Antimicrobial resistance in the microbiome of feedlot watering bowls and bovine respiratory disease associated pathogens.

A Thesis Submitted to the
College of Graduate and Postdoctoral Studies
In Partial Fulfillment of the Requirements
For the Degree of Master of Science
In the Department of Large Animal Clinical Sciences
University of Saskatchewan
Saskatoon.

By
DANIËL WILLEM KOS

PERMISSION TO USE STATEMENT

In presenting this thesis/dissertation in partial fulfillment of the requirements for a Postgraduate degree from the University of Saskatchewan, I agree that the libraries of this University may make it freely available for inspection. I further agree that permission for copying of this thesis/dissertation in any manner, in whole or in part, for scholarly purposes may be granted by the professor or professors who supervised my thesis/dissertation work or, in their absence, by the Head of the Department or the Dean of the College in which my thesis work was done. It is understood that any copying or publication or use of this thesis/dissertation or parts thereof for financial gain shall not be allowed without my written permission. It is also understood that due recognition shall be given to me and to the University of Saskatchewan in any scholarly use which may be made of any material in my thesis/dissertation.

Requests for permission to copy or to make other uses of materials in this thesis/dissertation in whole or part should be addressed to:

Head of the Department of Large Animal Clinical Sciences
Western College of Veterinary Medicine
52 Campus Drive
University of Saskatchewan
Saskatoon, Saskatchewan, S7N 5B4 Canada

OR

Dean
College of Graduate and Postdoctoral Studies
University of Saskatchewan
116 Thorvaldson Building, 110 Science Place
Saskatoon, Saskatchewan S7N 5C9 Canada

ABSTRACT

Bovine respiratory disease (BRD) is the primary disease of concern to beef production and is driven by stress, bacteria, viruses, and environmental and management practices. Antimicrobial use (AMU) to control BRD selects for antimicrobial resistance (AMR) genes (ARGs). Treatment failure due to AMR necessitates a rapid switch to an effective antimicrobial before a devastating amount of death loss occurs. Monitoring AMR in a herd through individual animals is impractical. There is a need for a fast, pragmatic, and scalable methodology to monitor AMR within cattle to guide effective AMU. Cattle watering bowls (WB) have been previously shown to harbour BRD pathogens and ARGs but have yet to be explored in the same context as wastewater AMR monitoring is for public health. This thesis outlines a pilot project of WB sampling as a proxy method to monitor the presence of ARGs of relevance to BRD associated pathogens.

The microbiomes within watering bowls of two feedlots were genotypically and phenotypically assessed through water, swab, and sediment sampling. A new feedlot was sampled (68 water, 63 swab) as pens filled (up to 9 WB) over a series of 8 weeks. The other, older, feedlot was only sampled in the 8th week (sediment, swab, water) from 20 WB. For phenotypic AMR detection, samples were inoculated into antimicrobial sensitivity test (AST) panels containing microdilutions of four antimicrobials (enrofloxacin, florfenicol (FFN), tulathromycin, oxytetracycline). Bacteria that grew on these plates were isolated (n = 78). A subset (n = 28 new feedlot, n = 3 old feedlot + *H. somni* from a deep nasopharyngeal swab) of these underwent further characterisation on an AST panel containing 10 different antimicrobials. The whole genome of isolates (n = 10, 6 new, 4 old) was sequenced and analysed for ARGs. These detected ARGs were compared to a custom dataset of 172 BRD genomes from NCBI. The genetic neighbourhood of *floR*, an ARG encoding for the efflux of FFN, within the WB isolates (n = 4) was also compared to pathogen genomes. For the culture-independent methodology, samples from one feedlot underwent amplified 16S rRNA sequencing (n = 122), while samples from the other underwent shotgun metagenomic sequencing (n = 11). The ARGs detected in WB isolates were compared to the ARGs detected within metagenomic sequencing, and previous datasets of feedlot water.

Multi-drug resistant bacteria were routinely isolated from WBs. When environmental samples from the last week of sampling were inoculated into media with FFN (>32 µg/mL), 93/96 samples had bacterial growth. By constructing a genotypic AMR profile from two feedlots and by consulting previous metagenomic datasets from feedlot water, the understudied resistome of the watering bowl microbiome was shown to provide outstanding coverage of the ARGs (15/16) detected within 172 BRD genomes. The overlap in genetic neighbourhoods between copies of *floR* in WB and BRD genomes confirms the possibility of horizontal gene transfer via a myriad of transposable elements between both populations. Though further studies are needed to reaffirm our findings, in this thesis the understudied environmental microbiome within feedlot watering bowls was found to serve as a potential source of ARGs for BRD pathogens and thus may serve as a proxy for relevant ARGs within feedlot environments.

ACKNOWLEDGEMENTS

Funding was obtained from the generous support of the Saskatchewan Ministry of Agriculture, Agriculture Development Fund, and the Saskatchewan Cattlemen's Association.

The nucleotide sequencing discussed in this thesis was performed at *Centre d'expertise et de services Génome Québec,* the national research council of Canada, and at the global institute for food security.

My utmost appreciation for my supervisors Dr. Murray Jelinski and Dr. Tony Ruzzini, for accepting me into this program and for their guidance, teachings, and patience.

I would like to thank my committee members and graduate chairs for facilitating the progression of my program.

I would also like to thank all members of the Jelinski and Ruzzini labs, during the duration of my program they were helpful and kind. Especially Jenny Liang, Elhem Yacoub, Brittany Schreiner, Richa Jain, and Danilo Wadt provided thoughtful discussion and support.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVATIONS

AWaRe	Access, Watch and Reserve
AMR	Antimicrobial resistance
ARGs	Antimicrobial resistance genes
AST	Antimicrobial sensitivity test
AMU	Antimicrobial usage
BRD	Bovine respiratory disease
CARD	The Comprehensive Antibiotic Resistance Database
CFU	Colony forming units
CTC	Chlortetracycline
DNS	Deep nasopharyngeal swab
DOF	Days on feed
ENR	Enrofloxacin
FFN	Florfenicol
GAM	Gamithromycin
GTA	Gene Transfer Agents
HGT	Horizontal Gene Transfer
TIL	Tilmicosin
TUL	Tulathromycin
TYL	Tylosin
MIC	Minimum inhibitory concentrations
NPET	Ribosomal nascent peptide exit tunnel
RT	Room temperature
SNP	Single-nucleotide polymorphism
OTC	Oxytetracycline
WB	Watering bowl
WHO	World Health Organization

1 Introduction and literature review

This literature review is intended to give an overview of the beef industry in Western Canada, its struggle with the devastating impact of bovine respiratory disease (BRD), antimicrobial usage (AMU) within the industry to control BRD, and problems related to antimicrobial resistance (AMR) and effective AMU within the industry. This literature review will also discuss AMR within environmental microbiomes and the current understanding of its relationship with AMR of pathogens. This provides background information on the experimental chapters that study AMR within the feedlot environment to provide a greater understanding of AMR and how we might improve AMU and treatment outcomes within the Canadian beef industry.

1.1 BACKGROUND OF WESTERN CANADIAN FEEDLOTS

The Canadian beef industry is an important economical sector of Canada, and to many Canadians a source of cultural pride. Calgary is a metropolis with a cowboy hat on its flag and is colloquially known as “Cowtown” and “Stampede city”. Calgary is an example of the cultural impact that the industry has had in Western Canada. The industry consists of a network of calving operations, markets, and feedlots and was estimated in 2012 to contribute $5.4 billion annually to the Canadian economy (1).

1.1.1 Overview of the Canadian beef industry

The beef industry in Western Canada is divided into economically connected sectors that function individually. Beef calves are typically born in cow-calf operations in the spring and pastured over the summer months. In the fall, they are weaned and either maintained for another year by the producers on pasture (Yearlings) or sold as newly weaned (Fall-placed calves) to feedlots.

The focus of this thesis is on feedlots. Feedlots are specialized production facilities that house cattle for fattening in a series of large, fenced off, outdoor areas known as pens. Feedlots acquire cattle via auction markets or directly from individual cow-calf operations. In the auctions, cattle are re-sorted based upon traits such as their weight, size, breed, gender, and colour. This effectively means that cattle purchased at auctions are geographically diverse groups from a range of cow-calf operations. These calves are then transported to the feedlot and placed into pens that typically hold 200 to 300 cattle, depending on the facility.

1.1.2 Sizes, distribution, and regions of beef industry operations

Though feedlot capacity varies between producers, feedlots hold the largest concentrations of cattle compared to the other sectors of the beef industry. In a recent survey, participating feedlots had an average of ~12,000 cattle per feedlot (2). Cow-calf operations are typically much smaller with a median herd size of around 200 in Western Canada (3). The two regions of beef operations in Canada are Western Canada (Manitoba, Saskatchewan, Alberta, and British Columbia) and Eastern Canada (Ontario and Quebec). Out of the 10 provinces in Canada, the largest concentration of beef cattle and largest herd sizes are in the provinces of Alberta and Saskatchewan, making Western Canada the largest beef production region in Canada, accounting for >85% of all beef cattle (4, 5). Management practices in Western Canada follow similar styles to large feedlot operations in the Western and Central continental United States (6). Beef industry management

practices differ greatly between North America and Europe where the beef industry is less specialized and often integrated with the dairy industry, and generally large scale feedlots are not used (6).

1.1.3 Veterinary oversight and antimicrobial class classification systems

In Canadian livestock production the administration of antimicrobials is performed by the producers with veterinary oversight and antimicrobials are prohibited for use as growth promoters. The World Health Organization (WHO) has made a series of recommendations for AMU in livestock. The WHO recommends that antimicrobials of medical importance should not be used to prevent disease in any animal that has not been clinically diagnosed by a veterinarian, and that the use of antimicrobials which are critically important to human medicine completely cease in livestock (7).

The WHO categorizes antimicrobials as critically important through a classification system that was recently updated in 2018 (8). This system uses two conditions to classify antimicrobials into one of three categories (8). Condition one is that the antimicrobial has the capacity to treat serious infections in humans and that there is a limited availability of alternative treatments (8). Condition two is the capacity to treat infection from non-human sources and bacteria with AMR from non-human sources (8). If both conditions are met the antimicrobial class is considered "critically important", if only one is met it is considered "highly important", and if neither are met it is considered "important" (8). Through this categorization system, a large number of antimicrobials are labelled as critically important, including antimicrobials used on a regular basis in beef cattle production and those utilized only as a last resort (9). While stewardship of all critically important antimicrobials is important, there is a need to exercise more caution for those with few alternatives. To emphasize this, the WHO has a secondary classification system that is parallel to the critically important list, Access, Watch and Reserve (AWaRe), which was last updated in 2021 (10). This system has a more utilitarian focus on three levels of usage, stewardship (access), restraint (watch) and last resort (reserve) (10). The AWaRe system is based on the availability of alternative treatment, the seriousness of treatable infection, and the likelihood of antimicrobial resistance development (10). Similar to the WHO AWaRe system, the system devised by Health Canada is based on the availability of alternative treatments and the seriousness of the treatable infection (Table 1.1) (11).

While the classification systems are logical in nature, the overall classification of antimicrobials as critically important by the WHO can lead to confusion. Macrolide usage is common in the livestock industry and this drug class is considered a category 2 antimicrobial of high importance by Health Canada and in the Watch category in WHO AWaRe (Table 1.2) (9-11). However, macrolides are considered critically important by WHO's critically important antimicrobial system, and thus WHO recommends a complete cease of the use of macrolides in the livestock industry (7, 8). If we compare that to fluoroquinolones, which are commonly considered a last resort antimicrobial that should be rarely used in the North American livestock industry, WHO also rates this class as critically important and it is in the Watch section of AWaRe, while Health Canada categorizes it as of very high importance (category 1) (8-11). The WHO's critically important antimicrobial list recommends the cessation of the use of both macrolides and

fluoroquinolones in the livestock industry, even though there are few alternatives to the broad spectrum fluoroquinolones but a range of alternatives to macrolides (12).

The European Medicine Agency has created an antimicrobial usage classification system designed specifically for veterinarians, based on recommendations from the WHO (13). The system also greatly differs from the WHO by accounting for simplicity (A to D system) and for the alternative antimicrobials that are available for use in veterinary medicine (13). The European Medicine Agency system will hopefully inspire changes to the antimicrobial classification systems in North America towards simplification and adaptation to the unique conditions of veterinary medicine.

Table 1.1 Brief description of antimicrobial class classifications by organization and criteria

Level	Description
WHO (AWaRe)	
Access	Wide range of susceptible pathogens
	Low capacity for resistance
Watch	Higher resistance potential
Reserve	Highest priority agents (Critically Important Antimicrobials)
	Reserved for treatment of multidrug resistant microbes
WHO (Critically important antimicrobial class list)	
C1 (Criteria)	Treats serious infections
	Limited alternatives
C2 (Criteria)	Treats infections from non-human sources OR with resistance genes from non-human sources
Important antimicrobials	Neither C1 nor C2 met
Highly important antimicrobials	Only C1 or C2 met
Critically important antimicrobials	Both C1 and C2 met
Health Canada	
Category I: Very high importance	Treats serious infections
	Limited to no alternatives
Category II: High importance	Treats serious infections
	Alternatives only in Category I
Category III: Medium importance	Alternatives are available in Category I and II
Category IV: Low importance	Not currently in use in human medicine

Table 1.2 Antimicrobial classes relevant to the livestock industry and their AMR group classifications

Antimicrobial class	Antimicrobial resistance classification system		
	WHO (AWaRe)	**WHO (Critically Important Antimicrobial list)**	**Gov. Canada**
Fluoroquinolones	Watch	Not listed (Quinolones listed as Critically Important Antimicrobial)	I - Very High Importance
Macrolides	Watch	Critically Important Antimicrobial	II - High Importance
Tetracyclines	Access Watch Reserve	Highly Important Antimicrobial	III - Medium Importance
Phenicols	Watch	Highly Important Antimicrobial	III - Medium Importance

1.2 BOVINE RESPIRATORY DISEASE

Bovine respiratory disease (BRD) is a leading cause of morbidity and mortality in feedlot cattle (14, 15). Newly weaned calves are most vulnerable to BRD upon arrival at the feedlot (16). The risk of BRD is increased from stress due to transportation, new environments, and new social hierarchies (commingling), hence the colloquial name, "Shipping Fever" (14). Monetary loss is associated with treatment costs, loss in production performance (lack of weight gain), and death (17-19). The total worldwide economic impact of BRD is likely to be in the billions of dollars annually. Not only is BRD an economic concern, but it is also a major animal welfare concern, causing respiratory issues, and in some instances polyarthritis and lameness (20).

1.2.1 Clinical signs and symptoms

Clinical signs are often the only criteria available to feedlot staff to determine if cattle need antimicrobial therapy for BRD beyond on-arrival metaphylaxis. Common clinical signs of BRD in cattle include fever and discharge from the eyes, nose, and ears (21, 22). Other BRD clinical signs include general lethargic behaviour, respiratory anomalies such as coughing and whistling, and a low hung head (21, 23). BRD leads to lesions in the lungs, which can be present even in cattle that otherwise do not exhibit any overt clinical signs of BRD, a condition referred to as subclinical BRD (24). Subclinical BRD, while less serious than clinical BRD, is still an animal welfare and financial concern for producers due to the cattle gaining less weight (24).

1.2.2 Etiology

Sometimes referred to as bovine respiratory disease complex, BRD is a multifactorial disease, the establishment of which in young feedlot cattle is thought to be due to stress, bacterial and viral infection, and other environmental and management factors. BRD is not considered a highly contagious epidemic or zoonotic disease as is the case for bird and swine flu. Rather, BRD is an industry specific problem created by conditions placed on the animals by industry practices (25, 26). For instance, the practice of mixing cattle from different sources, as often happens due to sorting at auction, has been linked with increasing mortality rates (27). BRD is more complex than a single viral or bacterial infection and is commonly associated with a set of viruses and opportunistic pathogens. Specifically, BRD is associated with four gram-negative bacteria, three of which are in the *Pasteurellaceae* family (*Pasteurella multocida, Mannheimia haemolytica, Histophilus somni*) and the fourth is not (*Mycoplasmopsis bovis*) (14, 28-30). Bovine viral diarrhea

virus type 1 and 2, bovine respiratory syncytial virus, and bovine herpes virus 1 are three viruses that are commonly associated with BRD (31).

There are three subspecies of *P. multocida* which are differentiated based on the types of carbon sources they utilize (32). A zoonotic opportunistic pathogen, *P. multocida* subspecies *multocida* causes respiratory disease in humans, domesticated pets (*i.e.*, snuffles in rabbits), and livestock, including swine, poultry, and cattle (33, 34). The other two subspecies *gallicida* and *septica* are considered less problematic (32). Highlighting that *P. multocida* is a common member of the respiratory tract microbiome of various animals, it has been isolated from the saliva of 81% of healthy dogs (35).

Formerly *Pasteurella haemolytica*, *M. haemolytica* is considered the most important bacteria of the BRD complex and is the most abundant BRD bacterial pathogen in respiratory samples of BRD mortalities (36). Compared to *P. multocida*, *M. haemolytica* is more exclusively associated with cattle respiratory tract microbiomes.

Thought to be also a primary contributor to BRD, *H. somni* can also infect other organs, such as the brain and heart (37, 38). It is understudied due to the difficulties in growing it in laboratory conditions compared to the other two *Pasteurellaceae* species. Growing in both aerobic and anaerobic conditions, with growth being more extensive in the latter, *H. somni* requires rich media supplements such as horse serum (39).

Mycoplasmopsis bovis belongs to the *Mycoplasmataceae* family, and differs compared to the *Pasteurellaceae* BRD associated pathogens, in that it lacks a cell wall and has a smaller genome (40). Chronic pneumonia and polyarthritis in cattle are attributed to *M. bovis* (20, 41-43). When pneumonia and polyarthritis occur concurrently, it is termed chronic pneumonia and polyarthritis syndrome (CPPS) (20, 44). Along with *M. bovis*, other mycoplasmas are periodically associated with BRD, including *Mycoplasmopsis dispar* (23).

The relative abundance of the four main BRD associated pathogens indicates they are well suited to exploit immunocompromised cattle (36). Not all four BRD bacterial pathogens are present in every case of BRD, and the presence of more than one is not required for a BRD diagnosis. However, it is still of interest to consider how the BRD associated pathogens interact with each other in the establishment of disease. BRD associated bacteria have been documented to form a biofilm on epithelial cells at body temperatures (45). Specifically, *H. somni* and *P. multocida* form a biofilm in various areas of the body of cattle challenged with *H. somni* (38). In contrast to *H. somni*, *M. haemolytica* has been shown to antagonistically impact *P. multocida* when cohabitants in *in vitro* biofilms (46).

1.2.3 Epidemiology

The Canadian beef industry is primarily located in Western Canada, with the majority of feedlots located in the province of Alberta, which are managed and operated similar to those in western and central USA. BRD is associated with industry specific stressors that cattle experience, thus it is inappropriate to describe the spread of prevalence and ‘outbreaks’ of BRD as is the case for a more classic epidemic disease (47).

The cumulative incidence rate of BRD at feedlots in Western Canada varies, in a study of 28 Alberta feedlots the rate was measured to be 16% in the summer and 66% in the fall (48). In the same study, of diagnosed animals, BRD accounted for 46% of cases (48). The majority of BRD cases occur after 10 days on feed (DOF), a peak time that is changed by metaphylaxis as in this case morbidities tend to occur later in the feeding period (48). While most cases of BRD occur within the first three weeks of DOF and severe cases can lead to mortality, there is also the chronic infection that often develops that can contribute to mortalities at later DOF (49). At packing plants, the occurrence of lesions in the lungs of cattle, even in those not diagnosed with BRD, suggests that subclinical BRD occurs in a large number of cattle (24). The death loss attributed to BRD is typically around 3% to 30% and about half of the total death loss is due to respiratory related diseases (48-50).

Problems with BRD are common wherever cattle are intensively managed. There are several factors as to why North American feedlots experience a higher rate of BRD than Europe and other areas of the world (Europe 2% incidence rate) (51). North American management practices are different to the European beef industry. Feedlots in North America, especially Western Canada, also experience larger temperature changes which are linked to increased BRD (52). Most European beef production does not occur in specialized facilities. In Europe, high-stress veal rearing facilities have the highest BRD incidence rate at 5% (53). However, the subclinical BRD rate of 50% (as measured by lung lesions) suggests that the incidence of BRD may not differ that much from North American beef production (54). Variation in BRD incidence and losses per feedlot, management style, and production system further add to the need for a greater understanding of factors specific to the feedlot environment that contribute to BRD and how they can be altered to prevent or treat the disease.

1.3 CLINICAL CONTROL OF BOVINE RESPIRATORY DISEASE

1.3.1 Vaccines

A range of vaccines have been developed to prevent morbidity and mortality due to BRD. Some are focused on the viruses associated with BRD and other vaccines are focused on the bacterial aspect of the disease. There are vaccines available for viruses associated with BRD that are commonly used in feedlots. In general, there currently is not an accepted or trusted commercial vaccine that targets the bacteria involved in BRD and is compatible with the current beef production practices in Western Canada.

There has been progress in vaccine development. A *M. haemolytica* vaccine was found to induce an antibody response, but vaccination upon arrival to the feedlot has thus far, not been shown to significantly and dependably decrease mortality rates due to BRD (55, 56). This has been hypothesized to be due to the stressed state of the animals upon arrival (56). The general hypothesis is that vaccines are administered too late for the animal's immune system to mount an effective response, even with the proper antibodies. Vaccines exist and are commercially available for *H. somni*, *M. haemolytica*, and *P. multocida*, yet there needs to be proof that vaccination of the cattle with these vaccines is worth the financial investment (57). Studies on these vaccines are often flawed. In a recent review, 183 peer-reviewed publications were assessed and of those, only 5 were statistically sound for inclusion in the review (57). Over a third were rejected from the review due

to a lack of blinding (57). A decrease in BRD mortalities as a result of immunization to BRD specific bacterial pathogens has been shown to be statistically significant (58). However, vaccination for BRD bacterial pathogens has yet to be shown to be clinically significant enough for the practice to be adopted by feedlot producers. Vaccine development for BRD pathogens is not a novel concept, but progress has been hampered by inconclusive studies.

1.3.2 Antimicrobial metaphylaxis

The definitions of the terms "prophylaxis" (prevention) and "metaphylaxis" (control) in herd health management have shifted over time, having been used interchangeably and to refer to separate treatment events (59). It has been stated that group treatment of asymptomatic cattle upon arrival to the feedlot should be referred to as prophylaxis and that metaphylaxis refers to antimicrobials administered to a cohort of animals in which some are displaying clinical signs of BRD (59). However, usage of the term "prophylaxis"/"preventative" may cause on-arrival treatment to be misinterpreted as an unnecessary and inappropriate use of an antimicrobial when communicated to the public. Additionally, the nature of BRD complex with its wide array of causes (including opportunistic pathogens) and undefined stages, as well as the stress induced immuno-compromised state of calves arriving at the feedlot, often means that calves have subclinical disease. The term metaphylaxis will be used to describe on arrival treatments in this thesis, which, in Canada, a veterinarian is required to prescribe.

Metaphylactic treatments used in the beef industry include drug classes such as phenicols, macrolides, and tetracyclines (9). However, long-acting macrolides such as tulathromycin are considered the drug class of first choice (9). After initial on-arrival treatment, there may be further treatments administered to cattle if they are deemed to be morbid. The treatment of choice will vary by feedlot and severity of disease (9). Cattle are often pulled from their home pens and placed in hospital pens, where they can be closely monitored and provided with additional treatments. Providing additional antimicrobial treatments for BRD is expensive, significantly reducing net returns per animal (17).

1.3.3 Antimicrobial treatment

While a variety of antimicrobials are utilized in the livestock industry in Canada, tetracyclines, β-lactams and macrolides are the most common (9, 60, 61). There have been gradual changes in what antimicrobials are used in the livestock industry. For instance, the use of macrolides in livestock has been increasing, though not as quickly as the use of tetracyclines has been decreasing (9). Tetracyclines contain a naphthacene base (4 series of six membered rings) with a variety of functional groups attached (Fig 1.1). Tetracyclines' mode of action is to prevent the production of proteins by targeting the interaction between amino acyl tRNA and mRNA (62). One of the first tetracyclines discovered was oxytetracycline (OTC), found in an environmental sample in the late 1940's and since then OTC has been commonly used in the livestock industry (63).

Macrolides are a class of broad-spectrum antimicrobials defined by their central macrolactone ring of 14-16 atoms (Fig 1.1). Macrolides target the ribosomal nascent peptide exit tunnel (NPET) of the 50s ribosomal subunit, blocking the protein manufacturing capacity of bacteria (64). Macrolides do not physically block the NPET, but instead bind to specific motifs on

the produced proteins and thus prevent the exiting/completion of these proteins (64). Macrolides are a common choice for BRD treatment, partially because they accumulate in the pulmonary epithelial lining fluid of the lungs, and many are designed as extended release formulations (65). Antimicrobials are often used across livestock species, such as tulathromycin (TUL), a macrolide that is used to treat respiratory diseases in cattle (such as BRD) and swine (66). TUL has been used in cattle in Canada since 2006 (2005 in USA) under one brand until recently mirroring the overall rise in usage of macrolides across the livestock industry (67, 68).

The WHO has recommended drug classes such as phenicols for livestock over macrolides, which are considered critical in human healthcare (69). The use of phenicols is less common in the industry than tetracyclines and macrolides, and fluoroquinolone use is even less common compared to the other classes (9). The first phenicol registered in Canada for use in cattle was chloramphenicol, however it was banned in July 1985 due to public health concerns (70). Subsequently, florfenicol (FFN) was registered for veterinary use in Canada in 1996 (71, 72). The mode of action of FFN is to bind to the V domain of the 23S rRNA of 50S ribosomal subunit, thereby inhibiting protein synthesis (73-75). FFN has additional protection from enzymatic degradation by substituting a fluoride for the hydroxy group found in chloramphenicol (Fig 1.1) (76).

Fluoroquinolones such as enrofloxacin target the initiation of DNA replication of bacteria by targeting DNA topoisomerases (77). Developed in the 1980's, enrofloxacin was the first fluoroquinolone, created by the addition of a fluorine atom to a quinolone, which improved the efficacy of this broad-spectrum antimicrobial (78, 79). Enrofloxacin is used in domestic animals and in the livestock industry and has been shown to prevent BRD, though it may be less efficacious in comparison to TUL (80, 81). Antimicrobial usage trends in feedlots come from decisions made by veterinarians based upon economic factors such as the expense of the drug, ease of administration/storage, and historical efficacy. Higher than average loss rates due to antimicrobial resistance (AMR) often leads to a switch in treatment regimens to another antimicrobial or antimicrobial class.

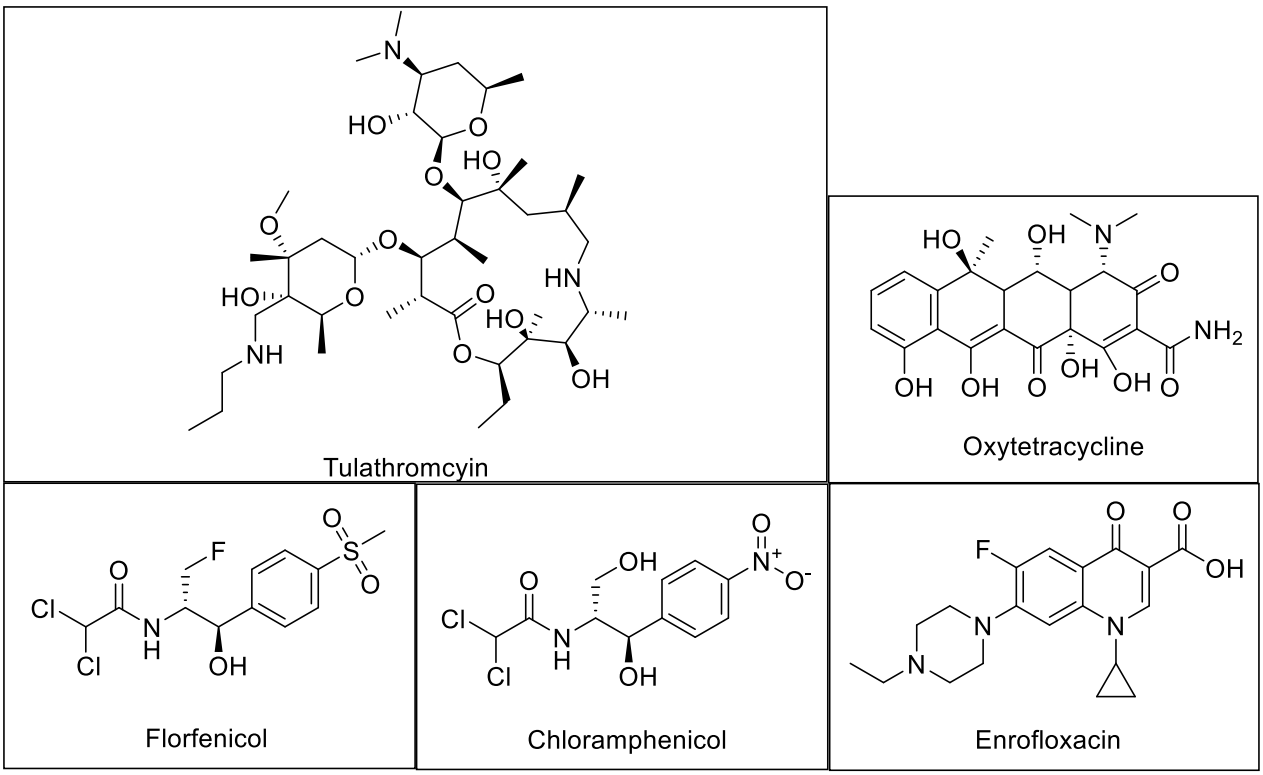

Figure 1.1 Two dimensional chemical structures of tulathromycin, oxytetracycline, florfenicol, chloramphenicol and enrofloxacin. Created on ChemDraw Prime (2022, PerkinElmer).

1.4 ANTIMICROBIAL RESISTANCE

The AMR crisis was recently predicted to cause 100 million deaths each year by 2050 (82). In 2019, there was an estimated ~5 million deaths associated with AMR and 1.3 million directly attributed to AMR (83). Those 1.3 million deaths in 2019 may have been prevented through successful antimicrobial treatments, if not for AMR (83). The increasingly high number of resistant pathogens found in humans and livestock by national surveillance programs is predicted to result in increasing mortalities (60). By mass, the livestock industry accounted for approximately 75% of all antimicrobials distributed in Canada in 2018 (including drug classes not utilized in human medicine) (60). By reducing the effectiveness of commonly used antimicrobials, livestock industry profits are decreased by AMR as a result of increases in the total use as well as a shift to increasingly expensive antimicrobials. AMR pathogens for which clinical symptoms are not resolved necessitates subsequent rounds of antimicrobials, further increasing both the cost and AMU (17).

While transmission of bacteria and thus AMR between humans and agriculture has been shown to be limited, studying AMR within the livestock industry is still beneficial to addressing the global AMR crisis (84). AMR surveillance can lead to the discovery of new and emerging antimicrobial resistance genes (ARGs) of clinical relevance, thereby improving the detection capabilities of culture-independent AMR monitoring (85). AMR monitoring in agriculture may also reveal zoonotic pathogens that are multi-drug resistant and at risk of moving from one host to another, as has been documented between humans and cattle (86, 87). The livestock industry,

which relies on AMU to treat large cohorts of livestock suffering from infectious disease, may benefit from a greater understanding of the emergence, transmission, and persistence of AMR within production environments.

1.4.1 General mechanisms of resistance

In broad terms the following mechanisms of AMR have been found in bacteria: (i) efflux of the drug through an efflux pump, (ii) modification of the drug, (iii) modification of the target binding site of the drug, and (iv) replacement of the target with a functionally equivalent molecule (88, 89). Efflux pumps can be general or drug specific. Drug modification includes inhibition of binding to the target or full drug degradation. Binding site modification occurs through a variety of ways, including a change in the ionic charge and blockage through bulky amino acids. Resistance through binding site modification is either a change in the gene that encodes for the target or through an ARG encoding for an enzyme that alters the binding site to prevent binding of the drug. Resistance through replacement of the target with a functionally equivalent protein occurs through producing a replacement that is not structurally similar enough to be targeted, but still retains function. If the replacement is less efficient than the target, then it can be produced in parallel to the target of the antimicrobial, or if the replacement is equally effective it can completely replace the target. It should also be noted that an intrinsic form of this strategy exists in which there is no paralogous second protein but modulation of target gene expression to overcome drug activity.

1.4.1.1 Intrinsic Resistance

Intrinsic resistance to antimicrobials is due to an inherit characteristic of the microbe. For example, cephalosporins inhibit transpeptidases and other enzymes involved in peptidoglycan synthesis, but have no effect on the *Mollicutes*, including *Mycoplasmopsis* spp., which lack a cell wall (90). This is an example of a characteristic that is multifactorial, and it is non-transferable and not acquired through horizontal gene transfer since it involves many complex and coordinated pathways (or the lack thereof). One of the main differences between intrinsic and acquired resistance, which is typically conferred by single or small collections of genes and mutations, is that intrinsic resistance is shared among all members of genetically similar groups or taxonomic clades. Acquired resistance on the other hand can vary even at the highest/lowest level of taxonomic classification, including subspecies-level variation in AMR phenotypes. In order to identify intrinsic resistance, it is common practice to compare AMR amongst isolates of the same species across different sites, though this is not a fully conclusive test. Genomic information that we possess for cultivable and non-cultivable bacteria can also be used to identify the presence and absence of antibiotic targets based on taxonomy and may also have value in predicting mutations at precise drug target interaction sites. AMR found across strains of the same species is an inconclusive indicator of the mechanism of resistance being intrinsic rather than acquired (91). Bacteria that are intrinsically resistant to commonly used antibiotics can complicate culture-dependent and independent approaches to monitor AMR.

1.4.2 Surveillance of ARGs in feedlots

Surveillance of AMR in feedlots provides insight into the AMR crisis, how it is affecting the livestock industry, and should be used to improve antimicrobial stewardship. Studies on the accumulation of ARGs in feedlots have focused on the microbiota in cattle through deep nasopharyngeal swabbing (DNS) and fecal sampling, often (though not always) focusing on AMR in pathogens of interest or sentinel species (92-99).

DNS samples often have opportunistic pathogens associated with BRD (100). While this provides a direct insight into ARGs present in the upper respiratory tract, performing DNS requires training and proper cattle restraint otherwise there is a risk of injury to the handler, especially in heavier cattle. Collectively, DNS studies have found that ARGs are less prevalent in cattle that are only a few DOF, with ARGs increasing with number of DOF (92, 97, 101). AMR surveillance methodology, especially the bioinformatic pipelines after sequencing, are not standardized between studies making it difficult to directly compare results between studies (97, 102). Future surveillance of AMR through purely DNA sequencing methods could provide more rapid analysis than conventional culture-based approaches and could theoretically be performed on-site to support rapid real-time decisions for AMU. However, there are several key obstacles that hinder its adoption in feedlots.

Surveillance of AMR through DNA sequencing methods is challenging in that individual genomes do not meet throughput needs and conventional (the most economical) metagenomic analyses cannot differentiate ARGs in non-pathogenic and pathogenic organisms. Recommendations for AMU by ARG detection also assumes complete knowledge of all the ARGs. However, we know that this is not the case. This was highlighted recently in the discovery of an unannotated macrolide ARG in 2023, which was found to be widely present in BRD pathogens (85). Another issue is that the lack of concordance between genotype and phenotype AMR results (103). Mismatches where the genotype indicates resistance but the phenotype is sensitive are common in BRD associated pathogens (103). The disadvantage of phenotypic AMR surveillance is the need for appropriate facilities, trained personnel, and the time associated with bacterial growth.

A culture-based AMR screen is typically performed using a standard antimicrobial sensitivity test (AST) using microdilution methodology that evaluates growth in nutrient broth supplemented with a range of antimicrobials at clinically-relevant concentrations (104). High-throughput formats, including 96-well plates, are used to simultaneously test an array of antimicrobials. These wells are then inoculated with a standardized concentration of the bacterium, measured in colony forming units (CFU) per millilitre. The growth within these wells is compared to a positive control with no antimicrobial in order to assess the minimum inhibitory concentration (MIC) to bacterial growth. Another common AST methodology is disk diffusion, where the placement of a disk impregnated with antimicrobial on an inoculated agar plate creates a diffusion gradient of the antimicrobial within the agar. Resistance and sensitivity can be measured via the standardized interpretation of the distance of the growth from the disk (104).

1.4.3 Known reservoirs of ARGs.

Bacteria within environmental microbiomes are the source of most antimicrobials and AMR mechanisms (105). The genomes of environmental bacteria have ARGs that encode for naturally produced antimicrobials, which allow the bacteria to exist within complex bacterial communities. A "pristine" environment free of antimicrobials or chemical warfare and the associated ARGs is unlikely to have existed or lasted for long as bacteria evolved to compete throughout the biosphere (106). However, not all ARGs are of clinical interest: that is to say, not all ARGs accumulate in pathogens to the same extent and many ARGs protect against antimicrobials that are not used clinically.

The anthropogenic impact of antimicrobial usage places a positive selective pressure on ARGs, which has caused an increase in distribution and concentration of ARGs of interest in the biosphere. Instances of anthropogenic impact through antimicrobial pollution into water and soil environments include improperly treated hospital wastewater and the use of manure as fertilizer from treated livestock (107, 108). There is also anthropogenic pollution directly from animal husbandry, whereby livestock metabolize and excrete antimicrobials and functionally active metabolites into the environment (109, 110). The term anthropogenic impact is an important distinction for referring to the increase in concentration of antimicrobials in addition to the "base" levels from naturally occurring antimicrobial-producing bacteria and fungi and others that reside within various environments.

Examples of previously studied environmental resistomes include wastewater, wildlife, manure, and food (99, 111, 112). The presence of ARGs in some of these locations can be explained by persistent selective pressure from anthropogenic induced antimicrobial pollution (113, 114). Yet ARGs of interest are also persistent in environments with minimal to no anthropogenic activity indicating that ARGs are maintained in environmental bacteria, even when not exposed to antimicrobials (115, 116). There is an increased metabolic cost of expression of ARGs that would theoretically create negative selection. In other cases, persistence of ARGs without positive selective pressure can be partially explained by cost reduction mechanisms that inactivate the expression of the ARGs through a small number of nucleotide changes (105, 117). That environmental microbiomes are potentially serving as a resistome (a reservoir of ARGs in the bacteria that make up the microbiome) may also extend to environments within feedlots (115, 118).

1.4.4 Spread of antimicrobial resistance genes.

Bacteria are capable of sharing genes. This is accomplished by horizontal gene transfer (HGT), and ARGs are not an exception, in fact, they are exemplary examples of how bacteria can acquire a new trait like AMR under pressure. Although HGT occurs more often intraspecies than interspecies, there are restrictions that reduce the rate of HGT (119, 120). HGT of ARGs can occur in numerous ways. The four routes of HGT are conjugation, transformation, transduction, and gene transfer agents (121). Conjugation is likely the most common way that whole functional genes are transferred and successfully expressed. It involves cell to cell contact and transfer of a plasmid (121). Within the route of conjugation there are finer details on how the DNA becomes a transferable plasmid. For instance, conjugative transposons are a series of regulatory elements in

the genetic code (122). These elements mediate the excision of DNA, which forms a plasmid that can integrate into another part of the same genome or into another bacteria's genome (122). In transformation, extracellular DNA is "picked up" by a bacterium in a state of competence that is then integrated into the genome (121). While competence is usually induced through artificial stressful conditions in the laboratory, naturally competent bacteria exist in the environment, and there is evidence that exposure to antimicrobials can facilitate natural transformation (121, 123). Transduction and the relatively understudied gene transfer agents (GTA) are very similar to each other, and essentially require a third actor that can either be a bacteriophage or bacteriophage-like particles (121).

Certain bacteria may form key hubs that are part of a large network of HGT between members of environmental microbiomes and pathogens. Species such as *Escherichia coli* and *Klebsiella pneumoniae* survive in both hosts and in various environments, and are often rich in ARGs that maybe shared across microbiomes within both (124, 125).

1.4.5 Known antimicrobial resistance genes and their mechanisms of resistance to drugs used in feedlots.

There are many ARGs of interest that provide resistance to drug classes used in Western Canadian feedlots. These ARGs further complicate the treatment of BRD by decreasing the efficacy of antimicrobials. The drug classes of particular interest are tetracyclines, macrolides, phenicols, and fluoroquinolones. Some ARGs that have shown to confer resistance to macrolides are *erm(42), ermF, ermB* and *msr(E)* (target site protection) as well as *mph(E)* (inactivation of antimicrobial) (126-130). Another form of macrolide AMR is enzymes that hydrolyze the macrolactone ring of macrolides, which creates a linear and thus inactive molecule (131). In addition to macrolide specific ARGs, others that result in cross-resistance to macrolide, lincosamides and streptogramin (MLS) also exist.

Resistance to fluoroquinolones such as enrofloxacin can be due to mutations in the binding site (DNA topoisomerases), decreased permeability, and efflux pumps, though ARGs to this drug class are likely under-identified and understudied (132, 133). There are two main forms of resistance to phenicols such as FFN, efflux pumps, and methylation of the binding site (134). In the last decade another mechanism, ribosomal protection, has also been found to confer resistance to FFN (135). All three forms confer resistance to both chloramphenicol and FFN, explaining why resistance to an antimicrobial that has been banned in the industry for over 35 years is still commonly identified (76). The efflux pump family *flo* contains the earliest known gene encoding FFN resistance, *pp-flo* as well as the more thoroughly studied, *floR* (134). The *floR* gene encodes for efflux pump mediated FFN resistance and has been previously found on plasmids and transposons in bacteria associated with the environment as well as in pathogens (136, 137). Due to how long tetracyclines have been in use, resistance to tetracyclines is well studied and a range of ARGs have been identified that target this drug class through drug inactivation, efflux, and ribosomal protection (138, 139). In fact, there are over 100 unique entries of tetracycline resistance genes in the highly curated Comprehensive Antibiotic Resistance Database (CARD).

1.4.6 Presence of AMR in BRD associated pathogens.

Phenotypic AMR to fluoroquinolones, phenicols, tetracyclines and macrolides has been previously found in BRD associated pathogens isolated from feedlot cattle (28, 103, 140). Resistance to phenicols such as FFN in BRD pathogens has increasingly become a concern at feedlots in North America (141). Reports of phenotypic resistance to macrolides such as TUL in the *Pasteurellaceae* species associated with BRD vary greatly, but generally macrolide resistance tends to be more frequent in *M. haemolytica* and *P. multocida* than *H. somni* (28, 103, 130). Resistance to TUL has been found to be widespread in *Mycoplasmopsis bovis*. In one study, 73% of the 126 *M. bovis* isolates showed resistance (142). It should, however, be noted that AMR in mycoplasmas is mediated by single-nucleotide polymorphisms (SNPs), and not by genes that encode for efflux pumps or enzymes that perform target modification or antimicrobial inactivation. Thus, HGT of ARGs between mycoplasmas and other BRD pathogens is unlikely to occur (143).

Even though North America struggles with a higher prevalence of BRD than Europe, AMR in BRD pathogens in Europe is still an issue (144). While there are many factors that lead to a higher incidence of BRD within feedlots, there are few tools available to feedlot managers to address BRD. Antimicrobials are important tools in combatting BRD, however AMR has been decreasing their effectiveness.

1.5 RATIONALE OF THE STUDY

A challenge facing beef producers is effective AMU and stewardship in the face of AMR. Decision timelines for treatment strategies are also narrow due to the seasonal aspect of beef production. Feedlots fill up in a short period of time and BRD cases spike within a few weeks of their arrival. Treatment failure due to suspected AMR can be hard to identify in time to adjust regimens within the narrow time window available and is not compatible with individual animal-level diagnostics (*e.g.*, DNS). Thus, there is a need for pragmatic and scalable AMR monitoring systems within the beef industry.

Antimicrobial treatments significantly impact the selection of ARGs in treated versus untreated cattle, but there is a lack of understanding of the initial origin of the ARGs found in problematic and resistant pathogens. It is known that ARGs exist at higher levels at later DOF, even in untreated cattle (92, 101, 145, 146). The source of ARGs in relevant BRD associated pathogens is likely more complex than a simple relationship of AMU selecting for a pre-existing subset of opportunistic AMR pathogens within the upper respiratory tract of cattle (28). The challenge of effective AMU for beef producers creates a need for a greater understanding of AMR within the industry. Part of addressing that need is identifying potential sources of ARGs within beef production systems. The sources of ARGs need to be identified before feedlot management practices can be modified to address them and improve treatment outcomes.

As discussed, environmental microbiomes are known to contain ARGs of clinical relevance and there are numerous environmental microbiomes with which the cattle interact with on a daily basis in their pens. The pen floors, insects, fecal matter from pen-mates and other animals, and the water source, including shared watering bowls are all potential reservoirs of ARGs. In this study the focus is on the pen watering bowls. An Australian study measured the impact of feedlot management practices on BRD and showed that cattle that shared a watering trough with cattle in

adjacent pens were 4.3 times more likely to develop BRD than cattle that did not share a watering trough. This was identified as the highest risk factor among management practices (26, 147). The Australian study did not suggest that water in the watering troughs was a vector, but *M. haemolytica* has been isolated from sheep watering bowls and ARGs have previously been detected within the water source of pens (148, 149). The introduction of organic matter in watering bowls from drinking animals, including mucus and feed, provides conditions for the formation of complex microbial communities. These communities could be a potential source of ARGs for BRD associated pathogens, and the degree to which they undergo HGT at these sites is unknown. The goal of the studies that follow constitute the first steps towards defining watering bowls as a potential reservoir of ARGs and assessing their relevance to three major BRD pathogens.

The first experiment was a pilot project on assessing the complexity of the microbiome within watering bowls and the associated ARGs of isolates within it using culture-dependent and independent approaches to broadly describe AMR and microbial communities in these under-studied environments. Significantly, this was performed at a newly established feedlot, as no cattle resided within the feedlot before the start of the study. For the second experiment the microbiome within watering bowls of a well-established feedlot was analysed using a standard shotgun metagenomic approach. To provide evidence that HGT of ARGs results in commonly observed genes and multigene loci in environmental and pathogenic bacteria, *floR*-containing gene clusters in resistant microbes found in the watering bowls and within BRD associated pathogens were compared.

1.6 HYPOTHESES AND OBJECTIVES

We hypothesize that the understudied environmental microbiomes of beef cattle feedlot watering bowls are a potential source of AMR found in BRD pathogens.

Objectives:

1. Define the microbial communities that live in feedlot watering bowls and isolate and characterize AMR members thereof.

2. Define the collection of ARGs found in water bowl microbiomes and begin to assess the potential for direct HGT between environmental and pathogenic bacteria.

Two experiments were conducted in support of objective 1. In experiment 1, watering bowl samples from a newly established feedlot underwent taxonomic characterization through culturing and microbial community profiling by 16S rRNA gene amplicon sequencing. In experiment 2, watering bowl samples from a well-established feedlot underwent phenotypic screening and genomic characterization, including whole genome and shotgun metagenomic nucleotide sequencing. In both cases, the ARGs identified in watering bowls were compared to those known to be present in the genomes of three BRD pathogens: *P. multocida*, *M. haemolytica* and *H. somni*. Industry-specific antibiotics and ARGs were the emphasis of this analysis with *floR* loci acting as a case study on HGT and the accumulation of common gene clusters between diverse organisms with varying impacts on feedlot animal health.

2 Experiment 1: Insight into antimicrobial resistance at a new beef cattle feedlot in Western Canada

The literature review from chapter 1 outlined the need for a greater understanding of the potential sources of ARGs within the feedlot. In chapter 2, there was characterisation of the microbiome within feedlot watering bowls of a newly established feedlot located on land that had not previously been used for beef cattle production and had not been fertilized with livestock manure. The AMR within members of the microbiome within feedlot watering bowls was studied to assess whether the ARGs within overlapped with those previously found within BRD genomes and environmental microbiomes of feedlots. Additionally, a community profile of the microbiome within the feedlot watering bowls was described. A unique novelty to this study was the opportunity to compare these environmental microbiomes before and after the arrival of cattle.

Author contributions: Conceptualization and Methodology from DK, ST, TM, MJ, and AR; Investigation by DK and BS; Visualization and Writing - Original Draft: DK, MJ, and AR; Writing – Review & Editing by DK, ST, TM, MJ and AR. Supervision by MJ and AR. Funding Acquisition by ST, TM, MJ, and AR.

Insight into antimicrobial resistance at a new beef cattle feedlot in Western Canada

Daniel Kos[1], Brittany Schreiner[1], Stuart Thiessen[2], Tim McAllister[3], Murray Jelinski[1*], and Antonio Ruzzini[4,5,*]

[1]Department of Large Animal Clinical Sciences, Western College of Veterinary Medicine, University of Saskatchewan, Saskatoon, SK, Canada, S7N 5B4

[2]Namaka Farms Inc, Outlook, SK, Canada, S0L 2N0

[3]Lethbridge Research and Development Centre, Agriculture and Agri-Food Canada, Lethbridge, AB, Canada, T1J 4B1

[4]Department of Veterinary Microbiology, Western College of Veterinary Medicine, University of Saskatchewan, Saskatoon, SK, Canada, S7N 5B4

[5]Department of Biochemistry, Microbiology and Immunology, College of Medicine, University of Saskatchewan, Saskatoon, SK, Canada, S7N 5E5

*to whom correspondence should be addressed

2.1 ABSTRACT

In North America, beef production relies on the administration of antimicrobials to manage disease. Bovine respiratory disease (BRD) is the most significant disease of beef cattle, and antimicrobial resistance (AMR) to conventional therapies presents an existential risk to animal welfare and food production. While AMR surveillance programs are poised to help facilitate antimicrobial stewardship and decision-making at feedlots, monitoring strategies for large numbers of animals at an individual or group-level is time-consuming and costly. Accordingly, we completed a pilot investigation of feedlot water bowls, which is an understudied interface between cattle and bacteria. By performing culture-dependent and independent studies, we demonstrate that water bowl-dwelling bacteria can act as sentinel organisms for clinically relevant antimicrobial resistance genes (ARGs), and that cattle have an impact on the microbial communities in the bowls. Moreover, by sampling water at a feedlot site before animal arrival, we detected resistance to two feedlot-specific antibiotics: florfenicol and tulathromycin. After just 4 weeks of operation, multi-drug resistant bacteria were routinely found in most water bowls. A comparison of ARGs encoded by 5 water bowl bacterial isolates, along with previously reported source and wastewater metagenomes, to those found in BRD pathogens confirmed the utility of using water samples for AMR surveillance.

2.2 IMPORTANCE

A better understanding of how environmental reservoirs of ARGs in the feedlot related to those found in animal pathogens will help inform and improve disease management, treatment strategies, and outcomes. Monitoring individual cattle or small groups is invasive, inefficient, expensive, and unlikely to gain adoption by the beef cattle industry. Wastewater surveillance has become standard in public health studies and has inspired similar work to better our understanding of AMR at feedlots. We derived our insights from sampling water bowls in a newly established feedlot which was a unique opportunity to observe AMR prior to animal arrival and to monitor its

development over 2 months. Importantly, the bacterial community of a single water bowl can be influenced by direct contact with hundreds of animals. Our results suggests that water bowl microbiomes are economical and pragmatic sentinels for monitoring relevant AMR mechanisms.

2.3 INTRODUCTION

Beef production in North America consists of a network of cow-calf operations, markets, feedlots, and packing plants. Generally, a relatively large number of cow-calf operations supply calves to feedlots either directly or through an auction process wherein animals are shipped to central auction sites and then sold to feedlots in assembled groups based on traits such as weight and sex. Additional commingling occurs at the feedlot where cattle from multiple auctions converge and may undergo additional sorting. The stress of shipping and commingling has been linked to higher incidences of disease within the first weeks of arrival to the feedlot. Specifically, animals are prone to bovine respiratory disease (BRD), colloquially referred to as shipping fever, which is a multifactorial disease complex associated with stress from shipping, commingling, weather, and processing of the animals upon arrival (150). This mixing, sorting, and resorting increases the exposure of individuals to a constellation of viral and opportunistic bacterial pathogens. Accordingly, BRD, which is the most common cause of morbidity and mortality in feedlot cattle, is managed through vaccination, antimicrobial metaphylaxis, and treatment (151, 152).

In Canada, β-lactams, phenicols, macrolides, streptogramins, tetracyclines and, as a last resort, fluoroquinolones are used to treat feedlot cattle (9, 60). Quinolones and macrolides are both considered category I antimicrobials by the WHO whereas Health Canada places quinolones in category I and macrolides in category II (8, 11). Metaphylaxis by macrolides is common practice in North America and has been suggested to be the most effective antimicrobial class to mitigate losses due to BRD (151). Nevertheless, the effective use of antimicrobials relies on an understanding of antimicrobial resistant determinants that accumulate in relevant pathogens, and the respective reservoirs that contribute to their dissemination. In the context of agriculture, this includes both animal-associated and environmental reservoirs, including everything in between. The effects of cattle manure deposition over decades, for example, can result in the accumulation of antimicrobial resistance genes (ARGs) (153), and evidence of positive selection for feedlot-specific ARGs exists in the form of decreasing resistome diversity during feeding in a pen-independent manner (99). Thus, while antimicrobial use begets resistance, the sources, transmission, and identities of ARGs at feedlots remains an active field of study.

Reliance on the widespread use of antimicrobials has resulted in the dissemination of ARGs throughout the biosphere. Environmental reservoirs of ARGs are ubiquitous and challenge the utility of antibiotics. Resistance determinants that confer resistance to multiple drug classes and those that provide resistance to both human and veterinary pharmaceuticals represent existential threats to healthcare systems and agricultural food production. Disease and AMR surveillance using wastewater sampling is now a conventional approach that can be adapted to a variety of related research questions. In fact, water sources and effluents have been studied at feedlots to monitor AMR (149, 154-156). Moreover, water has been associated with disease, specifically BRD, in both feedlot and dairy environments (147, 157). In one case, shared water troughs between

pens were the most significant risk factor for BRD (147), though direct surveillance of microbes and ARGs are still lacking from this feedlot niche. To our knowledge, the bacterial communities that reside in water bowls have yet to be systematically characterized and, therefore, represent an understudied reservoir of AMR at the interface between the water source and animal.

In the Fall of 2021, we were presented with a unique opportunity to study the microbiota present in the water bowls and changes to resident populations as cattle entered a new feedlot in Western Canada. We rationalized that targeting the water bowl would also offer a pragmatic and non-disruptive sampling strategy for the feedlot industry to gain insight into microbial communities, and potentially pathogen and AMR gene transmission. The results of applying both culture-dependent and culture-independent approaches to analyze the microbial communities in feedlot water bowls and their relationship to AMR observed in BRD pathogens are presented and discussed in the context of ongoing and future AMR surveillance platforms that will continue to inform antimicrobial use.

2.4 MATERIALS AND METHODS

2.4.1 Facility and animals.

A newly established cattle feedlot was the subject of our study. Mixed cross-bred beef calves or yearlings were administered tulathromycin (Draxxin®, Zoetis, Kirkland, QC, Canada) 2.5 mg/kg bodyweight (BW) or oxytetracycline 20 mg/kg BW (Oxymycin® LA 300, Zoetis) on arrival. Tylosin (Tylosin 40Bio Agri Mix, Mitchell, ON, Canada) was incorporated into the feed at a rate of 11 mg/kg/day. Cattle with clinical signs of BRD were administered one or more regimens of parenteral antibiotics: ceftiofur 6.6 mg/kg BW (Excede® 200, Zoetis), florfenicol 40 mg/kg BW (Florkem® Ceva Animal Health, Guelph ON, Canada), and marbofloxacin 10 mg/kg BW (Forcyl®, Vetoquinol, Lavaltrie, QC, Canada). In general, cattle arriving at the feedlot were transited through receiving pens (T1-3) before allocation to home pens (A1-6 and B1-7: see Figure 2.1A). Water was provided ad libitum via stainless-steel water bowls on an automated refilling system. Bowls were not emptied or cleaned during the collection period. Irrigation water was pumped to a reservoir, which supplied the watering bowls.

2.4.2 Sample collection.

Water and biofilm swabs were collected on Wk0, before the cattle arrived to the feedlot, and then on a weekly basis from October 20, 2021 (Wk1) until December 9, 2021 (Wk8; see Table 2.1A). Water samples (~500 mL) were collected in commercial plastic water bottles from the water bowls and near the shoreline of the reservoir. Cotton swabs (BD 220144) were used to sample the water bowl biofilms; alternating sides of the bowl were sampled each week.

2.4.3 Water and swab sample processing.

Water samples were passed through two filters: an autoclaved coffee filter to remove large particulates followed by a 0.2 μm Nalgene Rapid-Flow Sterile Single Use Bottle Top Filter (ThermoFisher Scientific). After filtration, the 0.2 μm filters were excised from their plastic cups, quartered, placed in 10 mL of M9 salt solution, and vortexed for 15 s. A 0.5 mL aliquot was used immediately for antimicrobial sensitivity testing (AST) and bacterial isolation, and the remaining 9.5 mL volume was centrifuged at 1,000 x *g* for 10 min, and resuspended in phosphate buffered

saline (PBS, pH 7.4) supplemented with 20% glycerol for cryopreservation at -80 °C (Figure A2.1). Swabs were processed by adding 3 mL of M9 salt solution to the transport unit, followed by vortexing for 10 s. Then, 250 μL was diluted and used immediately for AST and bacterial isolation while the remaining solution was centrifuged and stored as described above in PBS supplemented with 20% glycerol.

2.4.4 AST and isolation of bacteria based on AMR.

Water and swab-derived samples were used to inoculate 96-well microtiter plates containing a 2-fold dilution series of four antibiotics: enrofloxacin (ENRO: 0.12 to 64 μg/mL), tulathromycin (TUL: 0.25 to 64 μg/mL), florfenicol (FFN: 0.25 to 64 μg/mL) and oxytetracycline (OTC: 0. 5 to 256 μg/mL). All AST experiments were performed in tryptic soy broth (TSB) amended with 150 μg/mL cycloheximide to suppress fungal growth. All antibiotics were obtained as high-purity (>95%) powders from scientific research vendors. The AST plates were incubated at either room temperature (RT) or 37 °C overnight to 4 d. Growth was evaluated daily. Samples were collected from wells with the highest growth-permitting concentrations of antibiotics, and serial dilutions thereof (10^{-4} to 10^{-8}) were plated on TSB agar (TSA) containing a sub-inhibitory concentration of corresponding antibiotic for bacterial isolation. Well-isolated, morphologically distinct colonies were then sub-cultured in 5 mL of TSB for ~24 h before preparing 20% glycerol stocks. A total of 75 bacteria were taxonomically identified by comparing a ~400 bp region of their 16S rRNA gene to the NCBI 16S rRNA database. A subset of 28 isolates was subjected to additional AST using a panel of 10 antimicrobials that belong to 5 disparate classes that are commonly used in feedlot veterinary medicine as previously described (142).

2.4.5 DNA isolation from water and swab samples and microbial community profiling.

DNA was extracted and purified from water and biofilm swab samples utilizing GenElute bacterial genomic DNA (Sigma Aldrich) and Purelink Microbiome (ThermoFisher Scientific) kits. A total of 122 samples, including controls, were subjected to bacterial community profiling according to the PCR amplified V3 to V4 regions of their 16S rRNA genes. Sequencing was performed using the Illumina MiSeq PE300 platform at Genome Quebec (Montreal, QC, CA). The control samples and DNA extractions were performed on material processed as described above, including (i) the potable water from the plastic bottles used for bowl collection, (ii) unused sterile swabs, and (iii) a blank PCR amplification. Sequence data was assembled, taxonomically sorted, and analyzed using QIIME2™ with an 16S rRNA pretrained classifier (158-160). Data visualization was performed using QIIME2™, phyloseq and microViz (161-163).

The Shannon diversity index for each sample was generated in QIIME2™ with a sampling depth of 6000 and an analysis of variance (ANOVA) and subsequent pairwise comparisons (Tukey Honest Significant Differences) was performed on this data in R (4.2.2) (158-161). Importing the taxonomic data into a phyloseq object using qiime2R, a PERMANOVA with distance calculation Aitchison (9999 permutations), and principal component analysis (centered log ratio transformation) was generated from the genera of each sample with microViz in R (4.2.2) (161-163). A linear discriminant analysis (LDA) effect size (LEfSe) was performed on the 16S rRNA microbiome data using MicrobiomeMarker (164) in R (4.2.2). Samples were grouped by type

(swab and water), read counts were normalized to counts per million and the Kruskal–Wallis test (p-value < 0.05) was employed.

2.4.6 DNA isolation, whole genome sequencing and analysis of AMR bacteria.

Using a Purelink Microbiome kit (ThermoFisher Scientific), DNA was isolated from 6 bacterial monocultures obtained from water bowls (WB, named WB4-9) grown in TSB. PacBio SMRT HiFi nucleotide sequencing and assembly (hifiasm) were performed at the University of Saskatchewan's Global Institute for Food Security(165). Manual curation of the assembled data was performed to merge contigs with identical sequences. The detection of ARGs in assembled genomes and metagenomic data was performed using ABRicate (166) through comparison to protein sequences made available by the Comprehensive Antibiotic Resistance Database (CARD; April 2023) (167). In addition to the 6 WB genomes, the genomes of BRD associated pathogens (*P. multocida* n = 51, *H. somni* n = 23, and *M. haemolytica* n = 98) were selected based on their isolation from cattle from NCBI BioProjects PRJNA281531, PRJNA306895 and PRJNA340884. Water metagenomic sequencing data was downloaded from PRJNA529711 and PRJNA292471 and assembled using SqueezeMeta v1.6.2, March 2023 using Megahit (168, 169). Short contigs (<200 bp) were removed using prinseq (170). Initial positive identifications of ARGs were determined by 80% identity and coverage thresholds. After automated gene identification, the list was manually curated to unify hits with redundant/synonymous nomenclature, count multicomponent systems as single entries, and to remove regulatory proteins. A Venn diagram was then generated using nVennR in R (4.2.2) (171).

2.5 RESULTS AND DISCUSSION

2.5.1 Direct evaluation of aerobes living in water provides rapid insight into feedlot AMR status.

To overcome the limitations and impractical nature of individual and pooled cattle swabbing, we elected to sample and evaluate the antimicrobial susceptibility profiles of the animals' water source. Furthermore, sampling prior to any animals arriving to the feedlot (Wk0) provided a rare opportunity to study the accumulation of ARGs in a feedlot. By collecting water samples and swabbing visible biofilms at the air-water interface, we were able to assess for the presence and absence of aerobic AMR organisms. Four antimicrobials commonly used at beef cattle feedlots were selected for this experiment: enrofloxacin (ENR), tulathromycin (TUL), florfenicol (FFN) and oxytetracycline (OTC). Altogether, a total of 131 samples from 11 cattle pens over a 9-week period were collected (Figure 2.1A; Table A2.1). Direct inoculation of water and biofilm samples into 96-well plates containing one of the four aforementioned antibiotics revealed the presence of AMR prior to the cattle arriving to feedlot and their influence on AMR (Figure 1B). In the Wk0 samples we observed the growth of bacteria at room temperature (RT) in the presence of FFN and TUL at concentrations >32 μg/mL in samples from the feedlot's water reservoir and water bowls. Notably, AMR organisms were observed more routinely at RT than at 37 °C. The 37 °C temperature was expected to restrict growth and was selected as it mirrors animal body temperature more closely than the watering bowls, which were maintained at ~10 °C or greater throughout the sampling period. Upon animal arrival, resistance, which was defined as growth at >1 μg/mL ENR, >32 μg/mL FFN or TUL and >64 μg/mL OTC, was observed within 4

weeks to all four antibiotics in both water and biofilms. A single exception was that OTC resistance took longer to develop in biofilm samples inoculated at 37 °C. Nevertheless, it is reasonable to conclude that the impact of animals and feedlot practices, including the use of antimicrobials, contributed to the accumulation of AMR organisms in water bowls.

This crude dataset demonstrated the recovery of organisms resistant to FFN and TUL prior to the entry of cattle and the use of antimicrobials at the newly established feedlot site. The dataset also shows the recovery of AMR bacteria to all four antibiotics investigated occurred rapidly across the feedlot after animal entry and antimicrobial use. These antibiotics belong to four distinct classes: the fluoroquinolones (ENR), macrolides (TUL), phenicols (FFN) and tetracyclines (OTC). Pen-level observations of AMR occurred across all pens within 4 weeks of animal arrival. We suspect that both abiotic and biotic factors, including the dispersion of particulate matter (dust) across the site, shared fencing between adjacent pens, movement of animals within the feedlot, and the occurrence of disease and antimicrobial use throughout the feedlot, all contribute to the ability to recover AMR bacteria after 4 weeks.

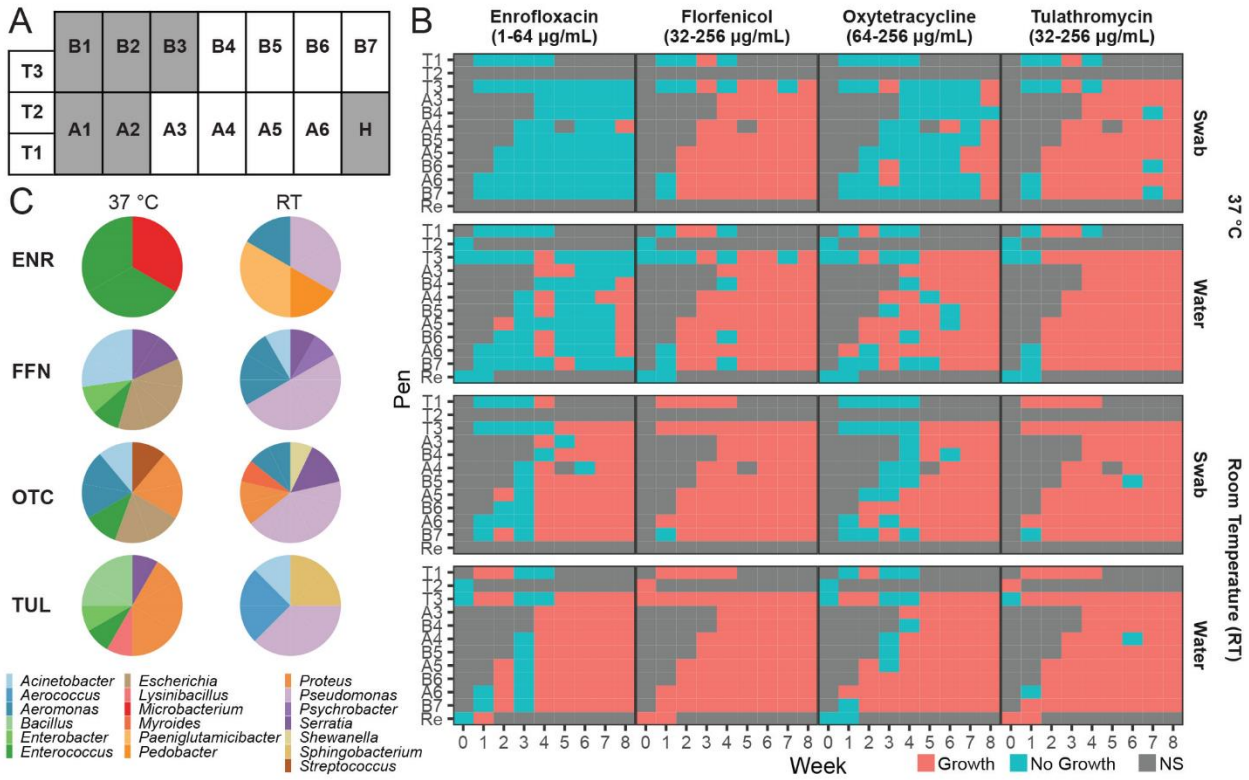

Figure 2.1 (A) A schematic overview of the feedlot. Individual pens are labeled, including home pens (A1-6; B1-7), transfer pens (T1-3) and a hospital pen (H). The pens that appear in white (A3-6, B4-7, and T1-3) were sampled during this study. **(B)** AMR profiles of water and swab samples taken from a feedlot for 8 weeks after animal arrival (week 0 indicates samples collected prior to animal arrival). The binary heatmap reports on the observation of growth (red) or no growth (green) in TSB supplemented with antibiotics. Grey boxes are used for timepoints that were not sampled (NS) because animals had yet to be homed in these pens. **(C)** The genetic identities of morphologically distinct bacteria isolated from water samples based on antibiotic selection (shown at the left).

2.5.2 Isolation of antibiotic resistant bacteria from water and bowl swabs.

To begin to assign the bacterial sources of AMR in the water, we selected four pens for culture on agar supplemented with the previously described four antimicrobials. Specifically, two pens used to temporarily house animals (pens T1 and T3) and two home pens (A6 and B7) were selected. We prioritized the isolation of bacteria from water samples rather than biofilm swabs because water bowl type (plastic or stainless steel) varies across the industry in Western Canada. A total of 13 water samples collected between weeks 1 and 8 resulted in the isolation of 75 distinct bacteria belonging to 19 genera (Figure 2.2A, Table A2.2). These bacteria were selected based on their ability to grow with either ENR, FFN, TUL or OTC. Individual isolates were selected based on distinct morphologies, resulting in 1 to 8 unique organisms per sample. Bacteria isolated from water samples incubated at 37 °C and RT differed in that *Acinetobacter*, *Escherichia* and *Enterococcus* were prevalent at 37 °C whereas *Pseudomonas* and *Psychrobacter* spp. dominated the isolates recovered from RT incubations.

The isolation of bacteria based on resistance to a single antibiotic under aerobic conditions resulted in a collection of multidrug resistant (MDR) organisms. In fact, further evaluation of the antimicrobial susceptibilities of the water bowl isolates to a panel of 10 drugs in 5 classes showed that all 28 isolates were resistant to at least 3 drug classes (Figure 2.2A, Table A2.2), when resistance was defined by MICs values >0.12 μg/mL for ENR, >16 μg/mL for tetracyclines, >32 μg/mL for FFN or macrolides and >8 μg/mL for penicillin.

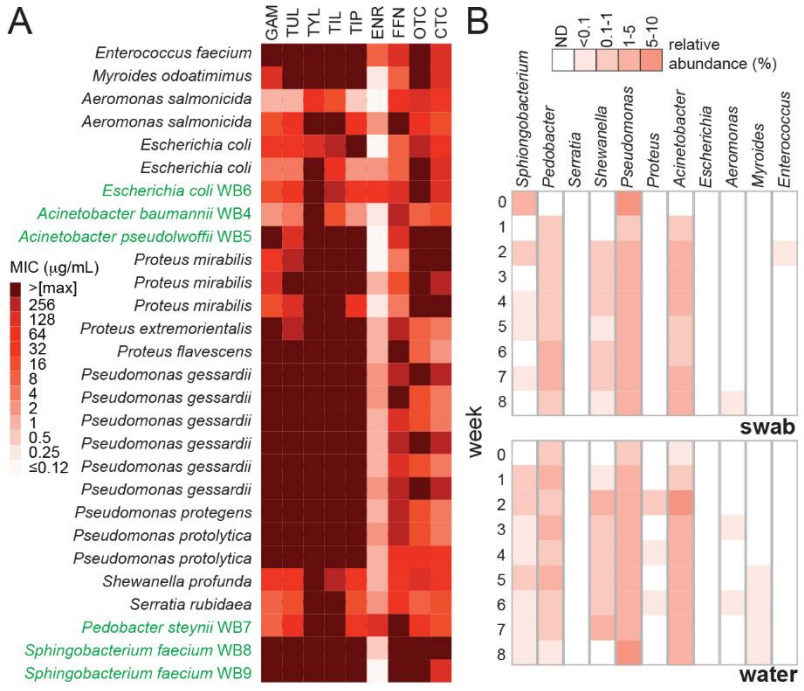

Figure 2.2 (A) Antimicrobial susceptibility profiles of 28 bacteria isolated from feedlot water bowls: 6 isolates (green, WB4-9) were further characterized by whole genome sequencing. **(B)** Heatmaps showing the relative abundance of isolated genera in culture-independent community profiles of water and swab samples before animal arrival at the feedlot and during the first 8 weeks of operation.

2.5.3 AMR genera are represented in water bowls community profiles.

To evaluate how representative our biased collection of AMR aerobic bacterial isolates was of the populations living in the water bowls, we performed microbial community profiling of both water and water bowl swabs. The same samples that were used to monitor AMR were subjected to community profiling using a region of the 16S rRNA gene. Overall, several genera were well-represented in both our 75-member isolate collection and the water communities. *Acinetobacter*, *Pseudomonas* and *Pedobacter* were detected throughout the sampling period whereas *Sphingobacterium* appeared sporadically based on amplicon sequencing data (Figure 2.2B). It is noteworthy that two of these genera, *Acinetobacter* and *Pseudomonas*, are known to possesses intrinsic and acquired resistance mechanisms relevant to human and veterinary medicine, though their impact on the latter has not been as extensively studied (172). A member of the genus *Sphingobacterium* was recently shown to encode for a macrolide esterase that hydrolyzes tylosin (85), which was used as a feed additive during the study. In contrast to these ever-present genera, *Escherichia*, *Enterococcus*, and *Serratia* were not routinely detected, though they were isolated from feedlot samples when culture was performed at 37 °C. The selection bias applied to our culture-based methods may enrich for low abundance genera and or those with a more transient existence in cold water. Nevertheless, these minor populations remain potential reservoirs of ARGs.

The water and swab sample communities varied in their composition (Figure 2.3; Figure A2.2). At the level of class, water and the presumed biofilm communities were readily distinguished: water was dominated by *Gammaproteobacteria* and *Bacteroidia* whereas the biofilms also contained *Alphaproteobacteria* at relatively high abundance based on sequenced amplicons. Notably, a reduction in the relative abundance of *Cyanobacteriia* was observed in the water bowls relative to the water source, where it was the third most abundant taxon. This is likely a reflection of differences between the biological niches: compared to the open reservoir, the water bowls are in direct contact with cattle and contain additional nutrients through the deposition of feed. Both animals and feed are likely to contribute growth substrates to the water bowl's resident bacterial populations. The overall alpha-diversity of the water and biofilm communities did not differ significantly during the study with few exceptions of water samples being less diverse sporadically and at the end of the study (Figure 2.4A). A reduction in diversity may be the consequence of antimicrobial use, as previously reported (97, 149) or due to climate. The ambient temperature shifts dramatically in the Fall (Table A2.1), though the water temperature is maintained in the bowls at ~10 °C to avoid freezing.

Bacterial communities living in water and biofilms were readily distinguished from each other. Beta-diversity analysis clearly demonstrated significant differences in the bacterial communities defined from swab and water samples (Figure 2.4B). Indeed, sample type variance (PERMANOVA: F = 20.8, R^2 = 0.13, p <0.0001) was greater than that observed between collection weeks (F = 3.2, R^2 = 0.13, p <0.0001) and pens (F = 1.8, R^2 = 0.10, p <0.0001), which account for relatively modest differences within sample types. Further inspection of bacterial demographics by linear discriminant analysis (LDA) revealed differentially abundant taxa based on sample type (Figure 2.4C). The influence of the animals was also apparent. In water, a relatively high abundance of *Moraxella*, which are common residents of the upper respiratory tracts of cattle

and account for ~50% of the bovine nasal microbiota (173), was remarkable and suggests transfer between the animal and the water bowl. These bacteria are also closely related to *Psychrobacter* and *Acinetobacter*, which are widespread in nature and were also enriched in both water communities and our collection of AMR isolates. Finally, the *Microbacteriaceae* Family, which includes members from the genera *Microbacterium* and *Paeniglutamicibacter*, were more abundant in water than in swab biofilm communities and were readily isolated from water using antibiotic selection, showing concordance between culture-independent and dependent approaches.

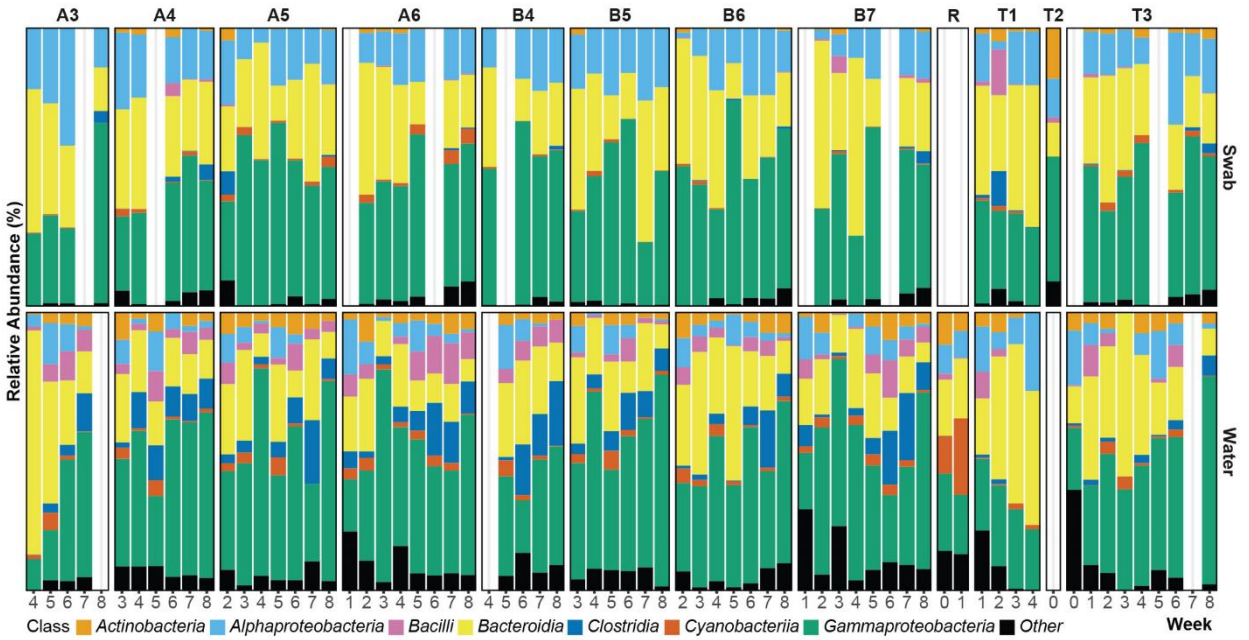

Figure 2.3 Seven most abundant bacterial classes present in water bowl swabs or water samples collected at a new feedlot. Pen IDs are provided at the top. Home pens (A3-A6 and B4-7) are separated from transfer (T1-3) pens by samples of the water source, a water reservoir (R) that was sampled before animal arrival and during the first week of operation. After animal arrival, the occupancy of pens T1-3 was variable (Table A1).

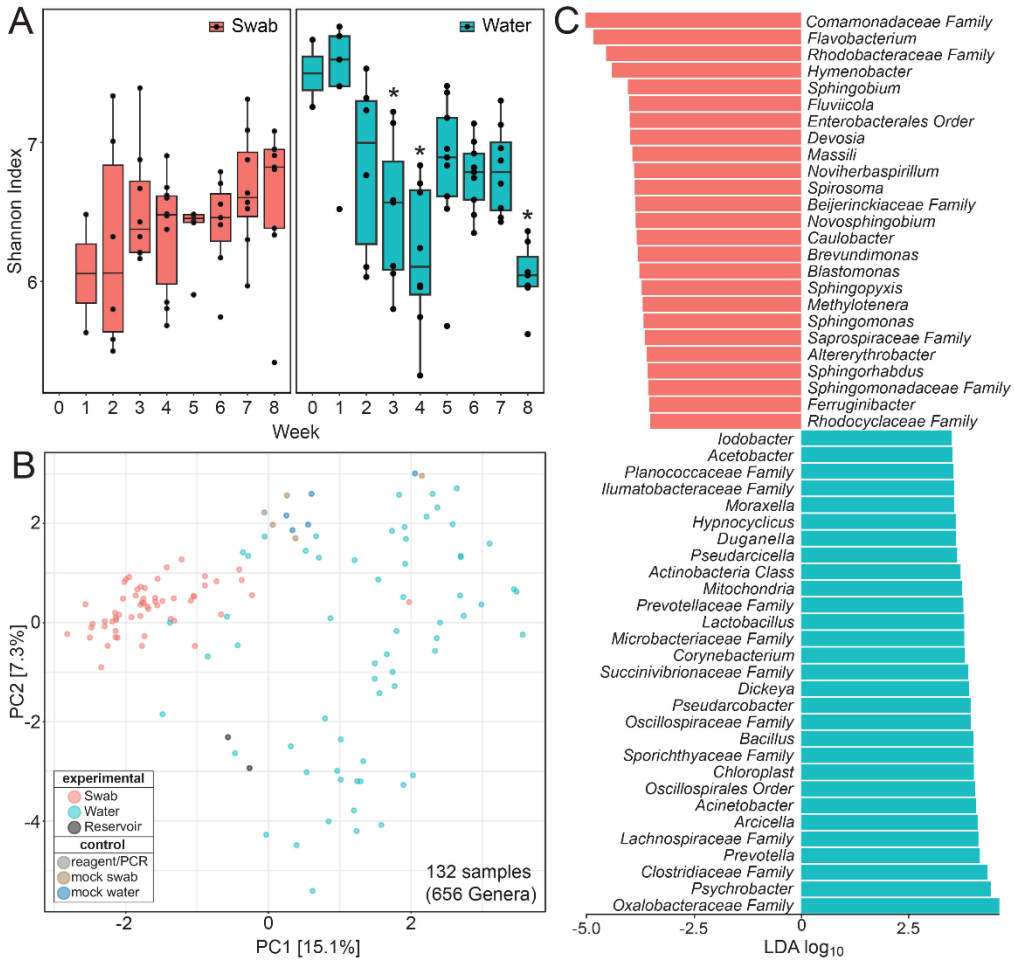

Figure 2.4 (A) Alpha-diversity of bacterial communities observed in swab and water samples represented weekly using Shannon diversity indices. The Shannon indices for swab communities did not differ significantly across samples. Significant differences between water samples collected before and after animal samples are indicated by asterisks (95% confidence, $p < 0.08$). **(B)** Beta-diversity of bacterial communities observed in swab and water samples, including analysis of the feedlot reservoir (the water source) and technical reagent and mock extraction controls. **(C)** Detected bacterial taxa that differ significantly in their abundances between water bowl swabs and water samples identified by linear discriminant analysis (LDA; >3.5 $\log_{10}$ taxa shown; $p < 0.05$).

2.5.4 Water bowl isolates are rich in ARGs, including those of clinical relevance.

While bacterial community profiles showed the influence of animals on water samples and revealed taxa that are notoriously associated with AMR (*e.g.*, *Acinetobacter*), the plasticity of bacterial genomes precludes definitive predictions of ARGs within communities based on taxonomic identity. We explicitly investigated the AMR genotypes of 6 MDR isolates using long-read whole genome sequencing (Table A2.4). Two of the six isolates were *Acinetobacter* spp., which were selected based on their divergent AMR phenotypes and, more generally, due to their relative abundance in the water community profiles. *Escherichia coli* and *Pedobacter steynii* were selected because they were either rarely (*E. coli*) or readily (*P. steynii*) detected in standard microbial community profiles and resistant to all 5 classes of antibiotics. Finally, two *Sphingobacterium faecium* isolates were selected based on this species' aforementioned role in harboring feedlot-specific macrolide resistance to tylosin, tilmicosin and tildipirosin (85).

Although the genome of *Pedobacter steynii* WB7 does not encode for known ARGs, a homology-based informatic approach resulted in the identification of 39 distinct resistance determinants from the remaining 5 water bowl isolates (Table A2.5).

To begin to assess the relationship between these four bacterial genera and other feedlot-associated environmental reservoirs of AMR, we compared their single ARGs and multi-gene systems to those reported from metagenomic studies of water and the whole genomes of *Pasteurellaceae* that are involved in BRD (*Histophilus somni*, *Mannheimia haemolytica* and *Pasteurella multocida*). This analysis revealed differences between sets of ARGs in distinct feedlot-associated niches (Figure 2.5A, B); however, it also demonstrated the specific ARGs accumulate across environments. In particular, the ARGs observed in both WB isolates and BRD pathogens were found to be physically clustered or in the case of *S. faecium* WB8 and WB9 on a ~56 kb conjugative plasmid (Figure 2.5C). A total of 6 ARGs (*aadA*, *APH(3")-1b*, *APH(6)-Id*, *sul2*, *floR* and *estT)* were found throughout water sample genomes and metagenomes as well as within a representative set of 172 BRD pathogens. Sulfonamides and aminoglycosides are not routinely used in feedlots (9), and were not used during the course of this study. Nevertheless, two aminoglycoside phosphotransferases and a sulfonamide resistant dihydropteroate synthase were observed in ARG clusters (Figure 2.5C). In fact, the observation of ARGs shared between environments and across taxa is explained by their presence within gene clusters of mobile genetic elements. A central role for conjugative plasmids in the dissemination of ARGs across distinct sequence elements and organisms has been proposed (174). Thus, while the presence of *floR* (a phenicol exporter) and *estT* (a macrolide esterase) can be rationalized by the use of both classes of antibiotics during the study period, the current global status of the mobile resistome should not be ignored. ARGs are found throughout the biosphere, particularly in biological wastewater treatment facilities which impact their patterns of dissemination in bacteria (175).

Our results show that antimicrobial use creates a niche, in this case within water bowls, that selects for MDR bacteria from the environment. Remarkably, the whole genome sequences of just 4 MDR isolates revealed 5/16 ARGs found in a much larger set of cattle pathogens. Furthermore, along with previously obtained water metagenomes, our limited dataset was used to identify 14/16 ARGs in the same 3 *Pasteurellaceae* that contribute to BRD. Only two ARGs were unique to the pathogen genomes analyzed, *APH(3')-1b* and *dfrA14*, though orthologs of the latter (*e.g.*, *dfrA12* and *dfrA17*) were observed in distinct datasets. Thus, combined culture-dependent and independent approaches using water for the isolation of both bacteria and DNA represents a pragmatic approach to gain insight into AMR at feedlots. Water bowls, which are in direct contact with animals, appear to be an excellent proxy for AMR surveillance.

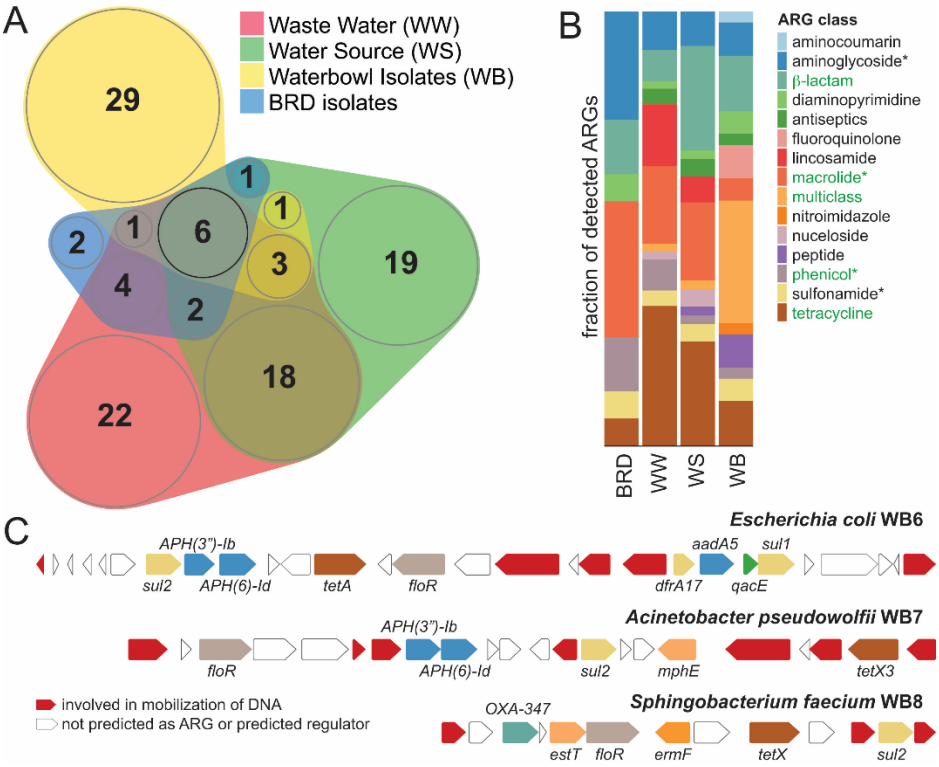

Figure 2.5 (A) Venn diagram showing ARGs that are commonly observed in distinct and overlapping environments. The ARGs identified in the whole genomes of 5 WB isolates were compared to the genomes of BRD pathogens (PRJNA281531, PRJNA306895 and PRJNA340884: 51 *P. multocida*, 98 *M. haemolytica* and 23 *H. somni*), feedlot source water and wastewater metagenomes (PRJNA292471 and PRJNA529711). **(B)** Comparison of identified ARGs, by antibiotic class, across four sample types. Antibiotic classes listed in green were used at the feedlot during the study. Asterisks indicate that specific ARGs to these classes were observed in both water sample type metagenomes as well as within WB isolate and BRD pathogen genomes. **(C)** ARG clusters carried by MDR water bowl isolates. The *S. faecium* WB8 and WB9 genomes have syntenous ARG clusters (WB9 not shown). ARGs are colored coded by antibiotic class and their identities are noted.

2.6 ACKNOWLEDMENTS

The authors thank Namaka Farms Inc. employees for assistance on site and Dr. Mike Jelinski of Veterinary Agri-Health Services Ltd. for helpful discussions and providing the feedlot's antimicrobial history. Funding for this research was provided by the Saskatchewan Cattlemen's Association, the Saskatchewan Ministry of Agriculture Development Fund, Saskatchewan Cattlemen's Association, and the Canadian Cattle Association's Beef Cattle Research Council.

2.7 CONFLICT OF INTEREST STATEMENT

Stuart Thiessen is the owner and operator of the feedlot that was sampled during the study, Namaka Farms Inc. located in Outlook, SK, Canada.

2.8 DATA AVAILABILTY

The nucleotide sequences of bacteria characterized in this study are available through GenBank BioProject PRJNA820789. Sequence data for the microbial community profiling is available through BioProject PRJNA976740.

3 Experiment 2: Environmental microbiomes and their role in florfenicol resistance in bovine respiratory disease pathogens.

The literature review from chapter 1 outlined the need for a greater understanding of the potential sources of ARGs within the feedlot and monitoring technique. In chapter 2 the microbiota within WB was phenotypically and genotypically characterised and an overlap was found between ARGs within watering bowl isolates and ARGs within BRD genomes. In this chapter, the relevance of these ARGs is investigated through assessing the potential for HGT between WB isolates and BRD pathogens. This was assessed by focusing on the genetic neighbourhood of a specific ARG and screening for it across species, the microbiome, BRD genomes, and beyond. Additionally, a different genotypic approach was taken to characterize the ARGs present in the WB microbiome as a whole.

Author contributions: Conceptualization and Methodology from DK, TM, MJ, and AR; Investigation by DK, RJ, and BS; Visualization and Writing - Original Draft: DK, MJ, and AR; Writing – Review & Editing by DK, TM, MJ and AR. Supervision by MJ and AR. Funding Acquisition by TM, MJ, and AR.

Environmental microbiomes and their role in florfenicol resistance in bovine respiratory disease pathogens.

Daniel Kos [1], Murray Jelinski [1], Richa Jain [2], Brittany Schreiner [1], Tim McAllister[3], Antonio Ruzzini [2]

[1]Department of Large Animal Clinical Sciences, Western College of Veterinary Medicine, University of Saskatchewan, Saskatoon, SK, Canada, S7N 5B4

[2] Department of Veterinary Microbiology, Western College of Veterinary Medicine, University of Saskatchewan, Saskatoon, SK, Canada, S7N 5B4

[3]Lethbridge Research and Development Centre, Agriculture and Agri-Food Canada, Lethbridge, AB, Canada, T1J 4B1

3.1 ABSTRACT

Treatment failure due to antimicrobial resistance (AMR) is a contributing factor to mortality related to bovine respiratory disease (BRD). A Western Canadian feedlot experiencing treatment failure after metaphylactic administration of florfenicol (FFN) was investigated. The objective was to define the resistome of the watering bowls (WBs), and to determine the potential role of horizonal gene transfer (HGT) occurring between WB microbiomes and BRD pathogens. Antimicrobial sensitivity testing (AST) was performed on samples of water, swabs of WB biofilms, and sediment samples (n=60) collected from 20 WBs. A subset (n=11) underwent shotgun metagenomic sequencing. When inoculated into media with FFN (>32 µg/mL), 57/60 of WB samples had bacterial growth. Three FFN-resistant WB isolates, *Sphingobacterium faecium*, *Psychrobacter sp.* and *Acinetobacter terrestris* as well as a *Histophilus somni* isolate from a deep nasopharyngeal swab obtained from a symptomatic animal, were phenotypically and genotypically characterized through AST and whole genome sequencing. A total of 94 unique ARGs were detected within the metagenomes of the 11 WB samples. WB isolates were resistant to combinations of FFN (>32 µg/mL), tulathromycin (>32 µg/mL), and oxytetracycline (>128 µg/mL). An ARG (*floR)* encoding for a FFN efflux pump was found in all three WB isolates and *H. somni*, a contributor to BRD. The loci of *floR* within the isolates were compared to each other and to publicly available genomes of BRD pathogens. Evidence that HGT is occurring via a series of transposable elements was observed from common genetic loci encoded in both environmental isolates and BRD genomes. Thus, WB microbiomes are a potential reservoir of ARGs and a means to monitor AMR in BRD pathogens.

3.2 IMPORTANCE

Bovine respiratory disease (BRD) or "Shipping Fever" is the leading cause of disease in feedlot cattle. The epidemiology of BRD is well-known, with most clinical cases occurring within weeks of arrival to the feedlot. Mitigation strategies such as the administration of antimicrobials on-arrival is a commonly used management tool for controlling BRD in high-risk groups of cattle. Florfenicol (FFN) is used in feedlots for metaphylaxis and as a BRD treatment. Antimicrobial use inevitably leads to antimicrobial resistance (AMR). FFN-resistance is typically mediated by the *floR* gene, which encodes for a phenicol-specific efflux pump. We found that *floR* loci detected in bacteria residing in feedlot watering bowls are similar to those in pathogens that commonly contribute to BRD. This discovery suggests that monitoring AMR in watering bowls captures mechanisms that are particularly relevant to feedlot pathogens.

3.3 INTRODUCTION

Bovine respiratory disease (BRD) is an economic concern to the beef industry with losses associated with treatment costs, decreases in production performance, and animal death (17). The latter is an animal welfare concern as cattle often suffer from chronic pneumonia and septic arthritis before succumbing to BRD (44). BRD is most prevalent in newly weaned calves that come off pasture in the fall. The animals are first transported to auctions where they are commingled with calves from other operations and sold in cohorts of variable origin. They are then transported to feedlots and sorted into pens. The common BRD moniker "Shipping fever" is based on the correlation between the stresses of transportation and the subsequent onset of this multifactorial respiratory disease. Accordingly, on-arrival metaphylactic treatment with antimicrobials is a common practice at feedlots to manage BRD (15). The BRD-complex has been attributed to animal stress, viruses, and opportunistic bacterial pathogens such as *Histophilus somni, Pasteurella multocida, Mannheimia haemolytica,* and *Mycoplasmopsis bovis* (176).

Antimicrobial resistance (AMR) has been shown to decrease the efficacy of antimicrobials used in feedlots such as phenicols, macrolides, tetracyclines, and fluoroquinolones (9). The most commonly used phenicol, florfenicol (FFN), inhibits protein synthesis by binding to the 23S rRNA component of the 50S large ribosomal subunit (9, 73-75). In feedlots, resistance to FFN is increasing, creating a need for a greater understanding of FFN AMR (141).

It is known that bacteria living within feedlots are exposed to a positive selective pressure from anthropogenic antimicrobial use (AMU) (109, 110). It is important to also appreciate that ARGs associated with clinically important antimicrobials have been found in the microbiota of wildlife and in an isolated subterranean cave (106, 115, 116). Thus, ARGs can be maintained and disseminated without direct or constant exposure to antimicrobials from anthropogenic activity. The environmental microbiomes within feedlots are exposed to an influx of ARGs from the biosphere from various sources and are under near constant selective pressure for AMR from AMU (177). Furthermore, bacteria that have adapted to a range of environments may act as intermediaries, trafficking ARGs between environmental microbiomes and cattle pathogens (124, 125, 178). In Western Canada, feedlot watering bowls (WBs) are typically filled with untreated water sourced from rivers, reservoirs, and wells. The WBs are lined with stainless steel or plastic and heated to prevent freezing in cold climates. They are also frequently contaminated with feed

and other organic matter, transferred by cattle. Thus, WBs can serve as a milieu for the growth of complex environmental microbiomes. In some cases, this can be observed at a macroscopic level when biofilms form at the air water interface. An Australian feedlot study identified shared pen water as the highest management-related risk factor for cattle developing BRD, while cohort size was not found to have an impact (26, 147). We speculate that this association may, in part, be related to horizontal gene transfer (HGT) of ARGs from WBs to BRD pathogens. The WBs may also be transient reservoirs for these pathogens and facilitate their spread between animals. Additionally, feedlot wastewater and pen water has previously been found to harbour ARGs and a FFN-resistance gene (*floR*) has been found on plasmids and transposons of environmental bacteria and cattle pathogens (136, 137, 149, 154).

We hypothesize that the commonly overlooked environmental microbiomes of WBs are a potential reservoir for relevant ARGs, such as *floR*. To test this, the *floR* loci were compared between FFN-resistant WB isolates and the genomes of BRD pathogens. Additionally, 11 WB-associated samples were subjected to metagenomic sequencing to catalogue the ARGs present within the WB microbiome. Finally, we then specifically identified and compared *floR* loci as a feedlot industry-specific test case to examine HGT. Close similarity of these loci confirmed that WBs are excellent sentinel systems to study AMR, and provided evidence that clusters of relevant ARGs appears to be shared between the WB bacteria and BRD pathogens.

3.4 MATERIALS AND METHODS

The study was conducted at a Western Canadian feedlot and spanned two production years. A production year was defined as the feeding period from when the cattle enter the feedlot (fall) until they are sold for slaughter. In Year 1, microdilution AST methods and whole genome sequencing were used to identify the genera and species of FFN-resistant WB bacteria. Whereas in Year 2, there was a greater emphasis on characterizing the phenotype and genotype of microbiomes of WB water, biofilms, and sediment taken from the bowls. All samples underwent AST using a custom designed panel (AST-4) containing 2-fold microdilutions of 4 antimicrobials. Figure 3.1 is the flowchart for Year 1 and Year 2 samples.

3.4.1 Feedlot facility and sample collection

The feedlot pens were separated by wooden porosity fences with each pen having a dedicated watering bowl. The capacity of each pen was approximately 300 head of cattle. Water, biofilm, and sediment samples were obtained from twenty pens, including sixteen home pens (labelled A#, B#, C#, D#, or G#) and four hospital pens (labelled H#).

Cattle were vaccinated on arrival with Bovi Shield Gold One Shot (Zoetis, Canada INC, Kirkland, QC, Canada) and Bovilis® Vision® 8 Somnus with Spur (Merck animal health Canada, Kirkland, QC, Canada). In year 1, metaphylaxis was an intramuscular injection of 40 mg/kg BW of FFN (Florkem® Ceva Animal Health, Guelph ON, Canada) administered on-arrival or shortly thereafter. Cattle with clinical signs of BRD (febrile, lethargy, ocular/nasal discharge, coughing, and gaunt) were treated with either oxytetracycline (OTC) 20 mg/kg BW (Oxymycine® LA 300, Zoetis), ceftiofur 6.6 mg/kg BW (Excede® 200, Zoetis), or marbofloxacin 10 mg/kg BW (Forcyl®, Vetoquinol, Lavaltrie, QC, Canada). Tylosin (Tylosin 40, Bio Agri Mix, Mitchell, ON, Canada) was incorporated into the feed at a rate of 11 mg/kg/day to control liver abscesses. In year

2, the metaphylaxis treatment protocol was switched to tulathromycin (TUL) (2.5 mg/kg of bodyweight, Draxxin©, Zoetis).

3.4.2 Year 1: Isolation and characterisation of FFN resistant isolates from watering bowls.

3.4.2.1 Sample collection and isolation of resistant WB isolates

A 10 mL water sample was collected in a sterile 15 mL container (Falcon, Corning INC, Corning, NY, USA) from 10 WB. Samples were transported on ice and stored at 4°C until processed. The initial processing step was centrifugation for 10 min at 1,409 x *g* (Sorvall ST16R), with the pellets re-suspended in 500 µL dH_2O, pooled and stored at 5 °C. This pooled sample was diluted with 2.5 ml dH_2O and mixed 1:1 with 2-times concentrated tryptic soy broth (2 X TSB) supplemented with FFN at eleven two-fold microdilutions (0.25-256 µg/ml). After approximately 18 h at room temperature (RT), bacteria growing in the presence of 16 µg/ml to 256 µg/ml FFN were pooled. The pooled sample was serially diluted and plated on TSB agar containing 8 and 16 µg/mL FFN. Three dominant morphologies were isolated and labelled water bowl (WB) isolate 1, WB2 and WB3.

3.4.2.2 Antimicrobial sensitivity testing of the WB isolates

The three FFN-resistant WB isolates were cultured in 2 mL of TSB (30 g/L) for approximately 18 h at RT. The OD at 600 nm was 0.217, 0.355, 0.158 for WB1, WB2 and WB3, respectively. Downstream AST was then performed with ten antimicrobials (AST-10); the AST-10 panel contained antibiotics relevant to feedlots and has been previously described (14). Each isolate was inoculated (1:1) into the following 10 antimicrobials in two-fold microdilutions: FFN (0.25-256 µg/ml), enrofloxacin (ENR, 0.12-128 µg/ml), gamithromycin (GAM, 0.25-256 µg/ml), tildipirosin (TIP, 0.12-128 µg/ml), tilmicosin (TIL, 1-256 µg/ml), TUL (0.25-256 µg/ml), tylosin (TYL, 1-128 µg/ml), chlortetracycline (CTC, 1-256 µg/ml), OTC (0.5-256 µg/ml), penicillin (2-8 µg/ml) or no antimicrobial (positive control) (14). The AST-10 plates were incubated for approximately 18 h at RT, and growth was scored in comparison to the positive control. The three FFN resistant isolates were standardised to an OD of 0.005 and tested in a series of checkerboard MIC assays created from all permutations of pairwise combinations of 16 µg/ml to 128 µg/ml of FFN, OTC, and TUL (diluted in 30 g/L TSB) on a 96-well plate. The ratio of TSB and antimicrobial mixture was 1:1. The panel had a complement of negative (TSB media) and positive controls. The plate was incubated overnight at RT and growth was scored in comparison to the positive control (no antimicrobial).

3.4.2.3 DNA isolation, sequencing, and bioinformatics

For DNA isolation of the three WB isolates, they were cultured in 100 mL TSB for 6 h, at RT, with shaking at 200 rpm on a C2 platform shaker (New Brunswick Scientific, NJ, USA). At the time of cell harvest by centrifugation, WB1, WB2 and WB3 had an OD at 600 nm of 0.15,1.43, and 0.1 respectively. DNA isolation was performed utilising the GenElute Bacterial Genomic DNA Kits from ThermoFisher (Waltham, MA, USA). Full genome nucleotide sequencing and assembly was performed with PacBio SMRT sequencing technology (Menlo park, CA, USA) at the Genomic Resource Center (Institute of Genome Sciences, University of Maryland, MD, USA). The taxa of WB isolates were identified through a PubMLST search (179). Sequences were

annotated using RAST and the loci (~4.8 kbp to ~6 kbp) containing the *floR* gene in the WB isolates underwent a blastn search (National Center for Biotechnology Information (NCBI)), which was restricted to finding matches (90% identity, 90% coverage) within a selection of BioProjects containing key terms related to the BRD associated bacteria, *P. multocida*, *H. somni,* and *M. haemolytica* and to BioProjects containing top results from a blastn search of the non-redundant database (*Riemerella anatipestifer, Salmonella enterica*, *Acinetobacter spp.*) (180-185).

3.4.2.4 *Histophilus somni* isolate

A research partner (Chinook Contract Research, Airdrie, Canada) provided a *H.* somni isolate recovered from a deep nasopharyngeal swab from an animal from the same feedlot and production year. This isolate was cultured in 10 mL TSB with 5% horse serum in a semi-anaerobic tube for 18 hr. Growth from this was diluted to an OD of 0.0005 at 600 nm to inoculate 1:1 into an AST-10 panel. The isolate was cultivated (as described) and DNA was extracted from the *H. somni* isolate, using a Purelink Microbiome DNA purification kit (Waltham, MA, USA). The DNA was sequenced using the PacBio SMRT Hifi long read nucleotide sequencing platform and genome assembly was completed by the Global Institute for Food Security (GIFS, Saskatoon, SK, Canada).

3.4.3 Year 2 sampling and sample processing

3.4.3.1 Sample collection, processing, and AST

Plastic commercial drinking water bottles ranging in size from 500 mL to 1000 mL were emptied and filled with WB water from 20 pens. In addition to the water, the biofilms at the water-air interface on the sides of each WB were swabbed (220144, BD, Franklin Lakes, NJ, USA). Sediment from the bottom of the WB was sampled by scraping the bottom with a 15 mL Falcon tube (Corning INC, Corning, NY, USA). The water was processed through two filters. First, samples were passed through a coffee filter to remove larger particulate, and then the effluent was passed through a 0.2 µm Nalgene Rapid-Flow Sterile Single Use Bottle Top Filter (595-4520, ThermoFisher Scientific, Waltham, MA, USA) under vacuum. The 0.2 µm filter paper was quartered and was vortexed in PBS to liberate retained bacteria. After centrifugation, bacteria were resuspended in PBS supplemented with 20% glycerol for storage or directly assessed in ASTs. Specifically, an aliquot of the filter retentate was used to inoculate TSB containing one of a panel of 4 antimicrobials (AST-4 tests). This included TUL (0.25-256 µg/mL), FFN (0.25-256 µg/mL), OTC (0.5-256 µg/mL), and ENR (0.12-64 µg/mL) as described in Experiment 1. To process the swabs of WB biofilms, PBS was added to their transport tubes and the swab and liquid were vortexed. The same processing steps as the water filter retentate, inoculating the AST-4 panel was then conducted as described above. Sediment samples were processed by adding 50 mL PBS and after thoroughly mixing were left to settle for 1 h, after which 15 mL of the top layer was centrifuged at 1,000 x g for 10 min and the pellet resuspended in 2 mL 20% glycerol PBS. A 5 mL aliquot of the top layer was used to inoculate the AST-4 panels.

3.4.3.2 DNA extraction, sequencing, and assembly

DNA was extracted using two commercial kits. The GenElute bacterial genomic DNA kit (NA2120, Sigma Aldrich) was employed according to the manufacturer's instructions with a modification: a FastPrep-24 (MPBio, Santa Ana, CA, USA) was used at 4.0 m/s for 60 s rather than a horizontal vortex adaptor to mix and disrupt cells. DNeasy PowerSoil kits (47016, Qiagen, Hilden, NRW, Germany) as per manufacturer's instructions. The commercial water samples from the emptied water bottles were used as control samples. DNA was subjected to paired end PCR-Free (Lucigen library preparation) shotgun Illumina nucleotide sequencing (NovaSeq 6000 PE150, Illumina, San Diego, CA, USA) at the *Centre d'expertise et de services Génome Québec* (Montréal, QC, Canada). Nucleotide sequences were assembled with SqueezeMeta v1.6.2 March 2023 running sequential assembly via Megahit (168, 169, 186). Short contigs (<500 bp) were removed using Contigtools and contig statistics were generated using Seqfu (187, 188). Average contig coverage was calculated by mapping the unassembled reads to the assembled contigs using BBmap (189). A local blastn search was performed on the assembled metagenomic data with the ~5 kbp *floR* loci for each WB *floR* loci (≥80% coverage and ≥99% identity was considered a hit) and visualized in R (4.2.2) (185).

3.4.3.3 Detection of ARGs

For comparative analysis, metagenomic sequencing data from feedlot water BioProjects PRJNA529711 and PRJNA292471 were assembled as described above. The genomes of BRD associated pathogens isolated from cattle from NCBI BioProjects PRJNA281531, PRJNA306895 and PRJNA340884 (*P. multocida* n = 51, *H. somni* n = 23, and *M. haemolytica* n = 98) were also included in the comparison. ARGs were detected in the three WB isolates, the *H. somni* isolate, the three metagenomic datasets and the BRD genomes utilising ABRicate, 80% minimum identity and coverage, referencing CARD (fetched April 2023, *estT* was manually included) (134, 166). After automated ARG detection, the list of detected ARGs was curated to remove gene regulators. The list of unique ARGs received additional curation to remove ARGs that were close homologs of one another (≥95% sequence identity overlap). A Venn diagram was created using nVennR in R (4.2.2) (171). The ~5-7 kbp loci of *floR* was aligned in a sequence list along with the WB isolates (Geneious Prime 2021.2, https://www.geneious.com).

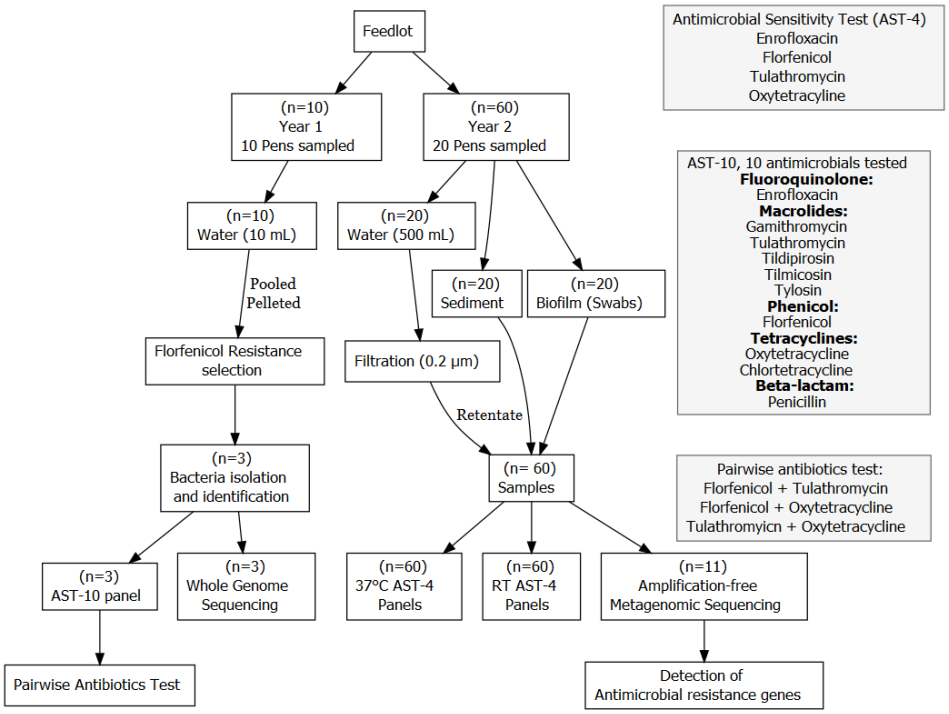

Figure 3.1 Workflow of the processing steps of the samples derived in Years 1 and 2. AST refers to the antimicrobial sensitivity test panels.

3.5 RESULTS AND DISCUSSION

Resistance to antimicrobials used at feedlots is well-established for BRD pathogens and other gastroenteric bacteria such as *E. coli* (76, 103, 110, 190, 191). However, this is the first report wherein environmentally isolated bacteria from feedlot WB were assessed as a potential source of AMR in BRD pathogens of concern through the analysis of the loci of an ARG. Previous studies of HGT of ARGs at agricultural food production complexes have been performed, yet these studies are specific to genera such as *Enterococcus spp.* or focused on intra-species transmission of AMR rather than inter-species transmission (94, 192, 193). *Histophilus somni* was found to have a similar *floR* locus as those found in WB2 and WB3, and there were matches to these *floR* loci in a wide range of BRD pathogens from North America and in other pathogens globally. Furthermore, the *floR* loci of WB2 and WB3 was detected in the majority of WB samples that underwent metagenomic sequencing. This study presents evidence that the *floR* gene is being exchanged between members of the microbiome of the watering bowl, whether in the watering bowl or otherwise, on a series of transposable elements through HGT and that this exchange, indirectly or directly, extends to BRD associated pathogens and beyond.

3.5.1 AMR in the WB isolates and *Histophilus somni*.

The impetus for finding FFN-resistant bacteria came from the feedlot switching the metaphylactic treatment from FFN to OTC after experiencing a spike in BRD treatment failure. The three WB bacteria showing FFN resistance were identified as *Sphingobacterium faecium* (WB1; 100% ribosomal Multilocus Sequencing Typing match), *Psychrobacter sp.*, (WB2; 85% match with *Psychrobacter maritimus*), and *Acinetobacter terrestris* (WB3; 96% match). The isolation of these taxa from a feedlot environmental sample was not unexpected, as *S. faecium* has been previously isolated from cattle feces and dairy milk (SAMD00169724, SAMEA81163918). *Psychrobacter* is associated with cold aquatic environments and *P. maritimus* is typically found in marine environments (194). A recently defined species, *A. terrestris*, is found in a wide range of terrestrial environments (195).

In addition to FFN resistance, all three isolates had a MIC >128 µg/ml for TUL, a macrolide, and OTC, a tetracycline (Table 3.1). Categorically, these organisms can be considered multi-drug resistant (MDR). There were, however, some differences in MICs with only the *Psychrobacter sp.* and *S. faecium* able to grow in 128 µg/mL of ENR whereas *A. terrestris* was susceptible to ENR. All three WB isolates also resisted the effect of pairwise antimicrobial combinations with MICs above the concentrations evaluated (>128 µg/ml for FFN, TUL and OTC; Table 3.1). For these MDR bacteria, treatment involving combinations of these antimicrobials would be as ineffective as individual antimicrobials.

The *H. somni* isolate from an animal at the feedlot was found to be resistant to GAM, TUL, OTC, CTC, and penicillin using the AST-10 screen. Resistance was based upon the breakpoints of the CLSI standards (Clinical and Laboratory Standards Institute, Wayne**,** Pennsylvania, USA) for *H. somni* isolated from cattle. The CLSI resistance breakpoints establish MICs of clinical relevance, where provision of an antimicrobial to treat an infection by an organism with an MIC past its corresponding breakpoint will be expected to be ineffective. While no CLSI resistance breakpoint exists for assessing TIL in *H. somni*, the TIL MIC was the same as GAM, suggesting resistance.

3.5.2 ARGs detected in WB isolates and *Histophilus somni*.

To characterise the AMR genotypes of the three WB and *H. somni* isolates, each underwent whole genome nucleotide sequencing. A plasmid with the *floR* gene was detected in each WB isolate including 2 copies of *floR* with unique loci in *A. terrestris*. Alongside *floR* there were 10 other unique ARGs detected in these plasmids (Table 3.2). In fact, ARGs that confer resistance to the tested drugs and explain the elevated MICs were readily detected in the bacterial genomes. For instance, the presence of *mphE*, a macrolide phosphotransferase, in *A. terrestris* and *H. somni* genomes explain the MIC of TUL required to inhibit *H. somni* and *A. terrestris*. Interestingly, a difference in the MIC for GAM between these two organisms was also observed, suggesting that the diversity of the *mphE* genes may impact the specificity of resistance between groups of similar macrolides (196).

There was also discordance between some of the phenotypes and genotypes. No detected ARG could explain the MIC of ENRO (> 128 µg/mL) found in *S. faecium* and *Psychrobacter sp*. There may be undescribed intrinsic resistance in these isolates or an unreported ARG may be responsible. The latter is a major concern as testing laboratories transition from culture-dependent to culture-independent AST, emphasizing the limitations of relying only on bioinformatic identification of ARGs. Along these lines, WB1 harboured an ARG that was discovered during the course of our recent feedlot studies. This ARG, named *estT*, encodes for a macrolide inactivating enzyme that belongs to the α-β hydrolase superfamily, and co-occurs in a cluster of ARGs on the *S. faecium* plasmid as well as in ARG clusters in BRD genomes (85). This commonly overlooked unannotated gene is now recognized as an important ARG encoding for tylosin, tilmicosin and tildipirosin resistance in BRD pathogens (85). There are likely other unannotated ARGs that need to be identified in order to have a truly comprehensive database, which are also biased by research that has historically and more intensively focused on human medicine and pathogens.

A similar discordance was the finding of the *floR* gene in *H. somni*, yet the strain was not observed to be resistant to FFN in laboratory ASTs. While the gene was present, it was clearly not expressed in the laboratory. This phenomenon has been previously reported. A study observed *floR* in *H. somni* in 11 isolates from cattle but reported no phenotypic resistance (103). The *estT* and *floR* genes highlight an important point. In the first instance, macrolide resistance was identified phenotypically using standard AST protocols. However, the ARG was unknown, and resistance could have easily been assumed to be associated with only *ermF*, a ribosome-modifying enzyme that is also present on the *S. faecium* plasmid. Conversely, the *floR* gene has been found in BRD pathogens, but phenotypically the isolates were sensitive to FFN. The latter raises the question as to whether AST is a reasonable and universal facsimile of what is occurring in the animal. If laboratory conditions impact gene expression and that causes a failure to report potential resistance in the animal, then DNA-based methods may fill in these gaps. In fact, genotypic and phenotypic characterization are complementary approaches, and these results highlight the need for additional research to continue to identify novel resistance genes and catalogue those that are known. Finally, for some important pathogens, an investment in identifying more appropriate conditions for ASTs may be necessary and help to better serve AMR surveillance efforts.

In addition to ARGs that confer resistance to commonly used feedlot drugs, we also detected genes encoding for resistance to drug classes not, or very rarely, used at feedlots. This includes ARGs that protect against aminoglycosides, sulfonamides, and diaminopyrimidines. The *H. somni* isolate harboured 7 unique ARGs on its chromosome, including *aadA8* and *sul2*, which encode for aminoglycoside and sulfonamide resistance, respectively. The aminoglycosides are a drug class that are not utilised to treat cattle in feedlots (9). This finding was not unique, as AMR to a wide range of antimicrobials in *E.coli* and *Enterococcus spp.* in cattle has been observed after administration of FFN, including to drug classes not used in cattle (110).

Table 3.1 MICs measured in an AST of 10 antimicrobials, and relevant ARGs detected for an *H. somni* isolate and feedlot WB isolates, *S. faecium*, *Psychrobacter sp*., and an *A. terrestris*. TUL, FFN and OTC were also tested in pairwise combinations of each other. ARGs were detected using ABRicate referencing CARD

		Sphingobacterium faecium		*Psychrobacter sp.*		*Acinetobacter terrestris*		*Histophilus somni*	
Class	Antimicrobial	MIC (µg/mL)	Gene	MIC (µg/mL)	Gene	MIC (µg/mL)	Gene	MIC (µg/mL)	Gene
Phenicol	Florfenicol (FFN)	>256	*floR*	>256	*floR*	>256	*floR*	1	*floR*
Fluoroquinolone	Enrofloxacin	>128		>128		16		≤0.12	
Macrolide	Gamithromycin	>256	*estT ermF*	>256		>256	*mphE msrE*	16	*mphE msrE*
	Tulathromycin (TUL)	>256		>128		128		128	
	Tilmicosin	>256		>256		>256		16	
	Tildipirosin	>128		>128		>128		2	
	Tylosin	>128		>128		>128		2	
Tetracycline	Chlortetracycline	>256	*tet(X)*	>256		128	*tet(X3) msrE*	16	*tet(H) msrE*
	Oxytetracycline (OTC)	>256		>256		128		32	
Penam	Penicillin	>8		>8		4		>8	
Pairwise antibiotics combinations									
Phenicol Macrolide	FFN + TUL	>128	*floR ermF*	>128	*floR*	>128	*floR, mphE*		
Phenicol Tetracycline	FFN + OTC	>128	*floR tetX*	>128	*floR*	>128	*floR tet(X3) msrE*		
Tetracycline Macrolide	OTC + TUL	>128	*ermF tetX*	>128		>128	*tet(X3) msrE mphE*		

> indicates growth in the highest tested concentration.

Table 3.2 List of ARGs detected, drug class they encode resistance to, in the plasmids or chromosome of the three environmentally isolated bacteria, *S. faecium*, *Psychrobacter sp.*, *A. terrestris* and an *H. somni* isolate. Whole genomes were searched with ABRicate referencing CARD

Gene	Isolate	Location	Resistance
aadA8	*H. somni*	Chromosome	aminoglycoside
ANT(3'')-IIa	*Psychrobacter sp.*	Plasmid	aminoglycoside
APH(3'')-Ib	*A. terrestris*	Plasmid	aminoglycoside
APH(6)-Id	*A. terrestris*	Plasmid	aminoglycoside
dfrA1	*Psychrobacter sp.*	Plasmid	diaminopyrimidine
ErmF	*S. faecium*	Plasmid	lincosamide; macrolide; streptogramin
estT	*S. faecium*	Plasmid	macrolide
floR	*A. terrestris*	Plasmid	phenicol
	A. terrestris	Plasmid	
	H. somni	Chromosome	
	Psychrobacter sp.	Plasmid	
	S. faecium	Plasmid	
mphE	*H. somni*	Chromosome	macrolide
msrE	*H. somni*	Chromosome	lincosamide; macrolide; oxazolidinone; phenicol; pleuromutilin; streptogramin; tetracycline
ROB-2	*H. somni*	Chromosome	cephalosporin; penam
SAT-2	*Psychrobacter sp.*	Plasmid	nucleoside
sul2	*A. terrestris*	Plasmid	sulfonamide
	H. somni	Chromosome	
	Psychrobacter sp.	Plasmid	
tet(H)	*H. somni*	Chromosome	tetracycline
tet(X3)	*A. terrestris*	Plasmid	glycylcycline; tetracycline
tet(X)	*S. faecium*	Plasmid	glycylcycline; tetracycline

3.5.3 The *floR* loci of the three isolated bacteria overlapped with those in BRD pathogens.

As not all genes may be able to be transferred to BRD pathogens, the relevance of the ARGs detected in the WB isolates was assessed through the investigation of the *floR* loci for evidence of HGT. This investigation was performed through a series of comparisons of the loci within this study and with publicly available genomes of BRD and other pathogens. The copies of *floR* shared ≥99% identity between the four isolates (Figure 3.2). One of the two *floR* loci of *A. terrestris*, the *floR* locus of the *Psychrobacter* isolate and the *floR* locus on the chromosome of *H. somni* were nearly identical, all three were flanked by a gene encoding for a putative regulatory protein, *lysR*, and a gene associated with the Type 4 secretory pathway. This 3-gene configuration was surrounded by genes encoding for mobile elements, except on one flank of the locus in *H. somni* indicating some distance. In *S. faecium* the *floR* gene was in a unique location with other ARGs: *tet(X)*, *estT* and the aforementioned *erm(F)*.

There were strong similarities (>90% match in coverage and identity) between the *floR* loci of *Psychrobacter sp.* and *A. terrestris* and *floR* loci in the genomes of BRD pathogens isolated from cattle across continental USA and some from Europe (Table 3.3). There were also some notable non-BRD matches, including a match for *S. faecium floR* loci with *Riemerella anatipestifer* a common pathogen of the poultry industry (197). There was also a strong match with the *Psychrobacter sp.* and *A. terrestris floR* loci in 33 of the 42 *Salmonella enterica* from infant fecal samples from Taiwan that contained *floR*. This is indicative of a global ubiquity of *floR* nested on this locus.

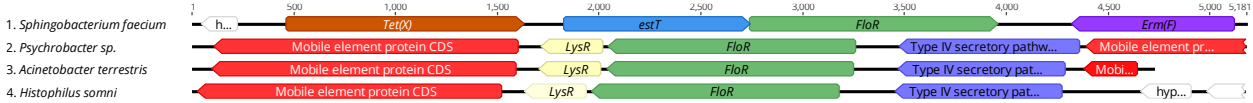

Figure 3.2 Sequence list of *floR* loci from the three environmentally isolated bacteria and the *H. somni* isolate from the same feedlot. Reverse compliment of the loci from *A. terrestris* and *H. somni*. Genes colour coded by annotation.

Table 3.3 NCBI blastn matches (≥90% Coverage, ≥90% Identity) of loci of *floR* identified in three environmentally isolated *S. faecium* (WB1), *Psychrobacter spp.* (WB2), and *A. terrestris* (WB3) bacteria from a feedlot watering bowl. Also listed are the source BioProject, the location listed in the BioProject or BioSample if available, the host animal (feedlot (F) and dairy (D) cattle) from which the sample was taken and the pathogen, the total number of that pathogen in the BioProject, and total *floR* gene hits within that number of pathogens.

Source	Locale	Host	Pathogen	(n) in Source	*floR* hits	Match to loci of *floR*		
						WB1	WB2	WB3
PRJNA497581	AZ, USA	Human	*Acinetobacter spp.*	100	8	0	8	8
PRJNA281531	KS, USA	Cattle (F)	*Histophilus somni*	7	5	0	4	4
PRJNA306895	CA, USA	Cattle (D)	*Histophilus somni*	16	7	0	4	4
PRJNA306895	CA, USA	Cattle (D)	*Mannheimia haemolytica*	29	2	0	1	1
PRJNA340884	North America	Cattle (F)	*Mannheimia haemolytica*	69	4	0	4	4
PRJNA433612	Denmark	Cattle	*Mannheimia haemolytica*	3	1	0	1	1
PRJNA774931	BB, Germany	Cattle	*Mannheimia haemolytica*	1	1	0	1	1
PRJNA281531	KS, USA	Cattle (F)	*Pasteurella multocida*	16	2	0	2	2
PRJNA306895	CA, USA	Cattle (D)	*Pasteurella multocida*	35	8	0	6	6
PRJNA339731	India	Buffalo	*Pasteurella multocida*	1	1	0	1	1
PRJNA433612	Denmark	Cattle	*Pasteurella multocida*	5	1	0	1	1
PRJNA660554	JS, China	Goose	*Riemerella anatipestifer*	14	10	2	0	0
PRJNA478278	Taiwan	Human	*Salmonella enterica*	50	42	0	33	33

3.5.4 Phenotypic and genotypic characterisation of the microbiome of watering bowls revealed the ubiquity of resistant bacteria.

To catalogue the ARGs in the WB microbiome at a feedlot that experienced treatment failure post FFN treatment, samples were collected and characterised by DNA sequencing experiments and culture-based phenotypic screens. A total of 11 samples were analyzed using an shotgun metagenomic approach. There was an average of 35M reads per sample and the duplicate read percentage ranged from 7% to 11%, suggesting high diversity within each sample (Figure 3.3A). The average fold coverage of all samples was 35x (Table 3.4). A limitation of metagenomic analysis is that appropriate sequencing depth in complex samples is challenging to evaluate. In the water sample this was evident, where higher read count led to a higher number of unique ARGs

detected, indicating that further sequencing would have resulted in the detection of additional ARGs (Figure 3.3D). Another limitation of the shotgun metagenomic approach is the requirement for a significant quantity of DNA, which for dilute samples such as water can be challenging to obtain from small volumes. A relatively small volume 0.5 to 1 L was used in this analysis, which accounts for 5-10% of the WB volume. This yield is much lower than what is typically found in wastewater which contains more biomass and can be sampled at higher volumes without altering the community. We expect that the communities in the water sample of the WBs to be quite dynamic since as water is consumed, new water is pumped into the bowls from a well or reservoir. Thus, a more targeted approach that looks only at clinically relevant ARGs, using a molecular enrichment technique such as probe capture is likely better suited for this kind of analysis.

In total, 94 different ARGs were detected including *floR* in 9 of 11 samples (Figure 3.4, Figure 3.3). Neighbouring ARGs to *floR* in *S. faecium*, *estT* (11 of 11), *tet(X)* (5 of 11) and *erm(F)* (4 of 11) were also detected. The *floR* locus of the *Psychrobacter* isolate was detected in 7 of 11 samples. As supported by the phenotyping and genotyping of individual resistant bacteria and previous environmental samples, ARGs are ubiquitous within feedlot water bowls (Figure 3.4) (149).

Of the ARGs detected in the water bowl, ~60% overlapped with previously performed water metagenomic sequencing (wastewater, water source) from other feedlots (Figure 3.5A). These ARGs encoded for a range of drug classes (Figure 3.5B). The distribution in drug class for which the ARGs encode resistance was also similar between metagenomic datasets (Figure 3.5B). While a lot of ARGs were detected, it is important to acknowledge that not all may be relevant to BRD pathogens. Metagenomic sequencing of the water bowl covered 12 of 16 detected ARGs across 172 BRD associated pathogen genomes from cattle, which are stored in NCBI BioProjects. The metagenomic sequencing datasets together covered 15 of 16 detected ARGs (Figure 3.5A). This is indicative of water sampling providing excellent coverage for ARGs, which are relevant to feedlot operation, even with the stated limitation of untargeted sequencing.

Table 3.4 Contig statistics and the total bases they were assembled from, of the 11 feedlot WB samples that underwent metagenomic sequencing.

Sample		Assembled Contigs (≥500 bp)							Unassembled data
Pen	Type	Total bases (Mbp)	n contigs	Avg. fold coverage	Avg. length (bp)	max length (bp)	N75	N90	Total bases (Mbp)
A6	Sediment	283.5	299,409	35	947	386,311	612	540	6164
A7	Sediment	268.9	157,655	46	1706	340,774	980	630	7324.9
B7	Sediment	120.7	83,205	70	1450	196,761	846	609	4653.9
C10	Sediment	240.9	150,778	34	1598	250,466	954	639	4513.2
D10	Sediment	1056.0	1,218,112	9	866	335,366	651	559	6489.7
H1	Water	418.0	270,609	21	1545	551,772	917	632	5214.5
H2	Water	543.3	335,107	20	1621	658,326	976	652	6134.3
H3	Sediment	210.6	119,920	57	1756	357,044	1,051	652	6436.1
H3	Water	424.3	264,070	22	1607	724,003	950	641	5371.4
H4	Sediment	222.6	134,139	54	1659	249,720	987	645	6489.1
H4	Water	518.1	300,944	23	1722	978,937	993	647	6693.2

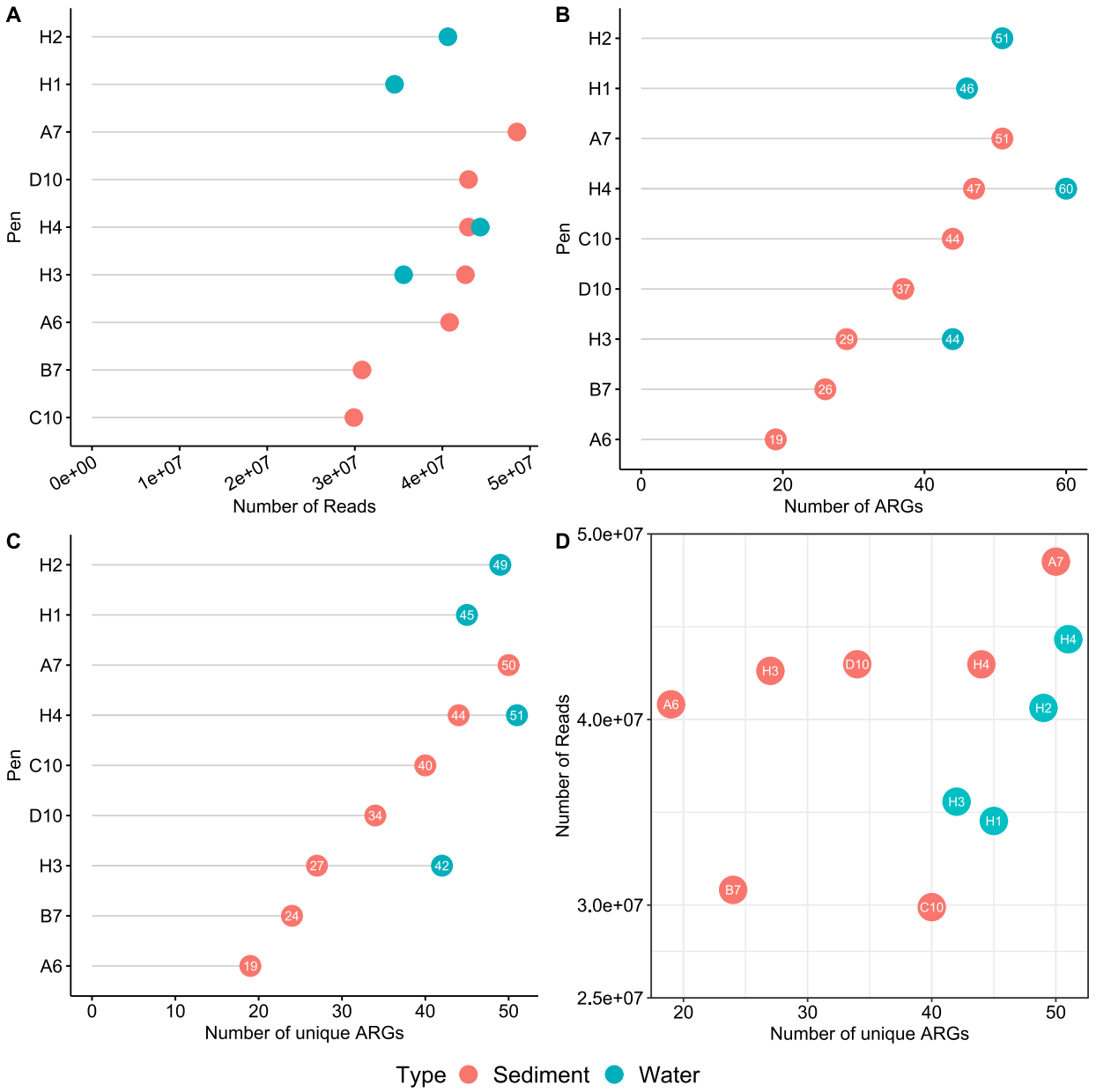

Figure 3.3 (A) Number of reads for each of the 11 watering bowl samples that underwent shotgun metagenomic nucleotide sequencing. (B) Number of antimicrobial resistance genes (ARGs) from each sample. (C) Number of unique ARGs detected from each sample. (D) Number of reads compared to number of unique ARGs detected.

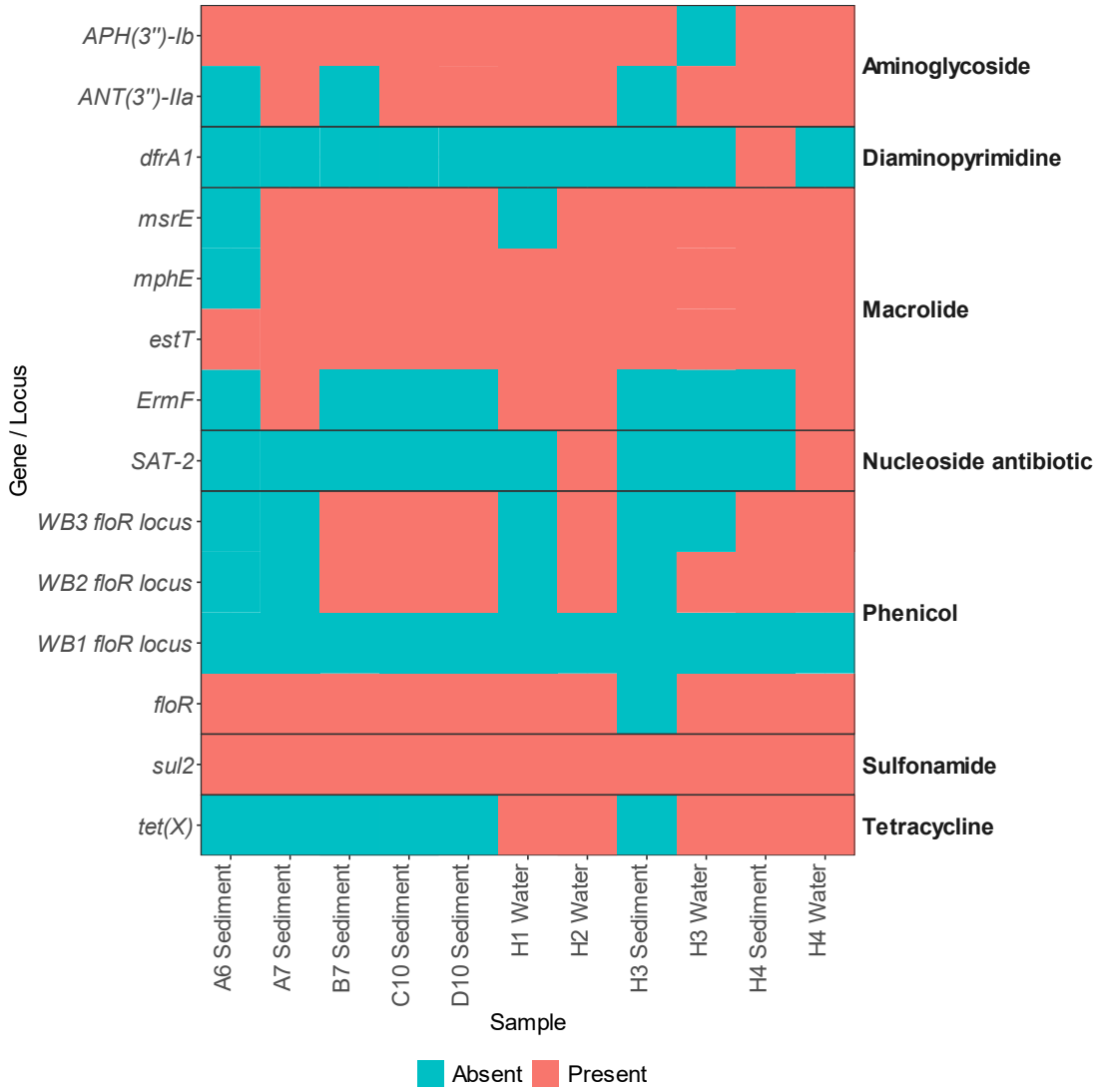

Figure 3.4 Antimicrobial resistance genes detected (≥80% coverage, ≥80% identity) in the feedlot watering bowl samples that underwent shotgun metagenomic sequencing that overlapped with genes in the same contig as *floR* in isolates from the same feedlot. Additionally listed is the detection (≥80% coverage, ≥99% identity) of the ~5 kbp *floR* loci found in the watering bowl isolates. The Comprehensive Antibiotic Resistance Database was used as the reference database (except for *estT*). A-D pens were home pens, the H pens were hospital pens.

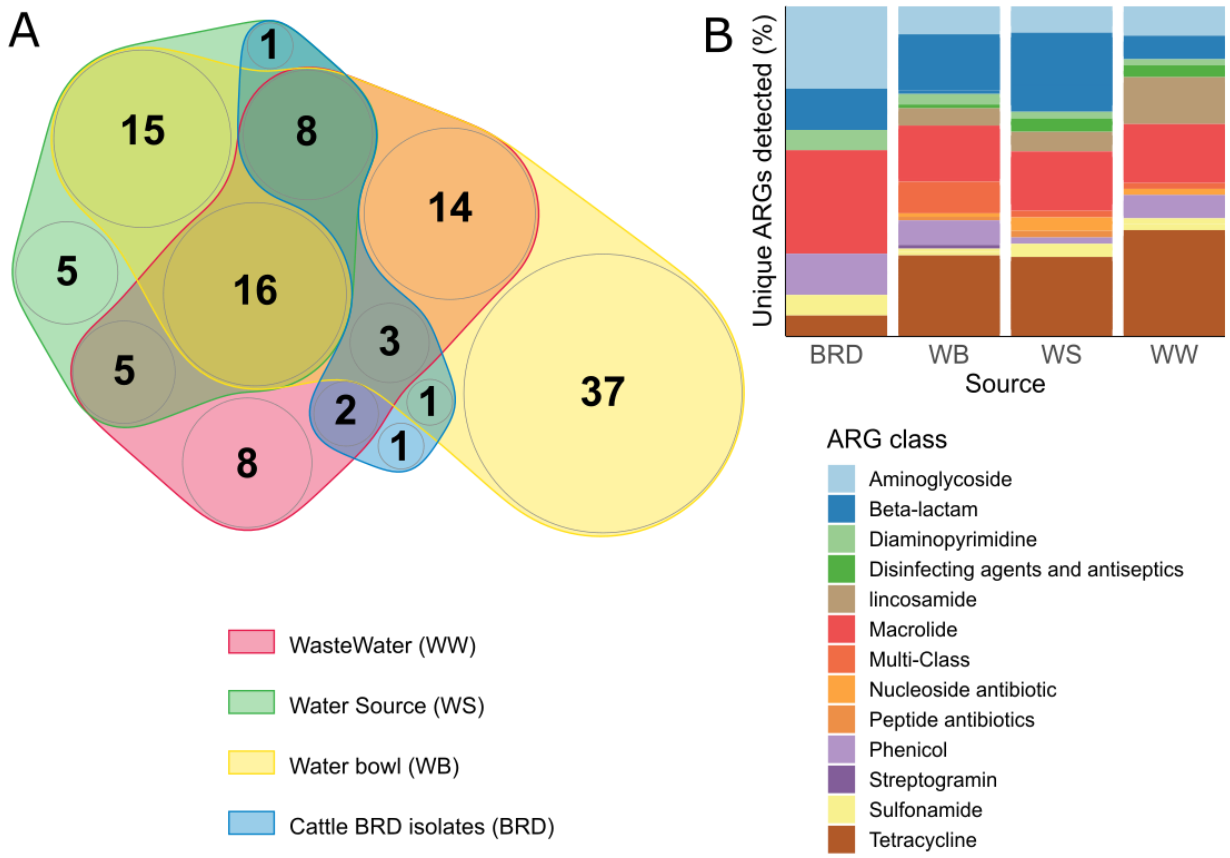

Figure 3.5 (A) Venn diagram showing ARGs detected. (B) Comparison of detected ARGs, by antibiotic class, across four sample types. The ARGs identified in the metagenomic sequencing of 11 samples was compared to the genomes of BRD pathogens (PRJNA281531, PRJNA306895 and PRJNA340884: 51 *P. multocida*, 98 *M. haemolytica* and 23 *H. somni*), feedlot source water and wastewater metagenomes (PRJNA292471 and PRJNA529711).

3.6 CONCLUSION

Three bacteria from a water sample were isolated based upon their resistance to FFN. It was then found that the gene encoding for this resistance, *floR*, was present in all three and the *floR* locus was highly similar between two of three. The *floR* locus was then found in an *H. somni* isolate and matched to contigs from metagenomic sequencing of water and sediment samples. We then extended our search for this locus to BRD pathogens and beyond. Identification of microbiota within the watering bowl having the capacity for indirect or direct HGT to pathogens has created an area of interest for future research. One avenue of research would be to investigate the impact of chemical water treatment on the microbiome of WB and treatment outcomes. In a comparison study, cattle provided with treated water had an increase in weight gain by 20% over cattle provided with untreated water in pasture-based cattle operations (198). With the overlap in ARGs shared between the isolates, metagenomic sequencing and BRD isolates, it is indicative that the HGT between environmental resistomes and pathogens extends beyond *floR*. At what time point the HGT occurred or what location facilitates this HGT is unknown. A limitation to this study was the lack of a comparison to the microbiome within cattle respiratory tracts. A future study that hopes to further establish this pragmatic approach could look at a pilot study that directly monitors the impact of cattle upon the watering bowl microbiome and vice versa. With that limitation in mind, this study confirmed that commonly overlooked bacteria present in the environmental microbiome of the pen WB have the potential to be a reservoir of ARGs such as *floR* for pathogens, and to be potentially used as sentinel bacteria for ARGs within the cattle population.

3.7 DATA AVAILABILITY

The nucleotide sequences of bacteria characterized in this study are available through GenBank BioProject PRJNA820789. Sequence data for the microbiomes is available BioProject PRJNA978540.

4 General summary and conclusion

4.1 DISCUSSION

The hypothesis of this thesis was that the understudied environmental microbiomes in feedlot WBs are a potential source of ARGs for BRD pathogens. In both experiments, WB samples were collected quickly, inexpensively, and in a manner that was not intrusive to the cattle. To investigate whether the microbiomes within WBs could be a source of AMR for BRD associated pathogens, the resistome within WBs was characterized using culture-dependent and independent approaches, and the analysis focused on antimicrobials utilized in the feedlot industry. Two experiments were conducted at two separate feedlots. The first involved a newly established feedlot, allowing sampling prior to arrival of cattle, while in the second experiment the feedlot had been in use for decades. An AMR profile was created at both feedlots with the same phenotyping approach, but each experiment had a unique approach for genotyping the WB microbiomes. Overall, ARGs of relevance to the industry were confirmed to be present in WB microbiomes.

Through culture-dependent characterization of the WB microbiomes, resistant bacteria belonging to a variety of genera were isolated through AMR selection. Isolation and genomic characterisation of resistant isolates from both feedlots focused on the clustering of ARGs of interest in mobile genetic elements and on plasmids. The observation of common ARG clusters in environmental and pathogenic organisms indicates that HGT facilitates their dissemination between organisms within the same and overlapping environmental niches. Isolates belonging to 19 different genera were isolated from the newly established feedlot, including *Psychrobacter, Acinetobacter,* and *Sphingobacterium,* which were also isolated from the older feedlot. At the level of bacterial taxa, our untargeted sampling approach provides an alternative and complementary strategy to the use of sentinel species such as *E. coli* for AMR surveillance.

While the WB isolates came from an environmental sample, AMR to antimicrobials of critical importance to human and veterinary medicine in these isolates should be taken seriously. For one, the AMR was widespread across genera indicating that highways of exchange of ARGs exist between these various bacterial species. The loci of *floR* in experiment 2 suggests that exchange of ARGs extends to BRD pathogens. This was evidenced by the fact that the transposons flanking the regions containing *floR* in the environmentally isolated bacteria matched that of the *floR* loci of the *H. somni* isolate and of many deposited genomes of BRD pathogens. Again, these results highlight the role of HGT in ARG dissemination and suggest that it may occur in the WBs. Another important point is that many of the genera had member species with the capability to cause serious diseases in livestock and in humans. *Klebsiella pneumoniae* and *Acinetobacter baumannii* were both isolated from the WBs and are both ranked in the top six leading pathogens for deaths associated with AMR (83). Thus, analyzing the resistome of the environmental microbiomes present within feedlots can give insights to AMR in these relevant bacteria within our shared biosphere.

Yearly antimicrobial cycling in metaphylactic treatment has been suggested as a form of AMR stewardship, which has been an ongoing discussion in clinical healthcare for at least 40 years

(199-201). The impact of AMU compared to no AMU in cattle has been previously compared and only a slight reduction in resistance capacity was seen in the cattle microbes (202). However, quantitation in this comparative study was not focused on feedlot specific ARGs such as *floR*. Plasmids carrying ARG clusters, as detected in both experiment 1 and 2, reduce the effectiveness of AMR prevention strategies that focus on reducing AMU of an in-use drug class. Positive selective pressure for resistance from antimicrobial treatment is not exclusive to ARGs encoding for resistance to that antimicrobial when ARGs are located on clusters or plasmids, the selective pressure extends to all ARGs on those genetic elements (203). A plasmid carrying a cluster of ARGs also reduces the amount of HGT events that need to occur before an ARG lacking bacteria acquires a wide range of ARGs (204). Furthermore, most antimicrobials are derived from antimicrobials produced by microbes. Therefore, it is probable that these naturally occurring antimicrobials provide a selective pressure in the environment to maintain the ARGs that have previously increased from anthropogenic pollution.

Long-term rotation of antimicrobials to reduce AMR is not a feasible strategy because there is an insufficient number of antimicrobial classes approved for use in veterinary medicine to support a rotation that lasts longer than a few years. It is also unknown what kind of timeframe would be sufficient to have a significant impact that restores the effectiveness of antimicrobials to previous levels. The other issue is that while ARGs may be resident in the biofilms within feedlots, newly arriving cattle are also a source of ARGs (145). Therefore, unless a feedlot is practicing an all-in and all-out production strategy, similar to the swine and poultry industry, it will be impossible to break the cycle of new ARGs being disseminated from one cohort of cattle to another and eventually across the entire feedlot. It is unclear how long it would take for the concentration of ARGs to decrease in the environment of a pen that is exposed to an influx from the greater biosphere, especially considering the discovery of an ARG encoding for macrolide resistance in a cave that was isolated for millions of years (106).

In experiment 2 a highly similar *floR* locus was detected in two of the WB isolates, the *H. somni* isolate, and in BRD associated pathogens from across North America. In addition, this same locus was found in *Salmonella enterica* isolates from the stool samples from infants in Taiwan. Together, these findings highlight a highway of genetic exchange that has global reach. In this interconnected biosphere of our pale blue dot, stopping the use of one antimicrobial in a feedlot that is exposed to dust, water, and other environmental influxes, in the hopes of restoring the effectiveness of that antimicrobial the next year seems futile (177). Future AMR stewardship efforts within feedlot management will likely only be effective in reducing AMR for new fully anthropogenic drug classes with no previous treatment history.

There are limitations to the conclusions that can be made from these series of studies. Both experiments were performed at feedlots under the same management, and these findings, especially the composition of the watering bowl microbiome, should be replicated at multiple feedlots with different management styles. Additionally, the route of transfer of bacteria from watering bowl to cattle upper respiratory tract and vice versa, and whether it occurs at all is not known. The evidence that HGT is occurring between members of the watering bowl microbiome is also not evidence that HGT is occurring when those members are in the watering bowl. While

the impact of beef production on the watering bowl is clear from the weekly sampling in experiment 1, it is not shown what the source of that impact is. The concentration of active antimicrobials within the water was not measured or directly linked to AMR in this work.

4.2 FUTURE RESEARCH

Displaying direct HGT to the BRD pathogens from the environmental bacteria and comparing the sequence differences overtime is outside of the scope of this research. The highway of HGT may include bacteria unculturable in a laboratory setting and there are various other complexities involved with reproducing HGT in a laboratory setting. It would however allow us to directly link detected ARGs in the environment and treatment effectiveness in a follow up animal study.

Studying environmental microbiomes through the selection for drug resistance within isolates can generate new novel future research ideas from the mismatches in the resistance recorded and the known ARGs detected in the genome, as evidenced in a previously published study which utilized that data from one of the feedlot watering bowl isolates from experiment 2 (85).

In terms of practical application, ideally the burden of AMR is reduced through the treatment of water provided to WBs. Water treatment strategies to limit the impact and interface of the resistome in the environmental microbiome and cattle pathogens and the cost versus benefit of that need further research. Alternatively, antimicrobial usage consultation for feedlot management could eventually use the ease of access of watering bowls as a proxy to estimate antimicrobial efficacy. For this, the protocol would need to be standardised at all levels to accomplish this. It would need to be decided on what data is generated as both culture-independent and culture-dependent data are likely needed to make conclusive decisions. This project provided data on the feasibility of sampling at scale from WBs for a pilot study that may eventually lead to a consultation tool that would be able to be used with relatively little interference to feedlot operation compared to sampling directly from the cattle. An obvious next step would be to compare metagenomic sequencing to targeted probe capture approaches that are focused on relevant ARGs, ensuring that this work is coordinated with discovery efforts related to previously unreported contributors to AMR in feedlots. A targeted approach would likely provide a higher sequencing depth and lower the amount of DNA required, which are both limitations of shotgun metagenomic sequencing encountered when genotypically analysing the environmental samples.

4.3 CONCLUSION

In conclusion, WB sampling is a fast, inexpensive, and non-intrusive methodology of detecting ARGs of medical importance. This study also provided evidence that ARGs within the WB microbiome have the potential to spread to pathogens of concern to the industry such as BRD associated pathogens. Much of the AMR is missed when the environmental microbiome, which the cattle interact with on a daily basis, is not included in AMR monitoring. In this thesis, a potential source within feedlots of ARGs in BRD pathogens was detected and methodology to study it was described.

5 References

1. Kulshreshtha S, Mondongo O, Florizone A. 2012. Economic impacts of livestock production in Canada: a regional multiplier analysis. Report Prepared for Canfax Research Services Canadian Cattlemen's Association Department of Bioresource Policy Business and Economics, University of Saskatchewan, Saskatoon.
2. Spooner JM, Schuppli CA, Fraser D. 2012. Attitudes of Canadian beef producers toward animal welfare.
3. Pearson JM, Pajor EA, Caulkett NA, Levy M, Campbell JR, Windeyer MC. 2019. Benchmarking calving management practices on western Canada cow–calf operations. Translational Animal Science 3:1446-1459.
4. Ugochukwu AI, Phillips PW. 2019. Canadian beef and dairy farmers' attitudes towards animal vaccines. Australian Journal of Agricultural and Resource Economics 63:814-840.
5. Government of Canada. 2023. Number of cattle, by class and farm type (x 1,000). StatisticsCanada, https://doi.org/10.25318/3210013001-eng.
6. Greenwood PL. 2021. An overview of beef production from pasture and feedlot globally, as demand for beef and the need for sustainable practices increase. Animal:100295.
7. Aidara-Kane A, Angulo FJ, Conly JM, Minato Y, Silbergeld EK, McEwen SA, Collignon PJ. 2018. World Health Organization (WHO) guidelines on use of medically important antimicrobials in food-producing animals. Antimicrobial Resistance & Infection Control 7:1-8.
8. World Health Organization. 2018. Critically Important Antimicrobials for Human Medicine.
9. Brault SA, Hannon SJ, Gow SP, Warr BN, Withell J, Song J, Williams CM, Otto SJ, Booker CW, Morley PS. 2019. Antimicrobial use on 36 beef feedlots in Western Canada: 2008–2012. Frontiers in veterinary science 6:329.
10. World Health organization. 2021. Access, Watch, Reserve (AWaRe) classification of antibiotics for evaluation and monitoring of use.
11. Government of Canada. 2009. Categorization of Antimicrobial Drugs Based on Importance in Human Medicine. Heatlh Canada, https://www.canada.ca/en/health-canada/services/drugs-health-products/veterinary-drugs/antimicrobial-resistance/categorization-antimicrobial-drugs-based-importance-human-medicine.html.
12. Wren CM, Cowper J, Greer N, Goldin L, Perry A. 2022. Effect of Reduced Fluoroquinolone Use on Cephalosporin Use, Susceptibilities and Clostridioides difficile Infections. Antibiotics 11:1312.
13. Gehring R, Mochel JP, Schmerold I. 2023. Understanding the background and clinical significance of the WHO, WOAH, and EMA classifications of antimicrobials to mitigate antimicrobial resistance. Frontiers in Veterinary Science 10:265.
14. Jelinski M, Kinnear A, Gesy K, Andrés-Lasheras S, Zaheer R, Weese S, McAllister TA. 2020. Antimicrobial sensitivity testing of Mycoplasma bovis isolates derived from Western Canadian feedlot cattle. Microorganisms 8:124.
15. Engler M, Defoor P, King C, Gleghorn J. 2014. The impact of bovine respiratory disease: the current feedlot experience. Animal health research reviews 15:126-129.
16. Kelly AP, Janzen ED. 1986. A review of morbidity and mortality rates and disease occurrence in North American feedlot cattle. The Canadian Veterinary Journal 27:496.
17. Blakebrough-Hall C, McMeniman JP, González LA. 2020. An evaluation of the economic effects of bovine respiratory disease on animal performance, carcass traits, and economic outcomes in feedlot cattle defined using four BRD diagnosis methods. Journal of Animal Science 98.
18. Cortes JA, Hendrick S, Janzen E, Pajor EA, Orsel K. 2021. Economic impact of digital dermatitis, foot rot, and bovine respiratory disease in feedlot cattle. Translational Animal Science 5:txab076.
19. Snowder G, Van Vleck LD, Cundiff L, Bennett G, Koohmaraie M, Dikeman M. 2007. Bovine respiratory disease in feedlot cattle: phenotypic, environmental, and genetic correlations with

growth, carcass, and longissimus muscle palatability traits. Journal of animal science 85:1885-1892.
20. Li C, Zaheer R, Kinnear A, Jelinski M, McAllister TA. 2022. Comparative Microbiomes of the respiratory tract and joints of feedlot cattle mortalities. Microorganisms 10:134.
21. McGuirk SM. 2008. Disease management of dairy calves and heifers. Vet Clin North Am Food Anim Pract 24:139-53.
22. Mahendran SA. 2020. Use of fever detection in combination with thoracic ultrasonography to identify respiratory disease, and compare treatments of antimicrobials and NSAID: a randomised study in dairy calves. Veterinary record open 7:e000415.
23. Tortorelli G, Carrillo Gaeta N, Mendonça Ribeiro B, Miranda Marques L, Timenetsky J, Gregory L. 2017. Evaluation of Mollicutes microorganisms in respiratory disease of cattle and their relationship to clinical signs. Journal of veterinary internal medicine 31:1215-1220.
24. Griffin D. 2014. The monster we don't see: subclinical BRD in beef cattle. Animal health research reviews 15:138-141.
25. Rice J, Carrasco-Medina L, Hodgins D, Shewen P. 2007. Mannheimia haemolytica and bovine respiratory disease. Animal Health Research Reviews 8:117-128.
26. Hay K, Barnes TS, Morton J, Clements A, Mahony T. 2014. Risk factors for bovine respiratory disease in Australian feedlot cattle: use of a causal diagram-informed approach to estimate effects of animal mixing and movements before feedlot entry. Preventive Veterinary Medicine 117:160-169.
27. Martin S, Meek A, Davis D, Johnson J, Curtis R. 1982. Factors associated with mortality and treatment costs in feedlot calves: the Bruce County Beef Project, years 1978, 1979, 1980. Canadian Journal of Comparative Medicine 46:341.
28. Timsit E, Hallewell J, Booker C, Tison N, Amat S, Alexander TW. 2017. Prevalence and antimicrobial susceptibility of Mannheimia haemolytica, Pasteurella multocida, and Histophilus somni isolated from the lower respiratory tract of healthy feedlot cattle and those diagnosed with bovine respiratory disease. Veterinary microbiology 208:118-125.
29. Griffin D, Chengappa M, Kuszak J, McVey DS. 2010. Bacterial pathogens of the bovine respiratory disease complex. Veterinary Clinics: Food Animal Practice 26:381-394.
30. Confer AW. 2009. Update on bacterial pathogenesis in BRD. Animal Health Research Reviews 10:145-148.
31. Kramer LM, Mayes MS, Fritz-Waters E, Williams J, Downey E, Tait Jr R, Woolums A, Chase C, Reecy JM. 2017. Evaluation of responses to vaccination of Angus cattle for four viruses that contribute to bovine respiratory disease complex. Journal of animal science 95:4820-4834.
32. Mutters R, Ihm P, Pohl S, Frederiksen W, Mannheim W. 1985. Reclassification of the genus Pasteurella Trevisan 1887 on the basis of deoxyribonucleic acid homology, with proposals for the new species Pasteurella dagmatis, Pasteurella canis, Pasteurella stomatis, Pasteurella anatis, and Pasteurella langaa. International Journal of Systematic and Evolutionary Microbiology 35:309-322.
33. Kluger MJ, Vaughn LK. 1978. Fever and survival in rabbits infected with Pasteurella multocida. The Journal of physiology 282:243-251.
34. Weber DJ, Wolfson JS, Swartz MN, Hooper DC. 1984. Pasteurella multocida infections. Report of 34 cases and review of the literature. Medicine 63:133-154.
35. Rollof J, Nordin-Fredriksson G, Holst E. 1989. Pasteurella multocida occurs in a high frequency in the saliva of pet dogs. Scandinavian journal of infectious diseases 21:583-584.
36. Klima CL, Holman DB, Ralston BJ, Stanford K, Zaheer R, Alexander TW, McAllister TA. 2019. Lower respiratory tract microbiome and resistome of bovine respiratory disease mortalities. Microbial ecology 78:446-456.
37. Goldspink LK, Mollinger JL, Barnes TS, Groves M, Mahony TJ, Gibson JS. 2015. Antimicrobial susceptibility of Histophilus somni isolated from clinically affected cattle in Australia. The Veterinary Journal 203:239-243.

38. Sandal I, Shao JQ, Annadata S, Apicella MA, Boye M, Jensen TK, Saunders GK, Inzana TJ. 2009. Histophilus somni biofilm formation in cardiopulmonary tissue of the bovine host following respiratory challenge. Microbes and infection 11:254-263.
39. Alhamami T, Low WY, Ren Y, Taylor K, Khazandi M, Veltman T, Venter H, Carr M, Turni C, Abraham S. 2022. Antimicrobial susceptibility and genomic analysis of Histophilus somni isolated from cases of bovine respiratory disease in Autralian feedlot cattle. Veterinary Microbiology:109460.
40. Razin S, Yogev D, Naot Y. 1998. Molecular biology and pathogenicity of mycoplasmas. Microbiology and molecular biology reviews 62:1094-1156.
41. Foster AP, Naylor RD, Howie NM, Nicholas RA, Ayling RD. 2009. Mycoplasma bovis and otitis in dairy calves in the United Kingdom. The veterinary journal 179:455-457.
42. Hananeh WM, Al Momani WM, Ababneh MM, Abutarbush SM. 2018. Mycoplasma bovis arthritis and pneumonia in calves in Jordan: An emerging disease. Veterinary World 11:1663.
43. Kanda T, Tanaka S, Suwanruengsri M, Sukmawinata E, Uemura R, Yamaguchi R, Sueyoshi M. 2019. Bovine endocarditis associated with Mycoplasma bovis. Journal of comparative pathology 171:53-58.
44. Krysak DE. 2006. Chronic pneumonia and polyarthritis syndrome in a feedlot calf. The Canadian Veterinary Journal 47:1019.
45. Boukahil I, Czuprynski CJ. 2016. Mannheimia haemolytica biofilm formation on bovine respiratory epithelial cells. Veterinary microbiology 197:129-136.
46. Boukahil I, Czuprynski CJ. 2018. Mutual antagonism between Mannheimia haemolytica and Pasteurella multocida when forming a biofilm on bovine bronchial epithelial cells in vitro. Veterinary microbiology 216:218-222.
47. Cusack P, McMeniman N, Lean I. 2003. The medicine and epidemiology of bovine respiratory disease in feedlots. Australian Veterinary Journal 81:480-487.
48. Davis-Unger J, Schwartzkopf-Genswein KS, Pajor EA, Hendrick S, Marti S, Dorin C, Orsel K. 2019. Prevalence and lameness-associated risk factors in Alberta feedlot cattle. Translational Animal Science 3:595-606.
49. Pollock C, Campbell J, Janzen E, West K. Descriptive epidemiology of chronic disease of calves in a western Canadian feedlot, p 152-153. *In* (ed),
50. Smith RA. 1998. Impact of disease on feedlot performance: a review. Journal of Animal Science 76:272-274.
51. Gay E, Barnouin J. 2009. A nation-wide epidemiological study of acute bovine respiratory disease in France. Preventive Veterinary Medicine 89:265-271.
52. Irwin M, MR I, JD C, GE W. 1979. Bovine respiratory disease complex: a comparison of potential predisposing and etiologic factors in Australia and the United States.
53. Pardon B, De Bleecker K, Hostens M, Callens J, Dewulf J, Deprez P. 2012. Longitudinal study on morbidity and mortality in white veal calves in Belgium. BMC Veterinary Research 8:1-15.
54. Leruste H, Brscic M, Heutinck L, Visser E, Wolthuis-Fillerup M, Bokkers E, Stockhofe-Zurwieden N, Cozzi G, Gottardo F, Lensink B. 2012. The relationship between clinical signs of respiratory system disorders and lung lesions at slaughter in veal calves. Preventive Veterinary Medicine 105:93-100.
55. Loan RW, Tung H-t, Payne JB. 1998. Comparative efficacy and duration of immunity of commercial Pasteurella haemolytica vaccines. The Bovine Practitioner:18-21.
56. Taylor JD, Fulton RW, Lehenbauer TW, Step DL, Confer AW. 2010. The epidemiology of bovine respiratory disease: what is the evidence for preventive measures? The Canadian Veterinary Journal 51:1351.
57. Capik SF, Moberly HK, Larson RL. 2021. Systematic review of vaccine efficacy against Mannheimia haemolytica, Pasteurella multocida, and Histophilus somni in North American cattle. The Bovine Practitioner 55:125-133.

58. Van Donkersgoed J, Schumann FJ, Harland RJ, Potter AA, Janzen ED. 1993. The effect of route and dosage of immunization on the serological response to a Pasteurella haemolytica and Haemophilus somnus vaccine in feedlot calves. The Canadian Veterinary Journal 34:731.
59. Baptiste KE, Kyvsgaard NC. 2017. Do antimicrobial mass medications work? A systematic review and meta-analysis of randomised clinical trials investigating antimicrobial prophylaxis or metaphylaxis against naturally occurring bovine respiratory disease. Pathogens and disease 75.
60. Government of Canada. 2020. Canadian Integrated Program for Antimicrobial Resistance Surveillance (CIPARS) 2018: Integrated Findings. PHAC, Geulph, Onataio.
61. Ebrahim M, Gravel D, Thabet C, Abdesselam K, Paramalingam S, Hyson C. 2016. Anitmicrobial Resistance (AMR): Antimicrobial use and antimicrobial resistance trends in Canada: 2014. Canada Communicable Disease Report 42:227.
62. Chopra I, Hawkey PM, Hinton M. 1992. Tetracyclines, molecular and clinical aspects. Journal of Antimicrobial Chemotherapy 29:245-277.
63. Chopra I, Roberts M. 2001. Tetracycline antibiotics: mode of action, applications, molecular biology, and epidemiology of bacterial resistance. Microbiology and molecular biology reviews 65:232-260.
64. Vázquez-Laslop N, Mankin AS. 2018. How macrolide antibiotics work. Trends in biochemical sciences 43:668-684.
65. Cox S, McLaughlin C, Fielder A, Yancey M, Bowersock T, Garcia-Tapia D, Bryson L, Lucas M, Robinson J, Nanjiani I. 2010. Rapid and prolonged distribution of tulathromycin into lung homogenate and pulmonary epithelial lining fluid of Holstein calves following a single subcutaneous administration of 2.5 mg/kg body weight. International Journal of Applied Research in Veterinary Medicine 8.
66. Villarino N, Brown SA, Martín-Jiménez T. 2013. The role of the macrolide tulathromycin in veterinary medicine. The Veterinary Journal 198:352-357.
67. United States Food and Drug Administration. 2021. FDA Approves First Generic Tulathromycin Products for Treating Disease in Certain Cattle and Swine.
68. Government of Canada. Drug Product Database.
69. Granja RH, de Lima AC, Patel RK, Salerno AG, Wanschel AC. 2012. Monitoring of florfenicol residues in fish muscle by HPLC‐UV with confirmation of suspect results by LC‐MS/MS. Drug Testing and Analysis 4:125-129.
70. Gilmore A. 1986. Chloramphenicol and the politics of health. CMAJ: Canadian Medical Association Journal 134:423.
71. Government of Canada. 2013. Product information NUFLOR DIN:02216558. Heatlh Canada, https://health-products.canada.ca/dpd-bdpp/info?lang=eng&code=43184#fn1.
72. Schering-Plough. 1996. NADA 141-063 Nuflor InjectableSolution, FDA approval document.
73. Kehrenberg C, Schwarz S, Jacobsen L, Hansen LH, Vester B. 2005. A new mechanism for chloramphenicol, florfenicol and clindamycin resistance: methylation of 23S ribosomal RNA at A2503. Molecular Microbiology 57:1064-1073.
74. Lobell RD, Varma KJ, Johnson JC, Sams RA, Gerken DF, Ashcraft SM. 1994. Pharmacokinetics of florfenicol following intravenous and intramuscular doses to cattle. Journal of Veterinary Pharmacology and Therapeutics 17:253-258.
75. Catry B, Duchateau L, Van De Ven J, Laevens H, Opsomer G, Haesebrouck F, De Kruif A. 2008. Efficacy of metaphylactic florfenicol therapy during natural outbreaks of bovine respiratory disease. Journal of Veterinary Pharmacology and Therapeutics 31:479-487.
76. White DG, Hudson C, Maurer JJ, Ayers S, Zhao S, Lee MD, Bolton L, Foley T, Sherwood J. 2000. Characterization of Chloramphenicol and Florfenicol Resistance in Escherichia coli Associated with Bovine Diarrhea. Journal of Clinical Microbiology 38:4593-4598.
77. Blondeau JM. 2004. Fluoroquinolones: mechanism of action, classification, and development of resistance. Survey of ophthalmology 49:S73-S78.

78. Grohe K, Zeiler H-J, Metzger KG. 1987. 7-amino-1-cyclopropyl-4-oxo-1, 4-dihydro-quinoline-and naphthyridine-3-carboxylic acids and antibacterial agents containing these compounds. Google Patents.
79. Wright DH, Brown GH, Peterson ML, Rotschafer JC. 2000. Application of fluoroquinolone pharmacodynamics. Journal of Antimicrobial Chemotherapy 46:669-683.
80. Robb EJ, Tucker CM, Corley L, Bryson WL, Rogers KC, Sturgess K, Bade DJ, Brodersen B. 2007. Efficacy of tulathromycin versus enrofloxacin for initial treatment of naturally occurring bovine respiratory disease in feeder calves. Veterinary Therapeutics 8:127.
81. Trouchon T, Lefebvre S. 2016. A review of enrofloxacin for veterinary use. Open Journal of Veterinary Medicine 6:40-58.
82. O'Neill J. 2016. Tackling drug-resistant infections globally: final report and recommendations.
83. Murray CJ, Ikuta KS, Sharara F, Swetschinski L, Aguilar GR, Gray A, Han C, Bisignano C, Rao P, Wool E. 2022. Global burden of bacterial antimicrobial resistance in 2019: a systematic analysis. The Lancet 399:629-655.
84. Thorpe HA, Booton R, Kallonen T, Gibbon MJ, Couto N, Passet V, López-Fernández S, Rodrigues C, Matthews L, Mitchell S. 2022. A large-scale genomic snapshot of Klebsiella spp. isolates in Northern Italy reveals limited transmission between clinical and non-clinical settings. Nature Microbiology 7:2054-2067.
85. Dhindwal P, Thompson C, Kos D, Planedin K, Jain R, Jelinski M, Ruzzini A. 2023. A neglected and emerging antimicrobial resistance gene encodes for a serine-dependent macrolide esterase. Proceedings of the National Academy of Sciences 120:e2219827120.
86. Richardson EJ, Bacigalupe R, Harrison EM, Weinert LA, Lycett S, Vrieling M, Robb K, Hoskisson PA, Holden MT, Feil EJ. 2018. Gene exchange drives the ecological success of a multi-host bacterial pathogen. Nature ecology & evolution 2:1468-1478.
87. Ward M, Gibbons C, McAdam P, Van Bunnik B, Girvan E, Edwards G, Fitzgerald JR, Woolhouse M. 2014. Time-scaled evolutionary analysis of the transmission and antibiotic resistance dynamics of Staphylococcus aureus clonal complex 398. Applied and environmental microbiology 80:7275-7282.
88. Sefton AM. 2002. Mechanisms of antimicrobial resistance. Drugs 62:557-566.
89. Munita JM, Arias CA. 2016. Mechanisms of antibiotic resistance. Microbiology spectrum 4:4.2. 15.
90. Gautier-Bouchardon AV. 2018. Antimicrobial resistance in Mycoplasma spp. Microbiology spectrum 6:6.4. 07.
91. Viana AT, Caetano T, Covas C, Santos T, Mendo S. 2018. Environmental superbugs: The case study of Pedobacter spp. Environmental Pollution 241:1048-1055.
92. Benedict KM, Gow SP, McAllister TA, Booker CW, Hannon SJ, Checkley SL, Noyes NR, Morley PS. 2015. Antimicrobial resistance in Escherichia coli recovered from feedlot cattle and associations with antimicrobial use. PloS one 10:e0143995.
93. Andrés-Lasheras S, Ha R, Zaheer R, Lee C, Booker CW, Dorin C, Van Donkersgoed J, Deardon R, Gow S, Hannon SJ. 2021. Prevalence and risk factors associated with antimicrobial resistance in bacteria related to bovine respiratory disease—a broad cross-sectional study of beef cattle at entry into Canadian feedlots. Frontiers in veterinary science 8:710.
94. Zaheer R, Cook SR, Barbieri R, Goji N, Cameron A, Petkau A, Polo RO, Tymensen L, Stamm C, Song J. 2020. Surveillance of Enterococcus spp. reveals distinct species and antimicrobial resistance diversity across a One-Health continuum. Scientific reports 10:1-16.
95. Checkley SL, Campbell JR, Chirino-Trejo M, Janzen ED, Waldner CL. 2010. Associations between antimicrobial use and the prevalence of antimicrobial resistance in fecal Escherichia coli from feedlot cattle in western Canada. The Canadian Veterinary Journal 51:853.
96. Checkley SL, Campbell JR, Chirino-Trejo M, Janzen ED, McKinnon JJ. 2008. Antimicrobial resistance in generic fecal Escherichia coli obtained from beef cattle on arrival at the feedlot and

prior to slaughter, and associations with volume of total individual cattle antimicrobial treatments in one western Canadian feedlot. Canadian Journal of Veterinary Research 72:101.
97. Doster E, Rovira P, Noyes NR, Burgess BA, Yang X, Weinroth MD, Lakin SM, Dean CJ, Linke L, Magnuson R. 2018. Investigating effects of tulathromycin metaphylaxis on the fecal resistome and microbiome of commercial feedlot cattle early in the feeding period. Frontiers in microbiology 9:1715.
98. Huebner KL, Martin JN, Weissend CJ, Holzer KL, Parker JK, Lakin SM, Doster E, Weinroth MD, Abdo Z, Woerner DR. 2019. Effects of a Saccharomyces cerevisiae fermentation product on liver abscesses, fecal microbiome, and resistome in feedlot cattle raised without antibiotics. Scientific reports 9:1-11.
99. Noyes NR, Yang X, Linke LM, Magnuson RJ, Cook SR, Zaheer R, Yang H, Woerner DR, Geornaras I, McArt JA. 2016. Characterization of the resistome in manure, soil and wastewater from dairy and beef production systems. Scientific reports 6:1-12.
100. McMullen C, Orsel K, Alexander TW, van der Meer F, Plastow G, Timsit E. 2019. Comparison of the nasopharyngeal bacterial microbiota of beef calves raised without the use of antimicrobials between healthy calves and those diagnosed with bovine respiratory disease. Veterinary microbiology 231:56-62.
101. Holman DB, Yang W, Alexander TW. 2019. Antibiotic treatment in feedlot cattle: a longitudinal study of the effect of oxytetracycline and tulathromycin on the fecal and nasopharyngeal microbiota. Microbiome 7:1-14.
102. Liguori K, Keenum I, Davis BC, Calarco J, Milligan E, Harwood VJ, Pruden A. 2022. Antimicrobial resistance monitoring of water environments: a framework for standardized methods and quality control. Environmental science & technology 56:9149-9160.
103. Owen JR, Noyes N, Young AE, Prince DJ, Blanchard PC, Lehenbauer TW, Aly SS, Davis JH, O'Rourke SM, Abdo Z. 2017. Whole-genome sequencing and concordance between antimicrobial susceptibility genotypes and phenotypes of bacterial isolates associated with bovine respiratory disease. G3: Genes, Genomes, Genetics 7:3059-3071.
104. Balouiri M, Sadiki M, Ibnsouda SK. 2016. Methods for in vitro evaluating antimicrobial activity: A review. Journal of pharmaceutical analysis 6:71-79.
105. Wright GD. 2010. The antibiotic resistome. Expert opinion on drug discovery 5:779-788.
106. Bhullar K, Waglechner N, Pawlowski A, Koteva K, Banks ED, Johnston MD, Barton HA, Wright GD. 2012. Antibiotic resistance is prevalent in an isolated cave microbiome. PloS one 7:e34953.
107. Sakkas H, Bozidis P, Ilia A, Mpekoulis G, Papadopoulou C. 2019. Antimicrobial resistance in bacterial pathogens and detection of carbapenemases in Klebsiella pneumoniae isolates from hospital wastewater. Antibiotics 8:85.
108. Czatzkowska M, Wolak I, Harnisz M, Korzeniewska E. 2022. Impact of Anthropogenic Activities on the Dissemination of ARGs in the Environment—A Review. International Journal of Environmental Research and Public Health 19:12853.
109. Call DR, Matthews L, Subbiah M, Liu J. 2013. Do antibiotic residues in soils play a role in amplification and transmission of antibiotic resistant bacteria in cattle populations? Frontiers in Microbiology 4:193.
110. Halleran JL, Minch R, Slyvester HJ, Jacob ME, Prange T, Baynes R, Foster DM. 2021. Comparison of the Intestinal Pharmacokinetics of Two Different Florfenicol Dosing Regimens and Its Impact on the Prevalence and Phenotypic Resistance of E. coli and Enterococcus over Time. Microorganisms 9:1835.
111. George A. 2019. Antimicrobial resistance (AMR) in the food chain: trade, one health and codex. Tropical medicine and infectious disease 4:54.
112. Beukers AG, Zaheer R, Cook SR, Chaves AV, Ward MP, Tymensen L, Morley PS, Hannon S, Booker CW, Read RR. 2018. Comparison of antimicrobial resistance genes in feedlots and urban wastewater. Canadian Journal of Veterinary Research 82:24-38.

113. Zhang H, Zhou Y, Huang Y, Wu L, Liu X, Luo Y. 2016. Residues and risks of veterinary antibiotics in protected vegetable soils following application of different manures. Chemosphere 152:229-237.
114. Huygens J, Daeseleire E, Mahillon J, Van Elst D, Decrop J, Meirlaen J, Dewulf J, Heyndrickx M, Rasschaert G. 2021. Presence of antibiotic residues and antibiotic resistant bacteria in cattle manure intended for fertilization of agricultural fields: a one health perspective. Antibiotics 10:410.
115. Ahmad I, Malak HA, Abulreesh HH. 2021. Environmental antimicrobial resistance and its drivers: A potential threat to public health. Journal of Global Antimicrobial Resistance 27:101-111.
116. Swift BM, Bennett M, Waller K, Dodd C, Murray A, Gomes RL, Humphreys B, Hobman JL, Jones MA, Whitlock SE. 2019. Anthropogenic environmental drivers of antimicrobial resistance in wildlife. Science of the Total Environment 649:12-20.
117. Levin BR, Perrot V, Walker N. 2000. Compensatory mutations, antibiotic resistance and the population genetics of adaptive evolution in bacteria. Genetics 154:985-997.
118. Berendsen BJ, Wegh RS, Memelink J, Zuidema T, Stolker LA. 2015. The analysis of animal faeces as a tool to monitor antibiotic usage. Talanta 132:258-268.
119. Sommer MO. 2014. Barriers to the spread of resistance. Nature 509:567-568.
120. Jiang X, Ellabaan MMH, Charusanti P, Munck C, Blin K, Tong Y, Weber T, Sommer MO, Lee SY. 2017. Dissemination of antibiotic resistance genes from antibiotic producers to pathogens. Nature communications 8:15784.
121. Von Wintersdorff CJ, Penders J, Van Niekerk JM, Mills ND, Majumder S, Van Alphen LB, Savelkoul PH, Wolffs PF. 2016. Dissemination of antimicrobial resistance in microbial ecosystems through horizontal gene transfer. Frontiers in microbiology 7:173.
122. Wozniak RA, Waldor MK. 2010. Integrative and conjugative elements: mosaic mobile genetic elements enabling dynamic lateral gene flow. Nature Reviews Microbiology 8:552-563.
123. Prudhomme M, Attaiech L, Sanchez G, Martin B, Claverys J-P. 2006. Antibiotic stress induces genetic transformability in the human pathogen Streptococcus pneumoniae. Science 313:89-92.
124. Wyres KL, Holt KE. 2018. Klebsiella pneumoniae as a key trafficker of drug resistance genes from environmental to clinically important bacteria. Current opinion in microbiology 45:131-139.
125. Graham DW, Bergeron G, Bourassa MW, Dickson J, Gomes F, Howe A, Kahn LH, Morley PS, Scott HM, Simjee S. 2019. Complexities in understanding antimicrobial resistance across domesticated animal, human, and environmental systems. Annals of the New York Academy of Sciences 1441:17-30.
126. Sutcliffe JA, Leclercq R. 2002. Mechanisms of resistance to macrolides, lincosamides, and ketolides, p 281-317, Macrolide antibiotics. Springer.
127. Weisblum B. 1995. Erythromycin resistance by ribosome modification. Antimicrobial agents and chemotherapy 39:577-585.
128. Halula MC, Manning S, Macrina FL. 1991. Nucleotide sequence of ermFU, a macrolide-lincosamide-streptogramin (MLS) resistance gene encoding an RNA methylase from the conjugal element of Bacteroides fragilis V503. Nucleic acids research 19:3453.
129. Yu L, Petros AM, Schnuchel A, Zhong P, Severin JM, Walter K, Holzman TF, Fesik SW. 1997. Solution structure of an rRNA methyltransferase (ErmAM) that confers macrolide-lincosamide-streptogramin antibiotic resistance. Nature structural biology 4:483-489.
130. Kadlec K, Brenner Michael G, Sweeney MT, Brzuszkiewicz E, Liesegang H, Daniel R, Watts JL, Schwarz S. 2011. Molecular basis of macrolide, triamilide, and lincosamide resistance in Pasteurella multocida from bovine respiratory disease. Antimicrobial Agents and Chemotherapy 55:2475-2477.
131. Zieliński M, Park J, Sleno B, Berghuis AM. 2021. Structural and functional insights into esterase-mediated macrolide resistance. Nature communications 12:1-9.
132. Hopkins KL, Davies RH, Threlfall EJ. 2005. Mechanisms of quinolone resistance in Escherichia coli and Salmonella: recent developments. International journal of antimicrobial agents 25:358-373.

133. Huguet A, Pensec J, Soumet C. 2013. Resistance in Escherichia coli: variable contribution of efflux pumps with respect to different fluoroquinolones. Journal of applied microbiology 114:1294-1299.
134. Alcock BP, Raphenya AR, Lau TT, Tsang KK, Bouchard M, Edalatmand A, Huynh W, Nguyen A-LV, Cheng AA, Liu S. 2020. CARD 2020: antibiotic resistome surveillance with the comprehensive antibiotic resistance database. Nucleic acids research 48:D517-D525.
135. Antonelli A, D'Andrea MM, Brenciani A, Galeotti CL, Morroni G, Pollini S, Varaldo PE, Rossolini GM. 2018. Characterization of poxtA, a novel phenicol–oxazolidinone–tetracycline resistance gene from an MRSA of clinical origin. Journal of Antimicrobial Chemotherapy 73:1763-1769.
136. Doublet B, Schwarz S, Kehrenberg C, Cloeckaert A. 2005. Florfenicol resistance gene floR is part of a novel transposon. Antimicrobial agents and chemotherapy 49:2106-2108.
137. Cloeckaert A, Baucheron S, Flaujac G, Schwarz S, Kehrenberg C, Martel J-L, Chaslus-Dancla E. 2000. Plasmid-mediated florfenicol resistance encoded by the floR gene in Escherichia coli isolated from cattle. Antimicrobial Agents and Chemotherapy 44:2858-2860.
138. Yang W, Moore IF, Koteva KP, Bareich DC, Hughes DW, Wright GD. 2004. TetX is a flavin-dependent monooxygenase conferring resistance to tetracycline antibiotics. Journal of Biological Chemistry 279:52346-52352.
139. Roberts MC. 2005. Update on acquired tetracycline resistance genes. FEMS microbiology letters 245:195-203.
140. Katsuda K, Kohmoto M, Mikami O, Uchida I. 2009. Antimicrobial resistance and genetic characterization of fluoroquinolone-resistant Mannheimia haemolytica isolates from cattle with bovine pneumonia. Veterinary microbiology 139:74-79.
141. Portis E, Lindeman C, Johansen L, Stoltman G. 2012. A ten-year (2000–2009) study of antimicrobial susceptibility of bacteria that cause bovine respiratory disease complex—Mannheimia haemolytica, Pasteurella multocida, and Histophilus somni—in the United States and Canada. Journal of Veterinary Diagnostic Investigation 24:932-944.
142. Kinnear A, McAllister TA, Zaheer R, Waldner M, Ruzzini AC, Andrés-Lasheras S, Parker S, Hill JE, Jelinski MD. 2020. Investigation of macrolide resistance genotypes in Mycoplasma bovis isolates from Canadian feedlot cattle. Pathogens 9:622.
143. Hata E, Harada T, Itoh M. 2019. Relationship between antimicrobial susceptibility and multilocus sequence type of Mycoplasma bovis isolates and development of a method for rapid detection of point mutations involved in decreased susceptibility to macrolides, lincosamides, tetracyclines, and spectinomycin. Applied and Environmental Microbiology 85:e00575-19.
144. Kroemer S, Galland D, Guérin‐Faublée V, Giboin H, Woehrlé‐Fontaine F. 2012. Survey of marbofloxacin susceptibility of bacteria isolated from cattle with respiratory disease and mastitis in Europe. Veterinary Record 170:53-53.
145. Stanford K, Zaheer R, Klima C, McAllister T, Peters D, Niu YD, Ralston B. 2020. Antimicrobial resistance in members of the bacterial bovine respiratory disease complex isolated from lung tissue of cattle mortalities managed with or without the use of antimicrobials. Microorganisms 8:288.
146. Zhang J, Wall SK, Xu L, Ebner PD. 2010. Contamination rates and antimicrobial resistance in bacteria isolated from "grass-fed" labeled beef products. Foodborne Pathogens and Disease 7:1331-1336.
147. Hay K, Morton J, Clements A, Mahony T, Barnes T. 2016. Associations between feedlot management practices and bovine respiratory disease in Australian feedlot cattle. Preventive veterinary medicine 128:23-32.
148. Burriel AR. 1997. News & Notes: isolation of Pasteurella haemolytica from grass, drinking water, and straw bedding used by sheep. Current microbiology 35:316-318.
149. Noyes NR, Yang X, Linke LM, Magnuson RJ, Dettenwanger A, Cook S, Geornaras I, Woerner DE, Gow SP, McAllister TA. 2016. Resistome diversity in cattle and the environment decreases during beef production. Elife 5:e13195.
150. Mosier D. 2014. Review of BRD pathogenesis: the old and the new. Anim Health Res Rev 15:166-8.

151. O'Connor AM, Hu D, Totton SC, Scott N, Winder CB, Wang B, Wang C, Glanville J, Wood H, White B. 2019. A systematic review and network meta-analysis of injectable antibiotic options for the control of bovine respiratory disease in the first 45 days post arrival at the feedlot. Animal health research reviews 20:163-181.
152. O'connor A, Hu D, Totton S, Scott N, Winder C, Wang B, Wang C, Glanville J, Wood H, White B. 2019. A systematic review and network meta-analysis of bacterial and viral vaccines, administered at or near arrival at the feedlot, for control of bovine respiratory disease in beef cattle. Animal health research reviews 20:143-162.
153. Zhang Y, Hao X, Thomas BW, McAllister TA, Workentine M, Jin L, Shi X, Alexander TW. 2023. Soil antibiotic resistance genes accumulate at different rates over four decades of manure application. Journal of Hazardous Materials 443:130136.
154. Zaheer R, Lakin SM, Polo RO, Cook SR, Larney FJ, Morley PS, Booker CW, Hannon SJ, Van Domselaar G, Read RR. 2019. Comparative diversity of microbiomes and Resistomes in beef feedlots, downstream environments and urban sewage influent. Bmc Microbiology 19:1-17.
155. Adator EH, Narvaez-Bravo C, Zaheer R, Cook SR, Tymensen L, Hannon SJ, Booker CW, Church D, Read RR, McAllister TA. 2020. A One Health comparative assessment of antimicrobial resistance in generic and extended-spectrum cephalosporin-resistant Escherichia coli from beef production, sewage and clinical settings. Microorganisms 8:885.
156. Agga GE, Arthur TM, Durso LM, Harhay DM, Schmidt JW. 2015. Antimicrobial-resistant bacterial populations and antimicrobial resistance genes obtained from environments impacted by livestock and municipal waste. PLoS One 10:e0132586.
157. Maier G, Love W, Karle B, Dubrovsky S, Williams D, Champagne J, Anderson R, Rowe J, Lehenbauer T, Van Eenennaam A. 2019. Management factors associated with bovine respiratory disease in preweaned calves on California dairies: The BRD 100 study. Journal of dairy science 102:7288-7305.
158. Bolyen E, Rideout JR, Dillon MR, Bokulich NA, Abnet CC, Al-Ghalith GA, Alexander H, Alm EJ, Arumugam M, Asnicar F. 2019. Reproducible, interactive, scalable and extensible microbiome data science using QIIME 2. Nature biotechnology 37:852-857.
159. Bokulich NA, Kaehler BD, Rideout JR, Dillon M, Bolyen E, Knight R, Huttley GA, Gregory Caporaso J. 2018. Optimizing taxonomic classification of marker-gene amplicon sequences with QIIME 2's q2-feature-classifier plugin. Microbiome 6:1-17.
160. Robeson MS, O'Rourke DR, Kaehler BD, Ziemski M, Dillon MR, Foster JT, Bokulich NA. 2021. RESCRIPt: Reproducible sequence taxonomy reference database management. PLoS computational biology 17:e1009581.
161. Bisanz JE. 2018. qiime2R: Importing QIIME2 artifacts and associated data into R sessions.
162. McMurdie PJ, Holmes S. 2013. phyloseq: an R package for reproducible interactive analysis and graphics of microbiome census data. PloS one 8:e61217.
163. Barnett DJ, Arts IC, Penders J. 2021. microViz: an R package for microbiome data visualization and statistics. Journal of Open Source Software 6:3201.
164. Cao Y, Dong Q, Wang D, Zhang P, Liu Y, Niu C. 2022. microbiomeMarker: an R/Bioconductor package for microbiome marker identification and visualization. Bioinformatics 38:4027-4029.
165. Cheng H, Concepcion GT, Feng X, Zhang H, Li H. 2021. Haplotype-resolved de novo assembly using phased assembly graphs with hifiasm. Nature methods 18:170-175.
166. Seemann T. 2016. ABRicate: mass screening of contigs for antibiotic resistance genes. Github.
167. Alcock BP, Huynh W, Chalil R, Smith KW, Raphenya AR, Wlodarski MA, Edalatmand A, Petkau A, Syed SA, Tsang KK. 2023. CARD 2023: expanded curation, support for machine learning, and resistome prediction at the Comprehensive Antibiotic Resistance Database. Nucleic Acids Research 51:D690-D699.
168. Li D, Liu C-M, Luo R, Sadakane K, 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.

169. Tamames J, Puente-Sánchez F. 2019. SqueezeMeta, a highly portable, fully automatic metagenomic analysis pipeline. Frontiers in microbiology 9:3349.
170. Schmieder R, Edwards R. 2011. Quality control and preprocessing of metagenomic datasets. Bioinformatics 27:863-864.
171. Pérez-Silva JG, Araujo-Voces M, Quesada V. 2018. nVenn: generalized, quasi-proportional Venn and Euler diagrams. Bioinformatics 34:2322-2324.
172. Lupo A, Haenni M, Madec J-Y. 2018. Antimicrobial resistance in Acinetobacter spp. and Pseudomonas spp. Microbiology spectrum 6:6.3. 01.
173. McMullen C, Alexander TW, Orsel K, Timsit E. 2020. Progression of nasopharyngeal and tracheal bacterial microbiotas of feedlot cattle during development of bovine respiratory disease. Veterinary Microbiology 248:108826.
174. Che Y, Yang Y, Xu X, Brinda K, Polz MF, Hanage WP, Zhang T. 2021. Conjugative plasmids interact with insertion sequences to shape the horizontal transfer of antimicrobial resistance genes. Proc Natl Acad Sci U S A 118.
175. de Nies L, Busi SB, Kunath BJ, May P, Wilmes P. 2022. Mobilome-driven segregation of the resistome in biological wastewater treatment. Elife 11:e81196.
176. Klima CL, Zaheer R, Cook SR, Booker CW, Hendrick S, Alexander TW, McAllister TA. 2014. Pathogens of bovine respiratory disease in North American feedlots conferring multidrug resistance via integrative conjugative elements. Journal of Clinical Microbiology 52:438-448.
177. Luiken RE, Van Gompel L, Bossers A, Munk P, Joosten P, Hansen RB, Knudsen BE, García-Cobos S, Dewulf J, Aarestrup FM. 2020. Farm dust resistomes and bacterial microbiomes in European poultry and pig farms. Environment international 143:105971.
178. Hua M, Huang W, Chen A, Rehmet M, Jin C, Huang Z. 2020. Comparison of antimicrobial resistance detected in environmental and clinical isolates from historical data for the US. BioMed research international 2020.
179. Jolley K, Bray J, Maiden M. 2018. Open-access bacterial population genomics: BIGSdb software, the PubMLST. org website and their applications. Wellcome open research 3.
180. Aziz RK, Bartels D, Best AA, DeJongh M, Disz T, Edwards RA, Formsma K, Gerdes S, Glass EM, Kubal M. 2008. The RAST Server: rapid annotations using subsystems technology. BMC genomics 9:1-15.
181. Overbeek R, Olson R, Pusch GD, Olsen GJ, Davis JJ, Disz T, Edwards RA, Gerdes S, Parrello B, Shukla M. 2014. The SEED and the Rapid Annotation of microbial genomes using Subsystems Technology (RAST). Nucleic acids research 42:D206-D214.
182. Brettin T, Davis JJ, Disz T, Edwards RA, Gerdes S, Olsen GJ, Olson R, Overbeek R, Parrello B, Pusch GD. 2015. RASTtk: a modular and extensible implementation of the RAST algorithm for building custom annotation pipelines and annotating batches of genomes. Scientific reports 5:1-6.
183. Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ. 1990. Basic local alignment search tool. Journal of molecular biology 215:403-410.
184. Wheeler DL, Barrett T, Benson DA, Bryant SH, Canese K, Chetvernin V, Church DM, DiCuccio M, Edgar R, Federhen S. 2007. Database resources of the national center for biotechnology information. Nucleic acids research 36:D13-D21.
185. Zhang Z, Schwartz S, Wagner L, Miller W. 2000. A greedy algorithm for aligning DNA sequences. Journal of Computational biology 7:203-214.
186. Puente-Sánchez F, García-García N, Tamames J. 2020. SQMtools: automated processing and visual analysis of'omics data with R and anvi'o. BMC bioinformatics 21:1-11.
187. Underwood A. 2022. Contig_tools. https://gitlab.com/antunderwood/contig_tools. Accessed 2023-06-14.
188. Telatin A, Fariselli P, Birolo G. 2021. Seqfu: a suite of utilities for the robust and reproducible manipulation of sequence files. Bioengineering 8:59.
189. Bushnell B. 2014. BBTools software packag. e.

190. Shin SW, Shin MK, Jung M, Belaynehe KM, Yoo HS. 2015. Prevalence of antimicrobial resistance and transfer of tetracycline resistance genes in Escherichia coli isolates from beef cattle. Applied and environmental microbiology 81:5560-5566.
191. Cameron A, McAllister TA. 2016. Antimicrobial usage and resistance in beef production. Journal of Animal Science and Biotechnology 7:1-22.
192. Rehman MA, Yin X, Zaheer R, Goji N, Amoako KK, McAllister T, Pritchard J, Topp E, Diarra MS. 2018. Genotypes and phenotypes of Enterococci isolated from broiler chickens. Frontiers in Sustainable Food Systems 2:83.
193. Inglis G, Morck D, McAllister T, Entz T, Olson M, Yanke L, Read R. 2006. Temporal prevalence of antimicrobial resistance in Campylobacter spp. from beef cattle in Alberta feedlots. Applied and environmental microbiology 72:4088-4095.
194. Romanenko LA, Lysenko AM, Rohde M, Mikhailov VV, Stackebrandt E. 2004. Psychrobacter maritimus sp. nov. and Psychrobacter arenosus sp. nov., isolated from coastal sea ice and sediments of the Sea of Japan. International Journal of Systematic and Evolutionary Microbiology 54:1741-1745.
195. Nemec A, Radolfová-Křížová L, Maixnerová M, Nemec M, Španělová P, Šafránková R, Šedo O, Lopes BS, Higgins PG. 2021. Delineation of a novel environmental phylogroup of the genus Acinetobacter encompassing Acinetobacter terrae sp. nov., Acinetobacter terrestris sp. nov. and three other tentative species. Systematic and Applied Microbiology 44:126217.
196. Pawlowski AC, Stogios PJ, Koteva K, Skarina T, Evdokimova E, Savchenko A, Wright GD. 2018. The evolution of substrate discrimination in macrolide antibiotic resistance enzymes. Nature Communications 9:112.
197. Leavitt S, Ayroud M. 1997. Riemerella anatipestifer infection of domestic ducklings. The Canadian Veterinary Journal 38:113.
198. Willms WD, Kenzie OR, McAllister TA, Colwell D, Veira D, Wilmshurst JF, Entz T, Olson ME. 2002. Effects of water quality on cattle performance. Rangeland Ecology & Management/Journal of Range Management Archives 55:452-460.
199. Bergstrom CT, Lo M, Lipsitch M. 2004. Ecological theory suggests that antimicrobial cycling will not reduce antimicrobial resistance in hospitals. Proceedings of the National Academy of Sciences 101:13285-13290.
200. McGowan JE. 1986. Minimizing antimicrobial resistance in hospital bacteria: can switching or cycling drugs help? Infection Control & Hospital Epidemiology 7:573-576.
201. Chatzopoulou M, Reynolds L. 2022. Systematic review of the effects of antimicrobial cycling on bacterial resistance rates within hospital settings. British Journal of Clinical Pharmacology 88:897-910.
202. Vikram A, Rovira P, Agga GE, Arthur TM, Bosilevac JM, Wheeler TL, Morley PS, Belk KE, Schmidt JW. 2017. Impact of “raised without antibiotics” beef cattle production practices on occurrences of antimicrobial resistance. Applied and environmental microbiology 83:e01682-17.
203. Gullberg E, Albrecht LM, Karlsson C, Sandegren L, Andersson DI. 2014. Selection of a multidrug resistance plasmid by sublethal levels of antibiotics and heavy metals. MBio 5:e01918-14.
204. Carattoli A. 2013. Plasmids and the spread of resistance. International journal of medical microbiology 303:298-304.

APPENDIX A – CHAPTER 2: Supplemental Material for Insight into antimicrobial resistance at a new beef cattle feedlot in Western Canada

Table A2.1. Summary of feedlot sampling

				Pen ID*											
Week	Date	Time	Temp	R	T3	T2	T1	A6	A5	A4	A3	B7	B6	B5	B4
0	15/09/21	15:00	12°C	4											
0	12/10/21	11:00	-1°C		1α †	1α †									
1	20/10/21	10:00	-2°C	4	2		2	1†				1†			
2	27/10/21	9:00	1°C		2		2	2	2			2	2		
3	03/11/21	9:30	-3°C		2α		2α	2	2	2		2	2	2	
4	10/11/21	9:30	1°C		2α		2α	2	2	2	2	2	2	2	1†
5	17/11/21	11:00	-8°C		1†			2	2	1††	2	2	2	2	1†
6	24/11/21	9:30	-14°C		2			1†	2	2	2	1†	2	2	2
7	1/12/21	9:30	4°C		1†			2	2	2	1†	2	2	2	2
8	8/12/21	9:30	-5°C		2			2	2	2	1†	2	2	2	2

R: Feedlot reservoir, two separate 4 x 500 mL water samples were pooled for analysis annotated as Week 0 and 1.

T1-T3: transfer pens used to temporarily hold animals (these pens are half the size/half the occupancy as home pens)

A & B: home pens, holding capacity of ~350 animals/pen. Not sampled prior to animal arrival at the feedlot.

*Values under the Pen ID heading refer to the number of samples collected: one water and one swab (2/week).

Filled grey boxes indicate that samples were not collected.

†: Not enough DNA yield from either a water or swab sample; ††: No swab sample collection due to ice

α: Cattle were not present in pen

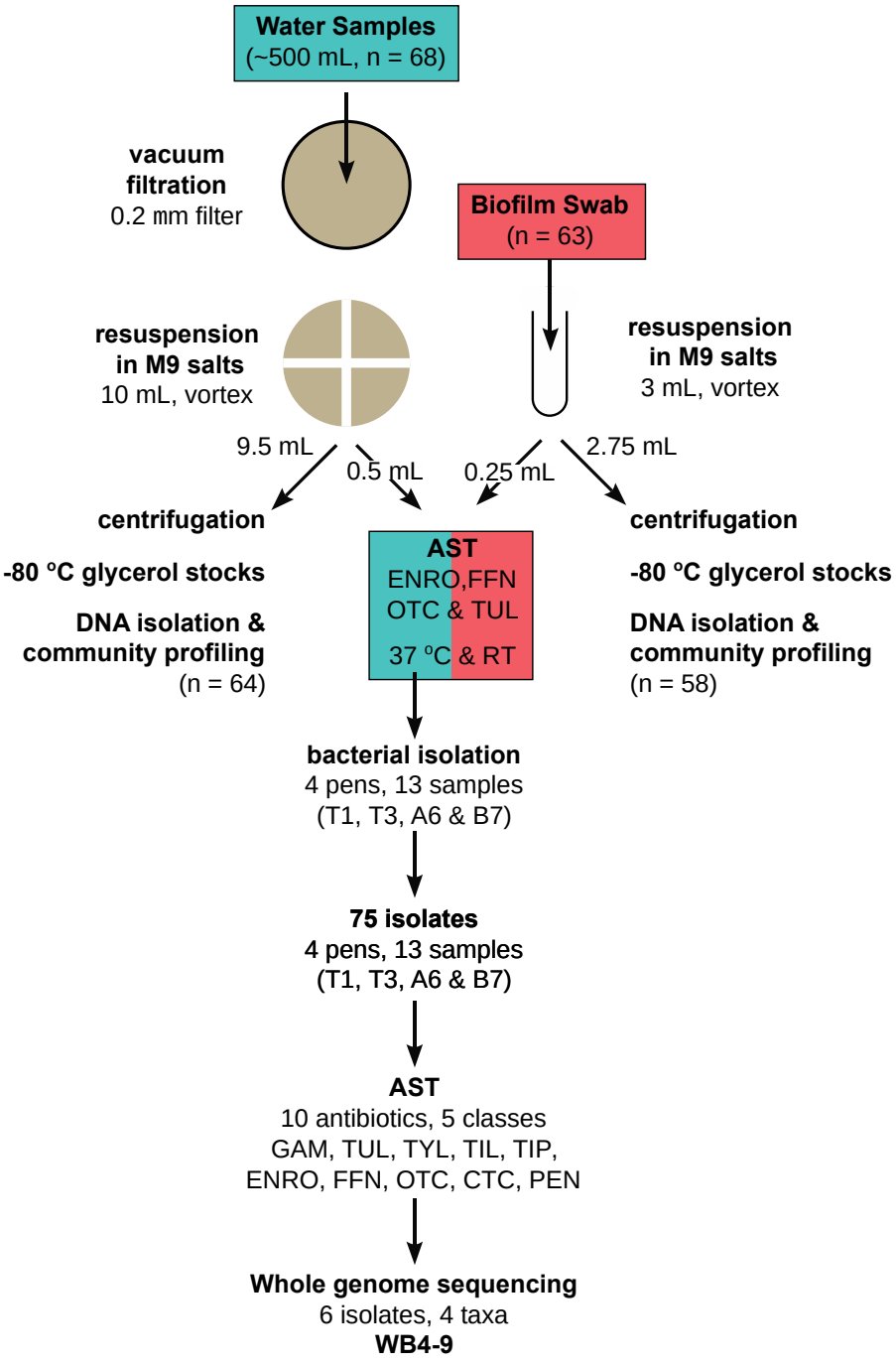

Figure A2.1. Workflow diagram of sample collection and processing of feedlot water bowl samples.

Table A2.2. Summary of 28 bacteria isolated from water bowls for which MICs were measured for 10 distinct antibiotics[a]

		Isolation Conditions			MIC (μg/mL)									
Pen	Week	Drug	Temp	Taxonomic ID[b]	GAM	TUL	TYL	TIL	TIP	ENR	FFN	OTC	CTC	PEN
T3	2	FFN	37 °C	*Acinetobacter baumannii WB4*	2	4	>128	16	2	0.25	256	8	16	>8
B7	5	FFN	RT	*Acinetobacter pseudolwoffii WB5*	>256	128	>128	>256	>128	≤0.12	128	>256	>256	>8
A6	5	FFN	37 °C	*Escherichia coli WB6*	16	32	>128	256	32	32	128	>256	128	>8
T3	8	ENR	RT	*Pedobacter steynii WB7*	8	64	>128	>256	32	128	>256	32	16	>8
B7	8	TUL	RT	*Sphingobacterium faecium WB8*	>256	>256	>128	>256	>128	≤0.12	>256	>256	128	>8
T3	6	TUL	RT	*S. faecium WB9*	>256	>256	>128	>256	>128	0.5	>256	>256	>256	>8
A6	8	FFN	RT	*Aeromonas salmonicida*	1	1	64	16	0.5	≤0.12	64	128	32	>8
A6	8	OTC	37 °C	*Aeromonas salmonicida*	16	64	>128	>256	64	2	>256	64	16	>8
A6	8	TUL	37 °C	*Enterococcus hirae*	>256	>256	>128	>256	>128	8	128	>256	128	>8
T3	1	OTC	37 °C	*Escherichia coli*	32	32	128	256	>128	≤0.12	8	256	64	>8
T3	2	OTC	37 °C	*E. coli*	4	4	>128	64	2	2	8	>256	128	>8
T3	6	OTC	RT	*Myroides odoratimimus*	128	>256	>128	>256	>128	0.25	8	>256	128	>8
A6	1	OTC	37 °C	*Proteus mirabilis*	32	256	>128	>256	>128	≤0.12	4	>256	>256	>8
A6	5	TUL	37 °C	*P. mirabilis*	128	>256	>128	>256	>128	1	16	>256	256	>8
T3	6	TUL	37 °C	*P. mirabilis*	16	128	>128	>256	64	0.25	4	>256	>256	>8
A6	8	OTC	RT	*Pseudomonas gessardii*	>256	>256	>128	>256	>128	2	256	>256	256	>8
B7	8	OTC	RT	*P. gessardii*	>256	>256	>128	>256	>128	2	256	>256	256	>8
T1	4	TUL	RT	*P. gessardii*	>256	>256	>128	>256	>128	1	128	16	4	>8
T3	1	OTC	RT	*P. gessardii*	>256	>256	>128	>256	>128	1	64	64	32	>8
T3	8	OTC	RT	*P. gessardii*	>256	>256	>128	>256	>128	1	256	>256	256	>8
T3	6	FFN	RT	*P. gessardii*	>256	>256	>128	>256	>128	2	>256	16	4	>8
T1	1	ENR	RT	*Pseudomonas proteolytica*	>256	>256	>128	>256	>128	2	256	16	4	>8
T3	1	ENR	RT	*P. proteolytica*	>256	>256	>128	>256	>128	1	128	16	4	>8
T1	1	TUL	RT	*Pseudomonas extremorientalis*	>256	256	>128	>256	>128	1	256	8	4	>8
B7	5	TUL	RT	*Pseudomonas flavescens*	>256	>256	>128	>256	>128	1	>256	8	2	>8
A6	1	FFN	RT	*Pseudomonas protegens*	>256	>256	>128	>256	>128	1	256	16	4	>8
T3	1	FFN	37 °C	*Serratia rubindaea*	8	16	>128	>256	16	2	32	8	16	>8
T3	5	OTC	RT	*Shewanella profunda*	32	32	>128	256	32	1	32	128	32	>8

a – the isolates highlighted in green were prioritized for whole genome sequencing experiment

b – top hits are presented based on at least 400 bp of the 16S rRNA gene sequence

Table A2.3. Alphabetical list of 47 additional water bowl-associated bacteria isolated based on antibiotic resistance

		Isolation Conditions		
Pen	Week	Antibiotic & Temp		Taxonomic ID[a]
T3	8	FFN	37 °C	*Acinetobacter baumannii*
T3	6	FFN	37 °C	*Acinetobacter baumannii*
T1	1	OTC	37 °C	*Acinetobacter indicus*
T3	2	TUL	RT	*Acinetobacter indicus*
B7	3	TUL	RT	*Aerococcus urinaeequi*
T3	2	TUL	RT	*Aerococcus viridans*
T3	8	FFN	RT	*Aeromonas enterica*
A6	8	OTC	RT	*Aeromonas rivipollensis*
A6	8	ENR	RT	*Aeromonas salmonicida*
B7	8	FFN	RT	*Aeromonas salmonicida*
B7	8	OTC	37 °C	*Aeromonas salmonicida*
T3	2	OTC	RT	*Aeromonas salmonicida*
B7	8	TUL	37 °C	*Bacillus haynesii*
T1	4	TUL	37 °C	*Bacillus paralicheniformis*
T1	4	TUL	37 °C	*Bacillus pumilus*
B7	1	FFN	37 °C	*Enterobacter hormaechei*
B7	1	TUL	37 °C	*Enterobacter cloacae*
B7	8	ENR	37 °C	*Enterococcus*
A6	8	OTC	37 °C	*Enterococcus hirae*
A6	8	ENR	37 °C	*Enterococcus hirae*
B7	1	FFN	37 °C	*Enterococcus mundtii*
A6	8	FFN	37 °C	*Escherichia coli*
T1	1	FFN	37 °C	*Escherichia coli*
T3	1	FFN	37 °C	*Escherichia coli*
B7	5	TUL	37 °C	*Lysinibacillus louembei*
B7	5	ENR	37 °C	*Microbacterium chocolatum*
B7	8	ENR	RT	*Paeniglutamicibacter antarcticus*
T3	2	ENR	RT	*Paeniglutamicibacter antarcticus*
A6	1	TUL	37 °C	*Proteus mirabilis*
T3	6	TUL	37 °C	*Proteus mirabilis*
A6	5	TUL	37 °C	*Proteus mirabilis*
B7	1	OTC	37 °C	*Proteus mirabilis*
B7	1	OTC	RT	*Proteus mirabilis*
T3	1	OTC	RT	*Proteus mirabilis*
T3	1	FFN	RT	*Pseudomonas edaphica*
T3	6	FFN	RT	*Pseudomonas fluorescens*
B7	1	FFN	RT	*Pseudomonas fulva*
T1	1	FFN	RT	*Pseudomonas gessardii*
T1	1	OTC	RT	*Pseudomonas gessardii*
T1	4	OTC	RT	*Pseudomonas gessardii*
A6	5	FFN	RT	*Psychrobacter maritimus*
B7	1	OTC	RT	*Serratia marcescens*
T1	4	FFN	RT	*Serratia marcescens*
B7	1	OTC	RT	*Serratia marcescens*
A6	1	FFN	37 °C	*Serratia rubidaea*
B7	1	TUL	37 °C	*Serratia rubidaea*
T3	5	OTC	37 °C	*Streptococcus equinus*

a – top hits are presented based on at least 400 bp of the 16S rRNA gene sequence

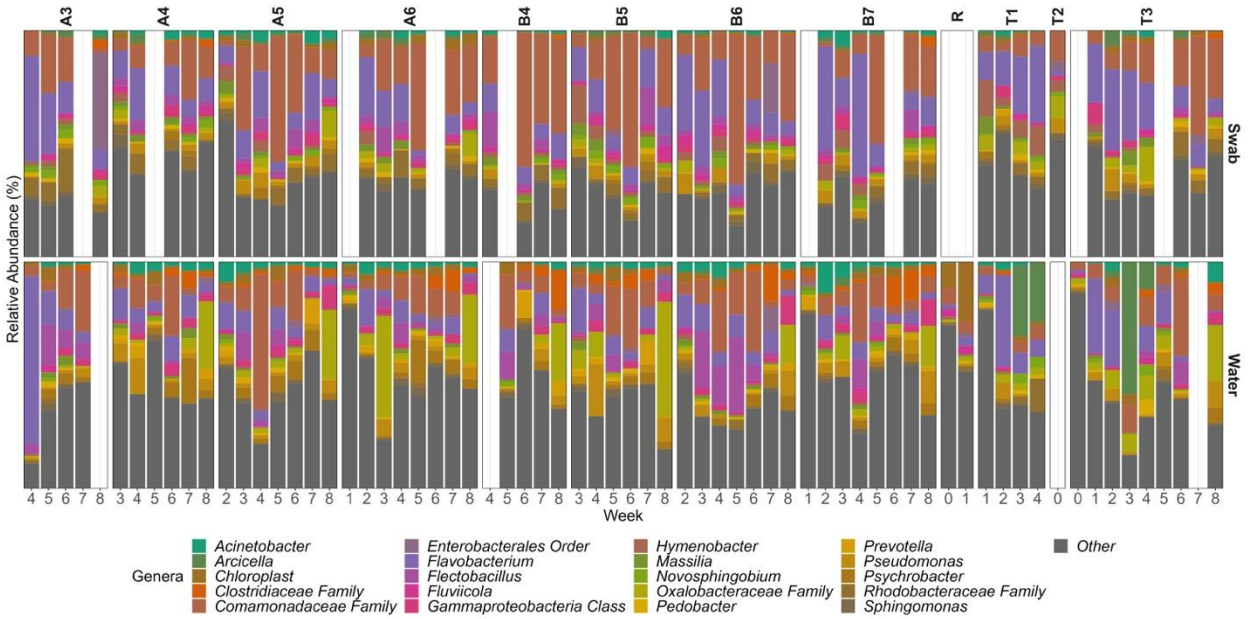

Figure A2.2. The composition of bacterial communities in water bowl swabs or water samples collected at a new feedlot represented using the 20 most abundant genera. Pen IDs are provided at the top. Home pens (A3-A6 and B4-7) are separated from transfer (T1-3) pens by samples of the water source, a water reservoir (R) that was sampled before animal arrival and during the first week of operation.

Table A2.4. Summary of water bowl isolate bacterial genome assemblies

	replicon	Genbank ID	length	GC (%)	no. of ARGs*
Acinetobacter baumannii WB4	chromosome	CP123854	3.80 Mb	39.0	9
Acinetobacter pseudolwoffii WB5	chromosome	JARXQN010000001	2.92 Mb	43.6	0
	plasmid	JARXQN010000002	163 kb	41.2	4
Escherichia coli WB6	chromosome	JARXQO010000001	5.04 Mb	50.6	26
Pedobacter steynii	chromosome	CP123860	6.19 Mb	40.6	0
Sphingobacterium faecium WB8	chromosome	CP123861	5.30 Mb	36.9	0
	plasmid	CP123862	57.8 kb	34.2	5
Sphingobacterium faecium WB9	chromosome	JARXQP010000001	4.62 Mb	37.3	0
	plasmid	JARXQP010000002	55.9 kb	34.4	5

*ARG detection based on protein sequences in the CARD of April 2023 + *estT*

Table A2.5. ARGs detected in 5 feedlot water bowl isolates using the CARD*

Class	*Acinetobacter baumannii* WB4	*Acinetobacter pseudolwoffii* WB5	*Escherichia coli* WB6	*Sphingobacterium faecium* WB8	*Sphingobacterium faecium* WB9
Aminocoumarin			*mdtABC,*		
Aminoglycoside		*APH(3")-Ib, APH(6)-Id*	*aadA, APH(6)-Id, kdpE*		
b-lactam	*ADC, OXA-695*		*EC-14, TEM*	*OXA-347*	*OXA-347*
Diaminopyrimidine			*dfrA12, dfrA17*		
Disinfecting agents and antiseptics			*qacEdelta1*		
Fluoroquinolone	*abaQ*		*emrA*		
Macrolide				*ermF, estT**	*ermF, estT**
Multi-Class	*abeS, abeM, adeFGH, adeIJK, AmvA*		*acrAB, acrEF, KpnEF, mdfA, mdtEF. mdtM, mdtNOP*		
Nitroimidazole			*msbA*		
Peptide antibiotic			*bacA, eptA, PmrF-ugd*		
Phenicol		*floR*	*floR*	*floR*	*floR*
Sulfonamide			*sul1, sul2*	*sul2*	*sul2*
Tetracycline	*adeABC*	*tet(X)*	*emrKY, tet(A)*	*tet(X)*	*tet(X)*

**estT* was not in CARD as of April 2023.

APPENDIX B – CHAPTER 3: Supplemental Material for: Environmental microbiomes and their role in florfenicol resistance in bovine respiratory disease pathogens.

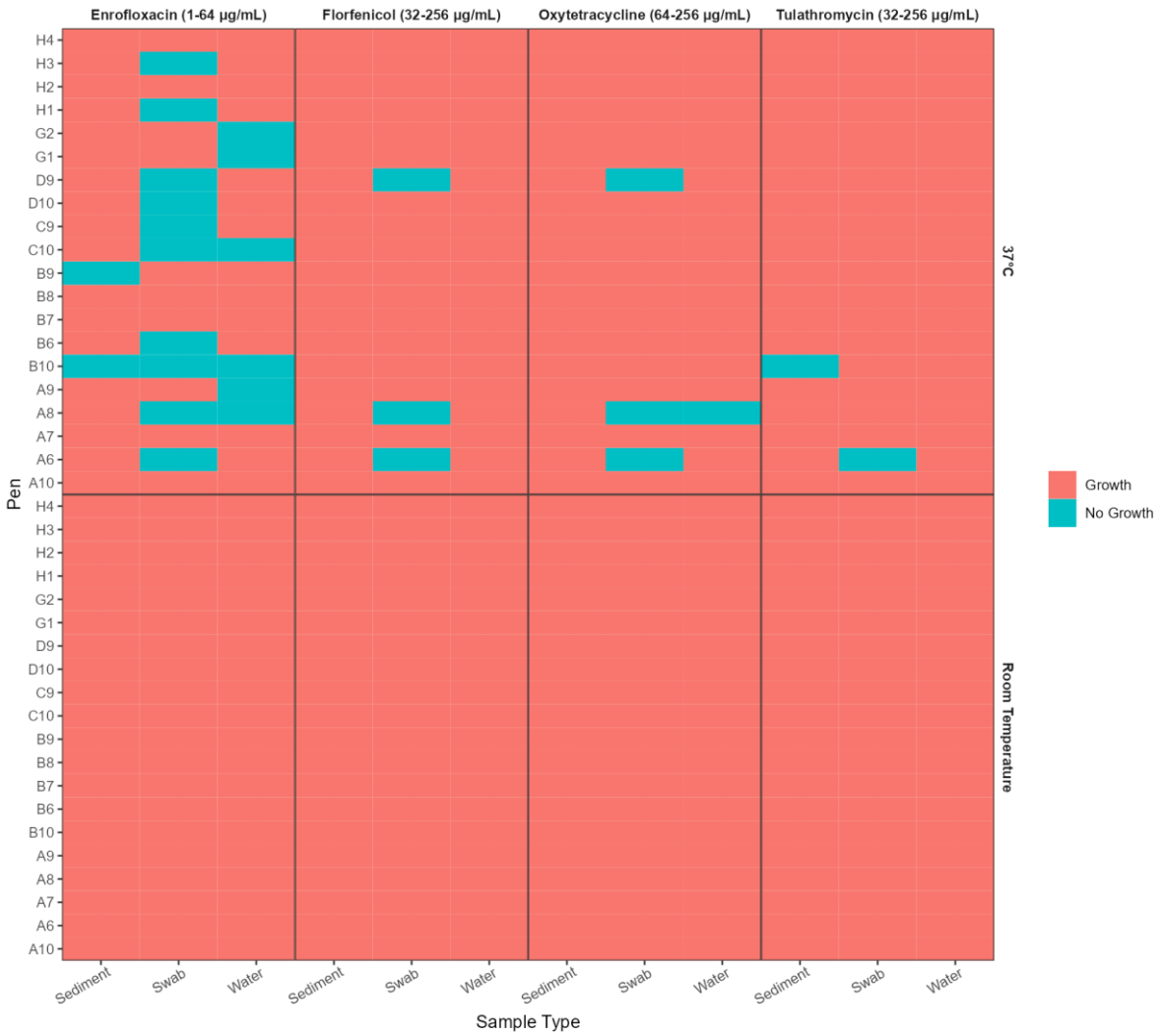

Figure B1. Heatmap summarising growth of inoculated environmental feedlot watering bowl samples in concentrations of antimicrobials above a threshold. Incubation at either 37°C overnight or room temperature for up to 96 hours for the four tested antimicrobials. Wells were inoculated with water, swab, or sediment samples.

Table B1. Assembly statistics of the WB isolates and the *H. somni* isolate broken down by contig.

Species	Replicon	Assembly	Coverage	Length	Circular	Mean QV	GC%
Sphingobacterium faecium	Chromosome	HGAP	12594x	5.0 mbp	Yes	93	36.8%
	pWB1		2951x	17.5 kbp	Yes	93	35.5%
Psychrobacter sp.	Chromosome	CANU	85x	3.2 mbp	Yes	93	43.3%
	pWB2		22x	44.9 kbp	Yes	93	44.8%
Acinetobacter terrestris	Chromosome	HGAP	15301x	3.0 mbp	Yes	93	41.2%
	pWB3		6425x	56.3 kbp	Yes	93	42.0%
Histophilus somni	Chromosome	hifiasm-meta	387x	2.3 mbp	Yes	98	37.5%