ANTIMICROBIAL USE, ANTIMICROBIAL RESISTANCE, AND THE MICROBIOME IN FOOD ANIMALS

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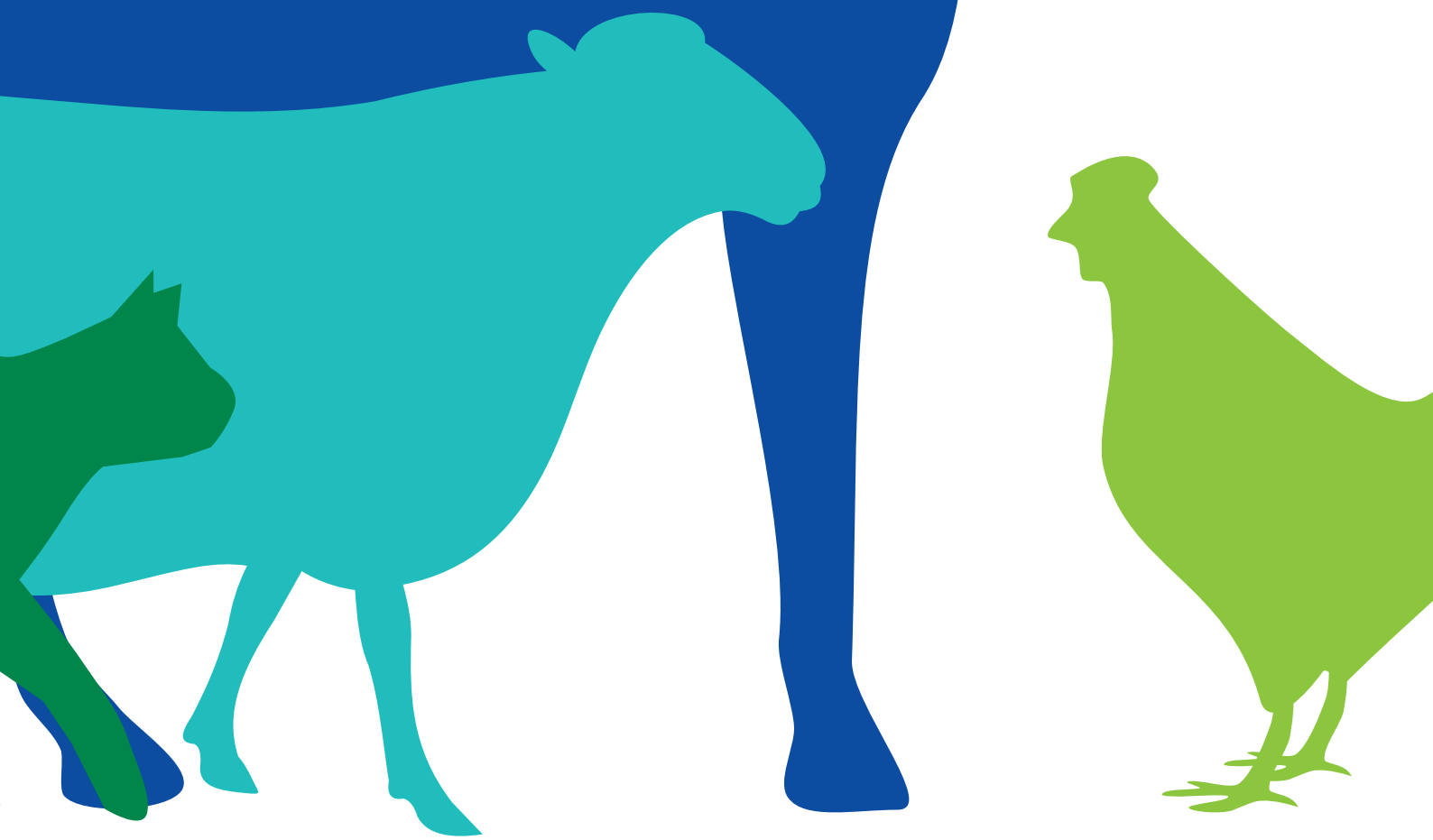

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ANTIMICROBIAL USE, ANTIMICROBIAL RESISTANCE, AND THE MICROBIOME IN FOOD ANIMALS

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EDITORIAL
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Editorial: Antimicrobial Use, Antimicrobial Resistance, and the Microbiome in Food Animals

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Editorial on the Research Topic

Antimicrobial Use, Antimicrobial Resistance, and the Microbiome in Food Animals

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AMU AND AMR

Despite the growing demand of animal protein, livestock, and poultry productions around the world are facing constraints including their consequences on environment, food safety, and animal health and welfare. No doubt that antibiotics significantly contributed to the increasing productivity of food-animals. Sir Alexander Fleming, who discovered the first antibiotic, was also the first person to warn about antimicrobial resistance (AMR) during his Nobel price speech in 1945. Nevertheless, nothing happened at that time. One of the first reports to seriously criticize our unnecessary and improper antimicrobial use (AMU) that led to some partial restrictions, was the Swann's report, released in the late 1960s. However, this was only dealing with the AMU as growth promoters and led only to partial restrictions: only non-therapeutically used antimicrobials could be used as growth promoters. It was only as late as in the late 1990s that a real movement started with the pressure for a total ban on the use of antimicrobial growth promotors which was adopted in the early 2000s, but only in Europe (1). Nevertheless, this was the start on our rethinking about AMU, and the first question was about the amount of antimicrobials used in both humans and animals. Indeed, we did not even know this. Articles in this Research Topic handled that subject (Gemeda et al.; Lardé et al.). This knowledge leads to a better understanding of the selection pressures and allowed to concisely make decisions on potential reductions, which came later in several countries. Of course, one should be aware that antimicrobials can have positive effects on certain syndromes and adverse effects of reducing AMU should be investigated so they can be counteracted by alternatives. One of these potential adverse effects was described by Davedow et al. however, they indicate possibility to reduce the tylosin use in feedlot cattle without impacting animal productivity.

MICROBIOTA AND MICROBIOME

The digestive tract is one of the largest immune organs responsible for a large proportion of immune responses, so optimal gut health in production animal is vital for their growth and performance. Gut health, which is linked to microbial community, can be achieved through a combination of nutrition, microbiology, immunology, and physiology approaches. When gut health is compromised, digestion, and nutrient absorption are affected which, in turn, can lead to a greater susceptibility to diseases that ultimately lead to an increase of antibiotics for treatments. It is also well-known that antimicrobials may have direct effects on the microbial communities. This has been investigated frequently on the selection of antimicrobial resistant bacteria but using mainly culture dependent methods. With the advent of the new high throughput sequencing methods, we can now have a better view on what happens in diverse ecosystems of the body. Turcotte et al., showed that short term effects are not to be expected on AMR, but several years are necessary. Meanwhile, alterations in the microbiome were also noted. Contrary, in the study on the nasopharyngeal microbiome and the effects of tilmycosin, minimal changes in the microbiome were detected (Zeineldin et al.). The selective effect and alteration of the microbiomes of antimicrobials is also influenced by the way the antimicrobial is administered, and likewise the reduction of resistance may be obtained (Ricker et al.) As such reduction and alternative applications of antimicrobials may also aid to breakdown resistance, albeit only at the long term. Perseverance is necessary. Nevertheless, more studies are needed to fully understand the total effects of antimicrobials on the selection of resistant bacterial, animal performance, and health as well as microbial communities and microbiome including the resistome.

ALTERNATIVES AND AMR

The clinical AMU will remain and a reduction in AMR, when reducing/eliminating unnecessary use, is taking time, but it works as shown in The Netherlands (2). Taking this into account, there is an urgent need for alternatives to antimicrobials and several strategies are under investigation. The road to alternatives is not paved smoothly and this was exemplified in the article of Kurt et al.. Apart from real agents killing bacteria, like phages, indirect strategies are also possible as exemplified in this Research Topic by Alizadeh et al., where immune stimulation was demonstrated by in ovo application of probiotic bacteria. Plant based alternatives were also handled and were found to improve performance, liver immunity, and intestinal health of broiler chicken (Das et al.). Not only plant-based products but also organic acid as formic acid may help in reducing the AMU and have been shown to reduce the prevalence of non-typhoid *Salmonella* (Ricke et al.). Non-typhoid *Salmonella* is still a major foodborne agent, frequently resistant to antimicrobials. Specifically, for *Salmonella*, several serotypes are resistant to multiple drugs, while others, are way more susceptible, though they are in the same ecosystem (Gu et al.). This is also an interesting finding as it may identify bacterial factors that inhibit the acquisition of resistance genes. Reduction of AMU can also be improved by better diagnostics, excluding viral infections leads to a lesser use of antibiotics as they have no effect on viruses. Isothermal tests described in this Research Topic seems allowing an easy, fast and on the field diagnosis of bacterial pathogens (Conrad et al.).

PERPECTIVES

It is clear that antimicrobial resistance is a complex problem for which multiple solutions need to be applied, though effects are only to be expected at a long term. Several pathways can be taken and some of them were presented in this Research Topic, though many others are under investigation. It is clear that we also should take into account of the microbiomes and more studies in this field are necessary to understand its roles and functions knowing that it is in part also shaped by bacteriophages. The role of the latter is in general a bit neglected, though interest is increasing, not only for their role in shaping the microbiomes but also as therapeutics. Furthermore, understanding how feeding programs including organic farming impact the beneficial microbes, pathogens, and AMR, will help guide dietary or management practices. Given the AMR complexity mentioned above, tackling this issue requires an "One Health" approach, which is based on the principle that human and animal health are interconnected in relation with a heathy environment. Efforts in one will not be sufficient to cover all current problems with AMR but also in the human and environmental ecosystems, measures should be taken to reduce its burden. The existence of high abundance of various antibiotic resistance genes (ARGs) in animal manure and subsequent environmental contamination may be avoided by treatment approaches, such as composting (thermophilic composting and vermicomposting) and anaerobic digestion.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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PERSPECTIVE
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Strategic Priorities for Research on Antibiotic Alternatives in Animal Agriculture—Results From an Expert Workshop

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The emergence, spread, and expansion of antibiotic resistance and increasing restrictions on the use of antibiotics in animal agriculture have created a need for efficacious alternatives that remains unmet. Prioritizing research needs in the development of alternatives is key to ensuring that scarce research resources are dedicated to the most promising approaches. However, frameworks to enable a consistent, systematic, and transparent evaluation of antibiotic alternative candidates are lacking. Here, we present such an evaluation framework.

Keywords: antibiotic resistance, animal agriculture, antibiotic alternatives, research prioritization, evaluation framework

INTRODUCTION

Traditional antimicrobial drugs, or antibiotics[1], are critical tools to promote human and animal health, yet their efficacy is increasingly threatened by antibiotic resistance[2]. Any exposure to antibiotics can select for resistant bacteria; therefore, their use in all settings must be carefully managed (1). In response to this global public health challenge and growing consumer concerns about food production practices, increasing numbers of food companies are voluntarily limiting the use of antibiotics in their supply chains (2).

For the purpose of this study, and consistent with other established definitions [see for instance (3, 4)], alternatives to antibiotics were broadly defined as any substance that can prevent the need for or be substituted for antimicrobial drugs. This includes a wide variety of substances including microbial-derived products (e.g., probiotics, bacteriophages, and bacteriophage-derived products), phytochemicals (e.g., essential oils), immune-derived products (e.g., antimicrobial peptides, immunomodulators), vaccines, enzymes, metals, minerals, and innovative animal drugs.

[1]Note that we use the term antibiotic in this paper to be consistent with common industry language and to avoid confusion with those alternatives that are antimicrobials; however, it is important to note that the terms often are used interchangeably, though technically antibiotics are a subset of antimicrobials.
[2]https://www.cdc.gov/antibiotic-use/stewardship-report/hospital.html

While many of the currently available alternatives enhance animal health and thus reduce the need for antibiotics, they cannot fully replace them. The need for effective alternatives that can more predictably prevent, control or treat disease has remained largely unmet (5)[3].

Public and private sector funding for research on antibiotic alternatives in animal agriculture is scarce (6). Prioritization is needed to ensure limited resources are dedicated to the most promising and impactful research areas and potential candidates (6). Ideally, the success or failure of an antibiotic alternative would be predictable early during the research and development (R&D) process. However, products may fail at many stages, including after they are fully commercialized. A framework to evaluate antibiotic alternatives early in R&D and enable the consistent and transparent prioritization of investments is sorely needed. This manuscript summarizes the outcomes of an expert workshop organized to address this need.

WORKSHOP GOALS AND OBJECTIVES

A pre-workshop survey of over 40 experts in animal agriculture identified antibiotic alternatives as the top research priority related to antibiotic stewardship, prompting its selection as the workshop topic (details available upon request). For the purposes of the workshop, antibiotic alternatives were defined broadly as animal feed additives (e.g., phytochemicals, pre- and pro-biotics, organic acids) as well as animal drugs (e.g., immune modulators) and veterinary biologics (e.g., novel vaccines, antibodies) that prevent, control, or treat infectious diseases. In contrast, management practices such as nutritional or backgrounding strategies, improvements in housing, or more stringent biosecurity were excluded from the discussion.

For the workshop, a panel of 23 subject matter experts from academia, industry, governmental, and non-governmental organizations convened for a 1 day in-person meeting in December 2018. The goal was to identify strategic priorities for funding research and development on antibiotic alternatives in animal agriculture. The workshop explored factors critical to the success or failure of new antibiotic alternatives and identified associated data gaps and research needs that, if addressed, could help grow the pipeline of safe and effective product candidates. The workshop consisted of two facilitated discussions to reach consensus on key factors important in the evaluation of research approaches and funding decision-making for antibiotic alternatives. This manuscript highlights key themes that emerged during the workshop and, in certain instances, develops them further. All workshop participants were given an opportunity to review this manuscript before publication.

[3]https://www.ars.usda.gov/alternativestoantibiotics/Symposium2016/ATAWorkshop2016.html

PREDICTING SUCCESS OR FAILURE OF AN ANTIBIOTIC ALTERNATIVE

Successful antibiotic alternatives solve a substantial real-world infectious disease problem and provide an economic and animal health benefit. An entity or entities must be willing to invest in scientific research to bring the product to market, and someone must be willing to purchase and use it. However, that alone is not sufficient for successful adoption. The ability of farmers and veterinarians to use the product relies on additional factors such as the logistics of delivery and storage and whether the product aligns with their own and their customers' values and expectations. Ultimately, many factors influence whether an alternative is successful. **Table 1** provides a framework for evaluating the potential success of an antibiotic alternative candidate, starting with an assessment of overall economic viability, followed by a more in-depth assessment of specific risks to product success.

Assessing the Economic Viability of the Project

Profitability is foundational to the success of an antibiotic alternative; farmers and veterinarians cannot adopt economically unsustainable products. Similarly, without a viable business model, investors and pharmaceutical companies are unlikely to provide sufficient funding to bring the concept to market. Economic viability is therefore the first framework criterion, although it can be difficult to predict. For instance, in 2018, <2 years after gaining FDA approval, the animal pharmaceutical company that developed Imrestor®, an antibiotic alternative addressing mastitis in dairy cattle, decided to suspend its commercialization (7, 8).

To determine economic viability, animal health companies and investors evaluate the potential product's expected revenue and probability of success, compared to anticipated costs and risks (9). These evaluations usually take a global perspective, and factor in relevant national and regional policies, such as current or likely future antibiotic use restrictions and the broader regulatory landscape.

Expected Project Costs

The initial discovery and development of a new animal health product typically incurs substantial costs, as outlined in **Table 1**. Product manufacturing, service, distribution, disposal and extensions to new species or indications can constitute substantial additional costs which may be challenging to predict during initial development stages (9). Uncertainty in the predicted project cost and associated risks, including the probability of regulatory success or public acceptance, will also discourage investment.

Expected Product Revenue

To predict product revenue, investors analyze both the market and the product's expected performance in it. The predicted market size for an antibiotic alternative ultimately depends on the number of farms and animals affected by the disease and on how likely the producer or veterinarian is to proactively take

TABLE 1 | Framework for evaluating the success of an antibiotic alternative.

Framework	Check-list items
1. Overall economic viability	
a. Expected project costs	- Product development • Research and Development (R&D) costs • Probability of regulatory approval success • Other feasibility considerations (e.g., Intellectual Property, manufacturability, existing data & models) - Product manufacturing and sales • Cost of materials • Sales and distribution, etc.
b. Expected product revenue	
i Market predictions	- Market size • Number of farms affected & geographic distribution • Disease incidence/prevalence on affected farms • Probability of treating affected animals • Short term economic/animal health impacts • Long-term impacts on animal productivity • Other related impacts (e.g., trade restrictions) - Market characteristics • Market accessibility • Industry structure • Global regulatory landscape • Existing market segmentation • Predicted market growth
ii Product-specific predictions	- Expected return on investment (ROI) for livestock producer/veterinarian • Animal health & productivity improvements • Number needed to treat (NNT) to impact one animal vs. number needed to harm (one animal, or person in case of public health) • Other benefits (e.g., enhanced market access) - Product competitiveness compared to substitutes
2. Specific project risks	
a. Product safety	- Food safety - Target animal safety - Microbial safety - Environmental safety
b. Product efficacy	- Effect type and size - Consistency under real-world conditions - Fitness for purpose
c. Product acceptability	
iii Farmers and veterinarians	- Product perception/mechanism of action - Attitudes, beliefs, perceived behavioral constraints - Trust in the product's consistent efficacy - Product performance relative to expectations
iv Society/consumers	- Consumer acceptance - Ease of explanation
d. Product practicality/ease of use	- Compatibility with current production practices - Administration mode (route, frequency, etc.) - Associated costs (e.g., labor costs, withdrawal times)

steps to address it through prevention, control, or treatment. Economic factors play a role here as well, including short term disease impacts as well as long-term consequences on animal health and productivity (10, 11). Mastitis in dairy cattle, for instance, persistently decreases milk yields in subsequent lactations (12). Transboundary infectious animal diseases can also inflict additional economic costs, for instance through trade restrictions and loss of export markets. In contrast, some animal diseases are controlled most effectively through culling, and in certain cases the animal health benefits associated with an intervention may not outweigh the costs. Given the considerable R&D costs, to be economically viable, antibiotic alternatives for food producing species must address relatively common health problems (i.e., endemic infectious animal diseases) that have substantial economic and animal health impacts.

Other market characteristics, such as segmentation of the existing market and predicted market growth, factor into the revenue calculation as well. In addition, market access may be greater in more highly integrated industries and for products with more internationally harmonized regulatory requirements.

Product specific considerations include the expected return on investment (ROI) for the livestock producer, and the competitiveness of the product compared to alternatives. As outlined in **Table 1**, several factors impact the ROI, making it potentially challenging to predict (9, 13, 14). Product competitiveness refers to the availability of "substitutes"—interventions that address the same health issue. Particular attention is given to less expensive, easier to administer or more effective substitutes, which often include existing antibiotics (5). Expectation of equal or superior performance for alternatives compared to existing antibiotics may be unrealistic. However, increasing regulatory and market-based restrictions on antibiotic use may render even less-effective alternatives highly competitive. Increasing bacterial resistance to antibiotics among target pathogens may further reduce the efficacy of currently available antibiotics (15–17). Currently, few signs point to this phenomenon as an important driver of demand for antibiotic alternatives.

Evaluating Project Risks

Even if these initial economic considerations are favorable, an antibiotic alternative candidate may fail for many reasons.

Product Safety

Product safety (see **Table 1**) is a prerequisite for the success of an antibiotic alternative and integral to the regulatory approval process but may be challenging to predict early in development. *In vitro* and *in silico* models have been developed to help assess the pharmacokinetics and predict the safety of veterinary drugs (18). The applicability of these models to antibiotic alternatives depends on the type of product, and can be influenced by the mechanism of action, host immune response, and potential for off-target effects. Ultimately, well-designed *in vivo* studies are critical for assuring end-users, regulators, and the public that a product is safe for animals, humans and the environment.

Product Efficacy

Antibiotic alternatives that do not meet customer expectations for efficacy in effect type—prevention, control and/or treatment,—as well as the magnitude and consistency of

the effect, are unlikely to be successful. The mechanisms by which alternatives exert their effects are diverse: for instance, they may enhance host immunity, induce cytotoxic effects in pathogenic organisms, block proteins that mediate cell entry or virulence through passive immunization, promote gut health, exert anti-inflammatory properties, or modulate microbial communities in the gut (19–27). In general, the magnitude of the effect is lower for alternatives compared to antibiotics, and tends to be more variable across settings (5). Clarifying customer expectations around some minimum threshold for efficacy (for instance, compared to antibiotics) for the alternative product candidate may prove useful.

Predicting product efficacy early in R&D can be challenging. *In vitro* data are often used to predict efficacy because they are easier to collect and do not require the larger investments needed for *in vivo* studies (28–30). However, predictions based on these data are less reliable than *in vivo* studies, which better capture genetic differences between animals and variations in host-pathogen interactions and environment. Key design questions for studies of *in vivo* efficacy include whether diseases are experimentally introduced in healthy animals (i.e., challenge studies) or else the rates of natural disease occurrence are observed, and whether animals are managed under real world conditions (i.e., experimental vs. field trials). More tightly-controlled studies—such as those experimentally infecting a small number of healthy, genetically homogenous animals with one pathogen strain at one point in time, can use smaller experimental group sizes for statistical significance than less closely controlled studies, but they often do not adequately capture population-level variations that can impact efficacy. For instance, the experimental animals may be more uniform with regard to factors such as age, breed, health status, management, and disease history than animals in commercial settings (31). Study complexity and cost also limit the ability to evaluate efficacy under different animal housing and management practices.

For many antibiotic alternatives, conclusive data from large, well-controlled *in vivo* studies are scarce—an issue that is compounded by lack of information regarding the products' mechanism of action (22, 32–34). Potential interactions across alternatives and efficacy under varying management and husbandry practices have also remained largely unexplored (5). In the swine industry, for instance, a range of alternatives have been studied with mixed results, yet a systematic assessment of this body of research and a definitive conclusion of overall impact on swine health remains a major need (35, 36). When evaluating efficacy, it is important to recognize that many antibiotic alternatives stimulate host immunity broadly, or else alter the microbial environment to be less conducive to pathogen adhesion or propagation, rather than directly kill pathogens or inhibit their growth.

Product Acceptability

The acceptability of new alternatives by farmers and veterinarians, who often have vast experience using antibiotics, is also key to success. Studies have shown that many farmers and veterinarians are skeptical about the efficacy of antibiotic alternatives (37–39). Behavioral and socio-economic factors such as prior experience and risk avoidance clearly impact decision-making regarding the use of antibiotics or alternatives (40–42). Behavioral studies related to the use of antibiotics and other medications in human health care and animal agriculture have identified attitudes toward the product, belief in its value, and perceptions of behavioral constraints such as economics, risk, trust in others, social norms (i.e., expectations of others) and moral obligation to treat animals under one's care as core behavioral drivers (40–43). Building trust in a new product usually requires, at minimum, evidence of clear and consistent product efficacy under field conditions. Independent third-party verification, for instance as part of a data clearing-house or a trial registry, could help address concerns about data dredging and cherry-picking of efficacy trials, although it is unlikely to solve all the underlying challenges and concerns.

The success of an alternative also requires that the animal products derived using the alternative are acceptable to consumers. Generally speaking, the biological function of an alternative should be easy to explain to a layperson and must align with consumers' beliefs and expectations concerning food production and their conceptualizations of risk and adulteration. Alternatives may be preferable over antibiotics to some consumers, to the extent that they alleviate concerns regarding their use (44). In fact, some consumers perceive foods derived from animals raised without antibiotics as more healthful or nutritious (45). Ultimately, consumer acceptance of new technologies is often highly context-specific and affected by a variety of factors including moral, social, political, economic, and religious values as well as geographical, ecological, and animal welfare concerns (46).

Product Practicality and Ease of Use

The widespread adoption of an antibiotic alternative requires that they be practical to use for farmers and veterinarians. This means any such product must be integrated into current production practices without causing major disruptions. Products that require disruptive shifts in the infrastructure or systems under which livestock commodities are raised are unlikely to succeed, at least in the shorter- to medium-term. In addition, products that are not readily compatible with current animal production practices—for instance, because of their application frequency, mode of administration, stability or timing of use—may face obstacles to adoption. Side-effects of the product or the stress associated with handling an animal to apply the product may also reduce adoption. As with antibiotics, farmers may need to observe specific withdrawal times that may limit their ability to market animal-derived products.

DISCUSSION

Antibiotic alternatives represent a major unmet need for the livestock sector. However, the factors predicting their success or failure are complex. Here, we outline a framework for the evaluation of alternative candidates that may empower federal agencies, philanthropic organizations, and other key stakeholders to consistently and transparently

prioritize investments in antibiotic alternatives. Our framework first considers the overall costs and benefits related to the new alternative, because economic viability is foundational to ultimate commercial success and this information may be readily available prior to or early during R&D.

Ultimately, bringing an alternative to market is an extremely complex process, involving evaluation of product safety, efficacy, acceptability and practicality. Therefore, the potential success of a new alternative may be best evaluated from multiple perspectives, an approach that we replicated in our original survey and workshop design and encourage in the evaluation of alternatives. Research funders may, for instance, start to involve farmers, veterinarians and farm advisors more closely in early funding decisions. Developing new antibiotic alternatives is a challenging issue but holds considerable promise for animal health and the fight to combat antibiotic resistance. This framework will empower research funders to evaluate alternatives early during R&D, and to dedicate scarce funding to the most promising alternatives.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this manuscript will be made available by the authors, without undue reservation, to any qualified researcher.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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Assignment of Canadian Defined Daily Doses and Canadian Defined Course Doses for Quantification of Antimicrobial Usage in Cattle

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Standardized units are essential to allow quantification and comparison of antimicrobial usage (AMU) between species and regions. In Canada, defined daily and course doses have not yet been harmonized for cattle. Our objective was to assign defined daily and course doses (named DDDbovCA and DCDbovCA, respectively) for cattle in Canada, by antimicrobial agent (AM) and by route of administration, based on the label of all products containing at least one AM, marketed and authorized in Canada for use in cattle. In April and December 2019, a systematic search was performed from the online Drug Product Database (DPD) of Health Canada to identify veterinary products containing at least one AM, marketed in Canada for use in cattle. Products were divided by route of administration (intramammary, intrauterine, injectable, oral, and topical). The monograph was retrieved for each product from the DPD, or from the Canadian Edition of the Compendium of Veterinary Products (CVP), and read completely to extract recommended dosages in cattle. Standard weights were applied to compute doses if required. DDDbovCA and DCDbovCA were assigned by calculating an average of daily and course doses, respectively, by AM and route of administration. Two products were excluded from calculations because of their claim as growth promotion or feed efficiency (no longer authorized in Canada for certain categories of AM). Overall, 39 injectable, 75 oral (including 23 medicated premixes), 8 intramammary (4 for lactating cows and 4 for dry cows), 5 intrauterine, and 4 topical products were used for calculations. DDDbovCA and DCDbovCA values were assigned successfully for each AM identified, by route of administration. These metrics will allow harmonized and transparent quantification of AMU in cattle in Canada.

Keywords: antimicrobial usage, antibiotic usage, animal infection, cattle, metrics, DDDbovCA, DCDbovCA, quantification

INTRODUCTION

With increasing interest in evaluation of the impact of antimicrobial usage (AMU) on antimicrobial resistance, international health organizations have highlighted the importance to monitor AMU in human and veterinary medicine, as well as in agriculture (1–3). Since, the early 2000s, countries have reported their AMU for animals (4–8). At the same time, problems of comparability between methods of quantification and between units of measurement were raised (9–12). Nowadays, standardization of indicators is targeted by public health authorities (13–15).

Quantities of antimicrobial agents (AMs) used can be reported in net mass or in number of standard doses per standardized biomass (16) or per animal or group of animals (17). To account for differences in potency and molecular weight between different AMs, standard doses are often preferred over net masses to report quantities. Different standard doses have been proposed: defined doses (18), used (or actual) doses (19), and prescribed doses (20). Cow Calculated Course is a recent metric conceived in the United Kingdom that stratifies AMU for young cattle (long-acting injectable and oral products) and adult cattle (intramammary and short-acting injectable products) by assuming certain products are only used in certain age groups (21). The use of one standard dose instead of another depends on the source of data collection and the aim of the report on AMU.

In this context, the European Medicines Agency (EMA), through the European Surveillance of Veterinary Antimicrobial Consumption (ESVAC) project, assigned defined daily and course doses for animals (DDDvet and DCDvet), by food-producing species (cattle, swine, poultry), route of administration (parenteral, oral, intramammary for lactating cows, intramammary for dry cows, intrauterine), and AM or combination of AMs (22). They followed principles already established by the World Health Organization (WHO) for assignment of Defined Daily Doses (DDD) for human medicines (23). Canadian defined daily doses for animals (DDDvetCAs) have recently been defined for poultry (broiler chickens and turkeys) and pigs (24). Defined doses have also been used for reporting on Canadian AMU in dairy cattle (25, 26) and in beef cattle (27), but are not harmonized between authors.

The objective of this research was, therefore, to assign defined daily doses (named DDDbovCA) and defined course doses (named DCDbovCA) for cattle in Canada, based on the labeled doses of all products containing at least one AM, that are marketed and authorized for use in cattle in Canada. Specifically, the aim of this work was to assign DDDbovCA and DCDbovCA values by AM and by route of administration, in order to quantify in a transparent way AMU in cattle in Canada.

MATERIALS AND METHODS

Database Search and Classification of Products by Route of Administration

The complete workflow for proposing DDDs and defined course doses is described in **Figure 1**. Health Canada, the federal institution responsible for regulating drugs to support public safety in Canada, provides an online Drug Product Database (DPD) updated nightly. Products defined as a drug under the Food and Drugs Act are identified by a unique Drug Identification Number (DIN), a computer-generated eight-digit number assigned by Health Canada to a drug product prior to being marketed (28). In April 2019, the DPD was searched by active antimicrobial ingredient to retrieve all products used for cattle containing at least one AM. The search was repeated in December 2019 to note any discrepancies. Only products with a status "marketed" were kept for further steps (this status refers to an active DIN that is currently sold in Canada). Then, for each product, the product monograph was consulted by following the link in the DPD. For products with no monograph available in the DPD, the Canadian Edition of the Compendium of Veterinary Products (CVP) was consulted (29). Products were separated according to their route of administration: systemic (oral or injectable) or non-systemic (intramammary, intrauterine, topical, or ophthalmic). Oral products were also classified according to their pharmaceutical form because of their diversity: "boluses, capsules, or tablets," "suspensions or solutions," "water soluble powders," and "medicated premixes." Subcutaneous hormonal implants containing an AM included as a local antibacterial for reducing the incidence of abscess formation at the implant site were excluded. Products containing an AM belonging to the Categories I, II, or III according to Health Canada (30), and having only growth promotion or feed efficiency indications were also excluded, as they are no longer marketed in Canada with this claim, since December 2018 (31).

General Rules Applied to Each Product for Extraction of Dosages and Doses From the Product Monograph

The terms "dose" and "dosage" are often used interchangeably. For the current work, though, we used the following definitions: a dosage corresponds to the amount of active substance applied per kilogram of body weight, whereas a dose corresponds to the amount of an active substance administered to a single animal (13). Daily dosages and course dosages were defined for systemically-used AMs, and were expressed in milligrams per kilogram per day and in milligrams per kilogram per course of treatment, respectively. All dosages were rounded to one (dosages $>$ 1 mg/kg) or two (dosages between 0 and 1 mg/kg) decimal place(s). Daily doses and course doses were expressed for systemically-used AMs in grams per animal per day and in grams per animal per course of treatment, respectively, rounded to two decimal places. Daily doses and course doses were expressed for non-systemically used AMs in milligrams per animal per day and in milligrams per animal per course of treatment, respectively, rounded to a whole number.

A combination of AMs in one product was analyzed as if each AM was found individually in different products. Exceptions were applied if the following three criteria were concomitantly encountered: the combination is always synergistic at the specific given ratio found in veterinary products; AND the combination is known to decrease the risk of antimicrobial resistance in comparison with the use of the individual AM; AND the AMs

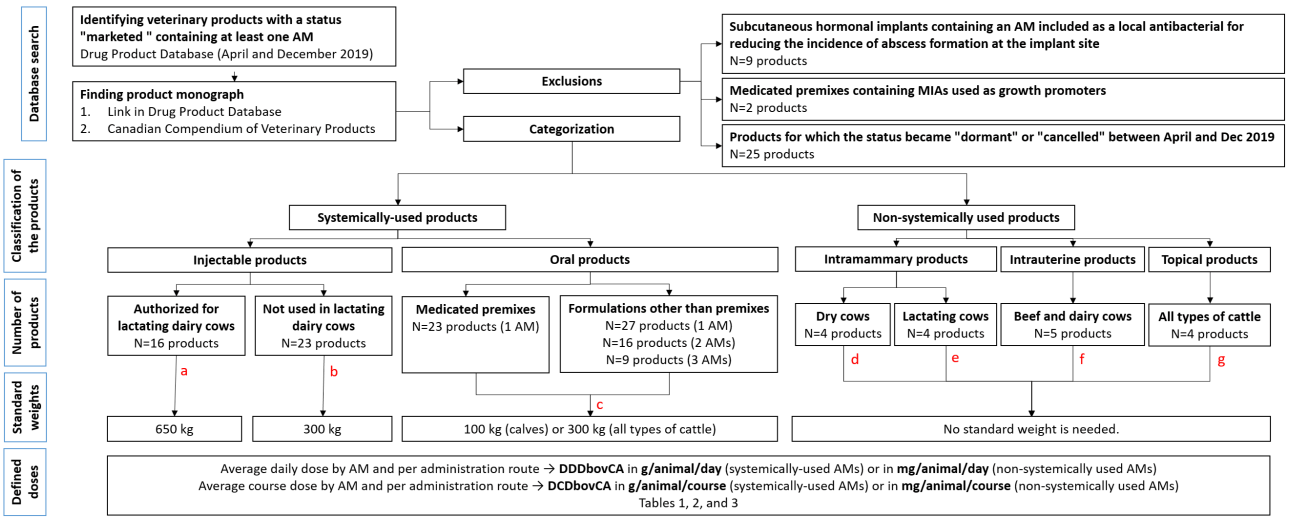

FIGURE 1 | Flow chart illustrating workflow for proposing DDDbovCA and DCDbovCA values for antimicrobial agents used for cattle in Canada. AM(s), Antimicrobial Agent(s); DDDbovCA, Canadian Defined Daily Dose for cattle; DCDbovCA, Canadian Defined Course Dose for cattle; MIA(s), Medically Important Antimicrobial(s) belonging to the Categories I, II, and III according to Health Canada. [a]Injectable products authorized for lactating dairy cows were assigned to a standard weight of 650 kg. [b]Injectable products not used in lactating dairy cows were assigned to a standard weight of 300 kg. [c]Oral products intended for "calves" or "calves up to 136 kg" were assigned to a standard weight of 100 kg. Oral products intended for all types of cattle or for "calves up to 360 kg" were assigned to a standard weight of 300 kg. [d]For intramammary products for dry cows, a complete treatment for a cow was defined as the infusion of four syringes (one per quarter) at drying off regardless of the product. The complete treatment (4 syringes) was assigned to a duration of action of 10 days meaning that the daily treatment was defined as the infusion of 0.4 syringe per cow, or 0.1 syringe per quarter. [e]For intramammary products for lactating cows, it was hypothesized that one quarter is infected (and then treated) at a time by cow. [f]For intrauterine products, when no indication could be found on the product monograph, a duration of action of 24 h was assigned by default. [g]For topical products, we assumed that 1 mL is sprayed per second (for sprays), 5 g of cream is used per application (for creams), or 5 g of powder is used per application (for powders for topical administration).

in the combination are never found alone in products marketed for cattle in Canada.

For long-acting products (i.e., products with duration of action or a duration between two administrations longer than 24 h), the daily dose was determined by dividing the amount of AM in one administration by the number of days between two administrations (for products with repeated administrations) or by the duration of action (in days) specified in the product monograph (for products with a single administration).

When both preventive and treatment dosages were indicated on the label, only the treatment dosage was used.

Conversion factors of 0.00012 and 0.00060 were applied to convert international units to milligrams for polymyxin B and penicillin G, respectively (32, 33). If a prodrug concentration was given in the product monograph, the prodrug was not converted into drug for calculations of dosages or doses, and was reported as such in tables.

Rules Specific to Products Used Systemically (Injectable or Oral Products)

For each product, a daily dosage and a course dosage were obtained from the monograph by AM, in milligrams of AM per kilogram of body weight per day and per course, respectively. To convert dosage to dose, the dosage was multiplied by a standard weight. Two standard weights were used for injectable products: 300 kg for products not authorized for lactating dairy cows, and 650 kg for products authorized for lactating cows. Two standard weights were used for oral products: 100 kg for products intended for "calves" or "calves up to 136 kg," and 300 kg for products intended for all types of cattle or for "calves up to 360 kg." The 650-kg weight for an adult cow was decided according to recent data recording the weight of mature cows in Canada (34). The 100- and 300-kg weights for a calf up to 136 kg and for "a lambda cattle," respectively, were the same weights used previously by Jensen et al. (18). The 100-kg weight represents the average weight between a newborn calf (around 50 kg) and a weaned calf (around 100 and 200 kg for a dairy and a beef calf, respectively). The 300-kg weight represents the average weight between a newborn calf and an adult cattle. It is also assumed to be representative of the average weight of a beef cattle entering a feedlot (200–300 kg for a feeder calf, and around 400 kg for a yearling).

For products with both a single-dose therapy and a multiple-dose therapy (danofloxacin, enrofloxacin, florfenicol), the course dose was determined by performing an average between both provided therapies. The daily dose was determined from the multiple-dose therapy only (duration of action easier to assess with repeated regimen).

For products with only a single-dose therapy, the course dose was equal to the dose provided. The daily dose was determined for beta-lactams by dividing the course dose (in g/animal/course) by the time (in number of days) the plasmatic concentration of the AM exceeds the Minimum Inhibitory Concentration (MIC) for pathogens targeted by the label (information read from

the product monograph), and for tetracyclines by dividing the course dose (in g/animal/course) by the time (in number of days) of sustained antibiotic blood level action. For macrolides and fluoroquinolones (from products with only a single-dose therapy), the daily dose was determined by dividing the course dose by an arbitrary duration of 7 days based on the most likely duration of action for treatment of bovine respiratory diseases (35).

For oral products, only the individual treatment was used when both individual and group treatments were present on the label, because doses were more accurately determined from the individual treatment (less approximations used for calculations). When a loading dose was indicated, followed by several days of treatment at a maintenance dose, a course dose was first calculated, then divided by the number of days of treatment to obtain the daily dose. A daily water intake of 10% of the body weight was used if the dosage was given in quantity of medicated water provided daily to the animal [same approximation used by the ESVAC project, Appendix 4 in European Medicines Agency, European Surveillance of Veterinary Antimicrobial Consumption (36)]. For medicated premixes, arbitrary decisions had to be made to determine daily and course doses: the period of exposition to the medicated feed was fixed to 3 months when no other information was provided on the label. A rounded month of 30 days was used. A standardized animal consuming daily 2% of its body weight (on a 100% dry matter basis) was used to provide estimates of dosages, if required [same approximation used by the ESVAC project, Appendix 4 in European Medicines Agency, European Surveillance of Veterinary Antimicrobial Consumption (36)].

Rules Specific to Products Used Non-systemically (Intramammary, Intrauterine, and Topical or Ophthalmic Products)

For each product, a daily dose and a course dose were obtained from the monograph by AM, in milligrams of AM per animal per day and per course, respectively. Some arbitrary decisions were made in order to assign a daily dose for every product. For intramammary products designed for lactating cows, it was hypothesized that one quarter at a time is infected (and thus treated) per animal. For intramammary products given at dry-off, the duration of action was set at 10 days. The actual duration of action was difficult to identify. Some data were available on persistence of antibiotic residues in milk: 8–21 days for cloxacillin benzathine (37, 38), 14–28 days for cephapirin benzathine (39), and 9 days for benzylpenicillin procaine (38), but no information was available on the time the antibiotics stay effective after drying-off at levels equal or higher than the MIC against the pathogens involved. Furthermore, the persistence of an antibiotic in the udder is affected by factors inherent to the product (such as the solubility of the antibiotic salt, the quantity of antibiotic infused, and the base in which it is formulated) (37) and, likely, by factors inherent to the cow (such as production at the time of drying-off, leaking milk after drying-off, chronic intramammary infection).

For intrauterine products, a duration of action of 24 h was assigned if no information was retrieved from the monograph. Finally, a duration of treatment of 5 days was hypothesized for topical products. These decisions were arbitrary made (but in agreement with the very scarce literature on this topic) in order to avoid missing values in assignment of defined doses. For topical products, it was assumed that 1 mL is sprayed per second as proposed by Postma et al. (40), and that 5 g of powder or of cream are applied on a wound per treatment.

Assignment of Defined Daily and Course Doses

When different products containing the same AM had different labeled doses, an average dose of the unique doses was calculated by route of administration. Antimicrobial agents from combinations were assigned different values than AMs found alone in products because the dose of a given AM is often lower when combined in a product than in a product where it is found alone. For oral AMs, a distinction was made between AMs originating from medicated premixes and AMs originating from other pharmaceutical forms. For oral AMs, an average dose was first calculated by type of formulation (an average for boluses, for suspensions and solutions, and for water soluble powders, respectively), then an overall average was calculated (each type of oral formulation represented one "weight" in the global average).

The average daily dosages and doses were called Defined Daily Dosages for cattle in Canada (dddbovCA, in mg/kg of body weight per day) and Defined Daily Doses for cattle in Canada (DDDbovCA, in mg (or g)/animal per day), respectively. The average course dosages and doses were called Defined Course Dosages for cattle in Canada (dcdbovCA, in mg/kg of body weight per course) and Defined Course Doses for cattle in Canada (DCDbovCA, in mg (or g)/animal per course).

Other Information Reported by AM and Route of Administration

The code in the Anatomical Therapeutic Chemical classification for veterinary medicinal products (ATCvet code) was searched in the ATCvet Index 2019 (41), and reported by product. The antimicrobial class and the category of the AM based on its importance in human medicine as defined by Health Canada (30) were also recorded for each AM. Four categories were described: category (I) AMs of "very high importance" (preferred option for treatment of serious human infections, without or with limited availability of alternative AMs); category (II) AMs of "high importance" (preferred option for treatment of serious human infections, but alternative AMs are available); category (III) AMs of "medium importance" (not the preferred option for treatment of serious human infections); and category (IV) AMs of "low importance" (AMs currently not used in human medicine).

RESULTS

Between April and December 2019, the status of 17 products (5 injectable products, 1 oral or intrauterine bolus, 7 water soluble powders, and 4 medicated premixes) changed from

"marketed" to "dormant" in the DPD. The status of 8 products (4 injectable products, 2 medicated premixes, and 2 topical sprays) changed from "marketed" to "canceled post market." These 25 products (**Supplementary 1.1**) were not used in the assignment of defined doses as they were not sold on December 2019 in Canada. No ophthalmic product was found with an indication for cattle. Eleven products (**Supplementary 1.2**) were excluded from calculations (9 subcutaneous implants containing oxytetracycline or tylosin, 1 medicated premix containing chlortetracycline and sulfamethazine and with only a growth promotion indication, and 1 medicated premix containing oxytetracycline and neomycin also with only a growth promotion indication).

A total of 131 products were retained for assignment of defined daily and course doses. For 101 and 30 products, the monograph was extracted from the DPD (Health Canada) and from the CVP, respectively. The last update of the monograph was <2 years for the DPD version, but generally was not indicated for the CVP version. The only combination of AMs that met the three criteria to be considered as one entity was the trimethoprim and sulfadoxine combination. This synergistic combination (42) was found in three injectable products at the fixed ratio of 1–5 (40 mg of trimethoprim and 200 mg of sulfadoxine per mL of injectable solution). Both trimethoprim and sulfadoxine were not found non-combined in any marketed products.

Injectable Route

Thirty-nine injectable products were identified (detailed in **Supplementary 2.1, 2.2**). Average calculations by AM are detailed in **Supplementary 2.3**, and the summary is presented in **Table 1**. Antimicrobial agents found in injectable products were: ampicillin, benzylpenicillin (benzathine or procaine), ceftiofur, danofloxacin, enrofloxacin, florfenicol, gamithromycin, marbofloxacin, oxytetracycline, tildipirosin, tilmicosin, tulathromycin, tylosin, and the combination of sulfadoxine and trimethoprim. The combination of benzathine benzylpenicillin and procaine benzylpenicillin was found in one long-acting product at the fixed ratio of 1 for 1 (150,000 international units per mL for both salts). Procaine benzylpenicillin was also found alone in six other products. Benzathine and procaine benzylpenicillin are two prodrugs

TABLE 1 | Assignment of DDDbovCA and DCDbovCA values for antimicrobial agents used systemically through the injectable route for cattle in Canada.

Antimicrobial agent	Combined with another antimicrobial agent in products?	Antimicrobial class	Category according to Health Canada	dddbovCA (mg per kg per day)	dcdbovCA (mg per kg per course)	DDDbovCA (g per animal per day)	DCDbovCA (g per animal per course)
Ampicillin	No	Penicillins with extended spectrum	II	6.0	30.0	3.90	19.50
Benzylpenicillin (Penicillin G) Benzathine	Combined with benzylpenicillin procaine	Beta-lactamase sensitive penicillins	II	0.9	5.4	0.27	1.62
Benzylpenicillin (Penicillin G) Procaine	Combined with benzylpenicillin benzathine	Beta-lactamase sensitive penicillins	II	0.9	5.4	0.27	1.62
Benzylpenicillin (Penicillin G) Procaine	No	Beta-lactamase sensitive penicillins	II	8.8	40.2	4.96	20.88
Ceftiofur	No	Third-generation cephalosporins	I	1.2	6.0	0.80	3.92
Danofloxacin	No	Fluoroquinolones	I	3.0	10.0	0.90	3.00
Enrofloxacin	No	Fluoroquinolones	I	3.8	12.5	1.14	3.75
Florfenicol	No	Amphenicols	III	10.0	40.0	3.00	12.00
Gamithromycin	No	Macrolides	II	0.86	6.0	0.26	1.80
Marbofloxacin	No	Fluoroquinolones	I	1.4	10.0	0.43	3.00
Oxytetracycline	No	Tetracyclines	III	5.9	18.4	2.62	7.29
Tildipirosin	No	Macrolides	II	0.57	4.0	0.17	1.20
Tilmicosin	No	Macrolides	II	1.4	10.0	0.43	3.00
Trimethoprim and sulfadoxine combination	No	Combinations of sulfonamides and trimethoprim	II	16.0	64.0	10.40	41.60
Tulathromycin	No	Macrolides	II	0.36	2.5	0.11	0.75
Tylosin	No	Macrolides	II	17.6	70.4	5.28	21.12

dddbovCA, Canadian Defined Daily Dosage for cattle (in mg/kg/day); DDDbovCA, Canadian Defined Daily Dose for cattle (in g/animal/day); dcdbovCA, Canadian Defined Course Dosage for cattle (in mg/kg/course); DCDbovCA, Canadian Defined Course Dose for cattle (in g/animal/course).

of benzylpenicillin not known to be synergistic; they were assigned separate defined doses. Products with both a single-dose therapy and a multiple-dose therapy contained danofloxacin ($n = 1$), enrofloxacin ($n = 1$), or florfenicol ($n = 3$). Products with a single-dose therapy only contained ceftiofur crystalline free acid ($n = 1$), gamithromycin ($n = 1$), marbofloxacin ($n = 1$), oxytetracycline dihydrate ($n = 7$), tildipirosin ($n = 1$), tilmicosin ($n = 3$), or tulathromycin ($n = 1$). For the products containing ceftiofur or oxytetracycline, the duration of effective concentration of the AM in plasma after administration was used to calculate a daily dose. For the products containing gamithromycin, marbofloxacin, tildipirosin, tilmicosin, or tulathromycin, the daily dose was estimated using the 7-day arbitrary duration of action. Antimicrobial agents identified in products authorized for lactating dairy cows as well as in products not for use in lactating dairy cows were: procaine benzylpenicillin, and oxytetracycline.

Oral Route

Fifty-two oral products other than medicated premixes were identified. Different types of formulations were available: boluses or tablets (12 products), suspensions or solutions (9 products), and water soluble powders (31 products). Twenty-seven, sixteen, and nine products contained one, two, and three AMs, respectively (detailed in **Supplementary 3.1, 4.1, 4.2**). Antimicrobial agents that could be found alone or in combination were neomycin, oxytetracycline, sulfamethazine, sulfapyridine, and tetracycline. Antimicrobial agents that were always found in combination in products were benzylpenicillin, streptomycin, succinylsulfathiazole, sulfaguanidine, sulfamerazine, sulfanilamide, and sulfathiazole. Sulfonamide-based products were numerous, and dosages varied widely from one product to another, depending on the type of sulfonamide, and the type of formulation. Aminoglycosides were reported under their sulfate form, and were kept as neomycin sulfate and streptomycin sulfate in calculations. Average calculations by AM are detailed in **Supplementary 3.2** (AMs used non-combined) and **Supplementary 4.3** (AMs used combined).

Twenty-three medicated premixes (detailed in **Supplementary 3.3**) were used for calculations and contained either ionophores (lasalocid, monensin, salinomycin), tetracyclines (chlortetracycline, oxytetracycline), or macrolides (tilmicosin, tylosin). No combination was identified. Only two products containing tilmicosin were designed for metaphylaxis purposes (reduction of morbidity in groups of feedlot beef cattle experiencing an outbreak of bovine respiratory disease). Other premixes were indicated for the prevention of diseases: foot rot (chlortetracycline), bacterial enteritis (chlortetracycline, oxytetracycline), liver abscesses (tylosin), and coccidiosis (lasalocid, monensin), or for growth promotion and feed efficiency (lasalocid, monensin, salinomycin).

The summary of defined daily and course dosages and doses for AMs used systemically through the oral route is presented in **Table 2**.

Intramammary, Intrauterine, and Topical Routes

Eight intramammary products (four for lactating cows, and four for dry cows) were authorized for use in Canada, all sold as 10-mL disposable single-use syringes (detailed in **Supplementary 5.1**). Three of the products indicated for lactating cows contained a single AM each (cefapirin, ceftiofur, or pirlimycin), and one product contained four AMs (procaine benzylpenicillin, dihydrostreptomycin, novobiocin, and polymyxin B). Three of the products indicated for dry cow therapy contained one AM each (cefapirin, ceftiofur, or cloxacillin), and one contained two AMs (procaine benzylpenicillin and novobiocin).

Five intrauterine products were authorized for use in Canada, marketed under different pharmaceutical formulations: disposable single-use syringes, injectable solutions, stable suspensions, and boluses (detailed in **Supplementary 5.2**). Three of them contained one AM each (cefapirin, gentamicin, or oxytetracycline). Two products contained a combination of two sulfonamides (sulfanilamide and sulfathiazole).

Four topical products were marketed in Canada for cattle (Detailed in **Supplementary 5.3**). Antimicrobial agents found in these products were: chlortetracycline, or a combination of two sulfonamides (sulfanilamide and sulfathiazole). Different formulations were available: sprays, creams, or powders.

The summary of defined daily and course doses for AMs used non-systemically is presented in **Table 3**.

DISCUSSION

Assignment of defined daily and course doses by species is an essential part of the evaluation of AMU. The main benefit of using dose-based metrics for AMU quantification is the ability to compare between different AMs, species, and regions, as it is the only metric that accounts for dose differences (and then for differences in animal weights). We used a reproducible method to assign DDDbovCA and DCDbovCA values for all AM currently used for cattle in Canada. This method will allow easy updates in the future to include new products in the calculations, or remove products that are no more sold.

DDDs were first described by the WHO in the seventies (43), and were aimed at providing an international measure system to quantify active substances found in human medicines. The WHO Collaborating Center for Drug Statistics Methodology updates annually their guidelines for DDD assignment (23). The DDDs are not intended to correspond perfectly to each regional specific usage of AMs, but with an internationally accepted metric, comparisons of AMU between regions in the world are easily performed.

A larger amount of long-acting veterinary medicines are available in comparison with human medicines. This observation explains the emergence of another unit for veterinary products: the defined course dose, first developed by the French Agency for Food, Environmental and Occupational Health & Safety (44) as ACD (Animal Course Dose), and adapted by the EMA in the ESVAC project as DCDvet (Defined Course Dose for Animals). The DDDvet (Defined Daily Dose for animals) and DCDvet values were assigned in 2016 (22) based on doses from

TABLE 2 | Assignment of DDDbovCA and DCDbovCA values for antimicrobial agents used systemically through the oral route for cattle in Canada.

Antimicrobial agent	Combined with another antimicrobial agent in products?	Antimicrobial class	Category according to Health Canada	dddbovCA (mg per kg per day)	dcdbovCA (mg per kg per course)	DDDbovCA (g per animal per day)	DCDbovCA (g per animal per course)
Antimicrobial agents used in oral products other than medicated premixes							
Benzylpenicillin (Penicillin G)	Combined with streptomycin	Beta-lactamase sensitive penicillins	II	5.3	13.2	0.53	1.32
Monensin	No	Ionophores	IV	0.52	49.8	0.34	32.40
Neomycin sulfate	No	Aminoglycosides	II	16.3	48.8	1.63	4.88
Neomycin sulfate	Combined with sulfonamides or tetracyclines	Aminoglycosides	II	14.1	51.1	1.41	5.11
Oxytetracycline	No	Tetracyclines	III	12.3	48.3	2.43	10.23
Oxytetracycline	Combined with neomycin	Tetracyclines	III	12.7	57.0	1.27	5.70
Streptomycin sulfate	Combined with benzylpenicillin	Aminoglycosides	II	27.3	68.1	2.73	6.81
Succinylsulfathiazole	Combined with neomycin	Sulfonamides	III	57.6	144.0	5.76	14.40
Sulfaguanidine	Combined with neomycin and sulfathiazole	Sulfonamides	III	29.8	104.2	2.98	10.42
Sulfamerazine	Combined with sulfonamides	Sulfonamides	III	4.5	27.7	1.35	8.31
Sulfamethazine (Sulfadimidine)	No	Sulfonamides	III	101.6	406.1	30.47	121.85
Sulfamethazine (Sulfadimidine)	Combined with neomycin or sulfonamides	Sulfonamides	III	59.0	195.8	13.57	44.35
Sulfanilamide	Combined with sulfonamides	Sulfonamides	III	91.4	91.4	27.42	27.42
Sulfapyridine	No	Sulfonamides	III	179.2	537.5	53.76	161.25
Sulfapyridine	Combined with sulfonamides	Sulfonamides	III	24.8	99.1	7.44	29.73
Sulfathiazole	Combined with neomycin and sulfaguanidine, or sulfonamides	Sulfonamides	III	44.2	142.6	12.67	40.69
Tetracycline	No	Sulfonamides	III	10.4	60.0	1.04	6.00
Tetracycline	Combined with neomycin	Tetracyclines	III	13.3	60.0	1.33	6.00
Antimicrobial agents used in medicated premixes							
Chlortetracycline	No	Tetracyclines	III	0.66	59.4	0.09	8.10
Lasalocid	No	Ionophores	IV	0.89	79.8	0.27	23.94
Monensin	No	Ionophores	IV	0.53	47.5	0.21	18.77
Oxytetracycline	No	Tetracyclines	III	1.1	99.0	0.09	8.33
Salinomycin	No	Ionophores	IV	0.33	30.0	0.10	9.00
Tilmicosin	No	Macrolides	II	12.5	175.0	3.75	52.50
Tylosin	No	Macrolides	II	0.22	19.8	0.07	5.94

*dddbovCA, Canadian Defined Daily Dosage for cattle (in mg/kg/day); DDDbovCA, Canadian Defined Daily Dose for cattle (in g/animal/day); dcdbovCA, Canadian Defined Course Dosage for cattle (in mg/kg/course); DCDbovCA, Canadian Defined Course Dose for cattle (in g/animal/course). Dosages and doses were not determined for combined chlortetracycline and sulfamethazine (1 premix), and for combined neomycin sulfate and oxytetracycline (1 premix) (see **Supplementary 1.2**).*

nine European countries for cattle, poultry, and swine, and are now used for comparison of AMU in Europe (8). Applying these values to Canadian AMU data, however, is very difficult because of notable differences between Europe and Canada, both in the types of AMs used, and in the doses they are used at.

TABLE 3 | Assignment of DDDbovCA and DCDbovCA values for antimicrobial agents used non-systemically through the intramammary, intrauterine, and topical routes for cattle in Canada.

Antimicrobial agent	Combined with another antimicrobial agent in products?	Antimicrobial class	Category according to Health Canada	DDDbovCA (mg per animal per day)	DCDbovCA (mg per animal per course)
Antimicrobial agents used through the intramammary route in cows during the lactation					
Benzylpenicillin (Penicillin G) Procaine	Combined with dihydrostreptomycin, novobiocin, and polymyxin B sulfate	Beta-lactamase sensitive penicillins	II	60	90
Cefapirin	No	First-generation cephalosporins	II	400	400
Ceftiofur	No	Third-generation cephalosporins	I	125	250
Dihydrostreptomycin	Combined with benzylpenicillin procaine, novobiocin, and polymyxin B sulfate	Aminoglycosides	II	100	150
Novobiocin	Combined with benzylpenicillin procaine, dihydrostreptomycin, and polymyxin B sulfate	Aminocoumarins	Not categorized	150	225
Pirlimycin	No	Lincosamides	II	50	250
Polymyxin B sulfate	Combined with benzylpenicillin procaine, dihydrostreptomycin, and novobiocin	Polymyxins	I	6	9
Antimicrobial agents used through the intramammary route in cows at drying-off					
Benzylpenicillin (Penicillin G) procaine	Combined with novobiocin	Beta-lactamase sensitive penicillins	II	48	480
Cefapirin	No	First-generation cephalosporins	II	120	1,200
Ceftiofur	No	Third-generation cephalosporins	I	200	2,000
Cloxacillin	No	Beta-lactamase resistant penicillins	II	200	2,000
Novobiocin	Combined with benzylpenicillin procaine	Aminocoumarins	Not categorized	160	1,600
Antimicrobial agents used through the intrauterine route in cows					
Cefapirin	No	First-generation cephalosporins	II	500	500
Gentamicin sulfate	No	Aminoglycosides	II	200	200
Oxytetracycline	No	Tetracyclines	III	2,500	2,500
Sulfanilamide	Combined with sulfathiazole	Sulfonamides	III	2,880	2,880
Sulfathiazole	Combined with sulfanilamide	Sulfonamides	III	480	480
Antimicrobial agents used through the topical route in cattle					
Chlortetracycline	No	Tetracyclines	III	147	441
Sulfanilamide	Combined with sulfathiazole	Sulfonamides	III	444	2,220
Sulfathiazole	Combined with sulfanilamide	Sulfonamides	III	444	2,220

DDDbovCA, Canadian Defined Daily Dose for cattle (in mg/animal/day); DCDbovCA, Canadian Defined Course Dose for cattle (in mg/animal/course).

This study highlighted differences between Europe and Canada in terms of AMs marketed: 2 (out of 8) intramammary products and 3 (out of 5) intrauterine products available in Canada had no equivalent in Europe. Three (out of 7) AMs from medicated premixes and 7 (out of 13) oral AMs (other than premixes) were sold in Canada but not identified in Europe. The

three AMs identified in Canadian medicated premixes but not in Europe were ionophores (lasalocid, monensin, and salinomycin) that are categorized as antimicrobials by Health Canada. All injectable AMs available in Canada were also listed in ESVAC reports. Moreover, for systemically-used AMs (injectable or oral), the comparison between European and Canadian daily doses showed that 74% of Canadian doses were lower than European doses (relative difference inferior by more than 10%), 11% of doses were relatively similar between Europe and Canada (relative difference between −10% and +10%), and 15% of Canadian doses were greater than European doses (relative difference superior by more than 10%). Because of the lower doses in general for Canada in comparison with Europe, and because the AMs were considered separately when identified in combinations, for an equal weight of AMs, the Canadian measure system will report a higher dose-based AMU (i.e., a higher number of DDDbovCA or DCDbovCA).

Main calculation differences between Europe and Canada concerned the oral products: we did separate medicated premixes from other oral formulations as it was assumed that their usage was really different (mass medication vs. individual treatment, duration of administration, type of cattle targeted by the medication). This was easily performed as most of the AMs found in premixes were different than AMs found in other oral formulations, with the exceptions of monensin and oxytetracycline that were identified in both premixes, and tablets (monensin), or soluble powders (oxytetracycline). Among oral formulations, AMs found in combinations were assigned separate DDDbovCA and DCDbovCA values than non-combined AMs. This latter decision was also different from that of ESVAC which used the same DDDvet and DCDvet values for an AM identified in oral combinations vs. oral single forms (36).

In injectable products, only two combinations were identified: combined trimethoprim and sulfadoxine, and combined procaine benzylpenicillin and benzathine benzylpenicillin. The combined trimethoprim and sulfadoxine in our system was the only combination that was kept undivided in assignment of defined doses. This combination is known to be synergistic because both AMs involve sequential inhibition of successive steps in the folate metabolism. Its usage as a combination is recommended instead of using just the trimethoprim or the sulfadoxine part (42).

Calculations were achieved by making some arbitrary decisions in order to propose defined doses for every AM marketed currently in Canada. The following decisions could be seen as limitations: need to use approximations of standard body weights, average daily intake (food, water), average cattle targeted by the label (beef/dairy, young/adult), and even approximation of the duration of action for long-acting products. Body weights and daily requirements vary depending on the age, sex, production type, and metabolic status of the animal. However, defining approximations was essential to obtain doses for every product. Three standard body weights were defined for systemically-used AMs: 650 kg for injectable products authorized for lactating cows, 300 kg for injectable products not for use in lactating cows and for oral products labeled for all types of cattle, and 100 kg for oral products labeled specifically for calves. The standard weights defined for Canada differ from the weights available from the ESVAC publications [425 kg for an adult cattle, 200 kg for a heifer, and 140 kg for a young cattle; Table A14 in European Medicines Agency, European Surveillance of Veterinary Antimicrobial Consumption (8)]. Nevertheless, in the current article, dosages (mg/kg) and doses (g/animal) were reported for systemically-used AMs. Reporting the total dose per animal is innovative as other publications generally only present dosages (22). Different standard weights could be applied to the dosages presented in this paper in order to obtain another set of doses more relevant for a specific context or to allow more direct comparisons with other countries.

Several durations of action were also defined: 10 days for dry-cow products, 24 h for intrauterine products, and 7 days for long-acting injectable macrolides and fluoroquinolones (when no other information was identified from the product monograph). These latter periods are not intended to be representative of the exact true duration of action for each product. They are approximations and were defined strictly for allowing assignment of daily doses for long-acting products in a transparent way. The ESVAC project did not assign doses for parenteral gamithromycin (daily and course), parenteral tildipirosin (daily and course), and dry-cow products (daily) (22). Without defined doses, these specific products are not quantified in reports using the DDDvet unit. One of our objectives was to propose defined doses for all AMs without exception in order to include them in reports on AMU using a daily-based indicator.

Defined doses are technical units; they are not intended to reflect recommended doses or to approximate actual doses. As an example, more than 80% of Canadian dairy producers reported off-label treatment for clinical mastitis (longer duration or higher frequency) in a recent study (45).

With an objective of harmonization between countries, the next step in assignment of veterinary defined doses would be to have just one set of defined values that could be used worldwide. Because the world market of antibiotics is not stable over time (new release of products, cessation of the sales of some products, etc.), defined doses should be updated regularly, as the WHO does for human drugs.

DDDbovCA and DCDbovCA can now be used to report on AMU in cattle in Canada. In the future, there will be great interest to compare defined vs. used and prescribed doses for the different Canadian provinces.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/**Supplementary Material**.

AUTHOR CONTRIBUTIONS

HL, SD, and DF contributed to the conception and design of the study. HL wrote the first draft of the manuscript. All authors listed have made a substantial, direct and

intellectual contribution to the work, and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2020.00010/full#supplementary-material

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Prevalence of *Salmonella* Isolates and Their Distribution Based on Whole-Genome Sequence in a Chicken Slaughterhouse in Jiangsu, China

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Salmonella has been known as the most important foodborne pathogen, which can infect humans via consuming contaminated food. Chicken meat has been known as an important vehicle to transmit *Salmonella* by the food supply chain. This study determined the prevalence, antimicrobial resistance, and genetic characteristics of *Salmonella* at different chicken slaughtering stages in East China. In total, 114 out of 200 (57%) samples were *Salmonella* positive, while *Salmonella* contamination was gradually increasing from the scalding and unhairing stage (17.5%) to the subdividing stage (70%) throughout the slaughtering. Whole-genome sequencing (WGS) was then performed to analyze the serotype, antimicrobial resistance gene profiles, and genetic relationship of all *Salmonella* isolates. The most common serotypes were *S*. Kentucky (51/114, 44.7%) and *S*. Enteritidis (37/114, 32.5%), which were distributed throughout the four slaughtering stages, and were also identified in the corresponding environments. The multilocus sequence typing (MLST) analysis revealed that seven sequence types (STs) were occupied by six different serotypes, respectively. Only *S*. Kentucky had two STs, ST314 was the predominant ST shared by 50 isolates, while the ST198 has 1 isolate. The antimicrobial resistance gene analysis demonstrated that most of the strains belonging to *S*. Kentucky (39/51, 76.5%) and *S.* Indiana (15, 100%) contained over five groups of antimicrobial resistance genes. Based on the core genome analysis, 50 *S.* Kentucky isolates were genetically identical, indicating that one *S.* Kentucky strain with the same genetic background was prevalent in the chicken slaughtering line. Although 37 *S.* Enteritidis isolates only had three different antimicrobial resistance gene profiles, the core genome sequence analysis subtyped these *S.* Enteritidis isolates into five different clusters, which revealed the diverse genetic background of *S.* Enteritidis in the slaughterhouse. The antimicrobial resistance phenotypes were consistent with the presence of the corresponding resistance genes of *S*. Kentucky and *S*. Enteritidis, including *tetA*, *floR*, *blaTEM-1B*, *strA/B*, *sul1*/*sul2*, and *gyrA* (D87Y). Our study observed

a high prevalence of *Salmonella* in the chicken slaughter line and identified the slaughtering environment as a main source of causing *Salmonella* cross-contamination during chicken slaughtering. Further studies will be needed to limit the transmission of *Salmonella* in the slaughterhouse.

Keywords: *Salmonella*, whole-genome sequencing, serovars, MLST, antimicrobial resistance

BACKGROUND

Salmonella is an important foodborne pathogen causing gastroenteritis in humans and animals (1, 2). In USA, 46,623 cases of culture-confirmed *Salmonella* infection were reported from 53 states and regional public health laboratories in 2016, in which summer was the high-incidence season (3). In Europe, 91,662 confirmed human salmonellosis cases were reported by all member states in 2017 (4). In China, ∼70–80% of foodborne pathogenic outbreaks are caused by *Salmonella*, and most of them are derived from animal-origin food products (5).

Salmonella are prevalent in domestic animals such as poultry, pigs, and cattle, and can be transmitted through the food chain by the animal-origin food products (6–8). Slaughter is considered as an important step causing *Salmonella* contamination in meat products (6, 7). A study demonstrated that the total isolation rate of *Salmonella* was 34.0% in a pig slaughterhouse in Hainan, China, and cross-contamination was also observed during the slaughtering process (9). In northern Italy, *Salmonella* was found in 12.3 and 11.2% of carcass samples from two pig slaughterhouses, respectively, indicating the potential transmission of *Salmonella* from slaughterhouse to retail meat (10). However, limited studies were conducted on the prevalence of *Salmonella* in chicken slaughterhouse in China.

The prevalent study had shown that the most common serotypes in *Salmonella* human cases in Europe were *S.* Enteritidis, *S.* Typhimurium, I 4,[5],12:i:-, *S.* Infantis, and *S.* Newport, while in the US, the most common serotypes were *S.* Enteritidis, *S.* Newport, *S.* Typhimurium, *S.* Javiana, and I 4,[5],12:i:- (3, 4). In China, *S.* Typhimurium were identified as the most common serotypes from humans followed by *S.* Enteritidis, *S.* Derby, and *S.* Indiana (11). Another research showed that the MLST of *S.* Enteritidis identified from humans was ST11 (12). The most common serotypes from the chicken were *S.* Enteritidis, followed by *S.* Indiana and *S.* Typhimurium, while the predominant MLST types were ST11, ST17, and ST19 in Shandong province of China (13, 14).

This study was to evaluate the distribution of *Salmonella* in different slaughtering stages/environments in a chicken slaughterhouse in summer and autumn. We selected four key slaughtering stages for sampling including scalding and dehairing, evisceration, pre-cooling, and subdividing. Based on whole-genome sequencing (WGS), we further analyzed the serotype, MLST, and antimicrobial resistance genes of all *Salmonella* isolates and evaluate the occurrence and distribution of *Salmonella* at different slaughtering steps and environments.

METHODS

Sample Collection and *Salmonella* Isolated

A total of 160 carcass swab samples and 40 environment samples were collected from a poultry slaughterhouse during August and October, 2018, in Jiangsu, China. Twenty carcass samples and five environment samples were collected at four different slaughtering steps including scalding and dehairing, evisceration, pre-cooling, and subdividing.

The isolation of *Salmonella* was performed as previously described (9). In brief, 100 ml of buffered peptone water (BPW) was added to cotton swab samples and incubated at 37°C overnight. Then, 1 ml of enriched BPW suspension was transferred to Rappaport-Vassiliadis R10 broth (RVR10), incubated at 42°C for 24–48 h, and further streaked on XLT4 agar plate and incubated at 37°C for 24 h for *Salmonella* selection. Presumptive *Salmonella* colonies were confirmed as *Salmonella* by PCR with the presence of the *stn* gene. The PCR program of *stn* gene was performed as previously described (15) the PCR results are shown in **Figure S1**.

WGS, Assembly, and Analysis

The genomic DNA of all *Salmonella* isolates were extracted by TIAN amp Bacteria DNA Kit (Tiangen, Beijing, China). All the genomes were fragment with an insertion size of 500 bp to construct the library, and the NEB Next Ultra DNA Library Prey Kit for illumina (NEB, Beverly, MA, USA) was used to generate sequencing libraries followed by the manufacturer's recommendation, and the WGS of libraries was performed by illumina platform Hiseq 2500. SPAdes version 3.10.0 was used to assemble the reads into contigs (16), and the information is shown in **Table S1**. The serotypes were analyzed by *Salmonella In Silico* Typing Resource (SISTR) (17). The multilocus sequence typing (MLST) of all isolates was conducted by Seemann MLST database (https://cge.cbs.dtu.dk/services/MLST/) (18). Antimicrobial resistance genes of each isolate were analyzed by ResFinder 3.2 database (https://cge.cbs.dtu.dk/services/ResFinder/) (19). WGS data of all *Salmonella* isolates were submitted to the European Nucleotide Archive with the accession number PRJEB34962.

Antimicrobial Susceptibility Testing (AST)

AST was based on the Clinical and Laboratory Standards Institute (CLSI 2018). The agar dilution method was performed to determine the minimal inhibitory concentration (MIC) of the *Salmonella* isolates to the antimicrobial drugs. The test antibiotics included tetracycline, chloramphenicol, ciprofloxacin,

ampicillin, cefazolin, cefotaxime, nalidixic acid, trimethoprim-sulfamethoxazole, and streptomycin. *Escherichia coli* ATCC 25922 was used for quality control strain.

Statistical Analysis

The proportions of *Salmonella* in different slaughtering steps of the two visits were based on ANOVA comparisons with SPSS statistical package (SPSS Inc., Chicago, USA). Statistical significance was set at $P \leq 0.05$.

RESULTS

Prevalence of *Salmonella* in a Chicken Slaughterhouse

A total of 114 (57.0%) *Salmonella* strains were isolated from 160 carcass swab samples and 40 environment samples at different slaughtering steps (**Table 1**). The *Salmonella* prevalence rate at different slaughtering steps showed no significant difference between the two visits ($P = 0.737$). The highest prevalence of *Salmonella* was observed at the subdividing link stage, in which 70% (28/40) of the samples were *Salmonella* positive, followed by pre-cooling with 65.0% (26/40) of positive samples and evisceration with 60.0% (24/40) of positive samples, respectively. The lowest prevalence of *Salmonella* was at the scalding and unhairing stage, in which only 17.5% (7/40) of samples were *Salmonella* positive. The result demonstrated that the prevalence of *Salmonella* in this slaughterhouse showed an increasing trend through the sequential processes. In addition, 72.5% (29/40) of the environment samples were *Salmonella* positive, and the prevalence rates showed no significant difference between the two visits, indicating the environment as an important arena for the cross-contamination of *Salmonella*.

Six different serotypes were identified from 114 *Salmonella* isolates based on WGS analysis (**Table 1** and **Table S2**). The most prevalent serotype was *S.* Kentucky (44.7%, 51/114), followed by *S.* Enteritidis (32.5%, 37/114), *S.* Indiana (13.0%, 15/114), *S.* Corvallis (6.1%, 7/114), *Salmonella* I 4,[5],12:i:- (2.6%, 3/114), and *S.* Hadar (0.9%, 1/114). Both *S.* Kentucky and *S.* Enteritidis were identified in the two visits. *S.* Indiana, *S.* Corvallis, and *S.* Hadar only appeared in the first visit, while *Salmonella* I 4,[5],12:i:- only appeared in the second visit. *S.* Kentucky and *S.* Enteritidis appeared in all four slaughtering steps and their related environments during the two visits, indicating the persistence of these two serotypes in the slaughtering line (**Figure 1**). Moreover, *S.* Indiana and *S.* Corvallis were found after the evisceration step for the first visits, indicating that contamination by these two serotypes may occur at this stage. *S.* Hadar was only observed in the slaughtering environment, indicating the low cross-contamination possibility of this serotype. MLST analysis showed that these 114 *Salmonella* isolates into seven STs (**Table 1**). Fifty out of 51 *S.* Kentucky strains were ST314 with only one isolate from ST198. All 37 *S.* Enteritidis isolates belonged to ST11, while all 15 *S.* Indiana isolates belonged to ST14. By correlating the STs to serotypes of

TABLE 1 | Prevalence of *Salmonella* isolated from carcass swab samples and environmental samples.

	Sample size per visit	Visit 1		Visit 2		Total ratio %	Serotype	Number		MLST
		Number	Ratio %	Number	Ratio %			Visit 1	Visit 2	
Scalding and Unhairing	20	0	0.0	7	35.0	17.5	*S.* Kentucky	-	4	ST314
							S. Enteritidis	-	3	ST11
Evisceration	20	17	85.0	7	35.0	60.0	*S.* Kentucky	5	4	ST314
							S. Enteritidis	4	3	ST11
							S. Indiana	7	-	ST14
							S. Corvallis	1	-	ST1541
Pre-cooling	20	13	65.0	13	65.0	70.0	*S.* Kentucky	5	6	ST314
							S. Enteritidis	4	5	ST11
							S. Indiana	3	-	ST14
							S. Corvallis	1	-	ST1541
							I 4,[5],12:i:-	-	2	ST34
Subdividing	20	14	70.0	14	70.0	70.0	*S.* Kentucky	5	7	ST314
							S. Kentucky	1		ST198
							S. Enteritidis	3	7	ST11
							S. Indiana	3	-	ST14
							S. Corvallis	2	-	ST1541
Environment	20	16	80.0	13	65.0	72.5	*S.* Kentucky	8	6	ST314
							S. Enteritidis	2	6	ST11
							S. Indiana	2	-	ST14
							S. Corvallis	3	-	ST1541
							I 4,[5],12:i:-	-	1	ST34
							S. Hadar	1	-	ST33
Total	100	60	60.0	54	54.0	57.0	**Total**	60	54	

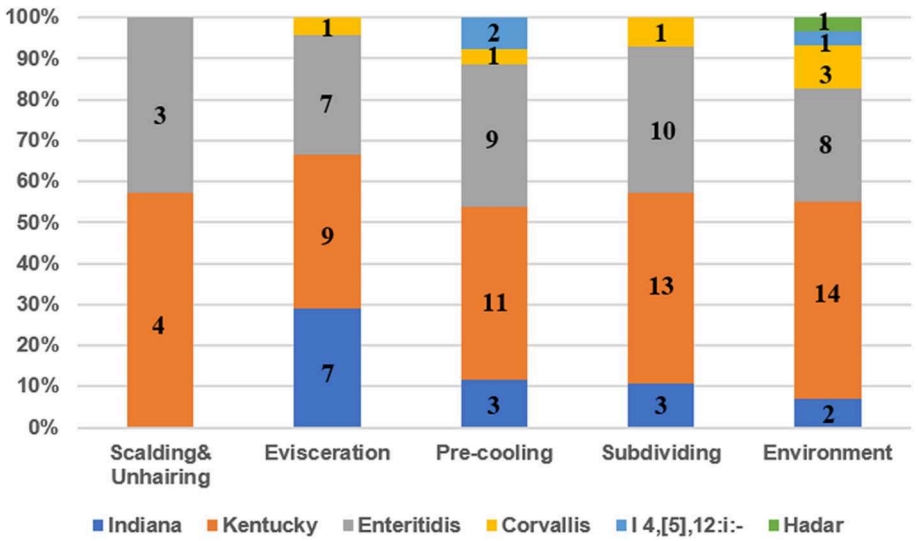

FIGURE 1 | The prevalence of serotypes of *Salmonella* isolates from different slaughtering stages and environments. Numbers represent the isolate numbers of different *Salmonella* serotypes in different steps.

all isolates, we observed a close relationship of these two typing results. These results indicate that one ST corresponds to one serotype, but different isolates belonging to one serotype may share multiple STs, which is consistent with previous studies (20).

Among the 13 plasmids identified in the 114 isolates, the most prevalent plasmid was IncX1 (55/114, 48.3%), followed by IncR (43/114, 37.7%), IncFIB(S)/IncFII(S) (32/114, 28.1%), IncQ1 (12/114, 10.5%), and CoI440I (9/114, 7.9%) (**Table S3**). In addition, the IncX1 plasmid was predominant in *S.* Enteritidis isolates, while IncR was the most prevalent plasmid in *S.* Kentucky isolates.

Antimicrobial Analysis

In total, 10 different groups of antibiotic resistance genes (ARG) were detected from 106 out of 114 *Salmonella* genomes. All of the ARGs and their frequency of occurrence in *Salmonella* isolates are listed in **Table S2**. 54.39% ($n = 62$) of isolates displayed ARGs related to the resistance to at least five groups of antibiotics, and 24.56% ($n = 28$) of isolates contained at least 8of the 10 groups of ARG. All 15 *S.* Indiana isolates, 3 *Salmonella* I 4,[5],12:i:- isolates, and 39 of 51 *S.* Kentucky isolates contained more than five classes of ARGs. Our results demonstrated a high prevalence of multidrug resistance *Salmonella* in the slaughter line and the related environments. The antimicrobial resistance genes were sporadically identified in the isolates, which are all listed in **Table S4**.

The resistant phenotype of quinolone was known to regulate by point mutant in the quinolone resistance-determining regions (QRDRs) of *gyrA*, *gyrB*, *parC*, and *parE* (21), and the plasmid-mediated quinolone resistance genes (22). The mutation of QRDRs in the *Salmonella* isolates is shown in **Table S5**. Interestingly, we also observed that different mutations in QRDRs were closely related to serotypes. All *S.* Indiana isolates, *S.* Hadar isolates, 35 of 37 *S.* Enteritidis, and 1 of 51 *S.* Kentucky isolates contained point mutations at *gyrA*, indicating that these isolates may be resistant to nalidixic acid and ciprofloxacin. Four quinolone-resistance-associated genes were identified in these isolates, in which *qnrB6* (33.33%, 38/114) was the most prevalent, followed by *qnrS1* (7.02%, 8/114), *oqxB* (2.63%, 3/114), and *oqxA* (1.75%, 2/114). Fifty of 51 *S.* Kentucky strains did not have the mutation of *gyrA*, whereas quinolone-resistance gene *qnrB6* was detected in 35 isolates.

Genomic Analysis of *S.* Kentucky Isolates

S. Kentucky ($n = 51$) was the most predominant serotype isolated in the two visits. The core genome sequence analysis divided the 51 strains into two clusters (**Figure 2**). Cluster I only contains one strain, while the remaining 50 isolates with the similar core genome sequences belong to cluster II (**Figure 2**). Interestingly, although only two clusters were shared by these *S.* Kentucky isolates based on the core genome sequences analysis, the antimicrobial resistance gene profiles are diverse in these strains (**Figure 2** and **Table S6**).

By WGS analysis, 18 antimicrobial resistance genes were identified in *S.* Kentucky isolates. The most prevalent antimicrobial resistance genes were *sul1* (78.43%, 40/51), followed by *aadA16* (76.47%, 39/51), *drfA27* (76.47%, 39/51), *mphA* (76.47%, 39/51), *ARR-3* (76.47%, 39/51), and *qnrB6* (68.63%, 35/51). 76.47% of the *S.* Kentucky isolates contained ARGs against five or more types of antibiotics (**Table S6**), while only eight isolates did not carry any antimicrobial resistance genes. The one *S.* Kentucky ST198 isolate contained the *strA*/*strB*/*aadA7*/*aac(3)-Id*, *tetA*, *sul1*, and *blaCTX-M-14* genes, which was very different from *S.* Kentucky ST314 isolates (**Figure 2**). The AST results confirmed that the *S.* Kentucky ST198 isolate was resistant to tetracycline (*tetA*), sulfamethoxazole (*sul1*), ampicillin/cefazolin/cefotaxime (*blaCTX-M-14*), streptomycin (*strA/B*), and nalidixic acid [*gyrA*

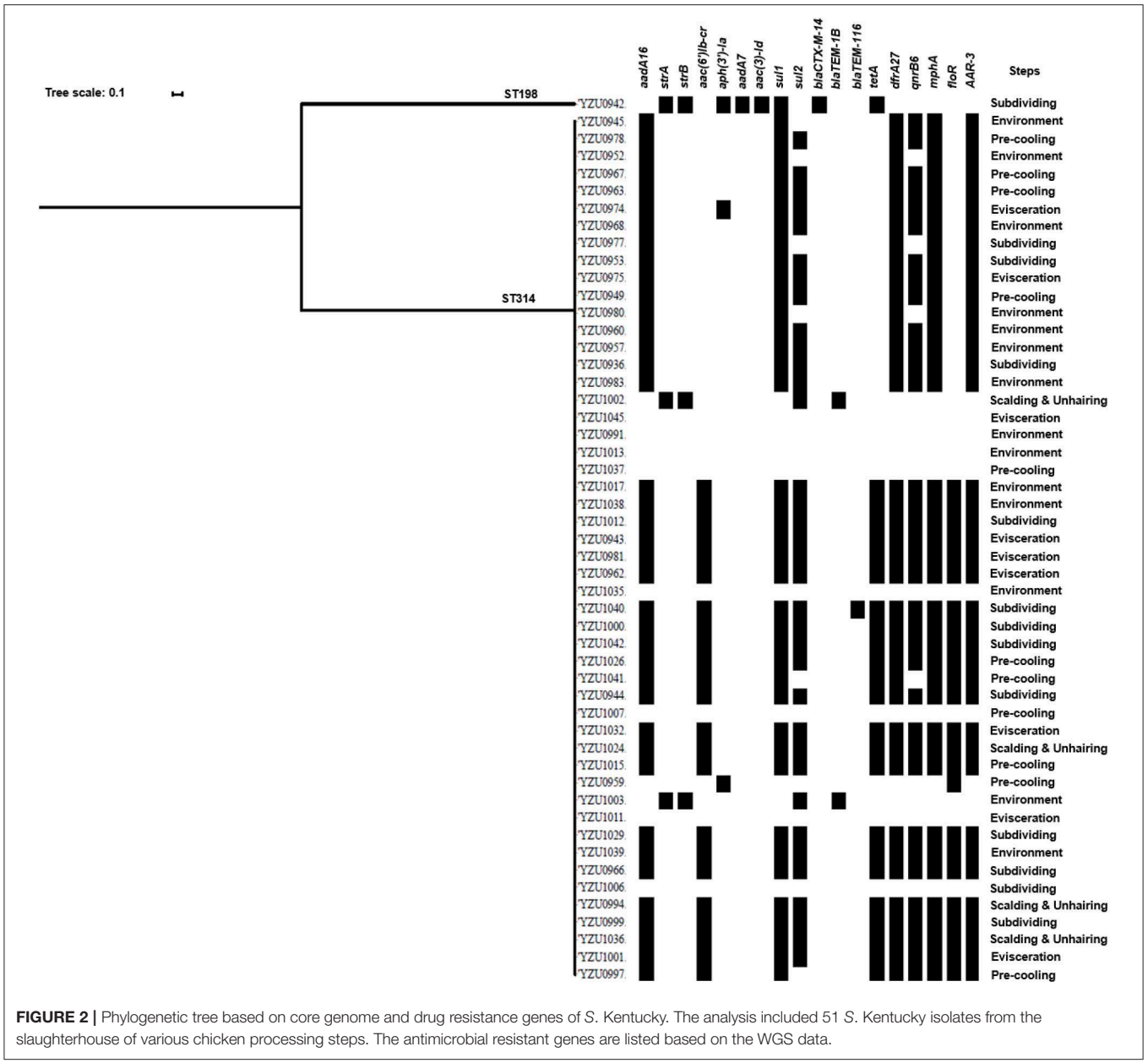

FIGURE 2 | Phylogenetic tree based on core genome and drug resistance genes of *S.* Kentucky. The analysis included 51 *S.* Kentucky isolates from the slaughterhouse of various chicken processing steps. The antimicrobial resistant genes are listed based on the WGS data.

(D87Y)]. Thirty-one of 51 *S.* Kentucky isolates were resistant to more than three antimicrobials. Moreover, 20 *S.* Kentucky isolates contained 10 antimicrobial resistance genes, mainly in the genotypes of the *S.* Kentucky that distributed among the four slaughtering stages and environments of the slaughterhouse. The strains carrying seven antimicrobial resistance genes were isolated from the evisceration, pre-cooling, and subdividing stages and environments (**Table S6**).

Genomic Analysis of *S.* Enteritidis

S. Enteritidis was identified as another prevalent serotype in the chicken and slaughterhouse, and 37 *S.* Enteritidis isolates were detected in this study. The phylogenetic tree analysis of *S.* Enteritidis isolates was constructed based on the core genome genes, which were divided into five clusters. The main cluster of *S.* Enteritidis contained 32 isolates, while the other clusters contained only one or two isolates (**Figure 3**). The main cluster of *S.* Enteritidis was detected from all four slaughtering stages and their related environments, while isolates from other clusters were only found at the pre-cooling and evisceration stages.

By WGS analysis, *S.* Enteritidis isolates were divided into three ARG profiles. Even though core genome sequences of *S.* Enteritidis isolates showed diversity, the majority of *S.* Enteritidis showed similar ARG profiles (**Figure 3**). Thirty-four of 37 *S.* Enteritidis isolates contained a four-ARG profile, which were *sul2*, *strA/strB*, and *blaTEM-1B*, and these strains were identified through the slaughterhouse (**Tables S4**, **S7**). The AST results of these isolates showed that the antimicrobial

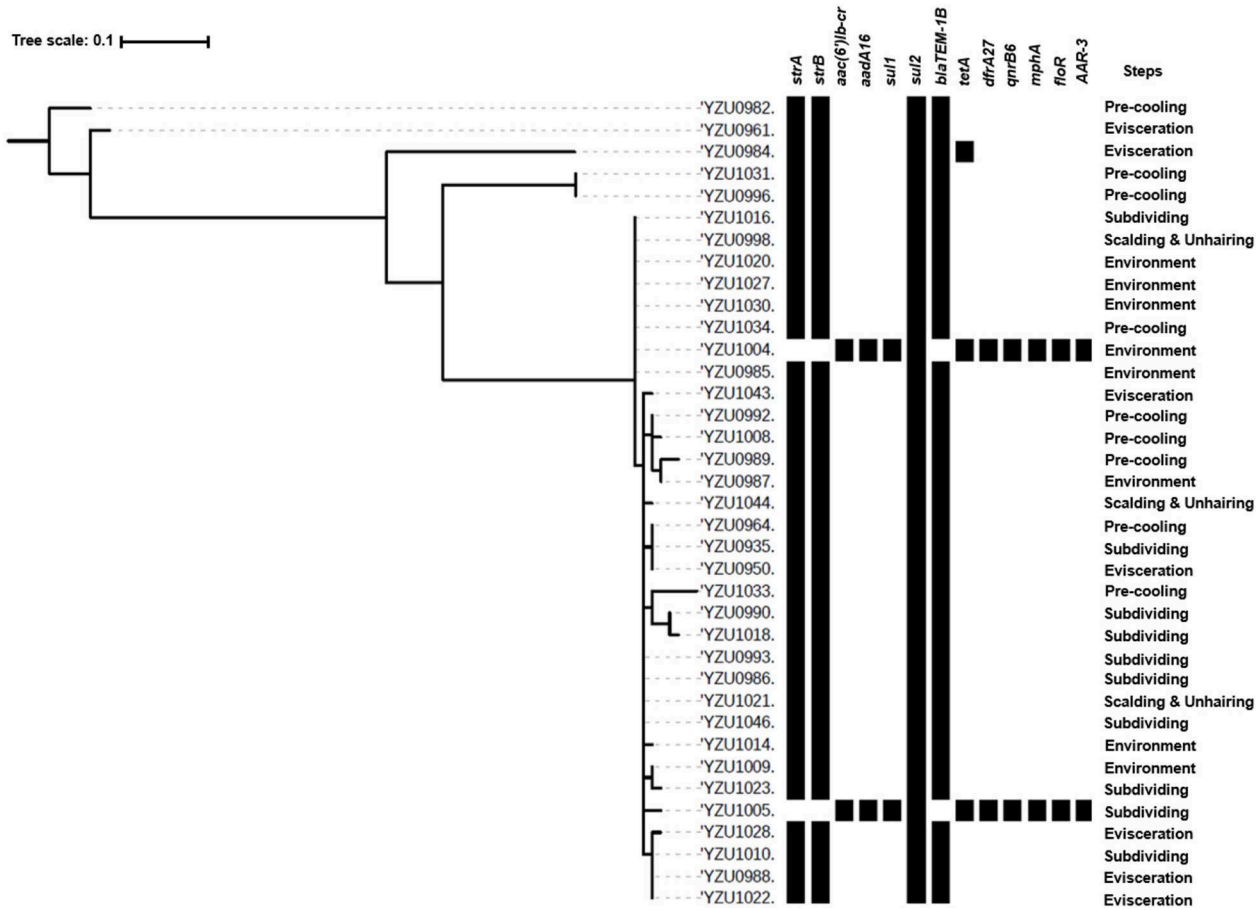

FIGURE 3 | Phylogenetic tree based on core genome and drug resistance genes of *S*. Enteritidis (ST11). The analysis included 37 *S*. Enteritidis isolates from the slaughterhouse of various chicken processing steps. The antimicrobial resistant genes were listed according to the WGS data.

resistance phenotypes were consistent with the presence of the corresponding resistance genes, including ampicillin (*blaTEM-1B*), streptomycin (*strA/B*), sulfamethoxazole (*sul2*), and nalidixic acid [*gyrA*(D87Y)]. One isolate from the evisceration step contained a very similar ARG profile as the 37 isolates mentioned above with five genes including *sul*2, *str*A/*str*B, *bla*TEM-1B, and *tet*A (**Figure 3** and **Table S7**), and this isolate showed resistance to tetracycline (*tetB*) besides the above antibiotics. Two isolates from subdividing stage and environment, respectively, contained the same ARG profile, which were distinctly different from other *S*. Enteritidis isolates including *aac(6′)Ib-cr*, *aadA16*, *sul1*, *sul2*, *tetA*, *dfrA27*, *qnrB6*, *mphA*, *floR*, and *AAR-3*. The AST results showed that both isolates were resistant to ciprofloxacin [*qnrB6*, *aac(6′)Ib-cr*], tetracycline (*tetA*), chloramphenicol (*floR*), trimethoprim, sulfamethoxazole (*sul1*, *sul2*, and *dfrA27*), and nalidixic acid [*gyrA*(D87Y)].

DISCUSSION

In recent years, the increased prevalence and antimicrobial resistance of *Salmonella* in food has frequently been reported in China, but the prevalence of *Salmonella* in chicken slaughterhouse located in Jiangsu province of China is rarely studied. This study analyzed 200 samples collected from a chicken slaughterhouse in Jiangsu province in 2018 and identified 114 *Salmonella* isolates, with a prevalence rate of 57% (**Table 1**), which was comparatively high than reported from other studies both globally and domestically. The prevalence rates of *Salmonella* were 30.0 and 9.4% in two different chicken slaughterhouses, respectively, in a study from South Korea (23), while the prevalence rate was 11.1% in a chicken slaughterhouse in the northeast of Algeria (24). A study from Brazil demonstrated that the prevalence of *Salmonella* was only 3.6% in a chicken slaughterhouse (25). In China, the isolation rate of *Salmonella* was 12.7% in chickens in Shandong province (13), while no *Salmonella* was detected in a chicken slaughterhouse in Sichuan province (26). However, in Guangdong province, the prevalence of *Salmonella* in chicken and pork meat at retail markets was 63.6 and 73.1%, respectively, and 62.86% of samples from slaughterhouse were detected to be positive for *Salmonella* (27, 28). In Jiangsu province, the prevalence of *Salmonella* in pig slaughterhouses and retail markets was 71.8 and 70.9%, respectively (20).

The isolation rate of *Salmonella* in our study is higher than the previous report in chicken slaughterhouses except that in Guangdong province, but less than that in pig slaughterhouses. These results indicated that the prevalence of *Salmonella* in Jiangsu province was more serious than that in other regions, which increased the potential transmission to humans. These results suggested that the contamination of *Salmonella* in the slaughterhouse should be concerned in control the transmission of *Salmonella*.

Among the various stages in the chicken slaughterhouse, 85 isolates with 17.5, 60.0, 65.0, and 70.0% of *Salmonella* were detected at scalding and dehairing, evisceration, pre-cooling, and subdividing stages, respectively (**Table 1**). The isolation rates in evisceration, pre-cooling, and subdividing stages were distinctly different from the scalding and dehairing stage, indicating that the evisceration stage was a source for *Salmonella* transmission. Therefore, this step may be the key point for the prevention and control of *Salmonella* contamination in this slaughterhouse. Besides, the isolation rate of *Salmonella* in the environment samples was 72.5%, which was much higher than the previous study with 20% of *Salmonella*-positive environment sample from other chicken slaughterhouses (24). This result demonstrates that the slaughtering environment is another key point for the spread of *Salmonella* in this slaughterhouse.

In total, 114 *Salmonella* isolates were subtyped into six serotypes with *S.* Kentucky and *S.* Enteritidis to be the predominant serotypes in the four slaughtering stages and environments (**Figure 1**) in the two visits, indicating that *S.* Kentucky and *S.* Enteritidis might be persistent throughout the slaughter line. Moreover, the chickens slaughtered at this abattoir were from different farms. Seven *S.* Corvallis isolates were isolated in the first visit, in which the serotype was also reported in chicken from Brazil with an isolation rate of 7.9% (29). In the present study, the prevalence of *S.* Kentucky and *S.* Enteritidis in the slaughterhouse was 44.7 and 32.5%, which was consistent with findings reported in Guangdong province (27, 28). However, the results were quite different from the results in Sichuan province, in which *S.* Derby and *S.* Typhimurium were identified as the most common serotypes (26). *S.* Enteritidis was reported as the most common serotype in human cases, which was mainly detected from laying hens, followed by broiler meat (4). *S.* Enteritidis was also the most common serotype of human *Salmonella* infections in the USA during 2011 and 2016 (3). In China, *S.* Enteritidis was recognized as the most frequently isolated *Salmonella* serotype in chicken meat (30, 31). The above data indicated that the *S.* Enteritidis was recognized as a dominant serotype worldwide. The most common ST of *S.* Enteritidis was ST11 in Hubei, Shanghai, and Shandong province, China, which was consistent with our study (32–34). In addition, the ST11 was also identified as the predominant ST of *S.* Enteritidis in Iran, Brazil, Denmark, Japan, and USA, indicating that the ST11 is probably an ancestral clone of *S.* Enteritidis successfully scattered in all of these geographically diverse countries (35).

S. Kentucky was identified as the most common serotype in this study (**Table 1**). Previous studies indicated that *S.* Kentucky was mainly found in North America, but that the isolation rate of *S.* Kentucky in retail meat was significantly increasing in China (27, 36). Human infection cases by *S.* Kentucky were reported in Europe and USA, and *S.* Kentucky was the seventh top serotype-causing human salmonellosis in Europe during 2017 (3, 4). ST314 (53/54) was predominant in the *S.* Kentucky isolates, while only one isolate belonged to ST198 (1/54) in this study. The most common ST of *S.* Kentucky isolates from Hubei province of China was ST314, while most of the isolates from Shandong province were ST198 (32, 34). Furthermore, ST198 was the most common clone among the *S.* Kentucky isolates from chicken in Vietnam and humans in USA (37, 38). Besides, the ST198 was considered as a worldwide-disseminated multidrug-resistant clone, which may originate outside of the North America (38), and our study also showed that the ST198 isolates could resistance to tetracycline, sulfamethoxazole, ampicillin/cefazolin/cefotaxime, streptomycin and nalidixic acid. By now, studies about the prevalence of *S.* Kentucky in chicken was limited and no infection casein humans was reported in China. However, our studies showed that the prevalence of *S.* Kentucky in chicken carcass was increasing, which indicated a potential risk of transmitting it to the public by the food chain in China. Further studies are required to explore the relationship between the recent and early isolates of *S.* Kentucky in China.

The antimicrobial resistance in *Salmonella* is one of the main concerns of its infection in humans. This study analyzed genotypes of antimicrobial resistance genes presenting in all 114 *Salmonella* isolates, which showed diverse relationship to the different serotypes. Based on the core genome analysis, the most prevalent serotype *S.* Kentucky was only divided into two clusters with a predominant cluster containing 51 isolates and one isolate to the other cluster. By correlating the core genome to the genotypes of antibiotic resistance genes, we observed a high diversity of the antibiotic resistance genes in the predominant cluster of *S.* Kentucky isolates (**Figure 2** and **Table S6**), indicating that the multidrug resistance of *S.* Kentucky was less related to the core genome. Previous studies showed that *S.* Kentucky were multidrug-resistance serotypes (38–41), while *S.* Kentucky isolates in this study contained antibiotic resistance gene from more than five different antibiotic groups. *S.* Enteritidis isolates in this study showed a close relationship of the core genome clusters to the genotypes of its antibiotic resistance genes (**Figure 3** and **Table S7**). Three types of the antimicrobial resistance genes of *S.* Enteritidis were identified, including the aminoglycoside resistance genes *strA*/*strB*, sulfonamide resistance gene *sul2*, and β-lactam resistance gene *blaTEM-1B*. These four genes were located in the IncX1 plasmid, which was predominant in *S.* Enteritidis. The IncX1 plasmid may mediate resistance genes transmission of *S.* Enteritidis in this slaughterhouse. Of 37 *S.* Enteritidis isolates, 35 contained the point mutant in *gyrA* gene for nalidixic acid resistance. A previous study showed that *S.* Enteritidis were highly resistant to nalidixic acid (91.3%), ampicillin (39.13%), and streptomycin (28.70%) in Jiangsu province, China (42), which were confirmed with our antimicrobial genotype analysis. Moreover, a study from Thailand also demonstrated similar results, in which *S.* Enteritidis showed highest resistance rates to nalidixic acid

(83.2%) and ampicillin (50.05%) (43). A previous study showed that aminoglycoside resistance genes *aadA5*, *aadA7*, and *aac(3)-Id*, and trimethoprim resistance genes *drfA14* and *drfA17* were only detected in isolates from human infection cases (44). However, these genes were also observed in our *Salmonella* isolates from chicken carcasses and the slaughter environments, indicating that these multidrug-resistant *Salmonella* isolates might have the risk to transmit from chicken meat to humans.

The predominant serotypes of *Salmonella* isolated from the food handlers' fecal matter in Jiangsu province, China, were *S.* Typhimurium (16.1%), followed by *S.* Derby (13.5%), *S.* Enteritidis (11.4%), and *S.* London (11.4%) (45). The high prevalence of *S.* Enteritidis in humans may be caused by chicken meat (46). Multidrug resistance rate among the strains was 73.4%, and the predominant phenotype among the MDR was Amp, Sul, and Tet resistance (47); we also found the genes responsible for these antibiotic resistance in this study, indicating the transmission of *Salmonella* from chicken to humans. Compared with the *Salmonella* isolated from humans in Hubei, Guangdong, and Zhejiang province of China, the *S.* Enteritidis was the common predominant serotype, indicating that the prevalence of *S.* Enteritidis was serious in Chinese people (32, 48, 49). Besides, almost all of the *S.* Enteritidis were multidrug resistance. The most common phenotypes of antimicrobial resistance in *S.* Enteritidis from Zhejiang province were nalidixic acid, sulfonamides, ampicillin, and streptomycin, and similar phenotypes were identified in Hubei and Guangdong province, which was consistent with our genotypes of AGRs in *S.* Enteritidis (32, 48, 49). These results indicate that these multidrug-resistant *Salmonella* isolates could be potentially transmitted from chicken meat to humans. This study calls for further attention in the prevention and control of foodborne disease caused by *Salmonella*, as well as improvement in the environment of food slaughterhouses.

CONCLUSIONS

This study investigated the overall prevalence of *Salmonella* in a chicken slaughterhouse in Jiangsu province of China. By WGS, serotypes and MLST types of all *Salmonella* isolates were analyzed, and *S.* Kentucky and *S.* Enteritidis were observed as the predominant serotypes in the slaughter line and environment. Meanwhile, a high prevalence of multidrug-resistant *Salmonella* was observed in chicken carcasses from all slaughtering steps and environment, indicating a potential risk transmission from chicken slaughterhouse to humans. Further studies will be needed to elucidate the extent to which human infections are caused by the *Salmonella* contamination from chicken slaughtering.

DATA AVAILABILITY STATEMENT

The datasets generated for this study can be found in the European Nucleotide Archive, accession number PRJEB34962: https://www.ebi.ac.uk/ena.

ETHICS STATEMENT

This study was carried out in accordance with the principles of the Basel Declaration and recommendations of the institutional administrative committee and ethics committee of laboratory animals, Animal Welfare and Ethics Committees of Yangzhou University. The protocol was approved by the Animal Welfare and Ethics Committees of Yangzhou University.

AUTHOR CONTRIBUTIONS

DG, YT, CM, and ZP contributed to the conception and design of this study. DG, ZW, and XC were responsible for the acquisition of the data analyzed in this study. DG, XK, ZP, and XJ were involved in the analysis and interpretation associated with this work. All the authors were involved in manuscript revisions and final approval of the version to be published.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2020.00029/full#supplementary-material

Figure S1 | Verification of *Salmonella* isolates by *stn* PCR. Lane 1 to 26 represent the PCR results of potential *Salmonella* isolates randomly picked single colonies that grew on the XLT4 agar plate. Lane M was DL2, 000DNA marker (Takara, Japan), the negative control used was *E. coli* DH5a, and the positive control was *Salmonella* Typhimurium LT2. PCR products were separated on a 1% agarose gel and stained with ethidium bromide.

Table S1 | The assembly information of whole-genome sequencing.

Table S2 | The analysis information of whole-genome sequencing.

Table S3 | Plasmid replicons.

Table S4 | Antimicrobial resistance genes of the *Salmonella* isolates.

Table S5 | Mutation of the QRDRs in different serotypes.

Table S6 | Antimicrobial resistant genes of *S.* Kentucky.

Table S7 | Antimicrobial resistant genes of *S.* Enteritidis.

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Antimicrobial Use in Extensive Smallholder Livestock Farming Systems in Ethiopia: Knowledge, Attitudes, and Practices of Livestock Keepers

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Antimicrobial resistance (AMR) is a major public health threat, and inappropriate antimicrobial use (AMU) in food animal production can contribute to the global burden of AMR in humans. This study was conducted to understand knowledge, attitude, and practice (KAP) of smallholder livestock owners regarding antimicrobial use, residue, and resistance in three agro-ecological zones and production systems in Ethiopia. A cross-sectional study based on structured interviews was conducted. Twenty-one items were used to assess farmers' KAP. Item response theory (IRT) model and Cronbach's alpha were used to assess the KAP measurement scales. Inferential analyses were used to compare the differences in the practices in terms of the farm and socio-economic characteristics. There was a difference in the type of antimicrobials reported use between agro-ecological zones and production systems. Pastoralists most commonly used antibiotics (86.7%) followed by anthelminthics (70.8%). Overall, tetracyclines (36.4%), aminoglycosides (31.3%), and trimethoprim-sulfonamides (6.2%) were the most frequently used classes of antibiotics across the study sites. Human preparation antibiotics (tetracyclines) were also being used for veterinary purposes by 18.5% of pastoralist households. About 81.6% of livestock owners surveyed reported to have access to veterinary drugs although access varied between agro-ecological zones and production system. About 72.3% of pastoralists administered antibiotics by not following through the full treatment course. Moreover, 70% of respondents were not aware of the recommended withdrawal periods of milk and meat after antibiotic treatment. It was noticed that around 80 and 70% of respondents had a tendency to give doses higher or lower than recommended of antimicrobials, respectively. The study confirms the need for interventions to increase knowledge among smallholder farmers to improve the way antimicrobials in general and antibiotics in particular are used in these settings. In addition, professional involvement, supervision, and guidance can

also lead to more efficient antimicrobial use by smallholder livestock owners. The study also highlights the need for research into the development of usable tools that measure antibiotic knowledge and attitudes.

Keywords: antimicrobial use, livestock, smallholders, knowledge, attitude

INTRODUCTION

Antimicrobials are applied in livestock farming for number purposes such as therapeutic (treating sick animals), metaphylaxis (control treatment of whole herd in case of disease outbreak), prophylaxis (preventive treatment), and growth promotion (1). The increasing demand for animal protein especially in developing parts of the world is causing an increase in animal production, and in connection with this, antimicrobial use in food-animal production was estimated to rise by 67% between 2010 and 2030 (2). Apart from the historical and the current positive contribution of antimicrobial use in animal health and production management, there exist a number of possible drawbacks associated with the use of antimicrobials in food-animals. Mis(use) of antimicrobials in food animals is potentially causing the emergence of antimicrobial-resistant bacteria strains by increasing selection pressure on bacteria to become resistant (2, 3). Other negative consequences associated with antimicrobial use in food animals is the occurrence of unacceptable level of drug residues in food of animal origin. The inappropriate use of antimicrobials in food animals can result in accumulation of toxic and harmful residues in animal products that can further affect the health of consumers largely by causing allergic reactions (4, 5). Therefore, the antimicrobial usage in food animals is indeed becoming a global issue associated with food safety and public health.

The growing concern regarding emergence of bacteria resistant to antimicrobials and their potential for transmission to humans via animal production has led various authorities worldwide to implement measures to decrease antimicrobial use in livestock production (6–9). Though some studies indicate the occurrence of naturally resistant bacteria, the substantial use of antimicrobial agents in animal production is suspected as one of the important factors driving the emergence of antimicrobial resistance in bacterial strains (10–12). Antimicrobial resistance is a major public health crisis (13, 14), threatening the return of untreatable infections and deaths on a massive scale if appropriate actions are not taken (15). To reduce the problem of human infections caused by resistant bacteria transferred from animals, there is continuous pressure to restrict the use of antimicrobials in animals (7, 9).

Apart from the public health impact, an increasing prevalence of antimicrobial resistance, particularly to frequently used antimicrobials in livestock, could also lead to reduced treatment options and increased animal disease and production losses (16). For instance, the World Bank (17) has estimated a 10% production loss in the livestock sector in low- and middle-income countries by 2050. In addition, infected animals may shed these bacteria, posing a threat to other farm animals, household pets, and humans, through direct contact or environmental contamination (11, 18). Infected animals may also act as a reservoir for resistant bacteria, which might enter the food chain (19).

Nowadays, several high-income countries monitor trends in AMU and AMR in livestock (20). These data, however, are generally scarce, particularly from low- and middle-income countries (LMIC) (2). Although access and usage of antimicrobials is improving in LMIC (21), information on actual AMU practices (volume, mode, and reasons for use) is lacking. Specifically, there is a huge gap in the availability of data that can be used to understand the trends over time and to evaluate the linkages between AMU and AMR. The availability of such data can potentially support informed decision-making process especially in connection with the framework of the global action plans formulated by international organizations such as the Food and Agriculture Organization (7), World Organization for Animal Health (9), and WHO (6).

Ethiopia has one of the largest livestock population in Africa with 60.4 million cattle, 31.3 million sheep, 32.7 million goats, and 1.4 million camels (22). Different production systems and agroecological zones coexist, making the process of nationally harmonized guidelines for livestock health and production challenging. This necessitates consideration of representative sampling considering the different agroecological zones of the country in research and development. Similar to many other developing countries, regulations on AMU in livestock in Ethiopia are poorly enforced and farmers have easy access to veterinary drugs; in the worst cases, the drugs may sometimes be falsified or substandard. Moreover, use of drugs in these settings is not commonly supervised by a trained veterinarian. Currently, information regarding AMU in livestock is scarce in Ethiopia, specifically the factors and incentives influencing the use of antimicrobial agents in animals at the farm level are poorly understood. Information on the knowledge, attitudes, and practices (KAP) of farmers regarding antimicrobials and their application will help in formulating strategies to maximize and preserve the benefits of AMU in livestock production with minimal jeopardy to public health. Therefore, we conducted a study to understand knowledge, attitude, and practice of smallholder livestock owners regarding antimicrobial use, resistance and residue in Ethiopia, which can serve as a case study for other comparable production systems.

MATERIALS AND METHODS

Study Area

This study was conducted in three, representative agro-ecological zones and production systems in the Amhara and Oromia regions in Ethiopia: (i) highland mixed crop-livestock production system (Menz Mama and Menz Gera district), (ii) lowland mixed

crop-livestock system (Abergelle and Zequwala district), and (iii) pastoral system (Yabello and Eleweya districts).

The highland agroecology with a mixed crop-livestock system is typical for areas above 2,200 m above sea level (masl) and is characterized as a system in which livestock husbandry and rain-fed cropping are closely interlinked. Livestock provide inputs (draft power, transport, and manure) to other parts of the farm system and generate consumable or saleable outputs (milk, meat, eggs, hides and skins, wool, hair, and manure). Crop residues are used as livestock feed; animals can be sold and revenues can be reinvested in agriculture or sold when the crop is failing because of weather or pests; cereals and most staple foods are produced in quantities that cover the needs of the family and excess is sold. The principal objective of farmers engaged in mixed farming is to gain complementary benefit from an optimum mixture of crop and livestock and spreading income and risks over both crop and livestock production (23).

The lowland agroecology with mixed crop-livestock system denotes elevation of ≤1,500 masl where farmers herd livestock in rangelands and produce crops on fertile land. The system is understood in a dual sense: firstly, it refers to farming systems entirely based on livestock but practiced in proximity to and perhaps functional association with cropping farming systems; secondly, it refers to the livestock subsystem of crop-livestock farming.

The lowland agroecology with the pastoral production system is characterized by sparsely populated pastoral rangelands, where subsistence of pastoralists is mainly based on livestock and livestock products. Livestock husbandry in this system is dominated by goats, cattle, sheep, and camels. Since the main source of food is milk, pastoralists tend to keep large herds to ensure sufficient milk supply and generate income by selling dairy products or live animals. The pastoral production system in some areas has been evolving into an agro-pastoral system (24).

Study Design and Sampling

A cross-sectional study was conducted with 379 smallholder livestock owners in 12 villages in six districts. The agro-ecological zones, districts, and villages were purposively selected to address the representation of different agroecological conditions and production systems. To determine the sample size required for the cross-sectional household survey, the sample size and power calculation tool of Epi InfoTM 7 (CDC, Atlanta, GA) was used. The required sample size of 374 was calculated (assuming allowable error of 6%; design effect of 1.4) and equally distributed to the clusters (agro-ecological zones and production systems). A sampling frame of all households from each of the selected villages was obtained from administration office and 423 households were randomly selected to account for non-participation of the selected households. Finally, the survey was conducted in 379 households. Five households were omitted from the final data analysis due to incomplete information. Each household was visited once.

Assessment Tool

The antimicrobial use assessment tool was developed and set up in Open Data Kit (ODK) on mobile tablet devices. The tool included open-ended and closed questions about household demographics, farm characteristics, management of manure, feed types, animal health constraints, disease prevention, animal health services, antimicrobial use, animal product consumption, and costs related to animal health. Prior to the study, veterinarians in the localities were trained as enumerators and the questionnaire was piloted with 40 livestock owners as a first step of validating the tool. Each interview took approximately 40 min to complete. Commonly available and used drugs at each study site were bought at the local veterinary drug stores and put in a demonstration box to facilitate interaction of enumerators with livestock keepers in gathering information on which drugs are used on the farm.

Data Analysis

Descriptive statistics were computed to describe household demographics and farm characteristics. Answers to open questions were coded into categorical variables and analyzed. Chi-square test was used to test potential associations between categorical variables and a $p < 0.05$ was considered as statistically significant.

Twenty-one items were used to assess farmers' knowledge ($n = 6$), attitudes ($n = 6$), and practices ($n = 9$) related to antimicrobial use and resistance. The outcomes concerning knowledge were initially multiple choice or "yes vs. no," and these were all reclassified as "correct" vs. "incorrect."

The attitude questions were either "yes vs. no" or on a five-point Likert scale "Strongly disagree" to "Strongly agree." The five-point Likert scale was grouped as follows: When a respondent indicated "strongly agree" and "agree" with a negative or "undesirable" statement, the response was classified as an "undesirable" attitude. The reverse was considered as a "desirable" attitude. Responses of "neither disagree nor agree" were not included in the analysis.

The response to questions regarding farmer practices were either "yes vs. no" or multiple choice, with the latter being dichotomized as "desirable" vs. "undesirable." Data were coded by giving 1 to correct or desirable answers and 0 to the wrong or undesirable response to a given question or item.

The percentages of "appropriate" answers (i.e., correct answers in the knowledge section, desirable attitude in the attitude question, and application of appropriate management practices in the practice section) were calculated for each KAP item.

Cronbach's alpha and the item response theory (IRT) model were used to assess the knowledge, attitude, and practice measurements. Internal consistency was evaluated using Cronbach's alpha, a parameter that describes the extent to which all the items in a test measure the same concept and it is thus connected to the inter-relatedness of the items within the test (25).

IRT analysis, which provides information on the discrimination and difficulty of each item across different levels of the underlying trait, was used. IRT is based on the assumption of unidimensionality [there is a single unmeasured (latent) trait underlying all items]. The assumption of unidimensionality was evaluated by subjectively evaluating the eigenvalues and factor loadings derived from an exploratory factor analysis

along with an evaluation of relationships among items within a correspondence analysis. Only questions related to practices met the assumption of unidimensionality.

A two-parameter logistic (2PL) model was used for practice items to calculate the probability that a person with a given level of management expertise would implement a specific item. This model is represented by the following equation (26):

$$Pij\ (ui = 1|\theta = t) = 1/1 + \exp\left[-1.7ai\left(t - bi\right)\right]$$

where a_i is the discrimination parameter for item i ($i = 1, \ldots, n$), b_i is the difficulty parameter for item i, u_i is the response of the person with trait level θ to item i, and 1.7 is a scaling constant.

The discrimination parameter is allowed to vary between items. Henceforth, the Item Characteristic Curve (ICC) of the different items can intersect and have different slopes. The steeper the slope, the higher the discrimination of the item, as it will be able to detect subtle differences in the management ability of the respondents. The difficulty parameter reflects how difficult it was for an individual to adopt the appropriate management practice (a high difficulty parameter would indicate that relatively few individuals adopted this practice).

A single composite trait (latent variable) called theta (θ) was used for description or analysis of the ability of person. Predicted values of theta were computed for each respondent based on their aggregate response to the practice questions. Inferential statistics (Mann–Whitney *U*-test) was used to compare the mean values of the predicted thetas across farm and socio-economic characteristics. A $p < 0.05$ was taken as significant for Mann–Whitney *U*-test.

Data was analyzed using Stata software version 14 (Texas, USA).

RESULTS

Sociodemographic and Farm Characteristics

Sociodemographic and farm characteristics are summarized in **Table 1**. Most of the respondents had long experience in keeping livestock but more than half of them reported that they had never been to school (**Table 1**).

Cattle and sheep were the main livestock species raised by the majority of the respondents (**Table 1**). The majority of the respondents had mixed type of livestock business with more than three livestock species kept at their farm. Only 16% of the respondents reported to have hired workers on the farm. The main income source for the households was most commonly small ruminant production and crop farming. Most of the respondents reported selling live animals, while sale of milk appeared to be less common. About 50% of respondents reported drinking cow or goat milk at least once per day. Children below 12 years of age were mentioned as the primary milk consumers by the family members in the 69.3% of the surveyed households.

Animal Diseases and Mortality

Table 2 summarizes the type of reported diseases in the past 12 months. Respiratory diseases were the most commonly mentioned diseases in cattle, sheep, and goat, followed by enteric illnesses. In addition, the proportion of respondents reporting an estimated mortality rate of more than 10% are included in **Table 2**.

Drug Use

From the livestock species present, livestock owners used drugs mostly for sheep, cattle, and goats. There was a difference in the type of drugs used between agro-ecological zones and production systems (**Table 3**). In the highland mixed crop livestock system, the most frequently reported use drugs were anthelmintics (95%), antibiotics (24%), and acaricides (4.7%). Pastoralists mostly used antibiotics (86.7%) followed by anthelmintics (70.8%) (**Table 3**). The proportion of anthelmintics usage was higher in highland mixed crop-livestock and pastoral than in the lowland crop-livestock system. The use of acaricides was less common compared to the use of other drugs in all agroecologies and production systems studied. Moreover, only 13% of the pastoralists did not have any antibiotic at hand during the survey. Drugs at hand were mostly stored under suboptimal conditions and exposed to change of temperature, sunlight, and dust. Human preparation antibiotics (tetracyclines) were also being used for veterinary purposes by 18.5% of pastoralist households, indicating high level of crossover use. Overall, tetracyclines (36.4%), aminoglycosides (31.3%), and trimethoprim-sulfonamides (6.2%) were the most frequently used classes of antibiotics across the study sites. Benzimidazoles (49.5%) were the most frequently used anthelmintic drugs followed by macrocyclic lactones (29.9%) and triclabendazole (24.6%). Triclabendazole and fenbendazole were only reported from highland mixed crop-livestock systems (**Table 4**).

Reasons for Use of Antimicrobials

Use of antimicrobials for prophylactic purposes was common. For the most frequently used drugs over the 12 months prior to the survey, antibiotics were mainly used for treatment purposes, whereas anthelmintics were used for disease prevention and livestock fattening purposes (**Figure 1**). Respiratory diseases and digestive/internal parasitic infections were the main reasons for therapeutic use of antimicrobials.

Access and Source of Veterinary Drugs

Overall, about 81.6% of livestock owners surveyed had access to veterinary drugs, although access varied between agro-ecological zones and production systems. Farmers in the highland mixed crop-livestock systems and the lowland pastoral systems reported access to veterinary drugs (97.7 and 93.3%, respectively), while the corresponding figure for livestock owners in the lowland mixed crop-livestock systems was 54%. The main source of veterinary drugs for livestock owners in both the highland and lowland mixed crop-livestock systems was the government or official veterinarian, whereas pastoralists most commonly accessed drugs from private suppliers (**Figure 2**).

Source of Information and Advice

Almost all respondents in the highland (99%) and 82% of respondents in the lowland mixed crop-livestock systems revealed that they received information and advice on veterinary

TABLE 1 | Household demographics and farm characteristics from a study of antimicrobial use in 374 households in 12 villages in six districts within three agro-ecological zones in Ethiopia.

Categorical variable	Category	Highland mixed crop-livestock (n = 128)		Lowland mixed crop-livestock (n = 126)		Mid/lowland pastoral (n = 120)		Total (n = 374)	
		n	%	*n*	%	*n*	%	*n*	%
Sex of the household head	Male	116	90.6	117	92.9	105	87.5	338	90.4
	Female	12	9.4	9	7.1	15	12.5	36	9.6
Sex of respondent	Male	109	85.2	105	83.3	83	69.2	297	79.4
	Female	19	14.8	21	16.7	37	30.8	77	20.6
Age of respondent	≤25	18	14.1	8	6.3	25	20.8	51	13.6
	25–55	90	70.3	95	75.4	67	55.8	252	67.4
	≥ 55	20	15.6	23	18.3	28	23.3	71	18.9
Education level	Never went to school	11	5.6	92	24.6	92	24.6	195	52.1
	Primary school	62	53	30	25.6	25	21.4	117	31.3
	Secondary school/College	55	88.7	4	6.5	3	4.8	62	16.6
Illiteracy level	Female	5	26.3	21	0	35	94.5	61	79
	Male	6	5.5	71	67.6	57	68.7	134	45
Type of livestock species	Cattle	128	100	115	91.3	110	91.6	353	94.39
	Sheep	127	99.2	110	87.3	115	95.8	352	94.12
	Goat	21	16.4	124	98.4	117	97.5	262	70.05
	Poultry	122	95.3	65	51.6	79	65.8	266	71.12
	Equine	111	86.7	119	94.4	40	33.3	271	72.45
Livestock species mix	Keep >3 species	116	90.6	100	79.4	86	71.7	302	80.75
	Keep ≤3 species	12	9.4	26	20.6	34	28.3	72	19.25
Hired worker on the farm	Yes	4	3.1	54	42.9	1	0.8	59	15.8
	No	124	96.9	72	57.1	119	99.2	315	84.2
Main income source for the household	Crop farming	90	70.3	36	28.6	47	39.2	173	46.3
	Cattle keeping	1	0.8	4	3.2	11	9.2	16	4.3
	Small ruminants	34	26.2	84	66.7	59	49.2	177	47.3
	Other	3	2.3	2	1.6	3	2.5	8	2.1
GRAZING MANAGEMENT									
Cattle beef (n = 353)	Zero grazing	65	50.5	33	28.7	0	0	98	27.7
	Fenced individual farm grazing	27	21.1	2	1.7	0	0	29	8.2
	Communal grazing	12	9.4	78	67.8	0	0	90	25.5
	Pastoral	0	0	2	1.7	88	80	90	25.5
Cattle dairy (n = 353)	Zero grazing	4	3.1	7	6.1	0	0	11	3.1
	Fenced individual farm grazing	69	19.5	3	0.8	0	0	72	20.4
	Communal grazing	55	43	104	90.4	0	0	159	45
	Pastoral	0	0	1	0.9	110	100	111	31.4
Small ruminant (n = 371)	Zero grazing	1	0.8	3	2.4	0	0	4	1.1
	Fenced individual farm grazing	63	49.6	3	2.4	0	0	66	17.8
	Communal grazing	63	49.6	118	94.4	1	0.8	182	49.1
	Pastoral	0	0	1	0.8	118	99.2	119	32.1
Poultry (n = 266)	Free range	116	95.1	27	41.5	79	100	222	83.5
	Housed	6	4.9	38	58.5	0	0	44	16.5
Equine (n = 271)	Zero grazing	0	0	17	15.5	0	0	17	6.3
	Fenced individual farm grazing	62	51.7	3	2.7	0	0	65	24

(Continued)

TABLE 1 | Continued

Categorical variable	Category	Highland mixed crop-livestock (*n* = 128)		Lowland mixed crop-livestock (*n* = 126)		Mid/lowland pastoral (*n* = 120)		Total (*n* = 374)	
		n	%	*n*	%	*n*	%	*n*	%
	Communal grazing	57	47.5	88	80	0	0	145	53.5
	Pastoral	1	0.8	2	1.8	41	100	44	16.2
Sale of milk	Yes	3	2.3	7	5.6	38	31.7	48	12.8
	No	125	97.7	119	94.4	82	68.3	326	87.2
Sale of live animals	Yes	128	100	125	99.2	117	97.5	370	98.9
	No	0	0	1	0.8	3	2.5	4	1.1
Continuous Variable		mean	sd	mean	sd	mean	sd	mean	sd
Size of the household		5.2	1.8	6.3	2.1	7.3	2.8	6.25	2.4
Age of respondent		39.9	12.9	41.7	12.1	40.9	16.4	40.9	13.9
Year of livestock keeping experience		19.9	11.4	20.7	11.1	22.5	15.2	21	12.7
Flock size	Cattle	4.5	1.6	4.9	5.7	15.9	20.5	8.3	13.2
	Sheep	18.8	12.1	16.8	16.1	26.2	39.5	20.5	25.5
	Goat	0.5	1.2	30.6	25.1	32.7	33.5	21	28
	Poultry	5.5	4.9	6.4	4.3	7.3	3.9	6.3	4.6
	Donkey	1.7	0.8	1.5	0.7	1.7	1.6	1.6	0.9

TABLE 2 | Owner reported occurrence of animal diseases from a total of 374 households in Ethiopia.

Disease	Cattle (*n* = 350)		Sheep (*n* = 352)		Goat (*n* = 262)	
	n	%	*n*	%	*n*	%
Respiratory diseases	95	26.9	147	41.7	100	38.2
Digestive tract/enteric illnesses	57	16.2	86	24.4	84	32.1
Reproductive diseases	2	0.6	5	1.42	5	1.9
Sudden death	6	1.7	8	2.3	5	1.9
Skin disease	17	4.8	2	0.6	1	0.4
Gastro-intestinal parasites	8	2.3	2	0.6	0	0
Neurological	0	0	17	4.9	20	7.6
Systemic disease	17	4.9	0	0	0	0
Other	16	4.57	11	3.1	9	3.44
No disease	132	37.4	73	20.7	38	14.5
Mortality >10%	34	9.7	131	37.2	121	46.2

drug use from a range of sources: veterinarians and animal health workers (78.3% of respondents), drug stores (9.4%), markets (2.7%), and other farmers (8.6%). Among the pastoralists, 74% reported not to depend on any of these sources and reported to commonly decide based on their own judgement on the kind of drugs to use, dose, and treatment duration.

KAP Related to Antimicrobial Use, Resistance, and Residue

Regarding the knowledge about antibiotic use, 84.2% of respondents were well aware that antibiotics are useful for treating and preventing infections. However, more than 50% of the respondents had inadequate understanding of antibiotics and they thought antibiotics could help to treat any kind of diseases, regardless of the cause. Moreover, a relatively high proportion of the respondents (>70%) were not aware of the recommended withdrawal periods of milk and meat after antibiotic treatment. Only 20% of livestock owners reported to have heard about antimicrobial resistance and at least 12% mentioned that they had experienced situations where drugs did not work.

About 82% of the respondents were aware that vaccines are generally administered as a preventive measure against infections. There was variation in livestock owners' knowledge of antibiotics between the different agro-ecological zones and production systems (**Table 5**).

Regarding the attitudes and perceptions related to antimicrobial use, around 50% stated that they would use antimicrobials more often if antimicrobials were more accessible and cheaper. It was noticed that around 80 and 70% of respondents had a tendency to use doses that were higher or lower than recommended for their animals during treatment, respectively.

About 69% were of the opinion that once the animal started to recover, there was no need to continue giving the full treatment course. Around 21.7% of the respondents had a tendency of keeping leftover antimicrobials at home, as they might be useful in the future (**Table 6**).

Regarding practices related to antimicrobial use (**Table 7**), a large proportion of the respondents reported that they commonly consumed milk (36.4%) and meat (51.8%) from animals that had just been treated with antimicrobials, although they assumed it might not be good for human health. The majority of pastoralists

TABLE 3 | Self-reported antimicrobial use from a total of 374 households in 3 agro-ecological zones in Ethiopia.

	Highland mixed crop-livestock ($n = 128$)		Lowland mixed crop-livestock ($n = 126$)		Mid/lowland pastoral ($n = 120$)		Total ($n = 374$)	
	Freq	%	Freq	%	Freq	%	Freq	%
Antibiotics	31_a	24.2	29_a	23	104_b	86.7	164	43.9
Anthelminthics	122_a	95.3	40_b	31.6	85_c	70.8	247	66
Acaricides	4_a	3.1	1_a	0.8	36_b	30	41	10.9

Each subscript letter denotes a subset of agro-ecological zones whose column frequency does not differ significantly from each other at the 0.05 level.

TABLE 4 | Common antimicrobial groups used by farmers from a total of 374 households in three agro-ecological zones in Ethiopia.

	Highland mixed crop-livestock ($n = 128$)		Lowland mixed crop-livestock ($n = 126$)		Mid/lowland pastoral ($n = 120$)		Total ($n = 374$)	
	n	%	*n*	%	*n*	%	*n*	%
Classes of antibiotics								
Tetracyclines	20_a	15.6	26_a	20.6	90_b	75	136	36.4
Trimethoprim-sulfonamides	3_a	2.34	1_a	0.79	20_b	16.67	24	6.24
Penicillins	0_a	0	0_a	0	5_b	4.17	5	1.34
Macrolides	0_a	0	0_a	0	17_b	14.17	17	4.55
Aminoglycosides	18_a	14.1	3_b	2.4	96_c	80	117	31.3
Groups of antihelimintics								
Albendazole/benzimidazole	84_a	65.6	40_b	31.8	61_c	50.8	185	49.5
Triclabendazole	92_a	71.9	0_b	0	0_b	0	92	24.6
Fenbendazole	6_a	4.69	0_b	0	0_b	0	6	1.6
Ivermectin (Macrocyclic lactones)	37_a	28.9	1_b	0.79	74_c	61.7	112	29.9
Imidazothiazole (Tetramizole, Tetraclozan, Clozasole)	65_a	50.8	1_b	0.8	0_b	0	66	17.6

Each subscript letter denotes a subset of agro-ecological zones whose column frequency does not differ significantly from each other at the 0.05 level.

(88.6% consumed milk and 98.3% consumed meat) reported this practice.

Overall, the majority of the respondents (70%) administered antibiotics as advised, but 72.3% of pastoralists administered antibiotics by not following through the full treatment course: "until the animal cured," "until package empty," "as long as they can afford," "one time treatment or continuously over extended period." All pastoralists self-administered antibiotics to their animals without any laboratory diagnosis. About 98% of pastoralists had good practice with regard to care of expired veterinary drugs, which they either disposed of by burying or returning to the vendor. Indeed, during data collection, 97% of the pastoralist households did not have any expired antimicrobial at hand.

Half of the respondents (50%) reported to have an isolation pen for sick animals and 40% indicated that they would allow animals currently receiving treatment to immediately freely graze with other animals without quarantine. Only 9% of the respondents implemented proper practices regarding disposal of dead animals, either through burial or incineration. The majority (97.5%) of the pastoralists and 4% of respondents from each of the highland and lowland mixed crop production systems revealed consumption of dead animals.

Assessment of the KAP Measurement Scales

Cronbach's alphas were poor for the knowledge (0.478) and attitude (0.319) scales, and the inter-item correlations were low. But the Cronbach's alpha was high for practice scale (0.816). "P4" from the practice scale presented a negative biserial coefficient and was therefore excluded from further analyses. The factor and correspondence analysis suggested that the knowledge and attitude scales were not unidimensional, and consequently, these scales were not used to develop IRT models. Based on a factor analysis of the practice scale, the assumption of unidimensionality seemed to be met. The first eigenvalue was 15 times larger than the second and accounted for 97% of the total variation.

The discrimination (a_i) and difficulty (b_i) parameters from the IRT analysis of the practice scale are presented in **Table 8**.

Most of the practice items have a similar discrimination level and a similar low level of difficulty except for the item "P8" with higher difficulty ($b_i = 2.59$), but low discrimination ($a_i = 1.66$). Items "P5" ($a_i = 4.53$) and "P9" ($a_i = 4.47$) had relatively high discrimination power, whereas "P7" had very low discrimination (a_i =0.59), suggesting that it contributed little to the scale (**Table 8**). On the basis of all this information, it appears that we

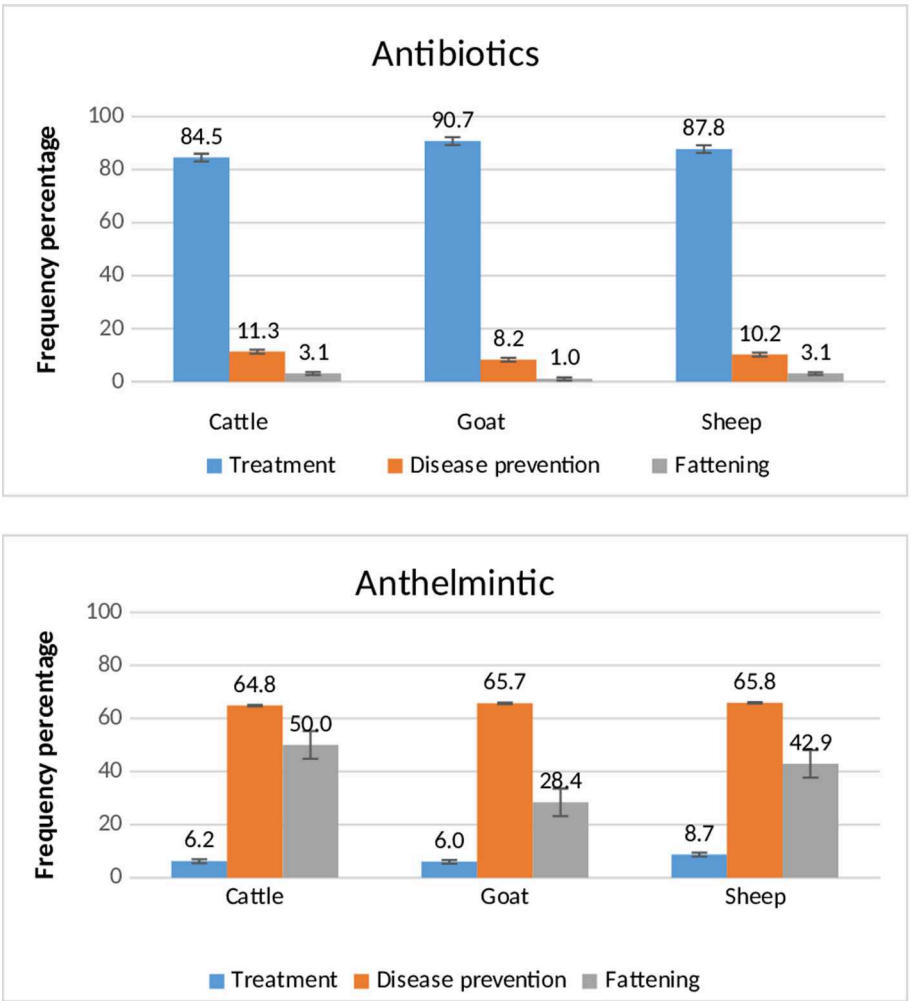

FIGURE 1 | Reason for the use of antibiotics and anthelmintics in different species reported by livestock owners from 374 households in 3 agro-ecological zones in Ethiopia (Frequency percentage and standard error bars).

can make a useful, unidimensional seven-item scale (P1, P2, P3, P5, P6, P8, and P9) (**Figure 3**). Therefore, the scale was able to differentiate among people with a level management expertise of theta between −1.5 to 1 (**Figure 4**), respectively, answering between 0 and 7 questions correctly.

Association of Household Demographics and Farm Characteristics, With Desirable Practices

A single composite trait or variable called theta (θ) was used to characterize the ability of person to perform desirable practices instead of a descriptive summative scale for practice. The composite variable provides an overall estimate of the quality being measured (management ability of person). It takes into account the difficult and discrimination values for each item and hence is a more reliable overall measure than a simple sum of the individual items in the scale. For each respondent, a theta (θ) score was computed and the mean theta of different groups, based on farm and socio-economic characteristics, were compared. Higher means indicated better desirable practice in a specific group of respondents (**Table 9**).

Among the variables, there were significant differences in the mean theta for agro-ecology/production system, education level, having hired workers on the farm, having more than 3 different livestock species, and household size ($p < 0.05$) (**Table 9**). Respondents from highland mixed crop livestock production system had a higher mean for theta than those of lowland mixed crop livestock and pastoral production system. However, there were no significant differences in the mean theta according to age group of the respondents or their livestock keeping experience.

DISCUSSION

Antimicrobial resistance (AMR) has been recognized as a global health problem. Monitoring of antimicrobial use (AMU) provides useful information for policy development to mitigate AMR risks and therefore has been recommended by international organizations (6, 7, 9).

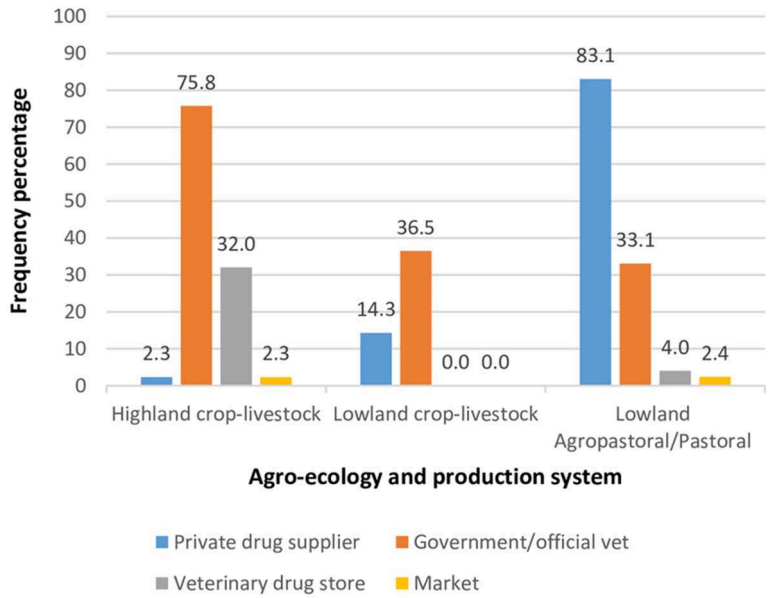

FIGURE 2 | Source of veterinary drugs used by 374 households in 3 agro-ecological zones and production systems in Ethiopia.

TABLE 5 | Knowledge about antibiotic use, resistance and residue ($n = 374$).

Questions	Levels	Responses	Highland crop-livestock ($n = 128$)		Lowland crop-livestock ($n = 126$)		Mid/lowland pastoral ($n = 120$)		Overall	
			freq	%	freq	%	freq	%	freq	%
K1_What does vaccination do?	Correct	Prevent animals from becoming sick	108_a	84.4	102_a	80.9	100_a	83.3	310	82.9
	Incorrect		20	15.6	24	19.1	20	16.7	64	17.1
K2_What do antibiotics do?	Correct	Cure sick animals and prevent animals from becoming sick	89_a	69.5	114_b	90.5	112_b	93.3	315	84.2
	Incorrect		39	30.5	12	9.5	8	6.0.7	59	15.8
K3_For how long should milk be avoided (in days) immediately after treatment of animals with antibiotics?	Correct	7–30 days depending on the label, as advised	81_a	63.3	20_b	15.9	2_c	1.7	103	27.5
	Incorrect		47	36.7	106	84.1	118	98.3	271	72.5
K4_For how long should meat be avoided (in days) immediately after treatment of animals with antibiotics?	Correct	7–30 days depending on the label, as advised	68_a	53.1	40_b	31.8	0_c	0	108	28.9
	Incorrect		60	46.8	86	68.2	120	100	266	71.1
K5_Have you ever heard about antimicrobial resistance?	Correct	Yes	38_a	29.7	14_b	11.1	$23_{a,b}$	19.2	75	20.1
	Incorrect	No	90	70.3	112	88.9	97	80.8	299	79.9
K6_Antibiotics help treat any kind of diseases.	Correct	No	95_a	74.2	46_b	36.5	43_b	35.8	184	49.2
	Incorrect	Yes	33	25.8	80	63.5	77	64.2	190	50.8

Each subscript letter denotes a subset of agro-ecological zones whose column frequency does not differ significantly from each other at the 0.05 level.

In Ethiopia, like other sub-Saharan countries, it is generally believed that antimicrobial agents are widely used in animal production systems; however, evidence on antimicrobial usage is limited and often anecdotal. We found only a single survey that evaluated the rational use of veterinary drugs, and it focused only on the college of veterinary medicine and agriculture veterinary teaching hospital and Ada district veterinary clinic of central Ethiopia (27).

This study characterized antimicrobial (includes anthelmintic) use knowledge, attitude, and practice in smallholder settings in three different agro-ecology and production system. To our knowledge, this study is the first

TABLE 6 | Attitudes and perceptions on antimicrobial use, resistance and residues ($n = 374$).

Questions	Levels	Responses	Highland crop-livestock ($n = 128$)		Lowland crop-livestock ($n = 126$)		Mid/lowland pastoral ($n = 124$)		Overall	
			freq	%	freq	%	freq	%	freq	%
A1_Is consuming milk or meat from animals who were just treated with antimicrobials good for human health?	Undesirable	Yes	2	1.6	12	10.3	8	7	22	6.2
	Desirable	No	125	98.4	104	89.7	106	93	335	93.8
A2_If antimicrobials were more accessible and at a lower price, would you use antimicrobials more often?	Desirable	No	67	52.3	79	62.7	44	36.7	190	50.8
	Undesirable	Yes	61	47.7	47	37.3	76	63.3	184	49.2
A3_To get a better response, I sometimes give more antimicrobials to animals than the dose advised by the veterinary clinician or pharmacist.	Desirable	Strongly disagree, disagree	8	6.3	49	41.2	14	12.8	71	20
	Undesirable	Strongly agree, agree	119	93.7	70	58.8	95	87.2	284	80
A4_It is advisable to always reduce the amount/dose of antimicrobial advised by veterinary clinician to avoid harming animals.	Desirable	Strongly disagree, disagree	9	7.1	76	63.9	19	17.8	104	29.5
	Undesirable	Strongly agree, agree	118	92.9	43	36.1	88	82.2	249	70.5
A5_Once the animal starts to feel better, there is no need to continue giving the full dose.	Desirable	Strongly disagree, disagree	8	6.3	21	17.2	81	75	110	30.8
	Undesirable	Strongly agree, agree	119	93.7	101	82.8	27	25	247	69.2
A6_I normally keep leftover antimicrobials for a long time at home because they might be useful in the future.	Desirable	Strongly disagree, disagree	18	14.1	24	20	35	33	77	21.7
	Undesirable	Strongly agree, agree	110	85.9	96	80	71	67	277	78.3

to investigate antimicrobial usage in livestock by smallholder farmers and pastoralists in Ethiopia. Most of the respondents were adults with many years of experience in keeping livestock.

We found that the use of antimicrobial agents in livestock production was very common among the livestock producers in the study areas. Antimicrobial use may vary widely between and within countries, species, production systems, and individual farms (28). This is also what we found in our study. The data on use of antimicrobial agents were not restricted to any particular livestock species but cut across mainly three livestock species (cattle, sheep, and goat) and equine and poultry in few cases. We observed large variation in the choice of drugs and proportion of respondents who had used antimicrobials among smallholder farmers in the three agro-ecology and production systems included in the study.

Livestock producers in mid/lowland pastoral systems appeared to use antibiotics more frequently than their counterparts in highland and lowland mixed crop-livestock systems. Tetracyclines, aminoglycosides, and trimethoprim-sulfonamides were the most dominantly used classes of antibiotics. Penicillins and macrolides were only reported to be used by the pastoral production systems. This is consistent with studies elsewhere that reported these antimicrobials to be frequently used in food animals in Africa (16, 27, 29–33). The penicillin, tetracycline, and aminoglycoside classes were also the most commonly reported antimicrobial usages across pig production systems in Thailand and Vietnam (34).

Despite known deficits in animal health services in Ethiopia, the livestock owners had good access to veterinary drugs. The main source of veterinary drugs in both the highland and lowland mixed crop-livestock systems was the government or official veterinarians, whereas the pastoralists most commonly accessed drugs from private suppliers. The study found that farmers tended to give higher or lower doses of antimicrobials to their animals than recommended. Medically irrational use of antimicrobials in food animals is known to contribute to the emergence, persistence, and spread of resistant bacteria from animals to humans (7). Regarding information and advice on antimicrobial usage in livestock, this study found that a high proportion of the pastoralists rely on their own judgment. Hence, it was not surprising that we found high levels of potentially wrong use of antibiotics. Access to antimicrobials without prescriptions results in increased risk for antimicrobial resistant pathogens, which has also been shown elsewhere in Africa (16, 30).

The inappropriate antimicrobial use by pastoralists might be linked with this ease of access and inadequate advice for farmers (35). Restricting access to antimicrobials by removing

TABLE 7 | Antibiotic use and related practices ($n = 374$).

Questions	Levels	Responses	Highland crop-livestock ($n = 128$)		Lowland crop-livestock ($n = 126$)		Mid/lowland pastoral ($n = 124$)		Overall	
			freq	%	freq	%	freq	%	freq	%
P1_Do you consume milk from animals who were just treated with antimicrobials?	Desirable	No	115	90.5	99	85.3	13	11.4	227	63.6
	Undesirable	Yes	12	9.5	17	14.7	101	88.6	130	36.4
P2_Do you consume meat from animals who were just treated with antimicrobials?	Desirable	No	102	80.3	68	58.6	2	1.8	172	48.2
	Undesirable	Yes	25	19.7	48	41.4	112	98.3	185	51.8
P3_How long do you use antibiotics in animals?	Desirable	As advised	127	100	99	79.2	33	27.7	259	69.8
	Undesirable	Until animal(s) cured; Until package empty; As long as I can afford; One time treatment; Continuously over extended period	0	0	26	20.8	86	72.3	112	30.2
P4_What do you do with expired veterinary drugs?	Desirable	Dispose of, Return to pharmacy; don't receive	117	91.4	56	45.5	118	98.3	291	78.4
	Undesirable	Give to other farmer; Use for intended treatment; Nothing	11	8.6	67	54.47	2	1.67	80	21.6
P5_How do you manage manure?	Desirable	Used as fertilizer; Use for fuel (incl. biogas); Sold for cash (fuel)	126	99.2	125	100	1	0.8	252	67.9
	Undesirable	Leave on farm; Open air; Discard into environment	1	0.8	0	0	118	99.2	119	32.1
P6_Do you have isolation pen for sick animals?	Desirable	Yes	87	68	72	57.1	21	17.5	180	48.1
	Undesirable	No	41	32	54	42.9	99	82.5	194	51.9
P7_Do you allow animals on treatment to immediately freely graze with other animals without quarantine for few days?	Desirable	No	97	75.8	66	52.4	62	51.7	225	60.2
	Undesirable	Yes	31	24.2	60	47.6	58	48.3	149	39.8
P8_What do you do if an animal dies from disease?	Desirable	Bury, burn	25	19.5	7	5.6	1	0.8	33	8.8
	Undesirable	Leave as it is; give to the dog; home consumption	103	80.5	119	94.4	119	99.2	341	91.2
P9_Who administers the antibiotics?	Desirable	Veterinarian; animal health practitioners	128	100	124	98.4	0	0	252	67.4
	Undesirable	Myself	0	0	2	1.6	120	100	122	32.6

over-the-counter sales has been identified as a potential route to better antimicrobial use in animals (6, 36).

Moreover, the reported frequent use of cow or goat milk in their meal coupled with a relatively high proportion of farmers not being aware of the recommended withdrawal periods of milk and meat after antibiotic treatment may lead to the potential hazard of repeatedly ingested residues altering the intestinal microbiome and promoting emergence and selection for resistant bacteria in the gastrointestinal tract of humans (37, 38). Withdrawal times are recommended in order to prevent the presence of drug residues in food products (39).

There is a possible risk of an infectious disease being transmitted from animals to human due to a habit of consumption of dead animals. The poor experience of isolating sick animals and improper disposal of dead animals by the majority of the farmers in this study illustrates the negligence of biosecurity practices and other precautionary measures to prevent infectious agents. However, infection prevention and control measures are crucial in order to reduce the incidence of infections and, therefore, reduce the need for antibiotics (40–42). Besides, the non-involvement of laboratory investigations in disease diagnosis prior to antimicrobial further fuels inappropriate use of antimicrobials, which may subsequently lead to the development and spread of AMR (43, 44), which definitely is a big challenge in Ethiopia.

TABLE 8 | Discrimination and difficulty values of the items in the practice scale (sorted by decreasing discrimination).

	Items	Coef.	Std. Err.	95% Conf. Interval	
Discrimination	P5_How do you manage manure?	4.53	0.58	3.379	5.685
	P9_Who administers the antibiotics?	4.48	0.58	3.334	5.685
	P1_Do you consume milk from animals who were just treated with antimicrobials?	3.51	0.51	2.492	4.52
	P2_Do you consume meat from animals who were just treated with antimicrobials?	3.18	0.49	2.196	4.154
	P3_How long do you use antibiotics in animals?	2.59	0.38	1.844	3.342
	P6_Do you have isolation pen for sick animals?	1.69	0.27	1.158	2.23
	P8_What do you do if an animal dies from disease?	1.67	0.92	−0.146	3.484
	P7_Do you allow animals on treatment to immediately freely graze with other animals without quarantine for few days?	0.59	0.12	0.344	0.853
Difficulty	P5_How do you manage manure?	−0.47	0.07	−0.611	−0.321
	P9_Who administers the antibiotics?	−0.45	0.07	−0.598	−0.308
	P1_Do you consume milk from animals who were just treated with antimicrobials?	−0.32	0.08	−0.478	−0.171
	P2_Do you consume meat from animals who were just treated with antimicrobials?	0.21	0.08	0.056	0.37
	P3_How long do you use antibiotics in animals?	−0.63	0.09	−0.814	−0.469
	P6_Do you have isolation pen for sick animals?	0.2	0.09	0.0153	0.396
	P8_What do you do if an animal dies from disease?	2.59	0.69	1.248	3,946
	P7_Do you allow animals on treatment to immediately freely graze with other animals without quarantine for few days?	−0.63	0.23	−1.092	−0.184

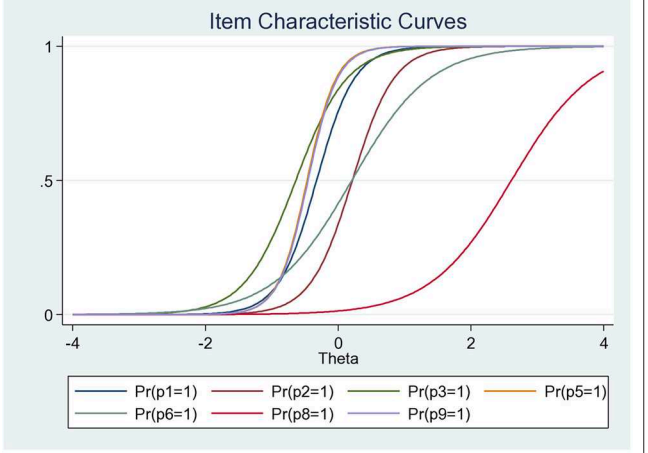

FIGURE 3 | Item characteristic curve for the 7 items used make up the scale related to antibiotics use practices.

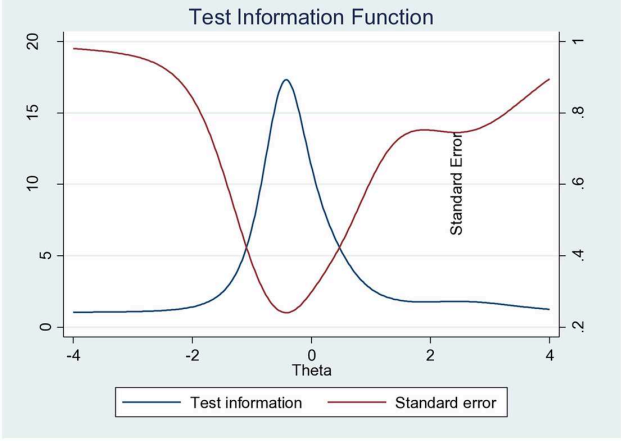

FIGURE 4 | Test information curve for the scale related to antibiotic use practices.

Despite the frequent use of antimicrobials by smallholder farmers to maintain good livestock health and production in the studied areas, there was overall poor knowledge about the purpose of antibiotics and their proper use. Poor knowledge may be the result of the fact that more than half of the smallholder farmers never went to school or have otherwise poor education. Farmers generally thought antibiotics could help treat any kind of diseases regardless of the causes. This could result in inappropriate antibiotic use with potential risks of antibiotic-resistant pathogens that will lead to treatment failures, increased mortality and production losses, and also possible human health risks (45). Founou et al. (46) also indicated that 86.6% of multidrug-resistant bacteria were detected in food animals at farms in Africa, which may be indicative of widespread use of antibiotics in farming practices, whereas 52.4% detected at abattoirs reflected bacteria surviving the processing stage and, therefore, able to reach the consumer.

Another finding of our study was the difference in the ability of respondents to give desirable response for practice on the basis of the agro-ecology and production system. Respondents

TABLE 9 | Comparison of household demographics and farm characteristics and ability to give desirable response for practice questions.

Description	*N* (374)	Theta Mean (SE)
Agroecology and production system		
Highland mixed crop-livestock production system	128	$0.84\ (0.04)_a$
Lowland mixed crop-livestock production system	126	$0.45\ (0.04)_b$
Pastoral/agro-pastoral production system	120	$-1.15\ (0.02)_c$
Sex of respondent		
Male	297	$0.13\ (0.05)_a$
Female	77	$-0.14\ (0.11)_a$
Education		
Never went to school	195	$-0.28\ (0.06)_a$
Primary school	117	$0.28\ (0.08)_b$
Secondary school/College	62	$0.78\ (0.08)_c$
Age		
Young (<30)	84	$-0.04\ (0.10)_a$
Medium (30–50)	204	$0.13\ (0.06)_a$
Old (>50)	86	$0.02\ (0.10)_a$
Livestock experience		
<= 5 year	30	$0.19\ (0.18)_a$
5–20	184	$0.02\ (0.07)_a$
>20 year	160	$0.10\ (0.07)_a$
Household size		
Small (<4 person)	37	$0.46\ (0.15)_a$
Medium (4–8 person)	279	$0.14\ (0.05)_a$
Large (>8 person)	58	$-0.55\ (0.12)_b$
Species mix		
3 and less species	72	$-0.29\ (0.11)_a$
More than 3 species	302	$0.16\ (0.05)_b$
Hired worker		
Yes	59	$0.62\ (0.06)_a$
No	315	$-0.03\ (0.05)_b$

Each subscript letter denotes a subset of agro-ecological zones whose column frequency does not differ significantly from each other at the 0.05 level (Mann–Whitney test).

from highland mixed crop livestock production systems were more likely to have higher ability to give desirable response for practice questions than those in lowland mixed crop livestock and pastoral production systems. There was a link between better ability of a person to perform desirable practices and higher education level. Besides the education barrier, limited professional supervision can also have an impact on a farmer's practice as reflected in the pastoral production system. Wrong public perception, attitudes, and beliefs about antibiotics are strong determinants of medically irrational use of antibiotics (43). It has been suggested that increasing knowledge and awareness about antibiotics and antibiotic resistance are key components of rational antibiotic use in human medicine (6, 7). While improving the knowledge and attitudes of smallholders can encourage them to practice medically rational use of antimicrobials, addressing the drivers for use is as important to achieve lasting behavior change.

IRT methods allow researchers to improve measurement scale construction and evaluate the quality of individual items. In this study, the 2PL logistic models fitted practice scales reasonably well. The Cronbach's alpha for knowledge and attitude scales was low, reflecting that the items were not internally consistent. But the corresponding value yielded for practice was high. The likely reason for low reliability may be that knowledge and attitude measurement items cover different dimensions like purpose of antimicrobial use, disease prevention, antimicrobial residue, and biosecurity issues and evaluate different concepts. The implications of these findings are that future research should focus on assessment of more extensive knowledge and attitude measurement scales toward AMU, AMR, and residues. Approaches that identify the quality of individual items that specifically measure one thing at a time for the knowledge and attitude scale construction should be attempted. The focus should be on the coverage of the content the instrument is supposed to measure. It is also necessary to include new items with high discrimination of knowledge and attitude and greater accuracy of measurement.

Findings of this study help to target future interventions to reduce antimicrobial use and resistance in the smallholder livestock systems of Ethiopia. While it is impossible to extrapolate data from this study to other sub-Saharan African countries, tools and methods used here can easily be applied elsewhere.

LIMITATION OF THE STUDY

Though the study was piloted with 40 participants, there was no instrument to objectively assess the honesty and recall ability of the participants. The training of enumerators on data collection and use of a demonstration box with drugs to facilitate the enumerator in gathering antimicrobial usage information helped to reduce this possible bias. In addition, as with most surveys, there is the possibility of social desirability bias that respondents may be over- or underreporting antimicrobial use.

The scale used to assess the knowledge and attitudes regarding antimicrobial use consists of a mix of different topics like disease prevention, drug residue, and biosecurity. The number of questions was also minimized with the intention of reducing the time taken to complete the questionnaire.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

ETHICS STATEMENT

Ethics approval (Certificate Ref. No: VM/ERC/01/07/10/2018) was obtained from the Addis Ababa University, College of Veterinary Medicine and Agriculture Animal Research Ethics Review Committee, and the Institutional Research Ethics Committee of the International Livestock Research Institute (ILRI-IREC2018-24). Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.

AUTHOR CONTRIBUTIONS

BG, BW, KA, and UM conceived and designed the study. BG, BW, HD, and GA followed up and monitored data collection. BG, ID, and GH analyzed the data. BG, BW, ID, GH, and KA conceptualized and drafted the paper. All authors read, commented on, and approved the final manuscript.

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Effects of Tilmicosin Treatment on the Nasopharyngeal Microbiota of Feedlot Cattle With Respiratory Disease During the First Week of Clinical Recovery

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While the nasopharyngeal (NP) microbiota is believed to be a key player in bovine respiratory health, there is limited published information about the change of NP microbiota associated with clinical recovery from bovine respiratory disease (BRD). The objective of this study was to evaluate the effect of tilmicosin treatment on the NP microbiota composition and diversity of BRD-affected calves during the first week of clinical recovery. Deep NP swabs were collected from diseased calves at the initial diagnosis of BRD, and again 7 days after the administration of a single dose of tilmicosin. As an experimental control, samples were collected from clinically healthy, pen-matched calves at the time of initial BRD diagnosis. In general, the NP microbiota from the control calves were more diverse than the NP microbiota from tilmicosin treated and BRD-affected calves. Principle coordinate analysis (PCOA) of Bray-Curtis and Jaccard dissimilarity also revealed that the overall composition of NP microbial communities in tilmicosin-treated calves closely resembled that of BRD-affected calves but differed significantly from pen-matched healthy calves. Overall, it appeared that there were only minor changes in NP microbial communities following tilmicosin treatment and, during the early phase of clinical recovery the NP microbiota in treated animals was disparate from that observed in healthy control calves. Understanding the potential impact of this prolonged recovery in mucosal microbiota would be important in optimizing the use of antimicrobials in health management programs in the feedlot industry.

Keywords: respiratory disease, feedlot, microbiota, tilmicosin, 16S rRNA gene

INTRODUCTION

Bovine respiratory disease is a common and costly health failure associated with a polymicrobial infection often occurring in newly transported feedlot cattle (1, 2). Various predisposing factors, such as neurohumoral stress, nutritional changes, environmental conditions, and upper respiratory mucosal damage from viral and bacterial pathogens have been implicated in the pathogenesis of BRD (3, 4). Clinical research shows that the most common bacterial pathogens associated

with BRD are known to be normally transient residents of the upper respiratory tract of healthy cattle that become opportunistic pathogens when viral infection and various management stressors combine to impair the host immune system (5, 6). The careful use of antimicrobials for prophylaxis, metaphylaxis and therapy has offered significant advances in BRD management in high-risk cattle (3). Tilmicosin is a long-acting macrolide with strong bactericidal action that can protect the cattle against BRD pathogens for up to 7 days (7) and, given its favorable bioavailability and broad efficacy against many BRD pathogens, is a popular option for the treatment and prevention of BRD (8). Feedlot cattle at high-risk of developing respiratory disease, and treated with tilmicosin, showed a distinct shift in the composition of NP microbiota during the first 10 days after arrival on the farm (7). Tilmicosin also markedly reduced the prevalence of microbes in the nasal secretions of BRD-affected calves for up to 6 days compared to control calves (9). With increasing concerns regarding the overall efficacy of current antibiotic treatment approaches and the growing emergence of antimicrobial resistance (10), new management strategies for optimizing mucosal health and immune defenses are required. Moreover, understanding the impact of infectious disease processes and antimicrobial agents on the respiratory microbial ecosystem is important clinically, since these communities appear to have a crucial role in maintaining mucosal health (11). The objective of this study was to evaluate the effects of tilmicosin treatment on the composition and diversity of the NP microbiota of BRD-affected calves during the first week of clinical recovery.

MATERIALS AND METHODS

Study Populations and Sample Collection

This study was a part of a larger experiment that examined the clinical and microbial predictors of susceptibility to BRD in beef cattle (12, 13). The use of the animals, and all experiments procedures were performed in accordance with relevant guidelines, and under the approval of, the University of Illinois Institutional Animal Care and Use Committee (IACUC Protocol: #15064). Briefly, a total of 135, 6 to 8-month-old, single source, Charolais feedlot calves (mean entry body weight 247 ± 33.8 kg) from the commercial and research university feedlot at South Farms Beef cattle and Sheep Field Laboratory (Urbana, IL, USA) were involved in this study. All calves were processed within 24 h after arrival to the farm. During the first month after arrival, all calves were monitored daily for signs of respiratory disease according to industry-standard protocols (anorexia, nasal discharge, change in respiratory pattern, rectal temperature $\geq$ 40°C and Whisper lung score $\geq$ 3) (12). Deep NP samples were collected with double-guarded sterile culture swab (Kalayjian Industries, Inc. U.S.A.) from calves diagnosed with BRD at the initial diagnosis and prior to treatment (BRD group, $n = 9$) according to published techniques (14). Equivalent NP samples were collected from clinically healthy, pen-matched controls calves (control group, $n = 9$) at the same time as the BRD-affected calves sampling. The BRD-affected calves were treated with a single dose of tilmicosin (10 mg/kg SC; Micotil, Elanco Animal Health) according to label instructions. Immediately after sample collection and treatment, each of the calves were returned to their original group pen. At day 7 post tilmicosin treatment, follow-up clinical examinations of the BRD-affected calves was performed, and disease recovery was characterized by the absence of respiratory signs, rectal temperature $\leq$ 39°C and Whisper lung score $\leq$ 2. A deep NP swab was collected from each tilmicosin-treated calf at this time (post-treatment group, $n = 9$). Following collection, all NP swabs ($n = 27$) were held on dry ice and transported to the laboratory where they were stored at −20°C pending further processing.

DNA Extraction, 16S rRNA Gene Sequencing and Bioinformatics

Extraction of DNA was performed from all NP swabs using power® Fecal DNA isolation Kit (MO BIO Laboratories, Inc., Carlsbad, CA) following the manufacturer's instructions (15). Total DNA concentration and purity were evaluated by optical density using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Rockland, DE, USA) at the wavelengths of 230, 260, and 280 nm, and the OD260/280 ratio of DNA ranged between 1.75 and 1.90. Genomic DNA was then transferred to the DNA Services lab at the W. M. Keck Center for Comparative and Functional Genomics at the University of Illinois at Urbana-Champaign for amplification and sequencing. The V1-V3 hypervariable regions of 16S ribosomal ribonucleic acid (rRNA) were amplified by Fluidigm access array amplification protocol (Fluidigm Corporation, South San Francisco, CA, USA) using the primer set F28-2-for (ACACTGACGACATGGTTCTACA) and R519-2-rev (TACGGTAGCAGAGACTTGGTCT) tagged with unique eight-base sequence barcodes. PCR reactions were performed on a Fluidigm Biomark HD™ PCR machine (Fluidigm Corporation, South San Francisco, CA, USA) using the default Access Array cycling program without imaging (**Table S1**). The final harvested products were quantified on a Qubit fluorometer and assessed using a Fragment Analyzer (Advanced Analytics, Ames, IA, USA) to confirm amplicon regions and sizes. The final pooled Fluidigm libraries were sequenced on the Illumina Miseq V2 platform according to the manufacturer's instructions (Illumina, San Diego, CA, USA).

The 16S rRNA gene sequences data obtained from the MiSeq sequencing were processed and analyzed with the Quantitative Insights Into Microbial Ecology (QIIME) algorithms using an operational taxonomic units (OTUs) approach (16). Sequences were quality-filtered using established guidelines (17). The open-reference OTU selection protocol (97% similarity) was conducted by QIIME using UCLUST clustering (18) and assigned taxonomy against SILVA reference database. Low abundance clusters and chimeric sequences were filtered and removed using USEARCH (19). Bacterial taxa that could not be assigned to a genus level, but were present in all NP samples, were still displayed based on the lowest taxonomic level that could be assigned to them. For subsequent bacterial diversity analysis, the OTUs table was randomly subsampled and rarefied to 3,037 sequences per sample using QIIME pipeline. The alpha

diversity indices were estimated using the Chao1 richness, phylogenetic diversity (PD) whole tree and Shannon diversity indices. Fastq data obtained in the current study were uploaded to the sequence read archive on the NCBI website to make the files available for public databases with a bio-project accession number PRJNA508519.

Statistical Analysis

Statistical analysis and graphing were performed using PAST version 3.13 and JMP® Pro 13 (SAS Institute Inc. Cary, NC, USA). For comparisons between the three groups, one-way ANOVA with all pair's comparisons using Tukey-Kramer HSD test were used to analyze data with a normal distribution and nonparametric Wilcoxon comparisons for each pair was used to analyze data that did not meet the assumptions of ANOVA. Differences between groups with $P < 0.05$ were considered statistically significant. A principal coordinate analysis (PCoA) of Bray-Curtis and Jaccard dissimilarity were performed on all samples using the relative abundance of higher taxonomic level taxa, and the significant difference between groups was analyzed using non-parametric multivariate analysis of variance (PERMANOVA) with 9999 permutations and Bonferroni corrected *P*-values in PAST version 3.13. To further quantify the overall microbial composition similarities between the different groups, unweighted pair group method with arithmetic mean (UPGMA) based on Bray–Curtis distance metrics were performed in PAST version 3.13. Finally, the Venn diagram representing the number of core shared microbiota between groups was generated.

RESULTS AND DISCUSSION

Overall Taxonomic Classification and Diversity of NP Microbiota

The composition and function of the respiratory microbial ecosystem is an extensive field of research (11, 20). The nasopharyngeal microbiota is believed to be a key player in the health of the upper respiratory tract, and has been shown to be significantly modified during episodes of immunological stress and clinical respiratory disease (21). As a result of these observations, it has been suggested that disturbances in NP microbial communities may contribute to the pathophysiology of BRD in feedlot cattle (12). Although several studies have investigated the bovine NP microbiota in the predisease and disease states (22–24), little information is available on the change of NP microbiota associated with clinical recovery from BRD. To help explore this gap in knowledge, we evaluated the effect of tilmicosin treatment on the NP microbiota of BRD-affected calves during first week of clinical recovery. Sequence analysis from all NP swabs resulted in a total of 410,615 filtered sequence reads. The mean sequence reads per sample was 15,207.963 (SD, 12,104.039) and comprised a total of 604 OTUs across all samples. In terms of relative abundance, taxonomic classification of OTUs revealed a total of 14 different bacteria phyla, and 182 bacterial genera, among all samples. Similar to previous 16S rRNA gene-based studies of the NP microbiota of feedlot cattle, the most abundant bacterial phyla across all sample were *Firmicutes* (27.07%), *Actinobacteria* (24.51%), *Tenericutes* (16.05%), and *Proteobacteria* (14.43%) (**Figure S1A**) (7, 22–25). These findings are similar to those reported in studies of the nasal microbiota of pigs (26, 27) and the upper respiratory tract of humans (28). All other classified OTUs belonged to bacterial phyla and comprised <1% of the total abundance represented as others/unassigned (**Figure S1A**). At lower taxonomic levels, the most prevalent bacterial taxa were *Mycoplasma* (18.73%), *Microbacteriaceae* (9.36%), *Acinetobacter* (7.35%), and *Corynebacterium* (6.36%) (**Figure S1B**). Our data analysis showed a high inter-individual variability in the composition of the NP microbiota across all the individuals. This was expected, especially in the type of feedlot husbandry system used in our study, since the upper respiratory tract is constantly exposed to many and various bacteria from the surrounding environment (22). This is also compatible with other studies that have explored the multifactorial determinants (genetic, epigenetic, environmental, age, sex, and dietary) that underlie the establishment of the mucosal microbiota (29). To measure the alpha diversity of the NP microbial communities among the three groups (control, BRD, and post-treatment), we used several metrics; Shannon, Chao1, and the PD whole tree indices, as depicted in (**Table 1**). None of the alpha diversity indices differed significantly between groups ($p > 0.05$), although the NP microbiota from the control calves were more diverse than in the NP samples from tilmicosin-treated and BRD-affected calves. Similarly, the NP samples from tulathromycin-treated calves showed a reduction in the bacterial diversity by one-week post treatment (24). While our study did not permit longer-term evaluation of microbial biodiversity, similar studies in other species studies have shown that antimicrobial treatment is often followed by a contraction in biodiversity of some taxa that can persist for several months (30). However, in order to better understand the potential health impact of these post-treatment fluxes in community structure, it is important to explore the dynamics of these changes over time in relation to disease

TABLE 1 | Bacterial diversity indices (Chao1, PD whole tree and observed species) measures for the nasopharyngeal microbiota of calves.

Bacterial diversity indices	Control	BRD	After treatment	*P*-value
Chao1 index	77.82 ± 16.40	44.34 ± 7.55	53.02 ± 13.75	0.174
PD whole tree	7.54 ± 1.10	5.76 ± 0.63	6.41 ± 0.84	0.213
Shannon index	3.66 ± 0.48	3.07 ± 0.28	3.11 ± 0.33	0.289

The data are presented as the mean ± standard deviation. There were no statistically significant differences in different bacterial diversity indices between the different groups ($p > 0.05$).

recovery and in association with different antimicrobial regimes. In addition, by understanding how mucosal microbiota respond to different management conditions, it should be possible to identify the mechanisms by which these communities contribute to mucosal recovery and the return of the respiratory system to a healthy state.

Comparison of NP Microbiota Across the Different Groups

The dynamics of change in the NP microbiota between clinically healthy calves and those that develop BRD were reported in detail in our previous published study (12). In this study, we compared the relative abundance of the most abundant bacterial phyla that accounted for more than 1% of the total (*Firmicutes, Actinobacteria, Tenericutes, Proteobacteria,* and *Bacteroidetes*) across all three groups. In tilmicosin-treated calves, we observed a significant increase ($P < 0.01$) in the relative abundance of *Firmicutes* compared to control and BRD groups (**Figure 1**). Both BRD-affected and treated calves showed significant decrease in the relative abundance of *Actinobacteria* ($P < 0.01$) compared to the healthy control calves (**Figure 1**). The relative abundance of *Tenericutes,* and *Bacteroidetes* did not show significant changes among the groups ($P > 0.05$). At the genus level, the relative abundance of *Microbacteriaceae* ($P < 0.001$), *Acinetobacter* ($P = 0.013$), *Pasteurella* ($P = 0.041$), *Lachnospiraceae* ($P = 0.021$), *Clostridium* ($P = 0.018$), *Solibacillus* ($P = 0.043$), and *Turicibacter* ($P = 0.026$) was significantly different among the three groups (**Figure 2**). It is notable that the administration of a single dose of tilmicosin for BRD treatment affected the bacterial composition of the NP microbiota. Most notably, the relative abundance of *Clostridium* and *Lachnospiraceae* were significantly increased in tilmicosin-treated calves compared to control and BRD-affected calves ($P < 0.05$; **Figure 2**). Interestingly, it has been recently shown that an abundance of *Clostridium* species is associated with antibiotic-associated colitis and influenza in humans (31, 32), and hemorrhagic diarrhea in feedlot cattle (15). The relative abundance of *Microbacteriaceae* and *Turicibacter* was significantly decreased in tilmicosin-treated and BRD-affected calves when compared to control calves ($P < 0.05$; **Figure 2**). In terms of the bacterial taxa commonly associated with BRD, the overall relative abundance of *Moraxella* and *Mannheimia* was not significantly different after tilmicosin treatment. Antimicrobial administration was recently demonstrated to be efficacious in treating dairy cattle that had been experimentally challenged with *Mycoplasma* via the respiratory tract (33). Interestingly, while the changes were not statistically significant, there were general trends in the relative abundance of *Mycoplasma* and *Acinetobacter* species in the BRD-affected calves one week following treatment. A possible explanation for this finding is that because *Mycoplasma* and *Acinetobacter* population are well-known to have resistance to multiple antimicrobials (34, 35), tilmicosin treatments could have decreased the presence of other bacterial inhabitants of the nasal cavity that potentially could promote the growth of those population. This microbial reshaping due to differential sensitivity to antibiotics might explain why resilience is not complete long after antimicrobial treatment.

Effect of Tilmicosin Treatment on the Overall NP Microbiota Composition and Core Microbiota

To evaluate the potential effect of tilmicosin antibiotic treatments on the overall NP microbiota composition of BRD-affected calves during the first week of clinical recovery, we compared the microbial community structure (beta diversity) between the three groups (Control, BRD and post-treatment) using Bray-Curtis and Jaccard dissimilarity. The PCoA plot of the Bray-Curtis and Jaccard dissimilarity revealed that the overall composition of NP microbial communities in tilmicosin-treated calves resembled that of the BRD-affected calves (PERMANOVA, $P > 0.05$; **Figures 3A,B**), and that both group differed significantly from pen- matched healthy calves (PERMANOVA, $P < 0.05$; **Figures 3A,B**). As the treatment groups did not differ significantly from the BRD groups ($P > 0.05$), it is conceivable that the tilmicosin treatment impaired recovery of the NP microbiota to a balanced homeostatic state. Unfortunately, since there was not a non-treated control group for the BRD-affected calves, the reality of a post-treatment inhibitory activity of the antimicrobial on microbiota recovery cannot be confirmed. A similar link between antimicrobial use and an altered microbial community structure in the upper respiratory tract of children up to six months after administration has been reported (36). To further evaluate the overall NP microbial similarities between the different groups, UPGMA cluster, based on Bray–Curtis distance metrics, were performed. Hierarchical clustering of the relative abundance of bacterial taxa of the NP microbiota was not evident in either the BRD or post-treatment groups. However, pen-matched clinically healthy control calves were generally clustered both closer together and further away from the BRD and post-treatment groups (**Figure 3C**). Additionally, a Venn diagram was generated to describe the unique and shared OTUs between the three groups (**Figure 3D**). With counts, the OTU distribution showed that there were 149, 69, and 114 unique OTUs identified in healthy control, BRD and post-treatment groups, respectively. Furthermore, a total of 121 OTUs, representing the core microbiota, were shared between the three groups (**Figure S2**). In combination, these results indicate there was little change in NP microbial communities following tilmicosin treatment, and that the initial difference in NP communities between the BRD-affected and healthy control calves remained in the early phase of clinical recovery. The absence of a non-treatment control for the BRD-affected calves prevented us from drawing any conclusions regarding the role of tilmicosin treatment in inhibiting the resolution of any BRD-related disturbances in NP microbial community structure. While tilmicosin has been implicated as being inferior to other antimicrobial treatment for control of BRD of high-risk cattle before the onset of signs of BRD (37), additional broader studies involving other types of antimicrobials, with the inclusion of the appropriate post-treatment control groups, would be required to evaluate the role of antimicrobial therapy

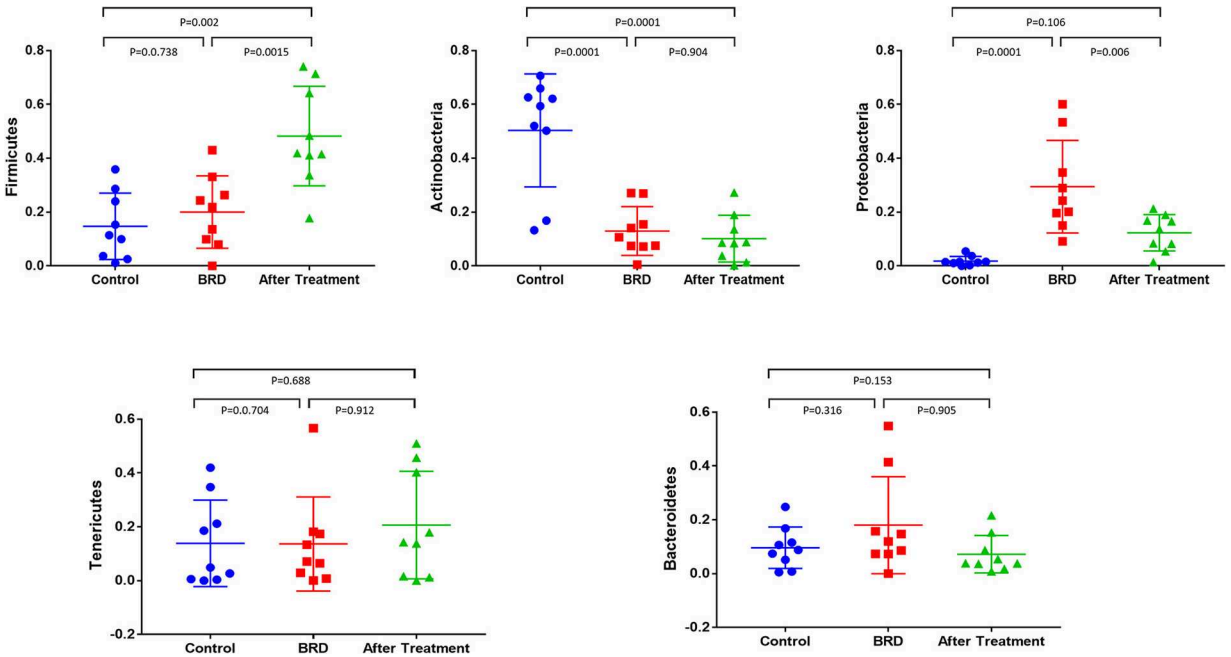

FIGURE 1 | Relative abundance of bacterial 16S rRNA gene sequences at the phylum level that showed the difference between tilmicosin-treated, BRD-affected and healthy control calves. Only those bacterial phyla represent those populations that averaged more than 1% of the relative abundance across all samples when sequencing V1-V3 hypervariable regions are displayed.

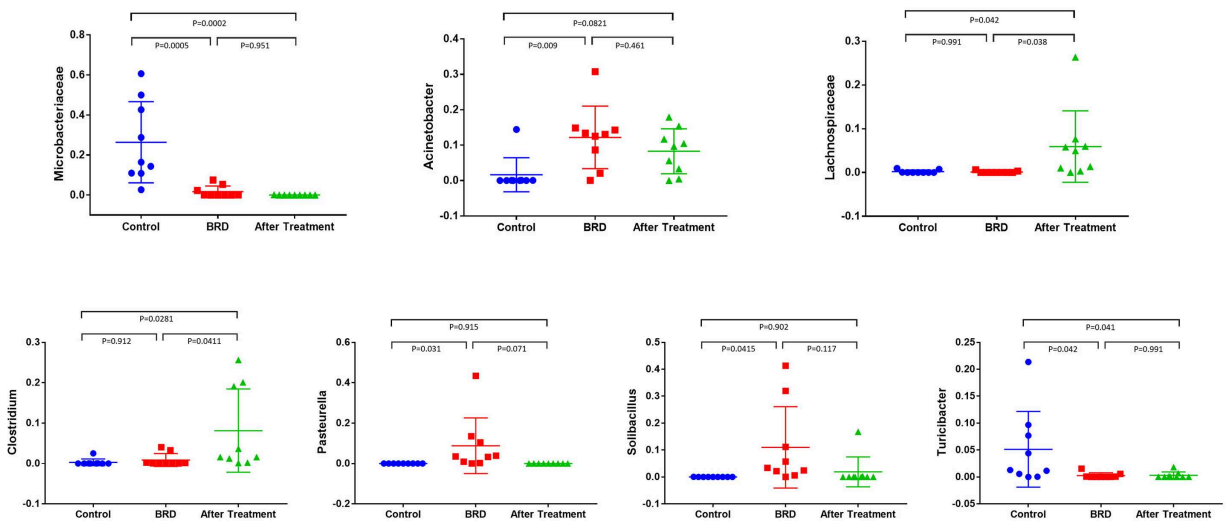

FIGURE 2 | Relative abundance of bacterial 16S rRNA gene sequences at the higher taxonomic level observed in the NP swab showed the difference between tilmicosin-treated, BRD-affected and healthy control calves. Only those bacterial taxa represent those populations that averaged more than 1% of the relative abundance across all samples when sequencing V1-V3 hypervariable regions are displayed.

on the rejuvenation of nasopharyngeal microbial community structure in cases of BRD.

CONCLUSION

In conclusion, the overall aim of this study was to examine changes in the NP microbiome during the clinical recovery of BRD-affected calves treated with tilmicosin. It appeared that there were no significant changes in NP microbial communities following tilmicosin treatment, and that the initial differences in NP microbial communities between healthy and BRD-affected calves, remained for the duration of the early phase of clinical recovery. Given the limitations of the present study (small number of treated calves, only one type of antibiotics, short term follow-up, lack of non-treated control group) further studies are necessary to evaluate the long-term effects

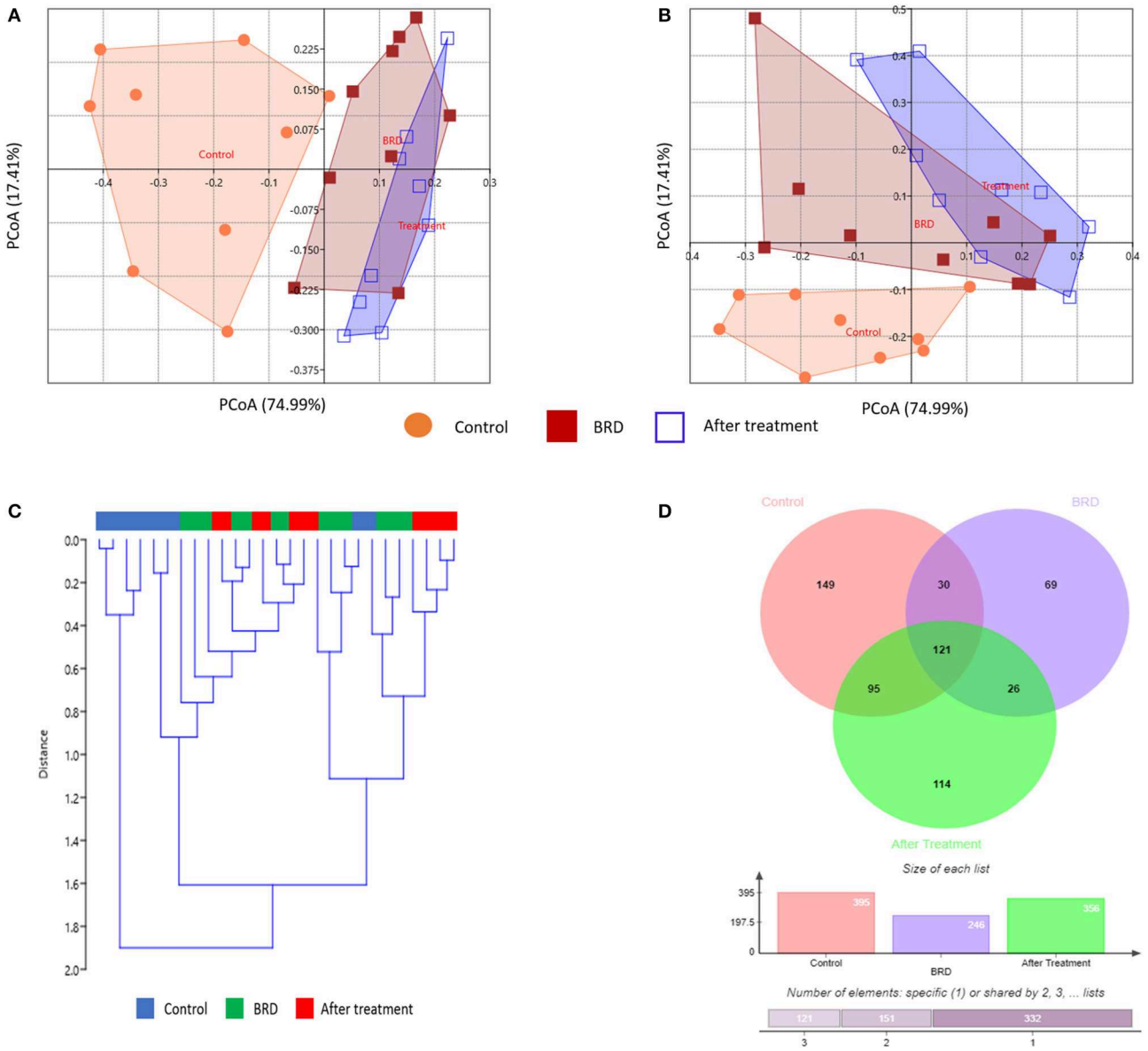

FIGURE 3 | Effect of tilmicosin treatment on the overall NP microbiota composition and core microbiota. Principal coordinate analysis (PCoA) depicting the Bray-Curtis **(A)** and Jaccard dissimilarity **(B)** for the NP swab in tilmicosin-treated, BRD-affected and healthy control calves. The percent variation explained by each principal coordinate is indicated on the axes. **(C)** UPGMA clustering of bacterial taxa profiles based on the Bray-Curtis dissimilarity. The color bars above the dendrogram indicate groups of samples, control (blue), BRD (green), and after tilmicosin treatment (red). **(D)** Venn diagram depicting the common and unique OTUs among the three groups (tilmicosin-treated, BRD-affected and healthy control calves).

of antimicrobial administration upon respiratory microbiota. Understanding the potential impact of the prolonged recovery in the mucosal microbiota will be important in optimizing the use of antimicrobials in health management programs in the feedlot industry.

DATA AVAILABILITY STATEMENT

Fastq data obtained in the current study were uploaded to the sequence read archive on the NCBI website to make the files available for public databases, with a bio-project accession number PRJNA508519.

ETHICS STATEMENT

The animal study was reviewed and approved by University of Illinois Institutional Animal Care and Use Committee (IACUC Protocol: #15064).

AUTHOR CONTRIBUTIONS

JL and BA designed the experiment. MZ and JL conducted the experiment. MZ performed the laboratory and data analyses. MZ and BA wrote the manuscript. All authors edited and approved the manuscript submission.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2020.00115/full#supplementary-material

Figure S1 | Relative abundance of bacterial 16S rRNA gene sequences at the phylum level **(A)** and higher taxonomic level **(B)** observed in the NP swab in tilmicosin-treated, BRD-affected and healthy control calves. Only those bacterial phyla represent those populations that averaged more than 1% of the relative abundance across all samples when sequencing V1-V3 hypervariable regions are displayed.

Figure S2 | Krona chart showing the relative abundance of bacterial taxa that represent the core microbiota and shared between the three groups (tilmicosin-treated, BRD-affected and healthy control calves).

Table S1 | Access Array cycling program without imaging (Fluidigm Biomark HD PCR machine) for amplifying the primer/sample combinations.

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Effects of *in ovo* Inoculation of Multi-Strain Lactobacilli on Cytokine Gene Expression and Antibody-Mediated Immune Responses in Chickens

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This study was conducted to investigate the effects of various doses of a multi-strain lactobacilli mixture (*Lactobacillus salivarius*, *Lactobacillus reuteri*, *Lactobacillus crispatus,* and *Lactobacillus johnsonii*) on the innate and adaptive immune responses in broiler chickens. At embryonic day eighteen, 200 eggs were injected with PBS, or three different doses of a multi-strain lactobacilli mixture (1×10^5, 1×10^6, and 1×10^7 CFU/egg, P1, P2, and P3 respectively) along with a group of negative control. On days 5 and 10 post-hatch, cecal tonsil, bursa of fabricius, and spleen were collected for gene expression and cellular analysis. On days 14 and 21 post-hatch, birds were immunized intramuscularly with both sheep red blood cells (SRBC) and keyhole limpet hemocyanin (KLH). Serum samples were collected on days 0, 7, 14, and 21 after primary immunization. The results demonstrated that lactobacilli inoculation increased the splenic expression of cytokines, including interferon (IFN)-α, IFN-β, IFN-γ, interleukin (IL)-8, and IL-12 on day 5 post-hatch compared to the control group (PBS). However, in cecal tonsils, lactobacilli treatment downregulated the expression of IL-6 on day 5 post-hatch and IL-2 and IL-8 on day 10 post-hatch. No significant differences were observed in the expression of cytokine genes in the bursa except for IL-13 which was upregulated in lactobacilli-treated groups P2 and P3 on days 5 and 10 post-hatch. Flow cytometry analysis showed that the percentage of KUL01, $CD4^+$ and $CD8^+$ splenocytes was not affected by treatments. In addition, no significant differences were observed for antibody titers against SRBC. However, lactobacilli treatment (P1, P2, and P3) was found to increase IgM titers on day 21 post-primary immunization compared to controls. Furthermore, *in ovo* injection of the highest dose of probiotics (1×10^7, P3) increased serum IgG titers

against KLH on day 7 post-primary immunization. In conclusion, this study demonstrated that that *in ovo* administration of lactobacilli can improve antibody-mediated immune responses and differentially modulate cytokine expression in mucosal and systemic lymphoid tissues of chickens.

Keywords: lactobacilli, *in ovo*, chickens, cytokines, antibody

INTRODUCTION

In the poultry industry, it is common for newly hatched chickens to experience delayed access to feed and water due to the time spent in the hatchery and during transportation to the production farm (1). This delay in feed and water intake may negatively influence post-hatch immune system function and bird performance (2). In addition, in broiler chickens, parents do not contribute to egg incubation, and development of the embryo occurs independently of its mother reducing parental influence on gut microbial development (3). Gut microbiota provides essential health benefits to the host by enhancing immune system development and maintaining and regulating intestinal immune homeostasis (4, 5). Recent studies have suggested that dysbiosis in gut microbiota is linked to the pathogenesis of a variety of intestinal disorders (6, 7). In chickens, the establishment of the gut microbiota occurs within 3 days post-hatch and the microbial composition remains relatively unchanged until 30 days of age (8). This indicates that early establishment of beneficial bacteria is very important and can further impact gut microbiota colonization and the development of barrier functions of the gastrointestinal tract (9–11). Therefore, pre-hatch colonization of chickens' gastrointestinal tracts with beneficial bacteria through *in ovo* technology may prevent pathogen colonization via competitive exclusion in addition to accelerating intestinal and immune system development (10). Different studies have reported the beneficial effects of probiotic bacteria on broiler growth performance, gut microbiota composition and immune system development (12–15). Among these probiotics, *Lactobacillus* bacteria have received considerable attention because of their immunomodulatory activities and intestinal health benefits (16–18). Lactobacilli are considered autochthonous residents in the chicken gastrointestinal tract and may contribute to the host gut health and immune system function through different mechanisms such as enhancement of the epithelial barrier, competitive exclusion of pathogenic microorganisms, production of antimicrobial substances, and interaction with immune system cells via stimulation of pattern recognition receptors (19, 20). Considering the vulnerability of newly hatched chicks toward various pathogens, pre-hatch administration of *Lactobacillus* bacteria via *in ovo* technology can be used as a strategy to strengthen immune responsiveness of chickens and reduce their susceptibility toward pathogens. Many studies suggest that different strains of lactobacilli can modulate multiple aspects of immune response including cytokine and chemokine expression, T lymphocyte populations and systemic antibody-mediated responses (21–23). In the present study, we hypothesized that one-time *in ovo* administration of a mixture of four *Lactobacillus* spp. (*L. salivarius*, *L. reuteri*, *L. crispatus*, and *L. johnsonii)* can modulate innate responses and thus, can accelerate the maturation of the immune system leading to enhanced antibody-mediated responses against thymus-dependent antigens. Therefore, this study was aimed at investigating the potential immunomodulatory effects of *in ovo* administration of lactobacilli on innate and antibody-mediated immune response in chickens.

MATERIALS AND METHODS

Chickens and Housing

Embryonated chicken eggs were obtained from the Arkell Poultry Research Hatchery (University of Guelph, ON, Canada). Newly hatched commercial broiler chicks housed in a separated floor pens per each treatment group, on clean wood shavings with free access to water and feed at Arkell Poultry Research.

Experimental Design

In this experiment, the selected *Lactobacillus* spp. including *L. salivarius, L. reuteri, L. crispatus,* and *L. johnsonii* were isolated from the intestinal contents of healthy broiler chickens as previously described (16). Two hundred embryonated broiler chicken eggs were incubated at 37°C at Arkell Research Station (Guelph, ON). On day 18 of incubation, 40 embryonated eggs were injected with one of three different doses of a selected mixture of *Lactobacillus* bacteria, including 1×10^5 CFU (P1), 1×10^6 CFU (P2), and 1×10^7 CFU (P3) of bacteria or phosphate buffered saline (PBS), all injections were 100 μL total volume. Each *lactobacillus* was grown separately and prepared at the certain dose from 1×10^5 to 1×10^7 cfu/ml in PBS and the strains were associated in equal amount within the multi-strain cocktail designated for this study. The remaining eggs (24) served as a non-injected untreated negative control, creating 5 groups. The lactobacilli cocktail was delivered precisely to amniotic fluid, where the negative pressure in abdominal cavity facilitates the passage of the intestinal content via peristaltic movement. Lactobacilli used in the present study have been recovered from the intestines of newly hatched chickens (unpublished data). This was assessed using a culture-based method and would be relevant to use in the future to use tagged bacteria for tracking them in the intestine.

Immunization, Serum Collection, and Tissue Sampling

To evaluate antibody-mediated immune responses, on days 14 and 21 post-hatch, birds were immunized intramuscularly with 0.25 mL of 2% SRBC (PML Microbiologicals, Mississauga, ON, Canada) in PBS and subsequently with 0.25 ml of PBS containing 100 μg keyhole limpet hemocyanin (KLH) (Sigma, Oakville, ON, Canada). The untreated, unimmunized group was injected with PBS. Blood samples (1–2 ml) were collected from the wing vein of 12 birds per treatment on days 0, 7, 14, and 21 post primary immunization. Blood samples were kept at room temperature for 2 h and then centrifuged at 580 × *g* for 10 min to isolate serum. Serum samples were stored at –20°C for antibody analysis. On days 5 and 10 post-hatch 6 birds per treatment were euthanized and bursa of Fabricius, cecal tonsils, and spleen tissues were collected, kept in RNA later and stored at −80°C for gene expression analysis. Spleen tissue was also kept on ice in 1 X Hanks' balanced salt solution (HBSS) (Gibco, Grand Island, NY) for analysis of splenocytes with flow cytometry.

Isolation of Spleen Mononuclear Cells and Flow Cytometry Analysis

Single-cell suspensions of mononuclear cells were prepared according to the procedure of Taha-Abdelaziz et al. (25). Briefly, spleen samples from 6 chickens per treatments were rinsed three times in HBSS and filtered through a 40-μm nylon cell strainer using the flat end of a 1 ml syringe plunger. Cells were resuspended in 5 ml RPMI (Invitrogen, Burlington, Ontario, Canada) containing 10% fetal bovine serum, 2.5% HEPES (Sigma Aldrich, St Louis, MO), 1% Penicillin-Streptomycin (Gibco, Grand Island, NY), 0.5% Gentamicin (Gibco, Grand Island, NY), and 0.05% 2-Mercaptoethanol (Sigma Aldrich, St Louis, MO) and they were overlaid on 4 ml Histopaque-1077 (Sigma, Oakville, ON) for density gradient separation, and mononuclear cells at the interface were harvested and washed twice in RPMI (Gibco, Grand Island, NY) media. Cells were counted using automated cell counter MOXI Z (Orflo, Ketchum, ID, USA) and 100 μL of each cell suspension was seeded in round bottom 96 well plates at density of 1×10^6 /ml in RPMI medium. Subsequently, cells were washed twice in FACS buffer (PBS containing 1% BSA) and stained for 30 min at 4°C in the dark with fluorescent monoclonal antibodies including mouse anti-chicken CD3-PB [CT-3], mouse anti-chicken CD4-PE [CT-4], mouse anti-chicken CD8-APC [CT-8], and mouse anti-chicken monocyte/macrophage-FITC [KUL01] (Southern Biotechnology Associates, Inc., Burlington, ON). The cells were washed twice in FACS buffer, fixed in 2% paraformaldehyde (PFA) and transferred to 5 ml polystyrene round-bottom tubes for analysis. Flow cytometry was performed using a FACS Canto II flow-cytometer (BD Bioscience, San Jose, CA, USA) and data were analyzed using FlowJo Software (v.10).

Serological Analysis

Detection of the total antibody responses to SRBC in sera was performed by a direct hemagglutination assay according to the procedure of Haghighi et al. (26). Serum samples were heat-treated at 56°C for 30 min. Then, 50 μL of PBS containing 0.05% of bovine serum albumin (BSA) was added into each well of a round-bottomed 96-well microplate, and 2-fold serial dilutions of serum samples were generated in duplicate. Subsequently, 50 μL of 1% SRBC in PBS was added to each well and the plates were shaken for 1 min followed by incubation for 24 h at 37°C. Positive result were recorded when at least 50% of SRBC agglutination was observed.

Detection of KLH-specific IgG and IgM titers in sera was performed by indirect enzyme-linked immunosorbent assay (ELISA). Briefly, each well of a flat-bottomed 96-well Maxisorp high binding microplate was coated overnight at 4°C with 100 μL of 1 μg/ml KLH in coating buffer (0.1 M $NaHCO_3$, pH 9.6) containing BSA (30 μg /ml). Wells were then washed 4 times with 200 μL of PBS with 0.05% Tween 20 (P137 Sigma Aldrich Inc., St. Louis, MO) (PBST) and were completely decanted between each washing step. Subsequently, 100 μL of blocking buffer (PBST containing 0.25% of gelatine) was added to each well and the plate was incubated for 2 h at room temperature. Washing was repeated and was followed by addition of 100 μL of chicken serum (diluted 1:200 v/v in blocking buffer) to each well. Plates were incubated 2 h at room temperature and then were washed 4 times with the washing solution. One hundred μL of detection antibody (goat anti-chicken IgG-Fc and IgM-Fc, Bethyl laboratories) conjugated with horseradish peroxidase (diluted in 1/5,000 of blocking buffer) was added to each well and incubated for 1 h at room temperature. Washing was repeated and was followed by addition of 100 μL ABTS [2,2_-azinobis (3 ethylbenzthiazolinesulfonic acid)] peroxidase substrate system (Mandel Scientific, Guelph, ON, Canada) to each well. Plates were incubated for 30 min at room temperature in the dark and absorbance was measured at 405 nm using the micro plate reader (Epoch, BioTek Instruments Inc., Winooski, VT). Positive and negative-control serum (fetal bovine serum) were included in each plate to justify the plate-to-plate variations. Sample/positive (Sp) ratios were calculated according to the following formula: (mean of test sample—mean of negative control)/(mean of positive control—mean of negative control).

RNA Extraction and Reverse Transcription

Total RNA was extracted from spleen, bursa of Fabricius and cecal tonsil tissues using Trizol as described by the manufacturer (Invitrogen Canada Inc., Burlington, ON, Canada). Total RNA was treated with DNase (DNA-free kit, Ambion, Austin, TX) and the quantity and purity of the RNA samples was measured by using a Nanodrop spectrophotometer (Thermo Scientific, Wilmington, DE). Reverse-transcription to cDNA was performed by using Superscript® II First Strand Synthesis kit (Invitrogen) according to the manufacturer's protocol.

Quantitative Real-Time PCR

Quantitative real-time (qRT) PCR was performed using the LightCycler® 480 II system (Roche Diagnostics GmbH, Mannheim, DE). Each qRTPCR reaction consisted of 10 μl of 2X SYBR Green Master mix (Roche Diagnostics), 1 μl of forward- and 1 μl of reverse-primer (5 μM), 3 μl PCR-grade water and 5 μl of target cDNA (1:10, diluted in nuclease free-water). The PCR cycling protocol included an initial denaturation step at 95°C,

TABLE 1 | Primer sequences used for real-time quantitative PCR[a].

Gene[b]	Primer sequence[c] (5′-3′)	Annealing temperature	GeneBank accession number
IFN-α	F: CGCTTAGGAGAGACAATCTGTGAA R: GCCTGTTTTAGGGATTTCAGAGAATTT	64	AB021154
IFN-β	F: AGTCTGAAATTGCTGAGCTCAAAT R: GCGACGTTAAGCCATGGAAG	64	GU119897.1
IFN-γ	F: TGGCGGCGGGAGGAAAAGTG R: CACCGTGCTCCAGCTCAGGC	60	NM_001030558
IL-2	F: GCAGGGCACGTTCAGGTGGG R: GCCACACAGCCTGGCTCCCT	58	NM_204153.1
IL-6	F: CTGAAGAACTGGACAGAGAG R: CACCAGCTTCTGTAAGATGC	60	NM_204628.1
IL-8	F: CTGAAGGTGCAGAAGCAGAG R: CCAGCTCTGCCTTGTAGGTT	64	AJ009800
IL-12p35	F: AGCAGATCAAGGAGACGTTC R: ATCAGCAGGTACTCCTCGAT	60	NM213588
IL-13	F: ACTTGTCCAAGCTGAAGCTGTC R: TCTTGCAGTCGGTCATGTTGTC	60	AJ621250.1
β-Actin	F: CAACACAGTGCTGTCTGGTGGTA R: ATCGTACTCCTGCTTGCTGATCC	58	X00182

[a] *The listed oligonucleotides were used to analyze gene expression via real-time quantitative PCR.*
[b] *IFN, Interferon; IL, Interleukin.*
[c] *F, forward; R, reverse.*

followed by amplification for 40–50 cycles consisting of 95°C for 10 s, an annealing step at a temperature described in **Table 1** for each of the primer pairs, and extension at 72°C for 10 s. The primers used were synthesized by Sigma-Aldrich (Oakville, ON), and their specific sequences and accession numbers are presented in **Table 1**.

Statistical Analysis

The expression levels of all genes were calculated relative to the housekeeping gene (β-actin) using the LightCycler® 480 software (Roche Diagnostics) and data were analyzed by using GLM procedure of SAS (SAS Institute Inc., Cary, NC). Differences among treatment means were determined using Tukey's multiple comparison test after log transformation when error deviations did not have homogenous variance across the treatments. *P*-value of <0.05 was considered statistically significant.

RESULTS

Hatchability

Hatchability was recoded on the day of the hatch. The results showed that in ovo inoculation of either PBS or lactobacilli did not influence hatchability of the chickens and 99.38% of eggs were hatched following *in ovo* injection.

Cytokine Gene Expression in Cecal Tonsils, Spleen, and Bursa of Fabricius

The results for gene expression of cytokines are presented in **Figures 1**–**3**. In the spleen (**Figure 1**), the expression of IL-2, IL-6, and IL-13 was not altered by treatment ($P > 0.05$). However, expression of IFN-α, IFN-γ, and IL-12 on day 5 and IL-8 on day 10 post-hatch was upregulated in the spleen of birds that received 10^5 CFU of lactobacilli (P1) *($P < 0.05$)*. In addition, lactobacilli-treatment of 10^6 CFU (P2) significantly upregulated the expression of IFN-γ and IL-12 on day 5 and IFN-*β* on day 10 post-hatch. In the cecal tonsils (**Figure 2**), expression of IFN-α, IFN-*β*, IFN-γ, and IL-13 was not affected by lactobacilli administration ($P > 0.05$) however, it led to downregulation of IL-6 on day 5 and IL-2 and IL-8 on day 10 post-hatch. In contrast, expression of IL-12 was upregulated in lactobacilli-treated groups on days 5 (P1 and P2) and day 10 (P3) post-hatch in the cecal tonsils. No significant differences were observed in cytokine gene expression in the bursa of Fabricius, except for IL-13, which was upregulated on day 5 (P1 and P2) and on day 10 (P2) post-hatch (**Figure 3**).

T Lymphocyte and Monocyte/Macrophage Populations

Results for the flow cytometric analysis KUL01 and T lymphocyte subpopulations in the spleen ($CD4^+$ and $CD8^+$) are presented in **Figure 4**. Inoculation of eggs with lactobacilli did not change the population of monocyte/macrophage and T cell subsets (single positive $CD3^+CD4^+$ and $CD3^+CD8^+$) in the spleen ($P > 0.05$).

Anti-SRBC and Anti-KLH Antibody Titres

The results for antibody-mediated immune responses against SRBC are presented in **Figure 5**. At 7, 14, and 21 days post-primary immunization, higher antibody titers against SRBC were observed in all immunized group compared to the non-immunized control group ($P < 0.05$). Nevertheless, inoculation of eggs with *Lactobacillus* bacteria did not affect serum anti-SRBC antibody titers ($P > 0.05$).

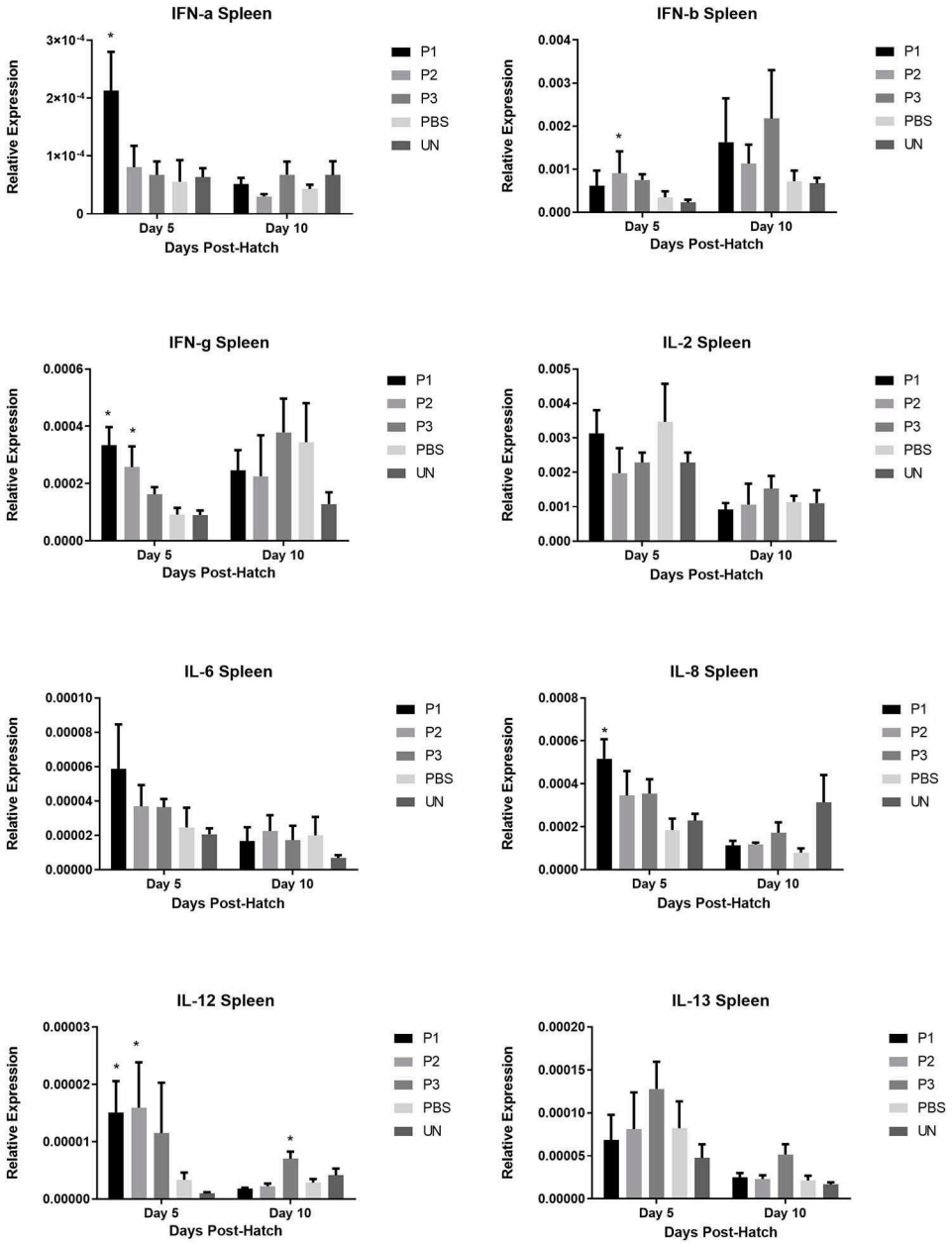

FIGURE 1 | Relative gene expression of IFN-α, IFN-β, IFN-γ, IL-2, IL-6, IL-8, IL-12, and IL-13 in the spleen of chickens at days 5 and 10 post-hatch. Samples collected from 6 birds per treatment. Treatment groups were as follows: P1, P2, and P3 received 1×10^5, 1×10^6, 1×10^7 CFU/egg of a selected mixture of *Lactobacillus* bacteria (*Lactobacillus salivarius*, *Lactobacillus reuteri*, *Lactobacillus crispatus,* and *Lactobacillus johnsonii)*, respectively (PBS, phosphate-buffered saline group; and UN, non-injected eggs). The reference gene (Beta-actin) was used for relative gene expression. Statistical significance among treatment groups was calculated using one-way ANOVA followed by Tukey's comparison test. Error bars represent standard errors of the mean. Results were considered statistically significant from the control group if $P < 0.05$. *Bars with asterisks differ significantly from control (PBS) group.

The results for antibody-mediated immune responses against KLH are presented in **Figure 6**. At 7, 14, and 21 days post-primary immunization, higher antibody titers against KLH were observed in all immunized groups compared with the non-immunized control group ($P < 0.05$). In addition, lactobacilli treatment at a dose of 10^7 CFU (P3) significantly enhanced serum IgG and IgM titers against KLH on day 7 and day 21 post-primary immunization, respectively.

DISCUSSION

In ovo technology was first introduced to the poultry industry several decades ago for vaccination against Marek's disease virus (27). This technique enables the delivery of various pharmaceuticals and biological supplements to chicken embryos during embryonation (28). One candidate supplement that can be administered *in ovo* to provide health benefits to the chickens

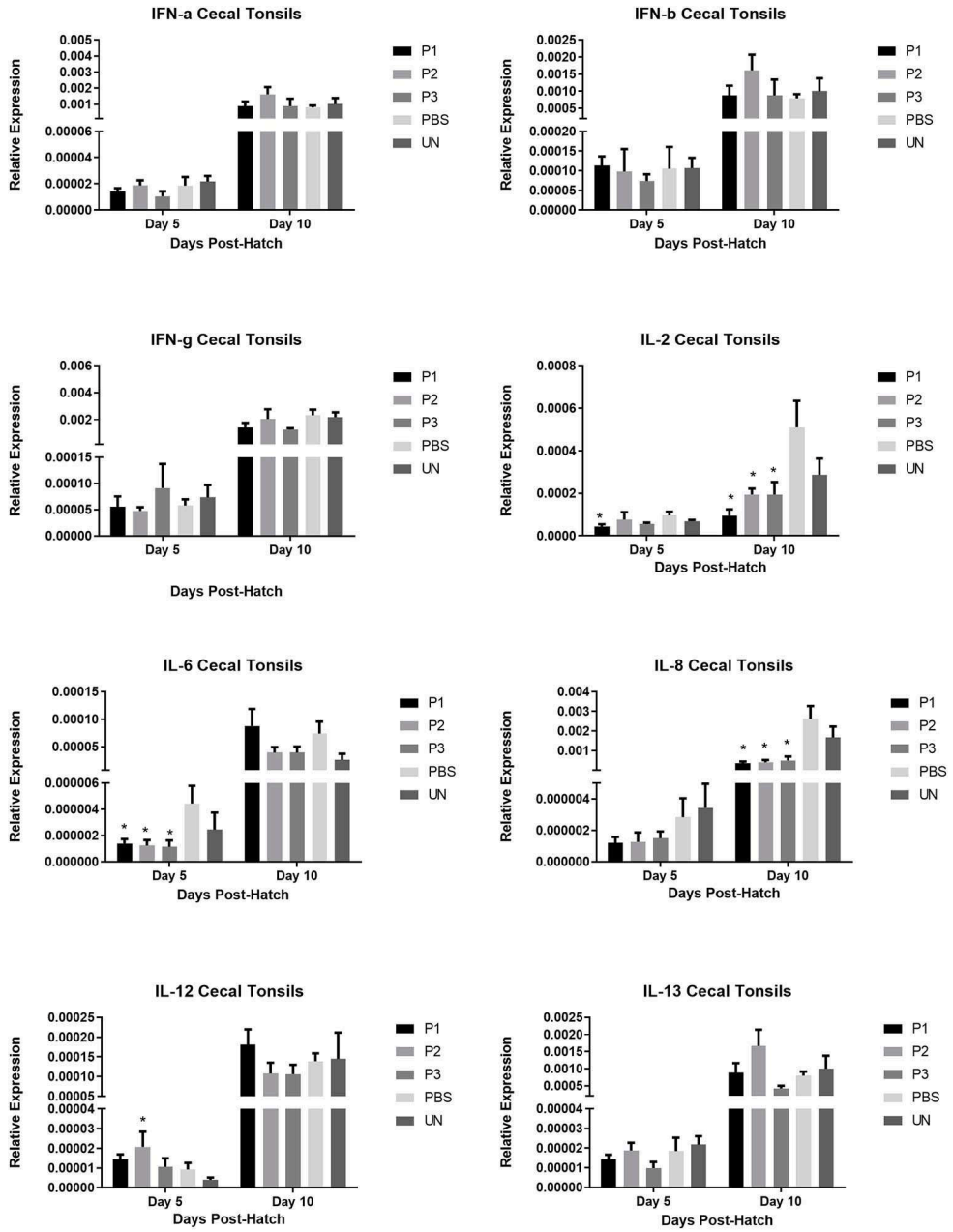

FIGURE 2 | Relative gene expression of IFN-α, IFN-β, IFN-γ, IL-2, IL-6, IL-8, IL-12, and IL-13 in the bursa of Fabricius of chickens on days 5 and 10 post-hatch. Samples collected from 6 birds per treatment. Treatment groups were as follows: P1, P2, and P3 received 1×10^5, 1×10^6, 1×10^7 CFU/egg of a selected mixture of *Lactobacillus* bacteria (*L. salivarius*, *L. reuteri*, *L. crispatus,* and *L. johnsonii)* respectively (PBS, phosphate-buffered saline group; and UN, non-injected eggs). The reference gene (Beta-actin) was used for relative gene expression. Statistical significance among treatment groups was calculated using one-way ANOVA followed by Tukey's comparison test. Error bars represent standard errors of the mean. Results were considered statistically significant from the control group if $P < 0.05$. *Bars with asterisks differ significantly from control (PBS) group.

are probiotics. It has been reported that the gut microbiota plays a critical role in development and regulation of the immune system (29). Probiotics may enhance immune responses and control pathogen infections in chickens by improving and restoring gut microflora (30). Several studies have reported the immunomodulatory activities of probiotics in chickens (16, 26, 31, 32). Therefore, the present study was conducted to evaluate the effects of *in ovo* inoculation of lactobacilli on innate and adaptive immune responses of chickens.

In the current study, expression of IL-2 was down-regulated in the cecal tonsils of lactobacilli-treated birds. IL-2 is mainly produced by activated T lymphocytes and is involved in the proliferation and activation of both T helper and cytotoxic T cells (33). Downregulation of IL-2 in lactobacilli-treated birds

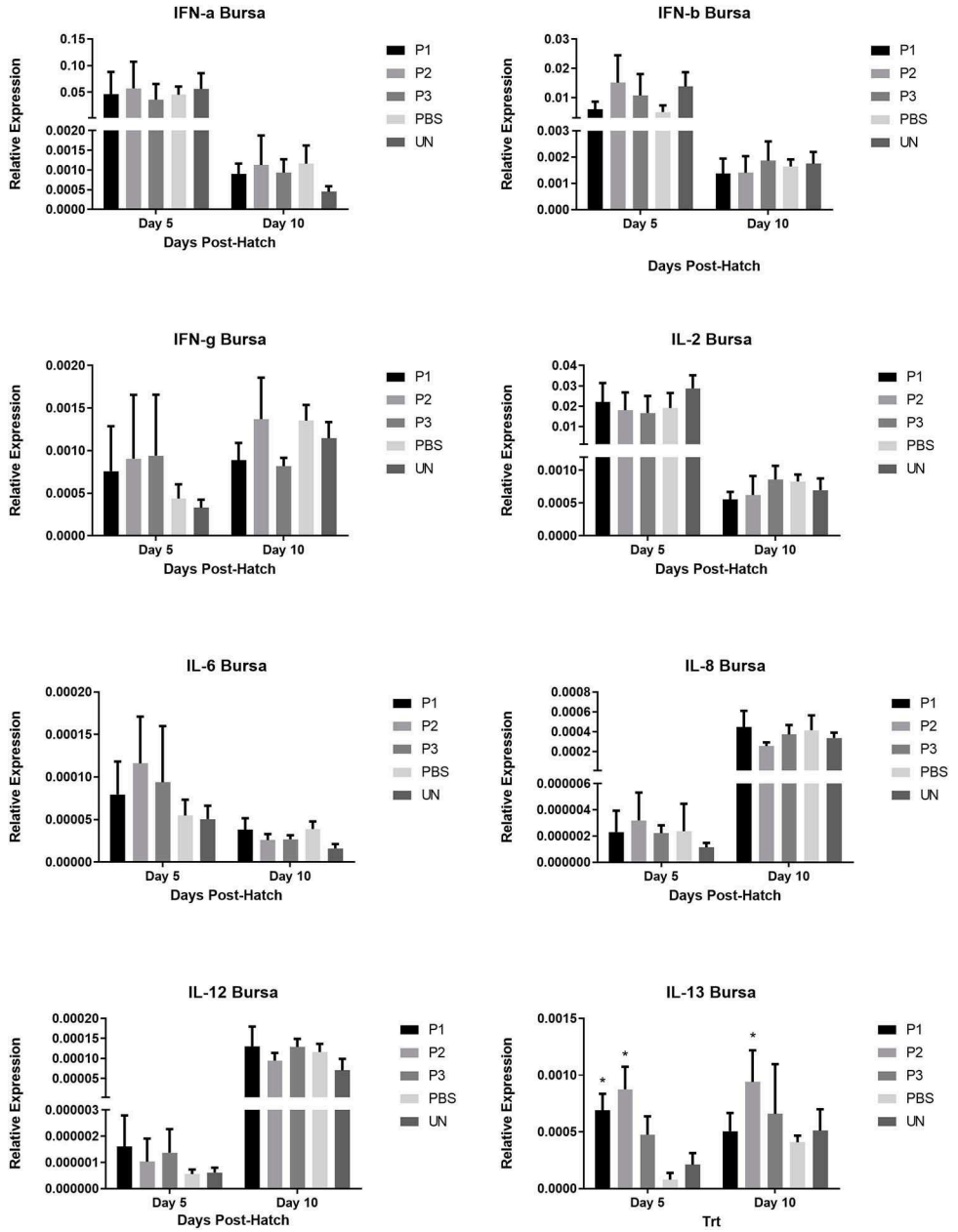

FIGURE 3 | Relative gene expression of IFN-α, IFN-β, IFN-γ, IL-2, IL-6, IL-8, IL-12, and IL-13 in the cecal tonsils of chickens on days 5 and 10 post-hatch. Samples collected from 6 birds per treatment. Treatment groups were as follows: P1, P2, and P3 received 1×10^5, 1×10^6, 1×10^7 CFU/egg of a selected mixture of *Lactobacillus* bacteria (*L. salivarius*, *L. reuteri*, *L. crispatus,* and *L. johnsonii)* respectively (PBS, phosphate-buffered saline group; and UN, non-injected eggs). The reference gene (Beta-actin) was used for relative gene expression. Statistical significance among treatment groups was calculated using one-way ANOVA followed by Tukey's comparison test. Error bars represent standard errors of the mean. Results were considered statistically significant from the control group if $P < 0.05$. *Bars with asterisks differ significantly from control (PBS) group.

suggests immunomodulatory properties of these bacteria in the absence of an infection. This suggestion can be supported by our observation that there was also a downregulation of IL-6 and IL-8 in the cecal tonsils of lactobacilli-treated birds, thus indicating that *Lactobacillus* bacteria might help maintaining immune homeostasis in the chicken intestine.

The results of previous studies indicate that dysbiosis of gut microbiota caused by a microbial challenge or an infectious disease is often associated with an activation of the immune system and upregulation of cytokines in secondary lymphoid organs in chickens (34, 35). Probiotics are thought to play a key role in maintaining the normal intestinal microbiota by reducing the population of pathogenic microorganisms though different processes, including competitive exclusion, inhibition of pathogen adhesion, and production of anti-pathogenic substances (19). In the present study, lactobacilli

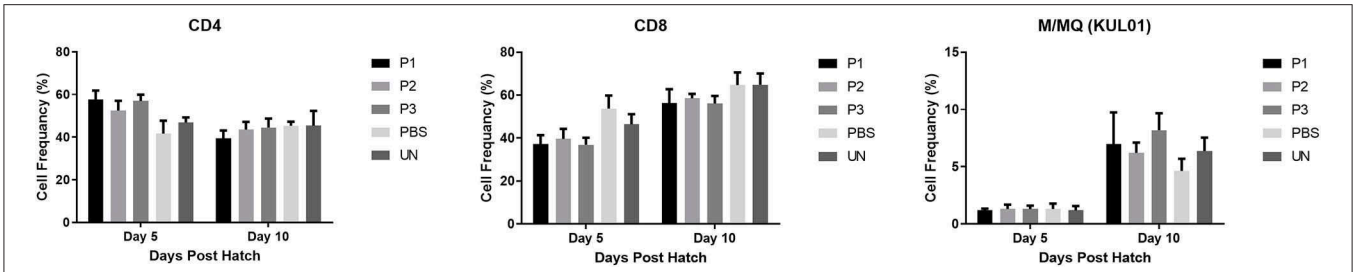

FIGURE 4 | T cell subsets and monocyte/macrophage (%) in the spleen of chickens following *in ovo* inculcation of *Lactobacillus* bacteria at days 5 and 10 post-hatch. Samples collected from 6 birds per treatment. Treatment groups were as follows: P1, P2, and P3 received 1×10^5, 1×10^6, 1×10^7 CFU/egg of a selected mixture of *Lactobacillus* bacteria (*L. salivarius*, *L. reuteri*, *L. crispatus,* and *L. johnsonii)* respectively (PBS, phosphate-buffered saline group; and UN, non-injected eggs). Statistical significance among treatment groups was calculated using one-way ANOVA followed by Tukey's comparison test. Error bars represent standard errors of the mean. Results were considered statistically significant from the control group if $P < 0.05$.

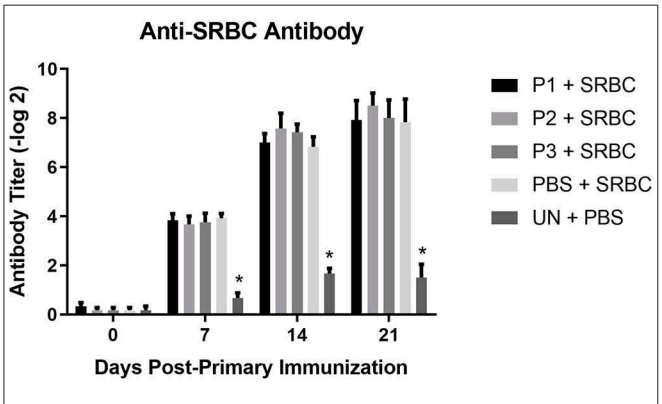

FIGURE 5 | Serum anti-SRBC antibody titers as determined by direct hemagglutination assay. Treatment groups were as follows: P1, P2, and P3 received 1×10^5, 1×10^6, 1×10^7 CFU/egg of a selected mixture of *Lactobacillus* bacteria (*L. salivarius*, *L. reuteri*, *L. crispatus,* and *L. johnsonii)* respectively and immunized with SRBC (P1 + SRBC, P2 + SRBC, and P3 + SRBC); chickens received 100 μl of phosphate-buffered saline/egg and were immunized with SRBC (PBS + SRBC); and chickens from non-injected eggs that were injected with PBS served as a control group (PBS). Serum samples collected from 12 birds per treatment. Statistical significance among treatment groups was calculated using one-way ANOVA followed by Tukey's comparison test. Error bars represent standard errors of the mean. Results were considered statistically significant from the control group if $P < 0.05$. *Bars with asterisks differ significantly from control (PBS) group.

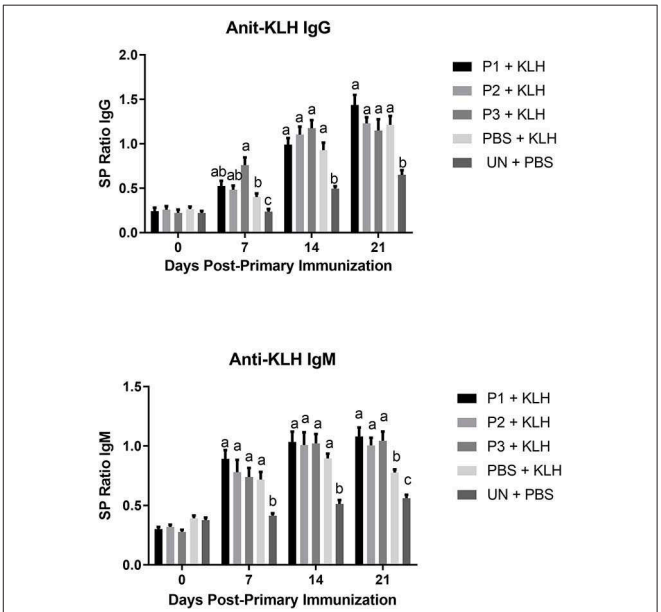

FIGURE 6 | Serum anti-KLH IgG and IgM titers as determined by indirect ELISA. Treatment groups were as follow: P1, P2, and P3 received 1×10^5, 1×10^6, 1×10^7 CFU/egg of a selected mixture of *Lactobacillus* bacteria (*L. salivarius*, *L. reuteri*, *L. crispatus,* and *L. johnsonii)*, respectively and immunized with KLH (P1 + KLH, P2 + KLH, and P3 + KLH); chickens received 100 μl of phosphate-buffered saline/egg and were immunized with KLH (PBS + KLH); and chickens from non-injected eggs that were injected with PBS served as control group (PBS). Serum samples collected from 12 birds per treatment. Statistical significance among treatment groups was calculated using one-way ANOVA followed by Tukey's comparison test. Error bars represent standard errors of the mean. Results were considered statistically significant from the control group if $P < 0.05$. (a–c) Means with no common superscripts differ significantly.

treatment downregulated the expression of cytokines (especially inflammatory cytokines) in cecal tonsils which are considered an intestinal lymphoid organs. This indicates that *Lactobacillus* bacteria might maintain microbial balance in the intestinal ecosystem by decreasing the population of pathogenic bacteria, thus preventing activation of the immune system. Decreased inflammatory responses to commensal bacteria within gut-associated lymphoid tissues (GALT) has been reported in previous studies, suggesting that although immune system cells in GALT can mount an inflammatory response toward pathogenic bacteria, they also remain slightly responsive to commensal bacteria (36).

Unlike in the cecal tonsils, the expression of cytokines was upregulated in the spleen, suggesting that lactobacilli might differentially modulate cytokine expression profiles in systemic (spleen) and local (cecal tonsils) secondary lymphoid organs. Gene expression in the bursa of Fabricius demonstrated that among all cytokines, only the expression of IL-13 was upregulated in lactobacilli-treated groups. Bursa of fabricius is considered as the primary lymphoid organs for B cell development and differentiation in newly hatched chick-s (37);

and IL-13 is a T helper type 2 anti-inflammatory cytokine with the function closely related to IL-4 including stimulation of activated B cells, and differentiation of B cells into plasma cells (38). Therefore, higher expression of IL-13 in the bursa of Fabrocius of lactobacilli-treated birds suggests the role of lactobacilli as beneficial commensal bacteria in B cell development. It has been previously reported that germ-free animals show impaired immune responses against different antigens suggesting the critical role of commensal bacteria in immune system development (39). In chickens, diversification of immunoglobulin mostly occurs during embryonic development, challenging the role of microbiota in pre-hatch B cells development and Ig diversification. However, it is reported that shortly after hatch, gut microbiota appears to influence the B-lymphocyte repertoire in bursa through transepithelial pinocytotic flow of intestinal contents into bursal follicles that occurred by M cell-like follicle-associated epithelium (24, 40). To this end, our observation of augmented IL-13 expression in the bursa can imply that *in ovo* administration of probiotic lactobacilli can influence bursal development of B cells.

In this study, we evaluated the effects of a mixture of *Lactobacillus* bacteria on $CD4^+$ and $CD8^+$ cell populations in chicken splenocytes. T helper cells ($CD4^+$) are involved in various immune system processes such as activation of B cells, macrophages and cytotoxic T cells (41). In addition, they play a key role in generating adaptive immune responses through interaction with major histocompatibility complex (MHC) class II molecules on antigen presenting cells (42). Inoculation of embryonated eggs with lactobacilli did not change the percentage of $CD4^+$ splenocytes on days 5 and 10 post-hatch. In contrast, Dalloul et al. (43) demonstrated that feeding lactobacilli to chickens increased the percentage of $CD4^+$ intestinal intraepithelial lymphocytes. Similarly, Noujaim et al. (22) showed that administration of a mixture of *Lactobacillus* bacteria including *L. acidophilus* and *L. reuteri* increased the number of $CD4^+$ cells in the small intestine of chickens. The percentage of $CD8^+$ T cells in the current study was not significantly affected by lactobacilli treatment. Asgari et al. (44) also observed no significant differences in $CD8^+$ cell counts in immune system organs (cecal tonsil and bursa of fabricius) of chickens treated with lactobacilli. However, Noujaim et al. (22) demonstrated that oral treatment of *L. reuteri* and *L. acidophilus* increased the number of $CD8^+$ cells in the epithelium and in the intestinal lamina propria of chickens. The inconsistent results observed in these studies could be attributed to the different types and dosages, including regimens of *Lactobacillus* bacteria in addition to differences in the route of administration used in different studies. The present results demonstrated that *in ovo* inoculation of eggs with lactobacilli enhanced serum IgG and IgM responses against KLH when a dose of 10^7 CFU was administered. In agreement with this result, previous studies have demonstrated that dietary/oral administration of probiotic bacteria enhances antibody responses against KLH, infectious bursal disease virus and avian influenza virus (16, 44, 45). Unlike KLH, lactobacilli treatment did not affect antibody production against SRBC. Similarly, Qorbanpour et al. (46) showed that dietary supplementation with multi-strain probiotics did not change antibody production against SRBC. In contrast, other studies demonstrated that dietary or oral administration of probiotic bacteria improves antibody response to SRBC (26, 47). In another study, Brisbin et al. (16) demonstrated that oral treatment of chickens with *L. salivarius* significantly increased serum antibody responses against SRBC compared to the control group; however, no such effect was observed when chickens were treated with *L. reuteri* and *L. acidophilus*. The conflicting results regarding the effects of lactobacilli on antibody-mediated immune response observed in different studies suggests that the immunomodulatory activities of *Lactobacillus* bacteria likely cannot be generalized at this point due to a number of factors such as the strain and dose of *Lactobacillus* bacteria, administration route, immunization regimen, timing of administration and experimental conditions.

In conclusion, the results of the current study demonstrated that *in ovo* inoculation of lactobacilli downregulated cytokine gene expression in the cecal tonsils, indicating the anti-inflammatory capacity of these bacteria in the intestine. However, elevated expression of cytokines observed in the spleen of *Lactobacillus*-treated birds suggested that lactobacilli may have different immunomodulatory activities in local and systemic secondary lymphoid organs. In addition, lactobacilli-treated groups, enhanced specific antibody-mediated immune responses against a highly immunogenic T cell-dependent antigen (KLH), suggesting the stimulatory effects these bacteria have on adaptive immunity. On the other hand, *Lactobacillus* bacteria did not have significant effects on T cell subsets in the spleen. Therefore, further studies are needed to investigate the effects of *in ovo* administration of lactobacilli on T and B cells population in the local and systemic immune system organs of chickens, in addition to further exploring the protecting effects of *in ovo*-inoculated lactobacilli against challenge with an infectious pathogen.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/supplementary material.

ETHICS STATEMENT

The animal study was reviewed and approved by Animal Care Committee, University of Guelph.

AUTHOR CONTRIBUTIONS

MA conceived and designed the project, collected and analyzed the data, and prepared the manuscript. BS, JA, KT-A, SK, JB, and RK helped for sample collection and reviewed the manuscripts, and provided suggestion and comments. SS provided intellectual input, approved the protocol, reviewed the manuscript and provided critical thinking, suggestion and comments.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Investigation of a Reduction in Tylosin on the Prevalence of Liver Abscesses and Antimicrobial Resistance in Enterococci in Feedlot Cattle

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Recent concerns over linkages between antimicrobial resistance in human pathogens and antimicrobial use in livestock have prompted researchers to investigate management strategies that reduce the current reliance on in-feed tylosin to control liver abscesses in feedlot cattle. A total of 7,576 crossbred yearlings were allocated to the study (~253 animals/pen, 10 replicate pens per treatment) and individually randomized to one of three treatments. Tylosin phosphate (11 ppm) was included in-feed (1) for the first 125 days on feed (DOF) (**FIRST-78%**), (2) for DOF 41 to 161 (**LAST-75%**), or (3) for the entire feeding period (**CON**; day 0–161). Fecal composites were collected from the pen floor on days 0, 81, and 160 of the finishing period. Serial dilutions were spread plated for enumeration of enterococci on Bile Esculin Azide (BEA) agar and BEA amended with 8 μg/ml erythromycin. Results indicated that although the proportion of Ery^R enterococci increased with DOF ($P < 0.01$), neither treatment ($P = 0.34$) or treatment × DOF ($P = 0.37$) affected antimicrobial resistance. Of the 538 isolates, 97% were enterococci, with mixed species isolated early in the feeding period and only *Enterococcus hirae* isolated at the end. Isolates were most frequently resistant to tylosin (86%), erythromycin (84%), and doxycycline (31%). Macrolide and tetracycline resistant isolates harbored *erm*(B), *msr*C, and *tet*(L), *tet*(M), *tet*(O) genes, respectively. Overall, the proportion of Ery^R enterococci increased ($P < 0.05$) in all three treatments over the feeding period. Compared to the control cattle, **FIRST-78%** cattle had more severe ($P < 0.05$) liver abscesses, while there was a trend ($P < 0.08$) for this response in **LAST-75%** cattle. There was no difference ($P > 0.05$) in total liver abscesses, growth performance, carcass traits, morbidity, or mortality among treatments. These results support the potential to reduce the duration and therefore quantity of tylosin administered to feedlot cattle during the feeding period without impacting animal productivity.

Keywords: Enterococci, antimicrobial resistance, tylosin, erythromycin, tetracycline, beef cattle

INTRODUCTION

Liver abscesses have a major economic impact on the North American beef cattle industry, with an average prevalence in feedlot cattle ranging from 12 to 32% (1), but it has been reported to be as high as 95% (2). Cattle with severely abscessed livers can exhibit compromised growth performance as a result of reduced feed intake and carcass weight (3, 4). In Canada, economic losses as a result of condemned and discounted livers are estimated at $60 million annually (5).

Antimicrobials are the primary tool used to prevent liver abscesses in cattle fed high-grain finishing diets. The macrolide, tylosin phosphate, is the most common antimicrobial included in feed to control liver abscess in beef cattle in North America (6), as it targets the causative agents, *Fusobacterium necrophorum* and *Trueperella pyogenes* (7). However, despite its use, the prevalence of liver abscesses in slaughter cattle still often exceeds 15% (5).

The use of antimicrobials in-feed has come under scrutiny by both the public and regulators over concerns that their use selects for antimicrobial resistance and poses a risk to public health (8). Tylosin belongs to the MLS_B superfamily (macrolide-lincosamide-streptogramin B) which are classified as a category II antimicrobial in terms of their importance for use in human medicine (9). Although tylosin is not used in human medicine, it cross-selects for resistance to other antimicrobials within this superfamily, including erythromycin, a macrolide widely used in humans (10).

It is essential to evaluate new strategies to manage liver abscesses in feedlot cattle while reducing reliance on medically important antimicrobials in livestock production. According to recently implemented restrictions in the United States (11) and Canada (12), all medically important antimicrobials require a veterinary prescription and cannot be used for growth promotion.

Enterococci are commensal bacteria of humans and animals that are often associated with serious hospital acquired infections (13). The most prevalent species associated with infections in human are *E. faecium* and *E. faecalis* (14), whereas *E. hirae* is the predominant species in cattle (15). Few studies have investigated the link between tylosin administration and antimicrobial resistance in enterococci in cattle. The most recent study in Canada, withdrew tylosin 28 days prior to slaughter in a small-scale (100 steers) trial and found a reduction in macrolide resistance in enterococci (16). Another feedlot study in the United States investigated the impact of intermittent (1 week on, 2 weeks off) and continuous administration vs. no tylosin on erythromycin resistance (Ery^R) in enterococci. They found no difference in the occurrence of liver abscesses between intermittent and continuous treatments, but there were more liver abscesses in cattle that did not receive tylosin (17). As such, it is important to continue to investigate ways to optimize tylosin use while promoting antimicrobial stewardship, supporting productivity, and working to minimize use of antimicrobials in livestock that are of importance in human medicine.

The present study investigated and compared the effect of tylosin administration in the first 78 or last 75% of the feeding period on antimicrobial resistance, liver abscess score, animal health, feedlot performance, and carcass traits of feedlot cattle.

MATERIALS AND METHODS

All procedures involving cattle were reviewed and approved by the Feedlot Health Management Services Ltd. (Okotoks, Alberta) and Lethbridge Research Center Animal Care Committees in accordance with guidelines of the Canadian Council on Animal Care (18). Informed consent for use of the cattle was received from the owners of the cattle.

Experimental Design

This study was conducted at a large commercial feedlot in southern Alberta over an 161-day finishing period. Cattle (n = 7,576) for this study were crossbred beef yearling steers and heifers (394 ± 5.49 kg) that arrived between June 11, 2018 and July 7, 2018. Upon arrival, cattle were randomly assigned to one of three treatments; **FIRST-78%**, **LAST-75%**, or **CON**. The experimental unit was the pen, with 10 pens (six steer, four heifer) allocated to each treatment. Average pen capacity was 253 ranging from 246 to 280 head/pen. Upon arrival, individual animals were managed as per standardized commercial Canadian feedlot practices, receiving an ear tag for identification, a hormonal growth promoter implant, a parenteral respiratory vaccine, a parenteral clostridial disease bacterin, and topical parasite control. No antimicrobials were administered to the cattle upon arrival. Cattle were randomly assigned to one of the three treatments and placed into a corresponding pen. Once a pen was full, then newly arrived cattle were allocated to a new pen for a second replicate of that treatment with this process continuing until all 10 pens per treatment were full.

Cattle were fed tylosin phosphate (Tylosin 40, Bio Agri Mix LP, Mitchell, ON) at an inclusion level of 11 ppm [100% dry matter basis [DM]] for: (1) the first 125 days of the 161-days feeding period (**FIRST-78%**), (2) the last 120 days of the feeding period (**LAST-75%)**, starting at an average of 41 days on feed (DOF) and continuing to slaughter at an average of 161 DOF, or (3) continuously throughout the 161-days feeding period (**CON**). Tylosin was administered at the concentration approved for the prevention of liver abscesses in beef cattle in Canada (19).

All diets were fed twice daily, and cattle were offered *ad libitum* access to feed and water. Using a series of four step-up diets, cattle were gradually transitioned to a high-concentrate finishing diet (dry matter basis) consisting of 85.8% concentrate, 11.5% roughage, and 2.8% supplement. The concentrate portion consisted of 70% corn with the remainder being tempered rolled barley / wheat. Monensin sodium was also included in diets at 33 ppm DM over the feeding period (Monensin Premix; Bio-Agri Mix LP, Mitchell, Ontario) according to the medicating ingredient brochure (19).

Sample Collection and Processing

Composite, fresh, pen-floor fecal samples from 20 different pats were collected from each pen using a standardized pen sampling plan. Samples were collected at allocation (0 DOF) before any tylosin was administered, in the middle of the feeding period

(avg. 81 DOF), and just prior to shipment for slaughter (avg. 160 DOF). Samples were collected in sterile Whirl Pak bags and stored at 4°C for an average of 1 day prior to transport to the Agriculture and Agri-Food Canada Lethbridge Research Center, Lethbridge, Alberta for microbial analysis. Samples were processed within 1 day of arrival at Lethbridge.

At the lab, each fecal sample was thoroughly mixed, weighed (1.0 g) and diluted 1:5 into 4.0 mL of sterile phosphate buffered saline and vortexed for 30 s. Samples were then 10-fold serially diluted and 100 μL of the appropriate dilution were plated in duplicate onto Bile Esculin Azide (BEA) agar containing no antimicrobials and BEA amended with erythromycin (8 μL/mL; BEA^E). The concentration of erythromycin added into the BEA plates was set at the breakpoint standards for defining resistance as described by the Clinical and Laboratory Standards Institute (CLSI) guidelines (20). After incubation for 48 h at 37°C, colonies that exhibited esculin hydrolysis (black precipitate) and morphology typical of enterococci were enumerated. Isolates that grew on BEA^E were considered resistant to erythromycin. The percentage of enterococci resistant to erythromycin was calculated according to Alexander et al. (21), in which: [(number of colonies on selective BEA^E plates / total colonies on non-selective BEA plates) × 100%].

For each sample, three enterococci colonies each from BEA and BEA^E plates (6 colonies in total) were subcultured onto their respective media and incubated for 48 h at 37°C, for purification and further characterization. To prepare template DNA for PCR, one colony from each plate was suspended in 100 μL of TE (10 mM Tris, 1 mM EDTA, pH 8.0) and heat lysed for 5 min at 98°C with shaking at 1000 RPM in an Eppendorf thermomixer (VWR, Missisauga, ON). Heat lysed cell suspensions were stored at −80°C for later use. Growth from subcultures was suspended in brain heart infusion (BHI) broth containing 15% glycerol and archived at −80°C for subsequent use.

Characterization of *Enterococcus* Species

A total of 538 presumptive enterococci isolates representing approximately six isolates from each pen on each sampling day were saved in TE as mentioned above. Tubes containing heat lysed cells were thawed and centrifuged at 10,000 × g for 5 min. The supernatant was used as the template DNA in a multiplex PCR to identify *Enterococcus* species. *Enterococcus*-specific *groES-EL* primers Ent-ES-211-233-F and Ent-EL-74-95-R (22) were used along with *Enterococcus hirae* muramidase gene (23) *mur2*-specific mur2h_F1 (5′-TATGGATACACTCGAATATCTT-3′) and mur2h_R (5′-ATTATTCCATTCGATTAACTGC-3′) primers were used in a multiplex PCR assay to distinguish *E. hirae* from other *Enterococcus* spp. Two microliters of template DNA was used in a 25 μL PCR reaction volume using HotStarTaq Master Mix Kit (Qiagen Canada, Inc., Mississauga, ON, Canada) as per manufacturer's instructions and with the following thermocycler conditions: 5 min at 95°C, followed by 45 cycles of 30 s at 94°C, 30 s at 49°C, 30 s at 72°C and a final extension for 10 min at 72°C. The PCR products were resolved on a 1.8% agarose gel. Isolates that were positive for both primer sets generated two PCR product bands and were identified as *E. hirae*, while single PCR products presumably originating from *groES-EL* positive, but *mur-2* negative (non-*E. hirae*) enterococci isolates were sent to Eurofins Genomics (Toronto, ON) for Sanger sequencing of the *groES-EL* intergenic region to identify species.

Antimicrobial Susceptibility Testing

A subset of 176 speciated isolates were randomly chosen to represent one isolate from each media type from all samples, with the exception of four isolates from the BEA plates that were not enterococci. Antimicrobial susceptibility testing for enterococci was performed against 12 antibiotics using disc diffusion methodology according to the CLSI guidelines for *Enterococcus* spp., documents M02-A12, M100-S26, and VET-01S (20, 24, 25). The panel covers medically important antibiotics that are classified as either medium, high or very high importance in human medicine (9). The antimicrobial panel, supplier, disk content, and zone diameter for determining break points are listed in **Supplementary Table 1**. *Staphylococcus aureus* ATCC® 25923 and *Enterococcus faecalis* ATCC® 29212 were used as standards and were included in each assay. Zone diameters were read using the BioMic V3 imaging system (Giles Scientific, Inc., Santa Barbara, CA, USA), and each enterococci isolate was classified as either susceptible, intermediate or resistant according to CLSI guidelines for 10 antimicrobials, or EUCAST for tigecycline (26). Tylosin does not have established interpretive criteria for *Enterococcus* spp., although there is an acceptable quality control range for 30 μg tylosin discs for *S. aureus* ATCC® 25923 set at 18–26 mm (24). For tylosin, previously published minimum inhibitory concentration (MIC) established in our lab (16) were used as breakpoints in the current study. Isolates that were resistant to three or more antimicrobials were defined as multidrug resistant.

Resistant Gene Determinants

The isolates displaying intermediate resistance or resistance to erythromycin or tylosin were screened by PCR for macrolide resistance genes *erm*(B), and *msr*C (27), using the primers of Chen et al. (28), and Beukers et al. (16), respectively. Reactions were processed as a multiplex PCR with an initial denaturation for 5 min at 95°C, followed by 35 cycles of denaturation for 30 s at 94°C, annealing for 30 s at 58°C and a final extension for 10 min at 72°C. Isolates displaying intermediate resistance or resistance to doxycycline were also screened by PCR for *tet*(L), *tet*(M), and *tet*(O) as previously described (29). All PCRs were prepared as a 20 μL reaction with 2 μL DNA template and resolved on a 1.5% agarose gel. Conventional PCR was performed using HotStarTaq Master Mix Kit, and multiplex reactions using the Mulitplex Master Mix Kit (Qiagen Canada, Inc., Mississauga, ON).

Animal Performance, Liver Abscesses, and Carcass Traits

Upon allocation, initial body weight (BW) and hip height were measured as baseline variables for each individual animal to assess homogeneity across treatments. Animal performance variables (final BW; daily dry matter intake, DDMI; average daily gain, ADG; feed-to-gain ratio, F:G) were calculated for each pen to describe feedlot performance. Final BW represented the

average net (shrink accounted for gut fill) live weight of cattle sold for slaughter. The DDMI was calculated by the total quantity of feed consumed divided by the number of days on feed and animals within a pen. The ADG was determined by the total net slaughter weight plus total weight of cattle shipped for salvage slaughter plus total weight of animals that died minus total allocation weight; divided by the number of days in the trial. Feed efficiency (F:G) was determined as DDMI divided by ADG on a live weight basis. Cattle were monitored twice daily by animal health personnel for evidence of disease. Individual cattle that were deemed "sick" were separated out of the pen and moved to a hospital facility for diagnosis and treatment. If cattle were housed in hospital pens, the feed was accounted for by proration to the home pen record as per standard procedures. An effort was made to avoid treating "sick" cattle with macrolides and they were returned directly to their home pen whenever possible. When this was not possible, their removal from the home pen was accounted for. Overall mortality was defined as the number of mortalities divided by the number of animals allocated.

All animals from this study were slaughtered at a single processing plant. Cattle from assigned pens were shipped for slaughter as a single lot as per finishing time as assessed by standard feedlot production practices. At slaughter, all livers were scored for severity and prevalence of liver abscesses by trained personnel, using a modified Elanco Liver Check System (Elanco, Greenfield, IN, USA). Livers that had no abscesses (normal healthy liver) were assigned a liver score of 0. Livers with one or two small active abscesses/scars or up to four abscesses with a diameter of <2.5 cm were assigned a liver score of A. Livers with one or more large abscesses (diameter > 2.5 cm) or more than four small/old abscesses of a diameter < 2.5 cm were assigned a liver score of A+ (severe).

Canadian quality grade (QG), yield grade (YG), and weight of each carcass were collected using the data capture system at the processing plant. The average carcass weight was determined by the total carcass weight at slaughter divided by the number of cattle sold for slaughter. The dressing percentage was calculated by the total carcass weight at slaughter divided by the total weight at slaughter expressed as a percentage.

Statistical Analysis

Data were analyzed using SAS® for Windows, Release 9.4 (SAS Institute Inc., Cary, North Carolina). Prior to analysis, microbial enumeration data were normalized by a $\log_{10}$ transformation and analyzed using the MIXED procedure of SAS with a completely randomized factorial arrangement with repeated measures. The treatments (**FIRST-78%**, **LAST-75%**, **CON**) and sampling days (0, 81, 160) and their interaction were analyzed as fixed effects with replicate as a random effect.

The baseline (initial BW and hip height), liver abscess score, feedlot performance, and carcass trait variables were analyzed using GLIMMIX in SAS. Baseline variables were tested as covariates of the feedlot performance variables and included in the model if statistically significant. Sex (steers or heifers) was included as a fixed effect in the models for feedlot performance and liver abscess score. Morbidity and mortality data were analyzed using the GENMOD procedure in SAS with Poisson regression in a log linear model for treatment effects and adjusted for clustering of disease (pen nested within replicate) with generalized estimating equations. For all tests, level of significance was set at $P < 0.05$.

RESULTS

CFU Counts of Enterococci and Proportion of Erythromycin Resistance

Enterococci were isolated from fecal composite samples from all 30 pens on all sampling days with the exception of four pens on day 81, where selected colonies were not enterococci. No difference ($P > 0.05$) was observed between **FIRST-78%**, **LAST-75%**, and **CON** cattle with regard to total enterococci, Ery^R enterococci (**Table 1**), or proportion of Ery^R enterococci within the total enterococci population (**Figure 1A**). However, there was a decrease ($P < 0.01$) in total enterococci with increasing days on feed. The proportion of Ery^R was highest on day 81 ($P < 0.01$) for all treatments. Compared to arrival, the proportion of Ery^R enterococci isolated just prior to slaughter increased by 52, 187, and 89% ($P < 0.01$) in the **FIRST-78%**, **LAST-75%**, and **CON**, respectively (**Figure 1A**).

Characterization of Enterococci

Of the 538 isolates collected throughout the trial, 97% were confirmed as enterococci by PCR. Speciation of 522 enterococci isolates revealed that 93.9% were *E. hirae* ($n = 490$), 3.3% were *E. villorum* ($n = 17$), 2.5% were *E. faecium* ($n = 13$), and 0.4% were *E. durans* ($n = 2$). Out of the 32 non-*hirae* enterococci isolated, 41% ($n = 13$) were collected from non-selective BEA, whereas 59% ($n = 19$) were isolated from selective BEA^E. The diversity of enterococci tended to be greater at arrival than later during the

TABLE 1 | Enterococci counts of the total population and Ery^R *enterococci* isolated from feedlot cattle feces from cattle fed tylosin for the **FIRST-78%**, **LAST-75%**, or continuously (**CON**) during the feeding period.

Item[a]	Treatments[b]			SEM	*P*–value[c]		
	FIRST-78	LAST-75	CON		T	D	T × D
No. of Enterococci ($\log_{10}$ CFU/g feces)							
Day 0	6.0	6.5	6.2	0.24	0.14	<0.01	0.12
Day 81	5.2	5.7	5.9				
Day 160	5.3	5.3	5.3				
No. of Ery^R Enterococci ($\log_{10}$ CFU/g feces)							
Day 0	4.4	4.5	4.9	0.41	0.18	0.02	0.98
Day 81	5.0	5.4	5.4				
Day 160	4.5	4.7	4.9				

[a] *Cattle were sampled upon arrival and after 81 and 160 days on feed. Total enterococci were enumerated on BEA, bile esculin azide agar; and erythromycin resistant (Ery^R) enterococci were enumerated on BEA^E amended with erythromycin (8 μg/ml).*

[b] *Tylosin inclusion at 11 ppm; FIRST-78% = tylosin in-feed from 0 to 125 days; LAST-75% = tylosin in-feed from d 41 to d 161; CON, control, continuous feeding of tylosin (0–161 days).*

[c] *T, Treatment; D, Days on feed; T × D, Treatment × Days on feed.*

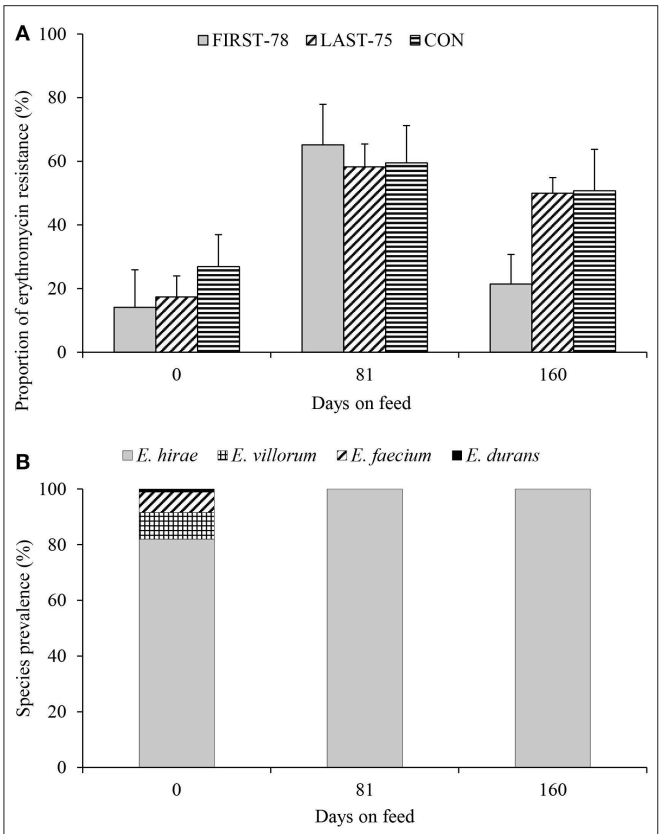

FIGURE 1 | Erythromycin resistance **(A)** and species prevalence **(B)** of enterococci isolated from the feces (sample 0, 81, 160 days) of feedlot cattle administered tylosin in the **FIRST-78%**, **LAST-75%**, or continuously (**CON**) during a 160 days feeding period. **(A)** Treatment group, $P = 0.34$; Treatment group X Days on feed, $P = 0.37$; Days on feed, $P < 0.01$. **(B)** Isolates are pooled across all pens, treatment groups and media type.

feeding period (**Figure 1B**), with *E. hirae* being the only species identified on day 81 and 160.

Antimicrobial Susceptibility Testing

Across all treatments, a total of 86% ($n = 151$), 84% ($n = 147$), and 31% ($n = 54$) of isolates displayed intermediate resistance or resistance to tylosin, erythromycin and doxycycline, respectively (**Table 2**). Ninety-five percent of the isolates ($n = 145/153$) that were not susceptible to macrolides displayed either intermediate resistance or resistance to both erythromycin and tylosin. In total, 16 antibiogram phenotypes were observed, ranging from no resistance (A1) to resistance to six antimicrobials (A16) (**Table 2**). No isolates displayed intermediate resistance or resistance to ampicillin, gentamicin, levofloxacin, or vancomycin; but at least one isolate was resistant to each of the other antimicrobials tested. The three most common antimicrobial resistance phenotypes across all treatments and days were A1 (No resistance), A5 (ERY-TYL), and A7(dox-ERY-TYL), representing 82% of all observed susceptibility patterns. Multidrug resistance (≥ 3 antimicrobials) occurred in 9.7% ($n = 17$) of isolates, and did not appear to be influenced by treatment.

Identification of Resistant Gene Determinants

Of the 153 enterococci isolates displaying intermediate resistance ($n_{Ery} = 8$; $n_{Tyl} = 7$) or resistance ($n_{Ery} = 139$; $n_{Tyl} = 144$) to erythromycin or tylosin, the *erm*(B) gene was detected in 144 (**Table 3**) with representatives of *E. hirae*, *E. faecium*, and *E. villorum*. Within these isolates, six [*E. hirae* ($n = 1$), and *E. faecium* ($n = 5$)] collected on day 0 were also positive for *msr*C. Nine isolates from BEA displayed intermediate resistance to either erythromycin or tylosin, but were negative for both macrolide resistance genes.

Within the 153 isolates screened for macrolide resistance genes, 39 displayed intermediate resistance and 15 were resistant to doxycycline. These isolates were further screened for tetracycline resistance genes, with 41 positive for both *tet*(M) and *tet*(L), and one positive for *tet*(M). Eleven isolates were positive for *tet*(O), with only one intermediate doxycycline resistant isolate being negative for all *tet* genes.

Liver Abscesses, Animal Performance, and Carcass Traits

Although the prevalence of severe liver abscesses (A+) for the **FIRST-78%** ($P < 0.05$) was or tended to be greater **LAST-75%** ($P < 0.08$) than **CON** (**Table 4**), the overall prevalence of liver abscesses (A and A+) was similar among treatments.

There were no significant differences detected between the **FIRST-78%** or **LAST-75%** and the **CON** for any of the morbidity or mortality outcomes (**Supplementary Table 2**). The incidence of morbidity was <3% and the overall mortality rate ranged from 0.9 to 1.4% for all treatments.

The treatments were homogenous ($P \geq 0.05$) at allocation with respect to average initial weight (kg) and average hip height (m) (**Table 4**). Growth performance of feedlot cattle did not differ ($P > 0.05$) between the **FIRST-78%** and **CON** or **LAST-75%** and **CON** for ADG or F:G (**Table 4**). Carcass weight was greater (absolute difference of 3.3 kg; $P = 0.04$) for cattle in the **FIRST-78%** compared to **CON** (**Table 4**). There was no difference detected between the **FIRST-78%** or **LAST-75%** and **CON** for dressing percentage (**Table 4**). Yield and quality grade also did not differ among treatments (**Table 4**).

DISCUSSION

For the purpose of this study, enterococci were chosen as the fecal indicator bacteria for assessing macrolide resistance, as *Escherichia coli* is intrinsically resistant to this antimicrobial family (30). Enterococci, notably *E. faecalis* and *E. faecium* are seen with increasing prevalence in clinical infections in humans (14). In the present study, *E. faecalis* was not detected, and *E. faecium* was only isolated from cattle upon arrival. Consistent with previous reports (16, 31, 32), there was a decrease in the diversity of enterococci over the feeding period, with *E. hirae* being the predominant species isolated from beef cattle feces, a species seldom associated with infections in humans (33). Beukers et al. (16) proposed that this shift in fecal enterococci

TABLE 2 | Antibiograms of enterococci ($n = 176$) isolated from feedlot cattle feces from cattle fed tylosin for the **FIRST-78%**, **LAST-75%**, or continuously (**CON**) during the feeding period.

Profile	Phenotype[c]	No. isolates (%) within treatments and days[a,b]									Total
		FIRST-78			LAST-75			CON			
		d 0 ($n = 20$)	d 81 ($n = 20$)	d 160 ($n = 20$)	d 0 ($n = 20$)	d 81 ($n = 18$)	d 160 ($n = 20$)	d 0 ($n = 20$)	d 81 ($n = 18$)	d 160 ($n = 20$)	
A1	No Resistance	6 (30.0)		2 (10.0)	6 (30.0)	1 (5.6)		5 (25.0)	1 (5.6)		21
A2	NIT			1 (5.0)							1
A3	Tyl						1 (5.0)		1 (5.6)	1 (5.0)	3
A4	ery-nit	1 (5.0)									1
A5	ERY-TYL	7 (35.0)	16 (80.0)	4 (20.0)	7 (35.0)	7 (38.9)	10 (50.0)	7 (35.0)	12 (66.7)	11 (55.0)	81
A6	nit-tyl						1 (5.0)	1 (5.0)			2
A7	dox-ERY-TYL	2 (10.0)	3 (15.0)	9 (45.0)	1 (5.0)	10 (55.6)	5 (25.0)	3 (15.0)	4 (22.2)	5 (25.0)	42
A8	ery-lin-NIT				1 (5.0)						1
A9	ERY-nit-TYL		1 (5.0)	1 (5.0)	1 (5.0)		1 (5.0)	1 (5.0)			5
A10	ERY-q-d-TYL	1 (5.0)		2 (10.0)			1 (5.0)				4
A11	ERY-str-TYL									1 (5.0)	1
A12	lin-NIT-TYL	1 (5.0)									1
A13	DOX-ERY-NIT-TYL	2 (10.0)		1 (5.0)	2 (10.0)		1 (5.0)	2 (10.0)		2 (10.0)	10
A14	ery-NIT-TIG-tyl							1 (5.0)			1
A15	dox-ERY-NIT-q-d-TYL				1 (5.0)						1
A16	dox-ery-lin-NIT-TIG-TYL				1 (5.0)						1

[a] *Enterococci were isolated from BEA and BEA^E media.*

[b] *Tylosin inclusion at 11 ppm; FIRST-78% = tylosin in-feed from d 0 to d 125; LAST-75% = tylosin in-feed from d 41 to d 161; CON, control, continuous feeding of tylosin (d 0 to d 161). Fecal samples were collected on d 0, d 81, and d 160.*

[c] *DOX, Doxycycline; ERY, Erythromycin; LIN, Linezolid; NIT, Nitrofurantoin; Q-D, Quinupristin-dalfopristin; STR, Streptomycin; TIG, Tigecycline; TYL, Tylosin. Upper case denotes complete resistance and lower case denotes intermediate resistance.*

TABLE 3 | Distribution of enterococci isolates from feedlot cattle feces grouped according to macrolide ($n = 153$) and tetracycline ($n = 54$) resistance genes and by cattle fed tylosin for the **FIRST-78%**, **LAST-75%**, or continuously (**CON**) during the feeding period.

Treatment[a]	No. Positive (%)[b]								
	Macrolide				Tetracycline				
	n	*erm*(B)	*msr*C	Negative	*n*	*tet*(L)	*tet*(M)	*tet*(O)	Negative
FIRST-78	51	49 (96.1)	1 (2.0)	2 (3.9)	17	13 (61.9)	13 (61.9)	4 (19.0)	0 (0)
LAST-75	51	48 (94.1)	3 (5.9)	3 (5.9)	21	18 (85.7)	19 (90.5)	2 (9.5)	0 (0)
CON	51	47 (92.2)	2 (3.9)	4 (7.8)	16	10 (62.5)	10 (62.5)	5 (31.3)	1 (6.3)
Total	153	144 (94.1)	6 (3.9)	9 (5.9)	54	41 (75.9)	42 (77.8)	11 (20.4)	1 (6.3)

[a] *Tylosin inclusion at 11 ppm; FIRST-78% = tylosin in-feed from d 0 to d 125; LAST-75% = tylosin in-feed from d 41 to d 161; CON = control, continuous feeding of tylosin (d 0 to d 161).*

[b] *Isolates pooled across all media types and sampling days.*

species may arise from the transition of cattle from a forage-based to a grain-based finishing diet during the finishing period. Others have proposed that it may also be influenced by age of the host (34, 35). In the present study, cattle were transitioned from a high (40%) to low (11.5%) forage diet over the first 20 days of the feeding period. Therefore, cattle pens sampled upon allocation had less concentrate in their diets compared to those sampled on days 81 and 160 when the high concentrate diet was fed.

Tylosin was administered to cattle at the concentration approved for the prevention of liver abscesses (19). Since this study revolved around the feeding regime of tylosin, the main focus was on Ery^R enterococci isolated from beef cattle feces. Antimicrobial susceptibility testing of enterococci indicated that all isolates initially collected from the selective BEA^E were resistant to erythromycin.

A small-scale study in Southern Alberta demonstrated that although tylosin did not reduce the overall prevalence of liver abscesses, severely abscessed livers tended to be lower in cattle fed tylosin (6.7%) than in those that did not receive it (negative control; 53.3%) (36). Due to the large number of animals enrolled in this study, and the importance of tylosin in liver abscess control (37, 38), a negative control group of cattle that did not

TABLE 4 | Growth performance, liver abscesses, and carcass traits of feedlot cattle from cattle fed tylosin for the **FIRST-78%**, **LAST-75%**, or continuously (**CON**) during the feeding period.

Item	Treatments[a]			SEM	*P*-values	
	FIRST-78	LAST-75	CON		FIRST-78 vs. CON	LAST-75 vs. CON
No. of cattle	2,525	2,526	2,525			
Growth[b]						
Initial Hip Height (m)	1.2	1.2	1.2	0.01	0.51	0.39
Initial BW (kg)	393.5	395.2	393.6	5.49	0.99	0.22
Final BW (kg)	681.0	680.0	677.5	9.25	0.25	0.40
DMI (kg/d)	11.9	11.9	11.8	0.14	0.80	0.22
ADG (kg/d)	1.8	1.8	1.7	0.03	0.25	0.69
F:G	6.7	6.8	6.8	0.07	0.23	0.70
Total liver abscesses(%)	61.0	64.2	61.9	0.4	0.81	0.53
Liver Score[c]						
0 (%)	39.0	35.9	38.1	3.64	0.81	0.53
A (%)	37.5	41.2	42.1	3.53	0.23	0.82
A+ (%)	23.5	23.0	19.8	3.92	0.05	0.08
Carcass Traits						
Carcass Weight (kg)	410.2	408.1	406.9	5.72	0.04	0.45
Dress Percentage (%)	60.2	60.0	60.1	0.1	0.20	0.61
Yield Grade						
Canada 1 (%)	21.9	21.6	20.9	3.91	0.74	0.82
Canada 2 (%)	35.9	39.2	39.0	2.11	0.11	0.92
Canada 3 (%)	42.2	39.3	40.2	5.35	0.55	0.80
Quality Grades						
Canada Prime (%)	1.0	0.8	1.2	0.25	0.62	0.25
Canada AAA (%)	69.2	64.4	66.7	2.69	0.31	0.35
Canada AA (%)	25.8	30.4	27.3	2.72	0.48	0.16
Canada A (%)	0.6	0.8	1.0	0.24	0.09	0.43
B4 (%)	3.3	3.4	3.6	1.33	0.75	0.80
Other (%)[d]	0.1	0.2	0.2	0.11	0.59	0.62

[a] *Tylosin inclusion at 11 ppm; FIRST-78% = tylosin in-feed from d 0 to d 125; LAST-75% = tylosin in-feed from d 41 to d 161; CON, control, continuous feeding of tylosin (d 0 to d 161).*

[b] *DMI, dry matter intake; ADG, average daily gain; F:G, feed-to-gain ratio, calculated as DMI divided by ADG (live weight basis).*

[c] *Liver score 0 = no abscesses (normal healthy liver); A = 1 or 2 small active abscesses/scars or up to 4 well organized abscesses >1 inch (2.5 cm) in diameter. A+ = 1 or more large active abscesses with surrounding zone of inflammation or more than 4 small/old abscesses >1 inch (2.5 cm) in diameter.*

[d] *Canada quality grades B2, B3, D2, D3, and E were combined into "Other" off grades category.*

receive tylosin was not economically feasible. As in the present study, several studies have shown that in-feed tylosin increases Ery^R enterococci in cattle as compared to those that do not receive this antimicrobial (16, 31, 39).

The amount of Ery^R enterococci did not differ among treatments at any of the three sampling days. However, between the time of allocation and mid-sampling, the proportion of Ery^R enterococci increased and then subsequently decreased at the end of the feeding period, an observation that coincides with Beukers et al. (16). In a smaller scale study, Beukers et al. (16) compared macrolide resistance in fecal enterococci in cattle fed tylosin for the first 197 days and after withdrawal 28 days prior to slaughter. They observed a reduction in macrolide resistance, just prior to and after the removal of tylosin. Müller et al. (17) explored the intermittent use (1 week on, 2 weeks off) of tylosin compared to continuous or no tylosin and found no difference in Ery^R enterococci between tylosin treatment at each time point. However, these researchers did record a higher percentage of Ery^R enterococci with increasing days on feed between day 20 and day 118. The beneficial effect of reducing tylosin in-feed on the degree of resistance is difficult to predict because antimicrobial resistant bacteria are present in nearly all environments (40). However, shortening the duration of tylosin administered could help reduce the selection pressure that exacerbates the occurrence of antimicrobial resistance (16). In relation to the present study, to realize the impact of the removal of tylosin on the reduction in macrolide resistance, a much longer duration than 25% of the feeding period may be required.

Cattle feces are a natural vector for the transmission of bacteria and their antimicrobial resistance genes into the environment (41). Enterococci are known as antimicrobial resistance gene traffickers because they can readily transfer and acquire antimicrobial resistance genes (42). Enterococci have emerged as a major public health concern, especially vancomycin resistant *E. faecalis* and *E. faecium* which are more difficult to treat (43). Of the 176 isolates screened for antimicrobial resistance, all were susceptible to vancomycin, a result that agrees with previous studies that have suggested that cattle feces are not a major source of vancomycin-resistant enterococci (16, 44). In the present study, resistance to tylosin, erythromycin and doxycycline was most prevalent among isolated enterococci. It has been proposed that the administration of tylosin may co-select for enterococci with resistance to tetracycline, even in the absence of tetracycline use (45). Müller et al. (17) reported increased proportion of Tet^R enterococci in cattle feces with increasing days on feed, but found no relationship between Tet^R occurrence and the administration of tylosin in feed. Although tetracycline was absent in the diet, Müller et al. (17) observed an initially high proportion of Tet^R enterococci in cattle feces at approximately 10% on day 0, with increases between day 20 (∼20%) and day 118 (∼40%). These results coincide with the present study, where initially a high number of enterococci isolates with intermediate or resistant phenotypes to doxycycline (23%) was detected, with this level only increasing slightly between days 81 (34%) and 160 (31%).

Resistance of enterococci to erythromycin and tetracycline are commonly encoded by *erm*(B), *msr*C, and *tet* (L), *tet*(M), *tet*(O) resistance genes, respectively (16, 44). The resistance gene *msr*C, is universally present in all *E. faecium* (27) and was detected in all isolates of this species as well as in one *E. hirae* isolate. Other Ery^R genes in enterococci include *erm*(A) and *erm*(C) (27), but we did not screen for these genes as they are infrequent in enterococci isolated from beef cattle (16, 31, 46). Nine isolates were negative for both macrolide resistance genes, suggesting that these isolates

contained unknown or other known macrolide resistance genes that were not screened (40, 46).

The occurrence of multiple resistance genes within a single isolate may suggest the presence of mobile genetic elements (MGE). Both *tet*(M) and *erm*(B) are known to be frequently associated with the *Tn*916 family of MGE that are common in enterococci (47). Therefore, feeding tylosin may create selective pressure for not only macrolide resistance, but also tetracycline resistance (45). Although erythromycin and tetracycline are seldom used to treat enterococcal infections, they are used to treat other bacterial infections in humans (48). If resistant enterococci serve as a reservoir of these MGE-associated antimicrobial resistance genes, they could present a public health risk (44).

Previous studies noted that liver abscesses, especially livers scored as severe (A+) result in reduced feed intake, and a lower final body weight (1). Tylosin is frequently administered in-feed throughout the entire feeding period and in the past was found to lower the prevalence of liver abscesses 40–70% (37). However, the incidence of liver abscesses in feedlot cattle has increased over time, even with the inclusion of tylosin in the diet (5). Brink (1) evaluated 12 experiments involving 566 cattle and found that on average, cattle finished at a final weight of 473.0 kg over 131 DOF had a prevalence of severe liver abscesses of 6% (Range 0–19%). Their study suggested that the risk of severe liver abscesses increase with increasing finishing weight and duration on feed. The reasons why tylosin does not completely control liver abscesses are unknown, but there are speculations it may promote the growth of opportunistic pathogens, select for resistance strains, or that its concentration in the rumen is too low to be affective against the causative bacteria (49). Although previous work has shown little evidence that exposure of *F. necrophorum* or *T. pyogenes* to tylosin promotes macrolide resistance (49–51).

In the current study, the proportion of severely abscessed (A+) livers was greater in the **FIRST-78%** ($P < 0.05$) and tended to be higher in the **LAST-75%** ($P < 0.08$) compared to the **CON**. However, the proportion of total liver abscesses was not affected when tylosin was administered for shorter durations during the feeding period. Despite the greater prevalence of severe liver abscesses with shorter duration tylosin programs, there was no difference ($P < 0.05$) between the **FIRST-78%** or **LAST-75%** and the **CON** for any of the morbidity or mortality outcomes. Overall, the mortality rate for the present study was <2% which is within the lower range (0–15%) of feedlot cattle in North America (52). The primary causes of mortality included bovine respiratory disease, lameness, metabolic disorders including bloat and acidosis. With the exception of metabolic disorders, all of the other causes of mortality were not treated with tylosin and the use of other macrolides was avoided.

Walter et al. (53) evaluated liver abscess prevalence in cattle ($n = 3{,}360$) fed tylosin during the first 42, first 84, last 84, and first 126 out of 162 days on feed compared to continuous or no tylosin administration. They observed a linear total decrease in abscessed and A+ livers as days of tylosin feeding increased. Cattle that were fed tylosin in the first 84 d had fewer A+ livers than cattle fed tylosin for the last 84 d, suggesting that a greatest risk of liver abscess formation and subsequent greatest efficacy if tylosin is administered early in the feeding period (53). However, in our study, the marginal difference of A+ liver score between **LAST-75%** and the **CON** suggests that there is still risk of severe liver abscess formation later in the feeding period. Similar to our study, Walter et al. (53) found a decrease in overall edible/healthy livers (score 0) with reduced tylosin administration. In the present study, the origin of the cattle was not recorded, but they were older yearling cattle. Therefore, the cattle may have had pre-existing or increased susceptibility to developing liver abscesses prior to their arrival at the feedlot. This or the fact that the feedlot diet contained a mixture of wheat and barley may account for the much higher prevalence of liver abscesses observed in our study as compared to Walter et al. (53). Using feedlot performance as a secondary indicator of animal health and welfare, no differences in mortality, ADG, F:G, hot carcass weight, marbling score or other carcass traits were observed.

CONCLUSION

Few studies have investigated the effect of reduced tylosin feeding in feedlot cattle. Based on the results of our study, shortening the duration of tylosin feeding is likely to result in slightly more severe liver abscesses, but the overall impacts on morbidity and mortality, animal performance and carcass traits may be minimal in cattle fed for ∼160 days. This study demonstrates that reduced feeding of tylosin either at the beginning or end of the feeding period is unlikely to significantly change the proportion of resistant enterococci in the feces at the time of slaughter. The measured levels of Ery^R and antimicrobial susceptibility patterns in enterococci only exhibited a modest relationship to the intermittent administration of tylosin to feedlot cattle. Additionally, *E. hirae*, was the predominant species of enterococci associated with feedlot cattle fed a high grain finishing diet, a species that is not commonly associated with infections in humans. Findings of this study support the potential for producers to reduce the administration of tylosin, a member of the macrolide class of antimicrobials that are considered important to public health. However, such practices are unlikely to reduce the amount of macrolide resistant enterococci excreted in beef cattle feces.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

ETHICS STATEMENT

All procedures involving cattle were reviewed and approved by the Feedlot Health Management Services Ltd (Okotoks, Alberta) and Lethbridge Research Center Animal Care Committees in

accordance with guidelines of the Canadian Council on Animal Care (18). Written informed consent was obtained from the owners for the participation of their animals in this study.

AUTHOR CONTRIBUTIONS

TM, RZ, SG, CB, SH, and CK conceived the project idea and devised a plan. TD coordinated laboratory level study implementation and conduced laboratory bench work with support from HS. TM, RZ, and CN-B were involved in planning and supervising the work. AB and CK coordinated feedlot-level study implementation and collected and delivered samples to the lab. AR-G performed the statistical analysis on the bacterial data. AB compiled animal health and performance data. CB and SH helped to verify final animal health and performance data and were involved in results interpretation. TD wrote the manuscript. All authors provided critical feedback and helped shape the research, analysis and manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2020.00090/full#supplementary-material

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Conflict of Interest: CB is part owner and managing partner of Feedlot Health Management Services Ltd. and Southern Alberta Veterinary Services Ltd.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

The reviewer MW declared a past co-authorship with one of the author TM to the handling editor.

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A Sensitive and Accurate Recombinase Polymerase Amplification Assay for Detection of the Primary Bacterial Pathogens Causing Bovine Respiratory Disease

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Rapid and accurate diagnosis of bovine respiratory disease (BRD) presents a substantial challenge to the North American cattle industry. Here we utilize recombinase polymerase amplification (RPA), a fast and sensitive isothermal DNA-based technology for the detection of four BRD pathogens (*Mannheimia haemolytica, Pasteurella multocida, Histophilus somni, Mycoplasma bovis*), genes coding antimicrobial resistance (AMR) and integrative conjugative elements (ICE) which can harbor AMR genes. Eleven RPA assays were designed and validated including: a) one conventional species-specific multiplex assay targeting the 4 BRD pathogens, b) two species-specific real-time multiplex RPA assays targeting *M. haemolytica*/*M. bovis* and *P. multocida*/*H. somni*, respectively with a novel competitive internal amplification control, c) seven conventional assays targeting AMR genes (*tetH*, *tetR*, *msrE*, *mphE*, *sul2*, *floR*, *erm42*), and d) one real-time assay targeting ICE. Each real-time RPA assay was tested on 100 deep nasopharyngeal swabs (DNPS) collected from feedlot cattle previously assessed for targets using either culture methods and/or polymerase chain reaction (PCR) verification (TC-PCR). The developed RPA assays enabled sensitive and accurate identification of BRD agents and AMR/ICE genes directly from DNPS, in a shorter period than TC-PCR, showing considerable promise as a tool for point-of-care identification of BRD pathogens and antimicrobial resistance genes.

Keywords: recombinase polymerase amplification, bovine respiratory disease, antimicrobial resistance, integrative conjugative element, competitive internal amplification

INTRODUCTION

Bovine respiratory disease (BRD) remains the most common and economically important disease affecting feedlot cattle, veal calves, weaned dairy heifers and beef calves (1, 2). Approximately 15% of cattle in North America are treated for BRD, accounting for 70% of morbidities and 40% of all cattle mortalities in feedlots (3, 4). Economic losses to the United States feedlot industry have been

reported to be as high as 1 billion dollars annually, due to losses in production, increased labor expenses, drug costs, and death (5, 6). As the clinical symptoms associated with BRD may be non-specific, subtle and exhibit a rapid onset, fast and accurate diagnosis of BRD presents a significant challenge (2). Often, cattle with BRD are detected late in the disease process or not at all (2).

BRD is characterized by complex interactions between the host's immune system, bacterial (i.e., *Mannheimia haemolytica*, *Pasteurella multocida, Histophilus somni*, and *Mycoplasma bovis*) and viral (i.e., Bovine Herpes Virus-1, Parainfluenza-3, Bovine Viral Diarrhea Virus, Bovine Respiratory Syncytial Virus) pathogens and management practices that increase stress such as weaning and transportation (4, 6–8). Although *M. haemolytica* is considered to be the predominant BRD agent (9), many of the bacterial species involved are ubiquitous and considered to be commensals of the bovine respiratory tract of healthy animals (4). However, suppression of the host immune system as a result of stress or viral infection can allow these pathogens to proliferate within the upper respiratory tract, spreading to the lower respiratory tract, resulting in lesions and acute pleuropneumonia (4, 6).

Controlling BRD is the primary reason for the use of antimicrobials in feedlot cattle (4). Often, metaphylactic administration of macrolides to asymptomatic animals in the presence of diseased animals is used to improve the welfare of cattle and to decrease financial losses as a result of morbidities and mortalities (4, 10). However, antimicrobial use selects for antimicrobial-resistant (AMR) bacteria, including pathogens as well as harmless bacteria that can potentially act as a genetic reservoir of AMR gene determinants (4, 11). Excluding *M. bovis*, the genomes of BRD pathogens often contain integrative conjugative elements (ICE), mobile genetic elements that can harbor multiple AMR genes and encode the conjugation machinery required for transfer of ICE between BRD pathogens and to other bacteria (4, 9). The resulting multi-drug resistance (MDR) among some BRD pathogens containing ICE presents a significant challenge for the efficacy of antimicrobial therapy as a treatment for BRDClawson et al. (12) found that the gene *tet(H)*, which confers tetracycline resistance was present in all AMR *M. haemolytica* strains isolated from confirmed BRD cases, and was also frequently found in *P. multocida* (13) and *H. somni* ICE (14). Furthermore, *tet(H)* was adjacent to the transposase gene *tnpA*, a core ICE gene associated with increased minimum inhibitory antimicrobial concentrations in *M. haemolytica, H. somni,* and *P. multocida* (15).

Isolation of BRD pathogens by traditional culture methods and PCR verification of bacterial isolates (TC-PCR) has long been used to confirm disease outbreaks, but with several limitations (16). Traditional culture methods are time-consuming, requiring several days to obtain bacterial isolates, and some species such as *M. bovis* and *H. somni* grow poorly, a characteristic that may result in an under representation of the role of these pathogens in BRD (16–18). Therefore, new technologies continue to be evaluated to improve the diagnosis, early detection, and prognosis of BRD (2). In this study, recombinase polymerase amplification (RPA) is proposed as an alternative diagnostic application for BRD because of its simplicity, flexibility, multiplexing capabilities and rapidity (19). Originally developed by Piepenburg (20), RPA is a sensitive, isothermal DNA-based technology which utilizes primers and recombination proteins to generate DNA amplicons, that can either be visualized by gel electrophoresis or evaluated in real-time using fluorescent probes.

The aim of this study was to utilize RPA for detection of the four main bacterial pathogens associated with BRD, as well as AMR genes and ICE, and to develop multiple real-time RPA assays containing a competitive internal amplification control (IAC) to identify false negatives (21–23). Real-time RPA assays were tested on bovine deep nasopharyngeal swabs (DNPS) collected from cattle at feedlot arrival, to determine accuracy and sensitivity of RPA in comparison to TC-PCR for detection of BRD pathogens, and to its suitability for field-based detection.

METHODS

DNA Extraction of Bacterial Strains

The strains used in this study are listed in **Table 1**. *M. haemolytica* and *P. multocida* strains were streaked onto tryptic soy agar containing sheep blood (TSA blood agar; Dalynn Biologicals, Calgary, AB, Canada) and incubated for 24 h at 37°C. *H. somni* strains were streaked onto TSA blood and incubated for 48 h at 37°C with 5% CO_2. *M. bovis* was cultured by inoculating 1.5 ml pleuropneumonia-like organism broth (PPLO; brain heart infusion broth at 17.5 g per l, yeast extract at 25 g per l, and heat inactivated fetal horse serum at 200 mL per l) with a loop of glycerol stock culture. This starter culture was incubated at 37°C with 5% CO_2 for 72–96 h. The entire 1.5 ml starter culture was then added to 30 ml PPLO broth and incubated for an additional 48 h.

DNA was extracted from cultured cells using the DNeasy Blood & Tissue Kit (Qiagen, Toronto, ON, Canada) using the animal tissues spin-column protocol. For *M. haemolytica, P. multocida*, and *H. somni*, lysis of the cells was completed in Qiagen tissue lysis (ATL) buffer with proteinase K at 56°C for 3 h, followed by storage at 4°C overnight. The following day the protocol was resumed according to kit instructions with an additional wash buffer 2 (AW2) wash step. For *M. bovis*, the lysis step was reduced to 2 min and the full protocol was completed without overnight incubation.

TABLE 1 | A list of control strains used in this study.

Species	Strain	RPA assay
Mannheimia haemolytica A1	ATCC BAA-410	*M. haemolytica (nmaA)*
Mannheimia haemolytica A6	ATCC 29697	*M. haemolytica (nmaA)*
Pasteurella multocida	CCUG 17976	*P. multocida (kmt1)*
Histophilus somni	ATCC 700025	*H. somni* (HS_0116)
Mycoplasma bovis	ATCC 25523	*M. bovis (uvrC)*
Mannheimia haemolytica	MH44 (9)	AMR, ICE (*tetH/tnpA*)
Pasteurella multocida	PM22 (9)	AMR, ICE (*tetH/tnpA*)
Histophilus somni	HS33 (9)	AMR, ICE (*tetH/tnpA*)

Preparation of Standard DNA

Extracted DNA was quantified using PicoGreen on the NanoDrop 3300 Fluorospectrometer (ThermoFisher Scientific, Ottawa, ON, Canada). The DNA was normalized to 10 ng/μl, and then to a 50,000 genome copies/μl stock and stored at −80°C. Calculation of DNA copy numbers per μl was based on the following formula: amount (copies/μl) = [DNA concentration (g/μl)/(bacterial genome length in base pairs × 660)] × 6.02 × 10^{23}. The following genome sizes were used: *M. haemolytica* 2.6 Mbp, *P. multocida* 2.3 Mbp, *H. somni* 2.3 Mbp, and *M. bovis* 1 Mbp.

Primer & Probe Design

Primers and probes were designed using Geneious 8.1.9 (Biomatters Ltd., Newark, NJ, USA) and verified using the NCBI BLAST nucleotide collection (nt/rt) reference sequence database (**Table 2**). The primers for *M. haemolytica* (*nmaA*) were designed for specificity to serotypes A1 and A6 because of their role as causative agents of BRD, while excluding serotype A2, a commensal of the bovine upper respiratory tract (12). Reference sequences used for primer design of each species-specific RPA include: *M. haemolytica* M42548 *nmaA* (GenBank: NC_021082.1), *H. somni* 2336 HS_0116 (GenBank: CP000947.1), *P. multocida Kmt1* (GenBank: FJ986389.1), and *M. bovis uvrC* (GenBank: AF003959.1).

The genomes of five MDR *M. haemolytica* (MH25, MH30, MH64, MH69, MH76) and one *H. somni* (HS31) from our collection, as well as the published sequences of *P. multocida* 36950 ICE*Pmu*1 (GenBank: CP003022.1), *M. haemolytica* M42548 ICE*Mh*1 (GenBank: NC_021082.1), and *H. somni* USDA-ARS-USMARC 63374 (GenBank: CP018808.1) were utilized during the design of the ICE RPA assay (**Figure 1**). While ICEs differ among strains, the presence of *tet(H)* (conferring tetracycline resistance) was found in 100% of AMR *M. haemolytica* strains associated with BRD (12). While the *tet(H)* gene itself is prevalent among genomes of numerous bacterial species, within ICE, *tet(H)* is located adjacent to a transposase (*tnpA*) with a conserved sequence among ICE-containing strains of *M. haemolytica, P. multocida,* and *H. somni*. Therefore, the ICE RPA was designed to span a region of both *tet(H)* and *tnpA,* allowing for specific detection of AMR ICE-containing strains of all three important BRD pathogens (**Figure 1**).

Species-Specific RPA Assays for BRD Pathogens & ICE

RPA reactions were performed in a total volume of 50 μl using the TwistAmp™ Basic Kit (TwistDX, Cambridge, UK). The reaction mixture included 420 nM each primer, 14 mM magnesium acetate, 29.5 μl rehydration buffer, 11.2 μl nuclease-free water, and 2 μl of bacterial DNA. A master mix was prepared containing all reagents except the DNA template and magnesium acetate, and then dispensed into 0.2 ml reaction tubes containing a dry enzyme pellet. Two microliters of DNA was added to each tube, followed by magnesium acetate into the tube lids and the lids were carefully closed. Reaction tubes were then vortexed and briefly centrifuged. Immediately thereafter, the reaction tubes were placed in an Eppendorf PCR thermocycler at 37°C to initiate the reaction. After 2 min the tubes were removed, briefly vortexed, centrifuged and then placed back into the thermocycler for another 28 min. Amplified RPA reactions were purified using the QIAquick PCR Purification Kit (Qiagen, Toronto, ON, Canada) automated on the QIAcube (Qiagen, Toronto, ON, Canada). Following purification, RPA products were electrophoresed on 2% (w/v) agarose gels containing ethidium bromide, and visualized using a fluorescence imager (FluorChem FC2; Alpha Innotech, San Leandro, CA, USA).

Each species-specific RPA assay, including multiplex and real-time assays were screened for inclusivity against 36 representative isolates of each of the four target species (n = 144). The *M. haemolytica* isolates represented both serotypes A1 and A6 and encompassed 35 different pulsed field gel electrophoresis (PFGE) profiles. Isolates were obtained from lung tissues of BRD mortalities, collected in both Canada and the USA (9, 15, 24). Arising from the same studies, *P. multocida* and *H. somni* isolates belonged to 31 and 21 PFGE types, respectively. The *M. bovis* isolates were collected from the Stanford et al. (15) study and consisted of 27 different PFGE profiles.

A total of 66 bacterial strains (**Table 3**) belonging to BRD pathogens, closely related species, or other species known to be present in the upper and lower bovine respiratory tract were used to test the specificity of the BRD target RPA assays using the basic kit (**Table 2**). Bacterial strains were purchased from the American Type Culture Collection (ATCC), Culture Collection University of Gothenburg (CCUG), or obtained from a collaborating laboratory (25). DNA was extracted using the DNeasy Blood & Tissue Kit (Qiagen, Toronto, ON, Canada) with appropriate protocols for Gram positive and Gram negative bacteria.

Similarly for the ICE RPA, reactions were prepared as described above. Specificity of the ICE target was evaluated using the three ICE control strains from our collection (**Table 1**) as well as an additional 22 sequenced strains (belonging to *M. haemolytica, P. multocida* and *H. somni*), 11 with and 11 without ICE.

TwistAmp™ Basic Kit Multiplex RPA Assay

A multiplex RPA using the TwistAmp™ Basic Kit (TwistDX, Cambridge, UK) was developed for the simultaneous amplification of all four of the BRD pathogens. Reactions were prepared as described (section Species-Specific RPA Assays for BRD Pathogens & ICE) with each of the 8 primers included at 120 nM.

AMR Gene RPA Assays Using TwistAmp™ Basic Kit

Seven RPA assays were designed for AMR genes (*tetH, tetR, msrE, mphE, sul2, floR, erm42*). Primers are listed in **Table 2** and reactions were prepared using the TwistAmp™ Basic Kit (TwistDX, Cambridge, UK) as described in section Species-Specific RPA Assays for BRD Pathogens & ICE. AMR gene RPA assays were verified using the sequenced strains listed in **Table 1** (9).

TABLE 2 | Primers and probes used in this study.

	Target	Gene	Forward primer sequence	Reverse primer sequence	Amplicon size	Exo probe sequence (F = fluorophore; H = tetrahydrofuran; Q = quencher)	RPA assay type[a]	RPAKit[b]
BRD targets	*Histophilus somni*	Hs_0116	CGTTTAATCCCATTGCGATCA TTCCCCATT	ATACTATTGCATTCGGC GATTTTTCCGCTT	342	TATTCAAGTAGATGCAGATGGGCAGCATAA FHQAATTGATGTCAAGAA	1	B/E
	Mannheimia haemolytica A1 and A6	*nmaA*	TCAAAATGGCTCCCTTAGTT GAGGGCTTTA	AGTGGTTGCTGTATCGCC ATGAACAAAAAT	254	TTCTGCTATTTTAGAAAAAATTCAACCTGT FHQTGCCGAATACAAAC	2	B/E
	Mycoplasma bovis	*uvrC*	ATGGTCCTTTTCCTTCTGG TTATGGAGCTA	TGGCTGCTTGATGCATTT TGTTAGTTAGTT	201	CAAAGACTATAACTTTTGGATTAATCAG TTFHAQAAAATTAAAGAAATT	2	B/E
	Pasteurella multocida	*kmt1*	GAACCGATTGCCGCGAAA TTGAGTTTTATG	CCAACAAAACTGTGCT TTTCTTTGCCACAA	132		S	B
	Pasteurella multocida	*kmt1*	GAACCGATTGCCGCG AAATTGAGTTTTATG	CGAACTCGCCACTTT TTGTTTCATTTGGAC	417	ATTATTTTATGGCTCGTTGTGAGTGG GCTTGFHGGQAGTCTTTTATTT	1	E
	ICE	*tetH/tnpA*	CATCCACTAACTACGGC GCTGACATATCAA	TTGGTCCCCTTTTATTTGC CTTTATTTATA	318	TTAAGGGGTTGAAATAACAGCTTT AGGTGFHGQTTTTCTTTGGTGAA	S	B/E
	IAC	NA	Refer to **Figure 2**	Refer to **Figure 2**	Varies	GGGACGTGTATTTAACGTACTCGGA GAAAAFHQTGATTTGAATGAACCG	1, 2	E
AMR targets	Tilmicosin/tulathromycin	*mph(E)*	TGGTATAAGTGAGCAATT GGAAACCCGCTA	TTGACCAATCAATAACG CCTGAAACAGCTC	155		S	B
	Tilmicosin/tulathromycin	*msr(E)*	AGTCGCTATAACTGGATCG AATGGAACAGG	TTGAATATCATTCGCT CCGATCCCCATTGA	238		S	B
	Trimethoprim-Sulfadoxine	*sul2*	GGCCTATCTCAATGATAT TCGCGGTTTTCC	GAATGCATAACGACGAG TTTGGCAGATGAT	90		S	B
	Florfenicol	*floR*	CTGGCGATGGATATTTATCT CCCTGTCGTT	ATCACCATATAGAGGCTCA ACGTGAGTTGG	101		S	B
	Oxytetracycline	*tet(H)*	CAAAATCTGTCGATGA TAATGCGCAAGGGA	ATAGCATAAAGTATTGCC CCCATCAGCCAT	166		S	B
	Tetracycline	*tetR*	CATTAAGCTCTATTGCGCA TTTTACATTAG	CTTTAATACTGTTTCAAG TCCAGAGATCAT	215		S	B
	Tilmicosin/tulathromycin	*Erm42*	GCCATGAATTTAAAAGTT CAAATGTGTCTA	TTGCTAAAGCTATGCAA TATGTTAGTTTTG	283		S	B

[a] *RPA assay type: 1 = multiplex, H. somni and P. multocida; 2 = multiplex, M. haemolytica and M. bovis; S = single-plex.*
[b] *RPA kit: B = TwistAmp™ Basic Kit (conventional); E = TwistAmp™ Exo Kit (Real-time).*

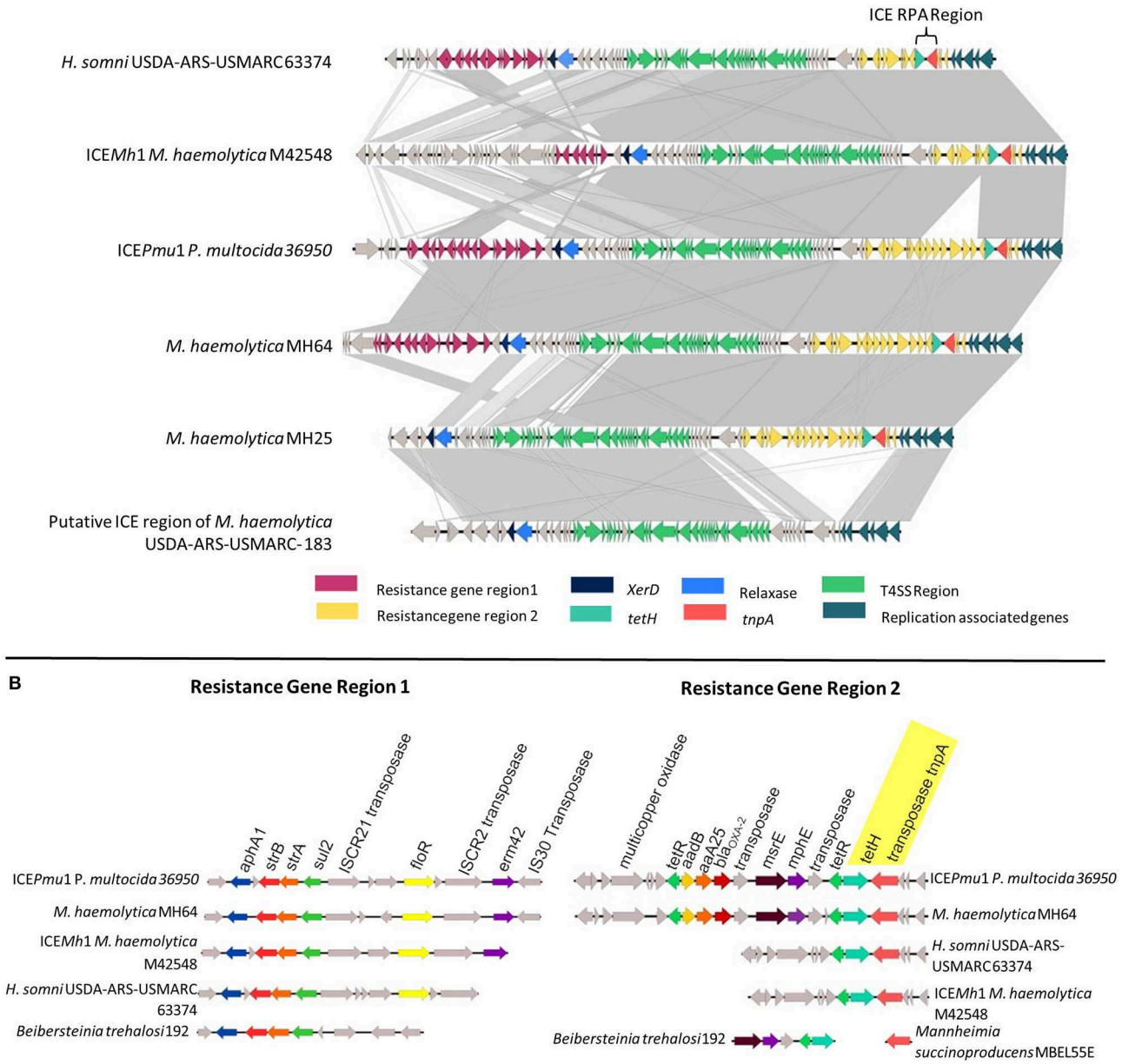

FIGURE 1 | (A) Genomic comparison of the integrative conjugative element (ICE) regions of *H. somni* USDA-ARS-USMARC 63374, ICE*Mh*1, ICE*Pmu*1, *M. haemolytica* MH64, *M. haemolytica* MH25, and the putative ICE region of *M. haemolytica* USDA-ARS-USMARC-183. Genes are represented as arrows, with the arrowhead indicating the direction of transcription. Areas between ICEs shaded in light gray indicate regions of ≥67% sequence identity, while areas shaded in dark gray indicate ≥99% sequence identity. **(B)** Comparison of resistance gene regions 1 and 2 in representative bovine respiratory disease species with alignments to cassettes found in other bacterial species.

Design of IAC for Multiplex Real-Time RPA

A competitive internal amplification control (IAC) was designed for use in multiplex real-time RPA and ICE RPA assays so that target primers also amplified the IAC, eliminating the need for additional primers specific for an internal control (**Figure 2**). Note that only one set of the target primers amplified the IAC, and therefore a positive control is still required as a verification for the other target primer set. The IAC template consisted of a sequenced region unique to *Bacillus atrophaeus* subsp. *globigii* (26, 27) containing a binding site for the IAC probe, and flanked by the primer sequences for *H. somni, M. haemolytica,* and ICE. The IAC was synthesized and inserted into a plasmid vector (pCR2.1) by Eurofins Genomics (Toronto, ON, Canada). The IAC plasmids were transformed into *E. coli* DH5α cells (Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA). Following plasmid purification using the QIAprep

TABLE 3 | A list of strains used for recombinase polymerase amplification specificity testing.

Target strains	Species	Strain/origin
	Mannheimia haemolytica A1	ATCC BAA-410
	Mannheimia haemolytica A6	ATCC 29697
	Pasteurella multocida	CCUG 17976B
	Histophilus somni	ATCC 700025
	Mycoplasma bovis	ATCC 25523
Non-target strains	*Mannheimia haemolytica A7*	ATCC 29698
	Mannheimia haemolytica A9	ATCC 29700
	Mannheimia haemolytica A2	ATCC 33396
	Mannheimia varigena (2 strains)	CCUG 38475, CCUG 38462
	Mannheimia	CCUG 38461
	Mannheimia granulomatis	CCUG 45422
	Mannheimia ruminalis (2 strains)	CCUG 38470, CCUG 38466
	Mannheimia glucosida (7 strains)	CCUG 28376, CCUG 38458, CCUG 38467, CCUG 38460, CCUG 28375, CCUG 38459, CCUG 38456
	Pasteurella canis	ATCC 43326
	Haemophilus influenza (2 strains)	ATCC 33391, ATCC 10211
	Haemophilus parasuis	ATCC 19417
	Mycoplasma bovirhinis	ATCC 27748
	Mycoplasma alkalescens	ATCC 29103
	Mycoplasma canadense	ATCC 29418
	Mycoplasma bovigenitalium	ATCC 19852
	Mycoplasma bovoculi	ATCC 29104
	Mycoplasma californicum	ATCC 33461
	Mycoplasma conjunctivae	ATCC 25834
	Mycoplasma arginini	ATCC 23243
	Mycoplasma canis	ATCC 19525
	Mycoplasma ovipneumoniae	ATCC 29419
	Trueperella pyogenes	ATCC 19411
	Moraxella bovoculi/lacunata	(25)
	Moraxella bovoculi/bovis	(25)
	Moraxella osloensis	(25)
	Psychrobacter pulmonis/faecalis	(25)
	Psychrobacter sanguinis	(25)
	Pseudomonas aeruginosa (2 strains)	ATCC 27853, ATCC 10145
	Acinetobacter baumannii	ATCC 17978
	Acinetobacter lwoffii	(25)
	Acinetobacter bouvetti	(25)
	Acinetobacter calcoaceticus/ oleivorans /juni	(25)
	Escherichia coli (2 strains)	ATCC 35218, ATCC 25922
	Streptococcus pneumoniae	ATCC 33400
	Streptococcus bovis	ATCC 33317
	Staphylococcus aureus (3 strains)	ATCC 35556, ATCC 29213, ATCC 29740
	Clostridium butyricum	ATCC 19398
	Clostridium difficile	ATCC 9689
	Actinobacillus succinogenes	ATCC 55618
	Bacillus atrophaeus	ATCC 9372
	Bacillus cereus	ATCC 10702
	Bacillus licheniformis	ATCC 14580
	Bacillus mycoides	ATCC 6462
	Bacillus subtilis	ATCC 6633
	Bacillus thuringiensis	ATCC 33679
	Leucobacter chromiireducens	ATCC BAA-1336
	Bibersteinia trehalosi (2 strains)	CCUG 27190, CCUG 37711

Spin Miniprep Kit (Qiagen, Toronto, ON, Canada), plasmid DNA was quantified by PicoGreen (Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA), normalized to 1×10^8 copies/μl and serially diluted to 5×10^2 copies/μl for use in real-time RPA assays.

Real-Time RPA Assays

Three real-time RPA assays were developed: (i) *P. multocida* and *H. somni* multiplex, (ii) *M. haemolytica* and *M. bovis* multiplex, and (iii) ICE RPA assay (**Figure 2C**). Real-time RPA was completed using the TwistAmp™ Exo Kit (TwistDX, Cambridge, UK). Reactions for ICE contained 420 nM of each ICE primer, 78 nM ICE probe, 24 nM internal control probe, 14 mM magnesium acetate, 29.5 μl rehydration buffer, 11.3 μl nuclease-free water, 1×10^3 genome copies per reaction internal control plasmid, and 2 μl of bacterial or sample DNA. Multiplex RPA reactions for *M. haemolytica* and *M. bovis* were prepared in the same way with the following modifications: 210 nM each primer, 45 nM each of *M. haemolytica* and *M. bovis* probe, and 30 nM internal control probe. Finally, for the *P. multocida* and *H. somni* multiplex RPA, reactions contained 190 nM *P. multocida* primers, 230 nM *H. somni* primers, 42.75 nM *P. multocida* probe, 52.25 nM *H. somni* probe, and 25 nM internal control probe, with all other reaction components being the same as for the ICE real-time assay. Reactions were prepared as described in section Species-Specific RPA Assays for BRD Pathogens & ICE with the following modifications: a magnetic bead was dispensed into each reaction tube immediately following the addition of master mix, and reaction tubes were placed in a T16-ISO instrument (TwistDX, Cambridge, UK) at 37°C for 33 min. Positive amplification was asserted when the fluorescence measured over 200 mV for 60 s.

The limit of detection (LOD) was determined for each real-time RPA using dilutions of genomic DNA (ranging from 1 to 1000 genome copies/reaction). Five reactions were prepared per DNA template concentration, with each run repeated 4 times, for a total of 20 reactions per dilution.

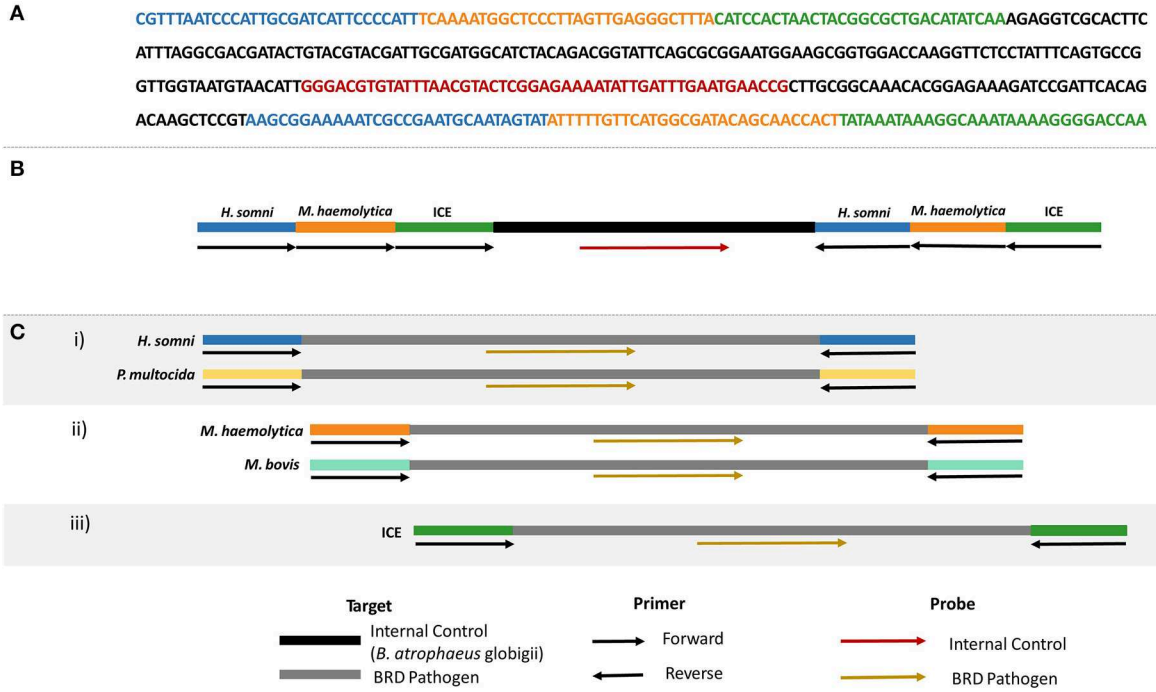

FIGURE 2 | (A) Nucleotide sequence of the internal amplification control (IAC), including primer binding sites for *H. somni* (blue), *M. haemolytica* (orange), and ICE (green), and the internal control probe binding site (red). Backbone sequence belonging to *B. atrophaeus* subsp. globigii indicated in black. **(B)** Schematic representation of the oligonucleotide primer and probe locations on the IAC. **(C)** Schematic representation of each real-time RPA assay to be used with the IAC.

Using RPA on Bovine Nasal Swabs

The ICE-specific real-time RPA assay, *M. haemolytica*/*M. bovis*, and *P. multocida*/*H. somni* multiplex real-time assays were tested using 100 DNPS collected from feedlot cattle, which were also screened for BRD pathogens using TC-PCR. Samples were obtained under the supervision of a trained veterinarian and the protocol was reviewed and approved by the Lethbridge Research Center Animal Care Committees in accordance with guidelines of the Canadian Council on Animal Care (28). Consent for sampling of the cattle was also obtained from the owners.

Swabs for RPA testing were selected based on PCR-verified culture data, including those positive for any combination of the four bacterial pathogens as well as samples which were culture negative for all four pathogens. Briefly, DNPS were placed into 1 ml brain heart infusion broth containing 20% glycerol (Dalynn Biologicals, Calgary, AB) and vortexed for 1 min. Methods for TC-PCR detection of *M. haemolytica, P. multocida*, and *H. somni* were identical to those described by Stanford et al. (15) with the following modifications: 100 µl of DNPS suspension was plated for *M. haemolytica* and *P. multocida*, 50 µl each of undiluted DNPS suspension and 10^{-1} dilution were plated for *H. somni* and incubated for 48 h. Methods for TC-PCR detection of *M. bovis* were completed as described by Andrés-Lasheras et al. (29). DNA was obtained from a 300 µl aliquot of DNPS suspension using the DNeasy Blood and Tissue kit (Qiagen, Toronto, ON, Canada). RPA reaction mixtures contained primers and probes at concentrations described in section Real-time RPA Assays, with 10 µl DNA sample, and 1.3 µl nuclease-free water.

Statistical Analysis

The LOD values for each RPA at a probability of detection of 95% were estimated by Probit regression analysis using Microsoft Excel (2016). Results of real-time, multiplex RPA and TC-PCR were compared by measuring the degree of agreement and kappa coefficient (*k*) (**Table 4**).

RESULTS

Using the TwistAmp™ Basic kit, RPA assays were optimized for ICE and each BRD species individually (*M. haemolytica, P. multocida, H. somni*, and *M. bovis*), as well as being used in a conventional multiplex containing all four BRD targets (**Figure 3**). RPA assays demonstrated 100% inclusivity and analytical specificity, as all 36 strains of each species were successfully identified in each species-specific RPA assay, and the 5 target strains were successfully detected (**Table 3**), while none of the 61 non-target strains were detected. Additionally, seven single-plex RPA assays were developed for AMR genes (*tetH, tetR, msrE, mphE, sul2, floR, erm42*). Positive and negative amplification was verified for each AMR gene assay using sequenced AMR strains (data not shown).

The real-time multiplex RPA assays are shown in **Figures 4A,B**, for *P. multocida*/*H. somni* and *M. haemolytica*/*M. bovis*, respectively. Each assay contained the IAC and the LOD was 161 and 40 genome copies, respectively, for *P. multocida*/*H. somni* and *M. haemolytica*/*M. bovis* assays. As few as 103 and

TABLE 4 | Comparison of traditional culture - PCR (T-PCR) and recombinanse polymerase amplificaton (RPA) for detection of bovine respiratory disease pathogens in deep nasopharyngeal swab samples.

	M. haemolytica			*M. bovis*			*P. multocida*			*H. somni*			Overall		
	TC-PCR+	TC-PCR−	Total	TC-PCR+	TC-PCR−	Total	TC-PCR+	TC-PCR−	Total	TC-PCR+	TC-PCR−	Total	TC-PCR+	TC-PCR−	Total
RPA +	32	2	34	44	14	58	28	7	35	27	13	40	131	36	167
RPA −	11	55	66	2	40	42	19	46	65	8	52	60	40	193	233
Total	43	57	100	46	54	100	47	53	100	35	65	100	171	229	400
	Agr: 87%	*k*: 0.728	89%*	Agr: 84%	*k*: 0.684	98%*	Agr: 74%	*k*: 0.470	81%*	Agr: 79%	*k*: 0.553	92%*	Agr: 81%	*k*: 0.611	90%*

+, positive; −, negative; Agr, agreement; k, kappa coefficient.
Agreement, [RPA positive, TC-PCR positive + RPA negative, TC-PCR negative]/total number of instances.
**Total % of instances of pathogen presence where RPA matched or exceeded detection by TC-PCR.*

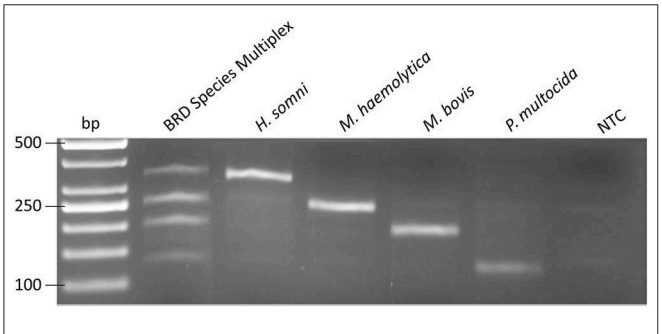

FIGURE 3 | Amplification by multiplex recombinase polymerase amplification for all 4 bovine respiratory disease pathogens, *H. somni*, *M. haemolytica*, *M. bovis*, and *P. multocida* at 5×10^2 genome copies. NTC, no template control.

7 genome copies, could be detected in 50% of cases for *P. multocida*/*H. somni* and *M. haemolytica*/*M. bovis*, respectively.

Figure 4C shows the real-time RPA assay for a region of the ICE specific to *M. haemolytica, P. multocida*, and *H. somni*, along with the IAC. The LOD for the ICE RPA was 134 genome copies per reaction (95% confidence interval). In 50% of cases, as few as 97 genome copies per reaction could be detected. **Figure 5A** illustrates the real-time RPA amplification of ICE using decreasing concentrations of genomic DNA template (1×10^4 to 1×10^2 copies/reaction).

Bovine DNPS samples ($n = 100$) were screened for ICEs and BRD pathogens using the ICE RPA and real-time multiplex RPA assays for BRD pathogens. RPA results were compared to data collected by TC-PCR for each BRD species. **Figure 5B** shows an example of the amplification results of the ICE RPA using DNPS samples collected from individual cattle upon arrival at the feedlot. The IAC successfully amplified in DNPS reactions (**Figure 5C**). Based on TC-PCR data, among the 100 bovine DNPS swabs selected for this study, each contained 0 to 4 of the selected members of the bacterial BRD complex, denoting a total of 131 instances of BRD pathogens. RPA exhibited 81% agreement (kappa coefficient, $k = 0.611$) with the TC-PCR data, while in an additional 36 instances, pathogens were detected by RPA, and in 40 instances detected by TC-PCR only (**Table 4**). The results showed that RPA had a positive rate that was similar to that of TC-PCR (**Table 4**), with detection of *M. bovis* and *H. somni* being higher by RPA, and *M. haemolytica* and *P. multocida* lower by RPA than as result of culture from DNPS. Positive rates were as follows, for TC-PCR vs. RPA, respectively: 43 vs. 34% for *M. haemolytica*, 46 vs. 58% for *M. bovis*, 47 vs. 35% for *P. multocida*, and 35 vs. 40% for *H. somni*. Agreement of RPA with culture data for *P. multocida* was 74% ($k = 0.470$), *H. somni* was 79% ($k = 0.553$), *M. bovis* was 84% ($k = 0.684$), and *M. haemolytica* was 87% ($k = 0.728$). Results in which RPA either agreed with or exceeded pathogen detection over culture methods accounted for 81, 89, 92, and 98% of cases for *P. multocida*, *M. haemolytica*, *H. somni*, and *M. bovis*, respectively. ICE was detected in 55% ($n = 55$) of the bovine nasal swabs tested. Of the swabs positive for ICE, 91% ($n = 50$) were also positive for one or more of the BRD-associated pathogens by RPA and/or TC-PCR.

DISCUSSION

In this study, RPA assays were developed to detect four bacterial BRD pathogens (*M. haemolytica, M. bovis, H. somni,* and *P. multocida*), seven AMR genes, and a region of ICE associated with BRD pathogens. Furthermore, detection of *M. haemolytica* was specific to serotypes A1 and A6, those most commonly associated with disease, while excluding all other serotypes, including A2 a common bovine commensal (30, 31). Beker et al. (13) developed a multiplex PCR assay targeting four conserved core genes required for integration and maintenance of ICE structures within the *Pasteurellaceae* family and demonstrated relevance of this assay to detecting these elements in *P. multocida* and *M. haemolytica* (13). Furthermore, RPA has recently been utilized for detection of *P. multocida* in cattle (32). However, to our knowledge, this is the first study to develop and apply RPA for detecting four major bacterial BRD pathogen species in multiplex and real-time formats, and BRD pathogen-associated with ICEs in bovine DNPS.

A conventional multiplex RPA assay was designed using the TwistAmp™ basic kit for simultaneous amplification of the four major BRD bacterial species. While this assay is useful for verification of presumptive positive isolates identified from

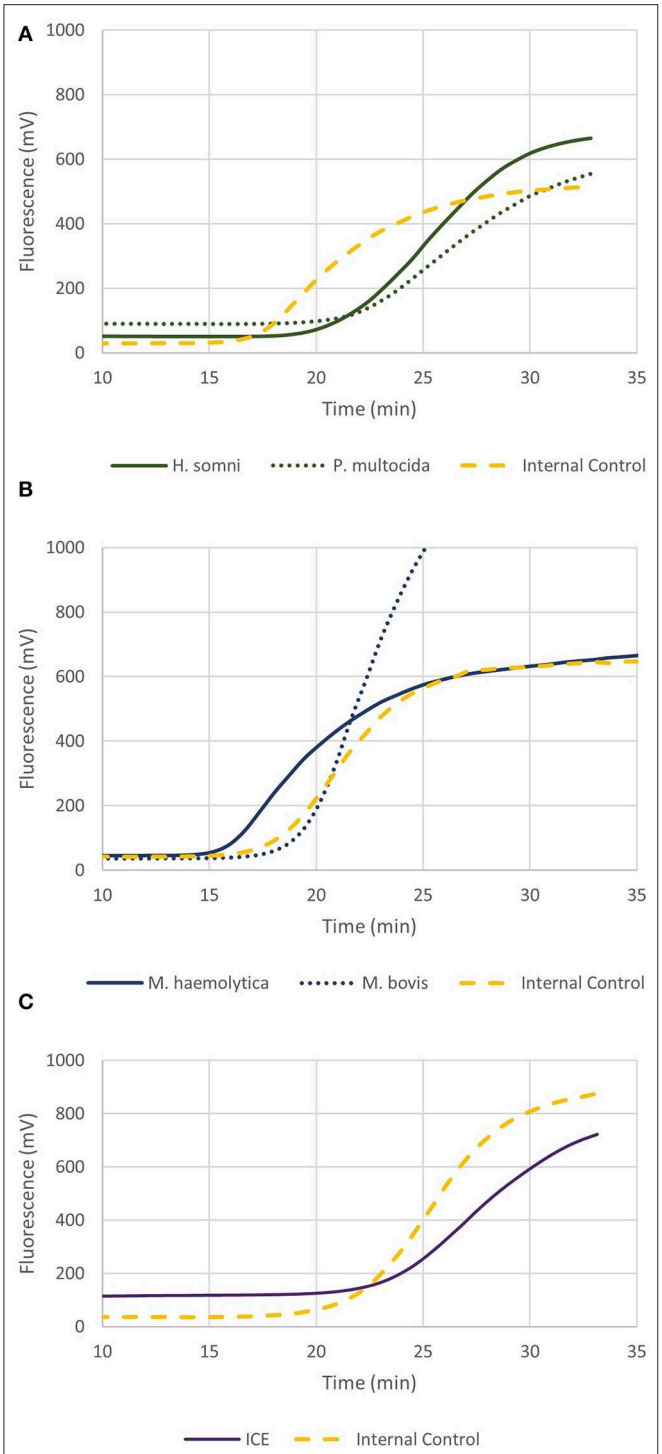

FIGURE 4 | Graphs depicting recombinase polymerase amplification (RPA) over time of 2 × 10^2 genome copies for **(A)** *P. multocida* and *H. somni* multiplex, **(B)** *M. haemolytica* and *M. bovis* multiplex, and **(C)** ICE RPA. Each assay included the internal amplification control (1 × 10^3 genome copies) designed in this study.

culture methods in a laboratory setting, all RPA assays using the TwistAmp™ basic kit require post-amplification clean up to remove excess proteins, and gel electrophoresis for visualization of amplified products, a procedure not easily achieved outside of a laboratory (33). In an effort to develop RPA assays for use in the field, RPA assays were modified for real-time detection using the TwistAmp™ Exo kit and T16-ISO instrument (TwistDX, Cambridge, UK). In comparison to real-time PCR, the RPA instrument cannot run as many reactions at a time, nor are the results quantitative. However, results are achieved within 20–30 min vs. 1.5–2 h with real-time PCR. The procedure exhibits similar sensitivity, and the instrument is substantially smaller and less expensive than a real-time PCR machine making it more suitable for a field application (33).

A real-time RPA assay for ICEs and two multiplex real-time RPAs were developed, each containing a competitive IAC. The addition of an IAC has been shown to avoid false-negatives (22, 23, 34). As opposed to a non-competitive IAC, a competitive IAC is co-amplified simultaneously with the target by the same primer set (23). By using a competitive IAC, the target and IAC are amplified by the same primers under the same conditions, reducing the need for an additional primer set, maximizing the quantity of the target primer. A competitive IAC also reduces the risk of undesirable interactions among the target primers and an additional control primer set (23). A limitation of this approach is the requirement for exogenous synthetic DNA.

The LOD was 161 and 40 genome copies per reaction for *P. multocida/H. somni* and *M. haemolytica/M. bovis* assays, respectively, and 134 genome copies for ICE. Limits of detection were similar to other published RPA and multiplex RPA assays (27, 32, 35, 36). Sensitivity of RPA depends greatly on primer and probe design, but design software and recommendations are currently lacking (19). As a result, several RPA primer and probe sets must be screened in order to determine the optimal combination (19). Multiplexing offers additional challenges, as competition among primer sets for recombinase proteins can result one target preventing the amplification of another (37).

The real-time RPA assay for ICEs amplified a region conserved among three of the four BRD pathogens targeted (*M. haemolytica, H. somni*, and *P. multocida*). An ICE is a mobile genetic element, transferred via conjugation between bacteria of the same or different species (9). ICEs may differ among species as well as within strains of the same species, containing as few as 1 to as many as 12 or more AMR genes (12). The gene *tet*(H), responsible for resistance to tetracycline has been associated with plasmids and chromosomal DNA, and also on a transposon-like element of *P. multocida* known as Tn5706 (38). The presence of *tet*(H) in ICEs is frequent among AMR *M. haemolytica, H. somni,* and *P. multocida* strains (12, 14, 39, 40). Within the ICE, *tet(H)* is located directly next to a transposase (*tnpA*) with a conserved sequence among ICE-containing strains of *M. haemolytica, P. multocida* and *H. somni*. Furthermore, *tet*(H) has only been reported in members of the *Pasteurellaceae* (39). Therefore, the ICE RPA was designed to span a region of both *tet(H)* and *tnpA* allowing for specific detection of three of the bacterial BRD bacterial pathogen that can potentially harbor AMR-ICE.

The bovine DNPS used in this study were collected from cattle upon arrival at the feedlot. Arrival at the feedlot is a particularly stressful period for cattle, which often involves transportation over long distances, and comingling of cattle,

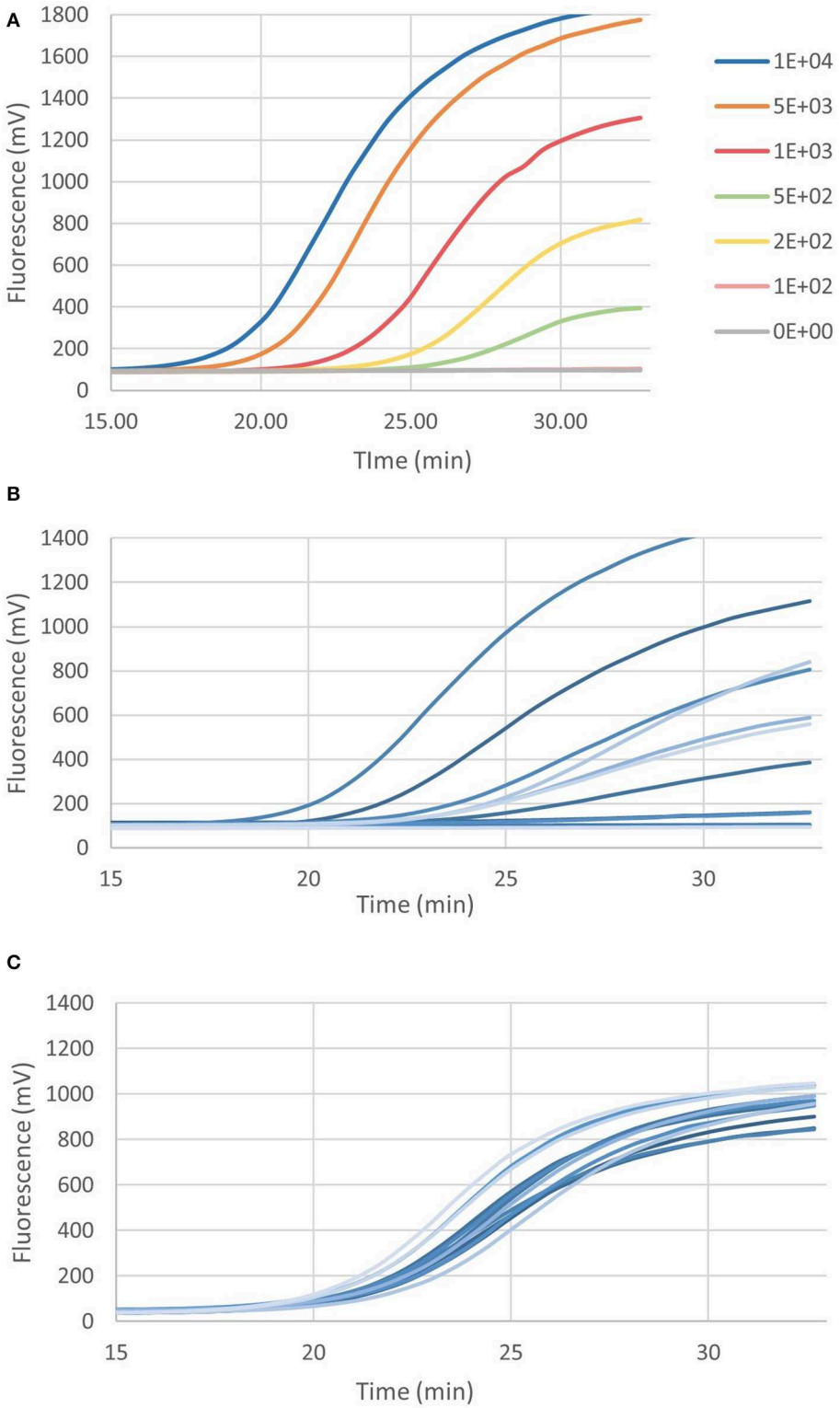

FIGURE 5 | (A) The recombinase polymerase amplification over time of ICE at decreasing genome copies (*M. haemolytica* MH44). **(B)** Example of ICE amplification from bovine nasal swabs, and **(C)** amplification of internal amplification control (1×10^3 genome copies) in bovine nasal swabs.

increasing transmission of BRD agents among members of the herd (6). While traditional culture methods are the standard for confirmation of BRD infection, they are not without limitations. Bovine nasal swabs inoculated onto agar plates can easily become overgrown by non-target bacteria, making it difficult to visually identify and isolate target species. Of the four bacterial BRD pathogens, *P. multocida* and *M. haemolytica* are most easily identified on the basis of morphology, however this approach is highly subjective. While *H. somni* also has a distinct morphology, it is difficult to culture and is easily overgrown as it requires twice the incubation period of *P. multocida* and *M. haemolytica* (16). *M. bovis* is even more challenging to culture as it requires a significantly longer to grow than other BRD pathogens, and must be cultured under humidified, microaerophilic conditions (16).

Detection of BRD species using multiplex real-time RPA showed a strong correlation with TC-PCR (90%). A greater number of swabs containing *M. bovis* and *H. somni* were detected by RPA than by TC-PCR, likely due to the aforementioned challenges associated with culturing these species in the laboratory. In contrast, fewer swabs were identified containing *M. haemolytica* and *P. multocida* by RPA than by TC-PCR. Likely, this is due to the ease with which these two species are cultured, and their distinct morphologies on laboratory media, aiding identification even when cell numbers are low. Culture-positive results for serotype A2 during TC-PCR were excluded as a positive result for *M. haemolytica* during data interpretation, and therefore is not a reason for the lower detection by RPA. However, RPA identified the presence of ∼10% more bacterial pathogens (36 instances) in swabs than TC-PCR, reflecting the greater sensitivity of RPA over traditional culture methods.

The ICE RPA assay was utilized to screen DNPS, because unlike the AMR gene RPA assays, this particular target is specific to all three BRD bacterial species, while also serving as an indicator of AMR and potential MDR. ICE was detected among 55% ($n = 55$) of the nasal swabs tested in this study. No BRD pathogens were detected in 9% of ICE-positive DNPS samples. Due to the transmissible nature of ICE, this suggests that BRD pathogens may be transferring ICE to other bacterial species (13, 41). A closely related species, *Bibersteinia trehalosi*, as well as *Moraxella* and *Acinetobacter* may also contain ICE (9, 31).

In this study, RPA was demonstrated to be a useful technology for detection of BRD pathogens and ICE from bovine nasal swabs. Advantages of RPA over polymerase chain reaction (PCR) and other isothermal technologies include simplified instrumentation amenable for field-based studies and reduced costs (19). Furthermore, detection by RPA is sensitive, and results can be obtained in real-time in <30 min (19). Similar to other molecular based techniques, detecting the AMR profile of BRD agents by RPA does not eliminate the need for culture methods. However, conventionally, it takes 2–5 days to confirm identity of BRD agents in a laboratory setting whereas RPA can accomplish this same feat in 1–2 h. Furthermore, RPA is more tolerant to inhibitors and background DNA than PCR (33). The robustness of RPA in the presence of traditional inhibitors facilitates amplification from crude extracts, which is not achievable using PCR (37).

Diagnosis of BRD in live cattle remains difficult, since there is no gold standard to define a BRD infection (2). Because many of the BRD pathogens are also commensals, their presence alone is not necessarily an indicator of disease without other predisposing environmental factors, physiologic stressors, or concurrent (viral) infections (6, 42). This affects the ability to accurately evaluate methods or technologies for diagnosis of BRD (2). A greater understanding of the virulence mechanisms of the infecting bacteria and pathogenesis is needed (6).

Further research is required to optimize RPA technology for BRD detection in the feedlot. Specifically, a method for obtaining a high yield and quality of nucleic acids from bovine nasal swabs without the use of a commercial kit will be required. Further refinement of RPA assays to enhance sensitivity and multiplexing capability would also be beneficial. Finally, a deeper understanding of the gene mechanisms associated with virulence and antimicrobial resistance of BRD pathogens may lead to identification of additional signature genes to further improve the utility of RPA.

CONCLUSION

RPA is a sensitive, specific and accurate method which detected 4 major BRD bacterial agents in deed nasal swabs collected from feedlot cattle. Furthermore, RPA was capable of detecting ICE from MDR *M. haemolytica, P. multocida*, and *H. somni* strains, which may contribute to dissemination of AMR and virulence genes among BRD pathogens. As compared to conventional approaches for detecting BRD pathogens, RPA is affordable, fast, and easily modified for real-time field-based detection. Further studies are required to evaluate performance of RPA in field settings. Additional study linking detected pathogens to clinical BRD as well as signature genes responsible for AMR profiles would enable RPA-guided selection of effective antimicrobial treatments by the beef industry, reducing antimicrobial usage by minimizing the need for repeated treatments due to AMR.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/supplementary material.

ETHICS STATEMENT

The animal study was reviewed and approved by Lethbridge Research Centre Animal Care Committees in accordance with guidelines of the Canadian Council on Animal Care (2009). Written informed consent was obtained from the owners for the participation of their animals in this study.

AUTHOR CONTRIBUTIONS

YN, KS, TA, SC, BR, and TM conceived and designed the study. RD, MB, MGB, and KA provided expertise in RPA technology and assisted in design and development of RPA assays. CC

performed laboratory activities. RZ and CC worked on analysis of the sequencing data. CC performed other statistical analyses and wrote the first draft of the manuscript. All authors revised the manuscript and gave approval for the final version to be published.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Gut Microbiota, Blood Metabolites, and Spleen Immunity in Broiler Chickens Fed Berry Pomaces and Phenolic-Enriched Extractives

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This study evaluated the performance, gut microbiota, and blood metabolites in broiler chickens fed cranberry and blueberry products for 30 days. A total of 2,800 male day-old broiler Cobb-500 chicks were randomly distributed between 10 diets: control basal diet; basal diet with bacitracin (BACI); four basal diets with 1 and 2% of cranberry (CP1, CP2) and blueberry (BP1, BP2) pomaces; and four basal diets supplemented with ethanolic extracts of cranberry (COH150, COH300) or blueberry (BOH150, BOH300) pomaces. All groups were composed of seven replicates (40 birds per replicate). Cecal and cloacal samples were collected for bacterial counts and 16S rRNA gene sequencing. Blood samples and spleens were analyzed for blood metabolites and gene expressions, respectively. The supplementation of COH300 and BOH300 significantly increased the body weight (BW) during the starting and growing phases, respectively, while COH150 improved ($P < 0.05$) the overall cumulated feed efficiency (FE) compared to control. The lowest prevalence ($P = 0.01$) of necrotic enteritis was observed with CP1 and BP1 compared to BACI and control. Cranberry pomace significantly increased the quinic acid level in blood plasma compared to other treatments. At days 21 and 28 of age, the lowest ($P < 0.05$) levels of triglyceride and alanine aminotransferase were observed in cranberry pomace and blueberry product–fed birds, respectively suggesting that berry feeding influenced the lipid metabolism and serum enzyme levels. The highest relative abundance of *Lactobacillaceae* was found in ceca of birds fed CP2 ($P < 0.05$). In the cloaca, BOH300 significantly ($P < 0.005$) increased the abundances of *Acidobacteria* and *Lactobacillaceae*. *Actinobacteria* showed a significant ($P < 0.05$) negative correlation with feed intake (FI) and FE in COH300-treated birds, whereas *Proteobacteria* positively correlated with the BW but negatively correlated with FI and FE, during the growing phase. In the spleen, cranberry products did not induce the release of any pro-inflammatory cytokines but upregulated the expression of several

genes (IL4, IL5, CSF2, and HMBS) involved in adaptive immune responses in broilers. This study demonstrated that feed supplementation with berry products could promote the intestinal health by modulating the dynamics of the gut microbiota while influencing the metabolism in broilers.

Keywords: broilers, cranberry and blueberry pomaces, blood metabolites, gut microbiota, spleen, immunity

INTRODUCTION

Necrotic enteritis (NE) caused by *Clostridium perfringens* and coccidiosis induced by *Eimeria* spp. are intestinal diseases that cause important economic losses to poultry production due to productivity losses, cost of treatments, and premature deaths (1). In conventional broiler production, antimicrobials such as bacitracin (BACI) and salinomycin are used in feed to prevent such intestinal diseases, resulting in an improvement of feed conversion and body weight (BW) gain. Antibiotic-free (raised without antibiotic: RWA) and organic poultry production are increasing in developed countries in response to consumers' demand for non-conventionally produced food, driven by issues including antimicrobial resistance (AMR) (2). Such production requires no use of traditional antibiotics in intensive production and/or free-range systems (outdoor access to pasture) in organic production. Accordingly, the Chicken Farmers of Canada (CFC) recently decided to eliminate the preventive use of Category II antibiotics in 2018 and of Category III antibiotics by the end of 2020 (https://www.chickenfarmers.ca/antibiotics). However, RWA and organic (antibiotic-free) production systems in some countries appear to increase the exposure to environmental pathogenic bacteria such as *C. perfringens* (3), avian pathogenic *Escherichia coli:* APEC (4), *Campylobacter* spp., and *Salmonella enterica* serovars (5) that pose a threat to birds' health and food safety. Antibiotic-free poultry production systems were reportedly associated with poorer feed efficiency (FE), reduced weight gain, and BW at slaughter, along with an increased incidence of clinical and subclinical NE cases (6). Thus, efficient and cost-effective methods for maintaining/improving birds' health, reducing food safety risks (foodborne pathogens), and lessening negative environmental impacts of production are warranted for antibiotic-free poultry production.

Feed additives have received much attention since the ban of in-feed antibiotics as growth promoters in Europe in 2006 and recent restriction of their use in North America (7). Feed additives may have a pleiotropic effect on poultry and are used to increase palatability, improve nutrient availability, reduce endogenous protein production and losses, reduce pathogenic microbial growth, reduce inflammation and gut permeability, increase binding of toxins, enhance intestinal recovery and function, increase colonization, and improve microbiota balance (8). The gut microbiota in chicken plays an important role in maintaining overall health and in the development of the immune system and intestinal homeostasis, and provides protection against pathogens (9). However, environmental conditions (such as housing, feed access, etc.) and host factors (line, sex, age, and disease conditions) significantly influence the composition of the intestinal microbiota (10).

The use of fruit pomaces in animal production is gaining popularity (11). The North American cranberry (*Vaccinium macrocarpon*) and wild blueberry (*Vaccinium angustifolium*) are characterized by their high phenolic acids, proanthocyanidins, anthocyanins, flavonoids, and other insoluble fiber contents. Bioactive compounds from berry pomaces and their extracts exhibited a wide range of biological activities, including antioxidant, anti-carcinogenic, anti-inflammatory, anti-neurodegenerative, and antiviral (12, 13). These products showed concentration-dependent effects by modulating gut microbiota (14, 15). Therefore, it is appropriate to explore these berry by-products as resources for different value-added applications.

Although feeding practices are known to impact animal health and productivity, very limited research has been done on the effects of berry by-products as feed supplements on gut microbiota composition and blood metabolites in broiler chickens. We previously reported the potential of berry products in food production including feeding strategies to modulate gut microbiota in food animals (15, 16), and demonstrated that cranberry products enhanced immuno-defense mechanisms of chickens against infections (17). Moreover, cranberry pomace fractions were reported to inhibit growth of AMR *Salmonella* serovars while affecting the metabolism and nutrient uptake as well as expression of virulence factors in *Salmonella* Enteritidis from broilers (18). These studies imply that both cranberry and blueberry products could be developed to maintain or improve poultry productivity and safety. In the present study, we evaluated the growth performance, intestinal health, as well as cecal and cloacal microbiota in broiler chickens receiving organic cranberry and wild blueberry pomace and their phenolic-enriched extractives (ethanolic extracts) in feed. The impact of berry product feeding on blood metabolites was estimated during growing and finishing periods. In addition, correlations between abundances of cecal bacterial taxa, performance parameters, and blood metabolite profiles were determined. Furthermore, we investigated the gene profile of the spleen to get insight into the potential immune response of broilers to dietary cranberry products, for which limited data exist.

MATERIALS AND METHODS

Animal Ethics

All experimental procedures performed in this study were approved (protocol #16-AV-314) by the Animal Care Committee of the Center de recherche en sciences animales de Deschambault

(CRSAD, Deschambault, QC, Canada) according to guidelines described by the Canadian Council on Animal Care (19).

Berry Products

Organic cranberry (CP: *V. macrocarpon*) and wild blueberry (BP: *V. angustifolium*) pomaces were prepared and characterized previously (13). Phenolic-rich pomaces were extracted with 80% ethanol from the CP and BP. After extraction, ethanol was removed from the CP and BP with a rotary evaporator and freeze-dried at −30°C to generate crude pomace extracts (COH and BOH) that were kept at −20°C until their use. Composition of the studied products including content in phenolic acids, tartaric esters, flavonols, anthocyanins, tannins, carbohydrate, lipids, proteins, and minerals such as Ca, Mg, Fe, Mn, and Cu has been previously reported (13).

Broiler Chickens and Housing

A total of 2,800 male day-old broiler Cobb-500 chicks were randomly distributed between 70 floor pens (40 birds/pen) at the CRSAD (Deschambault, QC, Canada). Before placement, all chicks were visually examined for health, and inferior chicks were not included in the trial. The concrete floor was covered with ∼3 in (7.6 cm) of clean softwood wood chips, and ventilation was provided by negative pressure with fans. Heat was provided by gas-fired brooders; water and feed were offered *ad libitum* through nipple drinkers and tube feeders, respectively. Birds were managed according to the Cobb recommendations (Cobb Breeder Management Guide and Vantress.com). The composition of the starter (days 0–10), grower (days 10–20), and finisher (days 20–30) diets included corn as the principal cereal, and soya and soybean cake as protein concentrates to meet the nutrient requirements for broiler Cobb-500 (20, 21).

Study Design

The 70 pens were assigned to 10 treatments (7 pens/treatment) using a complete randomized block design. The 10 treatments consisted of: control negative (CON: non-medicated basal feed); basal feed supplemented with BACI (55 ppm); two groups receiving basal feed supplemented with 1 and 2% cranberry pomace (CP1 and CP2); two groups receiving basal feed fortified with 1 and 2% blueberry pomace (BP1 and BP2); two groups receiving basal feed supplemented with 150 and 300 ppm of cranberry ethanolic extracts (COH150 and COH300); and two groups receiving basal feed supplemented with 150 and 300 ppm of blueberry ethanolic extracts (BOH150 and BOH300). All birds were vaccinated against coccidiosis. The tested products were applied from day 0 until day 30 of age. No additional anticoccidials or antibiotics were administrated to the birds throughout the trial.

Data Collection

Chicks were weighed at the start of the trial (day 0) and every week thereafter. Performance parameters including BW, feed intake (FI), and FE were measured at days 10 (phase 1), 20 (phase 2), and 30 (phase 3) from each pen (20). Birds were inspected at least twice daily. Any mortalities or culls were removed. The dates of removal and bird weights were recorded on a data capture sheet. Necropsies were performed by Services Vétérinaires Ambulatoires Triple-V Inc. (Acton Vale, QC, Canada) on all mortalities to determine the causes of death. Any birds showing signs of illness or distress were removed and humanely killed. During flock inspections, birds were observed for activities, and feed and water were checked to assure that each was always available.

Sample Collection, Bacteriology, and Necropsy

At days 21 and 28, two birds/pen (seven pens/treatment) were randomly chosen and weighed individually. Blood samples were collected from wing veins, and then birds were sacrificed by cervical dislocation. Cecal contents and cloacal (fecal) samples were aseptically collected from each bird and transferred to sterile Whirl-Pak plastic bags (Nasco, Fort Atkinson, WI) and test tubes, respectively; immediately frozen (−20°C); and transported to the laboratory for microbiota analysis. The collected cecal samples were analyzed using culture methods on selective media: *C. perfringens* on cycloserine supplemented tryptose sulfite cycloserine (TSC) agar media, *E. coli* on CHROMagar™, and *Lactobacillus* on MRS agar media. The results of cecal microbial enumerations were log transformed before statistical analysis. Necropsy and scoring of intestinal lesions due to coccidiosis (*Eimeria acervulina*, *Eimeria maxima*, and *Eimeria tenella)* and NE due to *C. perfringens* were performed on all sacrificed birds by Services Vétérinaires Ambulatoires Triple-V Inc. Intestines were longitudinally opened to score mucosa for NE lesions for each of the upper and lower gut (including ceca) as well as for coccidiosis as previously described (20). Birds were also monitored for yolk sac infection (omphalitis), trachea integrity, pododermatitis, gizzard ulceration, intestinal tonus, airsacculitis, metatarsal, femoral head necrosis, and bursal size.

Blood Serum Metabolites

Blood samples collected from birds on days 21 and 28 were allowed to clot at room temperature before centrifugation at 2,000 × g for 10 min for serum collection (15). Collected sera were transferred to sterile Eppendorf tubes and stored at −80°C until further analysis. Blood serum samples were assessed for 19 blood biochemistry parameters at the Animal Health Laboratory (University of Guelph, Guelph, ON, Canada) for: (1) enzymes: alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), amylase (AMY), lipase (LIP), and gamma-glutamyltransferase (GGT); (2) minerals: calcium (Ca), iron (Fe), magnesium (Mg), and phosphorus (P); (3) glucose, lipids, cholesterol (CHO), high-density lipoprotein cholesterol (HDLC), triglyceride (TRIG), and non-esterified fatty acids (NEFA); and (4) protein: total proteins (TP), albumin (ALB), globulin (GLO), and ALB–GLO ratio (AGR).

Phenolics in Blood Plasma by Liquid Chromatography–Mass Spectrometry

Individual blood samples from birds selected on day 21 were immediately centrifuged at 3,000 × g for 15 min at 4°C, and the plasma were collected and stored at −20°C. Chicken plasma

samples were transferred to Eppendorf microcentrifuge tubes, mixed with 4°C cold acetonitrile 1:4 (v:v), and centrifuged at 12,000 × g at 4°C for 15 min to remove precipitates; supernatant was transferred to a pre-balanced 33 μm polymer reverse phase 96-well-plate (60 mg/well) to remove residual proteins. The filtrates were analyzed by LC-MS/MS using a Thermo Scientific™ Q-Exactive™ Orbitrap mass spectrometer equipped with a Vanquish™ Flex Binary UPLC System (Waltham, MA, USA). Data were acquired using Thermo Scientific™ Xcalibur™ 4.2 software and Thermo Scientific™ Standard Integration Software (SII). The chromatographic separation was performed on a ZORBAX RRHD Eclipse Plus Phenyl-Hexyl HPLC column (2.1 × 150 mm, 1.8 μm, Agilent, Mississauga, ON, Canada). The binary mobile phase consisted of solvent A (99.9% H_2O/0.1% formic acid) and solvent B (99.9% ACN/0.1% formic acid). The following solvent gradient was used: 0–8 min, 0–24% B; 8–10 min, 24% B; 10–14 min, 24–60% B; 14–15 min, 60–100% B; 15–18 min, 100% B; 18–19 min, 100–0% B; 19–27 min, 0% B. The column compartment temperature was held at 40°C, the flow rate was set at 0.3 ml min^{-1}, injection volume was set at 2 μl, and peaks were monitored at 280, 320, 360, and 520 nm. Mass spectrometry data were collected in negative ionization mode using the Full-MS/$ddMS^2$ (TopN = 10) method, with NCE set at 30 and intensity threshold set at 1.0 e^5 counts.

Data were analyzed and visualized using Thermo FreeStyle™ 1.5 software. Automated sample analysis was performed using Compound Discoverer 2.0 software. A modified template, "Untargeted food research workflow with statistics," was used to perform sample grouping, peak detection, identification of unknowns, and differential analysis. The identification of compound in plasma was based on elemental composition prediction and subsequent ChemSpider database search (FullMS) as well as spectral matching of MS/MS data with the mzCloud library (MS^2). Statistical analysis performed on detected peaks included differential analysis where *P*-values and fold changes were visualized using Volcano plots; a principal component analysis (PCA) plot was also generated by Compound Discoverer™ software.

DNA Isolation for Microbiota Analysis

Genomic DNA for 16S rRNA sequencing was extracted from a cecal and cloacal sample using a QIAamp DNA Stool Mini Kit (Qiagen, Venlo, Netherlands) according to the manufacturer's instruction. DNA quality was checked by running on 1.0% agarose gel electrophoresis. DNA quantitation was performed using the Qubit® 2.0 Fluorometer (Life Technologies, Carlsbad, CA, USA) and the Qubit dsDNA HS assay kit (Life Technologies, Carlsbad, CA, USA). Sequencing libraries of the 16S rRNA gene were prepared according to the Illumina 16S Metagenomic Sequencing Library Preparation Guide. Briefly, the 16S V3–V4 hypervariable region was amplified using primers (5′-CCTACGGGNGGCWGCAG-3′) and Bakt_805R (5′-GACTACHVGGGTATCTAATCC-3′) containing Illumina overhang adapter sequences (5′-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG and 5′-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG, respectively) with KAPA HiFi HotStart ReadyMix (VWR), and purified with AMPure XP beads (Beckman Coulter). Sequencing adapters containing 8 bp indices were added to the 3′ and 5′ ends by PCR using the Nextera XT Index kit (Illumina) followed by a second purification with Ampure XP beads. Amplicons were quantified using the Quant-iT PicoGreen double-stranded DNA assay (Invitrogen), and equimolar ratios were pooled and combined with 10% equimolar PhiX DNA (Illumina) for sequencing on a MiSeq instrument, using the 600-cycle v3 kit (Illumina).

The data were analyzed by Quantitative Insights Into Microbial Ecology QIIME (version 1.9.1) (22). Paired-end reads (300 bp) were joined with fastq-join (23), and quality filtered and demultiplexed in QIIME using default settings. The reads were clustered at 97% sequence identity with UCLUST (24), and representative operational taxonomy units (OTUs) were picked using an open-reference approach (25). For both steps, the Greengenes representative OTU sequences (gg_otus_13_8), clustered at 97% identity, were used as reference. Taxa that could not be assigned a genus were presented as "unclassified" using the highest taxonomic level that could be assigned to them. The sequences were aligned against the Greengenes core set with PyNast (22), and a phylogenetic tree was constructed with FastTree (26). Alpha-diversity (within group) metrics were then calculated by QIIME, and a β-diversity (between group) distance matrix based on the unweighted UniFrac metric (27) was calculated, which was used for principal co-ordinate analysis (PCoA).

Spleen RNA Extraction

At day 21 of age, spleens from sacrificed birds were collected and placed in tubes containing an RNA stabilization solution (AM7021, ThermoFisher Scientific) before being frozen. Three spleen samples for every six treatments (control, BACI, CP1, CP2, COH150, and COH300) were defrosted at room temperature for RNA extraction. Total RNA was prepared using the RNeasy® Mini Kit (Cat. No./ID: 74104 Qiagen) according to the manufacturer's instructions. Briefly, 10–15 mg of spleen samples were cut with sterile forceps and surgical blades and transferred into 600 μl RLT buffer with 1% beta-mercaptoethanol (Fisher Scientific). Cells were homogenized using a Pro 200 homogenizer (Pro Scientific). The homogenates were centrifuged for 3 min at 13,000 rpm, and the supernatants were transferred to microcentrifuge tubes followed by the addition of 70% ethanol. RNA was eluted using RNeasy Mini column (Cat. No./ID: 74104 Qiagen). The RNA quality was checked on an agarose gel, and the quantity and purity were measured with a Nanodrop spectrophotometer (260 and 260/280 nm respectively). The absence of genomic contamination was confirmed by running RNA samples with the GAPDH housekeeping gene using real-time PCR.

Real-Time PCR

cDNA synthesis was performed using the Qiagen RT2 Profiler PCR Array Handbook 11/2018 according to manufacturer's instructions. In brief, 2 μg of total RNA of each sample was synthesized using RT2 First Strand Kit (Qiagen, Valencia, CA) and kept at −20°C until needed. cDNA samples were mixed

with molecular-grade water and RT^2 SYBR Green ROX qPCR Mastermix (Qiagen, Valencia, CA) according to manufacturer protocols and added to each well of the 96-well-plate purchased from the Chicken Innate & Adaptive Immune Response PCR Array (PAGG-052ZA, Qiagen). These plates were used to profile the expression of 84 genes involved in innate and adaptive immune response pathways. Gene expression was normalized using the housekeeping gene *ACTB* and *RPL4* selected by GeNorm assessment (28). Real-time PCR was performed using an Applied Biosystems 7500 Real-time PCR System with 7500 Software v2.3. Fold change in gene expression between the control and the remaining five treatments (BACI, CP1, CP2, COH150, and COH300) was calculated using the 2−ΔΔCt method, and *P*-value was calculated based on a Student's *t*-test between control and treatments at the significance level of 0.05.

Statistical Analysis

Statistical analyses on growth performance, relative abundance of bacteria taxa, and severity of intestinal lesions (scores) were conducted according to a randomized complete block design using the General Linear Mixed Model (GLMM) procedure of the Statistical Analysis System version 9.4 (SAS Institute Inc., 2016, Cary, North Carolina, United State) (29). Treatments and sample sources (ceca and cloaca) were used as sources of variation and the individual pens as experimental units (seven pens/treatment group). Relationship between performance parameters, blood metabolites, and microbial taxa were estimated by non-parametric correlation measurements. Least significance difference (LSD) was used to separate treatment means whenever the *F* value was significant. The association Cochran–Mantel–Haenszel test was used to determine the relationship between feed supplementation and the incidence of intestinal lesions using the FREQ procedures. The difference between treatments was considered significant at a $P < 0.05$.

RESULTS

Birds' Performance

Table 1 presents the composition of the all-vegetarian feed used in this study. Analyses of dry matter (DM), TP, amino acids, fatty acids, vitamins, and some of the most common minerals of the feed were performed at the Laboratory of Agro-Environmental Analysis (**Table 2**). Effects of the control and its supplementation with BACI, 1 and 2% organic cranberry (CP1, CP2) or wild blueberry (BP1, BP2) pomaces, as well as ethanolic extracts of cranberry (COH150, COH300) or blueberry (BOH150, BOH300) pomaces on BW, FI, FE, and mortality are illustrated in **Table 3**. The performance data obtained from this study showed improved phase dependent treatment effects on the BW and FE (**Table 3**).

During the starter phase, the highest BW ($P < 0.05$) was observed in birds treated with COH300, COH150, and CP2. A similar BW was found in birds fed BOH150, CP1, and BACI. No significant difference was found between the control birds and those fed BP1 and BP2, while the lowest BW was recorded in the BOH300 treatment. During the grower phase, the highest ($P < 0.05$) BWs were obtained with the BOH300 and BACI treatment, whereas no significant differences were observed between COH300, BOH150, control, BP2, and BP1. The lowest BW values were recorded in the COH150-, CP1-, and CP2-treated birds ($P < 0.05$). However, no statistical difference was found among the 10 treatment groups for the cumulative (overall) final BW.

TABLE 1 | Composition of the feeds used in the present study.

Ingredient (% of inclusion in diet)	Starter (days 0–10)	Grower (days 10–20)	Finisher (days 20–30)
Corn	58.04	61.56	63.34
Soya	10.00	15.00	20.00
Soybean cake granule	13.10	7.40	3.90
Dresses distillery	5.00	6.00	6.00
Corn gluten	4.60	3.40	1.50
Canola oilcake	4.80	2.00	–
Limestone	1.53	1.50	1.42
Monocalcium phosphate	1.25	1.19	1.08
Soybean oil	–	0.40	1.20
Lysine sulfate 70%	0.43	0.39	0.34
Sodium bicarbonate	0.36	0.27	0.28
Salt	0.25	0.24	0.24
Luzern concentrate	0.20	0.20	0.20
Methionine	0.18	0.18	0.23
Myco-curb liquid	0.10	0.10	0.10
Choline liquid 75%	0.06	0.06	0.05
Hy D premix (Vitamin D3)	0.03	0.04	0.04
Threonine 98%	–	0.01	0.04
OptiPhos 1,000ct 250 ftu (0.12%)	0.03	0.03	0.03
Vitamin E 100,000 IU	0.05	0.03	0.02

In the starter phase, COH300, COH150, and BACI feed treatments induced the lowest (improved) FE, while BP2, CP2, and BOH300 induced the highest (poorer) FEs ($P < 0.05$). No significant effect was observed between the control birds and those fed BP1, CP1, and BOH150. At day 30, COH150-, COH300-, and BACI-fed birds induced the best cumulative FE values among all feed treatments ($P < 0.05$). No significant effects of pomaces or their ethanolic extracts in feed were observed on the cumulative (days 0–30) FI or the mortality rate compared to control and BACI.

General and Intestinal Health

Gross examination revealed that the general heath was good; bone, cartilage, as well as muscle quality was adequate. At day 21 necropsy, few lesions on the internal organs, cases of retained yolks, and very slight airsacculitis were observed, with no evidence of active infection. Only three birds (4.3%) with airsacculitis were found in each of the BACI and BOH150 treatments. The bursae of Fabricius were in good condition with a satisfactory size, indicating a functioning immune system and absence of a health challenge.

At day 21, liquid and mucous intestinal content was observed in the majority of birds necropsied, which could be partially

TABLE 2 | Analyzed nutrient profile of the feeds used in the present study.

Nutrient	Starter (days 0–10)	Grower (days 10–20)	Finisher (days 20–30)
Calculated nutrient			
Granulometry (μ)	1,362.54	1,324.14	1,299.26
Gross protein (%)	21.00	19.28	18.07
AMEn poultry (kcal/kg)	2,989.03	3,086.25	3,177.03
Phosphorus available (%)	0.50	0.48	0.45
Total chloride (%)	0.21	0.21	0.21
Total sodium (%)	0.22	0.19	0.19
Choline added (mg/kg)	396.99	396.99	351.43
Vit.A added (IU/kg)	11,000.00	10,100.00	10,100.00
Vit.D added (IU/kg)	4,988.24	4,984.32	4,984.32
Vit.E added (IU/kg)	80.00	60.00	50.00
Arginine (%)	1.26	1.17	1.14
Lysine (%)	1.23	1.13	1.08
Meth and Cys (%)	0.90	0.84	0.83
Methionine (%)	0.53	0.50	0.50
Threonine (%)	0.79	0.74	0.72
Tryptophane (%)	0.24	0.22	0.22
Arg Dig V Vol (%)	1.16	1.07	1.05
Lys Dig V Vol (%)	1.08	0.99	0.95
M and C Dig V Vol (%)	0.80	0.75	0.74
Met Dig V Vol (%)	0.49	0.47	0.48
Thr Dig V Vol (%)	0.67	0.62	0.61
Try Dig V Vol (%)	0.21	0.19	0.19
ValDVV/LDV (ratio)	0.76	0.77	0.76
Calcium phytase (%)	1.00	0.96	0.90
Estimated nutrient			
Dry matter (%)	89.80	89.70	90.10
Total protein (%)	23.06	20.44	18.69
C (%)	41.00	41.10	41.40
N (%)	3.69	3.27	2.99
C/N ratio	11.10	12.60	13.90
P (mg/kg)	8,015.00	7,542.00	7,307.00
K (mg/kg)	9,519.00	8,470.00	8,631.00
Ca (mg/kg)	9,041.00	9,954.00	11,053.00
Mg (mg/kg)	2,127.00	1,842.00	1,845.00
Na (mg/kg)	1,898.00	1,957.00	2,195.00

explained by the effect of the all-vegetarian diet used. In general, subclinical (minor low lesion scores) NEs were observed, as shown in **Table 4**. The lowest ($P < 0.05$) prevalence of NE score of 1 (occasional lesions consisting of small areas of erosion, necrosis, or hemorrhage) was observed in birds treated with CP1 (21.4%) and BP1 (21.4%) compared to the BACI (42.8%) and control (42.8%) treatments. Only one bird with an NE lesion score of 2 (minor gross lesions consisting of occasional small areas of hemorrhage or necrosis at one to two lesions per 5 cm of the small intestine) was observed in the control group. In almost all necropsied birds independently of treatment, a duodenal congestion was notable, and in some birds, a lesion score of 1 or 2 due to *E. acervulina* was observed. The lowest

TABLE 3 | Effects of cranberry and blueberry pomaces and their extracts on broiler growth performance and mortality*.

Parameters	Control	Bacitracin	CP1	CP2	COH150	COH300	BP1	BP2	BOH150	BOH300	SEM	*P*-value
Bodyweight, g/bird												
Day 0	42.84	42.89	42.86	43.01	43.44	42.6	43.01	42.79	43.11	43.44	0.83	0.6
Day 0–10	241.41^{C}	$242.73^{B,C}$	$242.86^{B,C}$	247.49^{B}	248.76^{B}	250.57^{A}	241.01^{C}	240.21^{C}	$245.93^{B,C}$	238.86^{D}	0.001	0.01
Day 10–20	$856.44^{B,C}$	866.93^{B}	826.39^{C}	768.84^{D}	845.36^{C}	$858.07^{B,C}$	$853.24^{B,C}$	$856.01^{B,C}$	$857.37^{B,C}$	869.64^{A}	2.22	0.03
Day 20–30	1799.17	1817.47	1762.2	1762.2	1816.23	1815.47	1761.67	1797.09	1785.13	1810.24	1.37	0.22
Feed intake, g/bird												
Day 0–10	28.37	27.77	28.31	29.71	28.09	28.4	28.09	29.13	28.79	28.03	1.77	0.09
Day 10–20	81.53	77.01	81.19	82.4	72.25	74.51	82.61	86.27	82.53	82.9	0.78	0.64
Day 20–30	149.93	150.03	149.93	150.43	150.21	152.1	149.63	157.43	149.5	147.77	1.58	0.14
Day 0–30	928.57	900	928.57	928.57	900	885.71	957.14	985.14	928.57	942.85	0.002	0.25
Feed efficiency, FE												
Day 0–10	$1.29^{A,B}$	$1.26^{B,C}$	$1.28^{A,B}$	1.32^{A}	$1.24^{B,C}$	$1.23^{B,C}$	$1.29^{A,B}$	1.34^{A}	$1.28^{A,B}$	1.3^{A}	3.38	0.001
Day 10–20	1.46	1.36	1.54	2.07	1.34	1.35	1.49	1.57	1.49	1.45	1.37	0.22
Day 20–30	1.58	1.59	1.61	1.58	1.54	1.58	1.64	1.66	1.62	1.58	1.06	0.41
Day 0–30	1.5^{B}	1.47^{C}	$1.55^{A,B}$	1.59^{A}	1.44^{C}	1.46^{C}	1.55^{AB}	1.58^{A}	1.53^{B}	1.5^{B}	2.68	0.01
Mortality (%)	1.43	3.57	2.14	4.29	4.64	3.21	2.5	3.57	2.86	2.86	1.1	0.38

*Berry products and bacitracin were administrated via feed from 0 to 30 days. Data represent means ± SEM of seven replicates/treatment (n = 7 pens of at least 40 chickens/pen) arranged in a completely randomized block design. P-value was obtained by ANOVA. Different superscripted capital letters within a row indicate significant differences at $P < 0.05$.

TABLE 4 | Prevalence of birds presenting lesion scores: coccidiosis due to *Eimeria* spp. and necrotic enteritis (NE) caused by *Clostridium perfringens**.

Treatments	*Eimeria acervulina*		*Eimeria maxima*		*Eimeria tenella*		NE	
	1	2	1	2	1	2	1	2
Control	28.57	28.57	28.57	0	42.86	7.14	42.86	7.14
Bacitracin	42.86	21.43	21.43	0	35.71	14.29	42.86	0
CP1	42.86	21.43	7.14	0	28.57	7.14	21.43	0
CP2	42.86	28.57	14.29	0	7.14	14.29	78.57	0
COH150	50	14.29	21.43	0	0	21.43	78.57	0
COH300	57.14	7.14	14.29	0	7.14	7.14	28.57	0
BP1	42.86	28.57	21.43	0	35.71	7.14	21.43	0
BP2	50	7.14	0	7.14	35.71	7.14	50	0
BOH150	21.43	21.43	7.14	7.14	35.71	0	57.14	0
BOH300	42.86	28.57	7.14	0	28.57	7.14	50	0
P-value	0.88		0.94		0.58		0.01	

*Two birds per pen (14/treatment, 140 birds total) were sacrificed on days 21–22 for necropsy.

E. acervulina causing white plaques in the duodenum; the scores were scored on a scale of 0–4: "0"—normal, "1"—a maximum of five lesions per cm^2 mainly in the duodenum, "2"—several lesions in the duodenum and/or jejunum, but not coalescent.

E. maxima induces bleeding in the middle of the small intestines, scored from 0 to 4 as follows: "0"—normal, "1"—few petechiae on the serosal surface around Meckel's diverticulum, or in other areas of the intestine, "2"—several petechiae on the serosal surface, small petechiae on the mucosal side, watery contents, orange intestinal mucus.

E. tenella causing severe inflammation of ceca includes intestinal score from 0 to 4: "0"—normal, "1"—few petechiae on the cecal serosal and mucosal surfaces or little blood in the ceca and thick cecal contents, "2"—petechiae on the cecal serosal and mucosal surfaces or thick cecal wall or contents containing blood or fibrin and presence of grooves.

C. perfringens was scored on a scale of 0 to 3: "0"—no gross lesions, "1"—occasional lesions consisting of small areas of erosion, necrosis, or hemorrhage, "2"—minor gross lesions consisting of occasional small areas of hemorrhage or necrosis at one to two lesions per 5 cm^2 throughout the small intestine.

but not significant ($P = 0.94$) prevalence of intestinal lesion scores of 1 (few petechiae on the serosal surface around Meckel's diverticulum or in other areas of the intestine) by *E. maxima* was observed in birds treated with CP1, BOH150, and BOH300 (7.1% for each treatment) compared to the control- (28.6%) and BACI- (21.4%) treated birds. Among all treatments, the prevalence of *E. tenella* was lower in the CP2 and COH300 groups (7.1% for each treatment) than that in the control (42.8%). At day 28 of age, no necropsy was conducted, due to the general good health status of birds.

Blood Serum Metabolites

Nineteen blood serum metabolite levels were measured in birds at days 21 and 28 of age, which showed significant treatment effects for several biomarkers (**Table 5**). On day 21, BOH300 significantly reduced ($P < 0.05$) the serum enzymes ALT and LIP levels. The highest level ($P = 0.08$) of ALP was observed in birds treated with BACI and COH150. Compared to control birds, BOH300-treated birds showed 85 and 50% lower ALT and LIP content, respectively. Except the BOH300 treatment, the ALT levels decreased with age in all treatment groups, including the control. At day 21, significant treatment effects were observed for Ca, P, and Mg concentrations, with the lowest level of these three minerals (Ca = 1.58 mmol/L, P = 1.89 mmol/L, Mg = 0.93 mmol/L) being observed in CP2-fed birds ($P < 0.05$). Both levels of TRIG and NEFA were significantly decreased (∼20 and 16%, respectively) in all cranberry by-product–treated birds compared to control, with the lowest level of AGR being observed in birds treated with BACI, COH300, and COH150 ($P < 0.05$). Similar to day 21, ALT level was significantly low ($P < 0.001$) at day 28 in blueberry product–treated birds. Among minerals, only Mg was influenced ($P < 0.05$) by all cranberry by-products compared to control and the blueberry by-product treatments at day 28. Levels of TRIG and ALB (protein) were significantly low ($P < 0.05$) in birds treated with cranberry pomace compared to the other treatments in 28-day-old birds. Calculated AST:ALT ratio values were high on both days 21 and 28, with the highest values observed in blueberry pomaces and their ethanolic extract–fed chickens.

Plasma Metabolomics

The effect of feed supplementation with berry pomaces and their ethanolic extracts was evaluated on 140 chicken (two birds/pen) blood plasma samples (seven pens/treatment) at day 21 of age. Significant differences ($P < 0.05$) were noticed between treatments. Compared to control birds, all berry by-product–fed birds showed downregulation (green area) or upregulation (pink area) of the concentration of several metabolites as shown on the Volcano plot by differential analysis (**Figure 1A**). The blue dots in the upregulated area were identified and confirmed as quinic acid (QA) 1,3,4,5-tetrahydroxy-1-cyclohexanecarboxylic acid, concentrations of which were clearly higher in CP1 and CP2 compared to other treatments (**Figure 1B**).

Culture Dependent Bacteriology

Chicken ceca from all treatment groups were used for bacterial enumeration on selective media. In the ceca of 21-day-old birds, significantly lower populations of *C. perfringens* and *Escherichia coli* and higher counts of *Lactobacillus* spp. were observed in birds fed diets supplemented with berry by-products than control-fed birds except for COH300 feed groups (**Table 6**).

TABLE 5 | Blood serum metabolites of broiler chickens fed with organic cranberry (CP), wild blueberry (BP) pomace (1–2%), and their respective ethanolic extracts (COH150, COH300, BOH150, and BOH300 ppm) at days 21–28*.

Age (day)	Categories	Metabolites	Treatments											
			Control	Bacitracin	CP1	CP2	COH150	COH300	BP1	BP2	BOH150	BOH300	SEM	*P*-value
21	Serum enzymes (U/L)	Alanine aminotransferase (ALT)	8.67	8.50	9.14	7.50	7.86	7.86	1.57	1.57	1.86	1.29	0.57	<0.001
		Aspartate aminotransferase (AST)	322.17	288.17	239	210.17	298.29	239.71	234.86	314.86	248.86	247.57	33.03	0.255
		Alkaline phosphatase (ALP)	5,884	11,148.5	9,950	10,099.67	13,527.29	7,538.14	10,664.29	10,586.43	10,625.71	10,407.14	1,503.70	0.083
		Amylase (AMY)	917.33	1,332.83	1,091.43	691.17	1,075.14	775.57	1,034	850.71	883.86	779.43	176.38	0.362
		Lipase (LIP)	17.33	21.00	16.71	15.67	17.00	14.29	11.86	9.00	10.43	8.71	1.99	0.000
		Gamma-glutamyltransferase (GGT)	9.00	11.17	8.86	7.83	7.43	5.14	13.57	11.14	9.00	8.57	1.88	0.143
	Mineral (mmol/L)	Calcium (Ca)	1.92	1.94	2.06	1.58	2.05	1.95	1.83	2.12	1.95	2.08	0.11	0.057
		Magnesium (Mg)	1.07	1.03	1.07	0.93	1.16	1.03	1.00	1.11	1.11	1.07	0.06	0.047
		Phosphorous (P)	2.27	2.12	2.14	1.89	2.47	2.20	2.08	2.50	2.47	2.41	0.14	0.057
		Iron (Fe)	15.5	18.17	16.14	16.17	16.86	17.71	14.00	16.14	17.00	15.71	1.25	0.532
	Carbohydrate (mmol/L)	Glucose	14.68	14.48	13.97	13.12	14.3	12.93	14.00	13.31	14.61	14.83	0.74	0.567
	Lipid (mmol/L)	Cholesterol (CHO)	2.90	3.02	2.94	2.59	2.95	2.73	2.99	2.92	2.99	2.99	0.20	0.914
		High-density lipoprotein cholesterol (HDLC)	2.22	2.24	2.27	1.99	2.24	2.26	2.19	2.18	2.25	2.23	0.14	0.971
		Triglyceride (TRIG)	0.88	0.98	0.70	0.70	0.77	0.73	0.90	0.96	1.11	1.19	0.09	0.003
		Non-esterified fatty acids (NEFA)	0.62	0.70	0.53	0.53	0.71	0.52	1.04	0.93	1.20	1.08	0.09	<0.001
	Protein (g/L)	Total protein	22.83	25.17	23.43	22.00	23.29	21.71	22.71	23.57	24.00	23.57	1.35	0.860
		Albumin (ALB)	11.17	10.83	11.00	10.83	10.71	9.57	12.00	11.57	12.29	11.71	0.67	0.224
		Globulin (GLO)	11.67	14.33	12.43	11.17	12.57	12.14	10.71	12.00	11.71	11.86	0.80	0.215
		ALB–GLO Ratio (AGR)	0.97	0.77	0.90	0.96	0.85	0.79	1.13	0.99	1.05	1.01	0.048	<0.001
28	Serum enzymes (U/L)	ALT	5.60	6.40	6.20	3.80	4.60	3.80	1.40	1.00	1.60	1.60	0.74	<0.0001
		AST	215.75	236.40	273.80	205.2	220.80	229.00	229.40	232.40	210.80	274.00	26.25	0.592
		ALP	5,668.80	5,692.40	6,858.00	5,540.80	6,269.80	8,608.00	5,174.80	6,793.60	7,258.40	7,507.80	1,719.79	0.934
		AMY	1,131.80	767.00	849.40	856.40	643.20	473.20	931.00	801.80	761.60	921.40	267.46	0.908
		LIP	17.20	15.60	13.60	13.40	11.60	11.60	11.40	7.00	7.60	10.60	2,512.00	0.145
		GGT	8.20	10.40	9.80	12.20	9.60	14.00	13.40	14.60	13.40	10.80	1.99	0.337
	Mineral (mmol/L)	Ca	2.31	1.98	1.93	1.87	1.94	1.90	1.95	1.87	1.87	2.05	0.14	0.568
		Mg	1.04	0.96	0.86	0.82	0.88	0.84	0.94	0.92	1.00	0.94	0.05	0.044
		P	2.45	2.16	1.88	1.97	2.10	1.94	2.11	2.01	2.15	2.31	0.16	0.384
		Fe	17.4	17.2	15.2	13.8	16.80	15.00	18.2	17.8	15.40	17.20	1.38	0.389

(Continued)

TABLE 5 | Continued

Age (day)	Categories	Metabolites	Treatments										
			Control	Bacitracin	CP1	CP2	COH150	COH300	BP1	BP2	BOH150	BOH300	SEM	*P*-value
	Carbohydrate (mmol/L)	Glucose	14.44	13.96	13.24	12.80	15.02	14.00	13.50	14.52	11.46	14.28	1.05	0.486
	Lipid (mmol/L)	CHO	3.25	3.1	2.76	2.36	2.92	2.89	3.17	2.97	2.80	3.21	0.20	0.109
		HDLC	2.14	2.25	2.06	1.77	2.18	2.14	2.40	2.33	1.93	2.39	0.18	0.299
		TRIG	1.20	0.92	0.80	0.84	0.82	0.96	1.12	0.92	1.02	1.08	0.086	0.024
		NEFA	1.23	0.87	0.70	0.81	0.83	0.89	1.11	1.00	1.19	1.25	0.156	0.148
	Protein (g/L)	Total protein	26.60	24.00	22.80	21.60	23.00	22.60	26.60	24.60	25.80	25.20	1.77	0.460
		ALB	14.40	11.60	10.40	10.20	11.40	11.00	12.80	12.60	12.80	13.40	0.73	0.003
		GLO	12.20	12.40	12.40	11.40	11.60	11.60	13.80	12.00	13.00	11.80	1.21	0.945
		AGR	1.35	0.94	0.87	0.95	0.99	0.97	0.93	1.05	0.99	1.14	0.19	0.242

**Berry products were administrated via feed from 0 to 30 days. Data represent means ± SEM of seven replicates/treatment (n = 7 pens of at least 40 chickens/pen) arranged in a completely randomized block design. P-value obtained by ANOVA.*

The effect of CP2 appeared to be comparable to that of BACI at 55 ppm, especially in lowering *C. perfringens* and *E. coli* counts in the ceca. CP2 and COH300 significantly decreased the population of *E. coli*, whereas CP1, CP2, and COH150 significantly decreased *C. perfringens* counts, Higher counts of *C. perfringens* and *E. coli* were observed in blueberry pomaces and their by-products compared to both control (basal diet and bacitracin) and cranberry by-product treatment groups. The population size of *Lactobacillus* spp. was significantly higher in all birds fed blueberry products and CP2 ($P < 0.05$) than those of control and BACI treatment, but lower in the COH300-treated birds.

16S rRNA Gene Sequencing

After quality filtering, a total of 4,576,860 and 4,359,267 sequence reads were obtained from the cecal and cloacal samples, respectively, with the total reads per sample ranging from 29,449 to 110,189 in ceca and 30,460 to 98,801 in cloacae. On average, 62,381 sequence reads per sample were generated, averaging 356 and 252 OTUs in the ceca and cloacae, respectively (**Table 7**). The mean of Good's coverage (an alpha diversity index) for all samples was high (>98%), indicating that the majority of the microbial phylotypes in the ceca and cloacal samples were covered.

Species Richness and Diversity in Ceca and Cloacae

Alpha diversity indices of both cecal and cloacal data showed similar values for all 10 treatments. An increase in species richness and evenness was observed in the cecum, compared to cloaca, as indicated by both Chao1 and Shannon metrics. At the phylum level, the higher relative abundances of unassigned sequences, *Actinobacteria*, *Bacteroidetes*, *Proteobacteria*, and *Tenericutes* were observed in ceca compared to the cloacal microbial community ($P < 0.05$). Interaction between sample sources (cecum or cloaca) and treatments was only noticed at the phylum level for the unassigned and *Actinobacteria*. The PCoA plot using Permanova for Unifrac weighted β-diversity demonstrated no clustering ($P > 0.05$) either in the cecum or in the cloaca for any of the treatment groups at day 21 of age.

Cecal Microbial Population

The phyla with the highest relative abundances (≥1%) in 21-day-old broilers were *Firmicutes* (85.4%) and *Bacteroidetes* (11.1%), while *Proteobacteria* and *Tenericutes* were present at 1.8 and 1.1%, respectively. Significant effects ($P < 0.05$) were observed with BACI and CP2 treatments on the relative abundance of *Actinobacteria* (**Table 8A**). *Tenericutes* tended ($P = 0.08$) to be influenced by CP1 feeding (**Table 8A**). Across all treatments, the predominant bacterial families (≥1%) in ceca of 21-day-old broilers were *Ruminococcaceae* (48.6%), *Clostridiales* (18.3%), *Bacteroidaceae* (11.1%), *Lachnospiraceae* (10.9%), *Lactobacillaceae* (2.5%), and others (**Figure 2A**). Significant cranberry pomace effects ($P < 0.05$) were observed on the abundance of bacterial phyla *Eggerthella* (*Coriobacteriaceae_f)*, *Lactobacillus*

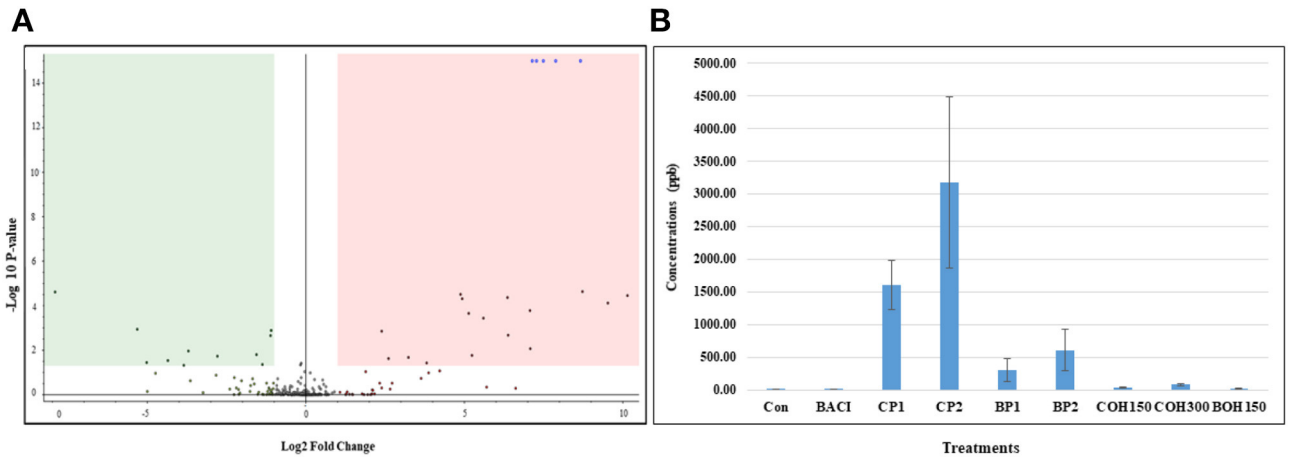

FIGURE 1 | (A) Volcano plot generated by differential analysis and showing a representative metabolomics profile of blood plasma of chickens treated with control and cranberry 2%. Volcano plot of fold changes (x-axis) and their associated $\log_{10}$ transformed *P*-values (y-axis) for phenolic compounds analyzed by LC-MS. The green and pink area indicated downregulation and upregulation, respectively. The blue dots in the upregulated area were identified as the quinic acid. **(B)** Quinic acid level in blood plasma of chicken fed with or without berry products. Data represent means ± SEM of seven replicates/treatment (two birds/pen = 14 birds/treatment).

TABLE 6 | Log_{10} bacterial numbers per gram of cecum samples from broiler chickens under berry pomaces and their ethanolic extracts.

Bacteria	Control	Bacitracin	CP1	CP2	COH150	COH300	BP1	BP2	BOH150	BOH300	SEM	*P*-value
Escherichia coli	3.89	1.67	3.60	1.25	3.06	1.03	4.04	3.92	3.96	3.98	0.528	<0.0001
Lactobacillus	6.62	5.99	6.98	7.27	6.16	1.03	7.45	7.35	7.23	7.09	0.528	<0.0001
C. perfringens	1.57	0.39	0.46	0.00	0.33	2.09	2.90	3.08	3.00	2.60	0.478	<0.0001

Standard error of means of seven replicates/treatment (n = 7 pens of 2 chickens/pen) arranged in a completely randomized block design.

(Lactobacillaceae_f), *Faecalibacterium (Ruminococcaceae_f)*, and *f__(Mogibacteriaceae)*. *Lachnospira* and *Coprococcus* (from the *Lachnospiraceae*_family) were affected by the blueberry pomace treatments, while *Oscillospira (Ruminococcaceae_f) and Erysipelotrichaceae_f* were affected by the BOH300 treatments (**Figure 3A**). *Lactobacillaceae* were significantly higher in the ceca of birds fed CP2 compared to BACI and the control-treated groups (**Figures 2A**, **3A**). The highest OTUs ($P < 0.05$) classified as *Lactobacillus agilis* (4.3%) and other unidentified *Lactobacillus* spp. (6.6%) known to include some isolates with probiotic activity were found largely in CP2-treated birds. BACI treatment affected the abundances of both *Clostridium* and *Eggerthella* (**Figure 3A**). *Enterococcus* spp., *L. agilis*, and *Blautia producta* were some unique bacterial species found only in the cranberry by-product treatments compared to BACI feed treatment. Accordingly, dietary COH300 was found to increase the abundance (8.0%) of *Enterococcus* compared to other treatments.

Cloacal Microbial Population

The most abundant phyla (≥1%) in 21-day-old broiler cloacal samples were *Firmicutes* (80.8%), followed by *Proteobacteria* (16.6%) and *Bacteroideteses* (1.9%), while other phyla were present at substantially lower levels (<1%). At the phylum level, a significant treatment effect ($P < 0.005$) was observed for BOH300, which increased the relative abundance of *Acidobacteria* compared to any other treatment (**Table 8B**). Similar to ceca, *Firmicutes* were the most abundant phyla, with the highest and lowest relative abundances being found with BOH300 (89.9%) and CP2 (65.4%), respectively (**Table 8B**). *Lactobacillales* (55.0%) and *Clostridiales* (24.5%) were the major orders found within *Firmicutes*, whose relative abundances slightly varied with different feed treatments ($P < 0.05$). Three *Clostridiales* families—*Clostridiaceae* (5.8%), *Lachnospiraceae* (2.4%), and *Ruminococcaceae* (7.1%)—predominated (**Figure 2B**), whereas *Lactobacillaceae* (43.9%) and *Enterococcaceae* (11.0%) were the most abundant orders in *Lactobacillales* group (**Figure 2B**). At the family level, BOH300 significantly ($P < 0.05$) affected the relative abundances of *RB41;f_*, *Bacillaceae*, and *Erysipelotrichaceae* (**Figure 2B**), while the population of *Anaeroplasmataceae* was influenced by the BACI treatment. The top genera belonging to the order *Lactobacillales* and *Clostridiales* were predominated by *Lactobacillus* (43.9%), *Enterobacteriaceae_* (15.9%), and *Enterococcus* (10.7%). A significant treatment effect was mostly observed with the blueberry by-product treatment; especially, BOH300 significantly ($P < 0.05$) increased the relative abundances of *Anoxybacillus kestanbolensis* and *Erysipelotrichaceae_f* (**Figure 3B**). *Acinetobacter*, *Pseudomonas*, *Comamonas*, and *Stenotrophomonas* were some of the unique bacterial groups found only in cloaca samples of CP2-fed birds compared to other treatment groups.

Correlation Between Cecal Taxa, Performance, and Blood Metabolites

Significant correlation was observed between cecal bacterial phyla, several performance parameters, and blood metabolites (**Figure 4**). As expected, a consistent negative correlation ($P < 0.05$) was observed between *Firmicutes* and *Bacteroidetes* regardless of treatments. Other bacterial phyla showed diverse significant correlations with each other ($P < 0.05$). For example, *Actinobacteria* were negatively correlated with *Proteobacteria*, *Tenericutes*, and *Cyanobacteria* in BACI, CP2, and COH300 treatment groups, respectively. However, in the BP2-treated group, significant positive correlations of *Actinobacteria* with *Bacteroidetes* and *Cyanobacteria* were observed. A significant negative correlation was observed between *Tenericutes* and *Proteobacteria* in both the BP2 and control treatments ($P < 0.05$).

Across all treatments, no consistent correlations were observed between bacterial phyla and performance. The cumulative (overall) FE (CuFE13) was positively correlated with *Actinobacteria* in control-, BACI-, and BP2-treated birds, which was not observed with the cranberry pomaces and its ethanolic extracts. A positive correlation was found between *Tenericutes*, initial BW (INBW2), and average daily FI (ADFI2) during the grower period ($P < 0.05$) in BACI, COH300 and BOH300 treatments; however, both berry pomace treatments resulted in a negative correlation between *Tenericutes* and cumulative FE (CuFE13). Interestingly, *Actinobacteria* was negatively correlated with mortality in birds fed control and cranberry by-products ($P < 0.05$), suggesting that increasing *Actinobacteria* could be beneficial by decreasing the mortality rate. On the other hand, *Bacteroidetes* and *Cyanobacteria* were positively correlated with the mortality rate in berry pomace and BACI treatments, respectively.

In control- and BACI-fed birds, the phylum *Firmicutes* was negatively correlated with blood metabolites such as serum enzymes, fats, and TP. Accordingly, LIP, AMY, GGT, glucose, CHO, HDLC, TRIG, TPRO, and GLO were all negatively correlated ($P < 0.05$) with *Firmicutes*. Similar correlation patterns were also found with the CP2 treatments, particularly for the minerals and proteins. As stated above, at day 21 of age, a significant treatment effect was observed for enzymes ALT and LIP in chicken serum of birds treated with blueberry products. ALT was negatively correlated with *Actinobacteria* and

TABLE 7 | Summary statistics of sequences analyzed including the number of average sequences after filtering but before operational taxonomy unit (OTU) picking, average reads after OTU picking, average OTU numbers, and microbial diversity covered.

Site	Treatment	Average reads/sample	Average OTUs	(% Good's coverages)
Cecum	Control	50,624.14	366.29	0.999
	Bacitracin	54,685.43	346.00	0.999
	CP1	51,922.57	364.86	0.999
	CP2	62,884.57	371.14	0.998
	COH150	79,487.57	369.71	0.999
	COH300	77,798.14	368.43	0.998
	BP1	77,517.14	365.14	0.999
	BP2	52,605.86	285.86	0.999
	BBOH150	62,950.29	378.71	0.999
	BBOH300	53,335.43	345.14	0.998
Cloaca	Control	55,710.57	259.00	0.998
	Bacitracin	72,261.14	303.43	0.998
	CP1	53,229.29	189.86	0.999
	CP2	54,064.86	241.43	0.998
	COH150	69,677.43	268.29	0.998
	COH300	71,845.29	254.14	0.998
	BP1	68,619.86	246.29	0.998
	BP2	49,943.43	254.86	0.998
	BBOH150	47,803.86	221.86	0.998
	BBOH300	57,764.86	290.29	0.998

For each of seven feed treatments, the sequencing reads were merged, and OTUs were clustered at >97% similarity using Quantitative Insights Into Microbial Ecology (QIIME).

TABLE 8 | Relative abundance of bacterial phyla treated with different feed supplements at **(A)** ceca and **(B)** cloacae of broiler chickens at 21 days of age[1].

Phylum	Control	Bacitracin	CP1	CP2	COH150	COH300	BP1	BP2	BOH150	BOH300	*P*-value
(A) Cecum (%)											
Unassigned	$0.24^{A,B,C}$	$0.20^{A,B,C,D}$	0.34^{A}	$0.26^{A,B}$	$0.23^{C,D,E}$	$0.11^{D,E}$	$0.13^{A,B,C}$	0.08^{E}	$0.19^{B,C,D,E}$	0.06^{E}	0.001
Actinobacteria	$0.11^{A,B,C}$	0.19^{A}	$0.15^{A,B}$	0.14^{A}	0.11^{c}	0.06^{C}	$0.03^{A,B,C}$	$0.03^{B,C}$	0.03^{C}	0.04^{c}	0.003
Bacteroidetes	4.93	2.6	9.46	11.52	4.36	14.18	18.67	12.36	12.62	20.27	0.327
Cyanobacteria	0.2	1.05	0.22	0.09	0.11	0.24	0.16	0.18	0.05	0.16	0.46
Firmicutes	92.07	94.54	87.07	83.45	92.75	80.11	78.74	84.72	83.98	76.78	0.286
Proteobacteria	1.11	0.73	0.81	3.57	1.16	4.4	1.12	2.43	2.09	1.1	0.35
Tenericutes	1.33	0.69	1.95	0.97	1.27	0.9	1.15	0.2	1.04	1.59	0.087
(B) Feces (%)											
Unassigned	0.36	0.31	0.25	0.25	0.19	0.42	0.34	0.17	0.25	0.13	0.07
Acidobacteria	0.01^{B}	0.00^{B}	0.00^{B}	0.00^{B}	0.00^{B}	0.00^{B}	0.03^{B}	0.01^{B}	0.02^{B}	0.12^{A}	0
Bacteroidetes	0.93	0.61	0.78	0.99	0.15	0.56	0.9	7.36	5.16	1.5	0.3
Cyanobacteria	0.23	0.11	0.21	0.78	0.38	0.41	0.21	1.06	0.51	0.28	0.32
Firmicutes	83.48	87.81	72.85	65.47	88.01	85.05	76.48	79.09	79.65	89.82	0.36
Proteobacteria	14.92	10.98	25.9	32.49	11.21	13.54	22.01	12.25	14.37	7.95	0.19
Tenericutes	0.06	0.16	0.02	0.02	0.06	0.01	0.03	0.04	0.03	0.19	0.09

[1]*n = 7 pens/treatment, 2 birds/pen: 14 birds/treatment.* $^{A-E}$*Means with different superscripts within a row differ significantly (P < 0.05).*

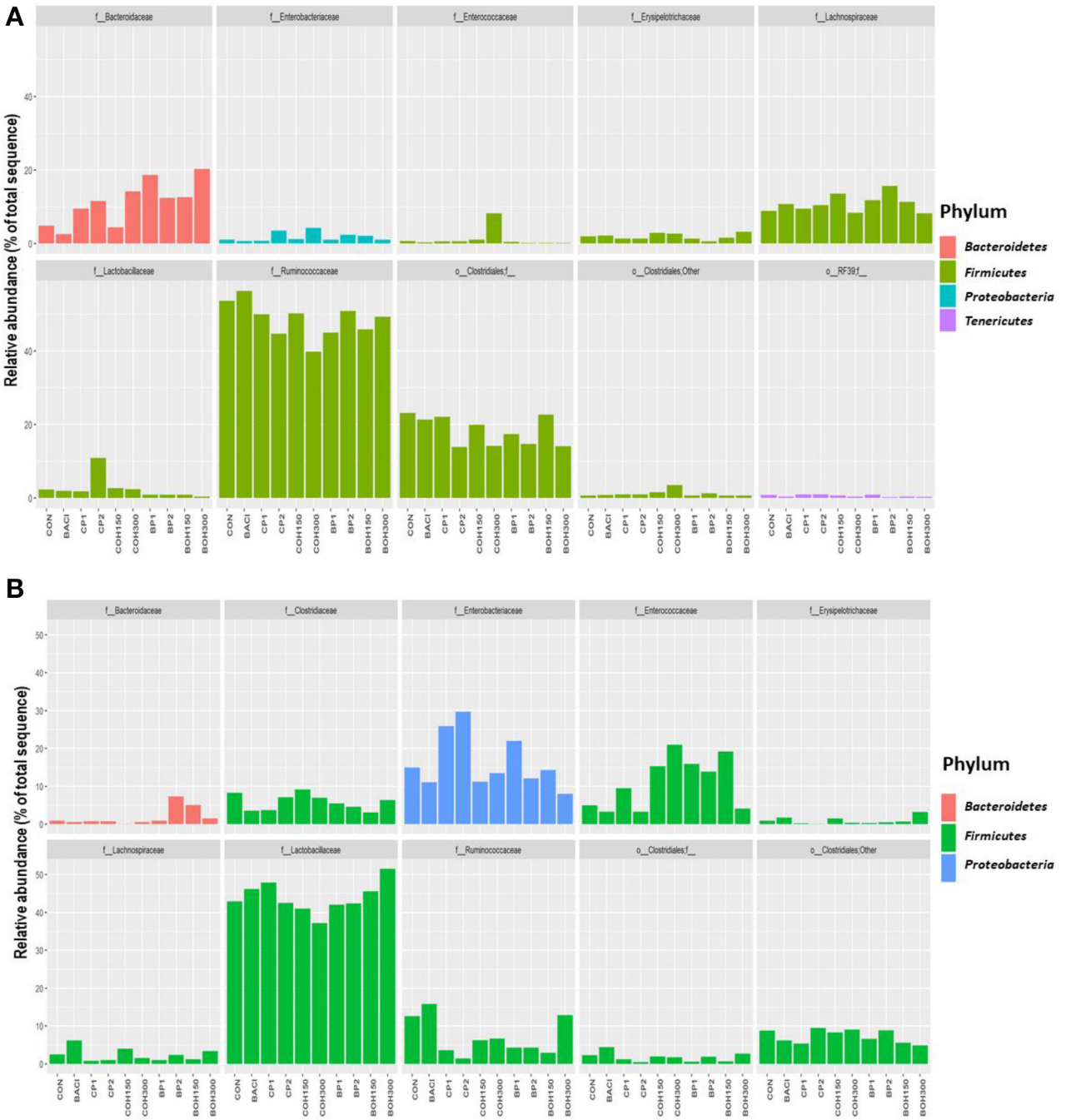

FIGURE 2 | (A) Relative abundances of the top 10 families averaged over all samples for the feed supplement groups for the ceca. **(B)** Relative abundances of the top 10 families averaged over all samples for the feed supplement groups for the cloaca.

Bacteroidetes, while *Firmicutes* showed a positive correlation with this ALT enzyme. Significant treatment effects for blood minerals (Ca and P), TRIG, and NEFA were found mainly in CP2-treated birds in blood. However, the correlation analysis showed positive correlations of *Actinobacteria* with either minerals or lipid and protein profiles. A positive correlation ($P < 0.05$) was observed between AGR and both *Actinobacteria* and *Firmicutes* in the BACI treatment.

Expression of Innate and Adaptive Immune Genes in Spleen

Since cranberry product feeding appeared to induce the shifts in gut microbiota toward potential beneficial bacteria, only spleens from 21-day-old birds fed cranberry by-products were analyzed for expression of 84 immune genes. Out of the 84 analyzed genes, 13 genes were upregulated, but the *MX1* was downregulated in the spleen of birds fed BACI and cranberry

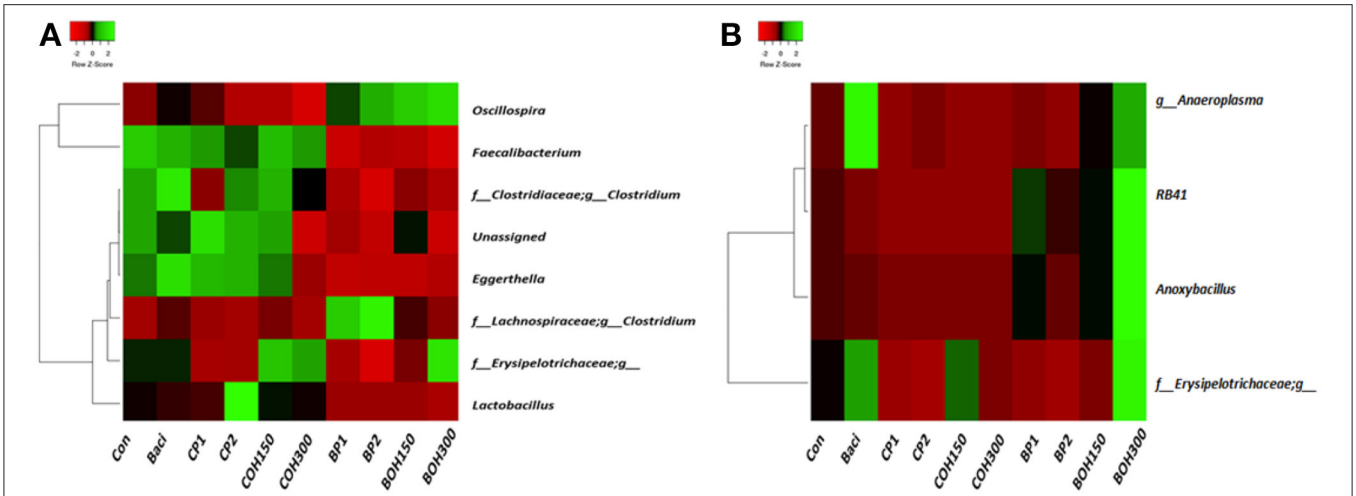

FIGURE 3 | Heatmap showing bacterial genera whose relative abundances were significantly ($P < 0.05$) changed by studied dietary treatments in **(A)** ceca and **(B)** cloacae.

TABLE 9 | Differentially expressed genes of innate and adaptive immune response pathway from chicken spleen tissue in response to feed treatments compared to control.

Gene symbol	Description	Fold change				
		BACI	CP1	CP2	COH150	COH300
CCR4	Chemokine (C-C motif) receptor 4	2.07*	1.93	1.38	1.6	1.64
CCR5	Chemokine (C-C motif) receptor 5	−1.2	2.07	1.71	1.35	1.52
CCR6	Chemokine (C-C motif) receptor 6	1.03	2.04*	1.89	1.5	1.85*
CD14	CD14 molecule	2.43	2.20*	2.09	2.25	1.93
CRP	C-reactive protein, pentraxin-related	6.20*	1.97	1.84	2.47	2.2
CSF2	Granulocyte-macrophage colony-stimulating factor	4.07	3.74*	2.97*	2.79*	2.82*
IL4	Interleukin 4	2.98	2.31*	2.14*	2.1	2.45*
IL5	Interleukin 5	9.42	12.65*	10.84*	11.58*	12.06*
IL13	Interleukin 13	2.08*	1.36	1.12	1.18	1.13
HMBS	Hydroxymethylbilane synthase	4.89	2.93*	2.20*	1.91	2.06*
MX1	Myxovirus (influenza virus) resistance 1, interferon-inducible protein p78 (mouse)	−1.75	−1.81*	−1.14	1.97	−2.00*
JAK2	Janus kinase 2	−2.16	1.62*	1.82*	1.57	1.57*
TLR15	Toll-like receptor 15	−1.29	1.95*	1.57	1.76*	1.84*

*Positive and negative numbers indicate upregulation and downregulation respectively. Red indicates fold regulation ≥2, and blue indicates fold regulation ≤ −2. *Indicates fold change values that are significantly different compared to control ($P < 0.05$).*

products compared to control group. Among the 12 upregulated genes, cranberry product treatments upregulated expression of the Th2 type immune response genes including those encoding IL4, IL5, CSF2, and HMBS compared to control. BACI treatment induced expression of genes coding for CCR4, CRP, and IL13 belonging to Th2. Moreover, genes coding for JAK2 and TLR15 appeared to be upregulated in the cranberry-treated birds while downregulated in the BACI-treated birds compared to the control ones ($P < 0.05$), but the levels of their expression were less than two-fold (**Table 9**). Interestingly, the levels of expression of genes for JAK2 and TLR15 in cranberry product–fed birds appeared to be oppositely expressed compared to BACI-treated birds (**Table 9**). Overall, no linear dose response effect was observed with cranberry product feeding; however, CP1 seemed to have consistently higher gene expression levels.

DISCUSSION

The use of plant extracts in human and animal feeding has been the subject of research due to their broad range of phytochemical compounds (16, 30). Thus, this study examines the effects of organic cranberry and wild blueberry pomaces and their ethanolic extracts in feed on performance, gut microbial community, blood metabolite profile, and spleen immune gene expressions in broiler chicken.

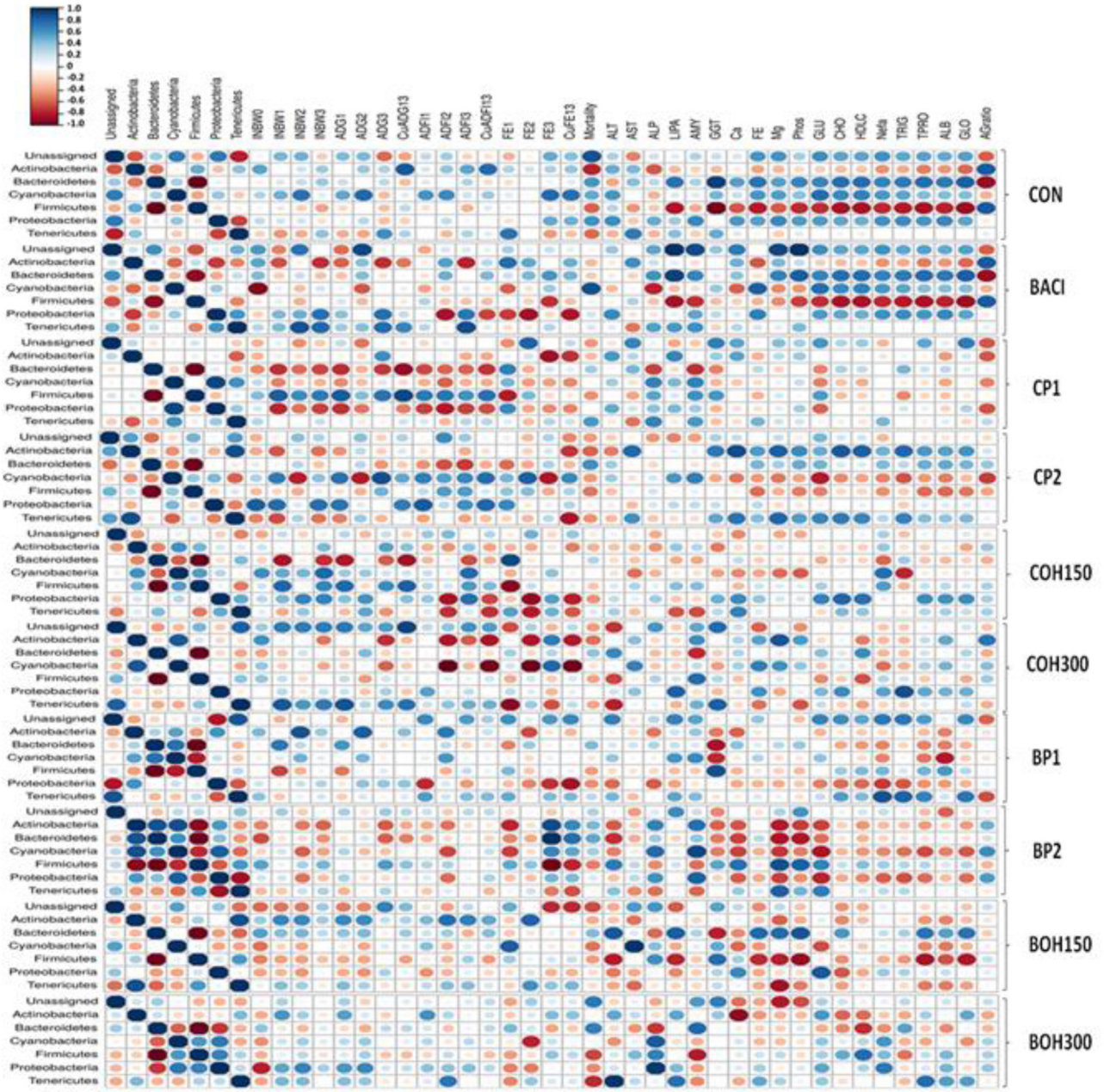

FIGURE 4 | Spearman non-parametric rank correlations among bacterial phyla, performances [individual body weight (INBW), average daily gain (ADG), average daily feed intake (ADFI), feed efficiency (FE) in the starter, grower, and finisher phases as well as during the overall raising period (cumulative)]; mortality; and different blood metabolites: enzymes, minerals, glucose, lipids, and protein. The scale colors indicate whether the correlation is positive (closer to +1, blue circles) or negative (closer to −1, red circles) between the taxa (phylum), performances and the blood metabolites. All correlations presented were statistically significant ($P < 0.05$). Large and small circles indicate strong and weak correlations, respectively.

The growth performance data showed that feeding birds with COH300 improved the BW and FE in the early age (day 10); however, BOH300 feed supplementation induced BW improvement at the grower period (days 10–20), while COH150 in feed improved the cumulative FE. Overall, the performance data showed random variation and little consistency without any evidence of dose responses, which is supported by previous research (15, 17, 31). It has been reported that dietary grape pomace did not influence the growth performance at a higher inclusion rate (6%); however, the FE was improved at a lower inclusion rate (3%) (32). In the present study, ethanolic extracts of cranberry pomaces showed a significant improvement of FE compared to control. The reason for a lower BW observed in birds fed pomaces compared to their ethanolic extracts needs

to be explored. The presence of a pure form of polyphenols in ethanolic extracts of berry pomaces compared to their pomace could be the reason for higher BW in chicken (13, 14, 33).

Blood serum enzymes such as ALT and AST, produced mainly by the liver, can be indicators of liver disease and the overall health, particularly for obesity and other metabolic syndromes (34). Moreover, there is an increase of lipid metabolism genes associated with the development of Wooden Breast (WB) disease in broiler chickens at 3 weeks of age (35). In our study, blueberry by-products showed a significant treatment effect in lowering ALT and LIP serum concentrations during the grower phase (day 21) of broilers probably due to their compositions. The major anthocyanins detected in the lowbush blueberry pomaces and ethanol extracts used in this study were peonidin 3-glucoside, malvidin 3-galactoside, malvidin 3-glucoside, and cyanidin 3-arabinoside (13). Lowbush blueberry has been reported to contain up to 332 mg/100 g fresh weight (FW) of total procyanidins (36). Sugiyama et al. (37) found that oligomeric procyanidins in apple pomace could be involved in the inhibition of LIP in mice and humans (37). A higher AST:ALT ratio and significantly lower concentrations of AST and ALT were found in the serum of fat birds (34). In the present study, higher AST:ALT ratios were also observed with the blueberry treatments; however, other biomarkers (HDLC) did not change significantly. Hence, further investigation is warranted to elucidate the effect of blueberry by-products on liver enzymes and the metabolism of fat. The low level of TRIG and NEFA observed in birds fed cranberry by-products indicated a decrease of fat deposition. More than 75% of the cranberry flavonols consist of quercetin (38) and have been associated with protection against cardiometabolic risk, such as lowering TRIG both in animal models and in humans (39). A possible mechanism proposed was that quercetin decreased the activity of microsomal TRIG transfer protein (MTP), resulting in the inhibition of intestinal apoB secretion (40). Moreover, proanthocyanidin was also reported to induce hypolipidemia by reducing TRIG in weaned pigs (41). Calcium and phosphorus are essential nutrients involved in many biological processes, and the studied wild blueberry pomaces have been reported to contain at least five times more Ca than in the used cranberry pomaces; however, both pomaces presented similar P content (13). Deficiencies, excesses, or imbalances in Ca and P can result in changes, including an increase or decrease in their absorption from the intestinal lumen. Magnesium has been reported to have several biological functions including muscle and bone growth and antioxidant properties; however, there are limited studies about its role in broilers. The actions of Mg seem to be linked to Ca and P; thus, the right inclusion rate of these minerals in diets can be important in poultry nutrition (42). The decrease of Ca, P, and Mg in the blood of birds fed the highest dose of cranberry pomace deserves more investigation to understand the mechanisms of modulation of these minerals by berry pomaces. In healthy birds, ALB represents the largest part of the protein fractions and reflects the nutrition status and immune system of chicken. While low ALB levels indicated a poor nutrition status, high GLO fractions can be related to a chronic inflammation (43). The reduced AGR in birds fed BACI and cranberry ethanolic extracts may indicate hypoproteinemia and acute or chronic inflammatory processes due to the elevation of GLO. Presently, long-term intense selection for improved BW, FE, and growth rate in broiler chickens result in higher abdominal fat deposition and metabolic changes that may impact the carcass quality. Data generated in the present study show that feeding berry products seems to influence lipid metabolism and serum enzyme secretion in broiler chickens. Necropsy revealed that none of the berry treatments significantly affects the appearance and weight of livers, indicating no liver function deficiency or fat deposition. In general, necropsy data suggested that dietary berry products did not affect the health status of the birds to any large extent.

QA is widely distributed in fruits including cranberry, blueberry, and lingonberry. After absorption from the intestinal tract to the serum, QA is converted into hippuric acid (an antimicrobial compound) or excreted unchanged in urine (44). QA has been found to be an antioxidant agent and an inhibitor of virulence factors of some pathogens such as *Streptococcus, Prevotella*, and *E. coli* (44–46). In the present study, feeding with cranberry or wild blueberry pomaces significantly increased the QA level in the plasma of chickens. Thus, feed supplementation with cranberry and blueberry products could reduce oxidative stresses and improved metabolic functions against reactive oxygen species damage in chickens due to synergistic effects of multiple-phytochemical combinations of both berries (47, 48).

In broiler chickens, it is known that preserving the gut health, which can be influenced by several factors including feeding practices, is important for bird growth performance and overall health. Dietary supplementation of CP1 and BP1 showed significant low NE incidences and lower colony counts compared to the BACI and control treatments. These results indicated that berry pomaces improve the gut heath of broilers by decreasing *C. perfringens* pathogenesis. Antibiotics appear to affect the gut microbiota by reducing the overall diversity, for example, reducing *Lactobacillus* and promoting *Clostridia* in the ilea (3). The microbial population varied in different sites as well as at different raising phases in broilers (49). In the present study, samples from day 21 collection were chosen for analysis based on the importance of this time point during birds' growth (vulnerable to infections), and on at this day, both ceca and cloacae showed similar predominances and abundances of *Firmicutes*. At the phylum level, *Firmicutes*, *Bacteroidetes*, and *Proteobacteria* were the core microbes in both sites, which is consistent with other studies (50, 51). An increased *Firmicutes*:*Bacteroidetes* (F:B) ratio has been considered as an indicator of obesity due to the improved energy harvesting capacity of *Firmicutes* species (52). Except COH150 treatment, berry pomace treatments reduced the F:B ratio compared to BACI and control; however, no consistent effect of F:B ratio on BW has been observed at 21-day-old broilers. Polyphenols in feed may increase the numbers of several bacteria, including *Bacteroidetes*, which tended to be higher with blueberry by-products in both ceca and cloacae as previously studied (15). These bacteria play an important role in breaking down complex carbohydrates to simpler compounds by encoding enzymes like polysaccharide lyases and glycoside hydrolases (41). BACI treatment resulted in lower abundances of *Proteobacteria*

compared to berry pomace treatments, which correlates with an increased population of *Firmicutes* and *Actinobacteria* and probably higher BW. *Actinobacteria* represent a small percentage of the gut microbiota; however, it has been able to maintain gut homeostasis (53). *Eggerthella lenta*, belonging to this phylum in the *Coriobacteriaceae* family, was abundant in the ceca of control birds and those treated with BACI and cranberry products (pomace and ethanolic extracts). However, feed supplementation with blueberry products (pomace and ethanolic extracts) significantly decreased the abundance of this species in the birds' ceca. *Coriobacteriaceae* have been found to be involved in the conversion of bile salts and steroids as well as the activation of dietary polyphenols (54). The tendency of cranberry products to maintain such bacteria compared to blueberry might be explained by the differences in their respective phenolic compounds (13, 55).

Berry pomace has a low pH and is composed of carbohydrates, proteins, lipids, and minerals with a high level of several phenolic compounds (flavonoids, anthocyanins, flavonols). In blueberries, anthocyanins are responsible for their 84% of the total antioxidant capacity, whereas quercetin and ellagic acids are the major flavonoids and total phenolic compounds of cranberries, respectively (55). It has been found that 95% of the total polyphenol intake may be accumulated in the colon and transformed by commensal bacteria into beneficial bioactive compounds (56). In the present experiment, birds fed berry pomace extracts exhibited increased cecal population of potential beneficial bacteria, such as *Enterococcus* and *Lactobacillus* (8.0% with COH300 and 10.9% with CP2, respectively—these counts were lower than 2% in the control). Similar beneficial effects of both berry pomace extracts were observed in the cloaca as well. These beneficial bacteria possess β-glucosidase activity and have the ability to metabolize berry anthocyanins into phenolic metabolites like *p*-couramic acid and benzoic acid (57). On the other hand, polyphenols in the berry pomaces may act as prebiotic support for growth of these beneficial bacteria, which produce lactate as the main fermentation product that can be assimilated in the cecum, serving as an energy source (58). Carbohydrates of berry pomaces could also stimulate the growth of these beneficial bacteria, which catabolize glycan, leading to the secretion of acetate, lactate, formate, and butyrate (59). Besides, iron-chelating activities of pomace compounds such as tannin could induce iron-poor conditions, which are favorable to *Lactobacillus*, as these bacteria do not require iron for growth (60). Accordingly, data of the present study showed an increase of butyrate-producing genera such as *Ruminococcus* and *Coprococcus* in cranberry pomace–fed broiler ceca similar to what was observed in broiler chickens fed chlortetracycline, virginiamycin, and amoxicillin prophylactically for growth promotion (61). The above changes induced by tested products in this study could explain, at least in part, the low prevalence of subclinical NE caused by *C. perfringens* and coccidiosis due to *Eimeria* species. These data indicate that berry pomaces could be developed as alternatives to traditional antibiotics in broiler production.

Overall, in all treatments, *Firmicutes* and *Actinobacteria* were negatively correlated with mortality, whereas *Bacteroidete* and *Cyanobacteria* were positively correlated with it. Increased cloacal *Firmicutes* facilitates nutrient absorption, whereas the opposite scenario has been observed with *Bacteroidetes* (62). In our cloacal samples, we found increased *Firmicutes* vs. *Bacteroidetes* with BACI, COH150, and COH300 feed treatments ($P > 0.05$), which may improve the nutrient absorption by the gut microbiota and resulted in a lower FE. However, we did not see a significant correlation between *Firmicutes* and FE. Rather, *Firmicutes* were negatively related in lowering some of the important blood metabolites like CHO, NEFA, and TRIG. Thus, feed supplementation by the cranberry products in broilers could improve production efficiency similar to BACI. Conversely, *Bacteroidetes* help to promote intestinal digestion, nutrient utilization, and hind gut fermentation of substrates to produce SCFA, as well as promoting the conversion of the absorbed SCFA to more complex compounds in the liver (63). We found that blueberry by-products significantly increased the abundances of *Bacteroidetes* more than any other treatments, particularly in the ceca samples, presumably related to the increase of BW during the growing period and to the reduction of blood serum enzymes like ALT and LIP.

High production performance can be harmful to immunity and intestinal integrity in broiler chickens. In the present study the prevalence of subclinical intestinal NE lesions due to *C. perfringens* was significantly low in birds fed cranberry pomace, along with high relative abundances of *Eggerthella*, *Ruminococcus*, and *Lactobacillus* in the gut. Moreover, cranberry pomace treatment significantly increased the QA level and influenced the lipid metabolism by reducing the level of TRIG and NEFA in blood. Gut microbiota play an important role in shaping immunity by influencing the balance between pro-inflammatory and immune regulatory responses to maintain immune homeostasis (64). The above observed biological activities with cranberry by-products led us to investigate its effects on broilers' spleen immunity. The spleen is a secondary lymphoid organ for both innate and adaptive immune response in chickens and therefore, its gene expression is commonly used as an indicator of immune response (65–67). Cranberry product treatment influenced the expression of genes encoding CD14, involved in innate immunity, and IL4, IL5, and CSF2, involved in adaptive immunity. These gene modulation effects could be related to effects on the enrichment of beneficial bacterial populations such as *Eggerthella* and *Lactobacillus* in the gut and accumulation of QA in the blood. Probiotic bacteria like *Lactobacillus* spp. were reported to reduce the production of pro-inflammatory cytokines like IL12 (64). The present study indicated that dietary cranberry products could reduce intestinal inflammation, while maintaining the intestinal homeostasis in broilers. Future investigations are warranted to establish the mechanisms involved in these processes.

Broiler production in Canada and in the United States of America is facing constraints. In fact, the consequences of broiler production for environmental, food safety, and animal welfare issues are forming the opinions of consumers, who are now demanding organic or antibiotic-free poultry products. Gut microbiota has been associated with wellness and diseases. Thus, understanding the molecular mechanisms by which these effects

occur in the host will be useful in designing strategies to modulate the gut bacterial composition. The present study showed that feeding with cranberry and wild blueberry products influenced lipid metabolism, mineral profile, and gut microbiota in broiler chickens. However, for most of the estimated parameters, no evidence of a dose-dependent response was noted. On some measured parameters, pomaces at 1% in feed seemed to be a more effective dose than 2%, which suggested a possible concentration-dependent response threshold. Phenolic-enriched extractives from cranberry pomace appeared to be the most effective products on FE. Therefore, more research on berry products would help in designing strategies to reduce the use of antibiotics and lessen antibiotic resistance in broilers.

DATA AVAILABILITY STATEMENT

The raw sequence read of bacterial 16S rRNA genes of the 140 (70 cecal and 70 cloacal) samples obtained in this study has been submitted to the Sequence Read Archive (SRA) database of the National Center for Biotechnology Information as FASTQ files under study accession number PRJNA273513.

ETHICS STATEMENT

The animal study was reviewed and approved (protocol # 16-AV-314) by the Animal Care Committee of the Center de recherche en sciences animales de Deschambault (CRSAD, Deschambault, QC, Canada) according to guidelines described by the Canadian Council on Animal Care.

AUTHOR CONTRIBUTIONS

MD and QD conceived and designed the experiments. DL, QD, XY, MI, HL, LM, JT, and MD performed the experiments and data analysis. MD, KR, YK, and HY contributed reagents and materials. QD and MD wrote the paper. MD, KW, MM, DL, JT, and MI reviewed and edited the manuscript. All authors read and approved the final manuscript.

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Toward Antibiotic Stewardship: Route of Antibiotic Administration Impacts the Microbiota and Resistance Gene Diversity in Swine Feces

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Oral antibiotics are a critical tool for fighting bacterial infections, yet their use can have negative consequences, such as the disturbance of healthy gut bacterial communities and the dissemination of antibiotic residues in feces. Altering antibiotic administration route may limit negative impacts on intestinal microbiota and reduce selective pressure for antimicrobial resistance genes (ARG) persistence and mobility. Thus, a study was performed in pigs to evaluate route of therapeutic oxytetracycline (oxytet) administration, an antibiotic commonly used in the U.S. swine industry, on intestinal microbial diversity and ARG abundance. Given that oral antibiotics would be in direct contact with intestinal bacteria, we hypothesized that oral administration would cause a major shift in intestinal bacterial community structure when compared to injected antibiotic. We further postulated that the impact would extend to the diversity and abundance of ARG in swine feces. At approximately 3 weeks-of-age, piglets were separated into three groups (n = 21–22 per group) with two groups receiving oxytet (one via injection and the second via feed) and a third non-medicated group. Oxytet levels in the plasma indicated injected antibiotic resulted in a spike 1 day after administration, which decreased over time, though oxytet was still detected in plasma 14 days after injection. Conversely, in-feed oxytet delivery resulted in lower but less variable oxytet levels in circulation and high concentrations in feces. Similar trends were observed in microbial community changes regardless of route of oxytet administration; however, the impact on the microbial community was more pronounced at all time points and in all samples with in-feed administration. Fecal ARG abundance was increased with in-feed administration over injected, with genes for tetracycline and aminoglycoside resistance enriched specifically in the feces of the in-feed group.

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Sequencing of plasmid-enriched samples revealed multiple genetic contexts for the resistance genes detected and highlighted the potential role of small plasmids in the movement of antibiotic resistance genes. The findings are informative for disease management in food animals, but also manure management and antibiotic therapy in human medicine for improved antibiotic stewardship.

Keywords: antibiotic usage, resistance, microbiome, oxytetracycline, swine

INTRODUCTION

Antibiotics are a critical tool for fighting bacterial infection in both human and veterinary medicine; yet there is increasing recognition of the need for judicious use of antibiotics to mitigate widespread resistance development. The relative contribution of antibiotic use in food animals to the human antibiotic resistance crisis is poorly defined; however, U.S. regulation on veterinary antibiotic usage has increased in the last few years. Efforts to improve antibiotic stewardship include defining appropriate judicious uses in animal agriculture, for example by disallowing the use of antibiotics in food animals for growth-promotion purposes. Disease treatment and prevention are currently the only approved label uses for antibiotics in food animals in the U.S. (1). Judicious practices may include treating only animals with clinical presentation as opposed to prophylactic or metaphylactic treatment to large numbers of animals.

The swine gastrointestinal microbiota harbors a diverse population of bacteria that play a role in pig health (2–4) but may also be a source of antibiotic resistance genes (ARG) (5). Disturbances to the gut microbiota may enhance ARG transfer and/or enhance abundance of antibiotic resistant bacteria shed from the animal (6, 7). Post-weaning piglets are highly susceptible to a number of diseases, and prophylactic oral antibiotics (in-feed or in-water) are commonly administered to prevent disease (8, 9). It is not uncommon for animals without clinical presentation to be treated with therapeutic antibiotics if other animals in the barn have been diagnosed with bacterial disease. Antimicrobials alter the microbial community throughout the swine gastrointestinal tract [reviewed in (10)]. Antibiotic driven shifts in the swine gastrointestinal microbiota vary in duration, and different taxa shift depending on antibiotic and intestinal segment (7, 11–17). Culture-independent methods (such as qPCR and shotgun metagenomics) are now commonly used to monitor ARG abundance in animal microbiota and the environment (18–20). The development of a common set of primers by Stedtfeld et al. (21) has facilitated the high-throughput analysis of a selection of common ARG across diverse samples. The ability to monitor multiple ARG simultaneously allows for the evaluation of previously unknown co-selection relationships within the microbiome that may influence gene persistence.

Antibiotics remain a necessary tool for limiting disease in food animals (22), and practices to minimize the abundance and persistence of ARGs in swine microbiota, and swine manure applied as fertilizer, is important for both veterinary and human health. Practices that maintain the ability to treat an animal but limit the disturbance to the gastrointestinal microbiota may be one component of antibiotic stewardship. Although oral antibiotic administration is less expensive and more convenient at the herd level, contact with the intestinal bacterial community may drive ARG abundance and mobility. Thus, to provide a method to treat an animal, but limit the impact on intestinal bacteria, we conducted a study to define the impact of injected vs. in-feed delivery of a therapeutic dose of commonly administered antibiotic in swine. The goal of our work was to determine whether the negative impacts of oral antibiotic administration on the gut microbiome, either community disturbance or increased resistance gene abundance, could be mitigated by changing the route of administration to intramuscular injection.

MATERIALS AND METHODS

Sampling Procedures

Animal studies were conducted in accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals. The animal experiments were reviewed and approved by USDA-National Animal Disease Center Animal Care and Use Committee. Ten sows were farrowed in environmentally controlled barns, with 65 piglets weaned at approximately 21 days-of-age and distributed across the three treatment groups to separate littermates (n = 21–22/group) (**Supplemental Table 1**). Two individual pens were established for each treatment in order to evaluate the impact of pen effect on observed differences. Oxytetracycline (oxytet) was used in this study because it is available in both an in-feed and injectable formulation, and is commonly used in the swine industry (23). One group was given oxytet in-feed for 7 days ("Feed" treatment group, Terramycin®100, Phibro) and the in-feed dose was formulated to 10 mg/lb of body weight daily (assuming 11 lb and 450 g feed per pig per day). A second group was given a single intramuscular oxytet injection at 9 mg/lb ("Inject" treatment group, Liquamycin LA-200®, Zoetis), using an estimated weaning weight of 11 lb to calculate injected dose. The third group received no antibiotic and was designated the non-medicated group ("NM" treatment group). Pigs in each group were necropsied on day 4 (n = 7/group), 7 (n = 7/group), and 14 (n = 7–8/group) for collection of ileal and cecal mucosal scrapings. Plasma and feces were collected as previously described (24) at timepoints indicated below to monitor oxytet levels. DNA was isolated from feces and mucosal scrapings for microbiota and ARG analysis (feces only). Feces were collected fresh and transported on ice, aliquoted for downstream applications, and stored at −80°C, as previously described (24). Colon mucosal samples were obtained by gently rinsing 2-inch

square sections of proximal colon tissue and then scraping the mucosa with a sterile cell lifter. Scrapings were transported on ice and frozen at −80°C until extraction. DNA was extracted using the PowerMag fecal DNA/RNA extraction kit (MoBio). Body weights were recorded on day 0 and at necropsy.

Oxytetracycline Concentrations in Tissue

Concentration of oxytetracycline was measured in feces and plasma (days 0, 1, 3, 4, 7, 9, 11, and 14), and intestinal samples collected at necropsy (days 4, 7, and 14) using high-pressure liquid chromatography (Agilent 1100 Pump, Column Compartment and Autosampler, Agilent Technologies, Santa Clara, CA, USA) with mass spectrometry detection (LTQ Ion Trap, Thermo Scientific, San Jose, CA, USA). Samples, spikes, QC's, and blanks (100 μL), were protein precipitated in 1.5 mL microcentrifuge tubes with 400 μL of acetonitrile/0.1% formic acid. An internal standard, demeclocycline, was incorporated into the acetonitrile precipitating agent at a concentration of 200 ng/mL. The samples were vortexed for 5 s after the addition of the acetonitrile and centrifuged for 20 min at 7,500 rpm to sediment the protein pellet. Following centrifugation, the supernatant was poured off into tubes and evaporated to dryness in a Turbovap at 48°C. The tube contents were reconstituted with 150 μL of 8% acetonitrile/0.25% formic acid and transferred to autosampler vials equipped with 300 μL glass inserts. The samples were centrifuged at 2,500 rpm prior to LC-MS analysis.

For LC-MS analysis the injection volume was set to 15 μL. The mobile phases consisted of A: 0.1% formic acid in water and B: 0.1% formic acid in acetonitrile at a flow rate of 0.275 mL/min. The mobile phase began at 5% B with a linear gradient to 95% B in 5.50 min, which was maintained for 1.75 min, followed by re-equilibration to 5% B. Separation was achieved with a HypersilGoldC18 column, 50 mm × 2.1 mm, 1.9 μm particles, Thermo Scientific, San Jose, CA, USA) maintained at 50°C. Oxytet and demeclocycline eluted at 3.43 and 3.82 min, respectively. Full scan MS with wideband activation was used for analyte detection and three fragment ions were used for quantitation of each analyte species. The fragment ions for oxytet were at 398, 408, and 426 m/z, while ions at 289, 430, and 431 m/z were characteristic of demeclocycline fragmentation. Sequences consisting of plasma blanks (porcine plasma), calibration spikes, QC's, and porcine samples were batch processed with a processing method developed in the Xcalibur software (Thermo Scientific, San Jose, CA, USA). The processing method automatically identified and integrated each peak in each sample and calculated the calibration curve based on a weighted (1/X) linear fit. Concentrations of oxytet in unknown samples were calculated by the Xcalibur software based on the calibration curve. Results were then viewed in the Quan Browser portion of the Xcalibur software. Twelve calibration spikes were prepared in blank porcine plasma covering the concentration range of 1 to 5,000 ng/mL. Calibration curves exhibited a correlation coefficient (r^2) exceeding 0.995 across the concentration range. QC samples at 7.5, 75, and 750 ng/mL were within a tolerance of ±15% of the nominal value. The limit of quantitation (LOQ) of the analysis was 2.0 ng/mL with a limit of detection (LOD) of 0.3 ng/mL.

Microbiome Sequencing and Statistical Analysis

Amplicons of the V4 region of the 16S rRNA gene were generated, sequenced, and analyzed in accordance with the Mothur SOP protocol [(25); https://www.mothur.org/wiki/MiSeq_SOP accessed March 2017], with the addition of removing singletons and doubletons using the split_abund command (cutoff = 2). Sequencing error rate was calculated by sequencing mock communities (26) and was found to be 1.2e-06 errors per basecall. The mothur output was analyzed in R using the phyloseq (27), vegan (28), and DESeq2 (29) packages. The total read counts for the ileal samples were deemed insufficient for further analysis and therefore the 16S bacterial diversity was only evaluated on fecal and colon mucosal samples. Community structure similarity analyses were performed by calculating Bray-Curtis dissimilarities on rarefied OTU tables (3,133 sequences per sample), and statistical testing was accomplished using vegan's adonis function with *post-hoc* comparisons being done with pairwise adonis tests using false discovery rate (FDR) *P*-value correction to account for multiple comparisons. Differential abundance was calculated using the DESeq2 package using Wald tests with parametric fits and FDR-corrected *P*-values. Prior to testing, OTUs with fewer than 10 counts globally were removed and the resulting unrarefied counts were used as the input for DESeq2, as the package recommends. OTUs were agglomerated at various taxonomic levels using phyloseq and these unrarefied agglomerated tables were used as inputs for DESeq2. Phyla level statistical significance was assessed using *T*-tests.

High Throughput Array-qPCR Analysis

In order to evaluate the impact of antibiotic administration route on ARG abundance within the fecal bacterial communities, DNA from both day 7 and day 14 samples were analyzed by high throughput array-qPCR on the Takara (formerly Wafergen) SmartChip system through Michigan State University using previously validated qPCR primers (21). Primers targeting a total of 48 different genes (resistance or mobility genes, **Supplemental Table 2**) were analyzed in duplicate. A Ct cutoff value of 28 was applied to all analyses, and the obtained values were analyzed using the delta CT method using 16S as the reference gene (30). In order to determine statistical differences between treatment groups, an ANOVA followed by Tukey's HSD *post hoc* test were performed for each gene. All calculated *P*-values were then corrected by the false discovery rate method.

Plasmid DNA Isolation and Sequencing

Alkaline lysis plasmid extraction was performed on 10 grams of feces for each animal collected at day 7 ($n = 7$ per group) and followed the protocol of Kav et al. (31) with the following exceptions: only one lysis protocol was used (see **Supplemental Methods**) and samples consisted of 10 grams of fecal material resuspended in 40 mL of extraction buffer. Neutralization was performed by adding 75 mL of 2 M Tris at pH 7.5 as opposed to adding 60 mL of 2 M Tris at pH 7. Samples were treated with plasmid-safe ATP-dependent DNase (Epicenter) and amplified with Genomiphi DNA polymerase (GE Healthcare) prior to sequencing. Although attempts were made

to degrade chromosomal content in these plasmidome samples, comparisons of 16S content before and after treatment indicated that this had variable effectiveness across samples and complete removal of chromosomal DNA was not achieved. Therefore, samples are referred to as plasmid enriched.

Short-read sequencing on each individual plasmidome-enriched sample was performed on the Illumina HiSeq 3000 (paired end and 150 bp high output mode). Sequencing depth ranged from 96 to 288 million reads per fecal sample and sequences were combined to a single assembly that corresponded to the complete plasmidome metagenome. The metagenome assembly pipeline included Trimmomatic v0.36 (32), digital normalization using digiNorm from the khmer package (33) and assembly with Megahit v 1.1.1 (34, 35). Quast v3.1 (36) was used to obtain assembly statistics on the final contigs. This assembly contained 1,877,620 contigs (881,559 contigs larger than 1,000 bp) with a total of 3,784,778,735 bp assembled and an N50 value of 3,653. Assemblies of individual plasmidome metagenomes for each treatment group were also performed using the same pipeline to determine resistance gene diversity by treatment. A single pooled sample was also submitted for long-read sequencing using the Pacific Biosystems RS II system and contigs were assembled using Canu v. 1.6 (37). Resistance genes were identified using the ABRicate software (https://github.com/tseemann/abricate) and Resfinder (38) database (updated 2018-Feb-23) with cut-off values of 98% sequence identity and 80% coverage.

Data Availability

Data and scripts are available through the Food Safety and Enteric Pathogens Research Unit github site (https://github.com/USDA-ARS-FSEPRU/FS1) and the sequencing data are available through the NCBI SRA (PRJNA553258).

TABLE 1 | Mean (+/–SE) oxytet concentration (ng/mL) by treatment for each tissue on day 4 of treatment as measured by LC/MS.

	Plasma	Ileum	Fecal
Non-Medicated (NM)	0.1 +/– 0	0 +/– 0	0 +/– 0
Feed	66.2 +/– 0.6	4,455.4 +/– 140.2	97,744.5 +/– 3,508.5
Inject	151.9 +/– 5.2	240.8 +/– 17.9	3,294.8+/– 283.9

Day 4 was used for this comparison since tissue samples were only collected at time of necropsy (days 4, 7, and 14). All concentrations are listed in ng/mL, consistent with the standards used for comparison.

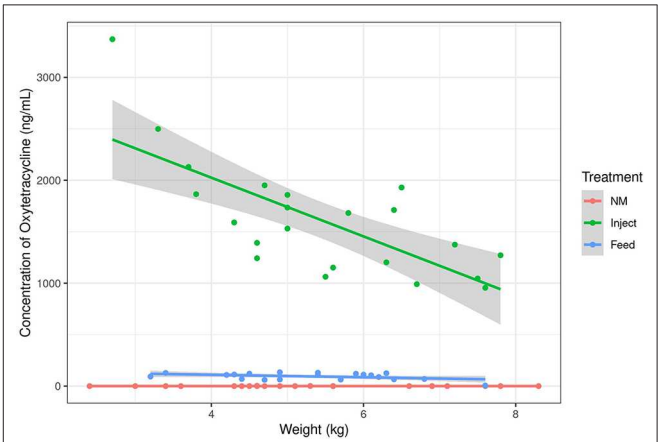

FIGURE 1 | Oxytetracyline concentration in swine plasma samples as measured by LC-MS. Oxytet concentration at day 1 is plotted relative to the animal's weight at initiation of treatment (day 0). The lines correspond to trends for each treatment group. "NM" = Non-medicated, "Inject" = injected oxytet, "Feed" = in-feed oxytet.

RESULTS

Route of Antibiotic Administration Impacted Antibiotic Concentrations in the Pig

Animals in the Inject group had a spike in oxytet concentration the day after injection (day 1) and the Inject group maintained higher plasma oxytet concentrations throughout the course of the experiment compared to animals in the Feed group (**Table 1**). Weighing individual pigs prior to administration of injected antibiotics would not be feasible in a production setting; therefore, the same dose of oxytet was administered to all pigs in the Inject group. **Figure 1** indicates the concentration of oxytet in the plasma on day 1 correlated with pig body weight measured on day 0 (linear regression model, $P = 0.000119$, $R^2 = 0.51$). Regardless of body weight, oxytet concentrations in the Inject group were an order of magnitude higher than the Feed group (**Figure 2**). The Inject group plasma concentrations on day 4 was also inversely correlated with weight at day 0 (P-value = 0.01122, $R^2 = 0.2447$; **Supplemental Figure 3A**); however, at this time point, overall oxytet concentrations in the Inject group were similar to the Feed treatment group (**Table 1**). Plasma concentrations for the pigs in the Feed group did not correlate with weight and ranged from ∼30 to 100 ng/mL during the 7 day course of treatment.

In contrast to plasma levels of oxytet in the Inject group, the ileum and fecal samples contained more oxytet for the Feed group compared to the Inject group (**Table 1**). Notably, samples from the Feed group had significantly lower oxytet levels in the plasma (mean 66 ng/mL), and instead, oxytet concentrations were much higher in the feces (mean 32,581 ng/mL). The combination of high fecal concentration and low plasma concentration compared to the Inject group illustrate that a substantial portion of the antibiotic received in-feed is directly excreted in fecal waste with limited systemic distribution in the host. Oxytet concentrations in the feces continued to be detected after cessation of treatment. The Feed group continued to have high oxytet (mean 5,030 ng/mL) in feces at day 9 (2 days after withdrawal of medicated feed) but had decreased to 88 ng/mL by day 11 and 38 ng/mL by day 14. The Inject group continued their gradual reduction in excretion, ranging from 454 ng/mL on day 9 to 92 ng/mL by day 14 (**Supplemental Table 3B**).

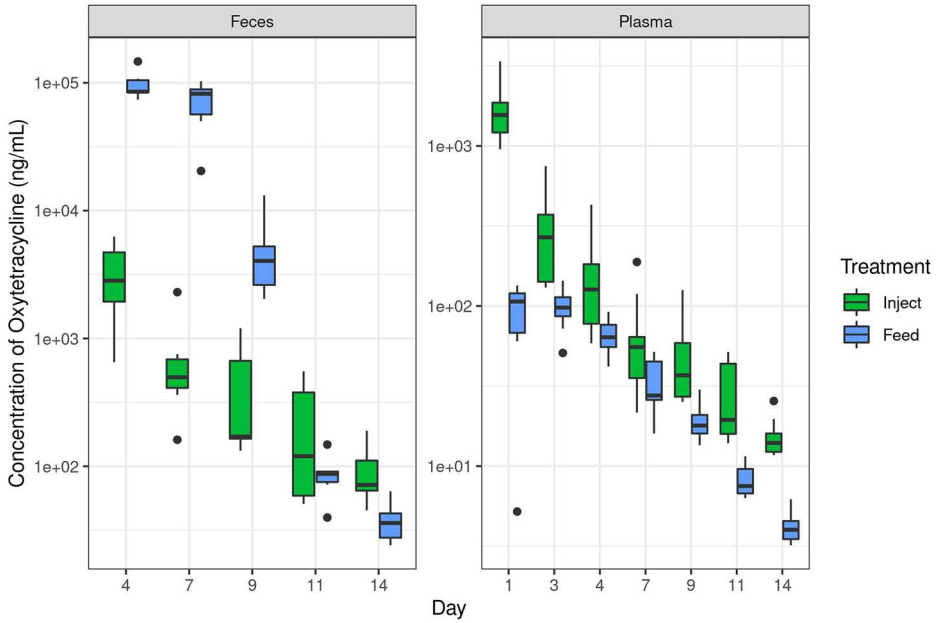

FIGURE 2 | Oxytetracycline levels in swine feces and plasma over time as measured by LC-MS. Samples were collected at the indicated time point and oxytet concentrations determined by LC-MS. The Inject group received a single therapeutic dose on day 0 and the Feed group received therapeutic dose in-feed up to day 7 as described in the methods. Data are plotted on a $\log_{10}$ scale. Note the different y-axis for each graph, necessary due to the broad range of concentrations observed in respective compartments. "Inject" = injected oxytet, "Feed" = in-feed oxytet.

Gut Bacterial Community Is Differentially Impacted by Route of Antibiotic Administration

16S rRNA gene amplicon analysis suggests the community structure of the fecal microbiota was strongly influenced by oxytet route of administration with the Feed group exhibiting the greatest changes relative to the NM group (**Figures 3A,C**). The microbiota shift was also evident in the Inject group but was only statistically significant at day 7 of study. The community wide changes were also appreciated in colon mucosal samples. Here bacterial communities from the Feed group differed significantly from the NM group at both day 4 and day 7 (**Figures 3B,D**), but were not different on day 14 (7 days after the end of treatment). Colonic mucosal bacterial community structure was not significantly different between the Inject group and NM group on any of the days evaluated.

To investigate how oxytet treatment impacted the abundance of specific bacterial taxa relative to the NM group, the fecal and colon mucosa samples were analyzed at both the phylum and order level at each time point. In the fecal samples, the phyla *Fibrobacteres* and *Proteobacteria* were significantly decreased in the Feed group compared to the NM group at both day 4 and day 7, and significant increases in *Euryarchaeota* and *Actinobacteria* were detected at day 4 within the Feed group relative to the NM group (**Supplemental Table 4**). Members of the *Actinobacteria* and *Euryarchaeota* phyla were also significantly increased when examining changes in specific orders within the fecal communities; however, the majority of the orders that decreased in abundance in the Feed treatment group belonged to the *Proteobacteria* (**Figure 4**). No significant difference at the phylum level, regardless of sampling day or location, was detected in feces of the Inject group compared to the NM group. Only two significant decreases at the order level were detected in the Inject group at day 4 (**Figure 4A**), both of which were members of the *Proteobacteria*. Overall, changes in the Inject treatment group were lower in magnitude and affected fewer orders than the Feed treatment group. The largest fold-change decrease seen in the fecal samples differed by day, with the order *Fibrobacterales* showing a 4-fold decrease relative to NM group animals on day 4 and unclassified *Delta-Proteobacteria* showing a 5-fold decrease at day 7, both of which occurred only in the Feed group (**Figure 4B**).

The colonic mucosa community had more significant changes at the phylum level than were seen in the fecal community. Changes in the ratio of *Firmicutes* to *Bacteroidetes* (F:B ratio) were detected in the colon mucosa at day 4 for both oxytet groups (**Figure 5A**) due to a significant increase in *Bacteroidetes* (Feed to NM $P = 0.001$, Inject to NM $P = 0.018$). At the phyla level, *Proteobacteria* were significantly reduced in the colonic mucosa of the Feed group on day 4 ($P = 0.032$), but not at day 7 or 14. Similar to shifts in feces, the colon mucosa had decreases in several orders of *Proteobacteria* with changes of greater magnitude evident in the Feed group (**Figure 5B**). There were fewer changes observed at day 7 and 14 in the colon mucosa, and only the *Firmicutes* were significantly impacted in the Inject group at day 14 ($P = 0.023$) (**Supplemental Table 4**).

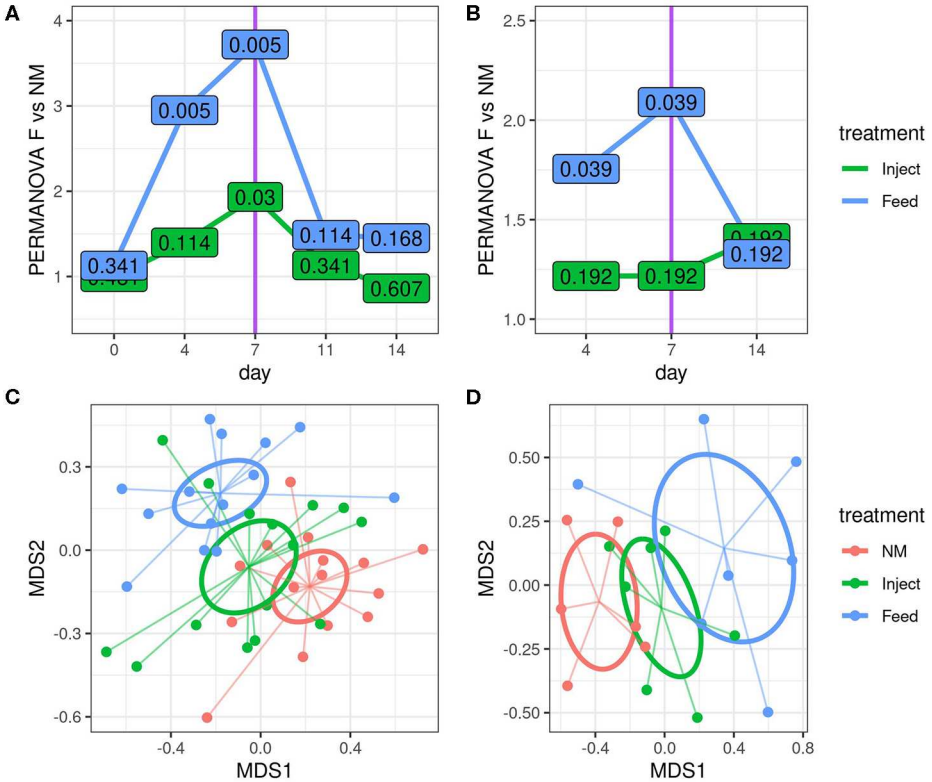

FIGURE 3 | Route of oxytetracyline administration impacted the magnitude of community disturbance relative to the non-medicated (NM) group, as determined by 16S rRNA gene amplicon analysis. Differences in community structure relative to the NM group were calculated using a series of pairwise PERMANOVA tests comparing each treatment to the NM group at indicated time point using Bray-Curtis dissimilarities for both fecal **(A)** and colon mucosa **(B)** communities. The y-axis displays the PERMANOVA pseudo F statistic (total intergroup dissimilarity divided by total intragroup dissimilarity); greater pseudo *F* values indicate greater differences between the group under consideration and the NM group. The values displayed at each point are the corresponding permuted FDR corrected *P*-values for each test. NMDS visualization of Bray-Curtis dissimilarities are also shown for fecal **(C)** and colon mucosa **(D)** microbiota communities on day 7. Ellipses represent the standard error of the centroid for each group.

Resistance Gene Abundance Impacted by In-Feed Administration Over Injected

Only two ARG had significantly higher prevalence in fecal samples at day 7 (**Figure 6**)—one encoding tetracycline resistance (primer label *tetW_191*) and the other encoding aminoglycoside resistance (primer label *aph2″-id_104*). Abundance of these genes was significantly higher in the Feed treatment compared to the NM group (Tukey's HSD test, FDR adjusted $P = 0.01$ for both) but not significantly higher in the Inject group. Overall prevalence of resistance genes, particularly to tetracyclines, continued to be high for all animals at day 14 regardless of treatment though shifts in abundance were detected through the course of treatment.

In addition to qPCR analysis, fecal plasmid DNA was sequenced using both long-read (Pacific Biosystems) and short-read (Illumina HiSeq) technologies to provide context to the resistance genes present. A master assembly for each technology combining all treatments was screened for matches to the genes found to be increased by qPCR analysis. For *tetW*, there was a single 4 kb contig from the PacBio assembly containing a match to the *tetW* gene as well as genes for a partial type IV secretion system, suggesting it could be part of a conjugative element (39). In addition, there were 14 contigs from the Illumina sequencing with complete or partial matches to the *tetW* gene. The longest of the 14 Illumina contigs was 15,397 bp; however, the closest match in the NCBI GenBank database had only 21% coverage. An examination of the region surrounding the *tetW* gene in our contig had top hits consistent with a plasmid mobilization protein (mob_pre) directly adjacent to *tetW*. Screening the Illumina contigs for the Mob protein confirmed that a small contig containing only the mob protein was also present in the assembly, suggesting multiple genetic contexts for this gene. Similarly, the aminoglycoside gene (*aph2*) identified through qPCR was present on 3 separate contigs in the Illumina assembly, all of which were 1.2 kb or smaller, indicating multiple genetic contexts for the gene which subsequently prevented assembly. The *aph2* gene was not identified in the PacBio assembled contigs. Mapping of the Illumina reads to the assembled contigs confirmed that the resistance genes had high coverage (2,000–5,000 reads per treatment; **Supplemental Figure 5**) indicating that the fragmented assemblies were not a result of insufficient coverage.

There was low overall prevalence of resistance genes within the PacBio assembled contigs; however, there were two resistance plasmids assembled that may give some insight into the transfer dynamics of both tetracycline and aminoglycoside genes within

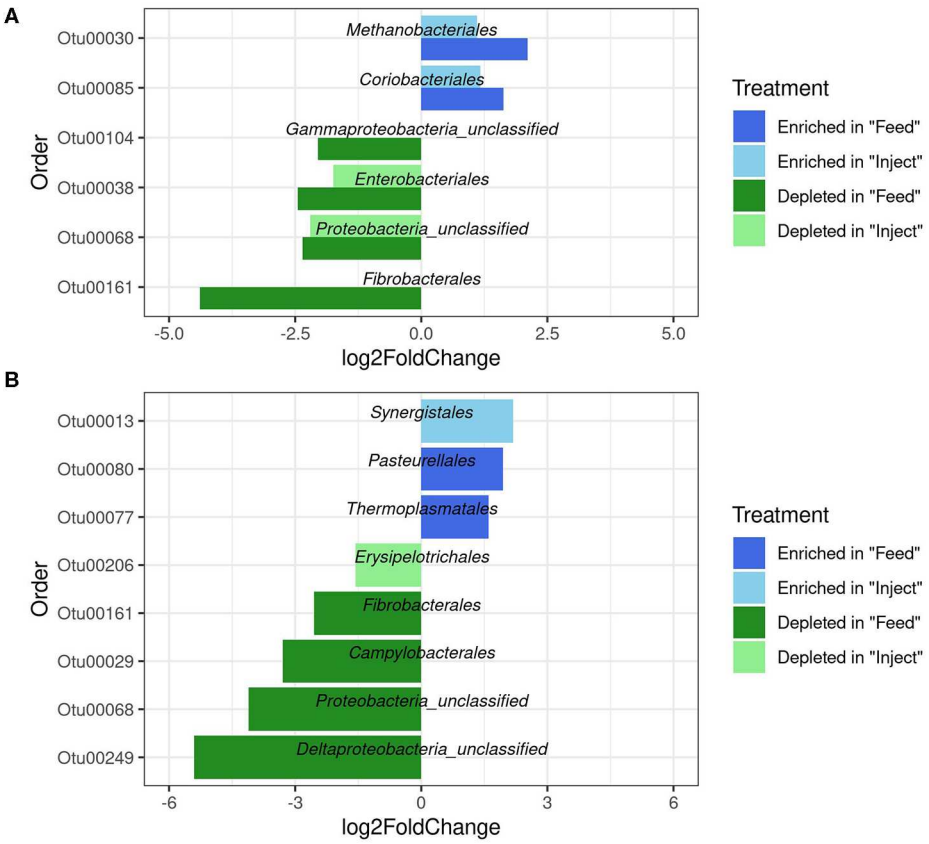

FIGURE 4 | Significantly differentially abundant microbial groups at the Order level ($p < 0.05$) in fecal microbiome after antibiotic administration via Feed or Injection on day 4 **(A)** and day 7 **(B)** via Feed or Injection. All comparisons are to the non-medicated (NM) group of animals.

the swine microbiome. Both contigs are 6 kb in size, carry the same plasmid backbone and carry distinct antibiotic resistance genes that were acquired in separate locations in the plasmid. Small plasmids, lacking any AMR genes, highly similar to the aforementioned plasmids were also identified in the assembly, suggesting a diverse population of small plasmids within the swine microbiome.

DISCUSSION

Oral antibiotic administration to pigs causes significant disturbances to intestinal microbial community, dependent on the antibiotic administered (10–13, 15–17, 40, 41). Disturbances are not limited to the bacteria in intestinal lumen (represented by fecal analysis), but also shift bacterial populations at the intestinal mucosa. Alterations to the structure of the bacterial community can have important implications for host metabolism (9, 15, 42, 43) as well as providing opportunities for the establishment of specific pathogens (44, 45). In a recent study, (46) specifically evaluated the impact of parenteral injection of five different antimicrobials currently used in swine production and identified antimicrobial-specific shifts in the microbial community during the course of treatment. Furthermore, a study looking at oxytetracycline administration in mice illustrated the differential impact of route of administration on the colonization and persistence of bacteria carrying resistance genes to the administered antibiotic (47). In this work we have expanded on these previous studies by evaluating the differential impact of a single antibiotic administered by two different routes at therapeutic level and evaluated these changes on the complete microbial community as opposed to an introduced strain.

Many factors drive shifts in the intestinal microbiota, including time, diet, and antimicrobials (48). In this study, the covariate with the greatest influence on microbial community structure was time, which correlated with dietary change of weaning to solid food (13). Although the time-driven shift in the community complicated analysis, the post-weaning time period was important to our experimental design since antibiotics are often administered at this stage to prevent post-weaning diarrhea. Antibiotic treatment caused similar changes in microbial communities regardless of route of administration; however, the impact on the microbial community was more pronounced at all time points and in all samples with in-feed administration. Decreased Proteobacteria at days 4 and 7 after treatment was a somewhat unexpected result, both due to the prevalence of tetracycline resistance in *E. coli* (a common member of the Proteobacteria) in swine, and published studies observing increases in *E. coli* abundance following antibiotic

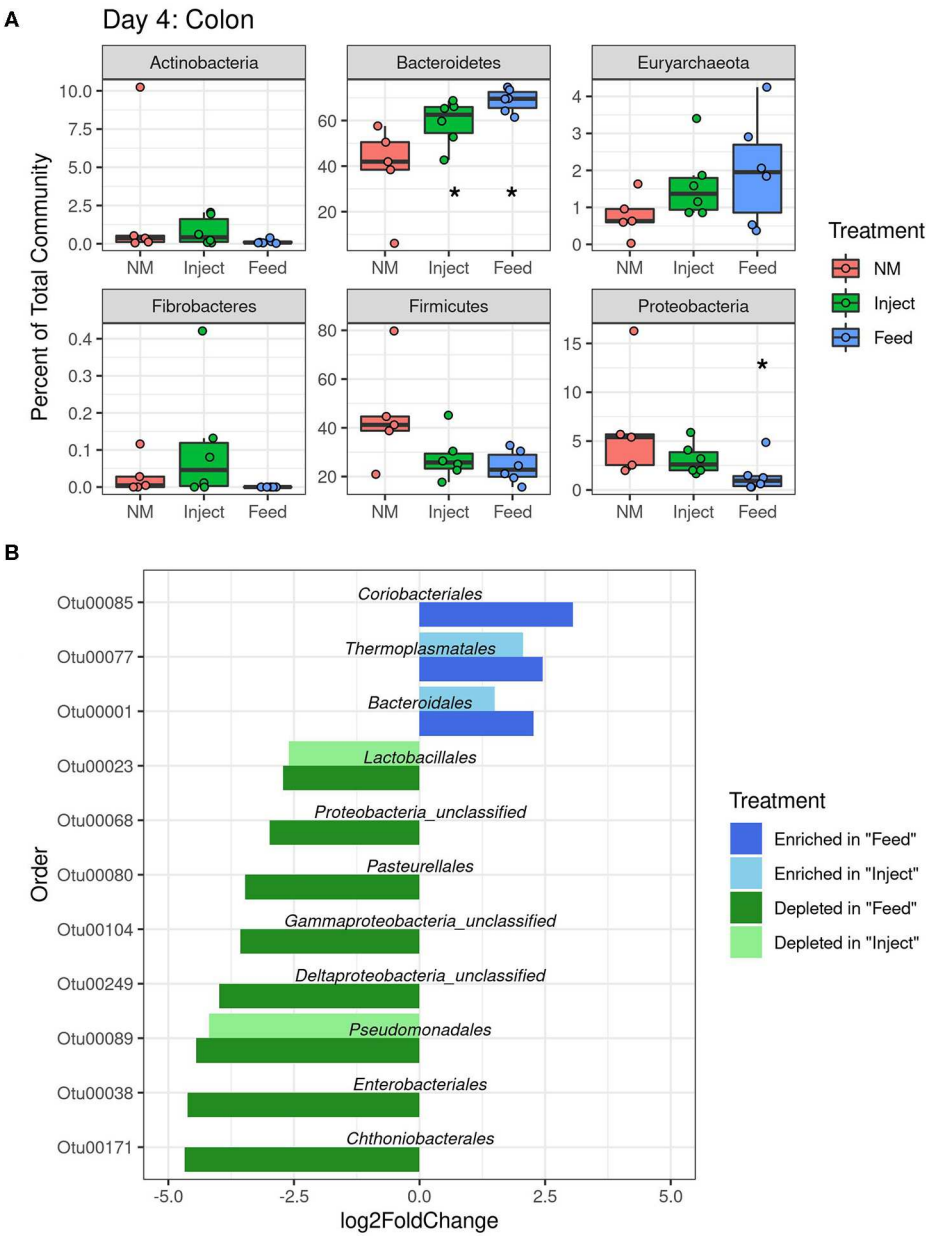

FIGURE 5 | Changes in relative abundance at colon mucosa due to oxytet administration route. Phylum level **(A)** analysis of taxonomic changes at day 4 ($P < 0.05$ indicated with an asterisks). Order level **(B)** changes in abundance ($P < 0.05$) for each treatment group compared to the non-medicated (NM) animals at day 4.

administration (7, 12, 16). Specifically, a recent study on oxytetracycline in swine (7) identified increases in abundance of *Escherichia/Shigella* OTU's in response to tetracycline treatment on day 8 after treatment. Our analysis was performed at the Order level as opposed to the OTU level, and therefore speaks to a broader impact on the Proteobacteria that may not be reflected in individual genera. Examining our data at the OTU level, the only significant change in the *Escherichia/Shigella* OTU was a decrease on day 4 at the colon mucosa ($P = 4.8 \times 10^{-6}$) and an increase in the feces at day 7 ($P = 0.03$), both of which occurred solely in the Feed treatment group (data not shown). Therefore, although there was an overall decrease in Proteobacteria observed at day 4 and day 7, *E. coli* abundance in feces was increased toward the end of oral oxytet treatment, consistent with other studies.

The decrease in endogenous Proteobacteria populations may have negative consequences for the host's resistance to colonization by opportunistic pathogens. In a recent study, Velazquez et al. demonstrate that endogenous *Enterobacteraceae* populations play a critical role in determining susceptibility to *Salmonella* colonization and infection in mice. *Enterobacteraceae* populations compete with *Salmonella* for the terminal electron acceptors that drive their respiratory metabolisms (49). Many

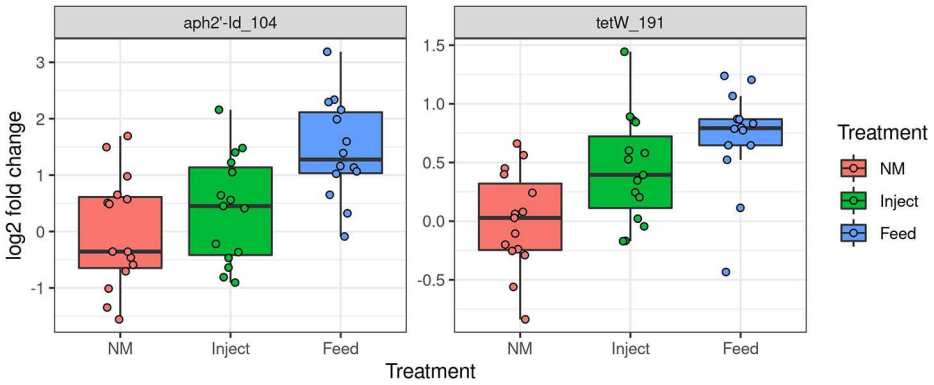

FIGURE 6 | Oxytet administration impacts abundance of antibiotic resistance genes in pig feces. Significant differences in abundance of antibiotic resistance genes in feces on day 7 based on high-throughput qPCR analysis. The Y axis is log2 fold change relative to the mean of the NM group.

important foodborne pathogens such as *E. coli*, *Salmonella*, and *Campylobacter* utilize respiratory metabolisms (50) and as endogenous (benign) populations using terminal electron acceptors are depleted, the compounds become available and can be used by various foodborne pathogens to assist in colonization of the host. While this hypothesis needs more rigorous investigation in other host species, there is some evidence that it occurs in pigs, as tetracycline treatment can increase *Salmonella* shedding from pigs (45). Our work suggests that the depletion of endogenous Proteobacteria may be unintended collateral damage with potential negative consequences for the host and that this collateral damage to the gut ecosystem may be mitigated by injecting oxytet as opposed to administering oxytet orally.

Bacterial community shifts give insight into the impact of antibiotic treatment on the overall swine gut community and disturbances may serve as a proxy for factors impacting intestinal health and ARG transfer; however, changes in resistance gene abundance can occur independent of community member shifts due to the selective elimination of susceptible community members (51) and potential horizontal ARG transfer within the community (6). Changes in ARG content separate from taxonomical distribution would be expected to be particularly relevant when the *Proteobacteria* are impacted, as this phyla carries the greatest diversity of mobile ARGs (52). We chose to examine changes in resistance gene abundance specifically in the fecal samples as it evaluates the resistome of the individual animals in a manner amenable to surveillance of fecal resistance genes that could be disseminated to the environment through field application of animal manure.

As noted above, tetracycline resistance in swine *E. coli* is highly prevalent, ranging from 79 to 100% of isolates (53–55). The observed decreases in Proteobacteria can therefore be expected to correlate solely with the tetracycline susceptible members of the community. Tetracycline resistance is commonly carried on plasmids and other mobile elements (56) and the role of plasmids in disseminating ARG has important implications to the overall risk of resistance gene evolution and spread [reviewed in (57)]. Genes associated with tetracycline resistance were more prevalent in feces of animals given oral oxytet, when compared to injected oxytet. There was also a significant increase in abundance of a gene involved in aminoglycoside resistance in feces of the Feed group, suggesting co-selection for bacteria with the gene. This is consistent with previous work highlighting an increase in aminoglycoside resistance with the use of unrelated antibiotics (12). The aminoglycoside gene identified in this case has been documented as transferring between *Enterococcus* and *E. coli* (58). Many ARG were detected in fecal DNA, even in the NM group (data available at https://github.com/USDA-ARS-FSEPRU/FS1/blob/master/wafergen_reanalysis_Oct2018.R), as noted in previous studies (16, 19). However, plasmid specific targets were not detected in any of the fecal DNA samples which indicates that we did not have robust detection of Gram-negative plasmids within the fecal community of these samples. This is also evident in the tetracycline resistance genes detected, as *tetB* was detected at low levels across all of the samples in contrast to *tetM* and *tetW* that were found in high abundance (and are more commonly associated with the Gram-positive strains that dominated the microbiota). Another limitation of using qPCR for resistome analysis is that the genetic context of the ARG cannot be determined. In order to address this limitation, plasmidome enrichment of the fecal samples was performed and the samples sequenced using both long- and short-read technologies. Although these methods gave only limited insight into the genetic context of the genes highlighted in the qPCR analysis, the detection of small (<7 kb) mobilizeable plasmids carrying tetracycline and aminoglycoside resistance genes provides a potential route of dissemination that has been underexplored. The possible role of small plasmids as gene capture platforms has also been identified by other researchers recently and merits further investigation (59–61).

The optimal route of administration of oxytetracycline for therapy may be dependent on the targeted pathogen. Both in-feed and injectable oxytet are labeled for the treatment of bacterial pneumonia caused by *Pasteurella multocida* and bacterial enteritis caused by *Escherichia coli*, though it's unclear if efficacy against each organism is the same regardless of administration route. Plasma concentrations of oxytet following injection were in agreement with previous reports for this formulation (62–64) and likewise, the low absorption of oxytet

into circulation after oral administration has been documented (65, 66). Oral oxytet administration to pigs may therefore be more effective against intestinal pathogens, as oral administration resulted in increased exposure of gastrointestinal bacteria to antibiotic, and large amounts of antibiotic in feces. However, it does require an animal to consume feed (or water), and anorexia during illness may limit uptake. Concentrations of OTC in nasal wash were higher in the Feed treatment group, likely as a result of the rooting behavior of swine, and this may provide increased protection against respiratory pathogens but also apply selective pressure for ARG in the nasal bacterial populations (67). For this reason, our group is currently investigating the effectiveness of each of these administration routes against a respiratory pathogen challenge and impact on respiratory microbiota. While we administered oxytet to non-infected pigs, and pharmacokinetics may differ during disease, oxytet is often administered to healthy animals when prophylactic treatment is initiated.

Route of antibiotic excretion and withdrawal times may be another consideration in selecting route of administration, as antibiotic contact with bacteria in the environment is also an important consideration related to resistance. Oxytet in feces ends up in manure pits, which may be spread onto fields and subsequently increase the diversity and abundance of ARG in both the treated animals and soils receiving manure from these animals (7, 18, 20, 68, 69). The bioavailability of oxytetracycline is dependent on the soil structure (35, 70); however, exposure in soil can impact microbial enzyme activity (71), functional microbial community structure (72), and increase the persistence of resistance genes following field application of manure (73). While injected administration led to less oxytet in feces, the amount of oxytet excreted in urine was not measured in the current study and may be the primary excretion site after injection. Between 40 and 60% of intravenous administered oxytet is excreted in urine (62, 74), and this is an important consideration for limiting oxytet in the environment. To limit oxytet residue in meat, oxytet must be removed at least 5 or 28 days prior to slaughter for in-feed and injected administration, respectively. Future studies examining all possible excretion routes would be beneficial to antibiotic stewardship efforts by identifying the optimal administration method to maximize the therapeutic effect of treatment while also minimizing unwanted side-effects, such as the release of antibiotic residues to the environment or the disruption of the healthy microbial communities. Collectively, many factors need consideration for treatment of animals with therapeutic oxytet.

Our results have important implications for antibiotic use in both food production animals and potentially also in human patients. In this study, the impacts of oxytet on the overall gut community, and on abundance of resistance genes, was reduced when the antibiotic was delivered by intramuscular injection as opposed to in-feed. In addition, the amount of oxytet in feces was high with in-feed administration and is an important consideration for selective pressure in the environment. Route of antibiotic administration may therefore be one critical control point for maintaining healthy gut communities and reducing selection for antibiotic resistance genes.

DATA AVAILABILITY STATEMENT

The datasets generated for this study can be found in the NCBI SRA (PRJNA553258).

ETHICS STATEMENT

The animal study was reviewed and approved by USDA-National Animal Disease Center Animal Care and Use Committee.

DEDICATION

This article is dedicated to the memory of Heather K. Allen.

AUTHOR CONTRIBUTIONS

NR, JT, AH, SB, CL, and HA designed and performed the experiments. NR, JT, PC, and JCh performed bioinformatic analyses. JT performed statistical analysis and generated figures. JJ and JL performed sequencing and qPCR preparation. JCo performed oxytetracycline analysis. NR, JT, CL, and HA wrote the manuscript. All authors read and approved the manuscript prior to submission. HA passed away on March 7, 2020 while the paper was under review.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2020.00255/full#supplementary-material

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REVIEW
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Formic Acid as an Antimicrobial for Poultry Production: A Review

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Organic acids continue to receive considerable attention as feed additives for animal production. Most of the emphasis to date has focused on food safety aspects, particularly on lowering the incidence of foodborne pathogens in poultry and other livestock. Several organic acids are currently either being examined or are already being implemented in commercial settings. Among the several organic acids that have been studied extensively, is formic acid. Formic acid has been added to poultry diets as a means to limit *Salmonella* spp. and other foodborne pathogens both in the feed and potentially in the gastrointestinal tract once consumed. As more becomes known about the efficacy and impact formic acid has on both the host and foodborne pathogens, it is clear that the presence of formic acid can trigger certain pathways in *Salmonella* spp. This response may become more complex when formic acid enters the gastrointestinal tract and interacts not only with *Salmonella* spp. that has colonized the gastrointestinal tract but the indigenous microbial community as well. This review will cover current findings and prospects for further research on the poultry microbiome and feeds treated with formic acid.

Keywords: formic acid, antimicrobial, food animals, foodborne pathogen, feed, gastrointestinal tract

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INTRODUCTION

Both food animal and poultry production industries are challenged to develop management strategies that achieve a balance between optimizing growth and performance while limiting food safety concerns. Historically, antibiotics fed at subtherapeutic levels were associated with improvements in animal health, welfare, and productivity of animals (1–3). Mechanistically, it has been suggested that antibiotics fed at subinhibitory concentrations mediated their animal host responses via modulation of the gastrointestinal tract (GIT) microbiota and, in turn, their interaction with the host (3). However, continuing concerns over the potential for proliferation of antibiotic-resistant food-associated pathogens and potential association with antibiotic-resistant infections in humans have resulted in the gradual removal of antibiotics for therapeutic use in food animals (4–8). Consequently, the development of feed additives and amendments that meet at least some of these requirements (improvements in animal health, welfare, and productivity of animals) has been an ongoing interest both from an academic research standpoint as well as a commercial development effort (5, 9). Numerous commercial feed additive products have entered into the food animal production market ranging from probiotics and prebiotics to a broad spectrum of essential oils and related compounds from botanical sources as well as chemicals such as aldehydes (10–14). Other commercial

feed additives common to the poultry industry are bacteriophages, zinc oxide, exogenous enzymes, competitive exclusion products, and acidic compounds (15, 16).

Among the available choices of chemical feed additives, aldehydes and organic acids have historically been the more extensively studied and utilized group of compounds (12, 17–21). Organic acids, particularly short-chain fatty acids (SCFA), are well-known antagonists to pathogenic bacteria. These organic acids have been employed as feed additives not only to limit the presence of pathogens in feed matrices but also potentially to be active toward general GIT function (17, 20–24). In addition, SCFA result from the fermentation of GIT microbiota harbored in the digestive tract and are believed to play a mechanistic role in the ability of certain probiotics and prebiotics to be antagonistic to pathogens entering the GIT (21, 23, 25).

Several SCFA have received interest over the years as feed additives. Specifically, propionate, butyrate, and formate have been the subject of numerous research studies and commercial applications (17, 20, 21, 23, 24, 26). While most early interest centered around controlling the occurrence of foodborne pathogens in animal and poultry feeds, the more recent focus has been directed toward animal performance and general promotion of GIT health (20, 21, 24). Acetate, propionate, and butyrate have received considerable attention as organic acid feed additives, with formic acid also being a viable candidate (21, 23). Most of the emphasis to date has focused on food safety aspects of formic acid, particularly on lowering the incidence of foodborne pathogens in livestock feed. However, other aspects of its potential utility are now being considered as well. The overall goal of this review is to discuss the historical and current applications of formic acid as a feed amendment for livestock use (**Figure 1**). As a part of this, the antimicrobial mechanism(s) attributable to formic acid will be examined. Further elaboration on how this impacts administration in animal and poultry agriculture, and potential approaches for improving efficacy will also be discussed.

BIOLOGICAL CONTAMINATION OF FEEDS

Food animal and poultry feed production is a complex operation with multiple steps, including physical processing of cereal grains such as grinding to reduce particle size, thermal treatment for pelleting, as well as supplementing the diet with numerous nutritional ingredients depending upon the specific nutrient requirement of the animal (27). Given this complexity, it is not surprising that during feed processing the opportunity to come in contact with numerous environments before the grains reach the feed mill, during feed milling, followed by delivery and feeding of the mixed feed ration occurs (9, 21, 28). Consequently, a highly variable set of microorganisms, including not just bacteria but bacteriophage, fungi, and yeast, have all been identified from feeds over the years (9, 21, 28–31). Some of these contaminants, such as certain fungi, can be problematic for animal health due to their production of mycotoxins (32–35).

Bacterial populations can be relatively diverse and are somewhat dependent on the corresponding methods used for isolation and identification of the microorganisms as well as the source of the samples. For example, microbial compositional profiles might be expected to be somewhat different before thermal processing associated with pelleting (36). While classical culture and plating methodologies have been somewhat informative, more recent applications of next-generation sequencing (NGS) of the microbiome based on the 16S rRNA gene offer a much more comprehensive evaluation of feed microbial communities (9). When Solanki et al. (37) examined the bacterial microbiomes of wheat grains stored over time in the presence of an insect fumigant phosphine, they concluded that the microbiomes were more diverse immediately after harvest and after 3 months of storage. In addition, Solanki et al. (37) demonstrated that Proteobacteria, Firmicutes, Actinobacteria, Bacteroidetes, and Planctomycetes were the dominant phyla among the wheat grains and *Bacillus*, *Erwinia*, and *Pseudomonas* as being the more predominant genera along with a lesser proportion of *Enterobacteriaceae*. Based on taxonomic comparisons, they concluded that phosphine fumigation altered the bacterial populations considerably but did not influence fungal diversity.

The microbiome-based detection of the genus *Enterobacteriaceae* by Solanki et al. (37) would suggest that feed sources can also harbor foodborne pathogens that could be of public health concern. Foodborne pathogens such as *Clostridium perfringens*, *Clostridium botulinum*, *Salmonella* spp., *Campylobacter*, *Escherichia coli* O157:H7, and *Listeria* have all been associated with animal feeds and silage (9, 31, 38). It is not clear how persistent other foodborne pathogens are in animal and poultry feeds. When Ge et al. (39) sampled over 200 animal feed ingredients, they were able to isolate *Salmonella* spp., generic *Escherichia coli*, and *Enterococcus* but did not detect *E. coli* O157:H7 or *Campylobacter*. However, matrices similar to dry feeds can serve as sources of pathogenic *E. coli*. In tracking the outbreak source of Shiga toxin-producing *E. coli* (STEC) serogroup O121 and O26 associated with human illness occurring in 2016, Crowe et al. (40) used whole-genome sequencing to compare clinical vs. food source isolates. Based on this comparison, they concluded that low moisture raw wheat flour from a flour processing facility was the likely source. The low moisture properties of the wheat flour would suggest that STEC could survive in low moisture animal feeds as well. However, as Crowe et al. (40) pointed out, there were difficulties with isolating STEC from flour samples, and an immunomagnetic-separation approach was required to retrieve sufficient bacterial cells. Similar diagnostic logistics could preclude the detection and isolation of rarely occurring foodborne pathogens in animal feeds as well. Detection difficulties could also be a challenge due to the long term persistence in these types of low moisture matrices. Forghani et al. (41) demonstrated that inoculated mixtures of enterohemorrhagic *E. coli* (EHEC) serogroups O45, O121, and O145 and *Salmonella* (Typhimurium, Agona, Enteritidis, and Anatum) in wheat flour held at room temperature were quantifiable at 84 and 112 days and remained detectable at 24 and 52 weeks, respectively.

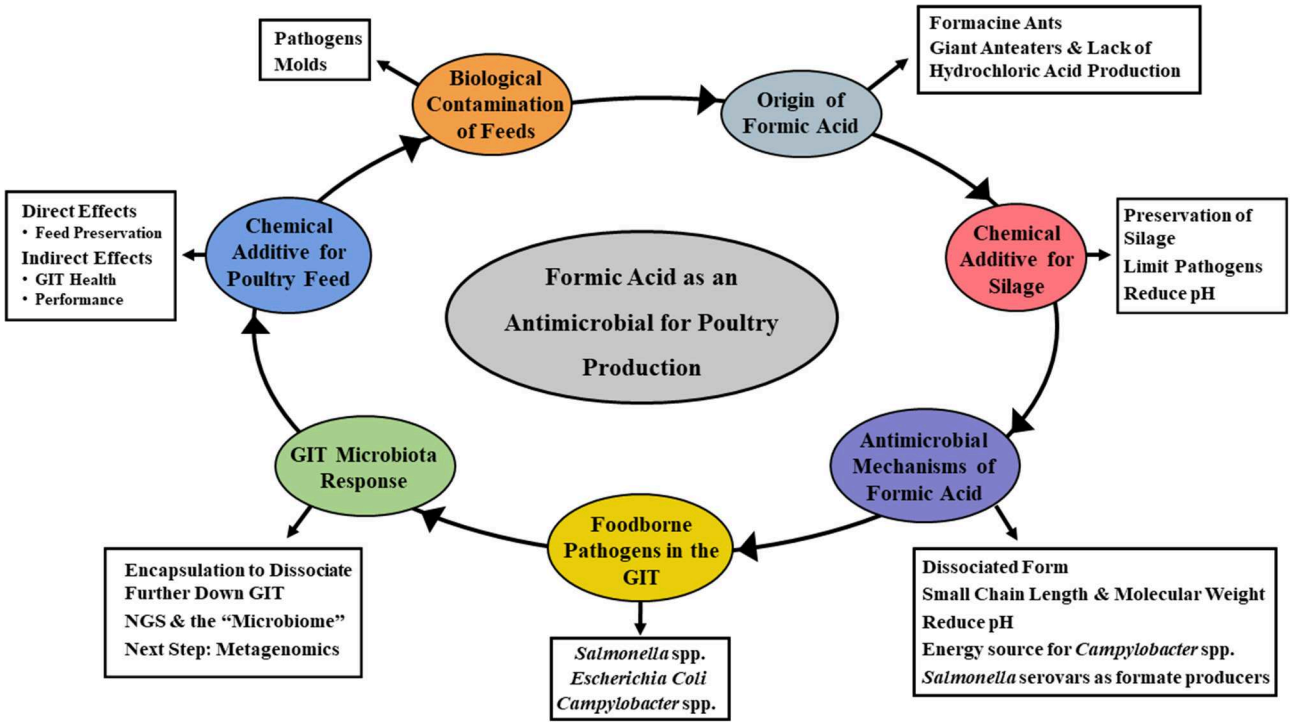

FIGURE 1 | Mind map of the topics covered in the current review. Specifically, focusing on the overall goal of describing the historical and current applications of formic acid as a feed amendment for livestock use, the antimicrobial mechanism(s) attributable to formic acid and how its administration impacts animal and poultry health, and potential approaches for improving efficacy.

Historically, *Campylobacter* species have not been isolated from animal and poultry feeds using conventional culture-based methods (38, 39) even though *Campylobacter* can be readily isolated from the poultry GIT and on poultry meat products (42, 43). However, feed as a potential source may still have some merit. For example, Alves et al. (44) demonstrated that the inoculation of starter and finisher poultry feed with *C. jejuni* followed by storage of the feed at two different temperatures for either 3 or 5 days resulted in the recovery of viable *C. jejuni* and in some cases multiplication. They concluded that *C. jejuni* could undoubtedly survive in poultry feeds and therefore could be a potential source for chickens.

Previously, *Salmonella* spp. contamination of animal and poultry feed has received most of the attention and remains a current focus for the development of detection methods suited explicitly for feeds as well as the pursuit of more effective control measures (12, 26, 30, 45–53). Numerous *Salmonella* spp. isolation and characterization survey studies have been conducted over the years on a wide range of feeds and feed mills (38, 39, 54–61). Collectively, these surveys have revealed that *Salmonella* spp. can be isolated from a diverse set of feed ingredients, feed sources, and types of feeds as well as feed mill operations. Level of prevalence and the predominant *Salmonella* serovar isolates also vary to some extent. For example, Li et al. (57) confirmed the presence of *Salmonella* spp. in 12.5% of the 2,058 total samples collected from complete animal feeds, feed ingredients, pet foods, pet treats, and pet supplements during a collection period from 2002 to 2009. In addition, of the 12.5% confirmed positive *Salmonella* samples, *S.* Senftenberg and *S.* Montevideo were the most prevalent serovars identified (57). In a survey of Texas ready-to-eat and animal feed byproducts, Hsieh et al. (58) reported that fish meal had the highest prevalence of *Salmonella* spp. followed by animal proteins with *S.* Mbanka and *S.* Montevideo being the most frequent serovars identified. Feed mills also represent several potential contamination sites for feeds during mixing and addition of ingredients (9, 56, 61). Magossi et al. (61) were able to demonstrate the potential for multiple contamination sites that occur throughout U.S. feed manufacturing. As a matter of fact, Magossi et al. (61) were able to identify at least one location (of the 12 tested sampling locations) at each of the 11 U.S. feed mills tested across eight states that were culture positive for *Salmonella* spp. Given the potential for *Salmonella* contamination during feed processing, transportation, and daily feeding, it is not surprising that numerous attempts have been made to develop feed additives that decrease microbial contamination and retain these reduced levels throughout the animal production cycle.

ANTIMICROBIAL MECHANISMS OF FORMIC ACID

Less is known mechanistically about *Salmonella*'s specific response to formate. Still, Huang et al. (62) noted that formate is present in the mammalian small intestine and that *Salmonella*

spp. are capable of producing formate. When Huang et al. (62) examined *Salmonella* virulence gene expression using a series of deletion mutants in critical pathways, they observed that formate could serve as a diffusible signal to induce *Salmonella* invasion of Hep-2 epithelial cells. More recently, Lü et al. (63) isolated a formate transporter, FocA in *Salmonella* Typhimurium, that acts as a specific formate channel at pH 7.0 but also serves as either a passive export channel at high external pH or a secondary active formate/hydrogen ion importer at low pH. However, this work was conducted exclusively on one serovar *S.* Typhimurium. The question remains as to whether all serovars mechanistically respond similarly to formic acid. This question remains a key research question that will need to be addressed in future studies. Regardless of the outcome, it is still prudent to employ multiple *Salmonella* serovars and perhaps even more than one strain for each serovar in screening experiments when general recommendations need to be made for the use of an acid additive to reduce *Salmonella* spp. in feed. Newer approaches such as the ability to genetically barcode strains to distinguish subpopulations of the same serovar (9, 64) offer opportunities to differentiate more subtle differences that could influence variances in conclusions and interpretation.

The chemical and dissociation form of formate may be important as well. In a series of studies, Beier et al. (65–67) demonstrated that inhibition of *Enterococcus faecium*, *Campylobacter jejuni*, and *Campylobacter coli* correlated with the amount of dissociated formic acid and not pH or undissociated formic acid. The chemical form of formate the bacteria are exposed to appears to matter as well. Kovanda et al. (68) screened several Gram-negative and Gram-positive microorganisms and compared Minimum Inhibitory Concentration (MIC) responses on sodium formate (500–25, 000 mg/L) and a blend of sodium formate and free formate (40/60 w/v; 10–10,000 mg/L). Based on the MIC estimates, they found that sodium formate was only inhibitory to strains of *Campylobacter jejuni*, *Clostridium perfringens*, *Streptococcus suis*, and *Streptococcus pneumoniae*, but not *E. coli*, *Salmonella* Typhimurium, or *Enterococcus faecalis*. Conversely, the blend of sodium formate and free formate was inhibitory to all the microorganisms leading the authors to suggest that free formic acid possesses most of the antimicrobial properties. It would have been interesting to examine different ratios of the two chemical forms to determine whether the range of MIC values correlated with the level of formic acid present in the blended formula vs. responses to 100% formic acid.

Gómez-García et al. (69), have screened essential oils in combination with organic acids such as formic acid against multiple isolates originating from swine, *Escherichia coli*, *Salmonella* spp. and *Clostridium perfringens*. They tested the efficacy of six organic acids, including formic acid and six essential oils, with formaldehyde as a positive control against the swine isolates. Gómez-García et al. (69) determined the MIC_{50}, MBC_{50}, and $MIC50/MBC_{50}$ of formic acid to *E. coli* (600 and 2,400 ppm, 4), *Salmonella* spp. (600 and 2,400 ppm, 4) and *Clostridium perfringens* (1,200 and 2,400 ppm, 2), with formic acid performing better out of all the organic acids against *E. coli* and *Salmonella* spp. (69). The explanation for the efficacy of formic acid against *E. coli* and *Salmonella* spp. is its small molecular size and chain length (70).

When Beier and coworkers screened *Campylobacter coli* strains isolated from swine (66) and *Campylobacter jejuni* strains originating from poultry (67), they concluded that the dissociated concentration for formate matched the determined MIC responses as seen with the other organic acids. However, caution was raised as to the relative effectiveness of these acids, including formic acid, since *Campylobacter* is capable of utilizing them as a substrate (66, 67). *Campylobacter jejuni*'s utilization of acids is not surprising as it has been characterized as having a non-glycolytic metabolism. As such, *Campylobacter jejuni* has a limited carbohydrate catabolic capacity and instead relies on gluconeogenesis from amino acids and organic acids for much of its energy metabolism and biosynthesis activities (71, 72). Early work by Line et al. (73) using a phenotype array with 190 carbon sources, noted that a *Campylobacter jejuni* 11168 (GS) could use organic acids as carbon sources, with most being intermediates of the TCA cycle. Further research by Wagley et al. (74) using a carbon utilization phenotype array approach noted that strains of both *Campylobacter jejuni* and *C. coli* examined in their study were able to grow with organic acids as carbon sources. Formic acid specifically serves as a primary energy source of *Campylobacter jejuni* by being a major electron donor for respiratory energy metabolism in *Campylobacter* (71, 75). *C. jejuni* is able to use formic acid as a hydrogen donor via a formate dehydrogenase membrane complex that oxidizes formate to carbon dioxide, protons, and electrons and serves as an electron donor for respiration (72).

FORMIC ACID AND ITS ORIGIN IN THE INSECT CLASS

Formic acid has a long history of being utilized as an antimicrobial feed amendment but also is generated by some insects for use as an antimicrobial defense chemical. Rossini et al. (76) suggested that formic acid was probably the constituent acid in the ant-generated acid juice described nearly 350 years ago by Wray (77). Since then, the understanding of formic acid production by formicine ants and other insects has evolved considerably, and this process is now known to be part of a well-orchestrated toxin defense system for insects (78). Several insect taxa including stingless bees, Oxytrigona (Hymenoptera: Apidae), carabid beetles (*Galerita lecontei* and *G. janus*), stingless formicine ants (subfamily Formicinae), and some moth larvae (Notodontidae, Lepidoptera) are known to produce formic acid as a defense chemical (76, 78–82).

Formicine ants are probably the best characterized and possess an acidophore, a specialized opening that allows them to spray their venom containing formic acid as the primary compound (82). The ant uses serine as a precursor and accumulates large quantities of formic acid in a poison gland that is sufficiently compartmentalized to protect the host ant from the cytotoxic levels of formate until it is dispersed as a spray (78, 83). The emitted formic acid spray can (1) be an alarm pheromone to recruit additional ants, (2) become a defense chemical against

competitors and predators, and (3) when combined with tree resin as part of their nest materials, serve as an antifungal and antimicrobial agent (78, 82, 84–88). The antimicrobial properties associated with formic acid production in ants suggests that it could also be applied externally as an additive compound. Brütsch et al. (88) demonstrated this when they added synthetic formic acid to resin resulting in a significantly increased antifungal activity. As further evidence of the potency of formic acid and its biological utility, giant anteaters that lack the ability to produce gastric hydrochloric acid consume ants containing formic acid to provide the concentrated formic acid as a substitute digestive acid (89).

FORMIC ACID AS A CHEMICAL ADDITIVE FOR SILAGE

The practical agricultural application of formic acid has been considered and examined for several years. Specifically, formic acid has utility as an additive for animal feed and silage. Both solid and liquid forms of sodium formic acid have been considered safe for all animal species as well as consumers and the environment (90). Based on their assessment (90), a maximum concentration of 10,000 mg formic acid equivalents/kg of feed was deemed safe for all animal species, while 12,000 mg formic acid equivalents/kg of feed were considered safe for swine. Application of formic acid as a feed amendment for animal nutrition has been examined for a number of years. It has been viewed as having commercial value as a preservative in silage and as an antimicrobial for animal and poultry feeds.

Chemical additives such as acids have been an essential element in the management of production and feeding of forage-based silages (91, 92). Borreani et al. (91) noted that achieving optimized, high-quality forage silage production requires stabilizing the forage quality while retaining the maximum amount of dry matter possible. The outcome of this optimization would be minimized losses during all stages of silage from initial aerobic conditions in the silo, followed by fermentation, storage, and reopening the silo for feeding. Specific methods for optimizing silage production in the field and the subsequent silo fermentation have been extensively reviewed elsewhere (91, 93–95) and will not be covered in detail in the current review. A primary concern is yeast- and mold-mediated oxidative deterioration while oxygen remains in the ensiled forage (91, 92). Consequently, biological inoculants, and chemical additives were introduced to counter the detrimental impact of deterioration (91, 92). Additional concerns for silage additives include limiting the proliferation of pathogens such as pathogenic *E. coli*, *Listeria*, and *Salmonella* spp. that may be present in the silage as well as mycotoxin producing fungi (96–98).

Muck et al. (92) have categorized acid additives in two distinct groups. Acids such as propionic, acetic, sorbic, and benzoic acids retain aerobic stability of silage while being fed to ruminants by limiting yeasts and molds (92). Muck et al. (92) delineated formic acid from the other acids as a direct acidifier that can suppress clostridia and spoilage microorganisms while preserving silage protein integrity. For the practical application of the acids, their corresponding salt form represents the more common chemical version employed to avoid corrosiveness of the non-salt versions of these acids (91). Formic acid has also been investigated as an acid additive for silage by numerous research groups. It is known for its rapid acidification potential and inhibitory action on the growth of undesirable silage microorganisms that reduce levels of silage forage protein and water-soluble carbohydrates (99). As such, He et al. (100) demonstrated the ability of formic acid to suppress coliforms and decrease the pH of the silage. Formic acid and cultures of lactic acid-producing bacteria have also been added to silage to promote acidification and organic acid production (101). In fact, Kuley et al. (101) determined that lactic and formic acid were produced in amounts exceeding 800 and 1,000 mg organic acid/100 g sample when silage was acidified with 3% (w/v) of formic acid. Muck et al. (92) have extensively reviewed the silage additive research literature, including studies focused on and/or including formic and other acids that were published since the year 2000. Therefore, these individual research studies will not be discussed in detail in the current review except to summarize a few key points regarding formic acid efficacy as a silage chemical additive. Both non-buffered and buffered formic acid have been examined, and in most cases clostridial spp. and their associated activities (consumption of carbohydrates, proteins and lactic acid, and the excretion of butyric acid) tended to decline along with decreases in ammonia and butyrate production and improved retention of dry matter (92). There were some limits to the impact of formic acid, but combinations with other acids as silage additive blends appeared to overcome some of these issues (92).

Formic acid may limit pathogenic organisms linked to human public health concerns. For example, Pauly and Tham (102) inoculated *Listeria monocytogenes* into small laboratory silos containing ryegrass at three different dry matter levels (200, 430 and 540 g/kg), followed by incorporating either formic acid (3 mL/kg) or lactic acid bacteria (8×10^5/g) with cellulolytic enzymes. They reported that either treatment reduced *L. monocytogenes* to non-detectable levels in the low dry matter silage (200 g/kg). However, in the medium-dry matter silage (430 g/kg), *L. monocytogenes* could still be quantified at 30 days in formic acid treated silage. The reduction in *L. monocytogenes* appeared to correspond to a lower pH, levels of lactic acid, and pooled undissociated acids. Therefore, Pauly and Tham (102) alluded to the fact that levels of lactic acid and pooled undissociated acids were especially important and were probably the reason why the reduction in *L. monocytogenes* was not observed in the formic acid treated medium in the higher dry matter silage. In the future, similar studies will need to be conducted with other common silage pathogens such as *Salmonella* spp. and pathogenic *E. coli*. A more comprehensive 16S rDNA sequence profiling of the entire silage microbial community could also help identify overall silage microbial population shifts occurring during the various stages of silage fermentation in the presence of formic acid (103). Generating microbiome data may provide analytical support to better predict the progress of silage fermentation as well as design optimal additive combinations to maintain high-quality forage silage.

FORMIC ACID AND ANTIMICROBIAL ACTIVITIES IN ANIMAL FEEDS

For cereal grain-based animal diets, formic acid has been employed as a feed antimicrobial to limit pathogen levels in a wide range of feed matrices originating from cereal grains as well as specific feed ingredients such as animal byproducts. Impact on pathogen populations in poultry and other animals can be broadly categorized as either direct effects on pathogen populations in the feed itself or the more indirect effect on pathogens colonizing the animal's GIT after the treated feed has been consumed (20, 21, 104). Obviously, these two categories are interconnected as a reduction of pathogens in the feed should lead to less colonization when the feed is consumed by the animal. However, several factors can potentially influence the antimicrobial properties of the particular acid introduced to a feed matrix such as feed composition, and form of the acid administered (21, 105).

Historically, much of the focus for the application of formic acid and other related acids has been on the direct control of *Salmonella* spp. in animal and poultry feeds (21). The results of these studies have been summarized in details in several reviews that have been published at different times (18, 21, 26, 47, 104–106) and therefore, only some of the key conclusions from these studies will be discussed in the current review. Several studies have indicated that the antimicrobial activity of formic acid in the feed matrix is dependent on the dose and exposure time of formic acid, the moisture content of the feed matrix, and the bacterial concentration of the feed and animal GIT (19, 21, 107–109). The type of feed matrix and the origin of animal feed ingredients are also factors. Consequently, several studies have indicated that level of *Salmonella* spp. recovered from animal byproducts may differ compared to their plant-based counterparts (39, 45, 58, 59, 110–112). However, some of these differences in response to acids, such as formate, may be related to serovar survival differences in feed and temperature of feed treatment (19, 113, 114). Serovar differences in response to acid treatment may also be a factor in poultry infection by contaminated feed (113, 115) and differences in virulence gene expression (116) could play a role. Differences in acid tolerance could in turn influence detection of *Salmonella* spp. on culture media if the acid that carries over from the feed is not adequately buffered (21, 105, 117–122). The physical form of the diet in terms of particle size may also contribute to the relative effectiveness of formic acid in the GIT (123).

Strategies to optimize the antimicrobial activity of formic acid addition to feed also appears to be critical. Application of acids at higher concentrations in feed ingredients that are at a high-risk of contamination prior to feed mixing has been suggested to minimize potential feed mill equipment damage and animal palatability issues (105). Jones (51) concluded that *Salmonella* spp. present in the feed before chemical decontamination might be more challenging to limit than those that come in contact with the feed after chemical treatment. Thermal treatment of feeds during feed mill processing is considered an intervention for limiting *Salmonella* spp. contamination in feeds but depends on feed composition, particle size, among other factors associated with the milling process (51). The antimicrobial activity of acids is also impacted by temperature, and increased temperature in the presence of organic acids can elicit a synergistic inhibition of *Salmonella* spp. as observed in liquid cultures of *Salmonella* (124, 125). Several studies on *Salmonella* spp. contaminated feed have supported the idea that increased temperature improved the efficacy of the acids incorporated in the feed matrix (106, 113, 126). Using a central composite design, Amado et al. (127) examined the interaction between temperature and acids (formic or lactic acid) on 10 *Salmonella enterica*, and *E. coli* isolates from various cattle feeds and inoculated into acidified pelleted cattle feed. They concluded that heat was the more dominant influential factor on microbial reduction with the type of acid and bacterial isolate also being a factor. Synergism with the acids still generally occurred, allowing for the potential to use lower temperatures and lower acid concentrations. However, they also noted that synergy did not always occur with formic acid, leading them to suspect that either volatilization of formic acid occurred at higher temperatures or buffering by feed matrix components was a factor.

IMPACT ON FOODBORNE PATHOGENS IN THE GASTROINTESTINAL TRACT

Limiting foodborne pathogens in the feed during storage prior to feeding animals is undoubtedly a means to control their introduction to the animal during consumption of the feed. However, acids in the feed have the opportunity as they enter into the GIT to continue to exhibit antimicrobial activities. Externally introduced acid antimicrobial activity in the GIT is potentially dependent on numerous factors including GIT acid concentration, GIT site of activity, level of GIT pH and oxygen, age of the animal, and the corresponding composition of microbial populations inhabiting the GIT as a function of GIT location and animal maturity (21, 24, 128–132). In addition, the resident GIT anaerobic microbial population, which becomes more dominant in the lower GIT sections of the monogastric animal as it matures, is actively producing organic acids via fermentation, which, in turn, are also potentially antagonistic to transient pathogens entering the GIT (17, 19–21).

Most of the early work focused on using organic acids, including formate, to limit *Salmonella* spp. in the poultry GIT, which has been discussed in detail in several reviews (12, 20, 21). From an overview of these studies, a few key observations have prevailed. McHan and Shotts (133) reported that feeding formic and propionic acid reduced cecal levels of *S.* Typhimurium inoculated in young chicks and quantified at 7, 14, and 21 days of age. However, when Hume et al. (128) tracked C^{-14} labeled propionate, they concluded that very little propionate in the feed likely reached the ceca. Whether this is true of formic acid remains to be determined. However, more recently, Bourassa et al. (132) did note that feeding formic acid at 4 g per ton

for a 6 week grow-out period in broiler chicks reduced cecal *S*. Typhimurium concentrations below detection levels.

The presence of formic acid in the diet likely influences other poultry GIT compartments. Al-Tarazi and Alshawabkeh (134) demonstrated that a formic and propionic acid mixture decreased the frequency of *S*. Pullorum in both the crop and the ceca. Thompson and Hinton (129) observed that a commercial blend of formic and propionic acid resulted in an increased concentration of these two acids in the crop and gizzard and, when representative crop conditions were simulated *in vitro*, were bactericidal to *S*. Enteritidis PT4. This is supported by *in vivo* data when Byrd et al. (135) added formic acid to the drinking water of broilers undergoing a simulated pre-transport feed withdrawal similar to that experienced by broilers prior to transit to the poultry processing plant. The presence of formic acid in the drinking water resulted in reduced *S*. Typhimurium crop and cecal populations along with a decrease in the frequency of *S*. Typhimurium positive crops, but not the number of positive ceca (135). Developing delivery systems that serve to protect organic acids as they enter the GIT to remain active in the lower compartments may help to increase efficacy. For example, protecting formic acid by microencapsulation for administration in feed has been shown to decrease *S*. Enteritidis in cecal contents (136). However, this may differ among animal species. For example, Walia et al. (137) did not see *Salmonella* spp. reduction in 28-day old pigs fed an encapsulated blend of formic acid, citric acid, and essential oils in either the cecal digesta or lymph nodes although *Salmonella* spp. shedding in the feces was reduced on day 14 but not on day 28. They did suggest that the horizontal transfer of *Salmonella* spp. among pigs was prevented.

While the majority of the research on formic acid as an antimicrobial in food animal production has focused on foodborne *Salmonella* spp., there have been some studies with other pathogens inhabiting the GIT. As indicated by the *in vitro* work of Kovanda et al. (68), formic acid may be effective against other GIT foodborne pathogens as well, including *E. coli* and *Campylobacter jejuni*. Early research indicated that organic acids, such as lactic acid and commercial blends that contained formic acids as one of several components, could lower *Campylobacter* levels in poultry (135, 138). However, employing formic as an antimicrobial agent against *Campylobacter* may need some caution exercised, as noted earlier by Beier et al. (67). This fact may be particularly problematic for poultry diet supplementation since formic acid serves as a major energy donor for *Campylobacter jejuni* respiration. In addition, it is believed that part of its ecological niche in the GIT is to metabolically cross-feed on the mixed acid fermentation products such as formic acid produced by GIT bacteria (139). There is some support for this. Because formic acid is a chemoattractant to *Campylobacter jejuni,* double mutants impaired in both formate dehydrogenase and hydrogenase display decreased cecal colonization in broilers compared to the wild-type *Campylobacter jejuni* strain (140, 141). It is not known how much external formic acid supplementation could influence *Campylobacter jejuni* establishment in the chicken GIT. Several variables could impact this as the actual GIT formic acid concentration could be lower due to catabolism of formic acid by other GIT bacteria or absorption of formic acid in the upper part of the GIT. Also, formic acid is a potential fermentation product generated by some GIT bacteria, and this could contribute to overall formic acid GIT levels. Quantitation of formic acid in GIT contents and metagenomics to identify formate dehydrogenase genes would potentially provide some clarity of formic acid microbial ecology.

Roth et al. (142) compared broilers fed either the antibiotic enrofloxacin or an acid blend of formic acid, acetic acid, and propionic acid on the prevalence of antibiotic-resistant *E. coli*. Total *E. coli* and antibiotic-resistant *E. coli* isolates were enumerated from pooled fecal samples of 1-day-old broiler chicks and cecal contents of 14- and 38-day-old broilers. *E. coli* isolates were screened for resistance to ampicillin, cefotaxime, ciprofloxacin, streptomycin, sulfamethoxazole, and tetracycline based on the breakpoint concentration for each respective antibiotic as previously defined. When the respective *E. coli* populations were quantified and characterized, neither the enrofloxacin nor the acid blend supplementation altered the total *E. coli* recovered from 17 and 28-day old broiler ceca. Birds receiving diets supplemented with enrofloxacin yielded increased levels of ciprofloxacin, streptomycin, sulfamethoxazole, and tetracycline-resistant *E.* coli in the ceca, but a decrease in cefotaxime resistant *E. coli*. The blended acids resulted in decreased numbers of ampicillin- and tetracycline-resistant cecal *E. coli* compared with both control and enrofloxacin-supplemented birds. The blended acids also resulted in fewer ciprofloxacin- and sulfamethoxazole-resistant *E.* coli in the ceca vs. the enrofloxacin supplemented birds. It is not clear mechanistically how acids could reduce antibiotic-resistant *E. coli* without reducing the total numbers of *E. coli*. However, the outcome of the study performed by Roth et al. (142) may be evidence for the reduction of dissemination of antibiotic-resistant genes among *E. coli,* such as the plasmid conjugation inhibitors described by Cabezón et al. (143). It would be interesting to conduct a more in-depth profile of plasmid-mediated antibiotic resistance in poultry GIT populations in the presence of feed additives such as formic acid and further develop this profile with an assessment of the GIT resistome.

INTERACTION OF THE NON-PATHOGEN GASTROINTESTINAL MICROBIOTA WITH FORMIC ACID

Developing optimal antimicrobial feed additives while targeting pathogens ideally should have minimal impact on the overall GIT microbiota, particularly microbial members that would be considered beneficial to the host. However, a deleterious impact on the resident GIT microbial population can occur in the presence of externally introduced organic acids and could, to some extent, offset their pathogen prevention benefits. For example, Thompson and Hinton (129) observed decreases in layer hen crop lactic acid in birds fed a formic acid-propionic acid blend suggesting that the presence of these external organic acids in the crop caused a decrease in the crop lactic acid bacterial population. The presence of lactic acid bacteria in the crop

is considered a barrier to *Salmonella* spp., so disrupting this resident crop microbiota could be problematic for achieving a successful reduction in *Salmonella* GIT colonization (144). Less impact may occur in the lower part of the avian GIT as Açikgöz et al. (145) did not detect differences in total intestinal bacteria or *E. coli* in 42-day-old broilers receiving formic acid acidified water. As the authors speculated, this might be due to the formic acid being metabolized in the upper part of the GIT as noted by others for externally introduced SCFA (128, 129).

The Case for Microencapsulation

Protection of formic acid via some form of encapsulation might offer a means to reach lower sections of the GIT. Willamil et al. (146) observed that microencapsulating formic acid significantly increased total SCFA in the ceca of pigs compared to those fed non-protected formic acid. This outcome led the authors to suggest that formic acid, if sufficiently protected, can effectively reach the lower GIT compartments. However, several other measurements, such as formic acid and lactate concentration, although higher than control diet-fed pigs, were not statistically different from non-protected formic acid-fed pigs. *Lactobacilli* populations were not changed by any of the treatments even though lactic acid was increased nearly three-fold in pigs fed either both unprotected or protected formic acid. It may be possible that differences would be more distinct with other lactic acid-producing cecal microorganisms (1) that were not detected with these methods and/or (2) whose metabolic activities were impacted to change fermentation patterns such that more lactic acid was being produced by the resident lactic acid bacterial population.

Enhanced Resolution—The Impact of Formic Acid on Poultry GIT Microbiota

To better delineate feed additive impact on the food animal GIT, microbiological identification methodologies with increased resolution are required. In the past few years, NGS of the 16S RNA gene for microbiome taxonomic identification and microbial community diversity comparisons (147) have made it possible to develop a better understanding of the interactions between dietary feed additives and the GIT microbiota of food animals such as poultry.

A few studies have incorporated microbiome sequencing assessment of the chicken GIT microbial consortia response to formic acid supplementation. Oakley et al. (148) conducted a study with 42-day-old broilers fed different combinations of formic, propionic, and medium-chain fatty acids administered either in the drinking water or feed. Seeder birds were inoculated with nalidixic acid-resistant *Salmonella* Typhimurium, and ceca were removed at 0, 7, 21, and 42 days of age. Cecal samples were prepared for 454 pyrosequencing and the sequence results assessed for taxonomic classification and similarity comparisons. In general, treatments had little impact on the cecal microbiome or levels of *S.* Typhimurium. However, in general, levels of recovered *Salmonella* spp. decline as the birds become older, and this was supported by the taxonomic microbiome analyses where the relative abundance of *Salmonella* sequences also declined over time. The authors noted that the most significant shifts in GIT microbiota occurred over time across all treatments as cecal microbial populations became more diverse over time as the broilers matured. In a more recent study, Hu et al. (149) compared drinking water and feed delivery of an organic acid blend (formic, acetic, and propionic acids and ammonium formate) with a Virginiamycin supplemented diet on broiler cecal microbiomes from samples collected during two phases (1–21 days and 22–42 days). While some cecal microbiome diversity differences among treatment were detectable in birds at 21 days, by the time birds reached 42 days of age, no differences in alpha or beta diversity were detected. The lack of differences at 42 days of age led the authors to suggest that growth performance benefits may be linked to the earlier establishment of an optimally diversified microbiota.

Microbiome analyses exclusively focused on the cecal microbial populations may not be reflective of where most of the dietary organic acid influence is occurring in the GIT. The upper GIT microbiome populations of broilers may be more likely impacted by dietary organic acids, as indicated by the results from Hume et al. (128). Hume et al. (128) demonstrated that most of the externally supplemented propionate is absorbed in the avian upper GIT. There are also more recent GIT microbial characterization studies that support this. Nava et al. (150) demonstrated that the combination of an organic acid blend [^{DL}-2-hydroxy-4(methylthio) butanoic acid], formic, and propionic acid (HFP) impacted the intestinal microbial populations and increased the *Lactobacillus* spp. colonization of the chick ileum. More recently, Goodarzi Boroojeni et al. (151) examined two levels (0.75 and 1.50%) of a formic and propionic acid blend fed to broiler chicks for 35 days. At the termination of the experiment, the crop, gizzard, distal two-thirds of the ileum, and ceca were removed and sampled for RT-PCR quantitation of specific GIT bacterial groups and GIT metabolites. In the crop, neither concentration of organic acids altered the *Lactobacillus* spp. or *Bifidobacterium* spp. populations, but did increase the *Clostridial* clusters. In the ileum, the only changes that occurred were decreases in *Lactobacillus* spp. and *Enterobacteria* vs. no changes in any of these bacterial groups in the cecum (151). Total lactate (D and L) concentrations were reduced for the highest level of organic acid additive in the crop, and both organic acid levels in the gizzard, the lower organic acid concentration in the cecum. No shifts occurred in the ileum. As for SCFA, only propionate was altered in the crops and gizzards of birds receiving organic acids. There was nearly a ten-fold increase of propionate in the crops of birds receiving the lower organic acid concentration and an eight- and fifteen-fold increase in the gizzard for the two levels of organic acids. There was less than a two-fold increase in acetic acid in the ileum. Collectively these data support the idea that most of the external organic acid additive influence occurs in the crop with minimal impact of organic acids on the lower GIT microbial populations and suggests that fermentation patterns may be altered in the resident populations of the upper GIT.

Clearly, more microbiome characterization is warranted to achieve sufficient elucidation of microbial responses to formic acid throughout the GIT. More emphasis on in-depth analyses of specific GIT compartmental microbial taxonomy, particularly

in the upper GIT sections such as the crop, could offer more explanations for understanding the selection of particular groups of microorganisms. Their metabolic and fermentation activities could also establish whether their relationship to pathogens entering the GIT would be antagonistic. It would also be of interest to conduct metagenomic analyses to see if more "acid-tolerant" resident bacteria are selected with exposure to acidic chemical additives that are fed to the birds over their lifetime and if either the presence and/or metabolic activity of these bacteria create additional barriers to pathogen colonization.

CONCLUSIONS

Formic acid has been used as a chemical animal feed additive and silage acidifier for several years. One of its main applications has been as an antimicrobial to limit pathogens in the feed and their subsequent establishment in the avian GIT. Formic acid has been shown to be a relatively effective antimicrobial against *Salmonella* spp. and other pathogens based on *in vitro* model studies. Still, it may be more limited in feed matrices due to the high organic matter and potential buffering capacity of the feed components. Once consumed with feed or through the drinking water, formic acid appears to be antagonistic to *Salmonella* spp. and other pathogens. Still, most of this occurs in the upper compartments of the GIT as the formic acid concentration probably diminishes in the lower GIT, as is known to occur for propionate. The concept of protection of formic acid via encapsulation offers a potential means for the delivery of more acid to the lower GIT. In addition, blends of multiple organic acids have been suggested as being more efficacious at enhancing bird performance rather than the administration of single acids (152). *Campylobacter* in the GIT may differ in its response to formic acid since it can use it as an electron donor, and thus the acid serves as a primary energy source. It has not been established whether increasing GIT formic acid concentration would favor *Campylobacter*, and this still may not occur depending on other GIT organisms that may be capable of using formic acid as a substrate.

More research needs to be conducted on the impact of GIT formic acid on non-pathogenic indigenous GIT microorganisms. Selective antagonism of pathogens without disruption of the members of the GIT microbial community considered beneficial to the host would be preferred. However, this requires more in-depth microbiome sequence analyses of these resident GIT microbial consortia. While some research has been reported on the cecal microbiome in birds fed formic acid, more emphasis needs to be placed on the upper GIT microbial communities. Identification of microorganisms and comparison of similarities among GIT microbial groups in the presence or absence of formic acid may not be the complete narrative. Other analyses, including metabolomics and metagenomics, are also needed to characterize the functional differences among compositionally similar populations. This characterization will be necessary for establishing linkages between the GIT microbial population and bird performance responses to the formic acid amendment. Combining methods to better define GIT function should lead to more effective organic acid supplementation strategies and, ultimately, better predictability for optimal bird health and performance while limiting food safety risks.

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SR wrote the review with assistance from DD and KR. All authors significantly contributed to the work of the current review.

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Impacts of Short-Term Antibiotic Withdrawal and Long-Term Judicious Antibiotic Use on Resistance Gene Abundance and Cecal Microbiota Composition on Commercial Broiler Chicken Farms in Québec

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The ever-increasing problem of antibiotic resistance makes routine use of antibiotics in animal production no longer considered as a reasonable and viable practice. The Chicken Farmers of Canada have developed and are implementing an Antimicrobial Use Reduction Strategy, which has the ultimate goal of eliminating the preventive use of medically important antibiotics in broiler chicken and turkey production. However, very little is known about the real overall impact of an antibiotic use reduction strategy in complex ecosystems, such as the bird intestine or the commercial broiler chicken farm. The main objectives of the present study were to compare the abundance of antibiotic resistance-encoding genes, characterize the intestinal microbiota composition, and evaluate the presence of *Clostridium perfringens*, in six commercial poultry farms adopting short-term antibiotic withdrawal and long-term judicious use strategy. Implementing an antibiotic-free program over a 15-months period did not reduce the abundance of many antibiotic resistance-encoding genes, whereas the judicious use of antibiotics over 6 years was found effective. The short-term antibiotic withdrawal and the long-term judicious use strategy altered the intestinal microbiota composition, with the *Ruminococcaceae* and *Lachnospiraceae* families being negatively impacted. These findings are in agreement with the lower production performance and with the increased *C. perfringens* populations observed for farms phasing out the use of antibiotics. Adopting a conventional rearing program on commercial broiler chicken farms selected for specific antibiotic resistance-encoding genes in many barns. This study highlights the potential impacts of different rearing programs in poultry production and will help guide future policies in order to reduce the use of antibiotics while maintaining production performance.

Keywords: resistance gene, microbiota, judicious antibiotic use, health, commercial broiler chickens, conventional program, drug-free program, antibiotic withdrawal

INTRODUCTION

In animal husbandry, antibiotics are used to prevent and to treat infections, as growth promoting claims for antibiotics are no longer permitted in Canada (1). However, the ever-increasing problem of antibiotic resistance makes the routine use of these medicines in animal production no longer considered as a responsible approach (2). To mitigate the development of antibiotic resistance, the commitment of stakeholders coming from different sectors, such as governmental agencies, the food-producing animal industry, and the medical field involving veterinarians and physicians is essential (3). In order to guide the veterinary use of antibiotics and to preserve the effectiveness of these compounds, the World Health Organization established the List of Critically Important Antimicrobials for Human Medicine in 2005 and is reviewed periodically (4). Comprising three different categories, this list classifies antibiotics as being important, highly important, or critically important for human health (4). Canada has established criteria for the categorization of antibiotics, from category 1 to category 4, according to their decreasing human medical importance (5). In September 2017, Canada launched its pan-Canadian action plan, which aims to harmonize the actions of all stakeholders who have a role to play in addressing the antimicrobial resistance problem (6). The Chicken Farmers of Canada are implementing an Antimicrobial Use Reduction Strategy designed to eliminate the preventive use of medically important antibiotics in broiler chicken and turkey productions (7). The preventive use of category 1 antibiotics was voluntarily banned in May 2014, followed by a prohibition on the use of category 2 antibiotics since the end of 2018. Furthermore, the ban on the preventive use of category 3 antibiotics is to enter into force for Canadian poultry producers at the end of 2020, but this date is currently being reviewed. When monitoring the impacts of these voluntary changes in antimicrobial use at the farm, slaughterhouse, and retail levels, available data from the Canadian Integrated Program for Antimicrobial Resistance Surveillance is extremely useful. Surveillance data revealed that stopping the preventative use of ceftiofur, a third-generation cephalosporin, in Canadian hatcheries was associated with a lower prevalence of *Escherichia coli* and *Salmonella* isolates resistant to ceftriaxone, an antimicrobial compound belonging to the same class of antibiotics (8). In addition, previously in Europe, several countries banned the use of non-essential antibiotics in animal production, such as growth promoters in order to reduce the selection of resistance genes forming the farm resistome. In Denmark, withdrawal of antibiotics as growth promoters has been associated with a decrease in antibiotic resistance in *Enterococcus feacium* chicken broiler isolates (9). Although these are encouraging observations in targeted indicator bacteria, the global impact of an antibiotic use reduction strategy in complex ecosystems, such as the bird intestine, or the commercial broiler chicken farm remains to be better documented.

The implementation of the Chicken Farmers of Canada's Antimicrobial Use Strategy has been associated with various challenges including production losses and disease issues, such as necrotic enteritis caused by *Clostridium perfringens*. Thus, the identification of antibiotic alternative strategies to keep disease challenges under control and to maintain production performances is essential (10). To date, none of the available alternatives has proven to be as effective as antibiotics in maintaining avian gut health on commercial farms and their contribution to the fight against antibiotic resistance is still to be documented. The contribution of antibiotics to the long-term shaping of microbial communities and to the resistome of the intestine of commercial birds and consequently of poultry houses needs to be better described. Understanding of antibiotic involvement would allow a proper assessment of the global impacts of the Chicken Farmers of Canada's Antimicrobial Use Reduction Strategy and to identify valuable replacement options.

A previous study conducted by our group on different commercial broiler chicken farms aimed to compare a conventional rearing program including an antibiotic and anticoccidial-based diet to a drug-free program that was implemented over a 15-months period. In the absence of in-feed antibiotics and anticoccidials, different alternatives were used including essential oil-based products added to the feed, organic and inorganic acids in the drinking water, and a coccidiosis vaccination approach at the hatchery level (10). Rearing broiler chickens using this drug-free program significantly impacted production performance, the frequency of occurrence of necrotic enteritis, and the abundance and richness of the *C. perfringens* populations (2, 10). Now, 6 years after the close of this field study, some of the participating farms are using antibiotics judiciously, whereas some other farms went back to a conventional rearing program after completion of the 15-months study period. Thus, there is now the opportunity to revisit these farms and compare the impacts of varied antibiotic use settings in a commercial context.

The objectives of the present study were to evaluate the abundance of antibiotic resistance-encoding genes, the presence of *C. perfringens*, and the composition of the intestinal microbiota in commercial poultry farms adopting either short-term antibiotic withdrawal (15 months) or long-term judicious antibiotic use strategy (6 years).

MATERIALS AND METHODS

Study Design

The farm selection was based on a previous study conducted by our group (10). Six (defined herein as farms A, B, C, D, E, and F) of the eight farms that took part of a previous 15-months study conducted 6 years ago agreed to participate in the current study.

In July 2012, at the end of the 15-months study, four farms (designated as farms C, D, E, and F) decided to reintroduce a conventional program (using antibiotics) in their drug-free barn (designated as "reintroduced" throughout the text), while the control barn on those farms was kept on a conventional program during both the 15-months study period and thereafter (designated as "continued" throughout the text). Those farms were then considered as having undertaken a short-term antibiotic withdrawal. The two other farms (designated as farms A and B or as "judicious" throughout the text) moved on

from a conventional rearing program and from a drug-free rearing program in their control and test barns, respectively, to a program for responsibly using antibiotics in both rearing facilities, meaning that antibiotics were kept only as a therapeutic option for birds when needed (**Supplementary Figure 1**).

Sample Collection

At the end of the 15-months study conducted between May 2011 and July 2012 (designated as sampling time point one throughout the text), 12 birds were randomly selected from each of the 12 participating barns, for a total of 144 birds. Birds harvested at the end of the rearing cycle were euthanized by cervical dislocation. The cecal content of the birds was sampled directly on the farm, frozen in liquid nitrogen, and transported to the laboratory. Samples were stored at −80°C for further analysis.

The same 12 barns were visited a second time in autumn 2018 (designated as sampling time point two throughout the text) for cecal sampling at the end of the rearing period. Using the same sampling protocol, 12 birds were randomly selected from each barn, for a total of 144 birds.

This protocol was approved by the Comité d'Éthique sur l'Utilisation des Animaux (CÉUA) of the Faculté de Médecine Vétérinaire of the Université de Montréal (project number 19-Rech-1970).

DNA Extraction From Cecal Samples

In a 2-ml screw cap tube containing 500 mg of 0.1-mm silica spheres (MP Biomedical, Solon, OH, USA), 200 mg of cecal content, and 700 µl of lysis buffer [Tris-HCl 500 mM pH 8, EDTA 100 mM pH 8, NaCl 100 mM, SDS 1% (w/v)] were mixed together. A 900-µl volume of lysis buffer was used as a negative control. A mechanical lysis step was performed using a FastPrep-24™ 5G Instrument (MP Biomedical) for three runs of 60 s each, at 6 m/s. Samples were kept on ice during 5 min between each run. A second step involving thermal lysis was carried out on the samples that were heated for 20 min at 95°C and kept for 5 min on ice at the end of the procedure. The supernatant was collected after a centrifugation at 18,000 × *g* for 15 min and a standard phenol/chloroform purification protocol was used to complete the DNA extraction (11). The DNA concentration of each sample was measured using a QFX Fluorometer (Froggabio, Toronto, ON), and the purity of those samples was assessed using a Nanodrop 1000 (Fisher, Ottawa, ON) device. DNA samples were stored at −20°C until analysis.

Detection of Gene Targets

DNA samples were screened for the presence of 12 antibiotic resistance genes for which the selection was either based on the use of antibiotics in commercial broiler chicken flocks in Canada or according to their importance for human medicine. The presence of the genes encoding the *C. perfringens* alpha toxin (*plc*) and enterotoxin (*cpe*) was also investigated in order to evaluate the impact of a short-term antibiotic withdrawal and of a long-term judicious use strategy on the presence of this animal and zoonotic pathogen. A total of 14 genes were investigated using different protocols (**Supplementary Table 1**). All gene targets were PCR amplified in a 25-µl reaction with 2.5 µl of 10× PCR buffer (Biobasic, Markham, ON), 0.2 µM of dNTPs (Biobasic), 1.5 or 2 mM of $MgSO_4$ (Biobasic), 1 or 1.25 U of Taq DNA Polymerase High Purity (Biobasic), template DNA, and different concentrations of specific primers (Invitrogen/Life Technologies, Burlington, ON). A Mastercycler® nexus thermocycler (Eppendorf Canada, Mississauga, ON), was used to carry out amplification reactions using cycling conditions as presented in **Supplementary Table 1**. A volume of 10 µl of each PCR product was subjected to gel electrophoresis using a 0.7–2% agarose gel (agarose concentration was established according to gene size) containing 0.01% SYBR Safe DNA gel strain (Fisher, Ottawa, ON). The PCR product was visualized under UV light using a 100-bp DNA ladder (Track it; Fisher).

Bacterial strains used as positive controls were grown overnight on 5% sheep blood agar plates (Fisher, Ottawa, ON) at 37°C under aerobic conditions for *Enterococcus faecium* [positive for *erm*(B) encoding for a 23S rRNA méthylase (12) and *vat*(D) encoding for a streptogramin acetyltransferase (13)], *E. faecium* [positive for *erm*(B) and *vat*(E) encoding for a streptogramin acetyltransferase (13)], *Salmonella* Heidelberg [positive for *Intl1* encoding for a class 1 integron-integrase (14)], *Enterococcus faecalis #7* [positive for *lnu*(B) encoding for a lincosamide nucleotidyltransferase (15)], and *Escherichia coli* ECL21264 [positive for *sul1* encoding for a dihydropteroate synthase (16)]. Under anaerobic conditions (AnaeroGen sachet, Fisher), *C. perfringens* c1261_A [positive for *bcrABDR* genes encoding for an ABC transporter and an overproduced undecaprenol kinase (17)] and *C. perfringens* AHL 155 (positive for *plc* and *cpe* genes) were grown overnight on 5% sheep blood agar plates (Fisher, Ottawa, ON) at 37°C. For DNA extraction, five colonies were suspended in 50 µl of a 6% Chelex solution (Bio Rad, Saint-Laurent, QC), heated at 56°C for 25 min and at 95°C for 10 min. The DNA-containing supernatant was collected after centrifugation at 18,000 × *g* for 5 min and used in PCR reactions. The positive control used for the *mcr-1* gene [encoding for a phosphoethanolamine transferase (18)] PCR amplification was DNA extracted from a French livestock *E. coli* strain expressing both a phenotype and a genotype of colistin resistance (19). The positive controls used for the PCR detection of *vga*(A) [encoding for ATP-binding proteins in active efflux (12)] and *vgb*(A) [encoding for a hydrolase (12)] was the plasmid pBluescript II SK+ (Biobasic) including the DNA fragment amplified with the primers of the resistance gene target.

Quantification of Resistance Gene Targets

The abundance of selected resistance gene targets was determined by qPCR as previously described (20–22). Gene targets *bcrA*, *bcrB*, *lnu*(B), and *vat*(E) genes were quantified using a Roche LC96 Real Time PCR thermocycler (Roche Canada, Laval, QC) with LightCycler® 96 System Software, version 1.1. The gene targets *erm*(B), *intl1*, and *sul1* were quantified using a Bio-Rad CFX96 real-time PCR instrument with Bio-Rad CFX Manager software, version 3.1. Primers (Invitrogen/Life Technologies), hydrolysis probes (Sigma-Aldrich, Toronto, ON), and cycling conditions are listed in **Supplementary Table 2**. Reactions were performed in 25-µl reaction volumes using the Brilliant II QPCR

Master Mix (Agilent, Toronto, ON) for the TaqMan PCR and the Brilliant II SYBR Green® Low ROX QPCR Master Mix (Agilent) for the SYBR Green PCR (Agilent). Two microliters of DNA template (10 ng of DNA) was added to each reaction, and sterile water was used to reach the final volume. Each reaction, including the negative control, was run in triplicate.

The abundance of each gene in all experimental samples was determined using a standard curve. For the *erm*(B), *intl1*, and *sul1* gene targets, respectively, the DNA fragment amplified with the primers of the gene target was cloned into the pSC-A-amp/kan plasmid using the StrataClone PCR Cloning kit (Agilent) and following the manufacturer's instructions before being used to transform *E. coli* competent cells from the StrataClone SoloPack (Agilent).

For *bcrA*, *bcrB*, *lnu*(B), and *vat*(E) gene targets, each standard curve was constructed using the plasmid pBluescript II SK+ (Biobasic) including the DNA fragment amplified with the primers of the gene target. For purification, the plasmid was linearized with the Not1-HF enzyme (New England Biolabs, Whitby, ON) for 2 h at 37°C and ran on a 1.5% agarose gel with SYBR Safe DNA gel stain (Fisher). The linearized plasmid was recovered using the QIAquick gel extraction kit (Quiagen, Montréal, QC). The plasmid DNA concentration was measured using a QFX Fluorometer (Froggabio), and the number of plasmid copies was calculated. The plasmid was diluted using a 10-fold serial dilution approach, and these dilutions were used for the standard curve construction.

16S rRNA Gene Amplicon Sequencing

Sequencing of the V4 region of the 16S rRNA gene was performed using the Illumina MiSeq platform. DNA was extracted from the cecal contents of all 288 birds. The 144 samples from the sampling time point one were multiplexed with controls for sequencing in one lane. The 144 samples from the sampling time point two and controls were sequenced in a separate lane. Libraries were prepared using a Mastercycler® nexus (Eppendorf Canada) with the forward primer 5′-ACACTGA CGACATGGTTCTACAGTGCCAGCMGCCGCGGTAA-3′ and the reverse primer 5′-TACGGTAGCAGAGACTTGGTCTGG ACTACHVGGGTWTCTAAT-3′ (Invitrogen/Life Technologies) (23). Following the manufacturer's instructions with some modifications, the amplification of the 292-bp segment was performed using 6 µl of 5× SuperFi™ Buffer (Fisher, Ottawa, Ontario), 6 µl of 5× SuperFi™ GC Enhancer (Fisher), 0.6 µl of 10 mM dNTP mix (Fisher), 0.9 µl of 20 µM primers (Invitrogen/Life Technologies), 0.6 µl of 20 mg/ml Pierce™ bovine serum albumin (Fisher), 0.3 µl of 2 U/µl Platinum SuperFi DNA Polymerase (Fisher), and 1.5 µl of DNA (15 ng) for a total reaction volume of 30 µl. Total volume was completed with sterile water. Sterile water was used as negative control, and the ZymoBIOMICS Microbial Community DNA Standard (Cedarlane, Burlington, ON) was used as positive control. Cycling conditions were as follows: a hot start step of 5 min at 95°C, followed with 23 cycles of 30 s at 95°C, 30 s at 55°C, and 3 min at 72°C, and a final elongation step of 10 min at 72°C. A volume of 10 µl of the PCR product was submitted to electrophoresis using 1.5% agarose gel containing 0.01% SYBR Safe DNA gel strain (Fisher). The PCR product was visualized under UV light using a 1-kb DNA ladder (Track it; Fisher).

Two libraries were prepared and sequenced separately. The first library was made up of 144 samples, six negative controls (one for each farm to validate the quality of the DNA extraction procedure), one negative control (sterile water), and one positive control (ZymoBIOMICS Microbial Community DNA Standard). The second library consisted of 144 samples, six negative controls (one for each farm to validate the quality of the DNA extraction procedure), two negative controls (sterile water), and one positive control (ZymoBIOMICS Microbial Community DNA Standard). Libraries were sent to the Génome Québec Innovation Centre (Montreal, QC) for DNA sequencing using an Illumina MiSeq PE250 platform (Illumina, San Diego, CA, USA).

As previously described by Larivière-Gauthier et al., with some modifications, the obtained sequences were cleaned using MOTHUR v. 1.14.3 (24). Reads that were too long or ambiguous were eliminated, and the Silva database v.132 was used to align unique sequences. Chimeras were discarded using the VSEARCH tool (25), and reads were clustered into operational taxonomic units, with a 3% dissimilarity (OTUs). Mothur-formatted Ribosomal database project trainset version 16 was used to classify the obtained OTUs. Further data analysis was done using RStudio (version 1.2.5033, 2019) with the following packages: phyloseq, vegan, dplyr, scales, grid, reshape2, igraph, ape, gplots, lme4, phangorn, plotly, tidyr, data.table, Maaslin2, ggplot2, stringr, and devtools.

In order to avoid the presence of OTUs found only in a single flock, sequences that were present in more than 12 samples for each sampling time point analyzed were retained for biomarker analysis. To characterize the microbial communities associated with the different rearing programs and sampling time points, MaAsLin2 (Multivariate Association with Linear Models) was used in RStudio (26).

Data Analysis and Statistics

For qPCR values, the detection limit for quantification was set at one copy per reaction. For values below this limit, a 0.9 gene copy value per reaction was chosen to calculate the average copy number of each sample ran in triplicate. This average was converted into a number of gene copies/ng of DNA, and resulting values were expressed on both a weight basis (raw values) and a ratio referenced to the total bacterial content of the sample according to the 16S rRNA gene copy number. GraphPad Prism (v8.0.2, GraphPad Software Inc., La Jolla, CA) was used to prepare the figures.

A first analysis using a linear mixed model measured changes in the mean of the log-transformed qPCR copy number of the 16S rRNA gene, considering the sampling time point, the rearing program, and the interaction between both as fixed effects and the farm as a random variable (27). Farms A and B were not included in this analysis as they were not using antibiotics at sampling time point two.

A second statistical analysis using a linear mixed model and considering the farm as a random effect analyzed the fixed effect of the rearing programs on the mean of the log-transformed qPCR raw values and ratios at sampling time point one for each

gene target. The same model was used for sampling time point two and also excluded farms A and B that did not use antibiotics.

A third analysis using a linear mixed model measured changes in the mean of the log-transformed qPCR raw values and ratios of each gene target, considering the sampling time point and program variables as fixed effects, and the farm as a random variable. Again, farms A and B were not included in this analysis. *A priori* contrasts were performed to compare mean values at each sampling time point and to compare means at sampling points one and two among programs. For these comparisons, the Benjamini–Hochberg sequential procedure was used to adjust the alpha level downward. The familywise error rate was set at 5% (28).

A fourth analysis considered each farm separately. A linear model was used to analyze changes in the mean of the log-transformed qPCR raw values and ratios for each gene target as a function of sampling points and rearing programs, followed by the use of *a priori* contrasts, as described above.

For the 16S rRNA amplicon metagenomic sequencing analyses, the alpha and the beta diversity indices were calculated using Rstudio. For alpha diversity analyses, the richness and the evenness were measured using diversity indices of OTU observed, Shannon, and inverse Simpson. To analyze the fixed effect of the rearing program on the mean of alpha diversity indices for sampling time points one and two, a linear mixed model with the farm as a random effect was used. To measure the effect of the sampling time point on the mean of alpha diversity indices, a linear mixed model was used considering the rearing program, the sampling time point, and the interaction between both as fixed effects and the farm as a random variable. Again, farms A and B were excluded from this analysis due to their different status regarding antimicrobial use. For farms A and B, a linear mixed model was also used considering the farm as a random variable and the rearing program as a fixed effect. For both analyses, *a priori* contrasts, as described above, were used. Statistical analyses were performed using SAS v.9.4 (Cary, N.C.). For the beta diversity analysis, distances between samples were displayed by non-metric multidimensional scaling (NMDS) graphs and calculated using the Jaccard and Bray–Curtis indices (24). Statistical differences between groups were calculated using the ADONIS test, with a significance level of 0.05.

RESULTS

Detection of Gene Targets

DNA samples were screened individually or as pooled samples for the presence of 14 genes. Based on the positive detection of *bcrA*, *bcrB*, *erm*(B), *intl1*, *lnu*(B), *sul1*, and *vat*(E), those gene targets were then quantified by qPCR (**Table 1**).

No quantitative approach was performed on *bcrR*, *vga*(A), *vgb*(A), *vat*(D), *mcr-1*, *cpe*, and *plc* genes. Pools were all found positive for the presence of *bcrA*, *bcrB*, and *bcrR* genes. Only *bcrA* and *bcrB* were submitted to the quantitative PCR approach as the presence of the *bcrR* regulator gene is not essential in conferencing a bacitracin resistance phenotype in bacteria carrying the bacitracin resistance operon (29). Attempts to evaluate the presence of *vga*(A) and *vgb*(A) genes were also made, but non-specific amplification issues have prevented the use of a quantitative approach to describe the presence of these genes. From a total of 72 DNA pools, the *vat*(E) gene was kept for the following quantitative analyses as 72% of the pooled samples were found positive for the presence of this gene, while only 4% of the samples screened were positive for *vat*(D). All the pools screened were negative for the presence of *mcr-1* (**Table 1**).

TABLE 1 | Sample treatment and PCR detection results.

Gene	Sample treatment	Detection result (%)
bcrA	From 288 samples pooled in groups of 6 or 4 samples	100
bcrB		
bcrR	From 288 samples pooled in groups of 4 samples	100
vat(D)		4
vat(E)		72
mcr-1		0
lnu(B)	From 288 individual samples	34
cpe		0
plc		17
erm(B)	From 246 individual samples	100
sul1	From 48 individual samples	69
intl1		92
vga(A)	From 12 individual samples	Non-specific amplification
vgb(A)		

Clostridium perfringens Detection Results

All the 288 individual samples screened were negative for the presence of the *cpe* gene. For the detection of the *C. perfringens* alpha toxin-encoding gene (*plc*), a total of 48 samples from the 288 tested individually were identified as positive (**Table 1**).

Quantification of Resistance Gene Targets

A quantitative PCR approach was used to establish the relative abundance of *bcrA*, *bcrB*, *erm*(B), *intl1*, *lnu*(B), *sul1*, *vat*(E), and 16S rRNA gene targets. After a short-term antibiotic withdrawal of 15 months, the relative abundance of *sul1* or *intl1* or both genes, decreased significantly in the drug-free flocks of four farms out of the six sampled (**Figure 1**).

For some of the flocks sampled from farms A and B at sampling time point two, a long-term judicious use strategy (6 years) was associated with a decrease in the relative abundance and the absolute copy number of some antibiotic resistance-encoding genes, namely, *bcrA*, *bcrB*, *erm*(B), *lnu*(B), and *vat*(E) (**Figures 2**, **3**). In contrast, routine use of antibiotics over a 6-years period on farms C, D, E, and F was associated, for some of the sampled flocks, with an increase in the relative abundance and in the absolute copy number of many of the resistance gene targets, namely, *bcrA*, *bcrB*, *erm*(B), *intl1*, *lnu*(B), *sul1*, and *vat*(E) (**Figures 2**, **3**).

Regarding the variability of the 16S rRNA gene target abundance according to rearing programs and sampling time points, a linear mixed model showed no differences. A second analysis grouping the six conventional flocks and the six drug-free flocks at sampling time point one showed an increase in the

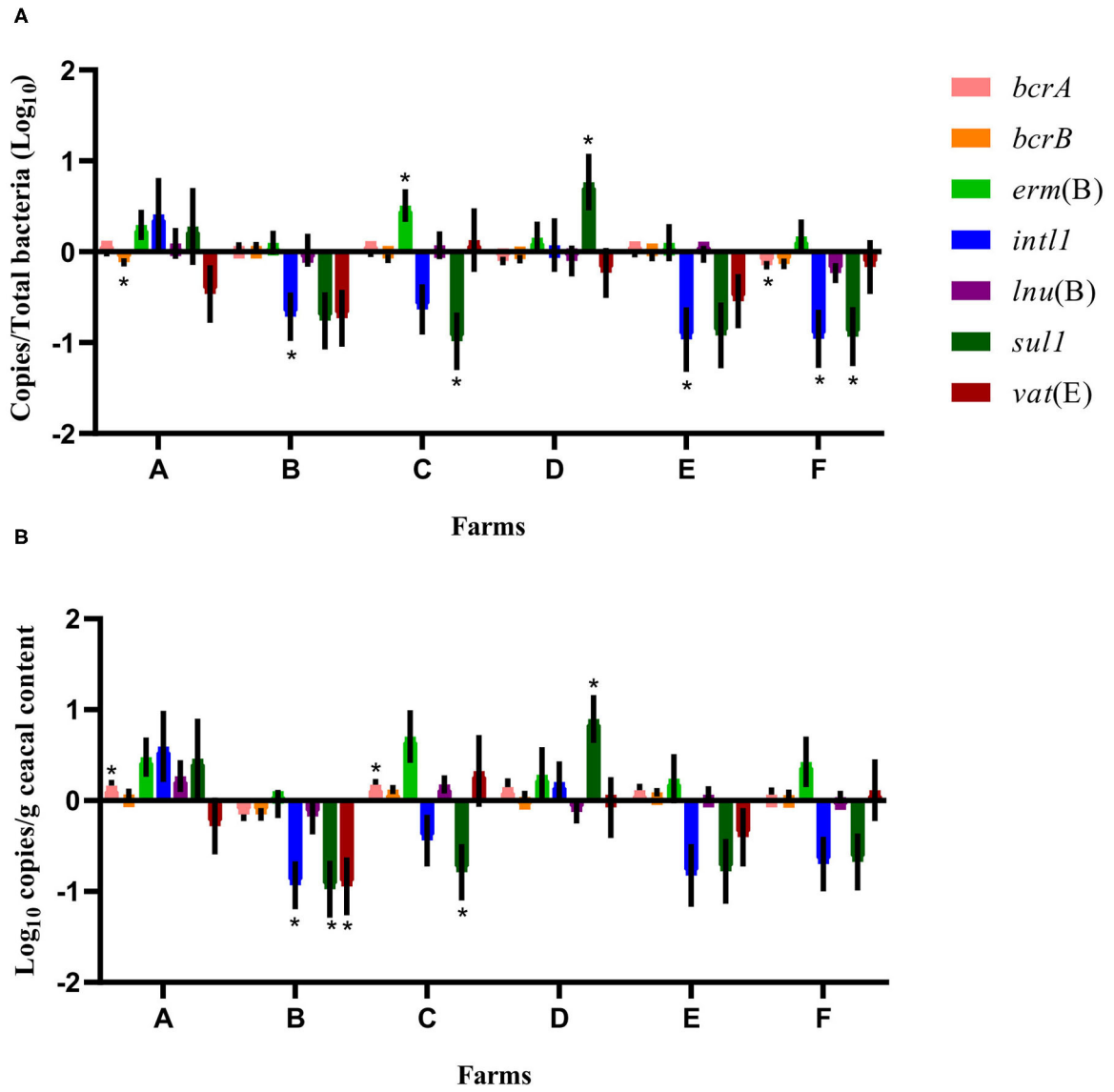

FIGURE 1 | Difference of resistance gene targets between drug-free and conventional flocks for each farm at sampling time point one. Negative results indicate a decrease in gene target, and positive results indicate an increase in gene target in drug-free flocks. Data are presented as the mean (SEM) of 12 replicates. For the quantitative approach, each sample was run in triplicate ($n = 3$). **(A)** Values are expressed on a ratio referenced to the total bacterial content of samples (16S rRNA). **(B)** Values are expressed on a weight basis (raw values). *Significant values are lower than the alpha level adjusted with the Benjamini–Hochberg method.

relative abundance ($p = 0.0074$) and the absolute copy number ($p = 0.0232$) of *erm*(B) in drug-free flocks. A third analysis investigating the impacts of a short-term antibiotic withdrawal and a long-term conventional rearing program (excluding farms A and B) on the abundance of antibiotic resistance-encoding genes showed that only the abundance of *bcrB* expressed as raw values increased ($p = 0.0082$) when using a long-term conventional program.

When considering the farm as the unit of analysis, the mean abundance of each gene target was compared between the conventional and drug-free flocks at sampling time point one for each participating farm. As presented in **Figure 1**, the relative abundance expressed as a ratio of the antibiotic resistance gene target to the 16S rRNA content of the samples showed, for drug-free flocks, a decrease in *bcrA*, *intl1*, and *sul1* on farm F, of *bcrB* on farm A, of *intl1* on farms B and E, and of *sul1* on farm C. In contrast, *erm*(B) and *sul1* increased on farms C and D, respectively. In the drug-free flocks sampled, raw values revealed a decrease in *intl1*, *sul1*, and *vat*(E) for farm B and a decrease for *sul1* only on farm C. In contrast, *bcrA* increased for farms A and C, whereas *sul1* increased for farm D.

Considering the farm as the unit of analysis and the barn that was on a drug-free program during the 15-months study period as the comparison reference unit, the mean relative

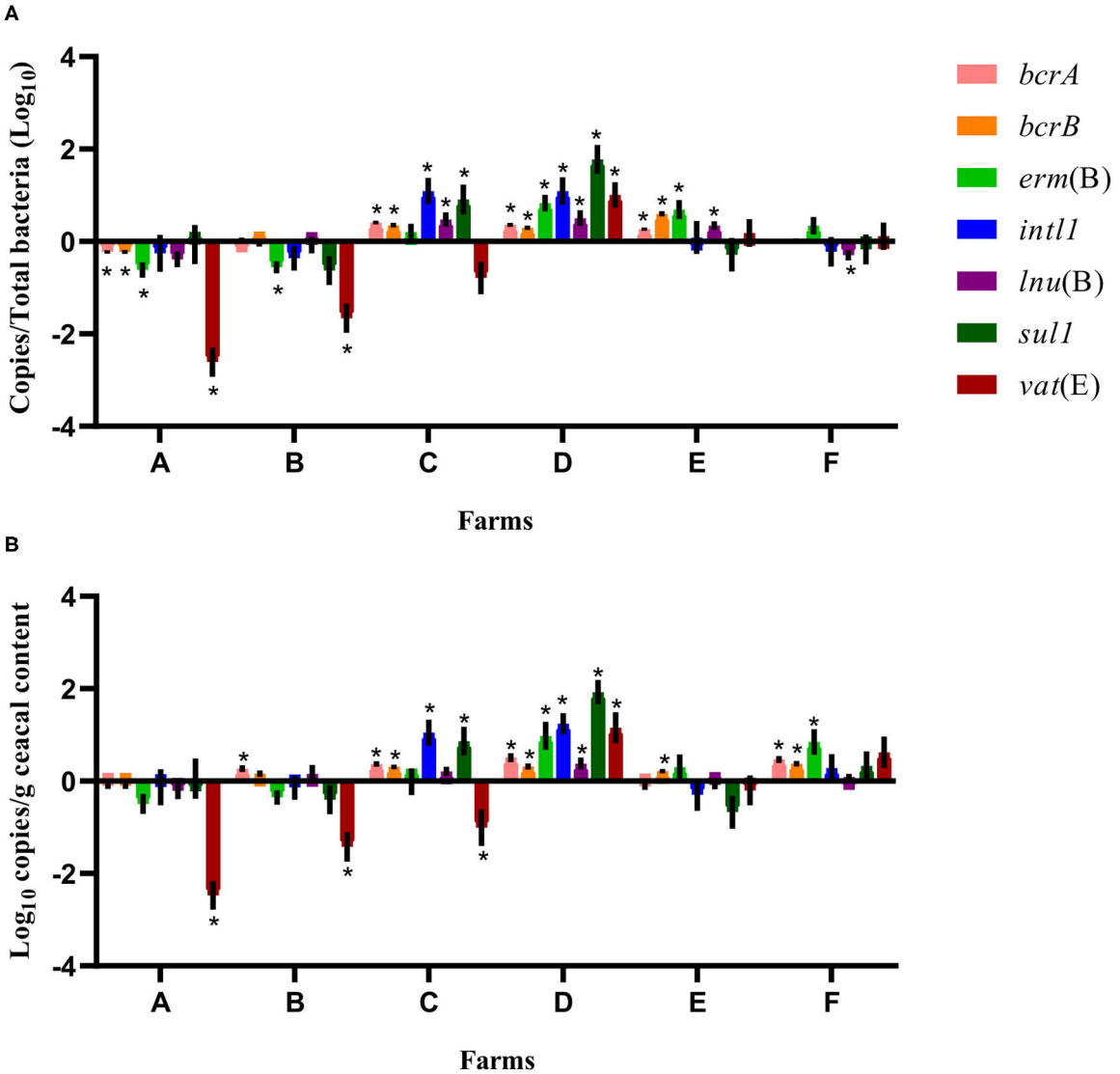

FIGURE 2 | Difference of resistance gene targets between flocks sampled from conventional barns at sampling time point one and the flock from the same barn at sampling time point two. At sampling time point one, conventional barns from farms C to F remained on a conventional rearing program after the 15-months study period, whereas barns from farms A and B moved to a program for judiciously using antibiotics. Negative results indicate a decrease in gene target, and positive results indicate an increase in gene target in the sampled flock at sampling time point two. Data are presented as the mean (SEM) of 12 replicates. For the quantitative approach, each sample was run in triplicate ($n = 3$). **(A)** Values are expressed on a ratio referenced to the total bacterial content of samples (16S rRNA). **(B)** Values are expressed on a weight basis (raw values). *Significant values are lower than the alpha level adjusted with the Benjamini–Hochberg method.

abundance of each gene target was compared at sampling time point two between flocks of the same participating farm that had adopted either a conventional rearing program or a program for judiciously using antibiotics after the completion of the 15-months study period (**Figure 4**). For farms A and B, ratios and raw values obtained for the antibiotic resistance gene targets measured showed a decrease in *vat*(E) for farm A. For farms C, D, E, and F, ratios and raw values showed a decrease in *bcrA*, *erm*(B), and *lnu*(B) genes and for *bcrA* and *lnu*(B), respectively, on farm C. As opposed, ratios and raw values showed an increase for *sul1*, and for *sul1* and *intl1*, respectively, on farm D.

Considering the farm as the unit of analysis, the mean relative abundance of each gene target was compared between sampling time points one and two, considering two categories of barns: barns using a conventional rearing program at both sampling time points (farms C, D, E, and F) and barns moving from a conventional program at sampling time point one to a program for judiciously using antibiotics after the 15-months study period

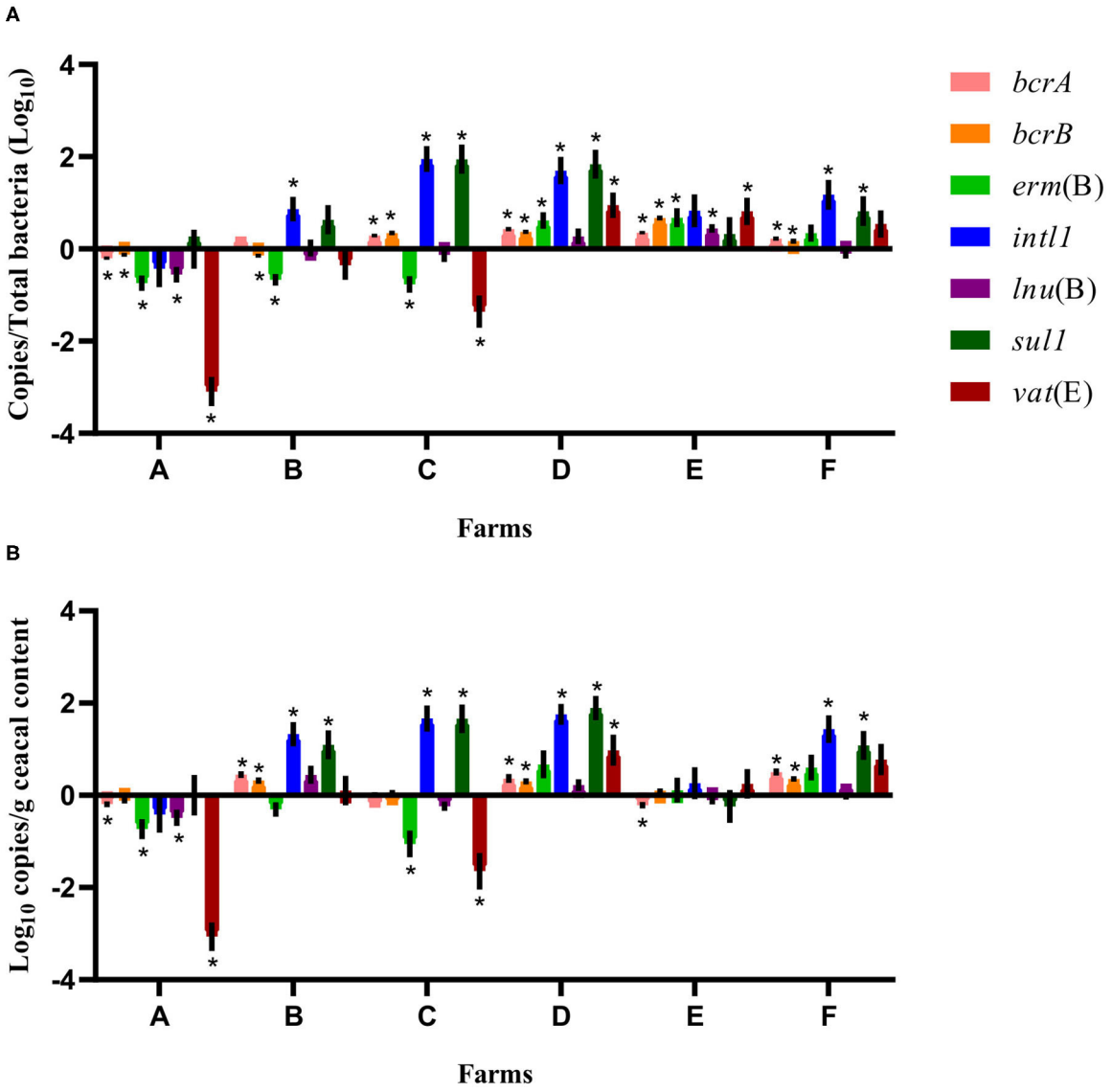

FIGURE 3 | Difference of resistance gene targets between flocks sampled from drug-free barns at sampling time point one and the flock sampled from the same barn at sampling time point two. Drug-free barns from farms C to F went back to a conventional rearing protocol after the 15-months study period, whereas drug-free barns from farms A and B moved to a program for judiciously using antibiotics. Negative results indicate a decrease in gene target, and positive results indicate an increase in gene target in the sampled flock at sampling time point two. Data are presented as the mean (SEM) of 12 replicates. For the quantitative approach, each sample was run in triplicate ($n = 3$). **(A)** Values are expressed on a ratio referenced to total bacterial content of samples (16S rRNA). **(B)** Values are expressed on a weight basis (raw values). *Significant values are lower than the alpha level adjusted with the Benjamini–Hochberg method.

(farms A and B). In **Figure 2**, for farms A and B, ratios showed a decrease for *erm*(B) and *vat*(E) on both farms and of *bcrA* and *bcrB* on farm A. For farms C, D, E, and F, the relative abundance increased for *bcrA*, *bcrB*, and *lnu*(B) on farms C, D, and E. The *intl1* and *sul1* genes increased on farms C and D, whereas an increase in *erm*(B) and *vat*(E) was noted for farms D and E, and farm D, respectively. As opposed, *lnu*(B) decreased on farm F. For farms A and B, raw values for *vat*(E) showed a decrease on both farms, whereas raw values of *bcrA* gene increased on farm B. Raw values also showed an increase for *bcrA* on farms C, D, and F, and for *bcrB* on farms C, D, E, and F. Raw values for *intl1* and *sul1*, and for *erm*(B) all showed an increase for farms C and D, and for farms D and F, respectively. An increase in *lnu*(B) and *vat*(E) genes was only observed for farm D. In contrast, raw values showed a decrease for *vat*(E) in farm C.

Considering the farm as the unit of analysis, the mean relative abundance of each gene target was compared between sampling time points one and two, considering two categories of barns: drug-free barns at sampling time point one going back to a conventional rearing program (farms C, D, E, and F) and drug-free barns at sampling time point one moving to a program

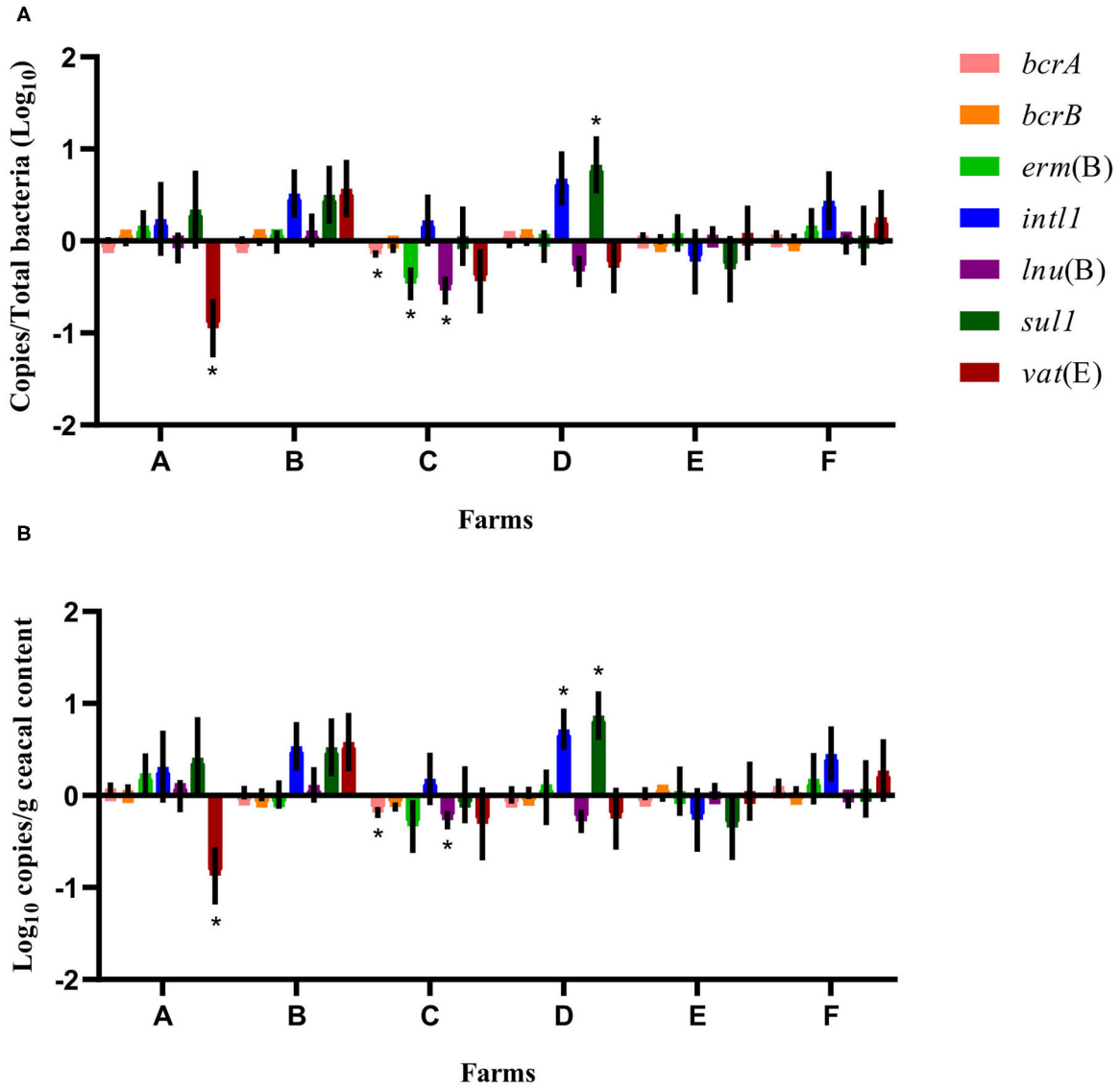

FIGURE 4 | Difference of resistance gene targets at sampling time point two between flocks of the same participating farm that adopted either a conventional rearing program or a program for judiciously using antibiotics after the completion of the 15-months study period, considering the barn that was on a drug-free program during the 15-months study period as the comparison reference unit. Negative results indicate a decrease in gene target, and positive results indicate an increase in gene target in the flock sampled at sampling time point two used as a reference unit. Data are presented as the mean (SEM) of 12 replicates. For the quantitative approach, each sample was run in triplicate ($n = 3$). **(A)** Values are expressed on a ratio referenced to total bacterial content of samples (16S rRNA). **(B)** Values are expressed on a weight basis (raw values). *Significant values are lower than the alpha level adjusted with the Benjamini–Hochberg method.

for responsibly using antibiotics (farms A and B) after the 15-months study period. As shown in **Figure 3**, for farms A and B, the relative abundance expressed as a ratio showed a decrease for *bcrB* and *erm*(B) on both farms and for *bcrA*, *lnu*(B), and *vat*(E) on farm A. In contrast, *intl1* increased on farm B. For farms C, D, E, and F, the relative abundance showed an increase for *bcrA* and *bcrB* on all four farms. Ratio values for *erm*(B) and *vat*(E) increased for farms D and E, for *intl1* and *sul1* on farms C, D, and F and for *lnu*(B) on farm E. As opposed, *erm*(B) and *vat*(E) decreased for farm C. Raw values of the antibiotic resistance gene target presented a decrease for *bcrA*, *erm*(B), *lnu*(B), and *vat*(E) on farm A, whereas an increase for *bcrA*, *bcrB*, *intl1*, and *sul1* was shown for farm B. For farms C, D, E, and F, raw values showed an increase for *bcrA*, *bcrB* on farms D and F, for *intl* and *sul1* on farms C, D, and F, and of *vat*(E) on farm D. In contrast, raw values presented a decrease for *bcrA* on farm E, and for *erm*(B) and *vat*(E) on farm C.

16S rRNA Gene Amplicon Metagenomic Sequencing

The two sequenced libraries, one for each sampling time point, were both analyzed at the same time on Mothur. Positive controls

TABLE 2 | (A) Comparison by column of mean (SEM) alpha diversity indices between conventional and drug-free flocks after the 15-months study (sampling time point one) using a linear mixed model.

	Observed	Shannon	InvSimpson
(A) SAMPLING TIME POINT ONE			
Conventional	409.45 (16.99)	4.44 (0.11)	38.87 (4.95)
Drug-free	421.12 (16.99)	4.36 (0.11)	32.98 (4.95)
p-value	0.13	0.11	0.03
(B) SAMPLING TIME POINT TWO			
Judicious	494.94 (28.88)	4,19 (0.07)	24,14 (2.77)
Reintroduced	524.08 (21.11)	4,26 (0.05)	24,85 (2.20)
Continued	573.10 (21.11)	4,56 (0.05)	38.67 (2.20)
***p*-values**			
*Judicious vs. reintroduced	0.42	0.40	0.84
*Judicious vs. continued	0.03[a]	<0.0001	<0.0001
*Reintroduced vs. continued	<0.0001	<0.0001	<0.0001

(B) Comparison by column of mean (SEM) alpha diversity indices, 6 years after the 15-months study (sampling time point two), between flocks from barns that adopted a long-term judicious use strategy (judicious), flocks that continued the conventional rearing program (continued), and flocks that reintroduced antibiotics after the 15-months study (reintroduced) using a linear mixed model.

[a]Not statistically significant after the alpha level was adjusted downward.

**The p-values are for the indicated pairwise comparisons.*

TABLE 3 | (A) Comparison by column of mean (SEM) alpha diversity indices, for farms C, D, E, and F, between barns sampled after the 15-months study (sampling time point one) and 6 years later (sampling time point two) using a linear mixed model.

	Observed	Shannon	InvSimpson
All sampling time points			
(A) FARMS C, D, E, AND F			
Sampling time point one			
Conventional	419.17 (17.56)	4.49 (0.09)	40.36 (4.40)
Drug-free	415.71 (17.56)	4.36 (0.09)	31.40 (4.40)
Sampling time point two			
Continued	573.10 (17.46)	4.56 (0.09)	38.67 (4.38)
Reintroduced	524.08 (17.46)	4,26 (0.09)	24,85 (4.38)
***p*-value**			
*Conventional vs. continued	<0.0001	0.53	0.72
*Drug-free vs. reintroduced	<0.0001	0.32	0.16
(B) FARMS A AND B			
Sampling time point one			
Conventional	393.05 (13.71)	4.35 (0.16)	35.89 (8.48)
Drug-free	438.05 (13.71)	4.36 (0.16)	36.92 (8.48)
Sampling time point two			
Judicious[a]	492.58 (13.13)	4.22 (0.16)	25.93 (8.42)
Judicious[b]	497.29 (13.13)	4.17 (0.16)	22.34 (8.42)
***p*-value**			
*Conventional vs. judicious[a]	<0.0001	0.21	0.04
*Drug-free vs. judicious[b]	0.002	0.05	0.003

(B) Comparison by column of mean (SEM) alpha diversity indices, for farms A and B, between barns at sampling time points one and two, using a linear mixed model.

[a]Was on a conventional rearing program at sampling time point one.

[b]Was on a drug-free program at sampling time point one.

**The p-values are for the indicated pairwise comparisons.*

corresponded to the theorical composition of the ZymoBIOMICS Microbial Community DNA Standard. A total of 192, 168, and 190 sequences were obtained for the three negative controls made from sterile water. The average of the sequences obtained for the 12 negative controls from DNA extraction was 1,893 sequences and the highest and lowest numbers of sequences obtained were 11,798 and 11. Positive and negative controls were excluded for the rest of the data analysis. Among the 288 samples left, an average of 34,661 sequences were obtained per sample and a total of 37,325 OTUs were detected. The highest and lowest numbers of sequence obtained in a sample were 64,131 and 10,107. Considering the distribution of sequences, 10 samples were excluded from the analysis due to a low number of sequences (below 15,000).

Comparing the conventional and the drug-free flocks sampled at sampling time point one (**Table 2**), the OTU observed and the Shannon indices showed no significant difference. In contrast, according to the inverse Simpson index, conventional flocks showed a higher alpha diversity when compared to drug-free flocks.

For sampling time point two (**Table 2**), the alpha diversity indices were compared between flocks sampled from barns using antibiotics judiciously, and barns that continued and reintroduced the antibiotics after the 15-months study. All indices showed that the conventional flocks that kept using a conventional rearing protocol after the 15-months study period had a higher alpha diversity than flocks that reintroduced antibiotics after a short-term antibiotic withdrawal. In addition, considering the Shannon and inverse Simpson indices, conventional flocks still using a conventional rearing protocol after the 15-months study period had a higher alpha diversity than flocks from farms that adopted a long-term program for judiciously using antibiotics. No differences were noted between flocks that reintroduced antibiotics after a short-term antibiotic withdrawal and flocks from farms A and B that moved to a long-term program for responsibly using antibiotics.

The alpha diversity indices were compared between the two sampling time points (**Table 3**). For all farms, results showed that the richness at sampling time point two, according to the OTU observed index, was greater than the sample diversity observed at sampling time point one. In contrast, the inverse Simpson index showed that the alpha diversity was greater at sampling time point one than at sampling time point two for farms A and B (**Table 3**).

For beta-diversity, sampled flocks were compared according to different rearing programs and sampling time points and visualized with an NMDS. Using the Jaccard (**Figure 5**) and Bray–Curtis (**Supplementary Figure 2**) indices, the ADONIS test was performed. Comparing the conventional and drug-free flocks at sampling time point one, the distance matrix showed the presence of two distinct groups. For sampling time point two, the NMDS showed a distinct structure for flocks sampled from barns that adopted a long-term strategy for judiciously using antibiotics, flocks that continued the conventional rearing

program, and flocks that reintroduced antibiotics after a short-term antibiotic withdrawal. In addition, the beta-diversity was measured between all samples from the two sampling time points. Regardless of the rearing program, samples collected from sampling time point one showed a pattern of aggregation, while samples collected at sampling time point two presented a scattered profile. Finally, for each distance matrix, using a different scale, it was possible to distinguish each sampled flock from a sampled farm and each farm from one another.

In order to identify associations with biomarkers, MaAsLin2 was used according to the rearing program at sampling time point one (conventional and drug-free barns) and two (continued, reintroduced, judicious barns). For sampling time point one, 92 OTUs were identified by MaAsLin2, from which 52 OTUs were positively associated with the drug-free program (**Supplementary Table 3**). At the genus level, nine taxa were significantly enriched with the drug-free program, whereas 10 taxa were significantly reduced with the drug-free program (**Table 4**). For sampling time point two, 258 OTUs were positively or negatively associated with the rearing programs (**Supplementary Table 4**). At the genus level, three taxa were significantly increased, whereas 11 taxa were significantly reduced with the judicious antibiotic use (**Table 5**). According to flocks from barns that reintroduced antibiotics after the 15-months study, *Sporobacter*, *Ruminococcus* 2, and *Odoribacter* were found to be positively associated, whereas *Lachnospiraceae* unclassified, *Romboutsia* and *Coriobacteriaceae* unclassified were negatively associated (**Supplementary Table 5**).

TABLE 4 | Bacterial members associated with the drug-free program after the 15-months study using MaAsLin2 at the genus level.

Drug-free program	Taxa	Coefficient	SE
Positively associated	*Holdemania*	0.0003	0.0001
	Anaerofilum	0.0004	0.0001
	Ruminococcus 2	0.0007	0.0002
	Proteobacteria unclassified	0.0009	0.0002
	Enterococcus	0.0067	0.0022
	Parasutterella	0.0111	0.0029
	Akkermansia	0.0178	0.0053
	Odoribacter	0.0611	0.0158
	Bacteroides	0.0669	0.0339
Negatively associated	*Bacteroidales* unclassified	−0.0328	0.0060
	Lachnospiraceae unclassified	−0.0243	0.0061
	Clostridiales unclassified	−0.0229	0.0072
	Firmicutes unclassified	−0.0186	0.0102
	Subdoligranulum	−0.0183	0.0086
	Clostridium IV	−0.0073	0.0040
	Anaeroplasma	−0.0038	0.0012
	Intestinimonas	−0.0012	0.0006
	Coriobacteriaceae unclassified	−0.0007	0.0001
	Anaerotruncus	−0.0002	0.0001

The positively associated genera are significantly more abundant in the drug-free than conventional program. The negatively associated genera are significantly less abundant in these drug-free flocks. Taxonomic assignment at genus level was not possible for unclassified members.

DISCUSSION

This study, conducted on six commercial broiler chicken farms in Québec, highlights the effects of a short-term antibiotic withdrawal and a long-term judicious use strategy, as well as the conventional antibiotic use, on the dynamics of antibiotic resistance genes and on the cecal bacterial community of broilers.

This study illustrated that for commercial broiler chicken farms, moving to a drug-free program over a 15-months period did not significantly reduce the relative abundance and the absolute copy number of many antibiotic resistance-encoding genes found in bird intestinal contents. Notwithstanding the decrease of *intl1* and *sul1* observed in some drug-free flocks that may be due to a higher fitness cost associated with the carriage of these genes (30). This decrease could also be attributed to a decrease in the selective pressure considering that the use of quaternary ammonium compounds and of sulfonamides may have influenced the persistence of class 1 integrons, which can carry both *sul1* and *qac* resistance genes (31). For instance, a Swedish work studied the impacts of the voluntary restriction on the use of trimethoprim-containing drugs over a 2-years period in Kronoberg County. The results showed a marginal effect on the trimethoprim resistance observed for *E. coli* strains from human urinary tract infections (32). The relative ineffectiveness of such an intervention to significantly impact the antibiotic resistance problem could be explained by the co-selection of antibiotic resistance-encoding genes through the use of other antibiotics or to the low fitness cost associated with the carriage of genes encoding trimethoprim resistance in bacteria (32). In environments, such as broiler chicken farms where the intestinal microbiota corresponds to a signature of the environmental bacterial communities, the fitness cost is considered as one of the most important factors guiding the reduction in the frequency of antibiotic resistance bacteria (30, 33). A decrease in the global antibiotic resistance problem at the community level is then predicted to be measurable years after antibiotic restriction (33). However, the acquisition of compensatory mutations improving fitness for bacteria can jeopardize the reversibility of antimicrobial resistance (30). Despite the lack of evidence on the rate at which resistant bacteria increase or decrease, according to the results of the current study, it could be hypothesized that stopping antibiotics at the farm level over a 15-months period is too short to observe a significant decrease in the abundance of antibiotic resistance genes in the complex ecosystems that are poultry barns for the evaluated genes. The low fitness cost of carrying resistance determinants, the occurrence of compensatory mutations in these bacterial communities, or the use of compounds co-selecting for some resistance determinants probably acted as main drivers. Also, the fact that participating farms of the current study followed the guidelines of the on-farm food safety program of the Chicken Farmers of Canada requiring that farmers wash and disinfect the barn only once a year might have influenced the dynamics of the measured resistance genes on those farms (34).

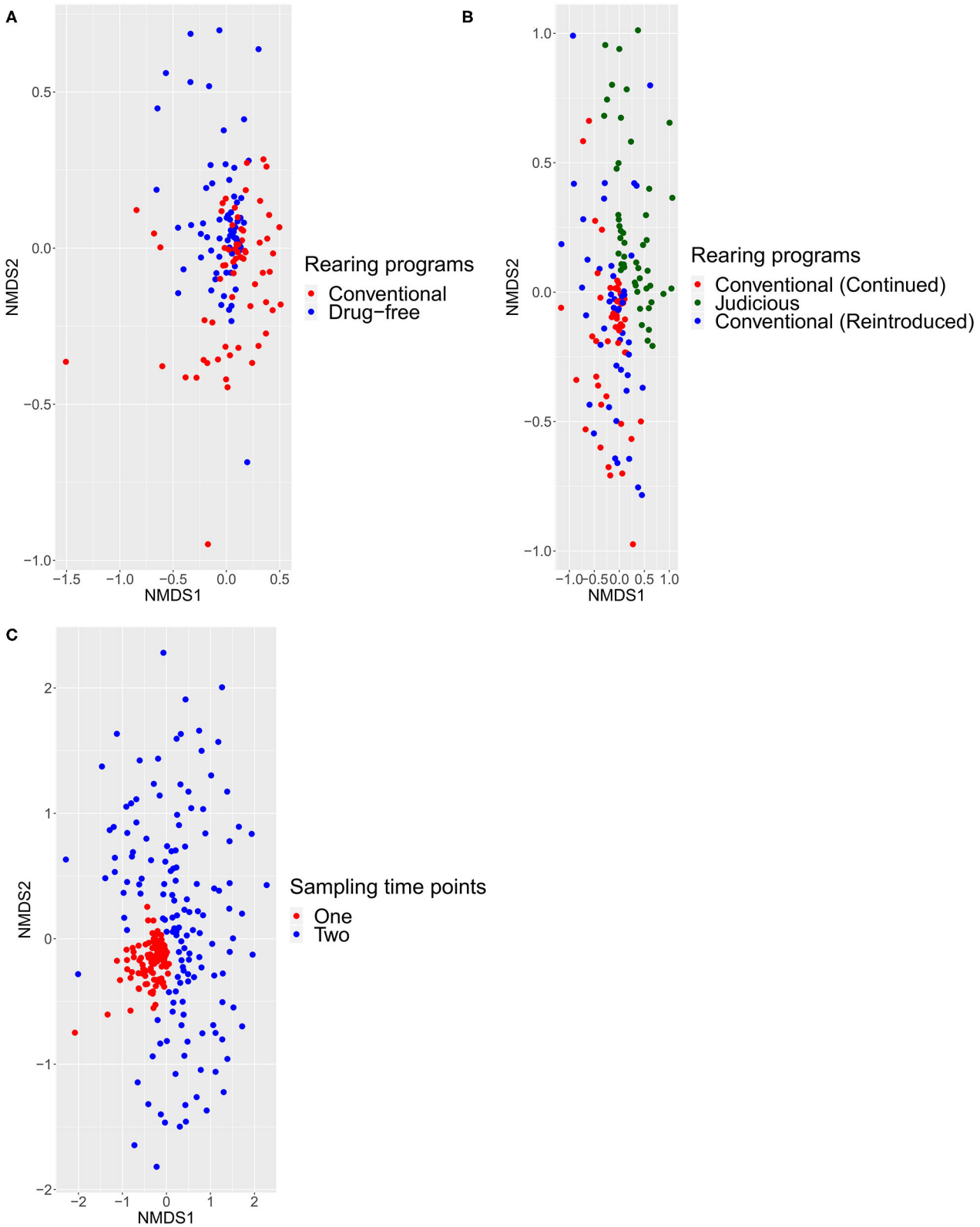

FIGURE 5 | Beta diversity calculated with the Jaccard index using ADONIS test with a significance level of 0.05. All comparisons were statistically significant ($p <$ 0.0001). Each point represented one bird sampled. **(A)** Differences between conventional and drug-free programs at sampling time point one. **(B)** Difference between flocks, at sampling time point two, from barns that adopted a long-term strategy for judiciously using antibiotics, barns that continued the conventional rearing program and barns that reintroduced antibiotics after a short-term antibiotic withdrawal. **(C)** Differences between sampling time points one and two.

TABLE 5 | Bacterial members associated with the judicious use of antibiotics, for farms A and B, 6 years after the 15-months study using MaAsLin2 at the genus level.

Judicious use of antibiotics	Taxa	Coefficient	SE
Positively associated	*Sporobacter*	0.0001	<0.0001
	Butyricicoccus	0.0124	0.0034
	Butyricimonas	0.0162	0.0010
Negatively associated	*Ruminococcaceae* unclassified	−0.0365	0.0119
	Blautia	−0.0086	0.0035
	Clostridium IV	−0.0068	0.0020
	Clostridium XIVb	−0.0056	0.0022
	Clostridia unclassified	−0.0052	0.0011
	Intestinimonas	−0.0038	0.0012
	Romboutsia	−0.0016	0.0007
	Anaeroplasma	−0.0011	0.0003
	Ruminococcus 2	−0.0004	0.0001
	Coriobacteriaceae unclassified	−0.0003	0.0001
	Coriobacteriaceae unclassified	−0.0002	0.0001

The positively associated genera are significantly more abundant with the judicious than conventional program (including barns that continued and reintroduced antibiotics after the 15-months study). The negatively associated genera are significantly less abundant in flocks using a judicious program. Taxonomic assignment at genus level was not possible for unclassified members.

It could also be hypothesized that the use of antibiotics, such as spectinomycin–lincomycin at the hatchery level during the 15-months study period could have contributed to the persistence of some genetic determinants encoding resistance to sulfonamides that are harbored on mobile genetic elements along with *aadA*, a spectinomycin resistance gene (35).

Consistent with previous studies (36, 37), birds submitted to either a conventional or to a drug-free program over a 15-months period did not show major differences for the alpha diversity. Only the inverse Simpson alpha diversity index was marginally increased in conventional flocks, illustrating the stability of the cecal microbiota (38). In contrast, alpha diversity analyses showed interesting changes when comparing flocks from barns using a long-term judicious program with a long-term conventional program (including continued or reintroduced) at sampling time point two. As shown in **Table 2**, the alpha diversity of birds from flocks raised using a conventional program during and after the 15-months study period was greater than the one observed for birds sampled from flocks that reintroduced antibiotics or adopted a judicious program after the 15-months study period. These observations are in agreement with a previous study where the use of bacitracin increased the richness and the evenness of the chicken cecal microbiota by reducing dominant microorganisms, such as *Lactobacillus* (39). However, no association between *Lactobacillus* and rearing program was documented in the present study. It could be hypothesized that the long-term use of a wide variety of antibiotics, as well as the rotation of these compounds in time, could have depleted some sensitive microorganisms, which in turn could have promoted the growth of other microorganisms. At sampling time point two, it is worth mentioning that no change in the alpha diversity was detected between flocks from barns that had adopted a judicious antibiotic use program and those that had reintroduced antibiotics after the 15-months study. It could be hypothesized that using a conventional program during a longer period of time would have allowed for the cecal microbiota to diversify further in flocks where antibiotics were reintroduced after the 15-months study.

For the cecal community structure, the beta diversity between conventional and drug-free flocks at sampling time point one was significantly different. In addition, at sampling time point two, the beta diversity was significantly different between flocks sampled from barns that adopted a long-term strategy for judiciously using antibiotics, those that continued the conventional rearing program, and those that reintroduced antibiotics after a short-term antibiotic withdrawal. These results are not surprising considering the previous observations of the antibiotic treatment effects on the bacterial community composition of the chicken cecum (39, 40). Results illustrated that a short-term antibiotic withdrawal and a long-term judicious use strategy mainly negatively affected *Ruminococcaceae* and *Lachnospiraceae*, which are the two main families forming the cecal microbiota (41). *Ruminococcaceae* and *Lachnospiraceae* members have the ability to ferment and digest carbohydrates and produce small-chain fatty acids, such as butyrate (41, 42). In a previous work, among 16 butyrate producers from the *Firmicutes*, the clostridial clusters IV and XIVa were associated with the largest production of butyrate (43). Butyrate is an important source of energy for the intestinal epithelium and helps maintain its barrier function by regulating the proliferation of enterocytes (44). In addition to having a negative effect on the colonization of *C. perfringens* (45), it was also found that butyrate enhances performances as evidenced by an increased body weight (46). These last findings were associated with a decrease in *Lactobacillus* and an increase in the ratio of villus height to crypt depth (46). For both sampling time points, antibiotic restriction significantly decreased *Clostridium* IV and *Intestinimonas*. The *Clostridium* cluster IV members includes *Clostridium*, *Eubacterium*, *Ruminococcus*, and *Anaerofilum* genera (47). These results are consistent with previous research work in which *Clostridium* IV members were enriched by the use of antimicrobial growth promoters (40). With regard to *Lachnospiraceae*, unclassified members at sampling time point one and *Blautia*, *Clostridium* XlVb, and *Ruminococcus* 2 at sampling time point two were significantly decreased with both a short-term antibiotic withdrawal and a long-term judicious use strategy. Thus, these members were more abundant in birds raised with a conventional program. These results are in agreement with those of Costa et al., who also observed that *Clostridium* XlVb was significantly enriched with enramycin, a polypeptide antibiotic used at growth-promoting doses (37). In addition, both short-term antibiotic withdrawal and long-term judicious use strategy were significantly associated with a decrease in *Anaeroplasma*, a member of the cecal microbiota for which the role remains unclear (42, 48). Overall, these results suggest that restricting the use of antibiotics tends to decrease the abundance of

bacterial populations producing butyrate, which could then affect bird performances and *C. perfringens* colonization. These assumptions are consistent with the results obtained during the previous 15-months study. Indeed, this previous study showed that raising commercial broiler chickens using a drug-free program was negatively impacting the production performance and significantly increasing the occurrence of necrotic enteritis (10). However, due to the low number of farms within each antibiotic use program, the current study did not try to correlate production performances to the microbiota composition at sampling time point two.

Findings of the current study showed significant changes in the abundance of many antibiotic resistance genes depending on both the rearing program and the sampling time point (**Figures 2**, **3**). For farms A and B, which adopted a long-term judicious antibiotic use strategy, a marked decrease in the abundance of various antibiotic resistance genes was observed, whereas this abundance increased for farms using antibiotics on a long-term basis. While minor changes, such as a decrease in *intl1* and *sul1* in some drug-free flocks were observed between barns using either a drug-free or a conventional program after the 15-months study, the 6-years period markedly influenced the abundance of many resistance genes, as predicted by Levin, who examined results of studies that used mathematical models to estimate the time needed for bacterial communities to show reversibility in their antibiotic resistance profile (33). Despite the fact that only two farms adopted a program for judiciously using antibiotics, results from these farms showed a potential impact of addressing the problem of antibiotic resistance by reducing the large-scale use of these compounds, as demonstrated by a systematic review and meta-analysis (49). In contrast, the long-term and routine use of antibiotics on four farms of the current study correlated with a global increase in the abundance of antibiotic resistance-encoding genes. These observations, believed to be influenced by the antibiotic selection pressure, are in agreement with some observations made at the bacterial strain level where the use of some antibiotics was associated with an increase in the prevalence of resistant bacteria to these antibiotics or other antibiotics by co-selection (31, 35). For example, since the voluntary ban on ceftiofur imposed by the poultry industry in Canada in 2014, a mix of spectinomycin and lincomycin was used at the hatchery level to prevent infectious diseases in chicks during the first few days of life (35). It has been reported that the co-selection and selection pressure generated by the preventive use of these two antibiotics at the hatchery level could have selected for gentamicin resistance (35). In addition, the genetic linkage between *vat*(E) and *erm*(B), previously identified in *E. feacium* from European poultry isolates could have contributed to the co-selection of these genes as these appear to be part of a same transposon (50, 51). Similarly, the use of quaternary ammonium compounds as sanitizers in poultry barns and of sulfonamides for the treatment of bacterial infections in commercial broilers could have contributed to the spread of class 1 integrons and could explain the significant increase in the abundance of both *intl1* and *sul1* in farms B, C, D, and F of the current study (**Figure 3**). Indeed, class 1 integrons can carry both *sul1* and *qac* genes, in addition to being able to capture other resistance genes, such as *aadA* (31, 35).

When comparing sampling time points one and two, a closer examination of the relative abundance and the absolute copy numbers of the targeted genes revealed that the abundance of *vat*(E) decreased markedly in farms A and B, an observation that was not made for the other genes measured. Encoding for an acetyltransferase resulting in streptogramin A resistance in the carrying microorganism, *vat*(E) is found in *Enterococcus feacium*, a microorganism that is part of the intestinal microbiota of broiler chickens (13, 52). As the *vat*(E) gene has been found on transferable plasmids (52), a great negative fitness cost associated with the carriage of this gene by a microorganism or a decrease in the rate of horizontal transfer for this mobile genetic element could both explain the decrease in the abundance of this gene observed on farms reducing the use of antibiotics. However, the rate of horizontal transfer of genes is difficult to predict, just as trends in horizontal antibiotic gene transfer according to the antibiotic exposure levels (53). For farm B (**Figure 3**), results showed an increase in genes associated with bacitracin and sulfonamide resistances. Considering that this farm had adopted a judicious antibiotic program for several years and that the restriction of the preventive use of antibiotics is a main predisposing factor for the occurrence of necrotic enteritis and of other concomitant bacterial infections (10), these results could reflect an increase in the therapeutic use of these compounds for the treatment of diseased commercial broilers (3). When comparing flocks between sampling time points one and two, results showed a significant increase in the abundance of five to seven targeted genes for farm D, and we could presume that this increasing trend would be attributed to the antibiotic regimen used on this farm over the past 6 years. In addition, the abundance of the *bcrA* and *bcrB* genes increased or decreased jointly in different farms (**Figures 2**, **3**). These observations can be explained by the fact that these two genes are found on the *bcrABDR* operon (29).

This study illustrated an increase in the richness in the samples between points one and two, as well as a marked dispersion of the samples on the NMDS, which was probably attributed to some changes in farm management practices after completion of the 15-months study. Indeed, as all participating farms that adopted a standardized protocol for chick, feed, water, and litter supply, and for coccidiosis management during the 15-months study went back to their previously highly diverse management practices once completing the study, this probably contributed to the changes observed in the cecal microbiota of broiler chickens. As previously described, many farm management factors can influence bird gut microbiota between flocks (54). According to the findings of the present study, it could be hypothesized that a standardization of farm management practices through a common rearing program could normalize the cecum bacterial community composition. Results pertaining to the detection of the *C. perfringens* alpha toxin-encoding gene were quite unexpected since only 17% of the samples were found positive for the presence of *plc*. In healthy broiler chickens, since the cecum is the main colonization site for *C. perfringens* and

because the alpha toxin gene is recognized as a hallmark of all *C. perfringens*, a 100% positivity rate was anticipated (55, 56). This low prevalence suggests that the number of *C. perfringens* in the samples screened was below the previously reported detection limit of 10^3 bacteria per gram of fecal content (56). Failure to detect the *cpe* gene can therefore be explained since only between 1 and 5% of *C. perfringens* population is known to be enterotoxigenic (57). Interestingly, as the *C. perfringens* population increases during a necrotic enteritis outbreak (55), more than half of the positive samples for the presence of the *plc* gene were identified from flocks experimenting short-term antibiotic withdrawal and long-term judicious use strategy that are recognized to increase the risk of occurrence for this disease.

In conclusion, results from the current study showed that moving to a drug-free program over a 15-months period does not seem to be sufficient to reduce the abundance of many antibiotic resistance-encoding genes, while the judicious use of antibiotics over many years seems to do so. The short-term antibiotic withdrawal and the long-term judicious use strategy changed the bird intestinal microbiota composition, where *Ruminococcaceae* and *Lachnospiraceae* families were negatively impacted, which could be correlated with negative performances and the increase in *C. perfringens* populations. Results also illustrated that adopting a conventional rearing program on commercial broiler chicken farms selected specific antibiotic resistance-encoding genes in many barns. This study highlights the potential impacts of different rearing programs in poultry production and will help develop future policies by guiding science-based decisions on how the use of antibiotics in broiler chicken production should be reduced while maintaining production performance. Reducing antibiotics and using them solely as a therapeutic option could help preserve the effectiveness of these precious tools by contributing to curb the global antibiotic resistance problem.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found at: https://www.ncbi.nlm.nih.gov/, PRJNA627503.

ETHICS STATEMENT

The animal study was reviewed and approved by Comité d'Éthique sur l'Utilisation des Animaux (CÉUA) of the Faculté de Médecine Vétérinaire of the Université de Montréal. Written informed consent was obtained from the owners for the participation of their animals in this study.

AUTHOR CONTRIBUTIONS

M-LG and AT elaborated the study design. CT, M-LG, and AT made the experiments. GB performed the statistical analyses. All authors analyzed the results, wrote this manuscript, and approved the publication of this work.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2020.547181/full#supplementary-material

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