Effects of Feed Additives and Mixed *Eimeria* Species Infection on Intestinal Microbial Ecology of Broilers[1]

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ABSTRACT Evaluation of digestive microbial ecology is necessary to understand effects of growth-promoting feed. In the current study, the dynamics of intestinal microbial communities (MC) were examined in broilers fed diets supplemented with a combination of antibiotic (bacitracin methylene disalicylate) and ionophore (Coban 60), and diets containing 1 of 2 essential oil (EO) blends, Crina Poultry (CP) and Crina Alternate (CA). Five treatments were analyzed: 1) unmedicated uninfected control; 2) unmedicated infected control; 3) feed additives monensin (bacitracin methylene disalicylate) + monensin (Coban 60; AI); 4) EO blend CP; and 5) EO blend CA. Additives were mixed into a basal feed mixture, and EO were adjusted to 100 ppm. Chicks were infected by oral gavage at 19 d of age with *Eimeria acervulina*, *Eimeria maxima*, and *Eimeria tenella*. Duodenal, ileal, and cecal samples were taken from 12 birds per treatment just before and 7 d after challenge; 2 samples each were pooled to give a final number of 6 samples total; and all pooled samples were frozen until used for DNA extraction. Denaturing gradient gel electrophoresis was used to examine PCR-amplified fragments of the bacterial *16S* ribosomal DNA variable region. Results are presented as percentages of similarity coefficients (SC). Dendrograms of PCR amplicon or band patterns indicated MC differences due to intestinal location, feed additives, and cocci challenge. Essential oil blends CP and CA affected MC in all gut sections. Each EO had different effects over MC, and they differed in most instances from the AI group. The cocci challenge caused drastic MC population shifts in duodenal, ileal, and cecal sections (36.7, 55.4, and 36.2% SC, respectively). Diets supplemented with CP supported higher SC between pre- and postchallenge MC (89.9, 83.3, and 76.4%) than AI (81.8., 57.4, and 60.0%). We concluded that mixed coccidia challenge caused drastic shifts in MC. These EO blends modulated MC better than AI, avoiding drastic shifts after a mixed challenge.

Key words: microbial ecology, *Eimeria* species, essential oil, denaturing gradient gel electrophoresis

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INTRODUCTION

The importance of understanding the dynamics of intestinal microbial ecology has been recognized for a long time (Savage, 1977). Microbes have profound effects on some of the physiological processes of their animal host (Ewing and Cole, 1994; Fuller and Perdigón, 2003). Factors such as diet composition and feed physical traits, animal immunological and physiological responses to stress and pathogens, and feed additives play significant roles in the dynamics of gut microflora (Apajalahti et al., 2001, 2004; Guo et al., 2004). Microbial communities (**MC**) are also affected by enteropathogen infections such as those caused by *Eimeria* spp. Apajalahti (2004) indicated that infection with *Eimeria maxima* changes MC and the patterns of fermentation in the ilea and ceca of broilers. Coccidiosis is still one of the most endemic enteric diseases in broiler production (McDougald, 2003; Williams, 2002, 2005). Coccidial stress consistently has been shown to sensitize broilers to enteritis, including necrotic enteritis (Van Immerseel et al., 2004; Williams, 2005).

Understanding the dynamics of gut MC is necessary to establish or develop strategies to improve feed efficiency and growth rate (Hays, 1991; Apajalahti and Bedford, 1999). Growth-promotant antibiotics are well known for the inhibition of undesired microbial populations and the negative effects of their metabolites (Anderson et al., 1999; Van Immerseel et al., 2004) and selection for beneficial bacteria (Engberg et al., 2000; Collier et al., 2003). Other products have been proposed as alternatives to growth-promotant antibiotics utilization (Thomke and Elwinger, 1998). One category of those relatively new feed additives is the specific essential oil (**EO**) blends. These

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products are mixtures of phytochemical compounds, such as carvacrol and thymol, with selective antimicrobial properties (Lee et al., 2004). Some specific EO blends have shown promising results toward the reduction of *Clostridium perfringens* colonization and proliferation (Mitsch et al., 2004), control of coccidia infection (Giannenas et al., 2003; Saini et al., 2003a) and, consequently, may help to reduce necrotic enteritis (Saini et al., 2003b).

The study of microbial succession is important to develop alternative methods to control intestinal clinical and subclinical disease and foodborne pathogens, especially when growth promotant antibiotics are not used. The utilization of molecular techniques has improved the analysis of complex intestinal microbial populations in poultry (Apajalahti et al., 2001, 2004; Van der Wielen et al., 2002; Hume et al., 2003; Amit-Romach et al., 2004). In the present study, PCR-based denaturing gradient gel electrophoresis (**DGGE**) of V3 *16S* rDNA was used to examine the in vivo effects of antibiotic, ionophore, and EO treatment on duodenal, ileal, and cecal microbiota during pre- and postperiods of a mixed coccidia challenge in broiler chickens.

MATERIALS AND METHODS

Bird Husbandry

All procedures involving birds were approved by the Institutional Animal Care and Use Committee. Day-of-hatch Cobb-500 male chickens (1,080) were placed in 30 floor pens (6 birds/pen; Oviedo-Rondón et al., 2006) in a tunnel-ventilated dark house and randomly assigned to 5 dietary treatment groups. Birds were placed in conjunction with birds in a concurrently run study to investigate the effects of performance enhancers, EO, and coccidia vaccination on infection and performance (Oviedo-Rondón et al., 2005). Placed birds were picked at 13 d of age for the current study and 49 d of age for the concurrently run study. Used litter, top-dressed with 5 cm of fresh pine wood shavings, was utilized as bedding. The previous flock housed in the facility was challenged with mixed coccidia oocysts. Broilers were fed with starter (1 to 13 d) and grower (13 to 26 d) diets, respectively, in the form of crumbles and pellets. Corn-soybean meal diets previously described (Oviedo-Rondón et al., 2005) were formulated to guarantee or exceed recommended nutrient requirements (NRC, 1994). One basal diet was mixed for each dietary period, and feed additives were blended in accordance with treatment distribution.

Chickens were divided into 5 treatment groups: 1) unmedicated uninfected control (basal diet and not infected with coccidia when birds were 19 d of age; **UU**); 2) unmedicated infected control (basal diet; **UI**); 3) antibiotic + monensin [basal diet supplemented with an antibiotic (bacitracin methylene disalicylate) at 50 g/ton and an ionophore monensin (Coban 60; Elanco Animal Health, Greenfield, IN) at 90 g/ton (**AI**)]; 4) Crina Poultry [**CP**; basal diet supplemented with the EO blend CP (Akzo Nobel Surface Chemistry LLC, Chicago, IL) at 100 ppm]; and 5) Crina Alternate [**CA**; basal diet supplemented with CA (Akzo Nobel Surface Chemistry LLC) at 100 ppm]. Birds were raised to 13 d of age in floor pens, at which time, birds from each pen were randomly selected and moved to battery cages (Petersime Incubator Company, Gettysburg, OH). This management guaranteed that birds had natural contact with litter microflora and recirculation of *Eimeria* oocysts during the prechallenge period. Chickens in the negative control treatments UU and UI were raised in battery cages from the first day of age to avoid cross contamination with oocysts. One additional unmedicated uninfected control group (**UUFp**) was raised up to 19 d of age in the floor pens for comparison with the other prechallenge treatments that were transferred to cages at 13 d of age. The *Eimeria* challenge was accomplished in batteries to facilitate comparisons with UU and UI control groups.

Mixed Eimeria Challenge

All broilers, except those in UU treatment, were challenged at 19 d of age with a standard oral inoculum of sporulated oocysts from *Eimeria acervulina*, *E. maxima*, and *Eimeria tenella* at 200, 100, and 50×10^3 viable oocysts/mL, respectively. Two chickens from each cage, for a total of twelve birds, and twelve UUFp birds from floor pens were euthanized just before the remaining chicks being challenged with *Eimeria*. Duodenal, ileal, and cecal samples were collected within 10 min after chickens were euthanized, frozen in liquid N, and kept at –70°C until analyses were performed. Postchallenge samples were collected 7 d after the coccidia challenge (26 d of age) from 2 chickens per cage for a total of 12 birds per group; therefore, differences in MC composition, as reflected by DGGE analyses, also took into consideration normal changes as chicks age and as influenced by treatment (Hume et al., 2003).

DGGE

Diversity of predominant cecal bacteria was determined by performing DGGE of *16S* ribosomal RNA gene PCR amplicons according to the methods of Muyzer et al. (1993) and Don et al. (1991). Bacterial DNA was collected (QIAamp Mini DNA Kit, Qiagen Inc., Valencia, CA) from the 6 pooled samples per group (see sample collection above), quantified, and stored at –70°C. Stored DNA from the 6 combined samples per group were pooled (41.7 ng each) for PCR. The PCR reaction mixture consisted of DNA, JumpStart ReadyMix (Sigma Chemical Co., St. Louis, MO), 50 pmol each of reverse and forward primers (IDT, Coralville, IA; Muyzer et al., 1993), 1 μL of BSA (10 mg/mL), to a total of 25 μL with PCR-grade water (Sigma Chemical Co.). Electrophoresis was carried out in a DCode Universal Mutation Detection System (Bio-Rad Laboratories Inc., Richmond, CA) for electrophoresis in 0.5× Tris acetate EDTA [20 m*M* Tris (pH 7.4), 10 m*M* Na acetate, and 0.5 *M* EDTA] at 59°C for 17 h at 60 V. Gels were stained with SYBR Green I (1:10,000 dilution; Sigma

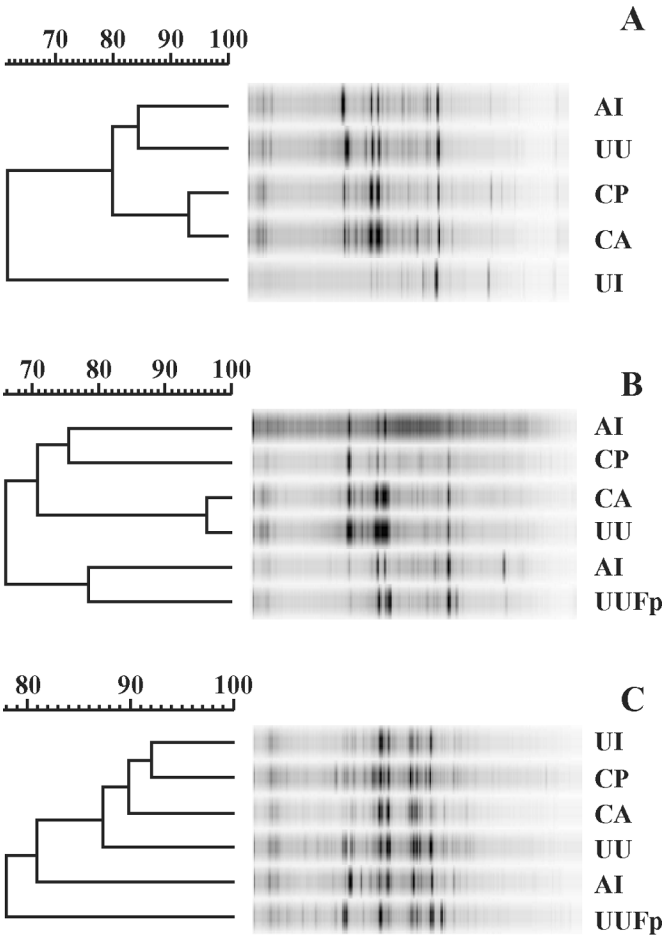

Figure 1. Denaturing gradient gel electrophoresis of duodenal (panel A), ileal (panel B), and cecal (panel C) microbial communities from broiler chickens at 19 d of age (prechallenge). Relative similarity of band patterns is indicated by their grouping on the dendogram and the percentage of similarity coefficient (bar). UU = unmedicated uninfected control; UUFp = unmedicated uninfected floor pen control; UI = unmedicated infected control; AI = bacitracin methylene disalicylate at 50 g/ton and monensin (Coban 60, Elanco Animal Health, Greenfield, IN) at 90 g/ton; CP = essential oil (EO) blend Crina Poultry (Akzo Nobel Surface Chemistry LLC, Chicago, IL); and CA = EO blend Crina Alternate (Akzo Nobel Surface Chemistry LLC).

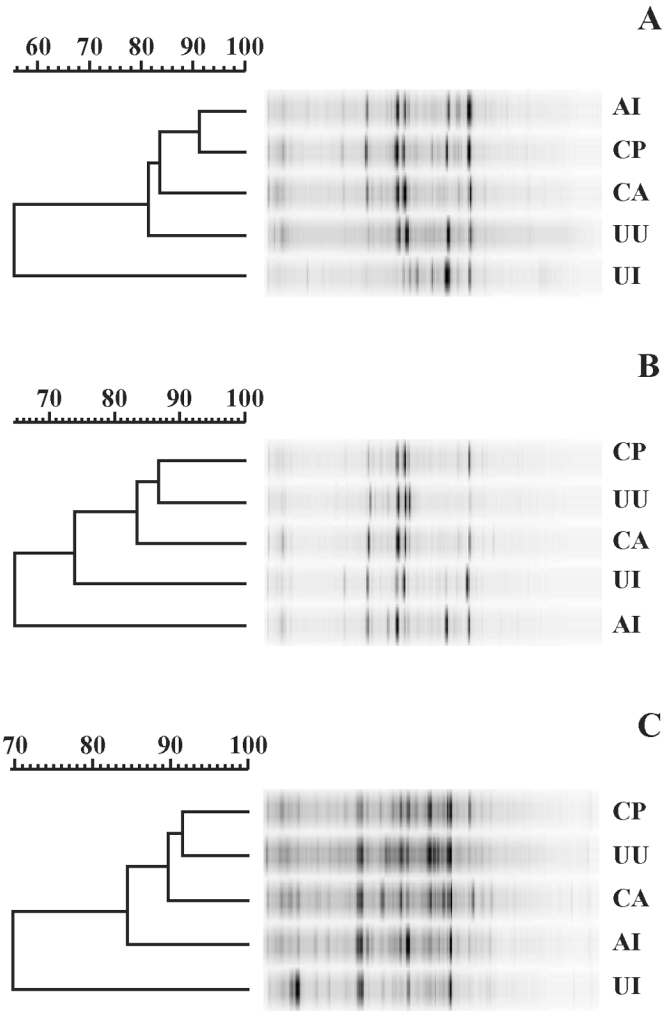

Figure 2. Denaturing gradient gel electrophoresis of duodenal (panel A), ileal (panel B), and cecal (panel C) microbial communities from broiler chickens at 26 d of age (postchallenge) and 7 d after mixed *Eimeria* spp. oral infection. Relative similarity of band patterns is indicated by their grouping on the dendogram and the percentage of similarity coefficient (bar). UU = unmedicated uninfected control; UI = unmedicated infected control; AI = bacitracin methylene disalicylate at 50 g/ton and monensin (Coban 60, Elanco Animal Health, Greenfield, IN) at 90 g/ton; CP = essential oil (EO) blend Crina Poultry (Akzo Nobel Surface Chemistry LLC, Chicago, IL); and CA = EO blend Crina Alternate (Akzo Nobel Surface Chemistry LLC).

Chemical Co.). Band patterns were analyzed for percentage of similarity coefficient (**SC**), and dendrograms were constructed using the Pearson product-moment correlation coefficient and unweighted pair group method using arithmetic averages for clustering (Molecular Analysis Fingerprinting Software, Version 1.6, Bio-Rad Laboratories).

RESULTS AND DISCUSSION

Compartment-specific factors play important roles in the development of broiler MC in each intestinal compartment (Van der Wielen et al., 2002; Hume et al., 2003) as well as site specificity exhibited by *Eimeria* spp. (McDougald, 2003). A molecular ecology approach (DGGE) was used in the current study to examine the digestive microbial composition and to determine community succession in the duodena, ilea, and ceca of broilers infected with *E.* spp. oocysts and fed a corn-soybean meal diet without feed additives or supplemented with either an antibiotic + anticoccidial (bacitracin methylene disalicylate + Coban; AI) or 2 specific EO blends.

Prechallenge Period in Birds at 19 d of Age

Chickens fed AI diets harbored similar (84.4% SC) duodenal MC to those in the UU control treatments (Figure 1, panel A). Duodenal MC of chickens fed diets with both EO were very similar (93.2%). However, the MC hosted by chickens in the UI treatment were very different from the other treatments (61.6% SC). In ileal content, the groups fed AI diets and left untreated in floor pens and in contact with litter flora, were very different (66.7%

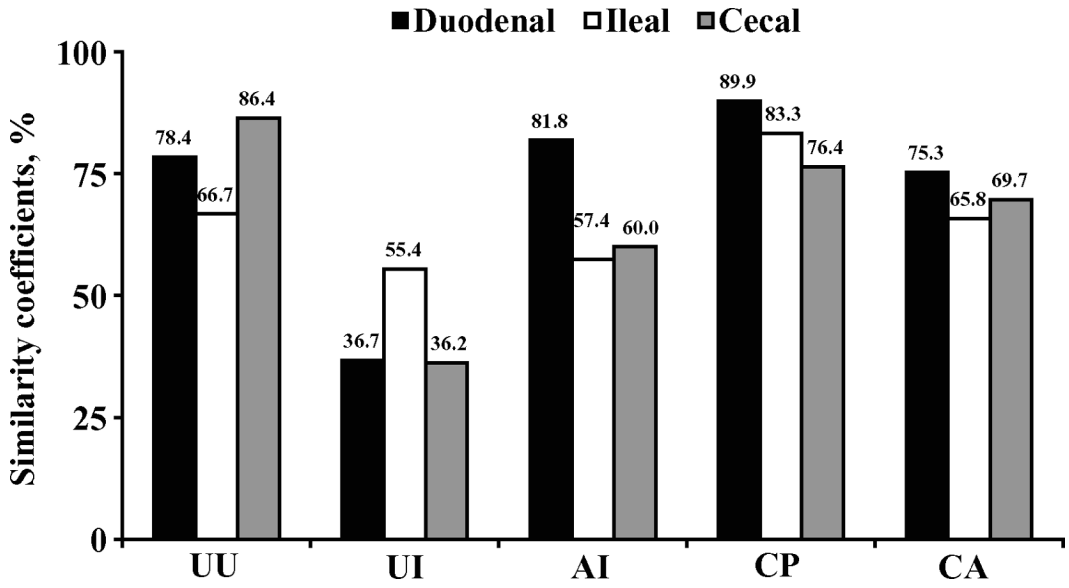

Figure 3. Percentage of similarity coefficients from comparisons of microbial communities in pre- and postchallenge samples within each treatment and intestinal compartment. UU = unmedicated uninfected control; UI = unmedicated infected control; AI = bacitracin methylene disalicylate at 50 g/ton and monensin (Coban 60, Elanco Animal Health, Greenfield, IN) at 90 g/ton; CP = essential oil (EO) blend Crina Poultry (Akzo Nobel Surface Chemistry LLC, Chicago, IL); and CA = EO blend Crina Alternate (Akzo Nobel Surface Chemistry LLC).

SC) from the other treatment groups (Figure 1, panel B). Chickens fed CA had ileal MC that were practically the same (96.3% SC) as those hosted by the UU control group. Broilers fed diets supplemented with CP had different cecal MC (Figure 1, panel C) than the other treatments (79.6% SC). The group fed AI diets and the group fed CA had very similar cecal MC (92.0% SC), which also were similar to the UI and UU controls (89.5 and 87.2% SC, respectively).

Although the UI and UU groups were both raised in batteries and treated the same until challenged, some differences in MC were observed between those 2 groups. This dissimilarity may be due to the high variability among individuals as they age and differences in feed and water consumption (Van der Wielen et al., 2002; Hume et al., 2003; Lu et al., 2003; Apajalahti et al., 2004). The MC in broilers raised in the floor pens (UUFp) were taken just before challenge and analyzed to observe the effect of housing (litter vs. cages) and stress of relocation on gut MC.

Postchallenge Period in Birds at 26 d of Age

The mixed *Eimeria* spp. challenge, along with changes brought on by the increased age of the birds, caused complete shifts in duodenal, ileal, and cecal MC (57.6, 74.0 and 69.8% SC, respectively) with comparisons to prechallenge samples. Duodenal MC in chickens fed AI diets were very similar (91.2% SC) to those in chickens fed CP, whereas these 2 groups were somewhat similar (81.3% SC) to MC in chickens given CA. However, ileal and cecal MC in chickens fed AI diets were either very different (64.4% SC in ilea) or similar (84.4% SC in ceca) to those given the other treatments. Chickens fed the EO blend CP had ileal and cecal MC that were similar (83.3 and 86.5% SC in ilea, respectively; 89.5 and 91.5% SC in ceca, resepctively) to the UU control treatments. Additionally, the MC from chickens fed CP were similar to the UU control treatment in ileal and cecal gut sections (86.7 and 91.5% SC, respectively). These relatively high similarities indicate some amount of modulation of the microbial ecology by these EO blends. Infection without anticoccidial treatment resulted in dramatic shifts in MC in all 3 compartments from those seen in chickens given the EO blends, AI diets, or who were untreated (Figure 2). The AI treatment in the ilea had an effect on the MC and resulted in a 64.4% SC to the other treatment groups.

General Comparison

Every treatment in the current study had a different effect over pre- and postchallenge MC in each section of the intestinal tract. Although the mixed coccidia challenge was associated with the greatest relative shifts in the postchallenge MC in all 3 sections of the intestine, independent of the treatment (UI), only in cecal samples was it possible to observe a clear difference between pre- and postchallenge MC for all treatments as well as the effect of coccidia challenge (UI; Figure 3).

Mechanisms involved in cecal and intestinal coccidiosis may be different (Gabriel et al., 2003; McDougald, 2003). Apajalahti et al. (2004) demonstrated differences in MC in ileal and cecal contents 7 d after an *E. maxima* challenge. In the experiment described here, each of the 3 *Eimeria* species included in the challenge inoculum affected each section of the intestine in a different manner (McDougald, 2003). The results observed in each intestinal compartment may be an effect of treatment in the previous compartment. Hume et al. (2003) discussed that similarities

in MC between adjacent digestive compartments are expected. It can be argued that the effects of coccidia on gut MC should be studied with individual species of *Eimeria*. However, all commercial vaccines available, at the time of this experiment, included at least the 3 *Eimeria* species used in this experiment (Williams, 2002) to address the lack of cross-immunity (McDougald, 2003; Dalloul and Lillehoj, 2005). Therefore, changes in MC and interactions with coccidia infection and immunomodulation were studied under conditions of mixed infection to be of practical application in the broiler industry.

The PCR-based DGGE methodology was useful in tracking shifts in MC caused by feed additives and *Eimeria* challenge. It was helpful to observe MC similarities across treatments and correlate them with some host responses. However, this methodology based on *16S* gene amplification has limitations to quantify and estimate true diversity when several amplicons of varied G + C content and primary sequences comigrate in the denaturing gel and also to detect minority populations that make up <1% of the total MC (Muyzer et al., 1993; Hume et al., 2003; Holben et al., 2004). In spite of these limitations, the technique is useful for studying the dynamics of microbial ecology, understanding changes in MC, and potentially pinpointing possible unknown bacteria involved in a complex infection similar to the one simulated in this experiment. Some specific bands visualized in the gels evaluated in the present experiment are candidates to search for MC correlated with differences in performance under these stress conditions. The cloning and sequencing of these individual fractions may help to identify specific taxa of interest (Apajalahti et al., 2004; Holben et al., 2004). On the other hand, due to the multiplicity of host-parasite interactions involved in the final response of the host, it is important to include markers of bacterial and host metabolism (Apajalahti et al., 2004) and mucosal immunity responses (Morris et al., 2004) to improve the understanding of this complex interaction between microbial ecology and coccidian pathobiology.

The present experiment indicated that feed additives modulate MC in coccidial challenges, although they do vary in their influences over MC in each intestinal compartment. Under the conditions of the present experiment, the specific EO blends CA and CP appear to be effective in modulating MC and avoid drastic changes in MC after a mixed coccidia challenge.

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