Short communication: No antimicrobial effects from one source of commercial dried distillers grains with solubles

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ABSTRACT

Because residual antibiotics in dried distillers grains with solubles (DDGS) could lead to inadvertent feeding of antibiotics to animals, the objective of our study was to determine if a commercial DDGS contained antibiotics. The DDGS used in a feeding study, and milk from cows fed the DDGS, were below the detection limits for at least 17 antibiotics. Additionally, we evaluated if DDGS had any antimicrobial effect against *Salmonella* Typhimurium, *Listeria innocua*, *Escherichia coli* ATCC 25922, *Staphylococcus aureus*, *Pediococcus acidilactici*, *Lactobacillus casei*, *Lactobacillus acidophilus*, *Bacillus licheniformis*, *Paenibacillus odorifer*, *Pseudomonas fluorescens*, and *Paenibacillus amylolyticus* using the disk diffusion seeded agar overlay method. Neither the buffered nor nonbuffered water-soluble fractions of DDGS yielded clear zones around disks, indicating that the water-soluble DDGS fraction had no antimicrobial properties against any of the microorganisms tested. The absence of antibiotic residues in DDGS and milk samples in this study confirmed that this source of DDGS can be used as livestock feed without fear of inadvertent feeding of antibiotics.

Key words: antibiotics, clostridia, milk, total mixed ration

Short Communication

Ethanol production from corn involves fermentation by yeast (*Saccharomyces cerevisiae*; US Grains Council, 2012). After fermentation and distillation of ethanol, whole stillage (water and solids) is produced as a co-product. Through centrifugation, whole stillage is separated into thin stillage and coarse solids or wet distillers grains (WDG, ~35% DM). These WDG are further heat-dried with air temperatures averaging 430°C

(Saunders and Rosentrater, 2009) in a rotary drier to form dried distillers grains (DDG). The mixture of syrup (30% DM), obtained after evaporation of thin stillage, and DDG results in the production of dried distillers grains with solubles (DDGS, ~90% DM). As an often economical energy, fiber, and protein source, DDGS have been used as feed for lactating dairy herds for over a decade (Schingoethe, 2007; Giuntoli et al., 2009; Hoffman and Baker, 2011; Testroet et al., 2015).

Virginiamycin, a streptogramin antibiotic, is used in industrial ethanol fermentations to control the growth of contaminating lactic acid bacteria that could interfere with desirable yeast fermentation (Hynes et al., 1997). Currently, virginiamycin is the only antibiotic that has been provided a “no objection letter” from the Food and Drug Administration (FDA; Nov. 16, 1993) for use in the ethanol fermentation process. Virginiamycin is effective against 7 strains of lactobacilli during alcoholic fermentation, but not against *Lactobacillus rhamnosus*, *Lactobacillus paracasei*, and *Lactobacillus plantarum* (Hynes et al., 1997). Virginiamycin also has been used in the livestock industry, particularly in ruminant diets, as a growth inhibitor of gram-positive bacteria. Although virginiamycin is not currently used in the US dairy or beef industry, previous research has shown that virginiamycin improves gain to feed ratios in beef cattle (Salinas-Chavira et al., 2009) and improves milk yield in lactating dairy cattle (Clayton et al., 1999). Current FDA recommendations discourage the use of antibiotics for improving production parameters in livestock, and instead, the FDA recommends judicious use of antibiotics in livestock to help prevent the development of antibiotic-resistant microorganisms (FDA, 2014).

Because antibiotics are used routinely in the production of ethanol, and consequently, the production of DDGS, feeding DDGS could potentially result in inadvertent feeding of antibiotics to ruminant animals. A study conducted by the FDA in 2012 (Fairfield, 2012) reported that 3 out of 28 (10.7%) samples of distillers grains (DG) had detectable antibiotic residues. Out of those positive samples, one contained 0.16 mg/kg of virginiamycin M1 residue; a second sample contained

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0.15 mg/kg of erythromycin; a third sample contained approximately 0.15 mg/kg of virginiamycin M1 residue and 0.24 mg/kg of penicillin G. Determination of the presence or lack of antibiotics in DG is important, but it is additionally important to determine the biological activity of these residual antibiotics. In a more recent study by Paulus Compart et al. (2013), antibiotic residue concentrations in DG (wet: 79 samples, dry: 80 samples) were found to be very low and less than the maximal concentrations approved by FDA in feed for food-producing animals.

Any residual antibiotics remaining in DDGS could be detrimental in animal feeding programs because misuse of antibiotics could lead to development of antibiotic resistance in bacteria. Consumption of DDGS that contains antibiotic residues by food-producing animals could lead to development of resistance to those antibiotics (US Grains Council, 2012). Inadvertent feeding of antibiotics to dairy cows through DDGS could have important implications for the dairy industry at large, particularly if any antibiotics in DDGS are transferred into milk. The objective of this study was to determine if one source of commercially available DDGS contained antibiotic residues and if these DDGS possessed any antimicrobial properties against select pathogens and milk spoilage microorganisms.

The DDGS used in this study were acquired from Heartland Cooperative (Prairie City, IA). Dietary treatments were formulated for a dairy cow feeding study reported separately (E. D. Testroet, S. Clark, and D. C. Beitz; unpublished data). Diets included (1) TMR with no DDGS, (2) TMR with 10% DDGS by dietary DM, and (3) TMR with 20% DDGS. To obtain a random sample representative of how feed is presented to the cows, samples of feed from all 3 treatments were collected by holding Ziploc bags (S. C. Johnson, Racine, WI) directly at the conveyer of the feed wagon. Sample bags from each of 3 mixed rations from 3 consecutive days of feeding were collected and pooled into a large rubber basin, minimally mixed by hand to avoid particle stratification, and subsampled randomly by hand and sent immediately to Dairyland Laboratories (Arcadia, WI) for proximate analyses by wet chemistry methods. Duplicate samples from all feed collected were frozen at -20°C until antibiotic/antimicrobial analyses were performed.

One sample for each dietary treatment was sent to the University of Nebraska–Lincoln Food Processing Center for microbial analysis. Aerobic mesophilic spore counts were determined on the feed samples as based on AOAC International, 1998; method 990.12. Feed samples were diluted (1:10 wt:wt) in Butterfield's phosphate buffer, heated to 80°C , and held at that

temperature for 12 min. Serial dilutions were plated on Standard Methods agar and incubated at 32°C for 48 h (Wehr and Frank, 2004). Plates were checked for growth, and visually distinct bacterial isolates were streaked for purity on Standard Methods agar.

Two representative samples of DDGS and 6 samples of milk (2 samples per treatment) collected from cows fed DDGS treatments underwent full drug screening tests at the Cyclone Custom Analyte Detection Service (CyCads) at the Iowa State University Veterinary Diagnostic Laboratory (Ames). Samples (approximately 1 kg) of DDGS were collected from the feed bin from both shipments of DDGS that were used in this study. Dried distillers grains with solubles samples were collected and stored in Ziploc bags (S. C. Johnson) at -20°C until antibiotic screening. Neither the dairy management nor the personnel at the DDGS supplier were aware of the collection dates or of the antibiotic testing. Milk samples (about 50 mL) were taken directly from freshly collected pooled milk of 3 sets of 10 cows (3 different diet treatments) in a feeding trial (Sankarlal et al., 2015), placed on ice, and delivered to CyCads for antibiotic screening where they were frozen at 20°C until antibiotic screening. Screening included quantification of β -lactams and cephalosporins, tetracyclines, sulfonamides, phenicols, macrolides/lincosamides/streptogramins, quinolones, aminoglycosides, and anthelmintic drugs by using liquid chromatography and mass spectrometry.

The antimicrobial properties of the water-soluble fraction of DDGS was tested by determining the zone of inhibition by using the disk diffusion agar overlay method as described in Xia et al. (2012). For this method, overnight pure cultures (10^8 cfu/mL) were used to create lawns on solid agar. Pour plates were made with 1.5% agar (Fischer Scientific, Fair Lawn, NJ) and allowed to cool/harden. Specifically, tryptic soy broth (BD Chemicals, Sparks, MD) was used for *Salmonella* Typhimurium ATCC 14028, *Listeria innocua* (ATCC 30090, Microbiologics (St. Cloud, MN)), *Escherichia coli* ATCC 25922, and *Staphylococcus aureus* (ATCC 25923, Microbiologics). MRS Lactobacillus Broth (BD Chemicals) was used for *Pediococcus acidilactici* (USDA NRRL, Beltsville, MD), *Lactobacillus casei* (Microbiologics), *Lactobacillus acidophilus* (USDA NRRL). Brain heart infusion broth (BHI Teknova, Hollister, CA) was used for *Bacillus licheniformis* (FSL J3-0143; Cornell University, Ithaca, NY), *Paenibacillus odorifer* (FSL H8-0237; Cornell University), *Pseudomonas fluorescens* FSL W5-0203; Cornell University), and *Paenibacillus amylolyticus* (FSL H7-0689; Cornell University). A 0.5-mL overnight culture suspension of each culture was added to 4.5 mL of tempered ($\leq 50^{\circ}\text{C}$) 0.75% overlay

agar of the same type and vortexed thoroughly before adding to each respective plate.

Dried distillers grains and solubles (25 g) were mixed with 225 mL of water or 0.1% BPW (225 mL; Fisher Scientific) and stomached (400C Seward, Davie, FL) for 30 s. The DDGS was mixed with 0.1% BPW to neutralize the pH of DDGS (~3.5). Blank sterile paper disks (BD Diagnostics, Sparks, MD) were saturated with the DDGS supernatant (around 20 μ L). Using sterile forceps, the disks were placed on the seeded agar overlay. After the disks attached to the surface of agar, the plates were inverted and incubated aerobically overnight at 35°C. The zone of inhibition experiments were conducted in duplicate. Tetracycline disks (BD Diagnostics) were used as positive controls, and water-saturated disks were used as negative controls.

The bacterial counts in DDGS were too low to count (<5 cfu/mL, data not shown). The high temperatures (up to 430°C) associated with the DDGS drying process likely only partially explain these results (Pfizer Animal Health, 2010). Although Pedersen et al. (2004) did not find microorganisms in wheat WDG samples collected directly out of the distillation column, storage in unclean environments leads to increases in microbial loads. Up to 8.4 cfu/g of lactobacilli (including species such as *Lactobacillus amylolyticus*, *Lactobacillus panis*, *Lactobacillus pontis*) were found in wheat WDG when stored more than 5 wk (Pedersen et al., 2004). Microorganisms naturally present in the environment contaminate DG during storage, and if antibiotics are also present in DG, such compounds could lead to antibiotic-resistant strains of bacteria resulting from incidental

contact during storage. Jacob et al. (2008) hypothesized that administering feed containing antibiotics to cattle would lead to development of antibiotic-resistant bacteria in the gut of cattle; however, they found no direct correlation between feeding DG containing antibiotics and development of antibiotic resistance in the gut of cattle. Based on their risk assessment review, Paulus Compart et al. (2013) concluded that the health risk to humans of antibiotic resistance from feeding DG to cattle is very minimal compared with other external factors such as prevalence of bacteria in feed that acquired antibiotic resistance genes from the environment. Because overuse and misuse of antibiotics can lead to antibiotic resistance and so-called superbugs, even minimal amounts of antibiotics in DDGS would be undesirable and potentially have negative effects.

In contrast to pure DDGS, in the present study, the total aerobic count in the TMR was 6.69 log₁₀ cfu/g. This is likely because TMR is not typically stored in a sterile environment. Bacterial isolates were not identified at the molecular level, but were determined to be, primarily, *Bacillus licheniformis* and *Bacillus brevis*, along with fewer amounts of *Paenibacillus* spp., *Bacillus pumilus*, and *Bacillus subtilis*.

Although utilization of antibiotics in ethanol production is strictly controlled by FDA, concerns are still present about antibiotic residues remaining in distillers co-products (Paulus Compart et al., 2013). However, in our study the concentrations of all tested antibiotics in DDGS (Table 1) and in the milk (Table 2) were below detection limits. Concentration of virginiamycin, the antibiotic commonly used in the ethanol produc-

Table 1. Concentration of antibiotics in dried distillers grains with solubles (DDGS) samples

Antibiotics	Limit of detection, mg/kg	Concentration in DDGS, ¹ mg/kg	
		DDGS 1	DDGS 2
Bacitracin methylene disalicylate	10	<10	<10
Bacitracin zinc	10	<10	<10
Bambermycins	2	DNT ²	DNT
Carbadox	0.125	<0.125	<0.125
Chlortetracycline	2	<2	<2
Florfenicol	0.5	<0.5	<0.5
Lincomycin	8	<8	<8
Naracin	1	DNT	DNT
Oxytetracycline	2	<2	<2
Penicillin	2	<2	<2
Sulfamethazine	1	<1	<1
Sulfathiazole	1	<1	<1
Tiamulin	0.125	<0.125	<0.125
Tilmicosin	1	<1	<1
Tylosin	0.5	<0.5	<0.5
Tylvalosin	0.5	DNT	DNT
Virginiamycin	10	<1	<1

¹DDGS 1 and DDGS 2 = 2 different samples of dried distillers grains with solubles.

²DNT = did not determine.

Table 2. Concentration of analytes in milk samples from cows fed dried distillers grains with solubles (DDGS) and TMR

Antibiotic/antiparasitic assay	Assay LOQ, ¹ µg/kg	Safe/tolerance level in milk, ² µg/kg	Assay result, ³ µg/kg
Beta-lactams and cephalosporins			
Amoxicillin	0.5	10	ND ⁴
Ampicillin	0.5	10	ND
Ceftiofur	0.5	100	ND
Desfuroylceftiofur	0.5	100	ND
DCCD (ceftiofur metabolite)	0.5	TNE ⁵	ND
Cephapirin	0.5	20	ND
Cloxacillin	0.5	10	ND
Penicillin G	0.25	5 ⁶	ND
Tetracyclines			
Chlortetracycline	2.5	300	ND
Oxytetracycline	2.5	300	ND
Tetracycline	2.5	300	ND
Doxycycline	2.5	TNE	ND
Sulfonamides			
Sulfachlorpyridazine	0.5	10 ⁶	ND
Sulfadiazine	0.5	10 ⁶	ND
Sulfamerazine	0.5	10 ⁶	ND
Sulfadimethoxine	0.5	10 ⁶	ND
Sulfapyridine	0.5	10 ⁶	ND
Sulfamethazine	0.5	10 ⁶	ND
Sulfaquinoxaline	0.5	10 ⁶	ND
Sulfathiazole	0.5	10 ⁶	ND
Macrolides, lincosamides, and streptogramins			
Erythromycin	2.5	50 ⁶	ND
Tulathromycin	2.5	TNE	ND
Tilmicosin	2.5	TNE	ND
Tylosin	2.5	50	ND
Lincomycin	0.5	TNE	ND
Pirlimycin	0.5	40	ND
Virginiamycin	2.5	TNE	ND
Quinolones			
Ciprofloxacin	0.5	TNE	ND
Enrofloxacin	0.5	TNE	ND
Norfloxacin	0.5	TNE	ND
Sarafloxacin	0.5	TNE	ND
Phenicals			
Chloramphenicol	NA ⁷	TNE	ND
Florfenicol	0.5	TNE	ND
NSAID ⁸ and miscellaneous			
Flunixin	0.1	2	ND
5-Hydroxyflunixin	0.1	2	ND
Meloxicam	0.1	TNE	ND
Bacitracin	2.5	500	ND
Tiamulin	0.1	TNE	ND
Ractopamine	0.1	TNE	ND
Tripelenamine	0.1	20	ND
Anthelmintic (antiparasitic)			
Doramectin	0.5	TNE	ND
Moxidectin	0.5	TNE	ND
Ivermectin	0.5	TNE	ND
Thiabendazole	2.5	50	ND

¹LOQ = limit of quantification.²Tolerance based on CFR 21 Part 556: Tolerances for residues of New Animal Drugs in Food. Tolerances are only applicable to milk.³One column indicates the assay result of all the 6 milk samples analyzed (2 samples from each of the 3 treatments: 0% DDGS, 10% DDGS, 20% DDGS).⁴ND = not detected.⁵TNE = tolerance not established.⁶Amounts are safe levels, not tolerances.⁷NA = not available.⁸NSAID = nonsteroidal antiinflammatory drug.

tion process, was less than 1 mg/kg. The FDA's Center for Veterinary Medicine recommended the maximal concentration of residual virginiamycin in distillers by-products be 0.2 to 0.5 mg/kg and about 2 to 6 mg/kg of virginiamycin should be allowed during the fermentation process (National Grain and Feed Association, 2009). The FDA-approved maximal concentration of virginiamycin in feed for finishing cattle is 20.4 mg/kg. In 2012, research conducted by the FDA found virginiamycin residues in 2 out of 28 DG samples (7.1%) at a concentration of approximately 0.16 and 0.15 mg/kg (FDA; Fairfield, 2010). The second sample contained penicillin G (0.24 mg/kg) along with virginiamycin. A more recent study conducted by Paulus Compart et al. (2013) found virginiamycin residues in 2 feed samples of DDG (0.6 and 0.5 mg/kg). Antibiotic residues in the previous and present work were below the current FDA-approved maximal concentrations. It should be noted, however, that even at low concentrations, toxic effects might result from the combined action of antibiotics. For instance, macrolide antibiotics such as erythromycin and virginiamycin may induce monensin toxicity by delaying its clearance in the liver (Basaraba et al., 1999). Basaraba et al. (1999) reported a case where monensin caused death of cattle fed DDG containing 50 to 1,500 mg of macrolide antibiotics (e.g., erythromycin, clarithromycin) per kg of DDG.

The supernatant of DDGS used in the study did not form any zones of inhibition against *L. innocua*, *Salmonella* Typhimurium, *E. coli* ATCC 25922, *Staph. aureus*, *Ped. acidilactici*, *L. casei*, *L. acidophilus*, *B. licheniformis*, *Paeni. odorifer*, *Pseud. fluorescens*, and *Paeni. amylolyticus*. *Bacillus licheniformis* and *Paenibacillus* spp. were the only organisms identified in the TMR. Thus, the antimicrobial activity of DDGS against other organisms found in TMR would need to be confirmed to ensure that DDGS did not have any antibiotic effect against any of the bacteria in the TMR. Similarly, zone of inhibition of DDGS against microorganisms that caused mastitis in the Iowa State University dairy farm cows would need to be performed to ensure that DDGS had no effect upon mastitis pathogens. In another recent evaluation of antimicrobial properties of DG, Paulus Compart et al. (2013) reported that out of 159 samples of DG tested (wet: 79 samples, dried: 80 samples), only one DG sample inhibited the growth of *E. coli* ATCC 8739. This DG sample, however, did not contain any detectable antibiotic residues. The source of antimicrobial effect against *E. coli* ATCC 8739 was unclear.

The commercial DDGS used for dairy cattle feeding research at Iowa State University did not possess a detectable concentration of antibiotics or antimicrobial

compounds that were active against *Salmonella* Typhimurium, *L. innocua*, *E. coli* ATCC 25922, *Staph. aureus*, *Ped. acidilactici*, *L. casei*, *L. acidophilus*, *B. licheniformis*, *Paeni. odorifer*, *Pseud. fluorescens*, and *Paeni. amylolyticus*. The DDGS used in the study will not likely contribute negatively to the health of the cows or to antibiotic resistance in the herd.

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