Comparison of In Vitro Fermentation and Molecular Microbial Profiles of High-Fiber Feed Substrates Incubated with Chicken Cecal Inocula

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ABSTRACT High fiber and nonstarch polysaccharide-based poultry diets have received more interest recently for retaining or promoting beneficial gastrointestinal microbial populations. The objective of this study was to investigate and compare the in vitro potential fermentability of high-fiber feed substrates (HFFS) by laying hen cecal microflora. Feed sources examined included soybean meal, soybean hull, beet pulp, wheat middlings, ground sorghum, cottonseed meal, 100% alfalfa meal, 90% alfalfa + 10% commercial layer ration, 80% alfalfa + 20% commercial layer ration, and 70% alfalfa + 30% commercial layer ration. Cecal contents and HFFS were incubated anaerobically in serum tubes at 39°C for 24 h. Samples from 2 trials were analyzed at 0 and 24 h for short-chain fatty acids (SCFA). Short-chain fatty acids in samples at 0 h were subtracted from 24-h samples to determine the net production of SCFA. In both trials involving HFFS incubations with cecal inocula, acetate production was highest followed by propionate and butyrate whereas isobutyrate and isovalerate production were in trace amounts. In trial 2, detectable valerate production appeared to consistently occur with alfalfa-based HFFS. It was clear that SCFA production was largely dependent upon HFFS, because cecal inoculum alone yielded little or no detectable SCFA production. For HFFS incubations without cecal inocula, acetate production was highest; propionate and butyrate were similar, and isobutyrate, valerate, and isovalerate production were in trace amounts. Polymerase chain reaction-based denaturing gradient gel electrophoresis results from both trials indicated 69 and 71% similarity for comparison of all feed mixtures in trials 1 and 2, respectively. All alfalfa-based HFFS yielded a higher similarity coefficient in trial 2 than in trial 1 with a band pattern of 90% similarity; diets containing 90% alfalfa + 10% commercial layer ration and 80% alfalfa + 20% commercial layer ration in trial 2 formed a subgroup with a 94% microbial similarity coefficient. These data suggest that high fiber sources may contribute to the fermentation and microbial diversity that occurs in the ceca of laying hens.

Key words: laying hen cecal inocula, in vitro fermentation, short-chain fatty acids, denaturing gradient gel electrophoresis, high fiber

INTRODUCTION Dietary fibers are predominantly a constituent of plant cell wall and also consist of nonstarch polysaccharides along with noncarbohydrates (Bach Knudsen, 2001). Upon ingestion, dietary fiber can alter gut microbial activities, rate of passage, metabolites, and digestive efficacy (Bach Knudsen, 2001; Wenk, 2001). In addition, certain dietary fractions including polysaccharides have been identified as potential prebiotics (Cummings and MacFarlane, 2002; Guo et al., 2003). Prebiotics are defined as nondigestible carbohydrate dietary fractions that stimulate the growth of one or more bacteria in the gastrointestinal (GI) tract that are beneficial to the host (Gibson and Roberfroid, 1995). Beneficial bacteria, including species of Lactobacilli and Bifidobacteria, have been identified as being inhibitory toward pathogens (Gibson and Roberfroid, 1995). It is known that certain GI tract microorganisms in many animal species have the potential to hydrolyze and ferment dietary fiber into oligosaccharides and other low molecular weight carbohydrates (Kass et al., 1980; Sunvold et al., 1995). It has been suggested that the ceca contain the largest number of microorganisms in the GI tract of poultry (Barnes et al., 1972, 1973; Barnes, 1979). More than 200 different bacteria have been isolated and most of these are strict anaerobes (Barnes, 1979). In poultry, extensive strict anaerobic activities including formation of short-chain fatty acids (SCFA) and methanogen-
esis occur in the ceca in birds fed a variety of diets (Ricke et al., 2004a). Studies with 4 species of birds indicated that ME obtained from SCFA production was equivalent to 5 to 15% of daily requirements for bird maintenance energy (Annison et al., 1968; Gasaway, 1976a,b). Microbial fermentation in the GI tract can also contribute to limiting foodborne pathogen colonization. It has been shown that anaerobic cecal bacterial culture from mature chickens or other competitive exclusion can be effective in limiting Salmonella invasion (Nurmi and Rantala, 1973; Ziprin et al., 1993; Nisbet, 2002). Short-chain fatty acids also have a bacteriostatic effect on some enteric bacteria including Salmonella Typhimurium, and do not inhibit beneficial GI tract bacteria such as Lactobacillus (Van der Wielen et al., 2000). McHan and Shotts (1993) reported that the in vitro toxic effect of SCFA to some Enterobacteriaceae showed a 50 to 80% reduction in Salmonella Typhimurium population in the presence of SCFA. It has been suggested that propionic acid was more effective in inhibiting pathogenic bacteria (Marounek et al., 1999), whereas others observed that acetate is more effective (Van der Wielen et al., 2000).

It is apparent that the cecal microbial ecology is an important factor in limiting pathogen colonization during dietary stress such as molting. In particular, feed withdrawal as a method for molting hens has been implicated and fermentable dietary fibers have been examined as potential alternative molting approaches (Seo et al., 2001; Ricke, 2003a; Woodward et al., 2005). The objectives of this study were to investigate and compare the in vitro potential fermentability of high-fiber feed sources (HFFS) that could be used to formulate a more refined alternative induced-molting diet that retains microbial diversity and fermentation capacity while potentially promoting the protective microflora in the GI tract of poultry.

**MATERIALS AND METHODS**

**Experimental Protocol**

Cecal contents were obtained from single comb Leghorn hens (>50 wk old) fed on a commercial layer ration. Cecal contents were diluted at 1:3,000 (Donalson et al., 2004) with anaerobic dilution solution (Bryant, 1973) to formulate the inocula. Ten HFFS, including soybean meal (S), soybean hulls (H), beet pulp (B), wheat middlings (W), ground sorghum (G), cottonseed meal (C), 100% alfalfa meal (A), 90% alfalfa + 10% commercial layer ration (A9), 80% alfalfa + 20% commercial layer ration (A8), and 70% alfalfa + 30% commercial layer ration (A7), were ground through a 1-mm screen in a Wiley mill (Arthur H. Thomas, Philadelphia, PA). Samples (0.5 g) of each feed substrate were placed in serum tubes with 10 mL of inoculum (I; to yield SI, HI, BI, WI, GI, CI, AI, A9I, A8I, and A7I) or 10 mL of dilution solution [feed + no inoculum (0); to yield S0, H0, B0, W0, G0, C0, A0, A90, A80, and A70] added anaerobically (Russell and Baldwin, 1979). A no feed + inoculum (NFI) control was included. There were 2 trials, and samples for analysis were conducted at 2 time periods (0 and 24 h). All 24-h serum tubes were capped with butyl rubber stoppers and crimp aluminum seals, pressurized using a CO2:H2 manifold (Balch and Wolfe, 1976), and incubated at 39°C. For each time point (0 and 24 h) samples were centrifuged (10,000 × g) for 10 min. The supernatants were aspirated, and stored at −20°C for determining SCFA concentration. The substrate pellets were used for molecular bacterial profile analysis.

**SCFA Concentration**

The concentration of SCFA from 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 a 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-butyric 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.

**PCR-Based Denaturing Gradient Gel Electrophoresis Procedure**

Genomic DNA was isolated from 1.0 mL of each sample using a QIAamp DNA mini kit according to instructions described in the manual (Qiagen, Valencia, CA). Denaturing gradient gel electrophoresis (DGGE) was conducted by the method of Muyzer et al. (1993) with modification, using bacterial-specific PCR primers to conserve the region flanking the variable V3 region of 16S rDNA genes. The PCR was run with a 50-µL total reaction volume. Primers (50 pmol of each per reaction mixture; primer 2, 5′-ATTACC CGGGCTGCTGG-3′, and primer 3 with a 40-bp G-C clamp (Sheffield et al., 1989; Muyzer et al., 1993), 5′-CAGCCCGCGCCGCGG CGGCGGGCGGCGACGGGGGCCTACGGAGGCAGCAG-3′), were mixed with Jump Start Red-Taq Ready Mix (Sigma Chemical Company, St. Louis, MO) according to kit instructions, with 250 ng (50 ng of DNA pooled from 3 samples each) of template DNA. Acetamide (5%, wt/vol) was added to eliminate preferential annealing (Reysenbach et al., 1992). Bovine serum albumin (10 mg/mL) and deionized water were 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-products (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°C for 1 min; 7) annealing at 58.0°C for 45 s; 8) repeat steps 6 to 7 for
Gel Electrophoresis

Polyacrylamide gels (8% vol/vol; acrylamide-bisacrylamide ratio of 37.5:1) were cast with a 35 to 60% urea-deionized formamide gradient. The 100% denaturing acrylamide was 7M 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 loaded in a DCode Universal Mutation Detection System (Bio-Rad Laboratories, Richmond, CA) for electrophoresis in 1× Tris-acetate EDTA (20 mM Tris, pH 7.4, 10 mM sodium acetate, 0.5 M Tris-acetate 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 1.610 (Bio-Rad Laboratories) based on the Dice similarity coefficient and the unweighted pair group method using arithmetic averages (UPGMA) for clustering. The Dice coefficient (values between 0 and 1) used was an arithmetic method, which determined the degree to which banding patterns were similar. Clusters (groups) were determined by sequentially comparing the patterns and constructing a dendrogram reflecting the related similarities. The amount of similarity was reflected by the relatively closeness or grouping and was indicated by the percentage similarity coefficient bar located above each dendrogram.

Statistical Analysis

Data for concentrations of SCFA were analyzed using the 1-way ANOVA subjected to linear regression using SAS software (SAS Institute, 2001). Differences between means were determined using least squares means and Tukey’s honestly significant test. Statistical variation was also estimated by the standard error of the mean. All statistical analyses were considered significant at $P < 0.05$.

RESULTS

SCFA Production

Short-chain fatty acid production results for HFFS incubated with cecal inocula are shown in Figure 1. The 2 trials were statistically different ($P < 0.05$) and were therefore, examined separately. In all HFFS incubations, acetate production (Figure 1a) was highest. Propionate (Figure 1c) and butyrate (Figure 1e) were next in order of magnitude whereas iso-butyrates (Figure 1b) and iso-valerate (Figure 1f) production were in trace amounts. However, detectable valerate (Figure 1d) production appeared to consistently occur with alfalfa-based HFFS. It was clear that SCFA production was largely dependent upon HFFS, because the treatment with no HFFS + cecal inoculum (NFI) yielded little or no detectable SCFA production. In trial 1 there were no significant differences for acetate production from the various feed substrates (Figure 1a) but in trial 2, HI, SI, AI, and A9I were significantly higher ($P < 0.05$) than all other feed substrates, whereas BI, WI, CI, A8I, and A7I were higher than GI. All feed substrates were higher than NFI. Propionate production (Figure 1c) in trial 1 ranged from SI, WI, GI, and CI being the highest ($P < 0.05$) to HI being the lowest, whereas in trial 2, SI, A9I, HI, BI, WI, and CI were the highest ($P < 0.05$) and AI, GI, A8I, and A7I were the lowest. Butyrate production (Figure 1e) in trial 1 was highest ($P < 0.05$) for A7I, GI, CI, A8I, AI, and WI compared with HI, BI, and A9I; in trial 2, A9I, A7I, SI, CI, A8I, AI, and WI were higher than HI, BI, and A9I.

Similar quantities of HFFS were incubated in anaerobic dilution solution without cecal content (treatments designated with “0”); Figure 2) during the same periods. Samples were processed similar to those with added inocula. The 2 trials were statistically different ($P < 0.05$) and were therefore examined separately. In all HFFS fermentations, acetate production (Figure 2a) was highest. Propionate (Figure 2c) and butyrate (Figure 2e) were next in order of magnitude (and similar), whereas iso-butyrate (Figure 2b), valerate (Figure 2d), and iso-valerate (Figure 2f) production were in trace amounts. In trial 1, acetate production (Figure 2a) from A0, B0, A90, A80, A70, W0, S0, and H0 were higher ($P < 0.05$) than G0 and C0. Treatment A0 was higher ($P < 0.05$) than all treatments except A90, and A80. In trial 2, H0 was higher than all other treatments except A80 and A70; these 3 treatments along with W0 yielded more acetate than B0, G0, and C0. Propionate production (Figure 2c) was only detectable for B0 in trial 1 and A0 and A70 in trial 2. For butyrate production in trial 1 (Figure 2e), W0 was higher ($P < 0.05$) than all other treatments except for A80. No butyrate was detected in G0, C0, and S0. In trial 2, W0 also yielded the highest level of butyrate with H0 and A70 being higher than S0, B0, A0, G0, A90, and A80.

PCR-Based DGGE

It was assumed that similar bacterial populations were obtained from both cecal inoculum and HFFS during in vitro fermentation; however, it was difficult to determine the percentage microbial contribution from either source. In trial 1, the amplicon profile of bacteria from cecal inoculum and HFFS fermentation in vitro revealed a developmental progression of microbial diversity (Figure 3). Bacterial diversity was divided into 4 distinct groups in trial 1: A9I, A8I, AI, and WI formed a cluster with 65% correlation, whereas SI, HI, GI, and BI formed a cluster with 73% correlation. Treatments A7I and NFI formed a group with an 85% similarity coefficient; CI was segregated from the other HFFS with a 45% similar-
Figure 1. Production of short-chain fatty acids (μmol/mL) during in vitro fermentation of cecal inoculum and different food substrates (trials 1 and 2). Feed substrates: SI = soybean meal, HI = soybean hull, BI = beet pulp, WI = wheat middlings, GI = ground sorghum, CI = cottonseed meal, AI = 100% alfalfa meal, A9I = 90% alfalfa + 10% commercial layer ration, A8I = 80% alfalfa + 20% commercial layer ration, A7I = 70% alfalfa + 30% commercial layer ration, and NFI = no feed + inoculum. Error bars represent standard errors of mean (n = 3); letters above error bars indicate significant differences (P < 0.05): *means within trial 1; **means within trial 2.
Figure 2. Production of short-chain fatty acids (μmol/mL) during in vitro fermentation of different food substrates with anaerobic dilution solution (i.e., no cecal inoculum; trials 1 and 2). Feed substrates: S0 = soybean meal, H0 = soybean hull, B0 = beet pulp, W0 = wheat middlings, G0 = ground sorghum, C0 = cottonseed meal, A0 = 100% alfalfa meal, A90 = 90% alfalfa + 10% commercial layer ration, A80 = 80% alfalfa + 20% commercial layer ration, A70 = 70% alfalfa + 30% commercial layer ration, and NFI = no feed + inoculum; R = reference amplicon. Error bars represent standard errors of mean (n = 3); letters above error bars indicate significant differences (P < 0.05): a–d means within trial 1; A–D means within trial 2.
The highest level of similarity (89.5%) was observed between A9I and A8I, groups that formed a subgroup in the aforementioned cluster. Treatments A7I and NFI also demonstrated high similarity (85%).

In trial 2, cecal inoculum and HFFS were also divided in the basis of microbial diversity (Figure 4). Three distinct groups were formed in trial 2, with HI, WI, and GI exhibiting 85% similarity in microflora banding patterns; A9I, A8I, AI, A7I, NFI, and BI forming a large cluster with an 84% correlation; and SI and CI forming a group with an 89% similarity coefficient. The overall band patterns for microbial similarity in trial 2 (72%) was much higher than in trial 1 (35%). All alfalfa-based HFFS fermentations exhibited a higher similarity coefficient in...
trial 2 compared with trial 1 with a band pattern at 90% similarity; treatments A9I and A8I in trial 2 formed a subgroup showing a 94% microbial similarity coefficient.

**DISCUSSION**

The microbial composition of the GI tract of avian species is not as extensively characterized compared with what is known about microorganisms in ruminants (Ricke and Pillai, 1999). A previous report (Zhu et al., 2002) indicated that only 10 to 60% of microorganism in the ceca could be propagated using anaerobic culture techniques. However, over 200 different bacteria have been isolated and characterized and are thought to be influenced by various factors including diet, health, and age (Barnes et al., 1972; Barnes, 1979; Ricke et al., 2004a). Such factors
become important during poultry production when particular management practices are introduced. In the poultry industry, feed deprivation is a procedure used to induce molting to achieve a rapid and economical new egg-laying cycle (Brake, 1993; Holt, 1995). Removal of feed can lead to changes in the nutritional status of the GI tract of poultry to alter the indigenous microbial population levels and fermentation activities that create sufficient colonization opportunities for pathogens such as Salmonella (Ricke, 2003a). It has been proposed that dietary fiber can be utilized preferentially by Lactobacillus and Bifidobacteria species (Kaplan and Hutkins, 2000), which leads to the production of lactic acid and SCFA. The presence of fiber can lead to the maintenance of a normal microbial population (Fuller and Turvey, 1971; Bird, 1999). High-fiber, low-energy diets including alfalfa and wheat middlings have been examined as alternative diets that could support sufficient microbial fermentation but reduce dietary energy enough to induce molt (Seo et al., 2001; Holt, 2003; Ricke, 2003a; Donalson et al., 2005; Landers et al., 2005a,b; Woodward et al., 2005).

The purpose of the current study was to use anaerobic in vitro incubations to screen the various fiber sources that have been examined in vivo as molt-induction diets for their capacity to support cecal microbial fermentation. In the current in vitro study, chicken cecal contents and anaerobic dilution solution (Bryant, 1973) were used to establish cecal inoculum to ferment 10 HFFS at 0 and 24 h. Levels of SCFA were compared because it is known that SCFA can inhibit Salmonella growth (Van der Wielen et al., 2001, 2002), thus serving as a potential indicator of the relative efficacy of HFFS. Based on the higher levels of SCFA from HFFS fermented with cecal microorganisms compared with HFFS without cecal microorganisms, it appears that cecal microorganisms are capable of fermenting a wide variety of fiber sources. Langhout and Schutte (1996) concluded that the concentration in cecal chyme were decreased when chickens were fed a diet containing high-methylated citrus pectin, whereas low methylated citrus pectin had no effect. Alfalfa, soybean, and soybean-based HFFS exhibited consistently high levels of SCFA production. Acetic acid was produced in the greatest amount with lesser quantities of propionic and butyric acid and trace amounts of other acids. This pattern is consistent with the observation from in vitro incubations of broiler cecal contents and carbohydrate fractions (Lan et al., 2005) and correlates with other in vitro studies demonstrating that dietary fiber fractions can be fermented by cecal microorganism to form end products such as SCFA, ammonia, CO₂, and methane (Jørgensen et al., 1996; Marounek et al., 1996, 1999; Jamroz et al., 2002; Guo et al., 2003; Lan et al., 2005; Saengkerdsub et al., 2006). Tsukahara and Ushida (2000) demonstrated that feeding chickens with a plant protein-based diet generated a higher concentration of SCFA than a diet of animal protein; those authors concluded that the difference in SCFA was due to a higher concentration of dietary fiber in a plant-based diet.

An examination of the digesta from different morphological regions of the GI tract of poultry revealed (Annison et al., 1968) that SCFA were present in the highest concentration in the ceca, including acetic, propionic, and butyric acids. Further investigation demonstrated that SCFA was not influenced by age (14 to 20 wk) and that there was a decrease in SCFA production when cecectomy was performed on birds. This suggested that the ceca might play a significant role in GI tract fermentation in poultry. Supporting evidence demonstrated that cecectomy leads to reduction in the digestibility coefficient for crude fiber including wheat and corn (Halnan, 1949).

**PCR-Based DGGE**

Historically, the isolation and characterization of commensal GI bacteria has been limited by traditional culturing techniques (Ricke and Pillai, 1999). More recent studies have demonstrated the efficacy of DGGE as a tool to examine complex microbial community (Ferris and Ward, 1997; Heuer et al., 1997; Muyzer and Smalla, 1998; Simpson et al., 1999; Hume et al., 2003; Ricke et al., 2004b). A difference in amplicon pattern and similarity scores of 16S rDNA when amplified can be used to determine overall microbial population variations. The current in vitro study indicated that bacterial populations were altered on the basis of different HFFS fermented with cecal inocula. This corresponds with the conclusion by Lan et al. (2005) that diet could be one of the major determinants factors in the bacterial diversity of the GI tract. For the most part microbial amplicon patterns of alfalfa-based HFFS in both trials demonstrated high similarity coefficients among microbial populations. Previous reports indicated that both the source of feed and local feed amendment can influence the bacterial profile of the intestinal microbial community significantly, whereas bacterial profiles of birds fed on identical feed regimens exhibited more closely aligned profiles (Apajalahti et al., 2001; Hume et al., 2003; Ricke et al., 2004b).

The current in vitro study was conducted to determine the efficacy of cecal microorganisms of chickens to ferment various HFFS. It appears that cecal microorganisms can ferment HFFS to produce acetate, propionate, and butyrate as major fermentation products and these are potentially important in limiting Salmonella colonization (McHan and Shotts, 1993; Ricke, 2003b). Based on the microbial amplicon patterns from DGGE in the current study it also appears that the cecal microorganisms can be altered by changes in dietary sources. Previous studies have demonstrated the value of in vitro fermentation systems for evaluating the prebiotic potential of dietary carbohydrates (Vulevic et al., 2004; Lan et al., 2005). Combining molecular profiling with fermentation characterization is a potentially useful tool to investigate microbial diversity in relationship to dietary shifts in poultry but in vivo comparisons of these dietary carbohydrates are needed for further validation.
ACKNOWLEDGMENTS

This research was supported by Hatch grant H8311 administered by the Texas Agricultural Experimental Station, USDA-NRI grant number 2002-02614 and US Poultry and Egg Association grant 485. Thanks to Albert Blanks (USDA-ARS, College Station, TX) for technical assistance and Cassie Woodward (Poultry Science Dept., Texas A&M University, College Station) for data analysis and helpful discussion for preparation of the manuscript.

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