FATE AND BIOLOGICAL ACTIVITY OF ANTIBIOTICS USED IN ETHANOL PRODUCTION

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DEVAN MARIE PAULUS COMPART

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ABSTRACT

Antibiotics are utilized in ethanol production to control unwanted bacteria from competing with yeast for nutrients during ethanol fermentation. However, there is no published scientific information on whether antibiotic residues in distillers grains (DG) co-products from ethanol production retain their biological activity. Therefore, the objective of this study was to quantify the concentration of various antibiotic residues in DG and determine if those residues are biologically active. Twenty distillers wet grains and 20 distillers dried grains samples were collected quarterly from nine states and 43 ethanol plants in the United States. Samples were analyzed for DM, CP, NDF, crude fat, S, P, pH, and titratable acidity. Samples were also analyzed for the presence of erythromycin, penicillin G, tetracycline, tylosin, and virginiamycin using liquid chromatography and mass spectrometry. Additionally, virginiamycin residues were determined using an FDA-approved method of analysis. Samples were further analyzed for biological activity by exposing sample extracts to varying levels of the sentinel bacteria *Escherichia coli* ATCC 8739 and *Listeria monocytogenes* ATCC 19115. Residues that inhibited bacterial growth were considered to have biological activity. Data were analyzed using the mixed procedure of SAS 9.2. Physiochemical characteristics varied among samples, but were consistent with previous findings. Ten percent of samples contained erythromycin residues at concentrations up to 0.87 ppm on a DM basis. Less than one percent of the samples contained penicillin G residues at concentrations up to 0.11 ppm on a DM basis. Less than one percent of samples

contained tetracycline residues at concentrations up to 1.12 ppm on a DM basis. None of samples contained tylosin residues. Additionally, 1.3% of samples contained virginiamycin residues at concentrations up to 0.6 ppm on a DM basis. Only one residue sample inhibited growth of *E. coli* at 10^4 CFU/g, but this sample contained none of the five antibiotic residues evaluated. No residues inhibited *L. monocytogenes* growth. These data suggest the likelihood of detectable residues in DG is low, and if they are present, they are found at very low concentrations. It appears that antibiotic residues in DG are inactivated during the production process or are present in sublethal concentrations. There is concern that low concentrations of antibiotics in DG may lead to development of antibiotic resistance in bacteria, especially if antibiotics are present in sublethal doses. However, the risk to human health appears to be minimal. Therefore, future studies on the utilization of antibiotics in ethanol fermentation should focus on the transfer of antibiotic-resistant genes through the ethanol fermentation process and resulting DG production.

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INTRODUCTION

Fuel ethanol production in the U.S. increased 75 fold from 1980 to 2010 (RFA, 2012a). This increase in fuel ethanol production also resulted in an increase in production of its primary co-product, distillers grains (DG). In 2010, 35 million metric tons of DG were produced and approximately 9,000 metric tons were exported (RFA, 2012b). In the U.S., a majority of the DG produced during fuel ethanol production is used as animal feed, with cattle consuming 80%, swine consuming 10%, and poultry consuming nine percent (RFA, 2012b). With such a large percentage of DG going into livestock diets, it is essential that DG be fully studied as to their nutritional value, as well as impacts on animal and human health and safety.

A major concern with the use of DG in food animal diets is the potential for the presence of antibiotic residues acquired during the ethanol fermentation process. If present, these antibiotic residues could theoretically lead to the development of bacterial resistance in animals consuming residues, as well as humans consuming tissue from those animals.

Thus, the aim of this study was to determine if residues from antibiotics used in the fermentation of corn to ethanol are present in the ethanol co-product DG. Furthermore, this study aims to determine the antimicrobial activity level of antibiotic residues found in DG samples.

REVIEW OF LITERATURE

Dry and Wet Grind Ethanol and Distillers Grains Production Process

Distillers grains (DG) are a co-product of ethanol production from corn or other grains. They are animal feed ingredients that come in the form of distillers dried grains (DDG), distillers wet grains (DWG), distillers dry grains with solubles (DDGS), distillers wet grains with solubles (DWGS), distillers dried solubles (DDS), or condensed distillers solubles (CDS) as defined by the Association of American Feed Control Officials Incorporated (McChesney, 2009). Ethanol can be produced in either dry or wet grind ethanol plants with over 67% being produced in dry grind facilities (Wheals et al., 1999). Ethanol fermentation averages 89% ethanol recovery efficiency; wet milled corn yields 9.84 L of ethanol per bushel (25.4 kg) and dry grinding of corn produces 10.60 L of ethanol and 7.7 kg DDGS per bushel (Wheals et al., 1999; Bothast and Schlicher, 2005).

Dry grind process of converting corn to fuel ethanol and DG can be done by either batch processing or continuous fermentation. Batch processing uses a single large fermentor which is filled, fermented, drained, and then cleaned for the next batch (Warner and Mosier, 2011). In this method, yeast is not recycled so fresh yeast must be added to each fermentation batch (Wheals et al., 1999). The advantage of batch fermentation is the reduced risk of contamination by bacteria (Warner and Mosier, 2011). Continuous fermentation is the most common method used to convert dry ground corn to ethanol and DG. In this method, a series of cascading tanks are used to ferment corn to

ethanol (Warner and Mosier, 2011). Liquid is continuously flowing through the tanks with new fermentation media continuously added to the front-end and ethanol continuously removed from the back-end (Warner and Mosier, 2011). There are several advantages to using continuous fermentation versus batch fermentation, including computerized control, “clean-in-place” technology, low labor costs, and reduced equipment down-time (Wheals et al., 1999). Furthermore, it has lower capital costs per liter leading to financial incentives for development of farmer-owned cooperatives as a form of ownership (Bothast and Schlicher, 2005). Regardless of which processing method is used, the resulting DG will contain all the nutrients from the processed corn minus the starch at concentrations at least three times greater than those of the incoming grain (Wheals et al., 1999). The starch component of the grain is converted to glucose and fermented to ethanol during the production process (Bothast and Schlicher, 2005).

The dry grind process of corn to ethanol with DG as one of the co-products consists of five basic steps: grinding, cooking, liquefaction, saccharification, and fermentation (Bothast and Schlicher, 2005). The process begins with whole corn kernels, initially cleaned of any foreign material, passing through a hammer mill and a 30 mesh screen to produce coarse flour. Coarse flour is then mixed with water to form a mash (also known as wort) with one bushel of corn producing 83 L of mash. The pH of the mash is adjusted with ammonia to the range of 5.0 to 6.0. Once pH is adjusted, alpha-amylase enzyme is added to the mash to begin the breakdown of starch polymers to soluble dextrins (long-chain sugars) via hydrolization of α 1-4 bonds, also called saccharification. Then a jet cooker raises the temperature of the mash above 100 °C. High

temperature and mechanical shear in the jet cooker allows starch molecules (especially those of high molecular weight) to cleave and rupture. The mash is kept at an elevated temperature for several minutes by pumping it through a holding tube with a backpressure valve. This elevated temperature helps to kill some unwanted acid-producing bacteria that may inhibit fermentation (Wheals et al., 1999; Davis, 2001; Bothast and Schlicher, 2005; Warner and Mosier, 2011).

The mash then proceeds from the holding tube into a flash tank. Mash temperature is allowed to drop down to 80 to 90 °C, and additional alpha amylase enzyme is added. In the flash tank, mash is liquefied for at least 30 min to allow for a significant reduction in size of the starch polymer. The dextrinized mash is then cooled, and its pH is adjusted to 4.5 with ammonia. This process is followed by the addition of glucoamylase enzyme at levels which allow for the breakdown of starch to glucose (saccharification) to proceed continuously throughout the fermentation at a speed that does not limit the rate of ethanol production. Saccharifying the liquefied starch prior to fermentation can reduce the amount of glucoamylase enzyme added to the mash (Wheals et al., 1999; Davis, 2001; Bothast and Schlicher, 2005; Warner and Mosier, 2011).

Once glucoamylase enzyme has been added, the mash is cooled to 32 °C and then transferred to fermenters where yeast and antibiotics are added. Yeast converts sugars in the mash to ethanol, while antibiotics prevent contaminating bacteria from competing with yeast nutrients. In continuous fermentation, the yeast is later removed from the fermentation medium via centrifugation and then washed in dilute sulfuric acid to reduce bacterial contamination for reuse in later fermentations. In addition to yeast, ammonium

sulfate or urea may be added to the fermentation as a nitrogen source for the yeast. Proteases may also be added to break down corn protein to free amino acids, another nitrogen source for the yeast. Additionally, sulfuric acid may be added to control pH during fermentation. Once all additions have been made to the fermenters, the mash is permitted to ferment for 48 to 72 h; fermenting mash is referred to as “beer”. During this time, pH in the mash drops below 4.0 due to an increase in activity of glucoamylase, and ethanol is produced in a final concentration of 10 to 12%.

An alternative method of dry grind ethanol production involves simultaneous saccharification and fermentation (SSF). In this method, there is reduced opportunity for microbial contaminants, a lower osmotic stress on yeast due to the avoidance of a concentrated glucose solution, an increase in ethanol yield of up to 8% more ethanol per bushel, and a decrease in energy use (Wheals et al., 1999; Davis, 2001; Bothast and Schlicher, 2005; Warner and Mosier, 2011).

After saccharification and fermentation, the beer is distilled through a distillation column. This allows for separation of the ethanol from solids and water in the mash. The distillation column uses heat to separate ethanol from water, in which alcohol vaporizes at 78 °C and water vaporizes at 100 °C. This method produces 95% pure alcohol and five percent hydrous alcohol. This hydrous ethanol is in the form of an azeotrope (constant-boiling mixture) that cannot be further separated by heat; thus, other methods must be employed to obtain 100% pure ethanol. These methods include the use of a molecular sieve system, absorption, or utilization of a third solvent to break the azeotrope, such as benzene or dehydrated carbohydrate. Finally, the resulting 100% pure anhydrous ethanol

is blended with about five percent denaturant, such as gasoline, to render it undrinkable. This prevents it from being served as a beverage and exempts it from alcohol beverage tax (Wheals et al., 1999; Davis, 2001; Bothast and Schlicher, 2005; Warner and Mosier, 2011).

After distillation, whole stillage remains at a volume 10 to 15 times greater than that of the ethanol produced. Whole stillage has both a solid and a liquid fraction and contains fiber, oil, protein, and non-fermented carbohydrate components of the grain. The liquid fraction of the whole stillage is referred to as “thin stillage”, while the solid fraction is called “wet cake”. The whole stillage is centrifuged into its two fractions with a decanter as it leaves the beer column. About 15 to 30% of the thin stillage fractioned out is returned to the dry grind process as backset. Backset reduces the amount of fresh water required for fermentation, and it contains nutrients for the yeast. The remaining thin stillage is concentrated further by evaporation and then mixed with residual solids from the fermentation to form a thick syrup called CDS. Condensed distillers solubles can then be mixed back with partially dried wet cake to create 65% DM DWGS, they can be dried to create DDS, or they can be fed alone. If no CDS is added, the partially dried wet cake is known as DWG. The DWG can be dried further to produce DDG. The most common practice is to dry DWGS to 10 to 12% moisture creating DDGS. Distillers wet grains with solubles can be used directly in animal feeds; however, the shelf life of DWGS is one to two weeks. Therefore, it is more economically feasible to dry DWGS and create more shelf-stable DDGS (Wheals et al., 1999; Davis, 2001; Bothast and Schlicher, 2005; Warner and Mosier, 2011). The final nutrient composition of DDGS is

90.3% DM, 30.4% CP, 10.7% EE, 6.9% crude fiber, 46.0% NDF, and 21.3% ADF (NRC, 1996). The nutrient composition of DG products can vary widely due to differences in production processes.

Wet milling of corn to ethanol differs from dry grind production process in that the corn kernel is divided up into several fractions and only the starch is used for production of ethanol (Wheals et al., 1999). Shelled corn delivered to a production facility is first steeped in 48 to 65 °C water with 0.1% sulfur dioxide for 30 to 50 h (Corn Refiners Association, 1999; Davis, 2001). This process softens the corn kernels and allows soluble nutrients to be absorbed by the water. Nutrients absorbed by the water are later concentrated by water evaporation to produce condensed corn fermented extractives. The remaining corn has the germ removed, which is further processed to produce oil and corn germ meal. Corn is then screened to separate the bran from the starch and gluten protein. The bran is combined with other co-product lines to produce corn gluten feed. The remaining starch and gluten protein slurry is then centrifuged to separate the two fractions. Gluten protein is then dried to produce corn gluten meal. Starch is washed and dried, or modified and dried. Some starch is then used in the food, paper, or textile industry, and the rest goes into the production of sweeteners or ethanol (Davis, 2001). Once separated from the kernel, the starch undergoes the same process that occurs in a dry grind facility beginning with liquefaction to produce ethanol (Bothast and Schlicher, 2005).

Potential Microbial Contaminants That Reduce Ethanol Yield and Methods to Reduce These Contaminants

Bacterial contamination during the milling of corn to ethanol and DG is a major problem encountered by the fuel ethanol industry which can lead to reduced ethanol yields. Bacteria can thrive during fuel ethanol production because the process is not sterile nor in pure culture conditions (Bischoff et al., 2009). Contaminating bacteria may be introduced to the grinding process via raw materials used to make ethanol (heat treatment of raw materials does not kill all contaminants), water used for pump and agitator seals, poorly stored backset, active dry yeasts, and yeast slurry used as inocula (Makanjuola et al., 1992; Skinner-Nemec et al., 2007; Heist, 2009; Leja and Broda, 2009; Muthaiyan and Ricke, 2010). Improper cleaning, especially of vessels and transfer lines, allows the bacteria to continue to thrive on equipment used in the dry grind process. The bacteria form biofilms, active bacterial colonies, which are possibly resistant to antibiotics and cleaning (Skinner-Nemec et al., 2007; Rich et al., 2011). Impediments to process flow can be a source of bacterial colonization as well (Heist, 2009).

The bacteria of greatest concern to the industry are lactic acid bacteria (LAB); but, other bacterial contaminants including *Bacteroides forsythus*, *Fusobacterium nucleatum*, *Propionicbacterium granulosum*, and *Clostridium aerotolerans* also have detrimental effects on ethanol production (Skinner and Leathers, 2004; Leja and Broda, 2009). Lactic acid bacteria are Gram positive anaerobes or facultative anaerobes that thrive in the anaerobic fermentation process (Muthaiyan and Ricke, 2010). The species of

greatest concern include species belonging to the following genera: *Lactobacillus* (primary contaminant), *Lactococcus*, *Leuconostoc*, *Pedicoccus*, *Bifidobacterium*, and *Weisella* (Skinner and Leathers, 2004; Leja and Broda, 2009; Muthaiyan and Ricke, 2010). Lactic acid bacteria are of concern because they compete with yeast for essential nutrients and growth factors, and they produce organic acids, including lactic and acetic acid, which inhibit yeast growth (Skinner and Leathers, 2004). In fact, lactic acid concentrations as low as one to four percent inhibited yeast growth, while 0.3% acetic acid concentration halted fermentation (Hynes et al., 1997; Weigel et al.). Lactic acid bacteria are especially problematic because they can tolerate high temperatures, low pH, and high ethanol concentrations encountered in the fuel ethanol production process. Furthermore, LAB have a rapid growth rate and reach high numbers of viable cells prior to the completion of yeast fermentation (Hynes et al., 1997; Bischoff et al., 2009; Leja and Broda, 2009). Failure to identify and control bacterial contamination can lead to a stuck fermentation. A stuck fermentation is one in which not all of the carbohydrates have been converted to alcohol. It results in a shutdown of the fermentor and loss of production time while the system is cleaned of contaminants and re-inoculated (Bischoff et al., 2009; Muthaiyan and Ricke, 2010).

The presence of LAB in the fermentation process results in several undesirable effects. First, the final pH is reduced due to the production of organic acids by LAB, resulting in a less efficient fermentation. Second, the final yeast count after fermentation is reduced due to the inability of the yeast to outcompete LAB for nutrients, as well as the inhibitory effects of the organic acids produced by LAB (Makanjuola et al., 1992; Heist,

2009). The reduction in final yeast count can lead to greater fuel ethanol production costs because less yeast is available for recycling in subsequent fermentations. Third, the presence of LAB often results in elevated and undesirable concentrations of residual carbohydrates because of the inhibited conversion to ethanol. Finally, the most critical effect of LAB on ethanol fermentation is the reduction in final ethanol yield (Makanjuola et al., 1992; Heist, 2009). Ethanol losses up to 30% were reported due to LAB contamination (Makanjuola et al., 1992; Chang et al., 1995; Bischoff et al., 2009).

Ethanol losses encountered when LAB are present in a fermentation are believed to be the result of several actions. One such action includes the presence of unused residual carbohydrates as mentioned above. Also, glucose needed for the production of ethanol is diverted to the production of lactic acid or is used by cellular constituents needed for LAB growth. In addition, low pH or higher lactic acid concentrations and other metabolic products inhibit alcohol production. An increase in yeast flocculation has also been noted in the presence of some lactobacillus strains. Flocculation is the forming of cloud-like aggregations that renders the yeast unusable by fermentation (Hynes et al., 1997). Finally, ethanol production can be reduced indirectly through the inhibition of glucoamylase which results from incomplete saccharification due to a reduction in pH by lactic acid (Hynes et al., 1997).

Detection of Bacterial Contamination

In order to combat bacterial contamination, producers of fuel ethanol must first be able to detect and identify bacterial contaminants. Currently, there are several methods developed for the quantification and identification of bacterial contaminants. The most basic method of bacterial detection is plating. In the plating method, a MRS agar plate is used to isolate bacterial contaminants. The population density of bacterial contaminants can be estimated by counting colony-forming units on the plate. In order to specify the medium for *Lactobacillus* species isolation, the reducing agent cysteine can be included with the MRS agar at 0.05%. This method is time-consuming, taking 24 to 48 h to complete; time during which increased contamination can occur and further delay of production results. However, plating is the most cost-effective method of determining bacterial contamination (Muthaiyan and Ricke, 2010).

A second method used to detect bacterial contamination is the use of a particle size distribution analyzer (PSDA). The PSDA distinguishes organisms based on their actual morphological differences; however, it cannot distinguish between viable and non-viable cells, making it difficult to determine course of treatment. In addition, PSDA may require addition filtration steps prior to use in order to remove any fermentation ingredients present in the sample because these may interfere with analysis (Muthaiyan and Ricke, 2010).

A third method of bacterial contaminant detection is fluorescence spectroscopy. This method is fast, simple, and reliable. There are two types of fluorescence

spectroscopy. The first is Fourier transform infrared spectroscopy, a method of fluorescence spectroscopy that utilizes fluorescence labeling along with chromatography dot-blot and fluorescence *in situ* hybridization (Muthaiyan and Ricke, 2010). Auto-fluorescence is a second fluorescence method that does not require labeling and is less time consuming, but does require the presence of a sufficient number of cells. In order to reach sufficiently high cell numbers for detection, cell multiplication would have to continue for 24 to 48 h. Waiting 48 h prior to detection would allow for increased bacterial contamination, which prevents application of auto-fluorescence in the fuel ethanol industry (Muthaiyan and Ricke, 2010).

Several methods utilize polymerase chain reaction (PCR) to detect bacterial contamination. Polymerase chain reaction is desirable because it directly detects bacteria at the nucleic acid level, which removes the need for culturing and enrichment steps and makes the procedure less time-consuming and more manageable. Standard PCR amplifies the product (in this case bacterial contaminants) from a single copy of a particular sequence. It, however, requires gel electrophoresis which is more laborious and carries the increased risk of contamination from carry-over PCR products. Real-time PCR, on the other hand, detects DNA amplification during the amplification process. To obtain the initial starting copy number, the signal produced from a given sample can be compared with a standard curve generated from serial dilutions of a known amount of the target DNA. With real-time PCR, the PCR products are detected via generation of a fluorescent signal. Real-time PCR also allows for multiplexing, which is the detection of more than one DNA fragment in a single reaction tube. Multiplexing is possible because of the

simultaneous monitoring of multiple fluorophores and melting curve analysis. This allows for the detection of multiple strains of bacterial contaminants simultaneously. Real-time PCR is advantageous because it only takes four to six hours for the entire procedure to be completed. However, it can accumulate non-specific gene amplification products, yielding false-positive results.

To prevent the possibility of getting false-positive results and to overcome the specificity limitations of traditional PCR detection, immuno-PCR can be utilized for contaminant detection. Immuno-PCR is designed on the basis of an antigen detection system using a specific antibody-DNA conjugate to detect antigens (microorganisms or other contaminants). It has the greatest sensitivity and specificity of any existing antigen detection system and could potentially be applied to detect a single antigen molecule. Immuno-PCR requires post-PCR evaluation of end products unless combined with real-time PCR. Real-time immuno-PCR detects and monitors the contaminant simultaneously during the exponential phase of PCR (Muthaiyan and Ricke, 2010).

In addition to the more traditional contaminant detection methods for bacterial contamination detection in the fuel ethanol industry mentioned above, there are several prospective electromagnetic engineering and nanotechnology detection methods being considered. For instance, the superconducting quantum interference device (SQUID), a highly sensitive detector of magnetic flux, is being considered for use (Muthaiyan and Ricke, 2010). It detects magnetically labeled bacteria and uses standard titration experiments to determine bacterial concentrations. Bioconjugated nanoparticles are also being studied for their application in the fuel ethanol industry. They provide a high

fluorescence signal for bioanalysis after being incorporated with biorecognition molecules such as antibodies. Antibody-antigen interaction and recognition can then allow for the identification of a variety of bacteria. This system has been reported to detect antibody-conjugated nanoparticles bound to single bacterial cells via a simple flow cytometry device (Muthaiyan and Ricke, 2010). Finally, a new, simple nanoparticle based platform is being developed (Muthaiyan and Ricke, 2010). It can rapidly detect pathogens in native biological samples with extreme sensitivity. In this system, bacteria are targeted by highly magnetic nanoparticles, concentrated into a microfluidic chamber, and detected by nuclear magnetic resonance. It is believed that LAB-specific antibody-conjugated nanoparticles could be generated for use in bioethanol fermenters to detect contaminating bacteria (Muthaiyan and Ricke, 2010).

Methods to Reduce Bacterial Contamination

Once bacteria are present and detected in the production line of a fuel ethanol facility, it is critical that their numbers be reduced. Primary methods of control include acid-washing yeast in a dilute sulfuric acid between fermentations, cleaning and sanitation of equipment, and the use of antibiotics such as erythromycin, penicillin, tetracycline, tylosin, and virginiamycin among others during the fermentation phase of ethanol production (Hynes et al., 1997; Heist, 2009; Olmstead, 2009; Muthaiyan and Ricke, 2010). In addition to the current primary methods of contaminant control, several new methods are under investigation for their ability to reduce or eliminate LAB in the

fuel ethanol production process. For instance, the use of the food additive Neupectin-L, a natural food additive made up of 10 molecules of galacturonic acid, eliminated LAB growth during long starch fermentations (Lee et al., 1993). Fluoride use has also been studied as a possible method to reduce the effects of LAB (Eisenberg et al., 1980). Fluorides can act as a proton conductor to discharge the pH gradient across cell membranes reducing the aciduric properties of the bacterium (Eisenberg et al., 1980). Chitosan, a biopolymer derived mainly from chitin deacetylation obtained mainly from crustaceous shells, is another product reported to reduce LAB. Chitosan and its derivatives demonstrated antimicrobial activity against a variety of bacteria and fungi groups. In addition, they have select antimicrobial activity against LAB isolated from beer production. In fact, as little as 0.1 mg/mL of chitosan effectively controlled *Bacillus megaterium, Lactobacillus* plantarum, and *Pediococcus* species without affecting yeast viability in brewing systems (Galvagno et al., 2007). Sulfites, including potassium metabisulfite, have also been extensively studied for their use against LAB (Muthaiyan and Ricke, 2010). Sulfites are chemical preservatives and antioxidants that control bacteria via molecular oxygen (Chang et al., 1997; Rojo-Bezares et al., 2007). When used in fuel production at high concentrations, sulfites effectively controlled bacteria while having no effect on yeast cells (Chang et al., 1997). In addition, when in combination with nisin, sulfites have a synergistic antibacterial effect (Rojo-Bezares et al., 2007). Nisin acts on the cytoplasmic membrane of gram positive bacteria by “forming transient pores which allow efflux of small hydrophilic compounds”, and low concentrations of ethanol (six percent) increases its antibacterial effects (Nilsson et al., 2000; Rojo-Bezares

et al., 2007). Bacteriocins, especially nisin, act alone or synergistically with sulfites to control LAB. Other, less well studied products for LAB control include 3,4,4'-trichlorocarbanilide, hydrogen peroxide, and chlorine dioxide. electroneutral Cl^-/OH^- exchange is carried out by the ionophore 3,4,4'-trichlorocarbanilide (TCC; Ahmed and Booth, 1983; Skinner and Leathers, 2004; Muthaiyan and Ricke, 2010). Hydrogen peroxide is an antimicrobial (Chang et al., 1997; Bischoff et al., 2007; Muthaiyan and Ricke, 2010). When used in fermentations at high concentrations, hydrogen peroxide does not affect viability of yeast; however, its effect on LAB varies based on the species and strain present (Chang et al., 1997; Bischoff et al., 2007; Muthaiyan and Ricke, 2010). Chlorine dioxide is an oxidizing and sanitizing agent with a broad biocidal activity against bacteria, yeast, viruses, fungi, and protozoa (Rajkovic et al., 2009). It causes a loss in permeability control in bacteria with efficiency dependent on the food product, concentration, and duration of treatment (Rajkovic et al., 2009).

Hops is a natural product that is being more extensively studied for bacterial control use in fuel ethanol production. The hop plant is a vine in the hemp family which is dioecious and blooms yearly. Antimicrobial hops acids (beta acids) are found in the inflorescence (Sakamoto and Konings, 2003). Hops beta acid, consisting of lupulone and colupulone, is a constituent of hops acids (Shen and Sofos, 2008). Having been stored at 4 °C, when combined with other antibiotics, and low pH, hops have inhibitory effects on gram positive bacteria (Sakamoto and Konings, 2003; Galvagno et al., 2007; Shen and Sofos, 2008). At high concentrations, hops beta acids had no significant effect on viable yeast cells (Galvagno et al., 2007). Currently, the USDA-FSIS approved the use of hops

beta acids as a safe antimicrobial product in meats (Shen and Sofos, 2008). Hops are currently used in the beer brewing industry to add flavor to beer; however, in the brewing industry, hops have little antimicrobial effect on LAB due to the development of resistance in the LAB. It is believed that resistance of LAB to hops in beer is the result of immunity acquired with prolonged contact with hops compounds under brewing conditions (Sakamoto and Konings, 2003). Resistance of LAB to hops is the result of the plasmid encoding hop resistant mechanism HorA (Galvagno et al., 2007). Over a year after serial sub-culturing in the absence of hops compounds, the LAB immunity to hops acids is reduced (Sakamoto and Konings, 2003). The extended time period of sub-culturing needed to diminish resistance indicates that resistance is a very stable property, once acquired. Therefore, the use of hops in the fuel ethanol industry could be short-lived if such resistance is acquired (Sakamoto and Konings, 2003). A final method used to reduce the inhibitory effects of LAB on fuel ethanol production is to adjust the rate of yeast inoculation. By altering yeast inoculation rate, yeast can out-compete the bacteria for nutrients (Narendranath and Power, 2004). Yeast inoculation rate is the only method beyond cleaning that does not require the use of additional products in the fuel ethanol production process.

Quality Control in the Ethanol Production Process – Utilization of Antibiotics to Manage Fermentation

Antibiotics have been used in fuel ethanol production for decades in order to improve production efficiency (Juranek and Duquette, 2007). Antibiotics are effective at reducing levels of LAB and other contaminating bacteria present during the fermentation of fuel ethanol, allowing for a more productive fermentation. There are two major concerns with the use of antibiotics in fuel ethanol. The first concern is the potential for bacteria to develop resistance, rendering antibiotics useless against them (Muthaiyan and Ricke, 2010). The second concern is the potential for antibiotic residues to end up in animal feeds and potentially animal tissues used for human consumption (Benz, 2007) through their presence in fuel ethanol production co-products such as CDS, DDGS, and DWGS.

Antibiotic resistance is thought to develop as a result of misuse of antibiotics (Muthaiyan and Ricke, 2010). This includes antibiotic overdosing when no effect is observed and underdosing when efficient control is observed in the ethanol industry (Muthaiyan and Ricke, 2010). Overdosing can lead to an increased chance that antibiotics will not be inactivated during the distillation process. This would result in their presence in co-products such as DG. Consumption of DG containing antibiotic residues by animals could result in animals as well as humans consuming food products from those animals, developing antibiotic resistance to antibiotics used in fuel ethanol production. Underdosing, on the other hand, leads to a lack of effectiveness of antibiotics. It also

carries a greater risk of causing resistance because bacteria can become accustomed to the antibiotic in low doses and thus, develop resistance mechanisms (Muthaiyan and Ricke, 2010). Three mechanisms can lead to the development of resistance in bacteria. The first is the lack of bacterial penetration by the antibiotic (alteration of permeability). The second is failure of the antibiotic to attach to the target on the bacteria. The third is biochemical inactivation of the drug by hydrolysis or coupling. Resistance can either be chromosomal or episomal (Cocito, 1979; Vannuffel and Cocito, 1996).

It is the responsibility of the Food and Drug Administration's (FDA) Center for Veterinary Medicine (CVM) to approve the use of drugs in animal feeds, as well as monitor and establish limits for feed contaminants (Benjamin, 2009; De Alwis and Heller, 2010). The use of antibiotics in fuel ethanol production could result in antibiotic residues in the DG produced as a co-product to ethanol. Thus, antibiotic residues in DG used as feed or feed ingredients are considered feed additives and regulated by the FDA (Benjamin, 2009).

Currently, the FDA has expressed several concerns with the use of antibiotics in the fuel ethanol industry. Their primary concern is that antibiotic residues may be present in DG and that those residues could be transferred to animal tissue upon ingestion. However, it is unlikely that antibiotic residues in DG would transfer into animal tissue due to the low concentrations present in DG, as well the pharmacology of the antibiotics used in fuel ethanol production. The second concern expressed by the FDA is the potential harm to humans who eat tissues containing antibiotic residues. The FDA is specifically concerned with the probability that consumption of contaminated animal

tissues could result in antibiotic resistance in humans. Many countries currently place maximum residue limits in foodstuffs of animal origin to combat this problem (Wang et al., 2008). Finally, the FDA expressed concern for the health of animals fed DG containing antibiotic residues (Juranek and Duquette, 2007).

The only antibiotic currently approved by FDA CVM for use in fuel ethanol production is virginiamycin. The CVM issued a letter of no objection for the use of virginiamycin in the fermentation phase of alcohol production at two to six parts per million (ppm). In addition, the CVM did not object to potential virginiamycin residues of 0.2 to 0.5 ppm in distillers co-products. This statement was based on calculating virginiamycin residues resulting from inclusion concentrations in ethanol production, estimated residues in DDGS, and on an animal diet containing no more than 20% DDGS. Moreover, it was stated that the CVM is unlikely to take regulatory action against DDG-containing feed with residual virginiamycin concentrations of virginiamycin below 0.5 ppm. Concentrations below 0.5 ppm pose no concern to broiler, turkeys, swine, or cattle consuming the feed, or to the humans consuming food derived from those animals (Benz, 2007).

Although virginiamycin is currently the only antibiotic that the FDA has addressed in regards to its use in fuel ethanol production, several others antibiotics are currently in use. The FDA does not currently regulate the use of antibiotics in fuel ethanol production.; thus, ethanol producers often utilize a variety of antibiotics at unknown concentrations.

Types and Modes of Action of Antibiotics That May Be Used In Ethanol Production

Several antibiotics are currently used in fuel ethanol production in order to control contaminating LAB. The main antibiotics in use that are relevant to this study include virginiamycin, penicillin, erythromycin, tetracycline, and tylosin. These antibiotics are all commonly used in the ethanol industry (Olmstead, 2009).These antibiotics can work in two ways. First, they can be bactericidal, meaning they kill bacteria *in vitro*. In contrast, the antibiotics can be bacteriostatic, meaning they slow or stop bacterial growth *in vitro* (Merck, 2004). The use of these antibiotics in fuel ethanol production is currently not regulated by the FDA.

Virginiamycin is a streptogramin antibiotic made up of two components, M and S (Vannuffel and Cocito, 1996). It is a bacteriostatic antibiotic when the components are not associated and a bactericidal antibiotic when the two components are associated (Vannuffel and Cocito, 1996; Hynes et al., 1997; Merck, 2004). The two components of virginiamycin are most active in a M:S ratio of 2:1 or 1:1 the M component being the first-limiting factor with a 15% concentration drop in M factor reducing antibiotic activity (Cocito, 1979). Together the two components work synergistically to decrease colony-forming capacity of bacteria; yet, each fraction alone only reduces viability of most bacteria after an exceedingly long incubation period. In fact, the activity of the two components together is 10 to 100 times greater than the activity of either individually (Cocito, 1979).

Upon the introduction of component S to component M, component S undergoes a 6-fold increase in affinity for the ribosome. The two components then pass through the

cell membrane of gram positive bacteria (Hynes et al., 1997). The M component of virginiamycin cannot permeate gram negative cell walls, thus gram negative bacteria are naturally resistant to virginiamycin (Islam et al., 1998). Once in the cytoplasm, the M component attaches to bacterial ribosomes inducing a conformational change that increases particle affinity six-fold for the S component. Once bound to the ribosome, the two components inhibit protein synthesis irreversibly (Cocito, 1979).

Virginiamycin is a narrow spectrum antibiotic effective at controlling gram positive bacteria including a majority of LAB (Cocito, 1979; Hynes et al., 1997; Islam et al., 1998). The effectiveness of virginiamycin against *Lactobacilli* species is dependent on strain and growth phase of the *Lactobacilli* species. Currently, some resistance to virginiamycin by several genera of gram positive bacteria, as well as breakdown of virginiamycin by *Lactobacilli* species, were reported (Hynes et al., 1997). In addition, reports of cross-resistance exist between macrolide antibiotics (e.g. tylosin and erythromycin) and virginiamycin in gram positive bacteria (Cocito, 1979). However, resistance to streptogramins, including virginiamycin, is less common than any other protein synthesis inhibitor (Vannuffel and Cocito, 1996).

Virginiamycin is effective for use in the fuel ethanol industry as it is stable at temperatures and pH encountered during alcohol fermentation (Hynes et al., 1997; Islam et al., 1998; Muthaiyan and Ricke, 2010). Initially, activity of virginiamycin dropped when exposed to lower pH (3.8 to 4.2); however, it stabilized thereafter with 86% of its activity present at 35 °C for pH 3.8 and pH 4.0 (Islam et al., 1998). In addition, virginiamycin is effective in controlling LAB, preventing ethanol yield reductions as

great as 11% in the presence of *Lactobacilli* species and yeast (Hynes et al., 1997). At low doses (2 mg/L), virginiamycin had no negative effects against yeast fermentation; however, at higher concentrations (20 mg/L), the rate of glucose uptake in yeast can be decreased. Thus, virginiamycin is most effective at doses lower than those which affect yeast glucose uptake rate (Hynes et al., 1997).

Consumption of DG containing virginiamycin residues by food-producing animals poses little or no harm to animal or human health (Juranek and Duquette, 2007). Several factors prevent virginiamycin in co-products of fuel ethanol production from being harmful to animals and humans. First, virginiamycin is inactivated during the ethanol distillation process (Hynes et al., 1997). Second, virginiamycin is not absorbed in the gut and was not found in the kidneys, liver, or muscle of chickens fed virginiamycin (Butaye et al., 2003; Juranek and Duquette, 2007). Third, virginiamycin has been fed to animals at much higher concentrations than FDA approved with no ill effects on the health of the animals. Finally, virginiamycin concentrations in DG (0.2 to 0.5 ppm) are much lower than those currently approved by the FDA for use in animal feeds (Table 1 and 2; (Juranek and Duquette, 2007; FDA, 2010a). Thus, virginiamycin is a safe and effective antibiotic choice for use in the fuel ethanol industry.

Penicillin, a beta lactam antibiotic, is another antibiotic extremely effective for the control of LAB (Day et al., 1954; Muthaiyan and Ricke, 2010). Beta lactam antibiotics, characterized by a beta lactam ring nucleus, exhibit both bacteriostatic and bactericidal effect on gram positive and some gram negative bacteria (Islam et al., 1998). For instance, they are ineffective against some *Escherichia coli* strains, but not others (Islam

et al., 1998; Bartlett et al., 2000). Their bactericidal activity is time-dependent; thus, increasing their concentration above the minimum inhibitory concentration (MIC) does not improve their effectiveness. Furthermore, penicillin activity *in vivo* is generally slow (Merck, 2004). Beta lactams act by binding to bacterial membranes irreversibly. They bind via penicillin-sensitive enzymes (synonymous with penicillin-sensitive proteins) which can be detected as those enzymes or proteins which bind to penicillin (Spratt, 1977). Once bound to the cell wall, beta lactams interfere with the incorporation of glutamic acid in the cell wall during cell division, inhibit mucopeptide synthesis in the cell wall, and inhibit the terminal stages of peptoglycan metabolism. This ultimately destroys the cell wall creating an osmotically unstable organism susceptible to cell lysis (Spratt, 1977; Islam et al., 1998; Bartlett et al., 2000; Merck, 2004). The actions of penicillin can be inhibited in some cells by the production of penicillinases, such as beta lactamase (Islam et al., 1998; Merck, 2004). The resistance shown by bacteria producing beta lactamase can be blocked by the use of beta lactamase inhibitor (Merck, 2004). However, beta lactamase inhibitor may only partially inhibit the actions of extended spectrum beta lactamases produced by some *Escherichia coli* strains (Merck, 2004).

Penicillin is an antibiotic commonly used in fuel ethanol fermentations (Day et al., 1954; Hynes et al., 1997; Islam et al., 1998; Olmstead, 2009). In one study, it was found that the 0.075 to 0.02 mL/ mL inclusion of penicillin is effective at preventing the growth of bacteria in fermentation mashes (Day et al., 1954). In addition, penicillin use at those concentrations results in the presence of more viable yeast cells at the end of the fermentation (Day et al., 1954). Penicillin inhibits acid and acrolein production and also

prevents destruction of alpha amylase (Day et al., 1954). Penicillin is often added at concentrations above 1.5 mg/L in fuel ethanol production due to the possibility of induced enzymatic degradation of the antibiotic (Hynes et al., 1997). This concentration is much lower than concentrations approved for use in food animals (Table 1 and 2; (FDA, 2010a). It has poor stability at pH below 5 and above 8, and it is less stable at higher temperatures (Hynes et al., 1997; Islam et al., 1998; Kheirolomoom et al., 1998). When penicillin is introduced to low pH, it initially undergoes either a rapid inactivation when pH ranges from 3.8 to 4.5, or a gradual inactivation when pH is 4.8 (Islam et al., 1998). This suggests that penicillin is inactivated by the final low fermentation pH and high temperature reached during distillation.

Erythromycin, a 14-membered lactone ring macrolide antibiotic, is another antibiotic in fuel ethanol production (Petropoulos et al., 2008; Olmstead, 2009). Macrolide antibiotics are bacteriostatic, binding to the 50S subunit of the ribosome in a ratio of one molecule per ribosome (Vannuffel and Cocito, 1996; Merck, 2004; Petropoulos et al., 2008). They reversibly inhibit protein synthesis by preventing both polypeptide synthesis and the translocation of peptidase chains (Vannuffel and Cocito, 1996; Bartlett et al., 2000; Merck, 2004). Secondarily, macrolides target ribosome assembly, inhibiting it in growing cells (Chittum and Champney, 1995). Erythromycin is effective against gram positive and some gram negative bacteria (Chittum and Champney, 1995). It is soluble in alcohol and insoluble in water. However, it becomes more unstable with higher alcohol concentrations. Furthermore, it degrades faster at higher temperatures and when dissolved in water. Its stability is also pH-dependent with

low pH causing it to become unstable; its optimal pH range is 7.0 to 8.0 (Brisaert et al., 1996). Like penicillin, erythromycin is likely inactivated by the low pH and high temperatures encountered during the fermentation and distillation of ethanol.

Once consumed, erythromycin is diffused well into body fluids, but food consumption decreases its absorption (Merck, 2004). It is currently approved for use in food animals (Table 1 and 2; FDA, 2010a). It is important to note that 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).

Tylosin, a commonly used antibiotic in ethanol production, is a macrolide antibiotic that targets bacterial protein synthesis (Omura et al., 1983; Petropoulos et al., 2008; Olmstead, 2009). Unlike erythromycin, it is a 16-membered lactone ring and is produced commercially by a strain of *Streptomyces fradiae* (Omura et al., 1983; Petropoulos et al., 2008). Like erythromycin it diffuses well into body. (Merck, 2004). Tylosin is most stable at pH of approximately 3.5 or 9.0. These pH points correspond to the salt and non-dissociated forms of the substance. Outside of those two pH ranges, there is significant inactivation of the antibiotic. In addition, increased temperatures and exposure periods can lead to inactivation (Aksenova et al., 1984). Currently, tylosin is approved to be fed in livestock (Table 1 and 2; FDA, 2010a). It is likely, however, that any residue in DG would be inactivated due to its low stability at pH and high temperatures of the fuel ethanol production process.

A final antibiotic of interest for this study, as well as one commonly used in fuel ethanol production, is the bacteriostatic antibiotic tetracycline (Moellering et al., 1972; Olmstead, 2009). Tetracycline acts by binding the 30S subunit of bacterial ribosomes, thus preventing bacterial protein synthesis (Merck, 2004). Tetracycline is unstable at low pH (pH < 2) and will form anhydrotetracyclines via the loss of water and proton transfer in strongly acidic conditions (Wang et al., 2008). Furthermore, tetracyclines degrade faster at low pH and high temperatures. In addition, its absorption is decreased with metal cations (Al, Ca, Mg, Fe), and it is antagonistic when co-administered with penicillin (Merck, 2004). Tetracycline is currently approved to be fed to livestock (Table 1 and 2). In the body, it penetrates most body tissues and fluids. Previous studies have looked at the effectiveness of heat sterilization of animal feedstuffs in order to reduce the level of active tetracycline residue. It has been reported that low-temperature, long-time treatments are most effective with conventional sterilization (121 °C for 20 minutes) reducing active residues to less than one percent (Hassani et al., 2008).

Bacteria Species Sensitive to Antibiotics

Antibiotics are routinely used to combat bacteria. They effectively suppress the growth of or kill bacteria. There are hundreds of antibiotics that are utilized to combat both Gram positive and Gram negative bacteria. These antibiotics work in a variety of ways to target bacteria and prevent their growth.

Two species of bacteria are under consideration for this study: *Listeria monocytogenes* and *Escherichia coli*. Both are commonly used when determining antibiotic activity.

Listeria monocytogenes are an intracellular, neutrophilic, Gram positive rod (Datta and Benjamin, 1997; Bartlett et al., 2000). *Listeria monocytogenes* responds to all five antibiotics under investigation in this study (Moellering et al., 1972; Hof et al., 1997; Georgnaras and von Holy, 2001). The beta lactam penicillin, the macrolides erythromycin and tylosin, and tetracycline have all been shown to have a bacteriostatic effect on *Listeria monocytogenes* (Hof et al., 1997). However, penicillin has also been shown to have a bactericidal effect on the bacteria. Multiple studies have examined the MIC and minimum bacterial concentration (MBC) values of penicillin and *Listeria monocytogenes* with MIC values indicating bacteriostatic action and MBC values indicating bactericidal action. The results of studies on the action of penicillin have been contradictory with some showing bacteriostatic activity and others reporting bactericidal activity (Moellering et al., 1972; Wiggins et al., 1978; Bartlett et al., 2000). Results from one study suggests that penicillin is bactericidal against most strains, but bacteriostatic action is the preferred intracellular situation (Safdar and Armstrong, 2003). In addition, *Listeria monocytogenes* has little resistance to erythromycin, penicillin, and tetracycline (Hof et al., 1997; Safdar and Armstrong, 2003).

Escherichia coli, the second bacterial species under investigation in this study, are highly acid-tolerant, facultative anaerobes, and gram negative rods (Datta and Benjamin, 1997; Islam et al., 1998; Bartlett et al., 2000). *Escherichia coli* are sensitive to all of the

antibiotics under investigation in this study; however, tetracycline and penicillim sensitivity is dependent on the *Escherichia coli* strain (Franklin and Godfrey, 1965; Franklin, 1967; Fernandez-Munoz et al., 1971; Spratt, 1977; de Bethune and Nierhaus, 1978; Omura et al., 1983; Cocito et al., 1997; Bartlett et al., 2000; Petropoulos et al., 2008). The macrolides erythromycin and tylosin affect bacteria by a two-step mechanism and are slow binding inhibitors of this bacterium. Each drug binds to the ribosome of bacteria with one molecule per ribosome and each has distinct side chains that show specific binding characteristics at both the transient and final binding site (Petropoulos et al., 2008). The binding of *Escherichia coli* ‘s ribosome and erythromycin is readily reversible under normal conditions. However, in the presence of alcohol, the reversibility of binding is extremely slow (Fernandez-Munoz et al., 1971). In fact, the affinity of erythromycin for *Escherichia coli*’s molecule is enhanced by the presence of alcohol (Fernandez-Munoz et al., 1971). Most *Escherichia coli* strains are also extremely sensitive to penicillin and posses six consistent binding sites for penicillin with an additional two detected on occasion. In addition, three penicillin sensitive enzymes, D-alanine carboxypeptidase 1, peptidoglycan transpeptidase, and peptidoglycan endopeptidase, have been detected in *Escherichia coli* (Spratt, 1977).

Methodology to Detect Antibiotics and Their Activity in Distillers Grains

A microbiological method to detect virginiamycin residues in chemical premixes, supplements, and complete feeds is currently the only FDA-approved method for use in

determining virginiamycin residues in DG. It was created by SmithKline Beecham Animal Health and was submitted, reviewed, and accepted by FDA in 1993. It has a detection limit of 0.3 ppm (Islam et al., 1998; Juranek and Duquette, 2007). The methods utilized in this method detect active virginiamycin residues containing both the M and S component. In addition, a liquid chromatography tandem mass spectrometric method developed by the FDA can also be utilized to detect the presence of virginiamycin. This method, discussed below, can only detect the M component of virginiamycin and can detect virginiamycin that is not active. However, it has a lower detection limit of at least 0.1 ppm.

A liquid chromatography tandem mass spectrometric method for the screening, determination, and conformation of 13 antibiotics residues in DG was developed and is currently in use by the FDA/CVM for routine screening studies (De Alwis and Heller, 2010; Heller and De Alwis, 2010). The 13 antibiotics for which this method screens include: ampicillin, penicillin G, tetracycline, oxytetracycline. chlortetracycline, bacitracin, virginiamycin M1, chloraphenicol, erythromycin A, clarithromycin, tylosin A, monensin A, and streptomycin. The method involves an initial solvent extraction followed by a two-track clean up and then concentration on hydrophilic polymeric or weak cation exchange solid phase extraction cartridges. Finally, the sample is analyzed by liquid chromatography ion trap tandem mass spectrometry. This method is employed to provide qualitative surveillance information while avoiding false positives, and to generate quantitative information on antibiotic residues in DDGS. This method can be utilized with a variety of DG-based matrices (De Alwis and Heller, 2010; Heller and De

Alwis, 2010). It should be noted that although developed for use by the FDA/CVM for screening purposes, this method is not yet approved by FDA for use in the screening of DG for antibiotic residues.

A method for the detection of tetracycline, oxytetracycline, and chloramphenicol in animal feedstuffs has been published (Wang et al., 2008). This method uses subcritical water extraction and high performance liquid chromatography followed by UV detection. The development study on this method reported that extraction was best performed at 100 °C and pH 2.0 with 5.5 mL of water for extraction for five min. A pH of 2.0 was ideal as most tetracyclines are unstable and will form anhydrotetracyclines by loss of water and proton transfer under strongly acidic conditions (pH < 2.0). The method was compared to typical ultrasonic extraction and found to have a higher extraction efficiency, better precision, shorter extraction time, is simplistic, avoids clean-up and concentration procedures, and significantly reduces any toxic organic solvents (Wang et al., 2008). In addition, a recovery and precision study on spiked samples of animal feeds presented good results (Wang et al., 2008). This method is not currently approved by the CVM for the use of detecting antibiotic residues in DG.

Minimum inhibitory concentrations (MIC) are generally utilized to determine the antimicrobial activity of a sample. In order to determine the MIC indicator bacteria species are exposed to the antimicrobial either on agar or in broth. With agar, a set number of bacterial cells are spotted onto an agar plate prepared with serial diluted antimicrobial concentrations. Growth of the bacterial colonies following incubation at conditions for optimum growth of the indictor bacteria demonstrate uninhibited growth.

With broth, a liquid medium is used with a set number of bacterial cells and increasing concentrations of the antimicrobial agent. After incubation, turbidity indicates growth of the organism. With both methods, the MIC is defined as “the lowest concentrations of the antimicrobial agent that prevents visible growth of a microorganism under defined conditions” (Wiegand et al., 2008).

Bacterial thresholds differ from MIC in that they expose varying concentrations of sentinel bacteria to a constant concentration of antibiotic. This method is more applicable to this study as the antibiotic residue concentration in distillers grains do not vary once they are in the product. However, the bacterial load the residues are exposed to changes based on the environment.

Published Surveys of Antibiotic Concentrations in Distillers Grains

Currently two surveys have been conducted by the CVM on antibiotic residues in DG. The first nationwide survey was completed in 2009 and the results are currently unpublished. News releases about the study reported that 24 of the 45 samples collected had antibiotic residues. Fifteen of the samples contained virginiamycin, 12 contained erythromycin, and five contained tylosin. The first survey conducted by the CVM used a multi-analyte method calibrated to only detect residues of virginiamycin, erythromycin, and tylosin (Olmstead, 2009; FDA, 2010b). The second survey was completed in 2012. The 2012 survey used the analytical method described by De Alwis and Heller to screen for 13 antibiotic residues (McChesney, 2009; De Alwis and Heller, 2010; Heller and De

Alwis, 2010). Of the total of 46 samples that were analyzed, three samples were determined to have detectable concentrations of erythromycin, virginiamycin, and penicillin. The first sample contained 0.58 ppm erythromycin. The second sample contained 0.24 ppm penicillin and 0.15 ppm virginiamycin. The final sample contained 0.16 ppm virginiamycin. Erythromycin had a detection limit of 0.5 ppm, penicillin had a detection limit of 1.0 ppm, and virginiamycin had a detection limit of 0.1 ppm (Luther, 2012). Although the second sample contained penicillin below the detection limit, the laboratory was able to accurately quantify it.

Mycotoxins and Their Effect on Antibiotics

Mycotoxins are toxic compounds produced by fungi and are undesirable contaminants of corn. Common contaminating mycotoxins include aflatoxins, deoxynivalenol (DON), fumonisins, T-2 toxins, and zearalenone. Mycotoxins found on corn can be especially detrimental to DG because the process of fermenting corn to ethanol results in DG containing approximately three time the amount of toxins as the original grain. It is the responsibility of the FDA to set regulatory concentrations and to monitor the concentrations of mycotoxins in feed ingredients (Zhang et al., 2009).

Currently little research has been conducted on the interactions between mycotoxins and antibiotics. However, aflatoxins have been reported to have antimicrobial effects. Studies have shown that although *Escherichia coli* is unaffected by aflatoxins, other bacteria, such as stains belonging to the *Actinomycetaceae* family, are sensitive. It

has been suggested that other mycotoxins beyond aflatoxin may also possess antimicrobial properties (Arai et al., 1967).

Overall there has been no major link between antibiotic activity and mycotoxins. Thus, it is unlikely that the presence of antibiotic residues in distillers grains would be affected by the presence of mycotoxins in the product.

Executive Summary

Distillers grains are an co-product of dry grind ethanol production. The dry grind production process goes through the following steps: grinding, cooking, liquefaction, saccharification, fermentation, and distillation. These steps convert whole corn to ethanol and a variety of distillers grains products. Bacterial contamination during ethanol fermentation can be detrimental to the ethanol production process. Bacteria utilize nutrients that would otherwise be utilized for the production of ethanol. This results in reduced ethanol yields and stuck fermentations. In order to combat bacterial contamination, ethanol producers utilize antibiotics. Several antibiotics are utilized by ethanol producers to combat bacterial contamination. However, the FDA has issued a letter of no objection only for the antibiotic virginiamycin.

Several concerns about the use of antibiotics in ethanol production have been cited by the FDA. First, they are concerned those antibiotic residues may be present in distillers grains and that those residues could be transferred to animal tissue upon ingestion. Second, the FDA expressed concern that animal tissues containing antibiotic residues could be harmful to the humans consuming them. Finally, the FDA expressed

concern for the health of the animals consuming distillers grains containing antibiotic residues.

To date, there have been no published surveys on the concentration of antibiotic residues in distillers grains. Moreover, there is no information on whether or not antibiotics are inactivated during the ethanol production process. Thus, it is uncertain as to how the use of antibiotics in ethanol fermentation will impact both humans and animals.

Table 1. FDA-approved concentrations of animal drugs for use in finishing livestock feed.

	Erythromycin thiocyanate			Penicillin G procaine			Oxytetracycline hydrochloride			Tylosin phosphate			Virginiamycin		
	Min	Max	Unit	Min	Max	Unit	Min	Max	Unit	Min	Max	Unit	Min	Max	Unit
Cattle	37.0	37.0	mg/hd	NA[2]	NA	NA	75.0	75.0	mg/hd	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	64.7	g/ton	50.0	50.0	g/ton[1]	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] In combination with chlortetracycline at 100 g/ton of feed plus sulfathiazole at 100 g/ton of feed.
[2] Not approved to be fed.

Table 2. Calculated parts per million concentrations of animal drugs for use in finishing livestock feed.[1]

	Erythromycin thiocyanate			Penicillin G procaine			Oxytetracycline hydrochloride			Tylosin phosphate			Viginiamycin		
	Min	Max	Unit	Min	Max	Unit	Min	Max	Unit	Min	Max	Unit	Min	Max	Unit
Cattle	3.7	3.7	ppm	NA[2]	NA	NA	7.5	7.5	ppm	8.8	11.0	ppm	17.6	24.8	ppm
Chicken	5.1	20.4	ppm	2.6	55.1	ppm	11.0	55.1	ppm	4.4	55.1	ppm	5.5	16.5	ppm
Swine	10.2	71.4	ppm	55.1	55.1	ppm	11.0	55.1	ppm	11.0	110.2	ppm	5.5	11.0	ppm
Turkey	10.2	20.4	ppm	2.6	55.1	ppm	11.0	55.1	ppm	NA	NA	NA	11.0	22.1	ppm

[1] Data calculations based on average DMI for listed species during the finishing period of feeding and FDA-approved feeding levels for each animal drug (listed in Table 1).
[2] Not approved to be fed.

FATE AND BIOLOGICAL ACTIVITY OF ANTIBITIOTICS USED IN FUEL ETHANOL PRODUCTION

D. M. Paulus Compart*, A. DiCostanzo*, G. C. Shurson*, G. I. Crawford*, R. C. Fink†, F. Diez-Gonzalez†

*Department of Animal Science, University of Minnesota, St. Paul, MN 55108

†Department of Food Science, University of Minnesota, St. Paul, MN 55108

SUMMARY

Antibiotics are utilized in ethanol production to control deleterious bacteria from competing with yeast for nutrients during ethanol 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 to determine whether residues were biologically active. Twenty distillers wet grains and 20 distillers dried grains samples were collected quarterly from nine states and 43 ethanol plants in the United States. Samples were analyzed for DM, CP, NDF, crude fat, S, P, pH, and titratable acidity 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 an FDA-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 ($\leq$ 1.12 ppm) antibiotic concentrations. Only one sample extract inhibited growth of *E. coli* at 10^4 CFU/g, but this sample contained no detectable concentrations of

antibiotic residues. No extracts inhibited *L. 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 one 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, distillers grains, ethanol production, antimicrobial

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).

In order to combat bacterial contamination, ethanol plants add antibiotics in relatively low concentrations during the fermentation stage of ethanol production (Juranek and Duquette, 2007). Currently, virginiamycin is the only antibiotic for which the U.S. Food and Drug Administration (FDA) has issued a letter of no objection for use in ethanol production (Benz, 2007).

There is concern that antibiotics added to ethanol fermentation remain in the ethanol co-product, distillers grains (DG), a common ingredient used 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 bacterial populations such as potential pathogens for animals and humans (Benz, 2007). It has been hypothesized that residues from antibiotics added during ethanol fermentation remain in DG. However, it is likely that the high pH and

temperatures encountered during the production process 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 the concentrations of various antibiotic residues in DG and to 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 one yr from January, 2011 through December, 2011. However, a distillers wet grains sample was not collected from the second quarter leading to a collection of 80 DDG (> 65% DM) and 79 DWG (< 65% DM). Samples were obtained from nine U.S. states and represented 43 ethanol plants. Samples were collected by nutrition consultants from various locations in the United States. Although this collection procedure potentially could result in some inconsistencies, and duplication, it was chose to guarantee random and blinded (by production source) 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 one another; therefore, they were retained as an individual observation in the antibiotic residue concentration and biological activity analyses dataset. Nutrient content and pH values of duplicate samples were averaged in

order to determine the main effect of DG type on sample physicochemical properties. Distillers grains samples were analyzed for pH and nutrient concentrations to characterize them and to reflect that they were representative of typical DG available in the feed ingredient market in the U.S.

Collection, handling, and shipment of samples

In order to collect DG 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 one-kg of DWG or DDG samples by collecting nine 0.5-cup (114 g) sub-samples from various locations in a DG pile. Sub-sampling 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 sub-samples were to be added to a bucket, mixed thoroughly, and then placed in a plastic bag (3.78 L). Consultants were expected to identify samples with the sample collection date, sample collection site, and location where the DG originated. They were also provided with all of the materials for collecting samples, as well as a written sampling protocol. In addition, 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 the date of collection. The amount of DDG samples collected ranged from 200 to 1,500 g. Distillers dried grains samples were immediately mailed to the University of Minnesota, Saint Paul Campus upon collection, and DWG samples were kept frozen from collection to delivery.

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 or using a Proctor Silex handheld mixer (Hamilton Beach Brands Incorporated, Southern Pines, NC). Samples were then subdivided using a measuring cup into at least four subsamples of at least 50 g each. All subsamples were stored in zippered plastic freezer bags. Subsamples were either utilized for nutrient or antibiotic residue analysis or they were refrozen in a -20° C freezer. Samples were thawed only once for sub-sampling to minimize any chemical changes in their contents form the original form collected.

Two subsamples from each sample were mailed to SGS North America Incorporated (Brookings, SD) for determination of nutrient content, antibiotic residue analysis, and biological activity analysis. Samples were placed in one of two 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 (Brookings, SD) including: moisture, DM, CP, crude fat, NDF, P and S. Moisture was determined using NFTA method 2.2.2.5. Dry matter was determined via calculation from moisture content. Crude protein was determined using AOAC method

990.03, crude fat was determined using AOAC method 945.16, and NDF and ADF were determined using an Ankom fiber bag (Ankom Technology; Macedon, NY). All minerals were determined using AOAC methods 968.08 and 935.13 by inductively coupled plasma spectrometry (ICP).

pH

Samples were analyzed for pH and titratable acidity at the University of Minnesota. In order to emulate the rumen of cattle, samples were mixed with deionized water to create a 20% DM mixture. After mixing, initial pH was determined using a pH probe (Corning pH Meter 345, Corning, Lowell, MA). Samples were then titrated with 1 M NaOH (Mallinckrodt) to reach a pH of 7.0. Samples were manually mixed constantly as the NaOH was added. Total acid added to each sample and total deionized water content of the mixture were used to calculate the titratable acidity of the sample.

Antibiotic Residue Analysis

Samples were analyzed by SGS for the concentration of virginiamycin M1 subunit, erythromycin, tetracycline, tylosin, and penicillin G using liquid chromatography and ion trap tandem mass spectrometry (LCMS) according to the methods described in De Alwis and Heller (2010). This method was created by the FDA specifically for determining multi-antibiotic residue concentrations in DG and was used by SGS North America, Inc. in the current study. This laboratory adheres to internal quality control

standards and is audited by the Internal Organization of Standardization every three yr to ensure that quality control standards are being met.

Initially, five g of DDG or DWG sample were weighed on an as-fed basis and added to a mixture of 1.5 mM ethylenediaminetetraacetic acid disodium salt (EDTA) and 1% trichloroacetic acid (TCA). The mixtures were shaken for 15 min, followed by 10 min of centrifugation at 4000 x g (Eppendorf Centrifuge 5810R, Hamburg, Germany). The supernatants were removed and diluted to 150 mL with water. Sample pellets were then extracted for 15 min with methanol. Samples were again centrifuged and the resulting supernatants were removed and added to the first extracts. The combined supernatant extract was diluted to 200 mL with water and mixed well. Aliquots of 10 mL were obtained from the extract mixture for use with solid phase extraction (SPE, De Alwis and Heller, 2010).

Solid phase extraction (SPE) columns (Oasis HLB, Waters, Milford, MA) were placed on a vacuum manifold and conditioned with methanol. 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. Samples 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 about one mL under the flow of nitrogen (N-Evap III, Organomation Associates, Inc., Berlin, MA). Diluent [87:13 (v/v) water:acetonitrile] was used to reconstitute the extracts to two mL. Lastly, the extracts

were centrifuged and added to an autosampler vial for analysis (De Alwis and Heller, 2010).

Autosampler vials were loaded into and analyzed by liquid chromatography (Dionex, Thermo Fischer Scientific, Minneapolis, MN) and ion trap tandem mass spectrometer (AB Sciex 4000 QTrap, Framingham, MA). Residue concentrations were determined via the ion trap tandem mass spectrometer software and reported in ppm. Values were adjusted from an as-fed basis to a DM basis after determination. The mass spectrometer used in this study had a minimum detection limit of 0.05 ppm for penicillin G, erythromycin, tylosin, and tetracycline, and virginiamycin (De Alwis and Heller, 2010). Limit of quantification, system suitability, and development and implementation of standard curves were as defined by FDA. Briefly, standard injections peaks were required to meet a signal-to-noise ratio of >3:1 low noise peak shapes. Blank controls were required to have no positive identification of any compound. Quality Control samples must meet screening/confirmation criteria as described below. All standard concentrations must be within 20% of target concentration or that standard was excluded, and limit of quantification (LOQ) adjusted. Quality control standards were required to be within 15% from target value. Limit of quantification was set > 3 fold greater than observed carryover in control extracts. Unknown sample peaks were required to have low noise and meet a signal-to-noise ratio of >3:1. Retention time of unknown sample was required to be within 2.5% of standard retention time; penicillin was required to be within 1% of standard retention time. Ion ratios must be at expected relative abundance percentages. Additionally, for quantitative acceptability, the sample value must be above

the LOQ. If the unknown sample is above the LOQ, but the linear regression of the standard curve has a $R^2 < 0.99$, it was re-analyzed.

Six standards (to develop a standard curve) and one quality control sample (internal standard) were analyzed with each set of 12 DG samples. About 16 mg of each antibiotic were dissolved in either water (erythromycin, penicillin G, and tylosin) or methanol (tetracycline and virginiamycin) to make 1,500 g/mL antibiotic stock 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 making up the volume with water. Intermediate standards were prepared by serially diluting the mixed standard with water to create standards ranging from zero to 50 µg/mL.

Working standards were prepared by weighing five g of a control DG (corn dried DG 08102, AAFCO, Champaign, IL), which was previously shown to contain no antibiotics, into a centrifuge tube and adding 500 µL of corresponding intermediate standard. Both 1.5 mM EDTA and 1% TCA were added to the control DG standard. Standards were shaken for 15 min and then centrifuged three min. Solution was collected and 30 mL methanol was added. Samples were shaken for 15 min and after shaking, standards were diluted to 175 mL with water. Using a scale, standards were further diluted to 190.2 g and shaken. Ten mL of standard were then collected for SPE extraction. The quality control sample was prepared using the same procedure as the working standards; however, 400 µL of 10 µg/mL standard was added to the control DG.

Limit of quantification, system suitability, and development and implementation of standard curves were as defined by FDA. Briefly, standard injections peaks were

required to meet a signal-to-noise ratio of >3:1 low noise peak shapes. Blank controls were required to have no positive identification of any compound. Quality Control samples must meet screening/confirmation criteria as described below. All standard concentrations must be within 20% of target concentration or that standard was excluded, and limit of quantification (LOQ) adjusted. Quality control standards were required to be within 15% from target value. Limit of quantification was set > 3 fold greater than observed carryover in control extracts. Unknown sample peaks were required to have low noise and meet a signal-to-noise ratio of >3:1. Retention time of unknown sample was required to be within 2.5% of standard retention time; penicillin was required to be within 1% of standard retention time. Ion ratios must be at expected relative abundance percentages. Additionally, for quantitative acceptability, the sample value must be above the LOQ. If the unknown sample is above the LOQ, but the linear regression of the standard curve has a $R^2 < 0.99$, it was re-analyzed.

A secondary set of residues was extracted using the above methods with the exception of phosphate buffered saline (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 utilized for microbiological procedures.

FDA-approved Virginiamycin Assay

Samples were also analyzed by Phibro EPG (Saint Paul, MN) 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. In contrast, liquid chromatography and ion trap tandem mass spectrometry can only detect the M component of virginiamycin. As a result, 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. The virginiamycin detected by LCMS could be inactive since both the M and S component are required for optimal antibiotic activity. Additionally, the method employed by Phibro EPG uses a biological assay to detect virginiamycin residues active against a specific bacterium. This differs from LCMS because it detects activity, not the presence of virginiamycin molecules. Initially, samples were extracted in a Soxhlet apparatus with a polar solvent and non-polar solvent. After extraction, the solvent was evaporated. This step was followed by a series of successive solvent extractions and evaporations, a de-fatting and de-waxing step, and by a solid phase concentration. Next, a methanol and phosphate buffer was utilized 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 rhizophilia*. Excess antibiotic spilled out of the wells creating a concentration gradient. The wells were incubated at 37° C for a pre-determined period of time. 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. The zone of inhibition was

directly proportional to the logarithm of the antibiotic concentration. The detection limit for this procedure was 0.3 ppm 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 plates and with all sample plates. The standards were made up of plates with six wells each. Three of the wells in each plate contained either one of the four calibration standards or a DG sample extract. The final three wells contained the reference standard. All antibiotic concentrations were based on the calibration standards and were 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. (Brookings, SD) 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. It has been noted in previous studies that methanol can affect the growth of some strains of the sentinel bacteria utilized in this study (Fried and Novick, 1979). Initially, the extracts were centrifuged and supernatants were filter sterilized using a 0.22 μm filter. In order to determine if antibiotic extracts were biologically active, they were exposed to 10^4 to 10^7 CFU/ mL of bacteria chosen as sentinel for this study: *Escherichia coli* ATCC 8739 and *Listeria monocytogenes* ATCC 19115.

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 and *L. monocytogenes* and tetracycline and *E. coli* (Wikler et al., 2007). *L. monocytogenes* ATCC 19115 was found to be susceptible to all five antibiotics under consideration. *E. coli* ATCC 8739 was found to be susceptible to all of the antibiotics except penicillin G. Penicillin G, erythromycin, and tetracycline produced 28, 28, and 29 mm zones of inhibition respectively when exposed to *L. monocytogenes*. Erythromycin and tetracycline produced 14 and 23 mm zones of inhibition, respectively, when exposed to *E. coli*. The MIC for tylosin was four μg/mL when exposed to *E. coli* ATCC 8739 and 125 μg/mL when exposed to *L. monocytogenes* ATCC 19115. The MIC for virginiamycin was 31 μg/mL when exposed to *E. coli* ATCC 8739 and 125 μg/mL when exposed to *L. monocytogenes* ATCC 19115.

Antibiotic extracts were mixed 1:1 with serially diluted 10^4 to 10^7 colony forming units (CFU) per mL liquid cultures of the individual sentinel bacteria in macro-dilution tubes. In this study, the antibiotic concentration remained constant and the bacterial counts were varied. This method is the opposite of a typical minimum inhibitory concentration (MIC) determination, and it determines the bacterial threshold of the antibiotic (Lambert and Pearson, 2001; Wiegand et al., 2008). The bacterial threshold is the concentration of bacteria required to eliminate bacterial inhibition by the antibiotic. This method differs from MIC because the bacterial concentration varies. The MIC is a measure of the concentration of antibiotic required to inhibit bacterial growth. When

determining an MIC, the bacterial concentration remains constant and the antibiotic concentration varies. Bacterial threshold was utilized in this study because the antibiotic residue concentration does not vary in DG, but the bacterial concentration that the residues encounter in the environment does. Thus, bacterial threshold more accurately represents the interaction with 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 μL 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 above procedure.

Statistical analysis of data

Nutrient profiles, pH, and titratable acidity concentrations 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 of SAS 9.2 (SAS Institute, Inc., Cary, NC). Main effects were DG type (wet or dry), DG ethanol plant of origin (EP), and the two-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 x 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, 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 was no repetition of EP x type. Moreover, 31 samples were duplicated by period, DG type, and ethanol plant of origin. These samples were averaged with their counterpart and analyzed as one sample. In total, 116 samples were analyzed. In order to determine the effect of period, an additional model was created using the MIXED procedure of SAS with the main effect of period and no random statement. Due to low incidence of antibiotic residues only descriptive statistics are reported.

RESULTS

A total of 159 samples were collected and examined in this study. Eighty of the samples were distillers dried grains (> 65% DM; DDG) and 79 were DWG (< 65% DM; Table 1 and 2). Sample physiochemical characteristics are shown in Tables 3 to 5. Crude protein ranged from 26 to 43%. Crude fat ranged from 5 to 14%. Neutral detergent fiber ranged from 26 to 47%. Phosphorus ranged from 0.44 to 1.27% and sulfur ranged from 0.30 to 1.08% across dry and wet DG samples.

Antibiotic Residues and Biological Activity

On a DM basis, a total of 3.8% of the DWG samples (n = 79) and 21.3% of DDG samples (n = 80) contained antibiotic residues (Figures 2 and 3). Although the M1 sub-unit of virginiamycin was quantified by the LCMS 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 only one sub-unit as is the case for the LCMS procedure, samples were measured for their biologically activity to inhibit growth of *K. rhizophila*. Two samples contained virginiamycin, the one sample contained of 0.6 ppm, and the second sample contained 0.5 ppm. Both samples were from DDG sources, from the same ethanol plant, and collected during the first quarter.

Results of the microbiological assays revealed that of the 159 samples, one sample inhibited the growth of *E. coli* ATCC 8739 at 10^4 CFU/mL. 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 the distillers grains produced in the U. S. (Rasco et al., 1987; NRC, 1996; Spiehs et al., 2002; Pedersen et al., 2007; Tangendjaja, 2007; Waldroup et al., 2007; Loy, 2008; Robinson et al., 2008; Bhadra et al., 2010; Buckner et al., 2011).

Antibiotic residues were found in 12.6% of the DG samples in this study with the majority (10.7% of the total) present in DDG samples. This observation may simply be an artifact resulting from conducting LCMS analyses in feeds varying in moisture concentration.

Preliminary results (unpublished) of research conducted by the FDA in 2010 revealed that 53% of DG samples contained antibiotic residues (FDA, 2010b). However, their study only examined samples for the presence of erythromycin, virginiamycin, and tylosin. In a 2012 FDA survey (unpublished), three out of 46 samples (6.5%) were found to contain detectable antibiotic residues. One sample contained virginiamycin M1 residues at concentrations of 0.16 ppm. The second sample contained erythromycin residues at a concentration of 0.58 ppm. The third sample contained virginiamycin M1 and penicillin G residues at concentrations of 0.15 and 0.24 ppm respectively (Luther, 2012). These concentrations are within the range (0 to 1.12 ppm) found in this study.

Concentrations of antibiotics residues in DG samples were low. Concentrations were lower than current FDA-approved antibiotic concentrations for finishing livestock and poultry use (Tables 8 and 9). It is important to note that penicillin is not approved for feeding in cattle (Tables 8 and 9). For a complete list of approved antibiotics for each class of domestic livestock and poultry, the reader is referred to Animal Drugs @ FDA (http://www.accessdata.fda.gov/scripts/animaldrugsatfda/). 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. The illness and death were the result of drug interactions between

monensin and DDG containing erythromycin. However, the DDG that caused death in those cattle (Basaraba et al., 1999) contained erythromycin concentrations that were several orders of magnitude higher (50 to 1,500 ppm) than erythromycin concentrations found in the current study. This report caused concerns about drug interactions resulting from the feeding of DG to livestock and poultry. Several of the 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 (Merck, 2004). Currently, the only report of negative DG antibiotic residues and therapeutic or growth-promoting antibiotic administration interactions is the one discussed above (Basaraba et al., 1999). The lack of additional reports of antibiotic residues affecting livestock or poultry suggests that the concentration of antibiotics in DG is generally too low to result in detrimental drug interactions.

Antibiotic residue concentrations observed in the current or FDA studies in DG appear to be at concentrations generally considered to be sublethal or may indeed be inactive. When exposed to bacteria in culture, antibiotic residues in were unable to inhibit bacterial growth except in three samples. Two were inhibitory of *K. rhizophilia,* indicative of virginiamycin, and one sample negative for antibiotic screened in this study demonstrated inhibition to the sentinel strain of *E. coli* chosen for this study. Presence of other microbial growth-inhibiting compounds including nutrient excesses or deficiencies

present in this sample or natural antimicrobials in the environment cannot be discounted by this procedure.

It is possible that the lack of inhibition in all other antibiotic-positive samples was due to antibiotic inactivation. Penicillin, erythromycin, and tylosin all degrade at the pH (< 4.0) reached during ethanol fermentation (Aksenova et al., 1984; Brisaert et al., 1996; Islam et al., 1998;). Moreover, penicillin, tetracycline, tylosin, and virginiamycin are degraded by the high temperatures (< 200 °C) reached during ethanol fermentation, distillation, and drying (Brisaert et al., 1996; Hynes et al., 1997; Kheirolomoom et al., 1998; Juranek and Duquette, 2007; Wang et al., 2008). Therefore, it is likely that under certain as yet uncharacterized conditions, all of the antibiotics examined in this study would degrade and become inactive during the production of DG due to the pH and temperatures they encounter. Because some antimicrobial activity remained in three samples, under other as yet unknown conditions, virginiamycin may not be degraded during the DG production process. Once produced, DG do not have measurable concentrations of bacteria (Pedersen et al., 2003; 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., 2003). Wet distillers grains have been found to have bacterial counts of 10^7 to 10^8 cells/ g dry mass within nine days of production (Lehman and Rosentrater, 2007). This suggests that bacteria in DG come from the environment and not from 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. Results from a study by Jacobs et al. (2008b) demonstrated the presence of monensin- and tylosin-resistant *Enterococcus* species in DG. A study by Martins da Costa et al. (2007) examined *Enterococcus* isolates in cereal co-products (corn gluten feed and DDGS) and found isolates resistant to tetracycline, erythromycin, rifampicin, gentamicin, chloramphenicol, nitrofurantoin, and ciprofloxacin. However, there is currently no link between antibiotic residues in DG and resistant bacteria. 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 made between the use of DG in cattle or swine diets and the development of bacterial resistance in the gastrointestinal tract of those species (Jacob et al., 2008b; Edrington et al., 2010). However, it has been found that swine naturally contain resistant bacteria, and the concentration of resistant bacteria increases in swine with the use of antibiotics (Sunde et al., 1998). Thus, there is risk for the concentration of resistant bacteria to increase in the swine gastrointestinal tract if active antibiotic residues are present in DG. In cattle, it has been found that adding DG to the diet can promote the growth of *E. coli* O157 (Jacob et al., 2008a). Additionally, rumen bacterial concentrations do not differ with the addition of DG to the cattle diet, but the populations can shift (Fron et al., 1995; Callaway et al., 2010). Thus, the risk of cattle developing bacterial antibiotic resistance is of little concern.

The presence of large bacterial loads in wet DG after several days of storage provides an opportunity for bacterial resistance to develop, and past research has shown that it can (Martins da Costa et al., 2007; Jacob et al., 2008b). When bacteria are exposed to antibiotics, antibiotic resistance can develop due to mutations and horizontal gene transfer (Bester and Essack, 2010; Kohanski et al., 2010). Furthermore, bacterial cross-resistance, the ability of bacteria to resist antibiotics they have not been exposed to, can develop (Kohanski et al., 2010). On the other hand, when bacteria are exposed to the degradation products of antibiotics, created during antibiotic inactivation, the opposite can occur. Antibiotic degradation products can actually select for non-resistant bacteria (Palmer et al., 2010). These two phenomena may explain why resistant and non-resistant bacteria co-exist. Thus, if antibiotic residues are degraded during the production of DG, their degradation products in DG could potentially promote the growth of non-resistant bacteria and help reduce the resistant bacteria populations.

If resistant bacteria were to develop in DG, those bacteria would have to make it through several barriers in order to become problematic to the human population. In the case of DG, the antibiotic would have to first end up in ethanol fermentation. That antibiotic would then have to be present and active in DG. Resistant genes would have to develop in bacteria present in the DG. Then the DG would have to be fed to animals. If no resistant genes developed in the DG prior to animal consumption, bacteria in the animal gastrointestinal tract would have to develop them. That resistant gene would have to leave the farm. The resistant gene would then have to remain on the animal carcass after harvest and survive on retail meat. The consumer would have to mishandle the

product and consume it. The consumer would become ill. Finally, the consumer would be treated with an antibiotic and the antibiotic would fail. Hurd et al. (2004) broke down this model from the administration of antibiotics to livestock to the failure of antibiotics in humans. He found that the risk of illness due to macrolide-resistant campylobacteriosis as the result of administering tylosin to swine was just less than 1 in 10 million for all meat commodities combines, 1 in 14 million for poultry, 1 in 53 million for beef, and 1 in 236 million for swine. 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.

Antibiotics are in abundance in the environment. They are present in soils, and are incorporated into soils after fertilization with manure from animals fed antibiotics (Jacobsen et al., 2004; Grote et al., 2007). They are also naturally produced by soil fauna (Riesenfeld et al., 2004; D'Costa et al., 2007). These antibiotics can then end up in the plants grown in those soils (Dolliver et al., 2007; Grote et al., 2007). Antibiotics are also present in the water supply, and they are heavily used in human medicine (Bester and Essack, 2010; Ma et al., 2011). Antibiotic resistance development is frequently due to the misuse of antibiotics in human health care (Bester and Essack, 2010). Poor water treatment can produce antibiotic resistant bacteria (Ma et al., 2011). Overall, bacteria develop antibiotic resistance in a wide variety of settings. Although there is some health risk to humans as a result of the use of antibiotics in ethanol production, several other

external factors pose a much greater threat to human health than antibiotic residues in DG.

CONCLUSIONS

Distillers grains contain antibiotic residues infrequently and at low concentrations. Moreover, the frequency of biologically active antibiotic residues in DG is extremely low (1.9%). These findings revealed that the DG production process inactivates the five antibiotic residues examined in this study. Likely, pH and temperatures encountered during DG production lead to elimination and inactivation of DG. However, when, in spite of a potentially negative environment encountered during DG production process, a low proportion of samples may retain antibiotic residues that are biologically active at sublethal concentrations. If they are biologically active at sublethal concentration, antibiotic residues in DG may lead bacteria to develop antibiotic resistance. However, if antibiotic residues do lead to the development of antibiotic resistance in bacteria, the health risk to humans is low relative to other external causes of bacterial resistance. More research is needed to determine if antibiotic residues in DG have biological activity in livestock and poultry species, and whether they contribute to the development bacterial resistance.

Table 1. Distribution of distillers grains samples analyzed for physiochemical properties by state-of-origin, collection period, and distillers grain type.

	Period							
	1		2		3		4	
DG Type	Dry	Wet	Dry	Wet	Dry	Wet	Dry	Wet
Iowa	6	5	5	3	5	3	3	6
Illinois	1	2	0	2	3	2	1	1
Indiana	0	1	1	0	2	0	1	0
Michigan	0	0	1	0	0	0	0	0
Minnesota	5	3	4	5	3	5	4	6
North Dakota	0	1	0	0	0	0	0	0
Nebraska	1	3	1	1	0	1	0	0
Ohio	0	0	0	0	1	0	0	0
South Dakota	2	3	2	3	2	3	1	2
Sample Total	15	18	14	14	16	14	10	15

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

	Period							
	1		2		3		4	
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

Table 3. Descriptive statistics of physiochemical properties of distillers dried grains samples on DM basis (n = 80).

	Mean	Standard Deviation	Coefficient of Variation	Minimum	Maximum
DM, %	90.01	1.32	1.0	86.49	93.29
CP, %	30.85	1.43	5.0	28.03	35.59
Crude Fat, %	11.53	1.35	12.0	7.33	13.44
NDF, %	31.49	3.85	12.0	25.98	47.01
P, %	0.87	0.15	17.0	0.44	1.15
S, %	0.64	0.18	28.0	0.30	1.08
pH	4.12	0.44	11.0	3.13	5.16
Titratable Acidity, g/L	4.50	3.17	70.0	1.82	25.03

Table 4. Descriptive statistics of physiochemical properties of distillers wet grains samples on DM basis (n = 79).

	Mean	Standard Deviation	Coefficient of Variation	Minimum	Maximum
DM, %	43.28	7.82	18.0	29.48	59.27
CP, %	31.00	2.62	8.0	26.07	42.58
Crude Fat, %	11.00	2.04	19.0	4.94	13.98
NDF, %	36.12	3.23	9.0	28.33	46.12
P, %	0.91	0.19	21.0	0.46	1.27
S, %	0.61	0.15	25.0	0.32	0.92
pH	4.02	0.39	10.0	3.22	5.17
Titratable Acidity, g/L	13.25	8.18	0.62	4.52	51.67

Table 5. Main effects of distillers grains type on physicochemical properties of distillers grains samples on DM basis.

	Treatment[1]			*P*-values[2]				
	DDG	DWG	SEM[3]	Type	Period	EP	Period*Type	Period*EP
DM, %	89.95	44.29	1.54	< 0.0001	0.07	0.81	0.85	0.12
CP, %	31.38	31.23	0.36	0.61	0.01	< 0.0001	0.85	0.54
Crude Fat, %	10.90	11.30	0.25	0.28	0.002	< 0.0001	0.04	0.01
NDF, %	31.76	36.01	0.60	< 0.0001	< 0.0001	0.04	< 0.0001	0.03
P, %	0.86	0.91	0.05	0.20	< 0.0001	0.05	0.61	0.79
S, %	0.64	0.63	0.05	0.78	< 0.0001	0.0003	1.00	0.77
pH	4.11	3.96	0.08	0.05	0.03	< 0.0001	0.42	0.60
Titratable Acidity, g/L	4.58	12.85	0.97	< 0.0001	0.86	0.08	0.41	0.88

[1]Treatments included dry distillers grains (DDG) or wet distillers grains (DWG)
[2]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 considered significant.
[3]Highest standard error of the mean reported.

Table 6. Descriptive statistics of antibiotic residue concentrations in distillers dried grains determined via liquid chromatography ion trap tandem mass spectrometry and reported on DM basis.

	Mean	Standard Deviation	Minimum	Maximum
Erythromycin, ppm	0.063	0.172	0.000	0.867
Penicillin G, ppm	0.001	0.012	0.000	0.107
Tetracycline, ppm	0.000	0.000	0.000	0.000
Tylosin, ppm	0.000	0.000	0.000	0.000

Table 7. Descriptive statistics of antibiotic residue concentrations in distillers wet grains determined via liquid chromatography ion trap tandem mass spectrometry and reported on DM basis.

	Mean	Standard Deviation	Minimum	Maximum
Erythromycin, ppm	0.009	0.055	0.000	0.356
Penicillin G, ppm	0.000	0.000	0.0000	0.000
Tetracycline, ppm	0.014	0.126	0.000	1.123
Tylosin, ppm	0.000	0.000	0.0000	0.000

Table 8. FDA-approved concentrations of animal drugs for use in finishing livestock and poultry feed.

	Erythromycin thiocyanate			Penicillin G procaine			Oxytetracycline hydrochloride			Tylosin phosphate			Virginiamycin		
	Min	Max	Unit	Min	Max	Unit	Min	Max	Unit	Min	Max	Unit	Min	Max	Unit
Cattle	37.0	37.0	mg/hd	NA[2]	NA	NA	75.0	75.0	mg/hd	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[1]	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]In combination with chlortetracycline at 100 g/ton of feed plus sulfathiazole at 100 g/ton of feed
[2] Not approved to be fed.

Table 9. Calculated parts per million concentrations of animal drugs for use in finishing livestock and poultry feed.[1]

	Erythromycin thiocyanate			Penicillin G procaine			Oxytetracycline hydrochloride			Tylosin phosphate			Viginiamycin		
	Min	Max	Unit	Min	Max	Unit	Min	Max	Unit	Min	Max	Unit	Min	Max	Unit
Cattle	3.7	3.7	ppm	NA[2]	NA	NA	7.5	7.5	ppm	8.8	11.0	ppm	17.6	24.8	ppm
Chicken	5.1	20.4	ppm	2.6	55.1	ppm	11.0	55.1	ppm	4.4	55.1	ppm	5.5	16.5	ppm
Swine	10.2	71.4	ppm	55.1	55.1	ppm	11.0	55.1	ppm	11.0	110.2	ppm	5.5	11.0	ppm
Turkey	10.2	20.4	ppm	2.6	55.1	ppm	11.0	55.1	ppm	NA	NA	NA	11.0	22.1	ppm

[1]Data calculations based on average DMI for listed species during the finishing period of feeding and FDA-approved feeding levels for each animal drug (listed in Table 7).
[2] Not approved to be fed.

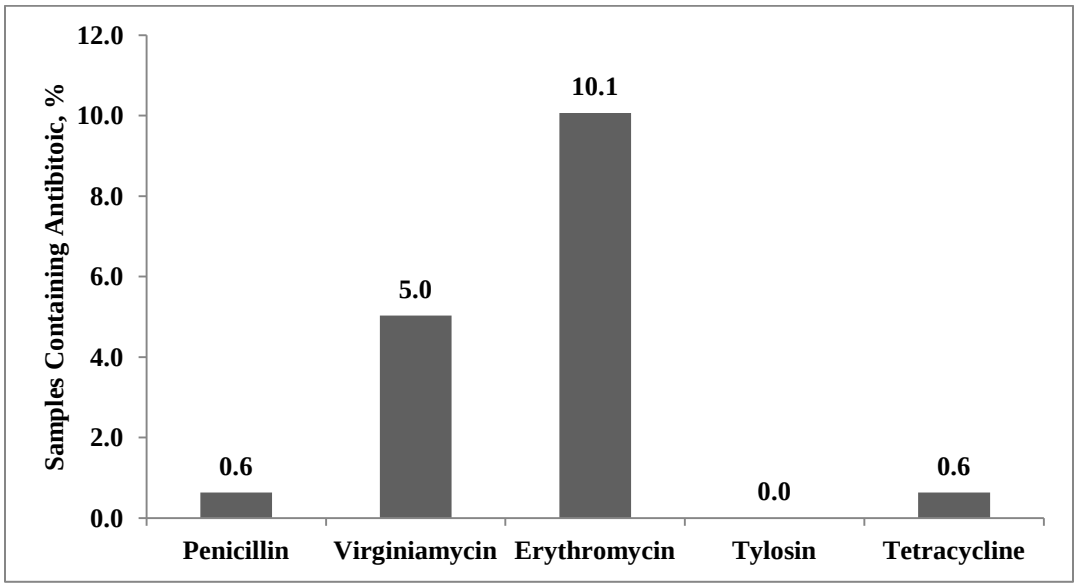

Figure 1. Percentage of both dried and wet distillers grains containing antibiotic residues determined via liquid chromatography ion trap tandem mass spectrometry and an FDA-approved assay.

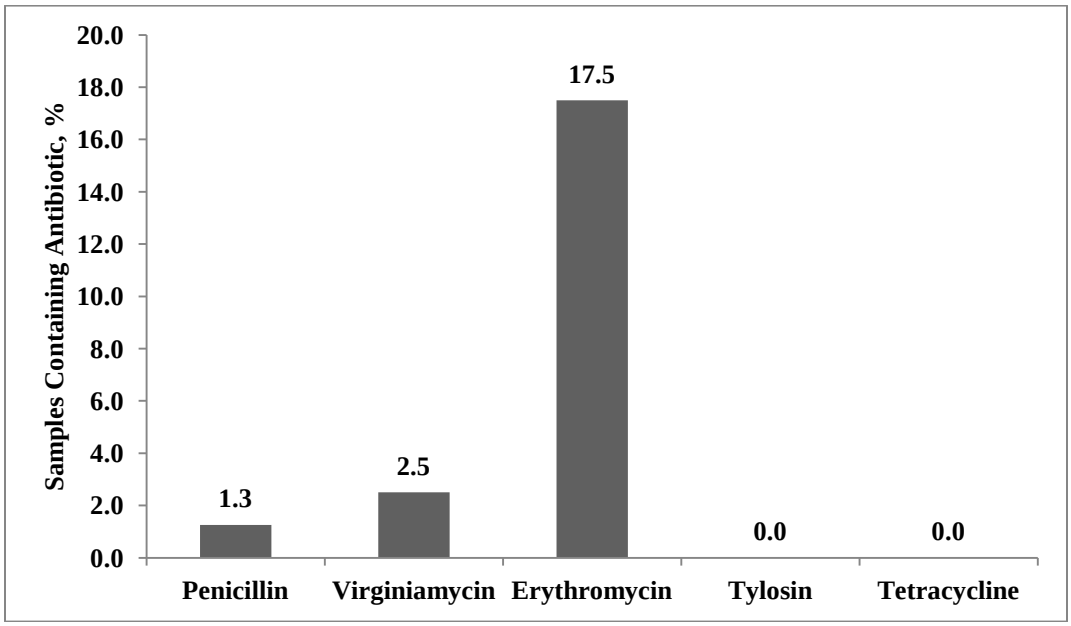

Figure 2. Percentage of distillers dried grains containing antibiotic residues determined via liquid chromatography ion trap tandem mass spectrometry and an FDA-approved assay.

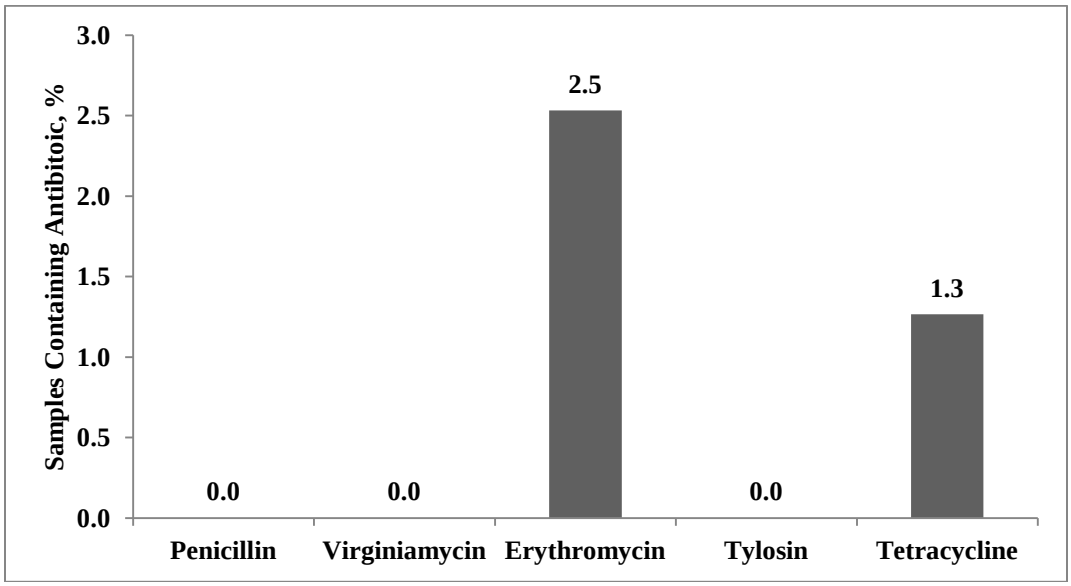

Figure 3. Percentage of distillers wet grains containing antibiotic residues determined via liquid chromatography ion trap tandem mass spectrometry and an FDA-approved assay.

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