COMPARATIVE METAGENOMICS IN LIVESTOCK PRODUCTION ANTIMICROBIAL RESISTANCE

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Bachelor of Science, University of Lethbridge, 2021

A thesis submitted
in partial fulfilment of the requirements for the degree of

MASTER OF SCIENCE

in

BIOCHEMISTRY

Department of Chemistry and Biochemistry
University of Lethbridge
LETHBRIDGE, ALBERTA, CANADA

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Date of Defense: December 4, 2023

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DEDICATION

To my fiancé, Daniel, who without his endless patience and support I would not have finished this thesis and to my Uncle Bob for inspiring me to pursue a career in science.

ABSTRACT

Antimicrobial resistance is a global health problem. It affects people suffering from infections and is also able to jeopardize livestock food security. With the rise of this problem, steps have been taken to reduce the use of antimicrobials in livestock production. “Raised Without” antimicrobial livestock production has been described to reduce antimicrobial resistance by removing the selective pressure which would reinforce resistance gene conservation in bacterial genomes. This thesis’ goal was to investigate the extent to which this statement is true. First, optimization of several different methods was used to detect resistance genes and associate them with mobile genetic elements to determine their transferability potential. Taking those methods and applying them to a beef cattle dataset revealed that an absence of antimicrobial use is likely not the cause of differences observed in antimicrobial resistance gene and microbiota abundance. Macrolide resistance genes are more likely to be stably conserved than tetracycline resistance genes. Expanding that analysis to include broiler chickens and swine both raised with and without antimicrobials, revealed there to be no difference between antimicrobial management practices unless at specific antimicrobial resistance gene group-levels.

PREFACE

This thesis is based on a series of publications with many co-authors. The analyses presented in this thesis would not have been possible if not for the extensive in-lab DNA extractions and sequencing that was performed by members of my lab and collaborators. Each contributing laboratory each had their own expansive team for sample acquisition, processing, sequencing, and data storage. I performed all bioinformatic based quality control, analysis, visualization, interpretation, and writing. Specific co-author contributions can be found at the beginning of each chapter and as follows. Abbreviations for names are found within the acknowledgements.

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Chapter 2 from section 2.1 to 2.4 inclusive, has been published as Lee, C., Ortega Polo, R., Zaheer, R., Van Domselaar, G., Zovoilis, A., and McAllister, T.A. (2023a). Evaluation of metagenomic assembly methods for the detection and characterization of antimicrobial resistance determinants and associated mobilizable elements. *Journal of Microbiological Methods***,** 106815. Study was conceptualized by R.O.P, R.Z., and T.A.M.. Provision, management, and access of bioinformatics tools and computing environment was facilitated by R.O.P. and G.V.D. Metagenomic assemblies were performed by C.L. and R.O.P. Data was analyzed by C.L. Interpretation of data was performed by C.L., R.O.P., and R.Z. Manuscript draft prepared by C.L. Manuscript reviewed and edited by all authors. Funding acquisition by R.Z., A.Z., and T.A.M.

Chapter 3 from section 3.1 to 3.5 inclusive, have been published as Lee, C., Zaheer, R., Munns, K., Holman, D.B., Van Domselaar, G., Zovoilis, A., and McAllister T.A. Effect of

Antimicrobial Use in Conventional Versus Natural Cattle Feedlots on the Microbiome and Resistome. *Microorganisms*. 2023; 11(12):2982. Study was conceptualized and designed by R.Z. and T.A.M. Collection of samples and metadata by K.M. Metagenomic DNA isolated by K.M. and R.Z. Assembly workflows provided by D.B.H. Data analysis was performed by C.L. Interpretation of data performed by C.L. and R.Z. Bioinformatics cluster facility availability provided by G.V.D Manuscript draft prepared by C.L. Manuscript reviewed and edited by A.Z., D.B.H., G.V.D., K.M., R.Z., T.A.M.; Funding acquired by A.Z., R.Z., and T.A.M.

Chapter 4 from section 4.1 to 4.5 inclusive, is intended for publication targeting the journal Microorganisms. Concept and design of the study done by D.P.-L., G.T., M.S.D., and T.A.M., Collaboration of read sequencing and data sharing by D.P.-L., G.T., K.M., M.S.D., R.Z., and T.A.M. Data analysis performed by C.L. Interpretation of data performed by C.L. and R.Z. Bioinformatics cluster facility availability provided by G.V.D Manuscript draft prepared by C.L. Manuscript reviewed and edited by A.Z., D.P.-L., G.V.D., K.M., M.S.D., R.Z., and T.A.M.; Funding acquired by A.Z., D.P.-L., G.T., M.S.D., R.Z., and T.A.M.

ACKNOWLEDGEMENTS

Thank you to all of my collaborators for their instruction, guidance, and willingness to share data. Thank you to my fellow Agriculture and Agri-Food Canada colleagues: Drs. Dominic Poulin-Laprade and Guylaine Talbot from Sherbrooke RDC; Dr. Moussa S. Diarra from Guelph RDC, Dr. Devin B. Holman from Lacombe RDC, and Rodrigo Ortega Polo and the entire McAllister lab from Lethbridge RDC. Thank you also to Dr. Gary Van Domselaar for access to the National Microbiology Laboratory's (Public Health Agency of Canada) bioinformatic infrastructure without which the analyses presented here would have been impossible. Thank you especially to Drs. Tim McAllister and Rahat Zaheer for their professional and personal support they have shown throughout my time as a Lethbridge RDC employee, from a Co-op summer student in 2018 to completing my MSc thesis in 2023. And finally, thank you to my committee members including Dr. Steve Wiseman and my co-supervisor Dr. Athanasios Zovoilis.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

AAFC	Agriculture and Agri-Food Canada
ACLAME	A CLAssification of Mobile genetic Elements
AMG	aminoglycoside
AMR	antimicrobial resistance
AMU	antimicrobial use
ANCOMBC	analysis of compositions of microbiomes with bias correction
ARG	antimicrobial resistance gene
AST	antimicrobial susceptibility testing
BAM	binary alignment map
BAT	Bin Annotation Tool
BLA	β-lactam
BLAST	basic local alignment search tool
BWA-MEM	Burrows-Wheeler Aligner-Maximal Exact Matches
CARD	Comprehensive Antibiotic Resistance Database
CAT	Contig Annotation Tool
CB	catch basin
CHL	chloramphenicol
CONV	conventional
EDTA	ethylenediaminetetraacetic acid
FC	fecal composite
HGT	horizontal gene transfer
ICE	integrative conjugative element
IRIDA	Integrated Rapid Infections Disease Analysis
MAG	metagenome-assembled genomes
MDR	multidrug resistance
MGA	melengestrol acetate
MGE	mobile genetic element
MIC	minimum inhibitory concentration
MLS	macrolide, lincosamide, streptogramin
NAT	natural
NCBI	National Center for Biotechnology Information
NMDS	non-metric multidimensional scaling
NML	National Microbiology Laboratory
OTU	operational taxonomic unit
PATRIC	Pathosystems Resource Integration Center
PCoA	principle component analyses
PCR	polymerase chain reaction
PERMANOVA	permutational multivariate analysis of variance
PHAC	Public Health Agency of Canada
RDC	Research and Development Centre

SAM	sequence alignment map
SDS	sodium dodecyl sulfate
SNP	single-nucleotide polymorphism
SRA	Short Read Archive
SUL	sulfonamide
TET	tetracycline
TMM	trimmed mean of M-values
TMP	trimethoprim
VFDB	Virulence Factors Database
WGS	whole-genome sequencing
β-ME	β-mercaptoethanol

CHAPTER 1

Literature Review

1.1 Introduction

Antimicrobial resistance (AMR) is a global health issue that impacts many different industries in complex and nuanced ways. One perspective to use in understanding this vast topic is the 'One Health' approach (Shallcross and Davies, 2014;McEwen and Collignon, 2018). One Health combines three different sectors – clinical health, agricultural health, and environmental health – into a single continuum which illustrates how each impacts the other in intrinsic ways (Figure 1; Behravesh et al. 2019). This model has been applied to infectious diseases where it is fundamentally related to the transfer of microorganisms and their risk of causing disease. In this way, One Health can be applied not only to the pathogenicity of microbial infections but also to the associated risks that antimicrobial resistance can confer to successful treatment. AMR is a concern to all sectors of One Health, but particularly in the clinical and agricultural sectors where over-prescription and misuse of antimicrobials are of major concern (Ramachandran et al. 2019; Xiong, Sun, and Zeng 2018). Knowing why and how antimicrobials are used is important in putting into context the risks of AMR evolution and proliferation. The current state of AMR across livestock production systems is an important starting point in generating strategies to continue surveillance and risk assessments that support approaches to ensuring the health of humans, animals, and the environment.

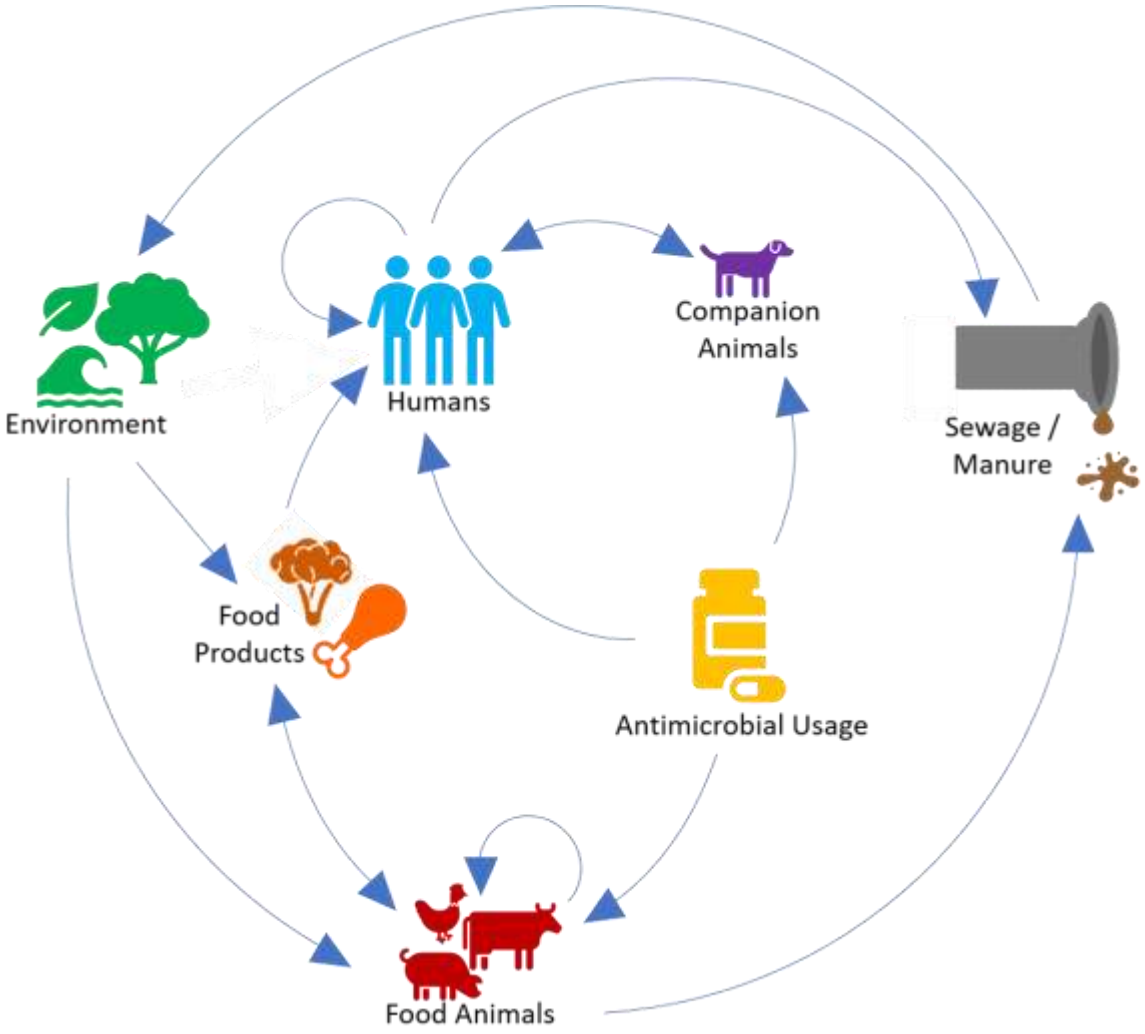

Figure 1. Network of interfaces at which ARGs can be transferred within the context of the One Health Continuum.

1.2 Uses of Antimicrobials

Antimicrobial use (AMU) has different roles in different sectors, but in all cases, antimicrobials are used to inhibit the growth or kill undesirable microorganisms. In the clinical sector of One Health, AMU is targeted at human or livestock pathogens and/or preventing the emergence of opportunistic pathogens (Leekha et al., 2011). This is primarily done through therapeutic treatment of patients presenting infections or as a prophylaxis for those at risk of

developing infections. Antimicrobials have been placed in categories based on their AMU in human health (Health Canada, 2009). Class I antimicrobials are of very high importance to human health. Some antimicrobials in Class I – such as vancomycin, third- and fourth-generation cephalosporins, and colistin – are often called 'last resort antimicrobials' due to these being the last prescribed therapeutic option for AMR or multidrug resistant (MDR) infections. Class II are high importance, but they generally have at least one alternative should they fail. Class III are of medium importance and are not the preferred option for treatment of serious human infections. Class IV antimicrobials are not used in human health at all. Broad-spectrum antimicrobials are used when antimicrobial susceptibility screening cannot or is not performed to identify a defined target bacterial species (Leekha et al., 2011). Specific or 'narrow spectrum' antimicrobials are more often used when such a screen has been performed.

There are two main AMU practices in the agriculture sector: livestock production and crop production. In livestock production there are four categories: therapeutic (single animal treatment), metaphylaxis (whole pen/herd treatment), preventative/prophylaxis (administered at times/to animals of high risk), and growth promotion. However, due to many factors, claims of AMU for growth promotion have been removed in recent years (Bengtsson and Wierup, 2006;Karavolias et al., 2018;Busch et al., 2020). The top antimicrobial classes used in livestock production are: tetracyclines, macrolides, and streptogramins for beef cattle; bacitracin, penicillin, and tetracycline in broiler poultry; and tetracyclines, β-lactams, and macrolides in swine (Public Health Agency of Canada, 2022a). Typically, broad-spectrum antimicrobials are used in livestock production as the feasibility of tailored treatment plans are often impossible. In crop production, the antimicrobials used are generally not antibiotics but fungicides, as fungi are the main crop plant pathogen, such as clubroot (*Plasmodiophora brassicae*) which infect the

family Brussicaceae (Javed et al., 2023) or tan-spot (*Pyrenophora tritici-repentis*) which infects wheat crops (Gourlie et al., 2022).

While there is no active broad-scale environmental AMU, there are still select instances such as in zoological and sanctuary settings where antimicrobials are administered. Rehabilitated wildlife that have received antimicrobials are often re-introduced to wild populations where their microbiota may interact. Generally, when addressing environmental health in an AMR One Health context, the environment may act as a reservoir or interface between other sectors to facilitate AMR proliferation in addition to acting as a reservoir of antimicrobial residues which exert their own selective pressures in the environment (Behravesh, 2019;Larsson and Flach, 2022).

1.3 Mechanisms of Antimicrobial Resistance Evolution

There are several strategies that bacteria possess that enable them to become resistant to an antimicrobial. For example, Enterococci are intrinsically resistant to β-lactams due to the expression of low-affinity penicillin-binding proteins that enable these bacteria to continue to undergo cell wall synthesis using peptidoglycan pentapeptide precursors (Sifaoui et al., 2001). There are four main strategies of acquired antimicrobial resistance in bacteria: 1) limiting the uptake of the drug to the bacterium through the cell wall / membrane, 2) modifying the target of the drug, 3) inactivating the drug with enzymes, and 4) active efflux of the drug out of the cell (Figure 2) (van Hoek et al., 2011;Reygaert, 2018). Non-lethal, spontaneous mutations can allow for a bacterium to alter an antimicrobial's target binding site enough that it disrupts the mechanism of action. For example, a single-nucleotide polymorphism (SNP) at Asp530 in the

*rpo*B gene at the rifampicin-binding site of the β subunit of RNA polymerase renders *Helicobacter pylori* resistant to rifampicin (Wang et al., 2001).

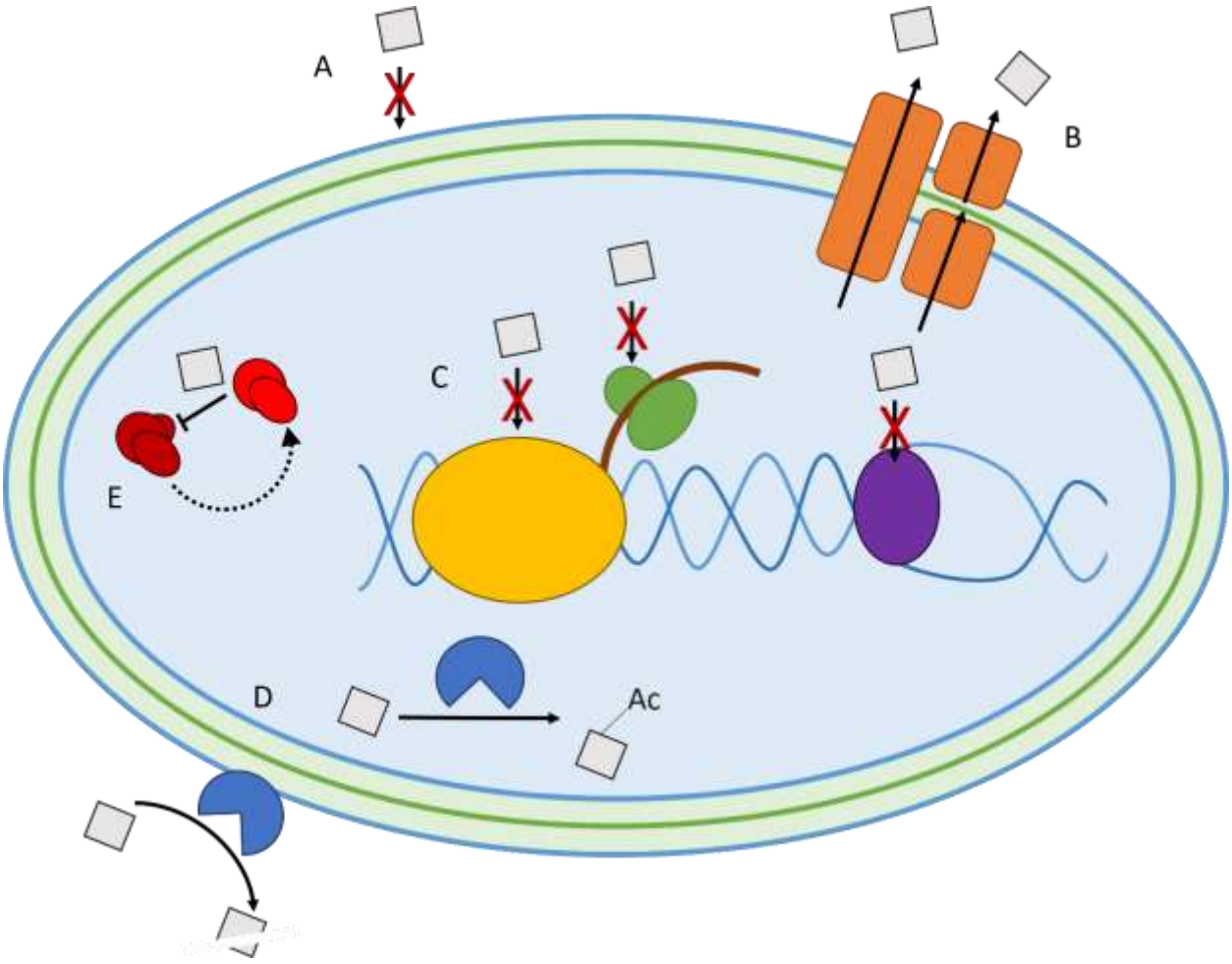

Figure 2. Strategies of acquired antimicrobial resistance in bacteria through A) impermeable barrier, B) efflux pumps, C) target modification mutation, D) drug inactivation, and E) alternative metabolic pathways. Adapted from Allen et al. (2010).

All AMR that is present in a given species in a population is spread in an identical manner through clonal growth, given that it follows simple asexual reproduction and provided that the trait is not inadvertently lost from the genome. However, when horizontal gene transfer

(HGT) occurs, AMR is transmitted in an inter-individual or inter-species fashion within a population. Plasmids, integrative conjugative elements (ICE), transposons, and integrons are all examples of mobile genetic elements (MGEs) that are able to harbour and transfer gene regions, including antimicrobial resistance genes (ARGs). MGEs of particular interest in the spread of ARGs include ICE and plasmids (Rozwandowicz et al., 2018;Botelho and Schulenburg, 2021).

1.4 Current Practices that Encourage AMR Proliferation

There are numerous AMUs that encourage AMR proliferation and are often labelled as 'misuse'. In humans, misuse is defined as 'overprescription' – the use of antimicrobials that are ineffective – or there is inadequate adherence to a course of antimicrobials (Byrne et al., 2019). There are a number of reasons for overprescriptions, some of which include preventing infections in at-risk populations that require therapeutics in advance of antimicrobial susceptibility tests (ASTs) (Liu et al., 2019a;Sloane et al., 2020). In animals, there is more nuance to 'proper' AMU, but nevertheless it has been shown that sub-therapeutic doses of antimicrobials – such as in prophylaxis and growth promotion – maintains the prevalence of ARGs within intestinal microbiota (Looft et al., 2012;Gullberg et al., 2014;Xiong et al., 2018)

1.5 Current Strategies to Mitigate AMR

There are various strategies that have been established to mitigate both the proliferation and spread of antimicrobial resistance. Mitigation is comprised of two components: research and surveillance of AMR with the aim of informing future actionable efforts and actionable responses. Surveillance initiatives are conducted at local, national, and global scales by various

government and research bodies. These bodies of information inform the ways that actions can be taken to respond to AMR. Actionable responses to AMR are further divided into proactive and reactive approaches.

AMR surveillance initiatives have been adopted into many national government policies including Canada (Public Health Agency of Canada, 2020;2022b), Australia (Australian Commission on Safety and Quality in Health Care, 2022), and India (Walia et al., 2019). Continental-wide surveillance initiatives have also been launched in Africa (Okolie et al., 2023) and Europe (Mader et al., 2021). Global cooperation has also been used in AMR surveillance, though this is primarily accomplished through collaborative research groups (Hendriksen et al., 2019;Zhou et al., 2022). While the majority of these programs focus on human related AMR, there are some that take a One Health approach to surveillance (Public Health Agency of Canada, 2020;World Health Organization, 2022;Zhou et al., 2022). Monitoring water (particularly urban wastewater) for AMR bacteria is an important interface between human and environmental health because of its ease of accessibility for sampling and how it is temporally tied to human AMU (Rizzo et al., 2013;Hendriksen et al., 2019).

Proactive responses to AMR include clinical (human and animal) ASTs and AMU alternatives such as gene editing. The use of ASTs allows for specific and targeted therapy towards a particular infection in both humans and animals. This practice of "test first" avoids the usage of broad-spectrum antimicrobials that are often associated with higher incidences of AMR and MDR (Johnson et al., 1995;Reid et al., 2007;Yoshida et al., 2022). Reactive responses to AMR include the use of plant bioactives and phage therapy as alternatives to using antimicrobials in treating AMR infections. The rationale behind using both of these strategies is that they represent therapeutic options beyond our known antimicrobials "of last resort", such as

vancomycin and third-generation cephalosporins. In some instances, plant bioactives can be used as a direct replacement for antimicrobials (Mosolygó et al., 2019;Nik Mohamad Nek Rahimi et al., 2022;Suvaithenamudhan et al., 2022) or as an adjuvant to make previously resistant bacteria sensitive to antimicrobials by lowering the minimum inhibitory concentrations (MICs) (Borges et al., 2016;Shriram et al., 2018;Shehabeldine et al., 2020;Yu et al., 2020). Phage therapy is used as a direct substitute for antimicrobials as they can lyse bacteria (Lin et al., 2017). This alternative requires a mass panel screening of phage libraries to find suitable phages for each specific individual infectious bacterium. However, both of these strategies face the same fundamental challenge as AMU, that microorganisms will develop resistance over time in the presence of a selective pressure. The goal of using these alternatives is to not only create additional therapeutic approaches, but to integrate therapeutic stewardship into their delivery.

1.6 Current State of AMR across Livestock Production Systems

There are a number of different factors that have been shown to have an impact on the current state of antimicrobial resistance within different livestock production systems. One of the most widely known is AMU, as illustrated by comparison of antimicrobial resistance in bacteria isolated from natural ("Raised Without" antimicrobials) or organic production systems as compared to conventional (use of antimicrobials) production systems. Several studies have been conducted to investigate the impact that lowered AMU has on prevalence of ARGs (Rovira et al., 2019;Nobrega et al., 2021;Weinroth et al., 2022). When investigating beef and dairy cattle production systems, there are specific classes of ARGs that differ between natural and conventional AMU. These ARG classes tend to be tetracyclines, multidrug ARGs, macrolides, β-lactams, and aminoglycosides (Rovira et al., 2019;Weinroth et al., 2022). However, a number of

factors contribute to the prevalence of ARGs including specific facility sites (Weinroth et al., 2022), diet, husbandry management, and cattle source (Rovira et al., 2019). A meta-analysis showed that the degree of AMU can influence ARG prevalence (Nobrega et al., 2021). Lowered AMU either had no effect, or in some instance the prevalence of some ARGs in cattle, swine, and poultry, including *van*A, *mec*A, $bla_{\text{CTX-M}}$, *mcr*-1, *aad*A2, *tet*(E), *tet*(P), *vat*(E), *sul*2, *dfr*A5, and *dfr*A13 actually decreased. Reducing AMU did not alter the prevalence of β-lactam ARGs in poultry or swine, whereas the prevalence of tetracycline resistance genes was highly variable in cattle, swine, and poultry.

Intensity of livestock production (i.e., time to market weight and number per facility) can be broken into two categories, intensive and extensive. Extensive production systems are typically associated with pastures, while intensive ones are associated with high density housing and high caloric diets. Intensive swine production systems are linked to increased ARG prevalence in addition to more plasmid-associated ARGs (Mencía-Ares et al., 2020). Higher density leads to increased proximity of animals, which facilitates the dissemination of ARGs via direct bacterial transfer and through an increase in the prevalence of mobile genetic elements (MGE).

Diet also plays in important role of the resistome of livestock. In dairy cattle, overall ARG abundance drops as calves are weaned and the diet is changed to solid feed, with the exception of tetracycline and MLS (macrolide, lincosamide, streptogramin) ARGs which were shown to increase (Liu et al., 2019). This pattern indicates that an adult-like diet is a factor that increases the presence of ARGs important to human health. Additionally, cattle fed forage were shown to have more chloramphenicol and microcin ARGs, while those fed concentrate had higher levels of aminoglycoside and streptomycin ARGs (Auffret et al., 2017). The possibility

that the addition of minerals to the diet alters ARG profiles has also been investigated. For example, addition of copper to the diet of swine was shown to decrease *Streptococcus, Lactobacillus, and Bifidobacterium* in fecal microbiomes, but did not impact the resistome (Brinck et al., 2023). Zinc supplementation has been shown to increase macrolide resistance in *E. coli* and *Enterococcus spp.* isolated from feces (Murray et al., 2021). One of the major reasons why diet change impacts resistome profiles, is due to the changes in the microbiota. A different microbiota composition will inherently have a different resistome profile as well.

1.7 Sequencing Methodologies

Understanding the technologies utilized for metagenomics is important in selecting a sensitive, yet cost-effective method to undertake in a study. Short-read sequencing is the cheapest of currently available sequencing technologies. There are two widely used short-read sequencing technologies; Ion Torrent and Illumina with the later being the most popular. The length of these reads ranges from 100 up to 600 bps, with the advantage of pair-end reads providing 2 copies of a single DNA fragment (Hu et al., 2021). All next-generation sequencing involves preparation of a library of DNA fragments (or RNA in the case of RNA-seq) with Illumina using a “bridge amplification approach” wherein a bead-linked transposase facilitates tagmentation, a process that simultaneously fragments and ligates DNA to a bead (Bruinsma et al., 2018). A single round of PCR (polymerase chain reaction) hybridization allows for sequencing adapters to be added to DNA fragments and removed for sequencing. Cyclic reverse termination is employed in the hybridization with 3′-blocked deoxynucleotides with fluorophores specific to each nucleic acid enabling the sequence of nucleotides to be determined (Bruinsma et al., 2018). While short-read sequencing is relatively accurate, one of its major limitations is the

time and resources required to achieve adequate coverage of metagenomic samples. Furthermore, using short-read data to identify MGEs often fails to provide assemblers with enough context to detect the highly repetitive regions and disproportionately under detects plasmids and genomic islands (Maguire et al., 2020).

An alternative method is long-read sequencing, including the platforms; Oxford Nanopore and PacBio. While these technologies are grouped together based on read size, they are markedly different in their length range and methods. PacBio can generate reads 10 – 300 kbps from a single DNA molecule and employs fluorescent subreads that are read through spectroscopy. Advanced algorithms are required to understand the complex mosaic of wavelengths and generate a consensus sequence for the single DNA molecule (Rhoads and Au, 2015). Oxford Nanopore is similar in that it sequences a single molecule, but it relies on reading the native electrical signal of each nucleic acid through a nanopore and translating that information into sequences that can be upwards of 3 Mbps using an advanced algorithm (Clarke et al., 2009). An advantage of these long-read technologies is their fast turn-around time and ability to cover an entire DNA molecule, overcoming some of the context issues found with short-reads. However, these technologies are more expensive than short-reads and have a higher error rate (Hu et al., 2021).

One strategy to overcome some of both short- and long-read technologies' limitations is to use a hybrid assembly approach. This method acquires reads from both short- and long-read sequencing methods and uses a *de novo* assembler algorithm to construct contigs with the scaffold of the error-prone long-read sequences combined with the more accurate short-reads to fill in the details. Tools that perform this function include hybridSPAdes (Antipov et al., 2016) and OPERA-MS which have been used specifically for metagenomic data (Bertrand et al., 2019).

While this method can generate “super contigs” it is an expensive and time-consuming method given the repetitive sequencing and computational complexity of bioinformatic pipelines needed to generate assemblies for down-stream analysis.

1.8 (Meta)genomic Strategies to Study AMR

An overview of all described (meta)genomic methods is visualized in Figure 3. The most established method for studying AMR is through the characterization of individual isolates (Pan American Health Organization, 2020) and has been used as the standard approach in ASTs and whole genome sequencing. This method yields phenotypic resistance information and detailed genomics which can help inform human and veterinary clinicians with regard to appropriate antimicrobial therapies. A drawback of this method is that it requires the bacteria and archaea to be culturable (Barer and Harwood, 1999), a limitation that is particularly relevant to characterizing AMR in diverse environments where it is conservatively estimated that 81% of microbial cells have yet to be cultured in the laboratory (Lloyd et al., 2018). This means that isolate-based genomics is unable to fully capture and quantify the diversity of bacteria/archaea and ARGs in complex environments.

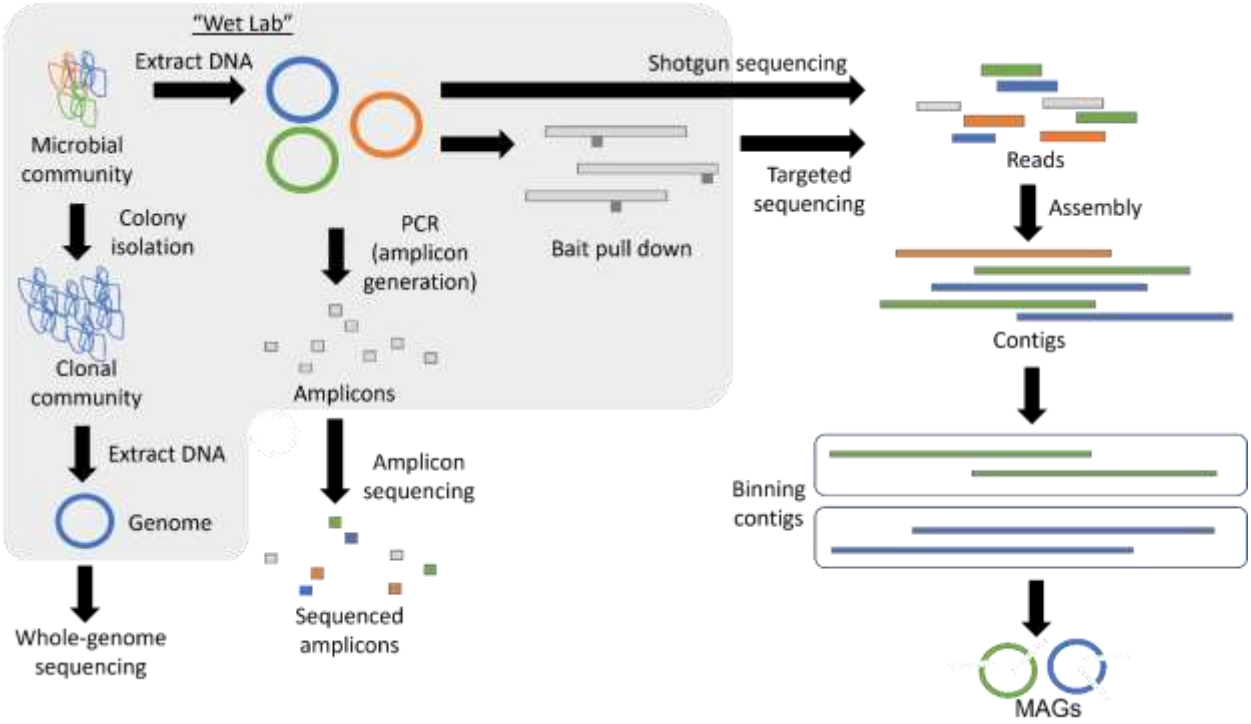

Figure 3. Overview of genomic and metagenomic methods to identify bacteria and antimicrobial resistance genes within microbial communities. Grey area represents in vitro steps of DNA detection while white represents in silico steps. (MAGs = metagenomically assembled genomes)

As an alterative to direct culture for identification, PCR has been used to amplify highly conserved 16S rRNA sequences to identify bacteria within communities (Amann et al., 1995). This technique has been expanded to include using the 18S rRNA gene (Kowalchuk et al., 1997) as well as internal transcribed spacer regions to identify protozoa, fungi and plants (Baldwin et al., 1995;Alvarez and Wendel, 2003;El Karkouri et al., 2007). While being more sensitive to underrepresented taxa as compared to isolation, this approach only generates phylogenomic information and does not enable the characterization of ARGs or MGEs. However, primers can be designed to detect individual ARGs, but such an approach can be laborious given the plethora of ARGs that are often associated with environmental samples. A technique used to simplify this approach is targeted bait capture hybridization which utilizes biotinylated oligonucleotides

("bait") to capture DNA molecules via hybridization (Gnirke et al., 2009). This technique is adaptable and highly customizable to specific ARGs of interest (Eramo et al., 2017;Raseala et al., 2020), but is limited to the number of probes that can be synthesized and is unable to capture the entire resistome. Such an approach is also only applicable to known ARGs.

Shotgun metagenomics is a procedure that is similar to targeted bait capture, with the exception of the bait pulldown step (Figure 3). This provides a more cost-effective approach to capture the entire metagenome, but it relies more heavily on bioinformatic analysis to identify key elements of interest (i.e., taxonomy, resistome, mobilome, etc.). There are a number of different avenues to study AMR using metagenomics: sequences can be aligned to AMR databases or assembled into contiguous sequences (contigs) which can be analyzed individually or be used to generate metagenomically assembled genomes (MAGs). Some of the available AMR databases that can used to identify ARGs include CARD (Comprehensive Antibiotic Resistance Database; (Alcock et al., 2023)), PATRIC (Pathosystems Resource Integration Center; (Antonopoulos et al., 2019)), NCBI (National Center for Biotechnology Information), and MEGARes (Doster et al., 2020). The advantage of read-based alignment for studying AMR, is the ability to survey all sequence fragments against a particular database, as large amounts of information are lost in the attempt to construct contigs and MAGs from contigs. The disadvantage is that relying solely on read-based methods results in the loss of the context of ARGs, making it difficult to assess information on gene mobility and potential associated risks.

1.9 Conclusions

Antimicrobial use has provided human and animal health systems with solutions to many infectious diseases. They can treat animals and humans presenting with infection as well as

prevent infections in at-risk populations. However, inappropriate AMU has contributed to the current global AMR crisis. Understanding the extent of the current situation can be holistically addressed with a One Health approach. Several surveillance programs have been deployed at local, national, and international scales using isolate- and metagenomics-based strategies to track AMR in the three components of One Health continuum, with a notable deficiency in the environmental sector. Many attempts to study metagenomes have failed to also account for the role of MGEs as facilitators of AMR and virulence transfer between bacterial species. Evaluation of the type of sequencing being performed on metagenomic samples is of importance: hybrid assembly of sequences is the most robust but when factoring in cost, short- or long-reads are more often to be the chosen technique. MAGs are one strategy to overcome any deficiency in context, but employing this on a surveillance level approach is difficult in terms of time and resources while also failing to truly survey the entire metagenome as many contigs fail to meet the quality standard for inclusion in analyses. Combination approaches in terms of sequencing and analysis types are essential for a complete study using metagenomics, especially in determining AMR transfer.

1.10 Project Overview

This project aimed to develop and employ bioinformatics methods to study AMR across principal livestock productions systems in Canada. With the goal of comparing “natural” raised without antimicrobial systems to “conventional” systems regularly using antimicrobials, this study investigated the fecal/cecal metagenomes of beef cattle, swine, and broiler chickens through the collaboration of three Agriculture and Agri-Food Canada Research and Development Centres (Lethbridge, Sherbrooke, and Guelph, respectively). Microbiomes and resistomes were

studied for all livestock production systems while beef cattle were further investigated for the co-localization of ARGs and MGEs. Datasets used in this project are outlined in Table 1. The first dataset was used to establish methods for detecting and associating ARGs and MGEs. The second dataset was used to compare the differences of ARGs and MGEs in fecal and catch basin water samples between beef cattle feedlots with and without the use of antimicrobials. The last dataset was used to compare the differences of ARGs and microbiomes between both livestock species and with or without AMU.

Table 1. Descriptions of datasets used in this project.

Dataset	Type of Samples	Region	Number of Samples	Related to Chapter
Agriculture-Environmental	Cattle fecal composite	Alberta	20	2
	Catch basin water		13	
	Manured soil		4	
	Wastewater influent		6	
Natural vs Conventional Feedlots	Cattle fecal composite	Alberta	60	3
	Catch basin water		13	
Canada-Wide Livestock Sectors	Cattle fecal composite	Alberta	60	4
	Swine fecal	Québec	66	
	Broiler chicken cecal	Ontario	16	

CHAPTER 2

Evaluation of Metagenomic Assembly Methods for the Detection and Characterization of Antimicrobial Resistance Determinants and Associated Mobilizable Elements[1]

[1] This chapter from section 2.1 to 2.4 inclusive, are from Lee, C., Ortega Polo, R., Zaheer, R., Van Domselaar, G., Zovoilis, A., and Mcallister, T.A. (2023a). Evaluation of metagenomic assembly methods for the detection and characterization of antimicrobial resistance determinants and associated mobilizable elements. *Journal of Microbiological Methods***,** 106815.. Study was conceptualized by R.O.P, R.Z., and T.A.M.; Provision, management, and access of bioinformatics tools and computing environment was facilitated by R.O.P. and G.V.D. Metagenomic assemblies were performed by C.L. and R.O.P. Data was analyzed by C.L. Interpretation of data was performed by C.L., R.O.P., and R.Z. Manuscript draft prepared by C.L. Manuscript reviewed and edited by all authors. Funding acquisition by R.Z., A.Z., and T.A.M.

2.1 Data Summary

The DNA sequence data used in this study was accessed from BioProject IDs PRJNA420682, PRJNA529711, PRJNA507800 and PRJNA482680 [DOI: 10.1186/s12866-019-1548-x (Zaheer et al., 2019)]. All analysis was performed using Agriculture and Agri-Food Canada's high performance computing cluster Biocluster, the National Microbiology Laboratory (Public Health Agency of Canada)'s high performance computing cluster Waffles, and their instance of Galaxy. All parameters for specific tools are described in the text.

2.2 Introduction

Antimicrobial resistance (AMR) is a global health issue that should be addressed using a One Health approach (Shallcross and Davies, 2014;McEwen and Collignon, 2018). One Health considers the health of three sectors, humans, animals, and the ecosystem as a single continuum (Behravesh, 2019). One Health can be applied to the pathogenicity of microbial infections and the associated risks of antimicrobial resistance causing treatment failure. AMR is a concern to all One Health sectors, but particularly in the human and animal sectors where over-prescription and misuse of antimicrobials are of major concern (Xiong et al., 2018;Ramachandran et al., 2019).

There are numerous ARG databases available, many with their own specific annotation tools. For example, CARD (Comprehensive Antibiotic Resistance Database) uses the Resistance Gene Identifier (Alcock et al., 2023), whereas Staramr (Bharat et al., 2022) uses ResFinder (Florensa et al., 2022). ABRicate (Seemann, 2020) identifies ARGs using a variety of reference sources, including MEGARes 2.0 (Doster et al., 2020) and ResFinder for the resistome. PlasmidFinder for the mobilome, and the Virulence Factors Database (VFDB) for virulence

determinants (Liu et al., 2022a). The AMR++ pipeline uses the MEGARes database for resistome discovery (Doster et al., 2020), but is unique in that it is a combination of ResFinder, CARD, and NCBI's Bacterial Antimicrobial Resistance Reference Gene Dataset (Feldgarden et al., 2019). These datasets encompass resistance genes and determinants to conventional antibiotic drugs, biocides, metals, and multi-class resistances. In this study, the MEGARes 2.0 database was selected due to its characteristic of using multiple databases, in addition to being implemented in the AMR++ pipeline.

Antimicrobial resistance genes (ARGs) are encoded within the genomes of both commensal and pathogenic bacteria (human, agricultural, and environmental). They can be transferred horizontally (Dröge et al., 1998) as mediated by mobile genetic elements (MGEs) (Frost et al., 2005). Integrative and conjugative elements (ICEs) are among the most common MGEs. They can integrate into the bacterial chromosome, enabling them to be replicated and transferred to daughter cells during cell division. However, ICEs can also excise from the chromosome and transfer to other bacteria through conjugation (Burrus et al., 2002). ICEs have been shown to harbour various cargo genes, including ARGs in bacteria of greatest health concern (Partridge et al., 2018;Botelho and Schulenburg, 2020). These pathogenic bacteria are most frequently members of the Firmicutes and Proteobacteria (Partridge et al., 2018;Farzand et al., 2019;Botelho et al., 2020;Botelho and Schulenburg, 2020). Other MGEs that can play a role in the transfer of ARGs include plasmids, transposons, and Class I integrons. Due to their importance in transferring ARGs from commensal to potentially pathogenic bacteria, understanding the linkage of MGEs and ARGs is integral to developing approaches to reduce AMR (Slizovskiy et al., 2020).

Integrating ARGs and their association with MGEs into AMR surveillance and risk assessment requires assessing the co-localization of the mobilome and resistome. This process is time consuming and expensive if done through clonal isolation and whole-genome sequencing (WGS). Alternatively, shotgun metagenomics can facilitate high-throughput analyses for AMR surveillance (Pillay et al., 2022;Sherry et al., 2023). Short-read sequencing technologies are much more affordable and have lower error rates than long-read approaches, making them popular for genomic surveillance. However, the read lengths achievable by these technologies limits their ability to characterize ARGs in their surrounding genomic context.

A variety of tools and strategies are being developed or proposed that utilize metagenomic data for AMR surveillance (Zankari et al., 2017;Juraschek et al., 2021;Nobrega et al., 2021;Cheng et al., 2022;Pillay et al., 2022;Sherry et al., 2023). Targeted and *de novo* assembly methods have been proposed, along with binning of metagenome-assembled genomes (MAGs) based on similar relative abundance and sequence composition. Targeted assembly uses an algorithm that constructs contiguous sequences (contigs) from sequenced reads via alignment to a guiding sequence scaffold. *De novo* metagenomic assembly constructs contigs without a guiding scaffold and provides a more comprehensive metagenome without database biases, but at the cost of reduced coverage. MAG binning is an additional construction step that generates hypothetical genomes to closely approximate the biological genomes in a metagenomic sample (Bowers et al., 2017). This study aims to use short-read sequences to determine which of these methods, assembled contigs (targeted or *de novo*) or binned MAGs is most conducive to determining the context of ARGs to MGE.

2.3 Materials and Methods

2.3.1 The Dataset

A previously generated dataset of sequenced metagenomic samples was selected for this study (Zaheer et al., 2019). This dataset originated from four different sample types: pen fecal composites (FC) collected at cattle feedlots, catch basin water at feedlots, soil from surrounding fields that had been fertilized with feedlot manure, and urban/municipal sewage influent from two municipal wastewater treatment plants. Samples were sequenced using Illumina HiSeq2000 to generate short, 100 bp paired end reads (Zaheer et al., 2019). Sequence Read Archive (SRA) accession numbers for paired-end short-read sequenced metagenomic samples (n=43, including FC n=20, catch basin n=13, soil=4, municipal sewage influent n=6) were accessed from the National Center for Biotechnology Information (NCBI) Short Read Archive (SRA) database from the BioProject IDs PRJNA420682, PRJNA529711, PRJNA507800 and PRJNA482680. For trial-and-error optimization of targeted assembly, *de novo* assembly as well as AMR and MGE co-localization, a single fecal composite sample (SRR6512893) was selected based on its high abundance of Proteobacteria and Firmicutes as these phyla tend to have a high abundance of MGEs (Partridge et al., 2018;Farzand et al., 2019;Botelho et al., 2020;Botelho and Schulenburg, 2020). Therefore, selecting a sample with a high number of MGEs with adequate coverage increased the likelihood of identifying co-localized MGEs and ARGs. The criteria for a successful method were: 1) number of assemblies was comparable to the literature, 2) co-localization was confirmed, and 3) automatable and replicable workflows to ensure reproducibility for large datasets. All samples were used to compare the overall microbiota composition and quality distribution of MAGs.

2.3.2 ARG and ICE Detection via Sequence Targeted Assembly

Sequence-targeted assembly of the metagenomic samples was accomplished with MetaCherchant v0.1.0 (Olekhnovich et al., 2018) with ICE and ARG sequences in the FC sample SRR6512893 assembled using ICEberg 2.0 (Liu et al., 2019c) and MEGARes 2.0 (Lakin et al., 2017) databases, respectively. Antimicrobial resistance that was linked to genes with a single-nucleotide polymorphism (SNP) and genes involved in the regulation of ARGs were excluded from the analysis.

2.3.3 De novo Assembly and Binning of MAGs

De novo assembly of all metagenomic samples was accomplished using the nf-core/mag pipeline v1.0.0 (Krakau et al., 2022), which also performs binning of MAGs. The nf-core pipeline (Ewels et al., 2020) is based on the Nextflow workflow manager (Di Tommaso et al., 2017), which uses two assemblers, MEGAHIT (Li et al., 2015;Li et al., 2016) and metaSPAdes (Nurk et al., 2017) to perform parallel assemblies and subsequent binning by metaBAT2 (Kang et al., 2019) (Figure 4). The two assemblers were used to compare assembly quality, particularly the number of quality filtered contigs and the completeness of subsequent MAGs. High-quality was defined as >90% completeness with <5% contamination; medium-quality was defined as >50% completeness with <10% contamination, with parameters below this considered to be of low-quality (Bowers et al., 2017).

The following parameters were supplied to nf-core/mag: a mean quality of 20 and trimming quality of 20 for fastp (Chen et al., 2018), Kraken 2 (Wood et al., 2019) database limited to 8 GB (constructed on 2019-04-01), the Contig Annotation Tool (CAT) (von

Meijenfeldt et al., 2019) (generated on 2020-06-18), and a minimum contig size of 1500 bp for both MEGAHIT and metaSPAdes. BUSCO (Simão et al., 2015) was used to identify contigs containing bacterial genes along with bacterial reference database "bacteria_odb9" which contained 148 single-copy bacterial genes from 3,663 species. All other tools included in nf-core/mag [Bowtie2 (Langmead and Salzberg, 2012), FastQC, PRODIGAL (Hyatt et al., 2010), QUAST (Gurevich et al., 2013), BUSCO (Simão et al., 2015), CheckM (Parks et al., 2015), MultiQC (Ewels et al., 2016)] were run using default parameters.

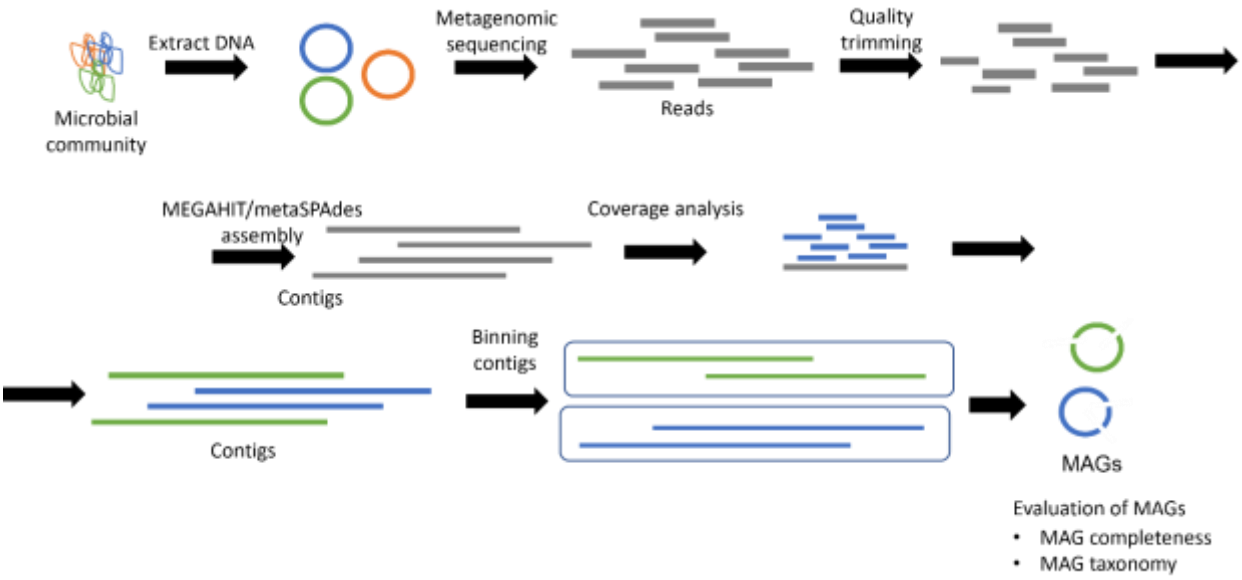

Figure 4. Workflow of the nf core/mag pipeline depicting steps from DNA extraction to the point of binning of metagenomics assembled genomes (MAGs).

2.3.4 ARG and ICE Detection via de novo Assembly

Metagenome-assembled genomes binned from the random FC sample SRR6512893 were locally aligned with ABRicate (Seemann, 2020) using default parameters to the MEGARes 2.0 (Doster et al., 2020) database to detect ARGs. These same MAGs were aligned with BLASTn [basic local alignment search tool – nucleotide (Johnson et al., 2008)] to the ICEberg

2.0 database to detect ICEs. The MAGs containing ARGs were taxonomically classified with the Bin Annotation Tool (BAT) (von Meijenfeldt et al., 2019). The MAG annotations containing both ARGs and ICEs were visualized using Geneious v10.2.5 to determine synteny. BLASTn-based methods like ABRicate to detect ARGs were compared to those of Staramr (with default parameters) that also integrate *k*-mer alignment (Bharat et al., 2022). Staramr was run using ResFinder (Florensa et al., 2022) and PlasmidFinder (Carattoli et al., 2014) databases. ARGs with the tag 'SNP-confirmation required' (single-nucleotide polymorphism) were filtered out as no SNP analysis was performed to identify point mutations in housekeeping genes. Similarly, ARGs with the 'regulator' tag were excluded because while regulatory genes modulate AMR expression, they do not result in resistance if ARGs are absent.

To detect colocalized ARGs and MGEs, all MAGs binned from the random FC sample SRR6512893 were supplied to the tool MOB-recon from the package MOB-suite (Robertson and Nash, 2018). The MOB-recon tool generated plasmid sequences along with information on predicted transferability, replicon family, and relaxase type. Reconstructed plasmid fasta files containing contigs were supplied to Staramr which included ResFinder and PlasmidFinder using default parameters. ResFinder identified ARGs that were colocalized on the previously identified plasmids.

2.3.5 Microbiota Comparison between Methods

Quality-filtered short read sequence data were taxonomically classified using Kraken 2 (Wood et al., 2019) to evaluate contig taxonomic classifications. The contig annotation tool (CAT) (von Meijenfeldt et al., 2019) was used to classify assembled contigs from MEGAHIT

and metaSPAdes. Taxon counts were generated using *phyloseq* (McMurdie and Holmes, 2013) in the R v4.0.1 statistical programming language. Non-metric multidimensional scaling (NMDS) was used to visualize ordination between all classification methods across all samples. NMDS was done at the phylum level to capture the majority of the diversity of the taxa present within the dataset.

2.3 Results

2.3.1 Targeted Assembly

MetaCherchant successfully identified 333 ARGs from the random single FC sample SRR6512893 (Figure 5). The most prevalent classes were tetracyclines (43.8%), MLS (macrolide, lincosamide, streptogramin; 18.3%), sulfonamides (17.4%), β-lactams (5.7%) and aminoglycosides (4.8%). Additionally, a total of 173 putative ICE were found to be associated with at least one ARG within this sample (Figure 6).

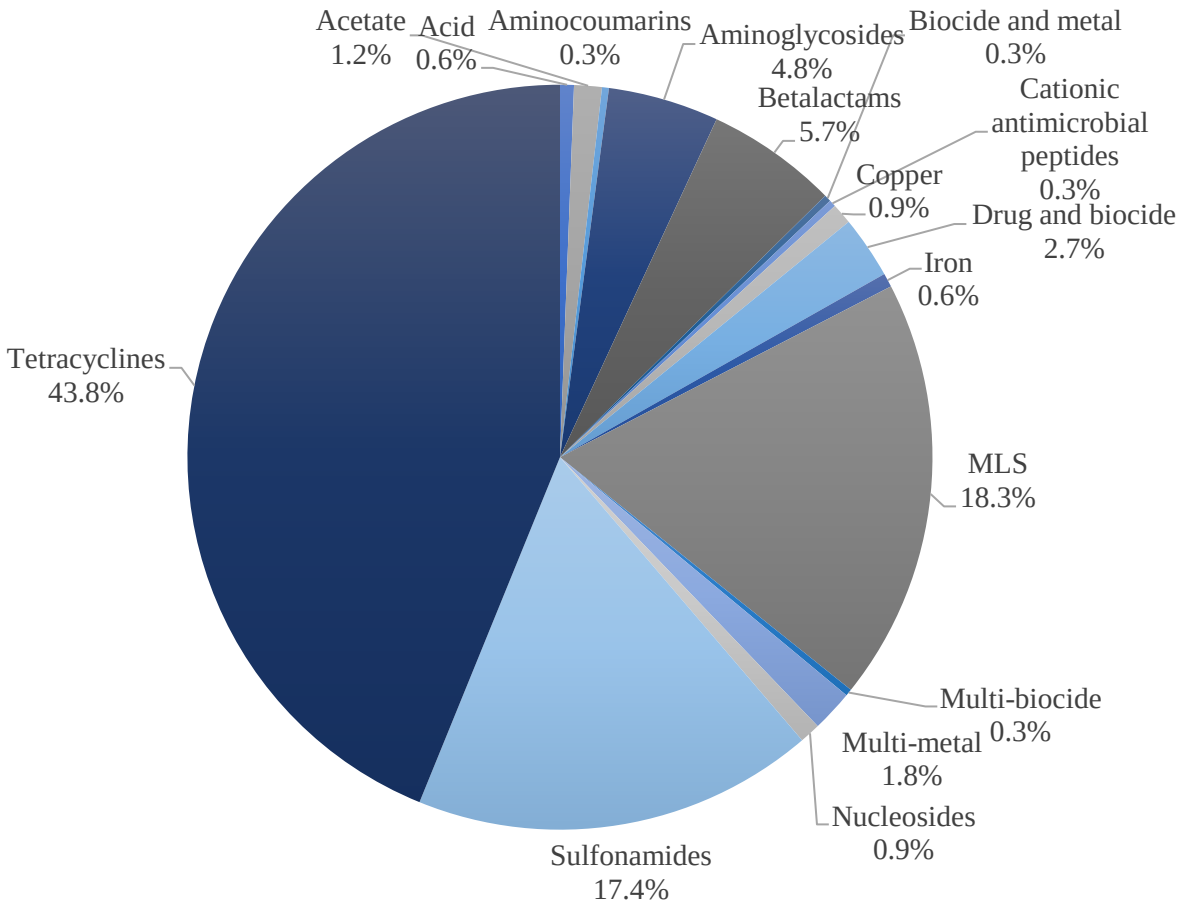

Figure 5. Distribution of antimicrobial resistant gene (ARG) classes from sequence targeted assembly. A total of 333 ARGs were detected in fecal composite sample SRR6512893 (n=1).

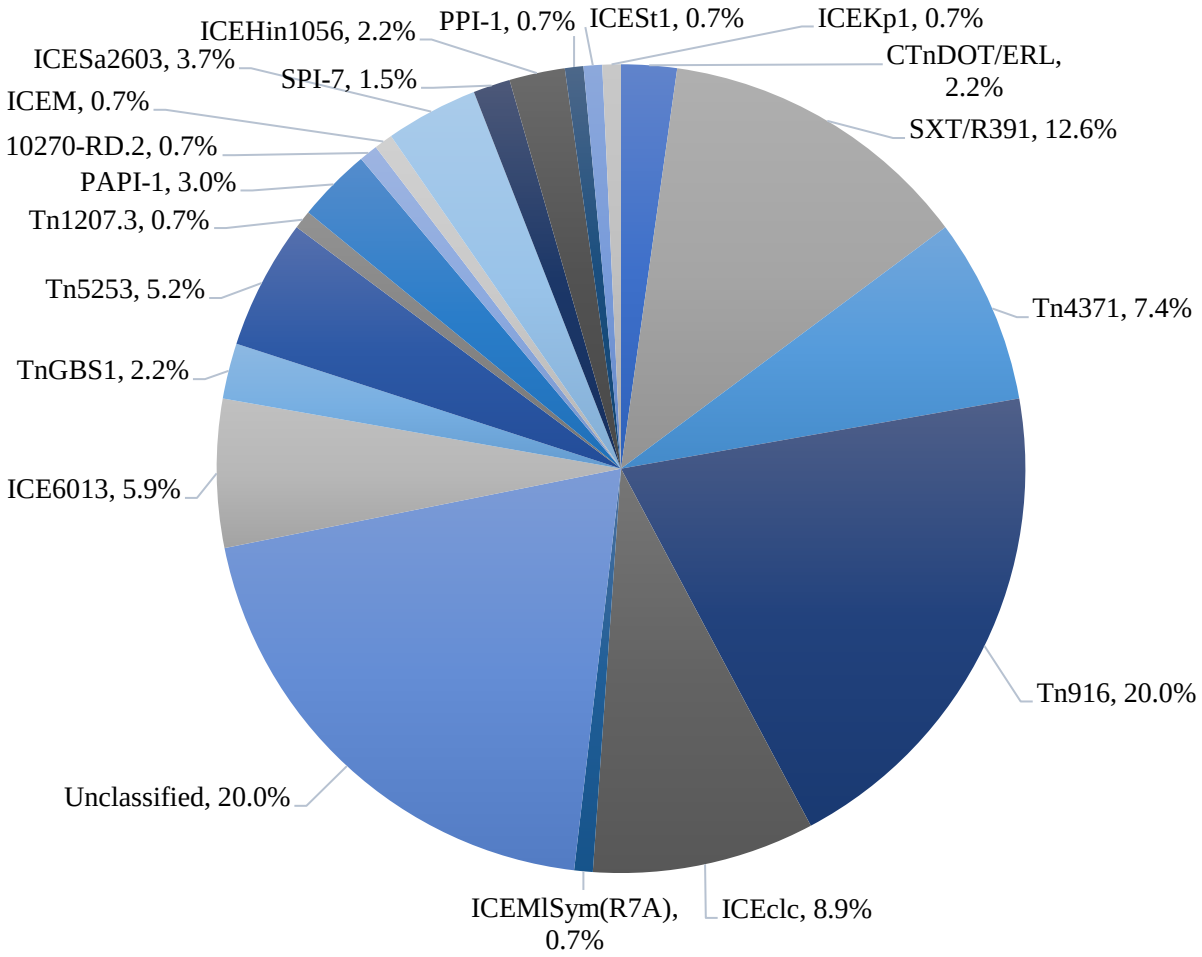

Figure 6. Distribution of 173 integrative conjugative element families associated with an antimicrobial resistant gene detected in fecal composite sample SRR6512893 from sequence targeted assemblies (n=1).

2.3.2 Taxonomic Comparison between Reads and Contigs

Non-metric multidimensional scaling of all fecal composite (Figure 7A), catch basin (Figure 7B) and soil samples (Figure 7C) revealed three distinct clusters with all three classification methods. In municipal sewage influent (Figure 7D), there was no distinction between the contig assembly methods, but read classifier Kraken was a distinct group.

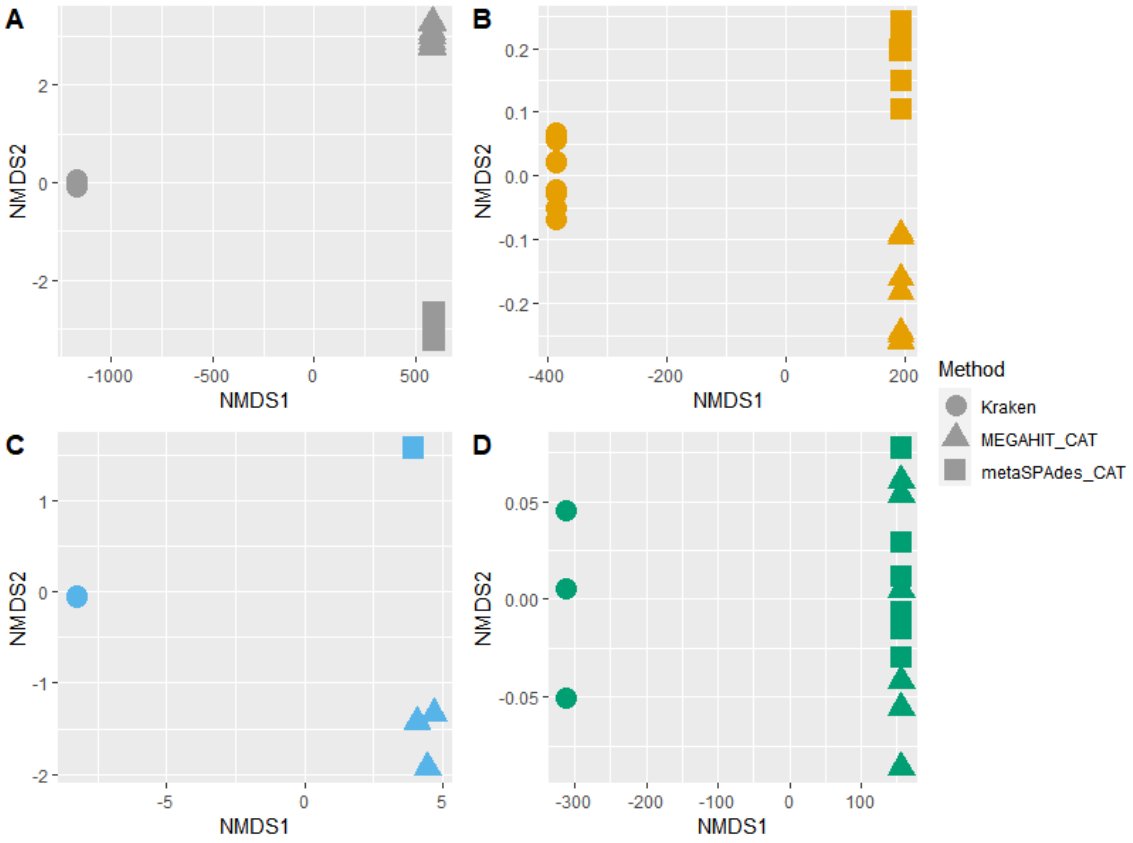

Figure 7. Non-metric multidimensional scaling of the phylum composition of each of the four environments' [A) fecal composite, n=20; B) catch basin, n=13; C) soil, n=4; and D) municipal sewage influent, n=6] prokaryotic communities compared by taxonomic classification method. Depicting Kraken 2's classification of short-reads (circles), CAT's (contig annotation tool) classification of MEGAHIT assembled contigs (triangle), and CAT's classification of metaSPAdes assembled contigs (square).

2.3.3 De novo Assembly and MAG Binning

We assessed the binning quality from all environments using all samples. MEGAHIT produced 327 high-quality and 1848 medium-quality MAGs, while metaSPAdes produced 267 high-quality and 1925 medium-quality MAGs. Across all environments and assemblers, there were more medium-quality than high-quality MAGs per sample (Figure 8). The fecal composite

and catch basin samples had the highest number of binned MAGs per sample. Fecal samples averaged 52.3 mean high-quality and 58.4 mean medium-quality MAGs, and catch basin samples averaged 47.2 mean high-quality and 49.6 mean medium-quality MAGs. Soil samples had the lowest mean number of recovered high-quality and medium-quality MAGs. Overall, the recovery rate of MAGs that were high quality were 8.4 and 7.0 MAGs per sample for MEGAHIT and metaSPAdes assemblers, respectively.

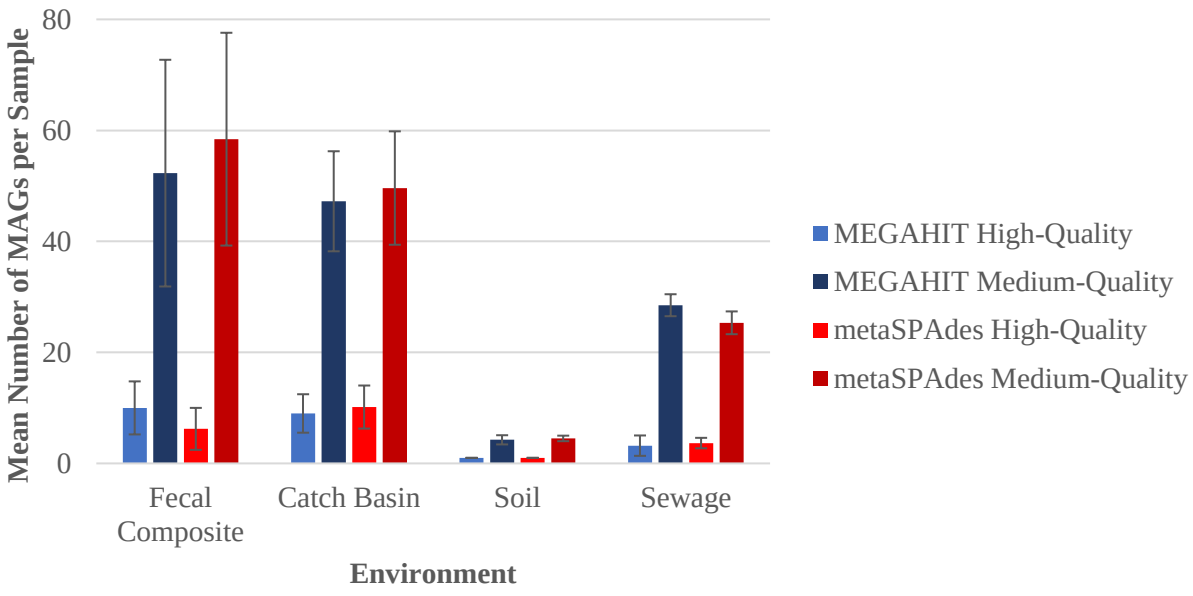

Figure 8. Mean distribution of high and medium-quality MAGs in fecal composite, catch basin, soil, and municipal sewage influent. Error bars represent standard deviation.

2.3.4 ARG and MGE Co-localization from MAGs

The same random FC sample, SRR6512893, used in section 3.1 was the subset of MAGs selected for further co-localization analyses to provide consistent comparison across all optimization methods. MEGAHIT assembled ten high-quality and 64 medium-quality MAGs. In

contrast, metaSPAdes assembled five high-quality and 69 medium-quality MAGs. Alignment of MAGs with BLASTn to the MEGARes database identified one high-quality and four medium-quality MAGs that contained at least one ARG (Table 2). Notably, only the medium-quality MAGs contained ICEs, regardless of their association with an ARG. One medium-quality MAG derived from MEGAHIT and one medium-quality MAG derived using metaSPAdes, both possessed *tetZ* and *sul1* and were classified as *Tessaracoccus sp.* One of the MAGs possessed 23 different ARGs. This MAG could not be classified beyond the Bacterial domain. The counterpart for this MAG was not found in the MEGAHIT assemblies.

Table 2. Antimicrobial resistance genes, mobile genetic elements\ and bin classifications of high-quality and medium-quality metagenome-assembled genomes binned from the representative fecal composite sample, SRR6512893.

Assembler	MAG Quality	MAG ID	ABRicate ARGs	MAG Taxonomy	ICE Aligns w/ ARGs	Staramr ARGs	MOB-recon Plasmid
MEGAHIT	High	29	*mphN*	Clostridiales	0	-	-
	Medium	72	*tetW*	Firmicutes	1 (no ARG align)	-	-
	Medium	30	*tetZ, sul1*	*Tessaracoccus sp.*	1 *tetZ*; 3 *sul1*	*tetZ, sul1*	-
metaSPAdes	Medium	74	*tetZ, sul1*	*Tessaracoccus sp.*	1 (no ARG align)	-	-
	Medium	149	23 ARGs – MDR[1]	Bacteria	2 (no ARG align)	-	pCM959, IncFIA

[1]MDR (multi-drug resistance; defined as resistance to >3 classes of antimicrobials).

Analysis of the MAGs with Staramr and ABRicate yielded different results (Table 2). Only MAG MEGAHIT-30 came back with an AMR-positive profile using Staramr. The same ARGs, *tetZ* and *sul1,* were detected with both methods. Staramr's output from PlasmidFinder

(Carattoli et al., 2014) showed that only metaSPAdes-149 contained the plasmid IncFIA. This finding was supported by the three 'non-mobilizable' plasmids reconstructed with MOB-recon, all of which were present in metaSPAdes-149, one of which was also IncFIA. No AMR-positive contigs were identified by Staramr, suggesting that the reconstructed plasmids did not contain ARGs. A combination of ABRicate and BLASTn yielded four more ARG- and MGE-positive MAGs than Staramr and MOB-recon.

2.4 Discussion

Targeted assembly methods to detect ARGs and MGEs like MetaCherchant (Olekhnovich et al., 2018) are more computationally efficient than *de novo* assembly approaches such as metaSPAdes or IDBA-UD (Slizovskiy et al., 2020;Calderón-Franco et al., 2022). Leveraging the efficient, focused approach of targeted assembly would simplify and streamline downstream data analysis. Targeted assemblers such as MetaCherchant (Olekhnovich et al., 2018) rely on a reference sequence database to generate assemblies. Targeted assembly methods can also provide context of flanking sequences in relation to reference sequences, which can be useful for defining the association of ARGs to MGEs. Comparing the resistome from targeted assemblies to the original read-based resistome of fecal samples revealed a remarkable similarity in the composition of ARGs (Zaheer et al., 2019). This comparison identified tetracyclines ARGs as being predominant, followed by those associated with MLS, β-lactams, and aminoglycosides. Sulfonamide ARGs were the only class to be detected in the targeted assembly, but not in the read-based resistome.

The reference-based assembly approach employed in this study identified 173 putative ICEs, but their linkage with ARGs could not be confirmed, due to the lack of overlapping reads

between ARG and ICE. Thus, this method failed to achieve the goal of co-localization of ARGs and MGEs in metagenomes, but larger sequences as obtained from *de novo* assembly and/or MAG binning were able to establish this linkage. Others have also been able to use MAGs derived from full-scale granular sludge along with human and cattle fecal samples to establish linkages between ICE and ARGs (Slizovskiy et al., 2020;Calderón-Franco et al., 2022).

Overall taxonomic classifications differed depending on the classifier tool used. The largest differences were seen between Kraken 2 and both CAT classifications (from MEGAHIT and metaSPAdes), as opposed to assembler type approaches (MEGAHIT vs metaSPAdes). Kraken 2 uses an exact alignment of *k*-mers to assign reads to the lowest common ancestor (Wood et al., 2019). In contrast, CAT and BAT use an open reading frame (ORF) algorithm to identify translated protein homologs and assign classification based on the NCBI non-redundant protein database (von Meijenfeldt et al., 2019). Another explanation for these differences could arise from the fact that Kraken 2 (MiniKraken) and CAT/BAT (NCBI-nr protein database) utilize different taxa classification databases. Our finding of the different classifications between the read-based and assembly-based approaches is not surprising, given that the coverage of taxa abundance differed between reads and contigs, with reads having higher coverage. A prior study found that MEGAHIT only assembled 55.8% of available reads (Li et al., 2015), whereas CAT and other contig-based classifiers had higher precision than the read-based classifier Kaiju [0.6-0.7 precision in contig- based versus 0.1-0.25 in read-based (von Meijenfeldt et al., 2019)]. This finding indicates that while assembly-based methods used in combination with contig classifiers may have higher precision than short-read classifiers, not all diversity is captured due to the substantial number of unincorporated reads. We observed this to be the case in our NMDS

analysis that showed clear clustering of read-based versus assembly-based taxonomic methods across all samples (Figure 7).

The recovery rate of MAGs that passed quality benchmarking (Bowers et al., 2017) was as expected (8.4 and 7.0 average MAGs per sample for MEGAHIT and metaSPAdes, respectively) and was comparable to another study that recovered 1,150 high-quality dereplicated MAGs at rate of 6.4 MAGs per sample from 179 metagenomic samples (Holman et al., 2022). Our study selected only MAGs with a cut-off minimum length of 1,000 bp, to filter out low-quality MAGs. This level of stringency should have allowed for the detection and association of even highly repetitive MGEs, ranging from small miniature inverted repeat transposable elements (50–800 bp) (Crescente et al., 2018) to prophages and transposons (6–358 kbp) (Gao et al., 2020). However, we found little to no co-localization between ARGs and MGEs, despite ARGs having sequences ranging from 237 bp (*dfr*B1) to 1,992 bp (*otr*(A)) (van Hoek et al., 2011). This finding could be due to the comparatively low recovery of MGE, whether co-localized with an ARG or not. While ARGs with the potential for mobilization were defined as being on the same contig as a MGE, those that were on the same MAG but on different contigs could also be potentially mobile as some MGEs exceeded 300 kbp, a size much larger than the average contig in this study.

We found a range of taxonomic classification levels within FC sample SRR6512893, due to the variability in the taxonomic specificity of the assembled contigs. This variable level of taxonomic assignment is comparable to other studies that looked at swine feces (Holman et al. 2022) and activated sludge (Zhao et al., 2020;Holman et al., 2022). The MAG metaSPAdes-149 was unique from the other assembled MAGs as it contained 23 different ARGs and had three non-mobilizable plasmids that did not contain any ARGs. ARGs tend to be more commonly

associated with MGEs and less so with the chromosome (Partridge et al., 2018;Yi et al., 2022), but we found all 23 ARGs to be associated with the chromosome, possibly within ICEs (Burrus et al., 2002). Some studies have found MDR MAGs with over 20 different ARGs (Yi et al., 2022), but the norm is closer to 1–6 ARGs, where a single ARG may confer resistance to multiple antimicrobials as is the case for multi-target efflux pumps (Zhao et al., 2020;Holman et al., 2022;Zhang et al., 2022). Given these observations, it is possible that MAG metaSPAdes-149 is an artifact of assembly and/or binning due to the repetitive elements that are within MGEs. If several MGEs contained numerous ARGs, then it is possible they might have been assembled/binned together into a single MAG. It cannot be conclusively investigated if this MAG is indeed an artefact or an uncultured bacterium due to the nature of how MAGs are binned or to the absence of sequence data on uncultured organisms.

The number of ARGs detected in the quality-filtered sequence read data of SRR6512893 were three orders of magnitude greater than that in MAGs, reflecting a loss in the coverage of ARGs. For this reason, metagenomic genomic surveillance of AMR using short-reads must still rely upon read-based methods as opposed to assembly-based approaches. However, to identify associations of ARGs and MGEs, assembly-based approaches are necessary, even though this approach generates an incomplete picture of relative abundance or the quantity of these elements.

There is a limitation to any AMR study that investigates any non-human sector of the One Health Continuum. All current AMR databases have a human-centric bias to them as they are primarily constructed from commensal or pathogenic bacteria isolated from humans, with an underrepresentation of ARGs from agricultural and natural environments. This bias would result in under detection of ARGs in agricultural and environmental samples, preventing a complete

picture of the resistome. A One Health-focused AMR database has yet to be developed, and until one is constructed, future studies will continue to have a human bias.

Detecting MGEs in metagenomes is challenging, as in addition to the absence of a comprehensive MGE database, no single tool encompasses all of algorithms needed to detect all types of MGEs (Pillay et al., 2022). The method employed in this study was to treat MAGs as incomplete genomes and screen them for MGEs using MOB-suite. This method failed to associate ARG with MGE within MAGs. While this may be a consequence of the low recovery of binned MAGs, it may also be due to a failure to detect ARGs. Methods for the detection of MGEs often involve BLAST alignment to a collection of MGE databases (Slizovskiy et al., 2020;Calderón-Franco et al., 2022), including ICEberg 2.0 for ICEs (Liu et al., 2019c); PlasmidFinder for plasmids (Carattoli et al., 2014); ACLAME (A CLAssification of Mobile genetic Elements) for phage genomes, plasmids, and transposons (Leplae et al., 2004); IS finder for insertion sequences (Siguier et al., 2006); and INTEGRALL for integrons, integrases, and gene cassettes (Moura et al., 2009). Another method proposed by Pillay *et al.* (2022) aligns to MGE databases using a mapping tool, such as BWA-MEM (Li and Durbin, 2009) or Bowtie2 (Langmead and Salzberg, 2012). This method of aligning to multiple databases could be more successful in the recovery of MGE in metagenomes as compared to aligning to a single database or using a single tool designed for whole-genome sequenced isolates such as MOB-suite.

Despite these numerous methods, MGEs such as plasmids and genomic islands have been shown to be disproportionally absent as compared to core chromosomal regions in MAGs generated using short-read assembled metagenomes (Maguire et al., 2020). This calls for the use of long-read assembly technologies that can span entire repeat regions that make the identification of MGEs difficult. Due to the high error rate (8-12%) associated with long-read

sequencing such as Oxford Nanopore Technologies (Morisse et al., 2021), it is preferable to couple the greater length of long-read sequencing with the higher accuracy of short-reads through hybrid assemblies (Zhang et al., 2020). Different hybrid assemblers have been outlined and compared, with Unicycler being more accurate and producing more contiguous assemblies (Chen et al., 2020). The authors of this study noted the lack of MGEs, particularly plasmids. Undertaking long-read sequencing and applying hybrid assembly to this data would increase the recovery of MGEs and allow for more accurate co-localization analysis.

In conclusion, targeted assembly methods such as MetaCherchant are not suitable for association studies on taxonomy, ARGs, and MGEs using 100 bp Illumina short-reads. *De novo* assembly approaches are likely needed for metagenomics when investigating association experimental questions, such as how the use of antimicrobials affects the prevalence of mobile ARGs in a given population as opposed to determining the specific relative abundance of elements. Second, MAG-based analyses into AMR do not provide comprehensive coverage of ARG diversity, relative abundance, or overall prevalence to adequately inform surveillance studies. Sequence read data is still required to investigate these attributes in metagenomic sequenced environmental samples. Increased sequencing depth, sequence read length and/or hybrid assemblies could overcome this limitation. Third, MGEs are too repetitive to be accurately detected in quality filtered sequence data (especially for short-reads) and need longer sequences gained from assembly to establish the surrounding context of other genes. Long-read technologies coupled with hybrid assembly could help circumvent this issue as well, though not to the same coverage as would be possible from read-based relative abundances. There are two possible recommended approaches to associate the resistome and mobilomes: a) use quality filtered read data (short or long-read) to get ARG relative abundances and MAGs for a more

comprehensive accounting of associations; and b) investigation of co-localized of ARGs and MGEs should be conducted using contigs. Both recommendations should be employed as each individually fails to provide a complete picture the state of AMR. The findings presented here help inform future projects that strive to use metagenomics to characterize AMR in a variety of environments.

CHAPTER 3

Effect of Antimicrobial Use in Conventional Versus Natural Cattle Feedlots on the Microbiome and Resistome[2]

[2] This chapter from section 3.1 to 3.5 inclusive, have been submitted for publication in Microorganisms as of October 30, 2023. Study was conceptualized and designed by R.Z. and T.A.M. Collection of samples and metadata by K.M. Metagenomic DNA isolated by K.M. and R.Z. Assembly workflows provided by D.B.H. Data analysis was performed by C.L. Interpretation of data performed by C.L. and R.Z. Bioinformatics cluster facility availability provided by G.V.D Manuscript draft prepared by C.L. Manuscript reviewed and edited by A.Z., D.B.H., G.V.D., K.M., R.Z., T.A.M.; Funding acquired by A.Z., R.Z., and T.A.M.

3.1 Introduction

Antimicrobial resistance (AMR) is a global health crisis that impacts clinical and animal health, food security, and the environment. By the year 2050, it is expected that antimicrobial-resistant infections will result in 10 million global deaths per year, overtaking cardiovascular disease and cancer as the leading cause of death (Review of Antimicrobial Resistance, 2016). Antimicrobial resistance surveillance programs and risk assessments have been launched in a number of countries, including Canada (Public Health Agency of Canada, 2020;2023), the United States (U.S. Center for Disease Control, 2022), the European Union (European Centre for Disease Prevention and Control, 2022), and at a global scale (Murray et al., 2022;World Health Organization, 2022;Zhou et al., 2022).

Identification of antimicrobial resistance genes (ARGs) using metagenomics is one approach to characterizing the nature of AMR across the One Health Continuum. Metagenomics can be used to compare information on phylogeny, ARGs, mobile genetic elements (MGE) and virulence factors across environments. If sequencing depth and read length are sufficient, the context of ARGs may also be established, providing information on horizontal gene transfer. Of these, ARGs are most frequently transferred via conjugation through the action of a number of MGEs including plasmids, integrative and conjugative elements (ICE), transposons, integrons and insertion sequences (Partridge et al., 2018).

There is a concern with regard to the contribution of AMU in livestock to overall AMR. In beef cattle, the majority of AMU takes place in feedlots as opposed to the more extensive cow-calf sector (Apley et al., 2023;Fossen et al., 2023). While feedlots can house over 40,000 head of cattle, which allows for higher meat production per head (Wagner et al., 2014), these high densities subject individuals to stressors that can encourage bacterial pathogens to

proliferate and rapidly transfer within a herd (Koyun et al., 2023). The increased incidence of morbidity requires much higher AMU to combat infections through therapeutic, prophylactic, and metaphylactic practices as compared to the more extensively managed cow-calf sector (Cameron and McAllister, 2016). As the majority of antimicrobials in beef cattle production are administered to feedlot cattle, it is a logical point to investigate AMR within this portion of the beef production cycle (Holmes et al., 2016). However, given the current knowledge about how AMU increases the prevalence of ARGs, some feedlots have opted to employ natural management practices that prohibit the use of antimicrobials except in therapeutic AMU wherein sick animal are removed from the general population into a sick pen. However, the effectiveness of this practice in limiting the prevalence of ARGs in feedlots is inconclusive (Rovira et al., 2019;Zaheer et al., 2019;Doster et al., 2022).

Shotgun metagenomics is one of the most comprehensive methods to study microbiomes, resistomes, and associated mobilomes (Lee et al., 2023a). Sequencing all of the DNA from a specific environment considers the contribution of the genomes of uncultured bacteria to the resistome, generating a perspective of all known ARGs within a sample or environment. In addition, the degree of AMU may also impact the colocalization of ARGs and MGEs. Some studies have aimed to compare the effect of no AMU in livestock production to the conventional use of antimicrobials on the resistome and found no impact on meat trimmings or fecal resistomes (Rovira et al., 2019;Weinroth et al., 2022).

The goals of this study were to investigate the effect that beef cattle raised without antimicrobials have on the microbiome, resistomes, and associated mobilome of beef feedlot-associated environments. We accomplished this by directly comparing fecal composite and catch basin water samples from conventional (raised with antimicrobials) and natural (raised without

antimicrobials) beef cattle feedlots in Alberta, Canada. We predicted that AMU in conventional beef feedlots would result in increased prevalence and abundance of ARGs and ARGs colocalized with MGEs as compared to natural feedlots.

3.2 Material and Methods

3.2.1 Natural and Conventional Feedlots

Conventional (CONV; n=2) and natural (NAT; n=2) feedlots in Alberta were included in this study. Cattle in one NAT feedlot were fed a typical North American backgrounding diet consisting of 62% corn silage, 27% barley grain, 7% vitamin mix and 4% canola meal and received no antimicrobials, ionophores or hormonal implants. Steers or heifers that required antimicrobials due to clinical illness were removed from the general population and quarantined in a hospital pen in the same feedlot until harvest. Both CONV and one NAT feedlot cattle were fed a finishing diet containing 85% barley grain, 10% barley silage, and 5% supplement. The CONV diet also contained the ionophore monensin at 48 ppm and the macrolide tylosin at 11 ppm (Elanco Animal Health, Greenfield, IN, USA). For heifers, the supplement also contained 0.045 ppm melengestrol acetate (MGA). For the last 40 days of the feeding period, ractopamine hydrochloride was included in the diet at 30 ppm. Cattle also received an implant containing 200 mg of trenbolone acetate and 20 mg of estradiol (REVALOR®-200, Merck & Co., Inc, Rahway, NJ, USA). An average of 180 head of cattle were housed in open pens and all cattle had free access to water and feed. Pen-level metadata (sex and age average) for animals used to collect fecal composites can be found in Table S1.

3.2.2 Fecal Sample Collection from Conventional and Natural Feedlots

Composite fecal samples (20 g) were collected from pens (n=10) in both CONV (n=2) and NAT (n=2) feedlots over two years (August 2016 – July 2018; CONV n=30 and NAT n=30). Each 20 g composite was generated by thoroughly mixing 1 g samples from 20 randomly selected fresh fecal pats within each pen. Composite fecal samples were placed in sterilized Whirl-Pak® bags (532 mL; Sigma-Aldrich, St. Louis, MO, USA) and transported on ice to the laboratory within 4 h of collection. Upon arrival, feces were homogenized, flash frozen in liquid nitrogen and stored at -80 °C in flat sheets.

3.2.3 Metagenomic DNA Extraction and Sequencing

To isolate metagenomic DNA from homogenized and frozen composite fecal samples, 325 mg of sample was transferred into a 2.0 mL sterilized safe-lock snap-cape with 0.4 g of sterilized zirconia beads (0.3 g of 0.1 mm and 0.1 of 0.5 mm sizes). Metagenomic DNA extraction and PCR inhibitor removal were performed following a previously described procedure (Zaheer et al., 2018). The quantity of the extracted DNA was determined by fluorescence at 480 nm using Quant-iT PicoGreen fluorometer (Thermo Fisher Scientific, Mississauga, ON, Canada), and the quality/purity was determined, using a NanoDrop spectrophotometer (Thermo Fisher Scientific), by measuring the ratios of absorbance at 260/280 nm and 260/230 nm wavelengths. Extracted DNA samples with absorbance ratios at 260/280 nm and 260/230 nm of 1.7-2.0 and 2.0-2.2 respectively, were considered acceptable. To evaluate the presence of PCR inhibitors undiluted and various dilutions of the extracted DNA were used as

PCR templates to amplify 16S rRNA gene amplifying with the universal bacterial 16S rRNA primers 27F and 1492R **(Lane, 1991)**.

Genome Québec Innovation Centre (Montréal, QC, Canada) performed all library preparations, sequencing, and quality control steps. Metagenomic sequencing libraries were prepared using a PCR-free shotgun DNA library preparation kit (Lucigen, LGC Biosearch Technologies; distributors: VWR International, Radnor, PA, USA). Prepared libraries were sequenced on an Illumina NovaSeq 6000 platform, with 45 samples multiplexed per sequencing lane to generate 2 × 150 base paired-end (PE) sequence reads. Each sequencing lane was spiked with the PhiX174 *sensu lato* virus genomic DNA library at ~1% concentration of the total DNA loaded per lane for the quality control of cluster generation and sequencing. Sequencing read data are available in the National Center for Biotechnology Information (NCBI) Short Read Archive (SRA) under the BioProject ID PRJNA420682.

3.2.4 Bioinformatics Resources

Metagenomic sequence data were stored on the National Microbiology Laboratory's (NML; Public Health Agency of Canada) high-throughput computing cluster, Waffles, and the Integrated Rapid Infectious Disease Analysis (IRIDA) Platform (Matthews et al., 2018) and was analyzed using a web-based instance of Galaxy (Galaxy Community, 2022) supported by the National Microbiology Laboratory, Public Health Agency of Canada (PHAC NML Galaxy). Microbiome taxonomic and resistome profiling was carried out using the workflow outlined below.

Trimmomatic v0.36 (Bolger et al., 2014) was used to trim adapters from PE reads and filter out low quality reads using the following parameters: leading and trailing adapters with "N" bases or a quality score < 3 were trimmed from sequence reads; a sliding window quality score filtered every four bases with a minimum Phred score of 15; sequences with < 36 nucleotides were discarded; adapters supplied in the TruSeq3 adapter sequence file with a maximum of two mismatches in the initial seed were removed; and if a match score of 30 was reached, the adapter was clipped. Singletons, whereby a read's matching pair failed quality control, were also included in downstream analysis.

In order to remove the Illumina PhiX spike-in control, reads were filtered against the *Escherichia* phage PhiX174 genome (GenBank accession NC_001422.1) using the minimum exact match (MEM) algorithm of the Burrows-Wheeler aligner (BWA) v0.7.17.1 (Li and Durbin, 2009). The sorted alignments were then processed with SAMtools v2.0.2 (Li et al., 2009) to retain only those reads that did not map to the PhiX174 genome. This was done using a flag value of 4 to extract the unmapped reads in binary alignment map (BAM) format. The PE reads that did not map to PhiX174 were then extracted from the alignment using the bamToFastq tool of BEDTools v2.27.0.0 (Quinlan and Hall, 2010). The PhiX-filtered reads were then classified with Kraken v2.0.8-beta (Wood et al., 2019) using the custom Kraken database bvfpa (Zaheer et al., 2018). Kraken2 results were then filtered using a confidence threshold of 0.05 to select for taxonomic assignments http://ccb.jhu.edu/software/kraken/MANUAL.html (accessed on 19 August 2021; Zaheer et al. (2018)). All Chordata reads were filtered out in downstream analyses.

Resistome analysis was conducted in parallel with the taxonomic classification as follows: trimmed PE reads were mapped to ARG sequences in the MEGARes database v2.00 (Doster et al., 2020) using BWA-MEM v0.7.17.1 (Li et al., 2009). The alignments in BAM

format were converted to a sequence alignment map (SAM) v2.0.2 format and post-processed with the Coverage Sampler tool (https://github.com/cdeanj/coveragesampler; accessed on 19 August 2021) using a 75% gene fraction threshold (Rizzo et al., 2013). These output matrices of the resistome and taxonomic composition were then analyzed on a local installation of R (v4.3.1; http://www.r-project.org/; accessed on 19 October 2023).

3.2.5 Assembly of Contigs

For metagenomic assemblies, adapters were trimmed from PE reads using fastp v0.20.1 with the following parameters: leading and trailing adapters were trimmed when "N" bases or quality scores were < 15; a sliding window quality score was filtered for every four bases with a minimum Phred score of 15; and sequences with < 100 nucleotides were discarded. Host and PhiX174 reads were filtered out using the *Bos taurus* (NC_037328.1) and PhiX (NC_001422.1) genomes concatenated with Bowtie2 v2.3.4.3 (Langmead and Salzberg, 2012), when minimum and maximum fragment lengths for valid PE alignments were 0 and 500, respectively. SAMtools flags '-f 12 -F 256' were used to convert SAM files into BAM (binary alignment map) files and only unmapped reads were included for downstream analyses. SAMtools was then used to sort the sequences in the BAM files to enable BEDTools to output PE fastq files. Contigs were assembled with the MEGAHIT v1.2.9 assembler (Li et al., 2015) with a minimum kmer length of 3 bp and minimum contig length of 1000 bp.

3.2.6 Microbiome and Resistome Analysis

Taxa abundances were normalized using the trimmed mean of M-value (TMM) method (Robinson and Oshlack, 2010) as per Pereira et al. (2018). For visualizations, phyla present at < 1% relative abundance were aggregated together into an 'Other' group. Species-level classifications were included in the diversity analyses. Alpha diversity was measured with the Shannon diversity index and beta diversity was measured using the Bray-Curtis dissimilarities calculated in R v4.3.1 with vegan in the phyloseq package v1.44.0. Log2 fold change was computed by the ANCOMBC (v2.2.2 R package treating CONV feedlots as the reference. Fold change was calculated and reported in tables. For decreases in NAT feedlots (i.e., fold change < 1) the inverse was taken to ease in text readability.

From the AMR++ v0.1 workflow, the tool MEGARes v2.0 (Doster et al., 2020) was aligned against a database containing ARGs to antibiotics, biocides, metals, and multi-compound resistance (an ARG conferring resistance to a combination of antibiotics, biocides, and metals). This database contained the following descending resistance classifications: type, class, mechanism, and group. Any reads associated with ARGs based on single nucleotide polymorphisms (SNPs) of house-keeping genes were removed. Additionally, any reads originating from ARG regulators were removed from the dataset due to the fact that in isolation a regulator gene would not directly result in a phenotypic resistance profile. The final analyses were created through local RStudio manipulation with the ggplot2 package v3.4.4 (Wickham, 2016). Abundant resistance classes were defined as those with > 25,000 TMM-normalized counts.

3.2.7 Resistome and Mobilome Colocalization on Contigs

A subset of fecal samples was selected to achieve even representation from differing ARG total abundances. Antimicrobial resistance gene TMM-normalized abundance was deemed as high (>30,000), medium (>10,000), or low (<10,000) at the ARG group level. Two of each abundance category were selected from each feedlot, resulting in a total of 24 fecal samples. Plasmids were predicted using MOB-Typer and reconstructed and extracted from assembled contigs using the MOB-recon tools from the MOB-suite package v3.0.0 (Robertson and Nash, 2018) using default parameters. Using the extracted plasmid contigs as input, Staramr v0.7.1 (Bharat et al., 2022) was used with default parameters to detect ARGs on plasmids. The output of both tools were subject to an in-house R script to correlate ARGs with their associated plasmids. Multidrug resistance (MDR) plasmids were defined as those having ≥ 3 ARGs from different classes of antimicrobials. Chromosomal ARGs were assumed to be any contig that was not identified on a plasmid. Associations were constructed with SankeyMATIC (https://sankeymatic.com; accessed on 20 October 2023) and the circlize R package v0.4.15 (Gu et al., 2014).

3.2.8 Statistical Analysis

Comparisons between NAT and CONV beef production systems and normalized microbial taxa counts of interest were made using the Analysis of Compositions of Microbiomes with Bias Correction (ANCOM-BC; (Lin and Peddada, 2020). False discovery rates were mitigated through p-value corrections (q-value) using the Benjamini-Hochberg method (Benjamini and Hochberg, 1995) with the R package ANCOMBC v2.2.2. This same test was

also applied to the normalized abundant (>25,000 TMM-normalized counts) ARG classes, mechanisms, and groups. For comparisons that were significant ($q < 0.05$), the fold change from CONV to NAT feedlots was calculated. Differences in Shannon diversity indices were determined with Wilcoxon signed rank test ($p<0.05$). Differences in Bray-Curtis distances were determined with PERMANOVA (permutational multivariate analysis of variance; $p<0.05$).

3.3 Results

3.3.1 Microbiota Composition Differences Between Conventional and Natural Systems

The phylum level microbiota composition of the fecal samples was explored through normalized total abundance (Figure 9A). For fecal samples, CONV feedlots had higher ($q < 0.05$) abundances of Bacteroidetes, Euryarchaeota, Proteobacteria, and Spirochaetes. Fecal samples from CONV feedlots also had lower ($q < 0.01$) abundances of Actinobacteria and Tenericutes, while there was no difference in Cyanobacteria or Firmicutes. In catch basin water, no phyla differed in abundance ($q > 0.05$) between CONV and NAT feedlots (Figure 9B). Only fecal samples had a raised alpha diversity from CONV to NAT feedlots ($p<0.0001$; Figure S1) and a difference of beta diversity between feedlots was observed ($p<0.001$; Figure S2).

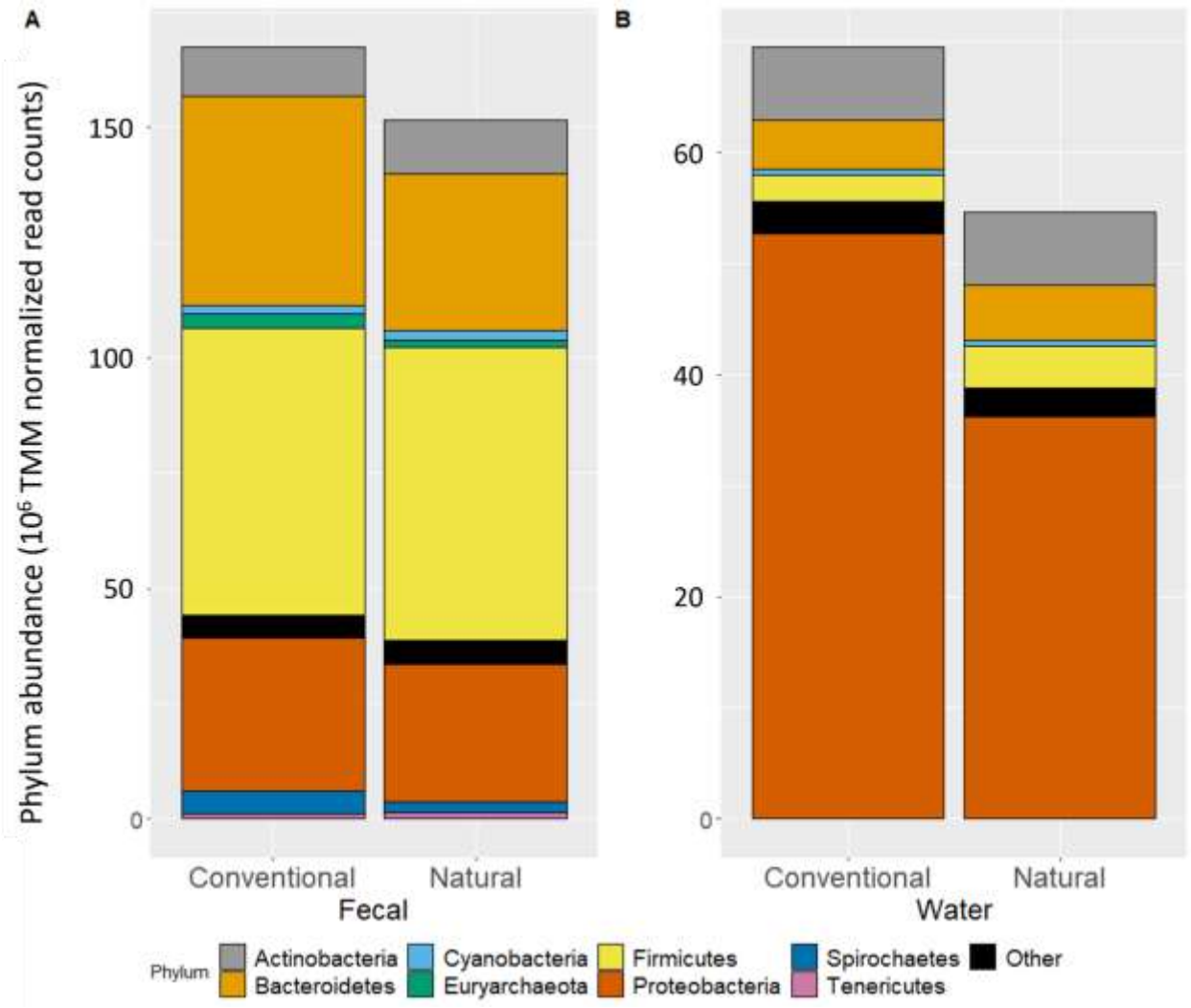

Figure 9. The abundance of phyla (> 1%) in fecal (A) and catch basin water (B) samples normalized with the TMM (trimmed mean of m-values) method across conventional and natural feedlot types.

To quantify differences in the microbiota between NAT and CONV feedlots, mean normalized abundances of taxa at higher resolution than phyla level were compared (Table S2). Of the 17 most relatively abundant (>1%) archaeal and bacteria classes in fecal samples, seven were significantly lower in CONV vs NAT feedlots. The abundance of Methanobacteria and Spirochaetia was higher by 1.7- and 1.5-fold, respectively, in CONV compared to NAT feedlots. For the catch basin water samples, classes did not differ between NAT and CONV feedlots.

The 20 most abundant genera in fecal and catch basin water samples are reported in Table 3. Two genera of note, *Methanobrevibacter* and *Treponema*, exhibited a 1.8- and 1.6-fold increase ($q < 0.01$) in CONV vs NAT production systems. A total of five genera (*Bacteroides*, *Chryseobacterium*, *Methanobrevibacter*, *Prevotella*, and *Treponema*; Figure 10 and Figure **11**) were lower ($q < 0.05$) when NAT vs CONV feedlot feces were compared, whereas seven genera (*Bacillus*, *Blautia*, *Clostridium*, *Eubacterium*, *Lactobacillus*, *Pseudomonas*, and *Streptococcus*; Figure 11) were higher in relative abundance ($q < 0.01$). Genera in catch basin water samples collected from CONV and NAT feedlots did not differ ($q > 0.05$). Five genera that were found to be abundant in both catch basin water and feces in both feedlot types were *Bacteroides*, *Clostridium*, *Flavobacterium*, *Prevotella*, and *Pseudomonas* (Table 3).

Table 3. Trimmed mean of m-value normalized mean relative abundance of the 20 most relatively abundant genera identified in fecal and catch basin water samples from natural and conventional feedlots of mean normalized abundance, interpreted significance of adjusted p-value ($q > 0.05$ = ns; $q < 0.05$ = *; $q < 0.01$ = **; $q < 0.001$ = ***; $q < 0.0001$ = ****), and fold change for significant differences from conventional (CONV) to natural (NAT).

Sample Type	Genus	CONV Mean Normalized Relative Abundance	NAT Mean Normalized Relative Abundance	Interpreted Significance	Fold Change
Fecal Composite	*Alistipes*	0.015	0.016	ns	-
	Bacillus	0.017	0.020	***	1.07
	Bacteroides	0.092	0.075	****	0.82
	Blautia	0.012	0.015	*	1.13
	Butyrivibrio	0.011	0.011	ns	-
	Chryseobacterium	0.014	0.011	****	0.80
	Clostridium	0.038	0.045	ns	-
	Eubacterium	0.013	0.016	ns	-
	Faecalibacterium	0.029	0.033	ns	-
	Flavonifractor	0.011	0.013	ns	-
	Lachnoclostridium	0.017	0.019	ns	-
	Lactobacillus	0.009	0.011	***	1.06
	Methanobrevibacter	0.015	0.006	****	0.55

	Oscillibacter	0.025	0.028	ns	-
	Paenibacillus	0.013	0.014	*	1.03
	Prevotella	0.071	0.051	*	0.77
	Pseudomonas	0.015	0.015	ns	-
	Ruminococcus	0.013	0.015	ns	-
	Streptococcus	0.009	0.011	**	1.04
	Treponema	0.028	0.012	**	0.63
	Acidovorax	0.014	0.017	ns	-
	Aeromonas	0.004	0.013	ns	-
	Allochromatium	0.018	0.003	ns	-
	Arcobacter	0.014	0.011	ns	-
	Bacteroides	0.004	0.013	ns	-
	Bordetella	0.012	0.009	ns	-
	Brevundimonas	0.011	0.005	ns	-
	Burkholderia	0.014	0.011	ns	-
	Clostridium	0.004	0.011	ns	-
Catch Basin	*Desulfomicrobium*	0.084	0.061	ns	-
Water	*Flavobacterium*	0.008	0.009	ns	-
	Hydrogenophaga	0.019	0.007	ns	-
	Marichromatium	0.014	0.002	ns	-
	Methylomicrobium	0.017	0.001	ns	-
	Polynucleobacter	0.010	0.067	ns	-
	Prevotella	0.001	0.012	ns	-
	Pseudomonas	0.058	0.053	ns	-
	Streptomyces	0.023	0.026	ns	-
	Thauera	0.026	0.012	ns	-
	Thiocystis	0.020	0.003	ns	-

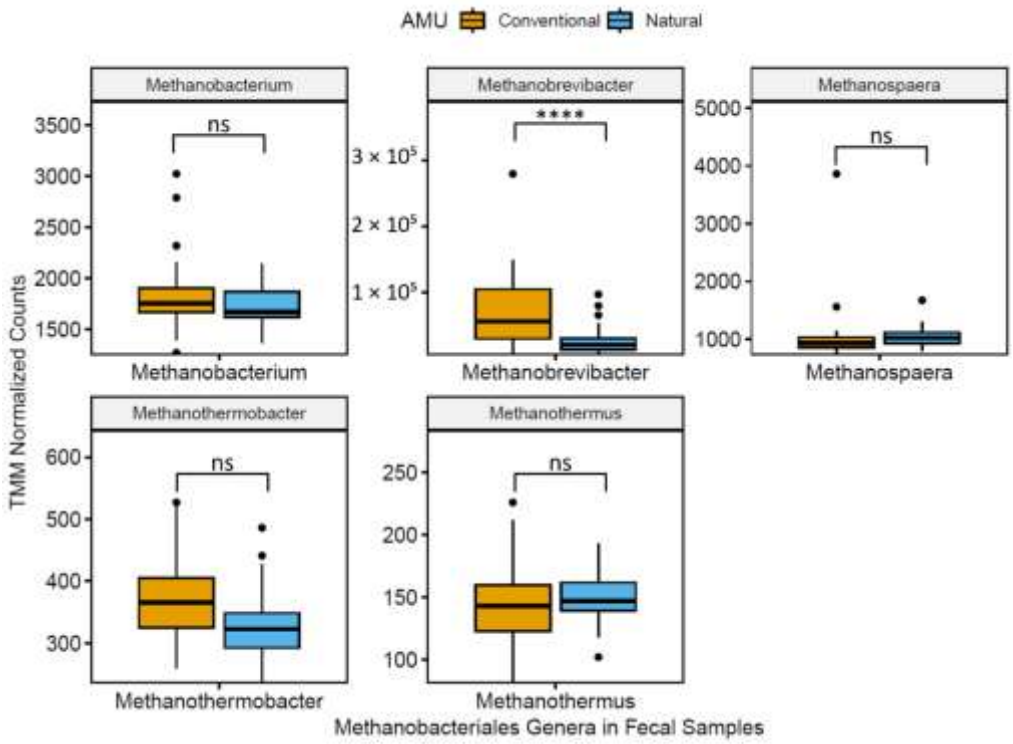

Figure 10. Boxplots of normalized TMM (trimmed mean m-value) abundance of prevalent (>1%) fecal genera within order Methanobacteriales (Analysis of Compositions of Microbiomes with Bias Correction with adjusted p-value significance via Benjamini-Hochberg method; q > 0.05 = ns; q < 0.0001 = ****).

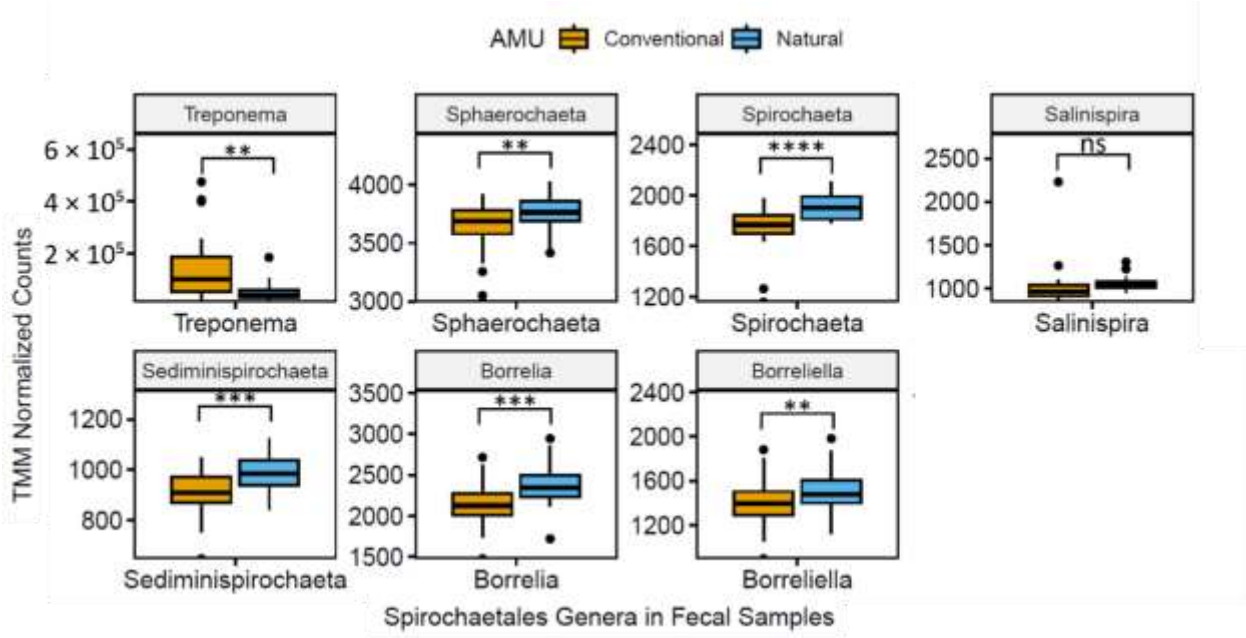

Figure 11. Boxplots of normalized TMM (trimmed mean m-value) abundance of prevalent (>1%) fecal genera within order Spirochaetales (Analysis of Compositions of Microbiomes with

Bias Correction with adjusted p-value significance via Benjamini-Hochberg method; q > 0.05 = ns; q < 0.01 = **; q < 0.001 = ***; q < 0.0001 = ****).

When determining the effect that diet may have had on the NAT microbiomes, only Spirochaetes increased (q < 0.0001) from backgrounding to finishing NAT cattle (Figure S3). When comparing the CONV vs NAT cattle on finishing diets, many of the same differences were observed as the overall CONV vs NAT feedlots except for no differences (q > 0.05) observed for Proteobacteria, Spirochaetes, and Tenericutes (Figure S3).

3.3.2 Resistome Differences Between Conventional and Natural Systems

When all antimicrobial resistance classes (inclusive of biocides and metals) in fecal samples were analyzed, only classes of the drug resistance type were >25,000 TMM-normalized counts. Any present ARG classes' overall abundance were visualized in Figure S4. All samples showed similar resistance profiles, with genes conferring resistance to tetracyclines, MLS (macrolides, lincosamides, streptogramins), β-lactams, and aminoglycosides being most frequently identified (Figure 12A). Tetracyclines were the most abundant ARGs with TMM-normalized counts exceeding 60,000. The MLS ARGs were the next most abundant with over 25,000 TMM-normalized counts. Aminoglycosides and β-lactams did not differ, with <10,000 TMM-normalized counts. There were no significant differences among antimicrobial classes in fecal samples from CONV vs NAT feedlots.

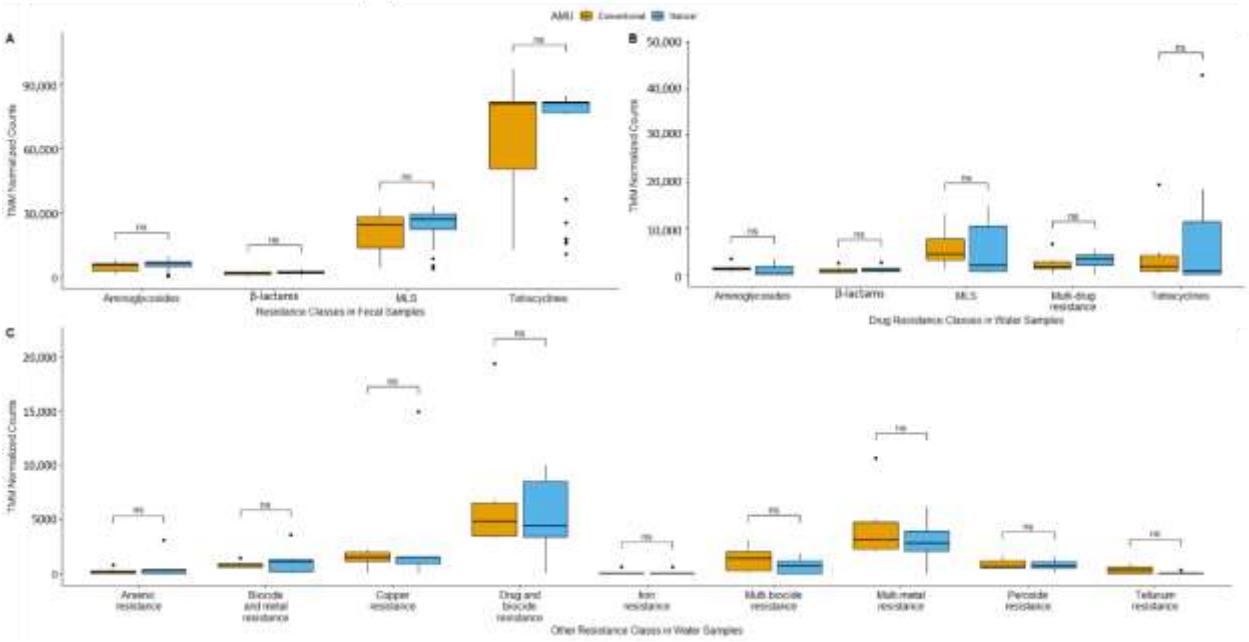

Figure 12. Boxplots of trimmed mean of m-value (TMM)-normalized gene abundance of resistance for abundant resistance classes (>25,000 normalized counts) in fecal samples (A); antimicrobial drug resistance classes in catch basin water samples (B); and biocide and metal resistance classes in catch basin water samples (C) (MLS =macrolide, lincosamide, streptogramin; Analysis of Compositions of Microbiomes with Bias Correction with adjusted p-value significance via Benjamini-Hochberg method; q > 0.05 = ns).

Catch basin water samples analysis revealed many more prevalent ARG classes than feces with abundance >25,000 TMM-normalized counts, including the presence of genes encoding for biocide and metal resistance (Figure 12B,C). Five drug resistance classes in catch basin samples were the same as those in fecal samples with the exception of the multi-drug resistance class being in catch basin water (Figure 12B). There were also nine biocide and metal resistance classes in catch basin water samples which included arsenic, multi-biocide and metal, copper, multi-drug and biocide, iron, multi-biocide, multi-metal, peroxide, and tellurium resistance (Figure 12C). Again, similar to the fecal samples, no differences (q > 0.05) were

observed in most of the catch basin water samples' resistance classes with tellurium resistance ($q < 0.0001$) being the exception.

The resistance groups from the most abundant classes were visualized as heatmaps with grouping according to resistance mechanism for fecal (Figure 13) and catch basin water samples (Figure 14). For both the CONV and NAT fecal samples, ARGs conferring resistance to tetracyclines were most abundant with *tet*W, *tet*Q, *tet*O, *tet*44, *tet*40, and *tet*32 predominating. Aminoglycoside O-nucleotidyltransferases were the next most abundant ARGs with *ant(9)* and *ant(5)* being the most common. There were a number of ARGs that were lower in NAT than in CONV feedlots that had an abundance of >10,000 TMM-normalized counts including: *mef*A ($q < 0.001$), *tet*40 ($q < 0.001$), *tet*O ($q < 0.05$), *tet*Q ($q < 0.0001$), and *tet*W ($q < 0.05$).

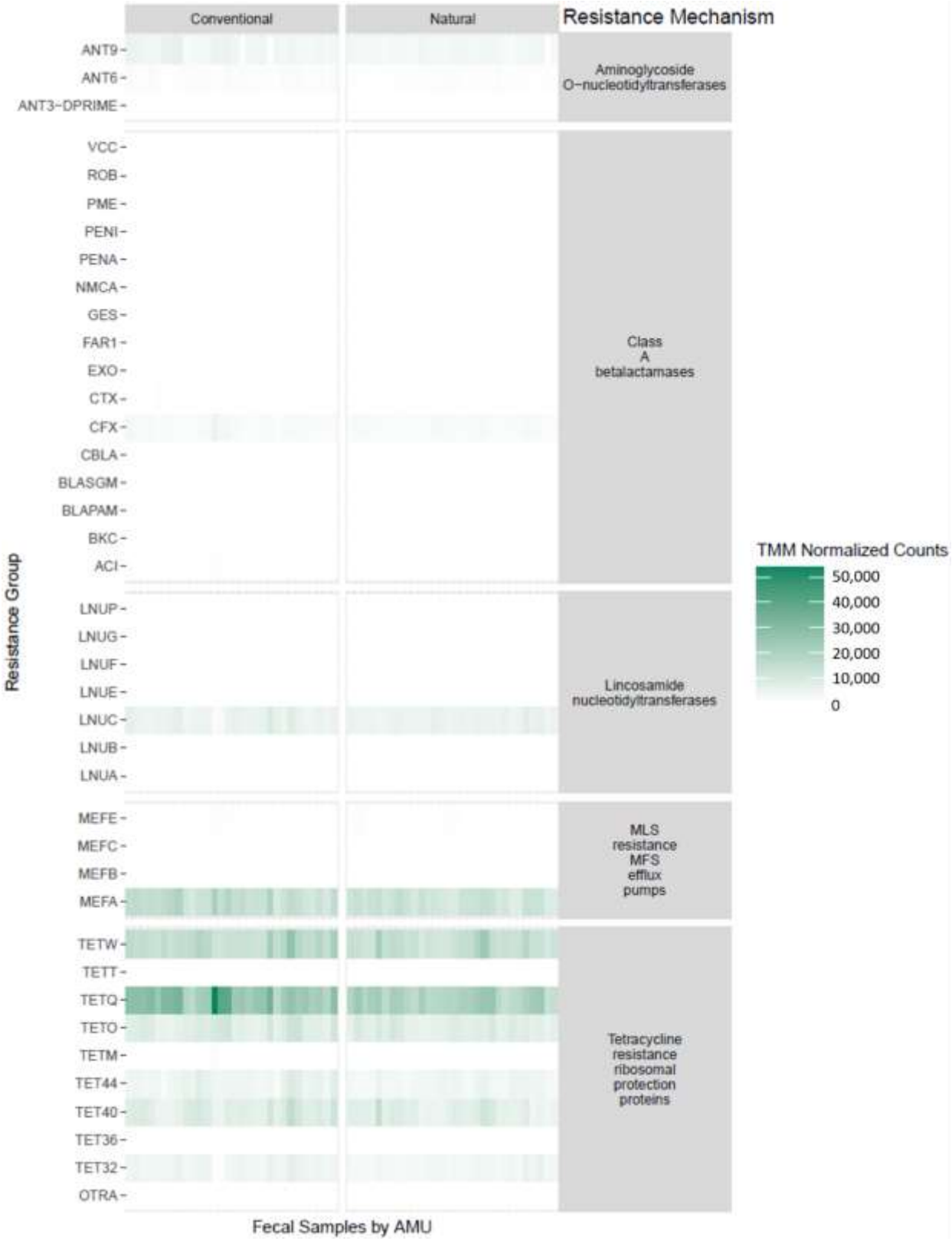

Figure 13. Heatmap of antimicrobial resistance groups from fecal samples stratified into resistance mechanism comparing conventional and natural feedlots.

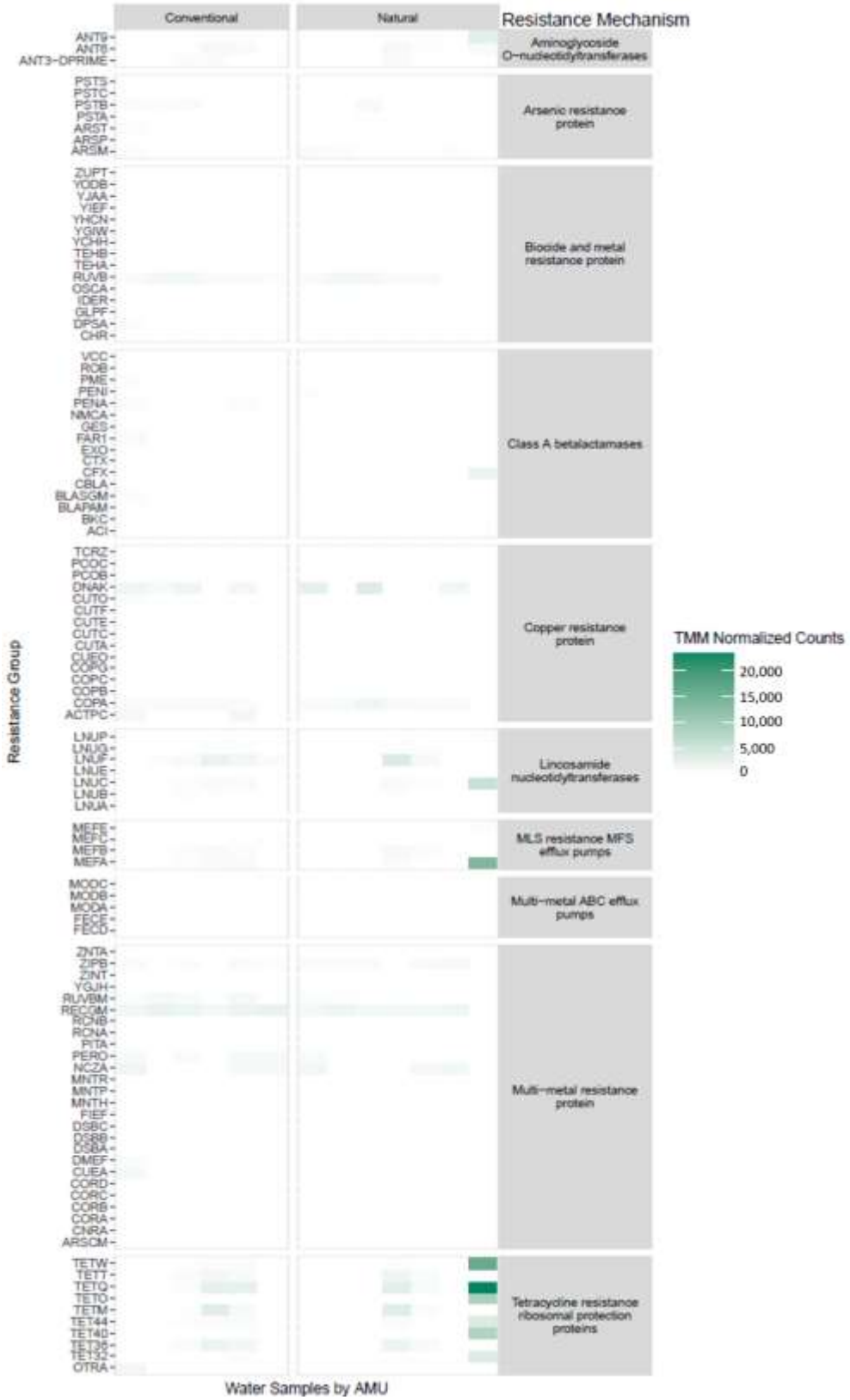

Figure 14. Heatmap of antimicrobial resistance groups in catch basin water samples stratified into resistance mechanism comparing conventional and natural feedlots.

For the catch basin water samples, the main variation in resistance group was associated with individual sample as opposed to feedlot management type. One outlier of note was a sample from NAT Feedlot B that had a catch basin water sample with the same resistance profile pattern as a fecal sample. Most CONV and NAT catch basin samples also possessed genes encoding for biocide and metal resistance proteins (*ruv*B), copper resistance proteins (*actPC*), lincosamide nucleotidyltransferases (*lnu*C), and multi-metal resistance proteins (*zipB, recGM*).

When determining the effect that diet may have had on the resistome of NAT cattle, backgrounding vs finishing NAT samples did not differ in ARG class abundance ($q > 0.05$; Figure S5). Likewise, for the finishing diets, CONV and NAT cattle did not differ in ARG class abundance ($q > 0.05$; Figure S5).

3.3.3 Mobilome and Resistome Colocalization on Fecal Metagenomic Assemblies

Out of 24 fecal samples, 22 had at least one plasmid (Table 4). The two samples that lacked plasmids were from the high-level resistance category (>15,000 normalized counts). There were two instances of MDR, both occurring in CONV samples (Table 4). There were three cases of multiple aminoglycoside resistance genes within a single sample including at least two of *ant(6)-Ia*, *ant*(6)-*Ia* , or *aph(3')-IIIa*.

Table 4. Summary of colocalized antimicrobial resistance gene-carrying plasmids from assembled contigs.

Level of Resistance[1]	AMU	Sample ID	No. of Plasmids with ARGs	No. of MDR[2]-Carrying Plasmids
Low	CONV	Con-Sum-A-05Sep17-MG44	1	-
		Con-Sum-A-26Apr17-MG7	3	1
		Con-Sum-D-26Jun18-MG19	1	-
		Con-Sum-D-26Jun18-MG20	1	-
	NAT	Nat-Sum-B-26Apr17-MG6	2	-
		Nat-Win-B-13Mar18-MG14	3	-
		Nat-Win-C-29Mar17-MG58	1	-
		Nat-Win-C-31Jan17-M7	1	-
Medium	CONV	Con-Sum-D-1Aug17-MG28	2	-
		Con-Win-A-11Dec17-MG55	1	-
		Con-Win-A-13Mar18-MG15	2	-
		Con-Win-D-2Feb17-M10	3	-
	NAT	Nat-Sum-B-26Apr17-MG5	1	-
		Nat-Sum-C-10Apr18-MG9	1	-
		Nat-Sum-C-26Jun18-MG17	1	-
		Nat-Win-B-25Oct16-M1	2	-
High	CONV	Con-Win-A-1Mar17-MG3	2	-
		Con-Sun-A-25Jul18-MG23	0	
		Con-Win-D-1Feb17-M9	4	1
		Con-Win-D-29Mar17-MG59	2	-
	NAT	Nat-Sum-B-28Jun17-M18	2	-
		Nat-Sum-B-29May18-MG45	0	-
		Nat-Sum-C-1Aug17-MG25	1	-
		Nat-Win-C-31Jan17-MG50	1	-

[1]Levels of resistance as TMM-normalized reads: high (>15,000), medium (>5,000), and low (<5,000).
[2]MDR: multi-drug resistance defined at resistance to ≥ 3 antimicrobial classes

There were four resistance profiles containing a single ARG within a plasmid (Figure 15A). The most prevalent AMR profile had genes conferring resistance to tetracyclines (36.8%) and aminoglycosides (18.4%). The next most prevalent resistance profile was a combination of both aminoglycosides and tetracycline resistance genes (15.8%). One of the instances of MDR

was the presence of aminoglycoside, β-lactam, sulfonamide, and tetracycline resistance genes on a single plasmid. The other instance was a plasmid which harboured ARGs encoding for aminoglycoside, chloramphenicol, sulfonamide, and tetracycline resistance. The first MDR plasmid is predicted to be novel (i.e. no match in any database) and conjugative with a host range within the family *Enterobacteriaceae*. The second MDR plasmid was coded as AC935 and was predicted to be non-mobilizable with a host range within the genus *Acinetobacter*.

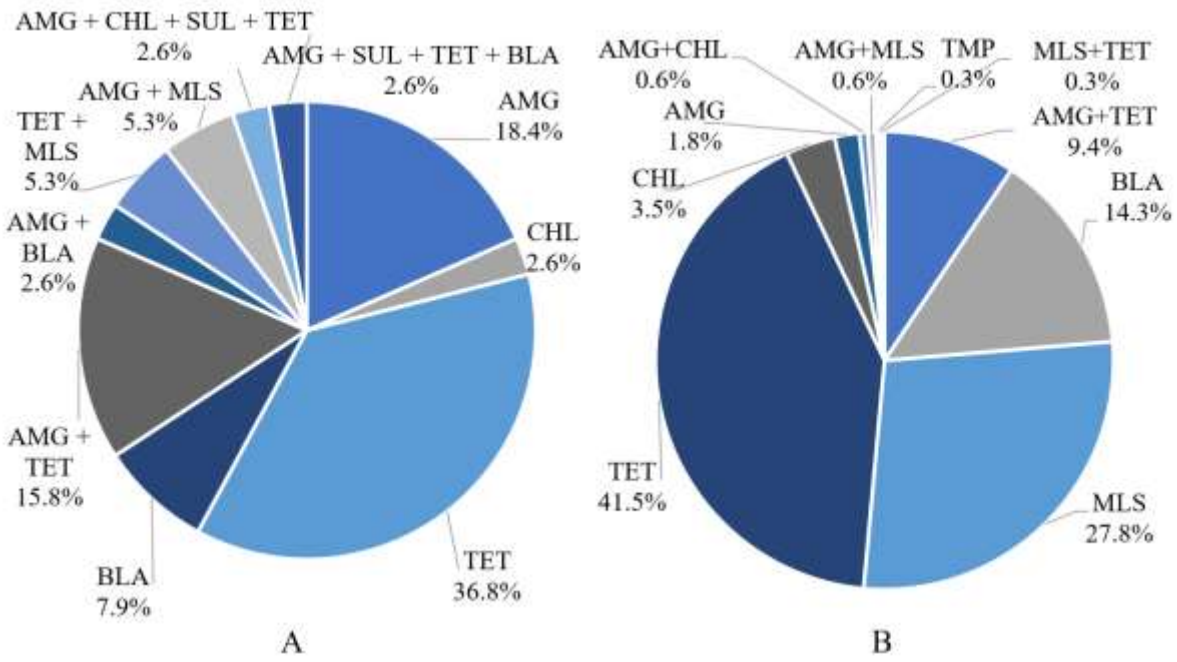

Figure 15. Fecal resistance profiles of ARG-carrying contigs from (**A**) 102 of plasmids (n=24 samples) and (**B**) 342 chromosomes (n=60 samples). AMG = aminoglycoside; BLA = β-lactam; CHL = chloramphenicol; MLS = macrolide, lincosamide, and streptogramin; SUL = sulfonamide; TET = tetracycline; TMP = trimethoprim.

In the chromosomal ARG-carrying contigs the most abundant resistance class was tetracycline (41.5%) followed by MLS (28.7%), β-lactam (14.3%), chloramphenicol (3.5%), aminoglycoside (1.8%) and trimethoprim (0.3%; Figure 15A). In most cases no two ARGs

belonging to the same class were associated with a contig, with the exception of two instances of two aminoglycoside ARGs, *ant(6)-Ia* and *aph(3')-III*, and one instance of two tetracycline resistance genes, *tet*(40), and *tet*(O).

For chromosomal ARGs, there was an even distribution between CONV and NAT feedlots, whereas more plasmid-derived ARGs in CONV as compared to NAT feedlots were identified (Figure 16). A larger proportion of aminoglycoside ARGs were found on plasmids compared to tetracycline resistance genes. Almost all MLS ARGs were chromosomally encoded. Both chloramphenicol and β-lactam ARGs were more likely to be found on the chromosome rather than on a plasmids.

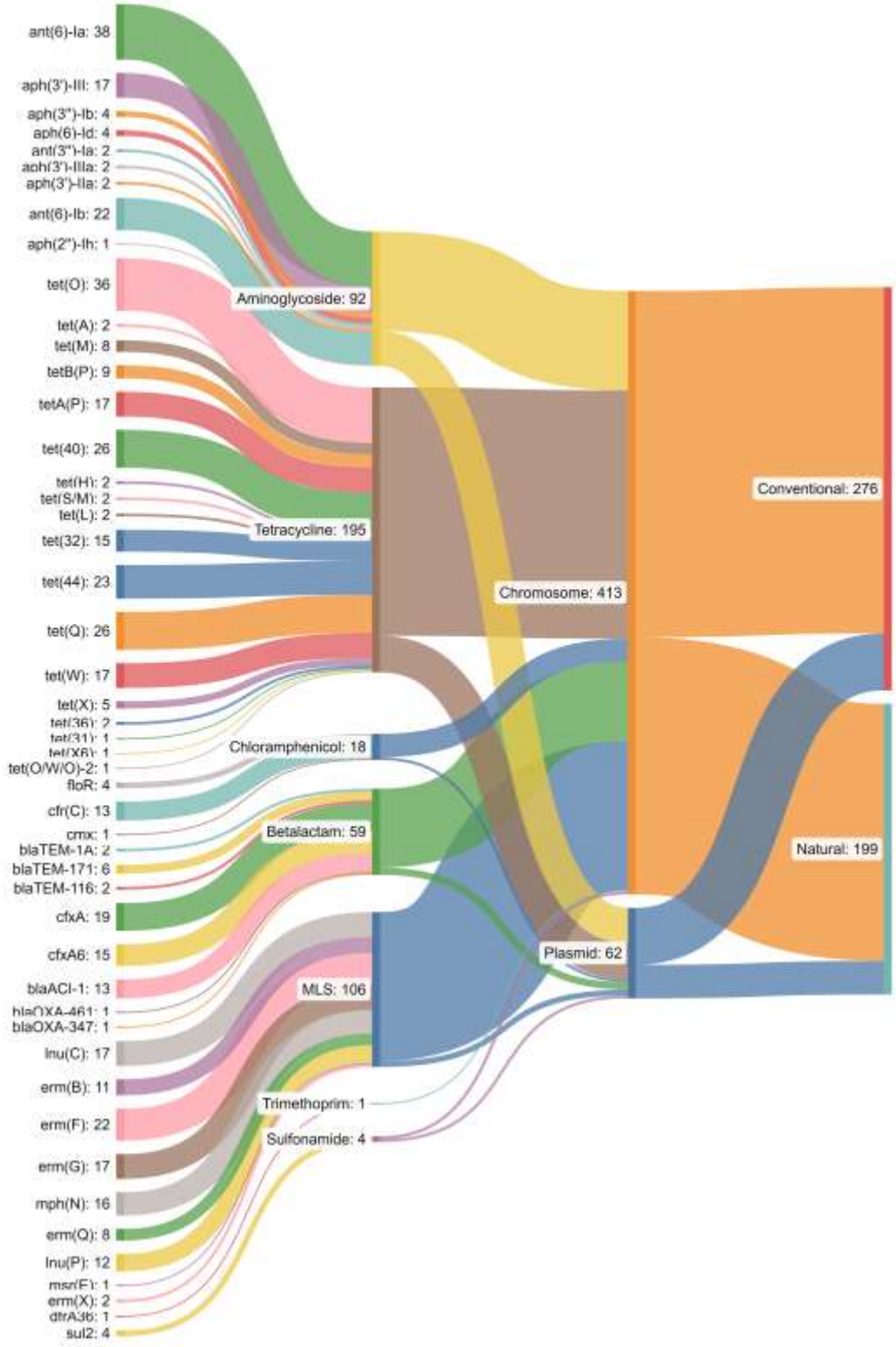

Figure 16. Sankey diagram depicting contigs with antimicrobial resistance genes (ARGs) and antimicrobial classes associated with their respective contig localizations and feedlot type origin.

For the ARG classes of interest, tetracyclines and aminoglycosides both saw a greater association with CONV than NAT feedlots, but MLS did not differ between production systems (Figure 17). For these same ARG classes, all three were more often chromosome- than plasmid-associated. Of all ARG classes, tetracycline and aminoglycosides were most commonly plasmid-associated. Chromosome-associated MLS ARGs were more prevalent than plasmid-associated ones in both CONV (20.3 times) and NAT feedlots (41.0-times). Tetracycline resistance genes exhibited a similar pattern, but of lowered magnitude with CONV being 5.6-times greater and NAT being 9.0-times greater for chromosome-associated compared to plasmid-associated ARGs.

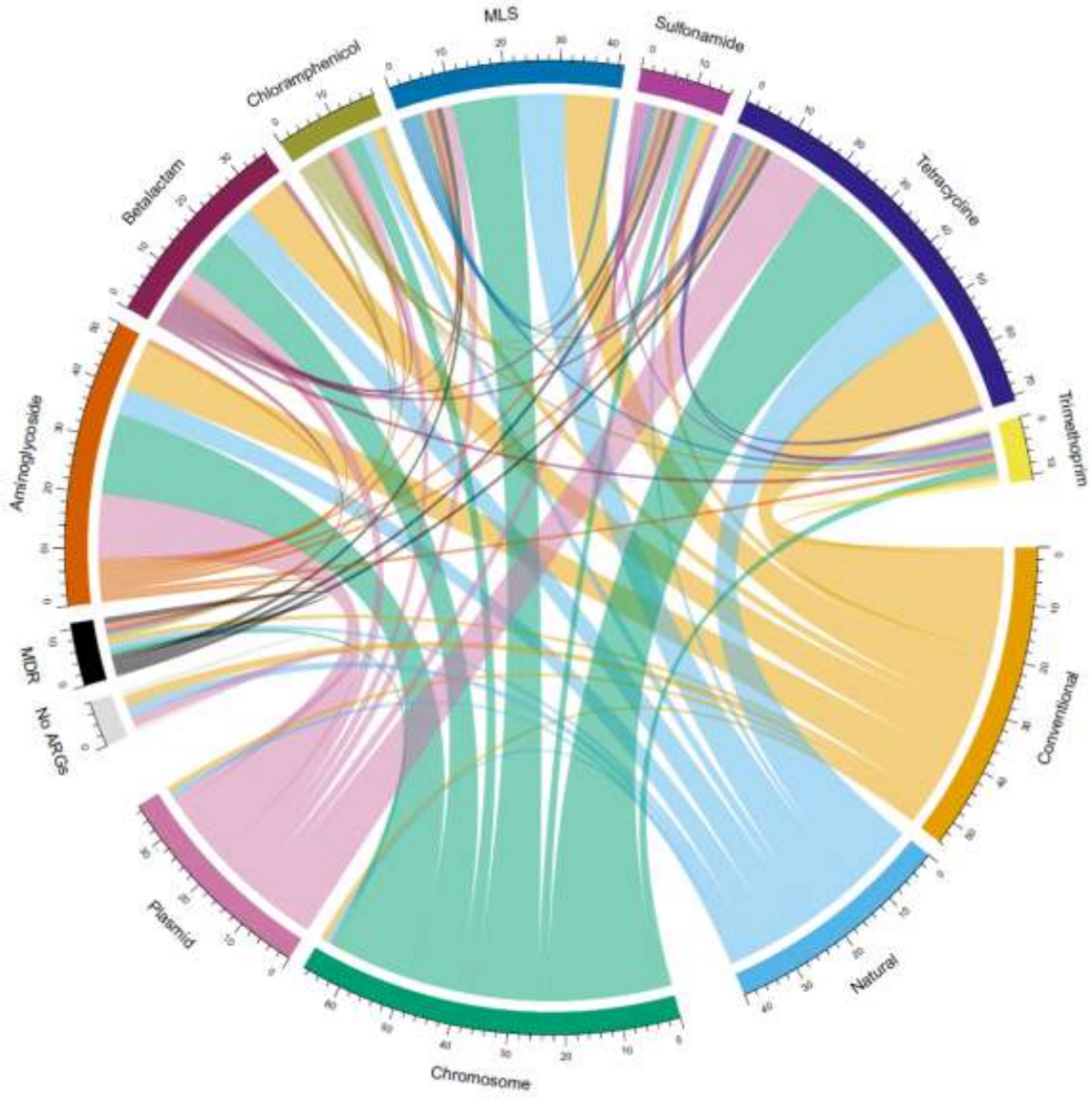

Figure 17. Chord diagram of associations between antimicrobial resistant gene classes in conventional vs natural feedlots, and association with chromosomes or plasmids. Associations within the same antimicrobial resistance gene (ARG) reflect that there are multiple ARGs on the same contig that confer resistance to a single antimicrobial class. (MDR = multidrug resistance from a single antimicrobial resistance gene).

3.4 Discussion

The aims of this study were to observe the effect antimicrobial use in beef cattle feedlots had on the microbiome and resistome using metagenomics. Through the use of shotgun short-read (i.e., Illumina HiSeq2000) sequencing, trends and changes in microbiome and resistome composition have been determined in fecal and catch basin water samples from both natural and conventional beef cattle feedlots. There is conflicting evidence on whether AMU impacts the phylum composition of cattle fecal microbiomes (Weinroth et al., 2019;Halleran et al., 2021;Doster et al., 2022). One study found that there was an increased relative abundance of Bacteroidetes, Firmicutes, and Spirochaetes in the feces of cattle treated with antimicrobials (Hammer et al., 2016). Another found a similar trend with increased relative abundance of Proteobacteria and Firmicutes in CONV cattle fecal microbiomes wherein, they used a model that only was able to account for 0.6% of variance due to antimicrobial exposure (Doster et al., 2022). This lack of model predictability, in addition to the many studies that failed to find large scale differences in the microbiomes of cattle treated with or without antimicrobials (Foditsch et al., 2019;Holman et al., 2019;Weinroth et al., 2019), suggest that antimicrobials may not be responsible for major shifts in bacterial populations within the gastrointestinal tract of cattle. Instead, the variations that our study observed between feedlot systems might be influenced by other factors that differ between feedlot management practices.

Studies have found that grain-based diets are a driver for phylum level microbiome instability (Lin et al., 2023) and that high concentrate diets tend to decrease the overall relative abundance of most taxa (Corrêa et al., 2021). These studies reflect the conditions of the finishing diets (80% barley grain, 15% barley silage, 5% supplement). Our study found that the CONV cattle feces had higher relative abundances of Bacteroidetes and Euryarchaeota than

backgrounding, while Corrêa et al. (2021) found increases of only Firmicutes. This discrepancy might be explained by the high degree of microbiota instability (ability to maintain composition with perturbations) and variation that was attributed to high grain-based diets (Lin et al., 2023).

Several classes were enriched in CONV fecal samples including Bacteroidia, Epsilonproteobacteria, Flavobacteriia, Gammaproteobacteria, Methanobacteria, Spirochaetia. These classes encompass both Gram-positive and Gram-negative bacteria along with archaea (Methanobacteria). The notion that macrolides select for certain bacterial taxa in bovine fecal samples is supported in the literature (Zaheer et al., 2013;Thomas et al., 2017;Stapleton et al., 2021). In particular, macrolide-resistant enterococci have been isolated from beef cattle feces in increasing proportions depending on the duration that tylosin is included in the diet (Zaheer et al., 2013;Stapleton et al., 2021). Enterococci belong to the class Bacilli, contradicting our study as Bacilli were more abundant in NAT feedlots. Another study found that the relative abundance of Bacteroides increased with tylosin and monensin in feed (Thomas et al., 2017). There are few studies that have investigated the effect of AMU on the class taxonomic level, with most being restricted to the phylum level.

Few differences were found in the phyla, classes, orders, and genera between catch basin water samples obtained from NAT vs CONV feedlots. This observation may be due to a dilution effect, as the members of the microbiota in catch masin water are not as directly influenced asby diet as those that reside in feces. Instead, the higher proportions of Proteobacteria that thrive is catchment basins are likely to reduce the differences between NAT and CONV feedlots.

The relative abundance of two genera, *Methanobrevibacter* and *Treponema*, was higher by >1.5-fold in CONV vs NAT production systems. *Methanobrevibacter* spp. are methanogens that are found primarily in the gastrointestinal tract of animals such as termites, ruminants, and

humans (Dighe et al., 2004;Janssen and Kirs, 2008;Grine et al., 2017) and produce methane as a by-product of cellular respiration (Blaut, 1994). Monensin has been shown to target Gram-positive bacteria that produce hydrogen and formate, which are substrates for methanogenesis (Bergen and Bates, 1984) It would have been predicted that the addition of monensin to the diets of cattle in CONV feedlots would result in a lower abundance of methanogens, such as *Methanobrevibacter* (Ogunade et al., 2018). Instead, in our study we found the opposite trend wherein a 1.8-fold increase in abundance of *Methanobrevibacter* was observed in CONV vs NAT feedlot types. This discrepancy highlights the impact of highly unstable microbiota compositions in CONV AMU that obscure any differences the addition of monensin might induce. Additionally, *Treponema spp*. are members of the Spirochaetes that are typically considered to be commensals but can also be associated with bovine digital dermatitis (Klitgaard et al., 2014). Avoiding practices that are associated with an increased abundance of *Treponema* could theoretically reduce the case rate of bovine digital dermatitis, and the attendant AMU. However, this study cannot conclusively identify the management practice(s) responsible for this proposed association.

As with the microbiome, in which the fecal and catch basin water samples were distinct, so too were the resistomes. At the resistance class level, there were differences between the fecal and catch basin water samples. Tetracyclines and MLS ARGs dominated fecal samples, whereas, in the catch basin samples, all ARGs were present at similar levels. There were more ARGs in fecal samples compared to catch basin water samples, as expected, by the higher microbial density in fecal samples. A previous study found that fecal composite samples and catch basin water samples shared 83% of ARG groups and separated into distinct clusters following nonmetric multidimensional scaling analysis (Rovira et al., 2019). The single catch basin water

resistome profile that resembled a fecal composite resistome profile should be treated as a source of “fecal contamination”, and likely arose as a result of a significant run-off event. This is encouraging, because it indicates that the resistomes associated with bacteria present in fecal composite samples are likely contained within the catchment basin.

Of the ARGs examined, the mechanisms that had significantly lower abundance in the NAT as compared to the CONV fecal samples were lincosamide O-nucleotidyltransferases, MLS resistance MFS efflux pumps, and tetracycline ribosomal protection proteins. While significant, the low fold-difference of ~1 indicated that there was essentially no difference between feedlots as a fold-difference of 1 has a ratio of 1:1 (i.e. 100% similarity). As phylum composition in the fecal microbiome may be attributed to diet, so too might the resistome. This conclusion is supported by evidence that certain ARGs tend to be found in a limited number of taxa, as even low-diversity communities can have high levels of ARGs (Pal et al., 2016). The transition of weaned calves onto a solid diet has been associated with increased ARG prevalence, with the exception of tetracycline and MLS ARGs, which were shown to increase (Liu et al., 2019). This suggests that starch digestion is associated with bacteria with a higher prevalence of MLS and tetracycline ARGs. A recent study that sampled NAT beef cattle fed a diet similar to ours (Ma et al., 2022) found that expressed ARGs in the rumen at slaughter were not associated with AMU, but rather with bacterial metabolic pathways.

Intrinsic ARGs are more likely to be contained within a chromosome than on a plasmid due to ‘intrinsic’ meaning belonging to all members of a species (i.e. being a part of the core genome) (Martinez et al., 2009). In a similar case, but not to the same extent, acquired ARGs will more likely be found on mobilizable MGEs rather than associated with the chromosome, such as ICE (Partridge et al., 2018;Botelho and Schulenburg, 2021). Given this trend, it can be

inferred that MLS resistance is more conserved than tetracycline resistance since there were more tetracycline ARGs found on plasmids than MLS ARGs (Zhao et al., 2020). As tetracyclines and macrolides have been both highly used historically in beef cattle, one might predict that they should both be mainly associated with the chromosome. It is unclear why this trend was not apparent for both ARG classes, but it may have to do with co-selection. If there was a highly conserved gene (e.g. heavy metal resistance gene) in proximity to the chromosomal MLS ARG locus, then the MLS ARG could also be highly conserved (Liu et al., 2022b). While this may explain some differences, it is difficult to validate through metagenomics. Nevertheless, these data suggest that tetracycline ARGs are more mobile than MLS ARGs and that tetracycline ARGs are retained even in the absence of tetracycline use. This may reflect the generational selective pressures that tetracyclines have presented to the beef cattle resistome or the use of tetracyclines in backgrounding feedlots or cow–calf systems.

The main limitation of this study is that the contig coverage of the entire metagenome will never be as comprehensive as that of the read coverage. This means that at the assembly step that is required from short-read data to investigate colocalizations and the overall anbundance of elements of interest will always lack precision. The employment of a hybrid assembly approach using short and long-read sequences would be one approach to improve the ability top define the colocalization of ARGs with specific MGE.

3.5 Conclusions

When comparing AMU practices, certain genera (*Treponema* and *Methanobrevibacter*) were enriched in fecal samples from cattle at feedlots that used antimicrobials. Few significant differences were observed in the microbiomes of catch basin samples from NAT vs CONV

feedlots. Contrary to our initial hypothesis, there were also no significant differences in the fecal resistome of cattle between the two feedlot management systems. It seems that generationally selected resistomes through decades of AMU persist even after antimicrobials are not used within the beef production system. The minute differences observed at the ARG group level may be explained by other differences than AMU and diet between CONV and NAT feedlots. More comprehensive investigations into the relationship between livestock diet and AMR should be conducted. Tetracycline selective pressure on the beef cattle industry is strongly established based on the historical use of this antimicrobial and the high abundance of tetracycline resistance genes present in the fecal microbiome. Our study suggests that short-term elimination of AMU is unlikely to substantially reduce the prevalence of ARGs in feedlot environments.

CHAPTER 4

Comparative Metagenomics Of Conventional and Natural Management Production Systems Reveals Few Changes in the Differential Abundance of Antimicrobial Resistance Gene Across Multiple Livestock Production Systems[3]

[3] This chapter from section 4.1 to 4.5 inclusive, is intended for publication targeting the journal Microorganisms. Concept and design of the study done by D.P.-L., G.T., M.S.D., and T.A.M., Collaboration of read sequencing and data sharing by D.P.-L., G.T., K.M., M.S.D., R.Z., and T.A.M. Data analysis performed by C.L. Interpretation of data performed by C.L. and R.Z. Bioinformatics cluster facility availability provided by G.V.D Manuscript draft prepared by C.L. Manuscript reviewed and edited by A.Z., D.P.-L., G.V.D., K.M., M.S.D., R.Z., and T.A.M.; Funding acquired by A.Z., D.P.-L., G.T., M.S.D., R.Z., and T.A.M.

4.1 Introduction

Antimicrobial resistance (AMR) is not only a global threat to human health and food security but also to environmental and animal health as well. The far-reaching impacts of AMR are complex and progressive. Understanding the extent of this threat can be achieved through a One Health lens – the idea that human, animal, and environmental health are all interconnected and influence one another. The interface between One Health sectors is an important point of study as it can provide insight into the transfer and proliferation of AMR bacteria across niches. Antimicrobial use (AMU) can be defined as appropriate or inappropriate for a given individual or population, in any of the One Health sectors (Spivak et al., 2016). Increases in AMR have been associated with misuse of antimicrobials in human, animal, and plant health (Chantziaras et al., 2014;Karakonstantis and Kalemaki, 2019).

Raised without antimicrobials has emerged as a new trend in intensive livestock production systems. This management practice has grown in popularity with the public (Bradford et al., 2022) as it is viewed as a 'natural' method of livestock production. Natural production has been proposed to mitigate the development and spread of AMR. A number of studies have used metagenomics to investigate AMR in dairy cows (Rovira et al., 2019), beef cattle (Doster et al., 2018), swine (Tunsagool et al., 2021), and broiler chickens (Gupta et al., 2021). However, these studies did not undertake a comparative study of AMR across livestock production systems using natural vs conventional production practices.

Using metagenomics in a cross-sector AMR study has the advantage of being able to determine differences in the fecal microbiome composition, the differential abundance of antimicrobial resistance genes (ARGs) present in a sample, and possibly the assemblage of metagenomic genomes (MAGs). MAGS can be used to investigate the association of ARGs with

specific bacterial species and their presence on mobile genetic elements (MGEs). Most cross-sector AMR studies have used specific indicator species such as *Escherichia coli* (Qiu et al., 2022) and *Enterococcus spp.* (Zaidi et al., 2023). These studies are limited to studying culturable microorganisms and fail to capture the entire landscape of diversity and prevalence of AMR in support of surveillance. Only a few studies have used metagenomics to investigate AMR from a One Health approach, and have typically only investigated a single livestock species in a comparative manner to human waste or agricultural soil (Zaheer et al., 2019;Maciel-Guerra et al., 2023).

To the authors' knowledge, there have not been any cross-livestock sector comparative metagenomic studies investigating the impact that natural vs conventional management practices have on the resistome and microbiome. Our study undertakes comparative investigation using fecal samples collected from beef cattle and swine, and cecal samples from broiler chickens raised under conventional and natural production systems. Based on our previous results in the beef cattle dataset (Lee et al., 2023b), we predict that within samples of each livestock production system there will be no difference in ARG abundance between conventional and natural systems, but there will be differences among livestock production systems.

4.2 Materials and Methods

4.2.1 Collection of Shotgun Metagenome Dataset

Overview. A total of 142 gut-associated shotgun metagenomes were included for comparative metagenomic analysis. These genomes originated from three livestock production system sources: (1) beef cattle feces (CONV n = 30, NAT n = 30) (Lee et al., 2023b), (2) swine

feces (CONV n = 23, NAT n = 43) (Poulin-Laprade, 2023), and (3) broiler chicken cecal contents (CONV n = 8, NAT n = 8) (Das et al., 2021;Yang et al., 2023).

Beef Cattle. All conventional (CONV) and half of natural (NAT, n=15) fecal composite samples were collected from pens of cattle that were fed a finishing diet of 85% barley grain, 10% barley silage, and 5% supplement with 48 ppm monensin and 11 ppm tylosin, while the other 15 NAT fecal composite samples were from animals fed a backgrounding diet of 62% corn silage, 27% barley grain, 7% vitamin mix and 4% canola meal with no antimicrobials while at the finishing feedlot. Metagenomic DNA was sequenced with Illumina NovaSeq6000 generating 150 bp paired-end reads (Lee et al., 2023b). These data are publicly available in the National Center for Biotechnology Information (NCBI) Short Reach Archive (SRA) under BioProject ID PRJNA420682.

Swine. CONV fecal samples were collected from piglets housed with sows that were reciving *per os* amoxicillin in water (300 mg/L) during the farrowing period (four days). Additionally, all CONV male piglets received an intramuscular injection of 1 mL of penicillin G procaine (300,000 U/mL) at three days of age. NAT piglets were reared in antibiotic-free commercial facilities. The feed for piglets up to ∼12 kg contained 2,500 to 3,000 mg/kg of zinc oxide and 29-277 mg/kg of copper sulfate, while the concentration of metals was reduced in feed formulated for finishers (100-232 mg/kg for zinc and 17-173 mg/kg for copper). Metagenomic DNA was sequenced with Illumina MiSeq generating 150 bp paired-end reads (Poulin-Laprade et al., 2021).

Broiler Chicken. CONV cecal samples were collected from 21-day old broilers fed a basal diet containing 55 ppm bacitracin, while NAT chicks were fed a standard basal diet that contained no antimicrobials. The basal diet for all broilers consisted of a starter diet from ages 0-

10 days, grower diet 11-20 days old, and a finisher diet 21-30 days old, as described in Das et al. (2021). Metagenomic DNA was sequenced with Illumina HiSeq2000 generating 100 bp paired-end reads (Das et al., 2021;Yang et al., 2023). These data are publicly available in the NCBI SRA under BioProject PRJNA666163.

4.2.2 Metagenomic Data Analysis

Sequence information was stored on the National Microbiology Laboratory's (Public Health Agency of Canada) high-throughput computing cluster, Waffles, and the Integrated Rapid Infectious Disease Analysis (IRIDA) Platform (Matthews et al., 2018). In addition, analyses were conducted on the web-based instance of Galaxy (Galaxy Community, 2022). Trimmomatic v0.38 (Bolger et al., 2014) was used to remove adapters, leading, and trailing nucleotides with a Phred score less than 30. With a sliding window every four nucleotides, reads were filtered out that were below a Phred score of 15 and contained less than 36 nucleotides after trimming. Microbiome classifications were generated using Kraken v2 (Wood et al., 2019) an the resistome was characterized using AMR++ v2.0 pipeline (Doster et al., 2020) combined with MEGARes v2.0 (Doster et al., 2020). The MEGARes 2.0 database classified ARGs based on: type, class, mechanism, and group. The output matrices of the resistome and taxonomic composition were then analyzed on a local installation of R (v4.3.1; http://www.r-project.org/).

4.2.3 Data Visualization and Statistical Analysis

Microbiome. The *phyloseq* R package (McMurdie and Holmes, 2013) was applied to the Kraken v2.0 output matrix to generate taxonomy and operational taxonomic unit (OTU) matrices

and associated metadata. All reads classified into the phylum Chordata were removed to eliminate host animal or human contamination. OTU abundances were normalized across samples using the trimmed mean of M-values (TMM) method (Robinson and Oshlack, 2010) as it has been shown to be appropriate for the normalization of shotgun metagenomics (Pereira et al., 2018). To be included in visualization, taxa were required to have a minimum of 1% relative abundance. Log2 fold-change comparisons of prevalent (>1% relative abundance) phyla were calculated as change from CONV to NAT management practices wherein values < 1 correspond to CONV enrichment and values > 1 correspond to NAT enrichment. Comparisons between NAT and CONV management practices and livestock production systems were made using the Analysis of Compositions of Microbiomes with Bias Correction (ANCOM-BC; Lin and Peddada (2020)). False discovery rates were mitigated through p-value corrections (q-value) using the Benjamini-Hochberg method (Benjamini and Hochberg, 1995) with the R package ANCOMBC v2.2.2.

Resistome. From the MEGARes v2.0 output matrix, in-house code was used to re-format the gene abundance matrix. TMM was also used to normalize the ARG abundance matrix. Any ARG that required single nucleotide polymorphism (SNP) confirmation was removed. Additionally, any ARG that contained the string “regulator” was removed as regulator genes alone do not confer phenotypic resistance. The final visualizations were generated using *ggplot2* R package. Statistics included the ANCOM-BC method with Benjamini-Hochberg p-value corrections.

4.3 Results

4.3.1 Comparative Microbiome

The general pattern of microbiome phylum composition was dictated by the host livestock species, albeit with variations between conventional and natural systems (Figure 18). In beef cattle, the most prevalent phyla in order of decreasing abundance were Bacteroidetes, Proteobacteria, and Firmicutes. In contrast, in broilers, Firmicutes were the most abundant followed by Proteobacteria and Bacteroidetes. Likewise in swine, the most prevalent were Firmicutes, but Bacteroidetes were more abundant that Proteobacteria. In no cases were there any difference in presence of abundant phyla between AMU practices within an animal's production system. The proportion of less abundant phyla were consistent across management practices, but some of the more minor phyla were livestock species specific. For example, Spirochaetes were more prevalent in beef cattle, while Euryarchaeota were not present at all in broilers.

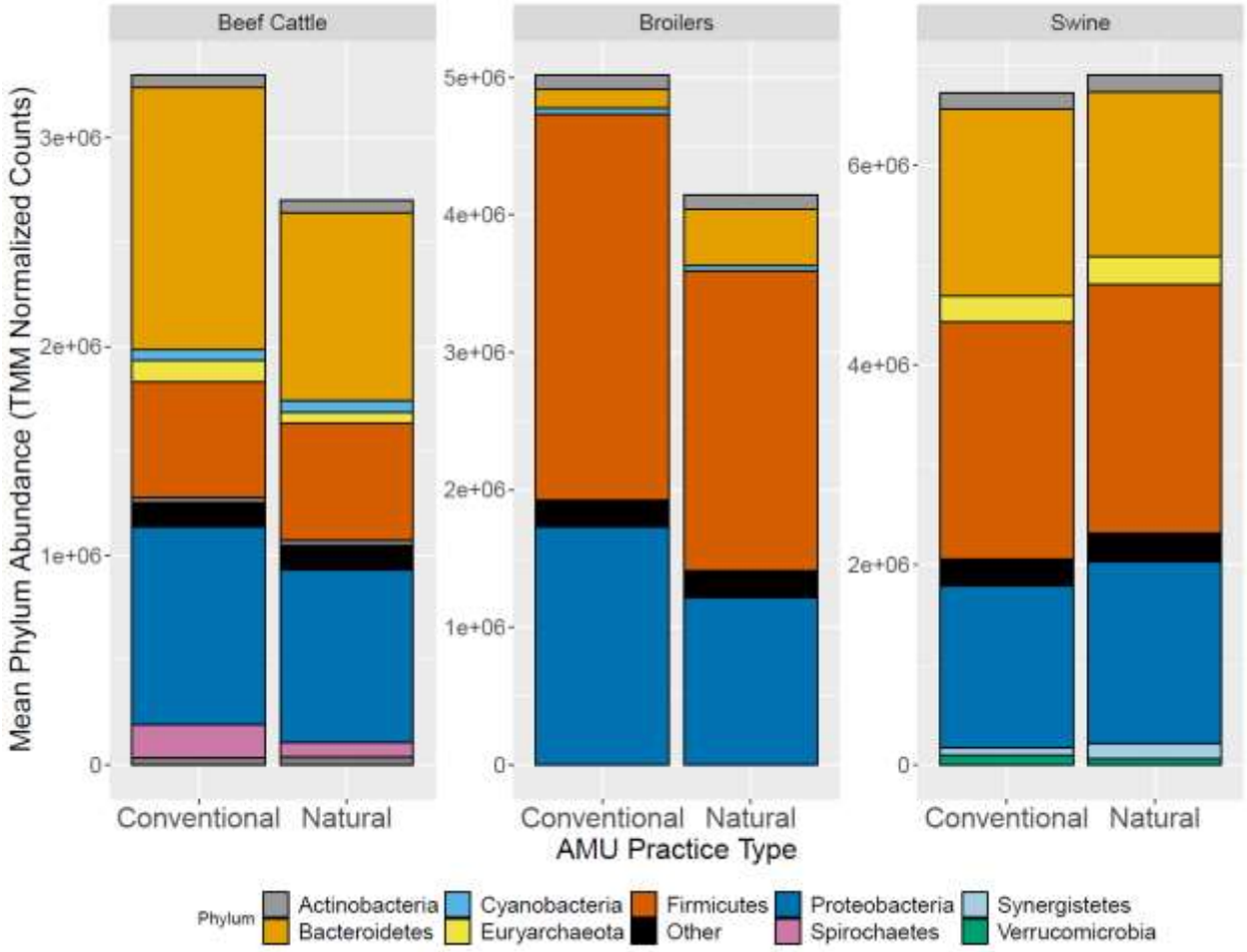

Figure 18. Stacked bar plots of prevalent (>1% relative abundance) mean trimmed mean M-value (TMM) normalized phyla read counts present in beef cattle fees, broilers cecal contents, and swine feces.

Quantitative comparisons of mean fold-changes of prevalent phyla from CONV to NAT systems revealed that only beef cattle phyla differed between AMU practices ($q < 0.05$; Figure 19). There were two >0.5 log2 fold decreases from CONV to NAT beef cattle ($q < 0.01$) for Euryarchaeota and Spirochaetes. However, when diet is taken into account, only Eyryarchaeota had decreased ($q < 0.0001$) by > 0.4 log2 fold change from CONV finishgin to NAT finishing cattle.

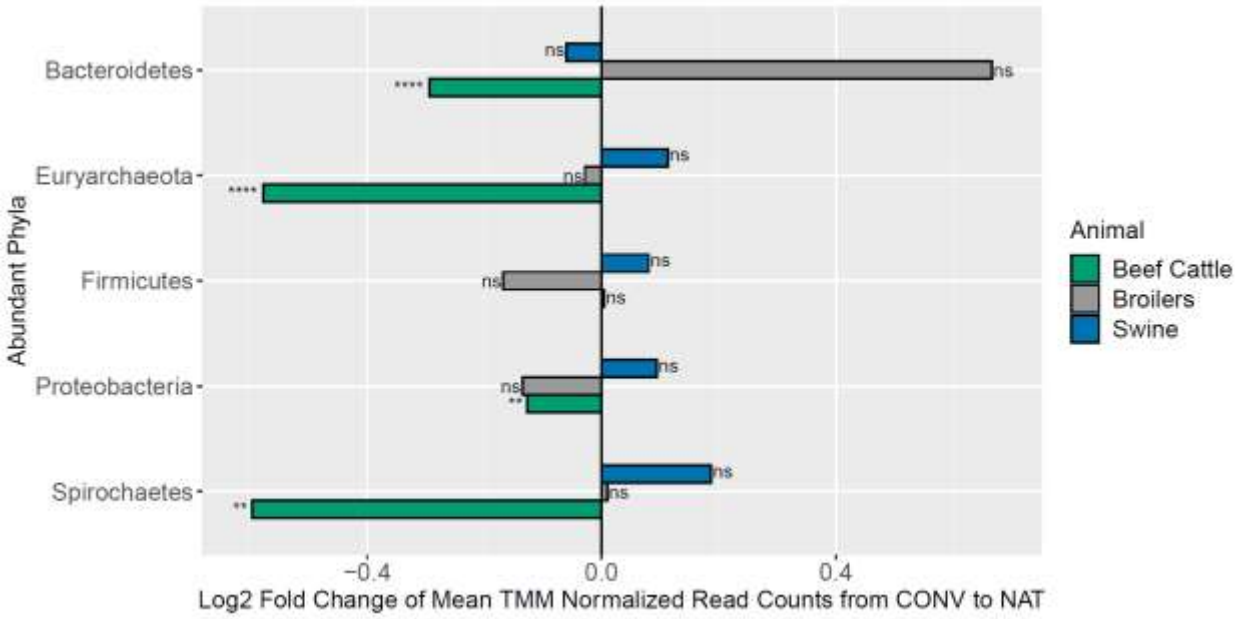

Figure 19. Bar plot comparisons of log2 fold changes of mean phylum abundance (TMM-normalized) from conventional (CONV) to natural (NAT) for each livestock animal species. Negative values represent natural enrichment and positive values represent conventional enrichment. (ANCOM-BC test with Benjamino-Hochberg o-value adjustments; $q > 0.05$ = ns, $q < 0.01$ = **; $q < 0.0001$ = ****)

4.3.2 Comparative Resistome

Across all ARG classes and livestock species, there were no comparisons between CONV and NAT systems that showed a change ($q < 0.05$; Figure 20). The most prevalent ARG classes were consistent across livestock species consisting of aminoglycosides, MLS, and tetracyclines. When comparing different ARG classes across livestock species, a number of differences were identifies ($q < 0.05$; Figure 21). Broilers and swine did not differ ($q > 0.05$) except with regard to β-lactam ARGs where they were higher in natural swine and lower in natural broilers, but all were present in low abundance. Tetracycline ARGs were more prevalent in beef cattle than broilers ($q < 0.001$) but not than swine ($q > 0.05$), regardless of AMU practice. Beef MLS ARGs were less abundant than in NAT broiler chicken ($q < 0.001$). No differences

between any livestock species or AMU practice were found in aminoglycoside or bacitracin ARG classes (Figures 20 & 21). MLS showed no differences ($q > 0.05$) in CONV management practices between livestock species.

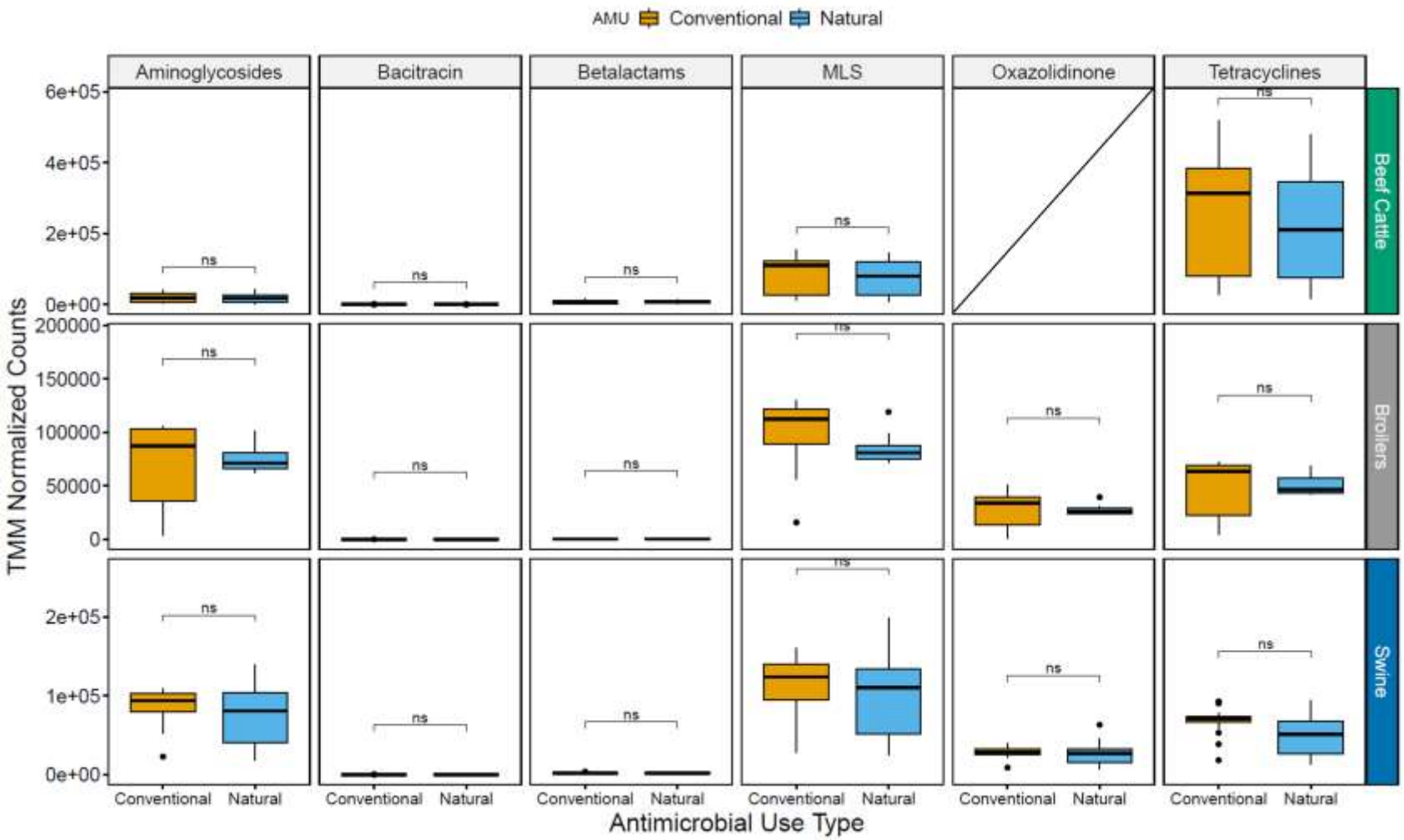

Figure 20. Boxplot comparisons of prevalent (>1%) ARG class TMM-normalized total abundances between AMU practices, separated by livestock species. (ANCOM-BC test with Benjamini-Hochberg p-value adjustments; q > 0.05 = ns)

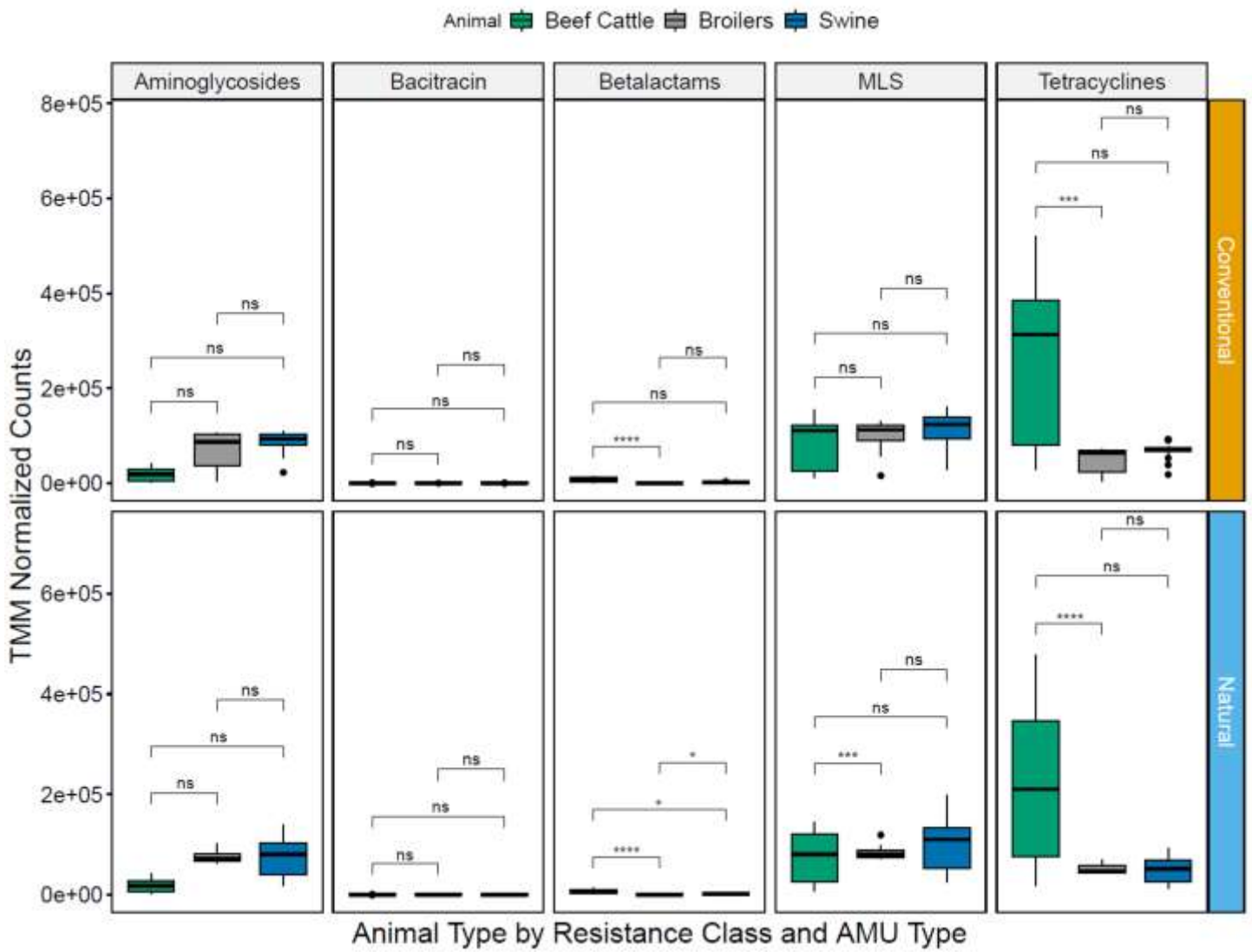

Figure 21. Boxplot comparisons of prevalent (>1%) ARG class TMM-normalized total abundances between livestock species, separated by AMU practice type. (ANCOM-BC test with Benjamini-Hochberg p-value adjustments; q > 0.05 = ns; q < 0.05 = *; q < 0.001 = ***; q < 0.0001 = ****)

Investigating the individual ARG groups within their ARG classes revealed there were few differences in the abundance of ARG groups between CONV to NAT AMU practices (Figure 22). Most ARG groups did not differ with AMU practice within livestock species ($q > 0.05$). For beef cattle, tetQ was most abundant, followed by tetW and mefA. For broilers, tetW was most abundant followed by ermB and tetO. For cattle and broilers, ARGs did not differ ($q > 0.05$) between CONV and NAT systems. For swine, the most abundant ARG groups in descending order were tetW, tetQ, and tetO with only tetQ being less prevalent in NAT than CONV systems ($q < 0.05$). The only MLS or β-lactam ARGs that were lower in NAT than CONC systems occurred in swine ($q < 0.05$). Interestingly, no bacitracin ARGs were greater than 1% relative abundance, especially in the broilers samples. In all livestock species, tetracyclines were the most prevalent.

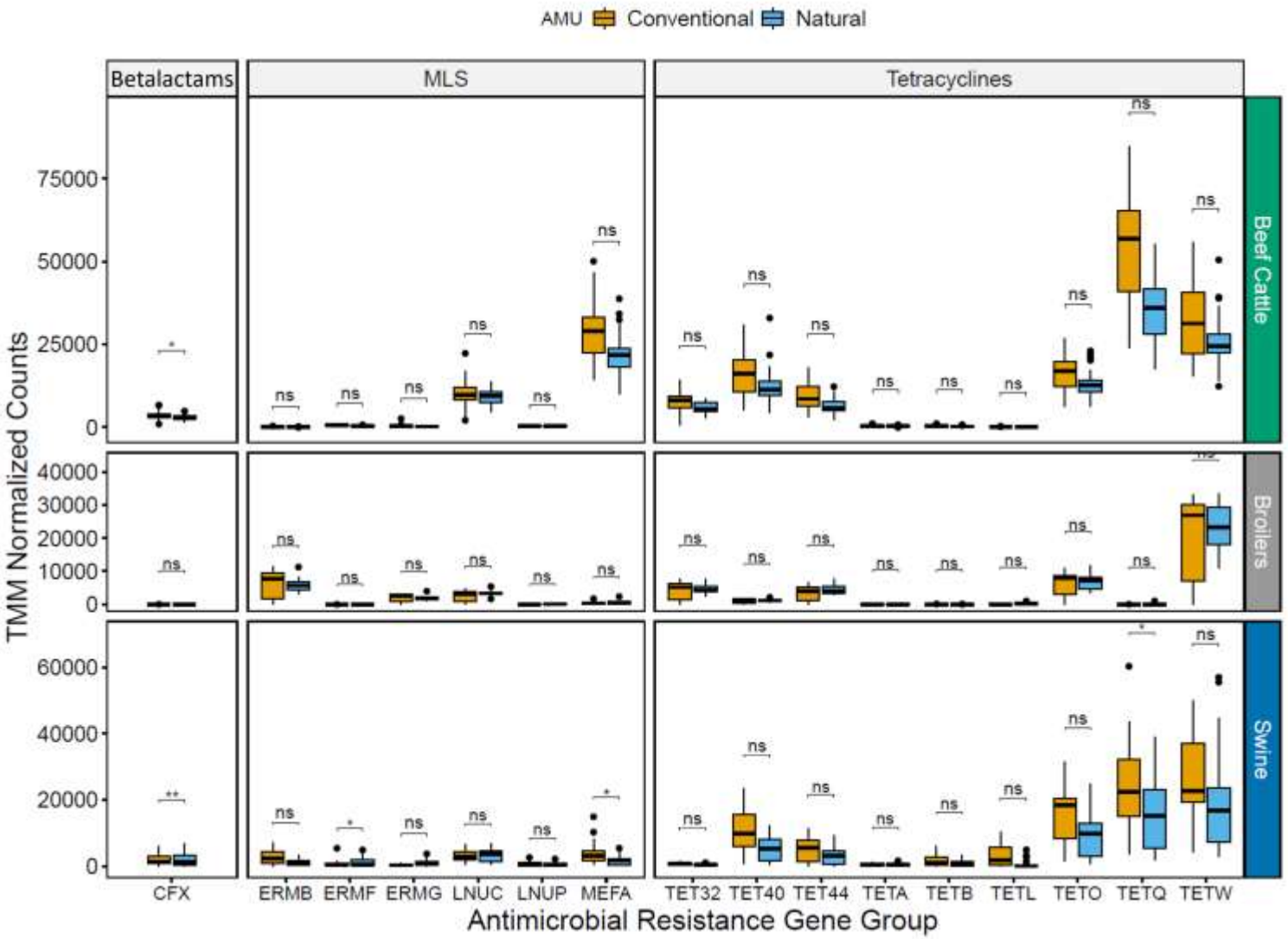

Figure 22. Boxplot comparisons of prevalent (>1%) ARG group TMM-normalized total abundances between AMU practices, separated by livestock animal species and ARG class. (ANCOM-BC test with Benjamini-Hochberg p-value adjustments; q > 0.05 = ns; q < 0.05 = *; q < 0.01 = **)

4.4 Discussion

Assessing the impact of differing AMU practices from a One Health perspective is a challenge given the many different factors involved. To ensure that no aspect of analysis is overlooked, metagenomics can be employed to account for all genetic determinants of current interest while conserving data for future re-analysis. In this study, this goal was accomplished through the collaboration of previous studies with similar design of livestock production systems assessed using a cross-sector approach.

Previous studies have investigated the fecal microbiome composition of livestock species. Cattle feces has been shown to have a high abundances of Firmicutes and Bacteroidetes (Shanks et al., 2011) which is reflected in our data in both CONV and NAT samples. The phylum Proteobacteria is generally not noted as being particularly abundant in beef cattle feces but it was the second most abundant phylum. Broiler cecal samples typically are very high in Firmicutes with some studies also being abundant in Bacteroidetes (Oakley et al., 2014). Our study found that Proteobacteria were more abundant than Bacteroidetes for broilers and swine. Swine fecal microbiomes tended to have Bacteroidetes and Firmicutes be the most abundant (Park et al., 2014). The presence of highly abundant Proteobacteria across all livestock species in our data is unusual among similar studies in the literature (Shanks et al., 2011;Oakley et al., 2014;Park et al., 2014). This higher than reported abundance of Proteobacteria may be attributed to other studies reporting relative abundance rather than absolute abundance. In Giuffrè et al. (2021), they found comparable proportions of Actinobacteria and Proteobacteria in swine. For broilers, Proteobacteria has been reported to be the second most abundant phyla, followed by Actinobacteria and Bacteroidetes (Proctor and Phillips, 2019). In select cases, Proteobacteria account for 20% of the relative abundance of bacteria in dairy cattle feces (Foditsch et al., 2019).

For all three livestock species, the abundances of prominent phyla are in line with the established literature.

When comparing CONV to NAT management practices, we found that only beef cattle differed in certain phyla ($q < 0.01$). If antimicrobials were the causal agent for certain phyla shifts, it could be expected that all livestock production systems would respond in a similar manner. One unique aspect of the beef cattle dataset is that the diets shifted between CONV and NAT management practices with the finishing diet having a higher starch content (Lee et al., 2023b). The basal diets for broilers did not differ between management practices, but there differences in beef cattle and swine. The predicted changes in prominent taxa abundance following diet shifts may not be consistent between livestock species since all three have very different digestive tract features and substantially different diets. One study found that Actinobacteria, Cyanobacteria, and Spirochaetes increased in abundance in swine fed highly as compared to lowly fermentable diets (Helm et al., 2021). A study in broilers determined that Actinobacteria abundance was higher with a low protein diet compared to basal diet, while Firmicutes and Proteobacteria decreased (De Cesare et al., 2019).

In no cases did we find that AMU practice impacted the abundance of ARGs at the class level. This supports the conclusion that factors other than AMU affect not only the microbiome but also the resistome. This may be expected since certain taxa tend to harbour the majority of ARGs (Pal et al., 2016). A study that compared swine raised-with and without antimicrobials found reduced levels of ARG classes in those farms that sis not use antimicrobials (Chekabab et al., 2021). While this study seems to contradict our findings, they did not report any information on the diets of either production system, only information of the therapeutic administration of antimicrobials. Another study failed to find a cause-effect relationship between AMU and ARGs

between cattle raised with and without antimicrobials (Rovira et al., 2019). They attributed this observation to ARGs being prevalent in any environment that uses therapeutic antimicrobials as those ARGs were identified regardless of production system. A study in broilers found there were no differences in ARG abundance at the resistance class level between controls that did not receive enrofloxacin and those that received therapeutic doses over time (Temmerman et al., 2022). However, that study did find many differences of ARG groups including increasing MLS, aminoglycoside, and tetracycline resistance gene abundances over time with the administration of enrofloxacin. Another study found differences at both the resistance class and mechanism level between swine with and without oxytetracycline therapeutic administration (Ghanbari et al., 2019). It seems that therapeutic doses of administered antimicrobials may affect the resistomes of livestock but sub-therapeutic doses as used in our study may not.

One interesting pattern observed in the resistomes was that regardless of production system, beef cattle had greater differential abundant ARGs than either broilers or swine, this being true for β-lactams, MLS, and tetracyclines. There were virtually no differences between broilers and swine ARGs across NAT and CONV production systems. This may be in part due to the highly complex nature of the ruminant digestive system. However, a study analyzed the resistome compositions and found the most similarity between veal calves and swine, though these models took into consideration ARG distribution as well as overall abundance (Duarte et al., 2021). It is important to note that at the calf life stage, cattle are most similar to monogastrics such as swine, until the rumen is properly developed (Diao et al., 2019). Another study compared high-throughput qPCR of 183 ARGs from manures applied to agricultural soils and found that in decreasing gene count abundance were cattle, swine, and chicken (Tyrrell et al., 2023). It is important to note that the presence of an ARG does not necessarily relate to a proportional

degree of phenotypic resistance. All ARG abundance data is just an indicator for the potential that a given microorganism or metagenomic community has to be resistant to an antimicrobial. Gene expression via transcriptomics is a way to accurately determine AMR in a high-throughput method.

4.5 Conclusions

Our results demonstrate that in a comparative cross-sector analysis of multiple livestock production systems, only a single taxon in beef cattle was affected by differing AMU management practices, likely being diet. Differences in diet in the beef cattle accounted for several of the differences initially observed, wile all broilers and swine samples had no difference between AMU while on the same diets. For a broad classification of resistance, AMU management practices had no effect on ARG class differential abundance. Only at specific ARG group levels, were there decreased ARG abundances present in NAT compared to CONV management practices. These ARG groups typically belonged to the class tetracyclines, despite the fact that no tetracyclines were used in our study. This observation in combination with the lack of variation in ARG classes may be explained by the high selective pressures that historical AMU has had on the livestock industry. Our study has demonstrated that differing management practices within a given livestock production system do not vastly impact the overall resistome or microbiome on the timescale of this study.

CHAPTER 5

Final Conclusions and Future Directions

5.1 Thesis Summary

This study aimed to apply the use of shotgun metagenomics to investigate the impact of AMU in livestock on the nature an prevalence of AMR. Method optimization was achieved in metagenomic workflows to address specific questions surrounding differing AMU. Different management practices (NAT and CONV) employed by the livestock industry (beef cattle, broiler chickens, and swine) to curb the spread of AMR were investigated as to their impact on the prevalence and distribution of microbiota, ARGs, and MGEs.

Due to the many different published tools and approaches for detecting microbiota composition, ARGs, and MGEs, optimization of a streamlined method was undertaken. There were several findings with regard to the use of different metagenomic methods. I found that targeted assembly approaches were not suitable for studies aiming to associate ARGs with specifi genetic elements. To achieve data suitable for those investigations, *de novo* assembly must be used if using short-read sequencing data, although read-based analysis may be possible with appropriate long-read sequencing data. To determine accurate abundance information in short-reads, read-based methods for ARG abundance and microbiota classification must be undertaken before *de novo* assembly. ARG detection tools must output a fasta file to serve as input to a MGE detection/reconstruction tool for the most streamlined analysis of associated contigs.

Using data for beef cattle, the microbiome fluctuations revealed only specific taxa were potentially impacted by the absence of AMU. This pattern was not observed at the resistome class-level but was in the ARG group-level. In the ARG group level, most differences observed were related to tetracyclines. The lack of correlation between the ARGs impacted and antimicrobial used in the study, suggests that other selective factors such as diet may have been

the causal agent in ARG profiles and abundance. Association of ARGs and MGEs on assembled contigs revealed that macrolide resistance genes were much more likely to be chromosome-associated than tetracycline resistance genes, suggesting that macrolide resistance genes may be more stably conserved in the core genome.

Comparative analysis of cross-sector livestock production showed that there were few shifts in the microbiome as a result of differences in AMU. Indeed, the only shifts were in beef cattle that had differing diets between treatment groups while swine and broiler chickens experienced no shifts and had identical basal diets. AMU management practice had no effect on ARGs at the class level, while there were differences at the ARG group level. An absence of AMU resulted in less abundant ARGs, mainly in the tetracycline class across all livestock production systems. Tetracycline resistance genes were the most prevalent in beef cattle as compared to the other livestock species. No tetracyclines were used in the timeframe of this study, indicating that historical tetracycline use has likely exerted strong selective pressure that continues to promote the persistence of these ARGs in livestock and their environment.

5.2 Final Conclusions

The management practice of ‘raised without antimicrobials’ has been advertised as a ‘natural’ production method to consumers but also has been described as a method to mitigate the spread of AMR. This study aimed to investigate the reliability of this description. I identified that absence of AMU did not have a major impact on the resistome, and had minimal impacts on the microbiome. While some fluctuations are detectable, they can be attributed to other factors such as diet composition. Given the results of this study, raised without antimicrobial meat

production is unlikely to have an immediate impact AMR. It is still inconclusive as to what extent multi-year/decade removal of AMU would impact AMR.

5.2 Future Directions

Association of ARGs and MGEs reveals the transferability of ARGs in the beef cattle dataset. Just as was done for the beef cattle dataset, assembly of contigs to associate ARGs and MGEs should be performed on the entire cross-sector dataset. This would reveal the impact that AMU broadly has on ARG and MGE associations, but also specific antimicrobial impacts, as all three livestock production systems used different antimicrobials. The comparisons used in this study should be applied to a broader One Health range of environments, to understand the impact that raised without antimicrobial production has on the surrounding environment. The most puzzling finding was the high abundance of tetracycline ARGs across all livestock species. It would be prudent to include farm worker and community (nearby wastewater) metagenomes to identify if the commonality for tetracycline ARG abundance is proximity to human interactions. The results of this study have shown that shotgun metagenomics is a powerful tool capable of analyzing large datasets for specific associations such as with microbiomes and ARGs, or even ARGs and MGEs. This thesis has demonstrated that the analysis of large datasets necessary for adequate One Health perspective can be done, and that multivariate analysis can be applied to specific metagenomics-based investigations.

REFERENCES

Alcock, B.P., Huynh, W., Chalil, R., Smith, K.W., Raphenya, A.R., Wlodarski, M.A., Edalatmand, A., Petkau, A., Syed, S.A., Tsang, K.K., Baker, S.J.C., Dave, M., Mccarthy, M.C., Mukiri, K.M., Nasir, J.A., Golbon, B., Imtiaz, H., Jiang, X., Kaur, K., Kwong, M., Liang, Z.C., Niu, K.C., Shan, P., Yang, J.Y.J., Gray, K.L., Hoad, G.R., Jia, B., Bhando, T., Carfrae, L.A., Farha, M.A., French, S., Gordzevich, R., Rachwalski, K., Tu, M.M., Bordeleau, E., Dooley, D., Griffiths, E., Zubyk, H.L., Brown, E.D., Maguire, F., Beiko, R.G., Hsiao, W.W.L., Brinkman, F.S.L., Van Domselaar, G., and Mcarthur, A.G. (2023). CARD 2023: expanded curation, support for machine learning, and resistome prediction at the Comprehensive Antibiotic Resistance Database. *Nucleic Acids Res* 51**,** D690-d699.

Alvarez, I., and Wendel, J.F. (2003). Ribosomal ITS sequences and plant phylogenetic inference. *Mol Phylogenet Evol* 29**,** 417-434.

Amann, R.I., Ludwig, W., and Schleifer, K.H. (1995). Phylogenetic identification and in situ detection of individual microbial cells without cultivation. *Microbiol Rev* 59**,** 143-169.

Antipov, D., Korobeynikov, A., Mclean, J.S., and Pevzner, P.A. (2016). hybridSPAdes: an algorithm for hybrid assembly of short and long reads. *Bioinformatics* 32**,** 1009-1015.

Antonopoulos, D.A., Assaf, R., Aziz, R.K., Brettin, T., Bun, C., Conrad, N., Davis, J.J., Dietrich, E.M., Disz, T., Gerdes, S., Kenyon, R.W., Machi, D., Mao, C., Murphy-Olson, D.E., Nordberg, E.K., Olsen, G.J., Olson, R., Overbeek, R., Parrello, B., Pusch, G.D., Santerre, J., Shukla, M., Stevens, R.L., Vanoeffelen, M., Vonstein, V., Warren, A.S., Wattam, A.R., Xia, F., and Yoo, H. (2019). PATRIC as a unique resource for studying antimicrobial resistance. *Brief Bioinform* 20**,** 1094-1102.

Apley, M.D., Schrag, N.F.D., Amrine, D.E., Lubbers, B.V., and Singer, R.S. (2023). Antimicrobial use in 20 U.S. beef feedyards: 2018–2019. *Frontiers in Veterinary Science* 10.

Auffret, M.D., Dewhurst, R.J., Duthie, C.-A., Rooke, J.A., John Wallace, R., Freeman, T.C., Stewart, R., Watson, M., and Roehe, R. (2017). The rumen microbiome as a reservoir of antimicrobial resistance and pathogenicity genes is directly affected by diet in beef cattle. *Microbiome* 5**,** 159.

Australian Commission on Safety and Quality in Health Care (2022). "Australian Passive Antimicrobial Resistance Surveillance – third-generation cephalosporin resistance in Escherichia coli and Klebsiella pneumoniae: prevalence of extended-spectrum β-lactamase (ESBL) phenotype.". (Sydney).

Baldwin, B.G., Sanderson, M.J., Porter, J.M., Wojciechowski, M.F., Campbell, C.S., and Donoghue, M.J. (1995). The its Region of Nuclear Ribosomal DNA: A Valuable Source of Evidence on Angiosperm Phylogeny. *Annals of the Missouri Botanical Garden* 82**,** 247-277.

Barer, M.R., and Harwood, C.R. (1999). Bacterial viability and culturability. *Adv Microb Physiol* 41**,** 93-137.

Behravesh, B.C. (2019). Introduction. One Health: over a decade of progress on the road to sustainability. *Rev Sci Tech* 38**,** 21-50.

Bengtsson, B., and Wierup, M. (2006). Antimicrobial resistance in Scandinavia after ban of antimicrobial growth promoters. *Anim Biotechnol* 17**,** 147-156.

Benjamini, Y., and Hochberg, Y. (1995). Controlling the False Discovery Rate: A Practical and Powerful Approach to Multiple Testing. *Journal of the Royal Statistical Society: Series B (Methodological)* 57**,** 289-300.

Bergen, W.G., and Bates, D.B. (1984). Ionophores: their effect on production efficiency and mode of action. *J Anim Sci* 58**,** 1465-1483.

Bertrand, D., Shaw, J., Kalathiyappan, M., Ng, A.H.Q., Kumar, M.S., Li, C., Dvornicic, M., Soldo, J.P., Koh, J.Y., Tong, C., Ng, O.T., Barkham, T., Young, B., Marimuthu, K., Chng, K.R., Sikic, M., and Nagarajan, N. (2019). Hybrid metagenomic assembly enables high-resolution analysis of resistance determinants and mobile elements in human microbiomes. *Nature Biotechnology* 37**,** 937-944.

Bharat, A., Petkau, A., Avery, B.P., Chen, J.C., Folster, J.P., Carson, C.A., Kearney, A., Nadon, C., Mabon, P., Thiessen, J., Alexander, D.C., Allen, V., El Bailey, S., Bekal, S., German, G.J., Haldane, D., Hoang, L., Chui, L., Minion, J., Zahariadis, G., Domselaar, G.V., Reid-Smith, R.J., and Mulvey, M.R. (2022). Correlation between Phenotypic and In Silico Detection of Antimicrobial Resistance in Salmonella enterica in Canada Using Staramr. *Microorganisms* 10**,** 292.

Blaut, M. (1994). Metabolism of methanogens. *Antonie van Leeuwenhoek* 66**,** 187-208.

Bolger, A.M., Lohse, M., and Usadel, B. (2014). Trimmomatic: a flexible trimmer for Illumina sequence data. *Bioinformatics* 30**,** 2114-2120.

Borges, A., Abreu, A.C., Dias, C., Saavedra, M.J., Borges, F., and Simões, M. 2016. New Perspectives on the Use of Phytochemicals as an Emergent Strategy to Control Bacterial Infections Including Biofilms. *Molecules* [Online], 21.

Botelho, J., Mourão, J., Roberts, A.P., and Peixe, L. (2020). Comprehensive genome data analysis establishes a triple whammy of carbapenemases, ICEs and multiple clinically relevant bacteria. *Microbial Genomics*.

Botelho, J., and Schulenburg, H. (2020). The role of integrative and conjugative elements in antibiotic resistance evolution. *Trends in Microbiology*.

Botelho, J., and Schulenburg, H. (2021). The Role of Integrative and Conjugative Elements in Antibiotic Resistance Evolution. *Trends Microbiol* 29**,** 8-18.

Bowers, R.M., Kyrpides, N.C., Stepanauskas, R., Harmon-Smith, M., Doud, D., Reddy, T.B.K., Schulz, F., Jarett, J., Rivers, A.R., Eloe-Fadrosh, E.A., Tringe, S.G., Ivanova, N.N., Copeland, A., Clum, A., Becraft, E.D., Malmstrom, R.R., Birren, B., Podar, M., Bork, P., Weinstock, G.M., Garrity, G.M., Dodsworth, J.A., Yooseph, S., Sutton, G., Glöckner, F.O., Gilbert, J.A., Nelson, W.C., Hallam, S.J., Jungbluth, S.P., Ettema, T.J.G., Tighe, S., Konstantinidis, K.T., Liu, W.-T., Baker, B.J., Rattei, T., Eisen, J.A., Hedlund, B., Mcmahon, K.D., Fierer, N., Knight, R., Finn, R., Cochrane, G., Karsch-Mizrachi, I., Tyson, G.W., Rinke, C., Kyrpides, N.C., Schriml, L., Garrity, G.M., Hugenholtz, P., Sutton, G., Yilmaz, P., Meyer, F., Glöckner, F.O., Gilbert, J.A., Knight, R., Finn, R., Cochrane, G., Karsch-Mizrachi, I., Lapidus, A., Meyer, F., Yilmaz, P., Parks, D.H., Murat Eren, A., Schriml, L., Banfield, J.F., Hugenholtz, P., Woyke, T., and The Genome Standards, C. (2017). Minimum information about a single amplified genome (MISAG) and a metagenome-assembled genome (MIMAG) of bacteria and archaea. *Nature Biotechnology* 35**,** 725-731.

Bradford, H., Mckernan, C., Elliott, C., and Dean, M. (2022). Consumers' perceptions and willingness to purchase pork labelled ‘raised without antibiotics’. *Appetite* 171**,** 105900.

Brinck, J.E., Lassen, S.B., Forouzandeh, A., Pan, T., Wang, Y.-Z., Monteiro, A., Blavi, L., Solà-Oriol, D., Stein, H.H., Su, J.-Q., and Brandt, K.K. (2023). Impacts of dietary copper on the swine gut microbiome and antibiotic resistome. *Science of The Total Environment* 857**,** 159609.

Bruinsma, S., Burgess, J., Schlingman, D., Czyz, A., Morrell, N., Ballenger, C., Meinholz, H., Brady, L., Khanna, A., Freeberg, L., Jackson, R.G., Mathonet, P., Verity, S.C., Slatter, A.F., Golshani, R., Grunenwald, H., Schroth, G.P., and Gormley, N.A. (2018). Bead-linked transposomes enable a normalization-free workflow for NGS library preparation. *BMC Genomics* 19**,** 722.

Burrus, V., Pavlovic, G., Decaris, B., and Guédon, G. (2002). Conjugative transposons: the tip of the iceberg. *Mol Microbiol* 46**,** 601-610.

Busch, G., Kassas, B., Palma, M.A., and Risius, A. (2020). Perceptions of antibiotic use in livestock farming in Germany, Italy and the United States. *Livestock Science* 241**,** 104251.

Byrne, M.K., Miellet, S., Mcglinn, A., Fish, J., Meedya, S., Reynolds, N., and Van Oijen, A.M. (2019). The drivers of antibiotic use and misuse: the development and investigation of a theory driven community measure. *BMC Public Health* 19**,** 1425.

Calderón-Franco, D., Sarelse, R., Christou, S., Pronk, M., Van Loosdrecht, M.C.M., Abeel, T., and Weissbrodt, D.G. (2022). Metagenomic profiling and transfer dynamics of antibiotic resistance determinants in a full-scale granular sludge wastewater treatment plant. *Water Research* 219**,** 118571.

Cameron, A., and Mcallister, T.A. (2016). Antimicrobial usage and resistance in beef production. *Journal of Animal Science and Biotechnology* 7**,** 68.

Carattoli, A., Zankari, E., García-Fernández, A., Voldby Larsen, M., Lund, O., Villa, L., Møller Aarestrup, F., and Hasman, H. (2014). In silico detection and typing of plasmids using PlasmidFinder and plasmid multilocus sequence typing. *Antimicrob Agents Chemother* 58**,** 3895-3903.

Chantziaras, I., Boyen, F., Callens, B., and Dewulf, J. (2014). Correlation between veterinary antimicrobial use and antimicrobial resistance in food-producing animals: a report on seven countries. *Journal of Antimicrobial Chemotherapy* 69**,** 827-834.

Chekabab, S.M., Lawrence, J.R., Alvarado, A.C., Predicala, B.Z., and Korber, D.R. 2021. Piglet Gut and in-Barn Manure from Farms on a Raised without Antibiotics Program Display Reduced Antimicrobial Resistance but an Increased Prevalence of Pathogens. *Antibiotics* [Online], 10.

Chen, S., Zhou, Y., Chen, Y., and Gu, J. (2018). fastp: an ultra-fast all-in-one FASTQ preprocessor. *Bioinformatics* 34**,** i884-i890.

Chen, Z., Erickson, D.L., and Meng, J. (2020). Benchmarking hybrid assembly approaches for genomic analyses of bacterial pathogens using Illumina and Oxford Nanopore sequencing. *BMC Genomics* 21**,** 631.

Cheng, H., Sun, Y., Yang, Q., Deng, M., Yu, Z., Zhu, G., Qu, J., Liu, L., Yang, L., and Xia, Y. (2022). A rapid bacterial pathogen and antimicrobial resistance diagnosis workflow using Oxford nanopore adaptive sequencing method. *Briefings in Bioinformatics* 23**,** bbac453.

Clarke, J., Wu, H.-C., Jayasinghe, L., Patel, A., Reid, S., and Bayley, H. (2009). Continuous base identification for single-molecule nanopore DNA sequencing. *Nature Nanotechnology* 4**,** 265-270.

Corrêa, P.S., Jimenez, C.R., Mendes, L.W., Rymer, C., Ray, P., Gerdes, L., Da Silva, V.O., De Nadai Fernandes, E.A., Abdalla, A.L., and Louvandini, H. (2021). Taxonomy and Functional Diversity in the Fecal Microbiome of Beef Cattle Reared in Brazilian Traditional and Semi-Intensive Production Systems. *Frontiers in Microbiology* 12.

Crescente, J.M., Zavallo, D., Helguera, M., and Vanzetti, L.S. (2018). MITE Tracker: an accurate approach to identify miniature inverted-repeat transposable elements in large genomes. *BMC Bioinformatics* 19**,** 348.

Das, Q., Shay, J., Gauthier, M., Yin, X., Hasted, T.-L., Ross, K., Julien, C., Yacini, H., Kennes, Y.M., Warriner, K., Marcone, M.F., and Diarra, M.S. (2021). Effects of Vaccination Against Coccidiosis on Gut Microbiota and Immunity in Broiler Fed Bacitracin and Berry Pomace. *Frontiers in Immunology* 12.

De Cesare, A., Faria Do Valle, Ì., Sala, C., Sirri, F., Astolfi, A., Castellani, G., and Manfreda, G. (2019). Effect of a low protein diet on chicken ceca microbiome and productive performances. *Poultry Science* 98**,** 3963-3976.

Di Tommaso, P., Chatzou, M., Floden, E.W., Barja, P.P., Palumbo, E., and Notredame, C. (2017). Nextflow enables reproducible computational workflows. *Nat Biotechnol* 35**,** 316-319.

Diao, Q., Zhang, R., and Fu, T. 2019. Review of Strategies to Promote Rumen Development in Calves. *Animals* [Online], 9.

Dighe, A.S., Jangid, K., González, J.M., Pidiyar, V.J., Patole, M.S., Ranade, D.R., and Shouche, Y.S. (2004). Comparison of 16S rRNA gene sequences of genus Methanobrevibacter. *BMC Microbiol* 4**,** 20.

Doster, E., Lakin, S.M., Dean, C.J., Wolfe, C., Young, J.G., Boucher, C., Belk, K.E., Noyes, N.R., and Morley, P.S. (2020). MEGARes 2.0: a database for classification of antimicrobial drug, biocide and metal resistance determinants in metagenomic sequence data. *Nucleic Acids Res* 48**,** D561-d569.

Doster, E., Pinnell, L.J., Noyes, N.R., Parker, J.K., Anderson, C.A., Booker, C.W., Hannon, S.J., Mcallister, T.A., Gow, S.P., Belk, K.E., and Morley, P.S. (2022). Evaluating the effects of antimicrobial drug use on the ecology of antimicrobial resistance and microbial community structure in beef feedlot cattle. *Frontiers in Microbiology* 13.

Doster, E., Rovira, P., Noyes, N.R., Burgess, B.A., Yang, X., Weinroth, M.D., Lakin, S.M., Dean, C.J., Linke, L., Magnuson, R., Jones, K.I., Boucher, C., Ruiz, J., Belk, K.E., and Morley, P.S. (2018). Investigating Effects of Tulathromycin Metaphylaxis on the Fecal Resistome and Microbiome of Commercial Feedlot Cattle Early in the Feeding Period. *Frontiers in Microbiology* 9.

Dröge, M., Pühler, A., and Selbitschka, W. (1998). Horizontal gene transfer as a biosafety issue: a natural phenomenon of public concern. *Journal of Biotechnology* 64**,** 75-90.

Duarte, A.S.R., Röder, T., Van Gompel, L., Petersen, T.N., Hansen, R.B., Hansen, I.M., Bossers, A., Aarestrup, F.M., Wagenaar, J.A., and Hald, T. (2021). Metagenomics-Based Approach to Source-Attribution of Antimicrobial Resistance Determinants – Identification of Reservoir Resistome Signatures. *Frontiers in Microbiology* 11.

El Karkouri, K., Murat, C., Zampieri, E., and Bonfante, P. (2007). Identification of internal transcribed spacer sequence motifs in truffles: a first step toward their DNA bar coding. *Appl Environ Microbiol* 73**,** 5320-5330.

Eramo, A., Delos Reyes, H., and Fahrenfeld, N.L. (2017). Partitioning of Antibiotic Resistance Genes and Fecal Indicators Varies Intra and Inter-Storm during Combined Sewer Overflows. *Frontiers in Microbiology* 8.

European Centre for Disease Prevention and Control (2022). "Antimicrobial resistance in the EU/EEA (EARS-Net) - Annual Epidemiological Report for 2021". (Stockholm, Sweden: ECDC).

Ewels, P., Magnusson, M., Lundin, S., and Käller, M. (2016). MultiQC: summarize analysis results for multiple tools and samples in a single report. *Bioinformatics* 32**,** 3047-3048.

Ewels, P.A., Peltzer, A., Fillinger, S., Patel, H., Alneberg, J., Wilm, A., Garcia, M.U., Di Tommaso, P., and Nahnsen, S. (2020). The nf-core framework for community-curated bioinformatics pipelines. *Nat Biotechnol* 38**,** 276-278.

Farzand, R., Rajakumar, K., Zamudio, R., Oggioni, M.R., Barer, M.R., and O'hare, H.M. (2019). ICEKp2: description of an integrative and conjugative element in Klebsiella pneumoniae, co-occurring and interacting with ICEKp1. *Scientific Reports* 9.

Feldgarden, M., Brover, V., Haft Daniel, H., Prasad Arjun, B., Slotta Douglas, J., Tolstoy, I., Tyson Gregory, H., Zhao, S., Hsu, C.-H., Mcdermott Patrick, F., Tadesse Daniel, A., Morales, C., Simmons, M., Tillman, G., Wasilenko, J., Folster Jason, P., and Klimke, W. (2019). Validating the AMRFinder Tool and Resistance Gene Database by Using Antimicrobial Resistance Genotype-Phenotype Correlations in a Collection of Isolates. *Antimicrobial Agents and Chemotherapy* 63**,** e00483-00419.

Florensa, A.F., Kaas, R.S., Clausen, P., Aytan-Aktug, D., and Aarestrup, F.M. (2022). ResFinder - an open online resource for identification of antimicrobial resistance genes in next-generation sequencing data and prediction of phenotypes from genotypes. *Microb Genom* 8.

Foditsch, C., Pereira, R.V.V., Siler, J.D., Altier, C., and Warnick, L.D. (2019). Effects of treatment with enrofloxacin or tulathromycin on fecal microbiota composition and genetic function of dairy calves. *PLOS ONE* 14**,** e0219635.

Fossen, J.D., Campbell, J.R., Gow, S.P., Erickson, N., and Waldner, C.L. 2023. Antimicrobial Use in Canadian Cow–Calf Herds. *Veterinary Sciences* [Online], 10.

Frost, L.S., Leplae, R., Summers, A.O., and Toussaint, A. (2005). Mobile genetic elements: the agents of open source evolution. *Nature Reviews Microbiology* 3**,** 722-732.

Galaxy Community (2022). The Galaxy platform for accessible, reproducible and collaborative biomedical analyses: 2022 update. *Nucleic Acids Res* 50**,** W345-w351.

Gao, R., Naushad, S., Moineau, S., Levesque, R., Goodridge, L., and Ogunremi, D. (2020). Comparative genomic analysis of 142 bacteriophages infecting Salmonella enterica subsp. enterica. *BMC Genomics* 21**,** 374.

Ghanbari, M., Klose, V., Crispie, F., and Cotter, P.D. (2019). The dynamics of the antibiotic resistome in the feces of freshly weaned pigs following therapeutic administration of oxytetracycline. *Scientific Reports* 9**,** 4062.

Giuffrè, L., Giosa, D., Galeano, G., Aiese Cigliano, R., Paytuví-Gallart, A., Sutera, A.M., Tardiolo, G., Zumbo, A., Romeo, O., and D'alessandro, E. (2021). Whole-metagenome shotgun sequencing of pig faecal microbiome. *Italian Journal of Animal Science* 20**,** 1147-1155.

Gnirke, A., Melnikov, A., Maguire, J., Rogov, P., Leproust, E.M., Brockman, W., Fennell, T., Giannoukos, G., Fisher, S., Russ, C., Gabriel, S., Jaffe, D.B., Lander, E.S., and Nusbaum, C. (2009). Solution hybrid selection with ultra-long oligonucleotides for massively parallel targeted sequencing. *Nature Biotechnology* 27**,** 182-189.

Gourlie, R., Mcdonald, M., Hafez, M., Ortega-Polo, R., Low, K.E., Abbott, D.W., Strelkov, S.E., Daayf, F., and Aboukhaddour, R. (2022). The pangenome of the wheat pathogen Pyrenophora tritici-repentis reveals novel transposons associated with necrotrophic effectors ToxA and ToxB. *BMC Biology* 20**,** 239.

Grine, G., Boualam, M.A., and Drancourt, M. (2017). Methanobrevibacter smithii, a methanogen consistently colonising the newborn stomach. *Eur J Clin Microbiol Infect Dis* 36**,** 2449-2455.

Gu, Z., Gu, L., Eils, R., Schlesner, M., and Brors, B. (2014). circlize implements and enhances circular visualization in R. *Bioinformatics* 30**,** 2811-2812.

Gullberg, E., Albrecht Lisa, M., Karlsson, C., Sandegren, L., and Andersson Dan, I. (2014). Selection of a Multidrug Resistance Plasmid by Sublethal Levels of Antibiotics and Heavy Metals. *mBio* 5**,** 10.1128/mbio.01918-01914.

Gupta, C.L., Blum, S.E., Kattusamy, K., Daniel, T., Druyan, S., Shapira, R., Krifucks, O., Zhu, Y.-G., Zhou, X.-Y., Su, J.-Q., and Cytryn, E. (2021). Longitudinal study on the effects of growth-promoting and therapeutic antibiotics on the dynamics of chicken cloacal and litter microbiomes and resistomes. *Microbiome* 9**,** 178.

Gurevich, A., Saveliev, V., Vyahhi, N., and Tesler, G. (2013). QUAST: quality assessment tool for genome assemblies. *Bioinformatics* 29**,** 1072-1075.

Halleran, J.L., Callahan, B.J., Jacob, M.E., Sylvester, H.J., Prange, T., Papich, M.G., and Foster, D.M. (2021). Effects of danofloxacin dosing regimen on gastrointestinal pharmacokinetics and fecal microbiome in steers. *Scientific Reports* 11**,** 11249.

Hammer, T.J., Fierer, N., Hardwick, B., Simojoki, A., Slade, E., Taponen, J., Viljanen, H., and Roslin, T. (2016). Treating cattle with antibiotics affects greenhouse gas emissions, and microbiota in dung and dung beetles. *Proceedings: Biological Sciences* 283**,** 1-7.

Health Canada (2009). *Categorization of Antimicrobial Drugs Based on Importance in Human Medicine* [Online]. Available: https://www.canada.ca/en/health-canada/services/drugs-health-products/veterinary-drugs/antimicrobial-resistance/categorization-antimicrobial-drugs-based-importance-human-medicine.html [Accessed 2023].

Helm, E.T., Gabler, N.K., and Burrough, E.R. 2021. Highly Fermentable Fiber Alters Fecal Microbiota and Mitigates Swine Dysentery Induced by Brachyspira hyodysenteriae. *Animals* [Online], 11.

Hendriksen, R.S., Munk, P., Njage, P., Van Bunnik, B., Mcnally, L., Lukjancenko, O., Röder, T., Nieuwenhuijse, D., Pedersen, S.K., Kjeldgaard, J., Kaas, R.S., Clausen, P.T.L.C., Vogt, J.K., Leekitcharoenphon, P., Van De Schans, M.G.M., Zuidema, T., De Roda Husman, A.M., Rasmussen, S., Petersen, B., Bego, A., Rees, C., Cassar, S., Coventry, K., Collignon, P., Allerberger, F., Rahube, T.O., Oliveira, G., Ivanov, I., Vuthy, Y., Sopheak, T., Yost, C.K., Ke, C., Zheng, H., Baisheng, L., Jiao, X., Donado-Godoy, P., Coulibaly, K.J., Jergović, M., Hrenovic, J., Karpíšková, R., Villacis, J.E., Legesse, M., Eguale, T., Heikinheimo, A., Malania, L., Nitsche, A., Brinkmann, A., Saba, C.K.S., Kocsis, B.,

Solymosi, N., Thorsteinsdottir, T.R., Hatha, A.M., Alebouyeh, M., Morris, D., Cormican, M., O'connor, L., Moran-Gilad, J., Alba, P., Battisti, A., Shakenova, Z., Kiiyukia, C., Ng'eno, E., Raka, L., Avsejenko, J., Bērziņš, A., Bartkevics, V., Penny, C., Rajandas, H., Parimannan, S., Haber, M.V., Pal, P., Jeunen, G.-J., Gemmell, N., Fashae, K., Holmstad, R., Hasan, R., Shakoor, S., Rojas, M.L.Z., Wasyl, D., Bosevska, G., Kochubovski, M., Radu, C., Gassama, A., Radosavljevic, V., Wuertz, S., Zuniga-Montanez, R., Tay, M.Y.F., Gavačová, D., Pastuchova, K., Truska, P., Trkov, M., Esterhuyse, K., Keddy, K., Cerdà-Cuéllar, M., Pathirage, S., Norrgren, L., Örn, S., Larsson, D.G.J., Heijden, T.V.D., Kumburu, H.H., et al. (2019). Global monitoring of antimicrobial resistance based on metagenomics analyses of urban sewage. *Nature Communications* 10**,** 1124.

Holman, D.B., Kommadath, A., Tingley, J.P., and Abbott, D.W. (2022). Novel Insights into the Pig Gut Microbiome Using Metagenome-Assembled Genomes. *Microbiol Spectr* 10**,** e0238022.

Holman, D.B., Yang, W., and Alexander, T.W. (2019). Antibiotic treatment in feedlot cattle: a longitudinal study of the effect of oxytetracycline and tulathromycin on the fecal and nasopharyngeal microbiota. *Microbiome* 7**,** 86.

Holmes, A.H., Moore, L.S., Sundsfjord, A., Steinbakk, M., Regmi, S., Karkey, A., Guerin, P.J., and Piddock, L.J. (2016). Understanding the mechanisms and drivers of antimicrobial resistance. *Lancet* 387**,** 176-187.

Hu, T., Chitnis, N., Monos, D., and Dinh, A. (2021). Next-generation sequencing technologies: An overview. *Human Immunology* 82**,** 801-811.

Hyatt, D., Chen, G.-L., Locascio, P.F., Land, M.L., Larimer, F.W., and Hauser, L.J. (2010). Prodigal: prokaryotic gene recognition and translation initiation site identification. *BMC Bioinformatics* 11**,** 119.

Janssen, P.H., and Kirs, M. (2008). Structure of the archaeal community of the rumen. *Appl Environ Microbiol* 74**,** 3619-3625.

Javed, M.A., Schwelm, A., Zamani-Noor, N., Salih, R., Silvestre Vañó, M., Wu, J., González García, M., Heick, T.M., Luo, C., Prakash, P., and Pérez-López, E. (2023). The clubroot pathogen Plasmodiophora brassicae: A profile update. *Mol Plant Pathol* 24**,** 89-106.

Johnson, M., Zaretskaya, I., Raytselis, Y., Merezhuk, Y., Mcginnis, S., and Madden, T.L. (2008). NCBI BLAST: a better web interface. *Nucleic Acids Res* 36**,** W5-9.

Johnson, S.V., Hoey, L.L., and Vance-Bryan, K. (1995). Inappropriate Vancomycin Prescribing Based on Criteria from the Centers for Disease Control and Prevention. *Pharmacotherapy: The Journal of Human Pharmacology and Drug Therapy* 15**,** 579-585.

Juraschek, K., Borowiak, M., Tausch, S.H., Malorny, B., Käsbohrer, A., Otani, S., Schwarz, S., Meemken, D., Deneke, C., and Hammerl, J.A. (2021). Outcome of Different Sequencing and Assembly Approaches on the Detection of Plasmids and Localization of Antimicrobial Resistance Genes in Commensal Escherichia coli. *Microorganisms* 9.

Kang, D.D., Li, F., Kirton, E., Thomas, A., Egan, R., An, H., and Wang, Z. (2019). MetaBAT 2: an adaptive binning algorithm for robust and efficient genome reconstruction from metagenome assemblies. *PeerJ* 7**,** e7359.

Karakonstantis, S., and Kalemaki, D. (2019). Antimicrobial overuse and misuse in the community in Greece and link to antimicrobial resistance using methicillin-resistant S. aureus as an example. *Journal of Infection and Public Health* 12**,** 460-464.

Karavolias, J., Salois, M.J., Baker, K.T., and Watkins, K. (2018). Raised without antibiotics: impact on animal welfare and implications for food policy. *Translational Animal Science* 2**,** 337-348.

Klitgaard, K., Nielsen, M.W., Ingerslev, H.C., Boye, M., and Jensen, T.K. (2014). Discovery of bovine digital dermatitis-associated Treponema spp. in the dairy herd environment by a targeted deep-sequencing approach. *Appl Environ Microbiol* 80**,** 4427-4432.

Kowalchuk, G.A., Gerards, S., and Woldendorp, J.W. (1997). Detection and characterization of fungal infections of Ammophila arenaria (marram grass) roots by denaturing gradient gel electrophoresis of specifically amplified 18s rDNA. *Appl Environ Microbiol* 63**,** 3858-3865.

Koyun, O.Y., Balta, I., Corcionivoschi, N., and Callaway, T.R. 2023. Disease Occurrence in- and the Transferal of Zoonotic Agents by North American Feedlot Cattle. *Foods* [Online], 12.

Krakau, S., Straub, D., Gourlé, H., Gabernet, G., and Nahnsen, S. (2022). nf-core/mag: a best-practice pipeline for metagenome hybrid assembly and binning. *NAR Genomics and Bioinformatics* 4**,** lqac007.

Lakin, S.M., Dean, C., Noyes, N.R., Dettenwanger, A., Ross, A.S., Doster, E., Rovira, P., Abdo, Z., Jones, K.L., Ruiz, J., Belk, K.E., Morley, P.S., and Boucher, C. (2017). MEGARes: an antimicrobial resistance database for high throughput sequencing. *Nucleic Acids Research* 45**,** D574-D580.

Lane, D.J. (1991). "16S/23S rRNA sequencing," in *Nucleic Acd Techniques in Bacterial Systematics*. (New York, N.Y.: John Wiley & Sonce, Inc).

Langmead, B., and Salzberg, S.L. (2012). Fast gapped-read alignment with Bowtie 2. *Nature Methods* 9**,** 357-359.

Larsson, D.G.J., and Flach, C.-F. (2022). Antibiotic resistance in the environment. *Nature Reviews Microbiology* 20**,** 257-269.

Lee, C., Ortega Polo, R., Zaheer, R., Van Domselaar, G., Zovoilis, A., and Mcallister, T.A. (2023a). Evaluation of metagenomic assembly methods for the detection and characterization of antimicrobial resistance determinants and associated mobilizable elements. *Journal of Microbiological Methods***,** 106815.

Lee, C., Zaheer, R., Munns, K., Holman, D.B., Van Domselaar, G., Zovoilis, A., and Mcallister, T.A. (2023b). Effect of Antimicrobial Use in Conventional Versus Natural Cattle Feedlots on the Microbiome and Resistome. *Microorganisms* 11**,** 2982.

Leekha, S., Terrell, C.L., and Edson, R.S. (2011). General principles of antimicrobial therapy. *Mayo Clin Proc* 86**,** 156-167.

Leplae, R., Hebrant, A., Wodak, S.J., and Toussaint, A. (2004). ACLAME: a CLAssification of Mobile genetic Elements. *Nucleic Acids Res* 32**,** D45-49.

Li, D., Liu, C.M., Luo, R., Sadakane, K., and Lam, T.W. (2015). MEGAHIT: an ultra-fast single-node solution for large and complex metagenomics assembly via succinct de Bruijn graph. *Bioinformatics* 31**,** 1674-1676.

Li, D., Luo, R., Liu, C.M., Leung, C.M., Ting, H.F., Sadakane, K., Yamashita, H., and Lam, T.W. (2016). MEGAHIT v1.0: A fast and scalable metagenome assembler driven by advanced methodologies and community practices. *Methods* 102**,** 3-11.

Li, H., and Durbin, R. (2009). Fast and accurate short read alignment with Burrows-Wheeler transform. *Bioinformatics* 25**,** 1754-1760.

Li, H., Handsaker, B., Wysoker, A., Fennell, T., Ruan, J., Homer, N., Marth, G., Abecasis, G., Durbin, R., and Genome Project Data Processing, S. (2009). The Sequence Alignment/Map format and SAMtools. *Bioinformatics* 25**,** 2078-2079.

Lin, D.M., Koskella, B., and Lin, H.C. (2017). Phage therapy: An alternative to antibiotics in the age of multi-drug resistance. *World J Gastrointest Pharmacol Ther* 8**,** 162-173.

Lin, H., and Peddada, S.D. (2020). Analysis of compositions of microbiomes with bias correction. *Nature Communications* 11**,** 3514.

Lin, L., Lai, Z., Zhang, J., Zhu, W., and Mao, S. (2023). The gastrointestinal microbiome in dairy cattle is constrained by the deterministic driver of the region and the modified effect of diet. *Microbiome* 11**,** 10.

Liu, B., Zheng, D., Zhou, S., Chen, L., and Yang, J. (2022a). VFDB 2022: a general classification scheme for bacterial virulence factors. *Nucleic Acids Res* 50**,** D912-d917.

Liu, C., Li, G., Qin, X., Xu, Y., Wang, J., Wu, G., Feng, H., Ye, J., Zhu, C., Li, X., and Zheng, X. (2022b). Profiles of antibiotic- and heavy metal-related resistance genes in animal manure revealed using a metagenomic analysis. *Ecotoxicology and Environmental Safety* 239**,** 113655.

Liu, C., Liu, C., Wang, D., and Zhang, X. (2019a). Intrinsic and external determinants of antibiotic prescribing: a multi-level path analysis of primary care prescriptions in Hubei, China. *Antimicrobial Resistance & Infection Control* 8**,** 132.

Liu, J., Taft, D.H., Maldonado-Gomez, M.X., Johnson, D., Treiber, M.L., Lemay, D.G., Depeters, E.J., and Mills, D.A. (2019b). The fecal resistome of dairy cattle is associated with diet during nursing. *Nature Communications* 10**,** 4406.

Liu, M., Li, X., Xie, Y., Bi, D., Sun, J., Li, J., Tai, C., Deng, Z., and Ou, H.-Y. (2019c). ICEberg 2.0: an updated database of bacterial integrative and conjugative elements. *Nucleic Acids Research* 47**,** D660-D665.

Lloyd, K.G., Steen, A.D., Ladau, J., Yin, J., and Crosby, L. (2018). Phylogenetically Novel Uncultured Microbial Cells Dominate Earth Microbiomes. *mSystems* 3.

Looft, T., Johnson, T.A., Allen, H.K., Bayles, D.O., Alt, D.P., Stedtfeld, R.D., Sul, W.J., Stedtfeld, T.M., Chai, B., Cole, J.R., Hashsham, S.A., Tiedje, J.M., and Stanton, T.B. (2012). In-feed antibiotic effects on the swine intestinal microbiome. *Proceedings of the National Academy of Sciences* 109**,** 1691-1696.

Ma, T., Zaheer, R., Mcallister, T.A., Guo, W., Li, F., Tu, Y., Diao, Q., and Guan, L.L. (2022). Expressions of resistome is linked to the key functions and stability of active rumen microbiome. *Animal Microbiome* 4**,** 38.

Maciel-Guerra, A., Baker, M., Hu, Y., Wang, W., Zhang, X., Rong, J., Zhang, Y., Zhang, J., Kaler, J., Renney, D., Loose, M., Emes, R.D., Liu, L., Chen, J., Peng, Z., Li, F., and Dottorini, T. (2023). Dissecting microbial communities and resistomes for interconnected humans, soil, and livestock. *The ISME Journal* 17**,** 21-35.

Mader, R., Damborg, P., Amat, J.-P., Bengtsson, B., Bourély, C., Broens, E.M., Busani, L., Crespo-Robledo, P., Filippitzi, M.-E., Fitzgerald, W., Kaspar, H., Madero, C.M., Norström, M., Nykäsenoja, S., Pedersen, K., Pokludova, L., Urdahl, A.M., Vatopoulos, A., Zafeiridis, C., Madec, J.-Y., and Eu-Jamrai, O.B.O. (2021). Building the European Antimicrobial Resistance Surveillance network in veterinary medicine (EARS-Vet). *Eurosurveillance* 26**,** 2001359.

Maguire, F., Jia, B., Gray, K.L., Lau, W.Y.V., Beiko, R.G., and Brinkman, F.S.L. (2020). Metagenome-assembled genome binning methods with short reads disproportionately fail for plasmids and genomic Islands. *Microb Genom* 6.

Martinez, J.L., Fajardo, A., Garmendia, L., Hernandez, A., Linares, J.F., Martínez-Solano, L., and Sánchez, M.B. (2009). A global view of antibiotic resistance. *FEMS Microbiology Reviews* 33**,** 44-65.

Matthews, T.C., Bristow, F.R., Griffiths, E.J., Petkau, A., Adam, J., Dooley, D., Kruczkiewicz, P., Curatcha, J., Cabral, J., Fornika, D., Winsor, G.L., Courtot, M., Bertelli, C., Roudgar, A., Feijao, P., Mabon, P., Enns, E., Thiessen, J., Keddy, A., Isaac-Renton, J., Gardy, J.L., Tang, P., The Irida Consortium, Carriço, J.A., Chindelevitch, L., Chauve, C., Graham, M.R., Mcarthur, A.G., Taboada, E.N., Beiko, R.G., Brinkman, F.S.L., Hsiao, W.W.L., and Van Domselaar, G. (2018). The Integrated Rapid Infectious Disease Analysis (IRIDA) Platform. *bioRxiv***,** 381830.

Mcewen, S.A., and Collignon, P.J. (2018). Antimicrobial Resistance: a One Health Perspective. *Microbiol Spectr* 6.

Mcmurdie, P.J., and Holmes, S. (2013). phyloseq: An R Package for Reproducible Interactive Analysis and Graphics of Microbiome Census Data. *PLOS ONE* 8**,** e61217.

Mencía-Ares, O., Cabrera-Rubio, R., Cobo-Diaz, J.F., Alvarez-Ordonez, A., Gomez-Garcia, M., Puente, H., Cotter, P.D., Crispie, F., Carvajal, A., Rubio, P., and Arguello, H. (2020). Antimicrobial use and production system shape the fecal, environmental, and slurry resistomes of pig farms. *Microbiome* 8**,** 164.

Morisse, P., Marchet, C., Limasset, A., Lecroq, T., and Lefebvre, A. (2021). Scalable long read self-correction and assembly polishing with multiple sequence alignment. *Scientific Reports* 11**,** 761.

Mosolygó, T., Mouwakeh, A., Hussein Ali, M., Kincses, A., Mohácsi-Farkas, C., Kiskó, G., and Spengler, G. 2019. Bioactive Compounds of Nigella Sativa Essential Oil as Antibacterial Agents against Chlamydia Trachomatis D. *Microorganisms* [Online], 7.

Moura, A., Soares, M., Pereira, C., Leitão, N., Henriques, I., and Correia, A. (2009). INTEGRALL: a database and search engine for integrons, integrases and gene cassettes. *Bioinformatics* 25**,** 1096-1098.

Murray, C.J.L., Ikuta, K.S., Sharara, F., Swetschinski, L., Robles Aguilar, G., Gray, A., Han, C., Bisignano, C., Rao, P., Wool, E., Johnson, S.C., Browne, A.J., Chipeta, M.G., Fell, F., Hackett, S., Haines-Woodhouse, G., Kashef Hamadani, B.H., Kumaran, E.a.P., Mcmanigal, B., Achalapong, S., Agarwal, R., Akech, S., Albertson, S., Amuasi, J., Andrews, J., Aravkin, A., Ashley, E., Babin, F.-X., Bailey, F., Baker, S., Basnyat, B.,

Bekker, A., Bender, R., Berkley, J.A., Bethou, A., Bielicki, J., Boonkasidecha, S., Bukosia, J., Carvalheiro, C., Castañeda-Orjuela, C., Chansamouth, V., Chaurasia, S., Chiurchiù, S., Chowdhury, F., Clotaire Donatien, R., Cook, A.J., Cooper, B., Cressey, T.R., Criollo-Mora, E., Cunningham, M., Darboe, S., Day, N.P.J., De Luca, M., Dokova, K., Dramowski, A., Dunachie, S.J., Duong Bich, T., Eckmanns, T., Eibach, D., Emami, A., Feasey, N., Fisher-Pearson, N., Forrest, K., Garcia, C., Garrett, D., Gastmeier, P., Giref, A.Z., Greer, R.C., Gupta, V., Haller, S., Haselbeck, A., Hay, S.I., Holm, M., Hopkins, S., Hsia, Y., Iregbu, K.C., Jacobs, J., Jarovsky, D., Javanmardi, F., Jenney, A.W.J., Khorana, M., Khusuwan, S., Kissoon, N., Kobeissi, E., Kostyanev, T., Krapp, F., Krumkamp, R., Kumar, A., Kyu, H.H., Lim, C., Lim, K., Limmathurotsakul, D., Loftus, M.J., Lunn, M., Ma, J., Manoharan, A., Marks, F., May, J., Mayxay, M., Mturi, N., et al. (2022). Global burden of bacterial antimicrobial resistance in 2019: a systematic analysis. *The Lancet* 399**,** 629-655.

Murray, S.A., Amachawadi, R.G., Norman, K.N., Lawhon, S.D., Nagaraja, T.G., Drouillard, J.S., and Scott, H.M. 2021. Effects of Zinc and Menthol-Based Diets on Co-Selection of Antibiotic Resistance among E. coli and Enterococcus spp. in Beef Cattle. *Animals* [Online], 11.

Nik Mohamad Nek Rahimi, N., Natrah, I., Loh, J.-Y., Ervin Ranzil, F.K., Gina, M., Lim, S.-H.E., Lai, K.-S., and Chong, C.-M. 2022. Phytocompounds as an Alternative Antimicrobial Approach in Aquaculture. *Antibiotics* [Online], 11.

Nobrega, D.B., Tang, K.L., Caffrey, N.P., De Buck, J., Cork, S.C., Ronksley, P.E., Polachek, A.J., Ganshorn, H., Sharma, N., Kastelic, J.P., Kellner, J.D., Ghali, W.A., and Barkema, H.W. (2021). Prevalence of antimicrobial resistance genes and its association with restricted antimicrobial use in food-producing animals: a systematic review and meta-analysis. *J Antimicrob Chemother* 76**,** 561-575.

Nurk, S., Meleshko, D., Korobeynikov, A., and Pevzner, P.A. (2017). metaSPAdes: a new versatile metagenomic assembler. *Genome Res* 27**,** 824-834.

Oakley, B.B., Lillehoj, H.S., Kogut, M.H., Kim, W.K., Maurer, J.J., Pedroso, A., Lee, M.D., Collett, S.R., Johnson, T.J., and Cox, N.A. (2014). The chicken gastrointestinal microbiome. *FEMS Microbiology Letters* 360**,** 100-112.

Ogunade, I., Schweickart, H., Andries, K., Lay, J., and Adeyemi, J. 2018. Monensin Alters the Functional and Metabolomic Profile of Rumen Microbiota in Beef Cattle. *Animals* [Online], 8.

Okolie, O.J., Igwe, U., Ismail, S.U., Ighodalo, U.L., and Adukwu, E.C. (2023). Systematic review of surveillance systems for AMR in Africa. *Journal of Antimicrobial Chemotherapy* 78**,** 31-51.

Olekhnovich, E.I., Vasilyev, A.T., Ulyantsev, V.I., Kostryukova, E.S., and Tyakht, A.V. (2018). MetaCherchant: analyzing genomic context of antibiotic resistance genes in gut microbiota. *Bioinformatics* 34**,** 434-444.

Pal, C., Bengtsson-Palme, J., Kristiansson, E., and Larsson, D.G.J. (2016). The structure and diversity of human, animal and environmental resistomes. *Microbiome* 4**,** 54.

Pan American Health Organization (2020). *Protocol for Enhanced Isolate-Level Antimicrobial Resistance Surveillance in the Americas. Primary Phase: Bloodstream Infections.* Washington, D.C., USA: Pan American Health Organization.

Park, S.-J., Kim, J., Lee, J.-S., Rhee, S.-K., and Kim, H. (2014). Characterization of the fecal microbiome in different swine groups by high-throughput sequencing. *Anaerobe* 28**,** 157-162.

Parks, D.H., Imelfort, M., Skennerton, C.T., Hugenholtz, P., and Tyson, G.W. (2015). CheckM: assessing the quality of microbial genomes recovered from isolates, single cells, and metagenomes. *Genome Res* 25**,** 1043-1055.

Partridge, S.R., Kwong, S.M., Firth, N., and Jensen, S.O. (2018). Mobile Genetic Elements Associated with Antimicrobial Resistance. *Clinical Microbiology Reviews* 31**,** e00088-00017.

Pereira, M.B., Wallroth, M., Jonsson, V., and Kristiansson, E. (2018). Comparison of normalization methods for the analysis of metagenomic gene abundance data. *BMC Genomics* 19**,** 274.

Pillay, S., Calderón-Franco, D., Urhan, A., and Abeel, T. (2022). Metagenomic-based surveillance systems for antibiotic resistance in non-clinical settings. *Frontiers in Microbiology* 13.

Poulin-Laprade, D. (2023). (Sherbrooke, QC, Canada: Sherbrooke Research and Development Centre (Agriculture and Agri-Food Canada)).

Poulin-Laprade, D., Brouard, J.S., Gagnon, N., Turcotte, A., Langlois, A., Matte, J.J., Carrillo, C.D., Zaheer, R., Mcallister, T., Topp, E., and Talbot, G. (2021). Resistance determinants and their genetic context in enterobacteria from a longitudinal study of pigs reared under various husbandry conditions. *Appl Environ Microbiol* 87.

Proctor, A., and Phillips, G.J. (2019). Differential Effects of Bacitracin Methylene Disalicylate (BMD) on the Distal Colon and Cecal Microbiota of Young Broiler Chickens. *Frontiers in Veterinary Science* 6.

Public Health Agency of Canada (2020). "Canada Integrated Program for Antimicrobial Resistance Surveillance (CIPARS) 2018: Design and Methods". (Guelph, Ontario).

Public Health Agency of Canada (2022a). "Canadian Antimicrobial Resistance Surveillance System Report". (Ottawa, Ontario).

Public Health Agency of Canada (2022b). Canadian Nosocomial Infection Surveillance Program. Healthcare-associated infections and antimicrobial resistance in Canadian acute care hospitals, 2016–2020. *Can Commun Dis Rep* 48**,** 308-324.

Public Health Agency of Canada (2023). "Pan-Canadian Action Plan on Antimicrobial Resistance". (Ottawa, ON, Canada).

Qiu, T., Huo, L., Guo, Y., Gao, M., Wang, G., Hu, D., Li, C., Wang, Z., Liu, G., and Wang, X. (2022). Metagenomic assembly reveals hosts and mobility of common antibiotic resistome in animal manure and commercial compost. *Environmental Microbiome* 17**,** 42.

Quinlan, A.R., and Hall, I.M. (2010). BEDTools: a flexible suite of utilities for comparing genomic features. *Bioinformatics* 26**,** 841-842.

Ramachandran, P., Rachuri, N.K., Martha, S., Shakthivel, R., Gundala, A., and Battu, T.S. (2019). Implications of Overprescription of Antibiotics: A Cross-Sectional Study. *J Pharm Bioallied Sci* 11**,** S434-s437.

Raseala, C.M., Ekwanzala, M.D., and Momba, M.N.B. (2020). Shared Extended-Spectrum β-Lactamase-Producing Salmonella Serovars between Agricultural and Aquatic Environments Revealed through invA Amplicon Sequencing. *Microorganisms* 8.

Reid, G.E., Grim, S.A., Aldeza, C.A., Janda, W.M., and Clark, N.M. (2007). Rapid Development of Acinetobacter baumannii Resistance to Tigecycline. *Pharmacotherapy: The Journal of Human Pharmacology and Drug Therapy* 27**,** 1198-1201.

Review of Antimicrobial Resistance (2016). "Tackling Drug-Resistant Infections Globally: Final Report and Recommendations", (ed.) J. O'neill. (London, United Kingdom).

Reygaert, W.C. (2018). An overview of the antimicrobial resistance mechanisms of bacteria. *AIMS Microbiol* 4**,** 482-501.

Rhoads, A., and Au, K.F. (2015). PacBio Sequencing and Its Applications. *Genomics, Proteomics & Bioinformatics* 13**,** 278-289.

Rizzo, L., Manaia, C., Merlin, C., Schwartz, T., Dagot, C., Ploy, M.C., Michael, I., and Fatta-Kassinos, D. (2013). Urban wastewater treatment plants as hotspots for antibiotic

resistant bacteria and genes spread into the environment: a review. *Sci Total Environ* 447**,** 345-360.

Robertson, J., and Nash, J.H.E. (2018). MOB-suite: software tools for clustering, reconstruction and typing of plasmids from draft assemblies. *Microb Genom* 4.

Robinson, M.D., and Oshlack, A. (2010). A scaling normalization method for differential expression analysis of RNA-seq data. *Genome Biology* 11**,** R25.

Rovira, P., Mcallister, T., Lakin, S.M., Cook, S.R., Doster, E., Noyes, N.R., Weinroth, M.D., Yang, X., Parker, J.K., Boucher, C., Booker, C.W., Woerner, D.R., Belk, K.E., and Morley, P.S. (2019). Characterization of the Microbial Resistome in Conventional and "Raised Without Antibiotics" Beef and Dairy Production Systems. *Front Microbiol* 10**,** 1980.

Rozwandowicz, M., Brouwer, M.S.M., Fischer, J., Wagenaar, J.A., Gonzalez-Zorn, B., Guerra, B., Mevius, D.J., and Hordijk, J. (2018). Plasmids carrying antimicrobial resistance genes in Enterobacteriaceae. *Journal of Antimicrobial Chemotherapy* 73**,** 1121-1137.

Seemann, T. (2020). "Abricate". (https://github.com/tseemann/abricate: Github).

Shallcross, L.J., and Davies, S.C. (2014). The World Health Assembly resolution on antimicrobial resistance. *J Antimicrob Chemother* 69**,** 2883-2885.

Shanks, O.C., Kelty, C.A., Archibeque, S., Jenkins, M., Newton, R.J., Mclellan, S.L., Huse, S.M., and Sogin, M.L. (2011). Community structures of fecal bacteria in cattle from different animal feeding operations. *Appl Environ Microbiol* 77**,** 2992-3001.

Shehabeldine, A.M., Ashour, R.M., Okba, M.M., and Saber, F.R. (2020). Callistemon citrinus bioactive metabolites as new inhibitors of methicillin-resistant Staphylococcus aureus biofilm formation. *Journal of Ethnopharmacology* 254**,** 112669.

Sherry, N.L., Horan, K.A., Ballard, S.A., Gonçalves Da Silva, A., Gorrie, C.L., Schultz, M.B., Stevens, K., Valcanis, M., Sait, M.L., Stinear, T.P., Howden, B.P., and Seemann, T. (2023). An ISO-certified genomics workflow for identification and surveillance of antimicrobial resistance. *Nature Communications* 14**,** 60.

Shriram, V., Khare, T., Bhagwat, R., Shukla, R., and Kumar, V. (2018). Inhibiting Bacterial Drug Efflux Pumps via Phyto-Therapeutics to Combat Threatening Antimicrobial Resistance. *Frontiers in Microbiology* 9.

Sifaoui, F., Arthur, M., Rice, L., and Gutmann, L. (2001). Role of penicillin-binding protein 5 in expression of ampicillin resistance and peptidoglycan structure in Enterococcus faecium. *Antimicrob Agents Chemother* 45**,** 2594-2597.

Siguier, P., Perochon, J., Lestrade, L., Mahillon, J., and Chandler, M. (2006). ISfinder: the reference centre for bacterial insertion sequences. *Nucleic Acids Res* 34**,** D32-36.

Simão, F.A., Waterhouse, R.M., Ioannidis, P., Kriventseva, E.V., and Zdobnov, E.M. (2015). BUSCO: assessing genome assembly and annotation completeness with single-copy orthologs. *Bioinformatics* 31**,** 3210-3212.

Slizovskiy, I.B., Mukherjee, K., Dean, C.J., Boucher, C., and Noyes, N.R. (2020). Mobilization of Antibiotic Resistance: Are Current Approaches for Colocalizing Resistomes and Mobilomes Useful? *Frontiers in Microbiology* 11.

Sloane, P.D., Tandan, M., and Zimmerman, S. (2020). Preventive Antibiotic Use in Nursing Homes: A Not Uncommon Reason for Antibiotic Overprescribing. *Journal of the American Medical Directors Association* 21**,** 1181-1185.

Spivak, E.S., Cosgrove, S.E., and Srinivasan, A. (2016). Measuring Appropriate Antimicrobial Use: Attempts at Opening the Black Box. *Clinical Infectious Diseases* 63**,** 1-6.

Stapleton, G.S., Cazer, C.L., and Gröhn, Y.T. (2021). Modeling the Effect of Tylosin Phosphate on Macrolide-Resistant Enterococci in Feedlots and Reducing Resistance Transmission. *Foodborne Pathog Dis* 18**,** 85-96.

Suvaithenamudhan, S., Ananth, S., Mariappan, V., Dhayabaran, V.V., Parthasarathy, S., Ganesh, P.S., and Shankar, E.M. 2022. In Silico Evaluation of Bioactive Compounds of Artemisia pallens Targeting the Efflux Protein of Multidrug-Resistant Acinetobacter baumannii (LAC-4 Strain). *Molecules* [Online], 27.

Temmerman, R., Ghanbari, M., Antonissen, G., Schatzmayr, G., Duchateau, L., Haesebrouck, F., Garmyn, A., and Devreese, M. (2022). Dose-dependent impact of enrofloxacin on broiler chicken gut resistome is mitigated by synbiotic application. *Frontiers in Microbiology* 13.

Thomas, M., Webb, M., Ghimire, S., Blair, A., Olson, K., Fenske, G.J., Fonder, A.T., Christopher-Hennings, J., Brake, D., and Scaria, J. (2017). Metagenomic characterization of the effect of feed additives on the gut microbiome and antibiotic resistome of feedlot cattle. *Scientific Reports* 7**,** 12257.

Tunsagool, P., Mhuantong, W., Tangphatsornruang, S., Am-In, N., Chuanchuen, R., Luangtongkum, T., and Suriyaphol, G. (2021). Metagenomics of antimicrobial and heavy

metal resistance in the cecal microbiome of fattening pigs raised without antibiotics. *Appl Environ Microbiol* 87.

Tyrrell, C., Do, T.T., Leigh, R.J., Burgess, C.M., Brennan, F.P., and Walsh, F. (2023). Differential impact of swine, bovine and poultry manure on the microbiome and resistome of agricultural grassland. *Science of The Total Environment* 886**,** 163926.

U.S. Center for Disease Control (2022). "2019 National Antimicrobial Resistance Monitoring System (NARMS) Update: Integrated Report Summary". (Atlanta, GA, USA: U.S. Department of Health and Human Services, CDC).

Van Hoek, A.H., Mevius, D., Guerra, B., Mullany, P., Roberts, A.P., and Aarts, H.J. (2011). Acquired antibiotic resistance genes: an overview. *Front Microbiol* 2**,** 203.

Von Meijenfeldt, F.a.B., Arkhipova, K., Cambuy, D.D., Coutinho, F.H., and Dutilh, B.E. (2019). Robust taxonomic classification of uncharted microbial sequences and bins with CAT and BAT. *Genome Biology* 20**,** 217.

Wagner, J.J., Archibeque, S.L., and Feuz, D.M. (2014). The Modern Feedlot for Finishing Cattle. *Annual Review of Animal Biosciences* 2**,** 535-554.

Walia, K., Madhumathi, J., Veeraraghavan, B., Chakrabarti, A., Kapil, A., Ray, P., Singh, H., Sistla, S., and Ohri, V.C. (2019). Establishing Antimicrobial Resistance Surveillance & Research Network in India: Journey so far. *Indian J Med Res* 149**,** 164-179.

Wang, G., Wilson, T.J., Jiang, Q., and Taylor, D.E. (2001). Spontaneous mutations that confer antibiotic resistance in Helicobacter pylori. *Antimicrob Agents Chemother* 45**,** 727-733.

Weinroth, M.D., Martin, J.N., Doster, E., Geornaras, I., Parker, J.K., Carlson, C.R., Metcalf, J.L., Morley, P.S., and Belk, K.E. (2019). Investigation of tylosin in feed of feedlot cattle and effects on liver abscess prevalence, and fecal and soil microbiomes and resistomes1. *Journal of Animal Science* 97**,** 4567-4578.

Weinroth, M.D., Thomas, K.M., Doster, E., Vikram, A., Schmidt, J.W., Arthur, T.M., Wheeler, T.L., Parker, J.K., Hanes, A.S., Alekoza, N., Wolfe, C., Metcalf, J.L., Morley, P.S., and Belk, K.E. (2022). Resistomes and microbiome of meat trimmings and colon content from culled cows raised in conventional and organic production systems. *Anim Microbiome* 4**,** 21.

Wickham, H. (2016). *ggplot2: Elegant Graphics for Data Analysis.* Springer-Verlag New York.

Wood, D.E., Lu, J., and Langmead, B. (2019). Improved metagenomic analysis with Kraken 2. *Genome Biology* 20**,** 257.

World Health Organization (2022). "Global Antimicrobial Resistance and Use Surveillance System (GLASS) Report 2022". Licence: CC BY-NC-SA 3.0 IGO. ed. (Geneva, Switzerland).

Xiong, W., Sun, Y., and Zeng, Z. (2018). Antimicrobial use and antimicrobial resistance in food animals. *Environ Sci Pollut Res Int* 25**,** 18377-18384.

Yang, C., Das, Q., Rehman, M.A., Yin, X., Shay, J., Gauthier, M., Lau, C.H., Ross, K., and Diarra, M.S. 2023. Microbiome of Ceca from Broiler Chicken Vaccinated or Not against Coccidiosis and Fed Berry Pomaces. *Microorganisms* [Online], 11.

Yi, X., Liang, J.-L., Su, J.-Q., Jia, P., Lu, J.-L., Zheng, J., Wang, Z., Feng, S.-W., Luo, Z.-H., Ai, H.-X., Liao, B., Shu, W.-S., Li, J.-T., and Zhu, Y.-G. (2022). Globally distributed mining-impacted environments are underexplored hotspots of multidrug resistance genes. *The ISME Journal* 16**,** 2099-2113.

Yoshida, H., Motohashi, T., De Bus, L., De Waele, J., Takaba, A., Kuriyama, A., Kobayashi, A., Tanaka, C., Hashi, H., Hashimoto, H., Nashiki, H., Shibata, M., Kanamoto, M., Inoue, M., Hashimoto, S., Katayama, S., Fujiwara, S., Kameda, S., Shindo, S., Suzuki, T., Komuro, T., Kawagishi, T., Kawano, Y., Fujita, Y., Kida, Y., Hara, Y., Fujitani, S., and Group, D.S.J. (2022). Use of broad-spectrum antimicrobials for more than 72 h and the detection of multidrug-resistant bacteria in Japanese intensive care units: a multicenter retrospective cohort study. *Antimicrobial Resistance & Infection Control* 11**,** 119.

Yu, Z., Tang, J., Khare, T., and Kumar, V. (2020). The alarming antimicrobial resistance in ESKAPEE pathogens: Can essential oils come to the rescue? *Fitoterapia* 140**,** 104433.

Zaheer, R., Cook, S., Klima, C., Stanford, K., Alexander, T., Topp, E., Read, R., and Mcallister, T. (2013). Effect of subtherapeutic vs. therapeutic administration of macrolides on antimicrobial resistance in Mannheimia haemolytica and enterococci isolated from beef cattle. *Frontiers in Microbiology* 4.

Zaheer, R., Lakin, S.M., Polo, R.O., Cook, S.R., Larney, F.J., Morley, P.S., Booker, C.W., Hannon, S.J., Van Domselaar, G., Read, R.R., and Mcallister, T.A. (2019). Comparative diversity of microbiomes and Resistomes in beef feedlots, downstream environments and urban sewage influent. *BMC Microbiol* 19**,** 197.

Zaheer, R., Noyes, N., Ortega Polo, R., Cook, S.R., Marinier, E., Van Domselaar, G., Belk, K.E., Morley, P.S., and Mcallister, T.A. (2018). Impact of sequencing depth on the characterization of the microbiome and resistome. *Scientific Reports* 8**,** 5890.

Zaidi, S.E., Zaheer, R., Poulin-Laprade, D., Scott, A., Rehman, M.A., Diarra, M., Topp, E., Domselaar, G.V., Zovoilis, A., and Mcallister, T.A. (2023). Comparative Genomic

Analysis of Enterococci across Sectors of the One Health Continuum. *Microorganisms* 11.

Zankari, E., Allesøe, R., Joensen, K.G., Cavaco, L.M., Lund, O., and Aarestrup, F.M. (2017). PointFinder: a novel web tool for WGS-based detection of antimicrobial resistance associated with chromosomal point mutations in bacterial pathogens. *Journal of Antimicrobial Chemotherapy* 72**,** 2764-2768.

Zhang, H., Jain, C., and Aluru, S. (2020). A comprehensive evaluation of long read error correction methods. *BMC Genomics* 21**,** 889.

Zhang, Z., Zhang, Q., Wang, T., Xu, N., Lu, T., Hong, W., Penuelas, J., Gillings, M., Wang, M., Gao, W., and Qian, H. (2022). Assessment of global health risk of antibiotic resistance genes. *Nature Communications* 13**,** 1553.

Zhao, R., Yu, K., Zhang, J., Zhang, G., Huang, J., Ma, L., Deng, C., Li, X., and Li, B. (2020). Deciphering the mobility and bacterial hosts of antibiotic resistance genes under antibiotic selection pressure by metagenomic assembly and binning approaches. *Water Research* 186**,** 116318.

Zhou, N., Cheng, Z., Zhang, X., Lv, C., Guo, C., Liu, H., Dong, K., Zhang, Y., Liu, C., Chang, Y.-F., Chen, S., Guo, X., Zhou, X.-N., Li, M., and Zhu, Y. (2022). Global antimicrobial resistance: a system-wide comprehensive investigation using the Global One Health Index. *Infectious Diseases of Poverty* 11**,** 92.

APPENDIX 1: Supplemental Tables and Figures Associated with Chapter 3

Table S1. Pen level Metadata of animals used to collect fecal composite samples. Sex listed without "Yearling" are calves.

Pen-level Fecal Composite Sample ID	Age/Sex
Nat-Win-B-25Oct16-M1	Yearling, Steers
Nat-Win-B-25Oct16-M2	Yearling, heifers
Con-Win-A-22Oct16-M4	Yearling, Steers
Nat-Win-C-31Jan17-M7	Steers
Con-Win-D-1Feb17-M9	Yearling, Steers
Con-Win-D-2Feb17-M10	Yearling, Heifers
Nat-Sum-B-28Jun17-M17	Yearling steers
Nat-Sum-B-28Jun17-M18	Yearling heifers
Nat-Win-B-1Mar17-MG2	Yearling, Heifers
Con-Win-A-1Mar17-MG3	Yearling, Not segregated
Con-Win-A-1Mar17-MG4	Yearling, Not segregated
Nat-Sum-B-26Apr17-MG5	Yearling, Steers
Nat-Sum-B-26Apr17-MG6	Yearling, Heifers
Con-Sum-A-26Apr17-MG7	Yearling, Heifers
Con-Sum-A-26Apr17-MG8	Yearling, Steers
Nat-Sum-C-10Apr18-MG9	Steers
Nat-Sum-C-10Apr18-MG10	Heifers
Con-Sum-D-10Apr18-MG11	Yearling, Not segregated
Con-Sum-D-10Apr18-MG12	Yearling, Not segregated
Nat-Win-B-13Mar18-MG13	Not segregated
Nat-Win-B-13Mar18-MG14	Not segregated
Con-Win-A-13Mar18-MG15	Yearling, Not segregated
Con-Win-A-13Mar18-MG16	Yearling, Not segregated
Nat-Sum-C-26Jun18-MG17	Steers
Nat-Sum-C-26Jun18-MG18	Heifers
Con-Sum-D-26Jun18-MG19	Yearling, Not segregated
Con-Sum-D-26Jun18-MG20	Yearling, Not segregated
Nat-Sum-B-25Jul18-MG21	Yearling, Steers
Nat-Sum-B-25Jul18-MG22	Yearling, Heifers
Con-Sum-A-25Jul18-MG23	Yearling, Not segregated
Con-Sum-A-25Jul18-MG24	Yearling, Not segregated
Nat-Sum-C-1Aug17-MG25	Heifers
Nat-Sum-C-1Aug17-MG26	Heifers
Con-Sum-D-1Aug17-MG27	Yearling, Heifers
Con-Sum-D-1Aug17-MG28	Yearling, Steers
Nat-Win-C-24Oct17-MG29	Heifers
Con-Win-D-24Oct17-MG31	Yearling, Steers
Con-Win-D-24Oct17-MG32	Yearling, Heifers
Nat-Win-C-29Jan18-MG33	Heifers
Nat-Win-C-29Jan18-MG34	Heifers
Con-Win-D-29Jan18-MG35	Yearling, Heifers
Con-Win-D-29Jan18-MG36	Yearling, Steers
Nat-Sum-B-05Sep17-MG41	Yearling, Steers
Nat-Sum-B-05Sep17-MG42	Yearling, Heifers
Con-Sum-A-05Sep17-MG43	Yearling, Steers
Con-Sum-A-05Sep17-MG44	Yearling, Heifers
Nat-Sum-B-29May18-MG45	Yearling, no data
Nat-Sum-B-29May18-MG46	Yearling, no data
Con-Sum-A-29May18-MG47	Yearling, Heifers
Con-Sum-A-29May18-MG48	Yearling, Steers
Con-Win-A-25Oct16-MG49	Yearling, Heifers
Nat-Win-C-31Jan17-MG50	Heifers
Nat-Win-B-11Dec17-MG53	Yearling, Steers
Nat-Win-B-11Dec17-MG54	Yearling, Heifers
Con-Win-A-11Dec17-MG55	Yearling, Steers
Con-Win-A-11Dec17-MG56	Yearling, Heifers
Nat-Win-C-29Mar17-MG57	Heifers
Nat-Win-C-29Mar17-MG58	Steers
Con-Win-D-29Mar17-MG59	Yearling, Heifers
Con-Win-D-29Mar17-MG60	Yearling, Steers

Table S2. Most abundant archaeal and bacterial classes (>1%) and orders (top 20) of fecal and catch basin water samples with mean normalized abundance, interpreted significance of adjusted p-value (q > 0.05 = ns; q < 0.05 = *; q < 0.01 = **; q < 0.001 = ***; q < 0.0001 = ****), and fold change for significant differences from natural (NAT) to conventional (CONV).

Taxa Level	Taxon	Fecal Samples				Catch Basin Water Samples			
		CONV Mean Normalized Abundance	NAT Mean Normalized Abundance	Interpreted Significance	Fold Change	CONV Mean Normalized Abundance	NAT Mean Normalized Abundance	Interpreted Significance	Fold Change
Class	Actinobacteria	0.055	0.066	**	1.09	0.096	0.122	*	0.99
	Alphaproteobacteria	0.041	0.045	ns	-	0.154	0.116	ns	-
	Bacilli	0.078	0.091	*	1.06	0.017	0.029	****	1.06
	Bacteroidia	0.210	0.168	***	0.81	0.019	0.041	****	1.01
	Betaproteobacteria	0.031	0.033	ns	-	0.229	0.251	ns	-
	Chlorobia	0.002	0.002	ns	-	-	-	ns	-
	Clostridia	0.271	0.307	ns	-	0.018	0.057	ns	-
	Coriobacteriia	0.012	0.014	ns	-	-	-	ns	-
	Cytophagia	0.014	0.014	ns	-	0.009	0.015	ns	-
	Deltaproteobacteria	0.016	0.017	ns	-	0.105	0.08	ns	-
	Epsilonproteobacteria	0.012	0.009	****	0.97	0.018	0.015	ns	-
	Flavobacteriia	0.039	0.038	*	0.93	0.030	0.041	****	1.13
	Gammaproteobacteria	0.096	0.091	*	0.91	0.249	0.161	ns	-
	Methanobacteria	0.014	0.006	***	0.58	0.000	0.002	****	1.18
	Mollicutes	0.007	0.009	**	1.11	-	-	ns	-
	Negativicutes	0.014	0.012	ns	-	-	-	ns	-
	Spirochaetia	0.029	0.015	**	0.69	-	-	ns	-
Order	Alteromonadales	0.009	0.008	*	0.88	-	-	-	-
	Bacillales	0.051	0.059	*	1.05	0.012	0.02	ns	-
	Bacteroidales	0.210	0.167	***	0.80	0.018	0.04	ns	-
	Bifidobacteriales	0.006	0.011	***	1.33	-	-	-	-
	Burkholderiales	0.023	0.024	ns	-	0.174	0.202	ns	-
	Campylobacterales	0.012	0.009	****	0.77	0.019	0.015	ns	-
	Caulobacterales	-	-	-	-	0.017	0.01	ns	-
	Chromatiales	-	-	-	-	0.085	0.016	ns	-
	Clostridiales	0.266	0.301	ns	-	0.017	0.055	ns	-
	Corynebacteriales	0.012	0.013	ns	-	0.018	0.022	ns	-
	Cytophagales	0.014	0.014	ns	-	-	-	-	-
	Desulfovibrionales	-	-	-	-	0.086	0.062	ns	-
	Enterobacterales	0.026	0.024	*	0.90	0.019	0.018	ns	-
	Erysipelotrichales	0.010	0.011	ns	-			-	-
	Flavobacteriales	0.040	0.039	*	0.92	0.031	0.042	ns	-
	Lactobacillales	0.026	0.030	*	1.04	-	-	-	-
	Methanobacteriales	0.014	0.006	***	0.57	-	-	-	-
	Micrococcales	0.009	0.010	ns	-	0.022	0.025	ns	-
	Nitrosomonadales	-	-	-	-	0.011	0.02	ns	-
	Pseudomonadales	0.020	0.020	ns	-	0.062	0.056	ns	-
	Rhizobiales	0.016	0.018	ns	-	0.052	0.045	ns	-
	Rhodobacterales	-	-	-	-	0.047	0.031	ns	-
	Rhodocyclales	-	-	-	-	0.038	0.023	ns	-
	Sphingomonadales	-	-	-	-	0.021	0.015	ns	-
	Spirochaetales	0.027	0.013	**	0.66	-	-	-	-
	Streptomycetales	0.009	0.010	ns	-	0.023	0.026	ns	-
	Xanthomonadales	-	-	-	-	0.018	0.016	ns	-

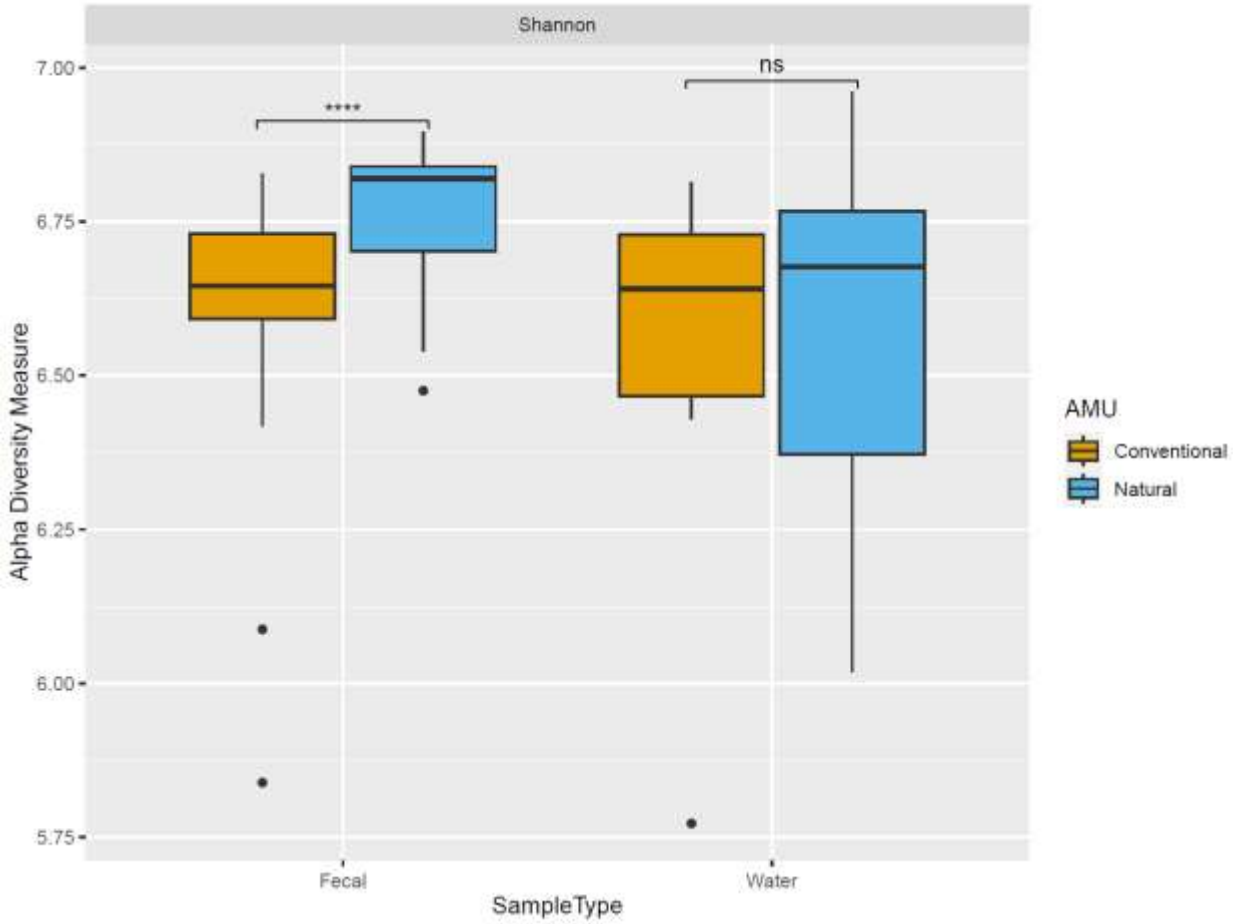

Figure S1. Boxplot of Shannon index comparisons between conventional and natural feedlots for fecal and catch basin water samples. (Wilcoxon signed rank test; $p > 0.05$ = ns; $p < 0.0001$ = ****)

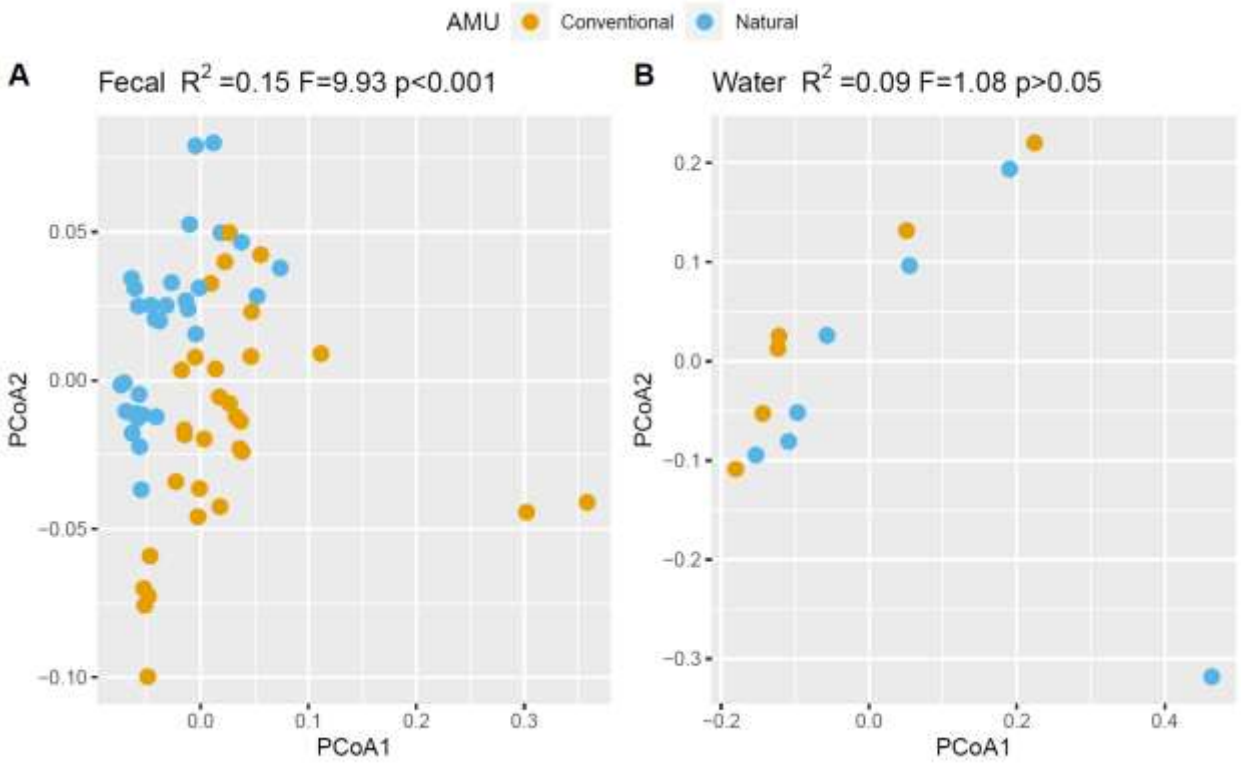

Figure S2. Principle component analyses (PCoA) of beta diversity differences using Bray-Curtis for fecal (A) and catch basin water (B) samples between conventional and natural feedlot types. (PERMANOVA test)

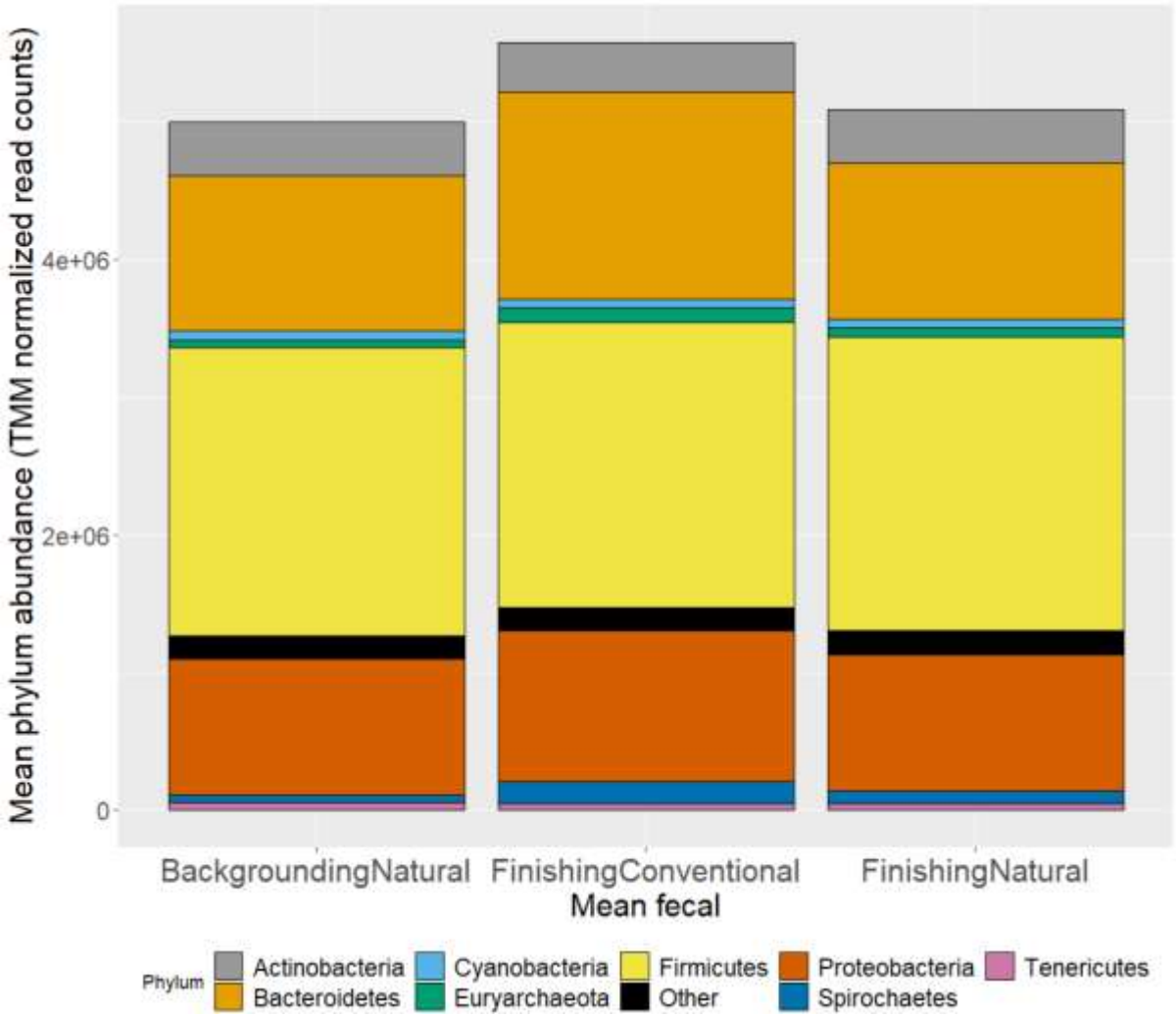

Figure S3. Stacked bar plots comparing TMM (trimmed mean of m-values) normalized mean read counts per sample of prevalent phyla (>1%) across fecal sample diet (Backgrounding vs Finishing) and feedlot management practices (Conventional vs Natural).

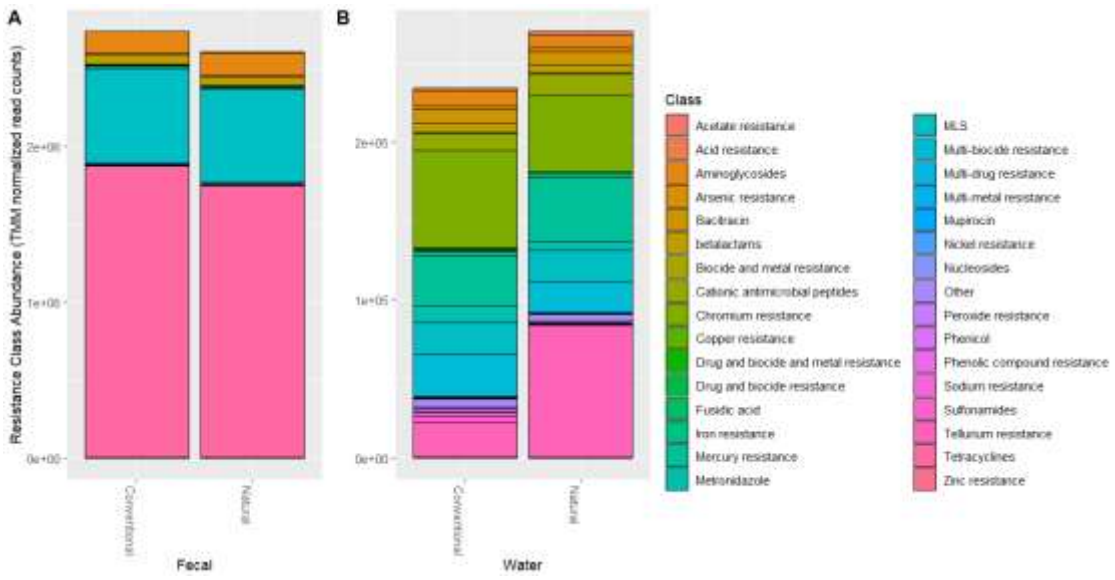

Figure S4. Stacked bar plots of TMM (trimmed mean of m-values) normalized read counts per feedlot management practice for fecal (**A**) and catch basin water (**B**) antimicrobial resistance gene classes.

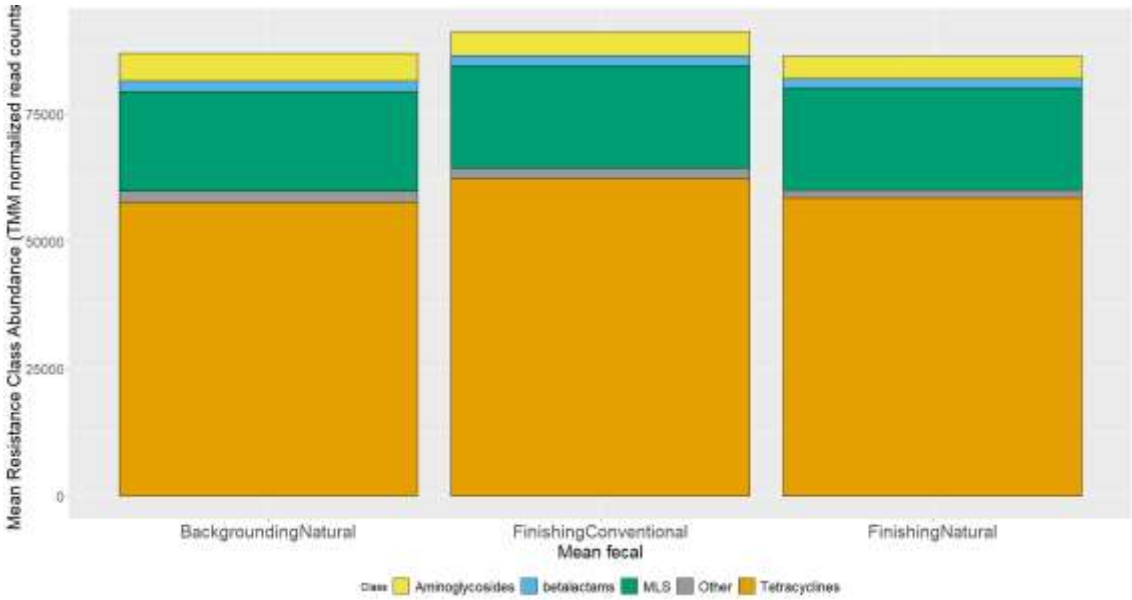

Figure S5. Stacked bar plots comparing TMM (trimmed mean of m-values) normalized mean read counts per sample of prevalent antimicrobial resistance gene classes (>1%) across fecal sample diet (Backgrounding vs Finishing) and feedlot management practices (Conventional vs Natural).