Project Title: Antimicrobial Resistance (AMR) Risk Assessment Data Gaps: AMR in Feces of Culled Market Cows and Bulls and Retail Meat Products from Animals Raised with and without Antibiotics

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Background

Food animal production has come under increased scrutiny for administration of antimicrobial drugs (AMD) for the treatment and prevention of disease in livestock due to the concern that this practice could increase the risk of antimicrobial resistance (AMR) in human health settings. While several published studies have demonstrated that AMD use has small to no effect on AMR levels in fed beef cattle, data on AMR for cull market cows is limited. This data gap needs to be addressed as approximately 19% of U.S. beef is sourced from cull market cattle. Three approaches (culture methods, quantitative PCR (qPCR), and shotgun metagenomics) were used to detect, compare, and characterize AMR. The combining of these three approaches produces more comprehensive data than using any one approach alone.

When complete, data will yield the best possible assessment of the impact of AMD use on the occurrence of AMR in cull cow colon content, on chilled cull cow carcasses, and beef trimmings derived from carcasses in the chilling cooler and from the fabrication of cull cow carcasses.

The objective of this study was to characterize and determine the extent of AMR in colon content and beef trimmings (collected from carcasses of culled cows during both chilling and fabrication) using culture, qPCR, and metagenomic tools.

Methodology

Two commercial processing plants were each visited 3 times for a total of 6 plant visits. Samples were collected from carcasses randomly identified in three treatment groups at each plant: i.) cull cows from organic dairies (hereafter termed “Organic”); ii.) cull cows from nonorganic dairies (hereafter termed “Dairy”); and iii.) cull cows from cattle cow- calf operations that supply beef feedlots with calves (hereafter termed “Beef”). For culture and qPCR approaches, the goal for each of the 6 plant visits was to obtain 30 colon content samples and 30 chilled carcass surface sponge swab samples from each of the 3 production systems (Beef, Dairy, Organic) for a total of 540 colon content samples and 540 chilled carcass surface sponge swab samples. The 535 colon samples and 534 chilled carcass surface sponge swab samples obtained deviated slightly from these goals because limited numbers of Dairy or Organic cattle available for sampling during some

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of the visits. For the metagenomic sequencing approach, the goal for each of the 6 visits was to obtain 3 composite colon samples and 3 composite trimmings derived from the chilling cooler from each three production systems (Beef, Dairy, Organic) for a total of 54 colon samples and 54 trimming samples. Additionally, one of the commercial processing plants fabricated Organic carcasses separately from Beef and Dairy carcasses. During each of the three visits to this plant, beef trimmings derived from the fabrication floor were collected from Organic and "Conventional" (since the plant fabricated Beef and non-organic Dairy carcasses together) treatment groups. During each of the 3 plant visits, 10 Organic and 10 Conventional samples of trimmings derived from fabrication were obtained for a total of 30 Organic and 30 Conventional samples to be used for culture and qPCR approaches. For metagenomic sequencing, during each of the 3 plant visits, 3 Organic and 3 Conventional samples from trimmings derived from fabrication were obtained for a total of 9 Organic and 9 Conventional samples.

Each colon, chilled carcass surface sponge swab sample, and beef trimmings derived from fabrication sample was cultured for the detection and quantification of the following bacterial taxa: generic *Escherichia coli*, tetracycline- resistant (TETr) *E. coli*, 3rd-generation cephalosporin resistant (3GCr) *E. coli*, generic *Salmonella enterica*, 3GCr *Salmonella*, generic *Enterococcus* spp., erythromycin-resistant (ERYr) *Enterococcus*, and vancomycin-resistant (VANr) *Enterococcus*. Additionally, each sample was cultured for the detection of methicillinresistant *Staphylococcus aureus* (MRSA). Bacterial detection rates were analyzed using the Generalized Linear Model Fit. Bacterial concentrations were analyzed using a two-way Analysis of Variance (ANOVA) test and compared using a Student's t test of least squares means differences.

The qPCR analysis of colon, chilled carcass surface sponge swab sample, and beef trimmings derived from fabrication sample to determine the presence and quantity of 10 antimicrobial resistance genes (ARGs) will begin after the retail ground beef qPCR included in this funding is complete.

For shotgun metagenomic and 16S sequencing samples, DNA was extracted directly from colon content and from rinsates of trimming derived from the chilling cooler and fabrication. Once DNA was extracted, the DNA was aliquoted into two separate library preparation methods, 16S and shotgun metagenomics. For 16S sequencing, standard library preparation was conducted and the V4 region of the 16S rRNA gene was sequenced. For shotgun metagenomic samples, library preparation was performed to decrease background DNA to gain a higher resolution of AMR genes; after this, libraries were sequenced. For 16S, alpha and beta diversity were assessed at the phylum and genus level was well as individual phylum and genus differences. For shotgun metagenomics, alpha and beta diversity was determined for AMR genes at the class level.

Findings

Culture.
Colon Content. Mean tetracycline-resistant (TETr) *E. coli* concentrations were impacted by production system ($P = 0.02$), but the differences (Organic = 4.92 $\log_{10}$ CFU/g, Beef = 5.08 $\log_{10}$ CFU/g, Dairy = 5.28 $\log_{10}$ CFU/g) were not biologically meaningful since their means differed by less than 0.5 $\log_{10}$ CFU/g. Furthermore, harvest location had a greater impact $P < 0.01$) on mean TETr *E. coli* concentrations. Production system impacted ($P < 0.01$) detection of 3rd-generation cephalosporin resistant (3GCr) *E. coli* (Organic = 48.3% Beef = 52.2%, Dairy = 67.6%). 3GCr *Salmonella* detection was low (Organic = 0.0%, Beef = 0.6%, Dairy = 1.7%). ERYr *Enterococcus* was more frequently ($P < 0.01$) detected in cull dairy cattle (Organic = 73.9%, Dairy = 75.4%) than in cull beef cattle (Beef = 56.7%). Vancomycin-resistant (VANr) *Enterococcus* results are pending. Methicillin-resistant *Staphylococcus aureus* (MRSA) was not detected in any

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sample.

Carcass swabs. TETr E. coli was detected on 6.7% of carcasses and detection was not impacted by production system (P = 0.61). 3GCr *E. coli* and 3GCr *Salmonella* were not detected. ERYr *Enterococcus* was detected on 0.7% of carcasses. VANr *Enterococcus* was not detected on any carcass. MRSA was not detected on any carcass.

Trimmings derived from fabrication. TETr *E. coli* was detected in 20.0% of samples but production system (P = 0.61) did not impact detection. 3GCr *E. coli* and 3GCr *Salmonella* were not detected. ERYr *Enterococcus* was detected in 1.7% of samples. VANr *Enterococcus* was not detected in any sample. MRSA was not detected in any sample.

Sequencing.
Resistome. In the chilling cooler, 89 gene accessions were identified, with resistance to tetracycline the most abundant (61%), followed by multi-drug resistance (21%) and beta-lactam resistance (13%). The production system cattle were raised in did not have an effect (P = 0.43) on overall composition of the meat resistome; however, harvest location did drive resistome differences (P = 0.001). In colon contents, 626 unique gene accessions were identified that could be classified as AMR genes. The most common classes of resistance included tetracycline (44%), multi-drug (36%), and aminoglycoside (9%). No colon content resistome differences were found between the production systems (P = 0.33) or the harvest location (P = 0.17). For a comparison through production, all treatments and locations were pooled and compared across colon content, trimmings derived from the chilling coolers and trimmings derived from the fabrication floor. The number of unique AMR genes found in colon content (richness) was higher (P < 0.05) than from trimmings obtained from carcasses during chilling and fabrication.

Microbiome. In the trimmings derived from the chilling cooler, the meat microbiome, alpha diversity did not differ by type of production system (P = 0.52) or harvest location (P = 0.09). Beta diversity did not differ (P = 0.66) between type of production system but was different (P = 0.01) between regions of harvest. For the colon content microbiome, alpha diversity did not differ by type of production system (P = 0.69) or region of harvest (P = 0.52). Beta diversity in colon content did not differ (P < 0.05) by region or production system.

Implications

These results provide a strong basis for a conclusion that cull cow production system has a minimal impact on AMR in colon content and no impact on AMR presence on carcasses and in trimmings derived from carcasses during either chilling or fabrication. The data does indicate that production system likely affects levels of a few specific antibiotic resistant bacteria and ARG. However, the magnitude of these specific impacts is small, and the overall broad colon content resistome is not impacted by production system. The impact of the few colon content AMR variations detected in this study on human exposure is likely small since the levels of AMR on carcasses and in trimmings derived from the chilling cooler and fabrication was very low. The low level of AMR detection on carcasses and in trimmings illustrates the effectiveness of plant sanitary interventions.

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