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Impact of Antibiotic Alternatives on Concentrations of Escherichia Coli Harbored by Broiler Chickens Challenged with Coccidiosis

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CHAPTER I

INTRODUCTION

Antibiotic growth promoters (AGPs) have been used in animal agricultural production systems in the United States since the 1940s, which includes the commercial poultry industry. AGPs are feed additives designed to improve bird performance and reduce the incidence of disease in broilers, chickens grown for their meat. Feed additives are products used in animal nutrition for purposes of improving the quality of feed and quality of food from animal origin, or to improve the animals' overall performance and health (Pirgozliev et al, 2019). In 1951, drug-resistant bacteria were discovered in turkeys for the first time, and by the 1980s, pathogenic bacteria resistant to multiple antimicrobial agents appeared worldwide (Dibner and Richards, 2005). This was followed by the suggestion to remove AGPs from poultry production entirely; as of 2013, the US Food & Drug Administration (FDA) recommended the phasing out of antibiotics for growth-promoting purposes in poultry (Salim et al, 2018; FDA, 2013). There is now a significant need to find viable alternatives to promote growth in broilers (Dibner and Richards, 2005).

A majority of commercial broilers in the US are produced under an intensive rearing system to meet the increasing demand of feeding the population. Keeping these birds in close proximity often increases the risk of disease, however (Sharma, 1999). Stress can also harm the immune status of broilers. Poultry are exposed to a number of stressors post-hatch, including transportation, processing at the hatchery and feed withdrawal before slaughter, among others.

Although these stressors serve important functions, such as feed withdrawal reducing intestinal volume and therefore lowering risk of carcass contamination during processing, they are still detrimental to bird health. Enteric diseases, like necrotic enteritis, are of major concern in the poultry industry because they lead to reduced productivity, increased mortality and food product contamination. The discontinuation of AGPs has reportedly led to increased incidence of enteric disease and animal performance issues (Park, 2016).

Animal welfare is also an area of concern as AGPs are removed from the poultry industry. Raising broilers without antibiotics may negatively affect animal welfare due to the increased risk of contracting diseases and the severity those diseases have on the flock as a whole. Antibiotics are employed in poultry to support good health and reduce or eliminate potential pain and suffering associated with disease and/or infection (Bengtsson and Greko, 2014). *Escherichia coli* is a type of bacteria prominently found in the poultry industry and is the cause of colibacillosis, an endemic disease. Colibacillosis is characterized by fibrinous lesions around the visceral organs; it can also lead to airsacculitis, pericarditis, cellulitis, perihepatitis and respiratory distress in the bird (Kemmett et al, 2013). In broiler chickens, *E. coli* is the most common cause of first week mortality, leading to significant economic loss every year (Poulsen et al, 2017). Without AGPs, broilers are also more susceptible to coccidiosis, a disease caused by microscopic parasites, coccidia, of the *Eimeria* genus which inhabit the intestinal tract (Zhai et al, 2018). Coccidiosis occurs in cattle, swine, sheep, goats and are especially prevalent in poultry. A mild form of coccidiosis results in diarrhea, while a more severe form can lead to death through a concurrent infection with necrotic enteritis (Karavolias et al, 2018).

A number of alternatives to AGPs are the subject of current research, including probiotics, essential oils and yeast cells. Probiotics are mono- or mixed cultures of living microorganisms which improve the properties of indigenous bacteria, benefitting the host (Huyghebaert et al, 2011). They are proven to improve intestinal microbial balance, provide protection against gut pathogens and modulate the immune system (Edens, 2003). In some cases, probiotics have been shown to enhance weight gain, increase feed conversion efficiency, increase egg and milk production, lower the incidence of disease and lower mortality in commercial animals (Khalique et al., 2020). Probiotics are also well established in the poultry industry and have been used since the 1960s. Their mode of action involves competing for space on the intestinal wall in addition to producing antimicrobial compounds. Probiotic bacteria promotion of gut health also shows its promise as a potential alternative to AGPs (Park et al, 2016).

Essential oils (EOs) are oily, aromatic liquids obtained from plant material such as flowers, buds, seeds, leaves, twigs and more (Yadav et al, 2016). They are complex mixtures of secondary plant metabolites and consist of low-boiling phenylpropenes and terpenes (Micciche et al., 2019). Recently, aromatic plants and their extracts have received attention as growth and health promoters (Diaz-Sanches et al., 2015). These extracts are residue-free, generally recognized as safe and are commonly used in the food industry (Si et al., 2006). EOs have been shown to enhance production of digestive secretions, stimulate blood circulation, exert antioxidant properties, reduce levels of pathogenic bacteria and may enhance immune status (Brenes and Roura, 2010).

Yeast cells have been utilized in a dried form in poultry nutrition for years. They are a well-known source of high-quality proteins and B vitamins. Yeast cells contain compounds with potential immunomodulatory effects, including β-1.3/1.6-glucans, mannan oligosaccharides (MOS), nucleotides, inositol and glutamine (Anwar et al, 2017). MOS, which are derived from yeast cell walls, are of particular interest as a possible AGP replacement because of their ability to agglutinate gram-negative pathogenic bacteria like E. coli (Świątkiewicz et al, 2014). The goal of using alternatives like yeast cell walls is to maintain performance and health while also minimizing mortality and morbidity (M'Sadeq et al, 2015). The objective of this experiment was to determine if the use of one of these AGP alternatives or a combination of them is capable of reducing *Escherichia coli* in the ceca.

CHAPTER II

MATERIALS AND METHODS

Birds, Diet and Management

In this study, 3,024 male Ross by Ross 708 broilers were acquired from a commercial hatchery and randomly distributed 28 birds/pen across 108 floor pens. These pens were divided by location into 10 blocks in an environmentally controlled grow-out facility. Lighting, temperature, and feed consumption followed Ross manual recommendations (“Ross Broiler Management Handbook”, 2018). A lighting schedule of 23 hours light (23L) and 1 hour dark (1D) photoperiod was followed from d1 to d7, and a 20L:4D photoperiod from d8 to d42. Aviagen’s recommended commercial temperature program was followed in the house. The birds were provided an industry standard basal diet in crumble form in the starter phase (d 0-14) according to Ross 708 Nutrient Guidelines. The grower (d 14-27) and finisher phases (d 28-42) contained a pellet diet. Diets were based on Ross 708 guidelines and contained corn, soybean meal and poultry fat and did not include antibiotics or anticoccidials (“Ross 708 Nutrition Specifications”, 2014). Feed and water were given ad libitum. A coccidiosis challenge was administered on day 14 of the grow-out through oral gavage with a 20X dosage of Coccivac®-B52 (Intervet Inc., Omaha, NE). It contained 5 live oocyst *Eimeria* species (*E. acervulina*, *E. maxima*, *E. maxima MFP*, *E. mivati* and *E. tenella*).

All bird management, handling methods and experimental procedures were approved by the Institutional Animal Care and Use Committee of Mississippi State University.

Treatments

The nine treatments were as follows: T1) a negative control without coccidiostat (NC), T2) a positive control with coccidiostat (Maxiban – 72 g/MT), T3) NC + ICC Essential Oil 300 g/MT, T4) NC + ICC Essential Oil 600 g/MT, T5) NC + Immunowall 0.50 kg/MT + ICC Essential Oil 300 g/MT, T6) NC + Immunowall 0.50 kg/MT + ICC Essential Oil 600 g/MT, T7) NC + Immunowall 0.50 kg/MT + ICC Essential Oil 300 g/MT + Levucell 10ME (100 g/MT = 1 billion CFU/g of feed), T8) NC + Immunowall 0.50 kg/MT + ICC Essential Oil 600 g/MT + Levucell 10ME, T9) NC + CRINA Poultry Plus (DSM) 300 g/MT.

Sample Collections and Microbiology

On days 14, 21, 28 and 42 of the grow-out, birds were euthanized using carbon dioxide exposure and intestinal samples taken for analysis. The small intestine, including the duodenum, jejunum and ileum, and the ceca were removed. One-inch sections of the ileum and right ceca were cut and placed in sterile whirl-Pak (Nasco, Saugerties, NY). These were weighed and recorded, and the amount of Peptone Buffered Saline (PBS) needed for each sample was calculated to obtain a 10-fold serial dilution. All tissues used for microbiology were homogenized (Stomacher 400 circulator, Seward, Worthing, UK) in PBS at a 1:10 weight/volume ratio and then 10-folded diluted with PBS. To obtain total coliform counts, 100 μL were spread onto Eosin-Methylene Blue media (EMB, Oxoid, Thermo Fisher Scientific, Waltham, MA). The plates were aerobically incubated for 24h at 37°C (VWR™ International, 1535 incubator, Cornelius, OR, USA). Counts were log transformed.

Statistical Analysis

All data collected were analyzed using SAS v. 9.4 (SAS Institute, Cary, NC). A randomized complete block design (10 blocks) with a split plot over the four selected days of grow-out (d 0, 7, 14, 21) was used to analyze log coliform counts. Fisher's Protected LSD was used to separate means, and significant differences were noted when $P \leq 0.05$ (Steel and Torrie, 1980).

CHAPTER III

RESULTS AND DISCUSSION

Effect of Day on E. coli

No similarities were found between grow-out days 14, 21, 28 and 42 in E. coli counts. As the grow-out progressed, log E. coli counts in the ceca steadily decreased. This is most likely due to prolonged exposure to the AGP alternatives. These results differ from those found in broilers given no antibiotics or their alternatives, which reported that E. coli counts increased with bird age (Northcutt et al, 2003). Another study compared the ceca microbial populations of birds consuming feed supplemented with mannan oligosaccharides (a prebiotic) and lignin (extracted from plant cell walls) to a positive control diet using AGPs. On day 28 of the grow-out, there was no significant difference between the AGP diet and others, but by day 42 both alternative diets had exceeded the positive control in lactobacilli, beneficial gut microbes, showing that prolonged exposure may contribute to the effects of AGP alternatives (Baurhoo et al, 2007).

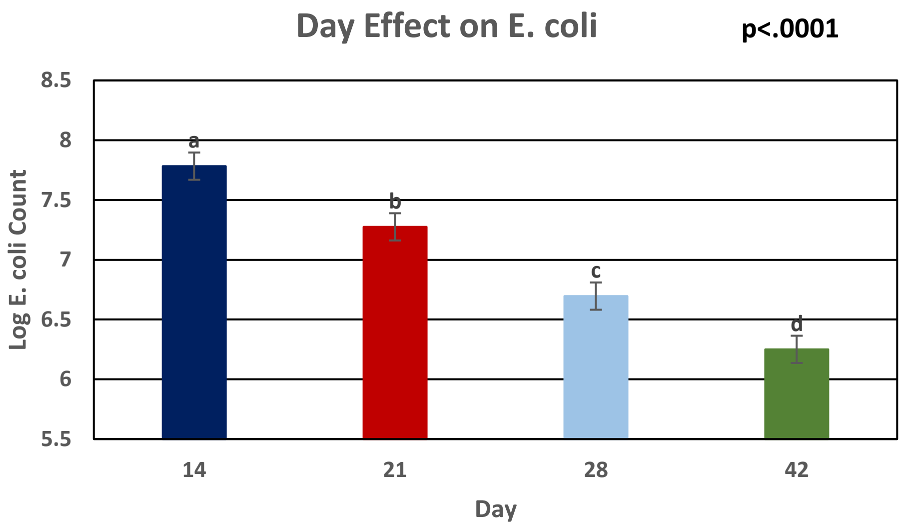

Figure 1. Day Effect on E. coli

Effect of Treatment on E. coli

There was a significant difference between treatments on the log counts of E. coli. Treatment 1, the negative control with no coccidiostat, had over 10^7 logs of E. coli and similarities with every treatment except 2, 7 and 8, which contained the positive control and doses of all three AGP alternatives, respectively.

Treatment 2 experienced a significant reduction in E. coli counts when compared to Treatment 1 and several others. This was to be expected considering it contained an antibiotic coccidiostat, Maxiban.

Treatment 3 contained even more log E. coli than Treatment 1 and appears to demonstrate that 300 g/MT of essential oils alone had no effect on E. coli content in the ceca. It also showed no similarity to Treatment 4, which contained a 600 g/MT dose of essential oils.

Treatment 4 expressed a difference of almost .4 logs from Treatment 3 and was very similar to Treatment 5.

Treatment 5 was a yeast cell wall program combined with 300 g/MT of essential oils. Combining these two programs reduced E. coli counts in the ceca but not a significant amount from the negative control. Treatments 4 and 5 were both similar to Treatment 6, another combination of yeast cell wall and essential oils. The only difference between 5 and 6 is the quantity of essential oils, which demonstrates that a 600 g/MT dose of essential oils did not reduce E. coli content and actually increased it compared to 300 g/MT. This coincides with the results of Ebani et al, who reported that essential oils were more active at a 0.5% concentration (Ebani et al, 2018).

Treatments 7 and 8 appear to be the most effective out of all the treatments containing AGP alternatives. These two treatments were combinations of all three AGP alternatives used in this experiment—the only difference, once again being the quantity of essential oils. Similarly to Treatments 5 and 6, the 300 g/MT measurement appears to have been more effective in combating E. coli than 600 g/MT of essential oils but only slightly.

Lastly, Treatment 9 did not seem to have any effect on log E. coli counts and actually contained the highest counts of all the treatments. It shared similarities with Treatments 1 and 3, with the difference being the addition of 300 g/MT of CRINA Poultry Plus, a blend of essential oils including thymol, eugenol and piperine.

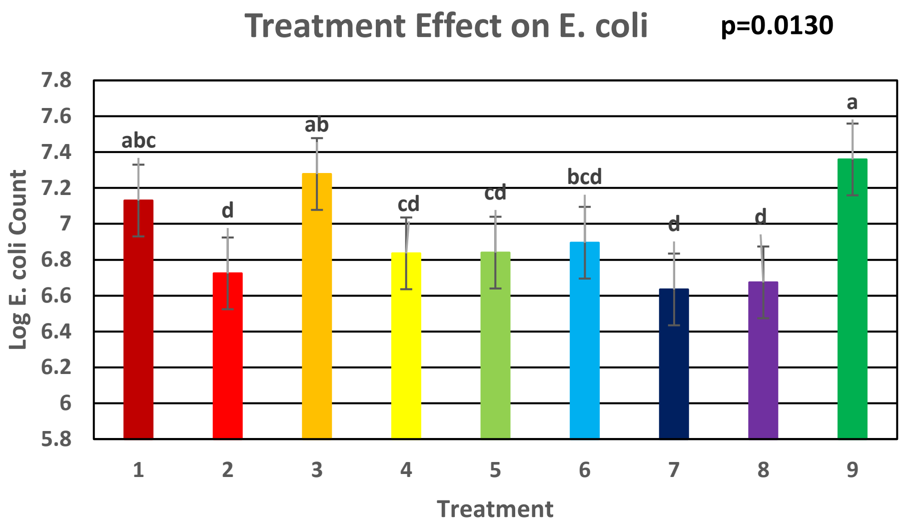

Figure 2. Treatment Effect on E. coli

A similar study conducted in 2004, used three mushroom and herb extracts as prebiotics and compared their effects on gut microbial populations of broilers infected with *Mycoplasma gallisepticum* to those of Apramycin, an antibiotic. They found that the prebiotic extracts reduced the number of harmful bacteria, including E. coli, in the gut while also increasing the numbers of potentially beneficial bacteria, like lactobacilli, compared to Apramycin (Guo et al, 2004). These results are similar to the current study's combination of AGP alternatives reducing E. coli counts, except the alternatives were not more effective than Maxiban.

A mixture of organic acids, another AGP alternative, has also proved to reduce the total as well as gram negative bacterial counts in broilers compared to the control basal diet. This was reportedly due to the pH-reducing properties of organic acids which work throughout the gastrointestinal tract. Pathogenic bacteria like some strains of E. coli grow at a considerably high pH. The effects of organic acids appear to lessen as they pass through the GI tract, however, as they are absorbed throughout the digestion process (Adil et al, 2010). The results agree with the current study, which also showed AGP alternatives reduced gram-negative counts compared to the negative control.

A 2005 experiment studied the effects of different concentrations of malic acid in drinking water against a control. In broilers, a 0.1% dose of malic acid showed increased E. coli counts compared to the control and 0.05% concentration of malic acid. The most concentrated dose of malic acid (0.15%), however, showed a significant decrease in log counts. These results differed from those seen in layer chickens, which experienced a significant reduction from the control in both the 0.1% and 0.15% doses (Moharrery and Mahzonieh, 2005). The study disagrees with the current study, which showed increased doses of AGP alternatives like essential oils were not always more effective at reducing log E. coli counts.

Antibiotics and their alternatives have both been shown to reduce total and gram-negative bacterial counts, as well as decrease muscularis thickness in the jejunum and ileum. These are thought to be connected because lymphocytes collect to fight pathogenic bacteria, causing inflammation. In one study, the bacterial counts in both the ileum and the ceca were reduced using antibiotics and a mixture of organic acids on days 21 and 42 of the grow-out (Gunal et al, 2006). These results agree with the current study because the study showed a steady decrease in bacterial counts over the grow-out using both an antibiotic coccidiostat and alternatives.

In a study by Ahmed et al in 2014, the use of *Bacillus amyloliquefaciens* as a probiotic did not affect the contents of potentially beneficial bacteria in the ceca, but it did significantly reduce the amount of E. coli. The E. coli counts steadily decreased as the dose of probiotics increased (Ahmed et al, 2014).

A mixture of essential oils has been shown to improve feed digestion and improve efficiency. It is believed that improved nutrient digestion may limit the number of pathogenic bacteria through a more balanced gut microflora. There was no significant difference between two doses of essential oils mixture (36 mg/kg and 48 mg/kg) in the results (Alcicek et al, 2004). The results agree with the current study because there was not a significant difference in log counts between essential oil doses.

Probiotics have the potential to boost the birds' immune system. In one study, chicks consuming a diet supplemented with *Lactobacillus bulgaricus* demonstrated improved antibody production against Newcastle disease vaccine antigen compared with a negative control. The optimum dose was found to be 60 mg/kg of *Lactobacillus bulgaricus* (Apata, 2008).

Ashayeriza et al, in 2008, reported that a mixture of probiotics and prebiotics added to the diet resulted in improved weight gain and a significantly better feed conversion ratio in broilers. They attribute this to a synergism between the two AGP alternatives, which could also apply to the current study. The mixture worked as well as flavomycin, the antibiotic used in the experiment (Ashayeriza et al, 2008).

Cho and Kim in 2013 reported a dosage of 0.2% lactulose had the same reducing effects on E. coli in the litter as a positive control of antibiotics. The 0.1% lactulose dose had no statistical differences from the negative control, which did not contain any antibiotics or alternatives. The 0.2% and positive control also significantly increased the levels of beneficial bacteria excretion (Cho and Kim, 2013). These results agree with the current study because a majority of treatments had no statistical differences from the negative control, and the ones that did were as effective as Maxiban.

CHAPTER IV

CONCLUSIONS

The current study results show that a combination of AGP alternatives—yeast cell wall, essential oils and probiotics—works best to reduce the content of *Escherichia coli* in the ceca of commercial broilers. In this instance, the combination was shown to be as effective as antibiotics but more research needs to be done to prove that. Essential oils appear more effective in smaller quantities. CRINA Poultry Plus did not reduce the E. coli counts and may have even raised them.

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