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J ANIM SCI 2013, 91:2395-2404.
doi: 10.2527/jas.2012-5714 originally published online March 5, 2013

Presence and biological activity of antibiotics used in fuel ethanol and corn co-product production[1]

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ABSTRACT: Antibiotics are used in ethanol production to control bacteria from competing with yeast for nutrients during starch fermentation. However, there is no published scientific information on whether antibiotic residues are present in distillers grains (DG), co-products from ethanol production, or whether they retain their biological activity. Therefore, the objectives of this study were to quantify concentrations of various antibiotic residues in DG and determine whether residues were biologically active. Twenty distillers wet grains and 20 distillers dried grains samples were collected quarterly from 9 states and 43 ethanol plants in the United States. Samples were analyzed for DM, CP, NDF, crude fat, S, P, and pH to describe the nutritional characteristics of the samples evaluated. Samples were also analyzed for the presence of erythromycin, penicillin G, tetracycline, tylosin, and virginiamycin M1, using liquid chromatography and mass spectrometry. Additionally, virginiamycin residues were determined, using a U.S. Food and Drug Administration-approved bioassay method. Samples were extracted and further analyzed for biological activity by exposing the sample extracts to 10^4 to 10^7 CFU/mL concentrations of sentinel bacterial strains *Escherichia coli* ATCC 8739 and *Listeria monocytogenes* ATCC 19115. Extracts that inhibited bacterial growth were considered to have biological activity. Physiochemical characteristics varied among samples but were consistent with previous findings. Thirteen percent of all samples contained low (≤1.12 mg/kg) antibiotic concentrations. Only 1 sample extract inhibited growth of *Escherichia coli* at 10^4 CFU/mL, but this sample contained no detectable concentrations of antibiotic residues. No extracts inhibited *Listeria monocytogenes* growth. These data indicate that the likelihood of detectable concentrations of antibiotic residues in DG is low; and if detected, they are found in very low concentrations. The inhibition in only 1 DG sample by sentinel bacteria suggests that antibiotic residues in DG were inactivated during the production process or are present in sublethal concentrations.

Keywords: antibiotic, antimicrobial, distillers grains, ethanol production

J. Anim. Sci. 2013.91:2395–2404
doi:10.2527/jas2012-5714

INTRODUCTION

Fermentation of corn starch to ethanol is highly susceptible to contamination by bacteria (Bischoff et al., 2009). Contaminating bacteria compete with yeast for nutrients during fermentation. This competition decreases conversion of sugars to ethanol by yeast and results in reduced ethanol yields (Skinner and Leathers, 2004). Reduction of ethanol yields of up to 30% have been reported due to contamination by lactic acid-producing bacteria (Makanjuola et al., 1992; Chang et al., 1995; Bischoff et al., 2009). To combat bacterial contamination, ethanol plants add antibiotics in relatively low concentrations during the fermentation stage of ethanol production (Juranek and Duquette, 2007).

The U.S. Food and Drug Administration (**FDA**) has the responsibility of evaluating, monitoring, and regulating antibiotics, and their use. The FDA has expressed concern that antibiotics added to ethanol fermentation may remain in the ethanol co-product, distillers grains (**DG**), a common ingredient used

[1]This research was financially supported by the Minnesota Corn Research and Promotion Council, Shakopee, MN.
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Received August 3, 2012.
Accepted February 11, 2013.

in livestock and poultry feeds. If biologically active amounts of antibiotic residues are present, they could theoretically have antimicrobial effects in livestock consuming DG. Unintended antibiotic residue consumption could also potentially lead to development of bacterial resistance in populations of potential bacterial pathogens of animals and humans (Benz, 2007).

Although it is possible that antibiotic residues may be present in DG, it is likely that the high pH and temperatures encountered during the production process may render these residues biologically inactive. Currently, no peer-reviewed studies have been published reporting the presence, concentration, or biological activity of antibiotic residues in DG. Therefore, it was the objective of this study to quantify concentrations of various antibiotic residues in DG and determine if those residues are biologically active.

MATERIALS AND METHODS

Twenty distillers dried grains (**DDG**) and 20 distillers wet grains (**DWG**) samples were collected quarterly for 1 yr, from January 2011 through December 2011. However, 1 DWG sample was not collected from the second quarter, leading to a total of 80 DDG (>65% DM) and 79 DWG (<65% DM) samples collected. Samples were obtained from 9 U.S. states, representing 43 ethanol plants, and were collected by nutrition consultants on farms using DG. Although this collection procedure potentially could result in some inconsistencies and duplication, it was chosen to guarantee random and blinded (by production origin) sampling.

Duplicate samples from the same ethanol plant occurred in each period; the number of duplicate samples ($n = 31$) represented 19% of the total samples collected ($n = 159$). Duplicate samples were collected independently of each other; therefore, they were retained as individual observations in the antibiotic residue concentration and biological activity data set used in the analysis. Distillers grains samples were analyzed for pH and nutrient concentrations to characterize them to reflect that they were representative of typical DG available in the U.S. feed ingredient market. Nutrient content and pH values of duplicate samples were averaged to determine the main effect of DG type on sample physicochemical properties.

Collection, Handling, and Shipment of Samples

To collect DWG samples, consultants used a 3.78-L bucket and 227-g scoop to gather a representative sample from on-farm feed piles. They were asked to obtain 1-kg samples of DWG by collecting nine 114-g subsamples from various locations in a DWG pile. Subsampling locations were to be at least 0.9 m apart, 152 mm above the floor, and 228 to 304 mm into the feed pile. All subsamples were added to a bucket, mixed thoroughly, and then placed in a plastic bag (3.78 L) that was sealed. Consultants identified and labeled samples with the collection date, site of sampling, and ethanol plant of origin. They were provided with all of the materials for collecting samples, as well as a written sampling protocol. Samples of DWG were kept frozen from collection to delivery. All DDG samples were provided by an independent nutrition consulting company. Distillers dried grains samples were mailed to the University of Minnesota, Saint Paul, in sealed plastic bags with identification of the ethanol plant of origin, as well as date of collection. The amount of DG samples collected ranged from 200 to 1500 g.

Once DG samples arrived at the University of Minnesota, they were stored in a –20°C freezer until further preparation. Samples were thawed in a refrigerator at 2.8°C for 24 to 48 h and then subdivided for further analyses. Each sample was thoroughly remixed, either manually (DDG) or using a Proctor Silex handheld mixer (DWG; Hamilton Beach Brands Incorporated, Southern Pines, NC). Samples were then subdivided, using a measuring cup, into at least 4 subsamples of at least 50 g each. All subsamples were stored in sealed plastic freezer bags. Subsamples were either used for nutrient or antibiotic residue analysis, or they were refrozen in a –20°C freezer. Samples were thawed only once for subsampling to minimize any chemical changes.

Two subsamples from each DG source of origin were mailed to SGS North America, Incorporated, Brookings, SD, for determination of nutrient content, antibiotic residues, and biological activity. Samples were placed in 1 of 2 Styrofoam coolers with cool packs, packaged in boxes, and mailed overnight to the laboratory. One additional set of subsamples was personally delivered to Phibro Ethanol Performance Group (**Phibro EPG**), Saint Paul, MN, for virginiamycin analysis.

Nutrient Concentrations

Samples were analyzed for nutrient composition by SGS North America, Incorporated, including moisture (NFTA method 2.2.2.5), CP (AOAC 990.03; Thermo Flash 2000, Thermo Scientific, Minneapolis, MN), crude fat (AOAC method 2003.06; petroleum ether, Soxtherm, Gerhardt, Bonn, Germany), and ADF and NDF, which were determined using an Ankom fiber bag method (Ankom Technology, Macedon, NY). Dry matter was determined via calculation from moisture content. All minerals were determined using AOAC methods 968.08 and 935.13, by inductively coupled plasma spectrometry (Optima 4300 DV, PerkinElmer, Waltham, MA).

pH

Samples were analyzed for pH at the University of Minnesota. Samples were mixed with deionized water, using the analyzed moisture content to create a 20% DM mixture. After mixing, initial pH was determined, using a pH probe (Corning pH Meter 345, Corning, Lowell, MA).

Antibiotic Residue Analysis

Samples were analyzed for concentration of virginiamycin M1 subunit, erythromycin, tetracycline, tylosin, and penicillin G, by SGS North America, Inc., according to the methods described in De Alwis and Heller (2010). SGS North America, Inc. adheres to internal quality control standards and is audited to meet requirements of the International Organization for Standardization every 3 yr to ensure that quality control standards are being met.

Samples were prepared in batches of 12 unknown samples, 6 matrix-extracted standards, and positive and blank quality control samples. Samples underwent sequential extraction, subsequent clean up by solid phase extraction (**SPE**), and final analysis using liquid chromatography and ion trap tandem mass spectrometry (**LCMSMS**).

Stock standards were prepared by dissolving ~16 mg of each antibiotic in either water (erythromycin, penicillin G, and tylosin) or methanol (tetracycline and virginiamycin M1) to make 1,500 g/mL antibiotic stock standard solutions. A 100-g/mL mixed standard was prepared by transferring 2,500 g of analyte from each stock solution into a 25-mL volumetric flask and bringing to mark with water. Intermediate standards were prepared by serially diluting the mixed standard with water to create standards ranging from 0 to 50 μg/mL.

Unknown samples were prepared by weighing 5 g of DDG or DWG (on an as-fed basis), and extracting with a mixture of 1.5 mM ethylenediaminetetraacetic acid disodium salt and 1% trichloroacetic acid. Samples were shaken for 15 min, followed by 10 min of centrifugation at room temperature (or 20°C) at 4,000 × *g* (Eppendorf Centrifuge 5810R, Hamburg, Germany). The supernatants were removed and immediately diluted with 175 mL of water to reduce acid concentration. Sample pellets were then extracted for an additional 15 min with methanol. Samples were again centrifuged (as described above) and the resulting supernatants were removed and added to the first extracts. The combined supernatant extract was diluted to 200 mL (by weight) with water and mixed well. Aliquots of 10 mL were collected from the extract mixture for use with SPE (De Alwis and Heller, 2010).

Solid phase extraction columns (Oasis HLB, Waters, Milford, MA) were placed on a vacuum manifold and conditioned with methanol and water. Reservoirs with polyethylene frit (Supelco, Sigma-Aldrich, Saint Louis, MO) were placed on top of the SPE columns. The 10-mL extract aliquots collected earlier were placed in the reservoir and allowed to pass through the column slowly. The SPE columns were dried for 5 min under vacuum, washed with water, and then dried for another 5 min. Analytes were then eluted with methanol and the eluates were evaporated down to ~1 mL under the flow of nitrogen (N-Evap III, Organomation Associates, Inc., Berlin, MA). Diluent [87:13 (vol/vol) water:acetonitrile] was used to reconstitute the extracts to 2 mL, samples were vortexed, and subsequently added to an autosampler vial for analysis (De Alwis and Heller, 2010).

Matrix-extracted working standards were prepared by weighing 5 g of a control DG (corn dried DG 08102, AAFCO, Champaign, IL), which was analyzed before use in the study to confirm no presence of antibiotics, into a centrifuge tube and adding 500 μL of the corresponding intermediate standard. Remaining extraction and the SPE procedure were applied in the same manner as previously described for the unknown samples. The quality control sample was prepared using the same procedure as the working standards; however, 400 μL of 10 μg/mL intermediate standard was added to the control DG sample.

Autosampler vials were loaded into the instrument and analyzed by reverse phase liquid chromatography (Dionex, Ultimate 3000, Thermo Fischer Scientific, Minneapolis, MN). Detection was measured using electrospray ionization on an ion trap tandem mass spectrometer (ABSciex 4000 QTrap, Framingham, MA). Residue concentrations were determined via the ABSciex software (ABSciex, Framingham, MA) and reported in mg/kg. Values were adjusted from an as-fed basis to a DM basis after determination.

System suitability, development and implementation of standard curves, and screening and confirmation criteria were as defined by FDA LIB 4438. For a batch to be acceptable, system suitability required that standard injection peaks meet a signal-to-noise ratio of >3:1 and have acceptable peak shapes. Blank controls were required to have no positive identification of any compound. Positive quality control samples were required to meet screening/confirmation criteria, as described below, and be within 15% of target values. Preparation of the standard curve required that all standard concentrations be within 20% of the target concentration and the linear regression of the standard curve must have an $R^2 > 0.99$, or that standard was excluded and limit of quantification (**LOQ**) was adjusted. Limit of quantification was set at threefold greater than observed carryover in control extracts.

For unknown sample data to be quantifiably acceptable, they must also meet the after screening and confirmation criteria. Peaks were required to have a signal-to-noise ratio of >3:1. Retention time of unknown sample

peaks was required to be within 2.5% of standard retention time; penicillin G retention time was required to be within 1% of standard retention time. Ion ratios were required to be within 20% of the expected relative abundance percentages. Additionally, for quantitative acceptability, the sample values were required to exceed LOQ. If an unknown sample was greater than LOQ but the linear regression of the standard curve had an $R^2 < 0.99$, it was reanalyzed or reported as an estimate (De Alwis and Heller, 2010). In this study, all positive samples reported met screening and confirmation criteria. The LOQ for this study was 0.05 mg/kg for penicillin G, erythromycin, tylosin, tetracycline, and virginiamycin M1.

A secondary set of residues was extracted, using the methods previously described, with the exception of PBS replacing methanol throughout the entire procedure. Phosphate-buffered saline was used to reduce any inactivation of antibiotic biological activity because many of the antibiotics examined in this study have been shown to degrade quickly in methanol (Liang et al., 1998). The PBS-extracted residues were used for microbiological procedures.

FDA-approved Virginiamycin Assay

Samples were also analyzed by Phibro EPG for virginiamycin quantity and activity, according to an FDA-approved procedure. This proprietary method detects active virginiamycin residues with both the M and S components of the molecule. Additionally, the method used by Phibro EPG uses a biological assay to detect virginiamycin residues active against a specific bacterium. In contrast, LCMSMS can only detect the M component of virginiamycin, giving only a measure of the presence of the molecule. The virginiamycin detected by LCMSMS could be inactive, because both the M and S components are required for optimal antibiotic activity. For this reason, we chose to use the Phibro EPG method as the definitive method for determining the presence and quantification of virginiamycin residues in samples used in the current study.

Initially, samples were extracted in a Soxhlet apparatus, with polar and nonpolar solvents. After extraction, the solvent was evaporated. This step was followed by a series of successive solvent extractions and evaporations, a defatting and dewaxing step, and solid phase concentration. Next, methanol and phosphate buffers were used for elution of the extracted virginiamycin from the solid phase.

After extraction, a measured quantity of antibiotic was distributed in wells punched in an agar medium seeded with a suspension of *Kocuria rhizophila*. Excess antibiotic diffused out of the wells, creating a concentration gradient. The wells were incubated at 37°C for a predetermined time period. After incubation, the wells were examined for bacterial growth. A zone of inhibition was determined to be the diameter around the well, in which no bacteria were able to grow, and was directly proportional to the logarithm of the antibiotic concentration. The detection limit for this procedure was 0.3 mg/kg virginiamycin.

Four virginiamycin standards spanning the range of the assay were used to calibrate the assay. A reference standard (at the midpoint of the assay) was analyzed with all calibration and sample plates. The standards were made up of plates with 6 wells each. Three of the wells in each plate contained either 1 of the 4 calibration standards or a DG sample extract. The final 3 wells contained the reference standard. All antibiotic concentrations were based on the calibration standards and adjusted for plate-to-plate variance, as calculated through the reference standards.

Biological Activity

Antibiotic extracts derived from PBS extraction were subjected to microbial testing by SGS North America, Inc. to determine their biological activity. Use of methanol-to-extract residues for the FDA-approved procedure was expected to affect microbial activity; therefore, PBS extraction was adopted to prevent this interference. Results from previous studies showed that methanol can affect the growth of some strains of sentinel bacteria used in this study (Fried and Novick, 1973). Initially, the extracts were centrifuged and supernatants were filter-sterilized, using a 0.22-μm filter. To determine if antibiotic extracts were biologically active, they were exposed to 10^4 to $^{10}7$ CFU/mL of sentinel bacteria chosen for this study: *Escherichia coli* ATCC 8739 and *Listeria monocytogenes* ATCC 19115. The sentinel bacteria were chosen for their sensitivity to the antibiotics under examination and societal concerns that they have the potential to become pathogenic.

Sentinel bacterial susceptibility to each antibiotic was determined using the Kirby Bauer method or broth dilutions, according to standard protocols (Andrews, 2001; Hudzicki, 2009). Susceptibility was determined according to the Clinical and Laboratory Standards Institute for penicillin G and *L. monocytogenes,* and tetracycline and *E. coli* (Wikler et al., 2007). *Listeria monocytogenes* ATCC 19115 was found to be susceptible to all 5 antibiotics under evaluation in this study. *Escherichia coli* ATCC 8739 was found to be susceptible to all antibiotics evaluated in this study, except penicillin G (Table 1).

Antibiotic extracts were mixed 1:1 with serially diluted 10^4 to 10^7 CFU/mL liquid cultures of the individual sentinel bacteria in macrodilution tubes. In this

study, antibiotic concentration remained constant and bacterial counts varied. This method is the converse of a typical minimum inhibitory concentration determination and it determines the bacterial threshold of an antibiotic (Lambert and Pearson, 2000; Wiegand et al., 2008). Bacterial threshold was used in this study because antibiotic residue concentrations do not vary in DG, but the bacterial concentration that residues encounter in the environment does. Thus, bacterial threshold more accurately represents the interaction of antibiotic residues in DG and environment. After incubation at 37°C for 18 to 24 h, tubes were examined for turbidity as an indicator of bacterial growth. Ten microliters from each tube were spread on blood agar plates and incubated at 37°C for 18 to 24 h. After incubation, colonies were counted to determine CFU/mL.

All samples were analyzed in duplicate. Additionally, a secondary set of antibiotic residues were autoclaved and tested according to the above procedure. Autoclaving was conducted to inactivate any antibiotic residues. Three control samples, consisting of culture broth, antibiotic extract, or sentinel bacteria with culture broth, were analyzed with each set of samples, according to the procedure previously described.

Statistical Analysis

Nutrient profiles and pH values, on a DM basis, were analyzed using the DG sample as the experimental unit in a randomized complete block design with repeated measures, using the MIXED procedure (SAS Inst. Inc., Cary, NC). Main effects were DG type (wet or dry), ethanol plant of origin (**EP**), and the 2-way interactions between DG type and collection period, and EP and collection period. The repeated measures statement within the MIXED model provided the error term to compare EP × DG type interactions between collection periods. The covariate structure SP (POW) of MIXED was used to test for repeated measures. The random statement within the MIXED model was used to quantify the effect of period. When significant ($P < 0.05$), model differences were noted and least square means were separated by the PDIFF function of SAS. Twelve samples were not included in the repeated measures analysis because no plants of origin were identified for the samples. Thus, there were no repetitions of EP × type. Moreover, 31 samples were duplicated by period, DG type, and ethanol plant of origin. These samples were averaged by EP and period, and analyzed as 1 sample for effects of EP, type, and period on physicochemical characteristics. In total, 116 samples were statistically analyzed. To determine the effect of period, an additional model was created, using the MIXED procedure of SAS with period as a fixed variable.

Table 1. Sentinel bacteria susceptibility to antibiotics determined using Kirby-Bauer method or broth dilution method to determine minimum inhibitory concentration (MIC)

	L. monocytogenes		*E. Coli*	
Item	Kirby-Bauer concentration/mm	MIC, µg/mL	Kirby-Bauer concentration/mm	MIC, µg/mL
Erythromycin	150 µg/28	–	15 µg/14	–
Penicillin G	10 IU/28	–	Not susceptible	–
Tetracycline	30 µg/29	–	30 µg/23	–
Tylosin	–	4	–	125
Virginiamycin M1	–	31	–	125

Due to a relatively low number of samples containing antibiotic residues and low concentrations of antibiotic residues, analyses of effects of EP, collection period, or type of antibiotic concentration was not conducted. Instead, the probability of finding a sample containing an antibiotic residue (regardless of antibiotic) was modeled with logistic regression, using GLIMMIX procedure of SAS. Independent variables were type of DG and period of collection; consultant was deemed to be a random independent variable. Means were separated by least square means procedures when *P*-value for the independent variable was <0.05.

RESULTS

A total of 159 samples were collected and examined in this study. Eighty of the samples were DDG (>65% DM) and 79 were DWG (<65% DM; Table 2). Sample physiochemical characteristics are provided to characterize samples and are shown in Table 3 on a DM basis. Crude protein ranged from 26 to 43%, crude fat (ether extract) ranged from 5 to 14%, NDF ranged from 26 to 47%, phosphorus ranged from 0.44 to 1.27%, and sulfur ranged from 0.30 to 1.08% among dry and wet DG samples. Samples of DG in this survey averaged a pH of 4, with a range of 3 to 5, regardless of type.

Antibiotic Residues and Biological Activity

Antibiotic residues were found in 12.6% (Table 4) of the DG samples in this study, with the majority (10.7% of total) present in DDG samples. On a DM basis, 3.8% of the DWG samples ($n = 79$) and 21.3% of DDG samples ($n = 80$) contained antibiotic residues (Table 4). Logistic regression determined this difference to be statistically significant ($P = 0.007$). Period of collection was not ($P = 0.61$) a significant source of variation.

Although the M1 subunit of virginiamycin was quantified by the LCMSMS procedure, and this procedure is sanctioned by FDA, only virginiamycin results determined using a FDA-approved biological assay were included in the data set. In contrast to measuring

Table 2. Distribution of distillers grains (DG) samples analyzed for antibiotic residues and biological activity by state of origin, collection period, and distillers grain type

	Period							
	Winter		Spring		Summer		Fall	
DG Type	Dry	Wet	Dry	Wet	Dry	Wet	Dry	Wet
Iowa	8	5	5	3	7	4	6	7
Illinois	1	2	0	2	3	2	1	2
Indiana	0	1	1	0	2	0	1	0
Michigan	0	0	1	0	0	0	0	0
Minnesota	7	3	6	8	5	6	11	6
North Dakota	0	1	0	0	0	0	0	0
Nebraska	1	4	1	1	0	2	0	0
Ohio	0	0	0	0	1	0	0	0
South Dakota	3	3	2	4	2	3	1	2
Origin unknown	0	1	4	1	0	3	0	3
Sample total	20	20	20	19	20	20	20	20

only 1 subunit of virginiamycin, as is the case for the LCMSMS procedure, samples were measured for their biological activity to inhibit growth of *K. rhizophila*, using the FDA-approved bioassay. Using the bioassay, 2 samples contained virginiamycin, with 1 sample containing 0.6 mg/kg and the second sample containing 0.5 mg/kg (data not shown). Both positive samples were DDG from the same ethanol plant and collected during the first quarter. Results of the microbiological assays revealed that of the 159 samples, 1 sample inhibited growth of *E. coli* ATCC 8739 at 10^4 CFU/mL (data not shown). This sample did not contain any detectable residues analyzed in this study. No residue extracts inhibited *L. monocytogenes* ATCC 19115.

DISCUSSION

Nutrient composition of samples in this study was comparable to previously reported values, suggesting that the samples in this study accurately represented DG produced in the United States (Rasco et al., 1987; NRC, 1996; Spiehs et al., 2002; Loy, 2007; Pedersen et al., 2007; Tangendjaja, 2007; Waldroup et al., 2007; Robinson et al., 2008; Bhadra et al., 2010; Buckner et al., 2011). Because DG samples were not separated before analyses by EP, some samples in this data set represent new fractionation and centrifugation technologies, wherein CP was greater, and crude fat was less than conventional DG samples. As these technological adaptations are incorporated into EP, results from this and future surveys will lead to greater ranges in these and other nutrients, as observed in the current survey.

In spite of various ethanol production processes represented in the current survey, average or range of pH in DDG samples was remarkably similar to those reported by Shurson and Alghamadi (2008). Greater coeffiecient of variation reported in the current survey simply reflected the greater variety of ethanol production processes represented.

The majority of antibiotic residues were present in DDG samples. Because sampling procedures did not permit collection of both DDG and DWG samples from each EP within each period, this observation may reflect a sampling bias. Also, the possibility exists that this observation may result from an artifact derived from conducting LCMSMS analyses in feeds varying in moisture concentration due to a dilution of DWG residues below LOQ.

Preliminary results (unpublished) of research conducted by FDA in 2010 (FDA, 2010) revealed that 53% of DG samples contained antibiotic residues (Fairfield, 2010). However, that study only examined samples for the presence of erythromycin, virginiamycin, and tylosin. In a 2012 FDA survey (unpublished), 3 out of 46 samples (6.5%) were found to contain detectable antibiotic residues. One sample contained virginiamycin M1 residues at concentration of 0.16 mg/kg, the second sample contained erythromycin residues at a concentration of 0.58 mg/kg, the third sample contained virginia-

Table 3. Descriptive statistics of physiochemical properties of distillers grains samples and main effects of distillers grains type on physicochemical properties of distillers grains samples on DM basis (n = 159)

	Distillers dried grains (DDG)		Distillers wet grains (DWG)		Treatment[1]			*P*-values[3]				
Item	Mean	SD	Mean	SD	DDG	DWG	SEM[2]	Type	Period	EP	Period × Type	Period × EP
DM, %	90.01	1.32	43.28	7.82	89.95	44.29	1.54	<0.0001	0.07	0.81	0.85	0.12
CP, %	30.85	1.43	31.00	2.62	31.38	31.23	0.36	0.61	0.01	<0.0001	0.85	0.54
Crude fat, %	11.53	1.35	11.00	2.04	10.9	11.3	0.25	0.28	0.002	<0.0001	0.04	0.01
NDF, %	31.49	3.85	36.12	3.23	31.76	36.01	0.6	<0.0001	<0.0001	0.04	<0.0001	0.03
P, %	0.87	0.15	0.91	0.19	0.86	0.91	0.05	0.2	<0.0001	0.05	0.61	0.79
S, %	0.64	0.18	0.61	0.15	0.64	0.63	0.05	0.78	<0.0001	0.0003	1	0.77
pH	4.12	0.44	4.02	0.39	4.11	3.96	0.08	0.05	0.03	<0.0001	0.42	0.6

[1]Treatments included DDG or DWG.

[2]Greatest SE of the mean reported.

[3]Effects of distillers grain type (type), collection period (period), sample ethanol plant of origin (EP), and their interactions (Period × Type; Period × EP). *P*-values < 0.05 were considered significant.

mycin M1 and penicillin G residues at concentrations of 0.15 and 0.24 mg/kg, respectively (Luther, 2012). These concentrations are within the range (0 to 1.12 mg/kg) found in this study.

A single bioassay procedure is approved by FDA for determination of virginiamycin. When comparing outcomes of the FDA-approved assay and the LCMSMS procedures in the current study, we detected the virginiamycin M1 subunit in 8 samples analyzed using LCMSMS. Using the bioassay procedure, only 2 samples had detectable concentrations of virginiamycin. Only 1 sample containing virginiamycin M1 was validated with the FDA-approved biological assay (0.33 vs. 0.5 mg/kg), whereas the other sample found to contain the M1 fraction was not detected through the bioassay method. All of the unconfirmed samples testing positive for the virginiamycin M1 by LCMSMS were also less than the LOQ of the bioassay. The LCMSMS detection method for the M1 faction of virginiamycin also resulted in a false negative result. General FDA guidance, for the industry regarding MS confirmation, recommends that methodology demonstrate ≤10% false negative rate at or above the tolerance level (Heller, 2010). A data set with a larger number of samples greater than the LOQ of the bioassay is needed to fully determine if that criterion is being satisfied by the LCMSMS procedure.

Samples of DG containing antibiotics had low concentrations of antibiotic residues. These values were also well below dietary concentrations of current FDA-approved antibiotic for finishing livestock and poultry feeds (Tables 5 and 6). It is important to note that penicillin is not approved for feeding in cattle (Tables 5 and 6). However, antibiotic residues in DG may be detrimental to livestock consuming them. A report by Basaraba et al. (1999) found that cattle fed monensin became ill or died after being fed DDG containing erythromycin residues. Illness and death were the result of drug interactions between monensin and DDG containing erythromycin. The DDG source that caused death in those cattle (Basaraba et al., 1999) contained erythromycin concentrations that were several orders of magnitude greater (50 to 1500 mg/kg) than erythromycin concentrations found in the current study. This report caused concerns about drug interactions resulting from feeding DG to livestock and poultry. Several antibiotic residues found in DG have antagonistic or toxic effects when combined. Erythromycin has an antagonistic effect when combined with virginiamycin or penicillin, and it can cause monensin toxicity due to delayed clearance or altered biotransformation of monensin when fed concurrently (Cocito, 1979; Hof et al., 1997; Basaraba et al., 1999). Additionally, tetracycline is antagonistic when co-administered with penicillin G (Merck, 2004). Currently, the only report of a negative outcome due to DG antibiotic residues and therapeutic or growth-promoting antibiotic administration interactions was published by Basaraba et al. (1999). The lack of additional reports of antibiotic residues affecting livestock or poultry suggests that the concentration of antibiotic residues in DG is generally too low to result in detrimental drug interactions.

Distillers grains antibiotic residue concentrations observed in the current study, as well as in unpublished FDA surveys, appear to be at concentrations generally considered to be sublethal or they may be biologically inactive. When exposed to bacteria in culture, antibiotic residues were unable to inhibit bacterial growth, except in 3 samples. Two were inhibitory of *K. rhizophila* (used in the approved Phibro bioassay), which were indicative of virginiamycin residues; and 1 sample that was negative for antibiotic residues screened demonstrated inhibition to the sentinel strain of *E. coli* chosen for this study. Presence of other microbial, growth-inhibiting

Table 4. Descriptive statistics for samples containing antibiotic residue concentrations in distillers grains and solubles, determined via liquid chromatography ion trap tandem mass spectrometry and reported on DM basis

	Distillers dried grains			Distillers wet grains		
Item	*n*	Mean	SD	*n*	Mean	SD
Erythromycin, mg/kg	14	0.35	0.26	2	0.35	0.01
Penicillin G, mg/kg	1	0.11	–	0	–	–
Tetracycline, mg/kg	0	–	–	1	1.12	–

Table 5. U.S. Food and Drug Administration-approved concentrations of animal drugs for use in finishing livestock and poultry feed[1]

	Erythromycin thiocyanate			Penicillin G procaine			Oxytetracycline hydrochloride			Tylosin phosphate			Virginiamycin		
Item	Minimum	Maximum	Unit	Minimum	Maximum	Unit	Minimum	Maximum	Unit	Minimum	Maximum	Unit	Minimum	Maximum	Unit
Cattle	37.0	37.0	mg/animal	NA[2]	NA	NA	75.0	75.0	mg/animal	8.0	10.0	g/ton	16.0	22.5	g/ton
Chicken	4.6	18.5	g/ton	2.4	50.0	g/ton	10.0	50.0	g/ton	4.0	50.0	g/ton	5.0	15.0	g/ton
Swine	9.25	64.75	g/ton	50.0	50.0	g/ton[3]	10.0	50.0	g/ton	10.0	100.0	g/ton	5.0	10.0	g/ton
Turkey	9.25	18.5	g/ton	2.4	50.0	g/ton	10.0	50.0	g/ton	NA	NA	NA	10.0	20.0	g/ton

[1]From Animal Drugs @ FDA (http://www.accessdata.fda.gov/scripts/animaldrugsatfda).

[2]Not approved to be fed.

[3]In combination with chlortetracycline at 100 g/ton of feed, plus sulfathiazole at 100 g/ton of feed

compounds or natural antimicrobials in the environment may have contributed to this inhibition.

It is likely that the lack of inhibition in all other antibiotic-positive samples was due to antibiotic inactivation. Penicillin, erythromycin, and tylosin all degrade at a pH < 4.0, reached during ethanol fermentation (Aksenova et al., 1984; Brisaert et al., 1996; Islam et al., 1999). The low pH values of DG may have contributed to the inactivation of these 3 antibiotics. Moreover, penicillin, tetracycline, tylosin, and virginiamycin are degraded by high temperatures (>200°C), which are reached during ethanol fermentation, distillation, and drying (Brisaert et al., 1996; Hynes et al., 1997; Kheirolomoom et al., 1999; Juranek and Duquette, 2007; Wang et al., 2008). Once produced, DG do not have measurable concentrations of bacteria (Pedersen et al., 2004; Lehman and Rosentrater, 2007). Over time, however, samples of wet wheat DG were found to contain up to 8.4 CFU/mL of lactobacilli (Pedersen et al., 2004). Wet DG have been found to have bacterial counts of 10^7 to 10^8 cells/g dry mass within 9 d of production (Lehman and Rosentrater, 2007). This suggests that bacteria in DG come from the environment and not the DG production system. Thus, bacterial resistance to antibiotic residues could potentially develop after DG has been produced and is exposed to the environment.

Results from previous research demonstrated that DG may contain antibiotic-resistant bacteria. Jacob et al. (2008) showed that monensin- and tylosin-resistant *Enterococcus* species were present in 1 source of DG. Furthermore, da Costa et al. (2007) examined *Enterococcus* isolates in cereal co-products (corn gluten feed and DDGS) and found resistance to tetracycline, erythromycin, rifampicin, gentamicin, chloramphenicol, nitrofurantoin, and ciprofloxacin. It is possible that antibiotic-resistant bacteria identified in DG from these studies gained antibiotic-resistant genes due to external environmental factors (i.e., from other bacteria in the environment).

Currently, no direct link has been shown between use of DG in cattle or swine diets and development of bacterial resistance in the gastrointestinal tract of those species (Jacob et al., 2008; Edrington et al., 2010). However, resistant bacteria can be found in all animals; their concentrations increase when feeding diets containing antibiotics (Sunde et al., 1998). Thus, there is risk for the concentration of resistant bacteria to increase in livestock gastrointestinal tracts if active antibiotic residues are present in DG.

If resistant bacteria were to develop in DG, those bacteria would have to make it through several barriers to become problematic to the human population (Hurd et al., 2004). Using a risk assessment, Hurd et al. (2004) found that the risk of illness due to macrolide-resistant campylobacteriosis, resulting from administering tylosin to swine, was <1 in 10 million for all meat commodities combined, 1 in 14 million for chicken, 1 in 53 million for beef, and 1 in 236 million for pork. This risk would decrease further, given the additional barriers involved in ethanol production. Based on this risk assessment and considering the low concentration of antibiotic residues in DG, the risk of bacterial antibiotic resistance developing and affecting the human population as the result of the use of antibiotics in ethanol production is extremely low. Overall, bacteria develop antibiotic resistance in a wide variety of settings. There is minimal health risk to humans as a result of the use of antibiotics in ethanol and DG production relative to several other external factors that pose a much greater threat to human health.

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Table 6. Calculated mg/kg concentrations of animal drugs for use in finishing livestock and poultry feed, based on average DMI for each species during the finish period of production[1]

	Assumed DM intake		Erythromycin thiocyanate			Penicillin G procaine			Oxytetracycline hydrochloride			Tylosin phosphate			Virginiamycin		
Item	Intake	Unit	Minimum	Maximum	Unit	Minimum	Maximum	Unit	Minimum	Maximum	Unit	Minimum	Maximum	Unit	Minimum	Maximum	Unit
Cattle	9.98	kg	3.7	3.7	mg/kg	NA[2]	NA	NA	7.5	7.5	mg/kg	8.8	11.0	mg/kg	17.6	24.8	mg/kg
Chicken	0.10	kg	5.1	20.4	mg/kg	2.6	55.1	mg/kg	11.0	55.1	mg/kg	4.4	55.1	mg/kg	5.5	16.5	mg/kg
Swine	2.72	kg	10.2	71.4	mg/kg	55.1	55.1	mg/kg	11.0	55.1	mg/kg	11.0	110.2	mg/kg	5.5	11.0	mg/kg
Turkey	0.15	kg	10.2	20.4	mg/kg	2.6	55.1	mg/kg	11.0	55.1	mg/kg	NA	NA	NA	11.0	22.1	mg/kg

[1]Data calculations based on average DMI for listed species during the finishing period of feeding and U.S. Food and Drug Administration-approved feeding levels for each animal drug (listed in Table 5).

[2] Not approved to be fed.

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