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Abundance of pathogens in the gut and litter of broiler chickens as affected by bacitracin and litter management

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ABSTRACT

Clostridium perfringens, *Salmonella* spp. and *Campylobacter* spp. are food-borne enteric pathogens that are commonly associated with poultry. The objective of this study was to investigate the effects of supplemental bacitracin and litter management (fresh vs. reused) on the abundance of these pathogens in commercial broiler chickens. Specific quantitative PCR (qPCR) assays were used to quantify *C. perfringens*, virulent *C. perfringens* that carried the genes encoding α-toxin (*cpa*) and NetB-toxin (*net*B), *Salmonella*, and *Campylobacter* in samples of ileal mucosa, cecal content, and litter. *Campylobacter* was not detected in any of the samples collected. The abundance of *Salmonella* was not affected by either bacitracin or litter condition. Generic *C. perfringens* was detected in the ileal mucosa at very low level at 10 days of age but was much higher at 35 days. Chickens reared on reused litter tended to have a lower abundance of generic *C. perfringens* compared with those reared on fresh litter. In the ileal mucosa, no *cpa* or *net*B was detected at day 10 but was detected at day 35 in the chickens that were not fed supplemental bacitracin. Chicks fed supplemental bacitracin had reduced abundance of generic *C. perfringens* as well as the *cpa* and *net*B genes in the ileal mucosa, cecal content, and litters. A strong positive correlation was found between the abundance of all three measurements of *C. perfringens*. The abundance of *Salmonella* spp. and *C. perfringens* was also shown to be correlated. This is the first study that has examined the effect of dietary bacitracin and litter conditions on the prevalence of these three common enteric pathogens. Unless contaminated from previous flocks, reused litter may not necessarily contain significantly greater abundances of *C. perfringens* or *Salmonella*.

1. Introduction

Species of *Campylobacter* and *Salmonella*, and *Clostridium perfringens* are important bacterial pathogens associated with chickens. These pathogens are typically carried in the gut of chickens but are also found in poultry products (Golden et al., 2009; Melero et al., 2012; Temelli et al., 2012). When consumed with poultry meat or other poultry products, these pathogens can cause serious infection (Altekruse et al., 1999; Warrell, 2003; Adzitey et al., 2012). In addition, both *C. perfringens* and *Salmonella* can infect chickens and cause considerable economic losses via increased mortality and reduced growth. For example, *C. perfringens* can cause necrotic enteritis, an costly enteric disease among young chicks and turkey (Van Immerseel et al., 2004). *Salmonella* can also cause mortality and reduced growth in young chicks.

In modern broiler and turkey production, antimicrobial growth promoters (AGP) have been used to prevent enteric diseases and enhancing performance (Coates et al., 1963; Miles et al., 2006). However, there is increasing concern and often contentious debate over the potential risk posed by increased development and dissemination of antimicrobial resistance (Witte, 2000; Hammerum and Heuer,

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2009; Marshall and Levy, 2011). Non-antibiotic alternatives are being evaluated to replace, at least partially, the AGPs (Huyghebaert et al., 2011). In a previous study, we reported that reused litter resulted in more intestinal bacteria of gut origin while fresh litter resulted in more gut bacteria of environmental origin (Cressman et al., 2010). Reused litter also increased coliform levels and coccidial outbreaks in poultry flocks (Stanley et al., 2004) and increased intestinal inflammatory response (Shanmugasundaram et al., 2012). In a preliminary study, we showed that reused litter effectively delayed intestinal *C. perfringens* colonization in chicks at 7 days of age. The objective of this study was to investigate the effects of litter management (reused; fresh) and supplemental bacitracin on abundance of *C. perfringens* (both generic and toxin-producing), *Campylobacter*, and *Salmonella* in broiler chickens.

2. Materials and methods

2.1. Animals and in vivo study design

The feeding experiment used a 2 × 2 factorial arrangement of treatments: litter management (fresh vs. reused litter) and dietary bacitracin (with vs. without) (Shanmugasundaram et al., 2012). The fresh litter came from fresh pine shaving and the reused litter was litter recycled from previous flocks in the same chicken house. Newly hatched chicks (1 day old) were randomly assigned to one of four treatment combinations: fresh pine shavings (referred to as fresh litter) and no dietary bacitracin (NF); fresh litter and bacitracin at 0.04% of feed (BF); reused litter and no dietary bacitracin (NR); reused litter and 0.04% dietary bacitracin (BR). Each treatment combination was consisted of six replicate pens with 36 chicks per pen at the start of the study. The eggs from which the chicks were hatched were not disinfected. In the hatchery, all chicks were subjected to the anti-coccidial spray vaccine (Coccivac-B, Schering-Plough Animal Health) at 87% of the recommended spray dose (21 ml per 1000 chicks). Chickens were fed standard corn–soybean-meal-based diets which met the NRC (1994) suggested nutrient levels. Ileal mucosa and cecal content samples were collected from five birds per pen at 10 and 35 days of age. The ileal mucosa samples were collected from the region between Meckel's diverticulum and the ileocecal junction, and cecal content samples were collected from each bird and pooled by pen as described by Cressman et al. (2010). Subsamples of litter were collected on day 35 from each of four locations within a pen: the brood area, beneath the water and feed lines, and along the walls and pooled to represent the average litter condition of each treatment. The study was repeated over three flocks with the litter being completely replaced in the fresh litter treatments. During the study all the birds were cared for following a protocol approved specifically for this study by the Institutional Animal Care and Use Committees of the Ohio State University.

2.2. DNA extraction

Microbial community DNA was extracted from the mucosal, cecal, and litter samples using the RBB + C method (Yu and Morrison, 2004). The DNA quality was evaluated using agarose gel (0.8%) electrophoresis, and DNA concentrations were quantified using a NanoDrop ND-1000 spectrophotometer (Thermo Scientific, Wilmington, DE). The concentration of an aliquot of the extracts was adjusted to 100 ng/μl using Tris–EDTA (TE, pH 8.0) buffer prior to PCR amplification. The DNA was stored at −20 °C before use.

2.3. Screening for enteric pathogens

Primer sets specific for the *inv*A gene of *Salmonella* spp., the 16S rRNA gene of the genus *Campylobacter*, the 16S rRNA gene of species *C. perfringens*, and the *cpa* gene and *net*B gene of virulent strains of *C. perfringens* were used to screen all the samples for the presence of the above pathogens using endpoint PCR amplification (Table 1). For each PCR reaction, 0.5 μl DNA (100 ng/μl) was added to a 24.5 μl master mix that contained 1× PCR buffer, 1.75 mM $MgCl_2$, 670 ng/μl bovine serum albumin, 200 μM dNTP, 500 nM of each primer, and 0.625 U Platinum Taq high-fidelity polymerase (Invitrogen Corporation, Carlsbad, CA). The PCR thermal program consisted of an initial denaturation step at 94 °C for 5 min; 40 cycles of a 30 s denaturation step at 95 °C, a 30 s annealing step at respective temperature (Table 1), and a 40 s elongation step at 72 °C; and a final extension step at 72 °C for 7 min before a 4 °C hold. The expected PCR amplification products were confirmed by agarose gel (1.0%) electrophoresis. No-template control and positive control samples were included in all the PCR screening.

2.4. Quantitative real-time PCR (qPCR)

The genomic DNA of *Salmonella enterica* and *Campylobacter jejuni* was used to amplify the gene fragment of *inv*A and 16S rRNA of *Salmonella* and *Campylobacter*, respectively, using their specific primer set (Table 1). Fragments of the 16S rRNA gene of species *C. perfringens*, *cpa* and *net*B of virulent strains of *C. perfringens* were amplified using each respective specific primer pair from the metagenomic DNA samples that were positive for each target (Table 1). Each PCR product was purified using a QIAquick PCR Purification kit (QIAGEN Inc., Valencia, CA) and then cloned using a TOPO TA cloning kit for sequencing (Invitrogen Inc., Carlsbad, CA). For each pair of primers, one clone was selected and linearized by *Nco*I restriction enzyme (New England Biolabs Inc., Ipswich, MA) that only cuts the vector region. Each of the linearized recombinant plasmids was purified using ethanol precipitation and dissolved in TE buffer. The concentration of each qPCR standard was determined using the Quant-it Kit (Invitrogen Corporation, Carlsbad, CA, USA). Copy-number concentration was calculated from the mass concentration and the length of each standard. Serial dilutions (1:10) were made in TE buffer prior to each qPCR assay. Each of the qPCR standards was also used as the positive control in the screening experiment (Section 2.4).

Each qPCR assay was performed using respective specific primers (Table 1) as described previously (Chen et al., 2007). Briefly, 0.5 μl of 100 ng/μl metagenomic DNA