Environmental Antibiotic Resistance in Surface Waters Near Concentrated Animal Feeding Operations in Michigan

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Abstract

This study addresses antibiotic resistance, recognized by the United Nations Environment Program (UNEP) as a threat to global health and sustainability. Concentrated Animal Feeding Operations (CAFOs), or "factory farms," administer low concentrations of antibiotics to animals through feed, a practice aimed at modestly enhancing growth rates and preventing infections (Hembach et al., 2022, p. 2). Such subtherapeutic antibiotic use allows pathogens to develop antibiotic resistance genes (ARGs), which can contaminate surrounding waters through runoff or manure spray (Martin et al., 2015). Nine surface water samples from sites near dairy and swine CAFOs in Michigan (MI) and two sites nearby were collected in the fall of 2023. Seven surface water samples from sites near dairy and swine CAFOs in MI and two sites nearby were collected in the spring of 2024. For both sets of samples, same-day culture-based analyses for fecal indicator bacteria using IDEXX Colilert 18, in the presence and absence of antibiotics. revealed elevated levels of *Escherichia coli*, total coliforms, and antibiotic-resistant coliforms. IDEXX also revealed a higher percentage of antibiotic-resistant *E.coli* and total coliform in spring samples relative to fall. Samples were further analyzed by qPCR for target ARGs and microbial source tracking genes (MSTs). In conjunction with qPCR analysis, this study utilized metagenomic analysis of shotgun-sequenced samples. A comparison between the three approaches will help standardize the antibiotic-resistant bacteria (ARB) surveillance process for communities near CAFOs. Results from this study will provide a more detailed understanding of environmental injustice manifested in the biased distribution of antibiotic resistance genes.

Introduction

Antibiotic use in animal feed is one of the most contentious agricultural practices in the United States. The Environmental Protection Agency of the United States (US EPA) outlines in Title 40, section 122.23 of the Code of Federal Regulations that an animal feeding operation is a livestock production facility where animals are confined for at least 45 days within a year. The classification of an operation as a concentrated animal feeding operation, or CAFO, depends on the number of animals present. For example, a dairy farm with more than 200 cattle would be considered a CAFO. In addition, the Clean Water Act (CWA), a key regulatory statute promulgated by the U.S. EPA, considers CAFOs as a point source of pollution.

Antibiotics administered to CAFO animals through feed serve two major purposes: to eradicate pathogens from livestock populations and to promote animal growth by increasing nourishment efficiency (Zhang et al., 2022). This process involves low-level dosing, which creates favorable conditions for bacteria to develop antibiotic resistance and proliferate, thus enabling the spread of antibiotic-resistant genes (ARGs) within the microbial community. Roughly 80% of antibiotics sales in the United States are allocated for application in animal agriculture, and approximately 70% of these antibiotics sold are categorized as "medically important," a classification reserved for drugs crucial to human patients (Martin et al., 2015). ARGs can potentially transfer from CAFO animals to human populations through various pathways, such as ingestion of ARG-contaminated produce or environmental ARG pollution. This poses a serious health concern as the spread of resistance genes in pathogens may render all drugs ineffective against infections (Hembach et al., 2022, pp. 1-3).

The fecal waste that hosts ARGs is rarely contained within CAFOs, as revealed by prior studies that identified high concentrations of microbial pollution in bodies of water near CAFOs.

Streams and rivers serve as hotspots for antimicrobial dissemination, providing dynamic environments for ARG transfers (Damashek et al., 2022). Livestock fecal matter entering surface waters can increase environmental ARGs (Paruch et al., 2022). Effluent runoff from feeding facilities, manure application on crops, and accidental contamination can all contribute to this issue (Heaney, 2015; Hubbard et al., 2020; Givens et al., 2016; Rieke et al., 2018; Graham et al., 2019). Heaney (2015) conducted a study on waters downstream of CAFO sites in North Carolina and found higher concentrations of swine-specific bacteria, especially during spring and summer or following rainfall (Heaney, 2015). Similar patterns were observed with poultry litter and groundwater contamination near concentrated poultry feeding operations (Hubbard et al., 2020).

Our practicum project investigated an issue that involves public health, agriculture, and the environment by recording the presence of ARGs near CAFOs. While a breadth of reports exist on occupational health hazards associated with CAFOs, community-based participatory research is limited.

Over the past year, we analyzed water samples for the presence and concentration of microbial markers of ARGs in residential areas adjacent to CAFO facilities in MI, using the genes *tetW, sul1, intl1, ermF,* and *blaSHV* as antibiotic resistance indicators, and *pig2Bac, CowM2, CowM3, blaCTX*, and *16S* as microbial source tracking (MST) genes. Our objective was to determine the concentrations of ARGs and MST genes in our samples, as well as to compare the resistome assessed through culture-based, qPCR, and metagenomic methods. Significant concentrations of ARGs and MSTs detected from a given area would indicate CAFOs as the most probable cause of contamination. Conversely, a high concentration of ARGs coupled with little to zero presence of MST genes detected would indicate another potential contamination source. Our community partners, Lynn Henning, the Director of the Water Rangers Program at

Socially Responsible Agriculture Project (SRAP), and Cole Dickerson, a scientist at SRAP, identified sampling sites in Michigan. Nine residential sites were near swine and dairy CAFOs, one site was not impacted by agriculture, and one was impacted by a wastewater treatment plant.

We created graphs to compare results from the two methods of ARG analysis, which illuminated the spatial and seasonal patterns of ARGs in surface waters. Our project highlighted that antibiotic resistance genes proliferate in CAFOs and infiltrate nearby environments, namely surface waters. Grave health repercussions experienced by nearby communities serve as one of a myriad of barriers to achieving environmental justice. We hope that our results will provide a nuanced understanding of the severity of risk faced by residents near CAFOs, offering valuable insights to guide public health policies and practices.

Methods

Site Description, Sample Collection, and Preservation

Our water sample collection was conducted in MI, specifically targeting areas near Concentrated Animal Feeding Operations (CAFOs). MI is home to numerous CAFOs, predominantly situated in rural areas where large-scale animal agriculture is prevalent. These CAFOs primarily consist of operations housing swine, cattle, and poultry, with some facilities accommodating thousands of animals at a single site. Informed by these characteristics and our connection with the Socially Responsible Agriculture Project, MI was chosen as the location for sample collection. All 11 sites were selected by Lynn Henning. Sites 1-9 are categorized as "CAFO-Impacted" sites due to their proximity to swine and dairy CAFOs or connection to swine and dairy CAFO discharge. Site 10 served as the "Non-agricultural Control." Site 11 is impacted by wastewater and is used as a comparison site.

Table 1A. Overview of sites

Site #	Site type	Location of site	Local CAFO(s) (upstream of site)
1A	Dairy	Hughes Hwy. Henning Drain	Hartland Farms, Inc.
2A	Dairy	Cadmus Rd S. Branch River Raisin	Bakerlads Farms, Hartland Farms, Inc.
3A	Dairy	Haley Road Rice Lake Drain	Hoffland Dairy LLC
4A	Dairy	Tomer Road Lake Hudson Bear Creek	Hartland Farms, Inc., Hoffland Dairy LLC, Rathmourne Dairy Medina
5A	Swine and Dairy	W.Ridgeville Road Silver Creek	SunRyz Dairy LLC, State Line Farms
6A	Swine and Dairy	W. Mulberry Road Silver Creek	SunRyz Dairy LLC, State Line Farms
7A	Dairy	Dillon Hwy. S. Durfee Branch	Rathmourne Dairy Medina
8A	Swine and Dairy	Lime Lake Road Lime Lake Inlet	Rathmourne Dairy Hudson, White Farms
9A	Swine and Dairy	Coman Road Lime Lake Inlet	Rathmourne Dairy Hudson, White Farms
10A	Non-agricultural control	Addison Township	N/A
11A	Wastewater treatment plant	Clinton Township	N/A

Table 1B. Overview of CAFOs near sites

CAFO	Animal type	Upstream of site(s) #	Reported year of establishment	Reported annual revenue (USD)[1]
Hartland Farms, Inc.	Dairy	1A, 2A, 4A	1968[1]	1,640,790
Bakerlads Farms	Dairy	2A	1976[1]	436,660
Hoffland Dairy LLC	Dairy	3A, 4A	2005[1]	586,566
Rathmourne Dairy Medina	Dairy	4A, 8A-10A	2015[2]	<500,000
SunRyz Dairy LLC	Dairy	5A, 6A	2016[2]	Unknown
State Line Farms	Swine	5A, 6A	2000[1]	80,000
White Farms	Swine	8A, 9A	1979[1]	128,779

1. Manta. (2024). Results driven online marketing agency.
2. OpenCorporates. (2024). The open database of the corporate world.

Surface water samples were collected from the 11 sites in MI during the fall of 2023 as well as the spring of 2024. Water samples were collected using a water sampler designed by a UCLA undergraduate student Tim Chen. The water sampler is composed of a PVC pipe frame filled with weighted rocks. A metal ring is attached perpendicular to the frame and is secured with zip ties. The ring can be tightened with a screwdriver to effectively fasten a sterile 2L sampling bottle. The rope secured at the top of the frame using a pipe hitch knot allowed sample collection from various heights. Using the water sampler, sample collection followed a procedure of flushing the 2L sampling bottle with site water three times before finally collecting a full bottle. Each water sample was kept on ice to maintain viability after each collection. Samples were delivered on ice immediately following collection and personally delivered to the UCLA Jay Laboratory within 24 hours for same-day IDEXX culture incubation and filtering in preparation for DNA extraction.

DNA Filtration and Extraction

Water samples collected from the designated locations were filtered using sterile 0.4µm pore size filter papers. The filter papers were then placed into sterile microcentrifuge tubes, and

1.5 mL of 50% ethanol solution was added to each tube to preserve the treesamples. The tubes were stored at -20°C for future use.

Microbial DNA was extracted from the samples using the MP FastDNA SPIN Kit. Extraction was performed according to the manufacturer's protocol. Extracted DNA from each site was sent to an external lab for shotgun sequencing to prepare for metagenomic analysis and kept frozen in the Jay Lab for use in qPCR experiments. The filters were removed using sterilized forceps, torn into small pieces, and transferred to Lysing Matrix E tubes. The remaining ethanol solution was centrifuged at 5000 g for 10 minutes, the ethanol was carefully discarded, and the pellet was resuspended in 978 µL Sodium Phosphate Buffer. The resuspended pellets were added to the Lysing Matrix E tubes. Following the MP FastDNA™ SPIN Kit for Soil protocol, 122 µL MT Buffer was added to each tube, and the samples were homogenized in a BeadBeater for 1.5 minutes twice with a 5-minute rest in between. The tubes were then centrifuged at 14,000 × g for 10 minutes to pellet debris. The supernatant was transferred to clean tubes, mixed with 250 µL PPS, and centrifuged again at 14,000 × g for 5 minutes. The resulting supernatant was combined with 1.0 mL of Binding Matrix suspension and mixed for 2 minutes. After allowing the mixture to settle for 3 minutes, 500 µL of the supernatant was discarded. The remaining mixture was transferred in portions to a SPIN filter and centrifuged at 14,000 × g for 1 minute. The filter was washed with 500 µL SEWS-M, centrifuged, and dried by an additional centrifugation step. Finally, the Binding Matrix was resuspended in 100 µL DES, optionally incubated at 55°C for 5 minutes, and centrifuged to elute the purified DNA.

Culture-based Method

IDEXX Colilert 18

To gauge the prevalence of viable ARGs from bacteria recovered from samples, surface water collected at sample sites was added to IDEXX Quanti-Tray/2000 System with Colilert 18 to monitor total coliform and *E. coli*, according to the manufacturer's instructions. In addition, the kit was modified with the addition of antibiotics to determine extended spectrum beta-lactamase (ESBL) total coliform, and ESBL *E. coli*.

Each site set included 6 IDEXX Quanti-Trays and 6 Nalgene bottles (100 mL), labeled as site-dilution-antibiotic. MilliQ water was added to the Nalgene bottles according to the IDEXX Dilution Sheet. Each bottle contained a dissolved IDEXX Colilert 18 powder packet and the appropriate antibiotic stock. After shaking a bottle of water sample and allowing it to settle, it was added to the Nalgene bottle using a pipette. The resulting solution was poured into the Quanti-Trays and placed upright in a metal basket. The Quanti-Trays were inserted into a rubber insert and then into the Quanti-Tray Sealer. The trays were incubated at 35℃ ± 0.5ºC for 18-22 hours, and the start time was recorded every time.

Molecular-Based Methods

q-PCR

Target ARGs *tet*W, *sul*1, *intI*1, *erm*F, bla_{SHV}, and MST genes pig2Bac, CowM2, CowM3, bla_{CTX}, and 16S were chosen for their relevance to human health and documented prevalence in the environment. qPCR assays for each target gene were performed on a StepOnePlus Real-Time PCR System using SYBR Green Master Mix or TaqMan Master Mix for MSTs. Master Mix was prepared using template DNA, water, forward and reverse primers, and the SYBR Green/TaqMan master mix. DNA extracts from each sample site were added in addition to

Master Mix into individual wells of a 96-well plate, with three biological replicates for each sample. A triplicate negative control and triplicate standard curves of 10-fold serially diluted standards of each target ARG were included on each plate. Known concentrations of plasmid DNA were prepared and included in each assay to establish a standard curve for quantification of target gene abundance in the samples. qPCR data was analyzed using StepOne Software (Thermo Fisher Scientific). Cycle threshold (Ct) values were determined for each sample, and the abundance of target ARGs will be quantified relative to the standard curve.

Shotgun Metagenomic Sequencing

Complementary DNA strands, known as "paired ends" in metagenomics, were sequenced for 300 cycles on the Illumina NovaSeq 6000 system by Mr. DNA, an external bioinformatics lab. To improve data-processing efficiency, low-quality reads were filtered and assembled using Trimmomatic on Galaxy. ARGs were identified and quantified from the assembled contigs using ARGs-OAP (normalized 16S subtype), an online pipeline that required coding in Python. The resistome risk score for each file was calculated on the MetaCompare website based on the number of ARGs, mobile genetic elements (MGEs), and pathogen abundance present. Taxonomic identification of bacteria present at genus and phylum levels was conducted on paired mates files using the platform NMDC-EDGE.

Results

IDEXX Cultures: Total Coliform and *E. coli*

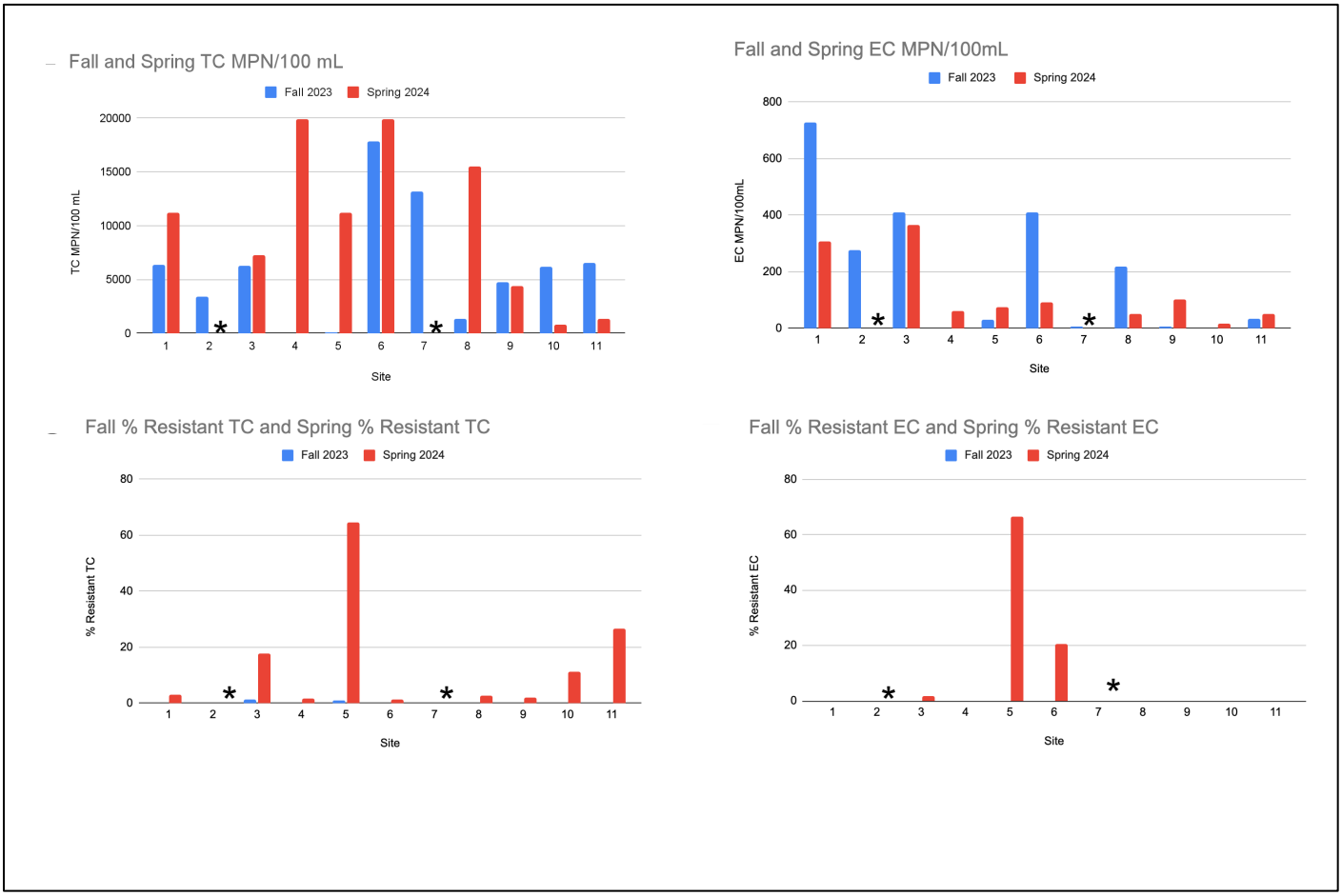

Figure 1: IDEXX Results Each site was cultured with and without the addition of antibiotics and incubated for 24 hours. Fall 2023 samples are presented in blue and Spring 2024 samples in red across all four graphs. **A)** Depicts the total coliform concentration per 100 mL of water sample. **B)** Depicts the concentration of *E. coli* per 100 ml water sample. **C)** While TC concentrations were comparable between fall and spring samples, the percentage of resistant total coliform detected by IDEXX was much higher in spring samples compared to fall. **D)** Depicts % of resistant *E. coli*.

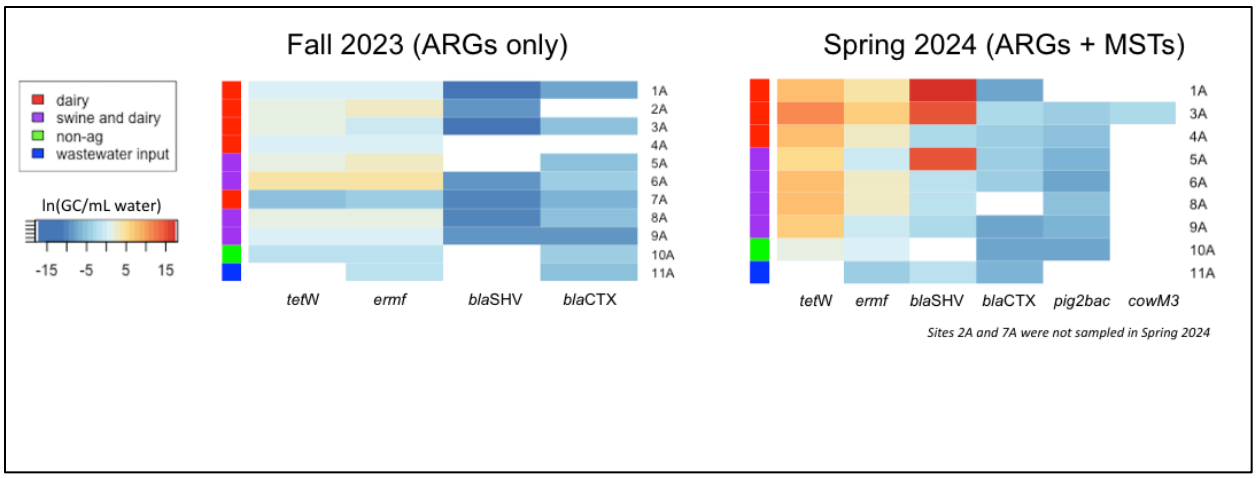

Figure 2. Seasonal qPCR Analysis of Antibiotic Resistance Genes (ARGs) and Microbial Source Tracking (MST) Markers. Markers were below detection limit in Fall 2023. The targeted ARGs—*tet*W, *ermf*, *bla*SHV, and *bla*CTX—were compared across seasons. Additionally, MST markers *pig2bac* and *cowM3* were detected in Spring 2024. This comparison highlights seasonal variations in the presence and abundance of specific ARGs and MST markers.

In Figure 1, while the concentrations of total coliforms (TC) and E. coli were comparable between fall and spring samples, as depicted in previous graphs, there was a notable difference in the percentage of resistant total coliforms detected by IDEXX, with a considerably higher proportion observed in spring samples compared to fall. One plausible explanation for this is the samples in the spring were taken soon after calving season where young animals could be exposed to more antibiotics in a feed in an effort to prevent disease and increase growth. Furthermore, in the Seasonal qPCR Analysis of Antibiotic Resistance Genes (ARGs) and Microbial Source Tracking (MST) Markers, conducted as part of this study, it was observed that markers were below the detection limit in Fall 2023. Subsequently, the targeted ARGs—*tet*W, *ermf*, *bla*SHV, and *bla*CTX—were compared across seasons, revealing significant variations. Additionally, MST markers *pig2bac* and *cowM3* were detected exclusively in Spring 2024.

qPCR Analysis: Antibiotic Resistant Genes and Microbial Source Tracking

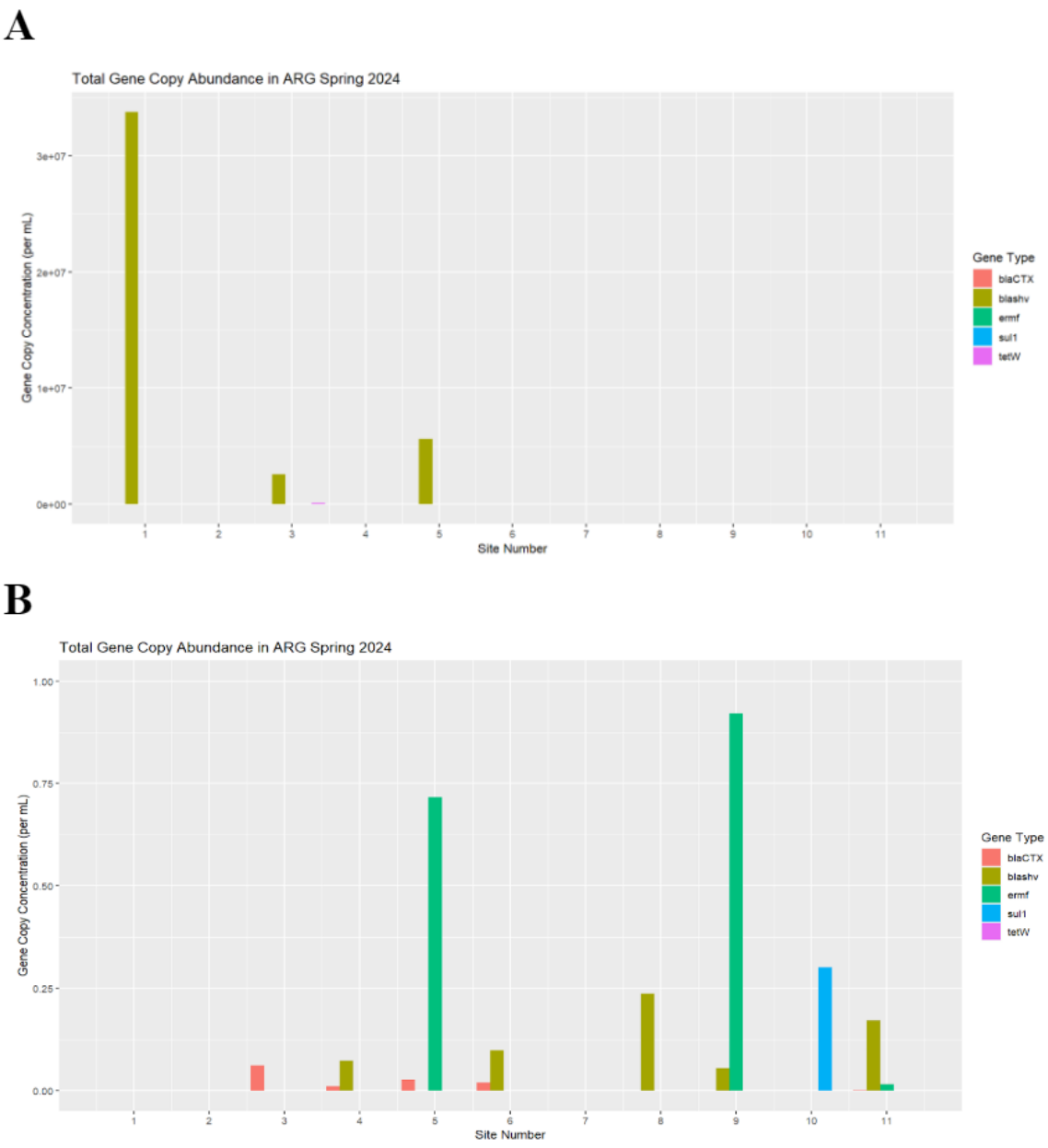

Figure 3. Spring QPCR ARG Results : Total Gene Copy Concentration Per Site in 2024 Samples. **A)** Concentrations of all samples illustrations limited by blashv results being exponentially larger than other genes. **B)** Close up of figure A. showing specific concentrations of other genes, not displayed due to differing scales of concentrations. Blashv was present in almost all sites at differing scales of concentrations.

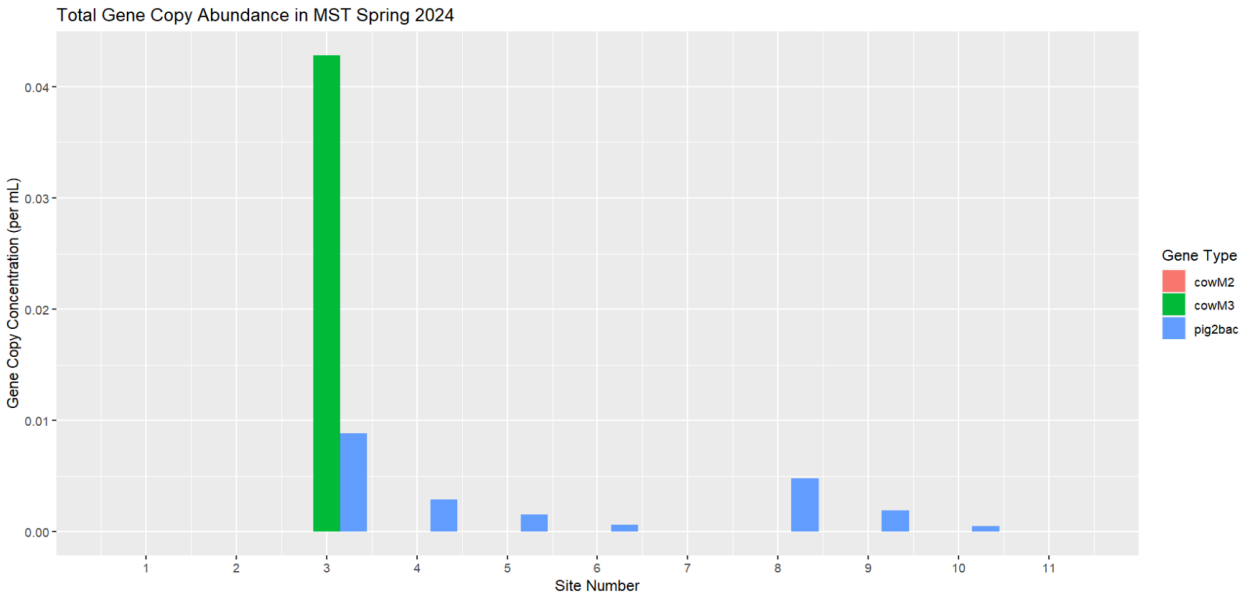

Figure 4 : Spring QPCR MST results. Sites where no gene detection resulted in no results were marked as 0 due to the technical workings of the graph formation. CowM2 and CowM3 were not detected in almost all sites. Pig2bac were detected in most of the sites tested.

Metagenomic Analysis

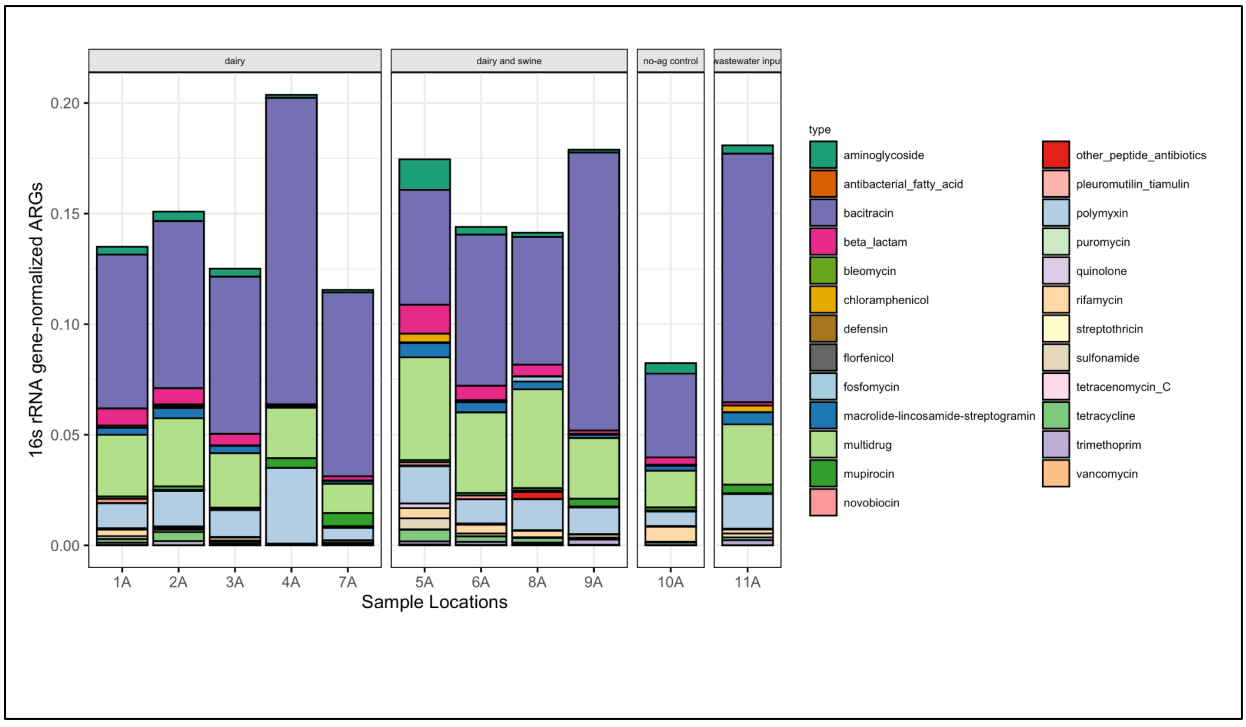

Figure 5. This graph shows ARGs gene abundance normalized against 16s rRNA abundance from samples from dairy, dairy and swine, non-agricultural control, and wastewater input sites. These results are from the ARGs-OAP pipeline, an online analysis workflow for antibiotic resistance genes detection from metagenomic data using an integrated structured ARG-database.

Table 2A. MetaCompare pipeline results from Fall 2023 sequenced sample sites

sample	# of contigs	# of ARG	# of MGE	# of PAT	risk score	source
1A	653924	1048	2654	626	21.46	dairy
2A	648888	1100	2042	1121	21.77	dairy
3A	1334968	2455	5556	860	22.13	dairy
4A	760340	2548	3365	1212	26.85	dairy
5A	542414	1491	2155	1944	24.98	dairy and swine
6A	602510	1596	2001	927	24.44	dairy and swine
7A	171484	239	1712	117	21.26	dairy
8A	643225	1345	2242	2033	22.96	dairy and swine
9A	807816	1966	2724	1158	23.89	dairy and swine
10A	447409	523	1134	248	20.33	no-ag control
11A	894197	3170	3188	1614	27.38	wastewater input

Table 2B. Source averaged MetaCompare pipeline results from Fall 2023 sequenced sample sites

source	mean risk score	mean # of contigs	mean # of ARG	mean # of MGE	mean # of PAT
dairy	22.6940	713920.8	1478.0	3065.8	787.2
dairy and swine	24.0675	648991.2	1599.5	2280.5	1515.5
no-ag control	20.3300	447409.0	523.0	1134.0	248.0
wastewater input	27.3800	894197.0	3170.0	3188.0	1614.0

Figure 5 illustrates that antibiotic resistance genes (ARGs) related to bacitracin resistance were the most prevalent, followed by those associated with multidrug resistance and polymyxin resistance. The total abundance of 16S rRNA gene-normalized ARGs ranged from 0.12 to 0.21 in CAFO-impacted sites. In wastewater-impacted sites, ARGs abundance was similar at 0.17 to this range, but it was significantly lower in non-agricultural control samples, at 0.08. Bacitracin,

commonly utilized in livestock, serves primarily as a feed additive aimed at promoting growth and enhancing feed efficiency. This application stems from its efficacy in inhibiting gram-positive bacterial infections, consequently bolstering the overall health and growth rates of the animals. Within agricultural contexts, bacitracin is administered at low concentrations (Merck). However, since bacitracin is minimally absorbed from the gastrointestinal tract of animals, its excretion via urine and feces can introduce resistant bacteria into the environment. This process constitutes an important pathway through which bacitracin-resistant bacteria disseminate in water bodies and soil, potentially transferring resistance genes to other bacteria (Merck).

Polymyxin antibiotics, categorized as cationic antimicrobial peptides, represent a last resort for treating multidrug-resistant Gram-negative bacterial infections and have been employed in veterinary medicine for several decades (Merck). Concerns over Polymyxin resistance have resulted in regulated use of the antibiotic in countries like Europe (Jansen et al., 2022), yet eight out of nine CAFO-impacted sites exhibited higher 16s rRNA gene normalized abundance compared to the non-agriculture impacted site, with site 4A's abundance polymyxin resistance making up 20% of ARGs. Widespread use of Polymyxin has led to recent research that emphasizes the risk of zoonotic transmission of polymyxin resistance (Scott et al., 2019).

Resistance to beta-lactam antibiotics, a class of antibiotics widely used in livestock (Coyne et al., 2019), was also more abundant in the majority of CAFO-impacted sites compared to both non-agricultural and wastewater-impacted areas. Similarly, tetracycline-resistant genes were prevalent in CAFO-affected regions compared to wastewater and non-agricultural impacted sites. Tetracycline, commonly used in the swine and dairy industries, has been associated with the development of resistance, as highlighted in various studies (Coyne et al., 2019).

Tables 1 and 2 showcase the outcomes of the MetaCompare pipeline analysis, illustrating the development of the "resistome risk" metric. This metric assesses the likelihood of ARGs associating with mobile genetic elements (MGEs) and transferring to pathogens, based on metagenomic data. Specifically, the analysis reveals that among the CAFO sources examined, both dairy and swine demonstrate a notably higher average risk score compared to dairy alone. Wastewater input exhibits the highest risk score, succeeded by dairy and swine, followed by dairy alone, and finally, a control group with non-agricultural impacted site.

Bacterial Community Analysis

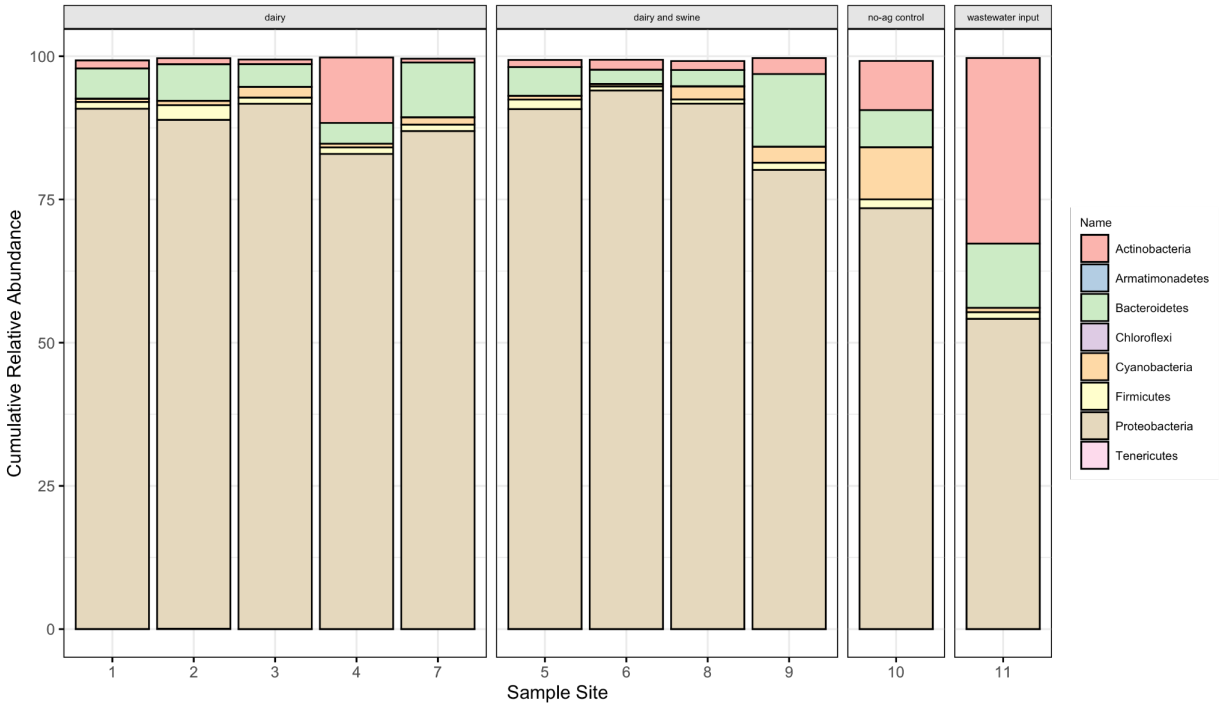

Figure 6: Top 8 Bacteria Phyla Abundance from Fall samples

Table 3. NMDC-EDGE Read: Overview of Genera Classification Results from Fall Samples

Site #	Site type	1	2	3	4	5	6	7	8	9	10
1A	Dairy	Pseudomonas	Flavobacterium	Limnohabitans	Acidovorax	Homo	Rhodoferax	Variovorax	Polaromonas	Hydrogenophaga	Dechloromonas
2A	Dairy	Arcobacter	Polynucleobacter	Flavobacterium	Pseudomonas	Acinetobacter	Acidovorax	Auranticrobium	Rhodoferax	Homo	Hydrogenophaga
3A	Dairy	Homo	Pseudomonas	Acidovorax	Rhodoferax	Variovorax	Dechloromonas	Hhydrogenophaga	Rubrivivax	Burkholderia	Geobacter
4A	Dairy	Polynucleobacter	Aurantimicrobium	Limnohabitans	Candidatus Planktophila	Pseudomonas	Rhodoluna	Flavobacterium	Rhodoferax	Homo	Acidovorax
5A	Swine and Dairy	Pseudomonas	Flavobacterium	Acidovorax	Rhodococcus	Homo	Rhodoferax	Variovorax	Streptomyces	Hydrogenophaga	Bradyrhizobium
6A	Swine and Dairy	Pseudomonas	Dechloromonas	Acidovorax	Streptomyces	Burkholderia	Leptothrix	Variovorax	Bradyrhizobium	Flavobacterium	Cupriavidus
7A	Dairy	Gemmobacter	Aurantimicrobium	Flavobacterium	Homo	Rhodobacter	Paracoccus	Novosphingobium	Limnohabitans	Polynucleobacter	Pseudomonas
8A	Swine and Dairy	Homo	Pseudomonas	Streptomyces	Aquaspirillum	Burkholderia	Raoultella	Variovorax	Acidovorax	Cupriavidus	Bradyrhizobium
9A	Swine and Dairy	Polynucleobacter	Flavobacterium	Auranticrobium	Limnohabitans	Homo	Acidovorax	Rhodoferax	Pseudomonas	Rhodoluna	Hydrogenophaga
10A	Non-agricultural control	Homo	Limnohabitans	Pseudomonas	Candidatus Planktophila	Mycobacterium	Anabaena	Mycolicibacterium	Hydrogenophaga	Acidovorax	Polynucleobacter
11A	Wastewater treatment plant	Candidatus Planktophila	Limnohabitans	Polynucleobacter	Flavobacterium	Rhodoluna	Pseudomonas	Candidatus Nanopelagicus	Acidovorax	Rhodococcus	Candidatus Methylopumilus

In Figure 6, the prevalence of *Proteobacteria* stands out as significantly higher compared to other bacterial phyla, with Actinobacteria and Bacteroidetes following behind. Notably, sites impacted by CAFOS exhibit distinct relative abundance patterns in comparison to wastewater

and non-agricultural profiles. Specifically, these CAFO-affected sites display elevated levels of *Proteobacteria* and considerable abundance of Actinobacteria in wastewater samples.This aligns Phylum analysis conducted by Hu et al. 2016 indicates that the transfer of antibiotic resistance between farm animals and humans primarily occurs through the dissemination of mobile ARGs, notably enriched in *Proteobacteria*, *Bacteroidetes*, *Actinobacteria*, and *Firmicutes*. Both Firmicutes and Bacteroidetes are prevalent phyla found in swine manure, but Zhang et al. 2019 found that the presence of *Bacteroidetes* and *Proteobacteria* is crucial in shaping the profiles of ARGs. The presence of such phylum in Figure 4 is consistent with these findings in that the microbial composition within swine manure could primarily facilitate the transfer of ARGs.

The above table (Table 3) displays the top 10 bacterial genera most abundant at each site. *Pseudomonas* (phylum *Proteobacteria*) was detected at every site and consistently ranked within the top one or two genera for sites near CAFOs. A notable zoonotic pathogen from this genus is *Pseudomonas aeruginosa,* a Gram-negative aerobic flagellated bacterial species (Alonso-Calleja et al., 2021, p. 2). It is commonly found in soil and water but also has been detected in produce and hospitals. A recent study published by Cravioto et al. (2024) found *P. aeruginosa* in cattle, sheep, goats, and horses, which aligns with our finding of greater abundance of *Pseudomonas* near CAFOs. Capable of infecting both plants and animals, *P. aeruginosa* is a highly adaptable pathogen that exhibits inherent and acquired multidrug resistance, where a singular bacterial strain can eradicate more than one drug from its system (Alonso-Calleja et al., 2021, p. 2). It can infect the respiratory system, urinary tract, blood, skin, and other bodily parts of livestock and humans (Jabłoński et al., 2019, p. 2).

Burkholderia (phylum *Proteobacteria)* was detected within the top ten genera for sites 3,6, and 8, located near CAFOs. These Gram-negative bacteria are normally found in soil and

water, while pathogenic species like *Burkholderia pseudomallei* and *Burkholderia cepacia complex* infect humans and animals, including domestic cattle (Ali et al., 2019, p. 2). Melioidosis, an infection of the host by *B. pseudomallei,* causes pneumonia, encephalitis, and other issues with the immune system, but is rarely transmitted through interspecific interactions (Currie et al., 2024, p. 155-156).

Streptomyces (phylum *Actinomycetota*) was detected within the top ten genera for sites 5, 6, and 8 only, all located near swine and dairy CAFOs. Species within this genus are thermophilic Gram-positive bacteria that form spores, a reproductive mechanism suited for harsh conditions (Khadayat et al., 2020). It is commonly found in soil, animal feed, and manure, and can survive in aquatic environments (Valdezate, 2022). Although rarely pathogenic, certain species are capable of infecting plants and animals. Watson et al. (2022) reported cases of mycetoma, a chronic skin infection, in Sudan, caused by *Streptomyces somaliensis and Streptomyces sudanensis.* Nonetheless, *Streptomyces* plays an integral role in modern medicine. Streptothricin, an ARG detected at a few sites in MI (Fig. 3), is one of many antibiotics derived from *Streptomyces* (Gopalakrishnan et al., 2020). Moreover, recent studies incorporated this hardy bacteria into swine manure fertilizer to examine its ability to extract nutrients and remove ARGs from agricultural fields (Chi et al., 2020; Sha et al., 2022).

Flavobacterium (phylum *Bacteroidota*) was detected at 8 sites near CAFOs and a wastewater treatment plant. These Gram-negative aerobic bacteria are found in various environments, including water (Enisoglu-Atalay et al., 2018). Flavobacteria are recognized as fish pathogens. While not a significant threat to human health, Wahli et al. (2018) observed a previously unidentified species of *Flavobacteria* in MI that may be the causal factor of diseases in fish populations. The myriad health implications posed by aquatic bacterial communities near

CAFOs demonstrate the gravity of antibiotic resistance occurrence, a repercussion of industrial farming to workers and local residents.

Discussion

Limitations

IDEXX Colilert-18

IDEXX can only detect culturable cells. As a result, viable but not culturable cells cannot be detected by this method (McLain et al., 2016, p. 437). This could result in an underestimation of the abundance of bacterial species, including those capable of spreading antibiotic resistance. Moreover, only a selective number of microbial species can be cultured in a laboratory environment, a phenomenon known as "culture bias" (McLain et al., 2016, p. 438). Therefore, IDEXX, a culture-based method, will not produce sufficiently representative results for a study of the environmental resistome.

qPCR

The real-time/quantitative polymerase chain reaction technique necessitates the selection of specific ARGs before sample analysis as it requires gene-specific primers. This selective process could prevent the detection of other ARGs in the sample (Liguori et al., 2022). Additionally, qPCR utilizes extracted DNA, and thus cannot discern between ARGs from viable hosts and those in non-viable hosts present in the sample.

Metagenomics

Metagenomic analysis, while comprehensive, lacks sensitivity as it examines the entire genome present (Liguori et al., p. 9152). It is thus less adept than IDEXX Colilert 18 and qPCR at detecting ARGs and other rare genes. Given this limitation, the actual resistome risk score at each site may be higher than our calculations. Moreover, previous studies found discrepancies in the diversity of genes calculated for samples based on the sampling method used (Liguori et al., p. 9152). For our research, the "grab" technique, where a single sample was collected from each

site in MI, was optimal given the time constraints and the risk of negative interactions with residents. Composite sampling, on the other hand, entails collecting multiple samples within a designated timeframe, hence the assumption that it would better represent diverse bacterial communities (Huijbers et al., p. 3). Therefore, our metagenomic analysis results may reflect an underestimation of bacterial diversity.

The costliness, time-consuming, and online nature of metagenomic analysis limits accessibility. Results may vary based on the platform used to analyze since different databases store different reference genes and species (Liguori et al., p. 9152). As novices to bioinformatics, our team felt challenged by the coding aspect of our project. Unfortunately, we could not conduct metagenomic analysis on samples collected in the spring, which have yet to be processed by Mr. DNA, the non-UCLA-affiliated DNA-sequencing lab.

Relevant Policies

Policy gaps and intentional allowances granted to CAFOs enable the contamination of water by animal fecal matter. For example, the USDA has a "zero tolerance policy" for fecal contamination of meat products. This policy, however, only applies to *visible* contamination, so the risks incurred by trace amounts of fecal matter go unmitigated (USDA, 2019). Specifically, the USDA's Food Safety and Inspection Service's Directive 6420.2, Revision 2, states that the zero tolerance standard applies to "visible fecal material" of which the individual examining the carcass determines that "both color and texture characteristics are identifiable" (U.S. Department of Agriculture, Food Safety and Inspection Service, 2020).

CWA requires point sources to file for a National Pollution Discharge Elimination System (NPDES) permit, granted to the permittee by individual state agencies. Moreover, point sources must not emit more than the "total maximum daily load" (TMDL) allowed, which refers

to the maximum level of chemical or microbial emissions permitted by the state. In 2020, the Michigan Farm Bureau (MFB) filed a lawsuit against EGLE (Department of Environment, Great Lakes, and Energy in MI), decrying the new NPDES permit conditions created for CAFOs. MFB accused EGLE of a lack of scientific evidence provided for allegations that CAFOs worsened water quality (ELPC, 2024). In addition, MFB claimed that EGLE violated the Administrative Procedures Act of 1969 (APA), an MI legislature that details specific requirements for rulemaking versus license programs. According to the plaintiff, EGLE should have sought public commentary per the APA for rulemaking processes. This lawsuit has undergone multiple appeals; if the MI Supreme Court rules in favor of MFB, the NPDES conditions in question will be invalidated as "unpromulgated rules" and could compromise EGLE's broader permit-issuing authority (Environmental Law & Policy Center, 2024). Such a localized process of pollution regulation is thus vulnerable to lobbyists who "help enact mechanisms that strip these communities of meaningful ways to fight" (Ren, 2022).

CAFOs can avoid NPDES permits if they do not discharge into "national" waters or repurpose fecal waste. When fecal waste is reused as manure, the CAFO producing that manure "...should not be held accountable for any discharge that is primarily the result of 'precipitation'"(Ren, 2022). The permit currently requires more transparency on how waste is transported between farms, especially in impaired watersheds. However, these requirements have not been viable in the contamination by CAFOs of public waterways; manure application in the wintertime, which serves no fertilizing purpose given the season, may still occur due to the permit's technicalities.

The Michigan Environmental Council reported that the demands of the existing permit "allow[s] risky management to continue," which raised concerns that health repercussions will

persist if amendments are not made to NPDES requirements (MEC, 2020). The Environmental Law and Policy Center (ELPC) recommended lowering TMDL for phosphorus and nitrates in the permit renewal (ELPC, 2024). However, the lack of suggestions for TMDL concerning *E. coli* and other microbial pollution exemplifies the general public's negligence of biohazards from CAFOs.

Implications

When cross-compared where applicable, results from IDEXX, qPCR, and metagenomic methods supported one another. For example, IDEXX results showed lower percentages of resistant *E. coli* and total coliform across fall samples compared to spring. qPCR results confirmed this trend, tending toward lower concentrations of selected ARGs in fall samples as compared to spring. While methods such as qPCR and metagenomics are valuable for their specificity, IDEXX, which cultures samples both with and without antibiotics at various dilutions, can reveal broader trends in the antibiotic resistome within 24 hours of sample submission to a lab. Therefore, IDEXX should be incorporated into ARB surveillance protocols as a primary screening tool. qPCR and metagenomic analysis, the traditionally used methods, should be incorporated when a more comprehensive analysis of specific ARGs, MSTs, or bacterial communities is desired.

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