Molting in *Salmonella* Enteritidis-Challenged Laying Hens Fed Alfalfa Crumbles. II. Fermentation and Microbial Ecology Response

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ABSTRACT The objective of this study was to examine microbial population shifts and short-chain fatty acid (SCFA) responses in the gastrointestinal tract of *Salmonella* Enteritidis-challenged molted and nonmolted hens fed different dietary regimens. Fifteen *Salmonella*-free Single Comb Leghorn hens (>50 wk old) were assigned to 3 treatment groups of 5 birds each based on diet in 2 trials: 100% alfalfa crumbles (ALC), full-fed (FF, nonmolted) 100% commercial layer ration, and feed withdrawal (FW). A forced molt was induced by either a 12-d alfalfa diet or FW. In all treatment groups, each hen was challenged by crop gavage orally 4 d after molt induction with a 1-mL inoculum containing 10^6 cfu of *Salmonella* Enteritidis. Fecal and cecal samples (d 4, 6, 8, 11, and necropsy on d 12) were collected postchallenge. Microbial population shifts were evaluated by PCR-based 16S ribosomal RNA gene amplification and denaturing gradient gel electrophoresis, and SCFA concentrations were measured. Total SCFA in fecal and cecal contents for FW molted hens were generally lower (*P* ≤ 0.05) in the later stages of the molt period when compared to ALC and FF treatment groups. The overall trend of SCFA in cecal and fecal samples exhibited similar patterns. In trials 1 and 2, hens molted with ALC diet generally yielded more similar amplicon band patterns with the FF hens in both fecal and cecal samples by the end of the molting period than with FW hens. The results of these studies suggest that ALC molted hens supported microflora and fermentation activities, which were more comparable to FF hens than FW hens by the end of the molting period.

Key words: *Salmonella* Enteritidis, microbial ecology, alfalfa, molting, denaturing gradient gel electrophoresis

INTRODUCTION

A full compliment of normal gastrointestinal (GI) tract microflora constitutes sufficient microbial complexity in adult chickens to be considered relatively resistant to enteropathogens (McNab, 1973; Ricke et al., 2004b). However, it appears that the poultry digestive microbial ecology can be altered enough during feed withdrawal-induced molting that the GI tract can become vulnerable to pathogen infection and colonization (Durant et al., 1999; Seo et al., 2001; Ricke, 2003). Corrier et al. (1997) reported that induced molted hens via feed withdrawal-induced molting that the GI tract can become vulnerable to pathogen infection and colonization (Durant et al., 1999; Seo et al., 2001; Ricke, 2003). Corrier et al. (1997) reported that induced molted hens via feed withdrawal exhibited sufficient changes in cecal microenvironment patterns, which corresponded with a significant increase in *Salmonella* Enteritidis colonization in ceca, liver, and in spleen of molted hens compared to fully fed hens. Conditions that reduce fermentation activity and ultimately decrease protective microflora in the GI tract are believed to facilitate *Salmonella* Enteritidis colonization (Ricke, 2003). Therefore, dietary molt induction regimens that promote short-chain fatty acid (SCFA) production and maintain GI tract microbial populations should reduce incidence of *Salmonella* Enteritidis colonization and infection (Ricke, 2003). Fermentable high-fiber diets such as alfalfa have been examined as potential molting dietary approaches that can retain normal microbial flora, hence, reduce proliferation of *Salmonella* Enteritidis (Woodward et al., 2005; McReynolds et al., 2005, 2006; Dunkley et al., 2007a) in the GI tract of molting hens. The use of alfalfa as a single dietary source would be expected to support formation of fermentative products including SCFA by chicken cecal microflora (Ricke, 2003; Woodward et al., 2005; Saengkerdsub et al., 2006; Dunkley et al., 2007a).

Molecular techniques have been used to compare bacterial diversity in chicken cecal microenvironment and for potential probiotic sources (Ricke and Pillai, 1999; Zhu et al., 2002; Hume et al., 2003; Ricke et al., 2004a; Saengkerdsub et al., 2007); however, some DNA isolation techniques have limitations regarding amplification.
and cloning and tend to favor the detection of certain bacteria and nucleic acid sequences. The PCR-based denaturing gradient gel electrophoresis (DGGE) has been widely used and facilitates unique visualization of PCR products (amplicons) that represent predominant diversity of digestive bacteria (Muyzer et al., 1993; Lee et al., 1996; Murray et al., 1996). Hume et al. (2003) reported that molecular-based DGGE techniques allowed qualitative comparisons in cecal bacterial communities during molt induction by feed withdrawal and hens supplemented with high levels of dietary Zn, low Ca, and alfalfa. Ricke et al. (2004a) also utilized molecular-based DGGE to detect changes in cecal and crop microbial communities of molted hens fed Zn acetate or Zn propionate-supplemented diets compared to feed withdrawal and fully fed hens. However, these studies profiled the fecal population only once at the end of the molt period and did not characterize microbial populations during initial introduction of the molt diet. The overall goal of this study was to monitor both fermentation activity and cecal population only once at the end of the molt period. The specific objectives of this study were to (1) determine the SCFA production profile of fecal and cecal samples of Salmonella Enteritidis-challenged laying hens on different molting regimes and (2) follow the microbial populations before and after Salmonella Enteritidis challenge by determining the microbial DGGE profile of fecal and cecal samples of Salmonella Enteritidis-challenged laying hens fed on different molting regimes.

**MATERIALS AND METHODS**

**Preparation of Inoculum and Sample Collection**

A Salmonella Enteritidis poultry isolate strain (phage type 13A) media was used to culture the resistant isolate and other procedures for bird infection are described elsewhere (McReynolds et al., 2005; Dunkley et al., 2007b). Fecal and cecal content samples were collected from 15 Single Comb Leghorn hens (>50 wk old) obtained from a local commercial laying flock. Hens were placed in wire layer cages (Agricultural Research Service, USDA, College Station, TX) and acclimated to their environment for a 2-wk period. Fifteen Salmonella-free Single Comb Leghorn hens (>50 wk old) were assigned to 3 treatment groups of 5 birds each based on diet in 2 trials: 100% alfalfa crumbles (ALC); full-fed (FF, nonmolted) 100% commercial layer ration; and feed withdrawal (FW). Specific details of molt procedures, diets, and experimental design are described elsewhere (Dunkley et al., 2007b).

On d 9, hens were euthanized, necropsied, and ceca were excised aseptically. One gram of cecal content from each hen was immediately suspended into a 15-mL Falcon tube (BD Biosciences, Franklin Lakes, NJ) containing 9 mL of PBS to yield a 9:1 (wt/vol) dilution ratio. Falcon tubes were capped, shaken thoroughly, and immediately stored in a −20°C freezer for use at a later date. Cecal samples were thawed, and 2 triplicate portions of 1.5-mL aliquot from each sample were transferred into sterile microcentrifuge tubes and were utilized for DNA extraction (DGGE) and SCFA determination. Fecal samples were collected on d 4, 6, 8, and 11. Aluminum foil sheets were placed under a total of 15 hens. Upon defecation, 1-g fecal samples were processed, stored, and thawed later for analysis in the same manner as described for the fecal samples.

**DGGE**

Fecal and cecal bacterial genomic DNA was isolated from 1.5 mL of each sample with a QIAamp DNA mini kit (Qiagen, Valencia, CA) by the method described in the kit. Denaturing gradient gel electrophoresis was conducted according to the method of Muyzer et al. (1993) with modification, using bacterial-specific PCR primers (Integrated DNA Technology Inc., Coralville, IA) to conserve regions flanking the variable V3 region of 16S ribosomal DNA genes. Polymerase chain reaction was run with a 50-μL total reaction volume. Primers [50 pmoL of each per reaction mixture; primer 2, 5′-AT-TACCAGCGG CTGCTGG-3′, and primer 3 with a 40-base G-C clamp (Sheffield et al., 1989; Muyzer et al., 1993), 5′-CGCCCCGCGCCGCGCGCGGCCGGGCCCCGGGGG GCCGCCCCGCTACGGGAGGCAGCA-3′ were mixed with Jump Start Red-Taq Ready Mix (Sigma Aldrich Co., St. Louis, MO) according to kit instructions, with 250 ng (50 ng of DNA pooled from 5 samples each) of template DNA. Acetamide (5%, wt/vol) was added to eliminate preferential annealing (Reysenbach et al., 1992); 10 mg/mL of BSA and deionized water was also added to make up a final 50-μL volume reaction. Amplification was done on a PTC-200 Peltier thermal cycler (MJ Research Inc., Waltham, MA) with the following program: 1) denaturation at 94.9°C for 2 min; 2) subsequent denaturation at 94.0°C for 1 min; 3) annealing at 67.0°C for 45 s, −0.5°C per cycle [touchdown to minimize spurious by-product formation (Don et al., 1991; Wawer and Muyzer, 1995)]; 4) extension at 72.0°C for 2 min; 5) repeat steps 2 to 4 for 17 cycles; 6) denaturation at 94.0°C for 1 min; 7) annealing at 58.0°C for 45 s; 8) repeat steps 6 to 7 for 12 cycles; 9) extension at 72.0°C for 7 min; and 10) 4.0°C final.

**Gel Electrophoresis**

Polyacrylamide gels (8% vol/vol; acrylamide-bisacrylamide ratio 37:5:1) were cast with a 35 to 60% urea-deionized formamide gradient; 100% denaturing acrylamide was 7 M urea and 40% deionized formamide. Amplified samples (4 μL of pooled template) were mixed with an equal volume of 2× loading buffer [0.05% (wt/vol) bromophenol blue, 0.05% (wt/vol) xylene cyanol, and 70% (vol/vol) glycerol], and 7 μL was placed in each sample well (16-well comb). Gels were placed in a DCode universal mutation detection system (Bio-Rad Laboratories, Richmond, CA) for electrophoresis in 1× TAE [20 mM Tris (pH 7.4), 10 mM sodium acetate,
0.5 M EDTA] buffer at 59°C for 17 h at 60 V. Gels were stained with SYBR Green 1 (1:10,000 dilution) for 40 min. Amplified fragment pattern relatedness of samples was determined with Molecular Analysis Fingerprinting Software, version 1.6 (Bio-Rad Laboratories) based on the Dice similarity coefficient and the unweighted pair group method using arithmetic averages for clustering. Dice coefficient (values from 0 to 1) is an arithmetic method that determines the degree to which banding patterns are alike. Clusters (groups) were determined by sequentially comparing the patterns and the construction of a dendrogram reflecting the related similarities. The amount of similarity was reflected by the relative closeness or grouping and is indicated by the percentage similarity coefficient bar located above each dendrogram.

**SCFA Concentration**

The fecal and cecal concentration of SCFA from tripli cate samples was determined by gas-liquid chromatography as previously described by Corrier et al. (1990). The analyses were conducted with a gas chromatograph equipped with flame ionization detector and peak profiles integration-quantification integrator (Shimadzu Corp., Columbia, MD). Each sample peak profile was integrated and quantified relative to an internal standard of methyl-butyl-acid placed in the same sample. Analyses were conducted at an oven temperature of 200°C and a flow rate of 85 mL/min. The concentration of each acid was expressed in micromoles per milliliter.

**Statistical Analysis**

Data from concentrations of SCFA were analyzed using the 1-way ANOVA subjected to linear regression using PC-SAS software (SAS Institute Inc., Cary, NC). Differences between means were determined using least square means and Tukey’s honest significance test. Statistical variation was also estimated by the SEM. All statistical analyses were considered significant at $P \leq 0.05$.

**RESULTS AND DISCUSSION**

**Fecal and Cecal SCFA Response**

In fecal samples, acetate production generally occurred in the greatest proportion (Figure 1a and b). Propionate (Figure 1c and d) and butyrate (Figure 1e and f) were next in order of magnitude, whereas only trace amounts of isovalerate, valerate, and isobutyrate (data not shown) were present. In trial 1, acetate concentrations for FW hens were lower ($P \leq 0.05$) compared to ALC and FF hens except when compared to ALC birds on d 8. In trial 2, acetate levels were initially highest ($P \leq 0.05$) for FW birds on d 4, but by d 8 and 11, ALC and FF birds exhibited the highest levels. In trial 1, ALC and FF propionate concentrations were similar and, except for d 8, both were higher ($P \leq 0.05$) compared to FW hens. In trial 2, ALC and FF propionate concentrations on d 8 and 11 were similar, and both were higher ($P \leq 0.05$) compared to FW hens. In general, butyrate concentrations were minimal except for FF birds on d 11 and were not detected in FW hens in trial 1, whereas in trial 2, similar trends occurred for all treatments except on d 4 for FW hens, which were higher ($P \leq 0.05$) than all other days in the 3 treatment groups. In trial 1, total SCFA concentrations for ALC and FF hens were higher ($P \leq 0.05$) compared to the FW treatment group except for ALC birds on d 8, whereas the highest total SCFA concentrations came from FF hens on d 11 (Figure 1g and h). In trial 2, FW hens on d 4 exhibited the highest ($P \leq 0.05$) total SCFA compared to FF and ALC hens but were the lowest for d 6, 8, and 11, whereas ALC and FF were similar except for the higher values from ALC birds on d 8.

Cecal acetate, propionate, and SCFA concentrations were similar for ALC and FF birds in trial 1 but were higher ($P \leq 0.05$) for FF birds in trial 2 (Figure 2a, b, and d). However, cecal butyrate levels for FF and ALC birds were similar in trial 2 but were higher ($P \leq 0.05$) in FF birds in trial 1 (Figure 2c). In both trials, the FF and ALC treatment groups generally produced higher ($P \leq 0.05$) acetate, propionate, butyrate, and total SCFA in the ceca than the FW hens except between ALC and FW butyrate concentrations in trial 1. The overall trends of SCFA in cecal and fecal samples were similar. In general, total SCFA production in ceca from FF and ALC hens was nearly doubled compared to fecal SCFA concentration in the FW and ALC groups. In addition, SCFA for FW hens in the ceca and fecal samples were both consistently low within the time span of molting period except for fecal samples on d 4 (prechallenged) in trial 2. Based on the trends for both cecal and fecal SCFA production, it appears that fecal SCFA represented trends that would be representative of cecal SCFA production and therefore could provide a reasonable noninvasive indicator for cecal fermentation.

These results indicated that induced molted hens by feed deprivation could influence production of SCFA in the ceca. By d 6, Corrier et al. (1997) observed that nonmolted hens yielded 1.5+, 1.5-, and 1.7-fold increases in total SCFA, acetate, and propionate concentrations in the cecal contents, respectively, compared to FW hens. Moore et al. (2004) observed no differences in acetic acid production among treatments when testing Zn layer ration combinations as molt induction diets. Woodward et al. (2005) noted that cecal acetate and propionate concentrations in FF hens were nearly doubled compared to feed deprivation birds. Woodward et al. (2005) suggested that decreased intake of alfalfa meal and decreased SCFA production could lower effectiveness in preventing Salmonella Enteritidis establishment in some birds. Based on these results, feed removal appeared to be accompanied by a decreased fermentation capacity in the ceca.
Figure 1. Production of short-chain fatty acids (SCFA) from fecal samples in *Salmonella* Enteritidis-challenged laying hens during molt. Error bars indicate SEM. *Means without a common letter differ significantly (*P* < 0.05). FF = full fed (nonmolted); ALC = alfalfa; FW = feed withdrawal; ND = not determined. Day 4 represents prechallenged sample; d 6, 8, and 11 represent postchallenged.

**PCR-Based DGGE**

The FF and ALC treatment groups exhibited higher percentage similarity coefficients (>90%) in trial 2 than in trial 1. Shifts in microbial populations should be reflected by differences in DGGE profiles due to dietary differences as well as by trial-to-trial and individual-bird variations. It was confirmed that the levels of *Salmonella* Enteritidis in fecal and cecal samples did not influence the dendrogram comparisons of microbial populations in diets (data not shown). In trial 1 (Figure 3a and b), the amplicon profile of bacterial diversity was divided into 4 distinct groups: 4FF, 8ALC, and 11FF formed a group with an 82.1% correlation; 6FW, 4FW, 4ALC, and 6ALC formed a group with an 84% correlation; 6FF and 8FF formed a group with a 92% similarity coefficient; and 11ALC, 12ALCCe, and 12FFCe exhibited a 68.1% coefficient similarity. Three feed withdrawal samples (8FW, 12FWCe, and 11FW) were segregated from the others with only 66.7, 61.4, and 54.3% similarity coeffi-
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Figure 2. Production of short-chain fatty acids (SCFA) from cecal samples in Salmonella Enteritidis-challenged laying hens during molt. Error bars indicate SEM. *a,b* Means without a common letter differ significantly (*P* < 0.05). FF = full fed (nonmolted); ALC = alfalfa; FW = feed withdrawal. Cecal samples collected on d 12 of molt via necropsy (postchallenged).

In trial 1, the similarity of ALC and FW treatment groups (Figure 3b) for prechallenged hens showed a 95.5% similarity (d 4); however, as molting progressed, their similarity coefficient began to shift by d 6 (84%) and eventually to 57.0% in the ceca at the end of the experiment (necropsy at d 12). A comparison of FF and ALC treatment groups at the initiation of the study (d 4) yielded a 79.7% similarity. In trial 2 (Figure 4a and b), four distinct groups were formed: 11FW and 12FWCe formed a group with an 82.3% similarity microflora band pattern, and 12FFCe and 12ALCCe formed a group with a 95.5% correlation similarity. Two large clusters were observed: 4ALC, 6FW, 6ALC, 8FW, and 4FW formed a group with a 68.3% similarity coefficient, and 6FF, 8ALC, 8FF, 4FF, 11FF, and 11ALC formed a group with a 67.7% similarity. Additionally, 11FF and 11ALC formed a subgroup of 98.0% similarity. The ALC and FF hens initially exhibited only a 30.9% amplicon similarity coefficient on d 4, but by d 12, a 95.4% similarity was observed for both in cecal samples.

Overall, it appears that there may be greater microbial population shifts occurring during the molt period between the ALC and the FW treatment groups compared to ALC and FF treatment groups. Alternative molting diets that retain fermentative microflora are believed to be instrumental in reducing Salmonella Enteritidis colonization in the GI tract (Ricke, 2003; Moore et al., 2004; Ricke et al., 2004a; Woodward et al., 2005). It is important to characterize if similar indigenous microflora are retained and therefore monitoring the gut populations by noninvasive collection of fecal material becomes necessary. In the current study, it was difficult to make a feasible comparison, because fecal samples were assessed on 4 d during the trial, whereas cecal samples were only assessed on d 12. In trial 1, fecal samples for d 4, 6, and 8 exhibited greater similarity within diets. Fecal samples for d 11 were segregated (ungrouped; unrelated). Cecal samples for FF and ALC (trial 1) were grouped with a 70% similarity compared to cecal samples for FW hens, which exhibited only a 61% similarity among amplicon bands. In trial 2, d 4 and 6 showed inconsistencies in amplicon band patterns, but patterns for d 8 and 11 fecal samples and d 12 cecal samples for the FF and ALC groups were similar.

Alfalfa as a dietary fiber may affect the GI tract by supporting microbial activities, decreasing rate of passage, metabolites, and digestive efficacy (Bach Knudsen, 2001; Wenk, 2001). Recently, certain dietary components...
Figure 3. a) Denaturing gradient gel electrophoresis gel showing bacterial 16S amplicon band patterns from *Salmonella* Enteritidis-challenged laying hens during molt (trial 1). b) Dendrogram and relative similarity coefficients of band from *Salmonella* Enteritidis-challenged laying hens during molt (trial 1). Band patterns are indicated by their grouping on the dendrogram and the percentage coefficient (bar): ≥ 92% are very related or the same, 85 to 91% are similar, 80 to 84% are somewhat similar, and ≤ 79% are unrelated. Numbers before treatment groups represent day of molt. Day 4 represents prechallenged sample; d 6, 8, and 11 represent fecal samples, postchallenged; and d 12 represents cecal samples collected via necropsy, postchallenged. FF = full fed (nonmolted); ALC = alfalfa; FW = feed withdrawal; Ce = cecal samples; R = reference lanes.
Figure 4. a) Denaturing gradient gel electrophoresis gel showing bacterial 16S amplicon band patterns from *Salmonella* Enteritidis-challenged laying hens during molt (trial 2). Numbers before treatment groups represent day of molt. Day 4 represents prechallenged sample; d 6, 8, and 11 represent fecal samples, postchallenged; and d 12 represents cecal samples collected via necropsy, postchallenged. b) Dendrogram and relative similarity coefficients of band from *Salmonella* Enteritidis-challenged laying hens during molt (trial 2). Band patterns are indicated by their grouping on the dendrogram and the percentage coefficient (bar): ≥92% are very related or the same, 85 to 91% are similar, 80 to 84% are somewhat similar, and ≤79% are unrelated. Numbers before treatment groups represent day of molt. Day 4 represents prechallenged sample; d 6, 8, and 11 represent fecal samples, postchallenged; and d 12 represents cecal samples collected via necropsy, postchallenged. FF = full fed (nonmolted); ALC = alfalfa; FW = feed withdrawal; Ce = cecal samples; R = reference lanes.
including polysaccharides were identified as having the potential to be utilized as prebiotics (Cummings and MacFarlane, 2002). The result of the current study illustrates the changes in the GI tract microenvironment during feed deprivation in molted hens that may help to create a more Salmonella Enteritidis colonization-susceptible state. Based on our results, FF and ALC in both fecal and cecal contents revealed generally higher SCFA than FW hens, which may also be reflected in the greater similarity in microbial amplicon band patterns of FF and ALC treatment groups when compared to the FW hens in the later times of the molt period. It appears that DGGE can be used to estimate changes in microbial diversity of fecal and cecal samples before and after Salmonella Enteritidis challenged during the time span of molting induction. Dietary trends at the initial stages of molt indicated inconsistencies, but as the molt period progressed, DGGE and SCFA for fecal samples collected at later times reflected consistencies with cecal samples on d 12, whereas FF and ALC hens exhibited greater similarity in molecular profiles and fermentation products than FW hens.

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