P < 0.05$).	2.8 vs 1.2 (PC vs EOs).	(160)
Thymol and carvacrol	0, 60, 120 or 240 mg/kg of feed	EO linearly reduce FCR ($P = 0.056$).	1.5 vs <0.5 (PC vs EOs).	(137)
Thymol and carvacrol	120 mg/kg of feed	Reduction in mortality (20% PC vs 4% EOs).	>2 vs <1 (PC vs EOs).	(138)
Thyme (thymol) and clove (eugenol)	Combination: Thyme 2.5 g/kg and Clove 1.25 g/kg of feed	EOs improve BWG in 200 g compared to PC. FCR 2.84 PC vs 1.88 EOs.	3.0 vs 1.0 (PC vs Eos).	(161)
Peppermint oil	0.5 or 0.25 ml/ml of water	Reduction in mortality (55 % PC vs 10% EOs). Improve BWG in 41 g	ND€	(162)
Thymol and carvacrol	120 mg/kg of feed	Reduction in mortality (20% PC vs 4% EOs).	>2 vs <1 (PC vs EOs).	(125)
Garlic nanohydrogel	100, 200, 300 or 400 mg/kg	EOs increase BWG 181g, 367g and 588 g (200, 300 and 400 mg/kg).	>2 vs <1 (PC vs 400 mg/kg EOs).	(163)
Thyme, savory, peppermint and black pepper	0.5, 1 or 2 g/kg of feed	Improvement in BW and FCR with 1 or 2 g/kg inclusion.	ND	(164)
Thymol and carvacrol	200 or 300 mg/kg of feed	Improve BWG during challenge period ($P < 0.05$).	ND	(165)
Eugenol and garlic tincture	100 mg/kg of feed	Overall FCR was improved 1.681 PC vs 1.645 EOs	0.5 vs <0.5 (PC vs EOs – only in males).	(132)

*PC: Positive control; €ND: Not determined

Figures

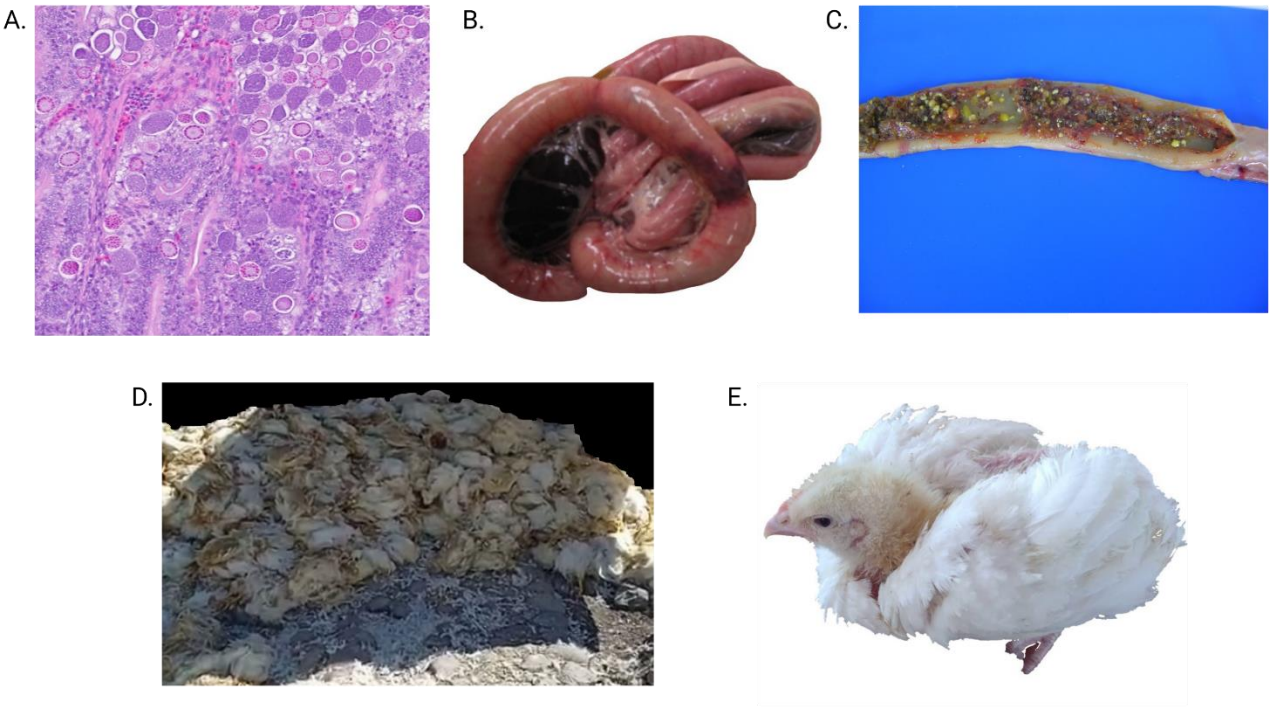

Figure 1. Even though necrotic enteritis is a multifactorial disease, *Eimeria maxima* has been considered a primary pathogen in the clinical and subclinical outbreaks of necrotic enteritis. A) Mucosal and submucosal jejunum with infiltration of inflammatory cells, ulceration, necrosis, and the presence of *E. maxima* oocysts. Hematoxylin and eosin staining. NE causes macroscopic B) ballooning of intestines and C) extensive sloughing of the intestinal mucosa and hemorrhaging. D) While clinical and acute outbreaks can induce severe mortality, the global economic losses are due to E) subclinical necrotic enteritis, affecting all performance parameters. (Created with BioRender.com)

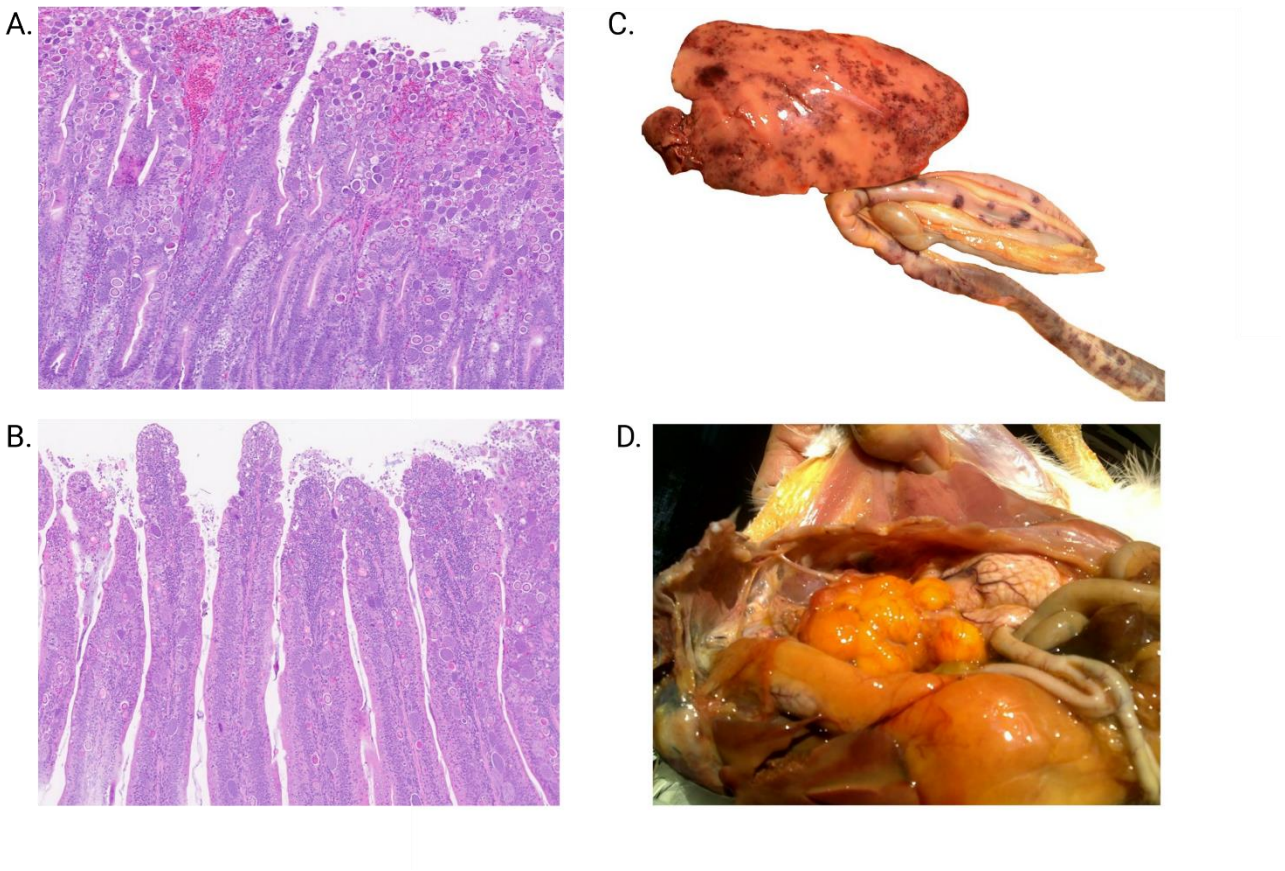

Figure 2. A) Mucosal and submucosal jejunum with infiltration of inflammatory cells, ulceration, necrosis, and the presence of *E. maxima* oocysts. Hematoxylin and eosin staining. B) Mucosal and submucosal duodenum with infiltration of inflammatory cells, ulceration, necrosis and the presence of *Eimeria acervulina* oocysts. Hematoxylin and eosin staining. C) Duodenum and jejunum of a layer hen with necrotic enteritis. The liver shows areas of necrosis and hemorrhages due to liver bacterial translocation and chronic systemic inflammation. D) Oviduct and ovary from the same layer hen showing hemorrhages and inflammation. (Created with BioRender.com)

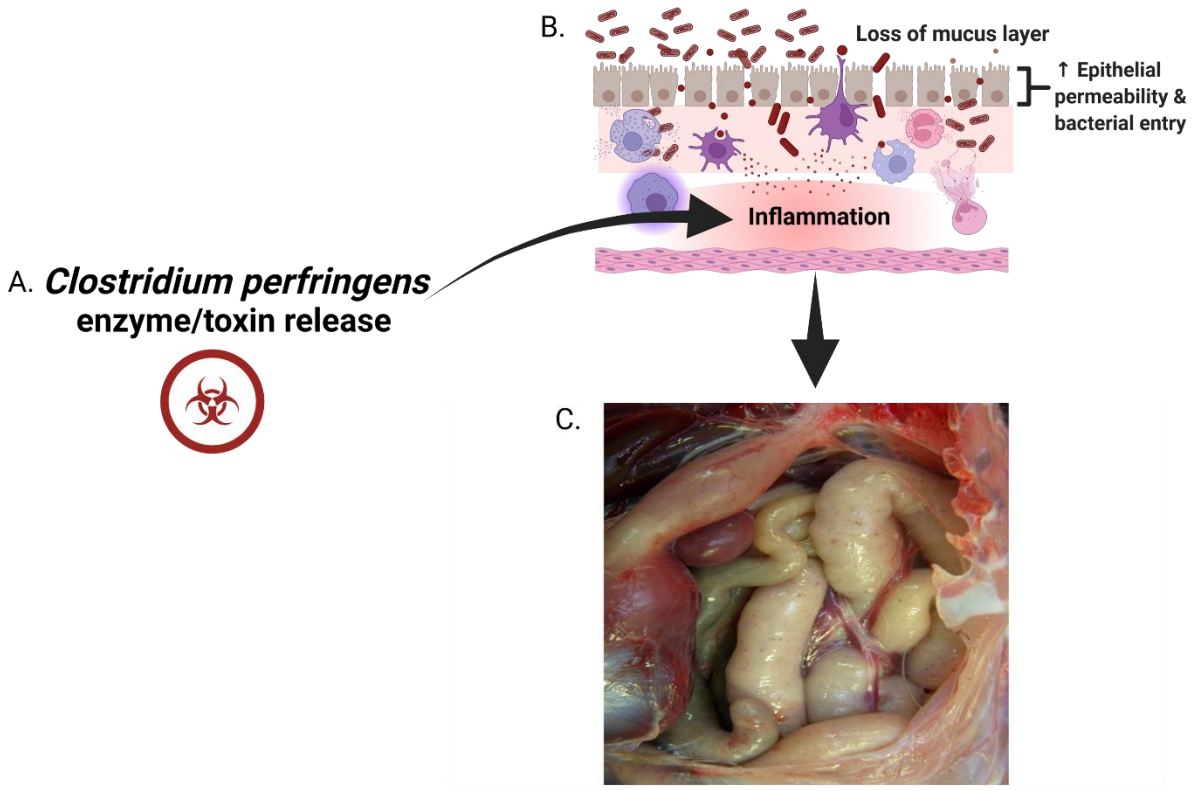

Figure 3. *C. perfringens* types A, C, and G release A) enzymes that break down host tissue causing B) inflammation, and disruption of the intestinal ecology, resulting in dysbiosis. NetB toxin, which has been established as a significant contributor in NE in broilers, is present in these lethal avian *C. perfringens* strains. Some strains, however, can produce NE without creating toxins, as proteolytic enzymes have been shown to cause severe damage to enterocytes as a result of a severe inflammatory response. C) Lesions caused by *C. perfringens* proliferation and toxin production can be observed macroscopically. (Created with BioRender.com)

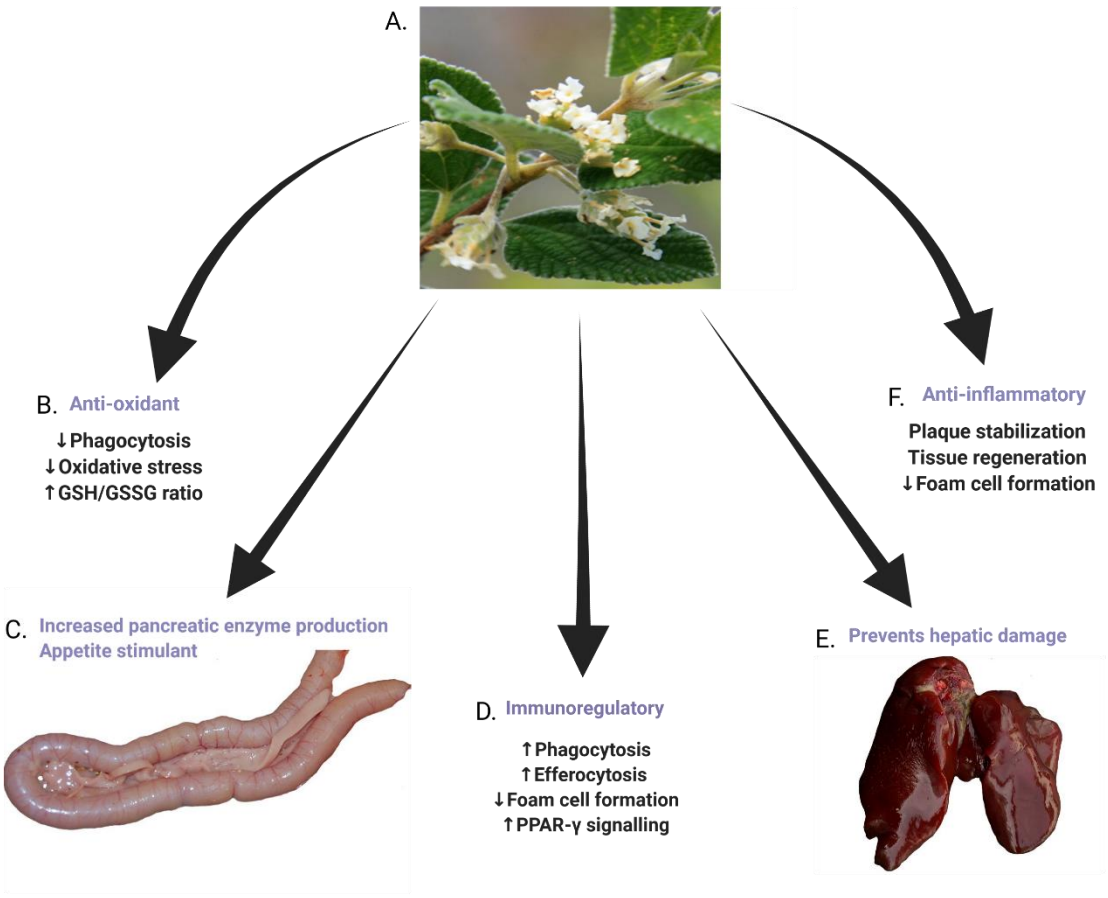

Figure 4. Carvacrol and thymol are two of the most abundant essential oils present in A) *Lippia origanoides*. Both essential oils have been extensively studied due to their B) anti-oxidant, C) appetite stimulant and increased pancreatic enzyme production, D) immunoregulation, E) ability to prevent hepatic damage, and F) anti-inflammatory properties. (Created with BioRender.com)

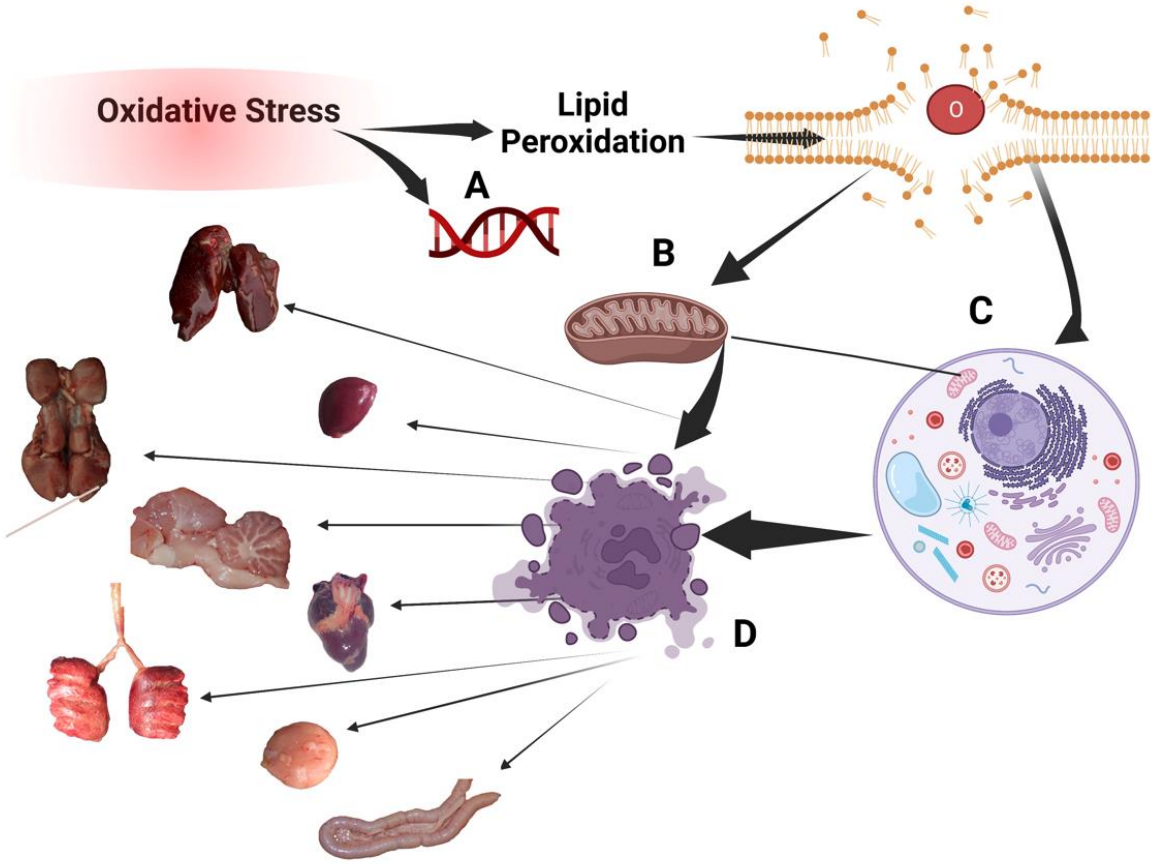

Figure 7. Oxidative stress causes A) DNA damage and lipid peroxidation in vital cellular components, such as B) the mitochondrial membrane and C) cell membrane. This triggers D) apoptosis, necrosis, inflammation, and multiple organ failure. (Created with BioRender.com).

Chapter III. Brief Research Report

Assessment of *Lippia origanoides* essential oils in a necrotic enteritis challenge model in broiler chickens

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Abstract

The objective of the present research was to evaluate dietary supplementation of essential oils from *Lippia origanoides* (LEO) on necrotic enteritis (NE). At day-of-hatch, chicks were randomly assigned to three groups. Group 1: negative control (NC); Group 2: positive control (PC) challenged with *Salmonella* Typhimurium (day 1), *Eimeria maxima* (EM, day 18), and *C. perfringens* (CP, days 22-23); Group 3: dietary supplementation with 37 ppm LEO, challenged in the same manner as the PC. At the end of the trial, at d 25 of age, serum samples were collected to evaluate FITC-d, SOD, IFN-γ, IgA. Chickens receiving LEO showed a significant improvement in BW and BWG when compared with the positive challenge control chickens. Chickens that received LEO had a significant improvement in FI compared with positive control chickens but similar FCR. Digested feed was utilized for *in vitro* assays. The digested feed supernatant, supplemented with LEO and inoculated with CP, showed a significant reduction in CP cfu/mL. Treatment Group 3 also exhibited a significant reduction in NE lesion scores compared with positive challenge control chickens. Chickens that received LEO showed a significant reduction in FITC-d compared with positive challenge control chickens. A significant increase in serum levels of SOD was also observed in chickens that received LEO compared with both control groups. However, chickens receiving LEO showed a significant reduction in serum levels of IFN-γ and IgA compared with positive control chickens. Further investigation to compare the effect of LEO and the standard treatment of clostridial NE is required.

Introduction

In recent years, a considerable attention to essential oils (EO) as nutraceuticals in livestock production has occurred, mainly as an alternative to antibiotic growth promoters (AGPs) worldwide. Essential oils are derived from various plants as secondary metabolites with well-

documented antibacterial, antiviral, antifungal, antioxidant, digestive stimulants, and immunomodulatory properties (1-5). Some EO are used in a combination with other phytochemicals to increase performance in poultry (6). Hence, EO have played an important role in controlling the increased incidence of coccidiosis and necrotic enteritis (NE) following the removal of ionophores and AGPs. Since NE is a multifactorial disease involving *Eimeria* spp. and *C. perfringens*, in many cases, EO are combined with other strategic products such as probiotics, prebiotics, organic acids, and enzymes to modulate the intestinal microbiota and immune system of the birds (7-9).

In poultry, NE is a disease caused by the anaerobe gram-positive bacterium, *Clostridium perfringens* (CP). Several virulence factors have been identified in the pathogenesis of NE, including toxins, proteolytic enzymes, bacteriocins, and adherent molecules produced by virulent strains of CP (10). In 2000, the annual worldwide economic cost of NE was estimated at over $2 billion (11, 12). However, the global number of chickens has increased from 14.38 billion chickens in 2000 to 23.70 billion chickens in 2018 (13). Based on those statistics, annual losses associated with mortality and decreases in performance caused by NE at the end of 2020 can be estimated at over $4 billion, especially in subclinical cases of NE (14,15). Several factors have been identified in the pathogenesis of this multifactorial disease such as coccidia infections, immunosuppression, dysbacteriosis, and removal of AGPs (7-10). Hence, several investigators have evaluated the use of several nutraceuticals that include EO, organic acids, probiotics, and plant extracts as an effort to reduce the economic impact that NE has in the modern poultry industry (8,15-24).

After two years of unsuccessful attempts to reproduce a laboratory NE challenge model, our laboratory inadvertently discovered the role of early *Salmonella* Typhimurium (ST) infection

as a predisposing factor for NE (25). Our unexpected finding came after working with recombinant *Salmonella* vaccine vectors containing *Eimeria maxima* (EM) epitopes (26). During those studies, control chickens receiving the empty vector at day 1, but challenged with EM at day 18, showed high mortality due to NE. Subsequent studies confirmed that day-old broiler chickens challenged with ST, followed by EM and CP challenge at day 18, caused enhanced NE development compared to an EM and CP challenge only (25). Puzzled by those results, we found that previous researchers demonstrated severe immune suppression and injury to intestinal integrity by persistent local inflammation caused by early ST infection in day-old chickens (27,28). Since then, we have used ST as a neonatal infection to induce early immunosuppression for consistent and reliable NE challenge in studies published by our laboratory (16,17, 29). The objective of the present research was to evaluate dietary supplementation of *Lippia origanoides* essential oils (LEO) on *in vitro* proliferation of *C. perfringens,* as well as performance, intestinal integrity, and NE lesions using early *Salmonella* Typhimurium (ST) infection as a predisposing factor for NE in broiler chickens (16, 17, 25, 29).

Materials and Methods

Essential oils from *Lippia origanoides*

The LEO was provided by Promitec S.A. (Bucaramanga, Santander, Colombia) and feed inclusion based on the manufacturer's recommendations. The product contains EO of *Lippia origanoides* microencapsulated with maltodextrin by spray drying with a diet inclusion rate of 37 ppm. Starter, grower, and finisher diets used in this experiment were formulated to approximate the nutritional requirements of broiler chickens as recommended by the National Research Council (30) and adjusted to the breeder’s recommendations (31). No antibiotics, coccidiostats or enzymes were added to the feed (Table 1).

Ethics

This study was carried out in accordance with the recommendations of the Institutional Animal Care and Use Committee (IACUC) at the University of Arkansas, Fayetteville, under protocol #21018.

Experimental design

In the present study, an experiment was conducted to evaluate the effect of *L. origanoides* LEO on a NE model previously described by our laboratory (25), which was confirmed and extended in more recent studies (16, 17, 29). Day-of-hatch Cobb 500 male broiler chicks (n= 300) were obtained from a commercial hatchery. At day-of-hatch, chicks were randomly assigned to one of three groups, with ten replicates and ten chickens in each replicate (n=10 replicates/group; 10 chickens/replicate). Group 1: negative control (NC); Group 2: positive control (PC) challenged with *Salmonella* Typhimurium (day 1), *E. maxima* (day 18), and *Clostridium perfringens* (days 22 and 23); and Group 3: 37 ppm final feed concentration LEO, challenged in the same manner as the PC

Body weight was recorded on days 0, 7, 14, 18, and 25. Body weight gain was recorded on d 0-7, 8-25, and 0-25. Feed intake (FI) and feed conversion ratio (FCR) per cage were recorded on d 0-7, 8-25, and 0-25.

Evaluation of serum levels of FITC-d, SOD, IFN- γ, and IgA

At the end of the trial, on d 25 of age, two random chickens per cage were selected (n=20) and orally gavaged with 8.32 mg/kg of body weight of fluorescein isothiocyanate-dextran (FITC-d, MW 3-5 KDa; Sigma-Aldrich Co). One hour after FITC-d administration, chickens were euthanized by CO_2 inhalation. Blood samples were collected from the femoral vein and centrifuged

(1000×g for 30 min) to separate the serum. Serum levels of FITC-d (ng/mL) were used as a biomarker to evaluate intestinal permeability as described by Baxter et al. (32). Commercial kits were used to evaluate serum levels of superoxide dismutase (SOD, U/mL), gamma interferon (IFN-γ, pg/mL), and immunoglobulin A (IgA, ng/mL) as described by (33).

Necrotic enteritis lesion score

Ileal NE lesion score (n=40 chickens/group) was evaluated as previously described (34) where 0 = no lesions; 1 = thin-walled and friable intestines; 2 = focal necrosis, gas production, and ulceration; 3 = extensive necrosis, hemorrhage, and gas-filled intestines; and 4 = generalized necrosis typical of field cases and marked hemorrhage.

Necrotic enteritis model: challenge organisms

The *Salmonella* Typhimurium (ST), *E. maxima* Guelph strain (EM- GS), and *Clostridium perfringens* (CP) isolate details and culture conditions used to induce NE are described in previous studies (16, 17, 25, 29, 35). One-day-old broiler chickens were weighed and challenged with $1x10^8$ cfu of ST per bird by oral gavage. At day 18, chickens in challenged groups were orally gavage with 40,000 oocyst per mL. The dose was selected based on a prvious trial conducted to determine a challenge dose causing sub-clinical coccidiosis and reduction of 35 % body weight gain as described previously (16, 17, 25, 29). The *C. perfringens* culture was administered on days 22 and 23 of age via oral gavage at a concentration of 1×10^9 cfu per bird and was also used in the *in vitro* proliferation assay (described below).

Clostridium perfringens proliferation using in vitro digestion assay

The *in vitro* digestion model used in this trial was centered on earlier studies (35, 36. The test was conducted using digested diets of the starter control feed or the control starter feed supplemented with LEO (37 ppm). An inoculum of 10^5 cfu/mL of CP was included to five replicates (n=5) in two groups containing: Group 1) 3mL TSB supplemented with THIO + 3 mL supernatant from digested control diet (positive control); or Group 2) 3mL TSB supplemented with THIO + 3mL supernatant from a digested diet supplemented with LEO. Supernatant from digested control diet without *C. perfringens* inoculation was included as a negative control group. Samples were incubated anaerobically at 40°C, with tubes set at a 30° angle with continuous shaking (200 rpm) for four hours. Following incubation, ten-fold serial dilutions were made from all three experimental groups, plated on TSA supplemented with THIO and incubated for twenty-four hours at 40°C, anaerobically. Results are reported as $\log_{10}$ cfu of CP per mL.

Data and statistical analysis

All data were subjected to analysis of variance as a completely randomized design, using the General Linear Models procedure of SAS (37). Significant differences among the means were determined by Duncan's multiple range test at $P < 0.05$.

Results

Effect of LEO on growth performance of chicken challenged with NE organisms

The evaluation of body weight, body weight gain, feed intake, and feed conversion ratio in broiler chickens consuming a diet supplemented with 37 ppm LEO on a necrotic enteritis challenge model are summarized in Table 2. Chickens that were challenged with ST at d 1 showed a

significant reduction ($P < 0.05$) in BW at d 7 when compared with negative control chickens. It was interesting to observe that chickens that received the diets with the inclusion of LEO had a similar BW compared with negative control chickens on d 14 and 18, before *E. maxima* challenge. At the end of the trial, chickens receiving LEO showed a significant improvement in BW and BWG when compared with the positive challenge control chickens (Table 2). As expected, negative control chickens showed a significant increase in FI and more efficient FCR as compared with chickens that were challenged. In this study, chickens that received LEO had a significant improvement in FI compared with positive control chickens but similar FCR (Table 2).

Improvement of intestinal integrity, antioxidant, and anti-inflammatory effect of LEO in chicken challenged with NE organisms

Figure 1 shows the results of the serum FITC-d levels, SOD, IFN-γ, and IgA in broiler chickens consuming a diet supplemented with 37 ppm LEO on a necrotic enteritis model challenge model. A significant reduction in FITC-d serum levels was observed in chickens in the negative control group when compared with chickens that were challenged. Interestingly, chickens that received LEO showed a significant reduction in leakage of FITC-d from the intestine into the bloodstream when compared with positive challenge controls (Fig. 1A). Furthermore, a significant increase in serum levels of SOD was also observed in chickens that received LEO compared with both control groups (Fig. 1B). Interestingly, chickens receiving LEO showed a significant reduction in serum levels of IFN-γ and IgA compared with positive control chickens (Fig. 1C and Fig. 1D).

Antimicrobial effect of LEO on Clostridium perfringens log_{10} count using in-vitro digestion assay

The supernatant from digested feed supplemented with LEO and inoculated with CP showed a significant reduction in log_{10} cfu/mL of CP when compared with the positive control feed without LEO Supernatant from digested feed without CP inoculation remain negative to CP (Table 3).

Protective effect of LEO against NE lesion in chickens challenged with NE organisms

Chickens that consumed LEO -supplemented feed had a significant reduction in NE lesion scores compared with positive challenge control chickens. No lesions were observed in negative controls (Table 3).

Discussion

It is estimated that EO are produced by over 17,500 species of plants; however, only a minor fraction are used commercially (38). The chemical composition of EO include several terpenes, terpenoids, and phenylpropanoids. Additionally, sulfur derivatives, fatty acids, aldehydes, alcohols, and oxides have also been identified (39, 40).

Due to their antimicrobial properties, the use of whole EO are more advantageous than purified components due to synergistic effects, multiple biological properties, and fewer probabilities to select for antimicrobial resistance (38, 40, 41, 42). One particular study has emphasized the antimicrobial effects of essential oils, even against multi-drug resistant bacteria (43). In another recent study, flow cytometry showed EO's antimicrobial properties are due to the increase in the permeability of the bacterial membrane and inhibition of the efflux pump activity

(44). In the same study, the immunomodulatory effect of EO on THP-1 cells was also demonstrated by gene expression profiles of pro-and anti-inflammatory cytokines (44). Of equal importance, EO biological properties have been associated with maintaining intestinal integrity, strengthening the mucosal barrier, and microbiota modulation (1, 4-6, 8, 18, 19).

Even at low concentrations, EO have shown substantial and superior antibacterial properties against *C. perfringens* and other pathogenic bacteria compared to antibiotics (45). Similarly, in the present study, feed inclusion of 37 ppm LEO significantly reduced the proliferation of *C. perfringens* in the digested supernatant of an *in vitro* digestive model compared to the positive control diet by 1.94 logs (Table 3). While EO have shown enhanced antimicrobial activity, the encapsulation of EO has shown higher antimicrobial activity compared to the use of EO alone by slowing down the degradation and reducing the organoleptic effects of EO (46, 47). In the present study, LEO was microencapsulated with maltodextrin through the spray dry process. At an inclusion rate of 37 ppm, this formulation showed a significant improvement in BW and a significant reduction in NE lesion scores compared with the positive challenge control group (Table 2 and Table 3). These findings are in agreement with the results published by other studies (20-24). Moreover, chickens that received LEO had a significant decrease in FITC-d serum levels compared to positive challenge control chickens (Table 3). Due to its high molecular weight (3-5 KDa), intestinal leakage of FITC-d has been reported to be a reliable biomarker to evaluate intestinal inflammation and disruption of tight junction proteins in several poultry models published by our laboratory (48-52). Other investigators showing a substantial improvement on intestinal integrity and permeability by oregano EO (53, 54) have described similar results.

Furthermore, there was a significant reduction in IFN-γ serum levels in chickens that received LEO compared with positive control chickens, suggesting a reduction in the inflammatory

response (33, 55, 56). Moreover, these serological results were also associated with a significant reduction in secretory IgA serum levels in chickens supplemented with LEO compared with the positive control (Table 3). Comparable results have been confirmed in previous studies conducted in our laboratory with the use of other phytobiotics, organic acids, and probiotics (16, 57, 58). As indicated by Staley et al. (59), secretory IgA is an excellent biomarker to evaluate stress and inflammation.

In addition to their antimicrobial properties, other assets of EO are their antioxidant activity and radical scavenging activities (60). In the present study, chickens fed the formulation of microencapsulated LEO at 37 ppm had increased SOD serum concentrations compared with the positive control chickens (Table 3). Free radical scavenging capacity protects the integrity of cellular and mitochondrial membranes from lipid peroxidation (61, 62). The EO from *L. origanoides* have shown to increase the antioxidant response, radical scavenging activity, and apoptosis, suggesting a unique mechanism by these compounds (63-68)**.**

Conclusions

In summary, the results of the present study are not new or novel. Rather, confirm what it has been published by multiple research groups describing the role of EO and other nutraceuticals as alternative tools to reduce the severity of NE (8, 15-24). Hence, in the present study, the antimicrobial, anti-inflammatory, and antioxidant properties of LEO were associated with improved performance and reduction of lesion scores compared to challenged control chickens in a NE model that includes an ST infection as a predisposing factor for NE in broiler chickens (16, 17, 25, 29). However, further investigation to compare the effect of LEO and the standard treatment of clostridial NE is required. It is fair to mention that this was a limitation in the present

study. Moreover, it is imperative to establish standardized protocols that consider individual and inter-sample variability and consider the utility of molecular tools and epigenetic adjustments underlying phytobiotics such as EO, where research has not yet been elucidated.

Conflict of Interest

Uribe Á.J., Martínez B.C., and Angel-Isaza J.A. are employed by Promitec S.A. The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Author Contributions

Tellez-Isaias G., Makenly E.C., and Forga AJ.: Conceptualization, Methodology, and Software. Uribe Á.J., Martínez B.C., and Angel-Isaza J.A.: Methodology and Visualization. Señas-Cuesta R., Graham BD, and Selby C.M.: Investigation and Data curation. Tellez-Isaias G., Makenly E.C., and Hargis B.M.: Supervision, Writing - Original draft preparation. Tellez-Isaias G. and Hernandez-Velasco X.: Reviewing and Editing. All the authors reviewed, edited, and approved the manuscript.

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Tables

Table 1. Ingredient composition and nutrient content of the corn-soybean diet used on an as-is basis

Item	Stater phase (d 1 to 7)	Grower phase (d 8 to 14)	Finisher phase (d 15 to 25)
Ingredients (%)			
Corn 9-14-18	51.80	57.81	59.64
SBM (45.16%)	37.66	31.62	27.23
DDGS 8.1% EE	4.00	4.00	6.00
Poultry fat	3.24	3.44	4.38
Limestone	1.08	1.06	1.03
Dicalcium phosphate	1.01	0.88	0.64
Salt	0.35	0.35	0.31
DL-methionine	0.29	0.25	0.22
L-lysine HCl	0.12	0.13	0.12
Waldroup TM Mix	0.10	0.10	0.10
Tyson 2x Broiler Vit	0.10	0.10	0.10
L-threonine	0.08	0.09	0.09
Choline chloride (60%)	0.06	0.06	0.05
Sodium bicarbonate	0.04	0.05	0.03
OptiPhos2000 (0.5lb/ton)	0.025	0.025	0.025
Se Premix (0.06%)	0.020	0.020	0.020
Santoquin	0.019	0.019	0.019
Total	100	100	100
Calculated analysis			
ME (kcal/ kg)	3015.00	3090.00	3175.00
Ether extract (%)	5.88	6.20	7.28
Crude protein (%)	22.30	20.00	18.70
Lysine (%)	1.18	1.05	0.95
Methionine (%)	0.59	0.53	0.48
Threonine (%)	0.77	0.69	0.65
Tryptophan (%)	0.25	0.22	0.20
Total calcium (%)	0.90	0.84	0.76
Total phosphorus (%)	0.63	0.58	0.53
Available phosphorus (%)	0.45	0.42	0.38
Sodium (%)	0.20	0.20	0.18
Potassium (%)	1.06	0.94	0.87
Chloride (%)	0.27	0.28	0.25
Magnesium (%)	0.19	0.18	0.17
Copper (%)	19.20	18.46	18.85
Selenium (%)	0.28	0.27	0.26
Linoleic acid (%)	1.01	1.13	1.16

Table 2. Evaluation of body weight (BW), body weight gain (BWG), feed intake (FI), and feed conversion ratio (FCR) in broiler chickens consuming a diet supplemented with 37-ppm *Lippia origanoides* essential oils on a necrotic enteritis challenge model*

Item	Negative Control	Positive Control*	*Lippia origanoides**
BW, g/broiler			
d 0	42.24 ± 0.36 [a]	41.87 ± 0.34 [a]	42.73 ± 0.30 [a]
d 7	156.08 ± 3.03 [a]	140.84 ± 1.96 [b]	147.50 ± 4.8 2[ab]
d 14	445.35 ± 8.98 [a]	415.24 ± 6.02 [b]	436.93 ± 9.71[ab]
d 18	690.13 ± 10.02 [ab]	659.58 ± 8.18 [b]	690.84 ± 12.0 5[ab]
d 25	1185.53 ± 12.80 [a]	828.82 ± 9.80 [c]	862.49 ± 13.39 [b]
BWG, g/broiler			
d 0-7	113.83 ± 3.14 [a]	98.97 ± 1.85 [c]	104.77 ± 4.68 [ab]
d 8-25	1000.12 ± 12.6 [a]	653.45 ± 10.77 [b]	649.07 ± 17.20 [b]
d 0-25	1135.09 ± 12.86 [a]	776.62 ± 10.16 [c]	800.44 ± 13.62 [b]
FI, g/broiler			
d 0-7	142.91 ± 10.07[a]	132.93 ± 2.58[a]	131.41 ± 3.10[a]
d 8-25	1180.75 ± 31.01[a]	983.39 ± 23.74[b]	901.94 ± 20.94[c]
d 0-25	1537.38 ± 20.30[a]	1295.70 ± 24.99[c]	1373.92 ± 64.61[b]
FCR			
d 0-7	1.25 ± 0.07[a]	1.35 ± 0.03[a]	1.28 ± 0.07[a]
d 8-25	1.37 ± 0.03[c]	1.73 ± 0.04[b]	1.87 ± 0.11[a]
d 0-25	1.36 ± 0.02[c]	1.67 ± 0.04[b]	1.75 ± 0.08[a]

*Day-old broilers were challenged with *Salmonella* Typhimurium (day 1), *E. maxima* (day 18), and *Clostridium perfringens* (days 22 and 23).

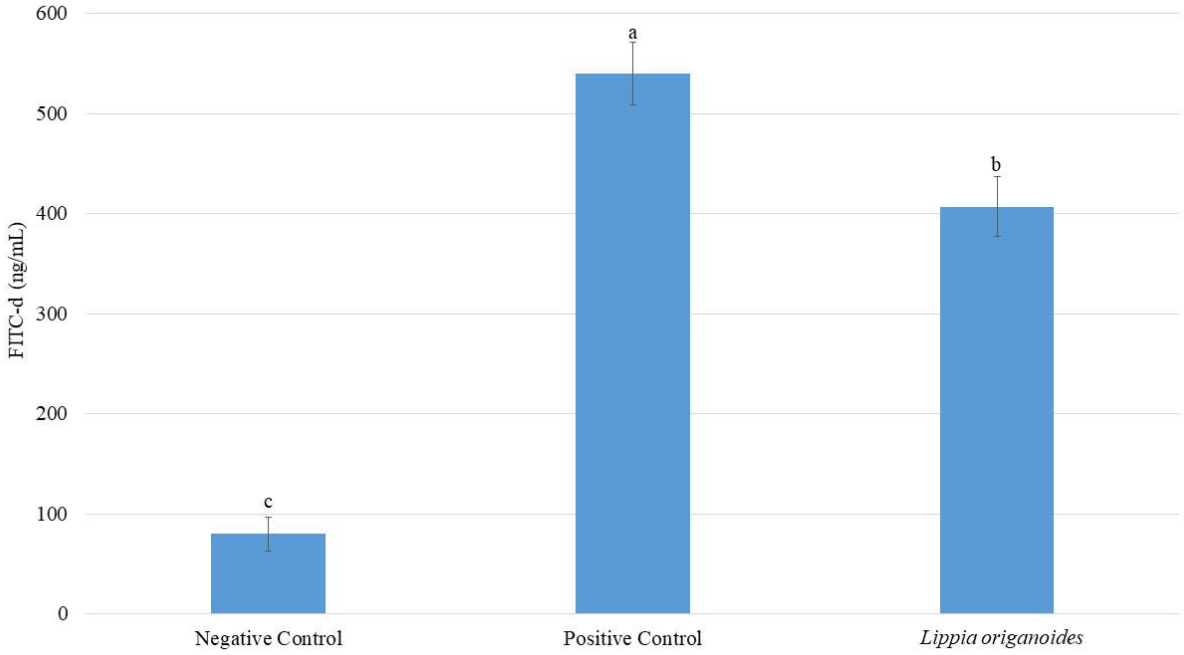
Data are expressed as mean ± SE. [a,b,c] Non-matching superscripts within rows indicates significant difference at $P < 0.05$.

Table 3. Evaluation of *Clostridium perfringens* proliferation using *in vitro* digestion assay and necrotic enteritis (NE) lesion scores in broiler chickens supplemented with *Lippia origanoides* essential oils on a necrotic enteritis model challenge model

Item	Negative Control	Positive Control	*Lippia origanoides*
C. perfringens (log_{10} cfu/mL)	0.00 ± 0.00 [c]	6.95 ± 0.20 [a]	5.01 ± 0.10 [b]
NE Lesion scores	0.00 ± 0.00 [c]	2.63 ± 0.05 [a]	1.76 ± 0.11 [b]

Data are expressed as mean ± SE.
[a,b,c] Non-matching superscripts within rows indicates significant difference at $P < 0.05$.

Figures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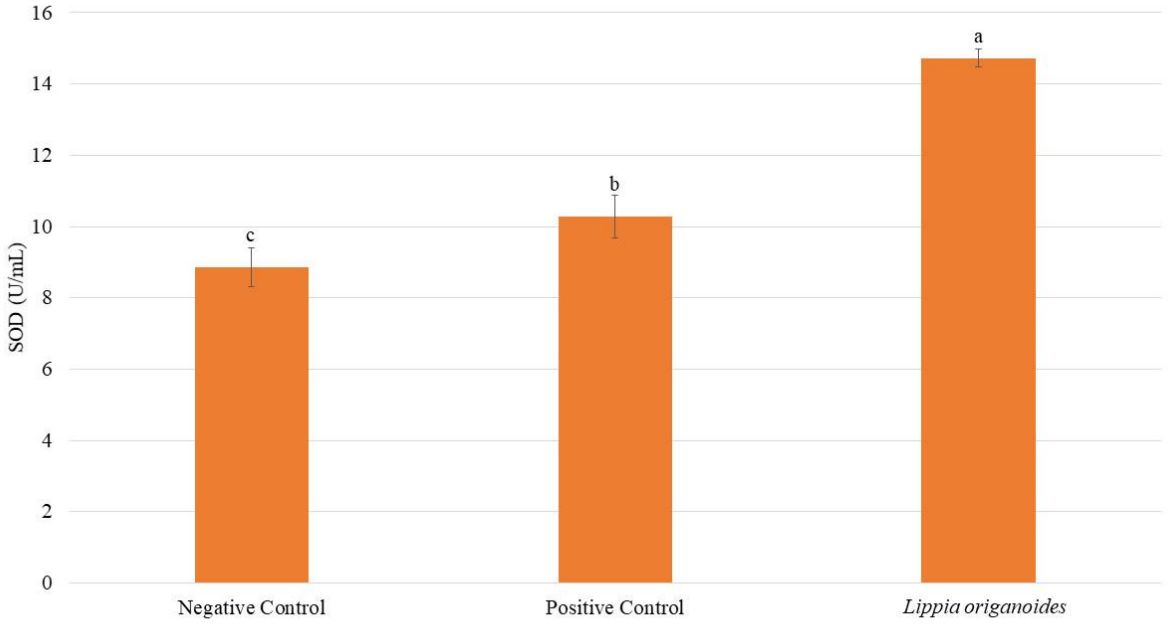
400
300
200
100
0
FITC-d (ng/mL)
a
b
c
Negative Control
Positive Control
Lippia origanoides


16
14
12
10
8
6
4
2
0
SOD (U/mL)
a
b
c
Negative Control
Positive Control
Lippia origanoides

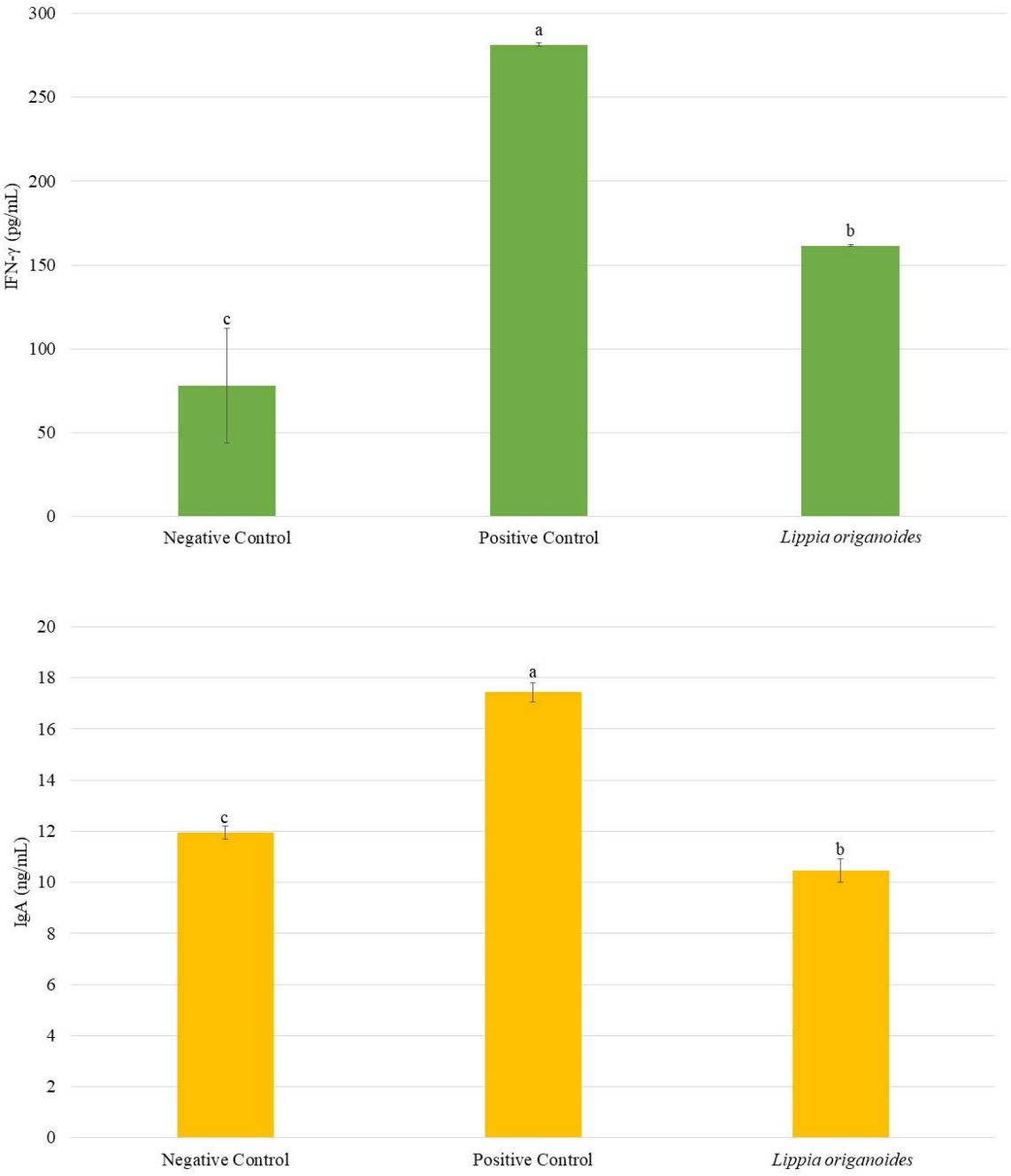


Figure 1. Evaluation of serum levels of fluorescein isothiocyanate dextran (FITC-d), superoxide dismutase (SOD), gamma interferon (IFN-γ), and IgA in broiler chickens supplemented with *Lippia origanoides* essential oils on a necrotic enteritis model challenge model. A) FITC-d, B) SOD, C) IFN-γ, and D) IgA. [a,b,c] Non-matching superscripts indicates significant difference at $P < 0.05$.

Chapter IV. Conclusion

There is still much to be learned about essential oils and their effect on the gastrointestinal tract (GIT) of poultry. In this study, the essential oil from *Lippia origanoides* (LEO) was seen to reduce necrotic enteritis lesions when compared to the positive control. It was also shown that the dietary inclusion of the essential oil improved all the performance parameters in comparison to the challenged untreated group. Additionally, administration of LEO reduced intestinal permeability and inflammation, as well as, enhanced the antioxidant activity in the supplemented animals. Moreover, when using an in vitro digestion model, it was also observed that LEO decreased the proliferation of *Clostridium perfringens*, further supporting the results observed during the in vivo study. These kind of results have been published multiple times by different research groups, all suggesting a reduction in necrotic enteritis lesions with no negative effects on bird performance. More research should be conducted comparing the LEO to the current standard treatment of clostridial necrotic enteritis. These results also validate the necrotic enteritis challenge model used in this trial. The *Salmonella* challenge on day of hatch predisposes the GIT to the Eimeria challenge on day 18. Both of the challenges set up the GIT for the opportunistic bacteria, *C. perfringens*, to colonize and induce necrotic enteritis. In the end, the inclusion of essential oils has the potential to be a reliable alternative to the use of antibiotics that the poultry industry can utilize to maintain production standards and a positive economic impact.

Appendix:

DIVISION OF AGRICULTURE
RESEARCH & EXTENSION
University of Arkansas System

To: Billy Hargis
Fr: Billy Hargis - Ag-IACUC Chair
Date: February 24th, 2020
Subject: IACUC Approval
Expiration Date: February 20th, 2023

The Division of Agriculture Institutional Animal Care and Use Committee (Ag-IACUC) has APPROVED your protocol # 21018 *Evaluation of various feed supplementation products on intestinal health permeability and inflammation.*

In granting its approval, the Ag-IACUC has approved only the information provided. Should there be any further changes to the protocol during the research, please notify the Ag-IACUC in writing (via the Modification form) prior to initiating the changes. If the study period is expected to extend beyond February 20th, 2023 you must submit a newly drafted protocol prior to that date to avoid any interruption. By policy, the Ag-IACUC cannot approve a study for more than 3 years at a time.

The following individuals are approved to work on this study: Billy Hargis, Guillermo Tellez-Isaias, Christine Vuong, Sami Dridi, Danielle Graham, Callie McCreery Selby, Makenly Coles, Cheryl Lester, Roberto Senas Cuesta, Elizabeth Greene, and Jared Ruff. Please submit personnel additions to this protocol via the modification form prior to their start of work.

The Ag-IACUC appreciates your cooperation in complying with University and Federal guidelines involving animal subjects.

BMH/tmp