Implementation and analysis of an on-farm surveillance system for enteric bacteria in Canadian dairy herds

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ABSTRACT

Antimicrobial resistance (AMR) is an increasing concern for public and animal health as it diminishes the effectiveness of antimicrobials against resistant bacteria and make treatments more difficult and expensive. The research documented in this thesis aimed to describe the development and implementation of CaDNetASR, an on-farm surveillance system focused on collecting data on antimicrobial use (AMU) and AMR in enteric bacteria (*E. coli*, *Campylobacter* spp., and non-typhoidal *Salmonella* serovars) from Canadian dairy farms. The research also included the use of the data collected through CaDNetASR surveillance to describe the phenotypic resistance patterns of *E. coli*, *Campylobacter* spp., and *Salmonella,* and explore the association between AMU and AMR in Canadian dairy herds. The surveillance was implemented in the fall of 2019, and the data were collected yearly from a convenience sample of 144 dairy herds from five different provinces in Canada. Fecal samples from pre-weaned calves, post-weaned heifers, lactating cows, and manure storage were collected. Additionally, herd health, herd management and AMU information were gathered. Fecal samples were cultured for *E. coli*, *Campylobacter* spp., and *Salmonella*. Susceptibility testing on the stored isolates was done using the broth microdilution system method. The proportion of farms positive for *Campylobacter* spp. was 95.7%, suggesting that these bacteria are widespread among Canadian dairy herds. For *Salmonella*, the proportion of positive farms was lower ranging from 12% to 17% from 2019 to 2021. No *Salmonella* Dublin was identified. A higher proportion of resistance to tetracycline than other antimicrobials was observed for all three bacteria. For *E. coli* a low proportion of resistance was observed to highly important antimicrobials; however, for *Campylobacter* spp., 19.9% of the isolates were resistant to ciprofloxacin. No resistance

was observed to highly important antimicrobials on *Salmonella* isolates, except for one isolate resistant to amoxicillin/clavulanic acid. The AMU was quantified in Defined Course Dose (DCD - the dose for a standardized complete treatment course on a standard size animal) and converted to a rate indicator - DCD/100 animal-years. The total AMU was split into systemic and intramammary routes of administration to explore the possible differences according to the administration route. Overall, the AMU varied substantially among the dairy farms. Regression models were built to explore the association of AMU and AMR in *E. coli* and *Campylobacter* spp. isolates. For *Campylobacter* spp., only the total AMU was associated with increased resistance to tetracycline. In *E. coli*, the systemic AMU was associated with increased resistance to nine antimicrobials; however, the intramammary AMU was not significantly associated with resistance. Overall, resistance in dairy farms was low compared to other food-producing animals such as poultry or swine. The findings documented in this thesis provided information that can be used in the future to develop interventions aiming to reduce the use of antimicrobials in dairy farms and promoting more sustainable and responsible husbandry practices.

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LIST OF ABBREVIATIONS

ADD	Animal daily dose
AMR	Antimicrobial resistance
AMS	Antimicrobial Stewardship
AMU	Antimicrobial use
ARG	Antimicrobial resistance genes
BTM	Bulk tank milk
CaDNetASR	Canadian Dairy Network for Antimicrobial Stewardship and Resistance
CIPARS	Canadian Integrated Program for Antimicrobial Resistance
CVMA	Canadian Veterinary Medical Association
DCD	Defined course dose
EFSA	The European Food Safety Authority
EP	Expert Panel
FAO	Food and Agriculture Organization of the United Nations
GCA	Garbage can audit
MDR	Multidrug resistance
PHAC	Public Health Agency of Canada
SC	Steering Committee
VAC	Veterinary Advisory Committee
WHO	World Health Organization
WOAH	World Organization for Animal Health

Chapter 1

1. Introduction and objectives

1.1. Introduction

Antimicrobial resistance (AMR) occurs when antimicrobials can no longer inhibit the growth of pathogens like bacteria, increasing the risk of spreading diseases, more severe illnesses, and death [1]. For those reasons, AMR has emerged as the leading public health concern worldwide, and unless action is taken, it is estimated that AMR could cause about ten million deaths by 2050 [2]. A systematic review from 2022 estimated that approximately 1.27 million deaths worldwide in 2019 could be directly attributed to antimicrobial-resistant bacteria [3]. Besides the public health perspective, such as the zoonotic transmission of AMR-bacteria through the food supply, AMR is also a concern for food-production animals, including dairy cattle, as resistant bacteria can make treatments less effective, affecting animal health and welfare and increasing production costs [4, 5].

Since 2010, awareness about AMR has increased, and action plans, guidelines and surveillance systems have been implemented in several countries [6]. Recognizing the importance of mitigating AMR in Canada, in 2017, the Public Health Agency of Canada (PHAC) launched the document "Tackling Antimicrobial Resistance and Antimicrobial Use: A Pan-Canadian Framework for Action," aiming to slow the rising trend of AMR. The overarching goal of the framework was to strengthen Canada's ability to mitigate AMR in a coordinated, multisectoral and effective manner. Additionally, this framework was grounded in a One Health approach, which recognizes the interconnectedness of humans, animals, and the environment [7]. In 2023, the "Pan-Canadian Action Plan on Antimicrobial Resistance" was released. The action plan is a five-year blueprint to coordinate an accelerated Pan-Canadian response to AMR, grounded on five main commitments: 1) research and innovation, 2) leadership, 3) surveillance, 4) infection

prevention and control, and 5) stewardship [8]. Other initiatives led by international organizations have been developed. In 2015, the World Health Organization (WHO) launched a global surveillance system to gather data on AMR. The surveillance system, called Global Antimicrobial Resistance and Use Surveillance System (GLASS), along with other strategies, provided help for countries in generating information to achieve better quality and representativeness of the data collected [9]. However, this initiative was more focused on human surveillance. To successfully tackle AMR, it is critical to address the problem by adopting the "One Health" approach which would allow for surveillance of AMR in humans, animals, and the environment [10]. In recognition of this, in 2018, the Food and Agriculture Organization of the United Nations (FAO), the World Organization for Animal Health (WOAH), together with the WHO formed a tripartite alliance (FAO-WOAH-WHO) to tackle AMR using a more integrated approach [11].

1.1.1. Public health implications of AMR in foodborne enteric bacteria

Enteric bacteria, such as *Campylobacter* spp., *E. coli*, and non-typhoidal serovars of *Salmonella enterica* ssp. *enterica*, have become a significant public health concern due to the potential for AMR associated with them. These foodborne pathogens are the leading causes of infections in humans worldwide, as they can contaminate food during production, processing, storage, or preparation, leading to a range of illnesses in humans, from mild gastroenteritis to severe diseases [12, 13]. The potential transmission of these pathogens to humans via direct contact with animals can also occur [14].

Efforts to reduce AMR in foodborne pathogens are crucial for maintaining food safety and preventing human infections with AMR-enteric bacteria. In Canada, a multi-partner

surveillance system facilitated by the PHAC was implemented to reduce the burden of enteric diseases in humans. The "FoodNet Canada" surveillance system includes four sentinel sites established in partnership with local public health units and provincial public health laboratories, monitoring local water, agriculture, and retail food sectors. The three sentinel sites are located in Ontario (Middlesex-London Health Unit), British Columbia (Fraser Health Authority), Québec (Montérégie Health Region), and Alberta (Alberta Health Services: Calgary and Central Zones) [15]. The data collected through this surveillance system facilitates in-depth investigations of foodborne and waterborne diseases and human exposure with a primary objective of source attribution. It is, therefore, critical to address AMR in these bacteria and take measures to enhance food safety and reduce the risk of spreading AMR-bacteria to humans.

1.1.2. Food-producing animals as a reservoir of AMR-enteric bacteria

In animals, *Campylobacter* spp., generic *E. coli*, and non-typhoidal *Salmonella* serovars can colonize the gastrointestinal tract asymptomatically and healthy animals can be reservoirs of AMR-enteric bacteria [16-20]. A study conducted in the United States analyzing resistance in *Campylobacter* spp. recovered from poultry, turkey, swine, and cattle reported higher proportions of resistance to antimicrobial classes such as tetracyclines, beta-lactams, aminoglycosides, macrolides, and quinolones [21]. Similarly, in *E. coli* and *Salmonella* spp. isolated from poultry, turkey, and swine, higher proportions of resistance were reported to tetracyclines, penicillins, and sulfonamides [22]. In Canada, previous studies also demonstrated the potential of food-producing animals to act as reservoirs of AMR-bacteria that could cause human infections [23-26]. Evidence in the literature also suggests dairy cattle as a potential reservoir of antimicrobial resistant *Campylobacter* spp., *E. coli*, and

Salmonella spp. that may be spread to humans through direct contact with animals or their environment [14, 27-29].

In addition to asymptomatic infections, antimicrobial resistant enteric bacteria might also cause disease in food-producing animals [30]. In dairy cattle, *Salmonella* Dublin can cause severe infections in young and adult animals, such as pneumonia, enterocolitis, and septicemia [18]. The increased trend of *Salmonella* harbouring AMR determinants makes controlling and treating these infections increasingly challenging and might affect herd health and productivity [30].

Additionally, generic *E. coli* can serve as a reservoir of antimicrobial resistance genes (ARGs) that can be horizontally transferred to other bacteria [31, 32]. A study from 2022 analyzing fecal samples from broilers, laying hens, turkeys, and pigs reported a shared resistance profile in *Campylobacter* spp., *E. coli* and *Salmonella* spp. suggesting that the horizontal transmission of ARGs between bacteria may have occurred; however, it was not reported which bacteria was the potential reservoir [22].

1.1.3. Risk factors associated with the occurrence of AMR in enteric bacteria in food-producing animals

E. coli is easily isolated from the gastrointestinal tract in healthy animals and is considered to be part of the normal flora in both humans and cattle [33]. Although most strains of *E. coli* are harmless to the host, some strains are pathogenic and can cause infections in humans and animals [34]. *Campylobacter* spp. is also considered a commensal bacterium for most domestic animals [16]; however, *Salmonella* is considered to be a pathogen. Specific husbandry practices might be associated with the occurrence of *Campylobacter* spp. and

Salmonella in food-producing animals. A study analyzing fecal samples from young beef cattle found that *Campylobacter* spp. were four times more likely to occur in samples from animals housed indoors than those housed outdoors [35]. In dairy cattle, prolonged confinement of calves in individual pens was associated with decreased odds of recovering *Campylobacter* spp. in these animals [36]. Additionally, the same study identified the presence of poultry as a risk factor for increased odds of recovering *Campylobacter* spp. from dairy cattle fecal samples. The occurrence and shedding of *Salmonella* in food-producing animals is usually less frequent than *E. coli* and *Campylobacter* spp. [33]. The lower prevalence of *Salmonella*, specifically in dairy cattle, makes risk factors analysis for its occurrence difficult. However, some risk factors, such as animal age (heifers above 1 year), season (first quarter of the year), and herd prevalence, have been associated with higher odds of shedding *Salmonella* Dublin, a host-adapted serovar, in dairy cattle [37].

Husbandry practices might also impact the occurrence of AMR in enteric bacteria. Numerous studies in dairy cattle have demonstrated that animal age (calves), herd size (larger herds), bedding management (farms that used recycled manure solids as a bedding material), and the number of culled animals (more culled animals) were associated with higher odds of resistance in *Salmonella* and *E. coli* [38-41]. The type of management system (antibiotic-free vs. organic vs. conventional) was another identified risk factor that could be potentially associated with AMR in *E. coli*, *Salmonella*, and *Campylobacter* spp. in other commodities (beef cattle, swine, and chickens) [42]. However, the association between these management systems and AMR is not yet fully understood and further research is required.

A critical management practice directly associated with AMR is how and when antimicrobials are used in food-producing animals. Antimicrobial use (AMU) is reported in the literature as a major risk factor associated with AMR [43, 44]. It is well described that AMU of a specific drug in food-producing animals is associated with resistance to that same active ingredient [43, 45-47]. In 2017, a systematic review and meta-analysis examined interventions aimed at reducing the use of antimicrobials in food-producing animals. The study also investigated the presence of antibiotic-resistant bacteria and found that limiting AMU in animals was associated with a reduction in AMR in both humans and animals [48]. Additionally, a risk assessment study published in 2020, demonstrated that withdrawing preventive ceftiofur use from poultry production reduced the probability of illnesses in humans caused by third-generation cephalosporin resistant-*Salmonella* Heidelberg [49]. These latter studies suggest that controlling AMU in animals could have a positive impact on public health by reducing the development and spread of AMR-bacteria. A study published in 2023 used the available AMU data to predict global trends of AMU in animals from 2020 to 2030. According to current trends, the study projected an 8% increase in the global consumption of antimicrobials intended to be used in animals by 2030 [50]. The results suggested by the latter study reinforces the importance of antimicrobial stewardship (AMS) strategies in food-producing animals.

1.1.4. On-farm surveillance for AMU and AMR

Given the well-established link between AMU in food-producing animals and the emergence and spread of AMR in enteric pathogens, it is essential to accurately quantify AMU at farm-level for developing and implementing stewardship measures aimed at

promoting prudent AMU. One common source used to obtain information on the quantity of antimicrobials intended to be used in animals is the sales data provided by pharmaceutical companies [51]. Sales data can provide a crude estimate of AMU at the national level, such as the data provided by the Veterinary Antimicrobial Sales Reporting System (VASR) in Canada [52]. However, a more precise source of AMU data is required to develop and quantify the impact of AMS strategies. To obtain reliable AMU data in food-producing animals, it is necessary to collect the data at the end-user or prescriber (farm-level) [53]. Gathering the AMU information at the farm-level is crucial as it not only provides the quantity of AMU, but also identifies the specific antimicrobials and their active ingredients being used. This knowledge is essential in developing effective AMS measures, as some antimicrobials hold greater importance than others in treating infections in humans and should be minimized in animal agriculture whenever possible. There are different sources of farm-level AMU data, such as on-farm treatment records, garbage bins audits, or veterinary pharmaceutical sale invoices, with the last two methods being more reliable than on-farm treatment records [54, 55]. Although garbage bin audits may provide useful and reliable information, they are often time-consuming and not sustainable as a long-term surveillance strategy. Therefore, a more practical and efficient approach to obtaining information about AMU in animals is through veterinary pharmaceutical sale invoices, which are a reliable source of data [53].

Implementing an on-farm surveillance system based on continuous information recording is necessary to monitor the AMU and the AMR, facilitating the development of AMS strategies. Most existing surveillance systems worldwide for food-producing animals follow the European Food Safety Authority (EFSA) recommendations, which include

guidelines on designing sampling strategies, identifying target animal and bacterial species, and determining which antimicrobials to test for each bacterium. The EFSA recommendations prioritize monitoring zoonotic enteric bacteria such as generic *E. coli*, *Campylobacter* spp., and *Salmonella* [56]. The primary objective of these surveillance systems is to monitor the emergence and spread of AMR in food-producing animals and to develop prudent AMU guidelines for effective treatment plans that minimize AMU while ensuring the health and welfare of both humans and animals. Some countries, such as Denmark and Netherlands, pioneered establishing a more integrated surveillance system (Danmap and Nethmap-Maran, respectively), including surveillance on humans, companion animals, and food-producing animals and a more comprehensive AMU data collection (on-farm) at the national level [57].

1.1.5. On-farm surveillance systems for food-producing animals in Canada

Due to the comprehensive nature of data collection required by on-farm surveillance, substantial investments in resources such as personnel, equipment, supplies, and technology might pose a challenge in its implementation. Moreover, implementing surveillance programs can face resistance from some stakeholders, such as farmers and veterinarians, who may perceive it as an added burden on their practices [58]. Additionally, regulations can vary across provinces, making it difficult to implement a standardized approach. To overcome these challenges, a coordinated effort is needed among various stakeholders, including government agencies, producers, veterinarians, and researchers, to develop standardized protocols, provide necessary resources and incentives, and increase awareness and education about the importance of responsible AMU.

In Canada, the Canadian Integrated Program for Antimicrobial Resistance Surveillance (CIPARS), an integrated surveillance system, was launched in 2002 to collect AMR and AMU data on humans and food-producing animals [59]. The CIPARS program is coordinated by the Public Health Agency of Canada in collaboration with federal, provincial/territorial, and private industry partners [60]. In the initial years, the system collected abattoir samples from cattle, chickens, and pigs for the animal component. In 2006, CIPARS implemented on-farm surveillance for swine. In 2013, the program extended the on-farm data collection to broiler chicken, and in 2014 and 2016, turkey and feedlot beef were added to the on-farm component, respectively [59]. A schematic flow diagram with the current CIPARS surveillance components is shown in Figure 1.1.

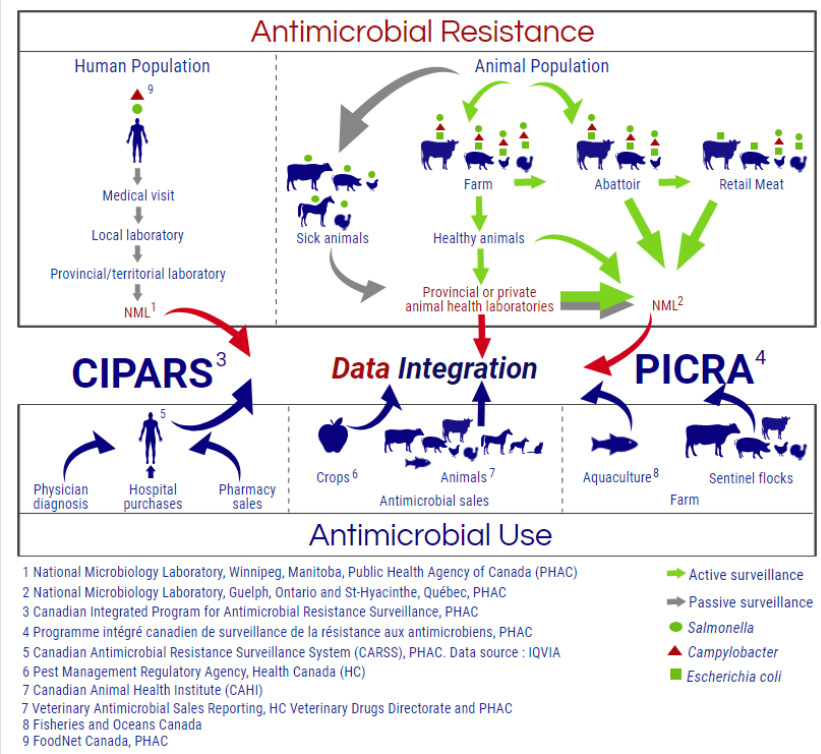

Figure 1.1 Canadian Integrated Program for Antimicrobial Resistance Surveillance (CIPARS), from the Public Health Agency of Canada [59].

The dairy industry is the second largest in the Canadian agriculture sector, and its milk and dairy products are recognized for their quality [61]. However, no comprehensive AMU and AMR surveillance system has been established to collect data for this commodity in Canada. As such, a comprehensive and effective on-farm surveillance system to monitor AMU and AMR in Canadian dairy herds was urgently needed.

1.2. Objectives

The research documented and reported in this thesis aimed to describe the development and implementation of the Canadian Dairy Network for Antimicrobial Stewardship and Resistance (CaDNetASR). CaDNetASR is an on-farm surveillance system for AMR of enteric bacteria and on-farm AMU in Canadian dairy herds that was developed to be comprehensive, efficient, and adaptable to change. This document also includes data gathered through CaDNetASR, to describe farm level AMR and investigate the association between AMU and AMR in generic *E. coli* and *Campylobacter* spp. Although CaDNetASR also collected samples for the detection of *Salmonella* spp., the sample size was not large enough to build epidemiological models, but information on the *Salmonella* serovars and their phenotypic resistance patterns associated with these isolates are described.

The specific research objectives of this thesis were:

1. Describe the development and implementation of the CaDNetASR surveillance system in Canada. A literature review of worldwide surveillance systems for AMU and AMR for food-producing animals, focusing on the dairy cattle, was performed, and is described in Chapter 2. The literature review in this chapter provided background about the AMR and AMU surveillance systems components, which

allowed us to identify the strengths and weaknesses of the CaDNetASR. This chapter also included preliminary results to determine the proportion of isolation of each target enteric bacteria.

2. Investigate risk factors associated with isolation and AMR in *Campylobacter* spp. (Chapter 3). The specific objectives also included estimating the proportion of *Campylobacter* spp. isolated from fecal samples, the proportion of AMR in *Campylobacter* spp, risk factors for isolating *Campylobacter* spp, and the association between AMU and resistance in *Campylobacter* spp.
3. Investigate risk factors associated with resistance in generic *E. coli* (Chapter 4). The specific objectives also included estimating the proportion of AMR in generic *E. coli* and the association between AMU and AMR in generic *E. coli*.
4. Determine the frequency of isolation and characterize the phenotypic resistance in nontyphoidal *Salmonella enterica* (Chapter 5). The specific objectives also included estimating the proportion of *Salmonella* isolated from fecal samples, the *Salmonella* serovars, and the phenotypic antimicrobial resistance pattern on these isolates.

   Together, these objectives represented the description and implementation of the CaDNetASR surveillance system, and the use of the data collected to determine the current state of knowledge on AMR in enteric bacteria in Canadian dairy herds and identify the potential association of AMU with AMR at the farm level.

Chapter 2

2. Canadian Dairy Network for Antimicrobial Stewardship and Resistance (CaDNetASR): An on-farm surveillance system

Mariana Fonseca, Luke C. Heider, David Léger, J Trenton Mcclure, Daniella Rizzo, Simon Dufour, David F. Kelton, David Renaud, Herman W. Barkema, and Javier Sanchez. Frontiers in Veterinary Science 2022, https://doi.org/10.3389/fvets.2021.799622

2.1. Abstract

Canada has implemented on-farm antimicrobial resistance (AMR) surveillance systems for food-producing animals under the Canadian Integrated Program for Antimicrobial Resistance (CIPARS); however, dairy cattle have not been included in that program yet. The objective of this manuscript was to describe the development and implementation of the Canadian Dairy Network for Antimicrobial Stewardship and Resistance (CaDNetASR). An Expert Panel (EP) of researchers was created to lead the development of the dairy surveillance system. The EP initiated a draft document outlining the essential elements of the surveillance framework. This document was then circulated to a Steering Committee (SC), which provided recommendations used by the EP to finalize the framework. CaDNetASR has the following components: (1) a herd-level antimicrobial use quantification system; (2) annually administered risk factor questionnaires; and (3) methods for herd-level detection of AMR in three sentinel enteric organisms (generic *Escherichia coli*, *Campylobacter* spp., and *Salmonella*) recovered from pooled fecal samples collected from calves, heifers, cows, and the manure pit. A total of 144 dairy farms were recruited in five Canadian provinces (British Columbia, Alberta, Ontario, Québec, and Nova Scotia), with the help of local herd veterinarians and regional field workers, and in September 2019, the surveillance system was launched. 97.1% and 94.4% of samples were positive for *E. coli,* 63.8%, and 49.1% of samples were positive for *Campylobacter* spp., and 5.0%, and 7.7% of samples were positive for *Salmonella*, in 2019 and 2020, respectively. *E. coli* was equally distributed among all sample types. However, it was more likely that *Campylobacter* spp. were recovered from heifer and cow samples. On the other hand, it was more common to isolate *Salmonella* from the manure pit compared to samples

from calves, heifers, or cows. CaDNetASR will continue sampling until 2022 after which time this system will be integrated into CIPARS. CaDNetASR will provide online access to farmers and veterinarians interested in visualizing benchmarking metrics regarding AMU practices and their relationship to AMR and animal health in dairy herds. This will provide an opportunity to enhance antimicrobial stewardship practices on dairy farms in Canada.

Keywords: dairy cattle; antimicrobial use; antimicrobial resistance; surveillance; Canada.

2.2. Introduction

Antimicrobial resistance (AMR) is a natural phenomenon that occurs when bacteria evolve and no longer respond to antimicrobial drugs that previously were efficacious. Major economic losses and animal health and welfare problems have been described as the consequences of AMR [62, 63]. Many AMR commensal and pathogenic bacteria have been described in food animals. For instance, a study conducted in North California demonstrated that all *Salmonella* Newport isolates recovered from dairy cattle fecal samples (symptomatic and asymptomatic animals) were multidrug-resistant (resistant to ≥ 3 antimicrobial classes) [64]. Infections caused by *Salmonella* Newport can cause economic losses due to treatment failure and increase mortality rates in animals [65]. Many bacterial organisms, including *Salmonella* Newport can be shared between human and animal populations. In humans, AMR can make treatment of bacterial infections more challenging, increase treatment costs, allow for increased disease spread, and increase the risk of mortality in people [66]. It is estimated that 700,000 deaths worldwide are caused annually by antimicrobial resistant bacteria and, by 2050, this figure may increase to 10 million [2]. For these reasons, AMR is considered one of the major challenges to public health [67].

To address the global problem of AMR, many countries have developed and implemented AMR surveillance systems for humans and animals. A surveillance system can be defined as "a system based on continuous information recording, making it possible to monitor the health status of a given population and the risk factors to which it is exposed, to detect pathological processes as they appear and study their development in time and space, and then to take appropriate measures to control them" [68]. The main objectives of an on-farm

AMR surveillance system are: (1) to determine the current prevalence of AMR (2) to describe AMR trends; (3) to detect the emergence of new types of resistance; and (4) to track a particular type of resistance [69].

In addition, this surveillance system should be able to provide estimates of the types and amounts of antimicrobials used on farms. Evidence suggests associations between using certain antimicrobials in animals with resistance in clinical bacterial isolates from humans [48]. Similar to the situation in humans, there is also a strong association between antimicrobial use (AMU) and AMR in the livestock sector [70-73]. In the dairy sector, the route of administration and the antimicrobial active ingredient seem to play an important role in the development of AMR. A study conducted in Canada demonstrated that the use of systemic antimicrobials was associated with resistance in non-aureus staphylococci isolated from milk, while intramammary treatments were not [74]. However, a study conducted in Ohio found that the use of cephalosporin-based dry cow therapy was associated with recovering a greater number of fecal coliform bacteria with reduced susceptibility to cephalothin and streptomycin in dairy cows [75].

Recognizing the interrelationship between AMU/AMR in humans and animals and the need for the standardization of methods between countries (e.g., AMU metrics, target pathogens, etc.), in 2018, the Food and Agriculture Organization of the United Nations (FAO), the World Organisation for Animal Health (WOAH), and the WHO formed a tripartite alliance (FAO-WOAH-WHO) focusing on a "One Health" approach to AMR [11]. The "One Health" approach includes surveillance of important AMR organisms and AMU in humans, animals, and the environment.

In support of this One Health approach to AMR, many countries developed surveillance systems to monitor AMU and AMR in food animal agriculture. Many of these surveillance systems report the proportion of antimicrobial resistant isolates of *Salmonella* spp., *Campylobacter* spp., and *Escherichia coli*, as these pathogens can be transmitted zoonotically through the food chain to humans [68].

Denmark and the Netherlands have comprehensive AMU surveillance systems (DANMAP and Nethmap-MARAN, respectively) [76]. In Canada, the Canadian Integrated Program for Antimicrobial Resistance Surveillance (CIPARS) was developed in 2002 to collect and analyze AMU/AMR data, and report trends in AMU and AMR from human, retail food, and food-producing animals [77]. In 2006, CIPARS implemented an on-farm component in grower-finisher pigs; then, in 2013, in broiler chicken and turkey [78], and in 2019, a surveillance system for feedlot cattle was started [79]. These national surveillance systems collect AMU data at the farm level to facilitate AMU benchmarking for farms and for developing interventions towards antimicrobial stewardship (AMS).

Reducing AMU in humans and animals is crucial to diminish the burden of AMR and prolong antimicrobial efficacy [80]. In Canada, initiatives led by the Canadian Veterinary Medical Association (CVMA) and the Public Health Agency of Canada (PHAC) have created guidelines to improve AMS. The CVMA defines AMS as "multifaceted and dynamic approaches required to sustain clinical efficacy of antimicrobials". In 2017, the PHAC released the document "*Tackling Antimicrobial Resistance and Antimicrobial Use: A Pan-Canadian Framework for Action'*. The framework's goal was to strengthen the ability to fight AMR in a coordinated, multisectoral and effective manner [81]. Antimicrobial stewardship was one of the components promoted to achieve the goal. However, despite

these initiatives, there are still challenges because the coordination of AMS leadership is sparse and inconsistent across the country [81].

In the dairy sector, some factors, such as dairy consumer perception, government requirements, and animal and human health are the main reasons for continuing to work on AMS programs [82]. Recognizing the knowledge gap on AMR and AMU in the dairy sector in Canada, the Canadian Dairy Network for Antimicrobial Stewardship and Resistance (CaDNetASR) was developed to help determine and improve AMU stewardship on Canadian dairy farms. This surveillance system will estimate AMU, determine how and why antimicrobials are used on dairy farms, and determine AMR patterns and trends in the Canadian dairy sector. This manuscript aims to describe the development and implementation of a national on-farm surveillance system (CaDNetASR), for an ongoing AMU and AMR data collection on Canadian dairy farms, towards improved AMS in this production sector.

2.3. CaDNetASR Surveillance Framework Development and Implementation

Research personnel from five veterinary colleges in Canada (University of Prince Edward Island, University of Guelph, University of Saskatchewan, University of Montreal, University of Calgary) and PHAC recognized the lack of information regarding AMU, AMR, and the importance of improving AMS in the Canadian dairy sector. Together they decided to develop a surveillance system to fill the knowledge gap. This diverse group of researchers had expertise in epidemiology, AMR, dairy production medicine, surveillance system development, and public policy.

In order to initiate the development of the surveillance system a five-year proposal was developed and funded by Dairy Farmers of Canada and Agriculture and Agri-food Canada, under the Dairy Research cluster 3 program, and by PHAC and the University of Prince Edward Island (UPEI). After the initial funding (2018-2022) the intention is to incorporate this system into CIPARS.

An Expert Panel (EP) was created to develop a farm-based surveillance framework for AMU, AMS, and AMR on dairy farms across Canada. The EP was composed of researchers from six Canadian universities (University of Prince Edward Island, University of Guelph, University of Saskatchewan, University of Montreal, University of Calgary, and Memorial University) and veterinary epidemiologists from the PHAC.

In the summer of 2018, members of the EP developed a draft of the surveillance framework. As part of the framework development, it was decided that the surveillance system should be deployed in five regions across Canada. These regions were the communities of Truro/Halifax in Nova Scotia, Montérégie region in Québec, London Middlesex in Ontario, Calgary-East in Alberta, and Fraser Valley in British Columbia, which are part of the sentinel sites from FoodNet Canada, a surveillance system focused on foodborne and waterborne diseases [15].

During the initial development phase of the surveillance framework, a Steering Committee (SC) was created, and the framework was sent to them for comments in January 2019. The SC was composed of relevant stakeholders from provincial and national milk boards (e.g., Dairy Farmers of Canada), veterinary organizations (e.g., Canadian Association of Bovine Veterinarians), PHAC, and dairy herd improvement organizations. The role of the SC was

to provide input on developing the surveillance framework for implementation in 2019 and ensure that the methods to collect farm samples and data were practical and sustainable. In addition, SC members were tasked with disseminating findings from the surveillance system to their respective organizations.

After the initial development of the framework, the EP and the SC, came together for a two-day meeting whereby the framework was introduced and discussed. Suggestions were offered to improve the quality of data generated and introduce the surveillance system to the Canadian dairy industry. The information generated from this meeting was used to refine and finalize the surveillance framework. A final framework was ready for implementation in the spring of 2019.

For the implementation of the surveillance system, an operation committee was created. The operations committee was composed of all EP members, regional project managers, regional field workers, technicians and graduate students involved in the system. The role of the operations committee was to provide feedback on the operational issues through monthly meetings after the surveillance implementation and contribute to potential refinements of the surveillance system.

Each of the five regions had one regional project manager responsible for overseeing herd selection, the data collection and supervising the regional field workers. The regional field workers scheduled the farm visits and conducted the sampling based on the protocols provided. The surveillance system (CaDNetASR) was implemented in September 2019 and continued for four years in the first round of funding. The development and implementation of CaDNetASR is illustrated in Figure 2.1.

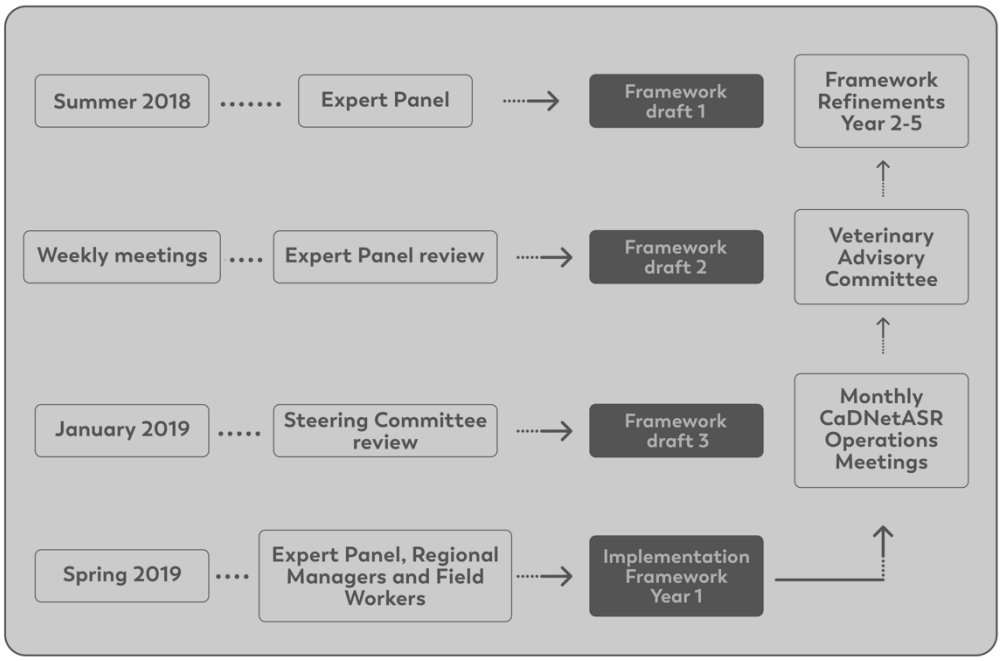

Figure 2.1 CaDNetASR framework development and implementation

2.4. CaDNetASR Surveillance Components

The CaDNetASR surveillance includes all the critical components for AMR and AMU surveillance, collecting, analyzing, and reporting AMR and AMU in dairy herds at the farm level. The components of CaDNetASR are described below and are illustrated in Figure 2.2.

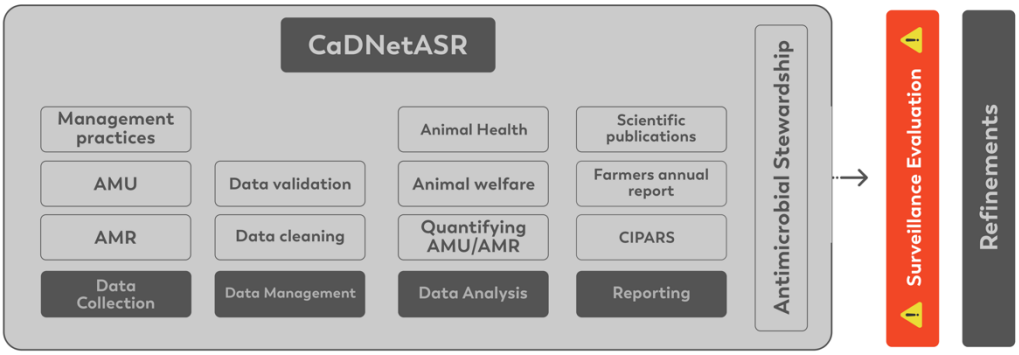

Figure 2.2 CaDNetASR surveillance system components

2.4.1. Farm Enrollment

As AMS was a key component in the surveillance system, the sample size was calculated to detect changes in the AMU based on the available data from AMU in dairy cattle in Canada [83]. In this study, AMU data were quantified in units of animal defined daily doses (ADD). The ADD (g/d) was defined as the average daily on-label dosage multiplied by the approximate weight of an adult dairy cow (body weight = 600 kg). The sample size was then calculated to estimate an AMU rate with a precision of +/- 0.3 for various antimicrobials based on the assumption that 95% of the farms have AMU rates between 0.001 and 4 ADD/1,000 cows [83]. Therefore, the goal was to select 30 farms from each of the five regions to participate in the research project. At implementation in 2019, a convenience sample of 144 dairy farms was enrolled. All regions enrolled 30 farms except Nova Scotia, where only 24 farmers agreed to participate. In 2020, three herds from British Columbia and one herd from Quebec dropped out of the program and were replaced with new herds. Farms should be representative of commercial dairy operations in each region. The following inclusion criteria were considered: (1) farms should be enrolled in ProAction/CQM (national mandatory certification program focused on several aspects of milk production) and DHI (dairy herd improvement organization responsible for milk recording, genetic evaluations and knowledge transfer in Canada); (2) minimum herd size of 50 animals except for Nova Scotia, that was minimum herd size of 40 animals; (3) raise their replacement heifers on-site; (4) Antimicrobial-free, organic or robotic herds should be enrolled proportional to their prevalence in a given region; (5) farmers should be willing to provide/share drug purchase information obtained from their veterinary clinics and feed mills. The only exclusion criteria were farms not planning to continue farming for

the next five years. To protect the identity of participating farms, each farm was assigned an identifier, and only the regional project managers recorded which farm was linked to the study identifier to maintain anonymity. All producers signed an informed consent form explaining the project objectives and their role as participants, at the beginning of the first year, which was reviewed with them annually. The summary of demographic information for the dairy farms enrolled in CaDNetASR is presented in Table 2.1.

Table 2.1 Summary of demographic information from dairy farms enrolled in CaDNetASR during 2019 and 2020.

	Province				
Characteristic	British Columbia	Alberta	Ontario	Quebec	Nova Scotia
Farms enrolled	30	30	30	30	24
Herd size* (mean)	175.3	170.4	159.8	86.3	101.1
% Free stall	100.0	96.6	87.1	21.4	62.5
% Tie-Stall	0.0	3.3	9.7	74.2	37.5
% Other housing	0.0	0.1	3.2	4.4	0.0
Milking parlour	57.1	76.6	48.4	21.4	37.5
Robotic	42.9	23.4	41.9	12.9	16.7
Pipeline	0.0	0.0	9.7	65.7	45.8
% Holstein	90.7	93.7	97.9	91.9	97.0
% Jersey	6.0	3.6	0.7	0.8	0.7
% Other breeds	3.3	2.7	1.4	7.3	2.3

* Number of lactating cows.

2.4.2. Data Collection, Data Management, and Reporting

On-farm data collection included annual collection of fecal samples, a bulk tank milk sample (BTM), administration of annual questionnaires to collect herd management practices, AMU, and risk factor information for AMR related projects/questions. The main sections of the questionnaires are presented in Tables S2.1 and S2.2 in Appendix A. Regional field workers collected annual pooled fecal samples from up to five pre-weaned calves, five breeding age heifers and five lactating cows and a single sample from the

manure storage system by pooling from three to five different locations in that system. Standardized sampling kits designed by PHAC were sent to each regional project manager.

Samples were stored in a cooler with ice and sent to be processed at the central laboratory at the Atlantic Veterinary College. Upon arrival at the laboratory, samples were processed for generic *E. coli*, *Campylobacter* spp., and *Salmonella* spp., in addition to preserving the raw sample following the protocol used by CIPARS [77]. A 1mL aliquot of each sample was saved for potential further processing. If there was growth on any of the three plates, then a single representative bacterial isolate was selected and stored. In 2019, a total of 560 fecal samples were collected and cultured. The proportion of samples positive for each target bacterial species were as follows: *E. coli* - 97.1% (544/560); *Campylobacter* spp. - 63.8% (357/560); and *Salmonell*a - 5.0% (28/560). In 2020, a total of 574 samples were collected and cultured. 94.4% (542/574), 49.1% (282/574) and 7.7% (44/574) of samples were positive for *E. coli*, *Campylobacter* spp. and *Salmonella*, respectively. The information is presented in Table 2.2. Susceptibility testing on the stored isolates was done using the broth microdilution system method (Sensititre™, ThermoFisher, Mississauga). *E. coli* and *Salmonella* were tested against 14 antimicrobials using the CMV2AGNF plate [84], and *Campylobacter* spp. was tested against eight antimicrobials using the CAMPY AST plate designed by the National Antimicrobial Resistance Monitoring System [85]. All results were extracted to a Microsoft Excel (office 16) spreadsheet by the laboratory technicians and uploaded into the central digital platform.

Table 2.2 Proportion (%) of fecal samples positive for target bacteria processed in 2019[a] and 2020[b]

Target bacteria	Calf		Heifer		Cow		Manure pit	
	2019	2020	2019	2020	2019	2020	2019	2020

Generic *E. coli*	97.9	98.6	99.3	99.3	99.3	100.0	92.1	79.7
Campylobacter spp.	31.4	21.5	82.9	66.4	84.3	72.2	56.4	36.4
Salmonella	3.6	3.5	2.1	4.9	2.9	4.9	11.4	17.5

[a]A total of 140 samples were analyzed by each production phase and manure pit.
[b]A total of 144 samples were analyzed by each production phase and manure pit.

During the initial phase of CaDNetASR, the garbage can audit (GCA) was implemented for a period of six months to quantify AMU. The farmers were advised to deposit all the empty antimicrobials vials (bottles, packages, and tubes) in the receptacles, which were placed strategically where antimicrobials might be administered around the farm. The contents of the receptacles were collected and recorded by the regional field workers. In addition, the regional field workers collected information on the antimicrobial inventory at the beginning and the end of the GCA period. The quantities of each antimicrobial were later converted to dose-based metric developed for Canadian dairy cattle as published by Lardé et al. [86]. For the following years, antimicrobial use will be estimated using veterinary clinic dispensing records. A Veterinary Advisory Committee (VAC) composed of three veterinarians was created to help understand how best to extract information from clinic electronic medical records. The surveillance components on AMU and AMR data are summarized in Table 2.3.

Table 2.3 Summary of the key activities of the CaDNetASR on-farm surveillance system.

	AMR	AMU	Questionnaire
Data collection	Annual bulk tank milk and composite fecal samples from: • Pre-weaned calves • Breeding age heifers • Lactating cows • Manure storage	Annual collection of dispensing veterinary records	Annual data collection on management practices (demographics, animal health, biosecurity, AMU)

Data management	Samples are shipped to one central laboratory and cultured for: • Generic *E. coli* • *Campylobacter* spp. • *Salmonella* Antimicrobial susceptibility test (MIC) Freeze-dried isolates bank The results from the laboratory are recorded and uploaded to the central digital platform. All the data is anonymized for privacy protection.	All AMU data are converted to the dose-based metric (DDD/DCD) and uploaded to the central digital platform after being validated by members of the operations committee. All the data is anonymized for privacy protection	Each regional field worker is responsible for recording the questionnaire information into a spreadsheet that is uploaded to the central digital platform after being validated by the regional managers.
Data analysis	Analysis of resistance profiles over time, regions, and sample types	Analysis of AMU converted to DDD and DCD/ 100 animals/year over time, regions, active ingredients, and administration routes	The questionnaires will provide information on potential risk factors that can contribute to the development of AMR, which can impact animal health and animal welfare
Data reporting	• Annual report with summary AMR results and AMU benchmarking for farmers and veterinarians • Scientific publications • CaDNetASR results integrated with CIPARS reports (integrated surveillance data reporting AMU and AMR		

	trends from animals and humans)
Antimicrobial Stewardship	• Development of decision support charts and guidelines for efficient use of antimicrobials • Develop decision support tools and educational material highlighting the importance of the prudent use of antimicrobials • Target interventions on management practices where the use of antimicrobials can be done more responsibly (e.g., dry-cow treatment, udder infections, etc.)
Antimicrobial Stewardship	• Development of decision support charts and guidelines for efficient use of antimicrobials • Develop decision support tools and educational material highlighting the importance of the prudent

	use of antimicrobials • Target interventions on management practices where the use of antimicrobials can be done more responsibly (e.g., dry-cow treatment, udder infections, etc.)

Data were managed through a collaborative and integrated computer system developed to store the data generated by the surveillance system efficiently. All data were standardized, validated, and uploaded to the central digital platform. All information stored in the digital platform was protected by restricted access. The data flow is illustrated in Figure 2.3.

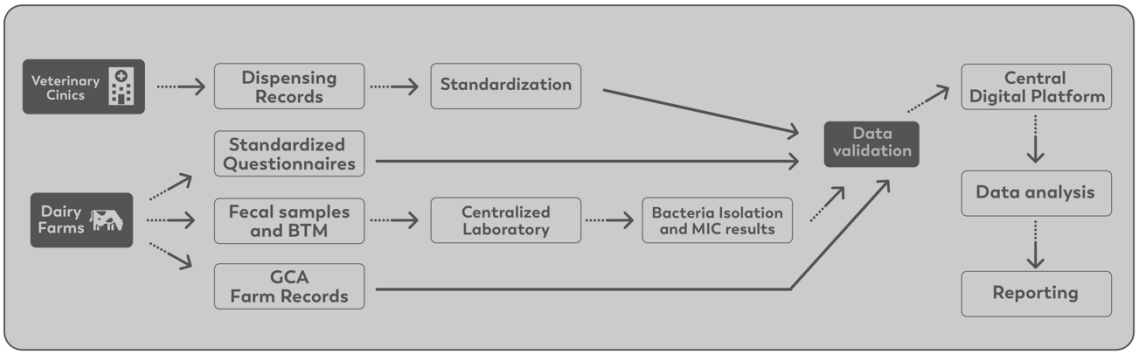

Figure 2.3 CaDNetASR communication policy and data flow. BTM: bulk tank milk; GCA: garbage can audit system; MIC: minimum inhibitory concentration test.

An important component for surveillance systems is knowledge dissemination. There is a diverse group of stakeholders interested in data regarding AMU and AMR in dairy cattle.

These include veterinarians, academia, industry, policymakers, producers, government, public, among others. After each year, summary findings on AMU and AMR are being sent to participating producers and their veterinarians (Figure S2.1 in Appendix A). Reports include benchmarking data on AMU at farm-level from a given year, which allow comparisons within participant farms. The report also includes a summary of AMR in the target pathogens. CIPARS publishes annual reports and will incorporate the dairy cattle data along with other animal species (e.g., pigs, poultry, and turkey). Peer-reviewed publications and abstracts for conferences are being prepared according to data availability.

2.5. Discussion

There is increasing pressure on animal agriculture to justify the use of antimicrobials to treat and prevent infections in animals. Antimicrobial use is the main driver of resistance in target and non-target bacteria in food animals, which can potentially pass to humans via the food chain [87]. In the United States, almost 70% of respondents from the general public believed that AMU in dairy cattle represented a moderate to high threat to human health [88]. In another study in Canada, 28% of the respondents from the general public reported that they preferred not to consume products from animals raised with antimicrobials [89]. The development of CaDNetASR provides AMR and AMU information for another major food animal production system in Canada.

Antimicrobial stewardship is a key factor for mitigating the effects of AMR, but changing how antimicrobials are used on farms can be challenging. To improve AMS in the food animal industries in Canada, all Medically Important Antimicrobials (MIAs) for veterinary use are sold by direction of a veterinary prescription. Additionally, to support AMS by

veterinarians, the CVMA launched the "SAVI" initiative (The Stewardship of Antimicrobials by Veterinarians Initiative). This initiative was supported by the government of Canada and the Canadian Agricultural Partnership. It consists of an electronic platform that has information on AMS and helps veterinary practitioners make informed decisions on AMU in their patients [90]. CaDNetASR will support these initiatives by collecting and analyzing AMU and AMR and determining any changes that may be occurring.

AMS initiatives can have substantial impacts on AMU and AMR on farms. For example, in the Netherlands there are compulsory and voluntary programs that affect AMS in farm animals, including dairy cattle. The RESET Mindset Model was a stewardship strategy used in the Netherlands in the dairy sector aiming to limit the use of critically important antimicrobials and to ban the preventive use of antimicrobials as in blanket dry cow treatment [91]. This model is a behavioural change intervention aimed at more rational use of antimicrobials by farmers and veterinarians and has proven to be effective at reducing AMU. These programs combined with new regulations have resulted in a 56% decrease in total AMU on participating farms between 2007-2012 [92, 93] . In Switzerland, interventions targeting management practices on udder health, uterine health, and calf health were implemented on farms that were followed for three years. The implementation of these interventions provided knowledge for evidence-based decisions that contributes to better AMU stewardship [94].

Most dairy farms in Canada are in the provinces of Quebec and Ontario, and the production of fluid milk is regulated in Canada using a quota system. Federal and provincial

organizations adjust quota to meet expected consumer demand. Milk produced in a province is frequently consumed within the province. Therefore, to ascertain AMR and AMU practices for Canadian dairy herds, it is necessary to conduct surveillance in as many provinces as possible. Each farm is visited annually for sample collection from three different age groups, which can aid in investigating AMR patterns in all stages of dairy production and may help target interventions where they are needed most. Additional data (herd demographic and farm management information) were collected on-farm using two questionnaires. All the information collected is standardized and stored in a central database. In the first two years, the questionnaires were administered using standardized spreadsheets that required manual data entry. In the process of validating these data, input errors were found, which had to be corrected. Automated processes for data entry are preferable to manual entry, and in future years, data will be uploaded from a hand-held device directly to a central database without the need for manual data entry.

The primary outcome of CaDNetASR is to inform the Canadian dairy industry, the general public, and policy decision makers on the level of AMU and AMR, and the impact that AMS practices have on AMU and AMR on Canadian dairy farms. Recently, fifteen countries collecting AMU data at the farm level were identified [53]. Among these countries, twelve have dairy surveillance programs monitoring AMU (Tables S2.3 and S2.4 in Appendix A), and only seven of these countries collect and report AMU at the farm level. A major feature of CaDNetASR is that AMU data is collected at the farm level for dairy cattle. Farm level AMU data results in better estimates of AMU as it can account for the number of exposed animals, exposed time, and biomass on individual farms and allows for benchmarking, which can be used to compare high and low users of antimicrobials [53].

High quality estimates of AMU from surveillance programs are essential to provide reliable results. AMU estimates can be made from a variety of sources. In Denmark, for instance, there is a national, centralized database (VetStat) that collects AMU data at the herd level. The VetStat was implemented in 2000, and the program estimates AMU by collecting antimicrobial dispensing records from pharmacies, veterinarians, and feed mills for individual farms [95]. In the Netherlands, estimation of farm level AMU started in 2004 with the implementation of MARAN (Monitoring of Antimicrobial Resistance and Antimicrobial Usage in Animals in the Netherlands). At the implementation, only a sample of farms was part of the program, and the experience gained with MARAN was used as a base for the development of a sectoral quality assuring system that collects AMU data nationally from the different animal sectors in Netherlands [53]. In 2010, the Netherlands Veterinary Medicines Authority (SDa) was established to receive and centralize the AMU information from the sectoral systems (veterinary prescriptions) and from national sales (pharmaceutical industry). All the AMU information is reported annually through the MARAN program.

Since 2018, the VASR system in Canada has provided an annual report regarding the sales of veterinary antimicrobials considered important for human medicine [96]. The information gathered by the VASR system provides crude estimates of the amount of antimicrobials used in animals in the different agricultural production sectors. This information is adequate to estimate AMU on a national scale but is not precise enough to estimate AMU at the farm level [97].

Efforts in Canada to improve farm-level estimates of AMU are ongoing. One method that has been used is the GCA, which is considered the reference test for farm-level AMU estimates. Garbage can audits are very labor intensive and time consuming, so other approaches for estimating AMU must be found. In Québec, a recent study investigated different methods of collecting AMU data at the farm level [54]. Garbage can audit was used as reference method and were compared with information collected through veterinary invoices, information from the Amélioration de la Santé Animale au Québec (ASAQ) Program (Provincial Government), and farm treatment records. It is important to mention, that in Québec, almost 90% of the veterinary clinics providing antimicrobials to dairy farms, use the same office management software (Vet-Expert software), which facilitates data standardization [54]. Veterinary invoices were found to have almost a perfect agreement with GCA and proved to be a reliable estimate of AMU. In the CaDNetASR system, the collection of veterinary clinics dispensing records was chosen to estimate farm-level AMU. This will demand standardization because of the variety of software packages used by veterinary clinics in Canada (other than the province of QC). To help with this process, 49 veterinary clinics that provided veterinary services, including sales of antimicrobials, to the 144 enrolled dairy herds were contacted and asked about their clinic software and how their AM sales were tracked. Responses from 23 clinics showed that only eight different electronic software systems were being used. Furthermore, there were also many differences in how sales were reported within each system. Consultations with the VAC helped CaDNetASR administrators understand the challenges associated with AMU data extraction from these different systems and to help determine the best approach to clinic engagement for data provision. Members of this group also provided preliminary herd-

level dispensing data, which were helpful in the development of automated routines necessary for the standardization of dispensing record data. This approach to AMU data collection and estimation will improve the quality of the dispensing record data received by CaDNetASR.

Antimicrobial use data collected by CaDNetASR, was transformed into a dose-based metric, to account for the different dosages among the different active ingredients. The dose-based metric divides the total amount of antimicrobial used (mg) by total animal weight and estimate daily dose for the antimicrobial [98]. There is no perfect metric, and the choice of a metric to be used should be made based on the surveillance objectives. Ten countries monitoring AMU at farm level use dose-based metrics to quantify AMU which allow for meaningful and comparable estimates of AMU within the different animal sectors [53]. A specific dose-based metric was developed for dairy cattle in Canada, and it is being used to estimate AMU in the CaDNetASR [86].

In addition to the amount of AMU on farms it is important to determine which antimicrobial is used as well. Some antimicrobials are more important than others in treating infections in humans and their use in animal agriculture should be minimized and used only when other antimicrobials are known to be ineffective. The WHO publishes a regularly updated document, classifying the antimicrobials according to their human importance [99]. In Canada, Health Canada's Veterinary Drugs Directorate (Government of Canada, 2009) has categorized the antimicrobials according to their importance in human and veterinary medicine [100]. These classifications can provide meaningful information to

be included in the AMS goals, aiming to decrease the usage of highly important antimicrobials for human medicine [101].

CaDNetASR is collecting AMR data from the following organisms: *Salmonella spp*., *Campylobacter spp., and E. coli*. These bacteria were selected because they are important zoonotic foodborne pathogens, where AMR is a concern or in the case of generic *E. coli*, it is thought to reflect the reservoir of resistance genes. These bacteria are monitored in other CIPARS surveillance programs [84] and have been recommended by the European Food Safety Authority (EFSA) [56] and the WHO [102]. By monitoring AMR in these target organisms, it may be possible to determine trends in resistance profiles. Ideally, after AMU interventions have been applied to surveillance farms, AMR in the target organisms will decrease and CaDNetASR would be able to measure these changes.

Not all countries report AMR in the same organisms. Among the thirteen countries listed in Table S4, only five provided information regarding AMR in bacterial isolates from dairy cattle in their national reports: Belgium, Denmark, Netherlands, Sweden, and United States. In the United States, NARMS monitors *Salmonella spp*., *Campylobacter spp., Enterococcus* spp., and the indicator *E. coli* from cecal samples of dairy cattle collected at the abattoir [103]. In Belgium (FASFC), Denmark (DANMAP), and Sweden (SVARM), only MRSA *Staphylococcus aureus* is targeted for AMR surveillance in dairy cattle. The most common MRSA clone in production animals is the Livestock Associated MRSA (LA-MRSA), which has been associated with pig production [104]. In Denmark and Sweden, the prevalence of LA-MRSA in dairy production remains low and it is not thought to be of concern in North America either [105, 106] . In Canada, the MRSA in dairy production also

has a limited occurrence. A study conducted in 91 herds across six provinces in Canada screened 1802 *Staphylococcus aureus* isolates for MRSA, and only one isolate was positive (0.05%) [107]. For this reason, the inclusion of MRSA in CaDNetASR was not considered. In the Netherlands (MARAN), annual surveillance for ESBL-producing *E. coli* from cattle fecal samples is reported. After the third year, CaDNetASR will be reporting recovery of ESBL-producing *E. coli* as well. Monitoring ESBL- producing enterobacteria is of critical importance as they pose a threat to human health [108]. To the author's knowledge, CaDNetASR is the only surveillance system for dairy cattle that monitors AMR in enteric bacteria in different production phases and from manure storage.

Another important feature of the CaDNetASR system is the development of an isolate bank. All bacterial isolates will be freeze-dried and stored for future analysis. Although currently whole genome sequencing (WGS) is being done only for *Salmonella* spp isolates, the idea is to expand to other isolates of interest, as it is anticipated that WGS will be routinely done in the future. The isolate bank will allow for the comparison of data from historical isolates to those collected in the future. In some European countries, WGS is being implemented gradually, and it will be mandatory after 2026 [56]. The WGS data can be used as a complementary tool to the phenotypic AMR surveillance data and provide more information on the AMR epidemiology. Another new approach used for AMR detection is the use of metagenomics. Shotgun metagenomics allows for the detection of the entire bacterial community in a sample. If using traditional culture methods only cultivable organism will be detected and some important data may be missed [109]. Whole genome sequencing also relies first on a culture and isolate of an organism, which is no longer required with metagenomic approaches. In the future, the inclusion of metagenomic

approaches to characterize the resistome of a sample will improve the monitoring of the spread of resistance genes and the association between resistance from animals and humans.

2.6. CaDNetASR Surveillance System Limitations

The development of a surveillance system requires an iterative process that will reduce data limitations and biases. Some of these limitations can be interpreted in the context of the main goals of the surveillance system. For instance, dairy farms were recruited by local veterinarians to participate in CaDNetASR. Therefore, the results from these farms should only be extrapolated to the study farms. Participating farmers might be more motivated and might have differing management practices and burdens of AMR compared to non-participating farms. According to the EFSA recommendations samples should come from randomly selected epidemiological units to avoid sampling bias [56]. CaDNetASR enrolled farms were not randomly selected, although, the samples collected within farms, were randomly selected from healthy animals, following the recommendations. Thus, it believed that findings can be extrapolated with caution to similar commercial operations. Data coverage is also a key factor that can affect the interpretation of surveillance results. Ideally monitoring would be conducted on as many farms as possible to obtain more precise results. Although CaDNetASR is not a full coverage system, it includes farms from five different provinces in Canada, and it could be used as a model to expand surveillance in the future.

The cross-sectional design implemented in CaDNetASR can bring disadvantages for supporting causal inferences; however, a major goal of the system is to benchmark AMR /

AMU patterns across years and regions rather than making a causal inference. Three other major limitations can be considered for this surveillance system. 1) Yearly sampling. This sampling scheme will limit the possibility of tracking seasonal variability; however, each farm is sampled during the same season, allowing comparisons over time. 2) Sample type. CaDNetASR is based on pooled samples from three different ages of cattle and samples from two areas of the farm (calves, heifers, cows, manure pit and BTM). In the future, CaDNetASR will evolve to genomic methods, detecting pathogens and AMR genes. Pooled individual samples have been recommended, as it provided optimal results measuring AMR genes at herd level [110]. The surveillance system might miss resistant bacteria occurring in other environments in the farm (e.g., feed, water) which could lead to a low diagnostic sensitivity [111, 112]. However, the sampling scheme used in CaDNetASR includes three age groups, the manure pit and BTM, which will increase the chances of detecting antimicrobial resistant bacteria. 3) Number of isolates. CaDNetASR has not established a required number of isolates to make inferences about the proportion of resistant bacteria. The initial years of CaDNetASR will provide the baseline trend information that will be used to develop sample size calculations for the ongoing surveillance.

Limitations can also occur in other two components of data collection in CaDNetASR: AMU and questionnaire information. AMU was initially estimated using a GCA system, which is time-consuming and prone to human errors. For this reason, all data were validated by each regional field worker to minimize errors. However, it is envisioned for the next years the AMU will be quantified using veterinary dispensing records. In Canada, all the antimicrobials are sold only with a veterinary prescription, thus, it is believed that

veterinary dispensing records can provide a reliable estimation of AMU at farm level. Inaccurate results can arise from questionnaires when response bias occur in data collected. The questionnaires applied during the visits are long, which can demotivate the responders. However, to avoid that, the answers were entered by the regional field workers, that were also responsible to contact again the farmers to fill missing questions or to revise answers. Thus, this procedure is expected to reduce bias.

2.7. Conclusions

In conclusion, the implementation and ongoing development of CaDNetASR are essential to guide AMS on dairy farms across Canada. It will also contribute to the Canadian program for AMR on animal health and public health. Finally, it will help stakeholders in the agricultural commodity groups to achieve more rational AMU on-farm, maintain and improve animal welfare, and support public health by diminishing AMR's burden.

Chapter 3

3. Antimicrobial use and its association with the isolation of and antimicrobial resistance in *Campylobacter* spp. recovered from fecal samples from Canadian dairy herds: A cross-sectional study

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3.1. Abstract

Campylobacteriosis is one of the most common zoonotic diseases in North America. As opposed to humans, animal infections caused by *Campylobacter* spp. are often asymptomatic. In this study, data collected through the Canadian Dairy Network for Antimicrobial Stewardship surveillance system were used to determine the proportion of *Campylobacter* spp. and antimicrobial resistant isolates recovered from dairy cattle herds. Additionally, the association of antimicrobial use (AMU) with fecal carriage and antimicrobial resistance (AMR) of *Campylobacter* spp. were investigated. Pooled fecal samples from 5 animals from each production phase (pre-weaned calves, post-weaned heifers, lactating cows), and a manure storage sample were collected from 140 dairy herds across Canada. Samples were cultured using selective media, and *Campylobacter* isolates were speciated using matrix-assisted laser desorption/ionization-time of flight mass spectrometry. Antimicrobial susceptibilities were determined using the minimum inhibitory concentration test, and interpretation was made according to the Clinical and Laboratory Standards Institute. Two multilevel logistic regression models were used to investigate the association between the AMU with the isolation and antimicrobial resistance in *Campylobacter* spp. Of 560 samples, 63.8% were positive for *Campylobacter* spp., and 96% of the participating farms had at least one sample source (i.e., calves, heifers, lactating cows, or manure storage) positive for *Campylobacter* spp. Overall, 54.3% of the *Campylobacter* spp. isolates were resistant to at least one antimicrobial. Resistance to tetracycline was observed in 49.7% of the *Campylobacter* spp. isolates, followed by ciprofloxacin (19.9%) and nalidixic acid (19.3%). The proportion of multi-drug resistant (≥3 antimicrobial classes) *Campylobacter* spp. isolates was low (0.3%); however, 15.6%

were resistant to two different classes of antimicrobials. Samples collected from lactating cows, heifers, and manure storage were more likely to be positive for *Campylobacter* spp. compared to calves. Total AMU was associated with a decreased probability of recovering *Campylobacter* spp. In addition, AMR to either tetracycline or ciprofloxacin had an interaction with antimicrobial use. The probability of resistance to tetracycline increased for each unit increase in the total AMU (Defined Course Dose/100 animal-years), while the probability of resistance to ciprofloxacin decreased. *Campylobacter coli* isolates were more likely to be resistant to ciprofloxacin and tetracycline when compared to *C. jejuni*. Our study demonstrated that *Campylobacter* spp. is widespread among Canadian dairy farms, and a higher proportion of resistance to tetracycline was identified. The total AMU was associated with increased resistance to tetracycline in *Campylobacter* spp. isolates; however, for ciprofloxacin the AMU was associated with decreased resistance.

Keywords: dairy cattle; antimicrobial use; antimicrobial resistance; *Campylobacter*; risk factors.

3.2. Introduction

Campylobacteriosis is one of the most common zoonotic diseases worldwide [113]. In the European Union, campylobacteriosis in humans were commonly reported [114]. In North America, the incidence of campylobacteriosis is also high. In 2018, the yearly incidence of human infections caused by *Campylobacter* spp. in Canada was higher than for *Salmonella* spp., comprising approximately 29 cases/100,000 population [115]. In the United States, it has been estimated that human infections caused by *Campylobacter* spp. lead to approximately 1.5 million illnesses every year [116]. *Campylobacter jejuni* is isolated from the large majority of infections in humans, with *Campylobacter coli* accounting for most of the rest of infections [116].

In humans, the clinical signs of campylobacteriosis are usually mild to moderate gastrointestinal symptoms, although in some situations, the development of more severe diseases, such as Guillain-Barré syndrome, may occur [117]. Infection in humans is commonly associated with waterborne, foodborne, or environmental exposure as well as direct contact with animals [118]. For instance, in Washington State, living or working on a dairy farm or having contact with cattle was associated with an increased risk for campylobacteriosis in humans [119]. In Canada, ingestion of poultry and beef products was associated with an increased risk for human campylobacteriosis [16].

As opposed to humans, infections with *Campylobacter* spp. in animals are often asymptomatic [17]. Most food-producing animals can be colonized by *Campylobacter* spp. and serve as reservoirs [16]. A meta-analysis using data from studies conducted in the United States and Canada reported that the prevalence estimates of *Campylobacter* spp. in North

American dairy cattle varied from 34% to 64% in individual and pooled fecal samples, respectively [120].

To better understand the reasons for *Campylobacter* spp. colonization in cattle, it is important to investigate risk factors associated with it. A study from Austria identified that the duration of individual housing for calves was associated with *Campylobacter* spp. colonization. In this latter study, calves that were housed individually for longer periods had a decreased odds for being *Campylobacter* spp. positive [36]. Other risk factors such as the presence of poultry and horses on farms lead to higher odds of recovering *Campylobacter* spp. from cattle fecal samples [35, 36]. On dairy farms in Québec, Canada, biosecurity measures, such as cleaning boots and washing or disinfecting the stalls, decreased the odds of recovering *Campylobacter* spp. from lactating dairy cows [121].

The emergence of antimicrobial-resistant *Campylobacter* spp. strains is a major concern, as it can carry resistance to several antimicrobial classes undermining the effectiveness of treatments with antimicrobials of choice (including macrolides and fluoroquinolones) [122]. Several studies have reported fluoroquinolone resistance in *Campylobacter* recovered from poultry, swine, sheep, beef cattle, and dairy cattle [28, 29, 123, 124]. In the United States, the prevalence of resistance to ciprofloxacin in *Campylobacter* spp. isolates recovered from cattle fecal samples ranged from 16.3% to 74.4% [29, 124, 125]. A higher proportion of tetracycline resistance was also observed in *Campylobacter* spp., ranging from 75% for *C. coli* to 88% for *C. jejuni* [124]. There is little information about the prevalence of AMR in *Campylobacter* spp. isolated from dairy cattle in Canada. A study conducted in Ontario in 2017 reported that 71% of the *Campylobacter* spp. isolates recovered from beef cattle,

swine, and dairy cattle were resistant to at least one antimicrobial out of the nine antimicrobials tested [125].

One of the major recognized risk factors for AMR development in livestock is the antimicrobial use (AMU) [70]. Several studies and surveillance systems reported the association between AMU and AMR [46, 48, 70, 126-128]. In these latter studies, a positive association between the use of fluoroquinolone and tetracycline antimicrobial classes and the development of resistance to these same classes was observed in *Campylobacter* spp. isolated from food-producing animals. To the authors' knowledge, there is no research investigating risk factors for AMR in *Campylobacter* spp. isolated from healthy dairy cattle in Canada.

The aims of this study were to: (1) describe the proportion of farms and sample sources positive for *Campylobacter* spp. isolated from fecal and manure storage samples collected on dairy farms from five dairy-producing provinces in Canada, (2) describe the proportion of AMR in *Campylobacter* spp. isolates recovered from the different sample sources in the dairy farms; (3) investigate the association between the AMU and the isolation of *Campylobacter* spp. (while accounting for confounders), and (4) investigate the association between AMU and AMR (while accounting for confounders) in *Campylobacter* spp. isolates.

3.3. Materials and Methods

The data of this study were collected by the Canadian Dairy Network for Antimicrobial Stewardship and Resistance (CaDNetASR), a surveillance system implemented in 2019 [129]. The recommendations for reporting observational studies were followed throughout the

text (STROBE-vet guidelines) [130]. The study was reviewed and approved by the University of Prince Edward Island Research Ethics Board and the Animal Care Committee on March 7, 2019 (file # 6008059).

3.3.1. Sample size, Farm Selection, and Sampling

Detailed information on sample size and farm enrollment was described elsewhere [129]. Briefly, a convenience sample of 140 dairy farms located in five Canadian provinces (British Columbia, Alberta, Ontario, Quebec, and Nova Scotia) were enrolled in CaDNetASR. Enrolled farms had a minimum of 50 animals except for Nova Scotia, which had a minimum size of 40 animals. Participating farms also needed to raise their replacement heifers on-site and provide antimicrobial purchase history, which was obtained from their herd veterinarian and feed mill. On each farm, pooled fecal samples (5 fresh fecal pats selected from different places on the floor) from each of 3 age groups (pre-weaned calves, post-weaned heifers, and lactating cows) and a manure storage sample were obtained in 2019. Samples were stored in coolers with ice packs and sent for processing at the University of Prince Edward Island. During the sampling visits, questionnaires were applied by CaDNetASR regional field workers to collect information on potential risk factors that were used in the regression analysis. The questionnaires obtained information on herd demographics, herd health, biosecurity, and AMU. Details on the questionnaires and sampling procedures were reported previously [129].

3.3.2. Antimicrobial Use

AMU data were obtained through a garbage can audit (GCA) [83, 131], except for the province of Québec, which obtained AMU information through veterinary invoices (Vet-Expert

software or Sysvet software), a method previously validated for this purpose [54]. AMU information was converted to a rate indicator – Defined Course Dose (DCD (dose for a standardized complete treatment course on a standard animal))/100 animal-years according to a previously validated metric for Canadian dairy cattle [86]. To explore the effect of AMU administered through different routes, total AMU was split into systemic or intramammary administration routes. Systemic use was defined as the sum of antimicrobials administered through oral, intravenous, intramuscular, and subcutaneous routes in DCD/100 animal-years. Intramammary use was defined as the sum of antimicrobials administered through this route in DCD/100 animal-years for lactating and dry cows. The AMU was also categorized based on their importance to human medicine according to the Public Health Agency of Canada (I-Very high importance for humans, II-High importance for humans, or III-Medium importance for humans) [132], and the World Health Organization (WHO) (HPCIA = highest priority critically important antimicrobials; CIA = high-priority critically important antimicrobials; HIA = highly important antimicrobials) [133]. Data on milk replacer and medicated feed were not captured by CaDNetASR. Details on AMU data collection are reported elsewhere [129].

3.3.3. Bacteriological Culture and Species Identification

Culturing of fecal samples for isolation of *Campylobacter* spp. was done according to the Canadian Integrated Program of Antimicrobial Resistance Surveillance (CIPARS) protocol [134]. Briefly, 25 g of feces from each sample was individually homogenized in 250 mL of buffered peptone water using a stomacher for 30 sec at 230 rpm. Thereafter, 1 mL of the homogenized sample was inoculated in a tube containing 9 mL Hunt's Enrichment Broth (HEB) and incubated under microaerophilic conditions at 35°C for 4 h. After 4 h of

incubation, 36 µL of cefoperazone was added to each tube and incubated under microaerophilic conditions at 42°C for 20-24 h. A loop of the HEB solution was streaked onto a modified charcoal-cefoperazone-deoxycholate agar (mCCDA) plate. A filter paper soaked with 1-2 drops of glycerol was added to each plate to prevent swarming, facilitating to pickup of isolated colonies. The plates were incubated under microaerophilic conditions at 42°C for 24 h. Colonies typical for *Campylobacter* spp. were transferred to another mCCDA plate and incubated for another 24 h. *Campylobacter* strain ATCC43501, and *Escherichia coli* strain ATCC25972 were used as quality control. Species identification was performed by matrix-assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF MS) using the Bruker Microflex MALDI-TOF MS (Bruker Daltonics, GmbH, Bremen, Germany). A single colony was transferred onto the target plate, air-dried at ambient temperature, and overlaid with alfa-cyano-4-hydroxycinnamic acid before being introduced into the MALDI-ToF mass spectrometer for automated measurement of mass spectra and comparison to the reference database (MBT 8468 MSP Library). Identification scores ≥ 2.0 were required for confident species identification.

3.3.4. Antimicrobial Susceptibility Testing

Antimicrobial susceptibility was determined using the Sensititre microdilution system using the *Campylobacter* CMVCAMPY plate (Trek Diagnostic Systems Inc., Westlake, OH, USA). The plate contained twofold serial dilutions of the following antimicrobials: azithromycin - AZM (0.015-64 µg/mL), ciprofloxacin - CIP (0.015-64 µg/mL), erythromycin - ERY (0.03-64 µg/mL), tetracycline - TET (0.06-32 µg/mL), florfenicol - FLR (0.03-32 µg/mL), nalidixic acid - NAL (4-64 µg/mL), clindamycin – CLI (0.03-16

μg/mL), and gentamycin - GEN (0.12-32 μg/mL). *Campylobacter* strain ATCC33560 was used as a quality control strain. The minimum inhibitory concentration (MIC) values were the lowest concentrations of antimicrobial that inhibited the visible growth of bacteria. If growth was observed at the highest antimicrobial concentration tested, the MIC was assigned to the next dilution. Interpretation of antimicrobial resistance was set according to the Clinical and Laboratory Standards Institute (CLSI) clinical breakpoints recommended by CIPARS [134].

3.3.5. Statistical Analysis

For laboratory data, the proportion of fecal and manure samples that were *Campylobacter* spp. positive were determined. Frequency distributions of MIC, MIC_{50}, and MIC_{90} were calculated for each antimicrobial for each isolate. Isolates with intermediate resistance were grouped with resistant isolates. Multidrug resistance (MDR) was defined as resistance to ≥ 3 antimicrobial classes [135]. For all statistical analyses, the unit of analysis was the sample obtained from a given sample source (calves, heifers, lactating cows, or manure storage). Each of these samples was defined by one *Campylobacter* spp. isolate. The five *Campylobacter lari* isolates were not included in the resistance and regression analysis due to their low frequency of isolation and because this species is infrequently associated with infections in humans [136].

3.3.5.1. Questionnaire variables

A total of 84 variables from the surveillance questionnaire (22 from the demographic section, 45 from herd health, and 17 from biosecurity) were explored to select a subset to use as predictors for the regression models. To select the variables to be included in the

analysis, an exploratory factor analysis was done. To do this, all continuous variables were temporarily transformed into binary variables. Continuous variables were re-scaled between 0 and 1 based on their median value. Unbalanced variables, with more than 95% of the values in one category were excluded from the analysis. Factor analysis using tetrachoric correlation was performed on the remaining, transformed variables to help identify patterns (variables representing the same information) and select the variables to be used in the regression models. After factor analyses, 11 transformed variables from the questionnaire were used for regression analysis and their selection was made based on the factor loadings after varimax rotation (variables with loadings ≥0.6 in each factor).

3.3.5.2. Risk factor modelling

A two-level, multivariable, logistic regression model with farm as a random intercept was used to determine the association between AMU with the probability of recovering *Campylobacter* spp. from a sample while accounting for confounding (model 1). In addition, a three-level, multivariable logistic model with farm and sample (two different antimicrobials per sample – tetracycline and ciprofloxacin) as random intercepts was built to explain the association between AMU with the recovery of ciprofloxacin and tetracycline resistant isolates from farms while accounting for confounding (model 2). For model 2, the other antimicrobials were not included in the outcome as the low prevalence of resistance (≤ 1.0%). As the resistance patterns of ciprofloxacin and nalidixic acid were similar (> 97% of isolates were resistant to both ciprofloxacin and nalidixic acid), only ciprofloxacin was retained in the model as a better estimation of the variance at the sample

level was obtained. The inferences made for resistance to ciprofloxacin can be made in parallel for nalidixic acid.

3.3.5.3. Variables selection for models 1 and 2

Chi-square tests were used to assess associations among the categorical predictors. Associations between continuous (AMU) and categorical variables were assessed using Kruskal-Wallis rank test. Correlation between the binary and continuous variables was assessed using Pearson correlation. Unconditional associations between the explanatory variables and the outcome were examined using the previously described multilevel, logistic regression models, but using only one predictor at the time. Only those variables with P values ≤ 0.20 were selected for inclusion in the multivariable models. Linearity for the continuous predictors was visually assessed using a scatter plot with a smoothed curve on the logit scale. Polynomial models were explored to further investigate the linearity assumption. Continuous variables that violated linearity and that were not significant in the polynomial models were then categorized based on their distribution frequency (quartiles). Two directed acyclic graphs (DAGs) including the main predictors for models 1 and 2 were built to help with the model building. The DAGs are illustrated in Figures S3.1 and S3.2 (Appendix B), for models 1 and 2, respectively. The main exposure in both models were the systemic and intramammary AMU (as two different predictors) converted to DCD/100 animal-years. Additional information on each active ingredients that were included in the total AMU can be found in the Appendix B (Tables S3.1 and S3.2). Season and sample source were included in both models. In addition, AMR by antimicrobial class and *Campylobacter* species were included in model 2. The *Campylobacter* species variable was included in model 2 as the objective was to investigate potential differences in resistance

at species level. Based on the factor analysis, 11 other independent variables, were examined in both models. These variables were province, herd size, barn type, frequency of veterinary visits, infected young stock, infected lactating cows, treated young stock, treated lactating cows, use of veterinary protocols, multiple species of livestock on the farm, and biosecurity practices. The description for each variable is presented in Table S3.3 (Appendix B). Model building was conducted as suggested by Dohoo et al. [137]. A backward manual selection was used to determine if any predictors were left out of the final model. Excluded potential confounding variables for AMU (main exposure) were reintroduced in the model and retained if a change of > 20% was observed in other coefficients. Biologically plausible interaction terms were explored. Predictors with Wald test *P*-value ≤ 0.05 were retained in the final model. Pairwise comparisons using Bonferroni's method were done for categorical predictors with more than two levels. Visual examination of the plot of residuals vs. predicted values did not show any significant pattern that could indicate lack of homoscedasticity. The residuals were also visually inspected for normality. Intraclass correlation coefficients (ICC) were calculated for the final model using latent variable approximation to estimate clustering within herds and within-sample type (model 2) [137]. The odds ratio was adjusted from cluster-specific to the population average using the formula below [137]:

$$\beta PA = \frac{\beta CS}{\sqrt{(1 + 0.346 * \sigma^2)}}$$

where βCS is the cluster-specific (CS) estimate to be converted to the population average (PA), and σ^2 is the sum of variances of the random effects. Statistical analyses were performed using Stata SE version 16.1 (StataCorp LLC, College Station, TX).

3.4. Results

3.4.1. Farm Characteristics and Sampling

The CaDNetASR surveillance system recruited 140 dairy farms across the five provinces in 2019. All provinces had enrolled 30 farms except Nova Scotia (n=24) and British Columbia (n=26). In 2019, 140 composite fecal samples each from pre-weaned calves, breeding-age heifers, lactating cows, and manure storage were collected, totaling 560 fecal samples.

3.4.2. Antimicrobial Use

A total of 131 participating farms provided information on AMU. The total AMU varied substantially among farms; however, all farms were using antimicrobials (Figure 3.1). The total AMU median value was 86.8 DCD/100 animal-years, ranging from 1.9 to 499.3 DCD/100 animal-years (Table 3.1). Median intramammary and systemic AMU DCD/100 animal-years were 49.8 and 25.0, respectively. Systemic and intramammary AMU represented 37.8% and 60.6% of the total use, respectively. The remaining 1.6% was the sum of intrauterine (1.4%) and topical (0.2%) AMU. The use of antimicrobials classified as the highest importance in human medicine (Health Canada category I and WHO HPCIA) represented approximately 18% and 20% of the total AMU, respectively (Table 3.2). Antimicrobial classes classified as highly important in human medicine included third-generation cephalosporins, polymyxins, fluoroquinolones, and macrolides.

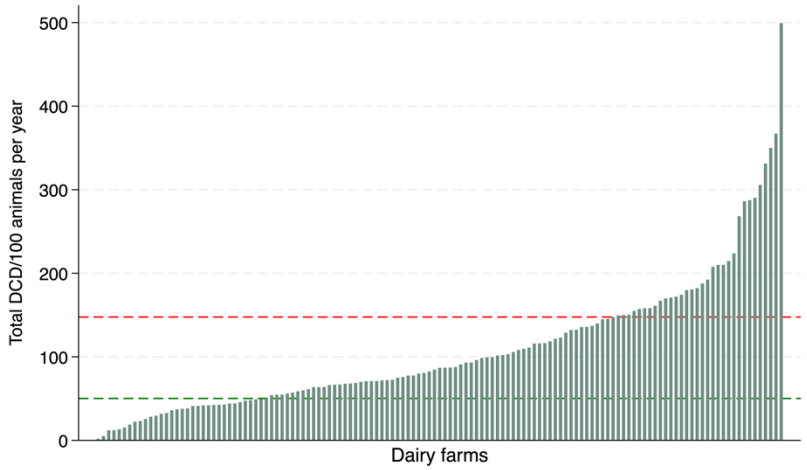

Figure 3.1 Total antimicrobial use variability among the 131 farms. The green line indicates the first quartile (50.2 Defined Course Doses/100 animal-years), and the red line indicates the third quartile (147.6 Defined Course Doses/100 animal-years).

Table 3.1 Total antimicrobial use in Defined Course Doses (DCD)/100 animal years of 131 dairy farms participating in CaDNetASR during 2019[a].

Province	No. farms	Mean	Min.	Percentile			Max.
				25th	50th	75th	
British Columbia	24	76.6	4.9	41.8	61.3	100.5	223.7
Alberta	30	136.6	18.8	71.7	125.8	180.6	367.1
Ontario	30	128.5	12.1	59.7	98.7	174.2	499.3
Quebec	27	76.7	1.9	42.4	63.8	93.1	350.0
Nova Scotia	20	116.7	12.0	66.4	111.1	147.4	305.7
Total	131	108.4	1.9	50.2	86.8	147.6	499.3

[a] Estimates were obtained using a garbage can audit, excepted for Québec farms.

Table 3.2 Antimicrobial use according to Health Canada categories and WHO in Defined Course Doses (DCD)/100 animal years from 131 dairy farms participating in CaDNetASR during 2019[a].

Health Canada	Min.	Mean	Percentile			Max.
			25th	50th	75th	
Category I – Very high importance	0.0	25.5	4.6	15.6	34.4	227.2
Category II – High importance	0.0	60.8	29.6	52.9	79.9	280.5
Category III – Medium importance	0.0	12.2	1.3	5.2	11.4	283.9
Category IV – Low importance	0.0	0.0	0.0	0.0	0.0	0.0
WHO						
HPCIA	0.0	28.6	6.1	17.3	37.3	227.2
CIA	0.0	8.8	0.0	1.8	14.6	69.4
HIA	0.0	56.2	27.2	48.3	74.0	297.8

[a] Estimates were obtained from a garbage can audit, except for Québec farms.
HPCIA = highest priority critically important antimicrobials; CIA = high-priority critically important antimicrobials; HIA = highly important antimicrobials.

3.4.3. Campylobacter spp. isolation

Out of 560 samples, 63.8% (357/560) were positive for *Campylobacter* spp. The most commonly identified species was *Campylobacter jejuni* (92.7%, 331/357), followed by *C. coli* (5.9%, 21/357) and *C. lari* (1.4%, 5/357). A higher proportion of *Campylobacter* spp. were isolated from samples from lactating cows (84.3%) and heifers (82.9%). The proportion of positive samples by sample source and province are summarized in Table 3.3. The proportion of farms with at least one positive sample for *Campylobacter* spp. was 95.7% (134/140). The number of positive samples for *Campylobacter* spp. per farm is presented in Figure 3. 2.

Table 3.3 Percentage (%) of *Campylobacter* spp.-positive samples over provinces by sample source in 2019.

Province	Calf[1] n=140	Heifer[2] n=140	Lactating Cow n=140	Manure storage n=140
British Columbia	11.5	84.6	88.5	38.5
Alberta	40.0	83.3	96.7	83.3
Ontario	36.7	80.0	93.3	63.3
Quebec	30.0	80.0	66.7	43.3
Nova Scotia	37.5	87.5	75.0	50.0
Total	31.4	82.9	84.3	56.4

[1]Pre-weaned calves
[2]Breeding age heifers

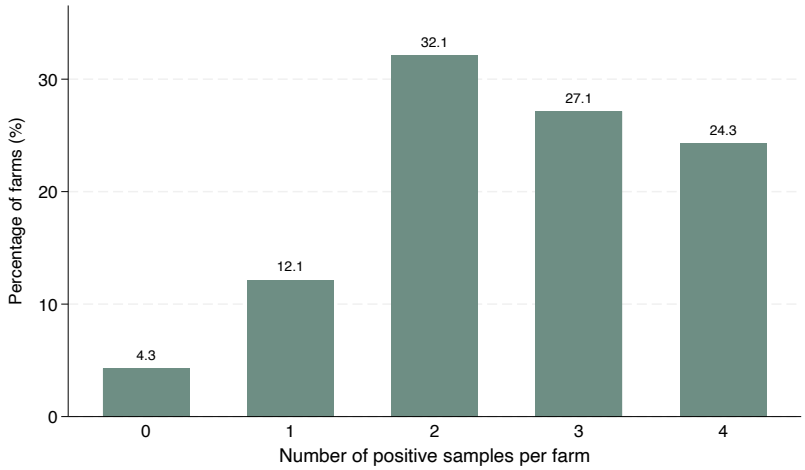

Figure 3.2 Number of positive samples for *Campylobacter* spp. per farm. On each farm, up to four samples were collected (pre-weaned calves, breading age heifers, lactating cows, and manure storage).

3.4.4. Antimicrobial Susceptibility

A total of 73.1% (98/134) of dairy cattle herds had at least one *Campylobacter* spp. isolate resistant to at least one of the antimicrobials included in the panel. Overall, 54.3% (191/352) of *Campylobacter* spp. isolates were resistant to at least one antimicrobial. Resistance to tetracycline was present in 49.7% of the *Campylobacter* spp. isolates, followed by ciprofloxacin (19.9%) and nalidixic acid (19.3%). The proportion of MDR *Campylobacter* spp. was low (0.3%); however, 15.6% of the isolates were resistant to two different classes of antimicrobials (tetracycline and quinolones or tetracycline and macrolide). The proportion of isolates pan-susceptible was 45.7%. The two most common resistant patterns were TET (33.8%) and CIP-NAL-TET (14.5%) (Table 3.4). Resistance to macrolides was very low (only two *C. jejuni* isolates were resistant to azithromycin). The proportion of resistant isolates for each antimicrobial by sample type is illustrated in Figure 3.3. The proportion of *Campylobacter* spp. resistant to each of the eight antimicrobials tested, and the MIC distribution is presented in Table 3.5. The results were

aggregated into 2 categories: *Campylobacter jejuni* (n=331) and *Campylobacter coli* (n=21). A heat map representing the resistance pattern, sample source and province where the 191 resistant-*Campylobacter* spp. isolates were recovered is illustrated in Figure 3.4.

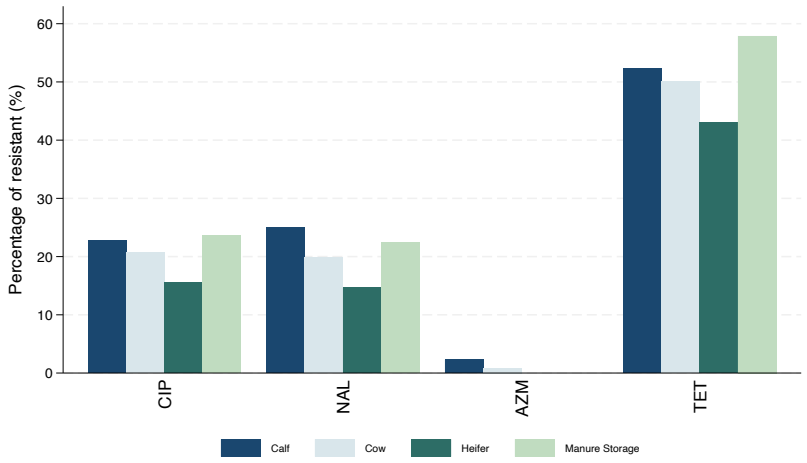

Figure 3.3 Percent of resistant isolates for each antimicrobial tested for the four sample sources. CIP: ciprofloxacin; NAL: nalidixic acid; AZM: azithromycin; TET: tetracycline. No resistance was observed for erythromycin, clindamycin, florfenicol, and gentamicin.

Table 3.4 Antimicrobial resistance patterns of 331 *C. jejuni* and 21 *C. coli* isolates recovered from dairy farms fecal and manure storage samples.

Antimicrobial pattern[1]	No. isolates (%)
Pan-susceptible	161 (45.7%)
TET	119 (33.8%)
CIP-NAL-TET	51 (14.5%)
CIP-NAL	16 (4.5%)
CIP-TET	2 (0.6%)
CIP-TET-AZM*	1 (0.3%)
TET-AZM	1 (0.3%)
NAL-TET	1 (0.3%)
Total	352 (100%)

[1]CIP: ciprofloxacin; NAL: nalidixic acid; TET: tetracycline; AZM: azithromycin

* Multidrug resistant isolates (resistant to ≥3 antimicrobial classes).

Table 3.5 Distribution of minimum inhibitory concentrations (MIC) for *Campylobacter jejuni* (n=331), and *Campylobacter coli* (n=21) recovered from dairy farms fecal samples in 2019.

Antimicrobial	Species	Range	% Res.[1]	0.01	0.03	0.06	0.12	0.25	0.5	1	2	4	8	16	32	64	128	MIC_{50}[2]	MIC_{90}[3]
GEN	*C. jejuni*	0.12-32	0.0				5.8	15.7	58.6	19.6	0.3	0.0	0.0	0.0	0.0			0.5	1.0
	C. coli	0.12-32	0.0				0.0	0.0	23.8	71.4	4.8	0.0	0.0	0.0	0.0			1.0	1.0
ERY	*C. jejuni*	0.03-64	0.0		0.0	2.1	7.9	54.4	33.5	1.8	0.3	0.0	0.0	0.0	0.0	0.0		0.25	0.5
	C. coli	0.03-64	0.0		0.0	0.0	0.0	0.0	4.7	33.3	52.4	0.0	0.0	0.0	0.0	0.0		2.0	2.0
AZM	*C. jejuni*	0.015-64	0.6	16.9	58.0	23.9	0.6	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.6	0.03	0.06
	C. coli	0.015-64	0.0	0.0	0.0	23.8	71.4	4.8	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0		0.12	0.12
CLI	*C. jejuni*	0.03-16	0.0		2.1	21.2	49.2	24.2	3.0	0.3	0.0	0.0	0.0	0.0				0.12	0.25
	C. coli	0.03-16	0.0		0.0	0.0	0.0	28.6	47.6	23.8	0.0	0.0	0.0	0.0				0.5	1.0
FLR	*C. jejuni*	0.03-32	0.0		0.6	0.0	1.2	6.9	20.9	68.6	1.8	0.0	0.0	0.0	0.0			1.0	1.0
	C. coli	0.03-32	0.0		0.0	0.0	0.0	0.0	0.0	52.4	47.6	0.0	0.0	0.0	0.0			1.0	2.0
CIP	*C. jejuni*	0.015-64	17.2	0.0	2.1	33.6	45.0	1.8	0.3	0.3	0.0	1.5	11.5	3.6	0.3	0.0		0.12	8.0
	C. coli	0.015-64	61.9	0.0	0.0	0.0	33.3	4.8	0.0	0.0	0.0	0.0	0.0	33.3	23.8	4.8		16.0	32.0
NAL	*C. jejuni*	4-64	16.6									57.1	25.1	1.2	0.6	2.1	13.9	4.0	128.0
	C. coli	4-64	61.9									0.0	14.3	23.8	0.0	0.0	61.9	128.0	128.0
TET	*C. jejuni*	0.06-32	48.6			2.7	16.3	25.4	4.8	1.2	0.3	0.6	0.6	2.1	5.5	16.3	24.2	1.0	128.0
	C. coli	0.06-32	66.7			0.0	0.0	0.0	4.8	28.6	0.0	0.0	0.0	0.0	0.0	4.8	61.9	128.0	128.0

Vertical lines indicate CLSI breakpoints. AZM: azithromycin; CIP: ciprofloxacin; CLI: clindamycin; ERY: erythromycin; FLR: florfenicol; GEN: gentamycin; NAL: nalidixic acid; TET: tetracycline.

[1]% of resistant isolates

[2] The MIC value that inhibits growth of 50% of the isolates

[3] The MIC value that inhibits growth of 90% of the isolates

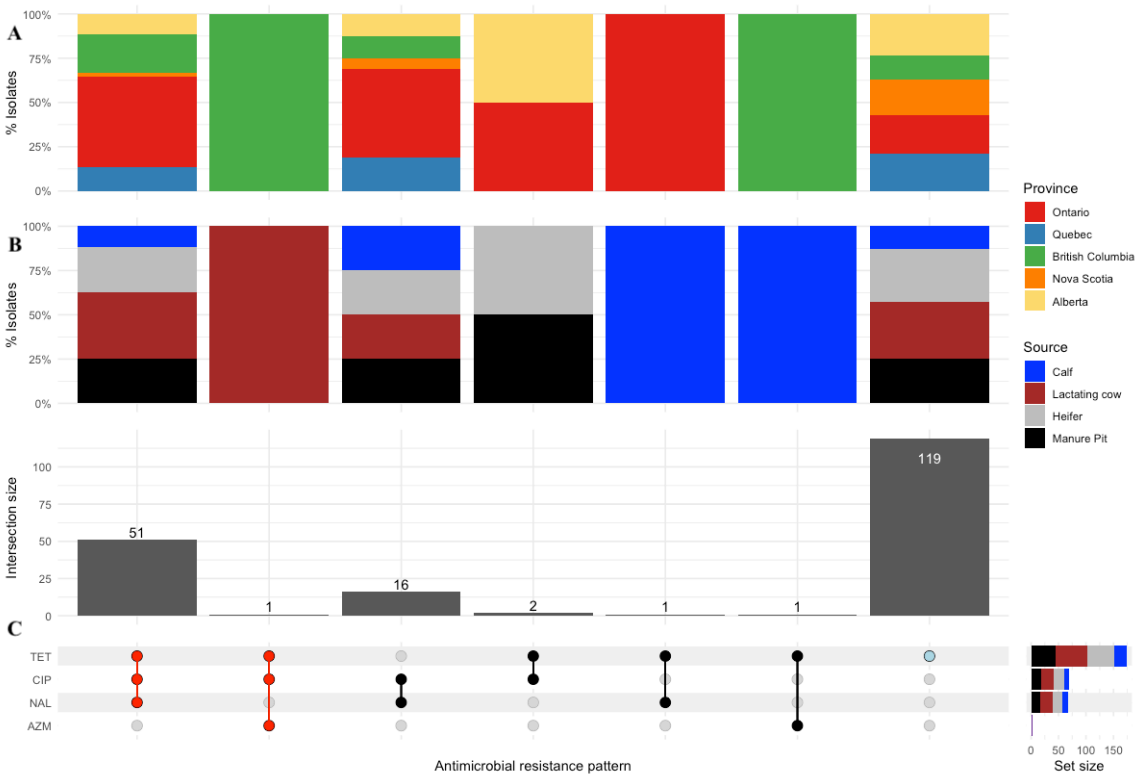

Figure 3.4 Heat map presenting the resistance patterns of the 191 *Campylobacter* spp. isolates with resistance to at least one antimicrobial that were recovered from fecal samples collected in the 140 dairy herds enrolled in CaDNetASR. A Province where the isolates were recovered; B Sample source where the isolates were recovered; C Number of isolates and their respective phenotypic resistance pattern. The different colors for the connected dots represent how many antimicrobials an isolate was resistant to: red= resistant to three antimicrobials; black=resistant to two antimicrobials; and light blue: resistant to one antimicrobial. TET: tetracycline; CIP: ciprofloxacin; NAL: nalidixic acid; AZM: azithromycin.

3.4.5. Factor Analysis and Multilevel Logistic Regressions

The variables created based on the results from the factor analysis are presented in Appendix B, Table S3.3.

3.4.6. Risk factors for Campylobacter spp. isolation (model 1)

The multilevel model examining risk factors for a sample being *Campylobacter* spp.-positive (model 1) included 131 of the 140 farms, as only 131 farms provided AMU information. Descriptive data for the variables considered in the model are presented in Tables 3.6 and 3.7.

Table 3.6 Unconditional association of the farm level (n=131) and sample level (n=524) categorical predictors with the isolation of *Campylobacter* spp. recovered from fecal samples collected from calves, heifers, lactating cows, and manure storage (model 1).

Section	Variable	Category	Frequency (n=131)	OR	Overall *P*-value
Demographics	Herd size	≤ 70 lactating cows	31	Baseline	0.096
		71-160 lactating cows	61	1.36	
		≥161 lactating cows	39	0.75	
	Barn type	Tie-stall	32	Baseline	0.200
		Free-stall	99	1.42	
	Province	British Columbia	24	Baseline	0.022
		Alberta	30	2.79	
		Ontario	30	1.86	
		Quebec	27	1.02	
		Nova Scotia	20	1.40	
Herd health	Veterinary visits	More visits for herd health and less visits for sick animals	40	Baseline	0.548
		Less visits for herd health or more visits for sick animals	65	0.75	
		Less visits for animal health and more visits for sick animals	26	0.91	

	Infected young stock (number of diseases reported)	≤ 2 diseases	33	Baseline	0.719
		3 to 4 diseases	73	0.79	
		5 diseases	25	0.79	
	Infected lactating cows (number of diseases reported)	≤ 1 disease	14	1.80	0.21
		2 diseases	26	0.77	
		≥ 3 diseases	91	Baseline	
	Treated young stock (record of treatment for a given disease) Treated	≤ 2 diseases	32	Baseline	0.644
		3 to 5 diseases	77	1.01	
		≥ 6 diseases	22	0.74	
	Treated lactating cows (record of treatment for a given disease)	≤ 2 diseases	30	Baseline	0.514
		3 to 4 diseases	50	0.89	
		5 diseases	51	0.71	
	Veterinary protocols for a given disease developed by a veterinarian	No protocol	56	Baseline	0.939
		Protocols for 1 to 5 diseases	41	0.91	
		Protocols for more than 6 diseases	36	0.92	
Biosecurity	Biosecurity	Only biosecurity practices other than vaccination	8	3.36	0.008
		At least one biosecurity practices and vaccines for 1 group	43	0.66	
		At least one biosecurity practices and vaccines for 2 groups	38	1.45	
		At least one biosecurity practices and vaccines for 3 groups	42	Baseline	
	Raise multiple livestock species	No	110	Baseline	
		Yes	21	1.55	0.188
Other	Season	Fall[1]	412	Baseline	0.889
		Winter[2]	112	0.95	

Sample Source	Calf	131	Baseline	<0.001
	Heifer	131	27.52	
	Cow	131	34.32	
	Manure storage	131	4.99	

OR: Odds ratio
[1]September to November
[2]December to March

Table 3.7 Unconditional association of the farm level (n=131) continuous predictors (AMU[1]) with the isolation of *Campylobacter* spp. recovered from fecal samples collected from calves, heifers, lactating cows, and manure storage (model 1).

Variable	No. farms	Percentile			OR[2]	Overall *P*-value
		25th	50th	75th		
Total AMU[3]	131	50.2	86.8	147.6	0.95	0.723
Systemic AMU[3]	131	15.1	25.0	38.6	1.11	0.713
Intramammary AMU[3]	124	23.9	49.8	99.1	0.89	0.515

[1]AMU in DCD/100 animals-year. Estimates were obtained from a garbage can audit, except for Quebec farms.
[2]OR: Odds ratio
[3]Estimates were multiplied by 100 before converted to odds ratio.

A total of 7 variables met the criteria to be included in the multivariable model (herd size, barn type, province, infected lactating cows, biosecurity, raise multiple livestock species, and sample source). The total AMU was highly correlated with intramammary AMU (correlation =0.86). Additionally, the intramammary and systemic AMU were not significant in the final model when modelled separately. The total AMU was chosen to be included in the final model (total AMU was forced in the final model as it was the main exposure) as it included all the active ingredients and the effect in the final model was stronger than intramammary AMU. The variable barn type was excluded from the final model as it was associated with herd size ($P \leq 0.01$) (Figure S3.1). The variable raise multiple livestock was not significant in the final model ($P=0.27$). Logistic regression model results are presented in Table 3.8. The total AMU was associated with a trend towards decreased probability of recovering *Campylobacter* spp. from the samples (borderline significant, $P=0.06$). For instance, movement from the first quartile to the third

quartile of total AMU (25th percentile= 50.2 DCD/100 animal-years to 75th percentile =147.6 DCD/100 animals-year), decreased the odds of recovering a *Campylobacter* spp. isolate from any sample type by approximately 30%. The odds of recovering a *Campylobacter* spp.-positive sample from lactating cows, heifers or the manure storage were higher compared to calves. Farms that did not use any vaccines were more likely to have a *Campylobacter* spp.-positive sample compared to farms that used vaccines for at least one group. There were no differences among farms using vaccines. The intraclass correlation of herd level was 0.29, meaning that there is a moderate clustering effect among farms.

Table 3.8 Final multilevel logistic regression model for the isolation of *Campylobacter* spp. from 524 fecal samples (131 farms) collected from calves, heifers, lactating cows, and manure storage (model 1).

	β	SE	OR[1]	95% CI		*P* value	Overall *P* value
Intercept	-0.92	0.69	-	-	-	-	-
Province							0.001
British Columbia	Ref.						
Alberta	1.88	0.57	4.73	1.93	11.59	0.002	
Ontario	1.14	0.57	2.56	1.05	6.25	0.039	
Quebec	-0.26	0.56	0.81	0.33	1.95	0.632	
Nova Scotia	0.46	0.60	1.46	0.56	3.82	0.439	
Sample Type							<0.001
Calves	Ref.						
Heifers	3.32	0.42	14.62	7.49	28.52	<0.001	
Lactating cows	3.53	0.44	17.43	8.71	34.87	<0.001	
Manure storage	1.62	0.33	3.70	2.18	6.30	<0.001	
Biosecurity[2]							0.002
No vaccine	2.12	0.84	5.75	1.47	22.48	0.012	
Vaccines for 1 group	-0.36	0.42	0.75	0.37	1.49	0.405	
Vaccines for 2 groups	0.95	0.44	2.19	1.08	4.44	0.029	
Vaccines for 3 groups	Ref.						
Total AMU[3,4]	-0.40	0.21	0.70	0.51	1.01	0.057	
Lactating herd size							0.006
36 to 70	Ref.						
71 to 160	0.14	0.44	1.11	0.55	2.26	0.756	
>160	-1.17	0.52	0.38	0.16	0.89	0.026	
Variance	Estimate	SE					
Herd level	1.36	0.57					

[1]OR: odds ratio adjusted to population average.

[2]Biosecurity: vaccines were group as follow: vaccines for calves, vaccines for adult animals, and vaccine for mastitis. The category vaccines for 3 groups means the farm was using vaccines for all above mentioned groups.
[3]Total DCD/100 animals-year.
[4]Estimates multiplied by 100.

3.4.7. Risk factors for resistance in Campylobacter spp. (model 2)

The model looking at the association between AMU and resistance to ciprofloxacin and tetracycline (model 2) included 125 of the 134 farms that had at least one *Campylobacter* spp.-positive sample, as nine of the farms did not provide complete information for either the risk factors variables or AMU and were excluded from the analysis. Descriptive data for the variables considered in model 2 are presented in Tables 3.9 and 3.10.

Table 3.9 Unconditional association of the herd level (n=125), sample level (n=331), and antimicrobial level (n=662) categorical predictors with resistance to tetracycline and ciprofloxacin (model 2).

Section	Variable	Categories	Frequency (n=125)	OR	Overall *P*-value
Demographic	Herd size	≤ 70 lactating cows	29	Baseline	0.962
		71 to 160 lactating cows	59	1.06	
		≥ 161 lactating cows	37	1.1	
	Barn type	Tie-stall	30	Baseline	0.460
		Free-stall	95	1.26	
	Province	British Columbia	23	Baseline	<0.001
		Alberta	29	0.57	
		Ontario	30	2.59	
		Quebec	25	0.84	
		Nova Scotia	18	0.57	
Herd health	Veterinary visits	More visits for herd health and less visits for sick animals	38	Baseline	0.393
		Less visits for herd health or more visits for sick animals	62	1.50	
		Less visits for animal health and more visits for sick animals	25	1.19	
	Infected young stock (number of diseases reported)	≤ 2 diseases	32	Baseline	0.151
		3 to 4 diseases	69	1.14	
		5 diseases	24	2.06	

	Infected lactating cows (number of diseases reported)	≤ 1 disease	14	0.97	0.363
		2 diseases	25	0.61	
		≥ 3 diseases	86	Baseline	
	Treated young stock (record of treatment for a given disease)	≤ 2 diseases	30	Baseline	0.158
		3 to 5 diseases	74	1.82	
		≥ 6 diseases	21	2.04	
	Treated lactating cows (record of treatment for a given disease)	≤ 2 diseases	29	Baseline	0.384
		3 to 4 diseases	47	1.22	
		5 diseases	49	1.59	
	Veterinary Protocols (protocol for a given disease developed by a veterinarian)	No protocol	51	Baseline	0.515
		Protocols for 1 to 5 diseases	41	1.39	
		Protocols for more than 6 diseases	32	0.99	
Biosecurity	Biosecurity	Only biosecurity practices other than vaccination	8	0.91	0.627
		At least one biosecurity practices and vaccines for 1 group	38	0.99	
		At least one biosecurity practices and vaccines for 2 groups	37	1.43	
		At least one biosecurity practices and vaccines for 3 groups	42	Baseline	
	Raise multiple livestock	No	105	Baseline	0.711
		Yes	20	1.14	
Other	Season	Fall[1]	261	Baseline	0.213
		Winter[2]	70	1.49	
	Sample Source	Calf	41	Baseline	0.110
		Heifer	109	0.56	
		Cow	108	0.77	
		Manure storage	73	0.97	
	Campylobacter species	*C. jejuni*	310	Baseline	0.002
		C. coli	21	3.82	
	Antimicrobial	Ciprofloxacin	331	Baseline	<0.001
		Tetracycline	331	7.18	

OR: Odds ratio
[1]September to November
[2]December to March

Table 3.10 Unconditional association of the farm level (n=125) continuous predictors (AMU[1]) with the outcome for resistance to tetracycline and ciprofloxacin (model 2).

Variable	No. farms	Percentile			OR[2]	Overall *P*-value
		25th	50th	75th		
Total AMU[3]	125	54.1	86.9	145.3	0.87	0.440
Systemic AMU[3]	125	15.9	25.2	40.1	1.46	0.243
Intramammary AMU[3]	118	23.9	49.8	97.6	0.86	0.084

[1]AMU in DCD/100 animals-year. Estimates were obtained from a garbage can audit, except for Quebec farms.
[2]OR: Odds ratio
[3]Estimates were multiplied by 100 before converted to odds ratio.

A total of 7 variables met the criteria to be included in the multivariable model (province, infected young stock, treated young stock, season, sample source, *Campylobacter* spp. species, and antimicrobial). As in model 1, the total AMU was highly correlated with intramammary AMU (correlation =0.86). When modelled separately, intramammary and systemic AMU were borderline significant (*P*=0.053 and *P*=0.051, respectively). The total AMU was chosen to be included in the final model as it included all the active ingredients and the effect in the final model was stronger than systemic or intramammary AMU. The variable treated young stock was not included in the final model as it was considered an intervene variable for infected young stock (Figure S3.2). The variables sample source and infected young stock were not significant in the final model (*P*=0.12 and *P*=0.08, respectively). Model results for the probability of resistance to tetracycline and ciprofloxacin are presented in Table 3.11. The interaction between antimicrobials and the total AMU in DCD/100 animal-years was significant, and a graphical representation of the relationship is presented in Figure 3.5. The odds of resistance to tetracycline increased for each unit increase in the total AMU, while the odds of resistance to ciprofloxacin decreased. For example, moving from the first quartile to the third quartile of total AMU doubles the odds of an isolate being resistant to tetracycline. *Campylobacter coli* isolates

were more likely to be resistant to ciprofloxacin and tetracycline when compared to *C. jejuni*. There was no difference in the probability of resistance among the different sample sources in the final model (*P*=0.12). The ICCs for farm level and sample type were 0.23 and 0.38, respectively, meaning the clustering effect was higher for sample level, but that moderate clustering occurred in both variables.

Table 3.11 Final multilevel logistic regression model for resistance to ciprofloxacin and tetracycline of 331 *Campylobacter* spp. isolates (125 farms) recovered from fecal samples collected from calves, heifers, lactating cows, and manure storage (model 2).

	β	SE	OR[1]	95% CI		*P* value	Overall *P* value
Intercept	-1.32	0.50	-	-	-	-	-
Province							< 0.001
British Columbia	Ref.						
Alberta	-0.82	0.51	0.53	0.25	1.14	0.106	
Ontario	1.20	0.51	2.52	1.17	5.44	0.018	
Quebec	-0.20	0.51	0.86	0.39	1.86	0.697	
Nova Scotia	-0.68	0.56	0.59	0.26	1.38	0.228	
Campylobacter species							
C. jejuni	Ref.						
C. coli	1.36	0.59	3.37	1.39	8.19	0.007	
Antimicrobial							
Ciprofloxacin	Ref.						
Tetracycline	1.19	0.40	2.49	1.37	4.58	0.016	
Total AMU[2,3]	-0.69	0.33	0.59	0.36	0.96	0.034	
Total AMU x Antimicrobial[2,3,4]	0.82	0.34					0.016
Tetracycline							
Variance	Estimate	SE					
Herd level	1.09	0.47					
Sample type	0.72	0.59					

[1]OR: odds ratio adjusted to population average.
[2]Total DCD/100 animals-year.
[3]Estimates multiplied by 100.
[4]Interaction term between antimicrobial class and total use of antimicrobials.

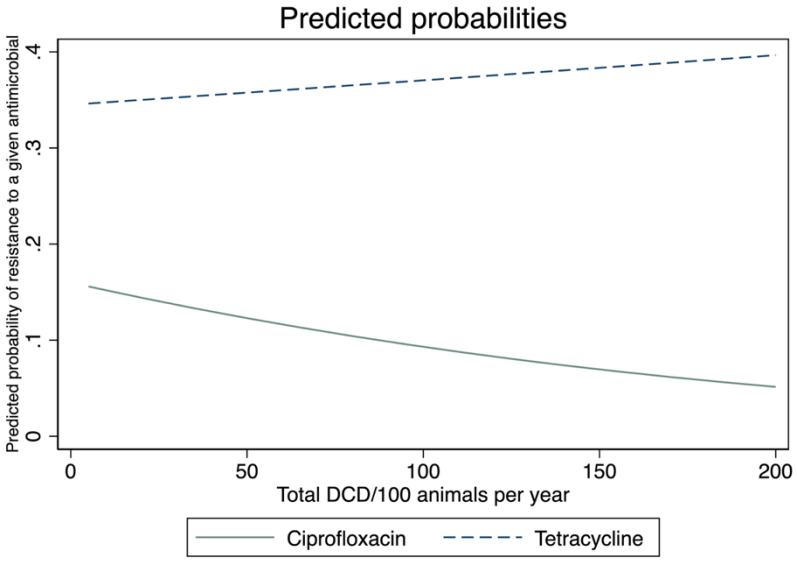

Figure 3. 5 Predicted probabilities for the interaction between AMU and antimicrobial class. The estimates are from isolates where all the predictors are set to the baseline.

3.5. Discussion

Overall proportion of *Campylobacter* spp. isolated from fecal and manure storage samples was 63.8%, whereas the proportion of dairy herds that were *Campylobacter* spp.-positive was 95.7%. These results corroborate a previous study from 2007 in the United States that found the overall prevalence for *Campylobacter* spp. was 51.2% and that 97.9% of dairy herds were positive for *Campylobacter* spp. [138]. It also agrees with a meta-analysis from 2021, which included studies from the United States and Canada reporting the prevalence of *Campylobacter* spp. recovered from pooled fecal samples from dairy cattle was 65% [120]. However, the meta-analysis also included studies published > 10 years ago, which suggests the proportion of *Campylobacter* spp. in dairy cattle in Canada might be stable in the last decades. The higher proportion of *C. jejuni* recovered from the fecal samples compared to *C. coli* in this study was also consistent with other studies in beef and dairy, suggesting that *C. jejuni* is the predominant species of *Campylobacter* in cattle [120, 139].

To investigate the association of AMU with the probability of recovering *Campylobacter* spp., we used total AMU, which includes the antimicrobials administered through systemic, topical, intrauterine, and intramammary routes. Total AMU was associated with a trend towards decreased probability of a sample being *Campylobacter* spp. positive. This association could be expected as systemic use of antimicrobials can impact the intestinal microbiota of young and adult animals and may prevent *Campylobacter* and other bacteria from colonizing the gastrointestinal tract of animals [140-142].

Our study also examined other potential risk factors for the probability of getting a positive culture for *Campylobacter* spp. from fecal and manure storage samples (model 1). The sample source was associated with the probability of recovering a *Campylobacter* spp. isolate. The odds of recovering a *Campylobacter* spp. isolate were higher for heifers (OR= 14.6), lactating cows (OR= 17.4), and manure storage (OR= 3.7) when compared to calf fecal samples (pre-weaned calves). A study from Austria reported that the longer dairy calves were housed individually, the lower were their chance of being *Campylobacter* spp. positive [36]. In Canada, most of pre-weaned dairy calves are housed individually [143, 144], and our results suggest that the exposure to *Campylobacter* spp. might increase in older aged animals as they tend to be housed in groups after the pre-weaned period. This hypothesis agrees with a study from South Korea published in 2018 that identified high animal density as a potential risk factor for the prevalence of *Campylobacter jejuni* on dairy farms [28]. However, other studies reported different findings. A study from Sweden investigated the occurrence of *Campylobacter* spp. in different types of fecal samples and did not find a difference in the proportion of positive samples among calves, heifers, and dairy cows. In this study, they considered as "calves" all animals younger than 12 months, which includes

post-weaned calves [145]. Additionally, two studies from France and one from the United States found a higher prevalence of *Campylobacter* spp. in calves compared to adult animals. These latter studies also considered as "calves" animals < 6 months of age, which also includes post-weaned animals with pre-weaned [146-148]. Post-weaned calves tend to be housed in groups (as opposed to pre-weaned), and a higher proportion of *Campylobacter* spp. found in fecal samples from calves in these latter studies might be attributed to the inclusion of post-weaned calves together with pre-weaned calves during the analysis. Other factors, such as different farm managements not controlled in our study, could also affect the results.

Region and herd size were included in the model as they might be potential confounders for AMU; however, only province was associated with the total AMU (Figure S3.1 in Appendix B). In other studies, herd size was not considered a risk factor for the recovery of *Campylobacter* from beef cattle or young cattle [28, 35, 36]. In this study, herd size was not the main exposure of interest, and the observed effect could be affected by unmeasured confounding factors, leading to a biased interpretation. For instance, demographic characteristics, such as barn type, were explored but were not included in the final model. During the unconditional analysis, barn type met the criteria to proceed to the final model (P=0.20), and it suggested a higher odds of recovering *Campylobacter* spp. in freestall barns. However, barn type was associated with the herd size (P<0.001) and it was not included in the final model (Figure S3.1 in Appendix B). Herd size was also associated with province. For those reasons, no inferences from differences among provinces and herd size should be made, as the estimates could be biased.

An interesting finding was regarding vaccine use by the farms (biosecurity variable). The odds of recovering *Campylobacter* spp. from fecal and manure storage samples from farms where vaccine use was not reported were higher (OR= 5.8) than farms reporting any vaccine use. To the authors' knowledge, no published studies have reported an association of vaccines commonly used in dairy herds with a decreased probability of recovering *Campylobacter* spp. However, an experimental study inducing *Campylobacter* spp. infection in murine models suggested colonization by this bacterium in animals with healthy gut microbiota occurred inconsistently and inefficiently compared to animals with limited gut microbiota [149]. Farms using more vaccines may have higher levels of biosecurity or different management practices that, together with the vaccines, help keep animals healthier compared to farms that do not. Healthy animals may have less *Campylobacter* spp. colonizing the intestinal tract, partially explaining why herds using vaccines were less likely to be *Campylobacter*-positive. According to the causal diagram (Figure S3.1 in Appendix B), this variable was included in the model as a possible confounder; thus, the estimates could be biased if influenced by unmeasured confounders or by an intermediate variable (AMU). If AMU is excluded from the model, the effect of vaccines on the outcome was stronger (data not shown).

Results from antimicrobial susceptibility testing of bacterial species produce reliable results which can be compared with different species and regions worldwide [150]. In this study, tetracycline was the most common antimicrobial to which *Campylobacter* spp. was resistant. The high proportion of isolates with resistance to tetracycline in this study (49.7%) agreed with another study from the United States, where resistance to tetracycline was 49% [138]. Additionally, a second study from the United States analyzing fecal samples

from feedlot cattle reported an even higher proportion of resistance for tetracycline (81%) [124]. Fluoroquinolones are another class of antimicrobials with high importance for public health. The proportion of isolates with resistance to ciprofloxacin (19.8%) was comparable to other studies using fecal samples from cattle in the United States and Sweden [29, 151]. However, a study from 2017 in feedlot cattle from the United States reported a higher proportion of resistance to fluoroquinolones, ranging from 36% for *C. jejuni* to 77% for *C. coli* [124]. Resistance to fluoroquinolones and tetracyclines has been reported at higher rates in other food-producing animals, like poultry and swine. Studies demonstrated that tetracycline and ciprofloxacin resistance in these food-producing animals was 88% and 64.5%, respectively [152, 153], demonstrating that *Campylobacter*'s affinity for acquiring fluoroquinolone and tetracycline resistance does not just occur in isolates from cattle.

The proportion of MDR *Campylobacter* spp. isolates recovered from our study was low (0.3%). Only one isolate (recovered from a lactating cow) was resistant to tetracycline, ciprofloxacin, and azithromycin. 15.6% of the isolates were resistant to two different classes of antimicrobials and resistance to tetracycline and quinolone was the most common identified (Table 4). A study from the United States, published in 2018, using data from feedlot cattle, demonstrated that the proportion of *Campylobacter* spp. isolates that were MDR or resistant to two different antimicrobial classes were 31.2% and 35.6%, respectively [124]. This result suggests that MDR in dairy cattle might be lower when compared to feedlot cattle. The lower proportion of MDR in dairy cattle could be related to different management practices compare to feedlot cattle that could be mitigating the spread of MDR-isolates.

The AMU is considered one of the main risk factors for developing AMR [48, 70]. For this reason, the total AMU was the main exposure investigated for a potential association with resistance in *Campylobacter* spp. isolates (model 2). Total farm AMU was positively associated with the presence of tetracycline resistance in *Campylobacter* spp. For instance, resistance to tetracycline were 2.1 times more likely to occur with an IQR (25^{th} =54.1 to 75^{th} =145.3) increase of the total AMU in DCD/100 animal-years. Similar results were reported for *Campylobacter* spp. isolated from swine, where the total AMU was associated with the increased proportion of tetracycline-resistant isolates [154]. It is well described that the use of a given antimicrobial can be associated with resistance to this same antimicrobial. In 2020, a study suggested that the use of tetracyclines in food-producing animals could be associated with resistance to tetracyclines in different bacteria (including *Campylobacter jejuni*) isolated from these animals [87]. Tetracycline is among Canada's five most frequently used antimicrobials in dairy herds [83, 131]. Among the herds included in model 2, 51.2% (64/125) used products containing tetracycline (Table S3.2 in Appendix B); however, tetracycline corresponded to only 8.2% of the total AMU. A study investigating tetracycline resistance in *Campylobacter jejuni* isolated from Canadian beef cattle, suggested that the use of chlortetracycline increased the carriage of tetracycline-resistant determinants (*tetB*, *tetC*, *tetM*, and *tetW*) [155]. Additionally, they observed that most of the *C. jejuni* carried *tetO* or *tetW* independently of the treatment [155]. According to our results, either lower amounts of tetracycline could contribute to selecting tetracycline-resistant *Campylobacter* isolates, or these determinants were already present regardless of the treatments and could be transferred through mobile elements (e.g., plasmids) among gut bacteria. As resistance to tetracycline was high, from a mitigation standpoint,

veterinarians should emphasize the importance of appropriate dosing (accurate animal weights) to avoid underdosing, which might contribute to selecting resistant isolates. To draw robust conclusions, conducting a whole genomic sequencing analysis is essential. By comparing genetic findings with phenotypic resistance observed in the present study, it will be possible to understand the resistance mechanisms involved.

Interestingly, there was an inverse association between total AMU and ciprofloxacin resistance. One possible explanation for this finding is the low proportion of enrolled farms using fluoroquinolones. Only 13 out of the 125 herds included in model 2 (Table S3.2 in Appendix B) used drugs that belonged to this class (enrofloxacin and danofloxacin), and the use of fluoroquinolones was low (median=0 DCD/100 animals-year), corresponding to only 0.2% of the total AMU. Thus, even farms with high total AMU might have a low proportion of resistance to ciprofloxacin. The persistence of isolates with fluoroquinolone resistance on farms with low AMU may have occurred due to unidentified management factors, which may exert enough selective pressure to impact the maintenance of resistant clones on those farms. Additionally, resistance to fluoroquinolones in *Campylobacter* spp. is usually conferred by a single point mutation in the *gyr*A gene, suggesting the resistance is not related only to the selective pressure exerted by AMU [156, 157].

Correlation between AMR of different sample types from the same farm was lower (ICC = 0.21) than the correlation of the resistance within a sample type (ICC = 0.35). Thus, if an isolate is resistant to one antimicrobial, it is more likely to be resistant to the other antimicrobial, which suggests within farm spread of resistance. Our findings demonstrated that 15.6% of the isolates were resistant to two different classes of antimicrobials

(tetracycline and quinolones or tetracycline and macrolides), corroborating the correlation found in the model.

The awareness of which antimicrobials are used and how they are administered in dairy farms is increasing. In the present study, first-generation cephalosporins, third-generation cephalosporins, and penicillins represented 14.3%, 14.8%, and 27.8% of the total AMU, respectively. Together, these antimicrobials accounted for 57% of the total AMU. Third-generation cephalosporins are antimicrobials considered highly important for human medicine [133, 158]. In the dairy industry, various formulations of ceftiofur (third-generation cephalosporin) are labelled for use in dairy cattle, and the emergence of resistance to this antimicrobial class is a important concern, particularly concerning Enterobacteriaceae such as *E. coli* [159]. However, it is important to acknowledge that the antimicrobial panel tested for *Campylobacter* spp. focuses on antimicrobials commonly used for treating human campylobacteriosis and does not include the antimicrobials that accounted for the higher proportion of total AMU on the enrolled farms.

Although our findings are interesting, it is possible that if we would use different metrics to quantify AMU, we might have found different results. Previous studies on poultry and swine have reported that using different metrics to quantify AMU can impact benchmarking of farm AMU or trends of AMU [160-162]. Different AMU indicators, such as weight-based and dose-based metrics, are frequently employed, and the selection of each can impact the results. For instance, a study from Canada using data from poultry and swine reported that the estimates could differ depending on the metric used to quantify AMU for studying the relationship between AMU-AMR [163]. No consensus has been reached on which metric to use to investigate the association between AMU and the development of

AMR [164]. The choice of AMU metric will depend on the study's objectives [165]. We used a newly developed metric to quantify AMU in the present study. This metric was specifically designed to account for all antimicrobial products marketed for use in dairy cattle, and for different dosages among different active ingredients, resulting in a more reliable and accurate quantification of AMU in Canadian dairy cattle (Lardé, et al., 2020).

In model 2, other potential risk factors for resistance to tetracycline and ciprofloxacin were explored. Compared to ciprofloxacin, resistance for tetracycline was higher (OR = 1.2); however, there was no significant difference in the resistance pattern among the sample sources (P = 0.12). Resistance patterns were different for *Campylobacter coli* and *jejuni. Campylobacter coli* was more likely to be resistant to either tetracycline or ciprofloxacin (OR = 3.4) than *C. jejuni*. This supports findings from several studies that also analyzed resistance patterns from *Campylobacter* from fecal samples in beef cattle, dairy cattle, and poultry [25, 124, 139, 166]. The province where farms were located was included in model 2 as a potential confounder for AMU. However, no inferences should be made as this variable was not the main exposure and unmeasured confounders could affect its estimates.

Some limitations may have affected our results. The enrollment of dairy farms through a convenience sample could have introduced selection bias. Extrapolation to other Canadian dairy farms other than the enrolled in the program should be made with caution as producers who agreed to participate may differ from the target population regarding the management practices (including AMU) and the burden of AMR on their farms. Additionally, this study had a cross-sectional design, which can bring some disadvantages for the causal inference in the relationship between AMU and AMR development. Both

the AMU and AMR data used in the present study were retrieved in 2019, and the resistance results might not fully represent the impact of the use of antimicrobials during the year 2019. Finally, the use of questionnaires and the methods of AMU data collection may have impacted our results. The questionnaires were lengthy and time-consuming; however, the regional field workers who collected this information were responsible for entering the producer responses into spreadsheets which should have decreased errors in reporting. We collected AMU data using two different methods. We conducted a GCA on all farms except those in Québec (QC). In QC, AMU was extracted from electronic veterinary invoices. However, a study conducted in QC comparing different quantification methods for antimicrobial use demonstrated that veterinary invoices and GCA provided similar results in quantifying AMU, suggesting that the impact of different AMU data sources in our study results might be negligible [54]. Additionally, collecting AMU data by conducting a GCA, which is laborious and time-consuming, can be prone to errors. The researchers validated all AMU data before being uploaded to the central database to prevent mistakes. This was done by carefully inspecting the data collected during the standardization and looking for inconsistencies before uploading it to the central database.

This study demonstrated that the overall proportion of *Campylobacter* spp. and the proportion of farms with at least one positive sample was high, indicating that *Campylobacter* spp. is widespread among Canadian dairy farms. *C. jejuni* isolates were more prevalent than *C. coli*, although the latter was more likely to be resistant to tetracycline and ciprofloxacin. Resistance to tetracycline, nalidixic acid, and ciprofloxacin were observed; however, it was lower when compared to other commodities. There was a significant association between the AMU and AMR, although the direction of the

association depended on the antimicrobial class. The total AMU was associated with increased resistance to tetracycline, while a decrease was observed for ciprofloxacin.

Chapter 4

4. Intramammary and systemic use of antimicrobials and their association with resistance in generic *Escherichia coli* recovered from fecal samples from Canadian dairy herds: A cross-sectional study

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4.1. Abstract

Antimicrobial resistance (AMR) in animals, including dairy cattle, is an important concern for animal and public health worldwide. In this study, we used data collected through the Canadian Dairy Network for Antimicrobial Stewardship and Resistance (CaDNetASR) to: (1) describe the proportions of AMR in fecal *E. coli*, and (2) investigate the relationship between antimicrobial use (AMU) (intramammary and systemic routes, while accounting for confounding by other variables) and AMR/multidrug resistance (MDR – resistance to ≥ 3 antimicrobial classes) in fecal *E. coli* from Canadian dairy farms. We hypothesized that an increase of the AMU was associated with an increase in AMR in *E. coli* isolates. A total of 140 dairy farms across five provinces in Canada were included in the study. Fecal samples from pre-weaned calves, post-weaned heifers, lactating cows, and farm manure storage were cultured, and *E. coli* isolates were identified using MALDI-TOF MS. The minimum inhibitory concentrations (MIC) to 14 antimicrobials were evaluated using a microbroth dilution methodology. AMU was quantified in Defined Course Dose (DCD - the dose for a standardized complete treatment course on a standard size animal) and converted to a rate indicator - DCD/100 animal-years. Of 1134 fecal samples collected, the proportion of samples positive for *E. coli* in 2019 and 2020 was 97.1% (544/560) and 94.4% (542/574), respectively. Overall, 24.5% (266/1086) of the *E. coli* isolates were resistant to at least one antimicrobial. Resistance to tetracycline was commonly observed (20.7%), whereas resistance to third-generation cephalosporins, fluoroquinolones, and carbapenems was found in 2.2, 1.4, and 0.1% of *E. coli* isolates, respectively. *E. coli* isolates resistant to two or ≥ 3 antimicrobial classes (MDR) was 2.7% and 15%, respectively. Two multilevel models were built to explore risk factors associated with

AMR with AMU being the main exposure. Systemic AMU was associated with increased *E. coli* resistance. For an increase in systemic AMU equivalent to its IQR, the odds of resistance to any antimicrobial in the model increased by 18%. Fecal samples from calves had higher odds of being resistant to any antimicrobial when compared to other production ages and farm manure storage. The samples collected in 2020 were less likely to be resistant when compared to samples collected in 2019. Compared to previous studies in dairy cattle in North America, AMR in *E. coli* was lower.

Keywords: dairy cattle, antimicrobial use, antimicrobial resistance, surveillance, Canada.

4.2. Introduction

Antimicrobial resistance (AMR) is a significant public health issue worldwide. A recent systematic review concluded that approximately 1.27 million deaths globally in 2019 could be attributed to bacterial AMR [3]. Food-producing animals have been scrutinized as a potential source of AMR that can be transmitted to humans through food products, direct contact with these animals or contact with the environment [167]. A systematic review published in 2018 suggested that antimicrobial resistant *Escherichia coli* from food-producing animals could be transferred to humans [168].

E. coli are ubiquitous commensal bacteria that colonize the gut of animals and humans, and most *E. coli* strains are harmless to the host. However, some strains are pathogenic and may cause infections in humans and animals [19]. *E. coli* is also a reservoir of AMR genes (ARGs) that can be horizontally transferred to other pathogenic bacteria [31, 32]. Food animals and products derived from them can be a source of antimicrobial-resistant bacteria for humans [168, 169]. Some recent studies demonstrated phylogenetic overlapping for a given AMR pattern in *E. coli* from food-producing animals and humans [170, 171]. For those reasons, *E. coli* is commonly used as an indicator of AMR in healthy animals.

To better understand the role of food-producing animals as the reservoir of antimicrobial-resistant bacteria, it is necessary to know the prevalence of resistance in *E. coli* in those animals. A study conducted in 2021, using genomes from public databases (from 1980 to 2018) assessing AMR in commensal *E. coli* isolates from food-producing animals showed a significant increase in the temporal trends for several antimicrobials, including penicillin, in combination with β-lactamase inhibitors, and extended-spectrum cephalosporins [172]. The

same study also demonstrated that the prevalence of multidrug resistance (MDR) in *E. coli* had doubled from 1980 to 2018 [172]. A study in the province of Quebec, Canada, in 2021 analyzed AMR in *E. coli* recovered from fecal and manure pit samples from healthy dairy cattle. They found high proportions of *E. coli* with resistance to tetracycline, sulfisoxazole and streptomycin, and 21.7% of the isolates were considered MDR [173]. Although this recent study provided valuable information on AMR in *E. coli* recovered from dairy cattle, there is still a knowledge gap for dairy cattle in other provinces in Canada.

The use of antimicrobials is known to be associated with the development of AMR in livestock [48, 70]. In 2021, the European Centre for Disease Prevention and Control (ECDC), European Food Safety Authority (EFSA), and European Medicines Agency (EMA) published the third joint inter-agency report on integrated analysis of the consumption of antimicrobial agents and the occurrence of AMR. This report observed associations between antimicrobial use (AMU) and the development of AMR in commensal *E. coli* in food-producing animals for antimicrobials such as tetracycline, aminopenicillins, and fluoroquinolones [45]. In Canada, a study using integrated data from broiler chicken, turkey, and swine identified associations between the increase of AMU and the decrease of susceptible generic *E. coli* to several antimicrobials [174]. Antimicrobial use has also been found to be associated with MDR in *E. coli* isolates on swine farms in the United Kingdom [175]. A study from Italy using *E. coli* isolates recovered from dairy calves demonstrated that animals treated with sulfonamides, fluoroquinolones, tetracycline, or oxytetracycline had a higher proportion of AMR and a higher expression of virulence genes on these isolates when compared to untreated animals. The study also reported a higher incidence of MDR in *E. coli* for the treated calves [176].

There is also a debate on the role of the route of administration of antimicrobials and their impact on resistance. A study conducted in the United States in 2010 found an association between cephalosporin-based dry cow treatment and reduced susceptibility to cephalothin and streptomycin in *E. coli* recovered from fecal samples [75]. However, a study conducted in Canada in 2018 found that the intramammary administration of antimicrobials was not associated with increased resistance in non-*aureus* staphylococci, while the systemic administration of penicillins, third-generation cephalosporins, or macrolides was associated with AMR in these bacteria [74].

The effects of management practices on the occurrence of AMR in bacteria have been explored in previous studies. For instance, a study on swine identified that biosecurity measures, such as disinfection and cleaning, were associated with less resistance to several antimicrobials in *E. coli* recovered from fecal, slurry, and environmental samples [177]. In dairy cattle, a previous study identified the use of waste milk to feed calves and daily cleaning of calf feeding equipment as risk factors for recovering ESBL/AmpC-containing *E. coli* in pre-weaned dairy calves [178]. Other studies in dairy cattle have identified age, season, and herd size as factors associated with increased levels of AMR in *E. coli* recovered from fecal samples [173, 179]. To the authors' knowledge, no Canada-wide studies investigated risk factors associated with resistance and MDR in generic *E. coli* recovered from dairy cattle. The present study aimed to (1) describe the proportions of AMR in generic *E. coli*, and (2) investigate the relationship between AMU (intramammary and systemic routes, while accounting for confounding by other variables) and AMR/MDR in fecal generic *E. coli* from Canadian dairy farms. We hypothesized that an increase on the AMU was associated with an increase in AMR in *E. coli* isolates.

4.3. Materials and Methods

The data were collected through the Canadian Dairy Network for Antimicrobial Stewardship and Resistance (CaDNetASR) surveillance system in 2019 and 2020 [129]. The current study was an observational cross-sectional design, conducted on commercial dairy farms. The recommendations for reporting observational studies were followed throughout the text (STROBE-vet guidelines) [130]. The study was reviewed and approved by the University of Prince Edward Island Research Ethics Board and the Animal Care Committee on March 7, 2019 (file # 6008059).

4.4. Sample size, Farm Selection and Sample Collection

The sample size calculation and farm enrollment were described previously [129]. Briefly, a convenience sample of 140 farms from five Canadian provinces (British Columbia, Alberta, Ontario, Quebec, and Nova Scotia) were enrolled. Enrolled farms had a minimum of 50 animals except for Nova Scotia, which had a minimum size of 40 animals. Participating farms also needed to raise their replacement heifers on-site and provide antimicrobial purchase history, which was obtained from their herd veterinarian and feed mill. On each farm, pooled fecal samples (5 fresh fecal pats selected from different places on the floor) from each of 3 age groups (pre-weaned calves, post-weaned heifers, and lactating cows) and a manure storage sample were obtained during the summer, fall, and winter of 2019 and summer and fall of 2020. Samples were stored in coolers with ice packs and sent to be processed at the University of Prince Edward Island. During the sampling visits, questionnaires were applied to collect information on farm demographics and

management practices. Detailed information from the questionnaire from which the risk factors variables were extracted has been previously reported [129].

4.5. Antimicrobial use

AMU data from 2019 were obtained through a garbage can audit system (GCA), except for the province of Québec, where veterinary invoices were used. The high correlation between the GCA and veterinary invoices was previously reported [54]. Antimicrobial use was quantified in Defined Course Dose (DCD - the dose for a standardized complete treatment course on a standard size animal) according to Lardé et al. and converted to a rate indicator - DCD/100 animal-years [86]. Total AMU was split into systemic and intramammary administration routes, the two main routes used in dairy cattle in Canada, to explore the effects of AMU administered through different routes. Systemic use was defined as the sum of antimicrobials administered through oral, intravenous, intramuscular, and subcutaneous routes in DCD/100 animal-years. Data on milk replacer and medicated feed were not captured by CaDNetASR.

4.6. Bacterial isolation and characterization

Fecal samples were cultured for generic *E. coli* according to the Canadian Integrated Program for Antimicrobial Resistance Surveillance (CIPARS) protocol (Government of Canada, 2018). Briefly, 25g of feces was homogenized in 250 mL of buffered peptone water for each sample using a stomacher for 30 seconds at 230 rpm. Using a sterile loop, 10µL of the homogenized sample was streaked onto a MacConkey Agar culture plate and incubated at 35°C for 24 hours. A colony typical for *E. coli* was subcultured to a new MacConkey Agar culture plate and incubated at 35°C for 24 hours. A colony from the pure

subculture was then streaked into a Luria-Bertani (LB) nutritional culture plate and incubated at 35°C for 24 hours. Reference strain *E. coli* ATCC 25922 was used as quality control. The species identification was performed by matrix-assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF MS) using the Bruker Microflex MALDI-TOF MS (Bruker Daltonics, GmbH, Bremen, Germany). A single colony was transferred onto the target plate, air-dried at ambient temperature, and overlaid with alfa-cyano-4-hydroxycinnamic acid before being introduced into the MALDI-TOF mass spectrometer for automated measurement of mass spectra and comparison to the reference database (MBT 8468 MSP Library). According to the criteria proposed by the manufacturer, identification scores ≥ 2.0 were required for confident species identification.

4.7. Antimicrobial Susceptibility Testing

Antimicrobial susceptibility was determined using the Sensititre™ microdilution system and the CMV4AGNF panel (Sensititre™, Thermo Fisher Scientific, MA, USA). The antimicrobial susceptibility test was performed in all isolates confirmed as *E. coli*. The plate contained twofold serial dilutions of 14 antimicrobials: amoxicillin/clavulanic acid - AMC (1/0.5-32/16 μg/mL), ampicillin - AMP (1-32 μg/mL), azithromycin - AZM (0.25-32 μg/mL), cefoxitin - FOX (0.5-32μg/mL), ceftriaxone - CRO (0.25-64 μg/mL), chloramphenicol - CHL (2-32 μg/mL), ciprofloxacin - CIP (0.015-4 μg/mL), gentamicin - GEN (0.25-16 μg/mL), meropenem - MER (0.06-4 μg/mL), nalidixic acid - NAL (0.5-32 μg/mL), streptomycin - STR (2-64 μg/mL), sulfisoxazole - SOX (16-256 μg/mL), tetracycline - TET (4-32 μg/mL), and trimethoprim/sulfamethoxazole - SXT (0.12/2.38-

4/76 μg/mL). Reference strain *Escherichia coli* ATCC 25922 was used as quality control. The minimum inhibitory concentration (MIC) values were the lowest antimicrobial concentrations that inhibited bacteria's visible growth. If growth was observed at the highest antimicrobial concentration tested, the MIC was assigned to the next dilution. Interpretation of antimicrobial resistance was made according to the Clinical and Laboratory Standards Institute (CLSI) clinical breakpoints, recommended by CIPARS [180].

4.8. Statistical Analysis

A descriptive analysis was performed to determine the proportion of fecal samples positive for generic *E. coli*, the proportion of isolates resistant to the antimicrobials included in the panel, and the proportion of MDR in the isolates. Multi-drug resistance was defined as resistance to three or more antimicrobial classes [135]. Frequency distributions of MIC, MIC_{50}, and MIC_{90} were calculated for each antimicrobial in the panel. Intermediate and resistant isolates were grouped and considered resistant. Each sample source was defined by one generic *E. coli* isolate. For model 1, some antimicrobials were not considered in the model due to the low prevalence of resistance (≤ 2.0%).

4.8.1. Multivariate multilevel logistic regression

Given the hierarchical structure of the data, a three-level multivariate, multilevel logistic regression model with farm and sample (where sample represented up to eight isolates for the two periods of data collection) as random intercepts (model 1) was built to investigate risk factors associated with the probability (resistant *vs. s*usceptible) of resistance in generic *E. coli* isolates. A multivariate model where each antimicrobial resistance was included as a separate binomial outcome was chosen because it allowed for better estimation of lower-

level variances than standard multilevel model [181]. The variance at the lowest level was provided as a matrix representing the correlations among the antimicrobials included in the model. The estimates from the multivariate model should be interpreted similarly to estimates from a multilevel model; however, with the population averaged interpretation. The analyses were carried out in MLwiN 3.05 [182].

4.8.2. Multilevel ordinal logistic regression

A two-level ordinal logistic regression with farm as a random intercept (model 2) was built to investigate risk factors associated with the probability of MDR in generic *E. coli*. The outcome was grouped as follows: 0 = fully susceptible; 1 = resistant to one or two different antimicrobial classes; and 2 = resistant to three or more antimicrobial classes. The random effects ordinal logistic regression accounts for both the hierarchical structure and the natural ordering structure of the outcome, while assuming proportional odds across the two cut-points (between categories 0 and 1, and between categories 1 and 2). This assumption can be relaxed in a partial proportional odds model for selected predictor effects, using the gllamm implementation for Stata [183, 184] . For each predictor, its effect was first expanded to allow for non-proportional odds, and the suitability of a proportional odds assumption was then assessed by a Wald test [185]. Predictors that violate the proportional odds assumption were kept in their expanded form with separate odds-ratios across the two cut-points. The analyses were carried out in Stata SE (16.1, StataCorp LLC, College Station, TX) [186].

4.8.3. Variable Selection

To assess the association among categorical predictors, a chi-squared test was used. Associations between continuous and categorical variables were assessed using the Kruskal-Wallis rank test. Correlations between the binary and continuous variables were assessed using Pearson correlation. Unconditional associations between the explanatory variables and the outcome were examined in the models previously described. Variables with *P*-values ≤ 0.20 were selected for inclusion in the multivariable model. The linearity of the continuous predictors was visually assessed using a scatter plot with a smoothed curve on the logit scale. To further investigate the linearity assumption the polynomial models were explored. The continuous variables that violated linearity and were not significant with the polynomial function were categorized based on their distribution frequency (quartiles). Two directed acyclic graphs (DAGs) were built to help with the model building (Figures S4.1 and S4.2 in Appendix C). The main exposures for both models were systemic and intramammary AMU (as two different predictors) converted to DCD/100 animal-years. Additional information on each active ingredient that was included in the total usage can be found in the supplementary material (Tables S4.1 and S4.2 in Appendix C). In addition, sample source, season, year, and antimicrobial by active ingredient (only model 1) were included. Another 11 variables (Table S4.3 in Appendix C) from the surveillance questionnaire were selected to be explored in the regression analysis. The model building was done using backward manual selection as suggested by Dohoo et al. [137]. Variables with *P*-values ≤ 0.05 were retained in the final model. The Wald test was used to test for the significance of predictors. Excluded potential confounding variables were reintroduced in the model and retained if a change of > 20% was observed in other

coefficients. Biologically plausible interaction terms were explored. The two models were evaluated by examination of residual plots at the highest level (farm) and influential observations [137]. Visual examination of the plot of residuals vs. predicted values did not show any significant pattern that could indicate a lack of homoscedasticity. The residuals were also visually inspected for normality, and no deviations of the normality assumption was observed.

4.9. Results

4.9.1. Antimicrobial use

A total of 131 farms provided AMU data and had a median total use of antimicrobials of 86.8 DCD/100 animal-years (range: 1.9-499.3). The median systemic and intramammary use was 25.0 and 49.8 DCD/100 animal-years, respectively (Figure 4.1). Systemic and intramammary AMU represented 37.8% and 60.6% of the total use, respectively, and are summarized by province in Table 4.1. The remaining 1.6% was the sum of intrauterine (1.4%) and topical (0.2%) AMU. The descriptive statistics for the antimicrobials according to Health Canada and WHO antimicrobial importance classifications for these farms is provided in Tables S1 and S2. The use of intramammary and systemic antimicrobials had a high variability among the farms (Figure 4.1). The enrolled farm's demographic information has been described in a previous study [129].

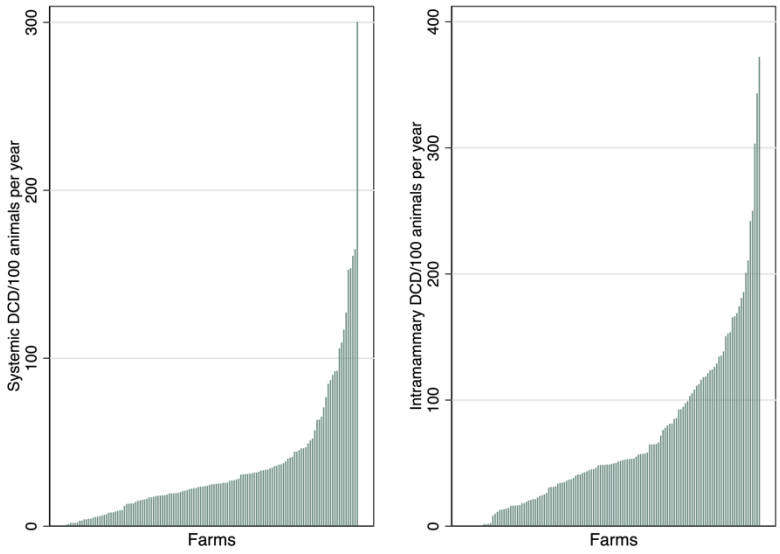

Figure 4.1 Systemic and intramammary antimicrobial use variability from data collected in 2019 among the 131 farms enrolled in CaDNetASR surveillance.

Table 4.1 Summary of systemic (Sys) and intramammary (IMM) antimicrobial use from 2019 in Defined Course Doses/100 animal-years from 131 CaDNetASR dairy farms by province. Estimates from a garbage can audit, except for Québec farms.

Province	N	Min.		25th		50th		75th		Max.	
		Sys	IMM	Sys	IMM	Sys	IMM	Sys	IMM	Sys	IMM
BC	24	3.3	0.0	14.8	19.5	23.1	35.4	36.2	52.7	153.6	201.0
AB	30	1.3	0.0	17.4	49.3	23.7	93.7	36.7	153.8	117.1	343.2
ON	30	2.0	0.0	17.9	43.5	31.0	57.8	47.0	111.3	164.9	372.1
QC	27	1.9	0.0	13.2	16.3	27.8	31.5	44.3	46.2	300.4	64.8
NS	20	0.8	8.3	7.6	41.6	19.5	80.7	31.7	117.1	90.1	303.3
Total	131	0.8	0.0	15.1	23.9	25.0	49.8	38.6	99.1	300.4	372.1

BC: British Columbia; AB: Alberta; ON: Ontario; QC: Québec; NS: Nova Scotia.

4.9.2. Resistance patterns in Escherichia coli

All the farms had at least one positive sample (from any source) for generic *E. coli* in 2019 and 2020. *E. coli* was recovered from 95.8% (1086/1134) of samples. The proportion of samples positive for generic *E. coli* was 97.1% (544/560) in 2019 and 94.4% (542/574) in

2020. Antimicrobial MIC susceptibility testing was performed on all *E. coli* isolates (n=1086).

The proportion of farms with at least one sample resistant to at least one antimicrobial in the panel was 70.7% in 2019 (99/140) and 64.6% in 2020 (93/144). A high proportion of farms had *E. coli* isolates resistant to tetracycline (20.6%), followed by streptomycin (16.0%), and sulfisoxazole (14.9%) than to other antimicrobials tested (Figure 4.2). The proportion of farms with at least one sample resistant to at least one of the 14 antimicrobials is summarized in Figure 4.2.

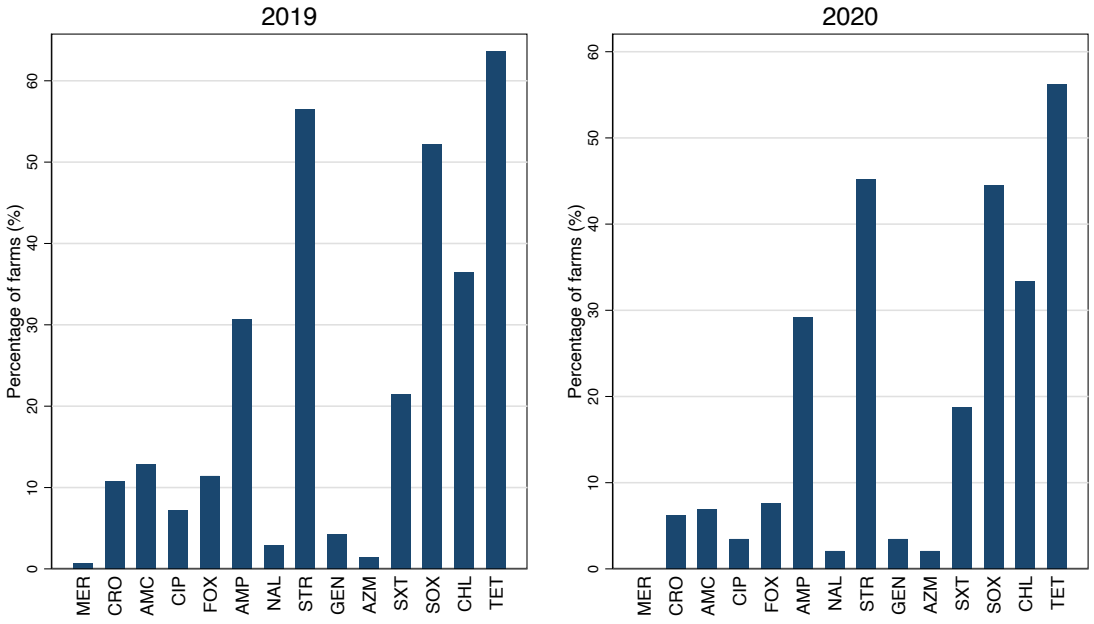

Figure 4.2 Percentage of farms with at least one sample resistant to each of the antimicrobials tested in the panel in 2019 and 2020. AMC: amoxicillin/clavulanic acid; AMP: ampicillin; AZM: azithromycin; FOX: cefoxitin; CRO: ceftriaxone; CHL: chloramphenicol; CIP: ciprofloxacin; GEN: gentamicin; MER: meropenem; NAL: nalidixic acid; STR: streptomycin; SOX: sulfisoxazole; SXT: trimethoprim/sulphamethoxazole TET: tetracycline.

Overall, 24.5% (266/1086) of the *E. coli* isolates were resistant to at least one antimicrobial. Resistance to third-generation cephalosporins, fluoroquinolones, and carbapenems was 2.2, 1.4, and 0.1%, respectively (Table 4.2). The total proportion of

isolates resistant to at least one antimicrobial in Alberta was 26.4%, followed by Québec (26.0%), British Columbia (25.5%), Ontario (25.1%), and Nova Scotia (18.5%). The proportion of isolates resistant to each antimicrobial by year and province is illustrated in Figure 4.3.

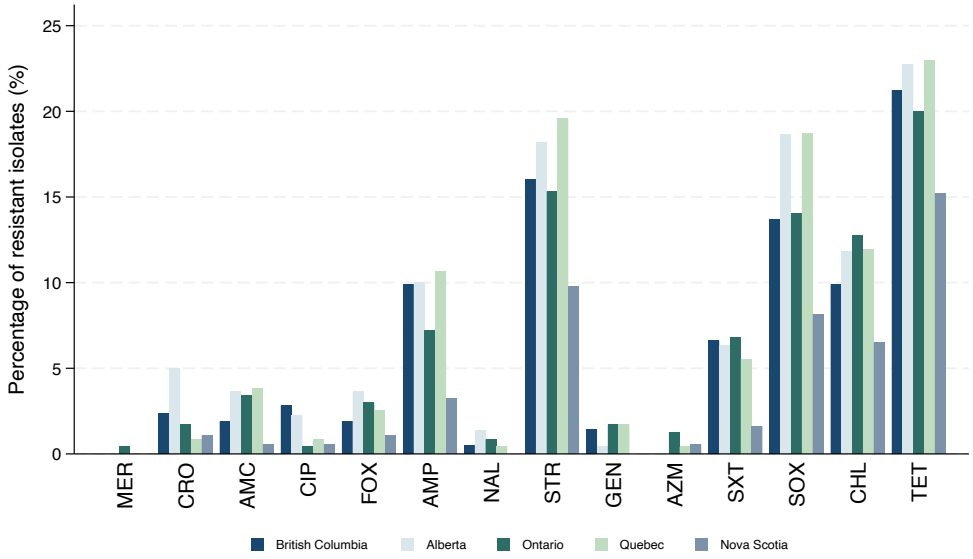

Figure 4.3 Percentage of resistant isolates for each antimicrobial tested in the panel by province. AMC: amoxicillin/clavulanic acid; AMP: ampicillin; AZM: azithromycin; FOX: cefoxitin; CRO: ceftriaxone; CHL: chloramphenicol; CIP: ciprofloxacin; GEN: gentamicin; MER: meropenem; NAL: nalidixic acid; STR: streptomycin; SOX: sulfisoxazole; SXT: trimethoprim/sulphamethoxazole TET: tetracycline.

Table 4.2 Minimum inhibitory concentrations (MICs) of 14 antimicrobials for 1086 generic *E. coli* isolates recovered from dairy herds fecal samples in 2019 and 2020.

				Distribution (%) of MIC (µg/mL)																	
Antimicrobial Class	**Antimicrobial**	Range	% Res.	0.015	0.03	0.06	0.12	0.25	0.5	1	2	4	8	16	32	64	128	256	512	[a]MIC$_{50}$	[b]MIC$_{90}$
Carbapenem	MER	0.06-4	0.1			99.8	0.1	0.0	0.0	0.0	0.1	0.0								0.06	0.06
Cephalosporin - 3rd generation	CRO	0.25-64	2.2					97.3	0.0	0.5	0.0	0.1	0.6	0.6	0.3	0.1	0.6			0.25	0.25
Penicillin-β-lactamase inhibitor combinations	AMC	1/0.5-32/16	2.8							1.2	15.8	66.6	13.5	0.7	1.5	0.6				4	8
Fluoroquinolone	CIP	0.015-4	1.4	97.3	1.2	0.1	0.2	0.8	0.2	0.0	0.0	0.0	0.2							0.015	0.015
Cephalosporin - 2nd generation	FOX	0.5-32	2.5						0.0	0.7	17.1	64.4	15.4	0.9	0.7	0.9				4	8
Penicillin	AMP	1-32	8.4							4.4	42.0	43.7	1.5	0.2	0.2	8.0				4	4
Quinolone	NAL	0.5-32	0.6						0.3	7.4	77.7	13.6	0.3	0.1	0.1	0.6				2	4
Aminoglycoside	STR	2-64	16.0								0.6	30.4	50.8	2.2	2.8	3.5	9.8			8	64
	GEN	0.25-16	1.1					4.2	69.5	24.4	0.7	0.1	0.1	0.1	0.9					0.5	1
Macrolide	AZM	0.25-32	0.5					0.0	0.1	0.7	10.3	58.0	29.5	1.0	0.1	0.4				4	8
Trimethoprim/sulfamethoxazole	SXT	0.12/2.38-4/76	5.5				90.5	3.5	0.5	0.0	0.0	0.0	5.5							0.12	0.12
Sulfonamide	SOX	16-256	14.9											84.4	0.7	0.0	0.0	0.0	14.9	16	512
Phenicol	CHL	2-32	10.8								3.8	42.6	42.9	0.9	0.0	9.9				8	16
Tetracycline	TET	4-32	20.7									79.4	1.6	1.2	6.0	11.9				4	64

Vertical lines indicate CLSI breakpoints. The horizontal lines from up to bottom divide the antimicrobials into category I, category II, and category III according to Health Canada classification [132]. Grey shade represents values outside concentrations included in the broth microdilution method. AMC: amoxicillin/clavulanic acid; AMP: ampicillin; AZM: azithromycin; FOX: cefoxitin; CRO: ceftriaxone; CHL: chloramphenicol; CIP: ciprofloxacin; GEN: gentamicin; MER: meropenem; NAL: nalidixic acid; STR: streptomycin; SOX: sulfisoxazole; SXT: trimethoprim/sulphamethoxazole TET: tetracycline.

[a] The MIC value that inhibits growth of 50% of the isolates

[b] The MIC value that inhibits growth of 90% of the isolates

A higher proportion of resistance (all antimicrobials tested, except for meropenem) was observed for *E. coli* isolates recovered from calf samples compared to other sample sources. *E. coli* isolates recovered from lactating cow samples tended to have a lower proportion of resistance to several antimicrobials (Figure 4.4).

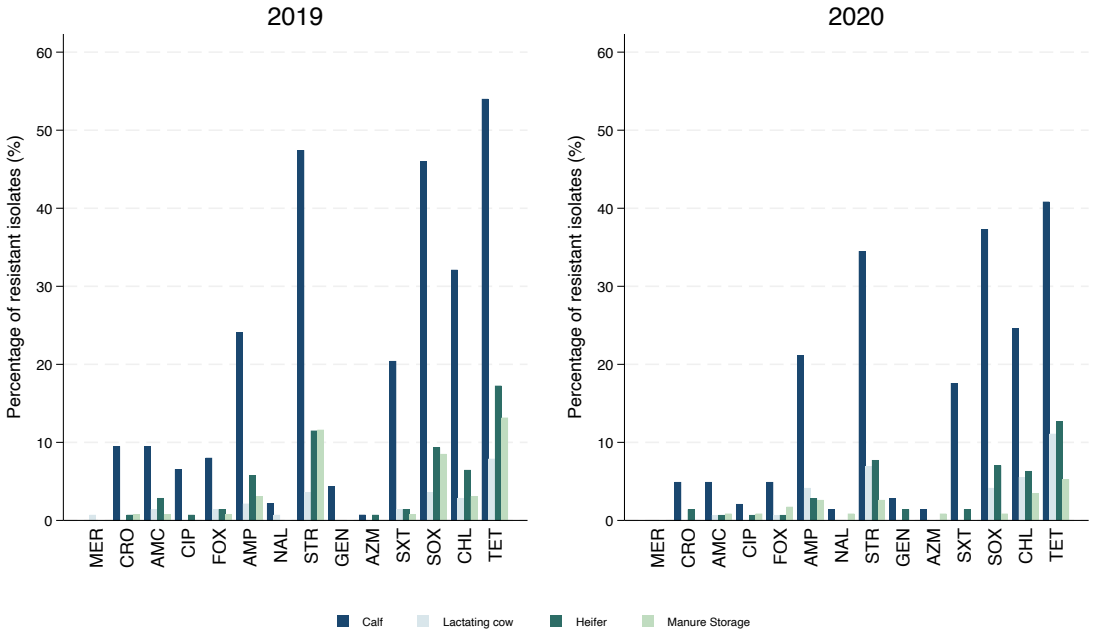

Figure 4.4 Percentage of resistant isolates for each antimicrobial tested in the panel by sample source in 2019 and 2020. AMC: amoxicillin/clavulanic acid; AMP: ampicillin; AZM: azithromycin; FOX: cefoxitin; CRO: ceftriaxone; CHL: chloramphenicol; CIP: ciprofloxacin; GEN: gentamicin; MER: meropenem; NAL: nalidixic acid; STR: streptomycin; SOX: sulfisoxazole; SXT: trimethoprim/sulphamethoxazole TET: tetracycline.

The proportion of *E. coli* isolates resistant to two different antimicrobial classes was 2.7%, whereas the proportion that were MDR (≥3 antimicrobial classes) was 15.0%. Compared to other groups, isolates recovered from calf samples were more likely to be MDR (Figure 4.5). A heat map representing the resistance pattern, sample source and province where the 266 resistant generic *E. coli* isolates is illustrated in Figure 6. The most prevalent

phenotypic resistance patterns were tetracycline only with 4.6% (50/1086), followed by sulfisoxazole-streptomycin-tetracycline with 2.5% (27/1086), and chloramphenicol-sulfisoxazole-streptomycin-tetracycline with 2.5% (27/1086). One *E. coli* isolate recovered from a lactating cow sample in Ontario was resistant to meropenem, amoxicillin/clavulanic acid, and ampicillin. Two isolates from calves (0.2%) were resistant to seven out of the nine antimicrobial classes tested, being considered extensively drug-resistant (XDR – resistant to all but two antimicrobial classes tested) [135] (Figure 4.6).

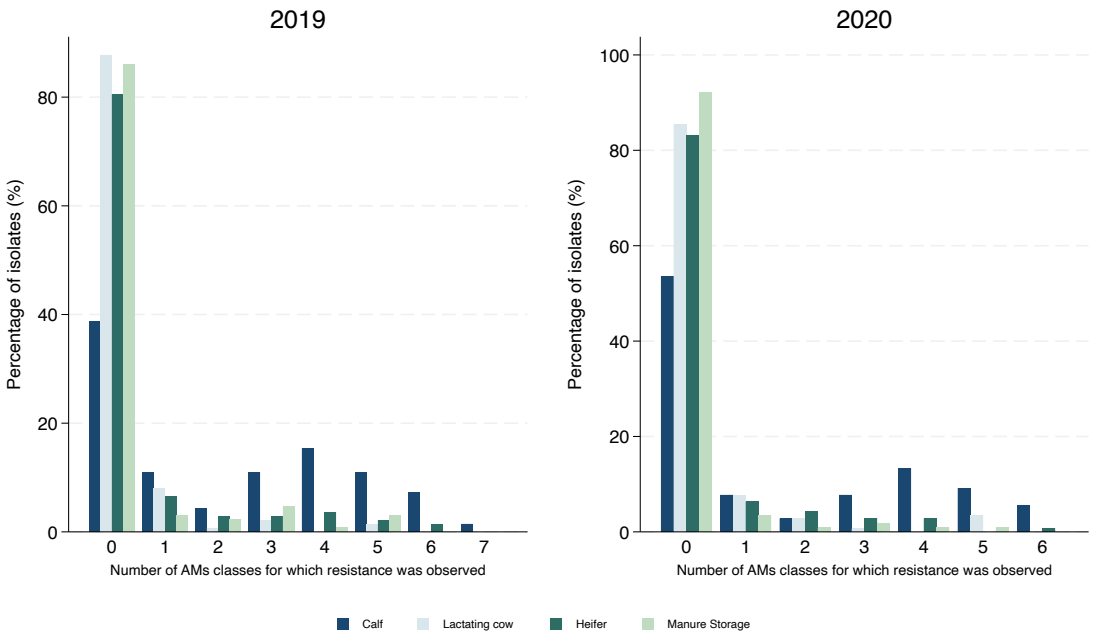

Figure 4.5 Percentage of isolates pan-susceptible or resistant to one or more antimicrobial classes by sample source in 2019 and 2020.

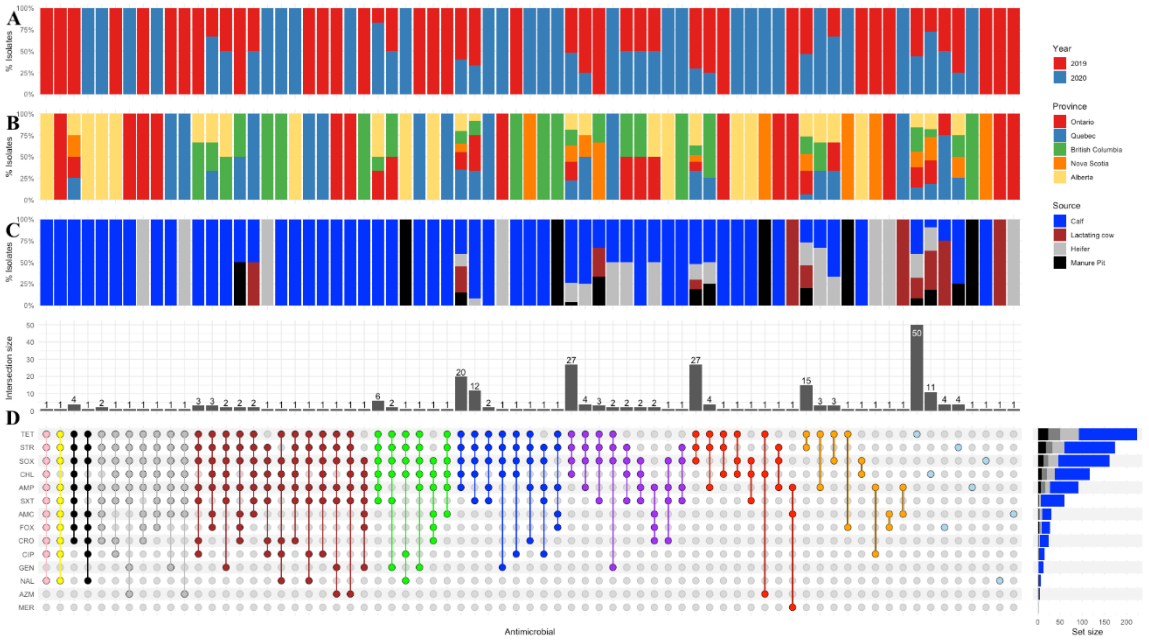

Figure 4.6 Phenotypic resistance pattern for the 266 *E. coli* isolates recovered from fecal samples that were resistant to at least one antimicrobial tested. **A** Year in which the isolates were recovered; **B** Province where the isolates were recovered; **C** Sample source where the isolates were recovered; **D** Number of isolates and their respective phenotypic resistance pattern. The different colors for the connected dots represents how many antimicrobials an isolate was resistant to: light pink= resistant to 11 antimicrobials; yellow=resistant to ten antimicrobials; black=resistant to nine antimicrobials; gray=resistant to eight antimicrobials; burgundy=resistant to seven antimicrobials; green=resistant to six antimicrobials; blue=resistant to five antimicrobials; purple=resistant to four antimicrobials; red=resistant to three antimicrobials; orange=resistant to two antimicrobials; and light blue=resistant to one antimicrobial. AMC: amoxicillin/clavulanic acid; AMP: ampicillin; AZM: azithromycin; FOX: cefoxitin; CRO: ceftriaxone; CHL: chloramphenicol; CIP: ciprofloxacin; GEN: gentamicin; MER: meropenem; NAL: nalidixic acid; STR: streptomycin; SOX: sulfisoxazole; SXT: trimethoprim/sulphamethoxazole TET: tetracycline

4.9.3. Risk factors modelling results

4.9.3.1. Risk factors for resistance in generic E. coli (Model 1)

Descriptive data and the unconditional associations for the variables considered in model 1 are presented in Tables 4.3 and 4.4. A total of 13 variables met the criteria to be included in the final model. Total AMU was highly correlated with intramammary AMU (correlation = 0.87) and moderately correlated with systemic AMU (correlation = 0.56). Systemic AMU and intramammary AMU were chosen to be included in the final model as they contributed approximately 98.4% of the total AMU. The variables "treated young stock" and "treated lactating cows" were considered intervening variables with "infected young stock" and "infected lactating cows", respectively (Figure S4.1 in Appendix C). The variable "infected young stock" was associated with "infected lactating cows"; thus, only the latter was included in the multivariable model. The variables "province" ($P = 0.52$), "veterinary visits" ($P = 0.47$), "season" ($P = 0.43$), and "intramammary AMU" ($P = 0.16$, OR = 1.0) were not included in the final model due to *P*-value above 0.05.

Table 4.3 Unconditional association of the herd level (n=131), and sample level (n=1000) categorical predictors with the resistance on *E. coli* recovered from fecal samples collected from calves, heifers, lactating cows, and manure storage (model 1).

Section	Variable	Category	Frequency	OR	Overall *P*-value
Demographics	Herd size	≤ 70 lactating cows	31	Baseline	0.671
		71-160 lactating cows	61	1.17	
		≥161 lactating cows	39	1.27	
	Barn type	Tiestall	32	Baseline	0.377
		Freestall	99	1.16	
	Province		24	Baseline	0.022
			30	1.17	

		British Columbia		30	1.04	
		Alberta		27	1.07	
		Ontario		20	0.72	
		Quebec				
		Nova Scotia				
Herd health	Veterinary visits	More visits for herd health and less visits for sick animals	40		Baseline	0.201
		Less visits for herd health or more visits for sick animals	65		1.30	
		Less visits for animal health and more visits for sick animals	26		1.37	
			33		Baseline	0.122
	Infected young stock[1] (number of diseases reported)	≤ 2 diseases	73		0.97	
		3 to 4 diseases	25		1.36	
		5 diseases	14		0.49	0.031
			26		1.07	
	Infected lactating cows (number of diseases reported)		91		Baseline	
		≤ 1 disease				
		2 diseases	32		Baseline	0.071
		≥ 3 diseases	77		1.18	
			22		1.61	
	Treated young stock[1] (record of treatment for a given disease)	≤ 2 diseases				
		3 to 5 diseases				
		≥ 6 diseases				
			30		Baseline	0.132
			50		1.29	
			51		1.46	
	Treated lactating cows (record of treatment for a given disease)	≤ 2 diseases				
		3 to 4 diseases				
		5 diseases	56		Baseline	0.330
			41		1.08	
		No protocol	34		0.82	
	Veterinary protocols for a given disease developed by a veterinarian	Protocols for 1 to 5 diseases				
		Protocols for more than 6 diseases				
Biosecurity	Biosecurity	Only biosecurity practices other than vaccination	8		0.70	0.755
		At least one biosecurity practices and vaccines for 1 group	43		0.87	
		At least one biosecurity practices and	38		0.93	

		vaccines for 2 groups	42	Baseline	
		At least one biosecurity practices and vaccines for 3 groups			
	Raise multiple livestock species		110	Baseline	0.716
			21	0.93	
		No			
		Yes			
Other	Season	Summer[2]	253	0.84	0.152
		Fall[3]	695	Baseline	
		Winter[4]	52	1.56	
	Sample Source	Calf	256	Baseline	< 0.001
		Heifer	258	0.21	
		Cow	260	0.14	
		Manure storage	226	0.13	
	Year	1	509	Baseline	0.029
		2	491	0.74	
	Antimicrobials*	AMC	1000	0.48	< 0.001
		AMP	1000	1.53	
		FOX	1000	0.44	
		CRO	1000	0.39	
		CHL	1000	2.06	
		STR	1000	3.32	
		SOX	1000	3.01	
		TET	1000	4.54	
		SXT	1000	Baseline	

OR: Odds ratio. *AMC: amoxicillin/clavulanic acid; AMP: ampicillin; FOX: cefoxitin; CRO: ceftriaxone; CHL: chloramphenicol; STR: streptomycin; SOX: sulfisoxazole; SXT: trimethoprim/sulphamethoxazole TET: tetracycline.
[1]Pre-weaned calves and heifers
[2]August to September 2nd
[3]September 2nd to December 20th
[4]December 21st to March 3rd

Table 4.4 Unconditional association of the farm level (n=131) continuous predictors (antimicrobial use - AMU[1]) with the resistance on *E. coli* recovered from fecal samples collected from calves, breeding-age heifers, lactating cows, and manure storage (model 1).

Variable	No. farms[2]	Percentile			OR[3]	Overall *P*-value
		25th	50th	75th		
Total AMU	131	50.2	86.8	147.6	1.22	0.016
Systemic AMU	131	15.1	25.0	38.6	1.15	< 0.001
Intramammary AMU	124	23.9	49.8	99.1	1.08	0.085

[1]AMU in DCD/100 animal-years. Estimates were obtained from a garbage can audit, except for Quebec farms.
[2]Number of farms using the respective route of administration.
[3]OR: Odds ratio corresponding to an increase of 1 IQR in the number of DCD/100 animal-years of the antimicrobials.

The multivariate multilevel logistic regression results are presented in Table 4.5. There was no interaction between antimicrobials and systemic AMU ($P = 0.32$). The use of systemic antimicrobials was positively associated with resistance, and the association was the same across the nine antimicrobials included in the model. For instance, moving from the first quartile to the third quartile of systemic AMU (25th percentile = 15.1 DCD/100 animal-years to 75th percentile = 38.6 DCD/100 animal-years) increased the odds of resistance to any antimicrobial in the model by approximately 17.8% (OR=1.18, 95% CI: 1.07-1.26). *E. coli* isolates recovered from calf fecal samples had higher odds of being resistant when compared to other production ages and manure storage. *E. coli* isolates recovered in 2020 were less likely to be resistant when compared to the ones recovered in 2019. For farms with the occurrence of fewer infectious diseases (≤1 disease reported) in lactating cows (the most common infectious diseases in lactating cows, such as lameness and mastitis, were excluded from the analysis as almost 100% of the farmers reported the occurrence of these diseases), the odds to have a resistant *E. coli* were lower compared to farms that reported the occurrence of three or more infectious diseases. There was no difference between farms that reported 2 diseases with farms that reported ≥ 3 diseases. At the sample

level, the highest correlation was between STR/SOX (0.84), followed by STR/TET (0.74), and SOX/TET (0.64) (Figure 4.7).

Table 4.5 Final multivariate multilevel logistic regression model for resistance of generic *E. coli* from 1000 fecal samples (131 farms) collected from calves, heifers, lactating cows, and manure storage in 2019 and 2020.

	b	SE	OR[1]	95% CI		Wald *P* value	Overall LRT *P* value
Intercept	-4.15	0.24	-	-	-	-	-
Sample Source							< 0.001
Calves	2.36	0.24	10.59	6.43	16.65	<0.001	
Heifers	0.45	0.28	1.57	0.90	2.67	0.105	
Lactating cows	0.07	0.29	1.07	0.60	1.89	0.790	
Manure storage	Ref.						
Year							0.023
2019	Ref.						
2020	-0.34	0.15	0.71	0.53	0.96	0.023	
Infected lactating cows							0.030
<=1 disease	-0.75	0.33	0.47	0.25	0.89	0.021	
2 diseases	0.19	0.20	1.20	0.81	1.79	0.357	
>=3 diseases	Ref.						
Antimicrobials							< 0.001
AMC	-0.79	0.21	0.46	0.30	0.69	<0.001	
AMP	0.48	0.15	1.62	1.20	2.19	0.002	
FOX	-0.86	0.23	0.42	0.27	0.66	<0.001	
CRO	-1.02	0.21	0.36	0.24	0.54	<0.001	
CHL	0.82	0.15	2.28	1.70	3.04	<0.001	
STR	1.40	0.13	4.05	3.12	5.25	<0.001	
SOX	1.28	0.12	3.59	2.82	4.58	<0.001	
TET	1.77	0.15	5.89	4.40	7.88	<0.001	
SXT	Ref.						
Systemic AMU*	0.70	0.05					< 0.001
Variance	Estimate	SE					
Herd level	0.09	0.09					

AMC: amoxicillin/clavulanic acid; AMP: ampicillin; FOX: cefoxitin; CRO: ceftriaxone; CHL: chloramphenicol; STR: streptomycin; SOX: sulfisoxazole; SXT: trimethoprim/sulphamethoxazole TET: tetracycline.

* Systemic use in DCD/100 animal-years. Odds ratio and CI provided in the text.

[1]Odds ratio

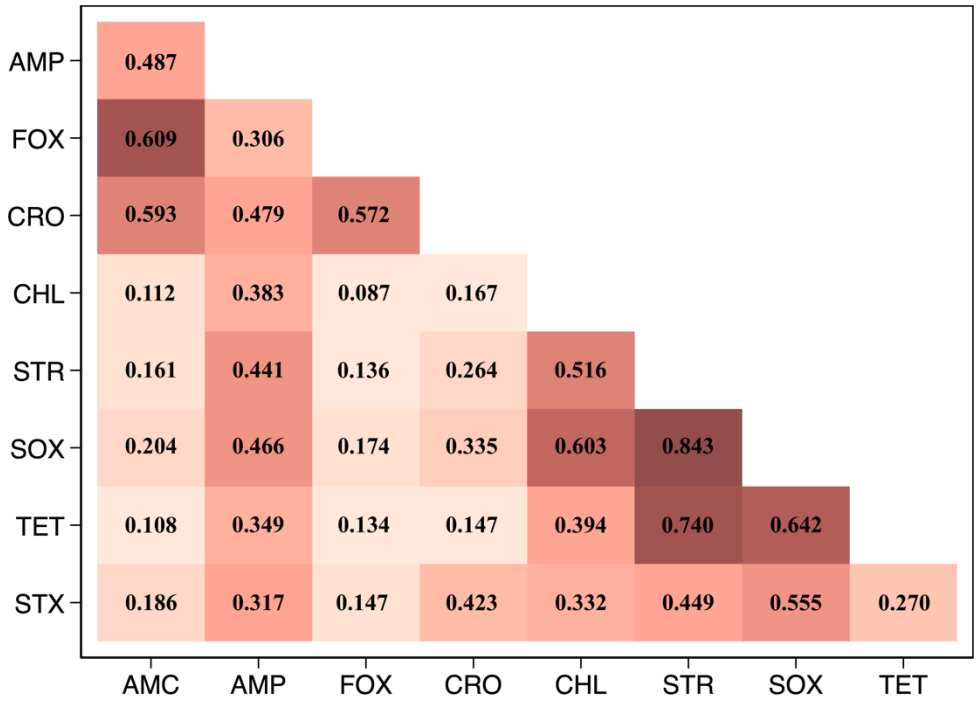

Figure 4.7 Estimated correlation results at the sample level from the multivariate multilevel logistic regression model for resistance of generic *E. coli* from 1000 fecal samples (131 farms) collected from calves, heifers, lactating cows, and manure storage in 2019 and 2020. AMC: amoxicillin/clavulanic acid; AMP: ampicillin; FOX: cefoxitin; CRO: ceftriaxone; CHL: chloramphenicol; STR: streptomycin; SOX: sulfisoxazole; SXT: trimethoprim/sulphamethoxazole TET: tetracycline.

4.9.3.2. Risk factors for MDR in generic E. coli (Model 2)

Descriptive data and the unconditional associations for the variables considered in model 2 are presented in Tables 4.6 and 4.7. As in model 1, the variables "treated young stock" and "treated lactating cows" were considered intervening variables with "infected young stock" and "infected lactating cows", respectively (Figure S4.2 in Appendix C). The variable "infected young stock" was associated with "infected lactating cows"; thus, only the latter was included in the multivariable model. The variables "raise multiple livestock" ($P = 0.20$), "season" ($P = 0.99$), "barn type" ($P = 0.65$), "infected lactating cows" ($P = 0.07$) and "intramammary AMU" ($P = 0.87$, OR = 0.99) were not included in the final model due to *P*-value above 0.05.

Table 4.6 Unconditional association of the herd level (n=131), and sample level (n=1000) categorical predictors with the multidrug resistance on *E. coli* recovered from fecal samples collected from calves, breeding-age heifers, lactating cows, and manure storage (model 2).

Section	Variable	Category	Frequency	OR	Overall *P*-value
Demographics	Herd size	≤ 70 lactating cows	31	Baseline	0.715
		71-160 lactating cows	61	1.05	
		≥ 161 lactating cows	39	1.18	
	Barn type	Tiestall	32	Baseline	
		Freestall	99	1.33	
	Province	British Columbia	24	Baseline	0.147
		Alberta	30	1.12	
		Ontario	30	1.01	
		Quebec	27	0.95	0.591
		Nova Scotia	20	0.74	
Herd health	Veterinary visits	More visits for herd health and less visits for sick animals	40	Baseline	0.415
		Less visits for herd health or more visits for sick animals	65	1.26	
		Less visits for animal health and more visits for sick animals	26	1.22	
	Infected young stock[1] (number of diseases reported)	≤ 2 diseases	33	Baseline	0.097
		3 to 4 diseases	73	1.03	
		5 diseases	25	1.52	
	Infected lactating cows (number of diseases reported)	≤ 1 disease	14	0.62	0.150
		2 diseases	26	1.10	
		≥ 3 diseases	91	Baseline	
	Treated young stock[1] (record of treatment for a given disease)	≤ 2 diseases	32	Baseline	0.084
		3 to 5 diseases	77	1.26	
		≥ 6 diseases	22	1.72	
	Treated lactating cows (record of treatment for a given disease)	≤ 2 diseases	30	Baseline	0.145
		3 to 4 diseases	50	1.17	
		5 diseases	51	1.47	
	Veterinary protocols for a given disease	No protocol	56	Baseline	0.432
		Protocols for 1 to 5 diseases	41	1.00	

	developed by a veterinarian	Protocols for more than 6 diseases	34	0.79	
Biosecurity	Biosecurity	Only biosecurity practices other than vaccination	8	0.77	0.890
		At least one biosecurity practices and vaccines for 1 group	43	0.97	
		At least one biosecurity practices and vaccines for 2 groups	38	0.92	
		At least one biosecurity practices and vaccines for 3 groups	42	Baseline	
	Raise multiple livestock species	No	110	Baseline	0.219
		Yes	21	0.83	
Other	Season	Summer[2]	253	0.68	0.209
		Fall[3]	695	Baseline	
		Winter[4]	52	0.92	
	Sample Source	Calf	256	Baseline	< 0.001
		Heifer	258	0.13	
		Cow	260	0.09	
		Manure storage	226	0.09	
	Year	1	509	Baseline	0.029
		2	491	0.74	

OR: Odds ratio.
[1]Pre-weaned calves and heifers
[2]August to September 2nd
[3]September 2nd to December 20th
[4]December 21st to March 3rd

Table 4.7 Unconditional association of the farm level (n=131) continuous predictors (AMU[1]) with the multi-drug resistance on *E. coli* recovered from fecal samples collected from calves, heifers, lactating cows, and manure storage (model 2).

Variable	No. farms[2]	Percentile			OR[3]	Overall *P*-value
		25th	50th	75th		
Total AMU	131	50.2	86.8	147.6	1.16	0.072
Systemic AMU	131	15.1	25.0	38.6	1.12	0.004
Intramammary AMU	124	23.9	49.8	99.1	1.04	0.620

[1]AMU in DCD/100 animal-years. Estimates were obtained from a garbage can audit, except for Quebec farms.
[2]Number of farms using the respective active ingredient.
[3]OR: Odds ratio corresponding to an increase of 1 IQR in the number of DCD/100 animal-years of the antimicrobials.

The results from the ordinal logistic regression are presented in Table 4.8. Systemic use of antimicrobials was positively associated with the odds of an *E. coli* isolate being resistant to one or two antimicrobial classes (*vs* fully susceptible), or to being MDR (*vs* 1 or 2 antimicrobial classes). For instance, movement from the lower quartile to the higher quartile of the systemic AMU IQR (25^{th} percentile = 15.1 DCD/100 animal-years to 75^{th} percentile = 38.6 DCD/100 animal-years) increased the odds of resistance for either comparison by 16.0% (OR=1.16, 95% CI: 1.06-1.27). Compared to all the other samples, *E. coli* isolates recovered from calf samples were more likely to be resistant to one or two classes of antimicrobials and to be multi-drug resistant. There was no difference among the other sample sources. The estimates from sample source (lactating cows) violated the proportional odds assumption, although the effect was in the same direction (being less likely to be resistant). *E. coli* isolates recovered from lactating cows' samples were less likely to be multi-drug resistant than isolates recovered from calves. *E. coli* isolates recovered from samples collected in 2020 were more likely to be in lower categories (susceptible or resistant to ≤ 2 classes) compared to the ones recovered from samples collected in 2019.

Table 4.8 Final generalized ordinal logistic regression mixed model for multidrug resistant isolates of generic *E. coli* from 1000 fecal samples (131 farms) collected from calves, heifers, lactating cows, and manure storage in 2019 and 2020.

	b	SE	OR[1]	95% CI		*P* value	Overall *P* value
Intercept_coefficient 1	0.18	0.17	-	-		-	-
Intercept_coefficient 2	-0.43	0.17					
Sample Source							
Calves	Ref.	-	-	-	-	-	<0.001
Heifers	-2.03	0.21	0.14	0.09	0.21	<0.001	
Lactating cows[2]	-2.32	0.23	0.10	0.06	0.15	<0.001	
Manure storage	-2.43	0.25	0.09	0.05	0.14	<0.001	
Year							
2019	Ref.						0.023
2020	-0.36	0.16	0.70	0.50	0.95	0.023	
Systemic AMU*[3]	0.63	0.2					0.002
Variance	Estimate	SE					
Herd level	0.17	0.14					

[1]Odds ratio.
[2]Estimate that violated the proportional odds assumptions is given, but the cut-points specific estimates for lactating cows were: coefficient 1= -2.18; coefficient 2=-2.97.
[3]Estimate multiplied by 100.

4.10. Discussion

This study focused on the proportion of resistant-*E. coli* recovered from dairy cattle and the risk factors associated with resistance in those isolates. Although all the farms enrolled had at least one sample type in each year positive for generic *E. coli*, there was significant variability in terms of AMR. The proportion of isolates resistant to at least one of the 14 antimicrobials was 24.5%, lower than the 42.2% reported from Canadian abattoir samples from cattle in 2016 [180]. Another study on dairy cattle in Quebec, Canada, reported that 30.2% of *E. coli* isolates were resistant to at least one antimicrobial, which is slightly higher than the 26.0% found in our study for the same province [173].

International organizations, such as WHO, have categorized antimicrobials according to their importance for human medicine [133]. In Canada, antimicrobials are categorized into four categories (I - very high importance to IV - low importance) based on their importance for human health [132]. Focus has been on AMU and AMR of Category I drugs on dairy farms. This study found low numbers of isolates with resistance to the Category I third-generation cephalosporins and fluoroquinolones (2.2% and 1.4 %, respectively) and only one *E. coli* isolate was resistant to meropenem. In Québec, the resistance to category I antimicrobials was also low, agreeing with the results from a previous study [173]. Since February 2019, the Québec government has enforced a new regulation restricting the use of Category I antimicrobials in food animals, which might contribute to the lower proportion of resistance to these drugs [187].

Higher proportions of resistance to category I antimicrobials were previously reported for presumptive ESBL *E. coli* isolates. A study conducted in Tennessee, reported that 30.8% of the *E. coli* isolates recovered from fecal samples were resistant to cefotaxime, a third-generation cephalosporin [188]. Additionally, a study from Pennsylvania also using selective media demonstrated a high proportion of resistance to third-generation cephalosporin in *E. coli* recovered from dairy herds, where the resistance to ceftiofur and ceftriaxone was 21.2% and 24.2%, respectively [38]. This latter study also investigated resistance in different sample sources (pre-weaned calves, post-weaned calves, dry cows, and lactating cows). The proportion of isolates resistant to third-generation cephalosporin (ceftriaxone and ceftiofur) in pre-weaned calves was 36.4%, which was higher than the other sample sources. Although the overall resistance to third-generation cephalosporin in our study was low (2.2%), the proportion of third-generation cephalosporin resistance in *E. coli* recovered

from pre-weaned calves (7.2%) was higher compared to the other sample sources. A review published in 2022 reported ceftiofur as being the drug of choice to treat diarrhea in pre-weaned , mastitis, and reproductive diseases, among others, in United States dairy herds [14]. In Canada, a study from 2022 on the management practices of dairy calves (also using CaDNetASR data) reported that the use of ceftiofur (third-generation cephalosporin) for treatments in dairy calves was low (1.1%) [189]. In our study, third-generation cephalosporin use (ceftiofur) accounted for approximately 15.5% of the total AMU (Table S1). However, most of the ceftiofur use was due to intramammary products. These results might suggest that the selective pressure exerted by the use of ceftiofur might not be the only factor contributing to the recovery of third-generation cephalosporin-resistant *E. coli* in dairy calves. For instance, a study from 2018 conducted in eight dairy farms in New Brunswick (Canada) reported that the recovery of extended-spectrum cephalosporin-resistant *E. coli* from calf fecal samples were 1.6 times more likely on farms feeding unpasteurized nonsalable milk to the dairy calves [190]. Additionally, a review including 19 studies on the effect of feeding waste milk to dairy calves also reported an increase in the shedding of extended spectrum beta-lactamase (ESBL)-producing *E. coli* [191]. According to a previous study in Canada also using CaDNetASR data, approximately 20% of the producers fed waste milk to the dairy calves [189].

Despite finding a low proportion of resistance to Category I antimicrobials in this study, higher proportions of resistance were observed for Category II and Category III drugs. In Canada, most of the drugs approved for use in dairy cattle belongs to category II. Resistance to streptomycin (Category II) and tetracycline (Category III) was 16.2% and 20.9%, respectively. This resistance pattern was confirmed by model 1, which indicated

higher odds of resistance for tetracycline and streptomycin (Table 4.5). Similar results with higher proportions of resistance to tetracycline and streptomycin were reported by CIPARS surveillance in beef cattle and a previous study from Quebec [173, 180]. A study from California also reported higher proportions of resistance to tetracycline and streptomycin in generic *E. coli* recovered from dairy cattle [192]. A higher proportion of resistance was reported for ESBL *E. coli* isolates in Pennsylvania dairy herds [38]. This higher proportion of resistance in this latter study was expected as previous studies indicated that ESBL isolates could carry ARGs contributing to co-resistance to other antimicrobial classes other than beta-lactams [188].

Tetracycline is one of the most used antimicrobials in food-producing animals in the last 50 years, so a higher proportion of resistance to this drug is expected [70]. A study published in 2011, using data from the major dairy provinces in Canada, reported that 64% (57/89) of the farms enrolled in the study were using tetracycline [83]. A study published in 2021, using data from Quebec, demonstrated that 45% (45/100) of the farms were using tetracycline [131]. In our study, 49% (64/131) of the enrolled farms were using tetracycline, suggesting that although tetracycline use has decreased compared to decades ago, the proportion of farms using tetracycline is still substantial. These results suggest that using tetracyclines could exert a selective pressure to select for resistance to this same drug. In Canada, although no longer available, a drug containing dihydrostreptomycin was commonly used to treat mastitis in dairy herds. Other antimicrobials belonging to the aminoglycoside class, such as gentamicin, are approved for intrauterine use in dairy cattle; however, intrauterine AMU accounted for only 1.4% of the total AMU and might have a very low contribution to selecting for resistance to streptomycin. Additionally, the use of

antimicrobials might not be the only factor contributing to the streptomycin resistance found on those farms. The ARGs transferred horizontally among bacteria in the gastrointestinal tract might contribute to the persistence of resistance even when an antimicrobial is no longer being used [193].

In agreement with the descriptive results, similar resistance patterns were found in model 1. Each antimicrobial was included as separate binomial responses, which provided a matrix with the correlations between each pair of antimicrobials considered in the model (Figure 4.7). The correlation of resistance at the sample level varied depending on the antimicrobials. The highest correlations were found between streptomycin/sulfisoxazole and streptomycin/tetracycline. A possible hypothesis to explain the high correlation between resistance from these two antimicrobials could be the acquired resistance through resistance genes to tetracyclines and streptomycin that can be transferred on plasmids or other mobile genetic elements like integrons [194, 195]. A study published in 2008 analyzed soil samples from dairy farms and demonstrated that some strains of *E. coli* recovered from these samples carried resistance genes for tetracycline and streptomycin [196]. Additionally, *E. coli* isolates carrying resistance to tetracycline, streptomycin and sulfadiazine were reported to be spread in dairy farms across Washington state, suggesting that resistance to more than one of these antimicrobials could commonly occur among dairy herds in North America [197].

As the major exposure in our study was the AMU, choosing the appropriate metric to quantify the use of these drugs on-farm is crucial to provide reliable results. Different metrics can be used to quantify AMU at the farm level, and the choice can be made according to the study's objectives, animal species, country, and others [57]. The metric used

in our study to quantify antimicrobials (DCD/100 animal-years) was developed for cattle in Canada [86]. This was done considering that this metric is widely used and can better account for long-acting or single-dose products [131].

It is well described in the literature that clinical mastitis is the main reason for AMU in dairy farms [198]. In our study, the intramammary and systemic AMU varied substantially among the enrolled dairy farms (Figure 4.1). A Canadian study published in 2012 quantified the antimicrobial usage across four different regions in Canada (Alberta, Ontario, Quebec, and Maritimes) and found that the AMU rate for systemic administration was higher than intramammary [83]. In our study, 94.7% of the farms across the five provinces used intramammary products, and this route of administration comprised 60.6% of the total AMU in DCD/100 animal-years. A 2021 study of Quebec dairy herds reported that intramammary AMU (lactating and dry cows) accounted for the highest proportion of the total AMU in DCD/100 cow-years (approximately 60.0% of the total use) [131]. In our study, the intramammary AMU data from Quebec represented 53.7% of this province's total use. The same pattern was observed for the other provinces, in which the intramammary AMU represented a higher proportion of the total usage (Table 4.1). These results suggest that the proportion of the total AMU represented by the intramammary administration route has increased when compared to results reported in Canada in 2012.

There is still a debate regarding the role of the route of administration of antimicrobials and its effect on AMR in enteric bacteria [199]. Despite contributing to the highest proportion of the total AMU in the enrolled dairy farms, no significant effect on resistance in generic *E. coli* was observed for antimicrobials administered through the intramammary route in the final models ($P = 0.16$ and $P = 0.87$ for models 1 and 2, respectively). A similar effect

was observed in an *in vitro* study published in 2022, investigating the effect of intramammary application of first-generation cephalosporins on resistance in *E. coli* recovered from fecal samples and manure slurry. In this latter study, no selection of ESBL *E. coli* was observed when using concentrations at or below the maximum concentration of these drugs after intramammary treatments [200]. The intramammary AMU is a local administration of antimicrobials, and these results suggest that the impact of those treatments on resistance in fecal bacteria such as *E. coli* might be low. These results also raise the debate if the effect of intramammary treatments on resistance could be different for udder pathogens isolated from milk, including *E. coli*. However, a study from Canada published in 2018 demonstrated that intramammary AMU was not significantly associated with resistance in non-*aureus* staphylococci recovered from milk samples [74].

A finding contradicting these latter studies was also published in the literature. A study conducted on dairy farms in the United States in 2010 suggested that farms using an intramammary first-generation cephalosporin-based dry cow treatment had a higher probability of recovering coliforms with reduced susceptibility to cephalothin [75]. This result could be related to the high use of blanket dry cow therapy, as a review published in 2022 discussing the use of selective treatment for cows at the dry-off period reported that 94.2% of 1,261 dairy herds from the United States were using blanket dry cow therapy (treating all animals at the dry-off period regardless of their infection status) [201]. Similarly, a study from 2018 which included dairy herds from ten Canadian provinces, reported that 84% of the farms (305/364) were using blanket dry cow therapy [202]. In our study, 91 farms (69.5%) used first-generation cephalosporins (either for DCT or during lactation), and its

use represented 13.9% of the total AMU; however, 50% of these farms used less than 4 DCD/100 animal-years (Table S3) and resistance to cefoxitin was low (2.5%).

Although IMM antimicrobials are the most used in dairy farms, other diseases such as reproductive and respiratory diseases might require systemic antimicrobials [198]. Additionally, young animals such as pre-weaned dairy calves may require systemic treatment. In Canada, a study from 2022, including dairy calf data from 5 provinces, demonstrated that the main reasons for receiving treatment in pre-weaned calves were respiratory diseases and diarrhea [189]. In our study, as opposed to the intramammary route, the systemic use of antimicrobials was significantly associated with AMR, including MDR, in the recovered *E. coli* isolates. For instance, in model 1, for an IQR increase in the systemic use of antimicrobials, the odds of resistance increased by approximately 17.8%. The same pattern was observed for the MDR model (model 2), where the IQR increase in systemic use increased the odds of being resistant to one or two classes or to be MDR by 16%. Most of the evidence on the association between AMU and AMR in food-producing animals is demonstrated using the total AMU or a given active ingredient and antimicrobial class; however, the route of administration is not often considered. A study published in 2013 investigated the impacts of systemic administration of tetracycline and ampicillin through the oral and injectable routes on the ARGs in fecal bacteria in mice with natural gut microbiota. The findings suggested that both oral and injectable routes increased the pool of ARGs for these two antimicrobials; however, the development of resistance was lower or delayed for injectable administration compared to oral using the same doses [199]. In our study, the systemic administration included the oral and injectable routes. The

injectable routes accounted for 85.8% of the systemic use, while the oral accounted for the remaining 14.2% (data not shown).

Although AMU is recognized as the main driver for AMR, other factors might contribute to the presence of AMR in dairy farms. We also investigated other variables that could contribute to understanding AMR on dairy farms. For example, AMR was associated with the sample source. Isolates recovered from calf samples were more likely to be resistant (OR = 10.6) when compared to isolates from other production ages or manure storage. The same pattern was confirmed by the ordinal model, where isolates recovered from calves were more likely to be resistant to one or two classes or be MDR compared to the other sample sources. Similar results were reported in the Quebec study in 2021 [173]. The Pennsylvania study also reported that MDR *E. coli* were more frequently recovered from pre-weaned calves compared to post-weaned calves, dry cows, and lactating cows [38]. As previously reported in the discussion, systemic AMU was associated to increased resistance in *E. coli* in our study. Given that dairy calves are typically treated with antimicrobials via systemic administration, it is reasonable to assume that this practice might contributed for the higher odds of resistance observed in this study among this production age group. A previous study using CaDNetASR data from 74 dairy herds, reported that 29.6% of the calves were treated at least once with antimicrobials [189] . The literature also suggested that other factors such as the diet during nursing and the housing system may also increase the risk of antimicrobial resistance in dairy calves. For example, a study by Liu et al. (2019) found that the colostrum fed to dairy calves may be a potential source of ARGs [203]. In addition, the housing system, whether individual or grouped, was found to influence the resistance to certain antimicrobials in *E. coli* recovered from dairy

calves [204]. We also hypothesize that the incompletely developed immune system in calves might also contribute to the higher proportion of resistance.

Our study included results from data collected in 2019 and 2020. The year in which the isolate was recovered was significant in the final models. In both models, isolates recovered in 2020 were less likely to be resistant than isolates recovered in 2019. Although the field sampling was scheduled to be done during the same period, in the first year of data collection (2019), the sampling was primarily done during fall. In the second year of data collection (2020), most of the sampling was done during the summer and early fall seasons. A study conducted in ten California dairy herds demonstrated that *E. coli* isolates recovered during the winter had a higher proportion of MDR when compared to isolates recovered during the summer [205]. However, another study from the United States, including *E. coli* isolates, suggested that AMR can be higher with an increase in temperature [206]. A third study from the United Kingdom also suggested that the ARGs are less likely to be detected in E. *coli* isolates during seasons when the temperature is lower [207]. In our study, the season was not significant ($P = 0.43$ for model 1 and $P = 0.99$ for model 2) in the final models; however, the sampling schedule was not planned to detect the effects of different seasons on the AMR.

In the present study, besides the main exposure, variables from the surveillance questionnaire were explored in models 1 and 2 as potential confounders for AMU. The occurrence of infectious diseases in lactating cows was found to be associated with AMR. Farms in which the farmers reported no occurrence of pneumonia, wound infections, diarrhea, and uterine infection, or the occurrence of only one of them, were less likely (OR = 0.47) to have a resistant *E. coli* isolate when compared to farmers that reported the

occurrence of three or all of these infectious diseases. In addition, it has been observed that the presence of infectious diseases can lead to increased use of antimicrobials [131]. A study quantifying AMU in Quebec dairy herds found that the total incidence rate of diseases was significantly associated with the total AMU rate. An increase in the AMU rate by a factor of 1.3 was observed for each increase of 10 diseased animals/100 animal at risk-years [131]. These findings support the importance of implementing good husbandry practices to reduce the incidence of infectious diseases as a way to reduce the use of antimicrobials on dairy farms. According to the DAG, systemic AMU was an intervening variable for infected lactating cows. If AMU was removed from the model, the effect of the variable "infected lactating cows" became even stronger (data not shown).

Some limitations in this study could have affected the results or inferences that were reported. For example, one potential limitation is the way in which the association between AMU and AMR was assessed. In cross-sectional studies it is difficult to imply if the AMU drove resistance or if the existing resistance in the farms led to increased use of antimicrobials. However, the association between AMU and AMR is well-established in the literature [45, 48, 70]. Additionally, the AMU data used in the study were retrieved in 2019, and the AMR data were retrieved in 2019 and 2020. Therefore, the resistance results from 2020 might not fully represent the impact of using antimicrobials during 2019. Another limitation could be associated with the validity of the study. Convenience sampling can introduce selection bias, as the farms enrolled may not completely represent the source population. Although the inferences from the results presented here should be made with caution to farms other than the ones included in the study, we believe the study farms are managed under comparable conditions to other commercial dairy herds in Canada. Another

limitation that could impact the results was the AMU data collection. The AMU information was obtained using the GCA system, except for the province of Québec, which collected information through veterinary invoices. A recent study from Québec on AMU quantification demonstrated that the data retrieved from veterinary invoices had similar results to GCA [54]. Thus, we believe that the impact on the results from using different methods is negligible.

Another potential source of bias that could have impacted our results is the information bias. During the on-site visits, we administered surveillance questionnaires to gather data on herd health, including the occurrence of various infectious diseases within the farm. It is essential to acknowledge the possibility of recall bias, which could have arisen if farmers provided inaccurate information about the occurrence of these diseases. Additionally, as the surveillance questionnaires were lengthy and time-consuming, all the information was collected by the regional field workers responsible for entering the answers in the spreadsheets to avoid entry mistakes and missing data. All data manipulation was done after being transferred to the statistical package to preserve the data entered in the spreadsheet.

Our study provided updated information on the proportion of AMR and MDR in generic *E. coli* recovered from dairy cattle fecal samples in Canada. The overall resistance was slightly lower compared to previous studies on beef and dairy cattle, and dairy farmers have the opportunity to implement measures to continue reducing the AMR. The AMU varied substantially among the farms, indicating that measures to promote prudent use of antimicrobials can be implemented. A higher proportion of resistance to tetracycline and streptomycin was observed, agreeing with other studies conducted in Canada. There was

no significant association between the intramammary AMU and the resistance; however, the systemic AMU was associated with increased resistance and MDR in the recovered *E. coli* isolates. Monitoring generic *E. coli* is an essential tool in surveillance systems as it is well established as a reservoir for antimicrobial resistance genes that can be spread to pathogenic bacteria.

Chapter 5

5. Frequency of isolation and phenotypic antimicrobial resistance of fecal *Salmonella enterica* recovered from dairy cattle in Canada

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5.1. Abstract

Salmonellosis is one of the leading causes of gastrointestinal infections in humans. In Canada, it is estimated that approximately 87,500 cases of salmonellosis occur every year in humans, resulting in 17 deaths. In the United States, it is estimated that 26,500 hospitalizations and 420 deaths occur every year. In dairy cattle, infections caused by nontyphoidal *Salmonella enterica* can cause mild to severe disease, including enteritis, pneumonia, and septicemia. Our study objectives were to determine the proportion of fecal samples positive for *Salmonella* in dairy cattle in Canada and determine the resistance pattern of these isolates. We used data collected through the Canadian Dairy Network for Antimicrobial Stewardship and Resistance (CaDNetASR). Pooled fecal samples from pre-weaned calves, post-weaned heifers, lactating cows, and manure storage were cultured for *Salmonella,* and the isolates were identified using matrix-assisted laser desorption/ionization-time of flight mass spectrometry. Antimicrobial susceptibilities were determined using the minimum inhibitory concentration test, and resistance interpretation was made according to the Clinical and Laboratory Standards Institute. A two-level, multivariable logistic regression model was built to determine the probability of recovering *Salmonella* spp. from a sample while accounting for province, year, and sample source. The percentage of farms with at least one positive sample were 12% (17/140), 19% (28/144), and 17% (24/144) for the sampling years 2019, 2020, and 2021, respectively. Out of the 113 *Salmonella* isolates, 23 different serotypes were identified. The occurrence of *Salmonella* appeared to be clustered by farms and provinces. The most common serovars identified were Infantis (14%) and Typhimurium (14%). Overall, 21% (24/113) of the *Salmonella* isolates were resistant to at least one antimicrobial. Resistance to tetracycline

was commonly observed (17%); however, very limited resistance to category I antimicrobials (categorization according to Health Canada that includes third-generation cephalosporins, fluoroquinolones, polymyxins, and carbapenems) was observed, with one isolate resistant to amoxicillin/clavulanic acid. The percentage of *Salmonella* isolates resistant to two and three antimicrobial classes was 3.5% and 8.8%, respectively. Our study provided valuable information on the proportion of fecal samples positive for *Salmonella*, the serovars identified, and associated resistance patterns across CaDNetASR herds, at regional and national levels.

5.2. Introduction

Salmonellosis, usually defined as a symptomatic infection caused by nontyphoidal *Salmonella enterica* in humans, is one of North America's leading causes of enteric diseases. Most infections in humans caused by nontyphoidal *Salmonella enterica* are self-limiting, and most infected people recover without specific treatment [208]. However, some people can develop severe symptoms that require hospitalization. In Canada, it is estimated that approximately 87,500 cases of salmonellosis occur every year, resulting in approximately 925 hospitalizations and 17 deaths [209]. There are approximately 1.35 million cases of salmonellosis annually in the United States, resulting in 26,500 hospitalizations and 420 deaths per year [210].

Food-producing animals are an important source of nontyphoidal *Salmonella* infections in humans [211, 212]. Approximately 80% of human salmonellosis cases in Canada are associated with foodborne transmission, with poultry meat being the most common source of infection [208]. Human infections were previously linked to cattle exposure. For instance, a review on sources of human salmonellosis suggested that infections can also occur through direct contact with infected dairy cattle or the consumption of raw milk [213]. Dairy cattle can become infected through horizontal transmission via the fecal-oral route by direct contact with other cattle or contaminated environment, contaminated feed, and through vertical transmission [18, 214].

Similar to what is observed in humans, gastrointestinal infections in dairy cattle caused by *Salmonella* can have no or mild clinical signs, making them a potential reservoir for nontyphoidal *Salmonella enterica* [18]. For instance, *Salmonella* Dublin is a cattle-adapted

serovar with the ability to persist in the cattle host without showing any clinical signs of infection [215]. However, *Salmonella* Dublin may cause severe diseases, such as pneumonia and septicemia, especially in dairy calves [18].

Antimicrobial resistance (AMR) in *Salmonella enterica* can threaten human and animal health, as it might affect the efficacy of antimicrobial treatment. Several studies from North America reported the resistance pattern from *Salmonella enterica* isolated from dairy cattle. One California study found a higher proportion of *Salmonella enterica* resistant to tetracycline, followed by ampicillin and ceftriaxone [40]. A second California study also reported the resistance pattern in *Salmonella* isolated from dairy cattle and found a higher proportion of resistance to streptomycin, followed by tetracycline and ampicillin [64]. In a 2018 Canadian study including eight dairy herds in New Brunswick, Canada, 16 *Salmonella* isolates were recovered from dairy calves and all isolates were susceptible to all antimicrobials tested, except for one isolate resistant to sulfisoxazole [190]. Another Canadian study including clinical isolates from 27 cattle operations (dairy and beef) in Alberta, reported moderate to high levels of resistance in *S.* Typhimurium and *S.* Dublin to the critically important beta-lactam antimicrobials ceftiofur, ceftriaxone, and amoxicillin-clavulanic acid [216]. To the authors' knowledge, there is no Canada-wide study reporting the resistance pattern of *Salmonella* isolated from dairy cattle. The primary objectives of this study were therefore to: 1) estimate the proportion of fecal samples positive for *Salmonella* and their geographical location; 2) identify the *Salmonella* serovars; and 3) determine the phenotypic resistance patterns of these isolates.

5.3. Materials and Methods

Data were collected through the Canadian Dairy Network for Antimicrobial Stewardship and Resistance (CaDNetASR) surveillance system in 2019, 2020, and 2021 [129]. The University of Prince Edward Island Research Ethics Board and the Animal Care Committee reviewed and approved the study on March 7, 2019 (file # 6008059).

5.3.1. Sample size, Farm Selection and Sample Collection

Sample size calculation and farm enrollment were described in chapter 2 [129]. Briefly, a convenience sample of 140 farms in 2019 and 144 in 2020 and 2021 from five Canadian provinces (British Columbia, Alberta, Ontario, Québec, and Nova Scotia) participated in this study. Enrolled farms had a minimum of 50 animals except for Nova Scotia, which had a minimum size of 40 animals. Participating farms also needed to raise their replacement heifers on-site. On each farm, pooled fecal samples (5 fresh fecal pats were selected from different places on the floor and combined) from each of three age groups (pre-weaned calves, post-weaned heifers, lactating cows) and a manure storage sample were obtained. Samples were stored in coolers with ice packs and sent to the Atlantic Veterinary College (Charlottetown, PE, Canada) for bacterial isolation and antimicrobial susceptibility testing. All *Salmonella* isolates were sent to Guelph Reference Services Unit for serovar identification.

5.3.2. Bacterial isolation and characterization

Fecal samples were cultured for *Salmonella* according to the Canadian Integrated Program for Antimicrobial Resistance Surveillance (CIPARS) protocol [217]. Briefly, 25 g from each

pooled feces sample or manure storage sample was homogenized in 250 mL of buffered peptone water (BPW) for each sample using a stomacher for 30 sec at 230 rpm and incubated at 35°C for 24 h. Modified Semi-solid Rappaport Vassiliadis (MSRV) agar was inoculated with 0.1 mL of the BPW mix using a sterile pipette. The plates were then incubated at 42°C for 24-72 h. After 24 h, the length of migration was measured, and if it was ≥ 20 mm, up to 2-3 colonies from outside the edge of the migration area were streaked onto a MacConkey culture plate and incubated at 35°C for 24 h. If migration was not evident or < 20 mm, the plates were incubated for up to 72 h and checked again for migration. A single colony typical for *Salmonella* (colourless) was subcultured onto a new MacConkey Agar culture plate and incubated at 35°C for 24 h. Isolates were confirmed to be *Salmonella enterica* by matrix-assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF MS) using the Bruker Microflex MALDI-TOF MS (Bruker Daltonics, GmbH, Bremen, Germany). A single colony was transferred onto the target plate, air-dried at ambient temperature, and overlaid with alfa-cyano-4-hydroxycinnamic acid before being introduced into the MALDI-ToF mass spectrometer for automated measurement of mass spectra and comparison to the reference database (MBT 8468 MSP Library). Identification scores ≥ 2.0 were required for confident species identification. Reference strain *Salmonella* Typhimurium ATCC 14028 was used as quality control for each target plate ran.

5.3.3. In Silico Serotyping

All *Salmonella* isolates were sent to the National Microbiology Laboratory at Guelph, Canada, for serotyping. These *Salmonella* isolates were serotyped using either the

traditional phenotypic serotyping method (isolates from 2019) or Whole Genome Sequencing (WGS)-based alternative method (SISTR - *Salmonella in silico* Typing Resource) (isolates from 2020 and 2021) [218]. The phenotypic serotyping method detects O or somatic antigens of the *Salmonella* isolates via slide agglutination. The H or flagellar antigens were identified with a microtiter plate well precipitation method [219]. Antigenic formulae and serovars of the *Salmonella* isolates were identified and designated as per White-Kauffmann-Le Minor (WKL) scheme. The SISTR detects the genes encoding surface O and H antigens and reports the corresponding *Salmonella* serovar following the existing WKL serotyping scheme [218].

5.3.4. Antimicrobial Susceptibility Testing

Antimicrobial susceptibility was determined using the Sensititre™ microdilution system and the CMV4AGNF (2019) or CMV5GNF (2020 and 2021) panels (Sensititre™, Thermo Fisher Scientific, MA). The plate contained twofold serial dilutions of the following antimicrobials: amoxicillin/clavulanic acid (AMC; 1/0.5-32/16 μg/mL), ampicillin (AMP; 1-32 μg/mL), azithromycin (AZM; 0.25-32 μg/mL), cefoxitin (FOX; 0.5-32μg/mL), ceftriaxone (CRO; 0.25-64 μg/mL), chloramphenicol (CHL; 2-32 μg/mL), ciprofloxacin (CIP; 0.015-4 μg/mL), gentamycin (GEN; 0.25-16 μg/mL), meropenem (MER; 0.06-4 μg/mL), nalidixic acid (NAL; 0.5-32 μg/mL), streptomycin (STR; 2-64 μg/mL), sulfisoxazole (SOX; 16-256 μg/mL), tetracycline (TET; 4-32 μg/mL), and trimethoprim/sulphamethoxazole (SXT; 0.12/2.38-4/76 μg/mL). In 2020, the antimicrobial streptomycin was replaced by colistin (COL; 0.25-4 μg/mL). Reference strain *Salmonella typhimurium* ATCC 14028 was used for quality control. The minimum inhibitory

concentration (MIC) values were the lowest antimicrobial concentration that inhibited visible bacteria growth. The MIC was assigned to the next dilution if growth was observed at the highest antimicrobial concentration tested. Antimicrobial resistance was interpreted according to the Clinical and Laboratory Standards Institute (CLSI) clinical breakpoints, recommended by CIPARS [220]. An isolate was considered multi drug resistant (MDR) if it was resistant to ≥ 3 antimicrobial classes [135].

5.3.5. Statistical Analysis

A descriptive analysis was performed to determine the proportions of *Salmonella*-positive farms and fecal samples. Frequency distributions of MIC, MIC_{50}, and MIC_{90} were calculated for all isolates and each antimicrobial in the panel. Isolates were also categorized as sensitive or resistant with intermediate sensitivity being categorized as resistant. Additionally, we determined the proportion of different serovars identified, the proportion of isolates resistant to each antimicrobial included in the panel, and the proportion of isolates that were MDR (defined as resistant to ≥ 3 antimicrobial classes). For all statistical analyses, the unit of analysis was the sample obtained from a given production phase (calves, heifers, lactating cows) or manure storage. Each of these samples was defined by one *Salmonell*a isolate. Given the hierarchical structure of the data, a two-level, multivariable, logistic regression model with farm as a random intercept was used to determine the probability of recovering *Salmonella* from a sample. Unconditional associations between the explanatory variables (province, year, and sample source) and the outcome were examined using the previously described multilevel, logistic regression model, but using only one predictor at the time. Chi-square tests were used to assess associations among these predictors. Only those variables with P values ≤ 0.20 were

selected for inclusion in the multivariable model. Predictors with *P*-value ≤ 0.05 were retained in the final model. Pairwise comparisons using Bonferroni's method were done for categorical predictors with more than two categories. Biologically plausible interaction terms were explored (province x sample source; province x year; and sample source x year). Visual examination of the plot of residuals at the higher level did not show any significant pattern that could indicate lack of homoscedasticity. The residuals were also visually inspected for normality. Intraclass correlation coefficients (ICCs) were calculated for the final model using latent variable approximation to estimate clustering within herds [137]. The odds ratio was calculated from regression coefficients and adjusted from cluster-specific to the population average using the formula below [137]:

$$\beta PA = \frac{\beta CS}{\sqrt{(1 + 0.346 * \sigma^2)}}$$

where βCS is the cluster-specific (CS) estimate to be converted to the population average (PA), and σ^2 is the variance of the random effect. Statistical analyses were performed using Stata SE (16.1, StataCorp LLC, College Station, TX).

Due to the low number of resistant-*Salmonella* isolates (24/113), no statistical test was conducted. Consequently, all comparisons were carried out by considering the numerically higher proportions.

5.4. Results

5.4.1. Farm characteristics

Detailed demographic information of the participating farms was previously described [129]. Briefly, mean herd size was 145 lactating cows, and free-stall was the most common barn type (75%). The study farms were comparable to other Canadian dairy farms, except for the proportion of free-stall herds in Ontario and Quebec, which was higher in the study farms. Additional information on the demographics by province are presented in Table 5.1.

Table 5.1 Demographics from the 144 dairy farms enrolled in the Canadian Dairy Network for Antimicrobial Stewardship and Resistance (CaDNetASR) surveillance (study farms) compared to the Canadian dairy farms statistics (without robotic system) in 2022[a].

	Study farms						Canadian farms					
Province	BC	AB	ON	QC	NS	Total	BC	AB	ON	QC	NS	Total
N. of farms	30	30	30	30	24	144	139	220	1,759	2,869	93	5080
Herd size (mean)												
Free-stall	173.9	186.3	178.8	132.4	137.8	145.1	207.6	174.1	141.3	146.6	142.9	162.5
Tie-stall	74.0	N/A	57.0	70.7	64.0	66.4	30.2	91.4	62.4	69.5	62.1	63.1
Barn type												
% Free-stall	96.6	100.0	90.3	33.3	52.2	75.4	95.7	95.5	32.4	10.9	51.1	57.1
% Tie-Stall	3.4	0.0	9.7	66.7	47.8	24.6	4.3	4.5	67.6	89.1	48.9	42.9
Breed												
% Holstein	90.7	93.7	97.9	91.9	97.0	94.2	-	-	-	-	-	93.0
% Jersey	6.0	3.6	0.7	0.8	0.7	2.4	-	-	-	-	-	4.0
% Other breed	3.3	2.7	1.4	7.3	2.3	3.4	-	-	-	-	-	3.0

BC: British Columbia; AB: Alberta; ON: Ontario; QC: Québec; NS: Nova Scotia.
The breed information was available only at national level [221].
[a]The demographic information included approximately 57% of Canadian dairy herds [222].

5.4.2. Salmonella isolation and serotyping

The percentages of farms with at least one positive sample were 12% (17/140), 19% (28/144), and 17% (24/144) for 2019, 2020, and 2021, respectively. A higher proportion

of farms had no positive samples for *Salmonella* in 2019 compared to 2020 or 2021; however, one farm had all samples positive in 2019 (Figure 5.1).

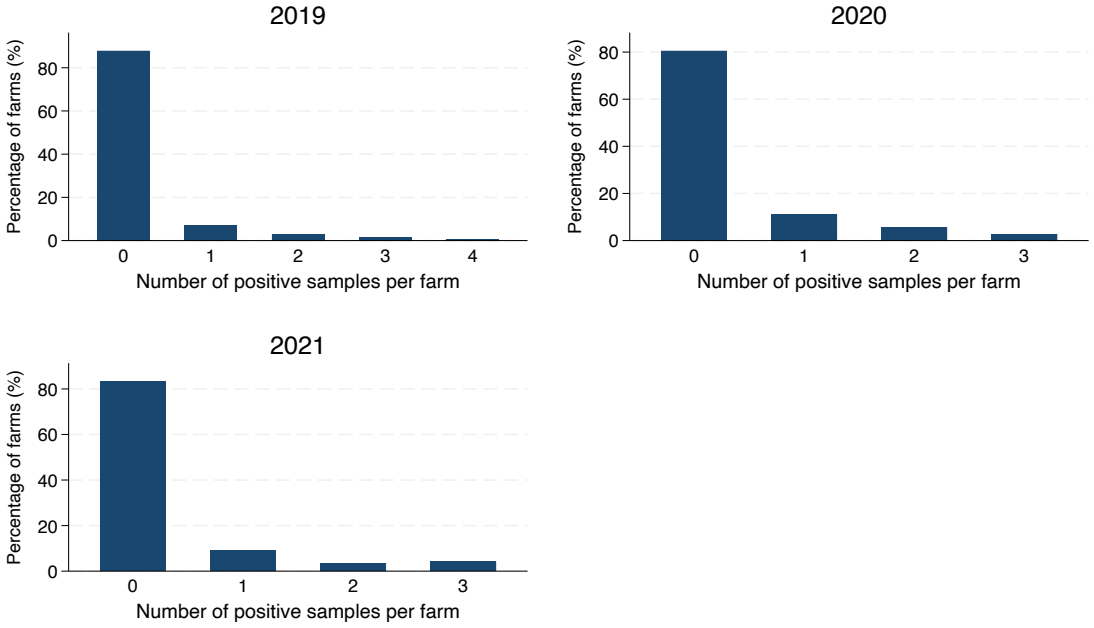

Figure 5.1 Number of positive fecal samples for *Salmonella* per farm. On each farm, up to four samples were collected (pre-weaned calves, post-weaned heifers, lactating cows, and manure storage).

Additionally, three farms in Ontario (H0070, H0082, and H0086) and one in British Columbia (H0012) had at least one *Salmonella*-positive sample each year (Figure 5.2). The overall percentage of *Salmonella* recovered from the fecal and manure storage samples was 6.6% (113/1709). In 2019, 2020, and 2021, the percentages of *Salmonella*-positive samples were 5.0% (28/560), 7.7% (44/574), and 7.1% (41/575), respectively. Farms in Ontario had a higher proportion of *Salmonella*-positive samples compared to British Columbia, Alberta, and Nova Scotia. Farms in Québec also had a higher proportion of *Salmonella*-positive samples compared to British Columbia and Alberta (Table 5.2). A higher

proportion of *Salmonella* isolates was recovered from manure storage samples compared to the other sample sources (Table 5.2).

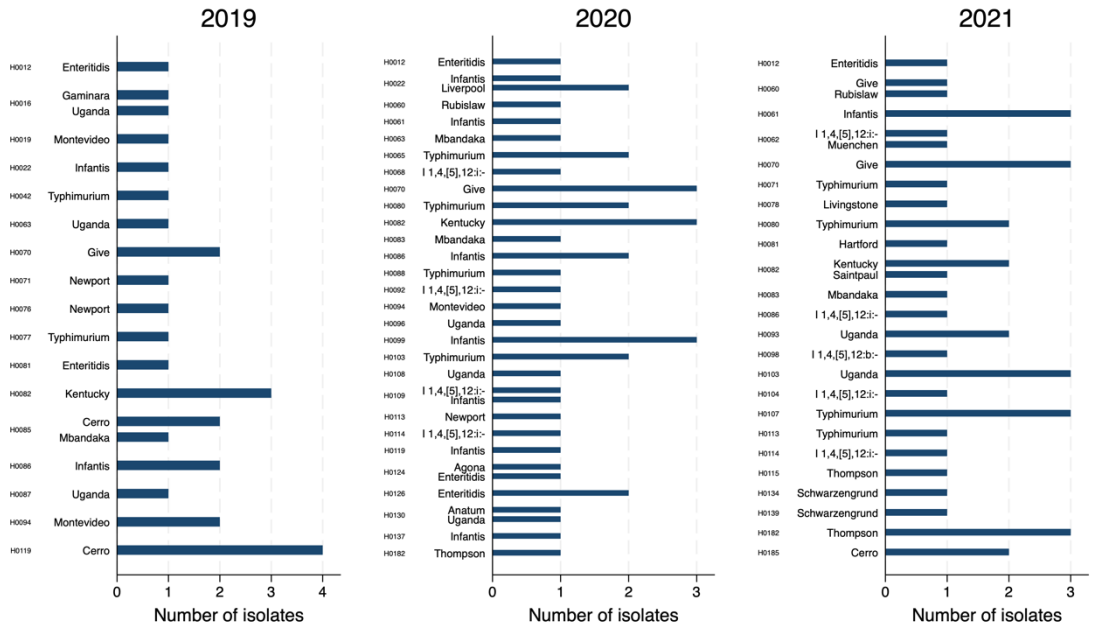

Figure 5.2 Dairy farms in which *Salmonella* was recovered in at least one sample from 2019 to 2021. On each farm, up to four samples were collected (pre-weaned calves, post-weaned heifers, lactating cows, and manure storage). Each farm is represented by the identifier H0XXX.

Table 5.1 Number and percentage (%) of *Salmonella*-positive samples over provinces by sample source from 2019 to 2021. A total of 1709 fecal samples were collected from Canadian dairy farms from 2019 to 2021 (140 dairy farms in 2019 and 144 dairy farms in 2020 and 2021).

Province	No. samples	Calves[1]	Heifers[2]	Lactating Cows	Manure storage	Total[3]
British Columbia	343	2 (2.3)	0 (0.0)	1 (1.2)	7 (8.2)	10[a] (2.9)
Alberta	359	0 (0.0)	1 (1.1)	1 (1.1)	2 (2.2)	4[a] (1.1)
Ontario	364	10 (11.0)	9 (9.9)	8 (8.8)	26 (28.6)	53[c] (14.6)
Québec	359	6 (6.8)	3 (3.3)	7 (7.8)	21 (23.3)	37[b,c] (10.3)
Nova Scotia	284	0 (0.0)	3 (4.2)	1 (1.4)	5 (7.0)	9[a,b] (3.2)
Total[4]	1709	18[a] (4.2)	16[a] (3.8)	18[a] (4.2)	61[b] (14.3)	113 (6.6)

[1]Pre-weaned calves
[2]Post-weaned heifers
[3]Provinces sharing the same letter are not significantly different at the 5% level (Bonferroni-adjusted comparisons after the final logistic regression model).
[4]Sample sources sharing the same letter are not significantly different at the 5% level (Bonferroni-adjusted comparisons after the final logistic regression model).

Out of the 113 *Salmonella* isolates, 23 different serovars were identified. No *Salmonella* Dublin was recovered. The most common serovars identified were Infantis (16/113; 14%), Typhimurium (16/113; 14%), Uganda (11/113; 10%), and Give (9/113; 8%) (Figure 5.3).

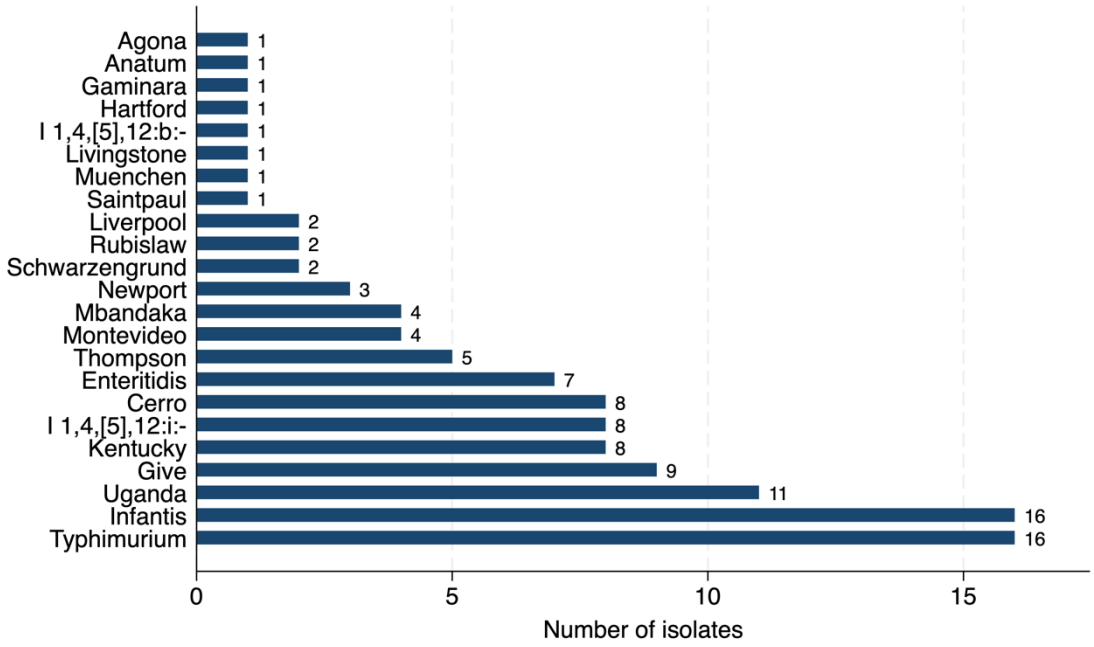

Figure 5.3 Number of each serovar identified for the *Salmonella* isolates recovered from fecal samples from 2019 to 2021.

Salmonella Infantis isolates were recovered from three different provinces; however, most were recovered in Ontario (n = 8, from two farms) and Québec (n = 5, from three farms). *Salmonella* Typhimurium were also recovered from three different provinces, again with most of them being recovered in Ontario (n = 9, from four farms) and Québec (n = 6, from three farms). *Salmonella* Uganda were recovered from four provinces, most of which were recovered in Québec (n = 7, from four farms). *Salmonella* Give was recovered in Ontario (n = 8, from the same farm) and Alberta (n = 1, from one farm). *Salmonella* Cerro was recovered in Ontario (n = 4, from the same farm) and Québec (n = 4, from the same farm)

(Figure 5.2, A and B). The number of each serovar identified in sample source is illustrated in Figure 5.4, A and B.

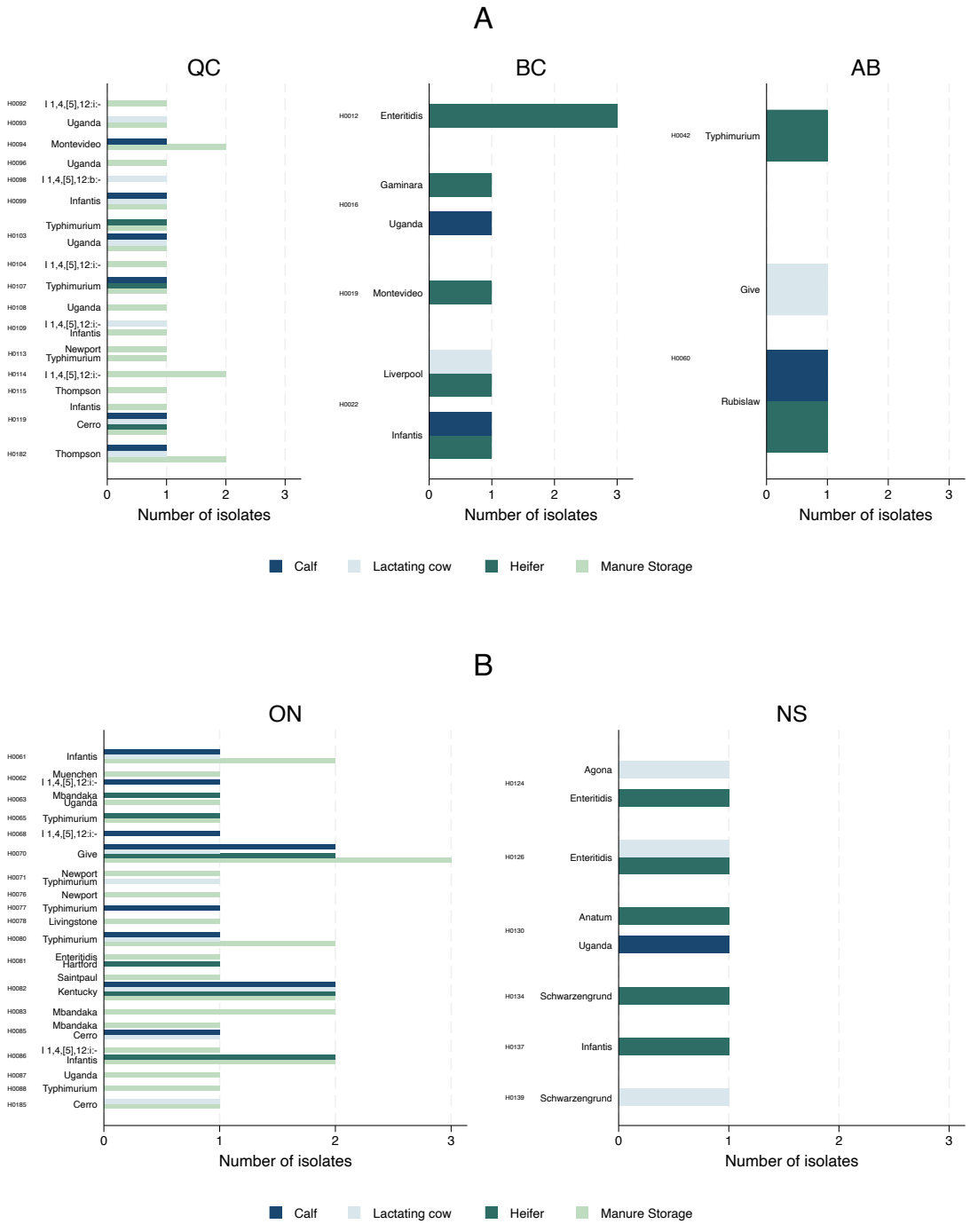

Figure 5.4 Number of *Salmonella* serovars isolated from each sample source from 2019 to 2021 by province. **A** QC: Québec; BC: British Columbia; AB: Alberta. **B** ON: Ontario; NS: Nova Scotia Each farm is represented by the identifier H0XXX.

5.4.3. Multilevel Logistic Regression

Descriptive data and the unconditional association results for the three variables considered in the model are presented in Tables 5.2 and 5.3. All the three variables (province, year, and sample source) met the criteria to be included in the multivariable model. Logistic regression final model results are presented in Table 5.4. The variable year was not significant in the final model (P=0.09). As demonstrated by the descriptive statistics, samples from Ontario dairy farms had a higher odds to be positive for *Salmonella* compared to British Columbia, Alberta, and Nova Scotia. Samples from Québec had a higher odds to be positive for *Salmonella* compared to British Columbia and Alberta. Manure storage samples had a higher odds to be positive for *Salmonella* compared to the other sample sources (Table 5.4). The intraclass correlation of herd level was 0.51, meaning that there was a high clustering effect among farms.

Table 5.2 Unconditional association of herd-level (n=144) categorical predictors with the probability of *Salmonella* isolation in fecal samples collected from Canadian dairy farms.

Variable	Categories	Frequency	OR	Overall *P*-value
Province	British Columbia	30	Baseline	<0.001
	Alberta	30	0.35	
	Ontario	30	10.88	
	Québec	30	6.86	
	Nova Scotia	24	1.58	
Sample source	Pre- weaned calves	427	Baseline	<0.001
	Post-weaned heifers	427	0.85	
	Lactating cows	428	0.99	
	Manure storage	427	6.39	
Year	2019	560	Baseline	0.122
	2020	574	1.74	
	2021	575	1.59	

OR: Odds ratio

Table 5.3 Final multilevel logistic regression model for the isolation of *Salmonella* from 1709 samples (144 farms) collected from calves, heifers, lactating cows, and manure storage from 2019 to 2021.

	β	SE	OR[1]	95% CI	*P* value	Overall *P* value

Intercept	-5.84	0.75	-	-	-	-	-
Province							<0.001
British Columbia	Ref.						
Alberta	-1.15	1.00	0.46	0.12	1.72	0.247	
Ontario	2.67	0.75	6.09	2.25	16.51	<0.001	
Québec	2.15	0.75	4.27	1.58	11.59	0.004	
Nova Scotia	0.53	0.85	1.43	0.46	4.43	0.533	
Sample Source							<0.001
Calves	Ref.						
Heifers	-0.16	0.39	0.90	0.53	1.51	0.682	
Lactating cows	-0.01	0.38	0.96	0.60	1.66	0.987	
Manure storage	1.85	0.33	3.50	2.25	5.46	<0.001	
Variance	Estimate	SE					
Herd level	3.42	1.05					

[1]OR: odds ratio adjusted to population average.

5.4.4. Resistance pattern in Salmonella

In total, 113 isolates (28, 44, and 41 in 2019, 2020 and 2021, respectively) had susceptibility testing and serotyping performed. The proportions of farms with at least one *Salmonella* isolate resistant to at least one antimicrobial in the panel were 4.3% (6/140), 4.9% (7/144), and 4.2% (6/144) in 2019, 2020, and 2021, respectively. Overall, 21% (24/113) of the *Salmonella* isolates were resistant to at least one antimicrobial. The proportion of *Salmonella* isolates resistant to at least one antimicrobial in 2019, 2020, and 2021 was 29%, 18%, and 20%, respectively. The most common antimicrobial for which *Salmonella* isolates were resistant was tetracycline (17%, 19/113), followed by sulfisoxazole (13%, 15/113) and streptomycin (12%, 8/113). Resistance to amoxicillin-clavulanic acid was observed in a single isolate. However, no resistance was detected for other very high important antimicrobials (according to Health Canada), including third-generation cephalosporins, fluoroquinolones, polymyxins, and carbapenems. Furthermore, no resistance was observed for macrolides, and one single isolate was resistant to second-generation cephalosporins (Table 5.5). The proportion of *Salmonella* isolates resistant to at least one antimicrobial was higher for Ontario (11.5%, 13/113), followed by Québec (6.2%,

7/113), Nova Scotia (1.8%, 2/113), and British Columbia (1.8%, 2/113). No resistance was observed in the four isolates from Alberta. A higher proportion of resistance to streptomycin, sulfisoxazole, and tetracycline was observed for the two isolates recovered from Nova Scotia (Figure 5.5A). Isolates from calves had a higher proportion of resistance to streptomycin (Figure 5.6A). The proportion of *Salmonella* isolates resistant to two different classes and MDR was 3.5% (4/113) and 8.8% (10/113), respectively. A higher proportion of MDR-*Salmonella* was recovered in Québec (4.4%, 5/113), followed by Ontario (3.5%, 4/113), and Nova Scotia (0.9%, 1/113). One *Salmonella* isolate from Ontario (calf sample) was resistant to five antimicrobial classes. No MDR isolates were recovered from British Columbia (Figures 5.5B and 5.6B).

Table 5.4 Minimum inhibitory concentrations of 14 antimicrobials for 113 *Salmonella* isolates recovered from dairy herds' fecal samples in 2019, 2020, and 2021.

				Distribution (%) of MIC (µg/mL)																	
Antimicrobial Class	**Antimicrobial**	Range	% Res.	0.015	0.03	0.06	0.12	0.25	0.5	1	2	4	8	16	32	64	128	256	512	[a]MIC$_{50}$	[b]MIC$_{90}$
Carbapenem[1]	MER	0.06-4	0.0			100.0	0.0	0.0	0.0	0.0	0.0	0.0								0.06	0.06
Cephalosporin - 3rd generation[1]	CRO	0.25-64	0.0					100.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0				0.25	0.25
Penicillin-β-lactamase inhibitor combinations[1]	AMC	1/05-32/16	0.0							93.8	2.7	2.7	0.0	0.9	0.0					1	1
Fluoroquinolone[1]	CIP	0.015-4	0.0	99.1	0.0	0.0	0.0	0.0	0.9	0.0	0.0	0.0								0.015	0.015
Colistin[1] (2020)	COL	0.25-4	0.0					89.6	0.0	10.4	0.0	0.0								0.25	1
Cephalosporin - 2nd generation[2]	FOX	0.5-32	0.0						0.0	14.2	62.0	22.1	0.9	0.9	0.0					2	4
Penicillin[2]	AMP	1-32	3.5							92.9	3.5	43.7	1.5	0.2	0.0	3.5				1	1
Quinolone[2]	NAL	0.5-32	0.9						0.0	0.0	33.6	65.5	0.0	0.0	0.9					4	4
Aminoglycoside[2]	STR	2-64	11.9								1.5	11.9	64.2	10.5	1.5	8.9	1.5			8	64
	GEN	0.25-16	0.0					66.4	0.0	33.6	0.0	0.0	0.0	0.0						0.25	1
Macrolide[2]	AZM	0.25-32	0.0					0.0	0.0	1.8	16.8	75.2	6.2	0.0	0.0					4	4
Trimethoprim/sulfamethoxazole[2]	SXT	0.12/2.38-4/76	1.8				98.2	0.0	0.0	0.0	0.0	0.0	1.8							0.12	0.12
Sulfonamide[3]	SOX	16-256	13.3											61.1	23.9	0.9	0.0	0.9	13.3	16	512
Phenicol[3]	CHL	2-32	1.8								5.3	38.1	54.0	0.9	0.0	1.8				8	8
Tetracycline[3]	TET	4-32	16.8									83.2	0.0	0.9	0.9	15.0				4	64

Vertical lines indicate CLSI breakpoints. AMC: amoxicillin/clavulanic acid; AMP: ampicillin; AZM: azithromycin; FOX: cefoxitin; CRO: ceftriaxone; CHL: chloramphenicol; CIP: ciprofloxacin; GEN: gentamicin; MER: meropenem; NAL: nalidixic acid; STR: streptomycin; SOX: sulfisoxazole; SXT: trimethoprim/sulphamethoxazole TET: tetracycline.

[a] The MIC value that inhibits the growth of 50% or more of the isolates.

[b] The MIC value that inhibits the growth of 90% or more of the isolates.

[1]Category I - Very high importance in human medicine.

[2]Category II - High importance in human medicine.

[3]Category III - Medium importance in human medicine.

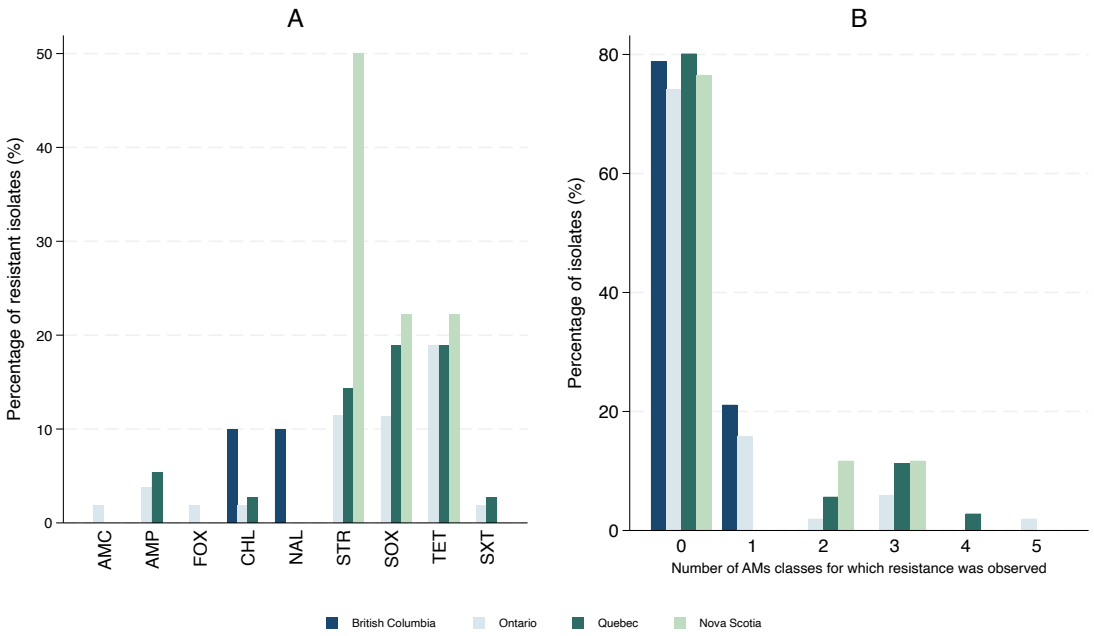

Figure 5.5 **A** Percentage of resistant *Salmonella* isolates for each antimicrobial tested in the panel by province from 2019 to 2021. AMP: ampicillin; CHL: chloramphenicol; NAL: nalidixic acid; STR: streptomycin; SOX: sulfisoxazole; SXT: trimethoprim/sulphamethoxazole TET: tetracycline. No resistance was overserved for amoxicillin/clavulanic acid, azithromycin, cefoxitin, ceftriaxone, ciprofloxacin, gentamicin, and meropenem. **B** Percentage of isolates pan-susceptible or resistant to one or more antimicrobial classes by province from 2019 to 2021. No resistance was observed for *Salmonella* recovered from Alberta.

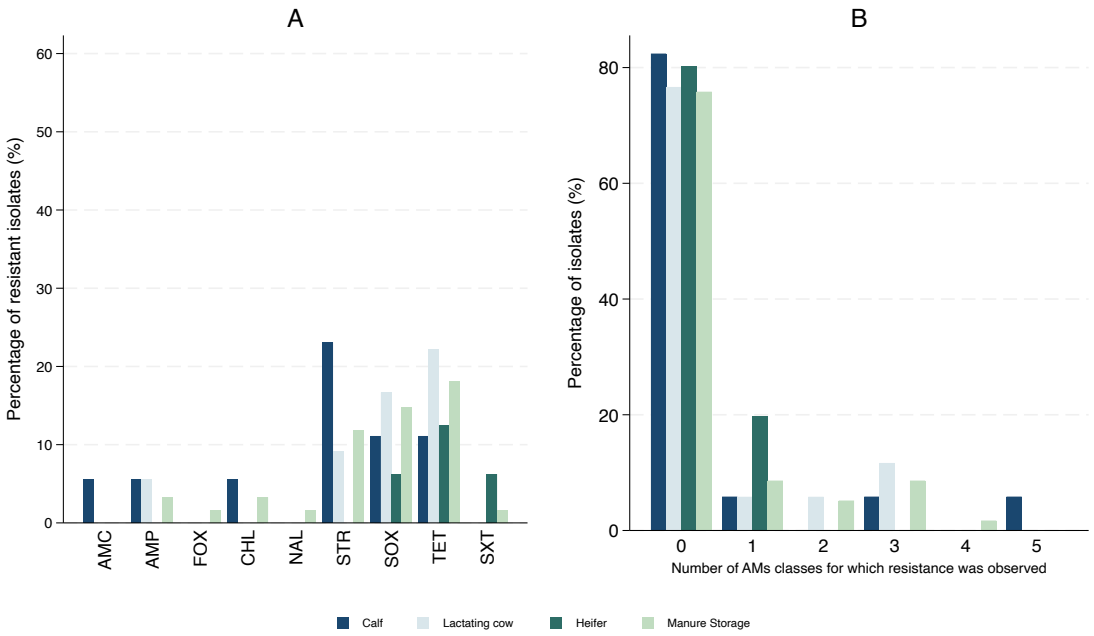

Figure 5.6 **A** Percentage of resistant *Salmonella* isolates for each antimicrobial tested in the panel by sample source from 2019 to 2021. AMP: ampicillin; CHL: chloramphenicol; NAL: nalidixic acid; STR: streptomycin; SOX: sulfisoxazole; SXT: trimethoprim/sulphamethoxazole TET: tetracycline. No resistance was overserved for amoxicillin/clavulanic acid, azithromycin, cefoxitin, ceftriaxone, ciprofloxacin, gentamicin, and meropenem. **B** Proportion of isolates pan-susceptible or resistant to one or more antimicrobial classes by sample source from 2019 to 2021.

In our study, the MDR pattern tetracycline-sulfonamide-aminoglycoside was the most frequently observed, accounting for 5.3% (6/113) of the isolates. This pattern was found in the serovars Uganda (n = 5) and Schwarzengrund (n = 1). The tetracycline-only resistance pattern was also common, accounting for 4.4% (5/113) of the isolates, and was most commonly associated with the serovar Infantis. Similarly, the tetracycline-sulfonamide resistance pattern was observed in 3.5% (4/113) of the isolates and was most commonly associated with serovar Uganda. One *Salmonella* isolate serovar Typhimurium was resistant to five antimicrobial classes (six antimicrobials), while one *Salmonella* isolate serovar I 1,4,[5],12:i:- was resistant to four classes (five antimicrobials), as presented in Figure 5.7.

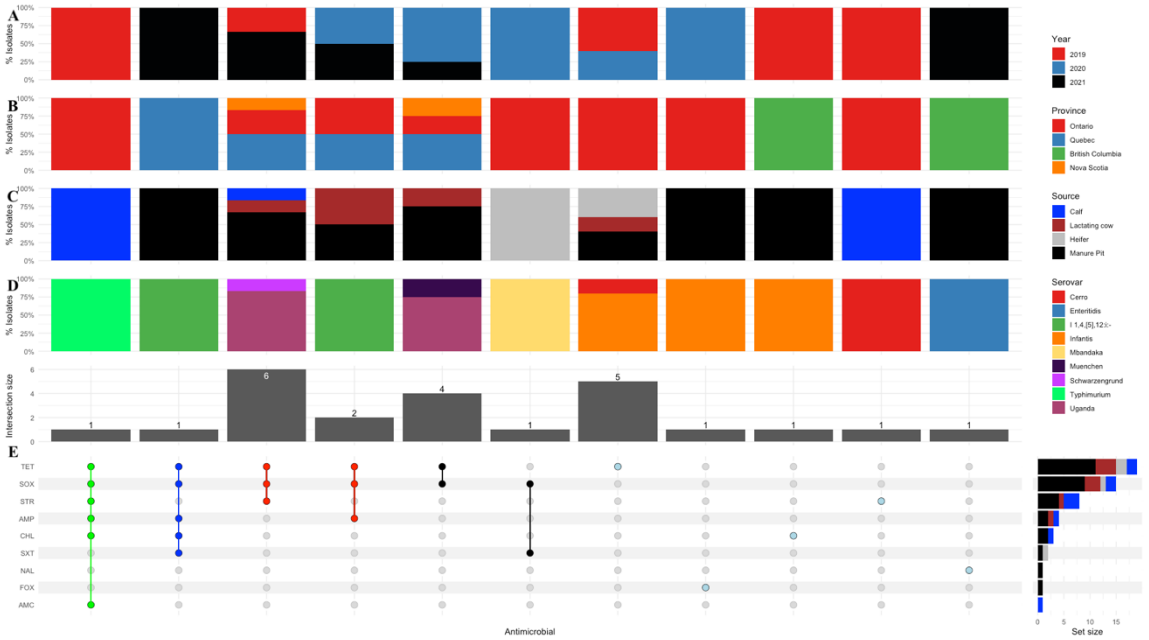

Figure 5.7 Phenotypic resistance pattern for the 24 of 113 *Salmonella* isolates recovered from fecal samples that were resistant to at least one antimicrobial tested. **A** Year in which the isolates were recovered; **B** Province where the isolates were recovered; **C** Sample source where the isolates were recovered; **D** *Salmonella* serovar; **E** Number of isolates and their respective phenotypic resistance pattern. The line connecting the coloured dots represents which antimicrobial an isolate was resistant to. The different colors for the connected dots represent how many antimicrobials an isolate was resistant to: green= resistant to 6 antimicrobials; blue= resistant to 5 antimicrobials; red= resistant to three antimicrobials; black= resistant to two antimicrobials; and light blue= resistant to one antimicrobial. TET: tetracycline; SOX: sulfisoxazole; STR: streptomycin; AMP: ampicillin; CHL: chloramphenicol; SXT: trimethoprim/sulphamethoxazole; NAL: nalidixic acid; FOX: cefoxitin; and AMC: amoxicillin/clavulanic acid. No resistance was observed in isolates from Alberta.

5.5. Discussion

Our study focused on *Salmonella* recovered from feces and manure on Canadian dairy farms and the antimicrobial resistance phenotypes of the isolates. The proportion of farms with at least one *Salmonella*-positive sample from 2019 to 2021 was relatively low, ranging from 12% to 19%. In addition, the multilevel logistic model indicated a clustering effect on the occurrence of *Salmonella* in our study farms, meaning that if a *Salmonella* was isolated from a farm, it was more likely to be isolated from multiple sample sources and on multiple years. [223]

In contrast to the low proportion of farms positive for *Salmonella* found in our study, according to the report from the National Animal Health Monitoring System (NAHMS), 39.7% of the dairy operations in the United States included in the survey (48/121) had a fecal-culture positive for *Salmonella* in 2007 [224]. A study from Pennsylvania, published in 2019, which also collected pooled fecal samples from 80 dairy farms, reported that 64% of the farms had at least one sample positive for *Salmonella* [38]. This disparity might be partially attributed to methodological differences, as the Pennsylvania study collected one pooled fecal sample from multiple sites within a pen (six to eight sites), which could increase the sensitivity in detecting a *Salmonella* isolate. Furthermore, according to a study published in 2019 using survey data to describe the management of dairy herds in Pennsylvania, it was possible to identify differences in demographic characteristics between our farms and those in Pennsylvania, which could impact the occurrence of *Salmonella* [225]. For instance, mean herd size in Pennsylvania farms was smaller (71 lactating cows) compared to our farms (145 lactating cows).

Additionally, the proportion of barn types differed, with tie-stall barns being the most common in Pennsylvania (34%), while our herds were predominantly free-stall barns. These variations in methodology, herd characteristics and management practices might have contributed to the differences observed in *Salmonella* occurrence between the studies. Furthermore, the higher proportion of *Salmonella*-positive farms in both the Pennsylvania study and the NAHMS report could potentially be attributed to the specific serovars detected within these research studies. A higher proportion of serovars Cerro e Kentucky was identified, both of which are commonly distributed across dairy operations within the United States [38, 224].

In the present study, the overall proportion of *Salmonella* recovered from fecal samples was 6.6%. The proportion of *Salmonella*-positive fecal samples in our study was similar to that reported in a 2005 study conducted in the United States, which included dairy farms from four states and reported an overall proportion of 4.9% [226]. The proportion of *Salmonella*-positive fecal samples was also similar to what was described in a meta-analysis published in 2019. This meta-analysis included studies from various countries; however, most studies were conducted in the United States. The meta-analysis reported an overall proportion of 9% (95% CI: 7-11%) for *Salmonella* in apparently healthy cattle (beef, dairy, and mixed), with a higher proportion of 16% (95% CI: 12-20%) when considering only studies from North America [227]. Other studies reported slightly higher proportions of *Salmonella* recovered from fecal samples in dairy cattle. A study using data from four states in the United States reported 10% of samples were positive for *Salmonella* [228]. Similarly, another study from Pennsylvania reported an overall proportion of 11.5% [38]. These variations in proportions might be attributed to differences in study design,

geographical location, herd management practices, sampling and laboratory techniques. Overall, the proportion of *Salmonella* recovered from fecal samples in our study seemed comparable to other studies conducted in North America.

Regional factors might also influence the occurrence of *Salmonella* in dairy cattle in Canada. To our knowledge, this study is the first to report the proportion of *Salmonella enterica* recovered from fecal samples collected on-farm in dairy herds across five provinces in Canada. In our study, the proportion of positive samples for *Salmonella* spp. was higher from dairy farms located in Ontario and Québec when compared to British Columbia, Alberta, and Nova Scotia. The province with a lower proportion of positive samples was Alberta (1.1%). A low proportion of *Salmonella* from dairy cattle in Alberta was also reported in a study from 2003, where pooled fecal samples were collected (2 to 3 cows for each sample) in 50 dairy farms across the province. They reported a proportion of 0.7% of positive samples out of the 750 pooled samples collected during the study [229]. In another Canadian study, which collected data from eight dairy farms in New Brunswick, 3.3% of 488 fecal samples collected from dairy calves, were *Salmonella*-positive [190]. In addition, three serovars were identified in this latter study: *Salmonella* Senftenberg, *Salmonella* Typhimurium DT02, and *Salmonella* Derby [190].In the present study, no *Salmonella* was recovered from pre-weaned calf samples in Nova Scotia (another Maritime province). Additionally, none of the serovars identified by Awosile et al. were detected in the positive samples for Nova Scotia. The demographics of the study farms were found to be similar to those of other Canadian dairy farms, with one exception. It was observed that the study farms from Ontario and Québec had a higher proportion of free-stall herds in comparison to the general distribution among dairy farms across Canada. The variation in

the proportion of *Salmonella*-positive samples across provinces could be attributed to other management factors not controlled by this study.

The effectiveness of *Salmonella* detection in dairy cattle varies depending on the type of sample collected, which can impact the sensitivity of detection for surveillance purposes. In our study, a higher proportion of isolates were recovered from manure samples, specifically the manure storage area. A study in the United States collected data from five states, including sampling from various sources such as the farm environment and direct rectal swabs from animals, including swine, dairy and beef cattle, and poultry. They observed a higher occurrence of *Salmonella* in samples collected from the environment than in rectal swabs. Specifically, the proportion of *Salmonella* recovered from rectal swabs in dairy cattle was 0.4%, whereas the environment samples ranged from 10% in feed to 15% in bedding material [230]. The findings for environmental samples aligned with our study, which also found a higher proportion of *Salmonella* in environmental samples (14%). However, other sampling methods might be more appropriate to estimate herd prevalence. For instance, a study from 2008 found a high correlation between composite fecal samples and milk filter samples collected at several time points to predict the herd prevalence of *Salmonella* spp. [231]. In our study, certain serovars were exclusively isolated from fresh fecal pats of animals. Consequently, the current sampling scheme, incorporating both fresh fecal pats and manure samples, appeared to offer advantages over relying solely on manure samples. In addition, although manure samples are more representative of the herd than fecal samples from a given production age, it might be influenced by external contamination from other animal sources, or the surrounding environment. As a result, the

Salmonella recovered from manure samples may not solely originate from the dairy farm itself.

Differences in the proportion of *Salmonella* recovered from different production ages in dairy cattle were previously reported. A study from Pennsylvania that collected fecal samples from different production ages in dairy farms reported a higher proportion of positive samples for *Salmonella* in lactating cows (64%), followed by dry cows (61%). In the same study, the proportion of *Salmonella* recovered from pre-weaned calves was 13%, indicating a lower proportion when compared to other production ages [38]. However, in our study, we did not observe any major variation in the proportion of *Salmonella*-positive samples among the different production ages. To the authors' knowledge, no studies report production age as a risk factor for *Salmonella*. Additionally, the probability of recovering *Salmonella* from lactating cows and dry cows can be affected by other risk factors. For instance, access of lactating or dry cows to surface water and herd size, have been previously identified as potential risk factors for *Salmonella* shedding in dairy cattle [226, 232]. Therefore, the variation in the proportion of *Salmonella* observed in different production ages across studies could be attributed to other factors beyond age alone.

In addition to determining the proportion of *Salmonella* recovered from dairy herds, identifying the specific serovars is important for a comprehensive understanding of the distribution of different *Salmonella* strains within the dairy industry. It is well known that *Salmonella* Dublin is a concern for the dairy industry in North America [233]. This serovar is host-adapted for cattle, is associated with subclinical shedding over animal life, and is usually associated with MDR [234]. In our study, the serovar Dublin was not recovered; however, it is important to mention that our sampling scheme was not designed to detect

Salmonella Dublin. We collected pooled fecal samples, which have been previously reported to exhibit a lower sensitivity in detecting this particular serovar [235, 236]. Other validated methods, such as testing bulk milk samples for the presence of antibodies, have been previously described as a convenient and cost-effective strategy for detecting *Salmonella* Dublin. However, the sensitivity of ELISA testing to detect *Salmonella* Dublin in bulk milk can vary, ranging from 40 to 100% in different studies [215, 237, 238].

While no *Salmonella* Dublin was identified, two other serovars of concern, Typhimurium and Newport, were identified. These two serovars can also be associated with subclinical shedding in dairy cattle, posing a contamination risk for people with direct contact with the animals, their feces, or ingesting contaminated raw milk [18]. Additionally, *Salmonella* Typhimurium is commonly associated with MDR and mortality in dairy calves [239, 240]. In our study, *Salmonella* Newport represented 2.7% of recovered isolates (two isolates from Ontario and one from Quebec). A study published in 2018 using whole genome sequencing (WGS) suggested that an outbreak from 21 states in the United States caused by *Salmonella* Newport could possibly be linked to dairy cattle sources (contaminated ground beef produced from slaughtered dairy cows) [241]. However, all three *Salmonella* Newport isolates came from manure storage samples and could be associated with external contamination. *Salmonella* Typhimurium was one of our study's most commonly isolated serovars (n=16), accounting for 14% of the recovered isolates (half of the isolates came from animal samples). Our findings suggested that this serovar was clustered within certain provinces and farms, as out of the 16 identified *Salmonella* Typhimurium, nine were identified in Ontario (across 4 farms) and six in Québec (across 3 farms). Another study conducted in Alberta, Canada, analyzed clinical *Salmonella* isolates from dairy cattle and reported that

Salmonella Typhimurium corresponded to 58.3% of the isolates (28/48) [216]. A WGS analysis from *Salmonella* recovered from humans and dairy cattle in New York and Washington states suggested that two *Salmonella* Typhimurium isolates from humans were remarkably similar based on their AMR gene sequences with an isolate from dairy cattle [27]. The AMR genes in all three isolates displayed 100% sequence identity, except for one gene, tet(RG), which exhibited a nucleotide difference at position 73 [27]. Additionally, a report documented an outbreak of MDR *Salmonella* Typhimurium in 2018 linked to the consumption of soft cheese in Mexico and beef in the United States [242]. These combined findings might suggest that dairy cattle and the dairy farm environment could serve as a potential source of *Salmonella* Typhimurium and Newport infections in humans.

Salmonella Infantis was the other most frequent serovar identified in our study (15%). Similar to *Salmonella* Typhimurium, our results suggested that this serovar was also clustered within specific provinces and farms, with eight isolates recovered from two farms in Ontario and five from three farms in Québec. According to the literature, this serovar is usually associated with poultry, and in 2016, an outbreak associated with exposure to raw chicken was reported in Canada, including cases across nine provinces [243]. Previous studies have highlighted the variability in the occurrence of *Salmonella* Infantis in dairy cattle among different geographic locations and farming systems [64, 223, 244, 245]. In North America, the proportion of isolation for this serovar was reported to be 1% and 8% in two different studies from the United States [223, 246]. In pasture-based farms in Australia, the occurrence of *Salmonella* Infantis was particularly high, accounting for 61% of the serovars recovered according to a study published in 2022 [244]. Out of the 16 *Salmonella* Infantis isolates, seven were recovered from animal samples.

In addition to the occurrence of *Salmonella*, we described the associated resistance patterns. Out of the 47 *Salmonella*-positive farms, only 16 had *Salmonella*-resistant isolates, indicating an even higher clustering within farm. Antimicrobial-resistant *Salmonella* were isolated from all the production phases and the manure storage. All provinces had at least one *Salmonella* resistant to at least one antimicrobial, except Alberta, where no resistance was observed. Among the antimicrobials tested, resistance to tetracycline was highest, accounting for 17% of the isolates, followed by sulfisoxazole (13%) and streptomycin (12%). Higher proportions of resistance to streptomycin and tetracycline were previously reported in North America. A study from California found that 51% of the *Salmonella* isolates from dairy cattle were resistant to streptomycin, and 50% were resistant to tetracycline [246]. A second study from California also reported higher proportions of resistance to tetracycline (39%) in *Salmonella* isolated from cull dairy cows [40]. Although resistance to sulfisoxazole in our study was the second most commonly identified, both studies from California found no resistance to sulfisoxazole. The absence of resistance to sulfisoxazole in California studies could be due to the fact that trimethoprim-sulfamethoxazole is not approved to be used in dairy cattle in the United States [247]. In Australia, a study from 2022 reported a higher proportion of non-wild type *Salmonella* isolates that were resistant to streptomycin (57%); however, all isolates were susceptible to all 16 tested antimicrobials using CLSI clinical breakpoints [244]. The differences in the resistance pattern could be attributed to different herd management, such as the choice of antimicrobials being used and other management that could affect the burden of AMR on those farms.

AMR involving antimicrobials considered highly important for human medicine are of particular concern, especially in food-producing animals [101]. In Canada, antimicrobials are classified into four categories; those very high important for human medicine are considered category I [248] . Our study observed no resistance for category I antimicrobials, except for one isolate resistant to amoxicillin/clavulanic acid. A study conducted in Alberta, Canada, reported higher proportions of resistance to category I antimicrobials. Resistance to ceftiofur, ceftriaxone, and amoxicillin/clavulanic acid was 43.6% for *Salmonella* Typhimurium. In addition, resistance to ceftiofur and ceftriaxone was 68.8% in *Salmonella* Dublin [216]. However, these *Salmonella* isolates were obtained from clinical samples, and these serovars are commonly associated with resistance to category I antimicrobials [234, 246]. Other studies from the United States have also reported resistance to these antimicrobials. For instance, a study from California reported 10, 9, and 5% of *Salmonella* resistant to ceftriaxone, amoxicillin/clavulanic acid, and ciprofloxacin, respectively [246]. Another study from California, including Salmonella isolates recovered from healthy and diseased animals, found that 40 and 46% of *Salmonella* was resistant to amoxicillin/clavulanic acid and ceftriaxone, respectively [246]. These findings highlight the variability in antimicrobial resistance patterns in North America and emphasize the need for continued surveillance.

Some *Salmonella* serovars are more frequently resistant to antimicrobials than others [246]. In our study, we identified MDR isolates representing four *Salmonella* serovars: Typhimurium, Uganda, Schwarzengrund, and I:4,[5],12:i:-. The predominant MDR pattern observed was tetracycline-streptomycin-sulfisoxazole, accounting for 5.3% (6/113) of the isolates, with Uganda being the most commonly associated serovar with this pattern of

resistance. The serovar I:4,[5],12:i:- was associated with two different MDR patterns: tetracycline-sulfisoxazole-ampicillin and tetracycline-sulfisoxazole-ampicillin-chloramphenicol- trimethoprim/sulphamethoxazole. While only one *Salmonella* Typhimurium isolate was identified as MDR, it displayed resistance to six different antimicrobials: tetracycline, sulfisoxazole, streptomycin, ampicillin, chloramphenicol, and amoxicillin/clavulanic acid. A study conducted on dairy cattle in 2009 reported MDR proportions of 60 and 39% for the I:4,[5],12:i:- and Typhimurium serovars, respectively [223]. A second study highlighted that all Typhimurium isolates were MDR [64]. Additionally, this same study reported 50% of *Salmonella* isolates being MDR, and the most common MDR pattern was amoxicillin/clavulanic acid-ampicillin-cefoxitin-ceftiofur-ceftriaxone-chloramphenicol-streptomycin-tetracycline (16%), which included resistance to category I antimicrobials. Furthermore, in 2019, a study from California reported an MDR proportion of 12% among *Salmonella* isolates, with certain MDR patterns displaying resistance to category I antimicrobials, such as third-generation cephalosporins and fluoroquinolones [40]. These findings highlighted the concerning presence of resistance to critically important antimicrobials in *Salmonella* isolates; however, our results suggested that such resistance is lower in Canadian dairy herds. Furthermore, it is worth noting that *Salmonella* Dublin is commonly associated with MDR [234]; thus, our study's absence of *Salmonella* Dublin might have influenced the lower proportion of MDR observed.

Some limitations applied to the present study. Convenience sampling could introduce selection bias if the farms in the present study did not represent the source population. Participants in our study were not blinded to the study's objective, and farmers who agreed to participate in the study could be the ones with higher levels of biosecurity,

underestimating the burden of *Salmonella* and resistance associated with these isolates. In addition, the proportion of free-stall herds in Ontario and Quebec study farms slightly differed from other commercial dairy herds in Canada as demonstrated in Table 1. Therefore, interpretation of results must take that into account, which could affect the external validity of the results to other dairy farms in Canada. Another limitation was the low number of *Salmonella*-resistant isolates (24/113) which precluded the logistic regression analysis. Finally, the ability to recover *Salmonella* from fecal samples can vary according to the culture method employed [249, 250]. As a result, the specific culture methods employed have the potential to selectively recover specific serovars of *Salmonella* which could introduce a bias against certain serovars that might be present within a given fecal sample. Therefore, the interpretation of the results and the comparisons with other studies should take that into consideration.

Our study findings revealed a clustering pattern in the occurrence and resistance of *Salmonella enterica* among farms and provinces. Understanding the diversity and occurrence of *Salmonella enterica* serovars in dairy cattle and the resistance pattern associated with these serovars provides important information to mitigate infections related to foodborne transmission and improve animal health. We identified a very low proportion of *Salmonella* isolates resistant to highly important antimicrobials compared to previous studies from North America.

Chapter 6

6. Conclusions and future directions

6.1. CaDNetASR implementation, evaluation, and future refinements

In the last decades, awareness regarding antimicrobial resistance (AMR) has been increasing worldwide. In 2019, a surveillance system for monitoring antimicrobial use (AMU) and AMR in dairy cattle in Canada (CaDNetASR) was implemented. The main objective of this surveillance was to collect on-farm data on AMU, AMR, and management practices, intending to promote antimicrobial stewardship on Canadian dairy farms. An extensive literature review on surveillance systems that included data collection on dairy cattle was done in chapter 2. Summarized information was included to help identify the strengths and weaknesses of CaDNetASR in the context of other surveillance systems worldwide. Among the 6 surveillance systems worldwide that included data collection in dairy cattle, CaDNetASR was the only surveillance that included data collection and sampling from different production ages (pre-weaned calves, post-weaned heifers, and lactating cows) and manure storage. The fecal samples were cultured for *Campylobacter* spp., *E. coli* and *Salmonella* spp., while phenotypic antimicrobial susceptibility testing to determine MICs (minimum inhibitory concentrations) was conducted to assess AMR. It is important to mention that CaDNetASR surveillance was a research project funded for four years. Including this dairy component as an ongoing farm program within the Canadian Integrated Program for Antimicrobial Resistance Surveillance (CIPARS) will require further funding to become sustainable. For that, changes to the sampling scheme might be necessary. Currently, CIPARS has funding to continue the sampling in Ontario, Quebec, and British Columbia dairy farms. There is also new research funding being applied for that would allow the continuation of the Nova Scotia and Alberta dairy farms for the next 3 years. However, relying solely on research funding for the continuation of a surveillance

program, like CaDNetASR, can present certain challenges and uncertainties. Research funding can be competitive and subject to fluctuations, which might leave the program vulnerable to gaps in funding or interruptions in data collection and analysis. In practice, a combination of research funding and government support may be the most sustainable approach. Research funding can help explore specific questions and innovations within the program, while government funding provides the necessary foundation for ongoing surveillance efforts. Building strong partnerships with both the research community and government agencies can help ensure the continued success of programs like CaDNetASR.

6.2. Public health implications of the CaDNetASR surveillance main findings

This thesis provided new insights into the proportion of positive fecal samples for *Campylobacter* spp., *E. coli*, and *Salmonella* in dairy cattle in Canada. The findings indicated that a substantial proportion of farms were positive for *Campylobacter* spp. and *E. coli*, with 95.7% and 100.0% of farms having at least one positive sample, respectively. The high proportion of *E. coli* was expected, as it is a commensal bacterium [19]. Although *Campylobacter* spp. is considered a human pathogen, it is also commonly recovered from healthy domestic animals [33]. These results confirmed that both *E. coli* and *Campylobacter* spp. were widespread among Canadian dairy farms. However, the overall proportion of farms positive for *Salmonella* was lower. The ICC (0.51) from the regression model on the probability of recovering a *Salmonella* from a sample suggested that the occurrence of *Salmonella* is more clustered by farms.

Regional differences have been reported in the recovery of *Salmonella*. Our research demonstrated that a higher proportion of *Salmonella* was found in Ontario dairy farms,

whereas Alberta dairy farms had the lowest proportion. The occurrence of *Salmonella* not only varied based on geographical location but also appeared to be influenced by the production system. For instance, while we found a lower proportion of *Salmonella* in Alberta dairy farms, according to the FoodNet report, a significantly higher proportion of *Salmonella* was recovered in broiler chicken farms in Alberta compared to Ontario and British Columbia [251]. Additionally, certain *Salmonella* serovars recovered from our dairy farms were also commonly reported in other commodities in Canada. In our study most of the serovars Typhimurium and Infantis were identified in a small number of farms in Ontario and Québec. Comparing these findings with the FoodNet report for Ontario revealed that the serovars Typhimurium and Infantis were also commonly identified in swine and broiler chicken farms on this province [251]. A meta-analysis on the epidemiology of *Salmonella* serovars published in 2019, reported that serovar Typhimurium was the most prevalent and disseminated serovar worldwide and it appeared to be most associated with swine and poultry in Europe and North America [252]. Our results indicated that *Salmonella* Typhimurium is one of the most common serovars in Canadian dairy cattle.

In addition to regional differences, the proportion of positive samples for *Salmonella* was significantly different among sample sources. A higher proportion of *Salmonella*-positive samples were found in the manure storage compared to the other sources. As the manure storage is usually located outside the barn, the higher proportion of *Salmonella* recovered from this sample source might be attributed to external contamination (e.g., birds and rodents). For instance, we found that out of the 7 *Salmonella* Enteritidis isolates identified (which is the most frequently identified serovar in birds), only one isolate was associated

with animal samples, suggesting the potential of external contamination in the manure storage.

Regional differences have also been observed in the recovery of *Campylobacter* spp. A higher proportion of positive samples was observed in Alberta when compared to British Columbia and Québec. No difference was observed for the other provinces. However, as for *Salmonella*, the differences seem to be due not only regional differences but also production systems. For instance, while in our study a higher proportion of *Campylobacter* spp. was detected in dairy farms in Alberta, a FoodNet Canada report from 2018 revealed that turkey farms in Alberta had fewer positive fecal samples for *Campylobacter* spp. than those in British Columbia [251]. In addition to regional differences, the proportion of positive samples for *Campylobacter* spp. among sample sources also differed. Heifer and lactating cow samples had a significantly higher proportion of *Campylobacter* spp. compared to calves and manure storage. The proportion of *Campylobacter* spp. in pre-weaned calves was low, which may be attributed to their housing conditions. Previous studies have shown that individually housed calves had a lower odds of *Campylobacter* spp. occurrence, and since most pre-weaned calves in Canada are housed individually, this might have contributed to the lower occurrence of *Campylobacter* spp. in calves [36, 143, 144].

As demonstrated in the previous paragraphs, the occurrence of *Campylobacter* spp. and *Salmonella* might not be solely influenced by geographical location of the dairy farms. Although some provinces had a higher occurrence of *Campylobacter* spp. or *Salmonella* compared to others, after comparing the results with other animal species, we observed that regions where we found higher proportions of *Campylobacter* spp. or *Salmonella* not

always had higher occurrence of these pathogens in other food-producing animals such as turkey and broiler.

Assessing the resistance pattern of *Campylobacter* spp., *E. coli*, and *Salmonella* isolates recovered from dairy cattle is important to evaluate their potential impact on public health. Farm-level resistance for *E. coli* and *Campylobacter* spp. appeared to be widespread. Specifically, 67.7% and 73.1% of the farms had at least one isolate that was resistant to at least one antimicrobial tested in the panel for *E. coli* and *Campylobacter* spp., respectively. While resistant *E. coli* and *Campylobacter* spp. isolates were widespread among farms and regions, for *Salmonella*, resistance patterns appeared to be more clustered by region and farms. Out of the 47 *Salmonella*-positive farms, only 16 had *Salmonella*-resistant isolates. Due to the limited number of resistant-*Salmonella* isolates (n=24), it was challenging to test regional differences. Ontario exhibited a higher numerical count of resistant *Salmonella* isolates (n=13); however, the occurrence of *Salmonella* was highly clustered by farms and might not have any association with a specific region. Additionally, describing AMR in *Salmonella* can be challenging due to the relatedness between serovars and resistance patterns [64]. For instance, in our study no *Salmonella* Dublin was recovered and this serovar is commonly associated with AMR and MDR [234]. The absence of *Salmonella* Dublin might have contributed to a lower overall proportion of AMR. Additionally, in our research, although we identified 23 serovars, only nine were associated with resistance to at least one of the tested antimicrobials.

Monitoring resistance in *E. coli* is an important component of surveillance systems as this bacterium can serve as a reservoir of antimicrobial resistance genes (ARGs) [253, 254]. A previous study from the United States reported an overlapping in tetracycline resistance

between *E. coli* isolates and *Salmonella* from the same sample (same animal) [39]. Another study from the United States analyzed animal cecal samples and concluded that the odds of *Salmonella* being resistant to antimicrobials significantly increased when *E. coli* isolated from the same sample was also resistant to the same antimicrobials evaluated [255]. In our study, we found some similarities in the resistance pattern for *E. coli* and *Salmonella*. For instance, the phenotypic multidrug resistant (MDR) pattern tetracycline-sulfisoxazole-streptomycin, was one of the most common MDR pattern identified for *E. coli* and *Salmonella.* Considering that *E. coli* is a commensal bacteria and might act as a reservoir of ARGs that could be horizontally shared with *Salmonella*, the herd-level prevalence of resistance in *E. coli* might be used to predict resistance in *Salmonella* [256]. However, in our study, we did not analyze simultaneous resistance from the same sample. Therefore, further investigation of overlapping resistance is necessary to drawn informed conclusions.

In addition, resistance in *E. coli* isolates have particularly importance regarding the spreading of clones between dairy farms. A study published in 2023 investigated the resistance profiles of *E. coli* isolates recovered from Québec dairy farms. The findings of this study not only confirmed the presence of clonal transmission of resistant bacteria between farms but also highlighted the occurrence of shared clones across geographically distant farming locations [257]. In our study, we found that the proportion of farms with *E. coli* isolates resistant to at least one antimicrobial exhibited minimal regional variation, ranging from 79.2% in Nova Scotia to 93.3% in Alberta. This suggests a consistent occurrence of resistance across different regions. In addition, we identified several shared phenotypic resistance patterns among *E. coli* recovered from different provinces. For instance, a shared MDR pattern was observed in *E. coli* isolates recovered from Alberta,

Ontario, Québec, and Nova Scotia. Similarly, we found that six more phenotypic patterns were shared among *E. coli* isolates recovered from all the five provinces. These findings are consistent with the observations made in Québec dairy farms, and further research on these specific isolates (e.g., WGS to investigate if they are clonal or closely related) could help supporting the clonal spreading hypothesis.

Given the established association between AMU and AMR, it was important to incorporate farm-level AMU data collection into the CaDNetASR surveillance program. Such data collection allowed for a reliable and accurate estimation of AMU on individual farms. Increasing awareness is given to the use and the resistance associated with antimicrobials, particularly in the context of antimicrobials that are deemed critically important for human medicine, as outlined by international and national organizations such as the World Health Organization and the Public Health Agency of Canada, respectively. Overall, our findings demonstrated that category I antimicrobials (very high important for human medicine according Health Canada) accounted for 22.4% of total AMU (sum of fluoroquinolone, third-generation cephalosporins, and polymyxin AMU – Table S4.1 in Appendix C). The median category I AMU varied across provinces, with Alberta and Ontario exhibiting higher median values (30.1 DCD/100 animal-years and 20.8 DCD/100 animal-years, respectively) compared to Québec (2.7 DCD/100 animal-years), which had the lowest median category I AMU. Despite the use of category I antimicrobials being common, *E. coli* isolates had very low resistance to these antimicrobials. Similarly, only one *Salmonella* isolate was resistant to amoxicillin/clavulanic acid. Third-generation cephalosporins, specifically ceftiofur, were the primary choice of category I AMU, accounting for 69.2% of the total use for this category. In addition, intramammary administration was the most

common route employed for the delivery of this antimicrobial. Moreover, the relatively low occurrence of *E. coli* strains exhibiting resistance to category I antimicrobials could potentially be attributed to the predominant use of intramammary administration for these antimicrobials. The findings from the regression model examining *E. coli* resistance patterns demonstrated a significant association between AMR and the administration of systemic antimicrobials, whereas intramammary administration did not show a significant association with AMR. As discussed in the chapter 4 this might be due to the intramammary route being a local treatment with minimal systemic absorption and less impact on AMR in enteric bacteria. The impact of administration route on AMR also varied depending on the bacteria analyzed. For *Campylobacter* spp., neither intramammary nor systemic antimicrobial AMU rate showed significant associations with AMR when assessed separately. However, in the final model, the total AMU rate was found to be associated with tetracycline resistance (P=0.057). These differing results could be attributed to intrinsic and acquired resistance in the different bacteria. Additionally, the significance in the *Campylobacter* spp. model was low. Overall, 19.9% of *Campylobacter* spp. isolates were resistant to ciprofloxacin (category I antimicrobial). A higher proportion of ciprofloxacin-resistant *Campylobacter* spp. was observed in Ontario (44.3%), although the use of fluoroquinolones was very low, and only two herds in Ontario were using this antimicrobial class. The presence of ciprofloxacin resistance in *Campylobacter* spp. strains found on farms with low or no fluoroquinolone usage raise important questions about the persistence of resistance and its potential sources. While reduced AMU could theoretically lower the selective pressure, the fact that resistance can still emerge suggests the involvement of other mechanisms. The persistence of ciprofloxacin resistance even on

farms with low AMU might be explained by two hypotheses. Firstly, the mechanism of fluoroquinolone resistance in *Campylobacter* spp. is usually conferred by a single point mutation in the *gyr*A gene, suggesting the resistance is not related only to the selective pressure exerted by AMU [156, 157]. Secondly, a more concerning mechanism is the horizontal transmission of ARGs associated with fluoroquinolone resistance through mobile elements like plasmids. In addition, there is the possibility of co-selection of AMR when ARGs are located in close proximity within a mobile genetic element. This phenomenon can contribute to the persistence of AMR even when an antimicrobial the bacteria is resistant to is no longer being used [258].

Although the main risk factor investigated for the association with AMR was the AMU, other variables from the surveillance questionnaire were explored in the regression models. An important variable investigated in the models was the source from where the isolates were recovered (production phases and manure storage). Interestingly, the effect of this variable was different for *Campylobacter* spp. and *E. coli*. There was no difference in the proportion of resistant-*Campylobacter* spp. isolates among different production ages and manure storage. However, a higher proportion of resistant *E. coli* was observed in isolates recovered from calves, and this pattern was observed in all provinces. As discussed in the chapter 3, this finding was commonly reported previously.

6.3. Directions for future refinements and further research

One of the primary objectives of CaDNetASR surveillance was to promote evidence-based antimicrobial stewardship (AMS) on Canadian dairy farms. While the development and implementation of AMS measures were not the primary objectives of this thesis, our

findings can serve as a basis for developing such measures. As a stewardship measure, it is essential to target both the quantity and selection of antimicrobials to effectively promote responsible AMU practices. In North America, many efforts have been made to decrease AMU in animals. In the United States, California became the first state to require a veterinary prescription for all medically important antimicrobials to be used for livestock [259]. Effective June 11th, 2023, a nationwide extension of the veterinary prescription requirement for all medically important antimicrobials has been implemented in the United States [260]. In Canada, a similar approach was done in 2018 by PHAC and Health Canada to improve AMS in the food animal industries, requiring veterinary prescription for all Medically Important Antimicrobials (MIAs) for veterinary use [261]. In Netherlands, government regulations and several activities were promoted to motivate veterinarians and farmers to decrease the use of antimicrobials. These activities included the implementation of herd health and treatment plans, and the forbiddance of blanket dry cow treatment from 2013 onwards [91]. In Denmark, the AMU was reduced in approximately 50% after an intervention study aiming to minimize the incidence of diseases and optimizing the selection criteria for AMU [262].

According to our results, the use of category I antimicrobials was considerably lower in Québec when compared to the other provinces. This is likely attributed to the provincial government's restriction on the use of category I antimicrobials for production animals, which was implemented in 2019 [187]. A study published in 2022 supported this claim, reporting a decline in the sales of category I antimicrobials following the implementation of the new regulation in Québec [263]. These findings suggested that governmental regulations can play a crucial role in reducing the use of antimicrobials considered highly

important for human medicine in animal production and ultimately contribute to minimizing the emergence and spread of antimicrobial-resistant bacteria. Additionally, our regression model indicated that the total systemic AMU in a farm was associated with increased resistance in *E. coli* isolates. This highlights the need for targeted antimicrobial stewardship measures that could reduce AMU in specific age groups and administration routes. Using the data generated by the surveillance system, herd veterinarians and dairy industry stakeholders can develop effective AMS measures and educate producers about responsible AMU. For instance, a study from Washington state demonstrated that policy changes aimed at reducing AMU for pre-weaned dairy calves led to a decrease in AMR for several antimicrobials [264]. These results emphasize the potential benefits of implementing evidence-based AMS measures in the dairy industry to mitigate antimicrobial resistance.

Another important future refinement for the CaDNetASR surveillance might be the sampling scheme. According to our results, no significant difference was observed for the isolation and the proportion of resistant-*Campylobacter* spp., *Salmonella*, and *E. coli* between post-weaned heifers and lactating cows. Thus, dropping post-weaned heifers from the sampling scheme might save financial resources that could be used to expand the surveillance to other provinces, such as Manitoba and Saskatchewan.

Finally, the CaDNetASR surveillance was developed to monitor enteric bacteria of public health importance. However, to further enhance its capabilities, it is worth considering expanding the target bacteria to include those with animal health implications, such as *Salmonella* Dublin. Furthermore, considering that milk production is the primary source of revenue for dairy farms, it becomes essential to address pathogens that directly impact milk production, such as *Staphylococcus aureus*. Targeting these pathogens can provide added

value to producers. Additionally, although these pathogens are less frequently associated with human diseases, it can also contribute to AMU in dairy farms. For instance, *Salmonella* Dublin is known to cause severe infections in dairy cattle and is commonly associated with multidrug resistance (MDR) [265]. Moreover, it can lead to economic losses in the dairy sector due to increased morbidity and mortality in animals [266]. Previous studies have validated the detection of *Salmonella* Dublin antibodies using ELISA testing in bulk milk samples, which offers a convenient strategy for herd-level detection. However, there has been high variability in the sensitivity of ELISA testing, with reported ranges from 40% to 100% [215, 237, 238]. Mastitis is the most common disease affecting dairy cattle in North America and therefore the main reason for treatments [198]. Mastitis can also lead to substantial economic losses within the dairy industry due to its direct influence on milk production. In Canada, it has been estimated that each affected cow incurs an annual cost of 662 CAD [267]. In addition, *S. aureus* was found to be the most prevalent pathogen associated with clinical mastitis in Canada [268, 269]. To screen for the presence of *S. aureus*, the implementation of culturing and PCR techniques on bulk tank milk samples has become a commonly employed practice [270-272]. Considering that the collection of bulk tank milk samples is already part of the CaDNetASR surveillance, the inclusion of *Salmonella* Dublin and *Staphylococcus aureus* surveillance could be considered in future iterations of the system. This would provide valuable insights into the prevalence and dynamics of these pathogens in dairy herds, contributing to the development of targeted control measures.

6.4. Limitations

Some limitations are worth to be mentioned. The herds enrolled in the surveillance were not randomly selected from the source population. Convenience sampling could impact the

results as the selected herds may not be representative of the source population, thus impacting the interpretation and extrapolation of the results to other dairy herds in Canada. As shown in Table 5.1, the proportion of free-stall dairy herds in Ontario and Quebec from our study differed from the other commercial dairy herds in Canada. Therefore, interpretation of results must take that into account, which could affect the external validity of our results. Another limitation that could affect the results is the study design. In cross-sectional studies it is not possible to allege if the AMR was a consequence of the AMU or if the AMR observed in the herds was already present, demanding higher amounts of antimicrobials. In addition, the AMU data were collected at herd level and the AMR data were from 5 pooled samples from each sample source once a year, which might not be a representation of the true AMR at herd level. All the information on herd health collected through the surveillance questionnaire was a self-report from the farmers. Information bias is a common source of bias that can affect the validity of questionnaire answers. In our study, participants were not blinded to the objective of the study, and the farmers could have provided a more desirable answer to the questions regarding infectious diseases in the herds, underestimating the risk parameters [273]. For future refinements, it might be interesting to use other source of data such as DHI records. Finally, the relatively low number of *Salmonella* isolates recovered in the first three years of CaDNetASR surveillance precluded the risk factor analyses for the association of AMU on AMR in this pathogen.

6.5. Conclusion

The knowledge generated by this thesis helped to fill knowledge gaps in the understanding of AMU and its potential impact on AMR in dairy cattle in Canada. Our results revealed

that resistance in *E. coli* and *Campylobacter* spp. was widespread among dairy herds, while for *Salmonella* spp., it appeared to be clustered by regions and farms. Our study also demonstrated that although intramammary AMU accounted for the highest proportion of the total AMU, only the systemic AMU was associated with AMR in *E. coli*. For *Campylobacter* spp., only the total AMU was associated with tetracycline resistance. For *E. coli*, higher proportion of resistance was observed for isolates recovered from pre-weanec calves' samples compared to the other sources. AMR is a complex issue that requires an integrated effort from human, animal, and environmental sectors. Considering that the development of new antimicrobials can take decades, and the potential increase of resistance in those currently being used, efforts should be continued to achieve a more rational use of antimicrobials.

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8. Appendix A

Table S2.1. Main sections of the herd level questionnaires applied at enrollment in CaDNetASR surveillance

Questionnaire 1	Questionnaire 2
- Breeds - Herd inventory - Non-dairy cattle species at the farm - Milking system - Type of housing for each production phase (pre-weaned calves, heifers, and lactating cows) - Farm treatment records (if and how individual treatments are recorded at the farm) - Veterinary services: number of veterinary visits for emergencies (e.g., sick animals), and number of preventive herd health visits - Drug use protocols (written protocols for the most common infectious diseases) - How the protocols are accessed (paper, electronically on a computer, electronically on a handheld device) - Antimicrobial drugs sources - Biosecurity (biosecurity practices, herd additions, and vaccination)	- Frequency of use of several antimicrobials' products (injectables, intramammary for lactating cows, intramammary for dry cows, intra-uterine, topical, feed, water, and oral bolus) - Reasons for AMU (reasons to use antimicrobials in different production phases to treat the most common infectious diseases) - Frequency of cases of the most common infectious disease in each production phase

Table S2.2. Main sections of the stewardship questionnaire applied at enrollment in CaDNetASR

Stewardship questionnaire	
Dry-off procedure	Calf management
- Frequency of tests for SCC - Use of teat sealant - Proportion of cows receiving teat sealant at dry-off - Use of SCC to decide the use of teat sealer - SCC cut-off to make the decision to use teat sealant - Proportion of cows receiving antimicrobials at dry-off - Use of SCC to select animals that needs antimicrobials at dry off - SCC cut off to make the decision to treat the animal	- Use of antimicrobials to treat calf diarrhea, and respiratory disease - Clinical signs considered to make the decision to treat the animal - Use of wasted milk to feed the calves

- Use of previous mastitis history to select animals to be treated at dry off - Proportion of clinical mastitis treated with antimicrobials - Proportion of clinical mastitis cases treated with intramammary antimicrobials - Proportion of clinical mastitis cases treated with systemic antimicrobials - Proportion of clinical mastitis cases treated with both intramammary and systemic antimicrobials	

Table S2.3. Modified farm-level AMU surveillance systems for dairy cattle identified by Sanders et al.

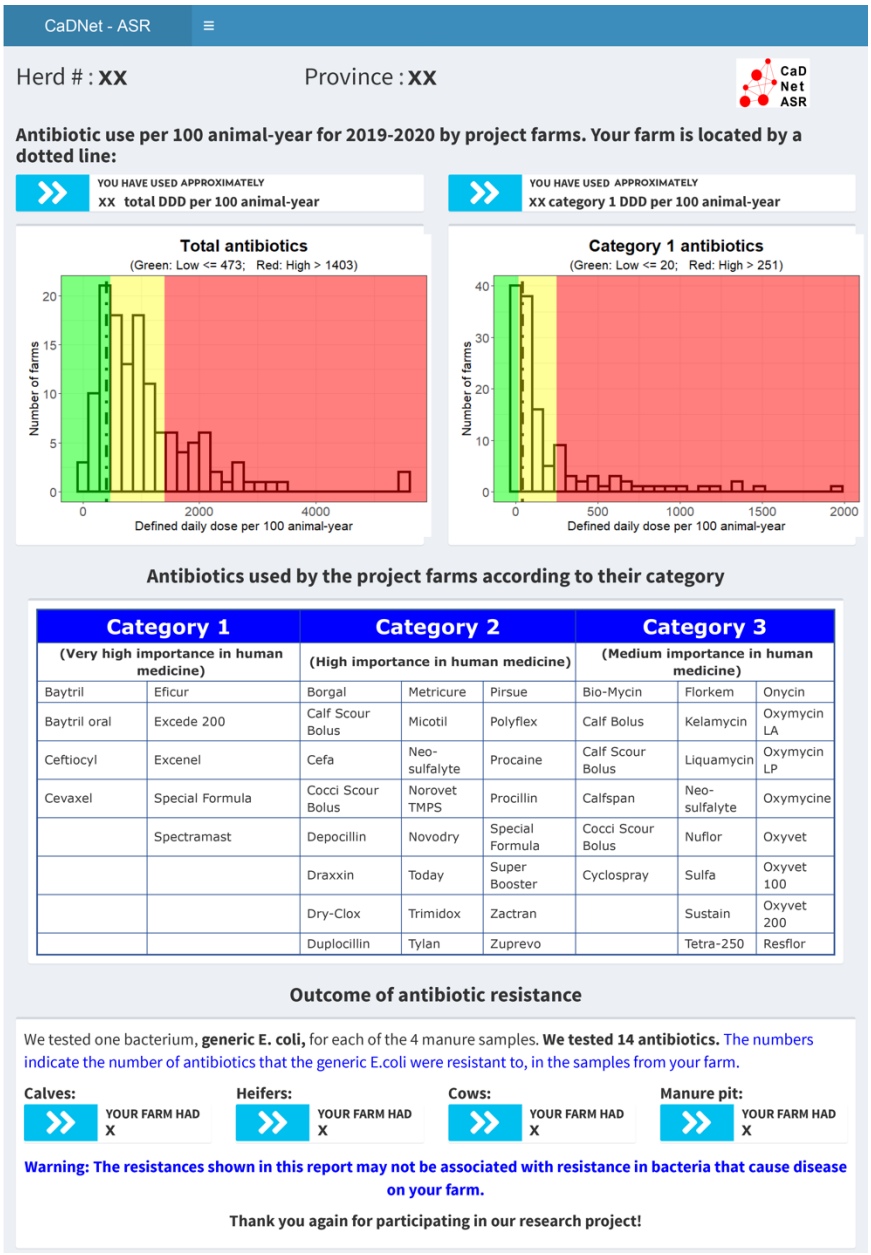
Country	AMU Surveillance for dairy cattle	Collects AMU at farm-level	Report AMU at farm-level	Coverage	AMU metrics
Austria	PHAROS	Yes	No	Full sector	Dose-based
Belgium	AB Register/BIGAME	Yes	Yes	Partial sector	Weight-based/Dose-based
Canada*	CIPARS/CaDNetAS R	Yes	Yes	Sample	Dose-based
Czech Republic	DLN cattle	Yes	Yes	Sample	Weight-based
Denmark	VetStat	Yes	Yes	Full sector	Dose-based
Germany	VetCAb-ID/VetCAb(-S)	Yes	Yes	Sample	Count-based
Italy	ClassyFarm	Yes	Yes	Sample	Dose-based
Netherlands	SQS|SDa	Yes	Yes	Full sector	Dose-based
Norway	VetReg	Yes	No	Full sector	Weight-based
Spain	NDVAP	Yes	No	Full sector	Weight-based
Sweden	SBA	Yes	No	Full sector	Weight-based
Switzerland	IS ABV	Yes	Yes	Full sector	Dose-based/Count-based
United States**	No	No	No	N/A	N/A

*Not included in Sanders et al.
**No AMU at farm-level, but it was included to allow comparison in North America

Table S2.4. AMR surveillance systems including data collection and report on dairy cattle

Country[a]	AMR surveillance food-producing animals	AMR Surveillance for dairy cattle	Type of Surveillance	Dairy Cattle subcategory	Material	Frequency of sampling	Target bacteria	MIC interpretation
AU	AURES	No	N/A	N/A	N/A	N/A	N/A	N/A
BE	FASFC	Yes	Active	Cows	Pool of nasal swabs	Every three years	MRSA *Staphylococcus aureus*	EUCAST
CA	CIPARS	Yes (CaDNetASR)	Active	Calves/Heifers/Cows/Manure storage	Feces/Bulk tank milk	Annual	*E. coli/Salmonella* spp./*Campylobacter* spp.	CLSI
CZ	No	No	N/A	N/A	N/A	N/A	N/A	N/A
DK	DANMAP	Yes	Active	Cows	Composite milk of 5 cows	First report on 2019	MRSA *Staphylococcus aureus*	EUCAST
GE	GERMVET	No	N/A	N/A	N/A	N/A	N/A	N/A
IT	ITAVARM - Reported only in 2003	No	N/A	N/A	N/A	N/A	N/A	N/A
NL	NethMap-MARAN	Yes	Active	Cows	Feces	Annual	ESBL-producing *E. coli*	EUCAST
NO	NORM-VET	No	N/A	N/A	N/A	N/A	N/A	N/A
SP	VAV - last report 2005	No	N/A	N/A	N/A	N/A	N/A	N/A
SW	SVARM	Yes	Active	Cows	Milk	Annual	MRSA *Staphylococcus aureus*	EUCAST
SZ	No information retrieved	No information retrieved	N/A	N/A	N/A	N/A	N/A	N/A
US	NARMS	Yes	Active	Cows	Cecal sample	Annual	*E. coli/Salmonella* spp./*Campylobacter* spp./*Enterococcus* spp.	CLSI

[a] AU: Austria, BE: Belgium, CA: Canada, CZ: Czech Republic, DK: Denmark, GE: Germany, IT: Italy, NL: Netherlands, NO: Norway, SP: Spain, SW: Sweden, SZ: Switzerland, US: United States; [b] N/A: Not applicable



Category 1 (Very high importance in human medicine)		Category 2 (High importance in human medicine)			Category 3 (Medium importance in human medicine)		
Baytril	Eficur	Borgal	Metricure	Pirsue	Bio-Mycin	Florkem	Onycin
Baytril oral	Excede 200	Calf Scour Bolus	Micotil	Polyflex	Calf Bolus	Kelamycin	Oxymycin LA
Ceftiocyl	Excenel	Cefa	Neo-sulfalyte	Procaine	Calf Scour Bolus	Liquamycin	Oxymycin LP
Cevaxel	Special Formula	Cocci Scour Bolus	Norovet TMPS	Procillin	Calfspan	Neo-sulfalyte	Oxymycine
	Spectramast	Depocillin	Novodry	Special Formula	Cocci Scour Bolus	Nuflor	Oxyvet
		Draxxin	Today	Super Booster	Cyclospray	Sulfa	Oxyvet 100
		Dry-Clox	Trimidox	Zactran		Sustain	Oxyvet 200
		Duplocillin	Tylan	Zuprevo		Tetra-250	Resflor

Figure S2.1. CaDNetASR annual reporting summarizing the findings at each farm. The first section of the report estimates the AMU in DDD/100 animals/year and benchmark the farm among the other farms enrolled in the program. It is also provided a table classifying the most common antimicrobials used at dairy farms according to their importance in human health. The last part of the report presents the results on generic *E. coli* susceptibility in each of the production phases and manure pit.

9. Appendix B

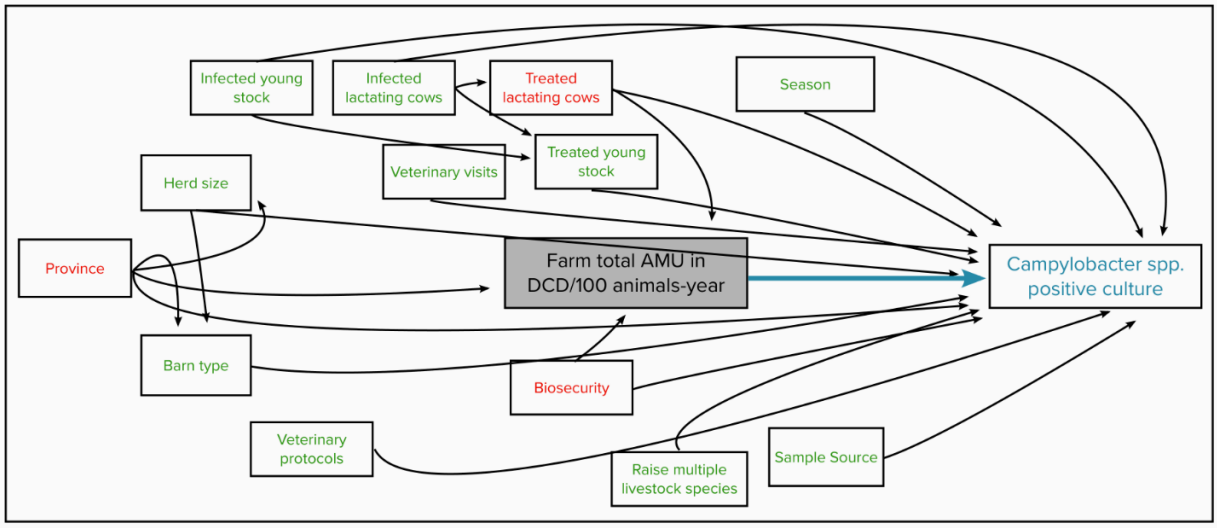


Figure S3.1. Revised (after the unconditional associations among predictors) directed acyclic graph (DAG) illustrating the main components of the causal assumptions among the study variables for the probability of recovering *Campylobacter* spp. from fecal samples. Red letters represent potential confounders; green letters represent competing exposures (not associated with the main exposure); blue letters represent the outcome, and the gray shaded box represents the main exposure.

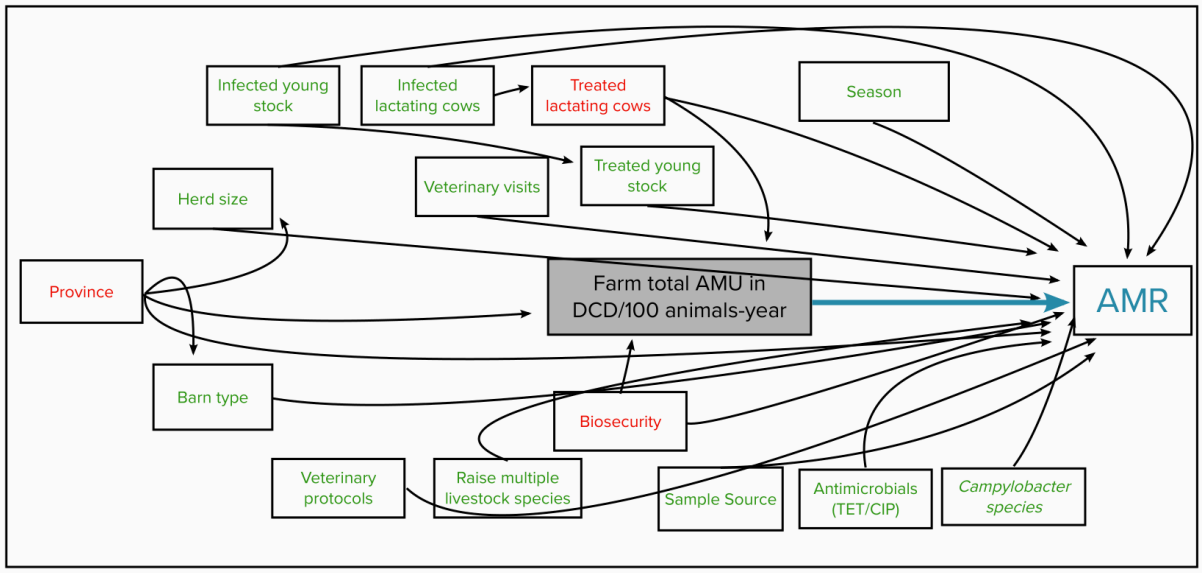


Figure S3.2. Revised (after the unconditional associations among predictors) directed acyclic graph (DAG) illustrating the main components of the causal assumptions among the study variables for the probability of resistance to tetracyclines and ciprofloxacin in *Campylobacter* spp. isolates. Red letters represent potential confounders; green letters represent competing exposures (not associated with the main exposure); blue letters represent the outcome, and the gray shaded box represents the main exposure. TET: tetracycline; CIP: ciprofloxacin.

Table S3.1. Descriptive statistics of each active ingredient (farm level, n=131) included in the total antimicrobial use in Defined Course Doses/100 animal-years* and their unconditional association with the probability of recovering a *Campylobacter* spp. from fecal samples collected from pre-weaned calves, post-weaned heifers, lactating cows, and manure storage (model 1).

Variable	Health Canada category	WHO[1]	No. farms[2]	% of total AMU[3]	Percentile			OR[4]	Overall *P*-value
					25th	50th	75th		
Fluoroquinolones	I	HPCIA	14	0.2	0.0	0.0	0.0	1.87	0.540
Third generation cephalosporins	I	HPCIA	117	15.5	2.2	8.2	22.8	0.99	0.085
Polymyxin	I	HPCIA	68	6.7	0.0	0.4	10.4	1.00	0.870
Macrolides	II	HPCIA	44	2.8	0.0	0.0	1.6	1.01	0.540
Penicillins	II	HIA	124	27.3	8.6	23.9	44.7	0.99	0.768
Aminoglycosides	II	CIA	76	8.1	0.0	1.8	14.6	1.00	0.968
First-generation cephalosporins	II	HIA	91	13.9	0.0	4.2	29.5	0.99	0.639
Lincosamides	II	HIA	49	0.7	0.0	0.0	0.0	0.98	0.775
TMS[5]	II	HIA	110	2.9	0.3	1.9	4.8	0.94	0.087
Sulfonamides	III	HIA	35	0.5	0.0	0.0	0.0	0.94	0.232
Tetracyclines	III	HIA	64	7.8	0.0	0.0	5.6	1.00	0.531
Amphenicols	III	HIA	93	3.0	0.0	1.7	6.0	1.01	0.810
Aminocoumarins[6]	N/A	N/A	95	10.6	0.0	2.8	20.6	1.00	0.889

*Estimates were obtained from a garbage can audit, except for Quebec farms.
[1]World Health Organization
[2]Number of farms using the respective active ingredient(s).
[3]% of the total AMU represented by each active ingredient averaged across farms.
[4]Odds ratio
[5]Trimethoprim-sulfamethoxazole
[6]Not categorized by Health Canada and WHO (currently not used in humans).

Table S3.2. Descriptive statistics of each active ingredient (farm level, n=125) included in the total antimicrobial use in Defined Course Doses/100 animal-years* and their unconditional association with the probability of resistance to tetracycline and ciprofloxacin in *Campylobacter* spp. isolates recovered from fecal samples collected from pre-weaned calves, post-weaned heifers, lactating cows, and manure storage (model 2).

Variable	Health Canada category	WHO[1]	No. farms[2]	% of total AMU[3]	Percentile			OR[4]	Overall *P*-value
					25th	50th	75th		
Fluoroquinolones	I	HPCIA	13	0.2	0.0	0.0	0.0	1.13	0.391
Third generation cephalosporins	I	HPCIA	113	14.8	2.6	8.1	20.2	1.00	0.495
Polymyxin	I	HPCIA	65	6.1	0.0	0.4	10.3	0.98	0.067
Macrolides	II	HPCIA	43	2.9	0.0	0.0	1.6	1.00	0.732
Penicillins	II	HIA	118	27.8	8.9	23.9	45.6	0.99	0.236
Aminoglycosides	II	CIA	73	8.2	0.0	2.0	13.0	0.99	0.210
First-generation cephalosporins	II	HIA	85	14.3	0.0	4.2	30.2	1.00	0.980
Lincosamides	II	HIA	45	0.7	0.0	0.0	0.0	0.93	0.238
TMS[5]	II	HIA	105	2.9	0.3	1.8	4.8	1.05	0.213
Sulfonamides	III	HIA	32	0.5	0.0	0.0	0.0	1.08	0.227
Tetracyclines	III	HIA	64	8.2	0.0	0.4	5.9	0.99	0.978
Amphenicols	III	HIA	89	3.1	0.0	1.7	6.3	1.08	0.046
Aminocoumarins[6]	N/A	N/A	89	10.3	0.0	2.6	20.1	0.98	0.075

*Estimates were obtained from a garbage can audit, except for Quebec farms.
[1]World Health Organization
[2]Number of farms using the respective active ingredient(s).
[3]% of the total AMU represented by each active ingredient averaged across farms.
[4]Odds ratio
[5]Trimethoprim-sulfamethoxazole
[6]Not categorized by Health Canada and WHO (currently not used in humans).

Table S3.3. Description for the independent variables considered in the regression models.

Variable	Explanation
Province	British Columbia, Alberta, Ontario, Quebec, and Nova Scotia
Herd size	≤ 70 lactating cows *vs*. 71-160 lactating cows *vs.* ≥161 lactating cows
Barn type	Tie-stall vs. Free-stall
Frequency of veterinary visits	We collected data on the reasons for veterinary visits on the farm (how many times the veterinarian visited the farm over the last 12 months). The categories were: scheduled veterinary visits for preventive/herd health and veterinary visits for sick animals/emergency. The variable was categorized as follows: More visits for herd health and less visits for sick animals *vs.* less visits for herd health or more visits for sick animals *vs*. less visits for animal health and more visits for sick animals
Infected young stock	Occurrence of infectious diseases in young stock (yes/no). The diseases included: pneumonia, arthritis, wound infection, navel infection, or pink eye in young stock (pre-weaned calves and post-weaned heifers). It was a self-report, so it does not represent the prevalence of these diseases. The variable was categorized as follows: ≤ 2 diseases reported *vs.* 3 to 4 diseases reported *vs.* 5 diseases reported
Infected lactating cows	Occurrence of infectious diseases in lactating cows (yes/no). The diseases included: pneumonia, wound infection, diarrhea, or metritis in lactating cows. It was a self-report, so it does not represent the prevalence of these diseases. Mastitis and lameness were excluded as all producers reported the occurrence of these two diseases. The variable was categorized as follows: ≤ 1 disease reported *vs.* 2 diseases reported *vs.* ≥ 3 diseases reported
Treated young stock	If the producer reported (self-report) a treatment (yes/no) with antimicrobials for pneumonia, arthritis, diarrhea, wound infection, navel infection, lameness, or pink eye in young stock (pre-weaned calves and heifers).

	The variable was categorized as follows: Reported treatment for ≤ 2 diseases *vs.* reported treatment for 3 to 5 diseases *vs.* reported treatment for ≥ 6 diseases
Treated lactating cows	If the producer reported a treatment (yes/no) with antimicrobials for pneumonia, lameness, diarrhea, wound infection, or metritis in lactating cows. The variable was categorized as follows: Reported treatment for ≤ 2 diseases *vs.* reported treatment for 3 to 4 diseases *vs.* reported treatment for 5 diseases
Use of veterinary protocols	Protocols developed by the herd veterinarian and producer for treatment of lameness, mastitis, metritis/retained placenta, heifer respiratory disease, cow respiratory disease, dry-off procedure, pink eye, calf diarrhea, and post-surgical care. The variable was categorized as follows: No protocol *vs.* farms with up to 5 protocols *vs.* farms with more than 6 protocols
Multiple species of livestock on the farm	Farmers that raise only dairy cattle vs. farmers who raise dairy cattle and other livestock such as chicken, horses, veal, or beef cattle
Biosecurity practices	Farms using one or more biosecurity practices other than vaccination, such as: biosecurity signage visible from the parking area closest to the main barn; restricted access onto the farm; regular pest control; if the farm provides boots or disposable boots for farm visitors and veterinarians; and on-farm isolation areas for new additions or sick animals. For farmers using vaccines, those were grouped into 3 groups: vaccines for adult animals (bovine viral diarrhea, respiratory diseases, and clostridiosis); vaccines for calves: (enteric and respiratory diseases); and vaccines for mastitis. The variable was categorized as follows: Only biosecurity practices other than vaccination *vs.* at least one biosecurity practice and vaccines for 1 group (any group) *vs.* at least one biosecurity practice and vaccines for 2 groups (any of the 2 groups) *vs.* at least one biosecurity practice and vaccines for 3 groups (all the three groups)

10. Appendix C

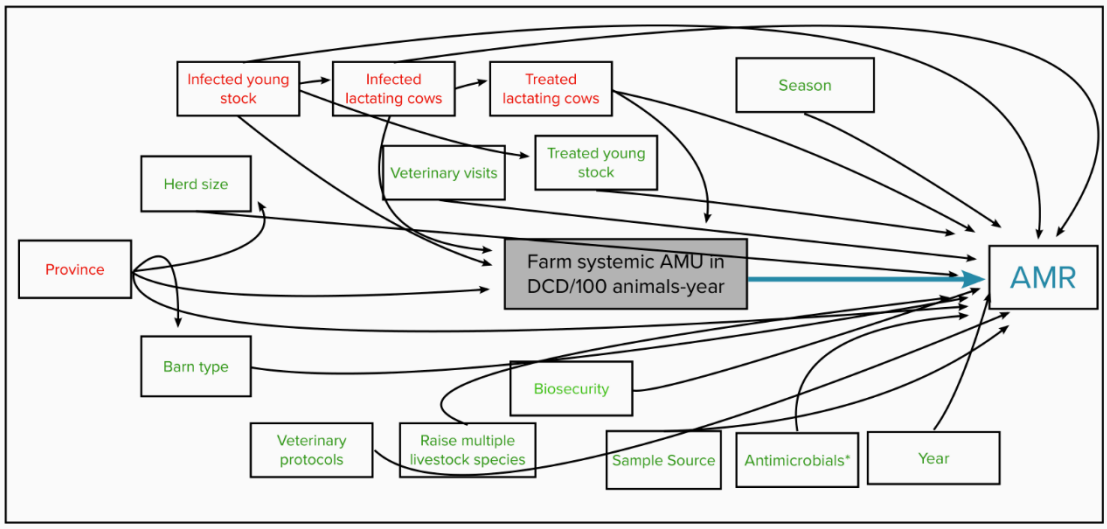


Figure S4.1. Revised (after the unconditional associations among predictors) directed acyclic graph (DAG) illustrating the main components of the causal assumptions among the study variables for the probability of resistance in *E. coli* isolates recovered from fecal samples. Red letters represent potential confounders; green letters represent competing exposures (not associated with the main exposure); blue letters represent the outcome, and the gray shaded box represents the main exposure. *Antimicrobial by active ingredients: Amoxicillin/clavulanic, ampicillin, cefoxitin, ceftriaxone, chloramphenicol, streptomycin, sulfisoxazole, tetracycline, and trimethoprim/sulphamethoxazole.

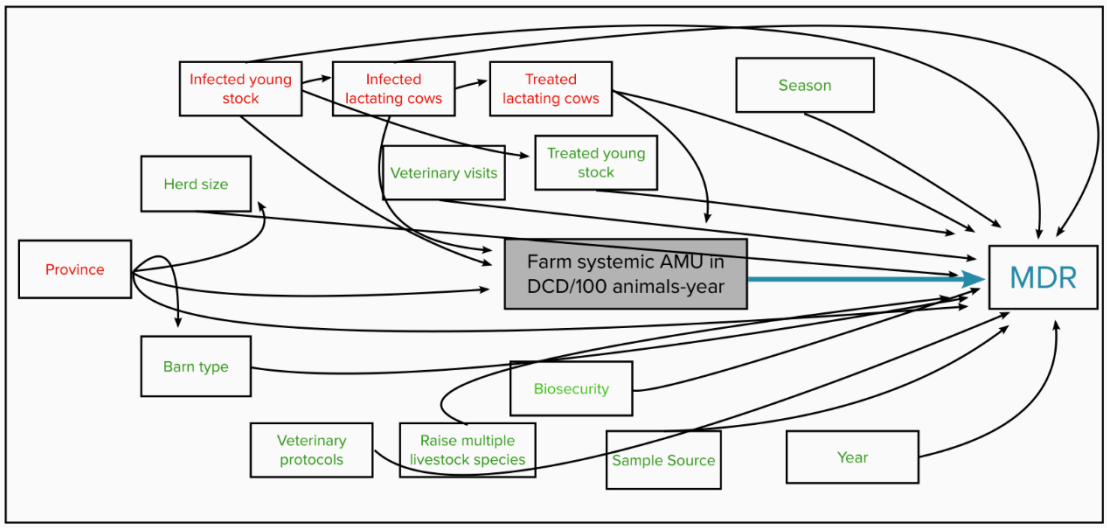


Figure S4.2. Revised (after the unconditional associations among predictors) directed acyclic graph (DAG) illustrating the main components of the causal assumptions among the study variables for the probability of MDR in *E. coli* isolates recovered from fecal samples. Red letters represent potential confounders; green letters represent competing exposures (not associated with the main exposure); blue letters represent the outcome, and the gray shaded box represents the main exposure.

Table S4.1. Descriptive statistics of each active ingredient (farm level, n=131) in DCD/100 animal-years[a] and their unconditional association with the probability of resistance to nine antimicrobials* in generic *E. coli* isolates recovered from fecal samples collected from pre-weaned calves, breeding-age heifers, lactating cows, and manure storage (model 1).

Variable	Health Canada category	WHO	No. farms[b]	% of total AMU[c]	Percentile			OR[d]	Overall *P*-value
					25th	50th	75th		
Fluoroquinolones	I	HPCIA	14	0.2	0.0	0.0	0.0	1.07	0.293
Third generation cephalosporins	I	HPCIA	117	15.5	2.2	8.2	22.8	1.00	0.738
Polymyxin	I	HPCIA	68	6.7	0.0	0.4	10.4	1.00	0.714
Macrolides	II	HPCIA	44	2.8	0.0	0.0	1.6	1.01	0.091
Penicillins	II	HIA	124	27.3	8.6	23.9	44.7	1.00	0.137
Aminoglycosides	II	CIA	76	8.1	0.0	1.8	14.6	1.00	0.793
First-generation cephalosporins	II	HIA	91	13.9	0.0	4.2	29.5	1.00	0.649
Lincosamides	II	HIA	49	0.7	0.0	0.0	0.0	0.97	0.379
TMS[e]	II	HIA	110	2.9	0.3	1.9	4.8	1.02	0.467
Sulfonamides	III	HIA	35	0.5	0.0	0.0	0.0	1.05	0.073
Tetracyclines	III	HIA	64	7.8	0.0	0.0	5.6	1.01	0.006
Amphenicols	III	HIA	93	3.0	0.0	1.7	6.0	1.05	0.011
Aminocoumarins[f]	N/A	N/A	95	10.6	0.0	2.8	20.6	1.01	0.318

[a]AMU in DCD/100 animal-years. Estimates were obtained from a garbage can audit, except for Quebec farms.

[b]Number of farms using the respective active ingredient.

[c]% of the total AMU represented by each active ingredient averaged across farms.

[d]OR: Odds ratio corresponding to an increase of 1 IQR in the number of DCD/100 animal-years of the antimicrobials.

[e]Trimethoprim-sulfamethoxazole

[f]Not categorized by Health Canada and WHO (currently not used in humans).

*Amoxicillin/clavulanic, ampicillin, cefoxitin, ceftriaxone, chloramphenicol, streptomycin, sulfisoxazole, tetracycline, and trimethoprim/sulphamethoxazole.

The antimicrobials were categorized based on their importance to human medicine according to the Public Health Agency of Canada (I-Very high importance for humans, II-High importance for humans, or III-Medium importance for humans)[132], and the World Health Organization (WHO) (HPCIA = highest priority critically important antimicrobials; CIA = high-priority critically important antimicrobials; HIA = highly important antimicrobials) [133].

Table S4.2. Descriptive statistics of each active ingredient (farm level, n=131) in DCD/100 animal-years[a] and their unconditional association with the probability of multi-drug resistance in generic *E. coli* isolates recovered from fecal samples collected from pre-weaned calves, breeding-age heifers, lactating cows, and manure storage (model 2).

Variable	Health Canada category	WHO	No. farms[b]	% of total AMU[c]	Percentile			OR[d]	Overall *P*-value
					25th	50th	75th		
Fluoroquinolones	I	HPCIA	14	0.2	0.0	0.0	0.0	1.87	0.540
Third generation cephalosporins	I	HPCIA	117	15.5	2.2	8.2	22.8	0.99	0.085
Polymyxin	I	HPCIA	68	6.7	0.0	0.4	10.4	1.00	0.870
Macrolides	II	HPCIA	44	2.8	0.0	0.0	1.6	1.01	0.540
Penicillins	II	HIA	124	27.3	8.6	23.9	44.7	0.99	0.768
Aminoglycosides	II	CIA	76	8.1	0.0	1.8	14.6	1.00	0.968
First-generation cephalosporins	II	HIA	91	13.9	0.0	4.2	29.5	0.99	0.639
Lincosamides	II	HIA	49	0.7	0.0	0.0	0.0	0.98	0.775
TMS[e]	II	HIA	110	2.9	0.3	1.9	4.8	0.94	0.087
Sulfonamides	III	HIA	35	0.5	0.0	0.0	0.0	0.94	0.232
Tetracyclines	III	HIA	64	7.8	0.0	0.0	5.6	1.00	0.531
Amphenicols	III	HIA	93	3.0	0.0	1.7	6.0	1.01	0.810
Aminocoumarins[f]	N/A	N/A	95	10.6	0.0	2.8	20.6	1.00	0.889

[a]AMU in DCD/100 animal-years. Estimates were obtained from a garbage can audit, except for Quebec farms.

[b]Number of farms using the respective active ingredient.

[c]% of the total AMU represented by each active ingredient averaged across farms.

[d]OR: Odds ratio corresponding to an increase of 1 IQR in the number of DCD/100 animal-years of the antimicrobials.

[e]Trimethoprim-sulfamethoxazole

[f]Not categorized by Health Canada and WHO (currently not used in humans).

The antimicrobials were categorized based on their importance to human medicine according to the Public Health Agency of Canada (I-Very high importance for humans, II-High importance for humans, or III-Medium importance for humans) [132], and the World Health Organization (WHO) (HPCIA = highest priority critically important antimicrobials; CIA = high-priority critically important antimicrobials; HIA = highly important antimicrobials) [133].

Table S4.3. The explanation for the risk factors variables considered in the regression models.

Variable	Explanation
Province	British Columbia, Alberta, Ontario, Quebec, and Nova Scotia
Herd size	≤ 70 lactating cows *vs*. 71-160 lactating cows *vs.* ≥161 lactating cows
Barn type	Tie-stall vs. Free-stall
Frequency of veterinary visits	We collected data on the reasons for veterinary visits on the farm (how many times the veterinarian visited the farm over the last 12 months). The categories were: scheduled veterinary visits for preventive/herd health and veterinary visits for sick animals/emergency. The variable was categorized as follows: More visits for herd health and less visits for sick animals *vs.* less visits for herd health or more visits for sick animals *vs*. less visits for animal health and more visits for sick animals
Infected young stock	Occurrence of infectious diseases in young stock (yes/no). The diseases included: pneumonia, arthritis, wound infection, navel infection, or pink eye in young stock (pre-weaned calves and post-weaned heifers). It was a self-report, so it does not represent the prevalence of these diseases. The variable was categorized as follows: ≤ 2 diseases reported *vs.* 3 to 4 diseases reported *vs.* 5 diseases reported
Infected lactating cows	Occurrence of infectious diseases in lactating cows (yes/no). The diseases included: pneumonia, wound infection, diarrhea, or metritis in lactating cows. It was a self-report, so it does not represent the prevalence of these diseases. Mastitis and lameness were excluded as all producers reported the occurrence of these two diseases. The variable was categorized as follows: ≤ 1 disease reported *vs.* 2 diseases reported *vs.* ≥ 3 diseases reported
Treated young stock	If the producer reported (self-report) a treatment (yes/no) with antimicrobials for pneumonia, arthritis, diarrhea, wound infection, navel infection, lameness, or pink eye in young stock (pre-weaned calves and heifers).

	The variable was categorized as follows: Reported treatment for ≤ 2 diseases *vs.* reported treatment for 3 to 5 diseases *vs.* reported treatment for ≥ 6 diseases
Treated lactating cows	If the producer reported a treatment (yes/no) with antimicrobials for pneumonia, lameness, diarrhea, wound infection, or metritis in lactating cows. The variable was categorized as follows: Reported treatment for ≤ 2 diseases *vs.* reported treatment for 3 to 4 diseases *vs.* reported treatment for 5 diseases
Use of veterinary protocols	Protocols developed by the herd veterinarian and producer for treatment of lameness, mastitis, metritis/retained placenta, heifer respiratory disease, cow respiratory disease, dry-off procedure, pink eye, calf diarrhea, and post-surgical care. The variable was categorized as follows: No protocol *vs.* farms with up to 5 protocols *vs.* farms with more than 6 protocols
Multiple species of livestock on the farm	Farmers that raise only dairy cattle vs. farmers who raise dairy cattle and other livestock such as chicken, horses, veal, or beef cattle
Biosecurity practices	Farms using one or more biosecurity practices other than vaccination, such as: biosecurity signage visible from the parking area closest to the main barn; restricted access onto the farm; regular pest control; if the farm provides boots or disposable boots for farm visitors and veterinarians; and on-farm isolation areas for new additions or sick animals. For farmers using vaccines, those were grouped into 3 groups: vaccines for adult animals (bovine viral diarrhea, respiratory diseases, and clostridiosis); vaccines for calves: (enteric and respiratory diseases); and vaccines for mastitis. The variable was categorized as follows: Only biosecurity practices other than vaccination *vs.* at least one biosecurity practice and vaccines for 1 group (any group) *vs.* at least one biosecurity practice and vaccines for 2 groups (any of the 2 groups) *vs.* at least one biosecurity practice and vaccines for 3 groups (all the three groups)