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IMMUNOLOGY, HEALTH, AND DISEASE

Intestinal Microbial Ecology of Broilers Vaccinated and Challenged with Mixed *Eimeria* Species, and Supplemented with Essential Oil Blends

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ABSTRACT Intestinal microbiota is an important component in the development of defense mechanisms in the gut mucosa. This project determined the dynamics of intestinal microbial communities (MC) of broilers vaccinated at first day of age with live oocysts of *Eimeria* species and fed diets supplemented with 2 specific essential oil (EO) blends, Crina Poultry (CP) and Crina Alternate (CA). Five treatments were analyzed: 1) unmedicated-uninfected (UU) control; 2) unmedicated-infected (UI) control; 3) vaccinated with Advent cocci-vaccine and without feed additive (COV) supplements; 4) vaccinated with Advent and supplemented with CP; and 5) vaccinated with Advent and supplemented with CA. The EO blends were added at 100 ppm to the same basal diets. Chicks were gavage-infected 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 the infection and 7 d after the challenge, pooled in 6 samples, and frozen. 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 amplicon patterns indicated MC differences due to intestinal location, feed additives, and cocci infection. The EO blends CP and CA did affect MC in all gut sections. The cocci-infection caused drastic MC population shifts in duodenal, ileal, and cecal sections (36.7, 55.4, and 36.2% SC, respectively). The CP-supplemented birds had higher SC between pre- and postchallenge MC in duodenal and ileal (73.3, 81.8%) than COV (66.4, 66.5%). However, COV broilers had the smallest changes in cecal MC after infection (79.5% SC). We concluded that cocci-vaccination causes small changes in intestinal MC, but challenge causes drastic shifts. The EO blend supplementation modulates MC in cocci-vaccinated broilers, avoiding drastic shifts after a mixed coccidia infection. Correlations between MC dynamics and host responses are discussed.

Key words: microbial ecology, *Eimeria* species, essential oil, cocci-vaccination

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INTRODUCTION

Digestive microflora populations affect broiler performance and health (Apajalahti and Bedford, 1999). These effects may be due primarily to the complex interactions that influence the intestinal environment and the development and responses of the host immune system against pathogenic and nonpathogenic antigens (Cebra, 1999; Kelly and Conway, 2005). The understanding and monitoring of the dynamics of gut microbial ecology are important to the development of alternative methods to modulate the microbial communities (**MC**) under situations of debilitating stress and disease, such as during coccidial infection in poultry.

Coccidiosis is one of the most endemic enteric diseases in broiler production. Additionally, coccidiosis is responsible for greater than $1.5 billion in annual losses to the poultry industry worldwide due to weight loss and poor feed use (Williams, 1999; Dalloul and Lillehoj, 2005). Currently, coccidia vaccines against *Eimeria* species infection in broilers have been developed to address the inefficiency of chemotherapy against coccidia and concerns toward antibiotic resistance (Williams, 2002; McDougald, 2003; Dalloul and Lillehoj, 2005). Nutrient immunomodulation, feed additives, and maintenance of normal gut flora are important considerations to obtain better responses to cocci-vaccination in broilers and to minimize the deleterious effects of coccidiosis (Dalloul and Lillehoj, 2005).

Feed composition and feed additives play significant roles in the modulation of gut microflora (Apajalahti et al., 2001; Apajalahti et al., 2004; Guo et al., 2004). One of those feed additives, essential oil (**EO**) blends, has shown promising results toward the reduction of *Clostridium perfringens* colonization and proliferation

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(Mitsch et al., 2004). Specific blends of EO appear also to control coccidia infection (Saini et al., 2003a) and consequently may help to reduce necrotic enteritis (**NE**; Saini et al., 2003b). Secondary enteritis in broilers might be caused by several factors including poor hygiene, management of bedding material, poor ventilation, draught, drastic changes in feed composition and low quality feed, or a combination of these. However, coccidial stress consistently has been shown to sensitize broilers to enteritis including NE (Williams, 2002; Van Immerseel et al., 2004).

Recently, molecular techniques have been used to profile intestinal MC in poultry (Apajalahti et al., 2001, 2004; Van der Wielen et al., 2002; Hume et al., 2003; Amit-Romach et al., 2004). This approach has allowed the evaluation of changes in MC caused by several factors including age, diet composition, management, and stress (Apajalahti et al., 2004). The 16S rDNA-based denaturing gel electrophoresis is applied in the current study to monitor the effects of feed additives and vaccination with live oocysts on gut MC to correlate responses under coccidia challenge with broiler performance. An eventual goal of this baseline study is to identify MC that may be correlated with enteric problems such as NE. The present project determined the dynamics of gut microbial ecology during pre- and postperiods of a mixed coccidia infection in broiler chickens vaccinated against *Eimeria* species and fed diets supplemented with specific EO blends.

MATERIALS AND METHODS

Animal Husbandry

All procedures involving animals were approved by the Stephen F. Austin State University Institutional Animal Care and Use Committee. One hundred eighty 1-d-old Cobb-500 male chickens were placed in 30 floor-pens (6 birds/pen) in a tunnel-ventilated dark house and randomly assigned among 5 dietary treatment groups (6 replicates per treatment). Used litter, top-dressed with 2 inches of fresh pine wood shavings, was utilized as bedding. The previous flock housed in the facility was challenged with mixed coccidia oocysts (*Eimeria acervulina, Eimeria tenella*, and *Eimeria maxima*). Broilers were fed with starter (1 to 13 d) and grower (13 to 33 d) diets in the form of crumbles and pellets, respectively. Diets 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 that were not cocci-vaccinated included groups: 1) the unmedicated-uninfected (**UU**) control, and 2) the unmedicated-infected (**UI**) control. Chickens in the last 3 treatments were vaccinated at 1 d of age with Advent cocci-vaccine (Viridus Animal Health LLC—Novus International Inc., St. Louis, MO) by spray in an automatic cabinet. These coccidia-vaccinated groups (**COV**) were fed diets 3) without feed additives (**WFA**); or supplemented with EO, 4) CP, or 5) CA. These specific EO blends were added to the basal diets at 100 ppm. Birds were raised to 13 d in floor pens and then moved to battery cages (Petersime Incubator Company, Gettysburg, OH). This management was to guarantee that birds had natural contact with litter microflora and recirculation of vaccinal and field oocysts during the preinfection period. The challenge was accomplished in batteries to facilitate comparisons with UU and UI control groups. 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 field or vaccinal oocysts. One additional control group (**CVFp**) vaccinated with Advent and fed diets WFA was raised to 19 d of age in floor pens for comparison with the other treatments and to evaluate effect of transportation.

Mixed Eimeria Challenge

All broilers, except those in the UU treatment, were challenged at 19 d of age with a standard oral inoculum of sporulated oocysts from *E. acervulina, E. maxima*, and *E. tenella* at 2.0, 1.0, and 0.5×10^5 viable oocysts/mL, respectively. Duodenal, ileal, and cecal samples were collected within 10 min after chickens were euthanized and were frozen in liquid nitrogen and kept at –70°C until analyses were performed.

Denaturing Gradient Gel Electrophoresis

Diversity of predominant digestive MC was determined by performing denaturing gradient gel electrophoresis (**DGGE**) of 16S ribosomal RNA (**rRNA**) gene PCR amplicons with modification of the methods of Don et al. (1991), Muyzer et al. (1993), and Hume et al. (2003). Template DNA was isolated from duodenal, ileal, and cecal contents of 12 chickens in each group. Contents from 2 chicks per pen were pooled before DNA isolation leaving a total of 6 pooled samples per treatment group. Following DNA isolation, 42 ng of DNA from each pool were combined for PCR.

Band patterns were analyzed for percentage of similarity coefficient, and dendrograms were constructed using Pearson product-moment correlation coefficient and unweighted pair group method using arithmetic averages (UPGMA) options in Molecular Analysis Fingerprinting Software, version 1.6 (Bio-Rad Laboratories, Hercules, CA).

RESULTS AND DISCUSSION

Intestinal compartment-specific factors play important roles in the development of broiler microbial communities (Van der Wielen et al., 2002; Hume et al., 2003) as well as site specificity exhibited by *Eimeria* species (McDougald, 2003), whether encountered in the form of vaccine, experimental challenge inoculum, or as the result of natural infection. A molecular ecological approach (DGGE) was used in the current study to ex-

amine the digestive microbial composition and to determine community succession in the duodenum, ileum, and cecum of broilers infected with *Eimeria* species oocysts by vaccination or infection or both, and fed a corn-soybean meal diet WFA or supplemented with 2 specific EO blends.

Prechallenge Period

The MC in the samples of broilers raised on the floor pens (CVFp) were collected just before the coccidia infection and were analyzed to observe the effect of housing (litter vs. cages) and stress of relocation on gut MC. The MC of CVFp duodenal samples were not too similar (79.9% SC) to MC from vaccinated broilers (COV) transferred to cages at 13 d, indicating that removing broilers from the litter into cages modifies microbial ecology.

The DGGE is examining the effects of nutritional and environmental factors on the makeup of microbial populations. Inherent to these population-altering factors are biochemical communications between microflora and eukaryotic host enterocytes and cross-talk between bacterial cells. Key elements stimulating and sustaining this intercellular communication are the nutritional signals detected by bacterial and host cells. A healthy plateau for the host animal is experienced during the establishment of luminal conditions supportive of a healthy microflora exclusive of bacteria threatening to a healthy mucosal integrity or disruptive of a healthy protective microflora. The technique does distinguish litter effects when comparisons are made against animals not raised on litter. However, differences caused by rearing environment may be neutralized or affected by contact with microflora contained in feed, air, and water, and through contact with equipment and handlers.

Two main comparative clusters were observed as a result of the effects of treatments on the MC in the duodenum (Figure 1a). Broilers from the control UI group had MC different from the other treatments (65.1% SC). All the other treatments, including the UU control resulted in MC that had some similarity (71.8% SC) to each other. In ileal contents (Figure 1b), the treatment groups vaccinated and supplemented with EO had very similar MC (93.3 %). The UU negative control treatment and the cocci-vaccinated broilers fed WFA diets had practically the same MC (94.5%), whereas UI and CVFp chickens had some similarity but had very different MC than other treatments (69.1%). Cecal MC from CVFp broilers were very similar (92.4%) to the MC in broilers vaccinated and supplemented with CA. The similarities observed among the MC from CVFp broilers and those from broilers fed diets supplemented with CA likely indicate a modulating effect of EO on the cecal environment in broilers moved to cages. Microbial communities in ceca (Figure 1c) were less affected by treatments, with an 85.9% cluster SC, than duodenal and ileal MC. The vaccinated treatment fed diets WFA had cecal MC that were the same or very similar (92.6%) to those in the UU controls.

Although the UI and UU groups were both raised in batteries and treated the same until the moment of the challenge, varying amounts of dissimilarity in MC were observed. This dissimilarity might be due to the high variability among individuals as they age, differences in feed and water consumption, and location within the batteries (Van der Wielen et al., 2002; Hume et al., 2003; Lu et al., 2003; Apajalahti et al., 2004).

Postchallenge Period

The mixed *Eimeria* species challenge caused complete shifts (49.6% SC) in duodenal MC (Figure 2a). Another cluster (80.5% SC) contained UU and broilers vaccinated and fed EO or WFA diets. Chickens fed WFA diets hosted similar MC (89.1% SC) to those observed in cocci-vaccinated birds fed both EO. However, it is important to mention that the vaccinated treatment and the one fed CA were the only ones to have significantly better ($P < 0.01$) feed conversion ratio than the UI groups (unpublished data).

In the ileal section, 2 main clusters with 67.5% SC were observed after mixed coccidia infection (Figure 2b). The ileal MC in the UI control were similar to those in vaccinated birds (80.5% SC) fed diets WFA. The treatments fed EO had ileal MC that were either similar or very similar (83.5 or 90% SC, respectively) with those in chickens from the UU control treatment. These high similarities indicate some type of modulation of the microbial ecology by these EO.

Challenge caused a relatively large shift in cecal MC (Figure 2c), resulting in the UI group being located separate from the main cluster (65.6% SC) containing the remaining profiles. The vaccinated groups given diets without feed additives (COV) were similar (84% SC) to both EO groups and the UU group. However, chickens fed CP hosted cecal MC that were closely related (91% SC) to the cecal MC found in UU. These observations suggest that CP was able to modulate cecal MC during the challenge and supported the maintenance of a MC similar to the ones observed in UU controls.

General Comparisons

Similarity coefficients were determined for MC in pre- and postinfection samples within each treatment (Figure 3). Every treatment had a different effect over MC in each section of the intestinal tract. Differences seen in MC for the treatment groups must take into consideration changes that occur as the broilers age and with maturation of the digestive microflora (Hume et al., 2003; Apajalahti et al., 2004). Stresses related to transport from floor pens to cages appear to have had comparatively minimal effect on MC status in broilers cocci-vaccinated at 1 d of age. However, it is important to take in consideration that samples analyzed in this experiment were taken at 19 d of age, when at least 2 *Eimeria* life cycles might have occurred (Williams, 2002; McDougald, 2003). Additionally, the host may have

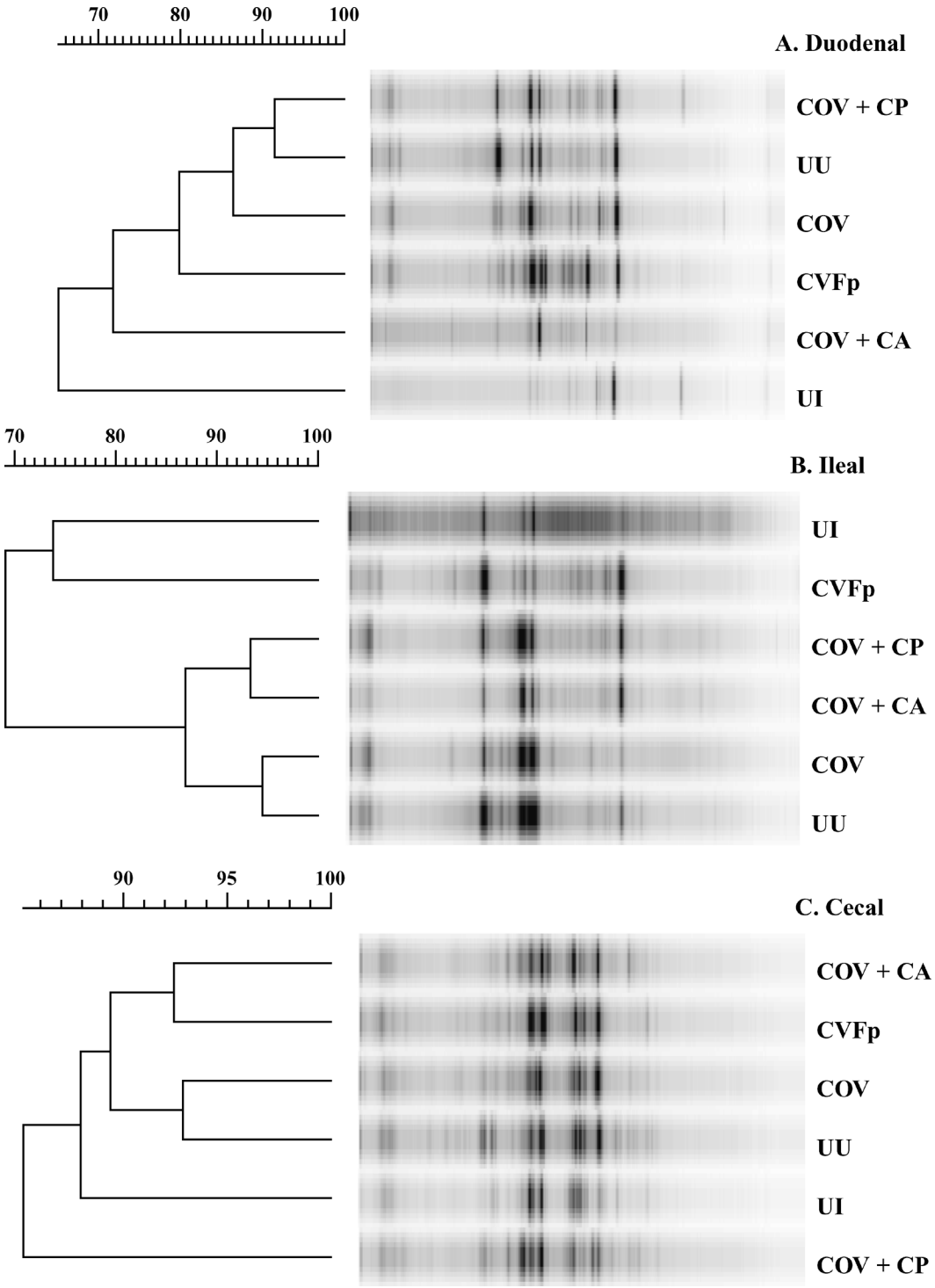

Figure 1. Denaturing gradient gel electrophoresis of A) duodenal, B) ileal, and C) cecal microbial communities from broiler chickens at 19 d of age (preinfection). Relative similarity of band patterns is indicated by their grouping on the dendogram and the percentage similarity coefficient (bar). UU = unmedicated-uninfected control; UI = unmedicated-infected control; COV = coccidia-vaccinated with Advent (Viridus Animal Health LLC—Novus International Inc., St. Louis, MO); CP = essential oil blend Crina Poultry; CA = essential oil blend Crina Alternate; CVFp = cocci-vaccinated floor pen.

adapted to the moderate infection caused by vaccination through crypt hyperplasia and increased turnover of epithelial cells (Morris et al., 2004). These host responses might help to balance the gut ecosystem. Apajalahti (2004) suggested that under commercial conditions, the changes in MC caused by *E. maxima* infection are continuous and less acute because oocyst recirculation and the host immune and physiological responses affect the gut environment. Apajalahti (2004) also presented data that showed that MC shifts caused by *E. maxima* challenge are temporary. The long-term deleterious effects observed in growth of cocci-infected broilers might be due

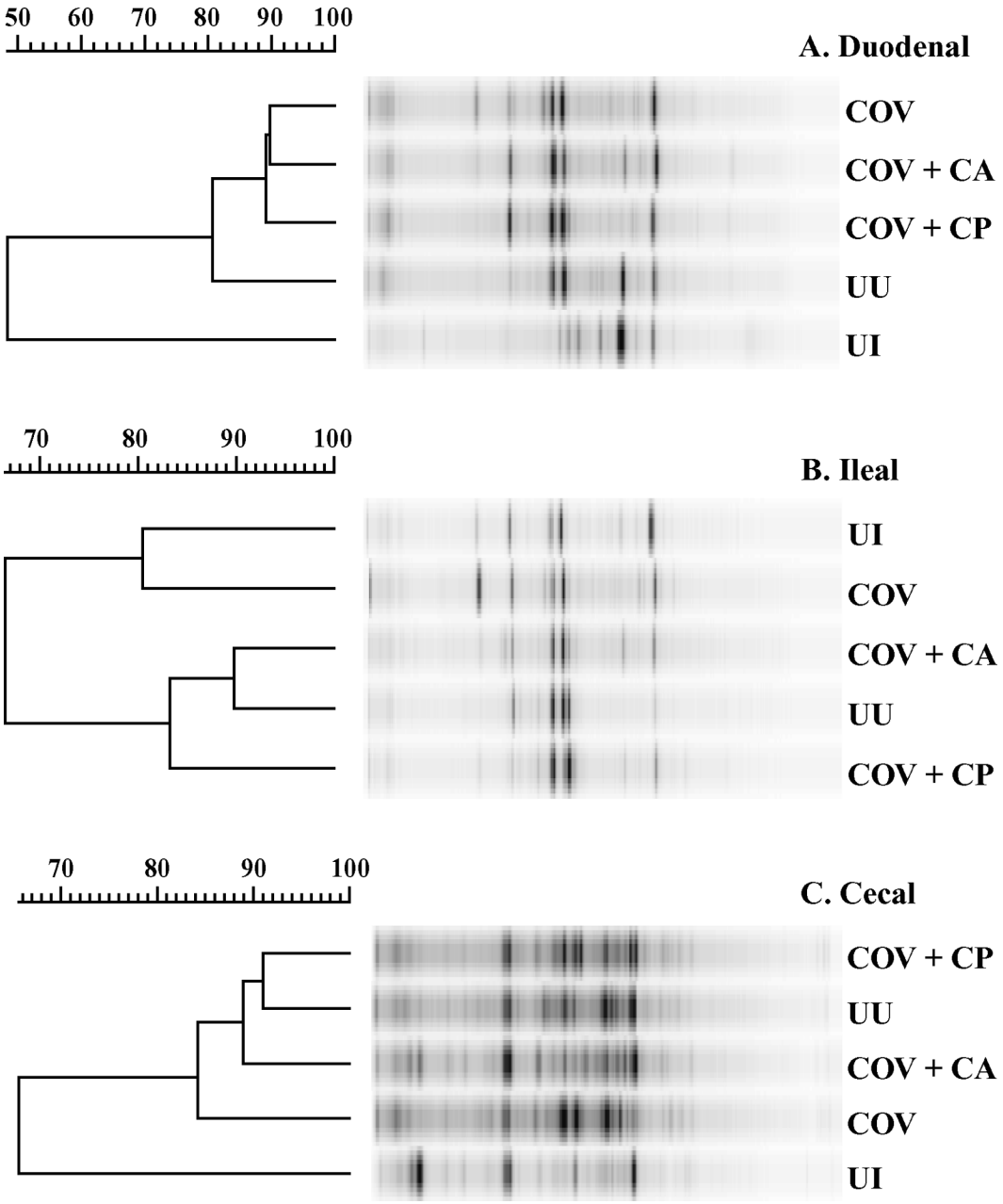

Figure 2. Denaturing gradient gel electrophoresis of A) duodenal, B) ileal, and C) cecal microbial communities from broiler chickens 7 d after mixed *Eimeria* species infection (26 d of age). Relative similarity of band patterns is indicated by their grouping on the dendogram and the percentage similarity coefficient (bar). UU = unmedicated-uninfected control; UI = unmedicated-infected control; COV = coccidia-vaccinated with Advent (Viridus Animal Health LLC—Novus International Inc., St. Louis, MO); CP = essential oil blend Crina Poultry; CA = Essential oil blend Crina Alternate.

to the temporary reduction in feed intake (McDougald, 2003) and redirection of nutrients to generate the immune and compensatory physiological response (Klasing and Calvert, 2000; Barnes et al., 2002).

Each of the 3 *Eimeria* species included in the vaccine and challenge inoculum affects each section of the intestine in a different manner (McDougald, 2003). The results in the current experiment observed in each intestinal compartment might be affected by the effect caused by 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 over gut MC should be studied with individual species of *Eimeria.* However, all commercial vaccines available include at least the 3 *Eimeria* species used in this experiment (Williams, 2002) to address the complication of lack of cross-immunity (McDougald, 2003; Dalloul and Lillehoj, 2005). Therefore, changes in MC and interactions with coccidia infection, vaccination responses, and immunomodulation were studied under conditions of mixed infection to be of practical application in the broiler industry.

The 2 EO blends appeared to influence MC in vaccinated birds. Cocci-vaccinated broilers fed CA were the

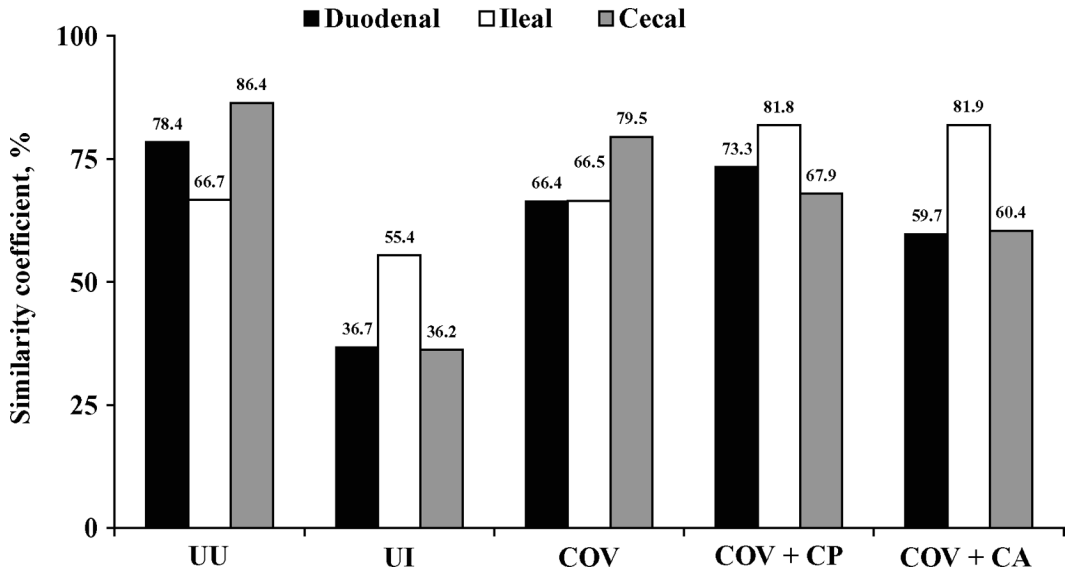

Figure 3. Similarity coefficients (%) comparing microbial communities in pre- and postchallenge samples within each treatment and intestinal compartment. UU = unmedicated-uninfected control; UI = unmedicated-infected control; COV = coccidia vaccinated with Advent (Viridus Animal Health LLC—Novus International Inc., St. Louis, MO); CP = essential oil blend Crina Poultry; CA = essential oil blend Crina Alternate.

only group that hosted duodenal MC very similar to those in the vaccinated group fed WFA diets (90.1 to 94.2% SC). These results indicate that all treatments had some effect over MC at the duodenal level. The EO blend CP was the feed additive with the highest modulation between pre- and postinfection in duodenal and ileal samples, respectively (Figure 3). Although, the duodenal and ileal MC of vaccinated birds fed WFA diets after the challenge were not similar to those of broilers in the UU control group, those vaccinated broilers had the best BWG and feed conversion ratio, and the lowest lesion score and oocyst indexes (data not shown). However, these birds had the lowest variation between pre- and postchallenge samples (79.6%) in cecal samples (Figure 3). An improvement in immunological responses of these broilers might have caused more favorable conditions that allowed the maintenance of a more stable cecal MC. In contrast, the cocci-vaccinated broilers fed diets supplemented with the EO CA had more negative performance than the cocci-vaccinated broilers fed WFA diets. The cecal MC of cocci-vaccinated broilers fed CP and CA diets changed more due to the challenge (67.9% SC for CP and 60.4% SC for CA), but MC in the ileal sections remained more stable (81.8% SC for CP and 81.9% SC for CA) compared with the group fed WFA diets (66.5%). More drastic changes in MC between pre and postchallenge cecal samples are correlated with lower host performance 7 d after the challenge.

The PCR-based DGGE methodology used in this experiment was useful to track shifts in MC caused by feed additives and 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 may co-migrate in the denaturing gel, and also to detect minority populations that make up less than 1% of the total MC (Muyzer et al., 1993; Hume et al., 2003; Holben et al., 2004). Underlying limitations are the inability of the technique to distinguish bacteria that are not represented in high numbers. Many of the undistinguished bacteria may play larger roles in helping to establish and maintain microenvironments supportive of majority populations. Additionally, the underrepresented and undistinguished organisms may potentially contribute by-products and metabolites that affect luminal and mucosal biofilm microbial populations and microbial interactions, as well as microbial metabolites that allow communication between intestinal bacteria and host cell systems.

The methodology used in this experiment does not by itself distinguish between native and transient organisms because the total contents were collected and include some digesta. This lack of discrimination may be considered a limitation of DGGE. Another potential limitation is that the technique does not distinguish bacteria by genus or species. The DGGE method would rely on additional techniques (e.g., cloning and fragment sequencing) to determine the identities of individual bacteria and to distinguish resident from transient bacteria.

In spite of these limitations, the technique is useful for studying the dynamics of microbial ecology and helps to understand the changes in MC and potentially pinpoint possible unknown bacteria involved in a complex infection, such as the infection simulated in the current experiment. Some specific bands visualized in the gels evaluated in the present experiment are potential candidates to search for MC correlated with differences in broiler performance under these stress conditions. The cloning and sequencing of these individual fractions can 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 bacteria and host metabolism (Apajalahti, 2004) and mucosal immunity responses (Kelly and Conway, 2005; Morris et al., 2004) to improve the understanding of this complex interaction between broiler physiology, microbial ecology, and coccidial pathobiology.

The present experiment indicates that cocci-vaccination with viable attenuated oocysts by itself causes small changes on intestinal MC, and that stresses and coccidia infection result in drastic MC shifts. Feed additives such us these specific EO blends CA and CP modulate MC in coccidial challenges and avoid drastic changes in MC after a mixed coccidia infection independent of vaccination, but they vary in their influences over MC in each intestinal compartment. The microbial ecology dynamics of the cecal compartment seem to be more related to the final broiler performance under conditions of stress.

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