Evaluation of feeding distiller’s grains, containing virginiamycin, on antimicrobial susceptibilities in fecal isolates of Enterococcus and Escherichia coli and prevalence of resistance genes in cattle

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ABSTRACT: Dried distiller’s grains (DG) produced from ethanol fermentations dosed with 0 (control), 2, or 20 mg/kg virginiamycin-based product or spiked with virginiamycin (VM) postfermentation were fed to cattle and effects on antimicrobial susceptibility, and prevalence of antimicrobial resistance genes in commensal bacteria was examined. Biological activity assays of DG (from each fermentation) indicated a concentration of 0, 0.7, and 8.9 mg/kg VM, respectively. Twenty-four crossbred beef steers were fed 1 of 4 diets (containing 8% of each of the different batches of DG) and a fourth using 8% of the control DG (0 mg/kg VM) + 0.025 g/kg V-Max50 (positive control) for 7 wk. Fecal samples were collected weekly throughout the experimental period and cultured for Escherichia coli and Enterococcus, and isolates were examined for antimicrobial susceptibility, antimicrobial resistance genes (vatE, ermB, and msrC in Enterococcus), and integrons (E. coli). No treatment differences (P > 0.05) were observed in antimicrobial susceptibility of the E. coli isolates. Enterococcus isolates were resistant to more antimicrobials; however, this was influenced by the species of Enterococcus and not treatment (P > 0.10). The prevalence of ermB was greater (P < 0.05) in the control isolates after 4 and 6 wk while at wk 7, prevalence was greater (P < 0.01) in the 0.7 and 8.9 mg/kg VM treatments. Taken together, the minor treatment differences observed for the presence of ermB coupled with the lack of effect on antimicrobial susceptibility patterns suggest that feeding DG containing VM residues should have minimal if any impact on prevalence of antimicrobial resistance.

Key words: antimicrobial resistance, cattle, distiller’s grains, virginiamycin

INTRODUCTION

The expansion of the fuel ethanol industry within the United States has produced a significant quantity of fermentation byproducts, generally referred to as distiller’s grains (DG), that are used extensively as cattle feed (National Agricultural Statistics Service, 2007; Vasconcelos and Galyean, 2007). There are concerns that antimicrobials (penicillin, virginiamycin [VM], streptomycin, tetracycline, and monensin), used to suppress bacterial growth during the fermentation process (Day et al., 1954; Narendranath et al., 2000), may result in residues in the DG. Originally, it was speculated that the distillation process destroys these antimicrobials, however, in 2009 and again in 2012, the United States Food and Drug Administration reported residues in domestic and imported samples of DG (McChesney, 2009; Luther, 2012).

Previous research attempted to examine the influence of feeding DG on antimicrobial susceptibility of fecal bacteria. Jacob et al. (2008) reported that feeding wet DG with solubles had no effect on the prevalence of antimicrobial resistance genes or antimicrobial susceptibility of Enterococcus and generic...
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Escherichia coli isolates. Similarly, we reported no effect of feeding wet DG on antimicrobial susceptibility patterns of fecal coliform isolates cultured from feedlot cattle (Edrington et al., 2010). However, both experiments suffered from a lack of antimicrobial susceptibility data before DG feeding; it was unknown if the DG were produced with antimicrobials in the fermentation process, and residue data were not reported; therefore, the effects of feeding DG on antimicrobial susceptibility were largely speculative. The objective of the current research was to examine the effect of feeding DG, produced in 3 separate fermentations using different levels of VM product and containing known concentrations of VM residues, on antimicrobial susceptibility patterns and the prevalence of resistance genes in the fecal Enterococcus and E. coli isolates in beef steers.

MATERIALS AND METHODS

Care, use, and handling of experimental animals were preapproved by the Animal Care and Use Committee of the Food and Feed Safety Research Laboratory, USDA.

Distiller’s Grains

Dried DG produced at the pilot plant facilities at the National Corn to Ethanol Research Center, Edwardsville, IL, were used in the feeding trial. Three lots were produced from different fermentations using no antimicrobial or dosed with 2 (a dosage recommended by the manufacturer to prevent and suppress bacterial contamination) or 20 mg/kg VM product (a dosage exceeding the manufacturer’s maximal recommended dosage of 6 mg/kg). Samples of the DG were extracted with a citric acid–acetone solvent (Hamdy et al., 1996) and analyzed for VM using a biological activity assay with Kocuria rhizophila ATCC 9341 as test organism. Bacteria were embedded in Antibiotic Medium #11 agar (Becton, Dickinson and Co., Franklin Lakes, NJ) arranged in a circle were placed on the surface of the cooled surface. Virginiamycin standard (a natural mixture of VM factors M and S purchased from RPI Corporation, Mt. Prospect, IL) or sample extract was applied to 3 of the discs in an alternating pattern with a reference solution of VM (2 μg/mL). Plates were incubated at 37°C for 18 to 24 h. Diameters for standards and samples were normalized to diameters for reference discs, and a standard curve produced by plotting normalized zone diameters versus the logarithm of VM concentration. Analysis indicated that DG produced from the 0, 2, and 20 mg/kg dosed ethanol fermentations contained 0, 0.7, and 8.9 mg/kg VM/g DG, respectively.

Cattle Feeding Experiment

Twenty-four crossbred Angus steers (average BW = 190 kg) were transported to the livestock research facilities at the USDA-ARS Food and Feed Safety Research Laboratory in College Station, TX. Upon arrival, steers were weighed, ear tagged, and housed in a large outdoor pen with access to shade. Over a 28-d acclimation period, steers were adapted to an 80% concentrate diet without DG (d 1 to 21) and pens (d 14 to 28) and trained to use individual feeders (Calan gates; American Calan, Inc., Northwood, NH) within each pen (d 14 to 28). Steers were randomly assigned to pens (outdoor and covered; 3 steers/pen) on d 14 where they remained throughout the remainder of the experimental period. Each pen was equipped with 4 Calan gates that allowed for individual feeding of steers within that pen. Steers were fed at 2.5% of their BW throughout the experimental period. Weekly adjustments were made in the amount of feed provided based on weight gain. Treatments (2 pens and 6 steers/treatment) were assigned to pen such that there were 2 consecutive pens of the same treatment separated by an empty pen to avoid animal to animal contact. Steers assigned to the positive-control treatment (described below) were housed in 2 pens that were approximately 50 m removed from the other pens. Foot baths containing an antimicrobial soap solution were placed in front of each pen entrance and used by workers before entering and immediately after leaving each pen. Pens floors all sloped slightly to an alley outside of the pens that transported waste (feces and urine) not collected by rake and shovel to a single collection point. This prevented cattle in one pen from coming into contact with feces from another pen. Pens were cleaned daily.

The DG was incorporated into a feedlot ration representative of what is used in the southwestern United States for feeding beef cattle (Table 1). Four diets (treatments) were evaluated: 3 using 8% of each of the different DG batches (0 [negative control], 0.7, and 8.9 mg/kg VM) and a fourth using 8% of the control DG + 0.06 mg/kg VM-Max50 (VM; positive control; Phibro Animal Health, Ridgefield Park, NJ). Experimental diets were initiated immediately following the 28-d adaptation period (d 1) and continued throughout the 49-d experimental period. Water was provided for ad libitum consumption throughout the experiment. Buckets and feed scoops, specific to each treatment, were used to deliver feed to each Calan gate feeder to avoid potential cross contamination of diets.

Fecal samples were collected on d 0, before initiation of experimental diets, and weekly thereafter throughout the 7-wk period and cultured for generic E. coli and Enterococcus species. Steers were restrained in a squeeze chute and feces collected via rectal palpation into a sterile palpation sleeve. All fecal samples were processed.
within 1 h of collection. Approximately 10 g of each fecal sample was mixed with 90 mL of tryptic soy broth (TSB) with phosphate (30 g TSB, 2.31 g KH$_2$PO$_4$, and 12.54 g K$_2$HPO$_4$/L, final pH 7.2) and a portion of the feces/TSB mixture plated directly onto CHROMagar E. coli agar (DRG International, Mountainside, NJ) for culture of generic E. coli or Enterococcus agar (Becton-Dickinson and Co., Sparks, MD). Plates were incubated at 37°C for 18 to 24 h. Generic E. coli isolates (3 per sample) were frozen for later analysis. Enterococcus isolates were confirmed and identified by species using API test kits (Rapid ID32 Strep; BioMerieux Inc., Durham, NC) and 3 isolates per fecal sample, representing the most prevalent species from each sample, were frozen for antimicrobial susceptibility testing and screening for antibiotic resistance genes described below.

**Isolate Testing**

Minimum inhibitory concentrations for antimicrobials were determined by broth microdilution as reported previously (Edrington et al., 2011). Enterococcus isolates ($n = 288$; 72 each from wk 1, 4, 6, and 7) were screened for the presence of Class 1 integrons and their gene cassettes using PCR primers (Bass et al., 1999).

**Statistical Analysis**

Data were analyzed using SAS version 9.02 (SAS Inst. Inc., Cary, NC). The PROC MIXED procedure was used to analyze antimicrobial resistance displayed by E. coli isolates, using steer as the experimental unit. Prevalence of the resistance genes was analyzed using the PROC GLIMMIX procedure with treatment, week, and treatment × week interaction included in the model statement. A chi-square analysis using the PROC FREQ procedure was used to examine the prevalence of different Enterococcus species, the prevalence of resistance genes within Enterococcus species, and the effect of treatment on gene prevalence within Enterococcus species. Differences in means were considered significant at a 5% level of significance.

**RESULTS**

A total of 576 E. coli and Enterococcus isolates were preserved for further analysis. However, due to the cost associated with susceptibility and PCR testing, a subset of the isolates was examined initially to determine if analysis of all isolates would provide meaningful information. As no significant differences were observed due to treatment in the isolates collected early in the experiment, not all weekly collections were analyzed for antimicrobial resistance or for the presence of resistance genes.

**Escherichia coli Isolates**

All of the tested E. coli isolates ($n = 288$; 72 each from wk 0, 7, 28, and 49) were susceptible to 8 of the 15 antimicrobials on the National Antimicrobial Resistance Monitoring panels and only a few resistant to ampicillin (6 isolates; 20 mg/kg treatment), chloramphenicol (4, 3, and 1 isolate in the negative-control and 0.7 and 8.9 mg/kg VM and positive-control treatments, respectively), and nalidixic acid (1 and 3 isolates in the negative-control and 8.9 mg/kg VM treatments, respectively). A substantial number of isolates demonstrated resistance to streptomycin, sulfisoxazole, tetracycline, and trimethoprim/sulphamethoxazole (Fig. 1). A treatment effect ($P < 0.05$) was observed in resistance to trimethoprim/ sulphamethoxazole and a treatment trend ($P = 0.06$) for isolates resistant to streptomycin (Fig. 1). Day had no affect ($P > 0.10$) with little change in the percentage of resistant isolates over the 7-wk experimental period (data not shown). Similarly, a treatment × day interaction was
not observed ($P > 0.10$). Multidrug resistance (MDR; resistance to 2 or more antimicrobials) was not influenced by treatment or day ($P > 0.10$) and was relatively constant throughout the experimental period. Isolates were generally resistant to 2 different antimicrobials with minimal change over the course of the experiment (Fig. 2). Class 1 integrons were detected in 2 $E. \text{coli}$ isolates and not affected by treatment (data not shown).

**Enterococcus Isolates**

The most predominant $Enterococcus$ species was selected from each fecal sample culture and was observed to vary over the course of the experimental period. In general, $E. \text{casseliflavus}$ and $E. \text{avium}$ were the most predominant species cultured in wk 1, to be replaced in wk 6 and 7 by $E. \text{gallinarum}$ and $E. \text{hirae}$. The number of different $Enterococcus$ species increased as the experiment progressed (Fig. 3). Resistance to individual antimicrobials as well as MDR (resistant to 2 or more antibiotics) was influenced by $Enterococcus$ species, which confounded treatment comparisons. All tested isolates ($n = 360$; 72 from each of wk 0, 7, 28, 42, and 49) were resistant to lincomycin and susceptible to vancomycin. The majority of resistance was to erythromycin, kanamycin, streptomycin, and tetracycline, with a few isolates also demonstrating resistance to gentamicin, nitrofurantoin, and quinupristin/dalfopristin (data not shown). The MDR isolates generally belonged to $E. \text{avium}$, $E. \text{gallinarum}$, $E. \text{faecium}$, $E. \text{fecalis}$, and on occasion $E. \text{hirae}$. Multidrug resistant $E. \text{fecalis}$ isolates were isolated only from negative-control steers (wk 4 and 7) and $E. \text{faecium}$ isolates from steers in the negative-control and 8.9 mg/kg VM treatments (wk 4 only). $Enterococcus \text{gallinarum}$ was cultured in all treatments, with the exception of control animals, later in the experiment (wk 6 and 7). $Enterococcus \text{hirae}$ was likewise cultured only in wk 6 and 7 of the experiment, in all treatments except 8.9 mg/kg VM. Feeding DG with VM residues did not appear to influence resistance to individual antimicrobials or the prevalence of MDR $Enterococcus$ isolates.

The prevalence of antimicrobial resistance genes associated with selection by VM was assayed in the $Enterococcus$ isolates ($n = 288$) and data presented in Fig. 4. Significant treatment and week effects were observed as well as a treatment × week interaction ($P < 0.05$). The prevalence of $\text{vatE}$ was decreased ($P < 0.05$) in the positive-control treatment compared to other treatments on d 42; otherwise no other differences ($P > 0.10$) were observed (Fig. 4A). Similarly, on d 14 and 42, the prevalence of $\text{ermB}$ (Fig. 4B) was increased ($P < 0.05$) in the negative-control and 0.7 mg/kg VM treatments compared to the 8.9 mg/kg VM and positive-control treatments, except on d 49 where the 0.7 and 8.9 mg/kg VM treatments had significantly higher prevalence. Although variable, no differences ($P > 0.10$) were observed for the prevalence of $\text{msrC}$ genes (Fig. 4C).

Similar to the antimicrobial susceptibility results, the presence of all 3 resistance genes differed among $Enterococcus$ species ($P < 0.05$). The $\text{vatE}$ gene was widely distributed in the various $Enterococcus$ species but most prevalent in $E. \text{avium}$ (Fig. 5). The $\text{ermB}$ gene was detected in 100% of the $E. \text{fecalis}$ isolates and over half of the $E. \text{gallinarum}$ and $E. \text{hirae}$ isolates. The presence of $\text{msrC}$ was found in 3 species and by far the most prevalent in $E. \text{gallinarum}$ (Fig. 5). Due to these species differences, data were analyzed by treatment within $Enterococcus$ species (Fig. 6). Feeding DG with VM residues tended to ($P < 0.10$) decrease the prevalence of $\text{vatE}$ in $E. \text{avium}$ isolates and likewise tended ($P < 0.10$) to decrease the presence of this gene in the $E. \text{casseliflavus}$ isolates, cultured from the 8.9 mg/kg VM and positive-control treatments. The
**DISCUSSION**

To our knowledge this is the first report investigating the feeding of DG, containing known levels of VM residues, on antimicrobial susceptibility profiles and prevalence of resistance genes in cattle. Before this, researchers speculated that residues could be present and looked for differences in susceptibility profiles of enteric bacteria between cattle fed DG and control animals. Previous work conducted in our laboratory reported no differences in antimicrobial resistance profiles of putative fecal coliform isolates in feedlot cattle fed diets with and without wet DG (Edrington et al., 2010). Similarly, Jacob et al. (2008) reported only minor differences in antimicrobial resistance to individual antibiotics in generic *E. coli* and no significant changes in susceptibility of fecal *Enterococcus* isolates in cattle fed wet DG.

Similar to these reports, the current research did not observe any significant effects of feeding DG, this time with known concentrations of VM residues, on antimicrobial susceptibility of *E. coli* or *Enterococcus* isolates. Quinupristin/dalfopristin, like VM, is a streptogramin and should serve as an indicator of selection for streptogramin resistance. Resistance was observed to this antibiotic: 27, 10, 12, and 8 isolates in the negative-control, 0.7 mg/kg VM, 8.9 mg/kg VM, and positive-control treatments demonstrated resistance, respectively. This resistance, however, was not influenced by feeding DG. *Enterococcus* isolates were examined for the following resistance genes that may have been selected for by VM: *vatE* (virginiamycin acetyltransferase), *ermB* (macrolide-lincosamide-streptogramin B resistance by methylation of 23S rRNA), and *msrC* (streptogramin B/macrolide efflux pump). Some minor treatment differences were observed but nothing consistently related to feeding DG containing VM residues.

Results herein suggest that feeding DG containing VM residues should have limited, if any, impact on antimicrobial resistance of enteric fecal bacteria or the prevalence of resistance genes conferring streptogramin resistance. This is certainly good news in regard to the potential spread of antimicrobial resistance from the ethanol to the cattle-feeding industry in regards to the use of DG. Future research would ideally seek to expand on this research by implementing a larger scale experiment in an experimental/com-
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Commercial feedlot and monitoring resistance among enteric bacteria throughout the entire feeding period in cattle fed DG with and without known antimicrobial residues.

LITERATURE CITED


Figure 5. Prevalence (%) of resistance genes (presented by Enterococcus species) detected in Enterococcus isolates cultured from cattle fed diets containing dried distiller's grains containing 0 (negative control), 0.7, or 8.9 mg/kg virginiamycin (VM) or the control diet with added VM (positive control).

Figure 6. Prevalence (%) of resistance genes (presented by treatment and Enterococcus species) detected in Enterococcus isolates cultured from cattle fed diets containing dried distiller's grains containing 0 (Neg-Control), 0.7, or 8.9 mg/kg virginiamycin (VM) or the control diet with added VM (Pos-Control). Percentages within a species and gene differ denoted by asterisks. (**P < 0.05; *P < 0.10).