Microbial Ecology Shifts in the Ileum of Broilers During Feed Withdrawal and Dietary Manipulations

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ABSTRACT

Broilers are withheld from feed for 8 to 24 h before processing to empty the gastrointestinal tract and reduce potential carcass contamination from gastrointestinal tract contents. Intestinal microbial changes during feed withdrawal (FW) have not been thoroughly defined. Two experiments were conducted to examine the effects of diet and FW on the microbial ecology in the small intestine. In experiment 1, 42-d-old broilers were fed diets containing no additive (control), 250 ppm of CuSO₄, or bacitracin (BMD; 30 ppm) and were also subjected to FW for 0, 10, and 24 h. Six birds from each dietary treatment were killed at each FW time point and ileal mucosa and digesta were collected. Microbial communities were determined by isolating bacterial DNA, amplifying the V3 region of 16S ribosomal DNA, and performing denaturing gradient gel electrophoresis. The mucosal microbial profiles from birds at 0 h of FW had higher similarity values than those at 24 h of FW, indicating that as FW time increased, uniformity of intestinal microbial populations decreased. Numbers of bands (an indicator of numbers of bacterial species present) at 0 h (9.38) were greater than those at 10 and 24 h (5.39 and 5.78, respectively), suggesting a reduction in microbial species and diversity as FW time increased. Copper-fed birds had greater similarity coefficients than either the control or BMD-fed birds, but BMD-fed birds had greater band numbers. No interaction between diet and FW was observed and no digesta differences were observed. In experiment 2, 62-d-old birds fed corn-soy diets in floor pens were subjected to 0, 8, 12, and 24 h of FW. Ileal mucosal tissue was collected and analyzed as in experiment 1. Mucosal microbiota similarities were greater at 0 h of FW than at 8, 12, or 24 h of FW and band numbers were reduced between 0 and 24 h of FW. Data from these studies suggest that FW and dietary treatments alter the microbial community of the intestine by decreasing bacterial diversity in the ileum.

Key words: antibiotic, broiler, copper sulfate, feed withdrawal, microbiota

INTRODUCTION

Carcass contamination in poultry processing plants poses both economic and product safety concerns, and occurs when the gastrointestinal tract (GIT) of the bird ruptures, causing the contents of the tract to come into contact with the carcass. Producers try to avoid this occurrence and thereby reduce carcass contamination in processing plants by withholding feed from broilers for 0 to 24 h before processing. This short period of feed withdrawal (FW) reduces the amount of ingesta in the GIT and also reduces the incidence of torn or ruptured gastrointestinal tracts, thereby decreasing the likelihood of carcass contamination (Wabeck, 1972; Bilgili, 1988; May and Deaton, 1989; Papa, 1991).

Although FW is one of the best known methods of decreasing contamination, it also causes increased pathogenic bacterial colonization of the broiler’s GIT. Research has demonstrated that healthy adult poultry are normally resistant to colonization by pathogens (Sadler and Edgar, 1968). However, there is increased Salmonella colonization of the GIT after a FW period (Ramirez et al., 1997; Corrier et al., 1999; Burkholder et al., 2003), suggesting that FW causes physical, chemical, and microbial changes in the GIT of chickens, which ultimately causes a breakdown in the body’s natural defense mechanisms. Additionally, birds left on litter after FW are prone to consume litter, thus increasing the possibility of greater Salmonella contamination of a GIT that is already compromised by fasting.

Although the effect of FW on intestinal characteristics has been well documented, there is a paucity of information regarding the effects of short-term FW on the microbial communities within the GIT. Hume et al. (2003) noted that molting for 10 d led to a significant reduction in the similarities of the cecal bacterial communities of molted and fed birds, an occurrence that indicates FW does affect the microbial communities. However, the effects of short-term FW periods are not well characterized, and it is of
interest to determine whether the alterations in bacterial communities observed in the studies of Hume et al. (2003) occurred over a short period relative to the 10-d FW. Additionally, because the indigenous microbiota protect against bacterial infection and contribute to intestinal function (Gong et al., 2002), any disturbances to the intestinal bacterial community during short-term FW may contribute to the increase in *Salmonella* colonization that occurs before processing.

In addition to measures taken to reduce carcass contamination during processing, other approaches have been used on the farm in an effort to reduce potential sources of carcass contamination before processing. Examples of such approaches include dietary additions of antibiotics and pharmacological levels of copper, which have been shown to influence the intestinal bacterial communities and improve growth.

In the past, direct-fed antibiotics have been the standard by which growth promotion and disease resistance were measured. Since the 1940s, antibiotics have been used as a method to enhance animal performance by increasing growth, improving feed efficiency, favorably altering intestinal bacteria, and reducing the incidence of disease. The addition of antibiotics to the diet may suppress the growth of known pathogens, an action that is useful for pathogen reduction before processing, and may diminish the potential for foodborne illnesses caused by carcass contamination at the plant. Supporting evidence for this can be found in the data of Bolder et al. (1999), who determined that dietary addition of flavomycin or salinomycin reduces the incidence of pathogens in preslaughter broilers while simultaneously reducing the degree and incidence of *Salmonella* shedding at 6 wk of age.

Because the future of antibiotics in agriculture is relatively uncertain at present, many other alternatives to antibiotics have been investigated. Among these is the use of pharmacological levels of dietary copper, which are known to have growth-promoting effects that are potentially mediated by alterations in the intestinal microbiota. Researchers hypothesize that copper favorably alters or inhibits certain bacterial populations within the intestinal microbiota, thus allowing for improved growth rates, although this theory has yet to be fully proven. This theory is based on evidence that copper inhibits bacterial growth and has a toxic effect on many microorganisms.

Currently, the effect of copper on bacterial communities has not been studied in any great detail. Various authors have reported that copper sulfate reduces intestinal populations of *Streptococcus* spp. (Fuller et al., 1960; Varel et al., 1987) and *Lactobacillus* populations while increasing coliform populations (Jensen, 1998). Other in vitro research has concluded that various bacterial species isolated from food animals are sensitive to copper sulfate as well (Aarestrup and Hasman, 2004).

Given the interaction between copper and bacteria, it is quite possible that pharmacological doses of copper do inhibit certain populations of bacteria, possibly reducing the pathogen load. Additionally, antibiotics may perform similarly, although the exact mechanisms are not currently understood. Regardless, bacterial shifts caused by dietary additives are not well characterized and warrant further investigation. Thus, 2 experiments were conducted to determine how short-term FW and dietary treatments affects gastrointestinal microbial communities.

**MATERIALS AND METHODS**

Two experiments were conducted to examine the effects of FW and diet on microbial communities within the GIT. All animal procedures were approved by the Purdue University Animal Care and Use Committee.

**Experiment 1**

**Birds and Experimental Design.** Ninety male Ross broilers were raised in floor pens (0.407 m²/bird) with wood shavings and ad libitum access to a standard industry corn-soybean meal diet and water. At 21 d of age, broilers were divided into 3 pens and fed 1 of 3 dietary treatments. A standard industry corn-soybean meal diet was mixed and divided into 3 separate portions. To the first portion, which served as the control, Sulkafloc (4.4 kg/909 kg; 0.5%) was added. Bacitracin, in the form of bacitracin methylene disalicylate (BMD, an antibiotic; 30 g/909 kg activity; Alpharma Inc., Fort Lee, NJ), was added to the second portion, and copper sulfate pentahydrate (250 ppm of Cu from CuSO₄; Potell Minerals and Ingredients, New Hamburg, Ontario, Canada) was added to the third portion. At 42 d of age, 6 birds from each treatment were randomly selected and killed with CO₂. Ileal digesta and mucosa were collected aseptically from 4 cm proximal to the ileocecal junction by obtaining 2 sections and squeezing the ileal digesta from the first section into a microcentrifuge tube. Ileal mucosa was collected by flushing the digesta from the second tissue section with PBS and squeezing the mucosa out of the tissue by moving a clean slide along the length of the tissue and into a microcentrifuge tube. Both tubes were immediately frozen at −20°C for future denaturing gradient gel electrophoresis (DGGE) analysis.

For the FW portion of the experiment, 24 birds (41 d of age) from each treatment were randomly selected and placed into 3 floor pens, for a total of 9 pens. Feed was removed from the first 2 pens (1 pen per dietary treatment) 24 h before sampling, and from the second pens 10 h before sampling, respectively. Birds remained on litter with access to water for the first 4 h of the FW period and were then placed in crates. At the conclusion of the trial, birds were killed with CO₂ and ileal mucosa samples were collected as described above. Digesta samples were collected but not analyzed because of the lack of any intestinal content at 10 and 24 h.

**DNA Isolation.** Ileal digesta and mucosa samples were thawed and homogenized with an UltraTurrax T25 Basic Homogenizer (Rose Scientific Ltd., Edmonton, Alberta, Canada), and genomic bacterial DNA was isolated by using an Ultraclean Fecal DNA kit (MoBio, Solana Beach,
of a 20% volume of 520 H9262 (vol/vol) 0.1 bromophenol blue, 0.025% (wt/vol) xylene cyanol, 47% phoresis in 0.5 System (Bio-Rad Laboratories, Hercules, CA) for electro-
were placed in a DCode Universal Mutation Detection
mix (dNTP); 520 H9262 complete strand separation during DGGE analysis; 0.75 GCG GCT GCT GG-3 1
primers capable of amplifying the variable V3 regions of
Conserved regions adjoining the variable V3 region of
Tris buffer.

PCR. The primers (Integrated DNA Technologies, Sko-
kie, IL) used in this study were the same as those used by Muyzer et al. (1993) and were designed to amplify the
conserved regions adjoining the variable V3 region of the
16S rDNA. The primers were designed as universal
primers capable of amplifying the variable V3 regions of
the majority of the bacteria found within the GIT. For
PCR amplification of the bacteria within the sample, 1
µL of template DNA (5 to 10 ng) was added to 49 µL of
a PCR mixture containing 34.75 µL of nuclease-free water;
1 µL of 0.02-nmol reverse primer (534r): 5′-ATT ACC
GCC GCT GCT GG-3′ and 1 µL of 0.02-nmol forward
primer (341fGC): 5′-CCG CCG CGG CGC CCG GCC GGC
GGG GCG GGA CGA CGG GCC GCC GCC GCC GCC GCC GCC
GCA CGG GGC GCC TAC GGG AGG CAG CAG-3′ with a guanine-cytosine clamp to prevent
complete strand separation during DGGE analysis; 0.75
µL of Promega Taq DNA Polymerase; 0.5 µL of nucleotide
mix (dNTP); 5 µL of 10× DNA polymerase buffer; and
6 µL of 25 mM MgCl2. The amplification reaction was
conducted by using a Gene Amp PCR System 9700 (P.E.
Applied Biosystems, Foster City, CA) with amplification
conditions as follows: 1 cycle of 94°C for 3 min; 30 cycles
of 94°C for 1 min, 65°C for 1 min (decreased by 0.5°C per
cycle), and 72°C for 1 min; 7 cycles of 94°C for 1 min,
55°C for 1 min, and 72°C for 1 min; 1 cycle of 72°C for 7
min, and held at 4°C. Products of the reaction were ob-
served by 1% agarose gel electrophoresis with ethid-
ium bromide.

DGGE. Denaturing gradient gel electrophoresis was
conducted according to the methods of Muyzer et al.
(1993) and with the modifications of Burkholder (2004).
To separate PCR fragments, polyacrylamide gels (8% acrylamide-bisacrylamide ratio 37.5:1) were cast in 40 to
60% urea-deionized formamide (Bio-Rad Laboratories,
Richmond, CA), of which the 100% denaturing acryl-
amide contained 7 M urea and 40% deionized formamide.
Amplified PCR samples (18 µL) were mixed with 2 µL of
a 20% volume of 5× loading buffer [0.025% (wt/vol)
bronophenol blue, 0.025% (wt/vol) xylene cyanol, 47%
(vol/vol) 0.1 M EDTA, and 47% (vol/vol) glycerol] and
20 µL was placed in each sample well (20-well comb). Gels
were placed in a DCode Universal Mutation Detection
System (Bio-Rad Laboratories, Hercules, CA) for elec-
rophoresis in 0.5× Tris-acetate EDTA buffer (20 mM Tris,
10 mM sodium acetate, 0.5 M EDTA) at 60°C for 10 min
at 200 V, followed by 16 h at 70 V. Silver staining was
performed according to the methods of Sambrook et al.
(1989). Briefly, gels were placed in a fixation solution (77%
ethanol, 4% glacial acetic acid, 16% dH2O) for 3 min,
followed by silver stain for 10 min (0.2% AgNO3, 99.8%
fixation solution). Gels were then developed for 15 to 20
min (0.001% BaBH4, 25% 1.5 M NaOH, 75% formalde-
hyde) and preserved in a preservation solution for 7 min
(27% ethanol, 12% glycerol, 61% dH2O).

Gels were analyzed for fragment pattern relatedness by
using Bionumerics Software, version 2.5 (Applied Maths,
Austin, TX). For each sample or lane, the number of bands
per sample and the similarity indices for bands between
samples were calculated. The number of bands per sam-
ple is a measure that is indicative of the number of bacte-
rial species within the sample. Similarity coefficients are
based on pairwise comparisons of numbers of bands and
positioning of bands between 2 samples within a treat-
ment, with all samples being compared. A similarity coef-
ficient of 100% would indicate that band numbers, posi-
tions, and densities are identical between birds. Compari-
sions of cross-products represent all pairwise comparisons
across treatments. Therefore, treatment similarity coeffi-
cients that are higher than the cross-product similarity
coefficients indicate that the homogeneity of bacterial
communities within treatment is greater than that be-
tween separate treatments. Similarity coefficients were
statistically analyzed by using the mixed model of SAS
(SAS Institute, Cary, NC), with Tukey-Kramer means sep-
oration to detect treatment differences. Analysis of band
numbers was performed with hours off feed as the treat-
ment effect, with linear, quadratic, and cubic contrasts
performed where appropriate.

Experiment 2

Birds. Thirty-two male Ross broilers were raised in
floor pens with ad libitum access to a standard industry
corn-soy diet and water. At 62 d of age, birds were ran-
domly selected and placed in 1 of 4 floor pens with 8
birds per pen. Feed was removed from the first pen 24
h before sampling and from the second and third pens 8
and 12 h before sampling, respectively. Feed was not
removed from birds in the fourth pen, which served as
the control treatment. Birds remained on litter with access
to water for the first 4 h of the FW periods and were then
placed in crates. At the conclusion of the trial, birds were
killed with CO2. Ileal mucosa was collected from 4 cm
proximal to the ileocecal junction and immediately frozen
at −20°C for future DGGE analysis. Samples were ana-
yzed by using the PCR, DGGE, and statistical methods
described for experiment 1.

RESULTS

Experiment 1

Amplicon profiles from the ileal digesta revealed that
dietary treatment had no effect on band numbers or simi-
arity coefficients. Likewise, band numbers of the muco-
sally associated bacteria were not affected by dietary
treatments (Table 1). Comparison of similarity coefficients
Table 1. Influence of diet on ileal mucosa- and digesta-associated bacteria in broilers at 0 h of feed withdrawal: Experiment 1

<table>
<thead>
<tr>
<th>Item</th>
<th>Mucosa-associated</th>
<th></th>
<th>Digesta-associated</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Band number</td>
<td>Similarity coefficient, %</td>
<td>Band number</td>
<td>Similarity coefficient, %</td>
</tr>
<tr>
<td>Dietary treatment</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>5.60</td>
<td>52.50ab</td>
<td>11.33</td>
<td>39.99</td>
</tr>
<tr>
<td>Antibiotic</td>
<td>8.51</td>
<td>40.38c</td>
<td>11.00</td>
<td>44.33</td>
</tr>
<tr>
<td>Copper sulfate</td>
<td>7.83</td>
<td>61.86a</td>
<td>10.00</td>
<td>48.34</td>
</tr>
<tr>
<td>Cross-products</td>
<td>—</td>
<td>45.00bc</td>
<td>—</td>
<td>46.04</td>
</tr>
<tr>
<td>SEM</td>
<td>0.77</td>
<td>2.99</td>
<td>1.40</td>
<td>7.4</td>
</tr>
<tr>
<td>Source of variation</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Treatment</td>
<td>0.30</td>
<td>0.02</td>
<td>0.77</td>
<td>0.82</td>
</tr>
</tbody>
</table>

**Table 1** Means in columns with differing letters are different ($P < 0.05$), as according to a Tukey-Kramer means comparison.

**Table 2** Means represent 6 birds per treatment.

**Table 3** The treatment similarity coefficients are means of all the within-treatment pairwise comparisons.

**Table 4** The cross-product similarities are the means of all between-treatment pairwise comparisons.

revealed that the mucosally associated bacterial communities of animals fed copper sulfate had higher similarity indices than either the control or BMD-fed birds (Table 1).

Feed withdrawal elicited a linear reduction in band numbers between 0 and 24 h of FW [linear equation: band number $= 7.10 - 0.1552(FW, h)$ ($R^2 = 0.44$)], and reduced similarity coefficients after 10 and 24 h when compared with 0 h (Table 2). Similarity coefficients of birds at 10 and 24 h were not different from those of the cross-products. No interactions were noted between dietary treatment and FW periods (data not shown).

**Experiment 2**

Band numbers were linearly decreased with FW time, with a corresponding linear regression equation [band number $= 9.30 - 0.2705(FW, h)$ ($R^2 = 0.45$)]. Additionally, similarity coefficient values decreased after 8, 12, and 24 h of FW when compared with 0 h (Table 2).

**DISCUSSION**

Because of the close association between the intestine and its bacterial microbiota, intestinal communities of bacteria are subjected to many of the same stressors that the intestine contends with on a daily basis. Intestinal bacterial communities, which were once considered a static tenant of the intestine that was subject to the environment created by the intestine, are now understood to contribute significantly to the intestinal environment, with far-reaching effects on intestinal development (Hooper, 2004), nutrient availability, mucus production...
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indicates that pharmacological concentrations of copper
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copper might suggest that the addition of pharmacologi-
munities more uniform than those in any other treatment.
less similar in all cases, with the exception of the birds
individual bacterial communities in birds appear to be
from the overall treatment comparisons. In this case, the
birds on the control diet had higher similarity coefficients
bacterial communities were affected by diet (Table 1). The
diversity of the mucosally associated bacterial commu-
nity, as measured by band number, was not affected by
dietary additives affected the bacterial communities located
in the mucosa of the ileum, although no differences were
observed in digesta bacterial communities. Although the
diversity of the mucosally associated bacterial community,
as measured by band number, was not affected by
dietary treatments, similarity coefficients between the
bacterial communities were affected by diet (Table 1). The
intestinal bacterial communities of birds fed copper were
more similar than those of birds on any other diet,
whereas the similarity coefficients of birds fed the BMD
diet were no different from the similarity indices of the
cross-products. Furthermore, the similarity coefficients
of birds on the control diet were similar to those of birds
fed copper and to the cross-product analysis. Therefore,
birds on the control diet had higher similarity coefficients
than those on the antibiotic diet but were no different
from the overall treatment comparisons. In this case, the
individual bacterial communities in birds appear to be
less similar in all cases, with the exception of the birds
fed CuSO₄, suggesting that CuSO₄ made bacterial communities
more uniform than those in any other treatment.
The high similarity coefficient observed in birds fed
copper might suggest that the addition of pharmacologi-
cal concentrations of dietary copper may select for popu-
lations of bacteria that are more copper resistant. Research
indicates that pharmacological concentrations of copper
inhibit bacterial growth and can potentially be toxic to
bacteria. Such copper toxicity can cause inactivation of
bacterial enzymes and alterations in bacterial membrane
integrity (Ohsumi et al., 1988). Another source of copper
toxicity is membrane-bound copper production of hydro-
peroxide free radicals (Rodriguez-Montelongo et al.,
1993), blocked functional groups of proteins, and the
“open gate” system (Nies and Silver, 1995), in which
cytoplasmic copper concentrations cannot be regulated
and thus become toxic. Furthermore, in anaerobic envi-
ronments such as the intestine, copper shifts from a Cu²⁺
to Cu¹⁺ oxidation state. This shift results in a substantial
increase in copper toxicity in bacteria such as Escherichia
coli, most likely because Cu¹⁺ is able to diffuse through the
bacterial cytoplasmic membrane, whereas Cu²⁺ cannot
(Beswick et al., 1976; Outten et al., 2001).
Because the birds were not started on a diet containing
high concentrations of copper on the first day of life, it
is likely that selection for copper-resistant organisms was
unnecessary. However, when the birds were switched to
a diet containing high levels of copper 20 d before sam-
pling and analysis, bacterial populations would have
been forced to up-regulate the transcription and transla-
tion of copper tolerance systems, such as the cue and cus
systems in E. coli (Outten et al., 2001), that would allow
the individual bacteria to survive in the environment.
Perhaps our observations reflect the shift in population
dynamics toward species of bacteria that are best
equipped to handle high concentrations of copper; there-
fore, similarity coefficients increased in the copper-
treated birds.
A paucity of data exist on the effects of copper on
intestinal bacterial populations. Varel et al. (1987) deter-
mined that 125 ppm of dietary copper sulfate caused
marked reductions in swine intestinal populations of
Streptococcus spp. Fuller et al. (1960) also reported similar
reductions in Streptococcus spp. populations in response
to copper sulfate in pigs. Lactobacillus populations are
also known to decline in the presence of copper, whereas
coliform populations are augmented by copper sulfate
(Jensen, 1998). In vitro research has demonstrated that
pure-culture isolates of Staphylococcus aureus and Staphylo-
coccus hyicus, E. coli, Enterococcus faecalis, and Enterococcus
faecium isolated from broilers, cattle, and pigs are sensitive
to concentrations of copper ranging from 2 to 24 mM
copper sulfate, as measured by the minimum inhibitory
concentration necessary to inhibit bacterial growth. Some-
what disturbingly, Salmonella isolates were less suscepti-
bile to copper sulfate and were inhibited at minimum
inhibitory concentrations of 20 to 28 mM copper sulfate.
If the results from the in vitro experiment simulate what
occurs in the gut, Salmonella would have an advantage
over other bacterial populations within the gut, particu-
larly when these compounds are used in feed. Last, the
Enterococcus populations displayed a bimodal response
to the copper sulfate, suggesting 2 populations: one popu-
lation that had not acquired copper-resistance mechani-
isms, and a population that had acquired those mecha-
nisms (Aarestrup and Hasman, 2004).
Copper resistance is not a new phenomenon and has
developed in pathogens as a means of contending with
the high levels of copper in various situations, such as
high levels of copper that are fed in livestock diets. For example, in 1993, Williams et al. (1993) identified copper-resistant strains of *E. coli* and *Salmonella* spp. in swine barns in the United Kingdom and Australia. Copper resistance has been investigated; Tetaz and Luke (1983) noted plasmid-controlled resistance to copper in *E. coli*, and numerous studies have confirmed these results in *E. coli* and other bacteria. In a study of *E. faecium* isolates, Hasman and Arestrup (2002) discovered that the genes encoding copper resistance are transferable between different bacterial isolates and, in some isolates, also correlate with increased resistance to antibiotics such as macrolides and glycopeptides. Interestingly, the authors also found correlations between dietary copper concentrations and copper resistance. In Danish pigs, which are commonly fed diets with copper concentrations ranging from 25 to 165 ppm, 44 of the 59 isolates (75%) were copper resistant. The authors also identified copper resistance in 10 of 29 isolates (34%) in Danish chickens, which are typically fed dietary copper concentrations of 20 ppm. Although we acknowledge that these studies do not represent the entire bacterial populations of the GIT, we can postulate that the increased similarity coefficients observed in the current experiment may also be a reflection of resistant bacteria within the broiler ileum.

Surprisingly, the similarity coefficients of the bacterial communities of birds fed antibiotics were lower when compared with either the control or copper sulfate similarity coefficients. In fact, the similarity coefficient of the antibiotic-fed birds was not different from the similarity coefficient of the cross-products, which demonstrates that the homogeneity of the bacterial community within the antibiotic treatment was no greater than the homogeneity of bacterial communities when compared across all treatments. When combined, these data suggest that dietary BMD did not reduce the overall numbers or diversity of bacterial communities within the intestine (as measured by band numbers) and simultaneously reduced the similarity coefficients of bacterial communities between birds. From these data, we can conclude that although the numbers of bacterial species did not change in response to BMD, the shifts in bacterial species (as measured by the similarity coefficient) were not uniform between birds. In effect, it would appear that individual host factors or individual bacterial communities were more influential factors in determining the response to dietary BMD in the current experiment.

Bacitracin, which belongs to the polypeptide class of antibiotics, has bactericidal effects against gram-positive bacteria and kills bacteria by forming a complex with and inactivating C55-isoprenyl pyrophosphate, which is a carrier of peptidoglycan precursors that are necessary for the synthesis of the bacterial cell wall (Stone and Strominger, 1971). Other research has indicated that bacitracin may interfere with other bacterial cellular processes as well (Pollock et al., 1994). Bacitracin is often used in the poultry industry as a means of improving growth and feed conversion as well as controlling necrotic enteritis in birds. Interestingly, bacitracin has also been reported to reduce the energy maintenance requirement (Bronsch and Manner, 1988) and improve the heat tolerance of laying hens (Manner and Wang, 1991). Numerous studies have reported positive growth responses to bacitracin in birds, and in 2000, bacitracin was used more frequently in starter and grower broiler diets than any other antibiotic (Chapman and Johnson, 2002).

In chickens, bacitracin has been shown to reduce populations of *Clostridium perfringens* (Engberg et al., 2000), an indigenous species of bacteria that is known to cause necrotic enteritis. Additionally, dietary bacitracin reduces intestinal *Enterococcus* populations (Barnes et al., 1978) and has bactericidal effects on populations of *Lactobacillus* spp. (Dutta and Devriese, 1981) and *Staphylococcus* spp. (Devriese, 1980). The effects of bacitracin on the intestinal bacterial community are not fully known at this point, but with the development of new molecular techniques to examine bacterial communities, it will be possible to better understand how bacitracin functions within the intestine.

Although not completely contrary to other reports, the data from the current experiment are a bit surprising, because most research supports the theory that antibiotics reduce the overall numbers or diversity of gut bacteria (Dibner and Richards, 2005). In the current experiment, neither bacterial numbers nor diversity was reduced in response to dietary BMD. Similar results were obtained by McCracken et al. (2001), who determined that the inclusion of 25 ppm of the broad-spectrum antibiotic cefoxitin to the drinking water reduced the similarity of intestinal bacterial communities but did not affect the number of bacterial species as measured by DGGE. However, Collier et al. (2003) observed that tylosin decreased duodenal and jejunal bacterial population diversity and that the bacterial communities of birds fed tylosin had greater similarities than those of birds not fed tylosin. The data also indicated that tylosin had a tendency to decrease total bacterial concentration, and significantly decreased numbers of *C. perfringens*. Other research by Knarreborg et al. (2002) determined that when combined, avilamycin and salinomycin caused shifts in the species of *Lactobacillus* found within the broiler ileum, as well as reducing plate counts of *C. perfringens*. In a comparison of bacitracin and salinomycin (Engberg et al., 2000), researchers concluded that neither antibiotic affected plate counts of broiler ileal anaerobic bacteria, lactose-negative enterobacteria, lactic acid bacteria, enterococci, or lactobacilli.

The lack of agreement among the currently published data can most likely be attributed to the fact that different antibiotics were used for each study. Data from numerous plating studies, combined with knowledge of the modes of action of individual antibiotics, indicates that different antibiotics elicit distinctive responses in intestinal bacterial communities. Additionally, the contributions of the individual host animals to bacterial populations cannot be disregarded when comparing separate experiments.

Despite the fact that the mucosa-associated bacteria were affected by dietary treatments, the digesta-associated bacteria did not respond to such treatments, as evi-
edenced by the lack of band number and similarity coefficient differences. Considering the transient nature of digesta within the tract, however, this result is not surprising. Mucosally associated bacterial communities are continuously exposed to dietary additives and are therefore able to adapt in response to the dietary additives, which was evidenced by changes in similarity coefficients. However, because digesta typically remains in the GIT of the chicken for 2 to 12 h (Tuckey et al., 1958), the bacterial populations associated with the digesta may not have time to respond to the dietary treatments. Nonetheless, one could argue that a portion of the digesta-associated bacteria are introduced to the intestine by way of the feed, and their existence within the feed before ingestion would require that the bacteria are capable of coexisting with the dietary additives. Even if this is the case, the bacteria would be accustomed to copper-resistance systems in an aerobic environment instead of an anaerobic environment, where copper has been shown to be far more toxic. Thus, on introduction to an anaerobic environment, and therefore greater copper toxicity, one would expect shifts in the population in response to the greater toxicity, but the 2- to 12-h feed passage time is not likely to be a sufficient amount of time for such shifts to occur.

Dietary additions of copper and BMD were not the only factors to shift the bacterial populations, because FW also affected the mucosally associated bacterial populations of the ileum in both experiments. As the FW period progressed from 0 to 24 h, we observed decreases both in band numbers and in similarity coefficients, with the reductions in band numbers being time dependent, as determined by linear regression analysis. In experiment 1, ileal amplicon profiles were reduced from 7 major bands or predominant species to 5 predominant species at both 10 and 24 h of FW. Simultaneously, the similarity coefficients were reduced from 57 to 48% in birds fasted for 10 and 24 h. Similar results were obtained in experiment 2, where FW reduced the bacterial diversity at 0 and 24 h of FW and also reduced the similarity coefficients of the 62-d-old broilers fasted for 8, 12, and 24 h. Comparison of these results indicates that regardless of age, short-term FW programs reduce the diversity of bacterial species found within the intestine while concurrently reducing the similarity indices.

If the reduction in bacterial species diversity is truly measured by the number of bands, the 24-h FW band number in experiment 2 is low. Lower band numbers were expected for the 24-h samples because the mucus layer, which is often part of the collected mucosa during most experiments, is significantly reduced at 24 h of FW (Thompson and Applegate, 2006). Plausibly, the reduction in mucus quantity also reduces the populations of bacteria. Additionally, DGGE procedures have inherent limitations that prevent scientists from visualizing every bacterial species within the intestine. For instance, 16S gene amplification fails to amplify bacterial species that exist in less than 1% of the population (Muyzer et al., 1993), and several amplicons have been shown to comigrate in the denaturing gradient gel, preventing completely accurate band number counts and similarity coefficient values (von Wintzingerode et al., 1997). Furthermore, Zhu and Joerger (2003) identified 243 different 16S rDNA bacterial sequences via amplicon cloning in chicken cecal content, but only 10 to 20 bands were visible on analysis by temperature gradient gel electrophoresis, a technique that is similar to DGGE (Hume et al., 2003). Consequently, the decline in the number of bands in the 24-h FW samples of the current experiment does not suggest that only one species of bacteria is present in the intestine. Instead, it may indicate that only a few species predominate in the ileum at 24 h of FW, and that the diversity of those species that constitute less than 1% of the entire population is increasing. In addition, the marked reduction in similarity coefficients implies that the emergence of the predominant species in response to FW differs among birds. As discussed previously in regard to the antibiotic diets, it appears that the individual host factors may be contributing to the shifts observed in response to FW.

Of further interest is that the reduction in band numbers and similarity coefficients of bacterial communities occurred between 0 and 10 h of FW in experiment 1. Experiment 2 yielded slightly different results in band numbers, in that they declined linearly to 24 h of FW, although similarity coefficients decreased by 8 h of FW with no subsequent change. Such a sudden decrease in species number and similarity between animals suggests that the bacterial response to FW is rapidly activated among bacterial populations within the ileum. Thus, we can postulate that much of the reduced similarity observed in the experiments of Hume et al. (2003), who examined cecal bacterial populations after 10 d of molting and reported that similarity coefficient values of roughly 40% may have occurred within the first day of molt. In that particular experiment, band numbers were not analyzed, but visual inspection of the published gels showed slightly fewer bands, all of which were considerably less dense than those of the nonmolting broilers. Additional reports from Hinton et al. (2000) indicate that FW periods up to 24 h caused increases in cecal Enterobacteriaceae populations and decreases in lactic acid bacteria of broilers.

Other experiments that examined molting in laying hens or total parenteral nutrition (TPN) in mammals have provided much of what is currently known regarding the effects of FW on gut microbiota. Unfortunately, as with the data of Hume et al. (2003), most of these experiments use FW periods that are far longer than 24 h of fasting, and the majority of the experiments examine how pathogens, not the indigenous bacteria, respond to periods of fasting. Three other studies have addressed how indigenous microbiota change in response to fasting periods. Schneider et al. (2000) conducted an experiment to define the effects of 6 wk of TPN on human fecal microbiota and determined that TPN causes significant decreases in both aerobic and anaerobic bacterial populations. Among the aerobes that were found to decrease in response to
TPN were *Streptococcus* spp., *E. faecalis*, *Staphylococcus* spp., *Lactobacillus* spp., and *E. coli* spp. Total parenteral nutrition also decreased populations of anaerobic bacteria such as *Clostridium* spp., *Bacteroides* spp., and *Prevotella* spp. Conversely, Nakasaki et al. (1998) observed that 2 wk of TPN leads to significant increases in the cecal aerobic (primarily *E. coli*) and anaerobic (primarily Enterobacteriaceae) bacterial populations in the rat.

The indigenous microbiota of the intestine are important contributors to animal health and well-being, particularly because the microbiota provide colonization resistance against pathogens. Unfortunately, externally controlled factors, such as diet and FW, have a sizeable impact on the intestinal bacterial populations, with reductions in bacterial diversity occurring in a time-dependent manner as FW periods increase. Currently, we do not fully understand the exact mechanisms by which diet and FW alter intestinal bacteria populations. However, with further research, it may be possible to develop intervention strategies that reduce pathogen colonization and shedding during FW periods.

**REFERENCES**


