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Evaluation of an Essential Oil Blend and a Novel Soybean Variety as Alternatives to Preventative Antibiotic Use in Monogastrics

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EVALUATION OF AN ESSENTIAL OIL BLEND AND A NOVEL SOYBEAN VARIETY AS ALTERNATIVES TO PREVENTATIVE ANTIBIOTIC USE IN MONOGASTRICS

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This thesis is approved as a creditable and independent investigation by a candidate for the degree, Master of Science, and is acceptable for meeting the thesis requirements for this degree. Acceptance of this thesis does not imply that the conclusions reached by the candidate are necessarily the conclusions of the major department.

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ABSTRACT

EVALUATION OF AN ESSENTIAL OIL BLEND AND A NOVEL SOYBEAN VARIETY AS ALTERNATIVES TO PREVENTATIVE ANTIBIOTIC USE IN MONOGASTRICS

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Increasing consumer pressure to minimize antibiotic use and the implementation of the Veterinary Feed Directive in 2017 has increased research on alternatives to preventative antibiotics. This thesis focuses on evaluation of Ralco's Microfused™ Essential Oils (MEO) fed to broilers and soybean meal from low allergenicity soybeans fed to weaned pigs and their potential use as alternatives to antibiotics. Essential oils are known to have antimicrobial, antioxidant, antiparasitic, and digestive stimulant effects. Low allergenicity (LA) soybeans were bred to contain significantly lower concentrations of the following anti-nutritional factors and allergenic proteins: Kunitz trypsin inhibitor, soybean agglutinin (lectin), and P34 (*Gly m* Bd 28 K; Schmidt et al., 2015).

For the poultry experiment, day old broilers (n = 768) were induced with a coccidiosis challenge and fed diets containing either MEO, antibiotics, or no additive for 41 days. The following parameters were measured: growth performance, jejunal villus height, jejunal crypt depth, breast meat pH, and whole breast and ground thigh meat color and lipid oxidation over seven days. Broilers fed MEO at 250 mg/kg performed similarly to the antibiotic-fed, but minimal differences were noted for jejunal histology. MEO did not affect breast pH, but impacted meat color and reduced lipid oxidation levels.

For the swine experiment, weaned pigs (n = 36) were housed in individual metabolism crates using three blocks of 12 pigs each. Pigs were fed either a high casein-starch diet (CAS), conventional soybean meal-starch diet (CON), or LA soybean meal-starch diet (LA) for 10 days and then euthanized. The following parameters were measured: growth performance, fecal energy digestibility, and ileal villus height, crypt depth, mast cell number, expression of IL-4 and IL-10 mRNA, and amino acid digestibility. CAS-fed pigs had significantly higher average daily gain compared to CON and LA-fed pigs (0.103, -0.008, and 0.36 ± 0.014 g; $P < 0.0001$). Minimal differences were noted for ileal villus height and crypt depth. Mast cell, IL-4, and IL-10 were unable to be quantified. Finally, CAS-fed pigs had greater digestibility of most ileal amino acids over LA-fed pigs and increased fecal energy digestibility over CON-fed pigs.

1.0 INTRODUCTION

There are a variety of environmental, nutritional, and immunological stressors that can adversely affect animal health and well-being (Proudfoot and Habing, 2015; Li et. al., 1990). When animals are hatched or weaned, they experience each of these stressors because there is a change in environment, change in diet, and change in antigen exposure. In addition to the social stress that can be caused by new pen mates (Proudfoot and Habing, 2015), animals also face nutritional stress due to a rapid change in the diet (Li et al. 1990). Prior to weaning when piglets are still consuming milk from the sow, they produce large amounts of the milk-specific enzyme lactase and low amounts of protease and amylase due to high lactose content of their diets. When pigs are transitioned to a corn-soybean meal diet after weaning, the secretion of lactase in the small intestine decreases and amylase and protease secretion increases to be able to digest the plant-based nutrients. Furthermore, in young pigs, anti-nutritional factors in soybeans have been linked to hypersensitivity, which may adversely affect piglet performance (Sun et al., 2008b; Li et al., 1990). When animals are exposed to stressors, there is an increase in nutrient demands of the animal to combat stressors, which results in decreased weight gain and a limited ability to create an immune response because not enough energy is available to meet the maintenance, growth, and immune system requirements. This is further exacerbated by lower a feed intake that is typically observed at weaning. This leaves the animal in an energy deficient state for growth and subsequent performance is reduced. Therefore, many young animals are administered antibiotics during this time of high-stress in order to prevent a disease breakout within the herd (Chirila et al., 2017).

With increasing consumer pressure for producers to minimize antibiotic use along with the implementation of the Veterinary Feed Directive by the Food and Drug Administration in 2017, more research is being conducted to find alternatives to preventive antibiotics. Some of these alternatives include prebiotics, probiotics, phytochemicals, organic acids, and resistant starch (Zeng et al., 2015). Antibiotic alternatives can be categorized into two broad categories: feed additives and feedstuffs.

1.1 Feed additives

Feed additives include, but are not limited to, antibiotics, coccidiostats, enzymes, buffers, organic acids, pre/pro-biotics, and phytochemicals (Jacela et al., 2009). Among the most commonly used additives to replace antibiotics and help manage disease stress are organic acids, pre/pro-biotics, and phytochemicals (Bagal et al., 2016; Dankowiakowska et al., 2013; Mathlouthi et al., 2011). Generally, feed additives are added to enhance the growth performance of animals, but some may be added to replace antibiotics for the prevention of disease in the diet. However, there is a large difference in the efficacy of feed additives.

Of the alternatives listed, phytochemicals, specifically essential oils (EOs), have been shown to have the most inconsistent results (Brenes and Roura, 2010). Essential oils are aromatic oils that can be extracted from plant material, typically by distillation. There are about 300 EOs that are commercially available and the inconsistency in results may be due to this wide variety of oils, or the fact that the chemical compounds in the oils can be affected by species, climatic conditions, harvest time, and plant part (Brenes and Roura, 2010). Not only is there a wide variety of oils, but each oil can contain different main components as well, further confounding the issue (Brenes and Roura, 2010).

However, EOs have been shown to have an impact on growth performance, gut health, and meat quality of the animals. According to Zeng et al. (2015), "EOs enhance the production of digestive secretions and nutrient absorption, reduce pathogenic stress in the gut, exert antioxidant properties and reinforce the animal's immune status." This wide variety of metabolic effects is supported by the fact that EOs are known to have antimicrobial, antioxidant, and digestive stimulant effects (Mathlouthi et al., 2012; Botslogou et al., 2002; Platel and Srinivasan, 2004).

1.1.1 Growth performance

Typically, the common way to assess the value of a feed additive is to analyze the growth performance of a group of animals fed the feed additive compared to a control group who have not received it. An increase in growth performance suggests that the feed additive is effective and has a positive response in the animal. In addition to being compared to a control group, a feed additive can also be tested against another treatment that is expected to have similar effects or at graded levels to determine optimal feeding level (Mahmoodi et al., 2014; Khattak et al., 2014). When comparing a feed additive to an alternative product, no differences in growth performance between the two products may indicate that the feed additive could serve as an appropriate alternative (Falaki et al., 2016).

Peng et al. (2016) concluded that oregano oil supplemented to broilers at 300 and 600 mg/kg significantly increased final body weight, ADG, and ADFI on d 42, but did not affect G:F. Furthermore, broilers fed oregano at 600 mg/kg had significantly increased ADG and ADFI over the positive control. Mathlouthi et al. (2012) reported that broilers fed avilamycin (44 mg/kg), rosemary (100 mg/kg), oregano (100 mg/kg), or a blend of EOs (1,000 mg/kg) had significantly greater weight gain and G:F compared to control-fed broilers. Additionally, Khattak et al. (2014) reported that broilers fed a blend

of EOs at 100, 200, 300, 400, and 500 grams per ton of feed had significantly higher weight gain and G:F versus those not fed the blend. Alp et al. (2012) concluded that broilers fed an anticoccidial (100 mg/kg) or oregano essential oil (300 mg/kg) had a significantly greater G:F over broilers on a control diet, but there were no differences in the final weight on day 42.

There is some research that indicates EOs having a negative or minimal effect on growth performance as well. Kirkpinar et al. (2011) fed broilers a control diet, garlic essential oil at 300 mg/kg, oregano essential oil at 300 mg/kg or a blend of oregano and garlic EOs at 150 mg/kg each. Broilers fed oregano essential oil had decreased ADG over control-fed and no differences were reported for ADFI or G:F. Basmacioglu et al. (2004) reported no differences in growth performance of broilers fed a control diet, oregano essential oil at 150 mg/kg or 300 mg/kg, rosemary essential oil at 150 mg/kg or 300 mg/kg, or a blend of the two containing 75mg/kg or 150 mg/kg of each oil.

There is also evidence that EOs increase the growth performance of swine, especially in young nursery pigs. Li et al. (2012) found a 22% increase in ADG and 19% increase in ADFI in nursery pigs fed a diet containing 100 mg/kg thymol and cinnamaldehyde. Manzilla et al. (2006) reported that EOs increased ADG and ADFI in nursery pigs, 33% and 26%, respectively. However, reports of enhanced growth performance are not as common in finisher pig studies. A study by Ranucci et al. (2015) on finisher pigs fed oregano essential oil and sweet chestnut wood extract for 155 days in the finisher period showed no differences in ADG. However, the carcass meat of the pigs fed the oregano essential oil and sweet chestnut wood extract had lower lipid oxidation levels than the carcass meat of the control-fed pigs. The lack of response to EO in older

pigs may be due to the higher stress levels of the animals or the limited ability for a newly weaned pig to create an immune response (Blecha et al., 1983). Following weaning, maternal immune protection that was received through the milk is removed. Therefore, piglets rely on their own immune system to recognize and properly respond to antigens (Baily et al., 2005). However, the authors also stated, "Despite this initial active response to fed proteins some form of tolerance is ultimately achieved, although this transition may take some time." Lee et al. (2016) stated that as pigs reach five to seven weeks of age, their immune system has reached adult maturity. This could be the reason antibiotic use is decreased in finisher pigs (Cromwell, 2006). As previously stated, newly weaned animals have a limited ability to create an immune response, so there is a greater opportunity to demonstrate the effects of the EOs.

Although the exact mechanism of EOs enhancing growth performance is not known, Jang et al. (2007) reported that broilers fed a blend of EOs had increased pancreatic amylase, trypsin, and maltase activity in the small intestine as compared to birds not fed EOs. Therefore, it may be a combined effect of the antimicrobial activity of EOs along with an increase in digestive secretions, which could result in increased nutrient absorption.

1.1.2 Small Intestine Health

A healthy small intestine is vital to the survival of livestock (Blikslager, 2010). Impairment of the small intestine leading to decreased resistance to pathogens or a decrease in nutrient absorption is detrimental to the health, and thus, survival of the animal. EOs can impact gut health in two major ways: shifts in gut microbiota and changes in the microscopic anatomy of the small intestine (Brenes and Roura, 2010;

Basmacioğlu et al., 2016). The combination of these two effects leads to an increased ability of the animal to combat disease by pathogenic organisms. However, research on the impact of EOs on the histology of the small intestine in poultry is limited, especially research on broilers that are under a coccidiosis disease challenge. Part of an experiment conducted by Basmacioğlu et al. (2016) looked at the influence of an essential oil blend on the histology of the ileum. Broilers fed the essential oil blend had increased villus height and lower crypt depth at 42 days of age over broilers fed a control diet. Oviedo-Rondón et al. (2006) investigated the impact of feeding EOs to coccidiosis-infected broilers on intestinal microbial ecology, but did not look at the impact on small intestine histology. However, they report that the essential oil blend impacted the microbial community in infected broilers by preventing drastic shifts in the microbial populations after the infection. Shifts in microbial populations can stimulate an immune response because new antigens are present (Baily et al., 2005). Furthermore, Evans et al. (2001) reported a decrease in the oocyst excretion from chicks fed a blend of EOs over those not fed the blend, but this has not been a consistent response in other trials. Christaki et al. (2004) found that feeding an essential oil product had no effect on oocyst excretion. The positive effects of EOs on the small intestine may be attributed to the ability of EOs to increase the health and integrity of the small intestine, and possibly its effect on gut microbial populations.

1.1.3 Meat Quality

Essential oils are also known to decrease the lipid oxidation of meat due to their antioxidant activity (Zeng et al., 2015). Oxidation causes rancidity, or spoiling of meat, and poultry meat is especially susceptible to lipid oxidation because of its high content of

polyunsaturated fatty acids (Al-Hijazeen et al., 2016). Essential oils, particularly oregano, sage, and rosemary, are known to have a high antioxidant activity (Brenes and Roura, 2010). Oregano is derived from *Origanum vulgare* and possesses high antioxidant activity due to its content of carvacrol and thymol (Economou et al., 1991; Yanishlieva and Marinova, 1995). Luna et al. (2010) found no differences in levels of lipid oxidation in poultry meat stored for 10 days from broilers fed butylated hydroxytoluene (BHT), carvacrol, or thymol. This suggests that carvacrol and thymol have the same antioxidant capacity as BHT, a commonly used synthetic antioxidant.

Furthermore, the effects of essential oils on lipid oxidation have been studied as both a feed additive and a direct additive through incorporation in meat products. Despite how EOs are used, the extent of lipid oxidation in the meat is generally measured using 2-thiobarbituric acid reactive substances (TBARS). 2-Thiobarbituric acid reactive substances measure the levels of malondialdehyde (MDA) in the meat, a secondary oxidation product (Salih et al., 1987), by creating a pink color complex with the MDA and 2-thiobarbituric acid. Besides measuring the extent of oxidation itself, undesirable changes in color, odor, and flavor are also indicators of oxidation, and subsequently, decreased shelf-life (Maraschiello et al., 1998). Therefore, these indicators of lipid oxidation can also be measured to understand the effects of lipid oxidation. While color is an objective analysis, measurement of odor and flavor can be subjective, even with well-trained taste panelists. The L* color value is a measure of the lightness of meat, a* is a measure of redness, and b* is a measure of yellowness (Al-Hijazeen et al, 2016). Al-Hijazeen et al. (2016) found that oregano oil infusion into chicken meat at 100, 300, and 400 ppm decreased lipid oxidation in both cooked and raw meat versus a control. Their

results showed a significant increase in the L* value in meat from the broilers fed the oregano treatments over control on day 7, but no differences in a*. Also, an increase in the b* value in the meat of broilers fed oregano at 300 and 400 ppm over control was observed on day 7. Meat from the broilers fed the EOs was lighter and yellower than meat from broilers fed the control diet. Botsoglou et al. (2002) found that dietary oregano essential oil addition in broiler diets decreased the level of lipid oxidation in both thigh and breast meat for both raw and cooked meat over the control birds. The authors concluded that the thighs had a greater extent of lipid oxidation compared to the breasts because of the higher fat content of thighs, and the cooked meat had a greater level of lipid oxidation versus the raw meat due to the cooking process. These results are supported by Tichivangana and Morrissey (1985), who stated that both cooking and mincing can increase the lipid oxidation potential of the meat.

1.2 Feedstuffs

Choice of dietary feedstuffs is also an option for maintaining or improving the health of animals. Good quality feedstuffs should always be used to prevent reduced performance or health issues from nutrient deficiencies or over-consumption of toxins (Greco et al., 2014). However, specialty feedstuffs can also be used as a replacement for similar types of feedstuffs, such as fermented or enzymatically treated soybean meal for traditional soybean meal, or a different type of feedstuff, such as fishmeal, for soybean meal. Generally, feedstuffs used for increasing pig health alter the digestive mechanisms in the animal. For example, resistant starch is added to diets because it is indigestible in the stomach and small intestine. However, it is fermented in the colon and produces short chain fatty acids that are known to increase satiety and prevent overgrowth of

pathogenic bacteria in the colon (Haenen et al., 2013). Not only is an allergic response to soybeans apparent in pigs, but it has been studied extensively in humans as soybean is one of the eight major foods that are known to be responsible for 90% of food allergies (L'Hocine and Boye, 2007). Glycinin, β-conglycinin, and *Gly m* Bd 28 K (P34) are soy proteins that have the potential to cause an allergenic effect, that can be identified by an abnormal immune reaction to the soy antigens (L'Hocine and Boye, 2007). In addition to allergenic proteins, soybeans also contain anti-nutritional factors, such as trypsin inhibitor, tannins, and phytate that cause decreased digestibility by rendering other nutrients indigestible (Zhou et al., 2010; Adeyemo and Onilude, 2013). Soybeans that have not been heat treated are especially high in trypsin inhibitor, which inhibits the activity of trypsin, a protease in the small intestine responsible for protein breakdown (Lei et al., 1981). Even though they are not present in large quantities, tannins can inactivate digestive enzymes and phytate can bind to minerals, reducing their bioavailability (Adeyemo and Onilude, 2013). The effect of anti-nutritional factors in soybean meal is especially evident in nursery pigs, and is one of the main reasons why alternate amino acid sources like porcine plasma and fishmeal are added to nursery diets.

1.2.1 Feeding Value

Fermentation or enzymatic treatment of soybean meal is shown to increase the digestibility of the soybean meal. These treatments increase the feeding value of soybean meal by improving protein utilization and reducing anti-nutritional factors (Zhou et al., 2010). The actual total levels of nutrients in the feed can be analyzed, but it may not be representative of the actual digestibility of those nutrients. This is important because pigs require available nutrients, not total nutrients, and nutritionists need to know the available

level of nutrients to ensure that the animal is receiving all of its required nutrients on a daily basis. Amino acid bioavailability of an assay feedstuff can be analyzed using the slope ratio assay by feeding graded levels of the test feedstuff versus a reference feedstuff that is nearly 100% digestible in the nutrient of interest. The ratio of the slope of the test ingredient versus the reference ingredient is used to determine the bioavailability (Stein et al., 2007).

Digestibility of nutrients can be reported as apparent ileal digestibility (AID), standardized ileal digestibility (SID), true ileal digestibility (TID), or total tract digestibility and can be determined by using an indigestible marker such as titanium dioxide (Stein et al., 2007). Zhou et al. (2010) studied the impact of feeding an enzymolytic soybean meal (ESBM) to weaned pigs by feeding increasing levels of ESBM from 5 to 15% of the total soybean meal content of the diet. As levels of ESBM increased, final weight, ADG, and G:F of the animals were improved. In addition to affecting growth performance, the authors also found that inclusion of ESBM at the 15% of total soybean meal level increased the digestibility of crude protein, digestible energy, calcium, and phosphorous. Song et al. (2010) fed graded levels of a fermented soybean meal (FSBM) as partial replacement for conventional soybean meal to weaned pigs. Results of the study demonstrated that pigs fed increased levels of FSBM had decreased diarrhea incidence compared to pigs fed conventional soybean meal. Reducing diarrhea would result in increased nutrient digestibility since the feed is spending a longer time in the small intestine, and there is more time for the enzymes to break down the feedstuffs (Song et al., 2010).

Additionally, Cervantes-Pahm and Stein (2010) analyzed the feeding value of fermented (FSBM), conventional, and enzyme-treated (ESBM) soybean meal as well as soy protein isolate, fish meal, and casein by determining amino acid digestibilities of each feedstuff. The feedstuffs were fed in six different diets along with a N-free diet to ileal cannulated pigs (10.9 ± 2.3 kg) to determine endogenous loss for AID calculation. The authors concluded that, with a few exceptions, the AID and SID of most amino acids (AA) in the conventional soybean meal, FSBM, and ESBM were not different from each other. They also concluded that the casein diet had the greatest SID of AA followed by soy protein isolate, and the lowest SID in the conventional SBM diet, fish meal, FSBM, and ESBM.

1.2.2 Small Intestine Health

The impact of feedstuffs on gut health can be determined through similar methods as feed additives: small intestine histology, microbial populations, and gut permeability (Li et al., 1990; Bakker et al., 1998; Ewaschuk et al., 2012). The method used depends upon the feedstuff of interest or question to be answered. Small intestine histology is a common method to assess gut health when feeding soybean meal because of the hypersentivity reaction soybean meal causes in the small intestine (Li et al., 1990). Zhou et al. (2010) stated, "Soybean meal high in protein-derived antinutritional factors, such as trypsin inhibitors, urease, and allergenic proteins, can cause gastrointestinal disturbances, intestinal damage, increased disease susceptibility, and reduced performances of piglets." Li et al. (1990) orally infused early weaned piglets with either dried skim milk or soybean meal from 7 to 14 days after birth. Following weaning at 21 days of age, the piglets were then fed either the dried skim milk or soybean meal again. The piglets fed the soybean

meal had decreased villus height but no differences in crypt depth. Dunsford et al. (1989) concluded that feeding either a soybean meal diet or corn-soybean meal based diet compared to a casein diet to pigs weaned at 21 days resulted in decreased villus height and deformed villi. The authors stated that increased villus height and decreased crypt depth are beneficial in the small intestine of pigs because decreased villus height and increased crypt depth can result in reduced absorption.

1.2.3 Immunological Response

It is well documented that the soy proteins in soybean meal cause a hypersensitivity reaction in the small intestine as well as a systemic allergic reaction (Li et al., 1990; Sun et al., 2008b). There are many compounds in the soybean that are known to cause an allergic response in humans and pigs, but the allergenic proteins found in greatest quantity are glycinin and β-conglycinin (Zhou et al., 2010). In addition to glycinin and β-conglycinin proteins, P34 (a soybean vacuolar protein) and lectins are also known to cause allergenic effects (L'Hocine and Boye, 2007).

According to L'Hocine and Boye (2007), there have been a few successful attempts to genetically alter soybeans to reduce allergenic compounds. Two examples are the Kunitz soybean which lacks trypsin inhibitor and the Tohuku 124 soybean which "lack[s] three of the known allergenic soybean proteins, the α and α' subunits of β-conglycinin, and the *Gly m* Bd 28 K" (L'Hocine and Boye, 2007). L'Hocine and Boye (2007) also stated that the allergenic capacity of lectins is unknown, but it does act as a carbohydrate binding protein.

The allergenic compounds in soybeans that affect the health and growth performance of weaned pigs transitioning to a corn-soybean meal diet have been well

researched. Friesen et al. (2014) concluded that even if pigs are fed a diet devoid of soybean meal at weaning and then transitioned to a corn-soybean meal diet 14 days after weaning, they will still exhibit similar loss in performance as compared to piglets fed soybean meal at weaning. The authors demonstrated that the pig's immune system has the capability to develop a tolerance to the soy protein.

The allergenic effect of soybean meal during this time of transition can be both local and systemic. Intestinal expression of inflammatory and anti-inflammatory cytokines and intestinal levels of IgE and IgA can be used to determine local allergenic effects. Sun et al. (2008b) found that weaned piglets fed 2-8% glycinin had increased levels of IgA, IL-4 (anti-inflammatory), and IL-6 (pro-inflammatory) in the jejunum as compared to the control piglets. However, no differences were noted in the levels of jejunal IgG and IgM between treatments. In another study by Sun et al. (2008a), IgE levels in the duodenum, jejunum, and ileum were increased with increasing glycinin inclusion levels. IgA is a mucosal antibody that plays a role in food allergies, IgE is an allergy-related antibody, and IgG is an antibody that can be specific to soy antigens. Unlike IgA, small quantities of IgG are secreted in the mucosa (Sun et al., 2008a; 2008b).

Serum expression of inflammatory and anti-inflammatory cytokines, serum levels of IgG and IgE, skin fold thickness after intradermal injection, and CD4+ and CD8+ subsets in the plasma can be used to determine systemic allergenic effects. Li et al. (1990) concluded that the piglets fed soybean meal had increased serum IgG titers to soybean protein, but no differences were noted in skin fold thickness after intradermal injection with saline, soy protein, or milk protein. Similar results were observed by Friesen et al. (2014) who fed weaned pigs either a corn-soybean meal or a corn-dried

skim milk-dried whey diet. The pigs fed the corn-soybean meal diet had increased serum IgG titers to soy protein, but no differences were found in skin fold thickness after intradermal injection of soy protein or casein on day 7 after weaning. Overall, both Sun et al. (2008a) and Sun et al. (2008b) concluded that the glycinin-induced allergenic response is predominantly a Th2-type immune response due to an increase in the CD4+ subsets in plasma, increased detection of IL-4, IL-6, IL-10 in serum, and increased number of intestinal mast cell numbers.

2.0 HYPOTHESIS and OBJECTIVES

Two studies were performed to determine the effects of a feed additive and novel feedstuff when fed during a time of high-stress to monogastrics. The objective of the feed additive study was to determine the effects of Ralco's Microfused™ Essential Oils (MEO) on growth performance, jejunal histology, and meat quality of coccidiosis-challenged broilers. The objective of the feedstuff study was to evaluate the feeding value of a soybean variety that is low in trypsin inhibitor, P34, and lectins for weaned pigs, and to determine its impact on the pig's small intestine and immune system.

The parameters investigated in the broiler trial were as follows:

(1) Broiler growth performance as measured by weight, average daily gain, average daily feed intake, and gain:feed in the starter (d0-16), grower (d17-27), finisher (d28-34), withdrawal (d35-41) and overall (d0-41) periods
(2) Jejunal histology as a measure of gut health using villus height, crypt depth, and villus height:crypt depth ratio during peak infection
(3) Meat quality as measured by pH, color, and the level of lipid oxidation using thiobarbituric reactive substances

The parameters investigated in the weaned pig trial were as follows:

(1) Feeding value as measured by feed nutrient analysis and energy and amino acid digestibilities.
(2) Small intestinal health as measured by villus height, crypt depth, and villus height:crypt depth ratio in the ileum
(3) Immunological response as measured by inflammatory cytokines and mast cell counts in the ileum.

3.0 EFFECTS OF DIETARY MICROFUSED™ ESSENTIAL OILS TECHNOLOGY ON GROWTH PERFORMANCE, JEJUNAL HISTOLOGY, AND MEAT QUALITY OF COCCIDIOSIS-CHALLENGED BROILERS

3.1 Abstract

An experiment was conducted to determine the effects of Ralco's Microfused™ Essential Oils (MEO) on growth, jejunal histology, and meat quality of coccidiosis-challenged broilers. Day old broilers (n = 768; 64 pens; 12 broilers/pen) were randomly allotted to one of 5 dietary treatments fed in four phases: starter (d0-16), grower (d17-27), finisher (d28-34), and withdrawal (d35-41). Diets were a corn-soybean meal basal diet (CON), CON+BMD®50/Coban®90 at 55/121, 250/550, 250/495 and 0 mg/kg (ANTI), CON+MEO at 375, 250, 125, and 100 mg/kg (MEOD), CON+MEO at constant 500 mg/kg (MEO500), and CON+MEO at constant 250 mg/kg (MEO250). Broilers were offered a 10X dose of a coccidiosis vaccine on d 3, 15, 22, and 29. On d 14, jejunal histology was measured. Two broilers per pen were harvested on day 41 and breast pH was measured at d 7 postmortem. Rancidity compounds (TBARS) were measured on whole breasts at d 7 postmortem and ground thighs on d 0, 3, 5, and 7 postmortem. ANTI- and MEO250-fed broilers had increased gain compared to the CON-fed broilers in the starter phase ($P < 0.01$) and ANTI-fed broilers had greater gain than CON-fed broilers ($P < 0.03$) in the grower phase. Overall, MEO250-fed broilers had a tendency for greater gain over CON-fed (63.6 vs 61.3 ± 0.87 g; $P < 0.08$). MEOD-fed broilers tended to have greater villus height compared to MEO500-fed (806.6 vs 716.3 ± 27.7 μm; $P = 0.09$. No differences were observed for pH or TBARS of breasts. There were differences in L*, a*, and b* color values of breasts and there was an interaction for color values of thighs. ANTI, MEO500, and MEO250 had decreased TBARS values for thighs

compared to CON at day 7 postmortem ($P \leq 0.05$). Overall, growth performance of MEO250 was similar to ANTI during peak challenge. MEO250 could improve growth performance and meat quality when broilers are subjected to a coccidiosis challenge.

3.2 Introduction

Consumer pressure for antibiotic-free meat products has led to increased research in the area of antibiotic alternatives, including essential oils. Essential oils have a wide variety of effects including antimicrobial, antioxidant, and digestive stimulant activities (Mathlouthi et al., 2012; Botslogou et al., 2002; Platel and Srinivasan, 2004). Essential oils have been demonstrated to positively impact growth performance, gut health, and meat quality, but the responses are inconsistent. The inconsistencies have been related to the species/subspecies of the plant, geographical location, harvest time, and plant part used that can affect the chemical composition of the oils (Brenes and Roura, 2010).

Microfused™ Essential Oils is a blend of oils, but is comprised mainly of oregano. The oils undergo a patented microfusion process that creates a surface area of oil droplets that is 20 times greater than other commercially available oils, increasing the stability and effectiveness of the oils. Due to its oregano content, MEO has a high antioxidant activity, which is attributed to its two main phenols, carvacrol and thymol (Economou et al., 1991; Yanishlieva and Marinova, 1995). Lipid oxidation affects meat quality by negatively impacting color, odor, flavor, and shelf-life (Maraschiello et al., 1998). Poultry meat is especially sensitive to lipid oxidation due to the high content of polyunsaturated fatty acids (Al-Hijazeen et al., 2016). Additionally, processes such as mincing and cooking can increase the oxidation potential of the meat (Tichivangana and Morrissey, 1985).

The objective of this experiment was to determine the effects of Ralco's Microfused™ Essential Oils (**MEO**) on growth performance, jejunal histology, and meat quality of coccidiosis-challenged broilers.

3.3 Materials and Methods

Experimental Design, Diets, and Animal Housing

Day old broiler cockerels (n = 768 + 20 for replacements) were transported from Cobb-Vantress Inc. in Siloam Springs, AK to Ralco's Poultry Research Facility in Lynd, MN. Chicks were left in shipping crates in the facility overnight with barn temperature maintained at 33°C. The following morning, chicks were wing banded, weighed, and randomly allotted to treatment. Treatments consisted of five diets that were fed as follows: a corn-soybean meal basal diet (**CON**), the CON diet with BMD®50/Coban®90 added at 500/605, 250/550, 250/495 and 0 mg/kg in the starter, grower, finisher, and withdrawal phase, respectively (**ANTI**), CON+MEO at 375, 250, 125, and 100 mg/kg added in the starter grower, finisher, and withdrawal phase, respectively (**MEOD**), CON+MEO added at 500 mg/kg in all phases (**MEO500**), and CON+MEO added at 250 mg/kg in all phases (**MEO250**). The MEO was supplied by Ralco Nutrition, Inc, in Marshall, MN. All diets were fed in mash form in four phases: starter (d0-16), grower (d17-27), finisher (d28-34), and withdrawal (d35-41) (Table 1). Samples of all experimental diets and the vitamin trace mineral premix were sent to Dairyland Laboratories, Inc. in Arcadia, WI for nutrient analysis.

The broilers (n = 12 per pen) were housed in two side-by-side battery brooders with four stacked rows and 32 pens (0.91 x 0.61 x 0.61 m) per brooder. There were 12 replications for the CON treatment and 13 replications for the remaining treatments.

Any mortality that occurred within the first five days was replaced with extra chicks that had been fed the CON diet. Prior to the removal of one broiler per pen for histological assessment on day 14, feeder space was 7.6 cm per broiler and stocking density was 0.028 square meters per broiler. After removal of one broiler, feeder space was 8.3 cm per broiler and stocking density was 0.031 square meters per broiler. Each pen was equipped with 3 nipple waterers.

Coccidiosis Challenge

A coccidiosis challenge was induced in order to stimulate the conditions of a disease stress. Broilers, including replacements on day 3, were offered a commercial coccidiosis vaccine containing viable oocysts of *E. acervulina, E. maxima,* and *E. tenella* at 10 times the dose on top of the feed in a gel carrier dyed green on days 3, 15, 22, and 29 to ensure a challenge was maintained. In order to determine when peak infection occurred, fecal samples were collected on days 6, 8, 13, 15, 20, 22, 28, 34, and 36 from each pen and pooled by treatment. Samples were immediately placed on ice and sent to Best Veterinary Solutions, Inc in Willmar, MN for analysis of oocyst counts.

Growth Performance

Individual bird weights were taken on day 0, 15, 26, 35, and 41 and a pen mean was calculated for statistical analysis. Pen feed disappearance was measured on days 15, 26, 35, and 42 at the end of each feeding phase. All feeders were emptied on the previously mentioned days and the new diet phase wasadded. Pens were checked daily for mortality or those needing to be euthanized due to leg or health problems. Any broilers that showed signs of disease and did not maintain or improve health over time were euthanized.

Jejunal Histology

On day 14, a randomly selected broiler from each pen was removed and euthanized using a CO_2 chamber. A 5 cm section of the jejunum, beginning 5 cm proximal to Meckel's diverticulum, was collected and placed into 10% formalin for histological assessment. Slides were prepared and stained with haematoxylin and eosin at a commercial pathology diagnostics lab (Animal Disease Research and Diagnostic Laboratory, Brookings, SD). Villus height and crypt depth was measured using a Nikon microscope (Tokyo, Japan) equipped with a DS2MV Nikon camera (Tokyo, Japan) and NIS Elements software (Tokyo, Japan). Due to an unexpected amount of damaged villi, measurements were taken on all viable villus and the crypt associated with each villi. The villus height:crypt depth ratio was calculated.

Carcass Fabrication and Color

On day 41, two broilers were randomly selected from each pen, leg banded, and transported to a small harvest facility in Pipestone, MN for harvest the following morning. Seventeen hours after removal from pens, broilers were stunned, eviscerated, and butchered. Individually bagged whole chickens were transported to the meat lab at South Dakota State University, Brookings, SD in a refrigerated trailer. The carcasses were placed in a cooler (1-3°C) overnight and fabricated the following morning. Breasts were removed, deskinned, deboned, and placed on a foam tray and overwrapped. Thigh meat was deskinned, deboned, and ground twice with a 3.18 mm die. Two pens (four carcasses) were pooled and utilized as one replication for color analysis and shelf life of thigh meat. The grinder was rinsed between replications. Each replication was split into four 113.4 g patties, placed on a black foam tray, overwrapped, and labeled as day 1, 3, 5,

or 7. Following tray overwrapping, both breasts and thighs were placed with one sample of each treatment per column on tables in an illuminated refrigerator at 4°C and rotated from front to back daily. Measurement of color using a Minolta colorimeter (CR-400; Minolta Corp., Ramsey, NJ; equipped with a 50 mm diameter measuring space and D65 illuminant) was taken on both breasts and thighs on day 0, 1, 2, 3, 4, 5, 6, and 7.

Lipid Oxidation

Rancidity compounds were measured using 2-thiobarbituric reactive substances (**TBARS**) on 2 broilers per pen on whole breasts at d 7 postmortem, and on ground thigh meat on d 0, 3, 5, and 7 postmortem. Thighs were removed from the refrigerator, vacuum sealed, and frozen (-80°C) as labeled on day 1, 3, 5, or 7. Breasts were removed, vacuum sealed, and frozen (-80°C) on day 7. In order to create a homogenous sample, breasts and thighs were powdered by placing small cubes of meat into liquid nitrogen. Once frozen, the cubes were placed into a chilled Waring blender and blended until it was powered. The powder was then placed into a Whirl-Pak® bag, vacuum sealed, and stored at -80°C. All samples for TBARS were run in duplicate and one spiked sample using **TEP** (97% 1,1,3,3, tetraethoxypropane, Sigma-Adlrich T9889) was used per plate to determine percent recovery. For each sample, 1 mL of 0.2 mg/mL butylated hydroxyl toluene (**BHT**; MP Biomedicals, LLC, cat# 101162) and 45.5 mL 10% trichloroacetic acid (**TCA**) were added to 5 g of sample. TCA was prepared using 96.26 g of o-phosphoric acid (Acros Organics, 389025000) and 400 g of trichloroacetic acid (Fischer Scientific, A322-500) into a total volume of 4000 mL with double distilled water. The spiked sample was prepared by weighing 5 g of sample, adding 1 mL BHT, 35.5 mL TCA, and 10 mL of 10 μM TEP. Each sample was homogenized using a IKA® T25

Digital Ultra-Turrax® (IKA® Works, Inc, Willmington, NC) for 1 minute and filtered through Whatman No1 filter paper into a 100 mL glass sample bottle. A clean culture tube was then used to mix 5 mL of filtrate with 5 mL of 0.02 M thiobarbituric acid (**TBA**; 2-thiobarbituric acid, Sigma Aldrich T5500). A standard curve was also prepared using 25 µM TEP, TCA, and TBA. The tubes were inverted five times and placed in a shaker at room temperature for 15-20 hours and then plated (250 µL) in duplicate and read using a plate reader (Molecular Devices SpectraMax 190, Molecular Devices, Sunnyvale, CA) at 530 nm (Witte et al., 1970). The procedure used by Tarladgis et al. (1960) was used for calculation of malondialdehyde (**MDA**) content using the percent recovery and absorbance values obtained.

pH

Two frozen, powdered, homogenous breast samples from each pen were used to determine pH. A volume of 90 mL of distilled water was added to 10 g of sample. The solution was then homogenized and read for pH, where pH 7 solution (Fischer Scientific, SB107-500) was used for a standard. The pH values were obtained using a Thermo Scientific Orion 370 Advanced PerpHecT® LogR® Meter (Chelmsford, MA). Samples were averaged for each pen and pen was considered the experimental unit.

Statistical Analysis

All statistical analysis was performed using the PROC MIXED procedure of SAS (Version 9.3, SAS Inst. Inc., Cary, NC). A completely randomized design was used with pen as the experimental unit for the performance and meat quality and bird was used as the experimental unit for histology. The control treatment had 12 replications for all results, except n = 5 for thigh TBARS and color. All remaining treatments had 13

replications for all results, except n = 6 for thigh TBARS and color. Repeated measures analysis was used for color and thigh TBARS and Tukey's adjustment for means separation was used where main effect of treatment was significant. Differences were considered significant when the *P*-value ≤ 0.05 and a tendency for significance when the *P*-value ≤ 0.10.

3.4 Results

In-feed antibiotics were intended to be included at manufacturers recommended levels; however, due to a mixing error, the levels of antibiotics in the starter phase were approximately 10% of the recommended inclusion rate. During transport to the harvest facility, one broiler fed the CON diet died, so no meat quality data was collected from that broiler.

Growth Performance

Growth performance results were divided by phases as follows: starter, grower, finisher, withdrawal, and overall. The ANTI and MEO250 supplemented broilers were heavier than CON at day 17, 28, and 35, respectively ($P < 0.02$; Tables 2-4). On day 41, MEO250-fed broilers had a tendency for increased weight over the CON ($P < 0.08$; Table 5). The ANTI- and MEO250-fed broilers had increased daily gain compared to CON-fed in the starter phase ($P < 0.01$; Table 2) and ANTI-fed had significantly greater daily gain compared to CON-fed ($P < 0.03$) in the grower phase (Table 3). Overall, ME250-fed broilers had a tendency for greater daily gain compared to CON-fed ($P < 0.08$; Table 6). There were no differences in ADFI, except in the grower phase, where MEO250-fed broilers had a tendency for increased intake over CON-fed ($P < 0.06$; Table 3). There were differences among treatments in the FCR in both the starter and grower phase

(Tables 2 and 3). In the starter phase, ANTI-, MEO500- and MEO250-fed broilers had decreased F:G over CON-fed ($P < 0.04$; Table 2), while ANTI-fed broilers had decreased F:G over MEO250 in the grower phase ($P < 0.02$; Table 3). For performance parameters where treatments were different ($P \geq 0.05$) MEOD and MEO500 performed intermediate to MEO250, ANTI, and CON.

Jejunal Histology

Jejunal collections were performed on day 14 and peak infection appeared to have occurred on day 15 according to both the pooled oocyst counts and visual signs of lethargy, fever, and weakness. Pooled fecal oocyst counts were 0, 14,053, 13,467, 1,052,533, 5,227, 1,734, 133, 0 and 0 on days 6, 8, 13, 15, 20, 22, 28, 34, and 36, respectively. The MEOD-fed broilers had a tendency for higher villus height than MEO500 ($P = 0.09$) and there were no differences in crypt depth or VH:CD ratio among treatments (Table 7).

Meat Quality

In order to determine meat quality from broilers, pH, color, and lipid oxidation were measured. There were no differences in pH among treatments for breasts at day 7 of retail display (Table 8). There were no treatment by day interactions for breast meat color. Therefore, main effects of treatment and day are reported in Table 8, Figure 1, Figure 2, and Figure 3. For analysis of color, there were differences observed for L* (lightness), a* (redness), and b* (yellowness) of breasts. The CON and MEOD treatments exhibited a lower L* value (overall average across all 7 days) compared to ANTI, MEO500, and MEO250 for breasts, while the ANTI group had a higher L* value over CON, MEOD, and MEO500 ($P < 0.0008$; Table 8). The value for L* of breasts did

not change from day 0 to 1, but decreased from day 1 to 7, with the exception of increases in L* value on day 4 and 6 ($P < 0.04$; Figure 1). During the seven day retail display period, the ANTI treatment exhibited a lower a* value compared to other treatments, while MEOD exhibited a higher a* value compared to CON, ANTI, and MEO250 ($P < 0.02$; Table 8). Breast a* values increased from day 0 to 1, did not change from day 1 to 4, decreased from day 4 to 5, and then plateaued from day 5 to 7 ($P < 0.03$; Figure 2). As an average value over all seven days, the ANTI and MEO250 treatments exhibited the lowest b* value, the CON treatment exhibited the highest b* value, and MEOD and MEO500 were intermediate ($P < 0.05$; Table 8). The b* value increased from day 0 to 4 and then plateaued from day 4 to 7 ($P < 0.0001$; Figure 3). Overall, breast meat decreased in lightness, decreased in redness after day 3, and increased in yellowness.

For thigh color, there was an interaction of treatment*day for L*, a*, and b*. The two treatments that did not change in L* value over time were MEOD and MEO250. The CON and ANTI treatments performed similarly in their L* values over time. Both treatments did not change from day 0 to 1, increased from day 1 to 3, and plateaued from day 3 to 7 ($P \leq 0.007$; Figure 4). MEO500 did not change in L* value from day 0 to 1, increased from day 1 to 2, did not change again from day 2 to 3, decreased from day 3 to 4, and then plateaued again from day 4 to 7 ($P \leq 0.05$). All treatments decreased in a* value from day 0 to 1 and increased from day 1 to 3 ($P < 0.02$; Figure 5). Following day 3, ANTI, MEO500, and MEO250 performed similarly, plateauing from day 3 to 7, while CON and MEOD a* value did not change from day 3 to 6 and then decreased from day 6 to 7 ($P < 0.03$). For b* of thighs, all treatments increased from day 0 to 2 ($P \leq 0.0001$;

Figure 6). The CON, MEOD, and MEO500 treatments did not change in b* value from day 2 to 7, while the ANTI and MEO250 treatments did not change from day 2 to 6 and then decreased from day 6 to 7 ($P < 0.0001$).

Differences among treatments were also noted for TBARS of thighs, but not breasts (Figure 7). All treatments had decreased levels of MDA per kg of wet tissue for ground thighs compared to CON on day 5 ($P \leq 0.05$). The ANTI, MEO500, and MEO250 treatments had decreased levels of MDA per kg of wet tissue for ground thighs compared to CON on day 7 ($P \leq 0.05$) indicating decreased levels of lipid oxidation.

3.5 Discussion

The objective of this experiment was to determine the effects of MEO on growth performance, jejunal histology, and meat quality of coccidiosis-challenged broilers. Furthermore, it was of interest to determine if MEO could be a suitable replacement for antibiotics in commercial poultry facilities. If the antibiotics would have been included at the appropriate inclusion rate in the starter, it would have been expected for the ANTI-fed broilers to have better performance than what was observed.

Based on performance measures, it is evident that MEO fed at a constant 250 mg/kg was the most effective method of combatting the negative effects of the coccidiosis challenge. One reason for these results could be that MEO at 500 mg/kg is too high, especially in the starter phase. Once the normal microbiota composition is altered, pathogens may be able to proliferate in the small intestine (Li et al., 2017). Therefore, with the strong antimicrobial potential of MEO, it is possible that MEO at 500 mg/kg negatively impacts the normal microflora of the gut. However, it can be noted that neither level of MEO nor the ANTI treatment significantly impacted ADFI. The effects

of MEO250 and ANTI treatments were most apparent during the starter and grower phases, and the differences started to diminish as the broilers started gaining immunity to the coccidiosis. However, the effects of these treatments on broiler weight were still present during the finisher and withdrawal phases. The ANTI- and MEO250-fed broilers had higher weights over CON-fed broilers at the end of the starter, grower, and finisher, but only the MEO250-fed broilers had a tendency for increased weight over CON-fed broilers at the end of the withdrawal phase. More than likely, this is due to the fact that the antibiotics were removed from the ANTI treatment in the withdrawal phase, so the increase in weight advantage was not maintained.

While there were positive effects on growth performance of MEO250-fed broilers, there was no effect of this treatment on jejunal histology. However, MEOD had a tendency for increased villus height over MEO500. Although MEO was fed at diminishing levels for the MEOD treatment, the only level the broilers received prior to histological assessment was 375 mg/kg.

The beneficial effects of MEO fed at 250 mg/kg were also shown in the meat quality data. There were no differences among treatments for pH of breasts, which suggests that no treatment has an impact on this meat quality measure. This is consistent with research performed by Simitzis et al. (2010) and Young et al. (2003), which found no impact on the pH of meat from finishing pigs fed oregano essential oil. In regards to color, the L* value of breasts decreased over time. Al-Hijazeen et al. (2016) also found that broilers fed oregano essential oil had decreased L* values for breast meat over time. The a* value of breasts peaked on day 2 and then decreased until day 7 and the b* value increased from day 0 to 7. However, Al-Hijazeen et al. (2016) found that both the a* and

b* value decreased from day 0 to 7. Moreover, the lower fat content of the breasts as compared to the thighs could be the reason there were no differences in the level of lipid oxidation among treatments for breasts, but there were differences in lipid oxidation of ground thighs. Not only is breast meat lower in fat content, but the breasts were analyzed whole and the thighs were ground (Kirkpinar et al., 2014). Processing such as mincing, grinding, and cooking of the meat will increase the lipid oxidation potential of the meat (Tichivangana and Morrissey, 1985). Furthermore, it was not expected for the antibiotic treatment to decrease the level of lipid oxidation in the meat to the same extent as the essential oil treatments. However, research performed by Knarreborg et al. (2004) found that supplementation of antibiotics (salinomycin, 40 mg/kg feed and avilamycin, 10 mg/kg feed) in broiler diets results in significantly increased plasma concentration of α-tocopherol, which is an antioxidant.

The objectives of the experiment were met and effects of MEO were quantified. Overall, MEOD- and MEO500-fed broilers performed intermediate to CON- and ANTI-fed broilers and MEO250-fed broilers performed similarly to ANTI-fed broilers. Based on this study, it appears that MEO250 is the optimal level to feed MEO. Microfused™ Essential Oils fed at 250 mg/kg has the potential to improve growth performance when broilers are experiencing a coccidiosis disease challenge and decrease lipid oxidation of ground thigh meat during illuminated storage.

Table 1. Composition of basal broiler diets for starter, grower, finisher, and withdrawal phases[1]

Ingredient, %	Starter	Grower	Finisher	Withdrawal
Corn	59.0	65.1	68.8	70.2
Soybean Meal, 46%	36.6	30.3	25.4	24.6
Limestone	1.25	1.28	1.30	1.30
Soy Oil	0.50	1.00	1.25	1.75
Monocal. Phosphate	1.35	1.18	1.10	1.13
Sodium Bicarbonate	0.30	0.30	0.29	0.29
DL-Methionine	0.28	0.26	0.27	0.21
Salt	0.24	0.23	0.24	0.24
L-Lysine	0.17	0.17	0.16	0.14
Choline 60 (dry)	0.08	0.07	0.06	0.06
L-Threonine	0.04	0.03	0.05	0.05
VTM Premix[2]	0.20	0.17	0.14	0.14
Phytase	0.01	0.01	0.01	0.01
Total	100	100	100	100

[1]Broilers were fed starter from d0-16, grower from d17-27, finisher from d28-34, and withdrawal from d35-41.

[2]Vitamin/trace mineral premix was formulated to contain the following: 42,047 KIU/kg vitamin A, 8,190 KIU/kg vitamin D, 275, 579 IU/kg vitamin E, 24, 683 mg/kg vitamin K, 10,441 mg/kg biotin, 7,976 mg/kg folic acid, 195,778 mg/kg niacin, 130,827 mg/kg D-pantothenic acid, 40,281 mg/kg riboflavin, 6,614 mg/kg thiamine, 6,612 mg/kg vitamin B_6, 198.4 mg/kg vitamin B_{12}, 1,667,000 mg/kg manganese, and 9,900 mg/kg selenium.

Table 2. Broiler growth performance results obtained during the starter phase (d0-16)[1]

	CON	ANTI	MEOD	MEO500	MEO250	Pooled SEM	P-value
Weight (g)							
Day 0	38.4	38.3	38.3	38.2	38.2	0.30	0.99
Day 17	494[b]	543[a]	525[a,b]	521[a,b]	545[a]	8.40	0.0003
ADG (g)	26.8[b]	29.7[a]	28.6[a,b]	28.4[a,b]	29.8[a]	0.50	0.0003
ADFI (g)	38.3	38.7	39.3	38.2	39.5	0.69	0.34
F:G	1.42[a]	1.30[b]	1.37[a,b]	1.34[b]	1.31[b]	0.02	0.0001

[1]Experimental diets were fed in the starter phase as follows: corn-soybean meal basal diet (CON), CON diet with BMD®50/Coban®90 added at 55/121 mg/kg (ANTI), CON+MEO added at 375 mg/kg (MEOD), CON+MEO added at 500 mg/kg (MEO500), and CON+MEO added at 250 mg/kg (MEO250). MEO = Ralco's Microfused™ Essential Oils.

[a,b] Values with different superscripts indicate a significant difference within rows ($P \leq 0.05$).

Table 3. Broiler growth performance results obtained during the grower phase (d17-27)[1]

	CON	ANTI	MEOD	MEO500	MEO250	Pooled SEM	P-value
Weight d28 (g)	1400[b]	1486[a]	1441[a,b]	1440[a,b]	1476[a]	18.74	0.001
ADG (g)	82.0[b]	85.4[a]	83.0[a,b]	83.2[a,b]	84.3[a,b]	0.99	0.04
ADFI (g)	120[y]	124[x,y]	123[x,y]	123[x,y]	125[x]	1.65	0.09
F:G	1.46[a,b]	1.46[b]	1.49[a,b]	1.49[a,b]	1.50[a]	0.01	0.01

[1] Experimental diets were fed in the grower phase as follows: corn-soybean meal basal diet (CON), CON+BMD®50/Coban®90 at 250/550 mg/kg (ANTI), CON+MEO added at 250 mg/kg (MEOD), CON+MEO at 500 mg/kg (MEO500), and CON+MEO at 250 mg/kg (MEO250). MEO = Ralco's Microfused™ Essential Oils

[a,b] Values with different superscripts indicate a significant difference within rows ($P \leq 0.05$).

[x,y] Values with different superscripts indicate a tendency to be significantly different within rows ($P \leq 0.1$).

Table 4. Broiler growth performance results obtained during the finisher phase (d28-34)[1]

	CON	ANTI	MEOD	MEO500	MEO250	Pooled SEM	P-value
Weight d35 (g)	1966[b]	2071[a]	2023[a,b]	2011[a,b]	2055[a]	19.75	0.003
ADG (g)	82.0	84.8	84.3	82.8	83.9	1.81	0.66
ADFI (g)	165	170	174	169	178	3.86	0.14
F:G	2.00	2.01	2.05	2.03	2.12	0.05	0.27

[1] Experimental diets were fed in the finisher phase as follows: corn-soybean meal basal diet (CON), CON+BMD®50/Coban®90 at 250/495 mg/kg (ANTI), CON+MEO at 125 mg/kg (MEOD), CON+MEO at 500 mg/kg (MEO500), and CON+MEO at 250 mg/kg (MEO250). MEO = Ralco's Microfused™ Essential Oils

[a,b] Values with different superscripts indicate a significant difference within rows ($P \leq 0.05$).

Table 5. Broiler growth performance results obtained during the withdrawal phase (d35-41)[1]

	CON	ANTI	MEOD	MEO500	MEO250	Pooled SEM	P-value
Weight d41 (g)	2552[y]	2617[x,y]	2591[x,y]	2572[x,y]	2646[x]	26.05	0.08
ADG (g)	96.7	90.4	94.9	93.4	97.3	2.41	0.20
ADFI (g)	179	180	179	183	183	2.87	0.74
F:G	1.87	1.98	1.86	1.98	1.86	0.03	0.02

[1]Experimental diets were fed in the withdrawal phase as follows: corn-soybean meal basal diet (CON), CON+BMD®50/Coban®90 at 0 mg/kg (ANTI), CON+MEO at 100 mg/kg (MEOD), CON+MEO at 500 mg/kg (MEO500), and CON+MEO at 250 mg/kg (MEO250). MEO = Ralco's Microfused™ Essential Oils.

[x,y] Values with different superscripts indicate a tendency to be significantly different within rows ($P \leq 0.1$).

Table 6. Broiler growth performance results overall for the entire experimental period (d0-41)[1]

	CON	ANTI	MEOD	MEO500	MEO250	Pooled SEM	P-value
ADG (g)	61.3[y]	62.9[x,y]	62.2[x,y]	61.8[x,y]	63.6[x]	0.63	0.08
ADFI (g)	98.9	102	102	101	103	1.33	0.13
F:G	1.61	1.62	1.65	1.64	1.63	0.02	0.69

[1]Experimental diets were fed as follows: a corn-soybean meal basal diet (CON), the CON diet with BMD®50/Coban®90 added at 55/121, 250/550, 250/495 and 0 mg/kg in the starter, grower, finisher, and withdrawal phase, respectively (ANTI), CON+MEO at 375, 250, 125, and 100 mg/kg added in the starter grower, finisher, and withdrawal phase, respectively (MEOD), CON+MEO added at 500 mg/kg in all phases (MEO500), and CON+MEO added at 250 mg/kg in all phases (MEO250). MEO = Ralco's Microfused™ Essential Oils. Broilers were fed starter from d0-16, grower from d17-27, finisher from d28-34, and withdrawal from d35-41.

[x,y] Values with different superscripts indicate a tendency to be significantly different within rows ($P \leq 0.1$).

Table 7. Measurements of villus height, crypt depth, and VH:CD[1] ratio of the jejunum from broilers collected on d14[2]

	CON	ANTI	MEOD	MEO500	MEO250	Pooled SEM	P-value
Villus Height (μm)	774.4[x,y]	752.1[x,y]	806.6[x]	716.4[y]	800.6[x,y]	27.7	0.09
Crypt Depth (μm)	103.0	100.7	111.8	108.7	103.4	4.9	0.42
VH:CD[2]	8.01	7.93	7.95	7.07	8.27	0.36	0.12

[1]Experimental diets were fed in the starter phase as follows (d0-16): corn-soybean meal basal diet (CON), CON diet with BMD®50/Coban®90 added at 55/121 mg/kg (ANTI), CON+MEO added at 375 mg/kg (MEOD), CON+MEO added at 500 mg/kg (MEO500), and CON+MEO added at 250 mg/kg (MEO250). MEO = Ralco's Microfused™ Essential Oils

[2]VH=villus height; CD=crypt depth

[x,y] Values with different superscripts indicate a tendency to be significantly different within rows ($P \leq 0.1$).

Table 8. Treatment averages for pH, L*, a*, and b* color values of raw whole chicken breasts stored in an illuminated refrigerator for seven days[1]

	CON	ANTI	MEOD	MEO500	MEO250	Pooled SEM	P-value
pH	6.08	6.04	6.04	6.07	6.08	0.027	0.54
L*	60.17[c]	61.97[a]	59.96[c]	61.32[b]	61.72[a,b]	0.12	< 0.0001
a*	9.97[b,c]	9.19[d]	10.44[a]	10.09[c]	9.67[b]	0.079	< 0.0001
b*	12.60[a]	11.82[c,d]	12.23[b]	12.10[c,b]	11.74[d]	0.094	< 0.0001

[1]Experimental diets were fed as follows: a corn-soybean meal basal diet (CON), the CON diet with BMD®50/Coban®90 added at 55/121, 250/550, 250/495 and 0 mg/kg in the starter, grower, finisher, and withdrawal phase, respectively (ANTI), CON+MEO at 375, 250, 125, and 100 mg/kg added in the starter grower, finisher, and withdrawal phase, respectively (MEOD), CON+MEO added at 500 mg/kg in all phases (MEO500), and CON+MEO added at 250 mg/kg in all phases (MEO250). MEO = Ralco's Microfused™ Essential Oils. Broilers were fed starter from d0-16, grower from d17-27, finisher from d28-34, and withdrawal from d35-41.

[a,b,c,d] Values with different superscripts indicate a significant difference within rows ($P \leq 0.05$).

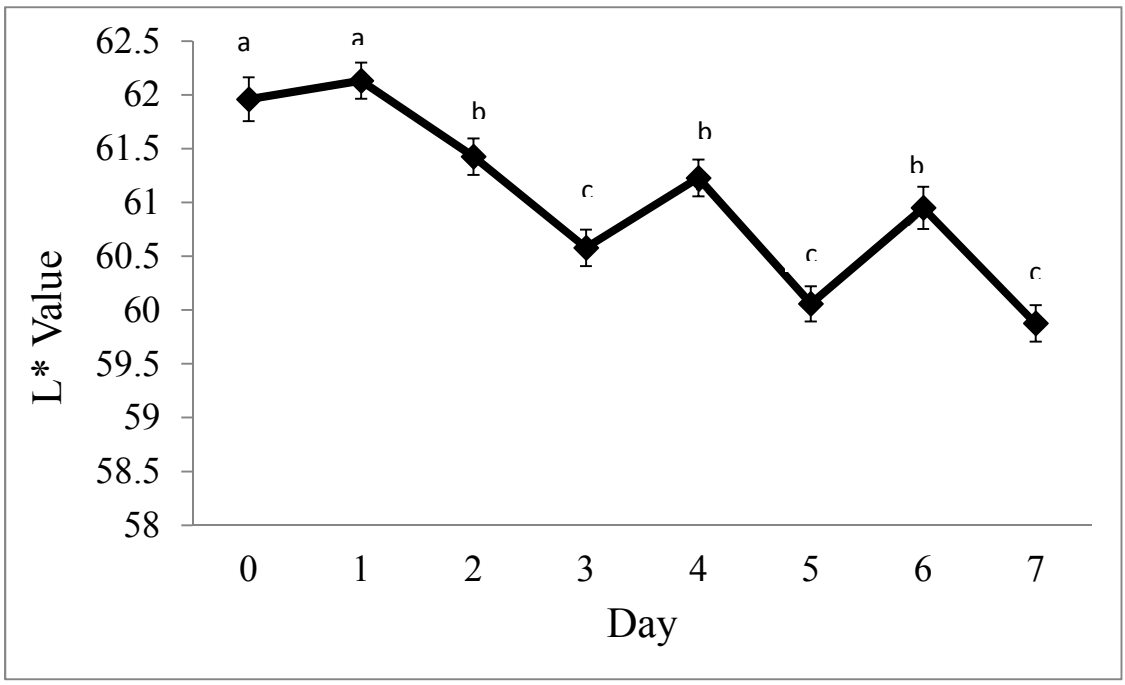

FIGURE 1. L* color value of raw whole chicken breasts stored in an illuminated refrigerator as an average of all treatments on day 0, 1, 2, 3, 4, 5, 6, and 7 ($P < 0.0001$)[1]

[1]Experimental diets were fed as follows: a corn-soybean meal basal diet (CON), the CON diet with BMD®50/Coban®90 added at 55/121, 250/550, 250/495 and 0 mg/kg in the starter, grower, finisher, and withdrawal phase, respectively (ANTI), CON+MEO at 375, 250, 125, and 100 mg/kg added in the starter grower, finisher, and withdrawal phase, respectively (MEOD), CON+MEO added at 500 mg/kg in all phases (MEO500), and CON+MEO added at 250 mg/kg in all phases (MEO250). MEO = Ralco's Microfused™ Essential Oils. Broilers were fed starter from d0-16, grower from d17-27, finisher from d28-34, and withdrawal from d35-41.

[a,b,c] Values with different superscripts indicate a significant difference ($P \leq 0.05$).

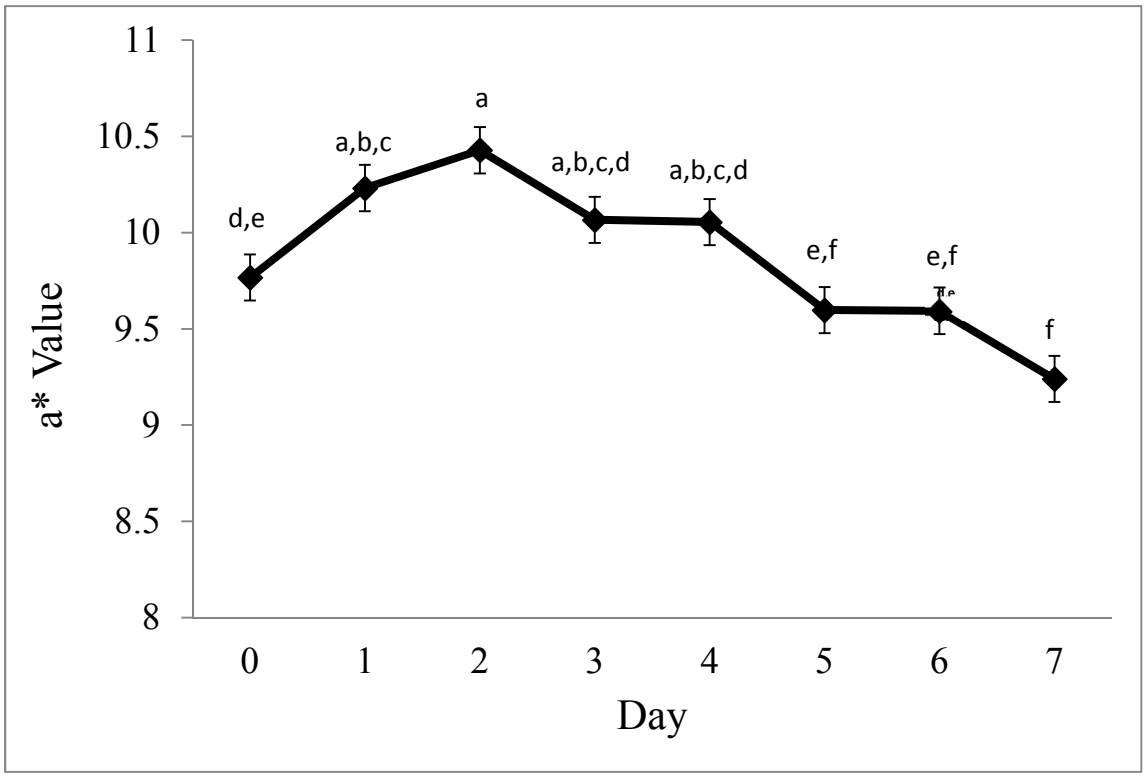

FIGURE 2. a*color values of raw whole chicken breasts stored in an illuminated refrigerator as an average of all treatments on day 0, 1, 2, 3, 4, 5, 6, and 7 ($P < 0.0001$)[1]

[1]Experimental diets were fed as follows: a corn-soybean meal basal diet (CON), the CON diet with BMD®50/Coban®90 added at 55/121, 250/550, 250/495 and 0 mg/kg in the starter, grower, finisher, and withdrawal phase, respectively (ANTI), CON+MEO at 375, 250, 125, and 100 mg/kg added in the starter grower, finisher, and withdrawal phase, respectively (MEOD), CON+MEO added at 500 mg/kg in all phases (MEO500), and CON+MEO added at 250 mg/kg in all phases (MEO250). MEO = Ralco's Microfused™ Essential Oils. Broilers were fed starter from d0-16, grower from d17-27, finisher from d28-34, and withdrawal from d35-41.

[a,b,c,d,e,f]Values with different superscripts indicate a significant difference ($P \leq 0.05$).

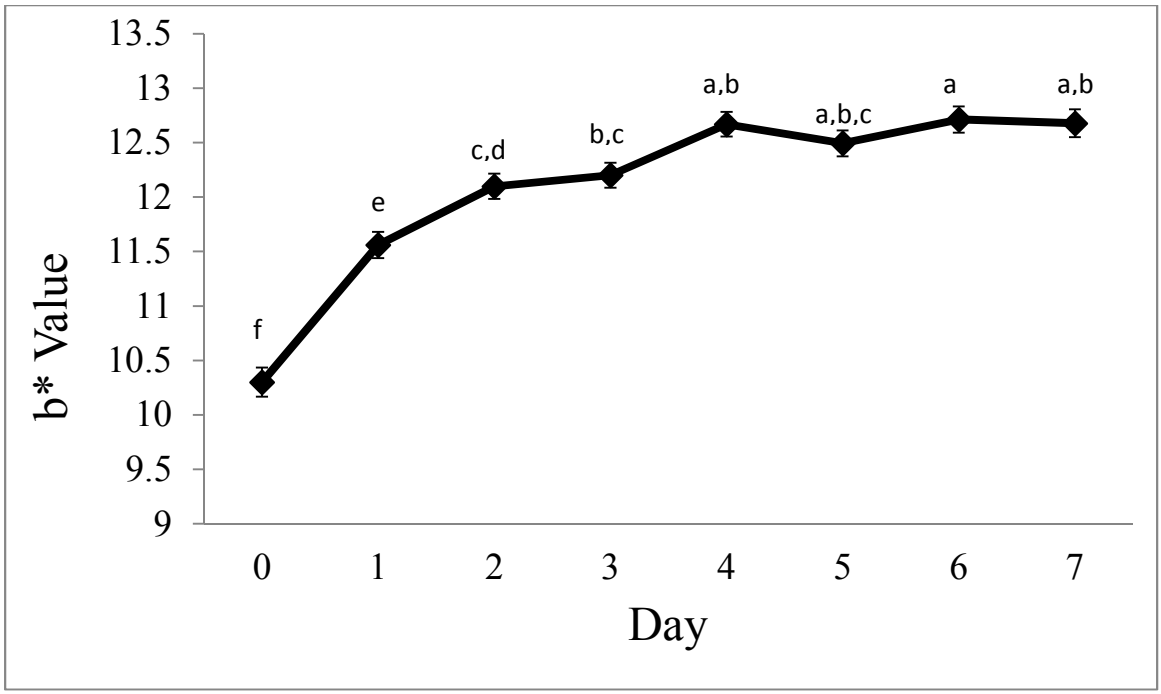

FIGURE 3. b*color values of raw whole chicken breasts stored in an illuminated refrigerator as an average of all treatments on day 0, 1, 2, 3, 4, 5, 6, and 7 ($P < 0.0001$)[1]

[1]Experimental diets were fed as follows: a corn-soybean meal basal diet (CON), the CON diet with BMD®50/Coban®90 added at 55/121, 250/550, 250/495 and 0 mg/kg in the starter, grower, finisher, and withdrawal phase, respectively (ANTI), CON+MEO at 375, 250, 125, and 100 mg/kg added in the starter grower, finisher, and withdrawal phase, respectively (MEOD), CON+MEO added at 500 mg/kg in all phases (MEO500), and CON+MEO added at 250 mg/kg in all phases (MEO250). MEO = Ralco's Microfused™ Essential Oils. Broilers were fed starter from d0-16, grower from d17-27, finisher from d28-34, and withdrawal from d35-41.

[a,b,c,d,e,f]Values with different superscripts indicate a significant difference ($P \leq 0.05$).

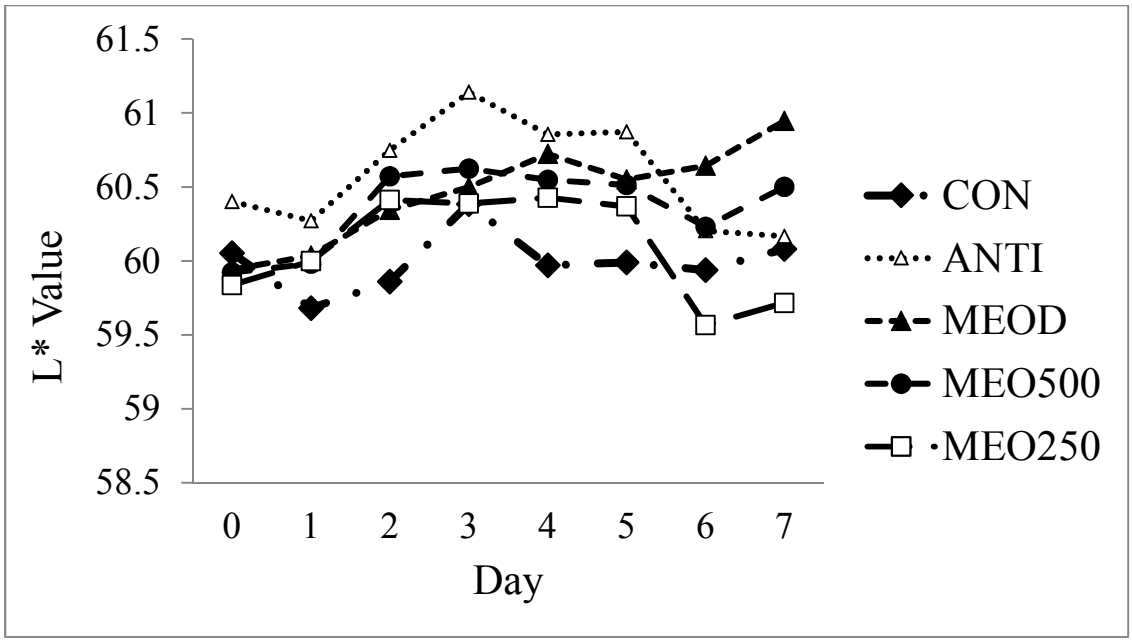

FIGURE 4. L*color values of raw ground chicken thighs stored in an illuminated refrigerator on day 0, 1, 2, 3, 4, 5, 6, and 7 (SEM = 0.52; Treatment*Day $P = 0.046$)[1]

[1]Experimental diets were fed as follows: a corn-soybean meal basal diet (CON), the CON diet with BMD®50/Coban®90 added at 55/121, 250/550, 250/495 and 0 mg/kg in the starter, grower, finisher, and withdrawal phase, respectively (ANTI), CON+MEO at 375, 250, 125, and 100 mg/kg added in the starter grower, finisher, and withdrawal phase, respectively (MEOD), CON+MEO added at 500 mg/kg in all phases (MEO500), and CON+MEO added at 250 mg/kg in all phases (MEO250). MEO = Ralco's Microfused™ Essential Oils. Broilers were fed starter from d0-16, grower from d17-27, finisher from d28-34, and withdrawal from d35-41.

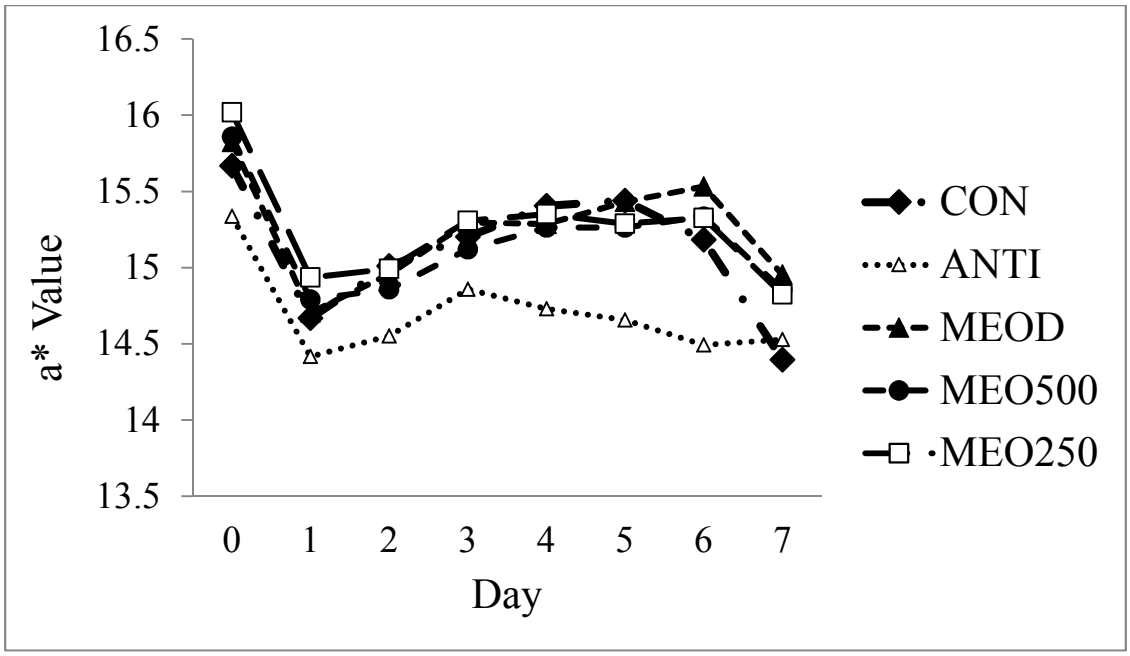

FIGURE 5. a* color values of raw ground chicken thighs stored in an illuminated refrigerator on day 0, 1, 2, 3, 4, 5, 6, and 7 (SEM = 0.29; Treatment*Day $P = 0.002$)[1]

[1]Experimental diets were fed as follows: a corn-soybean meal basal diet (CON), the CON diet with BMD®50/Coban®90 added at 55/121, 250/550, 250/495 and 0 mg/kg in the starter, grower, finisher, and withdrawal phase, respectively (ANTI), CON+MEO at 375, 250, 125, and 100 mg/kg added in the starter grower, finisher, and withdrawal phase, respectively (MEOD), CON+MEO added at 500 mg/kg in all phases (MEO500), and CON+MEO added at 250 mg/kg in all phases (MEO250). MEO = Ralco's Microfused™ Essential Oils. Broilers were fed starter from d0-16, grower from d17-27, finisher from d28-34, and withdrawal from d35-41.

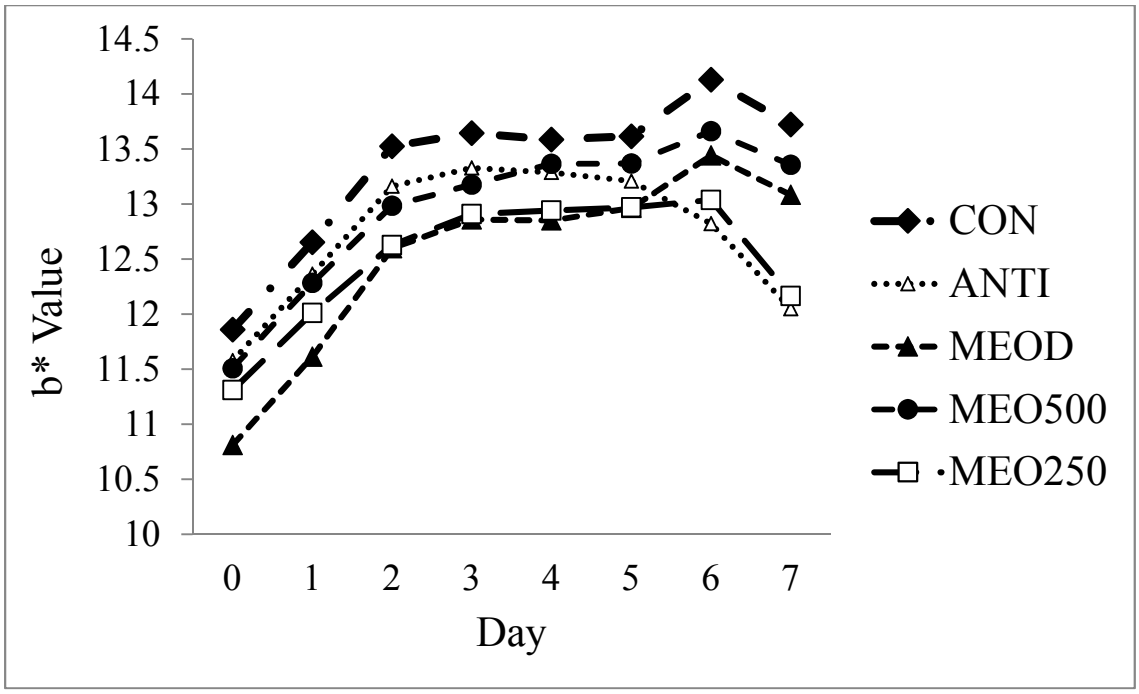

FIGURE 6. b* color values of raw ground chicken thighs stored in an illuminated refrigerator on day 0, 1, 2, 3, 4, 5, 6, and 7 (SEM = 0.29; Treatment*Day $P = 0.0006$)[1]

[1]Experimental diets were fed as follows: a corn-soybean meal basal diet (CON), the CON diet with BMD®50/Coban®90 added at 55/121, 250/550, 250/495 and 0 mg/kg in the starter, grower, finisher, and withdrawal phase, respectively (ANTI), CON+MEO at 375, 250, 125, and 100 mg/kg added in the starter grower, finisher, and withdrawal phase, respectively (MEOD), CON+MEO added at 500 mg/kg in all phases (MEO500), and CON+MEO added at 250 mg/kg in all phases (MEO250). MEO = Ralco's Microfused™ Essential Oils. Broilers were fed starter from d0-16, grower from d17-27, finisher from d28-34, and withdrawal from d35-41.

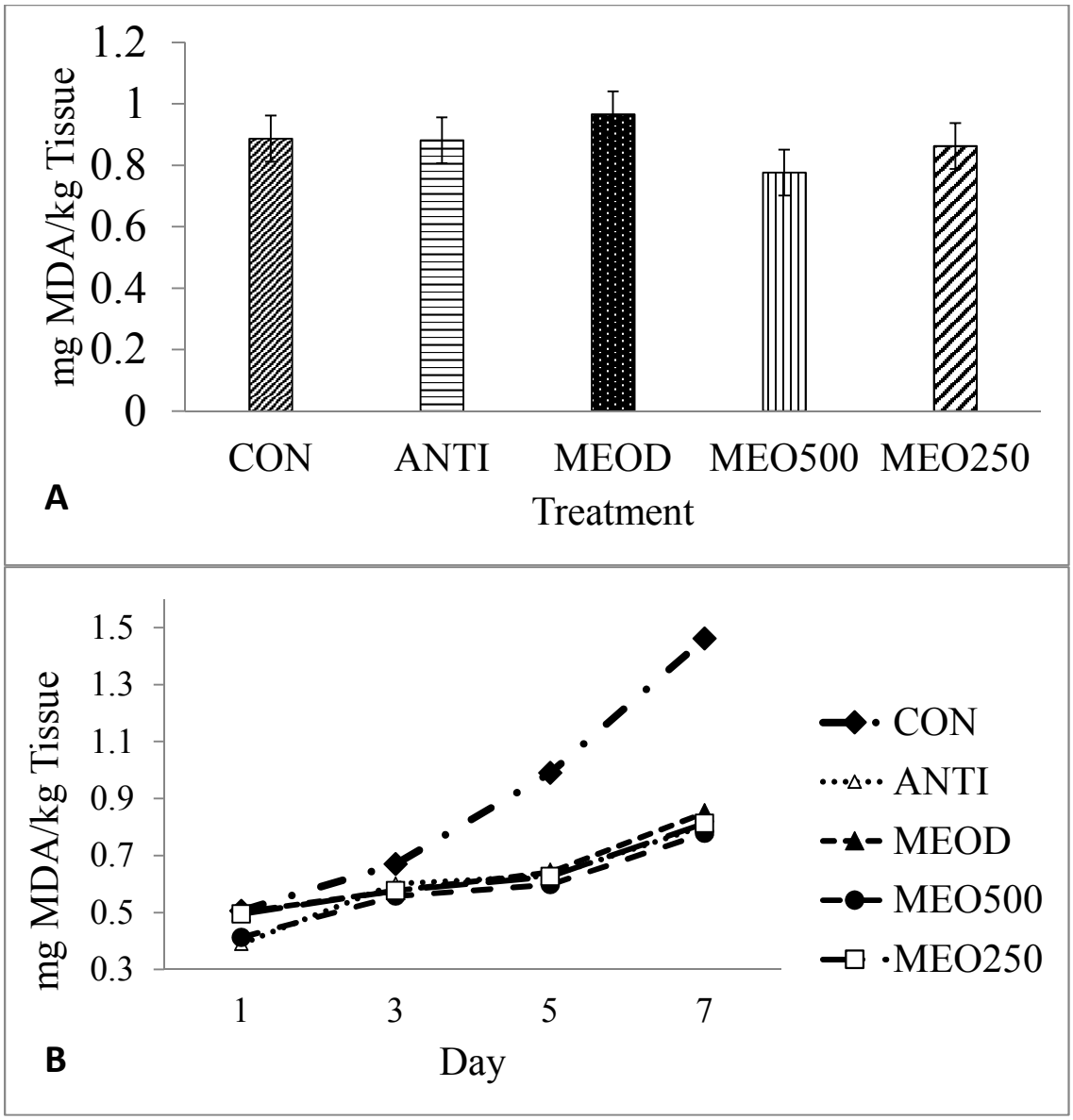

FIGURE 7. Measured levels of malondialdehyde (MDA) per kg of wet tissue in raw chicken breasts stored in an illuminated refrigerator for 7 days (A; SEM=0.08, $P > 0.05$) and in raw ground chicken thighs stored in an illuminated refrigerator for 1, 3, 5, or 7 days (B; SEM=0.06, Treatment*Day $P = 0.01$)[1]

[1]Experimental diets were fed as follows: a corn-soybean meal basal diet (CON), the CON diet with BMD®50/Coban®90 added at 55/121, 250/550, 250/495 and 0 mg/kg in the starter, grower, finisher, and withdrawal phase, respectively (ANTI), CON+MEO at 375, 250, 125, and 100 mg/kg added in the starter grower, finisher, and withdrawal phase, respectively (MEOD), CON+MEO added at 500 mg/kg in all phases (MEO500), and CON+MEO added at 250 mg/kg in all phases (MEO250). MEO = Ralco's Microfused™ Essential Oils. Broilers were fed starter from d0-16, grower from d17-27, finisher from d28-34, and withdrawal from d35-41.

4.0 IMMUNOLOGICAL RESPONSE AND AMINO ACID DIGESTIBILITY OF A NOVEL SOYBEAN VARIETY LOW IN P34 PROTEIN, TRYPSIN INHIBITOR, AND LECTINS FED TO WEANED PIGS

4.1 Abstract

An experiment was conducted to evaluate the feeding value of a soybean variety low in trypsin inhibitor, P34, and lectins for weaned pigs and determine its effect on the pig's small intestine and immune system. A completely randomized block design with weaned pigs housed in individual metabolism crates was used (n = 12 pigs/block; n = 3 blocks). Pigs were randomly assigned to one of three diets: high casein-starch diet (CAS), conventional soybean meal processed from locally grown soybeans-starch diet (CON), and soybean meal processed from low allergenicity soybeans-starch diet (LA). Fecal samples were collected on day 8 and 9 for analysis of total tract energy digestibility. On day 10, pigs were euthanized and ileal segments were collected for measurement of villus height, crypt depth, mast cell number, and expression of IL-4 and IL-10 mRNA. Ileal digesta was collected to determine AID of amino acids using titanium dioxide as a marker. There was no difference between weights on day 0 or 10, but CAS-fed pigs had higher average daily gain compared to CON- and LA-fed pigs (0.103, -0.008, and 0.36 ± 0.014 g, respectively; $P < 0.008$). Additionally, CAS-fed pigs had higher ADFI over CON-fed pigs (189.0 vs 127.7 ± 13.9 g; $P < 0.02$) and LA-fed pigs performed intermediate. CAS-fed pigs had a tendency for higher villus height than CON-fed (323.3 vs 278.0 ± 14.4 μm; $P < 0.08$) and there were no differences in crypt depth or VH:CD. Mast cells, IL-4, and IL-10 were unable to be quantified. CAS-fed pigs had greater digestibility of ASP, THR, SER, GLU, PRO, ALA, VAL, MET, ILE, LEU, PHE, LYS, HIS, ARG, and TYR ($P < 0.03$) versus LA-fed pigs and a tendency for increased total

tract energy digestibility (87.76 vs 82.98 ± 1.27%; $P < 0.07$) versus the CON-fed pigs. CON-fed pigs had AA digestibilities intermediate to CAS- and LA-fed pigs and LA-fed pigs had fecal energy digestibility intermediate to CAS- and CON-fed pigs. The feeding value of low allergenicity soybean meal is equivalent to conventional. No conclusion can be made on the effect low allergenicity soybean meal on the immune system.

4.2 Introduction

Soybeans serve as an excellent source of amino acids for pigs and are therefore commonly incorporated into swine diets as soybean meal. However, when fed to weaned pigs that have not been exposed to soybean meal as a main component of the diet, it causes a hypersensitivity reaction in the small intestine, a systemic allergic reaction, and reduced growth performance (Li et al., 1990; Sun et al., 2008b). Friesen et al. (2014) demonstrated that the pig's immune system has the capability to develop a tolerance to the soy protein; therefore, feeding soybean meal to finisher pigs does not have a negative impact.

Not only is an allergic response apparent in pigs, but it has been studied extensively in humans as soybean is one of the eight major foods that are known to be responsible for 90% of food allergies (L'Hocine and Boye, 2007). In addition to allergenic components, soybeans also contain antinutritional factors, such as trypsin inhibitor, tannins, and phytate that cause decreased digestibility by rendering other nutrients indigestible (Zhou et al., 2010; Adeyemo and Onilude, 2013).

A novel soybean was recently bred to contain significantly reduced levels of Kunitz trypsin inhibitor, soybean agglutinin (lectin), and *Gly m* Bd 28 K (P34) through the use of recessive alleles (Schmidt et al., 2015). Removal of the trypsin inhibitor and

P34 could potentially result in increased digestibility of the soybean meal, especially amino acids since trypsin inhibitor inhibits protein breakdown. Furthermore, P34 is a major allergenic protein known to cause soybean sensitivity in humans, especially neonates (L'Hocine and Boye, 2007; Schmidt et al., 2015). Therefore, the objective of the study was to determine amino acid digestibilities, gut health, and immunological response of weaned pigs fed a diet containing soybean meal processed from low allergenicity soybeans.

4.3 Materials and Methods

Experimental Design, Diets, and Animal Housing

The experiment was a Completely Randomized Block design using 12 metabolism crates and three replications with a total of 36 weaned pigs used. Maternal line gilts (6.98 ± 0.24 kg) weaned at 19 days of age were used. On the morning of day 0 for each block, 12 weaned pigs were transported 150 km to the research facility and weighed prior to being placed into individual metabolism pens. Pigs were randomly assigned to treatment and each placed in a raised pen equipped with an individual feeder, nipple drinker, visual access to another pig, 0.77 square meters per pig, and an individual heat lamp. Room temperature was maintained at 28.5°C. Block 1 pigs required metaphylactic treatment with Baytril® due to coughing upon arrival. The three dietary treatments (Table 9) consisted of different sources of protein: a high casein-starch diet (CAS), conventional soybean meal processed from locally grown soybeans-starch diet (CON), and LA soybean meal processed from low allergenicity soybeans-starch diet (LA; Schmidt et al., 2015). Pigs were fed their respective diets twice a day until the evening of day 9, when all feed was removed. During each feeding, pigs were fed slightly more than they were

expected to eat to try and achieve ad libitum feed intake. Intake as measured by disappearance of feed was determined daily. Four hours prior to being weighed and humanely euthanized using a captive bolt gun on the morning of day 10, each pig was offered 150g of feed to ensure appearance of digesta in the ileum during collection. Feed offerings were staggered to ensure that every pig had access to feed exactly four hours prior to being euthanized.

Soybeans were processed at the Northern Crops Institute in Fargo, ND in a micro-solvent extraction plant. Following cracking, the soybeans were heated to 79°C for 20 minutes and then flaked. Soy oil was removed from the flakes via hexane extraction. Following oil extraction, the defatted flakes were held at 110°C for 15 minutes to evaporate off the solvent and the hulls were added back prior to the final grinding process to obtain 46.5% crude protein. Once processing was complete, the soybean meals were used in the mixing of experimental diets (Table 9). All diets were formulated to be at 90% or greater than nutrient requirements according to the NRC (2012), with the exception of cysteine because it was not added as a synthetic amino acid. Cysteine was included at 60% of the requirement in the soybean meal diets and 23% of the requirement in the casein diet. Diets also contained 0.3% titanium oxide as a marker for analysis of digestibility. Samples of all experimental diets were sent to the University of Missouri (Columbia, MO) for analysis of amino acids (AOAC Official Method 982.30), crude protein (Combustion Analysis (LECO) AOAC Official Method 990.03), crude fat (By Ether Extraction, AOAC Official Method 920.39), crude fiber (AOAC Official Method 978.10), and ash (AOAC Official Method 942.05; Table 10). A Parr 1261 bomb calorimeter was used to measure the gross energy content of the diets using the protocol

recommended in the Parr manual (Parr Instrument Company, Moline, IL; Table 10). Additionally, all diets were sent to Eurofins Nutrition Analysis Center in Des Moines, IA for analysis of trypsin inhibitor, urease activity, and potassium hydroxide solubility (Table 10).

Histology

Following euthanasia, a 5 cm section of the ileum from each pig beginning 5 cm proximal to the ileocecal junction was collected and placed into 10% formalin for histological assessment. Slides were prepared and stained with haematoxylin and eosin at a commercial pathology diagnostics lab (Animal Disease Research and Diagnostic Laboratory (ADRDL), Brookings, SD). Villus height and crypt depth was measured using a Nikon microscope (Tokyo, Japan) equipped with a DS2MV Nikon camera (Tokyo, Japan) and NIS Elements software (Tokyo, Japan). Twenty five (5 sections of 5 villi each) villi and crypt measurements were planned to be taken for each slide, but due to unexpected damaged, villi measurements were taken on all viable villus and the crypt associated with each villi. The villus height:crypt depth ratio was calculated. Another set of slides were also prepared by ADRDL and stained using toluidine blue for mast cell counts (Sun et al., 2008a).

Gene Expression

A 10 cm section of the ileum was collected for analysis of gene expression starting at 10 cm proximal to the ileocecal junction. Samples were immediately placed in a tin foil pack and placed in liquid nitrogen. Following collections, the ileal samples were stored at -80°C. RNA was extracted using the protocol from Molecular Research Center, Inc. (MRC) for TRI Reagent® (TR 118). Bromochloropropane (MRC, BP-100) was used to

separate the homogenate into phases, and nuclease-free water was used to solubilize the RNA. Following RNA extraction, 260/280 ratio and concentration of RNA was determined using a Nano Drop 2000 spectrophotometer (Thermo Scientific). The RNA underwent DNAse treatment using the TURBO DNA-free™ protocol from Ambion® (AM1907). cDNA was then created from the RNA using a 100µL reaction as prepared by the protocol provided by Applied Biosystems® (4368813) and the MyCycler™ thermal cycler from Bio-Rad. Prior to plating, primers (Table 11) were used to create individual SYBR® Green master mixes containing a 300 nm concentration of forward and reverse primer (Qiagen, 204145). Also, 15 µL from each sample was pooled to create a cDNA master mix to ensure proper amplification of primer sets. Each plate well contained 4 µL of cDNA master mix and 21 µL of SYBR® Green master mix. Dilutions of cDNA of 16X, 8X, 4X, 2X, and 1X were used as standards. Plates were read and underwent 40 thermal cycles of 30 seconds at 95°C, 1 minute at 55°C, and 1 min at 72°C using Agilent Technologies Stratagene Mx300SP.

Digestibility

Fecal matter was collected on days 8 and 9 by collection of all freshly voided feces from the pen or rectal palpation where required. Some pigs were unable to be collected due to diarrhea. All samples were stored away from light and freeze dried in the Dura-Dry™ MP Microprocessor Control Corrosion Resistant Freeze Dryer by FTS Systems. Additionally, digesta contents from the small intestine were collected immediately after euthanasia on day 10 from 92 cm of the small intestine beginning at 20 cm proximal from the ileocecal junction. Digesta contents were placed into 20 mL glass jars and stored at -20°C. Prior to freeze drying, contents were transferred into a plastic

specimen cup. Once dried, samples were stored away from light in plastic specimen cups at room temperature. Ileal digesta, fecal matter, and diets were analyzed for titanium concentration.

Additionally, ileal digesta and diets were sent to the University of Missouri (Columbia, MO) for analysis of total amino acids (AOAC Official Method 982.30). The apparent ileal digestibility of amino acids and total tract energy digestibility was calculated using the indigestible marker method as reported by Stein et al. (2007). Energy content of fecal matter was obtained using bomb calorimetry (Parr 1261).

For analysis of titanium dioxide, 0.5 g of ileal sample, 0.3 g of fecal sample, and 5 g of feed sample were used. Samples were ashed in the Isotemp™ Programmable Muffle Furnace by Fischer Scientific at 525°C for 10 hours, rising 5°C per minute. Feed samples were sub-sampled into 0.04 g of ash in duplicates. Ash residue was acid digested with 0.8 g anhydrous sodium sulphate and 5 ml concentrated sulphuric acid for 24.5 hours at 120°C (Fischer Chemical, 5421-1; UN1830). Samples were diluted to 100 ml with double distilled water, inverted 11 times, and filtered using Whatman No.1 filter paper. For the color reaction, 5 ml of filtered sample was mixed with 0.2 ml of 30% hydrogen peroxide (Fischer Chemical, H325-500). Standards were used at concentrations of 0, 0.06, 0.12, 0.18, 0.24, and 0.3 mg/ml titanium dioxide and plates were read at least 15 minutes after the addition of hydrogen peroxide at 408 nm using the Molecular Devices SpectraMAX 190.

Statistical Analysis

All statistical analysis was performed using the PROC MIXED procedure of SAS (Version 9.3, SAS Inst. Inc., Cary, NC). A completely randomized block design was

used with pig as the experimental unit. Pig nested within block and treatment was the random variable. Tukey's adjustment for means separation was used where main effect of treatment was significant. Differences were considered significant when the *P*-value ≤ 0.05 and a tendency for significance when the *P*-value ≤ 0.10.

4.4 Results

Diets

Urease activity was negligible in the CAS diet and the soybean meal diets had similar activity (Table 10). Protein solubility was close to 100% for all diets. As expected, the CAS diet had minimal trypsin inhibitor and the LA diet had 61% lower trypsin inhibitor than the CON diet.

Growth Performance

All growth performance parameters are shown in Table 12. There were no differences among weights on day 0 or 10. However, CAS-fed pigs had significantly higher average daily gain compared to CON and LA-fed pigs ($P < 0.008$). The CON-fed pigs gained no weight over the experimental period. Additionally, the CAS-fed pigs had significantly higher ADFI over CON-fed pigs ($P < 0.02$) and LA-fed pigs performed intermediate to CAS- and CON-fed.

Histology

The CAS-fed pigs had a tendency for higher villus height than CON-fed ($P < 0.08$) and LA-fed pigs performed intermediate to CAS- and CON-fed. There were no differences in crypt depth or VH:CD among treatments (Table 12). Unfortunately, minimal to no mast cells were located in the ileum, so an assessment of mast cell numbers was not possible.

Gene Expression

Expression of the house keeping gene β-actin was successful as shown by appropriate amplification and an acceptable dissociation curve. However, minimal to no expression and an unacceptable dissociation curve was obtained for HPRT, IL-4, and IL-10. More than one set of primers as well as different concentrations and volumes of cDNA were used to try and obtain expression for IL-4 and IL-10, but attempts were unsuccessful. Therefore, we were unable to evaluate cytokine gene expression.

Digestibility

CAS-fed pigs had greater AA digestibility than LA-fed pigs for ASP, THR, SER, GLU, PRO, GLY, ALA, CYS, VAL, MET, ILE, LEU, PHE, LYS, HIS, ARG, and TYR ($P < 0.03$; Table 13). CAS-fed pigs had a tendency for greater glycine digestibility over LA-fed ($P < 0.09$; Table 13). There were no differences in the digestibility of cysteine among treatments. Additionally, there were no differences in ileal amino acid digestibilities between CON-fed and LA-fed pigs. A simple T-test was performed to determine if there was a difference in the content of apparent ileal digestible amino acids (g/kg) in CON soybean meal as compared to LA soybean meal. There were no differences for any amino acids except methionine and arginine. LA soybean meal was higher in digestible methionine and had a tendency to be higher in digestible arginine. Finally, CAS-fed pigs had a tendency for greater fecal energy digestibility over CON-fed pigs ($P < 0.07$) with LA pigs being intermediate (Table 13).

4.5 Discussion

The objective of the experiment was to determine amino acid digestibility, gut health, and immunological response of weaned pigs fed a diet containing soybean meal processed from low allergenicity soybeans. Analysis of the CAS, CON, and LA diets indicated that CAS should have the greatest quality protein and CON should have the

lowest protein quality with LA intermediate in quality based on trypsin inhibitor concentrations and protein solubility (Table 10). Trypsin inhibitor is an antinutritional factor that inhibits protein breakdown, so the lower levels of LA in the diet should result in increased digestibility of amino acids. Parsons et al. (1991) concluded that increased in vitro protein solubility can be linked to increased in vivo protein quality. Therefore, this suggests that LA soybean meal was of greater quality than CON soybean meal. There was minimal difference in the urease activity between the soybean meals.

For growth performance, there was no difference observed in pig weights at day 0 and 10. However, there was a difference in ADG, with CAS-fed pigs having increased gain compared the soybean meal treatments (Table 12). Furthermore, the CON-fed pigs, on average, did not gain weight throughout the experimental period of 10 days. This is more than likely due to the decreased ADFI as compared to the other treatments throughout the experimental period. Furthermore, the CON-fed pigs were the most difficult to get to consume feed after weaning. Furthermore, the increase in villus height of the CAS-fed pigs compared to the CON-fed indicates that CAS-fed pigs had superior gut health and greater villus surface area for nutrient absorption (Shen et al., 2014). Although it was expected to see a fair amount of mast cells, there were very few found per slide. Sun et al. (2008a) reported to have found anywhere from 12 to 47 mast cells/mm^2 in all segments of the small intestine with the number of mast cells increasing with increased glycinin intake. The results of the RT-PCR on IL-4 and IL-10 showed minimal to no expression of the mRNA for the interleukins in the small intestine. Sun et al. (2008b) were able to measure concentrations of IL-4 and IL-6 in the jejunum mucosa of pigs, but they did not analyze the expression of the mRNA for these interleukins, as

performed in this experiment. Sun et al. (2008a) were also able to quantify levels of IL-4 and IL-10 in the blood serum of pigs.

CAS-fed pigs had significantly greater AID of almost all amino acids than the LA-fed pigs. No differences were noted between CAS and CON-fed pigs, although digestibility values were numerically greater for CAS-fed pigs than CON-fed for all amino acids. An increase in the AID of amino acids was expected for the LA-fed pigs as compared to the CON-fed pigs; however, this was not observed in this trial. Although there was no statistical difference between the two soybean meal treatments, the LA-fed were numerically lower in digestibility of all amino acids. However, it is also important to note that digestibility is not equivalent to the bioavailability of the amino acids to the animal (Stein et al., 2007). Furthermore, there are many factors such as feed intake, amino acid content of feedstuff, and presence of antinutritional factors that can affect endogenous loss of amino acids, which in turn, affects AID values (Stein et al., 2007). With respect to fecal energy digestibility, the tendency for an increase in total tract energy digestibility of the CAS-fed pigs over CON-fed could be because the energy contained in the diet was more digestible or because the increase in villus height resulted in greater absorption of energetic compounds, therefore, resulting in greater digestibility.

Overall, the feeding value of low allergenicity soybean meal is equivalent to conventional and there were no differences in growth performance between the soybean meal treatments. No conclusion can be made on the effect of low allergenicity soybean meal on the immune system.

Table 9. Calculated composition of experimental diets fed to weaned pigs that contain different protein sources[1]

Ingredient, %	CAS	CON	LA
Starch	35.73	30.94	30.94
Sugar	35.72	30.94	30.94
Solka-Floc®	5	0	0
Soybean Oil	1	1	1
Casein	17.8	5	5
TiO2	0.3	0.3	0.3
CON Soybean Meal	0	28	0
LA Soybean Meal	0	0	28
Lysine	0	0.13	0.13
Methionine	0.16	0.16	0.16
Threonine	0.04	0.03	0.03
Vitamin premix[2]	0.05	0.05	0.05
Mineral premix[3]	0.15	0.15	0.15
Dicalcium Phosphate	1.4	1.4	1.4
Limestone	0.85	0.9	0.9
MgSO4	0.1	0	0
NaHCO3	0.35	0.4	0.4
NaCl	0.65	0.6	0.6
KHCO3	0.7	0	0
Total	100	100	100

[1]Dietary treatments consisted of the following: a high casein-starch diet (CAS), conventional soybean meal processed from locally grown soybeans-starch diet (CON), and LA soybean meal processed from low allergenicity soybeans-starch diet (LA; Schmidt et al., 2015).

[2]Provided per kg of complete diet: 11,002 IU vitamin A supplement, 1651 IU vitamin D_3 supplement, 55.1 IU vitamin E supplement, 0.044 mg vitamin B_{12} supplement, 4.4 mg menadione as menadione dimethylpyrimidinol bisulfite, 9.91 mg riboflavin supplement, 60.6 mg $_D$-pantothenic acid as $_D$-calcium, 55.1 mg niacin supplement, 1.1 mg folic acid, 3.3 mg pyridoxine as pyridoxine hydrochloride, 3.3 mg thiamine as thiamine mononitrate and 0.171 mg biotin.

[3]Provided per kg of the complete diet: 165 mg Zn as zinc sulfate, 165 mg Fe as ferrous sulfate, 43.5 mg Mn as manganese sulfate, 16.5 mg Cu as basic copper chloride, 0.36 mg I as ethylene diamine dihydriodide and 0.3 mg of Se as sodium selenite.

Table 10. Chemical analyses of experimental diets fed to weaned pigs and feedstuffs used in the diets[1]

	Experimental Diets			Feedstuffs		
Analysis	CAS	CON	LA	CAS	CON	LA
Crude Protein (%)	16.76	17.24	17.76	89.65	45.58	47.69
Crude Fat (%)	3.29	1.46	1.76	7.41	1.69	2.92
Crude Fiber (%)	2.17	1.61	1.57	0.12	5.77	6.38
Ash (%)	4.36	5.05	4.66	4.09	6.09	6.53
Aspartic Acid (%)	1.22	1.80	1.86	6.53	5.03	5.30
Threonine (%)	0.71	0.69	0.70	3.81	1.73	1.79
Serine (%)	0.84	0.80	0.82	4.49	2.02	2.10
Glutamic Acid (%)	3.64	3.32	3.55	19.53	7.98	8.69
Proline (%)	1.81	1.24	1.26	9.10	2.39	2.52
Glycine (%)	0.34	0.66	0.69	1.78	2.00	2.04
Alanine (%)	0.55	0.72	0.74	2.93	1.97	2.03
Cysteine (%)	0.10	0.21	0.22	0.47	0.68	0.69
Valine (%)	1.11	0.96	0.98	6.10	2.28	2.35
Methionine (%)	0.58	0.41	0.45	2.54	0.64	0.67
Isoleucine (%)	0.94	0.89	0.93	4.93	2.19	2.27
Leucine (%)	1.61	1.44	1.51	8.59	3.43	3.69
Phenylalanine (%)	0.80	0.90	0.94	4.52	2.29	2.46
Lysine (%)	1.37	1.36	1.33	7.33	2.97	3.06
Histidine (%)	0.47	0.47	0.49	2.60	1.17	1.25
Arginine (%)	0.59	1.06	1.12	3.34	3.16	3.46
Tyrosine (%)	0.78	0.65	0.66	5.00	1.61	1.61
Gross Energy (Cal/g)	4011	4038	4026			
Urease Activity (pH rise)	0.02	1.79	1.82			
Protein Solubility KOH (%)	100	96.73	98.75			
Trypsin Inhibitor (TIU/g)	<1000	16,700	6,500			

[1]Dietary treatments consisted of the following: a high casein-starch diet (CAS), conventional soybean meal processed from locally grown soybeans-starch diet (CON), and LA soybean meal processed from low allergenicity soybeans-starch diet (LA; Schmidt et al., 2015).

Table 11. Sequences of primers used for analysis of ileal cytokines of weaned pigs fed different protein sources using RT-PCR.

Primer	Sequence (5' to 3')
IL-4 Forward Set 2	CAACCCTGGTCTGCTTACTG
IL-4 Reverse Set 2	CTCCATGCACGAGTTCTTTCT
IL-4 Forward Set 3	AACACGACGGAGAAGGAAAC
IL-4 Reverse Set 3	TGGCTTCATGCACAGAACA
IL-4 Forward Set 4	TTCGGCACATCTACAGACAC
IL-4 Reverse Set 4	AGTGCTCTTCTTGGCTTCAT
IL-10 Forward Set 4	CTGATACCTCAGCTCCCATTTC
IL-10 Reverse Set 4	TTGAACACCATAGGGCACAC
β-Actin Forward	TCGCCGACAGGATGCAGAAGGA
β-Actin Reverse	AGGTGGACAGCGAGGCCAGGAT
HPRT Forward	AATGGGACTCCAGATGTTTCC
HPRT Reverse	GGCTATGCCCTTGACTACAAT

Table 12. Growth performance and small intestine health results of weaned pigs fed different protein sources for ten days[1]

	CAS	CON	LA	Pooled SEM	P-value
Weight d0 (kg)	6.78	7.17	6.99	0.24	0.54
Weight d10 (kg)	7.64	7.10	7.34	0.28	0.40
ADG (g)	0.103[a]	-0.008[b]	0.036[b]	0.014	<0.0001
ADFI (g)	189.0[a]	127.7[b]	155.0[a,b]	13.9	0.01
Villus Height (μm)	323.3[x]	278.0[y]	298.3[x,y]	14.4	0.09
Crypt Depth (μm)	78.4	71.0	73.0	3.1	0.24
VH:CD	4.33	4.17	4.37	0.19	0.71

[1]Dietary treatments consisted of the following: a high casein-starch diet (CAS), conventional soybean meal processed from locally grown soybeans-starch diet (CON), and LA soybean meal processed from low allergenicity soybeans-starch diet (LA; Schmidt et al., 2015).
[2]VH=villus height; CD=crypt depth
[a,b] Values with different superscripts of this type indicate a significant difference ($P \leq 0.05$).

Table 13. Apparent ileal digestibility of amino acids and total tract energy digestibility obtained from weaned pigs fed different protein sources for ten days[1]

%	CAS	CON	LA	Pooled SEM	P-value
Asparagine	97.37[a]	91.17[a,b]	86.60[b]	2.60	0.02
Threonine	97.25[a]	90.44[a,b]	86.59[b]	2.74	0.03
Serine	97.70[a]	90.75[a,b]	86.44[b]	2.60	0.02
Glutamine	97.80[a]	92.60[a,b]	88.41[b]	2.25	0.02
Proline	98.44[a]	91.08[a,b]	85.01[b]	2.60	.005
Glycine	93.55[x]	86.32[x,y]	80.54[y]	4.13	0.10
Alanine	97.13[a]	90.50[a,b]	86.84[b]	2.52	0.02
Cysteine	93.53	86.02	79.89	4.59	0.12
Valine	98.27[a]	91.28[a,b]	87.69[b]	2.38	0.01
Methionine	99.34[a]	95.54[a,b]	94.15[b]	1.16	0.01
Isoleucine	98.49[a]	91.85[a,b]	88.27[b]	2.26	0.01
Leucine	98.76[a]	91.81[a,b]	88.18[b]	2.26	0.009
Phenylalanine	98.63[a]	91.96[a,b]	88.21[b]	2.17	0.008
Lysine	98.62[a]	93.37[a,b]	90.76[b]	1.61	0.006
Histidine	98.79[a]	92.43[a,b]	88.90[b]	2.12	0.009
Arginine	98.03[a]	91.43[a,b]	88.12[b]	2.19	0.01
Tyrosine	98.99[a]	92.61[a,b]	89.03[b]	2.16	0.01
Energy	87.76[x]	82.98[y]	83.56[x,y]	1.27	0.05

[1]Dietary treatments consisted of the following: a high casein-starch diet (CAS), conventional soybean meal processed from locally grown soybeans-starch diet (CON), and LA soybean meal processed from low allergenicity soybeans-starch diet (LA; Schmidt et al., 2015).

[a,b] Values with different superscripts of this type indicate a significant difference ($P \leq 0.05$).

[x,y] Values with different superscripts of this type indicate a tendency to be significantly different ($P \leq 0.1$).

5.0 GENERAL DISCUSSION

The overall objective of these two experiments was to determine if either a blend of EOs that underwent a patented microfusion process or a novel soybean meal low in allergenic compounds and anti-nutritional factors could serve as an alternative to antibiotic use for the prevention of disease during times of high stress for monogastrics, such as disease or weaning.

The MEO were compared directly against antibiotics in broilers that were under a disease challenge. Parameters measured to determine if MEO could serve as an alternative to the antibiotics were: effects on growth performance, jejunal histology, and meat quality. It was observed that the MEO at 250 mg/kg was the most effective feeding rate based on the results of the study. MEO250-fed broilers performed similarly to ANTI-fed in regards to both feed and growth performance. However, the experiment should be completed with the proper level of antibiotics in the starter phase in order to obtain results that are more applicable to commercial production. If the antibiotics would have been included at the appropriate inclusion rate in the starter, it would have been expected for the ANTI-fed broilers to have better performance than what was observed. In order to obtain a better understanding of the mechanism of the EOs, more research should be performed on oocyst counts. The oocyst counts accurately reflected the intensity of the disease challenge, but not enough individual pen samples were obtained to determine differences in oocyst counts between treatments. This may lead us to understand if this blend of EOs has an anticoccidial effect. Furthermore, it was expected that if a treatment had increased gut health, they in turn, would also have increased growth performance due to increases in nutrient absorption. However, there were no

differences in villus height or crypt depth. Since this is a subjective measurement, the tendency for MEOD-fed to have increased villus height over MEO500-fed could be negligible. Finally, the effect of the EOs post mortem was apparent in the meat quality results. Although they did not have an impact on pH, MEO lowered lipid oxidation in the ground thighs on day 5 and 7 of storage and had an impact on the color over time. This also meets the objective of the experiment because MEO250-fed performed similarly to ANTI-fed broilers. However, to further ensure customer satisfaction of meat products from broilers fed MEO, a trained panel should be used to determine if there are any differences in odor or flavor. Further research is also warranted to determine how EOs have the ability to affect meat quality when fed through the diet; whether it is a direct effect of the essential oils or an increase in the compounds that combat oxidation, such as vitamin E.

The results of the novel soybean study were not as concise and clear as the MEO study, but that could be due difficultly getting piglets to eat the feed and individual housing. Another larger scale study should be performed on group housed pigs with either the same or increased number of replications in order to determine if significant differences between the two soybean meals would occur. Individually housed pigs are not in a "normal" environment as pigs like to socialize with each other. It is difficult to determine if the LA soybean meal could serve an alternative to antibiotics because there were no differences between soybean meal treatments and there were no pigs that received in-feed antibiotics. Furthermore, more research needs to be performed to determine if the numerically increased amino acid digestibilities for CON-fed pigs over LA-fed pigs would be consistent. Finally, the effect of LA soybeans on immune response

is also difficult to determine due to difficulties in methodologies. Once accurate immune response results are obtained, they may be able to be extrapolated into the human health sector. A positive impact on the immune system would suggest that the LA soybean meal could serve as an alternative to antibiotics. Additionally, serum IgG should also be analyzed to determine if there is a systemic response to the soybean meals.

Overall, the feed additive was more effective at serving as an alternative to preventative antibiotics in the feed. With further research, it may be possible to prove that LA soybean meal in weaned pigs diets could serve as an alternative to antibiotics by increasing the health of the animal during a time of increased stress and imminent disease threat.

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