# Soil Microbial Communities and Antibiotic Resistance in Cattle Farms in the United States: The Importance of Soils in Protecting Environmental Health

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## Abstract

Cattle farming is a large and evolving industry in the United States (US) with potential health effects in workers, consumers, and individuals living in areas surrounding these operations. Because cattle farming inherently generates a large amount of manure, it is considered a major contributor to antibiotic resistant (AR) genes and bacteria in nearby environments. This study sought to quantify AR genes and pathogens in cattle-associated soils, as well as describe the associated microbial communities. Microbial communities and AR genes were compared in soils within and outside of cattle pens. Soil was sampled from seven cattle farms from different states in the United States. From each farm, one soil sample was taken from within the cattle pens and one sample was taken from outside of the pens. Following DNA extraction, bacterial communities were analyzed via 16S rRNA sequencing. Droplet Digital PCR and real-time PCR were used to quantify three enteric pathogens, three AR genes, and three host-specific microbial source tracking (MST) markers, which were used to identify sources of fecal pollution. The family *Peptostreptococcaceae* was more abundant in pen soils, and *Rhodanobacteraceae* was more abundant in non-pen soils. The marker for ruminant fecal contamination (*Rum2Bac*) was abundant both in pen and non-pen soils, with no significant difference. However, the concentration of the human fecal contamination marker (*HF183*) was

lower in pen soils than non-pen soils. Soils from within cattle pens had higher levels of the pathogens *Campylobacter* and *Salmonella* ($p<0.05$). Cattle pen soils also had higher concentrations of two AR genes ($p<0.05$), *Klebsiella pneumonia* Carbapenemase (*KPC*) and s*ul1*, which offers resistance to sulfonamide antibiotics. This is the first study identifying *KPC* in livestock-associated soils in the US, an AR gene of concern because it is clinically relevant and spreads easily between bacteria. This study supports increased hazard associated with livestock soils for animal and human health, as well as environmental quality.

**Introduction**

Antibiotic resistance (AR) is a global public health threat. While AR is ancient and ubiquitous, the recent increase is attributed to anthropogenic sources (Finley et al., 2013), particularly the overuse and misuse of antibiotics (Davies & Davies, 2010). AR infections are linked to increased mortality, longer hospital stays, and increased costs (Huijbers et al., 2016). The industries that are the largest contributors to AR are medicine and agriculture (Davies & Davies, 2010). Antibiotics are the most reported pharmaceutical used on beef and dairy farms (USDA, 2017). Furthermore, most of the antimicrobials that livestock consume are released unaltered in their feces (Elmund et al., 1971), where they can enter soils. Cattle produce large amounts of waste, which total between 59-80lbs of manure per day (USDA, 1995). As a result, the major nonpoint source of AR to the environment is livestock waste (Felis et al., 2020). AR genes themselves have also been identified from cattle feces (Vikram & Schmidt, 2018; Bonardi & Pitino, 2019). Cattle-associated soils are an important context to study AR.

Cattle-associated soils are also a matrix of concern for zoonotic pathogen transmission. These are typically bacteria from fecal pollution that cause gastrointestinal (GI) distress in humans, such as indigestion, vomiting, and diarrhea (McDaniel et al., 2014). Infections from

cattle farms have been reported both occupationally, through direct transmission to farm workers, veterinarians, and laboratory workers (Guan & Holley, 2003; Klous et al., 2016), as well as indirectly through run-off to surrounding communities (Hoar et al., 2001; Klous et al., 2016). Most infections from the pathogens that are commonly found in cattle-associated soils are mild (McDaniel, 2014). However, some pathogens like Shiga toxin-producing *Escherichia coli* (STEC), which includes E. *coli* O157:H7, can result in severe health effects (Jay-Russell, 2013). Cattle-associated soils are a matrix of interest for One Health, or at the intersection of animal health, human health, and environmental quality (USDA, 2016).

The purpose of this study was to characterize cattle-associated ('pen') soils and compare to control ('non-pen') soils. The two goals were to (1) describe the differences between pen and non-pen soil microbial communities, and (2) quantify differences in marker genes for enteric pathogens, total bacteria, AR genes, and a mobile genetic element (MGE).

**Methods**

Soil samples were collected from 7 dairy and beef farms in the United States (US) in different states (California (CA), Georgia (GA), Iowa (IA), Kentucky (KT), Nebraska (NE), Pennsylvania (PA), Tennessee (TN)). Two superficial soil samples were taken from each farm, one from within a cattle pen, where cattle were present, and one at least 500m from the pen where workers indicated that the cattle had not been recently. Soil samples were collected with clean metal spoons on days it was not raining, and the ground was not muddy.

DNA was extracted using the PowerSoil kit (Qiagen, Valencia, CA, US) per manufacturer's instructions. Quantity and quality of DNA were assessed using Nanodrop spectrophotometer (ThermoFisher, Waltham, MA, US). To analyze microbial communities, the V1-V3 regions of the 16S rRNA gene was amplified using primers 27F and 518R (Ransom-

Jones et al., 2017) and sequenced via Illumina Miseq (Illumina, San Diego, CA, US). Initial sequencing and analyses were completed by Chunlab, Inc. (Seoul, Korea). PANDAseq v.2.9 and UCHIME algorithm were used for sequence processing. Taxonomic identification was done using the EzTaxon-e database at 97% similarity. Mothur and Shannon-ace-table.pl programs were used to calculate microbial community diversity. Differential abundance was analyzed between taxa in samples from California, Kentucky, Nebraska, and Tennessee, via QIIME2 (Bolyen et al., 2018). DADA2 was used for amplicon sequence variant (ASV) determination (Callahan et al., 2016). Taxa were assigned using the SILVA132 16S rRNA gene database (99%) (Quast et al., 2013). Differential abundance in taxa between pen and non-pen soils were determined using ANCOM (Mandal et al., 2015) and LefSe (Paulson et al., 2013).

Ten marker genes of interest were quantified using quantitative PCR, with PCR primers from prior literature. Total bacteria (16S rRNA gene) (An et al., 2018), AR genes for tetracycline (*tetQ*) (Klase et al., 2019), sulfonamide resistance (*sul1*) (Klase et al., 2019), and carbapenem resistance (*KPC*) (Subirats et al., 2017), and a MGE (*intl1*) (González-Plaza et al., 2019) were quantified using Droplet Digital PCR (ddPCR). Three marker genes for enteric pathogens, *Salmonella, Campylobacter* (Healy-Profitos et al., 2016), and STEC (*stx2*) (Ibekwe et al., 2002), and microbial source tracking (MST) markers for ruminant fecal pollution (*Rum2Bac*) (Mieszkin et al., 2010) and human fecal pollution (*HF183*) (Green et al., 2014) were quantified using real-time PCR.

Gene concentrations were calculated per gram of soil. Statistical analyses were completed in R 3.6.0 (R Core Team, 2019). Normality was assessed with QQ plots, density plots, and Shapiro-Wilk tests, which all demonstrated non-normality. As a result, non-

parametric Wilcoxon Ran Sum test were used to test for differences between pen and non-pen soils for each marker gene.

## Results

### *Microbial Community Results*

Nine phyla were identified in all soil samples, which included Acidobacteria, Actinobacteria, Bacteroidetes, Chloroflexi, Euryarchaeota, Firmicutes, Planctomycetes, Proteobacteria, and Verrucomicrobia, making up an average of 97% of each microbial community (Figure 1). Proteobacteria was the most abundant phyla, composing 20-40% of the community from each sample. There were no significant differences in diversity between pen and non-pen soils. Most paired state soil samples had similar microbial communities, which can be seen between the short distance between points on the PCoA plot (Figure 2). The samples from Iowa were the most different in microbial community composition. (Figure 2). There were no patterns in clustering or significant differences in beta diversity between pen and non-pen samples (Figure 2).

There were 2 taxa that were significantly different between pen and non-pen samples. The bacterial family *Rhodanobacteraceae* was more abundant in non-pen soil samples compared to pen samples, by LefSe testing. The family *Peptostreptococcaceae* was more abundant in pen soil samples than non-pen samples, which was identified by LefSe and ANCOM testing.

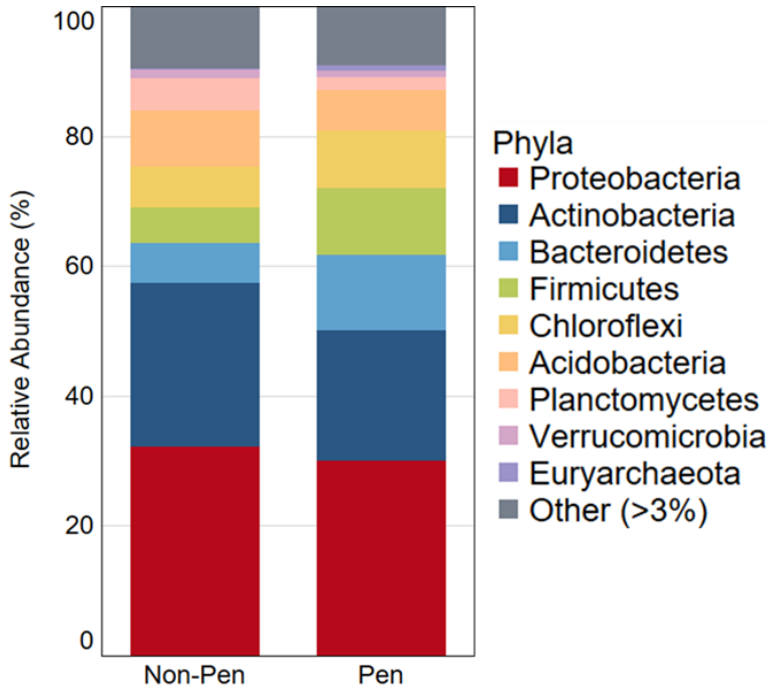


**Figure 1.** Microbial community composition at a phyla level averaged in pen and non-pen soil samples.

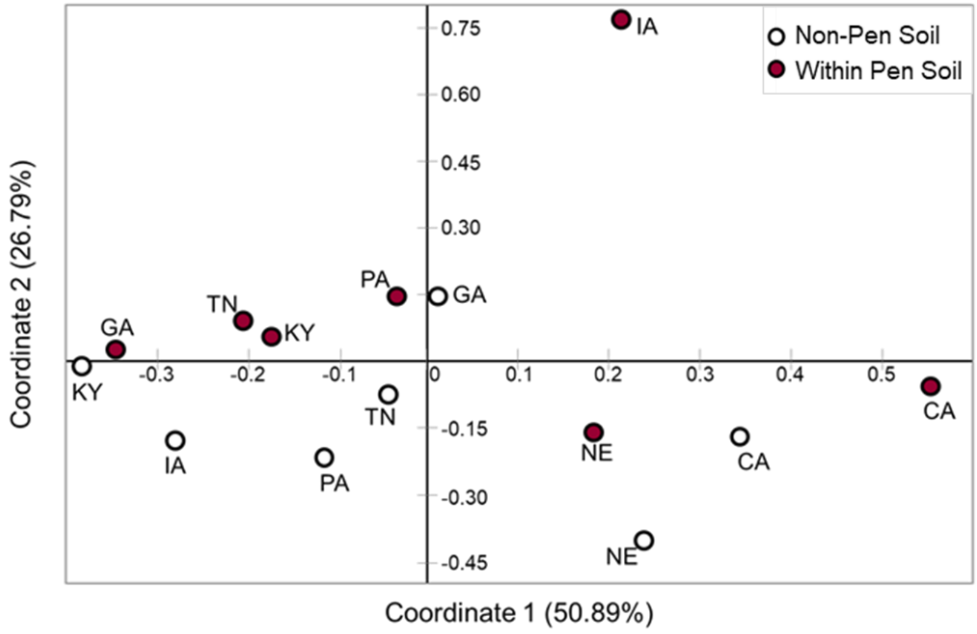


**Figure 2.** Principle coordinates analysis (PCoA) plotting dissimilarity in soil bacterial structure from each farm, labeled by state. Color indicates the difference in pen and non-pen samples.

*Marker Gene Results*

There were significantly higher concentrations of total bacteria, *Salmonella*, *Campylobacter*, *sul1*, and *KPC* in pen soil samples compared to non-pen samples (Figure 3). The human fecal marker was the only comparison that was significantly more abundant in non-pen soils than pen soils (Figure 3). Ruminant fecal contamination was abundant in both pen and non-pen soils.

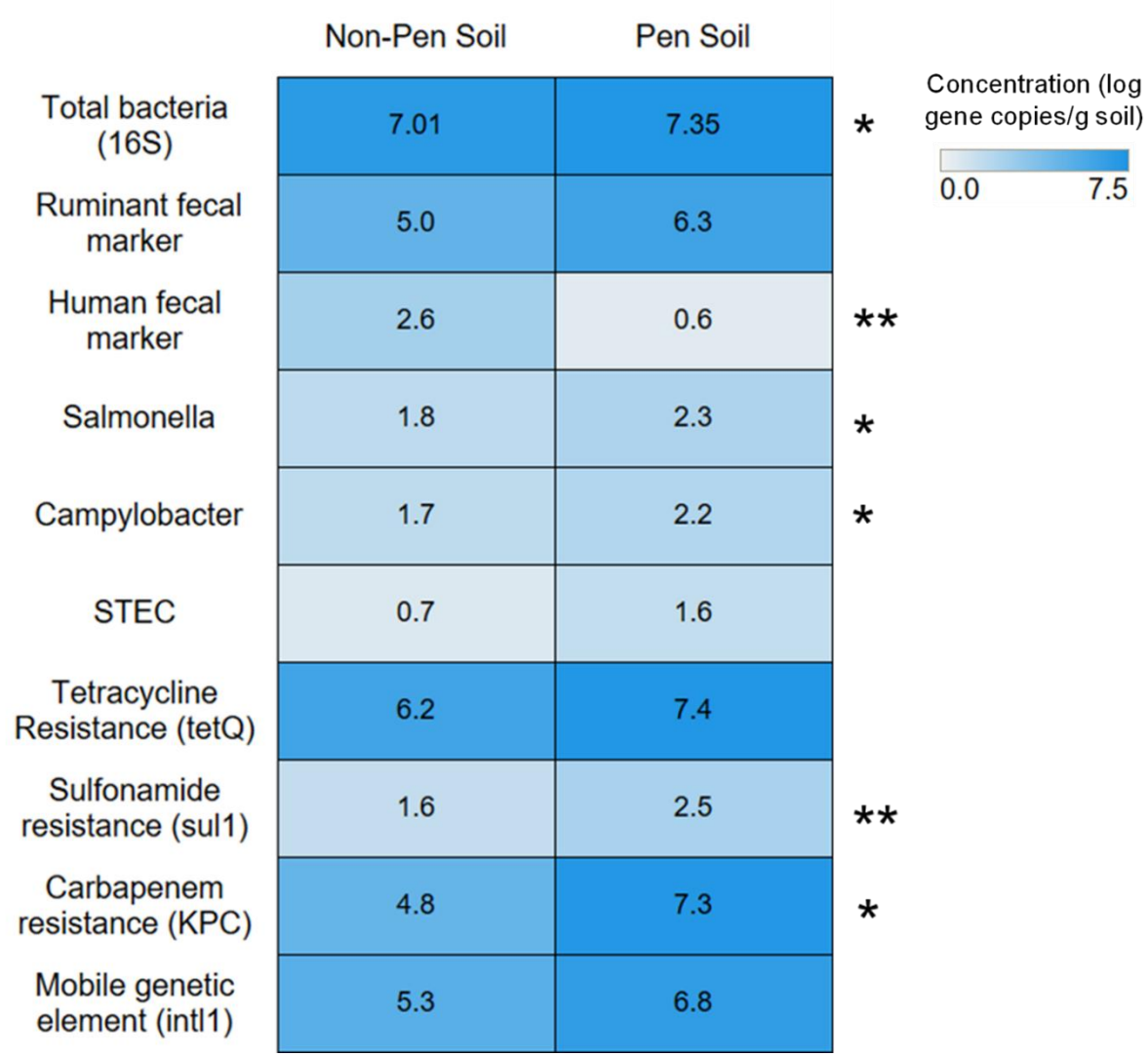


**Figure 3.** Average concentrations of each marker gene in log gene copies/g soil compared between pen and non-pen soils. *$p$<0.05 **$p$<0.01

**Discussion and Conclusions**

These analyses reveal that ruminant fecal pollution, likely from cattle, is abundant in cattle-associated soils. The choice of a 'control' soil was a limitation, as this was an area away from the cattle, but clearly was still under the influence of cattle manure, as demonstrated by the high concentration of *Rum2Bac* in non-pen soils (Figure 3). The cattle fecal pollution of non-pen samples likely indicates spread to nearby soils through runoff, transport by trucks or human traffic (shoes), or airborne spread by particulate matter. The choice of 'non-pen' soil samples as a control is a limitation of this study design, but it is challenging to find soil samples that are similar to the pen soils, with the only difference being presence of cattle. While cattle fecal pollution was greater than human fecal contamination of both pen and non-pen soils (Figure 3), human fecal pollution is still present in these soils, so any pathogens or genes identified could come from human or cattle feces. However, the overwhelming fecal pollution of these samples are from ruminants, such as cattle.

Among the differentially abundant families, *Rhodanobacteraceae* was more abundant in non-pen soils. This family contributes to nitrogen cycling (Li et al., 2014), which may indicate the loss of an important family in pen soils, but it likely just a difference in microbial communities under fecal stress. *Peptostreptococcaceae* was more abundant in pen soils, and this family is generally anaerobic and fermentative (Slobodkin, 2014), which could relate to the close relationship with cattle and direct application of their manure.

The differences in marker gene quantification indicate a potential for human health risk from cattle pen soils. The increased concentration of the zoonotic pathogens *Salmonella* and *Campylobacter* demonstrate this, particularly the risk of transmission to farm workers in close contact with these soils. Among the AR genes, tetracycline resistance was not different between

pen and non-pen soils. However, *tetQ* was the most abundant of the 3 AR genes tested in the soil samples (Figure 3). This could be tied to the high amounts of ruminant (cattle) pollution of the soils, which are also abundant in pen and non-pen soils. However, cattle manure has been identified as a reservoir of tetracycline, sulfonamide, and carbapenem resistance genes (Bonardi & Pitino, 2019; Vikram & Schmidt, 2018; Webb et al., 2016; Wittum et al., 2010). Sulfonamide resistance genes were more abundant in pen soils than non-pen (Figure 3), but had the lowest concentration of the three AR genes. It is unclear if the greater concentration of sulfonamide resistance genes in pen soils is due to the slight, non-significant increase in ruminant fecal pollution in pen soils, or unmeasured factors, such as antibiotic presence in soils. Antibiotic use on the farms could also be an important factor in the differences in the concentrations of *tetQ* and *sul1*, which was not collected. However, the most commonly used antibiotics on farms are tetracyclines, ionophores, penicillin, and macrolides (Economou & Gousia, 2015; Ghanbari et al., 2019). Sulfonamides are not likely as often used as tetracyclines, leading to less selective pressure for maintenance in cattle soils.

The greater concentration of *KPC* in pen soils was the most surprising finding in this study. Furthermore, *KPC* had a high concentration overall, and was comparable to tetracycline resistance (Figure 3). Tetracycline is commonly used on farms, but carbapenems are not. Carbapenems are not approved for livestock use in any countries, and are typically reserved for human use (OIE, 2015). Presence of *KPC* would be anticipated to be related to human fecal pollution, but human fecal pollution was greater in non-pen soils, which is the opposite trend as *KPC* (Figure 3). The high concentration of *KPC* and greater abundance in pen soils may be due to the mobile nature of this AR gene. *KPC* is plasmid-mediated and easily spread and maintained in bacterial communities (Potter et al., 2016; Wein et al., 2019). While the MGE

quantified was not significantly more abundant in pen soils, there was a slight increase compared to non-pen soils (Figure 3), which may indicate greater horizontal gene transfer (HGT) in pen soils. Furthermore, *KPC* lends cross-resistance to other antibiotic classes with the same mechanisms, and it is commonly co-resistant, so it is found on the same plasmid as other AR and metal resistant genes (Baker-Austin et al., 2006; Cantón & Ruiz-Garbajosa, 2011; Queenan & Bush, 2007). Prior study has provided rationale that identification of a different carbapenem resistant gene (*CTX*) in cattle feces could have been due to the selective pressure of another antibiotic used on the farm, Ceftiofur, for these same reasons (Webb et al., 2016). This could also be true in cattle-associated soils.

Cattle-associated soils are a complex environment to study AR and microbial communities. This study found that cattle soils contain ruminant fecal pollution (likely from cattle), clinically relevant AR genes, and enteric pathogens. While this study lacked a true control and had a limited sample size with no replicates, it can provide a valuable insight to this matrix for future study. Future study should include more farms, repeated sampling, and more careful consideration of 'control' sample selection. Recommendations from this analysis include limiting transmission of these AR genes and pathogens to farm workers by encouraging them to not wear work shoes inside their homes and frequently washing their hands.

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