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Influence of Feeding Anise Oil to Piglets and Broilers

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INFLUENCE OF FEEDING ANISE OIL IN PIGS AND BROILERS

A Dissertation

Submitted to the Graduate Faculty of the
Louisiana State University and
Agricultural and Mechanical College
in Partial fulfillment of the
requirements for the degree of
Doctor of Philosophy

in

The Interdepartmental Program in Animal and Dairy Sciences

by

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A mi madre (QDP) por tu amor eterno, enseñanzas y consejos. Tu apoyo siempre fue decisivo.
(To my mom (RIP) for her love, teaching and advising. Your support was always decisive).

A mi padre por darme mas alla de tus posibilidades para alcanzar mis metas.
(To my father for giving me more than you should to support my goals).

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(To my daughter Joanelle for teaching me that strength makes miracles).

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ABSTRACT

The objectives of this research were to determine 1) the effect of anise oil (AO) in pigs after exposure and re-exposure by dietary additions to sow and nursery diets; and 2) the maximum level of AO that could be fed to broilers and its effect on in-vitro growth of *Clostridium perfringens* (CIP); 3) to determine the effect of AO and diet type on growth performance of broilers after a challenge with CIP; and 4) to determine the effect of AO fed to broilers under commercial conditions. The results indicated that exposing lactating pigs to AO through the sow diet had a positive effect on pig feed intake after weaning, improved nursery feed efficiency, and had a positive influence on growth performance. Feeding broilers more than 4000 ppm AO in their diet reduced broiler feed intake, and more than 2500 ppm AO reduced growth. In an in-vitro Exp., the antibiotic Bacitracin and anticoccidial Salinomycin completely inhibited CIP growth, and AO linearly reduced CIP growth to undetectable levels. Thus, AO could have antimicrobial activity against CIP in-vitro. During a challenge with CIP (d 10 with 2×10^9 CFU/ml), broiler feeding AO reduced broilers ADFI and ADG during the first 10 days. Necropsy of all broilers indicated that feeding AO reduced the lesions of the jejunum (LS) related to CIP challenge. In a second CIP challenge (3×10^6 CFU/ml, on d 10 and 13), fish meal (FM) improved growth performance of broilers and had no effect on LS related to CIP challenge compared broilers fed vegetable protein. Feed intake was not affected by AO in this study. Broilers were fed a 3-phase feeding program in a floor pen study, and the treatment diets were antibiotic free, antibiotic added, or AO (1000 ppm). Feeding AO had a similar effect to feeding antibiotic added diet on growth performance of broilers. In summary, AO stimulated pig feed intake after weaning and growth performance after re-exposure. Also, AO had antimicrobial activity against CIP growth in-vitro, reduced jejunum lesions related to CIP in-vivo, and can be an alternative to antibiotics fed as growth promotants for broilers.

CHAPTER 1: INTRODUCTION

Pork and poultry are the most preferred meats around the world (Davis and Lin, 2005; DGARD, 2013; NCC, 2014). Both in Europe and the worldwide, pork has the greater per-capita consumption (DGARD, 2013), but in the U.S. poultry (Figure. 1.1) is the most consumed meat per-capita (Davis and Lin, 2005; NCC, 2014). Price, convenience, and healthy image are the main drivers for poultry consumption. Preference and price will maintain pork as the second choice worldwide. Beef and other red meats will continue to decline market share (Davis and Lin, 2005; DGARD, 2013). Although economics and health play important roles in choosing meats compared to other foods, population growth will be the most important factor for future demand. If the current meat consumption level is to be maintained at a constant rate, the U.S. will require an increase of at least 2.5 % in chicken and pork production on a yearly basis because of population growth (Davis and Lin, 2005; DGARD, 2013; NCC, 2014; World-Bank, 2014). However, there are several constraints to face in the coming years to be able to meet these demands.

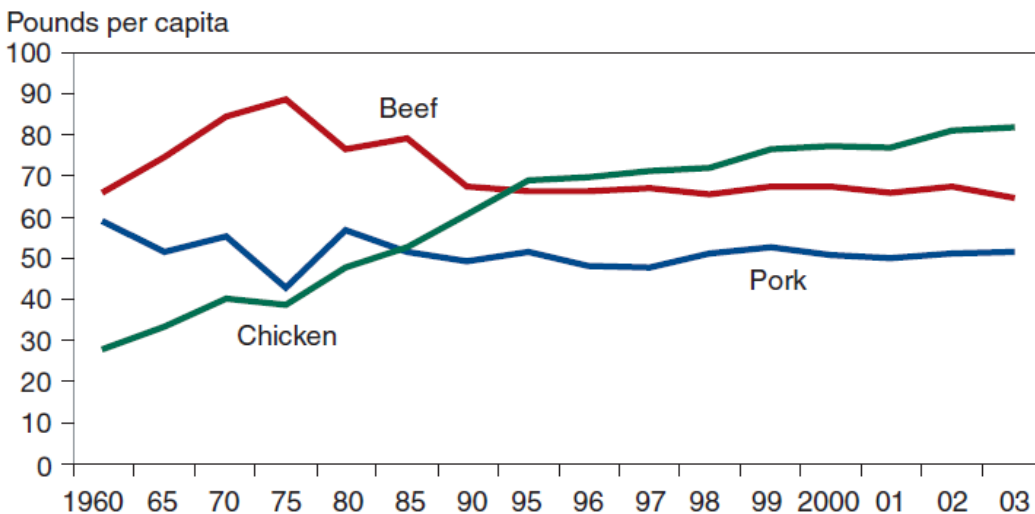


Figure 1.1 Historical meat consumption in the United States in pounds per capita. Adapted from Davis and Lin (2005).

Price and access to commodities will continue to have a direct impact on profitability of livestock production. However, new regulations in welfare and environmental factors will become important issues. In addition, markets for new products such as organics, cage free, or antibiotic free may become either challenges or opportunities for the animal protein industries (Dimitri and Greene, 2000). Europe has banned the use of antibiotics as growth promotants since 2006 (Casewell et al., 2003), and antibiotic use is under intense debate in the U.S. Antibiotics are added to diets as growth promotants to improve feed efficiency and increase carcass weight, as well as to regulate the gut microbiota (Belay and Teeter, 1996; Van Immerseel et al., 2009). In Sweden, the removal of antibiotics, as growth promotants, from diets has increased the incidence of enteric diseases in swine and poultry. For broilers, a higher incidence of necrotic enteritis has been observed and has led to an increase in therapeutic antibiotic use (Casewell et al., 2003). Van Immerseel et al. (2004) reported that withdrawing antibiotics from poultry diets represented losses of two billion dollars, worldwide. Several alternatives such as organic acids, probiotics, prebiotics, and phytochemicals have been proposed. None of those products have been consistently proven to be a viable alternative to antibiotic use in poultry.

Maintaining the health of the digestive tract is important to maximize nutrient uptake. Stress, associated with environmental changes and pathogens, compromises the intestinal lumen integrity (Pluske et al., 1997; Pié et al., 2004). Weaning is one of the most stressful events in a piglet's life. During the first few days post-weaning, feed intake is reduced in response to abrupt changes in feed form, environment and social interactions (Pluske et al., 1997). Although the presence of feed in the intestine of weanling pigs reduces intestinal damage, the management strategies can fall short of getting the pigs off to a good start. Creep feed consumption is highly variable for pigs during lactation, and has no effect on post-weaning feed intake. Newborn dogs,

rabbits, sheep, and humans have exhibited learning and food preference development when exposed to specific flavors in pre-natal and peri-natal stages (Marlier et al., 1998; Hepper and Wells, 2006). Therefore, these results suggest that similar strategies may smooth transition of piglets to solid feed. Anise oil has been used in pre- and peri-natal treatment in dogs with positive effects on feed intake. There is limited information on its effect in pigs. Additional research is needed to study the effect of feeding anise oil to sows during lactation and the subsequent effects on feed intake of piglets after weaning.

Although no specific mode of action has been accepted for antibiotics when used for growth promotion, the general theory is that improved growth performance is a result of modulation of gut microflora. The population of non-pathogenic bacteria is normally increased while pathogenic and fermentative populations are decreased. *Clostridium perfringens* is a pathogenic and ubiquitous bacteria of economic importance in poultry. In presence of coccidia, dietary changes, and other environment changes, the intestinal lumen is populated with coccidia and necrotic enteritis may occur.

Phytochemicals, also described as phytochemicals, are plant derivatives (mainly water and solvent extracts from flowers, leaves, seeds) with antibiotic, antioxidant, and other medicinal properties (Windisch et al., 2008). However, high concentrations of these compounds are required to exhibit antibiotic effects. In poultry, feed intake is reduced when feeding high concentrations of phytochemicals. Those changes may affect growth and the effects on feed efficiency are variable. Trans-anethole comprises 90% of anise oil (AO) and is widely used in the candy, drink, and healthcare industries (Newberne et al., 1999). It is not toxic at medium and high concentrations and is generally recognized as safe (GRAS). Antioxidant, anti-inflammatory, antifungal, anticarcinogenic, and antiviral effects are some of the pharmaceutical properties of

trans-anethole. Anise oil has antimicrobial effects against anaerobic, anaerobic facultative, and aerobic bacteria in-vitro. However, there is limited information about the levels required to produce antibiotic effects against *Clostridium perfringens*, the dosages tolerated by chickens, and its effect during a challenge under commercial conditions.

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CHAPTER 2: REVIEW OF THE LITERATURE

2.1 Introduction

Livestock productivity has been improved over time. Better husbandry practices and facilities have been developed. Animals grow faster, deposit more lean muscle, and have less fat than in the past. However, genetic selection for feed efficiency has not been as successful. From the nutritional standpoint, diets have been developed to accurately meet the requirement to maximize livestock output. Antibiotics and anticoccidials have been added to feeds to promote growth since 1950. Although there is no specific mode of action of these compounds, there is evidence that pathogenic and non-pathogenic microorganisms that cohabitate the digestive tract of animals are modulated. However, the trend is to limit their utilization to therapeutic purposes in livestock.

In general, a healthy digestive tract will maximize the absorption of nutrients from the diet. Stress, environment, bacterial load, and nutrition are the most important factors that can compromise intestinal health. Weaning is the most stressful event in piglet lives because they are exposed to abrupt societal and nutritional changes. Nutritionally, a dry and complex mixing of ingredients limits the intake of food and nutrients in pigs during the first days post-weaning. The stress and prolonged periods of anorexia and malnutrition in presence of gastric enzymes causes damage to the intestinal villi and lumen. These changes favor the colonization and growth of pathogenic bacteria and affect long term nutrient uptake. Therefore, it is important to develop methods to enhance pig feed intake early after weaning.

Clostridium perfringens (CIP) is one the agents of necrotic enteritis in chickens. It is present in the intestinal tract of both healthy and sick chickens. Changes in environment, the ingredients in the diets, or other microorganisms (coccidia) may trigger CIP growth in the small intestine. The addition of animal proteins to diets of broilers is another factor that may contribute

to necrotic enteritis development. Antibiotics and anticoccidials have been effective in overcoming this risk. It is suggested that phytochemicals, probiotics, prebiotics, or organic acids also can reduce this risk. Anise oil is an extract of aniseed and contains 90% trans-anethole. It is well tolerated by humans and animals. In addition to several pharmacological claims, aniseed has antimicrobial properties against several anaerobic and facultative anaerobic bacteria.

Therefore the objective of this study was to determine the effect of AO on post-weaning piglet feed intake, and as an alternative to antibiotics in broiler diets.

2.2 Weaning in piglets

Under natural conditions, weaning occurs between 9 and 22 week of age (Brooks and Tsourgiannis, 2003; Bolhuis et al., 2009). During this time, piglets are exposed to a gradual transition from milk to other dry dietary ingredients. Milk normally contains 20% dry matter (DM) compared to 15 – 30 % DM of food sources that are consumed during this process (Spreeuwenberg et al., 2001). Under commercial conditions piglets are weaned at an average age of 18-21 days (King and Pluske, 2003). At weaning piglets are separated from the sow, placed in a new housing system, mixed with unfamiliar pigs, and expected to abruptly transition from a liquid diet to a complex dry feed. In contrast to milk that contains 30 % protein, 40% fat, and 25% lactose on a DM basis (Spreeuwenberg et al., 2001), dry diets may contain 80 – 90 % DM with complex carbohydrates as the main source of energy.

There are other benefits associated with pig milk consumption before weaning. Passive immunity is transferred to pigs through colostrum during the first 36 hours of life and is essential for survival and performance (King and Pluske, 2003). Because the pig immune system develops from 21 days of age and does not become fully developed until 35 – 42 days of age, milk is a great source of immunoglobulins (Ig). The function of Ig (mainly IgA) is to protect the intestinal

lumen against pathogens (Partridge et al., 2001). Sow milk also contains epidermal growth factors, insulin-like growth factors, and polyamines which are peptides that contribute to the development of the intestinal tract (Dunshea, 2003). Although the milk is highly digestible (Mavromichalis et al., 2001), the amount produced by sows becomes limiting for maximizing piglet growth at 7 to 10 days of lactation (Dividich et al., 2001). Dunshea (2003) reported that piglets artificially reared did grow faster because they had ad-libitum access to supplemental feed. Therefore, weaning is necessary to ensure optimal piglet nutrition, as well as sow welfare and reproductive health.

The changes associated with the weaning process result in the up-regulation of pro-inflammatory cytokines, during the first two days post-weaning (Pié et al., 2004). These cytokines have an important role in the regulation of pigs' immunity. Other changes related to weaning include a decrease in villi height and an increase in crypt depth (Pluske et al., 1997). The alteration in intestinal morphology is more evident in weaned pigs at 14 days of age than in those weaned at an older age (21 days). The recovery of the intestinal lumen occurs from day five to eight after weaning, and it is frequently associated with the amount of feed intake. However, in order to minimize the damage of intestinal tissues, maximizing feed intake during the first week post-weaning is of great interest in swine production. The presence of feed is the most important stimuli for cellular proliferation in the intestine (Pluske et al., 1997). In contrast, villus height is decreased and crypt depth is increased by the absence of nutrients. Those changes are associated with reduced activity of the enzymes in the intestinal brush border, which reduces absorption capabilities in pigs (Pluske et al., 1997). With these considerations, it is suggested that a normal feed intake in the post-weaning period will reduce the damage to the small intestine and

increase the piglet digestive and absorptive capabilities. The result will be improved growth performance and health of older pigs.

2.2.1 Mammals food learning process

It is hypothesized that mammals develop a specific food preference as early as the fetal stage of life. Herz (2005) suggested that humans may learn to associate odor, and the emotional context in which odor was first encountered, by changing hedonic perception and odor-related behavior. For example, infants from mothers that consume volatiles from garlic, alcohol, or cigarette smoke during pregnancy or lactation showed preference for those smells later in life when compared to infants that were not exposed to those volatiles (Mennella and Beauchamp, 1993; Herz, 2005). Garlic volatiles did change the smell characteristics of amniotic fluid, when consumed by pregnant mothers (Mennella et al., 1995). Similarly, garlic and other volatiles change odor characteristics of milk (Mennella and Beauchamp, 1993; Mennella et al., 2001) which directly affects behavior and feeding patterns. Furthermore, those changes in response to likeness or aversion to certain odors and volatiles may affect the response in later stages of human and animal life (Mennella and Beauchamp, 1993; Mennella et al., 2001; Mennella and Beauchamp, 2002; Forestell and Mennella, 2007; Mennella et al., 2009). Similar results have been observed in rat pups (Galef and Henderson, 1972; Bronstein and Crockett, 1976), dogs (Birch, 1999; Hepper and Wells, 2006), kittens (Becques et al., 2010), lambs (Schaal et al., 1995), mice (Bouslama et al., 2005), and rabbits (Bilkó et al., 1994). Although all species seem to have been manipulated by pre- and post-natal exposure, pre-natal exposure has a greater impact on precocial animals. In contrast, animals with immature olfactory and brain systems at birth seems to have greater manipulation by post-natal exposure to odors alone (Oostindjer et al., 2009).

2.2.2 Pre-natal and peri-natal feed manipulation in pigs

In piglets a different approach has been used to manipulate early intake of solid feed. During lactation, creep feeding increases feed intake, growth rate, and body weight at weaning. Those pigs also have higher feed intake and growth rate after weaning. However, only a small percentage of piglets consume feed during lactation (Sulabo et al., 2010), the diets are costly, and there is a lot of feed wastage. Understanding the development of piglet behavior in regard to odor and flavor cues is important in addressing their aversion to solid feed at weaning. Morrow-Tesch and McGlone (1990) reported that piglets, independent of age after birth, are capable of identifying odors produced by their own mother. Those piglets also have an aversion to new odors like orange, citrus, vanilla, or odors from other mammal species. For instance, modifying the odor cues of maternal milk and fluids is suggested to be an alternative for piglets to learn about new food sources. Oostindjer et al. (2009) exposed pre- and post-natal piglets to novel odors, such as anise oil, by adding them to sow diets. As a result, when the piglets were exposed again to anise oil cues, they were attracted to the places where anise oil was present. Furthermore, those piglets that were pre-exposed to anise oil remained calm during a change to a new environment. A mix of aniseed and garlic also was provided to gestating and lactating sows to measure feed intake of creep feed and post-weaning feed intake. Although piglet creep feed intake was not affected by the dietary treatment, during nursing, it did increased feed consumption from days three to 10 after weaning (Langendijk et al., 2007). Similarly, using odor cues pre-natal and during lactation through the sow diet, did increased preference of piglets previously exposed to the odor (Figueroa et al., 2013). With these considerations, it is suggested that piglets may reduce neophobic reactions to solid feeds and increase feed intake if they are exposed to novel markers of odor that they can identify later after weaning.

2.3 The use and regulation of antibiotics in poultry

For more than 60 years antibiotics have been approved for use as growth promotants in poultry diets, without a prescription, by the United States Food and Drug Administration (FDA) (Castanon, 2007). Similarly, individual countries of the European Union (EU) allowed the use of antibiotics as feed additives in poultry diets. However, concerns about the possible increase of pathogen microbial resistance to antibiotics has been a concern in the EU since 1986. According to Casewell et al. (2003), Sweden banned the use of antibiotics as growth promotants, and Denmark banned the use of avoparcin in 1995 and virginiamycin in 1998. Following the same trend, the EU banned the use of avoparcin in 1997, and bacitracin, spiramycin, tylosin, and virginiamycin in 1999 (Casewell et al., 2003). Although there is no conclusive evidence of cross-resistance of pathogens in human there is an increasing social and political pressure to eliminate the use of antibiotics in livestock production in the US. Therefore, the FDA has started monitoring the safety and risk of resistance of human pathogens as a result of the use of sub-therapeutic levels of antibiotics in animal feeds (FDA, 2003). Recently, the utilization of “Judicious use of medically important drugs in food-processing animals” was suggested as a guideline to reduce the risk of increasing pathogen resistance (FDA, 2013). Although there is still a voluntary phase out of additive antibiotic utilization in livestock feeds, consumer demand for organic, free range, free cage, or antibiotic free products is increasing (Lusk et al., 2006). With this consideration, it is important to consider alternatives for antibiotics used as growth promotants. However, a decrease in feed efficiency and an increase in health issues, including necrotic enteritis, has been reported as a result of the ban of the use of antibiotics as growth promotants (Van Immerseel et al., 2009).

2.3.1 Benefits and mode of action of antibiotics in poultry

Direct effects of antibiotics added to poultry diets are mostly for feed efficiency (Dibner and Richards, 2005), although there are some authors that report greater growth rate when compared to non-antibiotic fed broilers (Dumonceaux et al., 2006). Although variable, other benefits include higher carcass and breast yield and improved meat quality (Costa et al., 2007). Because most of the antibiotics used as growth promotants are not absorbed by animals, it is generally accepted that they modulate the intestinal flora to produce increased growth rate of livestock (Vissek, 1978; Dibner and Richards, 2005). Early experiments conducted with germ-free animals indicate that there is no benefit of adding penicillin to their diets. However, under conventional environments, the inclusion of penicillin to animal diets resulted in increased growth rate. It was also reported that germ-free chickens grew faster than conventional chickens (Coates et al., 1963). Eyssen and De Somer (1963) reported that virginiamycin increased growth rate of broilers fed a complex source of energy (fat and carbohydrates). Since virginiamycin targets gram positive bacteria, it was suggested that the growth promotion effect was due to reduced gram positive bacteria. Dumonceaux et al. (2006) reported that virginiamycin was able to change the intestinal microbiota in different segments of the broiler intestine and to promote growth. In other studies, bacitracin sodium has increased the lactobacillus population and decreased the firmicutes and *C. perfringens* populations of intestines when fed to broilers. Thus, the sub-clinical effects of necrotic enteritis are reduced (Butaye et al., 2003). It also has been reported that antibiotics may change the morphology of the intestinal wall which may facilitate the absorption of nutrients, but also may result in energy and nutrient savings (Dibner and Richards, 2005). There are indications that antibiotics may reduce immune stress markers during

subclinical infection by reducing interleukin-1 production (Roura et al., 1992), which improves growth and feed efficiency.

2.3.2 Intestinal micro-biota and necrotic enteritis

There is evidence that the intestinal tract of broilers before hatching and piglets before birth is germ-free (Coates et al., 1963; Dibner and Richards, 2005). However, bacteria from the environment immediately colonize in the intestinal tract of young animals, as early as six hours after hatch or birth. At this time the population may reach 10^9 to 10^{10} CFU/g in the feces of pigs (Snel et al., 2002; Dibner and Richards, 2005). The increase of microorganisms is similar among several species including chickens, pigs, and humans (Mackie et al., 1999). However, the composition of the microbial population is more important. More than 500 species of bacteria have been identified in the intestine of animals, with total populations in the range of 10^{10} to 10^{12} /g of fecal material (Gaskins et al., 2002; Dibner and Richards, 2005). Their importance relies on the immunological, physiological, nutritional, and protective functions of the intestinal tract of the animals, which affects health and productive parameters of monogastric animals (Dibner and Richards, 2005).

Aerobes and facultative anaerobes (*Escherichia coli*, *Lactobacilli*, and *Streptococci*) are the groups that first colonize the small intestine (Smith and Jones, 1963; Mackie et al., 1999). However, they condition the gut environment for obligate anaerobes to colonize later. In this group the most important genera are *Bacteroides*, *Bifidobacterium*, and *Clostridium* (Smith and Jones, 1963; Mackie et al., 1999; Dibner and Richards, 2005). *Clostridium perfringens* toxinotype A is of economic importance in poultry because it is one of the causative agents of necrotic enteritis (Craven, 2000; Timbermont et al., 2010; Timbermont et al., 2011) and is able to produce NetB toxin (Lanckriet et al., 2010). Production losses, increased mortality, reduced

welfare of birds, and increased risk of contamination of poultry products have been associated with necrotic enteritis (Timbermont et al., 2011). Timbermont et al. (2011) reported that birds with necrotic enteritis have higher than 10^8 CFU/ml in their digestive tract, while healthy birds may have less than 10^5 CFU/ml (Timbermont et al., 2011). Both clinical and sub-clinical necrotic enteritis are a worldwide problem in poultry, and the estimated cost is five cents per bird. In 2000, these losses represented over \$2 billion per year (Hofacre, 2005). However, the growth of *Clostridium perfringens* and the onset of necrotic enteritis is dependent upon other factors such as sub-clinical coccidiosis (Lanckriet et al., 2010), stress, or dietary factors (Jia et al., 2009). Non-starch polysaccharides from wheat, barley, or rye, when added to broiler diets (Hofshagen and Kaldhusdal, 1992; Kaldhusdal and Hofshagen, 1992; Kaldhusdal and Skjerve, 1996), increase intestinal viscosity, reduce nutrient absorption, and influence gut microflora (Kaldhusdal and Skjerve, 1996; Jia et al., 2009). Manzanilla et al. (2009) also found that the amount and source of dietary protein affect the performance and gut microflora of young pigs. Similar results were reported when meat, poultry, or fish meal was added to poultry diets (Drew et al., 2004; Olkowski et al., 2006). The amino acids methionine and glycine are increased in diets containing high amounts of fish meal and *C. perfringens* growth was increased in the gut of broilers when high levels of these amino acids are added to diets (Drew et al., 2004; Dahiya et al., 2005; Wilkie et al., 2005; Olkowski et al., 2006). Due to the importance of necrotic enteritis in poultry, subclinical models have been developed to evaluate the effect of antimicrobial agents. Lanckriet et al. (2010) and Keyburn et al. (2006) reported that necrotic lesions are more frequently observed in the duodenum and jejunum. They also suggested that macroscopic lesions in subclinical necrotic enteritis are in the range of 0 (normal) to 2 (mild) on a scale from 0 to 4 (Figure 2.1) which is commonly used (Dahiya et al., 2005; Jia et al., 2009; Lanckriet et al.,

2010) in-vivo challenges. Focal necrosis and ulcerations, as well as less severe, extensive necrosis are typical signs of field cases, however those changes are not observed in induced models (Prescott et al., 1978).

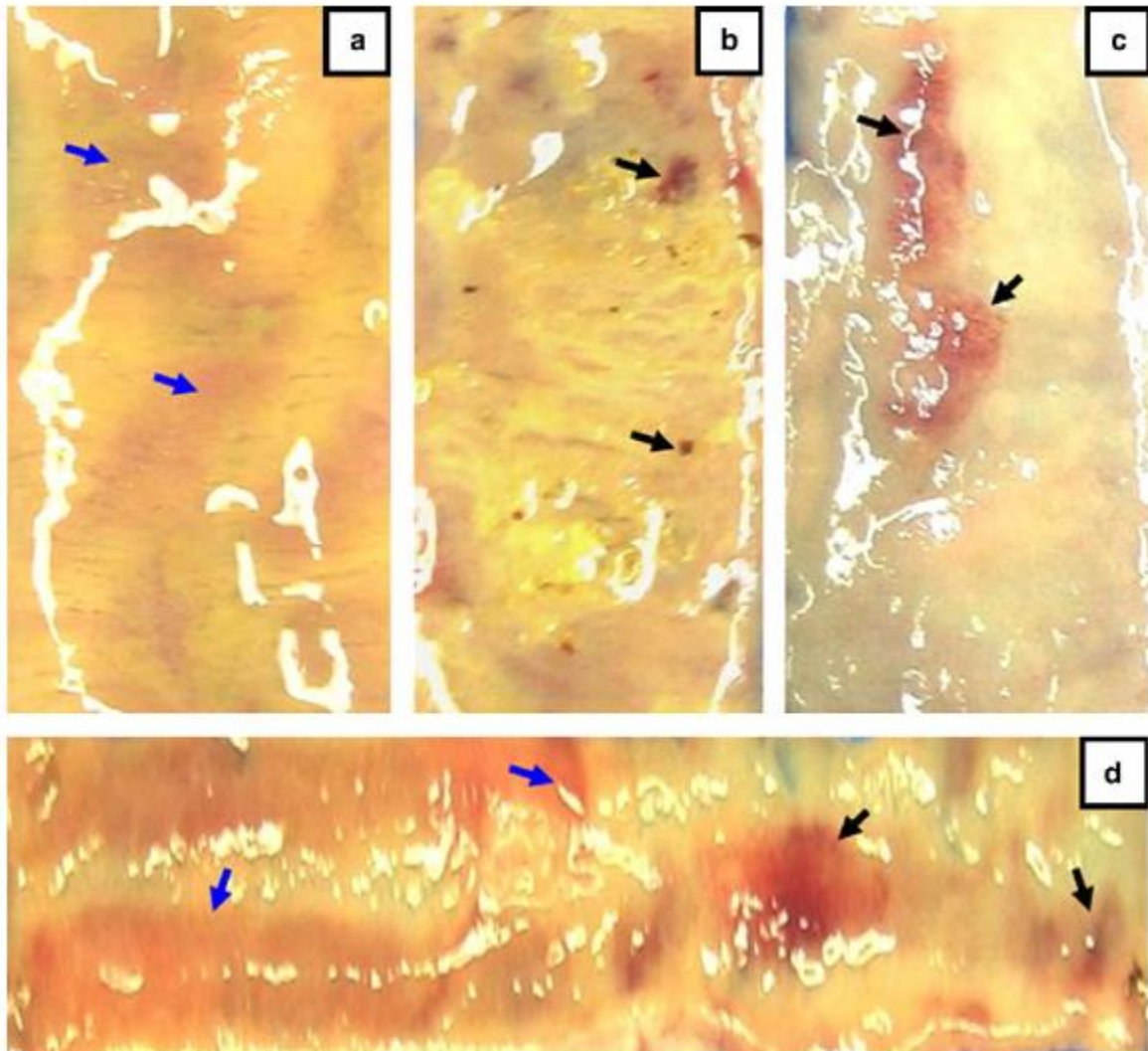


Figure 2.1 Mucosa of chickens orally challenged with *Clostridium perfringens*. Adapted and modified from Olkowski et al., 2006.

2.3.3 Feeding alternatives to antibiotic supplementation in diets

The ban and mandatory reduction of antibiotic use at sub-therapeutic levels to promote growth in livestock has increased research interest for antibiotic alternatives. Organic acids, probiotics, bacteriocins, bacteriophages, peptides, and phytochemicals are proposed as replacements for antibiotics (Joerger, 2003; Hume, 2011). These are designed to alter the microbial population in the digestive tract by increasing lactobacillus populations and decreasing the incidence of *E. coli* and *C. perfringens*. However, the results are not conclusive in terms of growth, feed efficiency, or meat yield and quality.

2.4 Phytochemicals

Phytochemicals, also called phytochemicals, are water or solvent extracts from plant roots, seeds, flowers, or leaves. Dry ground plant parts also are used as feed additives (Wang et al., 2011b). The compound's nomenclature varies depending upon its physical, functional, origin and processing characteristics (Windisch et al., 2008). In animals, they can have several functions such as antioxidants, anti-helminthic, anti-inflammatory, anti-bacterial, expectorant, etc. Most of the known activities are related to the concentration of phenolic terpenes or terpenoids, flavonoids, and anthocyanins. They normally have a strong odor. Species from the family rosmarinic, labiatae, zingiberaceae, umbelliferae, alliaceae, and capsiceae are the most utilized phytochemicals (Jamroz et al., 2003; Windisch et al., 2008; Amad et al., 2011; Maenner et al., 2011). They are used as feed additives in the diets of healthy animals during the whole period of production with nutritional enhancement purposes. In contrast antibiotics are currently regulated for use in therapeutic purposes in Europe (Windisch et al., 2008).

Many of these species have in-vitro antibacterial activity against a wide variety of pathogens (Deans and Ritchie, 1987; Hammer et al., 1999; Dorman and Deans, 2000) when used

at high concentrations. At high concentrations, those strong volatiles may compromise growth performance parameters. Feeding phytochemicals to broilers, in several cases, has reduced feed intake, but has not affected their growth rate or feed efficiency (Botsoglou et al., 2002; Alçiçek et al., 2004a; Denli et al., 2004; Cross et al., 2007; Bozkurt et al., 2009; Brenes and Roura, 2010). Broilers fed oregano, rosemary, cinnamon, and thyme extracts have decreased feed intake (Basmacioglu et al., 2004; Cabuk et al., 2006). Similarly, broilers and turkeys fed dried spices (garlic and oregano) have reduced feed intake (Bampidis et al., 2005; Sarica et al., 2005). Quail fed either black seed oil or coriander spice have increased feed intake (Denli et al., 2004; Güler et al., 2006). The rate of bird growth in most of the studies responded directly to feed intake, and ranged between -8 to 14% increase in body weight compared to birds fed a negative control (Alçiçek et al., 2004b; Sarica et al., 2005). These variable results are related to high variability in phytogenic extract composition, blends of known and unknown species, location, livestock systems, or environment (Alçiçek et al., 2004a, b; Hernández et al., 2004; Lee et al., 2004a; Cabuk et al., 2006). Modest improvements of feed conversion ratio have been reported for birds fed phytogenics (Botsoglou et al., 2002; Alçiçek et al., 2004a, b; Basmacioglu et al., 2004; Hernández et al., 2004; Lee et al., 2004a; Lee et al., 2004b; Cabuk et al., 2006). Terpenes and terpenoides present in essential oils are known to control *C. perfringens* growth in broilers (Mitsch et al., 2004; Cross et al., 2007). Specific strains of *C. perfringens* also were susceptible to thymol and cinnamaldehyde (Timbermont et al., 2010). Although there are no reported studies on the effect of anise oil in *C. perfringens*, there are studies that report that anise oil has antimicrobial activity against gram positive and negative anaerobic bacteria and facultative anaerobic bacteria (Deans and Ritchie, 1987; Kubo and Fujita, 2001; De et al., 2002; Gülçın et al., 2003; Wang et al., 2011a) in vitro.

In our laboratory anise oil was effective in reducing *C. perfringens* growth to less than 10^8 CFU/ml with low concentrations (Unpublished data). There are no reports in the literature on the in-vivo effect of anise oil on *C. perfringens* growth when fed to broilers. Timbermont et al. (2011) reported that birds with necrotic enteritis had higher than 10^8 CFU/ml in their digestive tracts, while healthy birds had less than 10^5 CFU/ml (Timbermont et al., 2011), which suggested a range of antimicrobial activity of anise oil. In Egypt, aniseed was fed to broilers with positive effects on body weight gain and blood cell parameters. There is still debatable data regarding the positive effect of not only anethole but other phytochemicals on bird growth performance and necrotic control. Several factors have added confounding effects, including phytochemicals and their methods of extraction, research methods, composition of experimental diets, and environment.

2.4.1 Anise oil properties and safety of use

Anise oil is the solvent extraction from aniseed and has approximately 90% trans-anethole. Anise oil is used as a flavoring substance in bakery products, candies, ice cream, chewing gum, and alcoholic beverages (Newberne et al., 1999). Anethole is “generally recognized as safe” (GRAS) by the FEMA Expert Panel (Newberne et al., 1999). Wang et al. (2011a) reported that aniseed has pharmacological properties as an antioxidant, antimicrobial, analgesic, expectorant, sedative, convulsive, and insecticidal. Anise oil, and its main component anethole, have exhibited some antimicrobial activity against aerobic and anaerobic bacterial growth (Kubo and Fujita, 2001; De et al., 2002; Gülçin et al., 2003; Wang et al., 2011b). However, the aerobic bacterial growth results are variable (Kubo and Fujita, 2001; De et al., 2002; Gülçin et al., 2003; Wang et al., 2011b) and there is limited information on anaerobic bacterial growth (ie. *Clostridium perfringens*). The advantage of AO is its suitability for feed and

food as evaluated in pigs and dogs (Wang et al., 2011a), which are more sensitive to flavors and smells than chickens (Sneddon et al., 1998). Humans may tolerate more than 100 ppm of anise oil if ingested, which makes it a safe product for human handling. Rats, mice, and other rodents can tolerate more than 1000 ppm of anise oil. Following metabolism of AO, more than 60% is excreted in the urine and through breath within four hours after consumption in several species (Newberne et al., 1999). However, there is no evidence of toxicity or the maximum level that can be fed to chickens. Similarly there is no information about its effect on chicken meat.

2.5 Summary

In summary, a healthy intestinal tract is important in maximizing the absorption and utilization of the nutrients present in diets for pigs and poultry. Reducing stress, improving environment, and modulating gut microbiota are management practices that can minimize the risk of compromised intestinal health. In pigs, weaning is the most stressful event in their productive life. A lack of feed intake not only reduces growth but also triggers proteolytic activities in the lumen of the intestine, which may enhance the propagation of pathogens. In poultry, the removal of antibiotics as growth promotants impacts the composition of microbiota in the gut. Other dietary factors also may predispose the changes on microbiota such as dietary protein or carbohydrates sources. Therefore, the aim of this research was to develop a method to familiarize piglets with the odor of AO through milk and feed of the sow, so that piglets will associate this with the AO odor in their nursery phase feed. For broilers, the objective of this research was to evaluate if AO was effective, at high dosages, in overcoming the negative effects of subclinical necrotic enteritis caused by *Clostridium perfringens* (an ubiquitous facultative anaerobe). Feed composition (fish meal inclusion) also was evaluated as a predisposing factor for the development of necrotic enteritis.

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CHAPTER 3: THE EFFECT OF ANISE OIL FED TO LACTATING SOWS AND NURSERY PIGS AS A FEED PALATANT, AND ITS EFFECT ON SOW FEED INTAKE, PIGLET PERFORMANCE, AND WEANLING PIG FEED INTAKE AND GROWTH PERFORMANCE

3.1 Abstract

Low or no feed intake usually is observed for piglets immediately after weaning. This study was conducted to determine if anise oil (AO) fed to lactating sows, and subsequently to nursery pigs immediately after weaning, would increase feed intake and growth performance of nursery pigs during the first week post-weaning. In addition, sow feed intake, productive performance, and reproductive performance were evaluated. After they were placed in farrowing stalls on d 110 of gestation, sows were fed either the control lactation diet or the control lactation diet + 50 ppm AO. At weaning, within lactation dietary treatment, piglets were allotted to either a nursery control diet or nursery control diet + 50 ppm AO. Pigs were fed a three phase nursery diet program with each phase lasting one wk. During nursery phase 1, feed intake was estimated on a daily basis. There was no effect of treatment on sow BW, BCS, or back fat loss ($P > 0.10$). Pigs weaned, pig BW, pig ADG, pig G:F, litter weight, and sow return to estrus were similar among sow treatment groups ($P > 0.10$). Nursery pigs exposed to AO during lactation had increased ADFI on d 2, 3, and 7 ($P < 0.10$) post-weaning. Feeding AO to nursery pigs also increased ADFI on d 1, 5, and 7 ($P < 0.10$) post-weaning. Pigs from sows fed AO had the lowest and highest ADFI on d 7 when fed the control or anise oil diet, respectively, during phase 1 ($P < 0.10$). During phase 1, pigs fed AO had increased ADFI ($P < 0.10$), but there was no effect on ADG or G:F ($P > 0.10$). During phase 2, ADFI was greater for pigs from sows fed AO and for nursery pigs fed AO ($P < 0.10$). Pigs fed AO only in nursery diets had increased ADG and G:F ($P < 0.10$). During phase 3, pigs from sows fed AO had the lowest and highest ADFI ($P < 0.10$) when fed the control or AO diet, respectively. There was no effect on ADG ($P > 0.10$) but G:F

($P < 0.10$) was decreased. Overall, pigs from sows fed AO had the lowest and greatest ADFI ($P < 0.10$) when fed the control or AO diet, respectively. Pig fed the AO diet had increased ADG ($P < 0.10$) and decreased G:F ($P < 0.10$). Pigs fed the AO diet were heavier at the end of the trial ($P < 0.10$). In conclusion, feeding sows AO does not affect sow performance, but increases ADFI in nursery pigs during the critical first days after weaning. Feeding AO to nursery pigs increased ADFI, ADG, G:F, and BW.

3.2 Introduction

Weaning is a major challenge for young mammals because sudden environmental, societal, and nutritional changes can lead to decreased feed intake. Selecting new, healthy, and nutritious food types by themselves is a slow learning process in pigs and may take between three to seven days (Naranjo et al., 2010). These short periods of anorexia and low feed intake compromise their intestinal integrity, further decreasing gut functionality and increasing the piglet's susceptibility to enteric diseases (Mahan, 1992; Mahan and Newton, 1993; Van Dijk et al., 2001; Naranjo et al., 2010). Dietary measures such as increasing L-tryptophan have improved but not alleviated the stress (Koopmans et al., 2006). Increasing protein quality (Van Dijk et al., 2001) and highly digestible carbohydrates has increased feed intake (Naranjo, 2010) in pigs, but not immediately after weaning. Other limitations of high density nutrition is its impact on feed costs. Early dietary manipulation may improve the weaning transition by enhancing feed preferences. Pigs and other mammals, including humans, have demonstrated sensory learning capabilities. Offspring may learn to select food by the exposure to olfactory markers produced by skin, feathers, fur, feces, diet, or breath (Galef and Henderson, 1972; Morrow-Tesch and McGlone, 1990; Bilkó et al., 1994; Oostindjer et al., 2009) of their parents.

Newborn mammals are able to recognize amniotic fluid from their own mother when exposed after birth (Marlier et al., 1998). Moreover, pre-natal and peri-natal exposure to plant aromatics increases acceptance of food with the same flavor in dogs (Hepper and Wells, 2006), humans (Mennella et al., 1995), rabbits (Coureaud et al., 2002), rats (Arias and Chotro, 2007), and pigs (Figueroa et al., 2013). However, peri-natal exposure to sensory flavors has not consistently increased preference (Bilkó et al., 1994) and may be dependent on the animal species (Oostindjer et al., 2009). Therefore, precocial animals (Seckl, 2001) may obtain more benefit from pre-natal exposure in contrast to altricial species (pigs, dogs, humans) that have immature and plastic brain and olfactory systems after birth (Oostindjer et al., 2009). Other benefits from early chemosensory modulations include the reduction of negative emotions (neophobia) and stress responsiveness after re-exposure to flavors (McCaffrey et al., 1993; Oostindjer et al., 2009).

Development of feed preference using plant extracts has been evaluated (Windisch et al., 2008). However, some of the research involves complex oil mixes. Under this circumstance, the animals may confound the identity of the oils, and subsequently may fail to recognize them. Furthermore, animals may take longer periods to identify smell/flavor markers (Wilson et al., 2004) when complex mixtures are used. Anise oil has been used as an aromatic in studies using a single botanical specie (Kreydiyyeh et al., 2003; Hepper and Wells, 2006; Figueroa et al., 2013), with positive recognition effects after re-exposure. Anise oil is listed as a generally recognized as safe (GRAS) substance by the Flavour and Extract Manufacturers Association (Newberne et al., 1999), and is approved for use in humans and other mammals at levels no greater than 120 mg/kg BW. Feed intake levels of approximately 695 mg/kg BW have shown acute toxicity in rats (Taylor et al., 1964). Historically, trans-anethole (~90% of the volatile components, as

analyzed in our laboratory) has been used as a flavoring substance in foods ranging from 2.5 ppm in gravies to 1500 ppm in gum (Newberne et al., 1999) with an average daily per-capita intake of 54 µg/kg BW.

Lambs and pigs have responded positively to pre-natal and peri-natal exposure to anise flavored feed. Although pigs responded positively to anise oil preference, indirect methods rather than direct methods were used to evaluate the response. Generally, flavor preference is assessed by double choice using the preference gate test, the Y maze test, the novel environment test, or the rooting test (Simitzis et al., 2008; Oostindjer et al., 2009). Therefore, these experiments measure behavior changes and time spent under flavored or unflavored environments with only limited measures of feed intake. The objective of this study was to determine whether peri-natal exposure to anise oil would improve feed preference by increasing feed intake during the first week post-weaning, as well as improve growth performance during the nursery phase.

3.3 Materials and methods

All experimental protocols used in these experiments were approved by the Louisiana State University Agricultural Center Animal Care and Use Committee.

3.3.1 Animals

3.3.1.1 Sows

Two consecutive groups of 12 gestating Yorkshire and crossbred (Yorkshire x Landrace or Yorkshire x Duroc) sows were obtained from the Louisiana State University Agricultural Center Swine Unit and allotted to two dietary treatments on d 110 of gestation. The treatment groups were balanced by breed, parity, and weight. The lactation treatment diets (Table 3.1) were corn and soybean meal diets: 1) control and 2) control +50 ppm of anise oil (AO). The diets were formulated to meet or exceed the requirements for lactating sows with anticipated

weight change of -10 kg and pig daily gain of 0.20 kg (NRC, 1998). Both diets contained 0.94 % total lysine and 3,265 kcal/kg of ME (as fed basis). The AA ratios suggested by Evonik-Degussa (Hess et al., 2006) were used to formulate the diets. Vegetable oil was used as a source of energy and as the carrier of the AO. Before allotment, all sows were fed a typical corn-soybean meal gestation diet that met or exceeded the requirements for gestating sows (NRC, 1998).

Table 3.1. Composition of the basal lactation diet, as-fed basis.

Ingredient	%
Corn	65.85
Soybean meal, 48% CP	27.00
Dry fat	2.76
Soybean oil	0.22
Limestone	1.00
Monocalcium phosphate	1.47
Salt	0.50
Choline chloride	0.10
Mineral premix ¹	0.10
Vitamin premix ²	0.50
Sodium bentonite	0.50
Calculated composition, %	
Ca	0.77
P	0.68
Total Lys	0.94
TSAA	0.58
Total Thr	0.68
Total Trp	0.21
Total Ile	0.74
Total Val	0.83
ME, kcal/kg	3265.00

¹Provided the following per kg of diet: Fe, 127 mg; Zn, 127 mg; Cu, 12.7 mg; Mn, 20 mg; I, 0.80 mg; and Se, 0.30 mg.

²Provided the following per kg of diet: vitamin A, 11,023 IU; vitamin D3 3,307 IU; vitamin E, 88 IU; niacin, 88 g; pantothenic acid, 50 mg; riboflavin, 13 mg; menadione, 8 mg; pyridoxine, 4 mg; thiamin, 4 mg; folic acid, 3 mg; vitamin B12, 61 µg; biotin, 441 µg; vitamin C, 110 µg.

On d 110 of gestation, sows were weighed and moved into the farrowing facility, and immediately started on the experimental diets. The facility was environmentally controlled and consisted of 28 individual farrowing stalls with hard plastic slotted floors and an under flush system. Each farrowing stall (1.5 x 2.1 m) had a stainless steel self-feeder, a nipple waterer inside for the sows, and a small nipple waterer for the piglets. Water was provided ad libitum. From d 110 to farrowing, the sows were fed approximately 2.8 kg/d (as fed basis) of their treatment diets. After farrowing, feed was gradually increased until the sows were fed ad-libitum. Feed additions were recorded for calculation of ADFI. Piglets were weighed, ear notched and received 1 mL of iron dextran (Ferrodex 100; Agri Laboratories Ltd., St. Louis, MO) within 48 hours after farrowing. At this time, piglets were cross-fostered within treatment group to adjust litters to approximately 10 pigs per litter. Pigs were weaned at 20 ± 2 d post-farrowing which was the same day regardless of date of farrowing. Sows and piglets were weighed at weaning, and the piglets were allotted to nursery dietary treatments. Changes in body weight (BW), 10th rib back-fat (BF), and body condition score on a scale of 1-5 (BCS) were assessed for the sows. After weaning all sows were checked twice daily for signs of estrus. Days to estrus was recorded when the sow stood to be mounted by a boar.

3.3.1.2 Piglets

Ninety-six (sow group 1) and 72 (sow group 2) mixed gender weanling pigs were housed (groups of 4 or 3, respectively) in a slotted-floor nursery building. Each pen (0.97 x 1.47 m in size) had one nipple waterer, and a 4-hole self-feeder. Feed intake was recorded daily at 0700 h during nursery phase 1 (d 1 – 7) and then weekly. Pigs were fed a three phase feed program that lasted 7 d per phase (Table 3.2.).

Table 3.2 Composition of the basal nursery diets, as-fed basis

Item, %	Phase 1	Phase 2	Phase 3
Corn	39.56	50.55	50.91
Soybean meal, 48% CP	20.79	25.00	35.68
Dried whey permeate	20.00	10.00	3.75
Fish meal	7.00	5.00	3.00
SDPP ¹	4.00	--	--
Dry blood cells	2.00	2.00	--
Dry fat	2.84	2.75	2.80
Monocalcium phosphate	0.42	1.15	0.90
Limestone	0.71	0.74	0.86
Sodium bentonite	0.50	0.50	0.50
Vitamin premix ²	0.50	0.50	0.50
Zinc oxide	0.28	0.28	--
Salt	0.10	0.36	0.11
Trace mineral premix ³	0.10	0.10	0.10
Antibiotic ⁴	0.25	0.25	0.25
Choline chloride	0.05	0.05	0.05
Biolys ⁵	0.24	0.28	0.21
DL-Methionine	0.22	0.16	0.13
L-Threonine	0.10	0.11	0.03
Soybean oil	0.22	0.22	0.22
L-Isoleucine	0.12	--	--
Calculated composition, %			
Total Lys	1.60	1.40	1.40
TSAA	0.96	0.84	0.84
Total Thr	1.04	0.91	0.91
Total Trp	0.28	0.27	0.28
Ca	0.90	0.90	0.80
P	0.80	0.80	0.70
ME, kcal/kg	3494.00	3433.00	3440.00

¹Spray-dried plasma protein (AP-920; American Protein Corp., Aimes, IA).

²Provided: vitamin A, 11,023 IU; vitamin D3 3,307 IU; vitamin E, 88 IU; niacin, 88 g; pantothenic acid, 50 mg; riboflavin, 13 mg; menadione, 8 mg; pyridoxine, 4 mg; thiamin, 4 mg; folic acid, 3 mg; vitamin B12, 61 µg; biotin, 441 µg; vitamin C, 110 µg per Kg of diet.

³Provided: Fe, 127 mg; Zn, 127 mg; Cu, 12.7 mg; Mn, 20 mg; I, 0.80 mg; and Se, 0.30 mg per Kg of diet.

⁴Provided: oxytetracycline, 165 mg and neomycin 116 mg per Kg of diet (Neo-terra 10/10; Nutra Blend LLC, Neosho, MO).

⁵Contains 50.7% Lys.SO₄ (Evonik-Degussa Feed Additives, Kennesaw, GA).

Diets were formulated to contain 1.6% lysine, 20 % CP, and 3494 Kcal/kg for Phase 1; 1.4% lysine, 18 % CP, and 3433 Kcal/kg for Phase 2; and 1.4% lysine, 16% CP, and 3440 kcal/kg for Phase 3. The nursery diets were corn and soybean meal based diets: 1) control and 2) control + 50 ppm of anise oil (Anise). The AA ratios suggested by Baker (1997) for 10-20 kg piglets were used to formulate the diets. Body weight and feed intake were determined weekly. Pigs were allotted within lactation treatment to two nursery dietary treatments in a randomized complete block design with dietary treatments arranged in a 2 x 2 split plot design. The main plot was lactation dietary treatment (control vs AO diet) and nursery diet within the plot. A total of 12 replicates were used per group. Feed in meal form and water was provided ad libitum. Pigs were checked at least once daily for any abnormalities.

3.3.2 Statistical Analysis

Sow data were analyzed as a randomized complete block design with lactation group as a random block. The nursery data, collected daily and weekly for feed intake and growth performance, were analyzed as a randomized complete block design with dietary treatment factors arranged in a 2 x 2 split plot design using the MIXED procedure of SAS (SAS-Institute, 2011). Pen was used as the experimental unit, weaning group as the block, and nursery dietary treatments were nested within lactation dietary treatment. Treatment responses were partitioned into the main effects of lactation, nursery, and lactation x nursery interaction. Treatment means were separated by the PDIF option of SAS at α level of $P < 0.10$.

3.4 Results

3.4.1 Sow and litter performance

Results for sow and litter performance during lactation are presented in Table 3.3. Sows fed the control diet or AO diet at 110 d of gestation had similar ($P > 0.10$) number of

Table 3.3 The effect of anise oil on sow, litter, and pig performance during lactation¹.

Item	Control	Anise Oil	SEM	<i>P</i> -Value
Sow performance				
Parity number	1.9	1.8	0.4	0.91
BW, kg				
110 d	220.4	223.2	10.5	0.84
Wean	211.4	214.1	10.8	0.87
Loss	-8.7	-12.8	5.5	0.41
BCS (1-5)				
110 d	3.5 ^a	3.02 ^b	0.17	0.06
Wean	3.25 ^a	2.77 ^b	0.16	0.05
Backfat, mm				
110 d	37.1	38.1	2.8	0.78
Wean	36.8	33.8	4.06	0.6
Loss	-3.05	-3.81	2.9	0.85
Feed intake				
ADFI, g	6083	6674	431.3	0.35
Waste, kg	2.34	0.43	0.99	0.81
Litter				
ADG, g	1648	1655	181.6	0.3
G:F, g/g	0.27	0.26	0.03	0.15
Size, number				
Pigs born alive	8.9	8.3	0.78	0.62
Pigs cross-fostered	9.6	9.5	0.4	0.75
Pigs weaned	8.9	8.7	0.68	0.69
Weight, kg				
Birth	13.97	13.02	1.16	0.53
Cross-fostered	15.06	14.74	1.12	0.72
Weaned	51.62	50.12	5.53	0.21
Pigs				
Weight, kg				
Birth	1.63	1.59	0.21	0.57
Cross-fostered	1.59	1.54	0.07	0.71
Weaned	5.85	5.81	0.51	0.26
ADG, g	185	188	22.7	0.28

¹The values are means of 12 replicated sows with their respective litter. BW = body weight, BCS = body condition score, ADFI = average daily feed intake, ADG = average daily gain, G:F = gain/feed.

^{ab}Means within a row with different superscripts differ ($P < 0.10$).

parities (2 ± 0.4 parities), BW (221.5 ± 10.5 kg), BCS (3.25 ± 0.17), and 10th rib BF depth (37.5 ± 3 mm). At weaning, sow feed consumption (6.35 ± 0.95 kg); litter ADG (1.65 ± 0.18 kg); and litter G:F ratio (0.265 ± 0.03) was similar among treatment groups ($P > 0.10$). Sows lost approximately 10 kg of BW and 3.5 mm of 10th rib BF, but the losses were similar for both treatment groups ($P > 0.10$). Control fed sows had higher ($P < 0.10$) BCS at the beginning and end of the study compared to AO fed sows, but the difference was constant.

At birth, pigs born alive (8.6 ± 0.78 pigs/sow), litter weight (13.50 ± 1.16 kg), and pig average weight (1.62 ± 0.10 kg) was similar among lactation treatment groups ($P > 0.10$). Similarly, after cross-fostering, litter size (9.6 ± 0.4 pigs/litter), litter weight (14.9 ± 1.12 kg), and pig average weight (1.57 ± 0.07 kg) was not different among treatment groups ($P > 0.10$). At weaning, there was no treatment effect ($P > 0.10$) for number of pigs weaned (8.8 ± 0.70 pigs), litter weight (50.9 ± 5.53 kg), pig average weight (5.83 ± 0.51 kg), or pig ADG (187 ± 22.8 g).

3.4.2 Nursery performance

The results for daily feed intake during nursery phase 1 (d 0 – 7) are in Table 3.4. After Table 3.4 Daily feed intake (g/day) of pigs exposed to or fed anise oil (50 ppm) during lactation and phase 1 of the nursery period, respectively¹.

Day	Lactation (L)		Nursery (N)		SEM	P-Value		
	Control	Anise	Control	Anise		L	N	L*N
1	17	23	16 ^a	24 ^b	9.8	0.34	0.1	0.54
2	150 ^a	190 ^b	165	175	19.8	0.05	0.61	0.32
3	236 ^a	267 ^b	248	255	15.5	0.05	0.64	0.85
4	342	362	345	360	25.7	0.2	0.33	0.66
5	349	328	322 ^a	356 ^b	23.6	0.6	0.08	0.64
6	293	313	298	308	23.7	0.22	0.42	0.91
7	310	327	302 ^a	336 ^b	15.7	0.08	0.01	0.10

¹Feed intake was measured each day at 0700, the values are the means of 12 replicates of 4 (Group 1) or 3 (Group 2) pigs.

^{ab}Means within a row with different superscripts differ ($P < 0.10$).

weaning, pigs fed AO in the phase 1 nursery diet consumed 50 and 10 % more ($P < 0.10$) feed on d 1 and 5, respectively, compared to pigs fed the control diet. Pigs from sows fed AO during lactation consumed 27 and 13 % more feed on d 2 and 3 after weaning compared to pigs from sows fed the control diet ($P < 0.10$).

An interaction between lactation and nursery dietary treatments was observed on d 7 (Table 3.5). Pigs fed the control diet, from sows fed the control diet, consumed 19 % less feed than pigs in the other groups ($P < 0.10$).

Table 3.5 Significant interactions of Lactation x Nursery diet on daily feed intake and average daily feed intake¹.

Lactation x Nursery		Daily feed intake, g	ADFI, g	
Lactation	Nursery	Day 7	Day 14-21	Day 0- 21
Control	Control	278 ^a	639 ^a	450 ^a
Control	Anise	326 ^{ab}	766 ^c	544 ^c
Anise	Control	342 ^b	675 ^b	493 ^b
Anise	Anise	330 ^b	679 ^b	510 ^b
MSE		16.1	23.3	20.6
P-Value		0.10	0.02	0.06

¹This table only reports the significant ($P < 0.10$) interactions for the nursery study.

Daily feed intake = feed intake recorder in daily basis, ADFI = average daily feed intake.

^{ab}Means within a row with different superscripts differ ($P < 0.10$).

The results for growth performance and feed efficiency during the nursery phase are presented in Table 3.6. Independent of sow dietary treatment during lactation, feeding AO in the phase 1 nursery diet increased ADFI ($P < 0.10$) by 13 %. However, the AO dietary treatment did not affect ADG or G:F ($P > 0.10$). In nursery phase 2 (d 7-14), pigs fed the AO dietary treatment consumed 8 % more feed than pigs fed the control diet ($P < 0.10$), as well as had greater ADG (+12 %; $P < 0.10$), and G:F (+6 %; $P < 0.10$). During this nursery phase, pigs from sows fed AO in the lactation diet, consumed 8 % more feed than pigs from sows fed the control diet ($P < 0.10$).

Table 3.6 Growth performance of pigs exposed to or fed anise oil (50 ppm) during the lactation and nursery period, respectively¹.

	Lactation (L)		Nursery (N)		MSE	P-Val		
	Control	Anise	Control	Anise		L	N	L*N
Weaning								
BW, kg	7.02	6.93	6.91	7.04	0.091	0.48	0.30	0.75
Day 0-7								
ADG, g	180	186	175	191	11.1	0.65	0.20	0.11
ADFI, g	252	269	243 ^a	273 ^b	10.5	0.38	0.04	0.28
G:F	0.71	0.67	0.69	0.69	0.06	0.71	0.91	0.13
Day 7-14								
ADG, g	375	395	361 ^a	409 ^b	16.2	0.45	0.01	0.39
ADFI, g	505 ^a	547 ^b	505 ^a	547 ^b	18.9	0.06	0.02	0.52
G:F	0.74	0.72	0.71 ^a	0.75 ^b	0.022	0.57	0.10	0.75
Day 14-21								
ADG, g	449	438	450	444	18.2	0.39	0.75	0.24
ADFI, g	703	677	656 ^a	722 ^b	16.4	0.3	0.02	0.02
G:F	0.65	0.65	0.67 ^a	0.62 ^b	0.031	0.98	0.03	0.39
Day 0-21								
ADG, g	320	334	318 ^a	343 ^b	12.7	0.31	0.07	0.12
ADFI, g	497	501	471 ^a	527 ^b	15.5	0.84	0.01	0.06
G:F	0.66	0.66	0.67 ^a	0.65 ^b	0.014	0.95	0.07	0.32
Final								
BW, kg	12.2	12.6	12.1 ^a	12.6 ^b	0.27	0.13	0.09	0.11

¹The values are the means of 12 replicates of 4 (Group 1) or 3 (Group 2) pigs. BW = body weight, ADG = average daily gain, ADFI = average daily feed intake, G:F = gain/feed

^{abc}Means within a row with different superscripts differ ($P < 0.10$).

During nursery phase 3 (d 14-21), there was an interaction (Table 3.5.) between lactation and nursery dietary treatment for ADFI ($P < 0.10$). Pigs from sows fed the control diet consumed the least nursery control diet and the most nursery AO diet (630 vs 766 g, respectively; $P < 0.10$). Pigs from sows fed the AO diet had an average consumption of either control or AO diet. Although ADG was greater in piglets fed the AO diet ($P < 0.10$), G:F ratio was lower ($P < 0.10$) compared to piglets fed the nursery control diet.

In the overall nursery period, there was an interaction (Table 3.5.) between dietary lactation treatment and dietary nursery treatment for ADFI ($P < 0.10$). Pigs from sows fed the

control diet consumed the least amount of nursery control diet and the most amount of nursery AO diet (450 vs 544 g, respectively), meanwhile pigs from sows fed the AO diet had an average consumption of either the control or AO diet during the nursery period. During the overall nursery period, piglets fed the AO diet had greater ADG ($P < 0.10$), but lower G:F ($P < 0.10$) compared to pigs fed the control diet. Pigs fed the AO diet also were 0.5 kg heavier at the end of trial ($P < 0.10$).

3.5 Discussion

Management practices in modern swine production include the weaning of piglets at approximately three weeks of age in the United States. At this point there is an abrupt change in piglet environment and nutrition. The signs of stress during this period are characterized by short periods of anorexia and low feed intake (Mahan et al., 2004; Naranjo, 2010; Oostindjer et al., 2010). In response to an under nutrition period, growth stasis is observed in pigs (McCracken et al., 1995). The lack of nutrient intake has a greater impact on reduction of intestinal integrity (villous atrophy and crypt hypertrophy) than diet changes and composition (Spreeuwenberg et al., 2001). Further effects of morphological changes at the intestinal level may cause lower nutrient absorption and poorer feed efficiency in later stages (Boudry et al., 2004). The focus of this research was to promote nutrient intake during the weaning transition period.

In this study feed intake was measured as an indicator of feed preference, which contrasts with methodologies used in other studies that measured the time it takes for neonatal or weanling animals to identify a scented environment compared to the environment that they were previously exposed to (Simitzis et al., 2008; Oostindjer et al., 2009; Oostindjer et al., 2010). Our research demonstrated that exposure of piglets to AO during lactation, by feeding sows diets supplemented with AO (50 ppm), increased pig daily feed intake after weaning. Also, feeding

weanling pigs nursery diets supplemented with AO (50 ppm) had a positive influence on feed intake immediately after weaning. Anise oil supplemented pigs also had a higher growth rate.

Anise oil was selected as a source of flavor and smell in the diets fed to sows because, as reported by Schaal et al. (2009), it is able to pass the uterus and mammary gland barrier and be present in the milk of mammals (Schaal et al., 2000). Anise oil also is suitable for food usage and has been used in highly sensitive animals, like dogs, with no negative effects (Hepper and Wells, 2006). As a feed supplement in our study, AO did not reduce feed intake. Independent of sow dietary treatment, AO increased feed intake of piglets after weaning, demonstrating that it was suitable for pig feed consumption at the levels evaluated in our study. Langendijk et al. (2007) fed lactating sows a mix of garlic and AO in sow diets and later in nursery diets. They also reported greater feed intake in piglets. However, in this study the pigs were exposed to intermittent suckling and creep feeding at two weeks of age, and they were weaned at > 28 d of age (Langendijk et al., 2007).

In pigs, growth rate is correlated with feed intake. Few studies have evaluated the effect of feeding plant oils on growth performance of pigs. Feeding a mix of garlic and AO stimulated higher feed intake but growth rates were similar to the control pigs (Langendijk et al., 2007) during the first 10 days after weaning. In our study, pigs fed AO after weaning consumed more feed during nursery phase 1, but growth rate was similar to the control group. However, AO stimulated growth in pigs during nursery phase 2 and the overall nursery period. Although small intestine integrity was not evaluated in this study, early feed consumption by pigs may improve absorption of nutrients in later stages, thus improving feed efficiency (Boudry et al., 2004). Feed efficiency was higher in the group fed the AO treatment during nursery phase 2.

In summary, this study evaluated the effect of peri-natal exposure of piglets to AO and its effect on feed intake after weaning. Anise oil added to nursery diets increased feed intake. Although growth rate was not affected during nursery phase 1, AO increased growth in later nursery stages, with a positive effect on feed efficiency. It also is important to note that feeding AO only during the nursery phase increased feed consumption but decreased feed efficiency. Overall, pigs fed AO were heavier at the end of the evaluation period. Additional research is needed to determine the benefits of using AO in sows and weanling pigs with a focus on metabolism of AO in the mammary gland of sows, as well as on the digestive tract integrity in piglets.

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CHAPTER 4: DETERMINING THE MAXIMUM IN-VIVO DOSAGE OF ANISE OIL FOR BROILER CHICKENS, AND ITS IN-VITRO EFFECT ON THE GROWTH OF CLOSTRIDIUM PERFRINGENS

4.1 Abstract

Two studies were conducted to determine an effective dosage of anise oil (AO) for commercial broilers from 0 to 17 d of age as an antibiotic growth promotant replacement, and its effect on in-vitro growth of *Clostridium perfringens* (CIP). In Trial 1, 648 Ross 708 male broilers were allotted to treatments with 9 replicates of 6 chicks per pen, and housed in battery cages. Bacitracin Methylene Disalicylate (BMD) and Salinomycin sodium (Sal) were used as antimicrobials. The dietary treatments were a negative control (NC, no antibiotics), a positive control (PC, Sal + BMD), and 10 AO levels of 500, 1000, 1500, 2000, 2500, 3000, 3500, 4000, 4500, or 5000 ppm. Feed intake and broiler health were carefully monitored. In Trial 2, based on the in-vivo trial, five levels of AO (500, 1000, 1500, 2000, or 2500 ppm; which did not compromise poultry growth performance), two levels of BMD (50 or 100 ppm), two levels of Sal (60 or 120 ppm), and distilled water (DW) for control were used to evaluate the in-vitro growth of CIP. The strain ATCC 12918 was used as reference and cultured two consecutive times for 24 h at 37 °C in anaerobic conditions. Three concentrations of CIP (10^9 , 10^8 , and 10^7 /ml) were inoculated in tubes containing nine mL of reinforced media and one ml of the carrier of the antibiotic concentration. All tubes were placed in an anaerobic environment and grown for 24 h at 37 °C. After 24 h tubes were analyzed for growth and then diluted 1:10 in distilled water. They were compared to McFarland opacity standards to estimate CFU/ml concentrations. In Trial 1, increasing levels of AO linearly reduced ADFI and ADG from d 0 – 10, d 10 – 17, and overall (d 0 to 17) when fed to broilers. Final BW also was reduced linearly with increasing levels of AO on d 17. Compared to NC and PC, feeding chicks AO at levels over 4000 ppm reduced feed intake, and feeding chicks AO at levels over 2500 ppm reduced ADG in d 0-10 broilers.

Although there was a treatment effect for overall G:F there was no direct relation with the AO level. In Trial 2 independent of the amount of CFU inoculated, in-vitro CIP grew up to approximately $1-2 \times 10^{10}$ CFU/ml in the control media. Growth of CIP was not detectable (ND) with both levels of BMD or Sal independent of inoculum concentration. Compared with control, AO linearly reduced CIP growth ($P < 0.05$; 9×10^9 , 7.5×10^9 , 3×10^9 , 1.5×10^9 , and ND CFU/ml with 500, 1000, 1500, 2000, and 2500 ppm of AO, respectively, when 1×10^9 CFU/ml of CIP was inoculated. Further reduction ($P < 0.05$) was observed when 1×10^8 CFU/ml was inoculated (3×10^8 , 3×10^8 , 1.5×10^8 , and ND CFU/ml with 500, 1000, 1500, and 2000 ppm of AO, respectively). Therefore, AO is suitable to feed to broilers at inclusion rates lower than 3000 ppm. Although AO linearly reduced the growth of CIP, it did not exhibit bactericidal effects with the levels studied. Both BMD and Sal completely inhibited CIP growth.

4.2 Introduction

The use of antibiotics at sub-therapeutic levels to produce a growth promoting effect is a common practice in livestock and poultry production. Despite the fact that there is no specific biological mode of action (Fuller, 1989; Gaskins et al., 2002), there is substantial information regarding the benefits of antimicrobials on feed efficiency, growth rate (Gaskins et al., 2002), and ultimately farm profitability when used under commercial conditions. However, general concerns are arising with the relationship of this practice and an observed increase in antimicrobial resistance of human pathogens (FDA, 2013). Therefore, several antibiotic groups have been banned for usage at sub-therapeutic dosages for producing animals in Europe (Casewell et al., 2003). Meanwhile, in the U.S. the FDA (2013) is requesting voluntary removal of antibiotics from livestock and poultry diets with the goal of limiting their use to only therapeutic purposes.

In the European Union, removal of antibiotics from poultry diets has led to an increase in animal health issues and a decrease in feed and system efficiency (Casewell et al., 2003). This compromises producer profit, as well as consumer safety and affordability of meats. There also is an increase in demand for differentiated foods (antibiotic free, free range, and organic foods). Many of those products are sold with a premium price as a marketing strategy (Harper and Makatouni, 2002), which represents an opportunity for the industry. Consequently, organic acids, probiotics, bacteriocins, bacteriophages, peptides, and phytochemicals are gaining interest as alternatives to antibiotics (Joerger, 2003; Hume, 2011). Phytochemicals, also called phytotherapeutics and phytogenics (vegetative parts, extracts, oils), are plant derivative compounds with a wide range of medical and physiological properties (Hume, 2011). Some plants have evolutionary developed antimicrobial properties for self-defense against pathogens which suggests they may be valuable as replacements for antibiotics. However, the composition of botanical additives is variable and dependent on species, part of the plant, and method of extraction (Burt and Reinders, 2003). This limits their practical utilization. Bacterial studies conducted in-vitro required high concentrations of botanicals to produce antibiotic effects (Deans and Ritchie, 1987; Guarda et al., 2011). Normally minimum inhibitory concentrations are reached with concentrations >500 ppm (Deans and Ritchie, 1987; Hammer et al., 1999; Dorman and Deans, 2000) which may compromise feed intake and growth performance in-vivo. Anethole, the main component of AO (~90% as analyzed in our laboratory), has been evaluated as an antimicrobial (Deans and Ritchie, 1987). The aerobic bacterial growth results are variable (Kubo and Fujita, 2001; De et al., 2002; Gülçin et al., 2003; Wang et al., 2011b) and there is limited information on anaerobic bacterial growth (ie. *Clostridium perfringens*, CIP). The advantage of AO is its suitability for feed and food as evaluated in pigs and dogs (Wang et al.,

2011a) which are more sensitive to flavors and smells than chickens (Sneddon et al., 1998). However, there is no evidence of the toxicity or maximum levels that can be fed to chickens. Therefore, the aim of this research was to determine the maximum level of AO that can be fed to broiler chickens without affecting growth performance. Also, this research was conducted to determine the antimicrobial effects of AO on CIP via in-vitro testing.

4.3 Materials and methods

The methodology used in this research was approved by the Louisiana State University Agricultural Center Animal Care and Use Committee, as well as by the Inter-Institutional Biological and Recombinant DNA Safety Committee (IBRDSC) for pathological bacteria use.

4.3.1 Trial 1, In-vivo

An experiment was conducted with 648 Ross x Ross 708 commercial broilers. On day of hatch the broilers were weighed, wing banded, and allotted to 12 treatments with nine replicates per treatment and six broilers per replicate. The trial lasted 17 days. The broilers were housed in temperature controlled batteries with wire floors and continuous lighting. Broilers received ad-libitum water and feed in mash form throughout the experimental period. Feed additions were recorded and broilers were checked twice daily. Broilers were weighed on d 10 and at the end of the trial. Feed leftovers also were weighed on d 10 and 17.

The diets were corn-soybean meal based and formulated to contain 1.29% total Lys (Table 4.1.). The ratios of TSAA and Thr to Lys were set at 0.72 and 0.70% respectively, which met or exceeded the suggested amino acid ratios (Baker, 1997) for broilers. All diets contained 3,200 Kcal of ME/kg, 1.05% Ca, and 0.50% non-phytate P, and met or exceeded the requirements of all other nutrients for broilers 0-21 d of age (NRC, 1994).

Table 4.1 Composition of the basal diet, as fed basis.

Item	%
Corn	46.12
Soybean meal, 48%	43.38
Soy oil	6.04
Monocalcium phosphate	1.73
Limestone, ground	1.36
Common salt	0.50
Mineral premix ¹	0.25
Vitamin premix ²	0.25
DL-Methionine ³	0.25
L-Threonine ³	0.07
Choline chloride ⁴	0.05
Calculated composition	
MEn, kcal/kg	3200
Lys, %	1.29
Met, %	0.62
Met+Cys, %	1.03
Thr, %	1.00
Ca, %	1.05
nPP, %	0.50

¹ Provided per Kg of diet: Cu (copper sulfate), 7 mg; I (calcium iodate), 1 mg; Fe (ferrous sulfate•H₂O), 50 mg; manganese (manganese sulfate), 100 mg; Se (sodium selenite), 0.15 mg; Zn (zinc sulfate), 44 mg.

² Provided per Kg of diet: vitamin A, 8,002.78 IU; vitamin D₃, 3003.8 IU; vitamin E, 25 IU; menadione, 1.5 mg; vitamin B₁₂, 0.02 mg; biotin, 0.1 mg; folic acid, 1 mg; niacin, 50 mg; pantothenic acid, 15 mg; pyridoxine, 4 mg; riboflavin, 10 mg; thiamin, 3 mg.

³ L-Threonine (99%), DL-Methionine (99%) were provided by Evonik Corporation, Kennesaw, GA.

⁴ Contains 750,000 mg/kg of choline.

The dietary treatments were: 1) Negative control (NC; no antibiotics), 2) Positive control (PC; Salynomicin, Sal + Bacitracin Methylene Disalicylate, BMD), and 3-12) anise oil (AO; 500, 1000, 1500, 2000, 2500, 3000, 3500, 4000, 4500, and 5000 ppm). No toxic levels were observed, therefore no removal of treatments was required for the welfare of the chicks.

4.3.2 Trial 2, In-vitro

Purified strains of *Clostridium perfringens* were obtained from American Type Culture Collection (ATCC 12918, Manassas, VA). An adaptation of the Methods for Antimicrobial Susceptibility Testing of Anaerobic Bacteria (NCCLS, 2012) as suggested by others (Stalons and

Thornsberry, 1975) was used in this experiment .The strain was rehydrated with reinforced broth and two serial dilutions were cultured for 24 to 36 h at 35 to 37 °C in brucella blood agar to verify purity and viability. From the culture described before, five colonies of at least one mm in diameter of growth were inoculated in reinforced brucella broth and allowed to grow for 12 to 14 h at 35 to 37 °C until adequate turbidity was obtained. After two consecutive growth cycles, three concentrations of CIP (1×10^9 , 1×10^8 , or 1×10^7 CFU/ml) were inoculated in duplicate to sterile tubes containing nine ml of reinforced anaerobic broth and one ml of the carrier containing the antibiotic. Based on the in-vivo research, five levels of AO (500, 1000, 1500, 2000, or 2500 ppm), two levels of BMD (50 or 100 ppm), two levels of Sal (BioCox; 60 or 120 ppm), and distilled water for control were used. All tubes were placed in an anaerobic environment and grown for 24 h at 35 to 37 °C. After 24 h, tubes were analyzed for growth and diluted 1:10 in distilled water. They were compared to BD McFarland opacity standards (Becton Dickinson and Co., Franklin Lakes, NJ) to estimate CFU/ml concentrations.

4.3.3 Statistical Analysis

In Trial 1, the in-vivo data were analyzed with the Mixed procedure of SAS (SAS-Institute, 2011) with treatment as the fixed effect in a completely randomized design. Linear and quadratic contrasts were determined for the effect of AO. In Trial 2, the in-vitro data were analyzed with the Glimmix procedure of SAS (SAS-Institute, 2011) for non-parametric variables with treatment as the fixed effect in a completely randomized design. Linear and quadratic contrasts were determined for the effect of AO.

4.4 Results

4.4.1 Trial 1, In-vivo

The results of the in-vivo trial indicated that during the 0 – 10 d period, feeding increasing levels of AO to broilers linearly reduced ($P < 0.05$) ADFI. Broilers fed more than 3000 ppm AO had lower ADFI compared to broilers fed NC or PC ($P < 0.10$). Similarly, ADG was linearly decreased by feeding increasing levels of AO to broilers ($P < 0.05$). Broilers fed more than 2500 ppm had lower ADG ($P < 0.10$) than broilers fed NC or PC. No linear or treatment effects were observed for G:F ($P > 0.10$) during this period. During the 10 - 17 d period, there was no effect of dietary treatment on ADFI, ADG, or G:F. However, there was a linear reduction in ADFI and ADG in response to increasing levels of AO in the diet ($P < 0.10$). There was no linear treatment effect on G:F during this period.

For the overall period (d 0 to 17), there was a linear reduction of ADFI and ADG with increasing levels of anise oil in broiler diets ($P < 0.05$). Although G:F was not affected by dietary treatment, broilers fed 2000 or 5000 ppm AO had the poorest G:F. Broilers fed 500, 1000, 2500, 3000, or 4000 ppm AO or PC had the best G:F. Broilers fed the other dietary treatments had intermediate G:F ($P < 0.10$). The results of the effect of increasing levels of AO on growth performance of broilers are presented in Table 4.2.

4.4.2 Trial 2, In-vitro

Based on the in-vivo results, an in-vitro study was conducted to evaluate the effect of AO on the growth of CIP (Table 4.3.). In this study, independent of the amount of inoculum, BMD and Sal inhibited growth of CIP with both dosages used in the study.

Table 4.2 The effect of anise oil (AO) on feed intake, growth, and feed efficiency of broilers from 0-17 d of age¹.

Day	ADG, g/d			ADFI, g/d			G:F, g/g			BW, g	
	0-10	10-17	0-17	0-10	10-17	0-17	0-10	10-17	0-17	0	17
Treatment ²											
PC	16.5 ^a	35.4	24.3	17.7 ^a	48.0	30.2	0.93	0.74	0.80 ^{abcd}	34.9	448.3 ^{ab}
NC	16.1 ^{ab}	36.3	24.4	17.6 ^a	50.0	30.9	0.91	0.73	0.79 ^{bcd}	34.9	449.9 ^{ab}
AO, 500	15.3 ^{abcd}	36.9	24.2	16.7 ^{abc}	49.0	30.0	0.92	0.75	0.81 ^{abc}	34.9	446.7 ^{ab}
AO, 1000	15.1 ^{abcd}	36.4	23.9	16.5 ^{abc}	48.4	29.6	0.92	0.75	0.81 ^{abc}	34.8	441.0 ^{ab}
AO, 1500	15.8 ^{abc}	37.2	24.6	17.2 ^{ab}	49.3	30.4	0.92	0.75	0.81 ^{ab}	34.9	453.5 ^a
AO, 2000	14.8 ^{abcd}	34.7	23.0	16.5 ^{abc}	48.4	29.6	0.90	0.72	0.78 ^d	35.5	426.9 ^{abc}
AO, 2500	14.9 ^{abcd}	33.9	22.7	16.5 ^{abc}	46.5	28.9	0.90	0.73	0.79 ^{abcd}	35.6	421.2 ^{abc}
AO, 3000	14.2 ^{cd}	35.9	23.1	15.9 ^{abc}	46.7	28.3	0.89	0.77	0.82 ^a	34.9	428.4 ^{abc}
AO, 3500	14.3 ^{bcd}	34.7	22.7	15.7 ^{bc}	45.7	28.0	0.91	0.76	0.81 ^{abc}	35.0	421.2 ^{abc}
AO, 4000	14.9 ^{abcd}	35.5	23.4	16.1 ^{abc}	48.6	29.5	0.93	0.74	0.80 ^{abcd}	35.0	432.5 ^{abc}
AO, 4500	13.8 ^d	32.8	21.6	15.1 ^c	45.0	27.4	0.91	0.73	0.79 ^{bcd}	34.3	401.6 ^c
AO, 5000	14.1 ^{cd}	33.7	22.2	15.6 ^{bc}	46.8	28.4	0.90	0.72	0.78 ^{cd}	34.9	412.3 ^{bc}
SEM	0.70	1.24	0.81	0.67	1.65	1.03	0.010	0.016	0.011	0.34	13.85
P-Val											
Trt	0.10	0.27	0.18	0.10	0.54	0.36	0.25	0.32	0.10	0.44	0.10
Linear	0.02	0.01	0.01	0.02	0.05	0.03	0.54	0.24	0.17	0.49	0.01
Quadratic	0.99	0.91	0.95	0.74	0.67	0.80	0.32	0.52	0.54	0.06	0.99

¹The values are the means of nine replicates of six broilers. ADG = average daily gain, ADFI = average daily feed intake, G:F = gain:feed, BW = body weight,

² PC = positive control (60 g/ton Bacitracin methylene disalicylate and 50 g/ton Salinomycin added), NC = negative control (no antibiotics added), AO = anise oil (10 levels ranging 50 – 1000 ppm).

^{abcd} Means within a column with different superscripts differ ($P < 0.10$)

Table 4.3 The effect of anise oil (AO) on in-vitro growth of *Clostridium perfringens*.

Treatment (Trt) ¹	CFU/ml inoculated			Overall
	10 ⁹	10 ⁸	10 ⁷	
Control	17 x 10 ⁹ ^a	16 x 10 ⁹ ^a	12 x 10 ⁹	15x10 ⁹ ^a
AO (ppm)				
500	9.0 x 10 ⁹ ^b	3.0 x 10 ⁸ ^b	ND	7.3 x 10 ⁸ ^b
1000	7.5 x 10 ⁹ ^{bc}	3.0 x 10 ⁸ ^b	ND	7.1 x 10 ⁸ ^b
1500	3.0 x 10 ⁹ ^c	1.5 x 10 ⁸ ^b	ND	4.2 x 10 ⁸ ^c
2000	1.5 x 10 ⁹ ^d	ND	ND	2.7 x 10 ⁸ ^c
2500	ND	ND	ND	1.7 x 10 ⁸ ^{cd}
Sal (ppm)				
60	NG	NG	NG	NG
120	NG	NG	NG	NG
BMD (ppm)				
50	NG	NG	NG	NG
100	NG	NG	NG	NG
MSE	1.5 x 10 ⁹	3.0 x 10 ⁸		1.5 x 10 ⁸
Concentration (C)				
10 ⁷				1.2 x 10 ⁸ ^c
10 ⁸				4.1 x 10 ⁸ ^b
10 ⁹				6.2 x 10 ⁸ ^a
MSE				8.0 x 10 ⁷
<i>P</i> -Val				
Trt				0.0001
C				0.001
Trt*C				0.41
AO Linear	0.0001	0.001	--	0.0001
AO Quadratic	0.05	0.001	--	0.03

^{abcd} Means within a row with different superscript differ ($P < 0.05$)

ND No detectable growth with McFarland standard after 1:10 dilution.

^{NG} No detectable growth of bacteria in direct media vials, which represents total control.

¹ Control = distilled water; AO = anise oil, Sal = Salinomycin sodium, BMD = Bacitracin methylene disalicylate.

In contrast, CIP grew up to 1.2, 1.5, and $> 1.7 \times 10^9$ in the control media when 1×10^9 , 1×10^8 , and 1×10^7 CFU/ml were inoculated ($P < 0.001$). The lowest CFU/ml of CIP inoculated failed to grow consistently in AO supplemented media. However, the growth of 1×10^9 and 1×10^8 CFU/ml CP was linearly ($P < 0.001$) and quadratically ($P < 0.05$) reduced by increasing

levels of AO. Although no interaction was observed between amount of inoculum and dosage of AO ($P > 0.10$), growth of the lower concentration inoculated required lower amounts of AO to arrest CP growth.

4.5 Discussion

Trans-anethole (4-methoxypropenylbenzene) is the main component of AO (~ 90% as analyzed in our laboratory) and is a flavoring substance that is GRAS (generally recognized as safe) in food (Newberne et al., 1999). Anethole metabolism and toxicology was evaluated in humans with dosages ranging between 0.015 to 12 mg/kg of BW (Caldwell and Sutton, 1988). Female mice were exposed to doses ranging from 50 to 426 mg and male mice were exposed to doses ranging from 0.05 to 1500 mg/kg of BW (Bounds and Caldwell, 1996). Bounds and Caldwell (1996) also exposed male and female rats to maximum dosage levels of 1039 and 1500 mg/kg BW, respectively. In our study, based on ADFI and BW of the chicks in the study, the range of AO consumption was from 0.45 to 4.5 mg/kg BW. These levels are well below the levels described in other species. However, those levels were greater than levels fed to pigs and dogs, which are considered highly sensitive to exogenous flavors and odors (Hepper and Wells, 2006; Langendijk et al., 2007). In other related studies, feed intake was reduced in mice when they were fed more than 426 mg/kg BW of trans-anethol (Newberne et al., 1999). In our study feed intake was linearly reduced in response to increasing levels of AO. No negative effect on growth performance or BW in response to feeding AO to animals has been reported. In our study feeding AO over 3000 ppm reduced feed intake, and feeding 2500 ppm AO reduced growth in 0-10 d old broilers. Although feed intake and growth were reduced linearly by increasing levels of AO in older chickens, no treatment effect was observed. However, the effect observed in young

chicks somewhat compromised overall performance. Feed efficiency was reduced in broilers fed more than 4000 ppm AO.

Several extracts and essential oils derived from spices exhibit antibacterial activity. In most of the cases they reduce bacterial growth (Oussalah et al., 2007). It also has been demonstrated that as an antibacterial, volatile oils have the biggest potential because spices and herbs, which are widely used in humans, are present in insufficient quantities to demonstrate significant antimicrobial activity (Oussalah et al., 2007). Deans and Ritchie (1987) reported that AO exhibits antibacterial effects against five anaerobes. The anaerobes are facultative bacteria species from the genus *Aeromonas*, *Brevibacterium*, *Brocothrix*, *Flavobacterium*, and *Leuconostoc*. Similar results have been obtained against *Aeromonas* and *Edwardsiella tarda* which are anaerobic facultative pathogens for salmonides (Parasa et al., 2012). As reported by Gülçin et al. (2003), water and ethanol extracts from aniseed also have in-vitro antibacterial activity against five gram positive and five gram negative bacteria that were facultative anaerobes or aerobes.

In poultry, CIP is associated with enteric disorders which can cause clinical manifestations such as intestinal lesions, necrotic enteritis, necrotic dermatitis, cholangiohepatitis, or gizzard erosion. *Clostridium perfringens* is a gram-positive, spore forming anaerobic bacteria present in soil, feed, litter, and the intestinal tract of diseased and healthy birds (Hafez, 2010). In agreement with previous research using anise extracts, in our study AO had antibacterial activity against CIP. However, as mentioned by Oussalah et al. (2007), there is no bactericidal effect of plant extracts at low dosages. Therefore, high concentrations of active volatile compounds are required to completely inhibit growth of bacteria. In our study, there was linear and quadratic reduction of bacterial growth with increasing levels of AO added to the

media. Although no interactions were observed when lower CFU/ml concentrations were inoculated, the growth is reduced to non-detectable levels with dosages above 1000 ppm AO. Whereas, CIP loads in field operations are low, AO may be an alternative to antibiotics to prevent disease or promote growth of broilers, but definitely is not a replacement of therapeutic interventions with major outbreaks.

In summary, these trials were conducted to evaluate the effect of AO on feed intake, growth, and feed efficiency of broilers from d 0 to 17, as well as to determine the maximum inclusion level of AO that does not compromise bird performance or welfare. The levels evaluated in this study were not toxic and they were tolerated by the broilers. However, broilers fed more than 3000 ppm AO had reduced feed intake. Also, broilers fed more than 2500 ppm AO had reduced growth, which was related to reduced feed intake. Feed efficiency was variable among treatments and there was no defined pattern of response to increasing levels of AO. In this study feeding AO up to 2500 ppm was determined to be a safe level to feed to broilers. In Trial 2 the in-vitro antibacterial effect of five different levels of AO from 500 to 2500 ppm was evaluated. They were evaluated for growth of CIP and compared to BMD and Sal. There was a linear reduction in growth by increasing levels of AO, which was independent of the CFU/ml concentration inoculated. However, there was no bactericidal effect as there was with BMD and Sal, which completely inhibited CIP growth.

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CHAPTER 5: THE EFFECT OF ANISE OIL AND DIETARY PROTEIN SOURCE ON GROWTH PERFORMANCE AND JEJUNUM LESIONS OF BROILERS DURING A CLOSTRIDIUM PERFRINGENS CHALLENGE

5.1 Abstract

The objectives of this research were to determine the effect of anise oil (AO) and fish meal on growth performance and jejunum lesions of broilers during a *Clostridium perfringens* (CIP) challenge. In Exp. 1, 360 Ross 708 male broilers were allotted to 8 treatments with 9 replicates and 5 birds per replicate. Salinomycin sodium (Sal) and Bacitracin methylene disalicylate (BMD) were used as antimicrobials. The treatment diets were 1) negative control (NC, no antibiotic), 2) Sal, 3) BMD, 4) positive control (PC, Sal + BMD), and 5-7) three levels of AO (500, 1000, 1500 ppm) and, 8) a NC treatment group was not challenged (NCU) with CIP. On d 10 birds were inoculated with 2×10^9 units of CIP. On d 18 birds were euthanized and necropsied. The jejunum was removed, cleaned, and scored. On d 0-10 and overall (d 0 – 18) broilers fed the AO diets (500, 1000, 1500 ppm) had reduced ADG ($P < 0.05$) and ADFI ($P < 0.10$), with no effect on G:F compared to broilers fed NC. Birds fed Sal had lower ($P < 0.02$) ADG and G:F from d 10 – 18 and d 0 – 18 compared to broilers fed NC. Birds that received AO had lower lesion scores (LS) than birds fed NC. In Exp 2, 350 broilers were allotted to 10 treatments with 7 replicates and 5 birds per replicate. The birds were fed a starter diet from d 0 – 18 with 5% fish meal except the broilers fed the all vegetable diet. The treatment diets were: 1) vegetable (Veg) protein, 2) NC, 3) PC, and 4-6) three levels of AO (500, 1000, 1500 ppm). Three treatment groups were fed NC from d 0-10 and 7) 500, 8) 1000, or 9) 1500 ppm AO from d 10-18. A 10) NC treatment group was not challenged (NCU). On d 10 and 13 broilers were infected with 3×10^6 units of CIP. During d 0 – 10, broilers fed the Veg diet had lower G:F ($P < 0.03$). After the challenge, broilers fed Veg had lower ADFI, ADG, and BW compared to broilers fed NC ($P < 0.09$). Broilers that received the AO had lower LS than broilers fed NC or

PC ($P < 0.01$). These results indicate that during a CIP challenge, broilers fed AO or antibiotics had similar growth, feed intake, and feed efficiency. Broilers fed AO had reduced LS, and adding fish meal to the diet had no effect on necrotic lesions.

5.2 Introduction

Necrotic enteritis and related subclinical diseases are of economic importance for the poultry industry. Production losses, increased mortality, reduced welfare of birds, and increased risk of contamination of poultry products have been associated with this disease (Timbermont et al., 2011). Both clinical and sub-clinical necrotic enteritis are a worldwide problem in poultry, and is estimated to cost five cents per bird. In 2000, these losses represented over \$2 billion per year (Hofacre, 2005). One of the causative agents is *Clostridium perfringens* (Craven, 2000; Timbermont et al., 2010; Timbermont et al., 2011) toxinotype A that is able to produce NetB toxin (Lanckriet et al., 2010). However, the onset of necrotic enteritis is dependent upon other factors such as sub-clinical coccidiosis (Lanckriet et al., 2010), stress, and some dietary factors (Jia et al., 2009). Non-starch polysaccharides from wheat, barley, or rye, when added to broiler diets (Hofshagen and Kaldhusdal, 1992; Kaldhusdal and Hofshagen, 1992; Kaldhusdal and Skjerve, 1996), increase intestinal viscosity, reduce nutrient absorption, and influence gut microflora (Kaldhusdal and Skjerve, 1996; Jia et al., 2009). Manzanilla et al. (2009) also found that protein source and amount affect performance and microflora in young pigs. Similar results were reported when meat, poultry, or fish meal was added to poultry diets (Drew et al., 2004; Olkowski et al., 2006).

Antibiotics and anticoccidials are commonly used to overcome the negative effects of necrotic enteritis (Lanckriet et al., 2010) and to promote growth when used at sub-therapeutic levels (Fuller, 1989; Gaskins et al., 2002). However, general concerns are rising with regard to

this practice along with the observed increase in antimicrobial resistance for human pathogens (FDA, 2003). Therefore, antibiotic use at sub-therapeutic dosages has been banned in Europe (Casewell et al., 2003). In North America the FDA is tightening regulations regarding the safe use of antibiotics, and the trend is to limit their use to therapeutic purposes (FDA, 2013). In poultry, there is evidence that removal of antibiotics at sub-therapeutic levels in the diet may decrease feed efficiency and system efficiency (Casewell et al., 2003). Consequently, organic acids, probiotics, bacteriocins, bacteriophages, peptides, and phytochemicals are gaining interest as alternatives to antibiotics (Joerger, 2003; Hume, 2011). Phytochemicals, also called phytotherapeutics (vegetative parts, extracts, oils), are plant derivative compounds with a wide range of medical and physiological properties (Hume, 2011). Some plants have developed antimicrobial properties for self-defense against pathogens (Brenes and Roura, 2010), which suggests they may be able to replace antibiotics. Bacterial studies conducted in-vitro with high phytochemical concentrations have produced antibiotic effects (Deans and Ritchie, 1987; Hammer et al., 1999; Dorman and Deans, 2000). However, the effect of phytochemicals in in-vivo studies has been variable, maybe related the wide range of methodologies utilized (Windisch et al., 2008; Brenes and Roura, 2010). In laboratory conditions (clean environment), antibiotics added to broiler diets have failed to produce growth promotant effects when compared to non-antibiotic diets. In this context, challenging studies with pathogenic bacteria or coccidia is the method suggested to evaluate the efficacy of antimicrobials and their alternatives (Kaldhusdal et al., 1999; Olkowski et al., 2006; Jia et al., 2009; Bozkurt et al., 2014). The production and reproduction of necrotic enteritis has been variable; however, infection with *Clostridium perfringens* (CIP), *Eimeria* spp., or a mix of both are commonly used in these models (Kaldhusdal et al., 1999; Craven, 2000; Olkowski et al., 2006; Jia et al., 2009; Bozkurt et

al., 2014). Anethole, the main component of AO (~90% as analyzed in our laboratory), has been evaluated as an antimicrobial (Deans and Ritchie, 1987). The results are variable with aerobic bacteria (Kubo and Fujita, 2001; De et al., 2002; Gülçin et al., 2003; Wang et al., 2011) and there is limited information on anaerobic bacteria such as CIP. The objectives of this study were to determine 1) the effect of AO on growth parameters and necrotic lesions in the jejunum of broilers challenged with CIP, 2) the effect of feeding fish meal during the challenge, and 3) the timing of feeding AO during the challenge.

5.3 Materials and methods

The methodology used in this research was approved by the Louisiana State University Agricultural Center Animal Care and Use Committee as well as by the Inter-Institutional Biological and Recombinant DNA Safety Committee (IBRDSC). The IBRDSC approved the use of pathological bacteria.

5.3.1 Bacterial cultures

In all experiments, *Clostridium perfringens* (CIP) Type A ATCC 12918 (American Type Culture Collection, Manassas, VA) was used. The bacteria was cultured anaerobically in thioglycollate (Becton, Dickinson and Co., Sparks, MD) medium for at least 20 hr at 35-37 °C. The culture was administered when it reached exponential growth of 2×10^9 or 1×10^8 colony forming units/mL (CFU/ mL) in Exp. 1 and 2, respectively. These procedures were performed in a Biosafety Level 2 facility (BSL) of the Food Microbiology Laboratory (LSU Agricultural Center, Chemistry Building).

5.3.2 Animals and management

The in-vivo CIP challenge of broilers was carried out in the Infectious Disease Isolation Facility (IDIF, LSU Agricultural Center) that exceeds controls for BSL-2. Brooder batteries were

disinfected and placed into IDIF. One day old birds were weighed, wing banded, and placed in brooder batteries. The birds were weighed again on d 10, and at the end of the trials on d 18. The broilers were provided ad libitum access to water and feed during the entire experiment. Diets were corn-soybean meal based in Exp. 1 and corn-soybean meal-fish meal in Exp. 2. Diets met or exceeded the broiler nutrient requirements (NRC, 1994) for the starter phase (d 0 – 21).

In Exp. 1, 360 unvaccinated one day old male Ross 708 commercial broilers were allotted randomly to one of eight treatments with nine replicates and five birds/replicate. Treatments (Table 5.1.) were 1) negative control diet (NC) containing no antibiotics, 2) Salinomycin sodium (Sal) added at 60 g/ton, 3) Bacitracin methylene disalicylate (BMD) added at 50 g/ton, 4) Positive control (PC) containing BMD and Sal at the levels described in treatment 2 and 3, 5) Anise oil 1 (AO1) added at 500 ppm, 6) Anise oil 2 (AO2) added at 1000 ppm, 7) Anise oil 3 (AO3) added to 1500 ppm, and 8) Negative control unchallenged (NCU, containing no growth promotant and not challenged). The broilers were fed their treatment diets beginning on the first day of the trial.

Table 5.1 Composition of the treatment diets used in Exp. 1 in as fed basis.¹

Item, %	NC	Sal	BMD	PC	Anise oil, ppm		
					500	1000	1500
Corn	49.84	49.27	49.84	49.27	49.84	49.84	49.84
Soybean meal, 48%	40.04	40.09	40.04	40.04	40.04	40.04	40.04
Soybean oil	5.49	5.69	5.49	5.69	5.49	5.49	5.49
Monocalcium phosphate	1.76	1.76	1.76	1.76	1.76	1.76	1.76
Limestone	1.24	1.06	1.19	1.06	1.24	1.24	1.24
Common salt	0.50	0.50	0.50	0.50	0.50	0.50	0.50
BMD ⁶	--	--	0.50	0.50	--	--	--
DL-methionine	0.29	0.29	0.29	0.29	0.29	0.29	0.29
Mineral premix ²	0.25	0.25	0.25	0.25	0.25	0.25	0.25
Vitamin premix ³	0.25	0.25	0.25	0.25	0.25	0.25	0.25
L-Lysine	0.18	0.18	0.18	0.18	0.18	0.18	0.18
L-Threonine	0.12	0.12	0.12	0.12	0.12	0.12	0.12
Choline chloride ⁴	0.05	0.05	0.05	0.05	0.05	0.05	0.05
Salinomycin ⁵	--	0.05	--	0.05	--	--	--

(Table 5.1 continued)

Treatment	NC	Sal	BMD	PC	500	1000	1500
Calculated composition							
MEn, kcal/kg	3,200	3,200	3,200	3,200	3,200	3,200	3,200
Ca, %	1.00	1.00	1.00	1.00	1.00	1.00	1.00
Non-phytate P, %	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Lys, %	1.29	1.29	1.29	1.29	1.29	1.29	1.29
Met, %	0.61	0.61	0.61	0.61	0.61	0.61	0.61
Met+Cys, %	0.93	0.93	0.93	0.93	0.93	0.93	0.93
Gly, %	0.96	0.96	0.96	0.96	0.96	0.96	0.96
Gly+Ser, %	2.163	2.163	2.163	2.163	2.163	2.163	2.163

¹ The dietary treatments were: NC = negative control; Sal = Salinomycin (50 g/ton); BMD = Bacitracin methylene disalicylate (60 g/ton); PC = positive control (commercial standard containing 50 g/ton Sal and 60 g/ton BMD); Three anise oil diets = containing 500, 1000, or 1500 ppm.

² Provided per kilogram of diet: Cu (copper sulfate), 7 mg; I (calcium iodate), 1 mg; Fe (ferrous sulfate•H₂O), 50 mg; manganese (manganese sulfate), 100 mg; Se (sodium selenite), 0.15 mg; Zn (zinc sulfate), 44 mg.

³ Provided per kilogram of diet: vitamin A, 8,002.78 IU; vitamin D₃, 3003.8 IU; vitamin E, 25 IU; menadione, 1.5 mg; vitamin B₁₂, 0.02 mg; biotin, 0.1 mg; folic acid, 1 mg; niacin, 50 mg; pantothenic acid, 15 mg; pyridoxine, 4 mg; riboflavin, 10 mg; thiamin, 3 mg.

⁴ Contains 750,000 mg/kg of choline.

⁵ Salinomycin sodium

⁶ Bacitracin methylene disalicylate

In Exp. 2, 350 day old male Ross 708 commercial broilers were randomly allotted to one of 10 treatments with seven replicates and five birds/replicate. The diets used in this experiment are presented in Table 5.2. Treatments were 1) Vegetable protein (Veg) containing no antibiotic, 2) Negative control (NC) containing no antibiotic, 3) Positive control (PC) containing BMD and Sal at levels described in Exp. 1, 4) Anise oil 1 (AO1, added at 500 ppm), 5) Anise oil 2 (AO2, added at 1000 ppm), 6) Anise oil 3 (AO3, added at 1500 ppm), treatment groups 7), 8), and 9) received NC from d 0-10, and then diets 5, 6, and 7, respectively, from d 10-18, and 10) NC unchallenged (NCU) containing no growth promotant and not challenged. All treatment diets except Veg had 5% fish meal.

Table 5.2 Composition of treatment diets used in Exp. 2 as feed basis.¹

Item	Veg	NC (5%)	PC	Anise oil (ppm)		
				500	1000	1500
Corn	49.215	50.766	50.142	50.766	50.766	50.766
Soybean meal, 48%	40.09	35.514	35.568	35.514	35.514	35.514
Fish meal	--	5.00	5.00	5.00	5.00	5.00
Soybean oil	5.691	4.962	5.164	4.962	4.962	4.962
Calcium phosphate	1.758	1.807	1.808	1.807	1.807	1.807
Limestone	1.059	0.588	0.403	0.588	0.588	0.588
Common salt	0.5	0.5	0.5	0.5	0.5	0.5
DL-Methionine	0.289	0.238	0.24	0.238	0.238	0.238
Mineral premix ²	0.25	0.25	0.25	0.25	0.25	0.25
Vitamin premix ³	0.25	0.25	0.25	0.25	0.25	0.25
L-Threonine	0.117	0.075	0.075	0.075	0.075	0.075
Choline chloride ⁴	0.05	0.05	0.05	0.05	0.05	0.05
L-Lysine	0.18					
Bacitracin ⁵	0.5		0.5			
Salinomycin ⁶	0.05		0.05			
Calculated composition						
MEn, kcal/kg	3200	3200	3200	3200	3200	3200
Ca, %	1.0	1.0	1.0	1.0	1.0	1.0
Non-Phytate P, %	0.5	0.5	0.5	0.5	0.5	0.5
Lys, %	1.29	1.27	1.26	1.27	1.27	1.27
Met, %	0.61	0.62	0.62	0.62	0.62	0.62
Met + Cys, %	0.93	0.93	0.93	0.93	0.93	0.93
Gly, % ⁶	0.98	1.12	1.12	1.12	1.12	1.12
Gly+Ser, % ⁷	2.16	2.31	2.30	2.31	2.31	2.31

¹The dietary treatments were: Veg = vegetable protein; NC = negative control; PC = positive control (commercial standard containing 50 g/ton Sal and 60 g/ton BMD); Three anise oil diets = containing 500, 1000, or 1500 ppm.

² Provided per kilogram of diet: Cu (copper sulfate), 7 mg; I (calcium iodate), 1 mg; Fe (ferrous sulfate•H₂O), 50 mg; manganese (manganese sulfate), 100 mg; Se (sodium selenite), 0.15 mg; Zn (zinc sulfate), 44 mg.

³ Provided per kilogram of diet: vitamin A, 8,002.78 IU; vitamin D₃, 3003.8 IU; vitamin E, 25 IU; menadione, 1.5 mg; vitamin B₁₂, 0.02 mg; biotin, 0.1 mg; folic acid, 1 mg; niacin, 50 mg; pantothenic acid, 15 mg; pyridoxine, 4 mg; riboflavin, 10 mg; thiamin, 3 mg.

⁴ Contains 750,000 mg/kg of choline.

⁵ Bacitracin Methylene Disalicylate

⁶ Salinomycin sodium.

⁷ Reported as total AA (%).

5.3.3 Challenge and pathological examination

In Exp. 1, the birds were orally inoculated with CIP (2×10^9 CFU/ml) on day 10. In Exp. 2, the birds were orally inoculated with a lower count of CIP (3×10^6 CFU/mL) on day 10 and 13. After the infection, the birds were checked twice a day for any sign of necrotic enteritis (i.e. huddling, diarrhea, depression, and mortality). Any bird that died at this time was necropsied to determine the cause of death. On d 18, all birds were euthanized by CO₂ inhalation. The intestinal tract was removed and examined for necrotic lesions. As suggested by Olkowski et al. (2006), most of the lesions caused by CIP were observed in the jejunum. Thus, the jejunum was scored for the presence of lesions. Scores used were: 0) normal and healthy jejunum, 1) multifocal, minimal to mild hyperemia, 2) multifocal hyperemia and hemorrhages, 3) moderate to severe, locally extensive, hyperemia and hemorrhages, and 4) disseminated severe hyperemia and hemorrhages. Similar to the study by Olkowski et al. (2006), in the specimens that had lesions, there was presence of a yellow or greenish, loosely adherent material lined over the intestinal mucosa.

5.3.4 Statistical analysis

Data analysis was conducted using the SAS software (SAS-Institute, 2011). The growth data were analyzed using the MIXED Procedure of SAS with treatment as fixed effect in a completely randomized design. The GLIMMIX Procedure for non-parametric (categorical) data was used to analyze the jejunum lesion scores with treatment as a fixed effect in a completely randomized design. The means separation was performed using the PDIFF function of SAS. Contrasts of AO and antibiotics against NC and PC were estimated. In Exp. 2, the contrast of Veg against NC also was estimated. The statements of significance were based on $P < 0.10$.

5.4 Results

From d 0-10, broilers fed AO diets (500, 1000, and 1500 ppm) consumed 11% less feed than broilers fed NC ($P < 0.01$). Broilers fed the other treatment diets consumed similar amounts of feed as broilers fed NC ($P > 0.10$). Broilers fed AO diets (500, 1000, and 1500 ppm) also had lower (11.4, 16.5, and 13.9 %; respectively) ADG than broilers fed PC or NC ($P < 0.05$). Feed efficiency was not affected by any of the dietary treatments during this period. After the bacterial challenge (d 10-18), ADFI was not affected by dietary treatment. However, broilers fed Sal had lower ADG ($P < 0.01$) than broilers fed NC. Feed efficiency also was lower in broilers fed Sal compared to broilers fed NC ($P < 0.01$). Overall (d 0 – 18), ADFI was reduced in broilers fed AO ($P < 0.05$) compared to broilers fed NC or PC. Broilers fed AO or Sal had lower ADG ($P < 0.02$) compared to broilers fed NC or PC. However, only broilers fed Sal had lower G:F ($P < 0.01$), than broilers fed NC or PC. The challenge with CIP did not affect ADFI, ADG or G:F as indicated by the comparison to broilers fed NC and NCU.

There was no mortality during this experiment related to the challenge with CIP. The birds displayed lower activity after the challenge, but feed intake was not reduced and the broilers were able to recover. During the post-mortem examination, no extreme signs of necrotic enteritis were observed (i.e. score 4) and most of the observed cases were less than a score of 3. Broilers fed AO or UNC treatment diets had lower jejunum LS ($P < 0.05$) compared to broilers fed NC or PC. Compared to broilers fed NC, broilers fed Sal or BMD also had lower LS ($P < 0.10$). Growth performance and LS results from Exp. 1 are presented in Table 5.3.

Table 5.3 The effect of anise oil on growth performance and jejunum lesion scores of broilers during a challenge with *Clostridium perfringens*¹.

Item/Day	BW, g		ADG, g			ADFI, g			G:F, g			Lesions ² scores
	10	18	0 – 10	10 – 18	0 – 18	0 – 10	10 – 18	0 – 18	0 – 10	10 – 18	0 – 18	
Treatment ³												
NC	267	617	22.5	50.3	34.2	24.1	61.8	39.5	0.94	0.80	0.87	1.3
Sal	280	600	23.7	42.7	31.9	25.4	59.2	39.9	0.93	0.72	0.80	1.1
BMD	263	614	22.0	46.8	34.0	24.1	59.4	38.9	0.92	0.78	0.85	1.0
PC	269	639	22.6	49.4	34.1	23.2	61.7	39.7	0.97	0.80	0.86	1.2
AO1	256	607	21.4	46.8	32.3	22.3	58.0	37.6	0.96	0.81	0.86	1.0
AO2	240	575	20.3	44.7	31.2	21.6	58.0	36.8	0.94	0.77	0.84	0.9
AO3	248	616	21.2	49.1	32.8	22.0	61.3	38.3	0.95	0.80	0.85	0.9
NCU	279	639	23.6	48.1	34.1	24.9	61.2	40.3	0.95	0.79	0.85	0.8
SEM	9.3	24.9	0.85	1.87	0.99	0.93	1.70	0.84	0.027	0.029	0.021	0.12
P-Value												
Treatment	0.0006	0.23	0.002	0.09	0.01	0.0006	0.53	0.06	0.56	0.04	0.04	0.05
NC vs BMD	0.62	0.89	0.58	0.20	0.84	0.99	0.32	0.62	0.46	0.54	0.43	0.10
NC vs Sal	0.18	0.50	0.15	0.007	0.02	0.15	0.30	0.74	0.84	0.005	0.001	0.09
NC vs PC	0.88	0.38	0.87	0.74	0.90	0.36	0.97	0.87	0.18	0.93	0.59	0.49
NC vs AO	0.01	0.40	0.03	0.13	0.01	0.008	0.18	0.05	0.53	0.72	0.34	0.006
AO vs PC	0.01	0.11	0.02	0.24	0.01	0.12	0.19	0.03	0.32	0.80	0.74	0.05
NC vs UnC	0.23	0.38	0.19	0.41	0.88	0.39	0.80	0.50	0.65	0.56	0.27	0.004

¹ The values are the means of 9 replicates with 5 birds per replicate. BW = body weight, ADG = average daily gain, ADFI = average daily feed intake, G:F = gain/feed.

² Scores: 0) normal and healthy jejunum, 1) multifocal, minimal to mild hyperemia; 2) multifocal hyperemia and hemorrhages; 3) moderate to severe, locally extensive, hyperemia and hemorrhages; and 4) disseminated severe hyperemia and hemorrhages

³ NC = Negative control, Sal = Salinomycin sodium, BMD = Bacitracin Methylene Disalicylate, PC = Positive control, AO1 = Anise oil 500 ppm, AO2 = Anise oil 1000 ppm, AO3 = Anise oil 1500 ppm, and NCU = Negative control unchallenged.

Experiment 2 was designed to determine if feeding animal protein increased the severity of necrotic enteritis lesions and to determine if feeding AO only after the challenge with CIP would be effective in reducing the lesions observed in Exp. 1. During d 0 – 10, ADFI and ADG were not affected by treatment. However, broilers fed Veg had lower G:F compared to broilers fed fish meal diets ($P < 0.03$). In this experiment, feeding AO from d 0 – 10 did not affect ADFI or ADG. After the challenge (d 10 – 18), broilers fed Veg consumed less feed ($P < 0.08$) and grew slower ($P > 0.09$) than broilers fed the other treatment diets. Feed efficiency was similar among treatments. Overall (d 0 – 18), broilers fed Veg had lower feed intake ($P < 0.10$) and lower ADG ($P < 0.03$) but similar G:F to broilers fed NC.

The challenge with CIP on d 10 and d 13 did not affect ADFI, ADG or G:F as indicated by the comparison to broilers fed NC and NCU. There was no mortality related to the challenge with CIP. The birds displayed lower activity after the challenges, but did not reduce feed intake and they were able to recover. During the post-mortem examination, no extreme signs of necrotic enteritis were observed (i.e. score 4) and most of the observed cases were lower than score 2.

Feeding AO after the challenge was as effective as feeding it throughout the evaluation period ($P > 0.10$). Broilers fed AO had lower lesion scores ($P < 0.10$) compared to broilers fed PC or NC. Feeding fish meal to broilers did not affect LS after the CIP challenge ($P > 0.10$) compared to broilers fed Veg. The results of treatment effect on growth, feed intake, feed efficiency, and jejunum LS are reported in Table 5.4.

5.5 Discussion

In this study, feeding 500 to 1500 ppm AO to broilers reduced feed intake by 10% from d 0 – 10, and 5% from d 0 – 18. Plant oils containing terpenes and terpenoids (Windisch et al., 2008; Brenes and Roura, 2010) have similar effects as AO on feed intake. Feeding 150 or 300

Table 5.4 The effect of vegetable protein, fish meal, and anise oil on growth performance and jejunum lesion scores of broilers during a challenge with *Clostridium perfringens*¹.

Item/Day	BW ² , g		ADG			ADFI			G:F			Lesions ² scores
	10	18	0 – 10	10 – 18	0 – 18	0 – 10	10 – 18	0 – 18	0 – 10	10 – 18	0 – 18	
Treatment ³												
Veg	217	564	18.0	43.4	29.3	19.8	57.3	36.1	0.91	0.76	0.81	0.65
NC	231	606	19.5	46.9	31.7	20.1	61.9	38.2	0.97	0.76	0.83	0.69
PC	218	598	18.1	47.6	31.2	19.0	59.0	36.8	0.95	0.80	0.84	0.64
AO1	218	585	18.1	45.9	30.5	18.7	59.1	37.0	0.97	0.78	0.82	0.40
AO2	235	599	19.9	45.5	31.3	20.4	60.3	38.5	0.95	0.75	0.81	0.65
AO3	233	613	19.7	47.4	32	20.0	63.2	38.7	0.98	0.75	0.83	0.40
NC+AO1	218	591	18.2	46.6	30.8	18.7	61.2	37.3	0.93	0.76	0.83	0.36
NC+AO2	230	586	19.3	44.6	30.5	19.9	64.8	38.6	0.97	0.72	0.79	0.38
NC+AO3	220	575	18.3	44.3	29.9	20.7	59.3	37.9	0.91	0.75	0.79	0.44
NCU	234	588	19.7	44.1	30.6	19.7	60.0	37.3	0.96	0.74	0.82	0.59
SEM	8.19	16.0	0.77	1.57	0.88	0.83	1.98	0.95	0.022	0.023	0.021	0.139
<i>P</i> -Val												
Treatment	0.31	0.39	0.30	0.49	0.40	0.66	0.23	0.52	0.16	0.37	0.55	0.07
Veg vs NC	0.14	0.03	0.14	0.09	0.03	0.83	0.08	0.10	0.03	0.92	0.52	0.82
NC vs PC	0.17	0.67	0.17	0.71	0.66	0.35	0.27	0.26	0.49	0.10	0.44	0.76
NC+AO vs AO	0.30	0.18	0.30	0.37	0.18	0.93	0.54	0.84	0.13	0.31	0.23	0.28
NC vs AO	0.72	0.63	0.72	0.71	0.63	0.77	0.98	0.77	0.67	0.41	0.69	0.01
PC vs AO	0.19	0.96	0.19	0.43	0.96	0.41	0.20	0.29	0.68	0.11	0.20	0.07
NC vs UNC	0.80	0.32	0.06	0.19	0.30	0.80	0.46	0.46	0.75	0.45	0.84	0.48

¹ The values are the means of 7 replicates with 6 birds per replicate.

² Scores: 0) for normal and healthy jejunum, 1) multifocal, minimal to mild hyperemia; 2) multifocal hyperemia and hemorrhages; 3) moderate to severe, locally extensive, hyperemia and hemorrhages; and 4) disseminated severe hyperemia and hemorrhages

³ Veg = Protein from vegetable sources, NC = Negative control, PC = Positive control, AO1 = Anise oil 500 ppm, AO2 = Anise oil 1000 ppm, AO3 = Anise oil 1500 ppm, NC+AO1, NC+AO2, and NC+AO3 were fed NC diet on d 0 – 10 d and anise oil diets on d 10 – 18, and NCU = Negative control unchallenged.

BW = body weight, ADG = average daily gain, ADFI = average daily feed intake, G:F = gain/feed.

ppm of oregano, rosemary, cinnamon, and thyme extracts reduced broiler feed intake by 2 and 8% (Basmacioglu et al., 2004; Cabuk et al., 2006). Feeding dried spices (garlic and oregano) also reduced feed intake of broilers and turkeys (Bampidis et al., 2005; Sarica et al., 2005). In contrast, AO had no effect on broiler feed intake (Ciftci et al., 2005; Simsek et al., 2007) when 400 ppm was fed. Mixing AO with other plant oils had a similar effect. Amad et al. (2011) fed incremental levels of a thyme and AO mixture with levels up to 1500 ppm AO, without affecting feed intake of broilers. In agreement, in our second experiment, feeding 1500 ppm AO, had no effect on feed intake. Feeding AO to older broilers (d 10 – 18) did not affect feed intake. These results indicate that feeding phytonutrients is not consistent in regard to feed intake. However, feeding AO had a limited effect on feed intake.

The literature indicates that feeding antibiotics as growth promotants to broilers has no influence on feed intake (Ciftci et al., 2005; Ertas et al., 2005; Simsek et al., 2007). The results of our study agree with the literature since feeding BMD alone, or feeding BMD and Sal together did not affect feed intake. However, feeding Sal did reduce feed intake of broilers. Similar results were reported by Bozkurt et al. (2014) in broilers older than 15 d. In contrast, Wheelhouse et al. (1985) reported an increase in feed intake improvement when Sal was fed to broilers. Those reports indicate that the effect of Sal on broiler feed intake is inconsistent.

Changes in feed intake may have a direct effect on growth rate of broilers. When feeding phytonutrients, growth rate is reduced, increased, or unchanged (Windisch et al., 2008; Brenes and Roura, 2010). Several authors reported no effect on growth rate of broilers (< 15 d old) fed AO (Alçiçek et al., 2004b; Ciftci et al., 2005; Ertas et al., 2005; Simsek et al., 2007; Amad et al., 2011). However, the same researchers reported that older broilers (> 15 d old) fed AO had greater growth rate compared to broilers not fed AO or antibiotic (> 15 d). In our study, feeding

broilers AO reduced their growth rate in Exp. 1 and had no effect in Exp. 2, compared to broilers fed NC or PC. Age of broiler (d 0 – 18 vs d 10 – 18) did not influence growth rate in response to feeding AO.

Modest improvements in feed efficiency were reported when broilers were fed phytogenics (Botsoglou et al., 2002; Alçiçek et al., 2004a, b; Basmacioglu et al., 2004; Hernández et al., 2004; Lee et al., 2004a; Lee et al., 2004b; Cabuk et al., 2006). In general, these observations were reported from field studies with 15 – 52 d old broilers (Alçiçek et al., 2004b; Ciftci et al., 2005; Ertas et al., 2005; Simsek et al., 2007; Amad et al., 2011). In our study there was no effect on feed efficiency of broilers fed AO. It is suggested that feed efficiency is improved by phytogenics due to increased digestibility of nutrients (Amad et al., 2011). Additionally, phytogenics may improve feed efficiency by directly affecting pathogenic bacteria in the intestine of broilers (Mitsch et al., 2004).

In studies challenging broilers with *C. perfringens*, it is suggested that a reduction in the pathogen population may reduce the number and severity of lesions in the intestinal tract. Lanckriet et al. (2010) and Keyburn et al. (2006) reported that necrotic lesions are more frequently observed in the duodenum and jejunum. In our study we found more accurate lesions in the jejunum. Most of the macroscopic lesions detected were in the range of 0 (normal) to 2 (mild) on a scale from 0 to 4, as described by others (Dahiya et al., 2005; Jia et al., 2009; Lanckriet et al., 2010). No necrosis typical of field cases (Prescott et al., 1978), characterized by focal necrosis and ulcerations, was found with the challenge in broilers. As expected, unchallenged birds had lesions since they were placed in the same batteries with challenged birds. Bacitracin methylene disalicylate, the antibiotic of reference, reduced the severity of lesions in broilers. As reported, BMD is a potent antibiotic used in the prevention and treatment

of necrotic enteritis and its causal agent *C. perfringens* (Prescott et al., 1978; Stutz et al., 1983; Samanta et al., 2010). In our study, Sal was not effective in reducing jejunum lesions in challenged birds. However, Sal is an ionophore anticoccidial with antibacterial activity against *C. perfringens* in-vitro (Kondo, 1988; Devriese et al., 1993; Martel et al., 2004). This has been confirmed in previous in-vitro research in our laboratory (Chapter 4). There was not an additive effect in controlling lesions when BMD and Sal were both added to the diet. Although, commercial diets normally contain both feed additives. Anise oil added to the diets was as effective as antibiotics in reducing the lesions in the jejunum related to the *C. perfringens* challenge. Timbermont et al. (2010) also reported the benefit in prevention of necrotic lesions when feeding phytogenic oil to broilers challenged with *C. perfringens*. This response may be explained by a direct antimicrobial effect of phytogenic oils on *C. perfringens* growth in the intestine (Mitsch et al., 2004; Cross et al., 2007). Previous in-vitro studies in our laboratory also demonstrated that AO had an antimicrobial effect against *C. perfringens* (Chapter 4). Phytochemicals also may reduce bacterial adhesion to the intestinal lumen (Olkowski et al., 2006), which should prevent intestinal lesions. Lesions were lower and less frequent in our second experiment, which is probably related to the lower CFU/ml used at the two challenge times. Using lower *C. perfringens* concentration during the challenge requires frequent exposure of the birds to reproduce a mild necrotic enteritis model (Olkowski et al., 2006; Timbermont et al., 2010). These findings indicate that a reduction in necrotic lesions is not a good indicator of a reduction in bacterial utilization of nutrients. Under commercial conditions (longer time periods), a healthy intestine should improve nutrient absorption and nutrient digestibility.

Dietary inclusion of meat meal, feather meal, or fish meal increases ileum and cecum *C. perfringens* populations (Wilkie et al., 2005; Olkowski et al., 2006). Both methionine and glycine

concentrations are increased when fish meal is added to the diets (Drew et al., 2004; Dahiya et al., 2005; Wilkie et al., 2005; Olkowski et al., 2006). These amino acids increase CIP population in the intestine of broilers (Dahiya et al., 2005; Dahiya et al., 2007). However, their diets contained twice the recommended levels of methionine and glycine. In our study, the addition of 5% fish meal increased dietary total Gly by 14% and total Gly + Cys by 6%. Fish meal did not increase the severity of lesions in the jejunum, and had a positive influence on feed intake, growth rate, and feed efficiency compared to broilers fed the corn-soybean meal diet. Our results indicate that at practical inclusion levels, fish meal does not increase the risk of necrotic enteritis development.

In summary, our results demonstrate that during an in-vivo challenge with CIP, AO fed to broilers reduced the severity of the lesions in the jejunum related to necrotic enteritis. Anise oil at the levels evaluated were safe to feed to broilers. The reduction in feed intake observed in Exp. 1 could be related to other factors. Fish meal added to the diet of broilers had no effect on the severity of lesions caused by the CIP. This suggests that in practice, low levels of animal protein do not affect the development of necrotic enteritis and can improve growth performance. The model used in this study was effective since it produced sub-clinical necrotic signs (lesions). For future research, accounting for the amount of alpha-toxins produced by the inoculum can help in developing reproducible necrotic models.

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CHAPTER 6: THE EFFECT OF ANISE OIL ON BROILER GROWTH PERFORMANCE, BREAST MEAT YIELD, AND BREAST FILLET SENSORY CHARACTERISTICS

6.1 Abstract

An experiment was conducted to compare the effect of anise oil (AO) and antibiotics on growth performance, breast yield, and meat sensory characteristics of broilers. Ross 708 males were randomly allotted to treatments on d 0, and the experiment lasted 42 d. The diets were a corn-soybean meal based, 3-phase feeding program similar to a commercial poultry industry program. Three diets were fed: 1) negative control (NC) with no antibiotics or anticoccidials added, 2) positive control (PC) with antibiotics and anticoccidials added, 3) the NC supplemented with 1000 ppm of anise oil (AO). Also, a group of broilers was fed AO during the starter phase, then NC during the grower and finisher phases; and another group was fed AO during the starter and grower phases, then NC during the finisher phase. Each treatment was replicated 6 times with 50 males per replicate. Daily gain, feed intake, and gain:feed were determined. On day 42, two birds per replicate were randomly selected for processing and collection of breast fillets. The breast fillets were stored at -28°C until analyzed to determine anethole. Solid phase microfiber extraction (SPME) and gas chromatography-mass spectrometry (GC-MS) were used for analysis of AO volatiles. There was no treatment effect on ADFI. From d 0 – 10, NC fed broilers had lower ADG than broilers fed PC or AO ($P < 0.10$). The NC fed broilers had the lowest G:F and AO fed broilers had better G:F than PC fed broilers ($P < 0.10$). From d 10 – 24, NC fed broilers had lower ADG than broilers fed PC or AO. Broilers fed AO in the starter and grower phases and then NC in the finisher phase had greater ADFI, but similar G:F to broilers fed NC ($P < 0.10$), which was lower than PC or AO fed broilers. During d 25 – 42, there were no treatment effects. Broilers fed NC during the overall period (d 0 – 42) had lower ADG and G:F ($P < 0.10$) than broilers fed PC or broilers fed AO during the starter

phase only. Breast yield was similar among treatments. Anethole volatiles were undetectable in breast fillets from broilers fed any of the dietary treatments. Anise oil can be used as an alternative to antibiotics without negatively affecting feed intake, performance, or breast fillet characteristics of broilers.

6.2 Introduction

Phytogenics, also called phytochemicals (plant derivatives), have been proposed as alternatives to antibiotic growth promotants in livestock feeds (Windisch et al., 2008; Brenes and Roura, 2010). Several herbal extracts containing mainly terpenes and terpenoids have antibiotic effects against a wide range of pathogens in-vitro (Deans and Ritchie, 1987; Hammer et al., 1999; Chang et al., 2001; Elgayyar et al., 2001; Friedman et al., 2002; Burt and Reinders, 2003). However, high concentrations are required to obtain antibiotic benefits (Oussalah et al., 2007). Feeding phytochemicals to broilers, in several cases, has reduced feed intake, but has not affected their growth rate or feed efficiency (Botsoglou et al., 2002; Alçiçek et al., 2004; Denli et al., 2004; Cross et al., 2007; Bozkurt et al., 2009; Brenes and Roura, 2010).

Anise oil is the solvent extraction from aniseed and has approximately 90% trans-anethole. Anise oil is used as a flavoring substance in bakery products, candies, ice cream, chewing gum, and alcoholic beverages (Newberne et al., 1999). Anethole also is “generally recognized as safe” (GRAS) by the FEMA Expert Panel (Newberne et al., 1999).

In previous studies conducted in our laboratory, we found that broilers could tolerate a maximum of 2500 ppm of dietary AO. Also, feeding AO reduced *Clostridium perfringens* (CIP) growth in-vitro (*Unpublished data*). *Clostridium perfringens* is one of the main agents of necrotic enteritis in broilers. The results of feeding anise oil to CIP challenged broilers indicate that AO is effective in reducing jejunum lesions related to the bacteria. Researchers have reported that

feeding AO to broilers has increased growth performance, feed efficiency, and meat yield, without affecting sensory characteristics (Ciftci et al., 2005; Ertas et al., 2005; Simsek et al., 2007; Amad et al., 2011). However, none of this research was conducted under US industry conditions. Therefore, the objectives of this study were to determine the effect of AO on broiler growth performance, breast yield, and breast fillet sensory characteristics; as well as to determine an AO withdrawal time if changes in sensory characteristics were detected.

6.3 Materials and methods

The methods used in this experiment were approved by the Louisiana State University Agricultural Center Animal Care and Use Committee.

6.3.1 Animals and treatments

Fifteen hundred broilers (Ross x Ross 708) were obtained from a commercial hatchery on d 0 post-hatching and sexed upon arrival at the LSU Agricultural Center Poultry Research Farm. Male broilers were randomly allotted to five treatments. Each treatment was replicated six times and there were 50 broilers per replicate. A three-phase feeding program equivalent to industry standard was used. The starting phase was fed from 0 – 10 d, the growing phase from 11 – 25 d, and the finishing phase from 25 – 42 d. The treatment diets were formulated to provide 1.30% total Lys and 3,025 kcal/kg ME for the starting phase, 1.10% total Lys and 3,150 kcal/kg ME for the growing phase, and 0.97% total Lys and 3,200 kcal/kg ME for the finishing phase. All other nutrients were formulated to meet the recommendations for Ross 708 broilers (Aviagen, Huntsville, AL). The basal diets used in this experiment are presented in Table 6.1.

The dietary treatments were: 1) negative control (NC) with no supplemental antibiotics or anticoccidials, 2) positive control (PC) supplemented with 50 g/ton Bacitracin Methylene Disalicylate and 60 g/ton Salinomycin sodium, and 3) a diet supplemented with 1000 ppm anise

oil (AO). Two additional treatments were: 4) AO fed during the starter phase, and NC fed in the grower and finisher phases, and 5) AO fed during the starter and grower phases, and NC fed during the finisher phase.

Table 6.1. Basal diets for the starter, grower, and finisher phases.

Ingredient, %	Starter	Grower	Finisher
Corn	51.94	55.14	62.27
Soy bean meal, 48%	39.55	35.80	29.60
Poultry fat	3.3	4.66	4.24
Common salt (NaCl)	0.50	0.50	0.50
DL-Methionine ¹	0.29	0.23	0.20
Biolys ¹	0.20	0.10	0.05
L-Threonine ¹	0.12	0.05	0.02
Mineral premix ²	0.25	0.25	0.25
Vitamin premix ³	0.25	0.25	0.25
BMD-3 ⁴	0.50	0.50	--
Biocox ⁵	0.05	0.05	--
Choline chloride ⁶	0.05	0.05	0.05
Ethoxyquin	0.05	0.05	0.05
Monocalcium phosphate	1.75	1.54	1.44
Limestone	1.20	0.93	1.08
Calculated values			
ME _n , kcal/kg	3025	3150	3200
nPP, %	0.50	0.45	0.42
Ca, %	1.05	0.90	0.85
Lys, %	1.30	1.10	0.97

¹ DL-Methionine (99%), L-Threonine (99%), and Biolys (L-Lys 50.6%) were provided by Evonik Corporation, Kennesaw, GA.

² Provided per kilogram of diet: Cu (copper sulfate), 7 mg; I (calcium iodate), 1 mg; Fe (ferrous sulfate•H₂O), 50 mg; manganese (manganese sulfate), 100 mg; Se (sodium selenite), 0.15 mg; Zn (zinc sulfate), 44 mg.

³ Provided per kilogram of diet: vitamin A, 8,002.78 IU; vitamin D₃, 3003.8 IU; vitamin E, 25 IU; menadione, 1.5 mg; vitamin B₁₂, 0.02 mg; biotin, 0.1 mg; folic acid, 1 mg; niacin, 50 mg; pantothenic acid, 15 mg; pyridoxine, 4 mg; riboflavin, 10 mg; thiamin, 3 mg.

⁴ Bacitracin methylene disalicylate.

⁵ Salinomycin sodium.

⁶ Contains 750,000 mg/kg of choline.

The experiment was conducted in a tunnel-ventilated house with concrete floored pens. The house consisted of 30 pens which are 1.52 x 3.05 m. Broilers were reared on built-up litter that was topdressed with approximately 2.5 cm of new wood shavings. The broilers had ad-libitum access to water by automatic nipple waterers, with 9 nipples per pen. The feed was provided by feed trays from 0 to 5 d and then by two hanging tube feeders per pen for the rest of the trial. The house was maintained between 29.4 and 32.2 °C for the first week and the temperature was decreased 2.8 °C every week until the house was between 23.9 °C and 26.7 °C, if the weather allowed. One infrared heat lamp per pen was used for the first week. The lighting was provided by incandescent lamps and was similar to a commercial lighting program: 24 h from 0 to 4 d, 20 h from 5 to 10 d, 18 h from 11-18 d, and 16 h from 19-42 d. The light intensity was 10.8 to 14 lx from 0 – 10 d post-hatching and then 2.2 – 2.3 lx until the end of the experiment.

At the end of each dietary phase and at the end of the experiment, the broilers and feed were weighed for determination of ADG, ADFI, and feed efficiency (gain:feed). On d 42, two birds per replicate were randomly selected for further processing and collection of the breast fillet (*pectoralis major*). The breasts were stored at -28 °C for six months until volatile components were analyzed.

6.3.2 Volatile component analysis

For volatile component analysis, the standards of trans-anethole (Sigma-Aldrich, St. Louis, MO), with a verified purity of 99.8%, and Hexane HPLC grade were used. Individual stock standard solutions of trans-anethole were prepared by weight in hexane and stored until use. As recommended for organic compounds, solid phase microfiber extraction (SPME) was used. A SPME 85 µm fiber with polydimethylsiloxane was used (Sigma-Aldrich, St. Louis,

MO). Before use, the fiber was conditioned in the GC injection port under helium flow at 250 °C. Thirty grams of ground breast fillet sample was placed in a 250 ml round bottom flask and mixed with 1 ml aliquot of standard solution. The flask was placed in a 60 °C water bath and was capped using a rubber cap. The fiber was inserted through the rubber cap to reach the neck of the flask. Then, the fiber was exposed to the sample headspace to perform volatile extraction for 30 min. After extraction the fiber was retracted to perform gas chromatography and mass spectrometry (GC-MS) analysis. The fiber was conditioned for 5 minutes at 250 °C before the next extraction.

The GC-MS system consisted of a Varian CP3800 GC and Saturn 2000 MS (Varian Inc., Walnut Creek, CA) with a SPB-5-fused silica capillary column (60 m \times 0.25 mm \times 0.25 mm film thickness; Supelco Inc., Bellefonte, PA). Helium was the gas carrier at a column linear flow rate of 1.5 ml/min. The extracted sample was injected at 250 °C in a split-less mode. The initial temperature of the GC oven was set to 50 °C and held for 5 min, then increased to 250 °C at a rate of 2 °C/min and maintained for 20 min. The volatile compound was identified by comparison of the mass spectra and retention time of internal standard to the trans-anethol standard and the National Institute Standards and Technology database library. The concentration of the volatile compound was calculated using the calibration curve of the relative response factors of the standard at three different concentrations.

6.3.3 Statistical analysis

Analysis of variance procedures for a completely randomized design using the MIXED procedures of SAS were used for data analysis. Replicate pen of broilers was the experimental unit. The model response contained treatment as the main effect. Treatment means were

separated using preplanned, pairwise comparisons (PDIF option of SAS). Means were considered different with $P < 0.10$.

6.4 Results

Results of broiler growth performance and breast yield are reported in Table 6.2.

Table 6.2 The effect of anise oil on growth performance and breast yield of 0 to 42 day old broilers.¹

Item	Treatments ²					SEM	P-Val
	NC	PC	Anise	Anise F2	Anise F3		
0 – 10 d							
ADG ³ , g	17.3 ^c	18.0 ^b	18.4 ^{ab}	18.5 ^{ab}	18.8 ^a	0.299	0.002
ADFI, g	21.3	21.3	21.0	21.1	21.2	0.295	0.91
G:F, g/g	0.81 ^d	0.85 ^c	0.86 ^b	0.88 ^{ab}	0.89 ^a	0.006	0.0001
10 – 24 d							
ADG, g	61.2 ^b	62.1 ^{ab}	63.1 ^{ab}	63.1 ^{ab}	63.5 ^a	0.47	0.06
ADFI, g	88.3 ^b	87.6 ^b	88.6 ^b	89.3 ^b	91.4 ^a	0.65	0.006
G:F, g/g	0.69 ^b	0.71 ^a	0.71 ^a	0.71 ^a	0.69 ^b	0.003	0.01
25 – 42 d							
ADG, g	101.4	102.1	101.3	100.7	102.5	0.635	0.27
ADFI, g	160.2	158.9	163.8	159.5	161.1	1.55	0.16
G:F, g/g	0.63	0.64	0.62	0.63	0.64	0.005	0.19
0 – 42 d							
ADG, g	67.3 ^b	68.4 ^a	68.2 ^{ab}	68.3 ^{ab}	69.1 ^a	0.401	0.05
ADFI, g	102.2	102.0	104.0	102.7	104.0	0.75	0.13
G:F, g/g	0.65 ^b	0.67 ^a	0.66 ^{ab}	0.66 ^{ab}	0.67 ^a	0.003	0.07
Final Wt, kg	2.89 ^b	2.92 ^{ab}	2.94 ^{ab}	2.93 ^{ab}	2.98 ^a	0.035	0.07
Breast yield ⁴ , %	17.7	18.8	18.7	18.5	18.5	0.05	0.39

¹The values are the means of 6 replicates with 50 birds/replicate for growth performance, and the means of 6 replicates of 2 birds randomly selected from each replicate for breast yield.

² NC = negative control; PC = positive control (antibiotics added); Anise = anise fed all phases, Anise F2, anise fed only starter phase; Anise F3 = anise fed starter and grower phase.

³ ADG = average daily gain; ADFI = average daily feed intake; G:F = gain/feed ratio.

⁴ Calculated as breast weight/total body weight.

^{abc} Values with different superscripts within the same row differ at $P < 0.10$.

During the starter phase (d 0 – 10), ADFI was similar among all the treatments. However, NC fed broilers had lower ADG than broilers fed PC or AO ($P < 0.10$). The NC fed broilers had the poorest G:F and AO fed broilers had better G:F than broilers fed PC ($P < 0.10$). From d 10 –

24, broilers that were switched to NC during this phase had greater ADFI than broilers fed the other treatments. They also had greater ADG than broilers fed NC ($P < 0.10$). However, broilers fed this treatment had similar G:F to broilers fed NC, which was lower than for broilers fed PC or AO ($P < 0.10$). During 24 – 42 d, there were no dietary treatment effects on ADFI, ADG, or G:F. Overall, there was no difference in ADFI among broilers fed any of the dietary treatments. The broilers fed NC for the entire trial had lower ADG ($P < 0.10$) and poorer G:F ($P < 0.10$) than broilers fed PC or AO during the starter phase only. Breast yield percentage was similar among all broilers fed any of the dietary treatments ($P > 0.10$).

The results of volatile compound analysis are reported in Table 6.3. and Figure 6.1.

Table 6.3 Test conditions for trans-anethole standard, added standard to raw breast fillets, and breast fillet samples.

Sample	Standard ¹		Standard + chicken ²		
	Anethole	Abundance ³	Anethole	Abundance ³	Recover, % ⁴
1	0.202	97578	0.202	83564	85.6
2	0.4	192427	0.576	233817	85.3
3	1.27	904421	1.1	711130	90.9
4	10.8	2333033	10.4	2246170	96.0
R-Sq		0.99		0.96	
NC ⁵				ND	
PC				ND	
Anise				ND	
Anise F2				ND	
Anise F3				ND	

¹ Standard anethole concentration in ppm analyzed. Sample (1 – 4) were incremental concentrations of standard anethole + hexane.

² Standard anethole concentration in ppm added and mixed with raw chicken breast filled, extracted and analyzed.

³ Abundance of gas-phase ions m/z (mass reflectance).

⁴ Recover percentage as abundance in Standard + chicken / Standard %.

⁵ NC = negative control; PC = positive control; Anise = anise diet fed during all phases; Anise F2 = Anise oil fed during starter phase only; Anise F3 = anise oil fed during starter and grower phases.

For volatile compound analysis, at least 85 % of trans-anethole was recovered when standard concentrations ranging from 0.2 to 10 ppm were added to raw chicken breast. The

standard curves generated with standard concentrations alone or added to chicken breast fillet have regression coefficients of 0.99 and 0.96, respectively. In the breast fillet from broilers fed NC, PC, or AO for all phases, anethole was not detected at any of the levels described for standards.

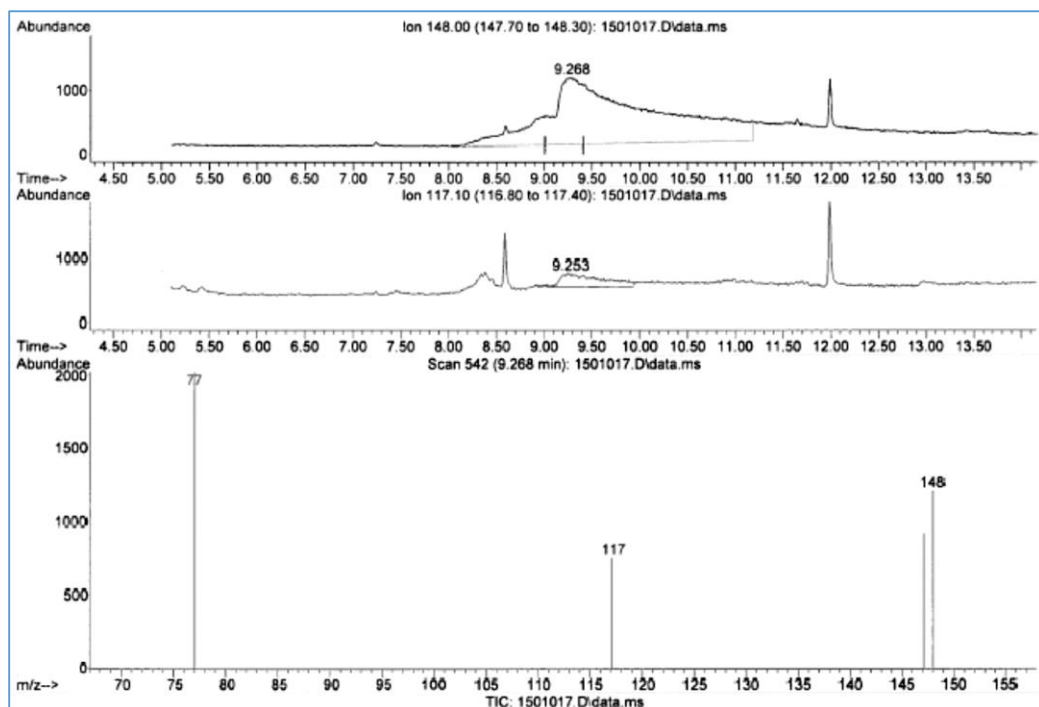


Figure 6.1 GC-MS chromatogram of standard trans-anethole (0.20 ppm) added to chicken breast fillet.

6.5 Discussion

In general, feeding 1000 ppm AO had no effect on broiler feed intake. These results are in agreement with other authors that fed AO added at 400 ppm to broilers diets (Ciftci et al., 2005; Ertas et al., 2005; Simsek et al., 2007; Amad et al., 2011). Ertas et al. (2005) reported an increase in feed intake in broilers fed AO; however, the AO in their experiment was mixed with clove and thyme oil. Ciftci et al. (2005) and Simsek et al. (2007) reported that broilers (week 2 – to market age) fed 200 ppm AO had greater ADG than broilers fed antibiotics. Similar results were reported when broilers were fed a mix of anise, clove, and oregano at 400 ppm (Ertas et al.,

2005). In our study, feeding an antibiotic and anticoccidial (PC) increased ADG compared to broilers fed NC during the period of evaluation. Feeding AO at 1000 ppm increased ADG compared to broilers fed NC in the starter phase (0 – 10 d). Feeding AO or antibiotics had a similar effect on broiler ADG during the grower and finisher phases, as well as for the overall period of study. Some authors have reported that feed efficiency is improved when feeding broilers 200 ppm AO compared to feeding PC (Ciftci et al., 2005; Simsek et al., 2007). The same researchers found that broilers fed low (100 ppm) or high (400 ppm) AO levels had similar feed efficiency to broilers fed PC. Broilers fed oil mixtures of anise with clove and oregano, or with thyme, also had improved feed efficiency (Ertas et al., 2005; Amad et al., 2011). Our results demonstrated that AO fed broilers were more efficient than broilers fed NC during the starter phase. Anise, and other herbal extracts, may improve broiler performance by affecting micro flora in the digestive tract and by stimulating enzyme production. Feeding a mix of thyme and star anise to broilers has increased nutrient apparent digestibility (Amad et al., 2011). In our laboratory, AO reduced growth of CIP in-vitro, and reduced necrotic lesions in broilers during a CIP challenge (*Unpublished data*).

Although recent studies have shown mixed results, it is generally accepted that feeding antibiotics as growth promotants increases carcass and breast yield (Belay and Teeter, 1996). Similar results are reported for phytochemicals fed to livestock. Bampidis et al. (2005) reported no effect of oregano on carcass weight and carcass yield when fed to turkeys. Similarly, Jamroz et al. (2003) reported that a blend of plant oils had no effect on broiler carcass weight, carcass yield, or breast yield. Rosemary fed to broilers reduced carcass weight, but dressing percent was similar to broilers fed the positive control diet (Loetscher et al., 2013). Simsek et al. (2007) reported that AO increased hot and cold carcass weight compared to broilers fed antibiotics, but

breast yield was similar among all broilers . Our results demonstrated that feeding antibiotics or AO had no effect on breast yield.

Off-odor in meats is a very important trait that affects consumer demand. Many changes in odor are related to changes in volatile compounds (Ahn et al., 2013). Anethole as well as other phytochemicals are mainly volatile compounds with strong and distinctive aromas (Newberne et al., 1999). Chaignaud et al. (2014) reported that a human panel was able to detect 167 OU (odor units) of anethole. Using the formula developed by Segura and Feddes (2005), this converts to 3.7 ppm. There is limited information regarding the presence of anethole or related metabolites in commercial poultry cuts after feeding AO to broilers. Based on feed intake and BW, broilers were fed 50 mg/kg/d of anethole until the day of breast collection. At this level of consumption, using SPME for extraction, trans-anethole was not detectable in breast fillets.

In summary, our results indicate that feeding 1000 ppm of AO had no effect on feed intake. The broilers fed AO had similar ADG and G:F as broilers fed antibiotics. Additionally, feed efficiency was better for broilers fed AO during the starter phase (d 0 – 10) than for broilers fed antibiotics. Final body weight and breast yield was not affected by treatment. Anise oil volatiles were undetectable in breast fillets. Anise oil may be used as an alternative to antibiotics in broiler diets.

6.6 Literature cited

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CHAPTER 7: SUMMARY AND CONCLUSIONS

Maintaining a healthy digestive tract is important to maximize the uptake of nutrients and feed efficiency. In modern swine production, weaning results in a sudden drop in feed intake, which reduces growth performance, increases susceptibility to enteric diseases, and damages the digestive tract. Research has been conducted to overcome those negative effects. Providing to piglets a high quality and nutrient dense pre-nursery diets during lactation has been suggested to improve feed acceptance. Research indicates that creep-fed pigs consume more feed than non-creep fed pigs. However, the application is limited because few pigs actually consume the creep feed. Since milk is high in lactose and fats, diets should contain dairy byproducts or milk products. However, these ingredients are expensive and increase the cost of the diets.

In broilers, necrotic enteritis is one of the main diseases that accounts for losses in productivity and profitability. Coccidiosis, environment, and diet are some of the factors creating necrotic enteritis. *Clostridium perfringens* (CIP) is the main pathogen involved in the development of clinical and subclinical lesions. Because of the ubiquitous characteristic of CIP, antibiotics are routinely added to broiler diets as growth promotants at sub-therapeutic levels. At those levels, antibiotics are capable of changing the anatomy of gut microflora. Generally, antibiotics increase the population of *Lactobacillus*, and reduce CIP and *Fermenters*. However, they have been banned for this purpose in Europe. In the United States the FDA has asked the poultry industry to remove sub-therapeutic levels of antibiotics. Alternative treatments are needed to prevent necrotic enteritis. Probiotics, prebiotics, and phytogenics have been proposed to stimulate gut health. Phytogenic compounds have antimicrobial activity against a wide range of bacteria. However, there is limited research on the specific effects on CIP under commercial conditions. Anise oil (AO) is the solvent extract from aniseed which contains 90% trans-anethole as its main component. Anise oil is currently used in human foods and healthcare products. In

animal studies, it is well tolerated at high dosages, which is important for safety and welfare of animals.

An experiment was designed to evaluate the influence of AO fed to sows during lactation and fed to the pigs during the nursery phase. Feed intake during the week after weaning was recorded. Growth performance also was monitored during the nursery phase. Results from this experiment indicate that AO increased feed intake of nursery pigs. Anise oil fed to sows during lactation improved feed efficiency of nursery pigs. Our results indicate that AO had a positive effect on piglet performance during the nursery phase. Additional experiments under commercial conditions are required to confirm these results.

In broilers, an experiment was conducted to determine the maximum dosage of AO that could be fed without compromising growth performance or exhibiting toxic effects. Feeding more than 4000 ppm AO to broilers decreased their feed intake, and feeding more than 2500 ppm AO decreased their growth rate. Feed efficiency was not affected by increasing levels of AO. In another experiment, the effect of AO on growth of CIP also was evaluated in-vitro. Growth of CIP was linearly decreased with increasing levels of AO to non-detectable levels. These results suggests that AO had antimicrobial activity against CIP. Additional experiments were conducted to determine the effect of AO and fish meal in the development of necrotic lesions and growth performance of broilers after a CIP challenge. In these studies, AO was effective in reducing the lesions in the jejunum related to the infection of CIP. The results also indicate that fish meal had beneficial effects on growth performance of broilers and no influence on the development of lesions associated with the infection with CIP. A field experiment also was conducted to evaluate the effect of AO on growth performance of broilers under commercial conditions. The results indicated that growth rate and feed efficiency of broilers fed AO was

similar to broilers fed PC (containing antibiotics as growth promotant) and greater than broilers fed NC (no antibiotics). These results indicate that AO is safe to handle and to feed to pigs and broilers. Anise oil improved feed intake and feed efficiency of nursery pig, and can be used as an alternative to antibiotics growth promotants in broilers. Additional experiments are needed to confirm these findings.

VITA

Jose W. Charal was born in Antigua, Guatemala, a colonial city located near the capital of Guatemala. He grew up in a small village surrounded by coffee farms and beautiful landscapes. Jose is the son of Vitalino Charal and Juana Cojolon (*RIP*), and is the oldest of four siblings: Jakeline, Ivone and Bryan. Growing up on a farm was the reason he was always inspired by nature and agriculture. He lived at home until he was fifteen years old, when he moved to Villa Nueva to attend a high school with Agriculture education (Escuela Nacional Central de Agriculture), where he spent three years. He always wanted to continue his undergraduate education at El Zamorano (Panamerican School of Agriculture) located in Honduras, however, he had to wait for a scholarship to attend. In the meantime, he worked as manager on a swine farm. In 2002, he received a BS in AgBusiness. His graduation project was developed with Cargill Animal Nutrition Central America, where he started working as a merchant and junior formulator. Although his role and vision was economic, he found in biological science the support to make the best business decisions, and that was the reason he became interested in pursuing graduate school in animal sciences. After a year as a visiting scholar at the University of Illinois and Maschhoffs LLC, he started his master of Science program at the University of Illinois in 2007. The same year he married his wife Marleny Mercedes, and his first daughter Joanelle was born one year later. In 2009, he obtained his MS degree in animal sciences under Dr. Michael Ellis' advice. In 2010, he moved to Baton Rouge, Louisiana, to continue his education in a doctoral program under the advice of Dr. Lee Southern. After Dr. Southern's retirement, Dr. Thomas Bidner became his advisor. In 2012, his second daughter, Madaelyn, was born. Currently, Jose works as research associate in animal nutrition in Dr. Theresia Lavergne's laboratory while he finishes his doctoral program.