Application of outcomes research in animal health and veterinary medicine

by

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B.S., Kansas State University, 2013
M.S., Kansas State University, 2015

AN ABSTRACT OF A DISSERTATION

submitted in partial fulfillment of the requirements for the degree

DOCTOR OF PHILOSOPHY

Department of Diagnostic Medicine and Pathobiology
College of Veterinary Medicine

KANSAS STATE UNIVERSITY
Manhattan, Kansas

2021

Abstract

While it has been well-established in human medicine, ‘outcomes research’ is a relatively recent field of research in animal health and veterinary medicine, hereafter referred to as the animal health industry. Outcomes research has applications in One Health systems, veterinary product development, post-licensure evaluation of veterinary pharmaceuticals and/or biologics, and economic analyses. The major themes of outcomes relevant to the animal health industry include, but are not limited to: health, production, economics, and marketing. Although broad-ranging in terms of animal species, objectives, research methodologies, design, analysis, value, and impact, research studies described herein are all united under the umbrella of outcomes research. Four research chapters are included in this doctoral dissertation, and a very brief summary of the objectives, findings, and impact follow. The objective of the first research chapter was to compare the efficacy of two antimicrobials administered for bovine respiratory disease (BRD) metaphylaxis in stocker calves backgrounded on pastures utilizing a randomized design to evaluate health, production, and economic outcomes. The second research chapter was also a comparative research study; however, canine acceptability of two chewable non-steroidal tablets for the management of canine osteoarthritis (OA) were evaluated. The final two research chapters were food safety studies focusing on Shiga toxin-producing *Escherichia coli* (*E. coli*) in cattle. For the third research chapter, the objective was to evaluate the effectiveness of a direct-fed microbial (DFM) product in reducing fecal shedding of *E. coli* O157:H7 in commercial feedlot cattle in Kansas and Nebraska prior to harvest. Whereas *E. coli* O157:H7 has been widely researched for over three decades, non-O157 STEC are not as thoroughly examined. Therefore, the objective of the fourth and final research chapter was to gather, integrate, and interpret data on the prevalence and concentration of the Top 6 non-O157 serogroups (O26, O45, O103, O111, O121, and O145) and associated virulence genes (*stx*$_1$, *stx*$_2$, and *eae*) in fecal, hide, and carcass samples of pre- and peri-harvest adult cattle worldwide, using a systematic review of the literature, meta-analysis, and meta-regression analyses. In summary, the chapters in this doctoral dissertation have impacted the fields of animal health, veterinary medicine, and One Health (via food safety research). The first research chapter compared two licensed antimicrobial products used in typical production conditions and management practices while measuring outcomes relevant to veterinarians and producers in the beef industry with externally valid research

findings. Similarly, the second research chapter supported the hypothesis that canine acceptability between two bioequivalent pharmaceutical products were comparable. The ease of voluntary prehension of chewable tablets by canines is conducive to long-term management of OA symptoms and increases pet-owner compliance to the treatment protocol, both key factors for long term efficacy and management of OA symptoms, in addition to the generic formulation being a more affordable option. In terms of food safety efforts, whereas the DFM product of interest in the third research chapter was not effective in reducing the prevalence and/or concentration of *E. coli* O157:H7 in cattle feces, the effectiveness of this DFM product in finishing feedlot cattle in the commercial environment was successfully evaluated. Lastly, the fourth research chapter generated data that contributes to quantitative microbial risk assessment models, provides evidence that is highly valued in expert panels, and offers robust estimates of the frequency of these non-O157 STEC pathogens, regionally and globally, while demonstrating the existing knowledge gaps for prevalence and concentration of these pathogens in hide and carcass matrices. Research studies presented in this doctoral dissertation highlight the versatility of outcomes research while emphasizing the widespread impact outcomes research has on the animal health industry globally.

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Acknowledgements

The work described in this doctoral dissertation would not have been possible without a huge support system.

First and foremost, my academic advisor, Dr. Natalia Cernicchiaro for her incredible amount of support, understanding, mentorship, and for constantly pushing me to be my best despite any challenges that arose. Thank you to Dr. David Renter, Dr. Barry Bradford, and Dr. Keith DeDonder for their mentorship in their respective disciplines and support as serving as a part of my PhD committee. Additional thanks to Dr. Michael Sanderson, Dr. Mark Mellencamp, Dr. Brandon Depenbusch, Dr. TG Nagaraja, and Dr. Jacob Hagenmaier for their collaboration on several projects and having a little bit of fun along the way. Last, but not least, I would like to express my gratitude to Dr. Kelly Lechtenberg and Dr. Charley Cull for their collaboration and supporting the efforts of work described in this dissertation and elsewhere—thank you—this opportunity would not have been possible without your support.

I would be remiss if I did not express my deepest gratitude for Dr. Adryanna Siqueira Drake and the Kansas State University, College of Veterinary Medicine for supporting counseling services for students in the college to seek mental health services, they have been truly life-saving and for that I am forever grateful. Additional thanks to more of my K-STATE FAMILY—Dr. Tim Rozell, Dr. Karol Fike, Dr. Megan Figger, Dr. Hannah Seger, Dr. Joaquin Baruch, Dr. Andrea Dixon, Dr. Christy Hanthorn, Dr. Leigh Ann George, Dr. Ashley Smith, Dr. Pius Ekong, Dr. Cameron Childress, Dr. Katie Hope, and Lucas Horton.

Lastly, thank you to my family and friends for the unconditional support. My pets deserve an honorary degree for their dedication to my academic and work schedule and making every single day better—Jade, Bennie, and Jasper are the real MVPs. To my parents, Tamara and Randy—I hope to continue to make you proud—thank you for supporting me and my academic ventures although you do not *really* "get it", it means a lot. To my two surviving grandparents—Grandpa Dewsbury and Grandpa Clemens, thank you for your unconditional love and support, especially during the hard times following the loss of Grandma Marsha and Grandma Rhoda—we know they are looking over us. My uncles' rule and I'd be lost without all of them—Jon, Bryce, Fred, James, and Graham, and their kids—love you all. To the one that has shown me what true love is supposed to look like—JJ, thank you for coming into my life when I least expected it and showing me the support and love I did not know existed. Additional thanks to Susette Porazik-Ball, Sheila Clemens, Sean Wacker, Bre Eulert, Tracy Koepke, and the Jacobs' family for their support. Finally, to my #KCMuscleFlossers—thank you for your unwavering support and being receptive to my scientific communication efforts during long runs, forcing me to take fun vacations, and being the most supportive group of friends that I have ever had, love you all and I am truly grateful for each and every single one of you.

Dedication

I am humbled to dedicate this dissertation work to my late grandma, Marsha Dewsbury. The woman who enjoyed every minute of life, gave her family her all, didn't take life too seriously, and always reminded me that 1) life is too short to be unhappy, and 2) not to work too hard. She is forever missed and I owe my new take on life to her.

Preface

The chapters included in this dissertation are formatted according to the respective journal guidelines in which they were published or intended to be published. Table and Figure numbers were modified to accommodate the designated dissertation chapter, otherwise content is unchanged from the publication.

Chapter 1 - The application, value, and impact of outcomes research in animal health and veterinary medicine

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(Formatted with intentions to submit to *Journal of American Veterinary Medical Association*)

Introduction to outcomes research

Outcomes research in human medicine has been defined, albeit inconsistently across the literature, as research concerned with the outcomes, or end results, of public health interventions and/or health services[1]. Of recent relevance are outcomes such as quality of life[2], individual preference[3], and cost-effectiveness[4]. While outcomes research has been a formal discipline for over two decades[5] it has been primarily practiced in human medicine. Therefore, outcomes research remains a relatively young discipline in the animal health industry. Described by the Animal Health Institute as the "business of keeping animals healthy", the animal health industry is a result of a collaborative effort between farmers, ranchers, livestock producers, government agencies, veterinarians, and other industry stakeholders (e.g., pharmaceutical companies, non-profits, private sector companies) where the overarching goal is "to ensure the health and safety of animals, humans, and the food supply[6]." Although the use of outcomes research in the fields of animal health and veterinary medicine has increased in popularity in the last decade, it has not been formally implemented or well-documented in the literature[7].

Traditional research includes assessing an outcome such as an intervention and/or service. In outcomes research, traditional research principles are combined with the evaluation of the value of the intervention and/or service to pet-owners, livestock producers, veterinarians, and/or clinicians in addition to the animal. This element of absolute and/or perceived value is what sets the discipline of outcomes research apart from an evidenced-based medicine approach, although they often work synergistically[1,5]. Commonly, value is associated with a reduction in cost; however, whereas economics are a metric of value, cost and value are not always synonymous[7]. Value can be represented by many factors, including products and/or services, that address functional, emotional, life-changing, and/or societal needs[8]. Considering the diversity in outcomes of interest and metrics used to assess value, the perceived value of those outcomes ultimately depends on the stakeholder. The perceived value of an intervention or service is likely to differ from the perspectives of a human patient, pet-owner, or production animal stakeholder. Inherent in human psychology, individual preferences are heterogeneous and multifaceted[3] further adding to the complexity of value perception. Compared to human health, the animal health industry faces not only having to manage different species, but also the fact that veterinary professionals must also satisfy and effectively communicate, collaborate, and work with the animal owners, managers, or producers.

Currently, there are many tools and guidelines available to design efficient and effective research trials to continue to maximize resources while yielding meaningful data to continue to propel the animal health industry forward. Looking ahead, the development of formal methodologies and implementation of novel methodologies currently utilized in human health, such as long-term cost-effectiveness models and dynamic decision analytical models, in the context of animal health are needed. Outcomes research has many applications in One Health systems, including comparative medicine, zoonotic and vector-borne diseases, food safety and security, and veterinary pharmaceutical and biological product development and post-licensure evaluation. However, despite the diversity of the discipline of outcomes research, the foundation of research principles within are universal across human and animal health industries. This review summarizes the potential utility of outcomes research in the animal health industry, highlighting key outcomes of interest and associated value metrics, discusses methods for study design, in addition to the application, and the potential impact of outcomes research in the animal health industry.

Key outcomes and value metrics in animal health and veterinary medicine

Key outcomes

The key themes of outcomes vital to the animal health industry can be distilled to: health, production, economics, and marketing. These four themes, and examples herein, are not intended to be an exhaustive list or mutually-exclusive categories as there are often points of overlap. Common health outcomes assessed in the animal health industry include morbidity, mortality, duration and quality of life, visual assessment score of pain, and blood parameters. In recent examples, health outcomes of interest include, evaluating the morbidity and mortality of Bovine Respiratory Disease (BRD) in stocker calves after metaphylaxis[9], assessing the impact of obesity on feline quality of life[10], and evaluating potential prognostic factors for survival of canines with diffuse large B-cell lymphoma treated with CHOP-based chemotherapy[11]. Production outcomes of interest include evaluating metrics for performance such as average daily gain (ADG), feed efficiency (F:G), hot carcass weight (HCW), carcass quality, carcass yield, and pounds of milk produced, in addition to reproductive performance metrics, such as: calving interval and days open, or production management strategies. Recent examples of production, reproductive, and management outcomes in the literature can be demonstrated by evaluating the role of medium-chain fatty acid supplementation in broilers on productive performance and meat quality[12], effect of individual versus group housing on the weaning-to-estrus interval on reproductive performance of sows[13], and evaluation of the effects of milk feeding strategies on short- and long-term productivity of dairy heifers[14], respectively.

Net return on investment, willingness to pay, and cost of intervention and/or service are economic outcomes of interest in animal health. In companion animal species, most economic outcomes of interest are cost to the pet-owner, willingness to pay, and overall affordability of veterinary care on an individual animal basis. However, food animals are commonly managed at the population level. Therefore, livestock producers are most interested in an overall low cost and/or an increased net-return on investment (i.e., financial incentive) in order to produce an affordable dairy, beef, pork, and/or poultry product consistently and sustainably. Conversely, marketing outcomes include any characteristic that can be used to market a product and/or service that demonstrates a clear value proposition, where value may represent a more affordable alternative and/or non-monetary metrics of value (see ‘Value metrics’). Measuring the safety and

efficacy of administration of oral and topically administered fluralaner in canines with sarcoptic mange[15], demonstrating canine acceptance of two bioequivalent carprofen chewable tablets[16], and evaluating equine treat palatability and associated owner preferences[17], are recent marketing outcomes examples. Intuitively, economic outcomes commonly overlap with health, production, and/or marketing outcomes. For instance, the use of gamithromycin for metaphylaxis in stocker steers was associated with better performance measured by ADG (production outcome), lower morbidity (health outcome), and greater net-return per head (economic outcome) compared to the competitor product—ceftiofur crystalline free acid (marketing outcome)[9]. This study demonstrates the quintessence of outcomes research by evaluating key outcomes of interest and value relevant to the veterinarian and cattle producer, while optimizing efficiency, demonstrating comparative efficacy, and maximizing resources in a single study[9]. Thus, all previously described outcomes are correspondingly those studied in traditional animal research studies. Without the addition of assessing value, evaluating these key outcomes alone do not constitute application of outcomes research, there must be an assessment of value.

Value metrics

Traditionally, veterinary research is focused on assessing an outcome, in example clinical efficacy of an orally administered pharmaceutical to felines for management of a chronic disease. The overarching goal of product development is to develop products that are both safe and efficacious. However, if the pharmaceutical is not palatable and/or easy to administer, then overall the product is not of great value to the pet and/or pet-owner. With difficulty in administration, potentially added stress for the pet and pet-owner, the pet-owners compliance to the treatment protocol will likely be poor and the pet's condition requiring treatment will subsequently remain untreated. In addition to evaluating efficacy, as in the example above, considering the functional needs of the pet-owner, such as ease of administration, affordability, and the ability to integrate this routine into normal daily activities must be considered. This consideration of functional needs, as an example, measures value in addition to product efficacy, and is paramount to the overall animal health industry in developing veterinary products that are effective, safe, and—valuable. Thus, the crux of what defines and sets outcomes research apart from other disciplines.

The perception of value depends on the species, research objective, study design and/or method used, and is ultimately up to the final decision maker/stakeholder to assess. For example, commercial cattle or dairy producers may perceive value differently than a companion animal owner. In addition to convenience of administration, other metrics of value may be evaluated through production outcomes, such as key performance metrics used to quantify monetary value and overall net-returns. While veterinarians and pet-owners may work together on treatment plans to determine the most effective and affordable option, economics are important in both production animal agriculture and companion animal health and management. However, with veterinary care of pets, value perception is more personified in terms of quality of life, functionality of treatment, and owner preferences on an individual pet basis rather than monetary outcomes and economic value on a large scale for a population.

Metrics of value are not mutually exclusive and multiple metrics may be represented within a study while addressing more than one stakeholder need. In fact, Almquist et al., state that the more elements of value that are conveyed, "the greater a customers' loyalty and the higher the company's sustained revenue growth[8]". The four types of needs—functional, emotional, life-changing, and/or societal[8]—used to represent value are determined by the stakeholder, and human perception plays a large role in the animal health industry. In addition to cost reduction, other potential functional needs may include interventions and/or services that reduce time and/or effort, avoid hassle, reduce risk of disease or outcome, and integrates easily into daily routines[8]. In addition to functional needs, value can address psychosocial or emotional needs such as improving quality of life, contributing to overall increase in wellness, providing therapeutic value, and is readily available to the stakeholder when needed. Lastly, 'life-changing' and 'social impact' needs that contribute to perceived value reflect aspects of a product/service that may provide hope and an organization that considers charity and gives back, respectively[8]. Philanthropy efforts can be seen in the work done by The Zoetis Foundation which has committed to providing $35 million dollars over 5 years to support communities and their animals, veterinary training, veterinary student scholarships, and to care for animals impacted by disaster[18]. Additional philanthropic efforts can be seen by Elanco's Healthy Purpose™ initiative to advance the well-being of animals, people, and the planet[19] and the MerckHelps™ assistance program to provide Merck medicines and vaccines for free to people who qualify[20]. The Zoetis

Foundation, Elanco's Healthy Purpose™, and MerckHelps™ are recent initiatives fulfilling stakeholder needs for life-changing and social impact through philanthropy efforts[18-20].

As noted in recent literature, the influence of human perception plays a large role in modifying behaviors when it comes to purchasing and giving food and/or treats to their companion animals[17, 21, 22]. In a recent study, data indicated that consumer acceptance and purchase intent did not accurately reflect horse preferences[17] thus demonstrating the importance of appealing to not only the target animal, but also the pet-owner. Additionally, the perception of pet-owners and motivation for giving treats to their canines was evaluated and demonstrated treat-giving was commonplace, used for positive reinforcement, and an expression of owner's affection as part of the human-animal bond rather than just simple nutritional merit[21]. Owner-reported behaviors and welfare for vegan and meat-based pet foods for canines and felines was reported by Knight et al. which shows the personification of human preferences on to companion animals[22]. Thus, in addition to meeting animal needs, we must also consider the necessity of appealing to the customer, which may be a pet-owner, veterinarian, and/or livestock producer, as their perception of value and preference often trumps that of their animal's.

Methods to design outcomes research trials

Overview of traditional methods

In veterinary vaccine and pharmaceutical development, experimental studies are often utilized in laboratory settings to demonstrate a proof of concept, establish challenge models to demonstrate efficacy, and field studies to demonstrate safety and/or efficacy of the product. Utilization of experimental studies in the field rather than in a controlled and/or laboratory result in greater external validity of research findings and opportunity to demonstrate value to the stakeholder. Recently, a double-blinded, placebo-controlled field safety and efficacy study of an orally administered pharmaceutical to prevent heartworms in client-owned canines was conducted in Denmark and Italy[23]. Similarly, field efficacy of direct-fed microbial products included in diets of finishing cattle to reduce *Escherichia coli* O157:H7 has been a key research area for pre-harvest food safety stakeholders[24-26]. Undoubtably, experimental research trials are the best approach to evaluate outcomes of interest as they allow for control of known and unknown confounders via randomization of study subjects and can assess causation. The use of experimental trials in the 'real-world' such as with client-owned animals or in commercial

production settings, allow for a more efficient use of resources, promote streamlining efforts for answering pertinent research questions, and generate data with greater external validity compared to a traditional laboratory experimental trial.

However, experimental studies are not always feasible or the most ethical and thus research questions may necessitate the use of an observational study design. Common observational study designs used in animal health research include cross-sectional, cohort, and case-control designs, in addition to hybrid designs with components representing more than one design[27]. Recently, a cross-sectional study design was conducted to evaluate laboratory animal welfare and human welfare assessing compassion fatigue[28]. A recent objective of cohort studies includes assessing risk factors associated with the development of non-infectious foot lesions in dairy cattle[29]. Case-control study designs are ideal for rare diseases such as evaluating the prevalence of *Bartonella* sp. in United States military working dogs with infectious endocarditis[30]. While the design and implementation of observational studies is challenging to control for bias, there is a valuable niche for observational studies in outcomes research.

With the increasing large body of research literature readily available, research synthesis methods such as scoping review, systematic review, and meta-analysis techniques can be used to synthesize data from multiple studies. Traditional narrative literature reviews are arguably a fundamental starting point for all research studies. However, they often lack a formal methodological process and researchers often have preconceived ideas on the topic they are reviewing[27]. Scoping and systematic reviews are more formal, transparent, and repeatable in nature than traditional narrative reviews[27, 31]. Recently, scoping reviews described the risks of introduction of transboundary animal disease (TAD) and the economic consequences associated with African Swine Fever, Classical Swine Fever, and Foot-and-Mouth Disease in the United States[32] or described non-antibiotic approaches for disease prevention and control in nursery pigs[33]. Rather than mapping a theme, systematic reviews focus on answering specific questions relating to disease prevalence or incidence[34], disease etiology and risk factors[35], diagnostic test accuracy[36], or evaluating interventions[37] in human and veterinary health care systems[38,39] and are commonly paired with meta-analysis. The use of meta-analysis in healthcare, veterinary and human alike, is an extremely valuable tool for topics in which there are many published studies available but the overall conclusions of the studies are contradictory and how to move forward with future research, client/patient recommendations, and/or modification of current practices

remains unclear. In example, scientific literature pertaining to the use of direct-fed microbials as a pre-harvest intervention to reduce *Escherichia coli* O157:H7 in cattle has been studied for decades often with conflicting findings. However, utilizing formal systematic review and meta-analysis techniques, there is evidence that direct-fed microbials reduce fecal shedding of *E. coli* O157:H7 in cattle[37]. Network analysis has been utilized to evaluate efficacy of teat sealants for preventing intramammary infections and mastitis in dairy cattle[40]. While labor intensive, when executed correctly, systematic reviews and meta-analytic techniques can effectively evaluate key outcomes valuable to stakeholders and decision makers at all levels by answering virtually any scientific question in any field.

Novel trends, methods, and technologies

The International Society for Pharmacoeconomics and Outcomes Research (ISPOR) published the 'Top 10 Health Economics and Outcomes Research (HEOR) Trends' for 2020[41]. While this report is focused on human healthcare, many parallels can be made between how these trends in human healthcare are shaping outcomes research for the animal health industry as well as how novel methodologies used in human healthcare can potentially be applied to animal health. Specifically, parallels in animal health can be made for HEOR trends such as drug pricing, overall healthcare spending, digital technologies, and precision medicine. As seen with human healthcare, the price of drugs and transparency of costs incurred for veterinary treatment is essential for building trust[42] with pet-owners and providing a spectrum of care[43]. Overall, spending in human healthcare is far greater than in veterinary medicine; however, while on a smaller scale, the animal health industry continues to grow exponentially with the increase in pet ownership and production animal agriculture expenditures. The use of digital technologies in animal health are also of recent interest for areas like willingness to pay for veterinary telemedicine[44] and assessing traceability of live animals and their products[45]. The trend of precision medicine, or personalized medicine is a growing field in human healthcare outcomes research[41]. Precision medicine utilizes big data[41] an area of interest for predictive technologies and algorithm development to identify the best treatment on an individual basis. Recently in the animal health industry, the use of WHISPER® on ARRIVAL technology demonstrated successful BRD treatment of individual cattle upon arrival that was similar in overall effect to traditional metaphylaxis, a practice used to treat groups of animals prophylactically rather than

making treatment decisions on an individual basis[46]. This predictive technology reduced antibiotic use in the cattle production environment while utilizing chute side technology to determine if the individual animal requires treatment or not[46] rather than a subjective decision to treat an entire population at the time of arrival. Thus, demonstrating the utility of this novel technology in animal health to improve antimicrobial stewardship and to subsequently reduce the costs associated with treatment[46].

Based on current trends in human healthcare, outcomes research in the animal health industry could benefit from considering two of the Top 10 HEOR trends: Real-World Evidence (RWE) and universal health coverage[41]. As defined by the United States Food and Drug Administration, Real-World Data (RWD) are data relating to patient health status and/or the delivery of healthcare, while RWE is the analysis of RWD regarding usage, benefits, and/or risk of a medical product[47]. The use of RWD and RWE has been utilized in recent vaccine licensure[48] in human medicine, and similarly could expedite the regulatory process in animal health. In example, using client-owned animals to prove concept of new animal drugs after the completion of necessary safety studies. However, admittedly there are concerns on relying solely on RWD and RWE for regulatory approval, mainly in comparison to the internal validity of a randomized controlled trial, the gold standard to evaluate product efficacy[49]. The advantages and limitations of the use of RWD and RWE in human healthcare product development have been reviewed in detail and overall show great promise in accelerating the product development process with results and findings more indicative of how the product performs in the real-world[49]. Lastly, the concept of universal health coverage in animal health, primarily for companion animals has been discussed as a vast majority of veterinarians agree that all pets deserve some standard level of veterinary care[50].

Long-term cost effectiveness models have been used in human medicine to evaluate impacts relating to cancer screening[51], but could also be utilized for long-term management of chronic diseases commonly seen for companion animals in the animal health industry. Cost-effectiveness models offer a methodology to evaluate the health effect in addition to costs associated with treatment[52]. Thus, cost-effectiveness models offer a better option than a cost-benefit analysis which simply monetizes a health effect while ignoring important aspects associated with treatment such as quality of life[52]. Similarly, decision analytical models provide evidence to guide decision making by use of mathematical techniques to synthesize data

comparing expected costs and consequences of potential decision options[53]. Decision analytic modeling could also be used to evaluate long-term outcomes as well as economic impacts to better inform decision makers[54]. For assessment of new oncology treatments, survival extrapolation to include general population mortality has been recently utilized[55] with methodologies that may translate well for evaluating oncology treatments in companion animals. While there are many trends in human healthcare outcomes research that mirror those trends observed in the animal health industry, a disconnect in methodologies remains present. The animal health industry is lagging behind the human health industry in terms of outcomes research methodologies and thus, a tremendous benefit could be gained by translating and applying these methods to current issues in animal health.

Key areas of application of outcomes research in the animal health industry

The Center for Disease Control and Prevention defines 'One Health' as "a collaborative, multisectoral, and transdisciplinary approach, working at the local, regional, national, and global levels, with the goal of achieving optimal health outcomes recognizing the interconnection between people, animals, plants, and their shared environment[56]". With the overarching goal of One Health to keep people and animals safe from disease (outcome) and improve quality of life (value)[56] the utility of outcomes research is clear. In the animal health industry, the areas of research that could benefit the most from the application of outcomes research are comparative medicine, zoonotic and vector-borne diseases, food safety and security, and veterinary product development and evaluation.

Comparative medicine

Comparative medicine has been documented as far back as the early 1900s where it was simply summarized that human and veterinary medicine are "two branches of one medicine", where there are similar problems, similar approaches to a solution, and more similarities than differences between humans and animals[57]. In human pharmaceutical and biological development, laboratory animal models are utilized to prove a concept, demonstrate efficacy, or safety, before utilizing non-human primate and/or human research subjects in clinical trials[58]. Traditionally in basic research, many animal models utilize rodents, primarily murine, however, inherent weaknesses and limitations have been demonstrated over the years with findings in

rodents not translating well into human medicine—"mice are not men[59]." When choosing which animal model is most appropriate, there are scientific, regulatory, and animal welfare considerations to contemplate prior to designing research trials[58]. While murine laboratory species have limitations, as do all laboratory species compared to humans, rodents remain integral in research as they are easy to handle, house, and are generally inexpensive compared to other species. However, in recent years, other laboratory animal models—including canine, feline, and swine models—have demonstrated extreme value in expediting advancements in human healthcare research and product development.

Canine and feline models are excellent representations for human disease as some genetic diseases are homologous to those found in human patients[60,61]. The use of purpose-bred canine and feline animals have led to successful approval of many therapies for many rare, yet extremely debilitating and lethal, genetic diseases in humans[61]. With diseases naturally occurring in canine and feline, these animal models offer a more efficient alternative and externally valid model than rodent models. For example, canine lymphoma and leukemia are more common in canines rather than humans but disease progresses in the same aggressive manner[60]. Therefore, researchers have utilized canines as pre-clinical models to evaluate new and modify current therapies for human hematopoietic neoplasia[60].

Beyond genetic diseases, comparative medicine is also applicable to infectious diseases[58, 62] and general anatomy and physiology research[63, 64]. Animal models are commonly used in the development of human and animal vaccines for infectious disease prevention and management[62]. In addition to companion animal species, swine have also demonstrated their usefulness as a biomedical model for humans in terms of metabolic, cardiovascular, digestive, and bone diseases[63-65]. Lelovas et al. (2014), discusses lessons learned over the last two decades of cardiovascular research utilizing animal models and the comparative anatomy of swine (commercial and laboratory breeds) to humans, deeming minipigs to be the most appropriate animals for cardiovascular research[63]. Similar to cardiovascular research, advantages and limitations associated with commercial swine breeds and minipigs has also been reviewed for swine models for metabolic, digestive, and bone disorders[66]. Additionally, commercial swine have also been used in biomedical research, most commonly nutrition and physiology studies where growing swine proved a suitable model for human metabolic studies in food research[64]. Overall, scientific findings associated with larger animal models such as canine, feline, and

swine translate well into into human medicine applications. Outcomes research in comparative medicine utilizes animal models for veterinary and human medicine evaluation, refinement, and ultimately approval of human and veterinary therapies, interventions, and overall improving One Health, and subsequently, quality and longevity of human and animal life simultaneously.

Zoonotic and vector-borne diseases

The human-animal bond has evolved over the years[67] from serving strictly in working and protection roles to now include a close companion role, even for non-traditional pets such as exotic animals, domesticated livestock, and domesticated wildlife. In the United States, 70% of households, or 90.5 million homes[68], own a pet and over half of pet-owners deem their pets to be family members[69], with canine and felines dominating in popularity. With such a close relationship with our pets, considering them family members, and all of the positive impacts they have on personal well-being, it may be challenging for some to consider their potential role in human disease transmission. Recently, Dalton et al., (2020) utilized a One Health approach reviewing the animal-human-environment interface in antimicrobial-resistant gram-positive healthcare associated infections in hospitals including mentioning the roles of pets in the home harboring the same bacterial infection as the humans they reside with[70]. Similarly, given the on-going COVID-19 pandemic, the role of zoonotic and reverse-zoonotic transmissions of COVID-19 and veterinarians was studied, demonstrating canine and felines as susceptible hosts to severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection[71]. Notwithstanding their potential to transmit SARS-CoV-2 to their owners, humans were extremely appreciative of their companion animals, specifically canines, as they aided in support of mental and physical health during the COVID-19 pandemic[72].

Whereas 60% of human pathogens originate in animals[73], arthropods play an essential role in transmission of many infectious agents resulting in devastating illnesses of One Health importance in the United States. While common arthropod vectors such as fleas, ticks, and mosquitoes are influenced by climate, represented by geography and season, vector-borne diseases are also influenced by socioeconomic, culture, pest management, and healthcare factors[74]. In the United States, endemic arthropod-borne human illnesses of relevance include West Nile Virus, Lyme disease, and Rocky Mountain spotted fever[74]. While arthropod-borne diseases are directly transmitted from the arthropod, companion animals can be susceptible to

disease and serve as definitive or intermediate hosts for causative agents of diseases such as leishmaniosis, borreliosis, bartonellosis, ehrlichiosis, rickettsiosis, and anaplasmosis[75]. Livestock also play a key role in zoonoses, such as in the transmission of foodborne pathogens including *Shiga toxin-producing Escherichia coli* and *Salmonella* spp. (discussed in the section 'Food safety and security').

Food safety and security

The World Health Organization (WHO) estimates that each year there are 600 million cases of foodborne illness resulting in 420,000 deaths yearly[76]. Furthermore, an estimated 820 million people suffer food insecurity, chronic hunger, and malnourishment globally[73]. By year 2050 the global human population is estimated to reach 9.7 billion[73]. With an increasing global population, limited resources, shortage of ranchers, farmers, livestock producers, and rural veterinarians, the challenge of ensuring food safety and food security remains a major global health concern [7, 73]. Through outcomes research, utilization of a One Health approach is paramount to understanding the complexity of foodborne pathogens. It has been documented that human pathogens such as *E. coli* O157:H7 can be harbored and shed by wildlife such as feral swine[77] and deer[78] in addition to domesticated species in the production setting such as cattle[79] and swine[80] resulting in potential for subsequent human foodborne illness. In the United States, nearly half of foodborne illnesses between 1998 and 2008 were attributed to produce, with approximately half of the outbreaks caused by Norovirus; however, more deaths were attributed to poultry, contaminated by *Listeria* or *Salmonella* spp., than any other commodity based on outbreak associated illnesses between 1998 and 2008[81]. Moreover, the use of antimicrobials in production animal agriculture and the increase in antimicrobial resistance remains a One Health concern globally for zoonotic diseases, including foodborne illness[82]. In addition to endemic food safety threats, transboundary diseases, such as African Swine Fever[83] or Foot and Mouth Disease[84], can have lasting impacts on human, animal, and environmental health in addition to resulting food insecurity and economic devastation[71].

Veterinary product development, licensure, and post-licensure evaluation

Veterinary pharmaceutical and biological products rely solely on research to prove safety, efficacy, field effectiveness, and other pivotal information under regulatory guidance in order to

receive approval for licensure from the respective agency in the United States. The federal agency responsible for oversight of development and product licensure for pharmaceutical and biological products are the United States Food and Drug Administration Center for Veterinary Medicine (FDA-CVM) and United States Department of Agriculture Center for Veterinary Biologics (USDA-CVB), respectively. Where pharmaceutical products are synthesized chemical compounds with known physical and chemical properties and biologicals are represented by a multitude of products such as vaccines, bacterins, antisera, diagnostic kits, and other products of biological origin utilized to elicit an immune or therapeutic (e.g., gene and cell therapies) response. The FDA-CVM is responsible for the oversight of veterinary medical devices. The last government agency relevant to animal health and veterinary medicine is the Environmental Protection Agency (EPA) which is responsible for veterinary products that kill insects and/or pests via external application and do not require absorption by the animal to achieve efficacy[85].

The overarching goal of all of the aforementioned regulatory agencies is to ensure quality, safety, and effectiveness of the veterinary products licensed. The culmination of poor oversight and malpractice yielded the Virus-Serum-Toxin Act of 1913 which authorizes the USDA to federally regulate industry to ensure biologics are pure, safe, potent, and efficacious[86]. Traditionally, the product development and licensure process requires many different experimental trials to demonstrate proof of concept, determine efficacy, and prove safety. To develop a veterinary pharmaceutical product, it is estimated to take five to 15 years from discovery to licensure with costs potentially exceeding $100 million[85]. Conversely, the time to develop and license a veterinary vaccine is approximately 5 to 8 years with costs estimated between $50 million to $100 million[87]. Time to licensure is significantly shorter for veterinary products compared to human products, and the input cost and profits are also reduced. The on-going COVID-19 pandemic highlighted the ability to rapidly develop a safe and effective human vaccine using mRNA technology (COMIRNATY®; Pfizer Inc., New York, NY)[48]. Although this example was an unusual pathway to market, born under extreme conditions, this rapid time to market amidst a global emergency shows there is room to improve efficiency in the licensure process for veterinary and human medicine moving forward. Through use of outcomes research the product development and licensure pathways have the potential to more efficiently utilize resources than traditional basic research studies by addressing the overall outcome, usually

efficacy or effectiveness, as well as added value such as ease of administration, storage or temperature requirements (e.g., cold chain), affordability, and/or net-return on investment.

The streamlining of research studies is not only needed to demonstrate safety and efficacy of veterinary products but could also be utilized to create value propositions by comparing to competitor products while in development. While this streamlining of research trials to tackle outcomes necessary to receive product approval and licensure, secondary outcomes such as cost, convenience of application, or acceptability, could also add increased value proposition while decreasing time to market and overall resources invested. Post-licensure evaluation is commonly conducted to compare against other licensed products to yield marketing information and/or to supplement technical bulletins further requiring additional time, labor, expertise, animals, and cost to generate supplemental data to prove value and the competitive edge over rival products.

Main areas of impact outcomes research in the animal health industry

Companion animal health and management

In the United States, the companion animal sector of animal health and veterinary medicine is arguably the largest growing. In 2020, Americans spent $103.6 billion on their pets, and for year 2021 it is projected to increase by another $6 billion[68]. Despite an increase in money spent on America's pets, many pet-owners struggle financially but with community programs[88], pet insurance[89], and willingness of veterinarians to provide a spectrum of care[43], these hardships can be alleviated. Through outcomes research focusing on clinical outcomes of importance such as efficacy as well as value metrics including cost, treatment administration ease for the pet-owner, and pet quality of life, veterinary products can be more efficiently developed, marketed, and of value to pet-owners and their pets. A fundamental component of veterinary product implementation is adherence of veterinary treatment protocol by the pet-owner, which is also paramount to treatment effectiveness[90]. The relationships between pet-owners and veterinarians in clinical decision making will also impact veterinary product implementation, compliance with veterinary protocols, and ultimately the management and success of pet health[43,90,91]. Recently, it has been documented that pet-owners are most responsive to a collaborative approach with the veterinarian, their most trusted source of pet health knowledge[91,92]. With 95% of veterinarians in agreement that all pets deserve some level of veterinary care[50], collaboration between

veterinarians and pet-owners may promote discussion of a spectrum of care options depending on their financial situations to offer affordable and effective care, although it may not be the most specialized or technologically advanced option[43]. Additionally, pet-owners appreciated when veterinarians suggested online resources to reference, ultimately improving their confidence and overall relationship with their veterinarian[92]. With accessibility of online resources and a collaborative relationship with the veterinarian, online marketing resources geared towards pet-owners would be a key outcome to emphasize during product development, licensure, and marketing.

Food animal production

With the said growing population, current status of food insecurity, food safety challenges, and threats of TADs—the future of production agriculture is in dire need of support, young professionals, researchers, and consumer acceptance. The future sustainability of production agriculture will be dependent on continuing to improve animal productivity and overall health while decreasing input costs and improving consumer trust[93]. The most impactful outcomes research in production animal agriculture include focusing on production outcomes via performance metrics, as well as their monetary value, both input costs and net returns, in the commercial food animal production systems. Additional considerations for focus of outcomes research could include concentrating on consumer-centric outcomes, such as animal welfare and perceptions of agricultural practices and subsequent animal protein food products, in order to better understand where to target focus in order to improve consumer perception and aid in marketability. In addition to consumer perception, the shortage of rural veterinarians, and farmers, livestock producers, and ranchers also poses a real threat in navigating the future growth and longevity of production agriculture, which unfortunately is not a new realization[94,95]. Production agriculture remains vital to animal health and veterinary medicine and the impact of outcomes research can greatly contribute to optimization of resources in research and contribute to generating data to improve consumer perception and trust and recruit future stakeholders.

Public perception and scientific communication

Public perception, especially as it relates to animal research and food animal production is an extremely sensitive topic area as it remains taboo for some and solutions to poor public

perception are rarely implemented although these issues are largely discussed behind closed doors. However, the best way to mitigate poor consumer perception in research is to communicate our scientific purpose and findings in a way that the layperson understands, sparing all of the scientific jargon and minute details while emphasizing the value of these efforts through addressing stakeholder needs. While some may still have unfavorable feelings about animal research or production management practices currently employed, increasing transparency and communicating rationale could help combat the negative publicity challenging production animal agriculture. Common misconceptions about hot topics widespread in food animal production in the United States such as the use of farrowing crates, feed yards, or impacts of antimicrobial use in food animals are widespread through viral videos, misinformation from activist groups, social media, and marketing seen on labels in the supermarket. Perhaps use of 'The Decision-Making Model for Agriculture and Natural Resource Science and Technology' theory described by Ruth et al (2018)[96] would offer an applicable solution to this challenge. Through use of this theory, the public could make more informed decisions as they relate to agriculture with a more complex understanding of current topics which would break the cycle of misinformation, poor decision making, and subsequently improve public perception[96]. Furthermore, exploration into public opinion through outcomes research may also provide insight that may come as a surprise, such as findings that the majority of American's had positive attitudes towards genetic modification of plants and animals, which is commonly used in biotechnology applications in medical and agricultural industries[97]. Moreover, and even less commonly discussed, is the necessary use of animals and especially companion animals in research. However, anecdotally using individuals' pets as an example to explain how we as researchers must rigorously evaluate the safety and efficacy of flea and tick preventative products, for example, prior to feeling confident to license a product for them to administer to their beloved 'Fido', often promotes understanding and realization of the associated value as a result of animal research efforts. Similarly, pet-owners are more likely to recognize the value of veterinary products when they see for themselves the great impacts successful treatment of chronic diseases such as osteoarthritis have on pets' quality of life. As evident in our current climate, utilizing the on-going COVID-19 pandemic as example, public mistrust in scientists, and the government and perceived political agenda is a very real and prevalent problem and ultimately poses an extreme threat to our global health. In alignment with this lack of trust, food

safety and antimicrobial use in food animals is also an area with extreme opportunity for education and increase in public awareness and trust. Additionally, to further complicate, there remains a fair amount of mistrust with the agenda of "big-pharma" companies and the perceived greed associated with large companies. Scientific communication and the associated public perceptions are paramount to the success of the future applications of outcomes research and resulting positive impact to the animal health industry.

Conclusions

Outcomes research is an essential multi-disciplinary approach in tackling many scientific challenges currently on-going. The increasing number of pets to care for, financial hardships associated with pet-ownership, food safety challenges, global food insecurity, threat of TADs, are some of the obstacles facing the animal health industry. While formally documented and utilized in human health for many years, outcomes research remains a relatively new discipline in the animal health industry. However, formal guidance and methodology resources in the context of the animal health industry are needed for newer methodologies such as long-term cost-effectiveness models and/or dynamic decision analytical models recently used in human healthcare research. Additional efforts to explore the use of RWD and RWE to supplement the product development licensure process, as practiced in human healthcare could be transformative in reducing the time to bring veterinary products to market. The fundamentals of outcomes research, outcome and value, are the crux of why this discipline is essential to the future successes of the animal health industry. Evaluating and considering value, in addition to assessing clinical or production outcomes of interest, is paramount to the success of any product, intervention, and/or service and what sets it apart from a competing product that may efficacious, but may not have proved as valuable to the stakeholder by meeting emotional, functional, societal, and/or life-changing needs. Moving forward, key areas with the most potential for impact by outcomes research is development and marketability of companion animal health products, and improvement of public perception through scientific communication efforts around production agriculture practices, safety of our global food supply, and increased trust in scientists. While the breadth and depth of outcomes research, including research approaches and methods utilized, key areas of application and overall impact have been briefly reviewed, it is clear the utility and unquantifiable value that this discipline contributes to animal and human

health worldwide. With an ever-growing population, limited resources, emerging and transboundary diseases, amidst a global pandemic, outcomes research has an opportune time to demonstrate its value to health worldwide.

Acknowledgements

We would like to acknowledge the College of Veterinary Medicine at Kansas State University.

Funding and support

There was no external funding provided for preparation of this manuscript. All authors state there were no conflicts of interest associated with the preparation and publication of this manuscript.

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Chapter 2 - A randomized trial comparing effects of respiratory disease metaphylaxis with gamithromycin or ceftiofur crystalline free acid on the health, performance, and economic return of auction market-derived stocker calves backgrounded on Missouri pastures

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(Published in *The Bovine Practitioner*, 2019, 53:1)

Abstract

This study's objective was to compare the effects of metaphylaxis with gamithromycin (GAM) and ceftiofur crystalline free acid (CCFA) for controlling impacts of bovine respiratory disease (BRD) in naturally exposed auction market-derived stocker steers. Steers (n = 240; initial BW = 537.54 ± 60.61 lb) were randomly allocated to 16 pastures randomized to two treatment groups, GAM or CCFA. Data were analyzed using linear models with means (± standard error) reported. Following metaphylaxis, 16 steers (GAM, n = 3; CCFA, n = 13) required treatment for BRD. Mean BRD morbidity was significantly higher ($P = 0.03$) in the CCFA group (10.83 ± 2.84%) compared to the GAM group (2.50 ± 1.43%). Eight steers died or were removed from the 59-day trial due to non-BRD health reasons. Average daily gain in steers finishing the study was greater ($P = 0.03$) in GAM (2.90 ± 0.09 lb) versus CCFA (2.57 ± 0.09 lb) steers. Mean net return per head for steers finishing the study was greater ($P \leq 0.01$) for GAM ($22.34 ± 6.75) versus CCFA

(-$6.67 ± 6.75) steers. Overall, steers administered metaphylaxis with GAM had lower morbidity, increased weight gain, and increased net revenue, compared to those given CCFA.

Key Words: Bovine, BRD, ceftiofur crystalline free acid, gamithromycin, respiratory disease

Introduction

With estimated costs exceeding $4 billion annually due to investments in prevention and treatment, as well as economic losses due to mortality and decreased productivity, bovine respiratory disease (BRD) is considered the most economically devastating disease facing the beef industry.[12] Although there are management strategies and products, both biological and pharmaceutical, to aid in the prevention and control of BRD, the beef industry structure in North America poses a challenge for overcoming BRD due to potential animal stresses and pathogen challenges.[23] The complex interaction of various bacterial and viral pathogens, as well as host, environmental, nutritional and management factors, creates inherent challenges for managing the BRD complex in a feeder cattle production environment.[11, 17]

Diagnosis of BRD is often subjective and accuracy can vary among observers. In addition, cattle are effective at concealing signs of illness.[17] For the common approach of using clinical observations for diagnosing BRD, White and Renter (2009) estimated diagnostic sensitivity and specificity to be 61.8% and 62.8%, respectively.[29] With these known shortcomings in BRD diagnosis and potential for high-risk of disease, metaphylaxis is an advantageous tool used to mitigate BRD in potentially high-risk populations. Metaphylaxis can decrease the potential for disease, and the subsequent severity and impacts, by treating the entire high-risk cohort at a single time point prior to the onset of illness (e.g., on-arrival to stocker or feedlot facility).[14, 17]

Metaphylaxis has been demonstrated to be efficacious in reducing the impacts of BRD in feedlot cattle.[1, 3, 16, 18, 19, 24] However, there are limited data comparing the impact of different antimicrobials administered metaphylactically in stocker calves.[2, 21] The objective of this study was to compare the field efficacy of two antimicrobials, gamithromycin and ceftiofur crystalline free acid, administered for BRD metaphylaxis in naturally exposed, potentially high-risk, beef stocker calves backgrounded in pastures over a 59-day period. Protocol-defined primary outcomes of interest for comparisons among treatment groups included standard health and

performance measures, as well as mean financial return per head estimated using a partial budget approach.

Materials and methods

Study design and cattle population

The study was designed as a double-blinded, positive control, clinical efficacy trial using a balanced randomized design with pasture as the experimental unit. The number of pastures (and animals within pasture) was optimized to detect a 10% difference in first treatment BRD morbidity, assuming that positive control group morbidity would be 20%. The level of significance (type 1 error) was set at a more liberal value of $P \leq 0.10$ due to limitations in the number of pastures available, and power was set at 80%. This study population was to represent a cohort of 450 to 650 lb (205 to 295 kg) auction market-derived beef stocker calves (steers) that were assumed to be at a high-risk of developing BRD. In October 2017, cross-bred beef steers were purchased from a livestock auction in southwest Missouri. The health histories of these steers were unknown. Steers were shipped in three truckloads to the research facility approximately 3.5 hours from the auction facility.

Processing and treatment allocation

Upon arrival to the facility all steers were commingled in holding pens for approximately 24 hours prior to processing. Prior to study enrollment and processing, purchased steers were observed for any abnormalities when unloading from the truck and again prior to processing; only calves with no observable clinical disease were enrolled. At processing, steers (n=240) received unique numbered tags in each ear and the following products (administered per label and dosed if applicable according to individual body weight): *Clostridium chauvoei-septicum-novyi-sordellii-perfringens Types C & D* bacterin-toxoid[a] (2 ml) administered subcutaneously (SC) in right neck (front of shoulder); modified-live Bovine Rhinotracheitis-Virus Diarrhea-Parainfluenza 3-Respiratory Syncytial Virus Vaccine with *Mannheimia haemolytica* toxoid[b] (2 ml) administered SC in left neck (front of shoulder); oxfendazole oral suspension[c] (1 ml/110 lb (50 kg) body weight) administered orally via drench applicator; eprinomectin (5 mg/ml) pour on[d] (1 ml/22 lb (10 kg) body weight) externally applied with pour on applicator; trenbolone acetate

(40 mg) and estradiol (8 mg) implant[e] administered SC in the backside of the left ear using implant gun.

Immediately following the standard arrival processing protocol, steers were allocated and administered one of two antimicrobials given as metaphylaxis for BRD prior to leaving the chute: gamithromycin (GAM; 150 mg/ml)[f] (2 ml/110 lb (50 kg) body weight) administered SC in left neck (front of shoulder), or ceftiofur crystalline free acid (CCFA; 200 mg/ml)[g] (1.5 ml/100 lb (45.5 kg) body weight) administered SC in the middle third or base or posterior aspect of the right ear.

Prior to study initiation, pastures (n = 16; experimental unit) were allocated to treatment by randomly assigning the first pasture to a treatment group and systematically assigning every other pasture to an alternate treatment group until all 16 pastures were assigned a treatment group. When calves were enrolled, the first 15 steers through the chute were randomly allocated to one of the 16 pastures and, consequently, to their pre-assigned treatment group. The same allocation order was used for each subsequent group of 15 steers through the chute until all enrolled calves were allocated to a pasture. Therefore, 120 steers were randomized to eight pastures for each treatment group, and each pasture housed 15 steers. All random numbers were generated in Microsoft Excel using the RAND function. Following enrollment in the study (day 0), the only additional processing was to collect individual body weights on study days 30 and 59. In the event steers were removed from the study prematurely, weights were to be obtained prior to removal if possible.

Housing and feeding

Steers were housed in approximately 54 ft x 54 ft grass lots attached to the assigned study pasture for five days following processing and metaphylactic treatment to allow for ease of observation and to acclimate the calves. Beginning on study day 6, calves had access to approximately 20-acre pastures joined to each of the grass lots. Each study pasture was equipped with at least one feed bunk and one waterer. Calves had *ad libitum* access to mixed grass hay, water, and minerals throughout the trial; additionally, calves were supplemented once daily with a creep feed ration at approximately 1% of the total body weight per pasture. Approximately halfway through the trial (day 31), all pastures received 90.2 lb (41 kg) of a

grain ration daily for the remainder of the study to optimize feeding logistics at the facility. Steers were housed and maintained on pasture for the duration of the trial.

Animal health

Prior to study initiation, the Boehringer Ingelheim Institutional Animal Care and Use Committee approved the care and use of cattle in this study as defined by the protocol. Steers were observed for clinical symptoms associated with BRD by trained personnel, who were masked to treatment allocation, twice daily from allocation (day 0) to day 13 and then once daily from day 14 until completion of the study on day 59. The clinical assessment included observations of the following symptoms: 1) increased respiratory rate, 2) depression, 3) nasal or ocular discharge, 4) cough and gait abnormalities. Based on these observations, steers were assigned a clinical assessment score (CAS) using a modified DART scoring system as follows[22, 30]: 0 = no symptoms associated with BRD were present, 1 = mild presentation of one or two symptoms, 2 = mild presentation of more than two symptoms or severe presentation of one or two symptoms, 3 = severe presentation of more than two symptoms, 4 = very severe presentation of several symptoms.

The post-metaphylaxis interval (PMI), defined as the period of time between metaphylaxis and when calves were eligible for further treatment, was designated as eight days for both treatment groups. Steers were first treated with a single-dose of florfenicol (300 mg/ml)[h] administered per label (6 ml/100 lb (45.5 kg)) SC in the neck if: 1) assigned a CAS of 1 or 2 and had a rectal temperature ≥104˚F, or 2) assigned a CAS of 3 or 4 regardless of rectal temperature. Following the PMI, steers assigned a CAS of 1 or 2 that had a rectal temperature of ≤ 103.9˚F were returned to their home pasture without BRD treatment. The post-treatment interval for florfenicol[h] was designated as four days. However, if after two days following treatment with florfenicol[h], a steer was assigned a severity score 3 or 4 then it could be administered a second BRD treatment. The second BRD treatment was a single-dose of enrofloxacin (100 mg/ml)[j] administered SC and dosed per label (1.1 to 2.3 ml/100 lb (45.5 kg) body weight) . If calves did not respond after treatment with enrofloxacin[j] they were to be pulled for further evaluation of respiratory disease and removed from the study if deemed chronic or unable to perform. Calves that exhibited illness with clinical signs not consistent with BRD were to be evaluated and

treated appropriately by the attending veterinarian. The attending veterinarian was masked to treatment group and thus granted clinical discretion in all instances to deliver appropriate care.

Measurements and calculations

The performance and clinical outcome variables of interest were average daily body weight gain (ADG), and BRD treatment morbidity, treatment success, mortality, and case fatality. All analyses were conducted at the pasture-level. The outcome variables were calculated (for each pasture) using the following general formulas:

$$\text{ADG (deads-out)} = \frac{\text{mean cattle weight at end – mean initial total cattle weight}}{\text{\# days on trial}}$$

$$\text{ADG (deads-in)} = \frac{\text{total cattle weight at end – initial total cattle weight}}{\text{\# head days on trial}}$$

$$\text{BRD morbidity} = \frac{\text{\# calves treated for BRD during trial period}}{\text{\# calves allocated to pasture}}$$

$$\text{Treatment success} = \frac{\text{\# BRD treated calves that were not retreated, BRD dead or chronic}}{\text{\# calves treated for BRD during the trial period}}$$

$$\text{BRD mortality} = \frac{\text{\# calves dead from BRD during trial period}}{\text{\# calves allocated to pasture}}$$

$$\text{Overall mortality} = \frac{\text{\# calves dead regardless of cause}}{\text{\# calves allocated to pasture}}$$

$$\text{Case fatality} = \frac{\text{\# calves treated for BRD that died of BRD}}{\text{\# calves treated for BRD}}$$

Economic assessment

The study protocol called for a comprehensive partial budget analysis, where standardized prices were used for all costs and revenues, and a corresponding net revenue was calculated for each pasture (experimental unit). Labor that was applied equally to all pasture groups, facilities, equipment, and other fixed costs were not included. A standardized purchase price for all study cattle (n = 240) of $161.08 per hundred-weight ($/cwt) was used, based on an average of USDA Agriculture Marketing Service (AMS) reports for 500-550 lb, medium to large frame steers sold in Missouri between September 15th and October 15th 2017.[25] Product costs were estimated from

an online distributer[28] for the following processing supplies and products: numbered ear tags, *Clostridium chauvoei-septicum-novyi-sordellii-perfringens Types C & D* bacterin-toxoid[a] , modified-live Bovine Rhinotracheitis-Virus Diarrhea-Parainfluenza 3-Respiratory Syncytial Virus Vaccine with *Mannheimia haemolytica* toxoid[b], oxfendazole oral suspension[c], eprinomectin pour-on[d] , trenbolone acetate and estradiol implant[e], gamithromycin[f] (GAM), ceftiofur[g] (CCFA), florfenicol[h], and enrofloxacin[j]. Additional standardized input costs included: a chute processing charge including product-deliver equipment and consumables such as needles, syringes, and implant guns ($2.00/head), grain cost ($219.65/ton; Twillman Feed Service), hay ($96.00/ton)[27] and mineral supplementation ($28.00/50 lb)[i], pasture lease costs ($10.50/head/month)[15], pull and temperature charge for animals identified as sick ($3.00/head), and a mortality disposal fee ($25.00). The value for individual animals that were removed prior to the study end for illness or lameness were assumed to be valued based on an average discount of 30% of the standardized purchase price.[4] A standardized sale price for cattle finishing the study of $140.86/cwt was an average price from USDA AMS reports for 650-700 lb, medium to large frame steers sold in Missouri between November 15th and December 15th 2017.[26] Associated total costs and revenues per pasture were calculated using the standardized prices indicated above, multiplied by pasture-level study measurements including weight and/or number of head per pasture, and then a total net return per pasture was expressed on a per-head enrolled basis (deads-in). In addition, a similar analysis, utilizing the same cost and revenue values, was performed with a dataset that excluded cattle that died or were removed during the study period (deads-out) since all deaths and removals were attributed to non-BRD causes. Net return values, on a per-head basis, for each pasture were used for statistical analysis as described below.

Statistical analysis

General and generalized linear models were used for all analyses. Data were coded so the data analyst (DR) was blinded during analysis. Data were formatted for pasture-level analyses. Models were fitted using binomial (e.g. health events) or normal (e.g. body weight, net return) distributions, maximum likelihood estimation, complimentary-log-log link, Kenward-Roger degrees of freedom and Newton-Raphson and Ridging optimization procedures (Proc GLIMMIX SAS 9.3). Fixed effects included the treatment structure. Treatment group means and standard

errors of the means (back-transformed to the original scale for generalized models) are reported. Per protocol, treatment effects were considered significant when *P* values were $\leq$ 0.10.

Results

Two hundred-forty steers, body weights ranging between 389.4 and 717.2 lb (177.0 to 326.0 kg) at allocation, were randomly allocated to 16 total pastures. Average cattle body weight at allocation, per pasture, was 537.5 lb (244.5 kg) and ranged from 504.5 to 572.1 lb (229.3 to 260.1 kg). At allocation, there was no evidence that treatment groups differed significantly with respect to day 0 body weight (Table 2-1). Means and standard errors of the means by treatment group are reported in Table 2-1 for allocation and performance variables.

Most of the allocated steers finished the 59-day study period (232 of 240 allocated); eight died or were removed from the trial prematurely due to health reasons (GAM, n=4; CCFA, n=4). The two mortalities, both in the GAM group, were attributed to non-BRD causes. One steer, found dead on study day 8, had a history of lameness and persistent diarrhea, and gross necropsy diagnosis was enterotoxemia. The other death occurred while the animal was being moved from pasture for evaluation and treatment. The diagnosis at necropsy was central nervous system disorder or cardiac failure; lung tissues were sent to the University of Missouri, Veterinary Medical Diagnostic Laboratory where culture and PCR results were found to be negative for all major bacterial (*Pasteurella spp., Mannheimia haemolytica, Histophilus somni*) and viral (Parainfluenza Type-3 Virus, Bovine Respiratory Syncytial Virus, Bovine Viral Diarrhea Virus, and Infectious Bovine Rhinotracheitis) BRD pathogens, respectively. Of the six steers removed (GAM, n=2; CCFA, n=4) from the study: four were due to lameness and two were due to persistent diarrhea. One steer, removed on day 14 for persistent diarrhea, depression, and anorexia, was sent to the University of Missouri Veterinary Medical Diagnostic Laboratory, euthanized and necropsied; post-mortem diagnosis was Bovine Viral Diarrhea (BVD) confirmed by PCR. Diagnostics were not performed on any other animals.

On study day 30, weights were collected from 234 calves (GAM, n=116; CCFA, n=118). The day 30 body weights for GAM and CCFA treatment groups were 632.07 lb (287.30 kg) and 612.02 lb (278.19 kg), respectively ($P = 0.20$). Including all steers on trial, from study days 0 to 30, ADG was 2.46 lb (1.12 kg) and 2.17 lb (0.99 kg), for the GAM and CCFA groups, respectively ($P = 0.41$). However, in steers that finished the study, excluding non-BRD removals

and deaths, from study days 0 to 30, ADG was 3.14 lb (1.43 kg) and 2.49 lb (1.13 kg), for the GAM and CCFA groups, respectively ($P = 0.04$). The final body weights (day 59) obtained included 232 calves (GAM, n=116; CCFA, n=116) and were 708.79 lb (322.18 kg) and 689.39 lb (313.35 kg) for the GAM and CCFA groups, respectively ($P = 0.18$). From study days 0 to 59, including all steers enrolled to the study, ADG was 2.55 lb (1.16 kg) and 2.23 lb (1.01 kg) for the GAM and CCFA groups, respectively ($P = 0.11$). Excluding removals and deaths due to non-BRD illness, the GAM group significantly outgained the CCFA group throughout the study; ADG was 2.90 lb (1.32 kg) and 2.57 lb (1.17 kg), for GAM and CCFA groups respectively ($P = 0.03$). Body weight means did not vary significantly between treatment groups on days 0, 30, and 59 (Table 2-1).

Treatment group means for health and economic outcome variables are reported in Table 2-2. Sixteen total steers (GAM, n=3; CCFA, n=13) were given an initial treatment (florfenicol[h]) for BRD; no BRD treatments were given after day 28 of the trial. The three steers in the GAM group received CAS of 1 (n=1) or 2 (n=2), and the 13 steers in the CCFA group received a CAS of 1 (n=5) or 2 (n=8); no calves received as CAS of 3 or 4. However, six febrile steers with clinical signs of respiratory disease were treated within the PMI period (3 each on study days 5 and 6) based on the clinical discretion of the masked veterinarian (GAM, n=1; CCFA, n=5). In steers treated for BRD, rectal temperatures for the GAM and CCFA groups ranged from 104.3 to 104.5˚F, and 104.1 to 107.1˚F, respectively. First treatment (BRD) morbidity was significantly different among groups with the mean for the CCFA group approximately 4-fold higher than the mean for the GAM group (Table 2-2). All calves treated for BRD recovered after the initial treatment; therefore, no calves were treated twice for BRD during the trial period, and treatment success and case fatality were numerically equal for both groups.

Overall mean net return per head, including removals and deaths unrelated to BRD, for steers in the GAM ($2.07 ± 6.83) and CCFA (-$15.70 ± 6.83) groups were significantly different ($P = 0.09$). Mean net return per head for steers that finished the study (excluding deaths and removals due to non-BRD reasons) was significantly higher ($P \leq 0.01$) for GAM ($22.34 ± 6.75) versus CCFA (-$6.67 ± 6.75) steers (Table 2-2).

Discussion

Results from this randomized trial of auction market-derived feeder steers backgrounded on pasture demonstrated differences in health, performance and economic outcomes between steers given metaphylaxis with gamithromycin and ceftiofur crystalline free acid for the control of BRD. Overall, BRD clinical morbidity was lower than expected, and the relatively few cattle that died or were removed were all attributed to non-BRD causes. Although BRD incidence following metaphylaxis was relatively low, it was significantly lower for the GAM steers compared to steers administered CCFA. Despite relatively low clinical morbidity, ADG in steers that finished the study was significantly greater for the GAM steers than those given CCFA; perhaps reflecting impacts of metaphylaxis on subclinical disease. The net economic return per head allocated to the study, which captures costs due to both health and performance, was greater for cattle given GAM versus those given CCFA, whether calculated on a "deads-in" or "deads-out" basis.

Relatively few cattle were observed with clinical BRD in this study, despite selecting a study population that was deemed to be high-risk for BRD and appropriate for receiving metaphylaxis upon arrival to a stocker facility. Ives and Richeson (2015) defined high-risk calves as: light-weight, recently weaned, highly commingled or of auction market origin, subjected to long transport time, and have an unknown health history.[14] The population of 240 mixed-breed beef steers for this study: ranged in body weight from 389.4 to 717.2 lb (177.0 to 326.0 kg), were commingled, were purchased from a livestock auction in southwest Missouri, were transported approximately 3.5 hours to the study facility, and had unknown health histories. While these study animals had known risk factors of BRD, the observed BRD morbidity and mortality following metaphylaxis were lower than expected. One of the health management challenges in this type of feeder cattle population is the uncertainty and variability in observed versus predicted BRD risks, which results in metaphylaxis often being an effective risk management tool.[14, 17] It is plausible that the relatively low BRD morbidity observed in this study could be due to decreased stressors or between-animal contact rates in pasture cattle compared to more intensively reared cattle in feedlot environments.[9, 17] It is also worth noting that both of these two antimicrobials, GAM and CCFA, may have been efficacious in reducing BRD, but in this study, that cannot be determined without a negative control group for comparison.

In this trial, sixteen steers were treated for BRD within the first 28 days following metaphylaxis, which is consistent with Buhman et al. (2000) who reported that approximately 91% of calves with BRD were diagnosed within the first 27 days after arrival.[7] Health and performance outcomes in this study were observed over a 59-day study period, which is reflective of common backgrounding periods in the industry, and beyond the time-frame when most cases of BRD occur.[10] The protocol-defined PMI for this trial was eight days, which would have inherently skewed the time distribution of initial BRD treatments and potentially impacted measures of severity or first treatment success. There were six febrile steers with clinical signs of BRD that were treated within the PMI at the clinical discretion of the attending veterinarian. The impact of those treatments relative to PMI and the distribution and severity of clinical disease within the population is unknown; however, by effectively masking the clinician to metaphylaxis groups, there would be no bias as to comparisons of observed outcomes between treatment groups.

There are relatively limited published data regarding effects of metaphylaxis for BRD in stocker cattle.[2, 21] However, metaphylaxis with GAM has been shown to reduce BRD morbidity in feedlot cattle, as compared to untreated animals or animals receiving other antimicrobials including oxytetracycline, tulathromycin, tilmicosin and CCFA.[2, 3, 16, 18, 19] Metaphylaxis with ceftiofur crystalline free acid in feedlot and stocker cattle has been compared to tilmicosin but there have been conflicting results in terms of relative efficacy.[5, 21] In a recent meta-analysis, the estimated odds of BRD morbidity were lower for GAM than CCFA metaphylaxis (odds ratio = 0.73, 95% credibility interval (0.29-1.55)) from day 1 to day 60; however, there was only one direct comparison between GAM and CCFA.[1] Amrine et al. (2014), directly compared GAM and CCFA in a study population comprised of both feedlot and stocker calves in multiple sites located in Missouri and Oklahoma.[2] Although that direct comparison of GAM and CCFA included a mixed population of calves in both feedlot and stocker production systems, the overall results observed in that study were consistent with what was observed in the present study.[2] They reported that calves administered metaphylaxis with GAM gained significantly more weight and resulted in fewer animals pulled for treatment than calves receiving CCFA for metaphylaxis.[2] In the study reported here, the significant difference in ADG for steers finishing the study was relatively substantial given the relatively low clinical burden of BRD in this study population.

To the authors' knowledge this is the first reported partial budget analysis directly comparing economic impacts of GAM and CCFA metaphylaxis. The partial budget approach for the economic analysis was defined *a priori* in the protocol, but given the lack of previously published data it was unknown whether a difference between the groups would be observed. However, given the observed differences in clinical morbidity, and weight gains in particular, it is perhaps not unexpected that economic differences were demonstrated. It has been well-established that BRD impacts performance and subsequently net returns in feedlot cattle.[6, 8, 13, 20] However, performance data relating to metaphylaxis for BRD in a stocker system are relatively sparse.[2] The economic differences observed in this study were relatively substantial and statistically significant, whether calculated on a deads-in or deads-out basis, and even though the magnitude of the estimated returns differed among the two approaches (as expected), both were consistent in that mean differences favored the group receiving GAM metaphylaxis.

Conclusions

This randomized trial was unique in that it directly compared health, performance, and economic impacts of metaphylaxis with gamithromycin and ceftiofur crystalline free acid for control of BRD in auction market-derived stocker calves backgrounded for approximately two months on pasture. Even with relatively low overall BRD morbidity, GAM was more effective than CCFA at reducing clinical morbidity. In addition, after excluding the few steers that died or were removed due to non-BRD reasons, steers receiving metaphylaxis with GAM significantly outperformed CCFA steers with respect to ADG over the entire study period. There was no evidence of significant differences in other health outcomes, but across the whole study population there were no deaths or removals attributed BRD and all steers with clinical BRD recovered after first treatment. The overall estimated net economic return per head was better for steers given GAM compared to CCFA whether removals and deaths unrelated to BRD were included in the analysis or not. Overall, steers in this study that were administered metaphylaxis with GAM had improved health, weight gain, and economic return as compared to those administered CCFA.

Table 2-1. Average daily gain of high-risk steers treated with gamithromycin or ceftiofur crystalline free acid for prevention of bovine respiratory disease.

Item	GAM[†]	CCFA[†]	*P* value
Day 0 Weight, lb	537.90 (7.08)	537.19 (7.08)	0.94
Day 30 Weight[‡], lb	632.07 (10.57)	612.02 (10.57)	0.20
Day 59 Weight[‡], lb	708.79 (9.83)	689.39 (9.83)	0.18
ADG Day 0-30, lb, (deads-out[≠])	3.14 (0.20)	2.49 (0.20)	0.04
ADG Day 0-59, lb, (deads-out[≠])	2.90 (0.09)	2.57 (0.09)	0.03
ADG Day 0-30, lb, (deads-in[±])	2.46 (0.24)	2.17 (0.24)	0.41
ADG Day 0-59, lb, (deads-in[±])	2.55 (0.13)	2.23 (0.13)	0.11

Sixteen pastures were randomly allocated to GAM (n = 8) and CCFA (n = 8). Two-hundred forty steers were randomized to treatment (GAM, n = 120; CCFA, n = 120), yielding 15 steers per pasture.

[†]GAM = Gamithromycin (Zactran®), CCFA = Ceftiofur crystalline free acid (Excede®)

[‡]Includes only the calves that were available for weight measures on days 30 and 59 (removals and dead cattle excluded)

[≠]Includes only the weights of calves that finished the study, excluding deaths and removals due to non-BRD reasons

[±]Includes all calves available for weight measures including deaths and removals due to non-BRD reasons

Table 2-2. Health and economic outcome variables of gamithromycin or ceftiofur crystalline free acid metaphylaxis treatment of high-risk stocker steers.

Item	GAM[†]	CCFA[†]	*P* value
First BRD treatment morbidity[‡], %	2.50 (1.43)	10.83 (2.84)	0.03
Second BRD treatment morbidity, %	0	0	-
First treatment success, %	100	100	-
BRD death loss, %	0	0	-
BRD case fatality, %	0	0	-
Overall death loss[≠], %	1.67 (1.17)	0 (0)	0.97
Non-BRD removals[±], %	1.67 (1.17)	3.33 (1.64)	0.43
Net return[γ] (deads-out[≠]), $	22.34 (6.75)	-6.67 (6.75)	0.01
Net return[γ] (deads-in[*]), $	2.07 (6.83)	-15.70 (6.83)	0.09

[†]GAM = Gamithromycin (Zactran®), CCFA = Ceftiofur crystalline free acid (Excede®)

[‡]Calves treated with florfenicol (Nuflor®)

[≠]Died of causes other than BRD (total = 2); 1 due to enterotoxemia, 1 due to central nervous system disorder or cardiac failure

[±]Removed for causes other than BRD (total = 6); 4 due to lameness, 2 due to persistent diarrhea.

[γ] Net return was estimated for each pasture using standardized prices for all costs and revenues, fixed costs were not included.

[≠]Includes net return only for the calves that finished the study, excluding deaths and removals due to non-BRD reasons

[*]Includes net return on all calves on trial including deaths and removals due to non-BRD reasons

Pharmaceuticals and manufacturers

[a]Alpha-7®, Boehringer Ingelheim, St. Joseph, MO

[b]PYRAMID® 5 + Presponse® SQ, Boehringer Ingelheim, St. Joseph, MO

[c]Synanthic® Suspension 22.5% Bovine Dewormer, Boehringer Ingelheim, St. Joseph, MO

[d]Ivomec® Eprinex® Pour-On, Boehringer Ingelheim, Duluth, GA

[e]Revalor®-G, Merck, Madison, NJ

[f]Zactran®, Boehringer Ingelheim, Duluth, GA

[g]Excede®, Zoetis, Kalamazoo, MI

[h]Nuflor®, Merck, Madison, NJ

[i]AMPT-A 54229™, ADM Animal Nutrition, Quincy, IL

[j]Baytril® 100, Bayer, Shawnee, KS

Declaration of conflicting interests

The author(s) declared the following potential conflicts of interest with respect to the research, authorship, and/or publication of this article: Drs. M. Liebstein and P. Thompson are employees of Boehringer Ingelheim®. Dr. D. Renter has had previous research or consulting paid by both Boehringer Ingelheim® and Zoetis®

Funding

This study was funded by Boehringer Ingelheim®.

Acknowledgements

The authors would like to thank the following individuals for their contributions to this research: Doug Ensley, D.L. Step, Jonatan Bader, Roxanne Gelven, Paul Dorr, Tim Underwood, Derrick Davidson, Grady Bartley, Courtney Williman, and Jeri Rippeto.

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Chapter 3 - A complete cross-over design evaluating canine acceptance of Carprieve® and Rimadyl® carprofen chewable tables in healthy dogs

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(Published in *BMC Veterinary Research*, 2019 15:394)

Abstract

Background: Osteoarthritis affects nearly 20% of all dogs greater than one year of age. Clinical signs include pain, discomfort, lameness, and ultimately lead to disability. Although there is currently no known cure, there are many therapeutic options that can slow the progression and alleviate the associated signs. There is ample supportive evidence demonstrating the efficaciousness of carprofen, a non-steroidal anti-inflammatory drug, in managing signs of osteoarthritis. Since the approval of the pioneer product (Rimadyl®, Zoetis; Kalamazoo, Michigan), the United States Food and Drug Administration (FDA) has assented to several other generic, bioequivalent products. The objective of this 2 x 2 complete cross-over design was to assess the acceptance of two bioequivalent carprofen liver-flavored chewable tablets (containing 25 mg carprofen), Rimadyl® and Carprieve® (Norbrook Laboratories Limited; Newry, Northern Ireland) in 37 healthy purpose-bred dogs.

Results: Overall, 73.0% (27/37) and 70.3% (26/37) of dogs voluntarily accepted Rimadyl® and Carprieve®, respectively. Considering acceptability tests paired by individual dog, 64.9% of

dogs (n = 24) voluntarily accepted both Rimadyl® and Carprieve® chewable tablets whereas 21.6% (8) of dogs denied or partially accepted both products offered. Three dogs (8.1%) fully accepted Rimadyl® but did not accept Carprieve®. Conversely, two dogs (5.4%) fully accepted Carprieve® but did not accept Rimadyl®. Canine acceptability did not significantly differ between Carprieve® and Rimadyl® carprofen chewable tablets ($P = 0.65$).

Conclusions: This study provides evidence that Carprieve® chewable carprofen tablets provide a similarly accepted bioequivalent formulation to the pioneer product, Rimadyl®.

Keywords: canine, carprofen, Carprieve, cross-over, dog, osteoarthritis, Rimadyl

Background

Osteoarthritis or degenerative joint disease is a complex syndrome that has been reported to affect approximately 20% of dogs over the age of one [1]. Clinical signs primarily include pain and discomfort which worsen over time ultimately resulting in lameness and disability [1]. Although there are currently no known cures, there are many treatments available to manage signs in dogs, including but not limited to non-steroidal anti-inflammatory drugs (NSAIDs), analgesics, nutraceuticals, functional foods, physical therapy, alternative therapies (e.g., stretching, acupuncture), and elective surgeries to slow progression or replace the joint entirely [2,3]. A systematic review synthesizing literature on therapeutic treatments for canine osteoarthritis found that NSAIDs, including carprofen, firocoxib, and meloxicam, effectively managed the symptoms associated with osteoarthritis [2]. Most of the published literature pertained to studies evaluating Rimadyl® (Zoetis; Kalamazoo, Michigan), the pioneer carprofen product [4-9]. In addition to Rimadyl®, the United States Food and Drug Administration (FDA) has approved several bioequivalent, generic carprofen products for commercial use [10].

Although the pharmacokinetics are considered bioequivalent between generic products and Rimadyl®, acceptance of the product and pet owner compliance to the treatment protocol, also crucial to drug efficacy, are not guaranteed [11]. Pet acceptability facilitates convenience of treatment administration and protocol compliance by the pet owner [12]. Veterinary drug products, including carprofen, come in a variety of presentations including, but not limited to: tablets, caplets, chewable tablets, and injectable solutions. While treats exist to house non-

chewable formulations or ease treatment administration to dogs resisting oral medication can be effective, those products add additional costs for the pet owner and may contribute to known causes of arthritis such as obesity. Developing highly palatable formulations, measured in terms of acceptance and preference, are at the forefront of pet food and orally administered veterinary drug product development. Canine acceptability, notably voluntary consumption, is especially important with medications that are administered daily for long periods of time for chronic conditions, such as osteoarthritis [12]. In products that are pharmacologically bioequivalent and are similarly accepted by the target species, the more affordable option may be more attractive depending on the financial means of the pet owner. Costs associated with veterinary care influence pet owner compliance to veterinary prescribed treatment protocols and ultimately, the quality of life of the pet. One in five pet owners admitted to taking one of these cost-cutting steps, 1) delayed purchasing of prescribed prescriptions, 2) used a less than recommended prescription dose, or 3) declined purchasing a medication their pet was prescribed altogether [13]. Ultimately, a more affordable veterinary product offers a better alternative than a pet being under-dosed or withheld treatment due to the costs of the pioneer product.

Carprieve® (25 mg carprofen; Norbrook Laboratories Limited; Newry, Northern Ireland) chewable tablets are an approved generic of Rimadyl® chewable tablets to treat symptoms associated with osteoarthritis and manage pain following surgery in dogs. While the safety, efficacy, and bioequivalence of the carprofen products was demonstrated prior to receiving initial FDA approval, canine acceptance between Rimadyl® and Carprieve® chewable tablets has not been directly evaluated. Therefore, the objective of this study was to evaluate and compare the acceptability of two liver-flavored carprofen products (Carprieve® and Rimadyl® 25 mg chewable tablets) in 37 healthy purpose-bred dogs using a 2 x 2 complete cross-over design.

Methods

Study population and study design

The study population consisted of healthy purpose-bred dogs at least one year of age; there were no restrictions on breed, weight, or sex (spayed female, neutered male, intact female/male). Dogs were sourced from an internal research colony at the Veterinary and Biomedical Research Center, Inc. (VBRC, Inc.; Manhattan, KS). Prior to study enrollment, all

dogs were physically examined by the attending veterinarian and a chemistry panel to screen for liver and/or kidney abnormalities. Dogs were randomly assigned to Group I or Group II, and thus assigned two different types of carprofen chewable tablets for acceptance tests on day 0 and day 7. Randomization was performed using a random number generator in Microsoft® Excel® 2016 (Windows 10).

The study was designed as a 2 x 2 complete cross-over design (AB/BA design) where each dog was randomly offered Carprieve® or Rimadyl® on day 0 and, after a seven-day "wash-out" period, offered the alternate carprofen chewable tablet. All dogs were weighed prior to acceptability testing on study day -1 to determine appropriate dose for testing. To avoid potential overdose and adverse events, the attending veterinarian recommended using the twice daily dose (2.2 mg/kg) as a single dose for this study. Further, it was determined that doses should be rounded to the nearest half or whole tablet, in this way the division of tablets would be minimized to no more than one division with the assumption being that the number of divisions could potentially confound the acceptance of the tablet. Adverse events associated with administration of carprofen per label include vomiting, diarrhea, changes in appetite, lethargy, behavioral changes, and constipation. Therefore, dogs were offered either a half (12.5 mg; bodyweight ≤ 10.2 kg) or whole carprofen chewable tablet (25.0 mg; bodyweight >10.3 kg) according to their body weight on day 0. Dogs were not reweighed prior to study day 7 and were offered the same dosage for both brands of chewable tablets. General health observations were performed twice daily by animal care staff on all dogs for the seven-day study period.

Animal care and housing

This study was conducted as a non-good laboratory practice (non-GLP) study at VBRC, Inc., a GLP compliant and fully accredited Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) facility. The study protocol was internally reviewed and approved by the Norbrook Laboratories Limited Research and Development personnel. Additionally, the study protocol was submitted to the VBRC, Inc. Institution for Animal Care and Use Committee (IACUC) where the protocol received approval prior to study initiation. Dogs were housed indoors, individually or paired with the same sex, in raised, stainless-steel kennels with access to a resting pad, water, food, and toys for enrichment. Indoor facilities were maintained according to the Guide for the Care and Use of Laboratory Animals, with an ambient

temperature of 10.0˚C to 26.7˚C and a 12:12 hour light:dark light cycle throughout the study [14]. All dogs received human interaction, as one form of provided enrichment, at minimum, twice per day. A commercial dry-food diet was fed twice daily with at least 8 hours between feedings, based on body weight; dogs housed in same-sex pairs were separated for acceptance testing and feedings. Water was provided *ad libitum*.

Acceptance test

Acceptance testing was conducted approximately one hour prior to the morning feeding time outlined per testing facility site standard operating procedures. The carprofen products were stored in a padlocked safe ensuring the products kept dry, out of direct-sunlight, and were maintained at room temperature (20˚C to 25˚C). The product labels were covered with a handwritten label containing an "A" or "B" by an unmasked individual (KD) so the product could not be identified by study personnel. Prior to acceptability testing, the appropriate number of chewable tablets were halved by an unmasked individual (KD). The whole or half tablets were removed from their relabeled original container, using a pair of forceps with one pair dedicated to each brand by the unmasked individual (KD) and placed into the gloved right hand of the acceptability test administrator who was blinded to treatment (DV). Gloves were changed between each individual acceptance test to keep acceptability tests consistent and unbiased for all dogs with no potential for carryover of scent or taste from the previous test article or dog. Acceptance of the tablets was assessed separately for each individual dog by offering the carprofen chewable tablet (Rimadyl® or Carprieve®) in a clean bowl and giving the dog the opportunity to voluntarily prehend and ingest the tablet. The dogs were given 60 seconds, measured with the use of a handheld stopwatch, to ingest the tablet. If the tablet was not completely consumed after 60 seconds it was then offered by the right gloved-hand of the test administrator (DV) for an additional 60 seconds without encouragement or coercion to ingest the offered tablet. Testing was terminated if the dog did not voluntarily ingest the tablet in the two minutes allotted and the remaining tablet was disposed of. Acceptability outcomes were recorded as "full" or "partial/none". Acceptability was recorded as "full" if the dog completely consumed the tablet offered from 1) the bowl within 60 seconds, and if not accepted from the bowl, 2) the right gloved-hand within 60 seconds. If the dog did not completely consume the offered tablet or

did not prehend the tablet at all when offered in the bowl or by gloved-hand, the acceptability outcome was recorded as “partial/none”.

Sample size determination

A total of 74 dogs, or 37 in cross-over design, were required to detect a difference of 15% or greater in acceptability between two products (Rimadyl® and Carprieve®) with a 95% (α = 0.05) certainty that the difference is real and not due to chance alone with a type II error rate of 20% (β = 0.80), as calculated using a predicted acceptability of 95% [15].

Statistical analysis

Data were managed and stored in Microsoft® Excel® 2016. Carprofen chewable tablet brands were coded prior to statistical analysis, hence the individual (DV) performing analysis was blinded to treatment groups. The individual dog was considered the experimental unit. The outcome of the acceptability test consisted of “full” or “partial/none” for each carprofen tablet for each dog. Descriptive statistics were summarized using two-way frequency tables presenting acceptability by study day, and by carprofen product. To account for the cross-over design, acceptability test results were matched by dog and classified into one of the four categories: 1) neither tablet accepted, 2) both tablets accepted, 3) only Carprieve® accepted, or 4) only Rimadyl® accepted. A McNemar's Chi-squared (χ^2) test was performed in Stata® 12.0 (StataCorp LP, College Station, Texas), using the calculated frequencies of the four categories previously described, accounting for the 1:1 paired data. Odds ratios and exact Fisher confidence intervals were obtained. Differences in acceptability were considered significant if McNemar’s χ^2 $P \leq 0.05$.

Results

Study population demographics

Thirty-seven cross-bred Beagles, including 18 females and 19 males, were enrolled in this study. At enrollment, all dogs were clinically healthy as determined by pre-study physical exams and collection of a blood sample for a chemistry panel to ensure normal liver and kidney function. All study dogs had normal serum chemistry results. All study dogs were sexually-intact. This cohort represented purpose-bred dogs for use in research studies and were uniquely

identified via microchip technology. Dogs were single-sourced and housed at the study facility for approximately 12 months prior to study initiation and participated in other unrelated, non-terminal research studies. On average, dogs weighed 10.6 ± 1.7 kg (range = 7.9 to 13.6 kg) and were 1.7 ± 0.5 years of age (range = 1.0 to 2.5 years). All dogs remained healthy throughout the study period and no signs of gastrointestinal upset were observed. Overall, 37 individual acceptability tests were completed for each chewable carprofen tablet.

Acceptance test

The study was initiated on September 20, 2018 (day 0) and was completed on September 27, 2018 (day 7). Study population characteristics (e.g., dog identification, sex, age, weight), carprofen tablet size administered (e.g., half or whole), carprofen dose administered (mg/kg), and group allocation for all dogs are presented in Table 3-1. On study day 0, 19 and 18 dogs were offered Rimadyl® (Group II) or Carprieve® (Group I), respectively. After the seven-day "wash-out" period, 18 dogs were offered Rimadyl® (Group I) and 19 dogs were offered Carprieve® (Group II). Individual acceptability outcomes for days 0 and 7 for each dog are presented in Table 3-2. On study day 0, 67.6% (25/37) of dogs fully consumed the carprofen tablet offered, either Rimadyl® or Carprieve®, whereas 32.4% (12/37) of dogs did not accept either product (Table 3-3A). Similarly, on study day 7, 75.7% (28/37) of dogs fully consumed the carprofen tablet and 24.3% (9/37) of dogs did not accept either product (Table 3-3A). The majority of dogs fully consumed Rimadyl® (73.0%; 27/37) and Carprieve® (70.3%; 26/37) tablets, whereas 27.0% (10/37) and 29.7% (11/37) dogs did not accept Rimadyl® and Carprieve®, respectively (Table 3-3B). The McNemar's χ^2 test indicated that acceptability did not significantly differ between Carprieve® and Rimadyl® carprofen tablets (McNemar's χ^2 $P = 0.65$; Fisher exact test $P = 1.00$) (Table 3-3C). Although not significantly different ($P = 1.00$), dogs offered Rimadyl® were 1.5 times (OR = 1.50; OR 95% confidence interval = 0.17 – 17.96) more likely to accept the tablet than dogs offered Carprieve®.

Discussion

In this study, we demonstrated that canine acceptance did not significantly differ between Rimadyl® and Carprieve® carprofen chewable tablets when administered to healthy purpose-bred dogs in a 2 x 2 cross-over design. Palatability testing of orally administered veterinary

pharmaceuticals is at the forefront of product development and marketing. Palatability testing includes two main categories: acceptance testing and preference testing. Acceptance testing is designed to assess voluntary intake and consumption whereas preference testing evaluates if the animal prefers one product over another. The most important measure, in terms of palatability, in veterinary pharmaceuticals is acceptability. Acceptability testing directly measures voluntary consumption and, subsequently, offers a measure of compliance to the treatment protocol by the pet owner [12]. Currently, there are no standardized methods for acceptability testing of veterinary pharmaceuticals; consequently, palatability studies are largely based on principles outlined by the pet food industry [12,16,17].

Cross-over designs are preferred in acceptability testing to optimize sample size and allow for an unbiased evaluation of multiple tablets using the same individual. Canine palatability, acceptance and/or preference, of carprofen chewable tablets has been evaluated using cross-over designs previously; with all studies involving Rimadyl® compared to other carprofen formulations of various presentations (e.g., chewable tablet, caplet, tablet) [18-20]. In one study, Rimadyl® was compared to two other carprofen products, Carprodyl® tablets (Ceva Animal Health; Amersham, United Kingdom) and Carprieve® caplets (formerly known as Norocarp® tablets), using acceptance and preference tests [18]. Following a complete cross-over design, 43 mixed breed dogs, aged between one to ten years old and weighing at least 10 kg, were randomly administered a carprofen tablet over two consecutive days [18]. Although not necessary to evaluate acceptability, and not included in other similar acceptability studies [18-20], a seven-day "wash-out" period was included in the present study to minimize the chance of conditioning the dogs to administration of the tablet, a presumed treat, so negative or favorable experiences did not interfere with observing the true acceptability of each tablet individually. Therefore, due to our study population and design limitations, we did not assess acceptability over a multiple day dosing regimen as would be typical for long-term osteoarthritis treatment in pets. To evaluate long-term acceptability outcomes typical of pets treated for osteoarthritis for Carprieve® and Rimadyl® chewable tablets, future research is warranted.

Payne-Johnson *et al.*, found that of 43 dogs, 90.7 and 48.8% voluntary accepted Rimadyl® chewable tablets and Carprieve® caplets, respectively [18]. Additionally, in the comparison between Rimadyl® chewable tablets and Carprieve® caplets, the acceptance tests were conducted using 75 mg and 50 mg carprofen chewable tablets, respectively [18]. It has

been documented that the concentration of active ingredient in the formulation, in this case—carprofen—can influence palatability [17]. While significant differences in acceptability and preference were observed in the previous study between Rimadyl® chewable tablets and Carprieve® caplets ($P < 0.005$), based on the product presentations compared, chewable tablets versus caplets, is not surprising [18]. In our study, we compared formulations of the same chewable tablet presentation formulated at 25 mg. In the present study, canine acceptance of Rimadyl® chewable tablets and Carprieve® chewable tablets was 73.0 and 70.3%, respectively. The chewable tablets in this study were formulated at 25 mg per tablet, however, the dose administered for acceptability testing was less than the recommended daily dose of 4.4 mg per kg of body weight (dosage administered ranged from 1.2 mg/kg to 2.4 mg/kg) but was approximate to the labeled halved daily dose of 2.2 mg/kg. Due to animal welfare concerns, given that our study population was healthy, we elected to not administer a complete target dose of carprofen, consistent with other carprofen acceptability studies in healthy dogs [18-20]. Thus, acceptability data should be interpreted with caution in the event where multiple chewable tablets would need to be given as treatment, as this study only administered half or whole tablets which may be more indicative of a dose given to a smaller dog.

In the present study, the study population of 37 dogs was very homogeneous in terms of age (1.7 ± 0.5 years of age) and breed (cross-bred Beagles) thus minimizing variability between dogs. Although this colony was readily available and purpose-bred for research, it has been documented, although anecdotally, that Beagles are a poor choice for use in palatability, namely preference studies; however, other extraneous factors such as inadequate acclimatization, laboratory versus in-home settings, and cultural differences such as use of treat rewards may outweigh any breed influence on palatability testing outcomes [12]. This study population may not be representative of typical pets or the target population of dogs experiencing a painful condition due to surgery or osteoarthritis but it does offer an unbiased estimate of acceptability of these two products. Dogs suffering from osteoarthritis, or recovering from surgery, may have a loss in appetite due to pain and stress which may ultimately impact acceptability compared to healthy, pain-free dogs [12]. Previous research (Norbrook Laboratories Limited, unpublished internal data) evaluated acceptability between Carprieve® and Rimadyl® 50 mg carprofen chewable tablets in 103 pet dogs with clinical symptoms requiring treatment by NSAIDs (e.g., hip dysplasia, spinal pain, osteoarthritis). Acceptability was assessed after a single administration

and no difference in acceptability was observed as 71.7 and 68.0% of dogs fully accepted Carprieve® and Rimadyl® chewable tablets, respectively (Norbrook Laboratories Limited, unpublished internal data). While these findings are comparable to our present study in healthy purpose-bred dogs, these chewable tablets were formulated at a higher dose (50 mg) and were offered to dogs experiencing a painful condition.

Carprieve® chewable tablets are an FDA approved bioequivalent product to Rimadyl® chewable tablets; therefore, Carprieve® has analogous pharmacokinetic properties, in addition to satisfactory safety and efficacy compared to Rimadyl®. A survey conducted by PetCareRx.com representing 1,100 pet owners from 440 households noted the impact of pet healthcare costs influencing veterinary care and treatment [13]. The current study provides evidence that acceptability to Carprieve® chewable tablets did not differ from Rimadyl® chewable tablets; however, Carprieve®, as a generic, is generally marketed at a price point below that of Rimadyl® [21]. Although the majority of pet owners (82%) admit they would consider paying almost any amount of money to keep their pets healthy, 21% of dog owners said they have scaled back on veterinary visits due to costs [13]. Additional findings reported that 20% of owners take cost-cutting measures in terms of veterinary prescribed medications by purposely under-dosing the pet or by delaying or refusing purchasing the medication altogether to save money. Annually, it is estimated that pet owners spend on average $611 per pet, and $935 when pets have a chronic condition [13]. If orally administered veterinary pharmaceuticals are palatable, easy to administer, and affordable, pet owners will be more likely to provide the necessary medication to their pets as prescribed ultimately improving the dogs and owner's quality of life.

Conclusions

In this 2 x 2 complete cross-over experimental study including 37 healthy cross-bred Beagles, canine acceptability did not significantly differ between Carprieve® and Rimadyl® chewable tablets. To be representative of acceptability of long-term NSAID treatment, future research is needed to evaluate acceptability between these two products when administered at the recommended daily dose over a longer duration to best represent acceptability and pet owner compliance.

List of abbreviations

NSAIDs: non-steroidal anti-inflammatory drugs

FDA: Food and Drug Administration

Declarations

Ethics approval and consent to participate:

Not applicable

Consent for publication:

Not applicable

Availability of data and material:

All data analyzed for this study are included in this published article.

Competing interests:

Authors KD and DR are employed by VBRC, Inc., the contract research organization where the study was conducted. NC is employed by Kansas State University. DV is co-employed by VBRC, Inc. and Kansas State University.

Funding:

This study was co-funded by Norbrook Laboratories Limited which manufactures and markets Carprieve® and VBRC, Inc. Representatives from Norbrook Laboratories Limited were involved in the study design but did not contribute to data collection, statistical analysis, or manuscript preparation.

Authors' contributions:

KD calculated sample size and served as the study investigator and attending veterinarian. DR contributed to sample size calculation and study design. DV performed the acceptability tests, analyzed the data (blinded to treatment), and significantly contributed to manuscript preparation. NC provided input for study design, oversaw the statistical analysis, and

significantly contributed to the manuscript preparation. All authors have contributed, read, and approved the submitted manuscript.

Acknowledgements

The authors would like to thank the animal caretakers, Beth Heimerman and Sevastian Bañuelos, for providing quality care to the dogs used in this study. Additional thanks to Breanna Kyle for data entry and coding for analysis.

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Table 3-1. Demographic characteristics, carprofen dose administered, and group allocation of the study population.

Group*	Dog ID	Sex±	Age, years	Weight, kg	Carprofen, mg≠	Dose, mg/kg
I	315-974	Female	1.0	10.4	25.0	2.4
I	439-468	Male	2.1	13.0	25.0	1.9
I	439-470	Male	1.2	11.6	25.0	2.2
I	440-118	Male	2.1	7.9	12.5	1.6
I	452-270	Female	1.5	8.1	12.5	1.5
I	540-556	Female	2.1	11.7	25.0	2.1
I	597-230	Male	2.0	12.7	25.0	2.0
I	597-674	Male	2.0	10.9	25.0	2.3
I	597-892	Male	1.1	11.3	25.0	2.2
I	600-010	Female	2.0	9.8	12.5	1.3
I	600-014	Female	2.0	9.4	12.5	1.3
I	600-236	Male	2.1	9.3	12.5	1.3
I	600-344	Male	2.0	9.8	12.5	1.3
I	600-816	Female	1.5	9.7	12.5	1.3
I	600-836	Male	1.0	13.3	25.0	1.9
I	600-934	Female	2.1	8.6	12.5	1.5
I	601-472	Female	2.1	8.4	12.5	1.5
I	601-663	Male	1.8	13.0	25.0	1.9
II	312-683	Male	1.0	11.4	25.0	2.2
II	312-987	Male	1.0	11.5	25.0	2.2
II	323-648	Female	2.5	9.3	12.5	1.3
II	439-977	Male	1.0	13.0	25.0	1.9
II	440-023	Female	2.0	11.9	25.0	2.1
II	440-104	Female	1.5	8.9	12.5	1.4
II	453-072	Male	1.0	13.6	25.0	1.8
II	597-303	Female	2.1	10.1	12.5	1.2
II	597-340	Female	1.6	9.4	12.5	1.3
II	597-362	Female	1.6	8.5	12.5	1.5
II	600-104	Male	2.1	10.3	25.0	2.4
II	600-324	Female	2.1	8.6	12.5	1.5
II	600-454	Male	1.0	12.7	25.0	2.0
II	600-779	Female	1.6	8.4	12.5	1.5
II	600-904	Male	2.1	9.9	12.5	1.3
II	601-341	Male	2.0	11.0	25.0	2.3
II	601-482	Female	2.0	10.7	25.0	2.3
II	601-928	Male	1.2	13.0	25.0	1.9
II	603-754	Female	2.1	10.3	25.0	2.4

*Group I was offered Carprieve® on day 0 and Rimadyl®; Group II was offered Rimadyl® on day 0 and Carprieve® on day 7.

±All dogs were unaltered (i.e., sexually intact)

≠25.0 mg indicates a whole tablet was offered, 12.5 mg indicates a half tablet was offered

Table 3-2. Acceptability testing results for individual dogs on days 0 and 7.

Dog ID	Day 0		Day 7	
	Treatment	Acceptability	Treatment	Acceptability
315-974	Carprieve	Full	Rimadyl	Full
439-468	Carprieve	Partial/none	Rimadyl	Full
439-470	Carprieve	Partial/none	Rimadyl	Partial/none
440-118	Carprieve	Partial/none	Rimadyl	Full
452-270	Carprieve	Full	Rimadyl	Full
540-556	Carprieve	Partial/none	Rimadyl	Full
597-230	Carprieve	Partial/none	Rimadyl	Partial/none
597-674	Carprieve	Partial/none	Rimadyl	Partial/none
597-892	Carprieve	Full	Rimadyl	Full
600-010	Carprieve	Full	Rimadyl	Full
600-014	Carprieve	Full	Rimadyl	Full
600-236	Carprieve	Full	Rimadyl	Partial/none
600-344	Carprieve	Full	Rimadyl	Full
600-816	Carprieve	Full	Rimadyl	Full
600-836	Carprieve	Full	Rimadyl	Full
600-934	Carprieve	Partial/none	Rimadyl	Partial/none
601-472	Carprieve	Full	Rimadyl	Full
601-663	Carprieve	Partial/none	Rimadyl	Partial/none
312-683	Rimadyl	Full	Carprieve	Full
312-987	Rimadyl	Partial/none	Carprieve	Full
323-648	Rimadyl	Partial/none	Carprieve	Partial/none
439-977	Rimadyl	Full	Carprieve	Full
440-023	Rimadyl	Full	Carprieve	Full
440-104	Rimadyl	Full	Carprieve	Full
453-072	Rimadyl	Full	Carprieve	Full
597-303	Rimadyl	Full	Carprieve	Full
597-340	Rimadyl	Full	Carprieve	Full
597-362	Rimadyl	Full	Carprieve	Full
600-104	Rimadyl	Partial/none	Carprieve	Partial/none
600-324	Rimadyl	Full	Carprieve	Full
600-454	Rimadyl	Full	Carprieve	Full
600-779	Rimadyl	Full	Carprieve	Full
600-904	Rimadyl	Full	Carprieve	Full
601-341	Rimadyl	Full	Carprieve	Full
601-482	Rimadyl	Full	Carprieve	Full
601-928	Rimadyl	Partial/none	Carprieve	Partial/none
603-754	Rimadyl	Full	Carprieve	Full

Table 3-3. Results of acceptability testing: A) Number of dogs fully or partially (or not) accepting either tablet by study day, B) Number of dogs fully or partially (or not) accepting a tablet by product, and C) Paired analysis of acceptability results

A. Number of dogs fully or partially (or not) accepting either tablet by study day

Study day	Acceptability Outcome		Total
	Partial/none	Full	
0	12	25	37
7	9	28	37
Total	21	53	74

B. Number of dogs fully or partially (or not) accepting a tablet by product

Treatment	Acceptability Outcome		Total
	Partial/none	Full	
Rimadyl®	10	27	37
Carprieve®	11	26	37
Total	21	53	74

C. Paired analysis of acceptability results by product

Carprieve®	Rimadyl®		Total
	Full	Partial/none	
Full	24	2	26
Partial/none	3	8	11
Total	27	10	37

Chapter 4 - Effectiveness of a direct-fed microbial product containing *Lactobacillus acidophilus* and *Lactobacillus casei* in reducing fecal shedding of *Escherichia coli* O157:H7 in commercial feedlot cattle

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(Published in *Foodborne Pathogens and Disease*, 2020, DOI: 10.1089/fpd.2020.2828)

Abstract

The objective of this study was to evaluate the effectiveness of a direct-fed microbial (DFM) product in reducing fecal shedding of *Escherichia coli* (*E. coli*) O157:H7 in finishing commercial feedlot cattle in Kansas (KS) and Nebraska (NE). Utilizing a randomized complete block design within feedlot (KS, n=1; NE, n=1), cattle were randomly allocated to 20 pens grouped in blocks of two based on allocation date, and then, within block, randomly assigned to treatment group (DFM or negative control). The DFM product was included in the diet at a targeted daily dose of 1 x 10^9 CFU *Lactobacillus acidophilus* and *Lactobacillus casei* combination per animal for at least 60 days prior to sampling. Feedlots were sampled for four consecutive weeks; weekly sampling consisted of collecting 20 pen-floor fecal samples per pen. Fecal samples were subjected to culture-based method for detection and isolation of *E. coli* O157, and positive samples quantified using real-time PCR. Primary outcomes of interest were fecal prevalence of *E. coli* O157:H7 and *E. coli* O157 super-shedding ($\geq 10^4$ CFU/ g of feces) prevalence. Data for each feedlot were analyzed at the pen-level using mixed models accounting for the study design features. Model-adjusted mean *E. coli* O157:H7 fecal prevalence (standard

error of the mean (SEM)) for DFM and control groups were 8.2% (SEM=2.2%) and 9.9% (SEM=2.5%) in KS, and 14.6% (SEM=2.8%) vs 14.3% (SEM=2.6%), in NE; prevalence did not differ significantly between treatment groups at either site (KS, *P*=0.51; NE, *P*=0.92). Mean *E. coli* O157 super-shedding prevalence for the DFM and control groups were 2.2% (SEM=0.7%) vs 1.8% (SEM=0.7%) in KS (*P*=0.66), and 6.7% (SEM=1.5%) vs 3.2% (SEM=1.0%) in Nebraska (*P*=0.04). In conclusion, administering the DFM product in the finishing diet of feedlot cattle did not significantly reduce *E. coli* O157:H7 fecal prevalence or super-shedding prevalence in study pens at either commercial feedlot.

Running title: DFM impact on *E. coli* O157:H7 fecal shedding in cattle

Key words: cattle, direct-fed microbial, DFM, *E. coli* O157:H7, feedlot, *Lactobacillus casei*, *Lactobacillus acidophilus,* summer, super-shedding.

Introduction

Seven Shiga toxin-producing *Escherichia coli* (STEC) serogroups, including serotype *E. coli* O157:H7, are considered adulterants in raw, non-intact beef products by the Food Safety and Inspection Service (USDA-FSIS, 2012). Based on United States data from 2000 to 2006, it is estimated that approximately 33% of human foodborne illnesses due to *E. coli* O157:H7 are attributed to ground beef (Withee *et al.,* 2009). Cattle shed these bacteria in their feces, and fecal material may contaminate the hides of cattle in the production environment, during transport, and/or in lairage, which then can serve as the primary source of carcass and subsequent beef product contamination (Fox *et al.,* 2008; Jacob *et al.,* 2010a; Loneragan and Brashears, 2005). Therefore, reducing the fecal shedding prevalence and concentration of *E. coli* O157:H7 in cattle conceivably reduces the risk of contamination of beef products.

A subset of cattle, termed “super-shedders”, shed *E. coli* O157:H7 at high concentrations ($\geq 10^4$ CFU/g of feces) and these animals have been shown to be associated with most of the within-pen transmission of *E. coli* O157:H7 in the cattle production environment (Omisakin *et al.,* 2003). However, super-shedding has been described as transient or intermittent, not continuous, in individuals over time (Munns *et al.,* 2014; Williams *et al.,* 2014); therefore, deeming individual animals as “super-shedders” may be a mischaracterization. While the role of

these super-shedding "events" in the feedlot environment is not completely understood, it is clear that they pose a risk to beef safety. Targeting *E. coli* O157:H7 in the bovine reservoir prior to harvest, offers an opportunity to decrease the bacterial load in the host and in the environment while reducing the potential for contamination of hides and subsequent food products.

Pre-harvest interventions to reduce *E. coli* O157:H7 fecal shedding in cattle have been at the forefront of beef safety research for over two decades (LeJeune and Wetzel 2007; Marder *et al.*, 2018). Evaluated pre-harvest interventions include diet interventions and management, direct-fed microbials (DFM), antimicrobials, and vaccines (Callaway *et al.*, 2013; Loneragan and Brashears, 2005). Despite conflicting research findings of various DFM products, a meta-analysis demonstrated that DFMs, including many strains of bacteria, yeast, molds, and combinations, may be effective as a pre-harvest intervention in reducing fecal prevalence of *E. coli* O157:H7 in beef cattle (Wisener *et al.*, 2015). Currently, there are limited data evaluating the impact of DFM products on the prevalence and concentration of *E. coli* O157:H7 in the production environment (Arthur *et al.*, 2010; Brown *et al.*, 2020; Stephens *et al.*, 2007a; Cernicchiaro *et al.*, 2010; Cull *et al.*, 2012). Thus, the objective of this study was to evaluate the effectiveness of a commercially available DFM product, containing a proprietary blend of *Lactobacillus acidophilus* and *Lactobacillus casei,* in reducing fecal shedding of *E. coli* O157:H7 and super-shedding events in finishing pens of commercial feedlot cattle in Kansas (KS) and Nebraska (NE) during summer months.

Materials and methods

Study population, design, and sample collection

Commercial feedlots were selected based on their willingness to conduct research, ability to feed a DFM product and a control diet concurrently, and capacity to fill 20 pens with cattle on a finishing diet during the summer. The study population was comprised of cross-bred beef cattle in 40 study pens, 20 pens per feedlot (10 per treatment group), with projected harvest dates between August and September 2018 at two commercial feedlots. One feedlot was located in KS (feedlot capacity = 30,000 cattle) and the other was located in NE (feedlot capacity = 16,000 cattle). Study pens were enrolled between April and May 2018. Cattle were procured, processed, and managed according to the standard operating procedures of each feedlot. Standard operation procedures of the commercial operations were followed to prevent mixing of the two study diets.

However, cattle were managed under typical commercial conditions and thus, DFM and control pens could share fence lines and waterers. Study pens in NE were confined to a single alley and treatment groups commonly shared fence lines and waterers. In contrast, at the KS site, blocks of cattle were housed in close proximity to each other, but study blocks were distributed throughout the feedlot, and the majority of study pens did not share fence lines with other study pens.

This field trial consisted of a randomized complete block design with pen as the experimental unit and repeated sampling. In each feedlot, cattle were randomly allocated, within arrival dates, to pens grouped in blocks of two (DFM or control); within block, pens were randomly allocated to DFM (n = 10) or control groups (n = 10). The total number of pens (20 per treatment group) were estimated based on design parameters and previous data described in Cull *et al.* (2012) assuming a mean *E. coli* O157:H7 prevalence of 40% and 25% for control and DFM groups, respectively ($\alpha = 0.05$, $\beta = 0.20$). Cattle in the DFM pens were administered the DFM product in their feed at a targeted daily dose of 1×10^9 CFU *L. acidophilus* and *L. casei* combination (50 mg per animal per day of BactaShield™; Legacy Animal Nutrition, LLC; Wamego, KS) whereas, cattle in the control pens received no DFM product. Feed testing was not conducted to determine as-fed DFM concentrations. Study pens were fed the allocated diet for at least 60 days prior to the first sampling.

Each of the enrolled pens were sampled weekly for four consecutive weeks with 20 fecal samples collected weekly from each pen. The KS feedlot was sampled during the first four consecutive weeks and the NE feedlot was sampled the following four consecutive weeks. A sample was comprised of approximately 30 g of freshly eliminated feces obtained off the pen-floor and collected in individual plastic bags (WHIRL-PAK®; Nasco, Fort Atkinson, WI) using plastic spoons. All fecal samples were placed in a cooler with ice packs, and transported to the Pre-harvest Food Safety Laboratory at Kansas State University for processing within 24h. One sampling crew collected all fecal samples for this study. At the time of sampling, data on pen conditions and weather also were documented using a standardized data capture form. Observed pen conditions were classified as dry/dusty, normal, wet, and very wet. Weather data from the National Weather Service mobile application included temperature (ºF), precipitation (yes or no), and humidity at the time of sampling for each pen.

Detection and quantification of *E. coli* O157:H7

Culture methods including the immunomagnetic separation (IMS) technique utilized have been described in detail previously (Dewsbury *et al.,* 2015). Following enrichment, IMS, plating on Sorbitol MacConkey agar supplemented with cefixime and potassium tellurite (CT-SMAC), and overnight incubation at 37˚C, putative colonies were tested for the O157 antigen by latex agglutination. If all subcultured colonies, maximum of six, tested negative for the O157 antigen by latex agglutination the sample was considered negative for *E. coli* O157:H7 and no further testing was done. If a subcultured colony tested positive for the O157 antigen by latex agglutination, it was tested by conventional multi-plex PCR assay targeting the *rfb*E, *fliC*$_{H7}$, *eae*, *stx*1, *stx*2, and *ehx*A genes (Bai *et al.,* 2010). A sample was considered *E. coli* O157:H7 if the isolate tested positive for *rfbE*, *fliC*$_{H7}$, *eae*, *stx*1 and/or *stx*2. Pre-enriched samples were subjected to quantitative PCR (qPCR; Noll *et al.*, 2015) to identify super-shedding events only if the enriched sample was positive for *E. coli* O157:H7. A sample was considered a super-shedding event if the pre-enriched sample yielded a qPCR (Noll *et al.*, 2015) average end-point threshold cycle for the *rfbE* gene that was less than or equal to 37.8 (*E. coli* O157 concentration $\geq 10^4$ CFU/g of feces).

Statistical analysis

Unadjusted *E. coli* O157:H7 sample-level prevalence was calculated as the number of fecal samples that tested positive for *E. coli* O157:H7 divided by the total number of samples subjected to culture methods. Similarly, unadjusted *E. coli* O157 super-shedding prevalence was calculated as the number of fecal samples with estimated concentrations $\geq 10^4$ CFU *E. coli* O157/g of feces divided by the number of samples subjected to culture methods.

All analyses were performed at the pen-level (experimental unit) with data analyzed for each study site separately. Outcomes of interest consisted of pen-level fecal prevalence and pen-level super-shedding prevalence which were modeled as the number of positive samples in each pen divided by the total number of samples collected in each pen at each sampling visit (events/trials). Models were fitted using generalized linear mixed models in Proc Glimmix (SAS 9.3; SAS Institute Inc., Cary, NC) with a binomial distribution, restricted pseudo-likelihood estimation, logit link, Kenward-Roger degrees of freedom, and Newton-Raphson and Ridging optimization procedures. In the model, treatment group (DFM or control), sampling visit (1, 2,

3, 4), and a treatment by sampling visit interaction were included as fixed effects. A random effect for block and a first-order autoregressive covariance structure at the pen-level to account for repeated measures also were included in the model. Tukey-Kramer methods were used to adjust for multiple comparisons. Treatment effects were considered significant when *P*-values were ≤ 0.05. Additionally, for all blocks a simple t-test was performed to evaluate if mean days on treatment differed between study sites; similar analyses were performed at the pen-level to evaluate if mean number of animals per pen, and mean initial body weights differed between KS and NE. Descriptive statistics were tabulated for observed pen conditions and weather data.

Results

Study population and sample collection

Study population and sampling data are provided for each study site in Table 4-1. At enrollment, the average body weight ± standard deviation (SD) of KS (379.2 ± 46.4 kg; range = 257.2 to 437.3 kg) and NE (365.5 ± 27.2 kg; range = 326.6 to 403.2 kg) study cattle were not significantly different ($P = 0.27$). The average pen size at the KS study site was significantly greater than the NE study site ($P < 0.01$); the average number of animals per study pen in KS and NE, was 129.8 (SD = 35.3 animals/pen; range = 59 to 192 animals/pen) and 78.7 (SD = 6.6 animals/pen; range = 70 to 90 animals/pen), respectively. Study sites differed in rations fed; notably the amount of wet distillers' grains (WDG) included in KS and NE were 22% and 44%, respectively.

At the time of the first sampling at the KS and NE feedlot, study pens were on the allocated treatment diet for an average of 90 days (SD = 13.8 days; range = 68 to 102 days) and 60.0 days (SD = 0.0 days; range = 60 to 60 days), respectively; mean days on treatment significantly differed between the study sites ($P < 0.01$). At the KS feedlot, 14 of 20 pens were sampled for the entire study period; however, six pens were only sampled for three consecutive weeks as they were sent to harvest prior to the fourth sampling visit. All 20 pens were sampled the entire four-week period at the NE feedlot. Overall, 3,080 fecal samples were collected (KS, n = 1,480; NE, n = 1,600). In KS, pen conditions were normal on sampling of most pens (64.9%; 48/74) or very wet (33.8%; 25/74), whereas in NE the majority of the pens were wet (43.8%; 35/80) or very wet (32.5%; 26/80) at the time of sample collection throughout the four-week

sampling period. The observed pen conditions and weather data are summarized for each sampling week by study site in Table 4-2.

E. coli O157:H7 fecal prevalence

Overall, 436 of 3,080 (14.2%) fecal samples tested positive for *E. coli* O157:H7. Unadjusted cumulative fecal prevalence in the KS and NE feedlots were 10.8% (160/1,480) and 17.3% (276/1,600), respectively. Model-adjusted mean *E. coli* O157:H7 pen-level prevalence and standard errors of the means (SEM) are reported by treatment, sampling visit, and treatment by sampling visit for KS and NE study sites in Table 4-3. Effects of DFM on prevalence of *E. coli* O157:H7 did not significantly differ by sampling visit in KS ($P = 0.77$) or NE ($P = 0.32$); i.e., the treatment by sampling visit interaction terms were not significant. In KS, mean *E. coli* O157:H7 fecal prevalence for DFM and control groups were 8.2% (SEM = 2.2%) and 9.9% (SEM = 2.5%), respectively ($P = 0.51$). At the NE study site, mean *E. coli* O157:H7 fecal prevalence for DFM and control groups were 14.6% (SEM = 2.8%) and 14.3% (SEM = 2.6%), respectively ($P = 0.92$). Model-adjusted mean *E. coli* O157:H7 prevalence estimates significantly differed among sampling visits for data from both KS ($P < 0.01$) and NE ($P < 0.01$).

E. coli O157 super-shedding prevalence

There were 130 (4.2%) of 3,073 fecal samples that tested positive for *E. coli* O157 at a concentration $\geq 10^4$ CFU per gram of feces. Of samples positive for *E. coli* O157:H7, 29.8% (130/436) contained super-shedding concentrations of *E. coli* O157. Unadjusted cumulative *E. coli* O157 super-shedding prevalence was 2.3% (34/1,473) and 6.0% (96/1,600) for the KS and NE study sites, respectively. Seven samples (DFM, n = 3; control, n = 4) from sampling visit 4 at the KS feedlot were culture positive for *E. coli* O157:H7, but pre-enrichment broth samples were unavailable for quantification. Model-adjusted mean *E. coli* O157 super-shedding pen-level prevalence and SEM are reported by treatment, sampling visit, and treatment by sampling visit interaction for KS and NE study sites in Table 4-4. Effects of treatment on the mean prevalence of *E. coli* O157 super-shedding did not significantly differ by sampling visit in KS ($P = 0.97$) or NE ($P = 0.14$). Thus, mean *E. coli* O157 super-shedding prevalence was not significantly reduced by feeding the DFM at either feedlot. Mean *E. coli* O157 super-shedding prevalence estimates were significantly higher ($P = 0.04$) for the DFM group in NE (6.7% ± 1.5%)

compared to the control group (3.2% ± 1.0%). Super-shedding prevalence did not significantly differ between treatment groups in KS ($P = 0.66$). Model-adjusted mean *E. coli* O157 super-shedding prevalence estimates did not significantly differ across sampling visits ($P = 0.18$) in KS but did in NE ($P < 0.01$).

Discussion

The findings of this field study indicated that the prevalence of *E. coli* O157:H7 fecal shedding and *E. coli* O157 super-shedding were not significantly reduced by including this DFM product in the finishing diet of these commercial feedlot cattle in KS and NE. Mean fecal shedding prevalence of *E. coli* O157:H7 significantly differed by sampling visit in KS and NE demonstrating the variability in *E. coli* O157:H7 fecal shedding patterns over time. The well-documented seasonal and intermittent shedding pattern of *E. coli* O157:H7 creates inherent challenges in understanding the ecology of the pathogen and potential effectiveness of interventions in cattle production environments (Besser *et al.*, 1997; Hancock *et al.*, 1997; Sargeant *et al.*, 2000). Due to the lack of understanding of the competitive exclusion mechanism of action in the gastrointestinal tract, and with the complexity of *E. coli* O157:H7 in cattle and feedlot production environments, DFM efficacy has been largely characterized as inconsistent (Callaway *et al.*, 2008). However, given the public health importance and shift away from antibiotic usage in production agriculture, DFM products offer a potential alternative to antibiotics to decrease pathogenic bacterial populations in the host.

For over a decade, DFM studies have been inconsistent in demonstrating effectiveness and repeatability as a pre-harvest intervention under commercial field conditions. While not always beneficial in reducing fecal prevalence of foodborne pathogens when included in the diet, DFMs may benefit cattle weight gain and feed efficiency (Cull *et al.*, 2015; Hanford *et al.*, 2011; Vasconcelos *et al.*, 2008). The most widely researched DFM products in the field are *Lactobacillus*-based, particularly those including *L. acidophilus*, formulated at various dosages and administered for a range of durations. While many studies have demonstrated *L. acidophilus* DFM products to be effective (Brashears *et al.*, 2003; Stephens *et al.*, 2007a,b; Tabe *et al.*, 2008; Younts-Dahl *et al.*, 2004, 2005) in the commercial production environment, others have not (Cull *et al.*, 2012; Luedtke *et al.*, 2016; Stephens *et al.*, 2007a, 2010). The lack of effectiveness in reducing *E. coli* O157:H7 shedding in this study could be due to many reasons, including

study limitations, environmental conditions, and/or a true lack of product effectiveness in commercial feedlot settings.

A priori sample size calculations were estimated based on parameters from Cull *et al.* (2012) assuming a mean *E. coli* O157:H7 prevalence of 40% and 25% for control and DFM treatment groups, respectively. In KS and NE, the mean observed control group *E. coli* O157:H7 prevalence estimates were 9.9% and 14.3%, respectively; therefore, the overall pathogen level observed in our study was much lower than expected, which may have limited our ability to demonstrate intervention effectiveness given the study design. In this current study, super-shedding events were compared by dichotomizing qPCR results for estimated *E. coli* O157 concentrations ($\geq 10^4$ or $<10^4$ CFU/g of feces; Noll *et al.*, 2015) within only culture positive samples, rather estimating specific concentrations within all samples. Although a more quantitative evaluation of specific concentrations of *E. coli* O157:H7 in all fecal samples may have provided additional information relative to estimates of pathogen loads, the semi-quantitate method employed was useful for addressing this study's primary objective of comparing pen-level prevalence estimates between treatment groups administered different interventions.

A notable factor observed in this study was the amount and difference in rainfall during the sampling periods between the two study feedlot locations (US Climate Data, 2019). The KS feedlot site received approximately 15.2 inches of rain between April and August 2018 and nearly five inches of rainfall during the sampling period (July to August 2018) with observed pen conditions ranging from dry and dusty to wet (Table 4-2). However, the feedlot in NE had an even more uncharacteristically wet summer, and resulting pen conditions during the sampling period were very muddy and contained large amounts of standing water, in some areas spanning between pens. At the NE site, nearly 23 inches of rainfall were received during the period of March to September 2018, with nine inches of rainfall during the two months sampled (August and September 2018). It has been documented that ambient temperature and moisture are associated with longer survival of *E. coli* O157:H7 in the environment, promoting re-exposure, and subsequently leading to higher fecal prevalence within pen (reviewed by Smith 2014). The large amount of rainfall during this study, particularly for the NE study site, likely affected the prevalence and distribution of *E. coli* O157:H7 within and among the study pens, perhaps due to the longer survival of *E. coli* O157:H7 in the environment, lack of complete independence and separation between pens due to standing water/slurry, and re-exposure to the organisms from the

environment and/or grooming contaminated hides. Environmental conditions likely impacted observed prevalence and may have negatively impacted the ability to demonstrate treatment effects among the study pens that received different treatments, but were all located within the same production environment and thus similarly exposed to drivers of prevalence.

Additional factors influencing fecal shedding of *E. coli* O157:H7 include breed, sex, diet, and stocking density (Callaway *et al.*, 2009; Jeon *et al.*, 2013; Smith *et al.*, 2001). Due to utilization of a randomized complete block design, the distribution of known and unknown risk factors and management factors should be similar across treatment groups within study blocks and feedlot sites. However, several differences existed between feedlots, including differences in sex, pen size, days fed DFM product, WDG included in the diet, and the aforementioned differences in environmental conditions. In KS and NE, the amounts of WDG fed in total mixed rations were 22% and 44%, respectively. It has been demonstrated that cattle fed 40% WDG in their diets were associated with significantly higher fecal prevalence of *E. coli* O157:H7 and super-shedding prevalence compared to cattle fed 20% WDG (Jacob *et al.*, 2008b). In addition, sex of study cattle differed between KS and NE study sites as did average pen size and days fed DFM product. Thus, the observed variability between KS and NE feedlots' results for *E. coli* O157:H7 prevalence (Table 4-3) and super-shedding prevalence (Table 4-4) should not be surprising. The variability among these factors, particularly diet and environmental conditions, as well as the variability of prevalence and shedding over time, are all rather typical observations for commercial feedlot production settings and are inherent challenges for evaluating pen-level interventions for reducing *E. coli* O157:H7 shedding. Given the potential for pathogen survival and dissemination in the environment and between pens, and "herd immunity" issues that result in control cattle having pathogen levels biased toward the levels in the treated cattle (Dodd *et al.*, 2011; Peterson *et al.*, 2007), future studies of pre-harvest interventions in commercial feedlots may warrant approaches other than the typical side-by-side pen-level study design.

Conclusions

This pen-level field trial did not demonstrate effectiveness for the DFM product reducing *E. coli* O157:H7 shedding in commercial feedlot cattle. There were no significant reductions in fecal prevalence of *E. coli* O157:H7 or super-shedding prevalence for cattle fed the DFM product versus cattle fed a negative control diet. However, there were significant differences in

shedding over time, and variability between study sites with regards to cattle and environmental data. While the exact reason(s) for the lack of effectiveness remain unknown, this study illustrates the challenges in demonstrating DFM products as effective pre-harvest interventions for reducing the prevalence of *E. coli* O157:H7 in commercial feedlot environments.

Acknowledgements

The authors would like to acknowledge the participating feedlot personnel, field sampling crews (Hannah Seger, Christy Hanthorn, Joaquin Baruch, Pius Ekong, and Tariku Jibat Beyene), laboratory personnel (Leigh Ann Feuerbacher, Xiaorong Shi, and Neil Wallace), and undergraduate students (Monica Anderson, Katie Hoch)—for their efforts on this project.

Author Disclosure

None of the authors have conflicts of interest. This study was funded by Legacy Animal Nutrition, LLC; however, Kansas State University employees were responsible for the study design, sampling, laboratory work, statistical analysis, results interpretation and manuscript preparation.

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Table 4-1. Feedlot, study population, and sampling characteristics by study site.

Characteristic, unit	Kansas	Nebraska
Feedlot capacity, # animals	30,000	16,000
Average enrollment pen size (range), # animals	130 (59-192)	79 (70-90)
Sex	Heifer	Steer
Average enrollment weight (range), kg	379 (257-437)	366 (327-403)
Average days on treatment diet at first sampling (range)	90 (68-102)	60 (60-60)
Sampling visit, date		
1	7/23/2018	8/20/2018
2	7/30/2018	8/27/2018
3	8/5/2018	9/3/2018
4	8/12/2018	9/10/2018
Sampling visit, # pens sampled (# samples collected)		
1	20 (400)	20 (400)
2	20 (400)	20 (400)
3	20 (400)	20 (400)
4	14 (280)	20 (400)
Total pens sampled	20	20
Total samples collected	1,480	1,600

Table 4-2. Pen conditions and weather data by sampling visit for each study site.

State	Sampling visit	Pens sampled*	Pen conditions, # pens (% pens sampled)				Average temperature (range), °F	Average humidity (range), %	Raining
			Dry/dusty	Normal	Wet	Very wet			
Kansas									
	1	20	1 (5.0)	17 (85.0)	0 (0.0)	2 (10.0)	74 (69-80)	81 (64-93)	No
	2	20	0 (0.0)	0 (0.0)	0 (0.0)	20 (100.0)	66 (61-72)	84 (66-93)	No
	3	20	0 (0.0)	17 (85.0)	0 (0.0)	3 (15.0)	73 (66-80)	63 (52-78)	No
	4	14	0 (0.0)	14 (100.0)	0 (0.0)	0 (0.0)	65 (58-71)	77 (59-90)	No
	Overall	74	1 (1.4)	48 (64.9)	0 (0.0)	25 (33.8)	70 (58-80)	76 (52-93)	
Nebraska									
	1	20	0 (0.0)	0 (0.0)	20† (100.0)	0 (0.0)	59 (58-60)	93 (89-96)	No
	2	20	0 (0.0)	19 (95.0)	0 (0.0)	1 (5.0)	67 (63-73)	88 (75-96)	No
	3	20	0 (0.0)	0 (0.0)	5 (25.0)	15 (75.0)	66 (66-67)	100 (99-100)	Yes
	4	20	0 (0.0)	0 (0.0)	10† (50.0)	10† (50.0)	60 (58-65)	93 (83-97)	No
	Overall	80	0 (0.0)	19 (23.8)	35 (43.8)	26 (32.5)	63 (58-73)	94 (75-100)	

*The same pens were sampled at each sampling visit; however, six pens were sent to harvest prior to sampling visit 4 in Kansas and were unable to be sampled.

†During fecal sample collection on sampling visits 1 and 4 in Nebraska some pens were wet and very muddy, and at visit 4 some pens (n = 10) contained large pools of standing water (very wet).

Table 4-3. Model-adjusted* mean *E. coli* O157:H7 fecal prevalence (standard error of the mean; SEM) by treatment, sampling visit, and treatment by sampling visit interaction for Kansas and Nebraska study sites.

Variable	*Kansas*			*Nebraska*		
	Mean Prevalence†, %	**SEM, %**	***P*-value**	**Mean Prevalence†, %**	**SEM, %**	***P*-value**
Treatment			0.51			0.92
Control	9.9	2.5		14.3	2.6	
DFM	8.2	2.2		14.6	2.8	
Sampling visit			**<0.01**			**<0.01**
1	3.8[a]	1.5		28.7[a]	4.3	
2	14.4[b]	3.4		19.7[ab]	3.6	
3	12.4[b]	3.1		13.2[b]	2.9	
4	9.1[ab]	2.9		5.2[c]	1.7	
Treatment × sampling visit interaction			0.77			0.32
Control – sampling visit 1	3.6	1.9		25.0	5.1	
Control – sampling visit 2	14.6	4.2		16.0	4.2	
Control – sampling visit 3	15.6	4.4		15.6	4.1	
Control – sampling visit 4	10.6	4.0		6.2	2.6	
DFM – sampling visit 1	4.1	2.0		32.6	5.7	
DFM – sampling visit 2	14.2	4.1		24.0	5.1	
DFM – sampling visit 3	9.8	3.3		11.1	3.5	
DFM – sampling visit 4	7.7	3.4		4.3	2.1	

*Presented results are from a GLMM modeling *E. coli* O157:H7 fecal prevalence with a binomial distribution and logit link including treatment, sampling visit, and treatment by sampling visit interaction as fixed effects, a random effect accounting for block, and an AR (1) covariance structure for repeated measures.

†Estimates with differing superscripts are significantly different at $P < 0.05$, estimates with shared letter superscripts do not differ significantly at $P < 0.05$.

Table 4-4. Model-adjusted* mean *E. coli* O157 super-shedding prevalence (standard error of the mean; SEM) by treatment, sampling visit, and treatment by sampling visit interaction for Kansas and Nebraska study sites.

Variable	*Kansas*			*Nebraska*		
	Mean Prevalence†, %	**SEM, %**	***P*-value**	**Mean Prevalence†, %**	**SEM, %**	***P*-value**
Treatment			0.66			**0.04**
Control	1.8	0.7		3.2[b]	1.0	
DFM	2.2	0.7		6.7[a]	1.5	
Sampling visit			0.18			**<0.01**
1	1.5	0.7		10.5[a]	2.2	
2	3.7	1.1		2.9[b]	1.4	
3	2.5	0.9		4.4[ab]	1.4	
4	1.1	0.7		3.4[b]	1.2	
Treatment × sampling visit interaction			0.97			0.14
Control – sampling visit 1	1.5	1.0		7.4	2.4	
Control – sampling visit 2	3.5	1.5		1.0	0.9	
Control – sampling visit 3	2.5	1.3		4.9	2.0	
Control – sampling visit 4	0.7	0.8		2.9	1.5	
DFM – sampling visit 1	1.5	1.0		14.8	3.4	
DFM – sampling visit 2	4.0	1.6		8.3	2.6	
DFM – sampling visit 3	2.5	1.3		3.9	1.7	
DFM – sampling visit 4	1.5	1.2		3.9	1.7	

*Presented results are from a GLMM modeling *E. coli* O157 super-shedding with a binomial distribution and logit link including treatment, sampling visit, and treatment by sampling visit interaction as fixed effects, a random effect accounting for block, and an AR (1) covariance structure for repeated measures.

†Estimates with differing superscripts are significantly different at $P < 0.05$, estimates with shared letter superscripts do not differ significantly at $P < 0.05$.

Chapter 5 - A systematic review and meta-analysis of published literature on prevalence of non-O157 Shiga toxin-producing *Escherichia coli* serogroups (O26, O45, O103, O111, O121, and O145) and virulence genes in feces, hides, and carcasses of pre- and peri-harvest cattle worldwide

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(Accepted with minor revisions in *Animal Health Research Reviews*, 2021)

Abstract

Objective: The objective of this study was to summarize peer-reviewed literature on the prevalence and concentration of non-O157 STEC (O26, O45, O103, O111, O121, and O145) serogroups and virulence genes (*stx* and *eae*) in fecal, hide, and carcass samples in pre- and peri-harvest cattle worldwide, using a systematic review of the literature and meta-analyses.

Data synthesis: Seventy articles were eligible for meta-analysis inclusion; data from 65 articles were subjected to random-effects meta-analysis models to yield fecal prevalence estimates. Meta-regression models were built to explore variables contributing to the between-study heterogeneity.

Results: Worldwide pooled non-O157 serogroup, STEC, and EHEC fecal prevalence estimates (95% confidence interval) were 4.7% (3.4-6.3%), 0.7% (0.5-0.8%), and 1.0% (0.8-1.1%), respectively. Fecal prevalence estimates significantly differed by geographic region ($P < 0.01$) for each outcome classification. Meta-regression analyses identified region, cattle type, and specimen type as factors that contribute to heterogeneity for worldwide fecal prevalence estimates.

Conclusions: The prevalence of these global foodborne pathogens in the cattle reservoir is widespread and highly variable by region. The scarcity of prevalence and concentration data for hide and carcass matrices identifies a large data gap in the literature as these are the closest proxies for potential beef contamination at harvest.

Running title: Systematic review and meta-analysis of non-O157 STEC in cattle worldwide
Key-words: cattle; *Escherichia coli*; non-O157; pre-harvest; prevalence; review; Shiga toxin; STEC

Introduction

Rationale

Globally, Shiga toxin-producing *Escherichia coli* (*E. coli*; STEC) are foodborne pathogens of public health importance (FAO and WHO, 2019). A subset of STEC, enterohemorrhagic *E. coli* (EHEC), are known to cause severe disease in humans such as hemorrhagic colitis and hemolytic uremic syndrome (Caprioli *et al.*, 2014). The Center for Disease Control and Prevention (CDC) estimates that out of approximately 265,000 human illnesses each year, approximately 3,600 patients are hospitalized and subsequently 30 deaths are attributed to these pathogens in the United States (CDC, 2016). EHEC causes severe human disease in part due to the intimate attachment of the bacterium to the host cell, mediated by intimin, which is encoded by an *eae* gene, in addition to at least one Shiga toxin gene (stx_1 and/or stx_2). Cattle are a known reservoir of STEC and EHEC as they harbor these pathogens in their gastrointestinal tracts and shed them in their feces (Bettelheim *et al*., 2000; Pihkala *et al*., 2012). When the source of illness was known, beef products were the most frequently attributed source of STEC-associated human illness worldwide (FAO and WHO, 2019).

Cattle feces contaminate cattle hides in the production environment, during transport, and/or in lairage increasing the potential for cross-contamination of beef carcasses, and subsequent beef products, at the harvest facility (Ekong *et* al., 2015; Fox *et al.*, 2008; Jacob *et al.,* 2010; Loneragan and Brashears, 2005). Therefore, cattle fecal, hide, and carcass STEC and EHEC prevalence estimates are a proxy for the potential risk at slaughter (Renter *et al.,* 2008), whereas concentration estimates quantify the risk these pathogens represent at harvest. In the last decade, EHEC of public health importance have been categorized into 'O157' and 'non-O157' serogroups. Each year in the United States, the CDC has estimated that O157 and non-O157 pathogens are responsible for approximately 95,400 and 169,600 human illnesses, respectively (CDC, 2016). Whereas *E. coli* O157, specifically *E. coli* O157:H7, has been widely researched over the last 30 years, including the publication of systematic reviews for *E. coli* O157 prevalence in cattle in North America (Ekong *et al*., 2015) and globally (Islam *et al*., 2014), research regarding non-O157 serogroups, and specifically the "top 6", including O26, O45, O103, O104, O111, O121, and O145, has only been prominent during the last decade. As a result, there is limited information about key risk factors, geographic distribution, and serogroup-specific estimates of the top 6 in cattle prior to harvest.

Prevalence and concentration estimates of non-O157 pathogens are crucial to assess the distribution and load of bacteria in the cattle reservoir and to implement targeted mitigation strategies for lowering the risk of these foodborne pathogens in the beef supply. Therefore, our overarching goal was to compile evidence on global estimates of prevalence and concentration of non-O157 serogroups in the cattle reservoir.

Objective

The objective was to gather, integrate, and interpret scientific data on the prevalence and concentration of the top 6 non-O157 serogroups (O26, O45, O103, O104, O111, O121, and O145) and virulence genes (*stx*$_1$, *stx*$_2$, and *eae*) in fecal, hide, and carcass samples of pre- and peri-harvest adult cattle globally using a systematic review of the literature and meta-analysis. Meta-regression models were employed to evaluate the sources contributing to the variability of the prevalence estimates obtained.

Methods

Protocol

The systematic review methodology employed was in accordance with procedures outlined by O'Connor and Sargeant (2014). Preferred Reporting Items for Systematic Reviews and Meta-Analyses Protocol (PRISMA and PRISMA-P) guidelines (Liberati *et al*., 2009; Moher *et al*., 2015; Page *et al.,* 2021) were followed for reporting purposes.

Eligibility criteria

Peer-reviewed, primary research published in English that reflected the inclusion criteria (Table 5-1) were considered eligible. Non-peer reviewed, gray literature, and peer-reviewed literature pertaining to experimental studies, *in vitro* experiments, simulation studies, or non-primary research (e.g., literature reviews, short communications) were excluded.

The research question was: What is the prevalence and concentration of the top 6 non-O157 serogroups (O26, O45, O103, O104, O111, O121, and O145) and virulence genes (*stx*$_1$, *stx*$_2$, and eae) in fecal, hide, and carcass samples of pre- and peri-harvest adult cattle globally? The initial protocol was modified from a restricted search of North America to include all regions worldwide. Specific components of the research question included:

> *Population (P):* Healthy, pre- and peri-harvest adult cattle (older than 8 months of age). Pre-harvest cattle were defined as cattle in their production environments before being sold or shipped to slaughter. Peri-harvest was defined as the time after cattle leave the farm until after stunning and hide removal, but prior to the application of any carcass interventions.
>
> *Outcomes (O):* Prevalence and concentration of non-O157 serogroups (O26, O45, O103, O111, O121, and O145) and associated virulence genes (*stx*$_1$, *stx*$_2$, and *eae*) in fecal, hide, and carcass samples. Prevalence and concentration data were extracted according to three different outcome classifications, depending on the virulence gene combination: 1) "serogroup" refers to samples that tested positive for an *E. coli* serogroup gene of interest (O26, O45, O103, O111, O121, or O145), 2) "STEC", refers to samples that tested

positive for a specific *E. coli* O serogroup and at least one Shiga toxin (*stx*$_1$ and/or *stx*$_2$) gene, and 3) "EHEC" refers to samples that tested positive for an *E. coli* O serogroup, at least one Shiga toxin gene, and the intimin (*eae*) gene.

Information sources

Electronic databases accessed through the Kansas State University Library on 21 March, 2019 included: Agricola, Web of Science, and PubMed. Retrieved titles and abstracts were imported into a bibliographic management program (EndNote^{X9}, Clarivate Analytics). In addition, reference lists of articles considered to be landmark publications on the subject, were also reviewed (i.e., hand-searched) for inclusion.

Search

In order to generate a complete list of all primary literature relevant to our research question, search terms were created to account for the population and outcomes of interest. The search algorithm used included the following terms: "(Beef OR Dairy OR Cattle OR Cow) AND (*Escherichia coli* OR STEC OR Shiga toxin OR Shiga toxin producing OR non-O157) AND (hide OR fecal OR carcass) AND (prevalence OR concentration)".

The search was restricted to articles published 01 January 2000 to 21 March 2019, with the assumption that diagnostic protocols used in articles published prior to year 2000 were generally less sensitive than the methods currently used. No language restrictions were set on the original search, however, after the retrieval of full-text articles, articles were excluded if they were not available in English due to budgetary constraints. Duplicate articles were removed using the EndNote ^{X9} software (EndNote^{X9}, Clarivate Analytics) as well as manually checked after importing from online databases due to miscellaneous spaces or typos that did not promote the use of automated removal of duplicates.

Study selection

The title and abstract of articles identified through electronic databases and hand searches were screened for eligibility by a trained reviewer (DD) based on preset inclusion and exclusion criteria (Table 5-1). A second reviewer (NC) validated the first reviewer's work. If the abstract

did not include enough details to assess eligibility, full text articles were retrieved and the entire article was screened. If the abstract, or article, was deemed eligible based on our criteria, full text articles were retrieved and subjected to the risk of bias assessment.

Data extraction protocols and tools were developed, pre-tested by all reviewers (DD, NC, and MS), and implemented for each step of the review process using spreadsheets created in Microsoft Excel (Microsoft Windows, 2016). Data were extracted from all articles that met four key risk of bias assessment quality criteria (see "Risk of bias in individual studies" for further details).

Data collection process

A data extraction spreadsheet tool was developed in Microsoft Excel (Microsoft Windows, 2016), where each column represented a variable when extracting data from the full papers. The data extraction form was pre-tested by all reviewers using a sample of 10 full-text articles. Data extraction was performed independently by two reviewers (DD and NC or MS). Disagreements were resolved by consensus or a third reviewer's input. Data were extracted for the different non-O157 *E. coli* O serogroups of interest (O26, O45, O103, O111, O121, and O145) reported at various hierarchical levels (e.g., sample, animal, pen, feedlot, and/or processing plant). Outcomes of interest were further classified into three outcome classifications—serogroup, Shiga toxin-producing *E. coli* (STEC), or Enterohemorrhagic *E. coli* (EHEC)—to assess the prevalence of specific serogroup and virulence gene combinations.

In the event that articles presented information on prevalence or concentration for different outcome classifications and/or O groups, the data were extracted in individual rows as unique events (hereafter defined as a "study") in the data extraction form. Therefore, an article (a peer-reviewed publication describing prevalence or concentration of non-O157 in cattle fecal samples eligible for data extraction) could contain more than one study. Each study reflected one outcome classification (e.g., serogroup O26, STEC O45, EHEC O103), at a single time point (e.g., day, month, season, year), as classified by a laboratory method, representing one cattle type, at different hierarchical levels (e.g., pen, feedlot) for a specified matrix (e.g., fecal, hide, carcass).

If data from a study were not explicitly presented but enough information was available (e.g., prevalence and number of samples tested), reviewers conducting the data extraction imputed the required values (e.g., number of positive samples). In addition, if the authors stated that they tested for serogroups and/or virulence genes of interest but did not detect them, it was recorded as a data point equal to zero for the respective outcome classification with the provided denominator. Conversely, if authors did not mention specific serogroups of interest, it was assumed that they were not tested for and data were neither extracted nor assigned a zero. Additionally, retrieved articles presenting hide prevalence and/or concentration data for non-O157 serogroups detected in commercial plants following hide wash interventions (e.g., cabinet wash or chemical application) and/or articles that did not state clearly at which stage of the harvest process the hide/carcass sample was collected were excluded from this review. Experimentally inoculated fecal, hide, and/or carcass studies were also excluded from this review. Although considered a peri-harvest intervention, articles reporting hide prevalence data after the application of bacteriophage in lairage pens or water post-stunning were deemed eligible and data were extracted. Authors were not contacted to identify additional studies or inquire about additional information, only the full-text articles were considered.

Data items

Publication information extracted from each article and study included: first author, title, and year of publication. Key study characteristics extracted were as follows: region (Africa, Asia, Australia/Oceania, Europe, Middle-East, North America, South America), time of harvest (pre-harvest or post-harvest), cattle type (beef, dairy, beef and dairy, or unknown), outcome classification (serogroup, STEC, or EHEC), non-O157 O gene of interest (O26, O45, O103, O111, O121, O145), diagnostic methodology (culture, culture + immunomagnetic separation (IMS), polymerase chain reaction (PCR) only, or other), specimen matrix (fecal, hide, or carcass), specimen type (pen-floor, rectal grab, rectal swab, cecal, unknown, or sponge sample), number of positive samples, number of samples tested, prevalence or proportion positive, and hierarchical level of data reported (sample, animal, pen, feedlot, or processing plant). For specimen type, rectal grab samples typically referred to samples collected pre-harvest but also included peri-harvest samples obtained from fecal material removed from the rectum prior to

evisceration as these were considered similar specimen types *a priori*. Additional data that were extracted, if provided, included: month(s) study was conducted, year(s) study was conducted, season, country of study, breed, age, stage of production (e.g., finishing period, at calving), study design (e.g., cross-sectional, longitudinal; as determined by reviewers), and repeated measures (yes or no).

Study risk of bias assessment

A set of seven quality criteria (Table 5-2) was designed, based on guidelines described by Sargeant *et al.* (2006) and Higgins *et al.* (2019). These criteria were modified from the risk of bias assessment used by Ekong *et al.* (2015). The purpose of the risk of bias assessment was to evaluate internal and external validity, and overall study design and execution, prior to extracting data from relevant articles by evaluating criteria (C) representing three domains (Sanderson *et al.*, 2007). The key domains evaluated include: 1) design-specific sources of bias (C1, C6), 2) appropriateness of population based on inclusion criteria (C2, C3, C4), and 3), methods for measuring outcome variables (C5, C6, C7). Sample size calculation (C1) and cattle type (C2), represented internal validity-related factors. Whereas, animal production setting (C3) and study catchment area (C4) served as external validity criteria. Additionally, criteria representing the outcome with a number of positives, clear number of samples, and/or ability to calculate a prevalence (C5), for a specified period of time (C6), for a specific serogroup (C7).

Four criteria (C2, C3, C5, C7) were deemed crucial to meet internal and external validity characteristics and needed to proceed with data extraction. Articles failing to meet one or more of these criteria were excluded. In some instances, cattle type (C2) was not explicitly stated, but if there was enough information (e.g., breed, age, diet, and/or housing) provided to indicate that the study population referred to healthy, adult cattle, the article was still considered for data extraction. If authors stated a specific breed and/or production purpose, reviewers assigned the breed to a cattle type category (e.g., beef or dairy). Criterion 3 posed a challenge regarding articles published from countries where animal production practices were not familiar to the reviewers; therefore, unless the authors specifically stated that the animals were housed in a research farm, it was assumed that animals were housed in representative field conditions for that region.

The protocol for assessing risk of bias (Table 5-2) was pre-tested on a set of 10 abstracts that were reviewed for relevance by two reviewers (DD and NC) to determine reproducibility. For all retrieved full-text articles, two reviewers (DD and MS or NC) independently evaluated the risk of bias (Table 5-2). Disagreements were resolved by consensus or a third reviewer's input.

Summary measures

For analysis purposes, data on fecal prevalence and calculated standard errors were logit transformed using R version 3.6.1 (R Core Team, 2019). Numerators with a zero value were assigned a value of 0.5 prior to the logit transformation. The final pooled logit results (including their 95% confidence intervals) obtained from the meta-analyses models were back-transformed and expressed as percentages.

Synthesis of results

Hide and carcass prevalence and concentration data for all matrices were summarized using qualitative methods. Fecal prevalence results presented at the sample-level were analyzed quantitatively using meta-analysis. Using EpiTools (Sergeant, 2015), prevalence estimates obtained from pooled fecal samples were adjusted to compute individual sample-level prevalence estimates using the pooled prevalence calculator for fixed pool size and assuming a perfect test; otherwise, only crude estimates were used in the analysis.

Meta-analysis

Data were separated into two datasets prior to analysis: 1) worldwide data by outcome classification, and 2) North American (Canada, Mexico, and USA) data by outcome classification. Random-effects meta-analyses were fitted to estimate the prevalence of non-O157 serogroup, STEC, and EHEC outcome classifications in cattle fecal samples, using the inverse variance method. All data were analyzed using R version 3.6.1 (R Core Team, 2019) using the *meta* package (version 4.9-9; Balduzzi *et al.,* 2019) unless otherwise stated.

Meta-analyses and subgroup analyses were used to determine serogroup-specific fecal prevalence estimates for each outcome classification (function 'metaprop'), in the: 1) worldwide dataset by region, 2) worldwide dataset by O gene, 3) North American dataset by O gene, and 4)

North American dataset by country. Following a logit transformation, the following specifications were used for each model: DerSimonian-Laird estimator for between-study variance (τ^2; DerSimonian and Laird, 1986), and Hartung-Knapp adjustment for random-effects (Knapp and Hartung, 2003; Viecthbauer, 2010a). The final pooled logit results (including their 95% confidence intervals) obtained from the meta-analysis models were back-transformed and expressed as percentages.

Between-study heterogeneity was quantified using the Cochrane's chi-square test of homogeneity (*Q*) and the I^2 statistic (Higgins *et al.,* 2019). Cochrane's Q statistic was used to evaluate whether the variation between studies exceeds that expected by chance and is used to compute the I^2 statistic; $I^2 = \left[\frac{Q - degrees\ of\ freedom}{Q}\right] \times 100$ (Higgins *et al.,* 2019). *P*-values less than 10% ($P < 0.10$) indicated significant between-study heterogeneity. The Higgins' I^2 statistic represents the percentage of the total variability in a set of effect sizes due to true heterogeneity rather than chance (Higgins *et al.,* 2019). Using the scale suggested by Higgins *et al.* (2019), I^2 values between 30-60%, 50-90%, and 75-100% may indicate moderate, substantial, and considerable heterogeneity, respectively. Causes of heterogeneity were explored using subgroup analysis and meta-regression techniques.

Additional analyses

Meta-regression

Uni-variable and multi-variable meta-regression models were built (using 'metareg') to examine the contribution of specific variables to the between-study heterogeneity of the worldwide and North American pooled fecal prevalence estimates obtained for each outcome classification. Explanatory variables of interest were: time of harvest (pre- or peri-harvest), cattle type (beef, dairy, beef and dairy, or unknown), laboratory method (PCR only, culture, culture + IMS, other), specimen type (cecal, rectal grab, pen-floor, rectal swab, or unknown), and region (Asia, Australia/Oceania, Europe, North America, or South America). Initially, uni-variable meta-regression models were fit to explore the association between each of the explanatory variables and the fecal prevalence for each outcome classification.

Variables with $P < 0.10$ in the uni-variable screen were included in the multi-variable meta-regression models. Based on our causal web diagram constructed *a priori*, specimen type is an intervening variable through harvest time and therefore, either specimen type or harvest time, not both, were eligible for inclusion in the multi-variable model (Supplementary Material, Appendix A). There were no plausible interactions between variables of interest based on our causal diagram and therefore no interactions were evaluated. A backward elimination procedure was followed for removal of non-significant variables. Variables with *P*-values less than or equal to 5% ($P \leq 0.05$) were deemed significant and were kept in the multi-variable meta-regression models. The final pooled logit regression coefficients and their 95% confidence intervals were back-transformed.

Risk of bias across studies

Although subjective, funnel plots allow visual interpretation of whether the association between prevalence estimates and a measure of study size (e.g., standard error) is greater than what may be expected to occur by chance (Sterne *et al.*, 2000). To assess potential publication bias, we generated funnel plots using the function 'funnel'. A formal asymmetry test (using 'metabias' and 'lingreg') was used to evaluate the presence of small study effects for non-O157 serogroup, STEC, and EHEC outcome classifications worldwide and for specific serogroups in North America (Egger *et al.*, 1997). This regression-based test for detection of skewness determined whether the intercept deviated significantly from zero in a weighted regression of standardized prevalence estimates (on a logit scale) against their precision (e.g., standard error) (Egger *et al.*,1997; Steichen, 1998). *P*-values less than 5% ($P < 0.05$) indicated funnel plot asymmetry.

Results

Study selection

The number of research articles retrieved at each step of the process is presented in Figure 5-1. Initially, a total of 3,241 articles were obtained from three electronic databases. Of the articles initially retrieved, 1,063 were duplicates and 1,952 were excluded based on the title and abstract screening. Two hundred sixteen full text articles were retrieved; however, 65 articles were excluded as they did not meet our inclusion criteria (Table 5-1). A total of 168 articles were

subjected to the risk of bias assessment (Table 5-2) and 98 articles were subsequently excluded. Data were extracted from 70 articles.

Study characteristics

In this systematic review, of the 70 articles retrieved, 65 articles reported the fecal prevalence of non-O157 serogroups and virulence genes in pre- and peri-harvest cattle. Few articles were retrieved for hide (n = 8) and carcass (n = 4) matrices worldwide. Five articles provided prevalence data for more than one matrix of interest: fecal and hide (n = 1; Midgley and Desmarchelier, 2001), hide and carcass (n = 2; Stromberg *et al.*, 2015, Svoboda *et al.*, 2013) and fecal, hide, and carcass (n = 2; Thomas *et al.*, 2012; Stromberg *et al.*, 2016b). Concentration data were scarce for all matrices. Three articles presented fecal concentration data (Murphy *et al.*, 2016; Shridhar *et al.*, 2016, 2017) and one article presented hide and carcass concentration data (Thomas *et al.*, 2012). Due to limited data, hide and carcass prevalence data and concentration data for all matrices were not subjected to meta-analysis. Fecal prevalence data, however, were analyzed using meta-analysis and meta-regression models.

Risk of bias within studies

Articles that were eligible for data extraction following the risk of bias assessment are tabulated by criteria in Table 5-2. The majority of data extracted were from articles presenting data for cattle housed in commercial farming conditions typical of their respective region (92.9%; 65/70) rather than research farms (7.1%; 5/70). Less than 20% of articles (12/70; 17.1%) included a sample size justification in the manuscript. The majority of articles (62.9%; 44/70) represented a study design that included multiple sites, whereas 37.1% (26/70) were conducted at a single site. The length of the study was not known for the majority (71.4%; 50/70) of the articles as only cumulative prevalence estimates were presented. For articles that presented study duration (28.6%; 20/70), six studies (30.0%; 6/20) reported to last less than three months whereas 14 studies (70.0%; 14/20) reported to last longer than three months.

Results of individual studies

Fecal prevalence and concentration

Fecal prevalence data for non-O157 serogroups and associated virulence genes of interest were extracted from 65 articles from seven regions (Africa, n = 3; Asia, n = 11; Australia/Oceania, n = 6; Europe, n = 17; Middle East, n = 1; North America, n = 21; South America, n = 6) worldwide. Although data from these 65 articles were eligible for inclusion in the worldwide fecal prevalence meta-analysis, due to limited data per respective outcome classification in each region, five articles and subsequently three regions were excluded from the worldwide meta-analysis by outcome classification: Africa (n = 3; serogroup: Musa *et al.,* 2012; STEC: Adamu *et al.,* 2018; EHEC: El-Gamal *et al.,* 2016;), Middle East (EHEC, n = 1; Mohammed *et al.,* 2015), and South America (serogroup, n = 1; Vicente *et al.,* 2005). Additionally, three articles were excluded from the worldwide fecal prevalence meta-analysis because they only presented farm-level, rather than sample-level, fecal prevalence data (n = 2; Australia/Oceania: McAuley *et al.,* 2014; Middle East: Rehman *et al.,* 2014) or contained redundant data with previously published literature (n = 1; North America: Shridhar *et al.,* 2016). Therefore, 57 articles were eligible for inclusion in the worldwide fecal prevalence meta-analysis by region. The sample denominator extracted from these 57 articles ranged from ten to 78,705 fecal samples. In two articles (Dargatz *et al.,* 2013; Stanford *et al.,* 2016), sample-level prevalence estimates were obtained from pooled fecal samples (range = 785 to 78,705) using EpiTools pooled prevalence calculator (Ausvet 2015). All other extracted data were unadjusted prevalence estimates (range = 10 to 6,086 fecal samples).

With respect to outcome classifications, most articles presented data for both EHEC and STEC classifications (n = 16), followed by EHEC only (n = 15), STEC only (n = 8), serogroup only (n = 8), STEC and serogroup (n = 1), and EHEC and serogroup (n = 1). Eight articles presented data for all outcome classifications, EHEC, STEC, and serogroup. Articles included in the worldwide fecal prevalence meta-analysis are reported by key study variables in Table 5-3. Fecal prevalence data were synthesized using meta-analyses to obtain worldwide fecal prevalence estimates by region (see Synthesis of Results), whereas fecal concentration data were much more limited and their results are presented below.

In addition to the worldwide results, we further explored fecal prevalence estimates in North America. Fecal prevalence estimates however, were largely represented by data from the USA (n

= 13; Agga *et al.,* 2017; Bai *et al.,* 2012; Baltasar *et al.,* 2014; Cull *et al.,* 2017; Dargatz *et al.,* 2013; Dewsbury *et al.,* 2015; Ekiri *et al.,* 2014; Paddock *et al.,* 2012; Schneider *et al.,* 2018a; Shridhar *et al.,* 2017; Singh *et al.,* 2015; Stromberg *et al.,* 2016b; Thran *et al.,* 2001). Canada was represented by five studies (n = 5; Hallewell *et al.,* 2016; Karama *et al.,* 2008; Renter *et al.,* 2007; Schurman *et al.,* 2000; Standford *et al.,* 2016), however, no data were obtained from Mexico. Fecal prevalence data by O gene for each outcome classification, obtained from North America, represented by the USA and Canada, were synthesized using meta-analyses (see Synthesis of Results).

Fecal concentration data for non-O157 serogroups of interest were limited (Murphy *et al.,* 2016; Shridhar *et al.,* 2016, 2017). Two articles represented beef cattle in the USA (Shridhar *et al.,* 2016, 2017) and one represented lactating dairy cattle in Ireland (Murphy *et al.,* 2016). These three articles utilized a variety of laboratory methods for quantification, including real-time PCR, multiplex quantitative PCR, and spiral plating. Murphy *et al.,* (2016) reported concentration data for O26 in two Irish dairy herds, represented by 40 lactating cows per herd, sampled via recto-anal mucosal (RAM) swabs, longitudinally over the course of one year. Three (0.6%) of 529 RAM swabs subjected to quantitative real-time PCR were classified as EHEC O26 high-shedding positives (defined as $\geq 10^4$ CFU/swab; Murphy *et al.,* 2016).

The remaining two articles (Shridhar *et al.,* 2016, 2017) presented fecal concentration data for all non-O157 serogroups of interest, from fed beef cattle housed in commercial USA feedlots sampled prior to harvest, and quantified utilizing multiplex quantitative PCR (mqPCR) and spiral plating methods. Five-hundred and seventy-six pen-floor fecal samples were subjected to mqPCR; the proportion of samples harboring super-shedding concentrations ($\geq 10^4$ CFU/gram of feces) were 7.1, 6.4, 5.0, and 0.4%, for O45 and O103, O121, O26, O145 and O111, respectively (Shridhar *et al.,* 2016). Similar trends were observed for the top 6 serogroups in another observational feedlot study comparing spiral plating (SP) and mqPCR methods (Shridhar *et al.,* 2017) where the most frequently quantified serogroups at high-shedding concentrations were O103 (SP: 7.5%, 86/1152; mqPCR: 18.2%, 210/1152) and O26 (SP: 1.6%, 18/1152; mqPCR: 6.9%, 80/1152). The proportion of quantifiable samples for the top 6 serogroups ranged from undetected to 7.5% for the SP method and 0.4 to 18.2% for mqPCR (Shridhar *et al.,* 2017).

Hide prevalence and concentration

Data on non-O157 serogroup and virulence gene prevalence and concentration were limited for cattle hides and are reported descriptively for all outcome classifications (Table 5-4). Eight articles containing hide prevalence data were retrieved from five countries (Australia, Honduras, Ireland, Nicaragua, and the USA). A single article presented hide concentration data (Thomas *et al.*, 2012).

Two articles, represented by five studies, reported data for non-O157 serogroups O26, O103, O111, and O145. These non-O157 serogroups were detected on peri-harvest beef cattle hides ranging from undetected to 27.1%. The two serogroups most frequently detected from beef cattle hides were serogroups O26 and O103, with reported prevalence estimates of 6.0 and 27.1%, respectively. Furthermore, Thomas *et al.* (2012) quantified serogroup O103 on cattle hides at harvest yielding estimates for six samples, out of the 130-sample subset tested, between 10 and 110 CFU/cm^2, the other 124 samples contained colony counts too low to estimate by direct plating methods (Table 5-4).

Hide prevalence estimates were obtained for all six non-O157 STEC of interest from three articles. Represented by 11 studies, non-O157 STEC hide prevalence estimates in peri-harvest beef cattle ranged from undetected to 0.3%. Only STEC O26 and O103 were detected on cattle hides. Other non-O157 STEC (O45, O111, O121, and O145) were tested for but not detected on peri-harvest cattle hides.

Seven articles containing hide prevalence data, representing 55 studies, presented non-O157 EHEC hide prevalence data. Prevalence estimates reported ranged from undetected to 47.0, 57.5, 35.9, 29.3, 46.0 and 49.0% for EHEC O26, O45, O103, O111, O121, and O145, respectively.

Carcass prevalence and concentration

Data on pre-intervention carcass prevalence and study characteristics are presented in Table 5-5. Four articles reported top 6 prevalence data and a single article (Thomas *et al.*, 2012) presented concentration data for pre-intervention carcasses. Serogroup prevalence estimates for the top 6

ranged from undetected to 13.8% on peri-harvest beef carcass samples. Serogroup O111 was not detected on peri-harvest carcasses in any of the retrieved articles. Thomas *et al.* (2012) reported a serogroup O103 carcass prevalence of 5.5%, but did not detect quantifiable concentrations of serogroup O103 on the corresponding cattle carcasses. Moreover, Thomas *et al.*, 2012 presented STEC prevalence data on pre-intervention beef carcasses where STEC O26, O103, O111, and O145 were undetected and STEC O45 and O121 were not tested for. Three articles (Stromberg *et al.*, 2015, 2016b; Thomas *et al.*, 2012) presented data for non-O157 EHEC. The top 6 EHEC prevalence on pre-intervention cattle carcasses ranged from undetected to 4.0%; EHEC O111 and O121 were not detected.

Synthesis of results

Worldwide meta-analysis of fecal prevalence by outcome classification and O gene

Pooled fecal prevalence estimates significantly differed among regions worldwide for the top 6 serogroups, STEC, and EHEC outcome classifications (Table 5-6). The worldwide serogroup meta-analysis was comprised of 18 articles, representing 165 studies. Studies from four regions, Asia, Australia/Oceania, Europe, and North America, were included in the analysis. Due to limited data, South America was not included in the worldwide serogroup meta-analysis. The estimated worldwide pooled non-O157 serogroup prevalence was 4.7% (95% confidence interval (CI) = 3.4-6.3%). Pooled fecal prevalence was highest for North America (6.4%, 95% CI = 3.7-10.8%) with respect to the serogroup outcome classification**.** The most prevalent serogroup reported worldwide was O103 (11.4%, 95% CI = 4.7-25.2%) followed by O45 (7.9%, 95% CI = 3.2-18.1%), O26 (6.6%, 95% CI = 4.2-10.4%), O121 (2.7%, 95% CI = 0.9-7.6%), O111 (1.6%, 95% CI = 0.8-2.9%), and O145 (1.3%, 95% CI = 0.5-3.6%). The worldwide STEC fecal prevalence meta-analysis included 33 articles, representing 191 studies. The estimated worldwide STEC pooled fecal prevalence was 0.7% (95% CI = 0.5-0.8%), with Australia/Oceania (1.3%, 95% CI = 0.7-2.5%) yielding the highest regional estimate worldwide. In this review, STEC O26 (1.0%, 95% CI = 0.7-1.4%) and STEC O103 (0.8%, 95% CI =0.5-1.4%) were the most frequently detected STEC globally. The global prevalence estimates for STEC O45, STEC O111, STEC O121, STEC O145 were 0.4 (95% CI = 0.2-0.8%), 0.4 (95% CI = 0.2-0.5%), 0.7 (95% CI = 0.3-1.4%) and 0.7% (95% CI = 0.4-1.2%), respectively. Worldwide EHEC pooled fecal prevalence estimates were summarized from 40 articles, representing 369

studies. The pooled EHEC fecal prevalence estimate was 1.0% (95% CI = 0.8-1.1%) with the highest observed regions in this review being Europe (1.3%, 95% CI = 1.0-1.7%) and North America (1.2%, 95% CI = 0.9-1.5%). Globally, as noted in global STEC prevalence, EHEC O26 (1.3%, 95% CI =0.9-1.8%) and EHEC 0103 (1.4%, 95% CI = 1.0-2.1%) were the most prevalent. Followed by EHEC O45 (0.9%, 95% CI = 0.5-1.8%), EHEC O111 (0.9%, 95% CI = 0.6-1.4%), EHEC O121 (0.4%, 95% CI = 0.3-0.6%), EHEC O145 (0.9%, 95% CI = 0.6-1.3%). In the present study, North America yielded the highest pooled fecal prevalence estimates for the serogroup outcome, and second highest worldwide for the STEC and EHEC outcomes—North America data were further evaluated by O gene for each outcome classification and by country. As there was evidence of between-study heterogeneity (I^2 statistic) in this worldwide meta-analysis, meta-regression analyses were conducted for all outcome classifications by key variables of interest.

North America meta-analysis of fecal prevalence by O gene

Overall, North American pooled fecal prevalence estimates were 6.4, 1.1, and 1.2% for the serogroup, STEC, and EHEC outcome classifications, respectively (Table 5-7). Serogroup-specific estimates were estimated from eight articles including 73 studies. The most prevalent serogroups reported were O103 (19.6%, 95% CI = 5.6-50.2%) and O26 (15.1%, 95% CI = 4.1-42.7%) whereas the least prevalent was O111 (1.0%, 95% CI = 0.2-5.8%). Estimates for STEC fecal prevalence in North America were obtained from eight articles, including 79 studies. Similar to the serogroup-specific estimates, STEC O103 (1.6%, 95% CI = 0.7-3.7%) was the most prevalent O gene, whereas STEC O111 (0.6%, 95% CI = 0.3-1.3%) was the least prevalent. Meta-analysis for EHEC fecal prevalence in North America included 10 articles representing 170 studies. As observed for the serogroup and STEC outcome classifications, fecal prevalence estimates remained highest for EHEC O103 (2.8%, 95% CI = 1.6-4.9). The lowest fecal prevalence estimate obtained was EHEC O121 (0.5%, 95% CI = 0.3-0.8%) in North America. Heterogeneity among North American studies were explored through meta-regression analyses for all outcomes by key variables of interest: time of harvest, cattle type, laboratory methods, and specimen type.

North America meta-analysis of fecal prevalence by country

To further explore fecal prevalence in North American cattle, random-effects meta-analyses were conducted to obtain pooled fecal prevalence estimates for the USA and Canada for each outcome classification. Meta-analysis for serogroup fecal prevalence in USA and Canada included six and two articles representing 61 and 12 studies, respectively. Top 6 serogroup prevalence for the USA and Canada were 4.8% (95% CI = 2.6-8.4%) and 9.4% (95% CI = 1.7-38.8%), respectively. Fecal prevalence estimates for the serogroup outcome classification did not significantly differ by country ($P = 0.40$). Estimates obtained for STEC fecal prevalence in USA and Canada were extracted from six and two articles representing 74 and 5 studies, respectively. Whereas, estimated fecal prevalence for the top 6 STEC in pre- and peri-harvest cattle was significantly higher ($P < 0.05$) in the USA (1.3%, 95% CI = 0.9-1.8%) compared to Canada (0.2%, 95% CI = 0.1-0.4%). EHEC-specific estimates were estimated from eight and two articles representing 166 and 4 studies, from the USA and Canada, respectively. As observed for STEC, fecal EHEC prevalence was significantly ($P < 0.05$) higher in the USA (1.2%; 95% CI = 1.0-1.6%) compared to Canada (0.1%; 95% CI = 0.0-0.3%). Although there was evidence of between-study heterogeneity in these models, due to the limited number of studies per country, meta-regression analyses were not attempted for outcome classifications by country within North America.

Additional analysis

Meta-regression

Worldwide meta-regression analyses of fecal prevalence by outcome classification

There was evidence of considerable between-study heterogeneity in the worldwide random-effects meta-analysis model based on the I^2 statistic for all outcome classifications. Worldwide serogroup uni-variable meta-regression analyses identified region, time of harvest, cattle type, laboratory methods, and specimen type as factors significantly ($P < 0.10$) contributing to between-study heterogeneity of non-O157 serogroup fecal prevalence estimates in cattle worldwide (Table 5-8). In the multi-variable model, region, cattle type, laboratory methods, and specimen type were significant ($P < 0.05$) factors contributing to between-study heterogeneity of non-O157 serogroup prevalence estimates in cattle worldwide. The covariates included in the

multi-variable meta-regression model explain 42.1% (pseudo R^2) of between-study heterogeneity in the worldwide serogroup fecal prevalence meta-analysis.

Worldwide STEC uni-variable meta-regression models identified all factors (region, time of harvest, cattle type, and specimen type) except laboratory methods to contribute significantly ($P < 0.10$) to between-study heterogeneity (Table 5-9). In the multi-variable meta-regression model, all factors except time of harvest remained significant ($P < 0.05$) contributing to between-study heterogeneity. This multi-variable model explained 36.9% (pseudo R^2) of the between-study heterogeneity of the STEC outcome classification worldwide.

With respect to the EHEC classification, evidence of heterogeneity was identified between-studies of all regions with the exception of Asia and Australia/Oceania ($I^2 = 0.0\%$). All of the factors were identified as contributing significantly ($P < 0.10$) to between-study heterogeneity in the uni-variable meta-regression analyses (Table 5-10). Region, cattle type, laboratory methods, and specimen type remained as significant factors contributing to between-study heterogeneity in the multi-variable model. Covariates in the multi-variable meta-regression models explained 44.3% (pseudo R^2) of the between-study heterogeneity for the EHEC outcome classification worldwide.

North America meta-regression analyses of fecal prevalence by outcome classification

In the uni-variable meta-regression model, cattle type, laboratory method, and specimen type significantly ($P < 0.10$) contributed to the between-study heterogeneity in the serogroup outcome classification for North America (Table 5-11). Only laboratory method and specimen type remained in the multi-variable meta-regression model as contributing significantly ($P < 0.05$) to between-study heterogeneity. These covariates multi-variable explained 44.0% (pseudo R^2) of between-study heterogeneity in North American serogroup prevalence outcome.

For the STEC outcome classification, uni-variable meta-regression analyses identified time of harvest, cattle type, and specimen type as variables contributing significantly ($P < 0.10$) to between-study heterogeneity (Table 5-12). In the multivariable meta-regression, time of harvest

and cattle type remained significant ($P < 0.05$) and accounted for 26.3% (pseudo R^2) of between-study heterogeneity in North American STEC prevalence outcome.

Time of harvest, cattle type, and laboratory methods were contributing significantly ($P < 0.10$) to between-study heterogeneity in uni-variable meta-regression analyses for EHEC fecal prevalence in North America (Table 5-13). However, time of harvest and laboratory methods were the only variables significant ($P < 0.05$) in the multi-variable meta-regression accounting for 33.7% (pseudo R^2) of between-study heterogeneity in North America EHEC fecal prevalence outcome.

Risk of bias across studies

Asymmetry in the funnel plots for serogroup, STEC, and EHEC outcomes, worldwide and in North America, indicated potential publication bias was present (i.e., risk of bias across studies; data not shown). Bias coefficients using the Egger's test indicated that small study effects were present in worldwide and North America fecal prevalence meta-analyses. Bias coefficients (*P*-values) for serogroup, STEC, and EHEC worldwide prevalence outcomes were 0.53 ($P = 0.54$), –1.54 ($P < 0.01$), and –2.60 ($P < 0.01$), respectively. Similar to the worldwide meta-analysis, bias coefficients (*P*-values) for North America fecal prevalence for serogroup, STEC, and EHEC outcomes were 1.93 ($P = 0.31$), –2.69 ($P < 0.01$), and –3.93 ($P < 0.01$), respectively, indicate the presence of small study effects. Bias coefficients from the Egger test indicate that fecal prevalence estimates from smaller studies were lower than the fecal prevalence estimates from the larger studies for STEC and EHEC outcomes, but not for the serogroup outcome classification in both the worldwide and North America fecal prevalence meta-analysis.

Discussion

Summary of evidence

Following a systematic review process, we identified 70 relevant articles that met the risk of bias assessment on prevalence and concentration of non-O157 STEC in different bovine matrices worldwide and data were extracted. Most of the retrieved articles in this review represented non-O157 STEC and EHEC prevalence data in cattle feces. Results from the worldwide meta-analyses for non-O157 STEC (range = 0.3 – 1.3%) and EHEC (range = 0.2 – 1.3%) fecal outcomes indicated that cattle harbor and shed these organisms in regions across the globe at

relatively low frequencies. Whereas concentration data were limited, when detected and reported in fecal and hide matrices, STEC and EHEC concentrations were at high-shedding concentrations ($\geq 10^4$ CFU/gram or $\geq 10^4$ CFU/cm^2) albeit for a limited number of cattle sampled. Likely, based on the limit of detection of available diagnostic methods for quantification, we are better at detecting samples with higher concentrations than those with a lower load. This review included a single article reporting quantification on pre-intervention carcasses, and there were no top 6 serogroups detected. Although limited in this review, concentration data offer a crucial piece of information when evaluating food safety risk along the beef continuum. In the literature, it has been documented that even at extremely low concentrations, fewer than 10 cells, pathogenic *E. coli* can induce human illness (Hara-Kudo and Takatori, 2011) thus demonstrating the pathogenicity of these organisms and their threat to public health via the cattle reservoir.

The pooled fecal prevalence estimates from the worldwide meta-analysis models significantly varied by region with non-O157 serogroup, STEC, and EHEC estimates being the highest in North America. Further, top 6 STEC and EHEC estimates of fecal prevalence were significantly greater in cattle in the USA compared to Canada, thus demonstrating variation between countries within the region. It is likely that prevalence estimates will vary also between countries in other regions.

In this review, the most prevalent EHEC O group in North American cattle feces was O103, which is the second most frequently reported non-O157 O group associated with culture-confirmed human STEC infections (15.6%) in the USA (CDC, 2018). Although we cannot directly attribute these clinical human STEC infections to cattle feces and/or contaminated beef, our data support cattle as a reservoir of these foodborne pathogens associated with human illness and demonstrate the potential threat of these non-O157 STEC of clinical importance to public health and food safety. From this review, limited conclusions can be drawn from hide and carcass results reported due to the low number of articles retrieved and the large variation between articles. Though peri-harvest hide and carcass prevalence and concentration data are the most crucial, as they are the best indicators of the contamination burden before carcasses are subjected to antimicrobial interventions at the harvest facility, these were the most limited data, regardless of the region (Arthur *et al.*, 2009; Brichta-Harhay *et al.*, 2008; Stephens *et al.*, 2009).

Limitations of the body of literature

In this review, the main limitations when reviewing the body of literature retrieved included: lack of standardization of the case definition, unclear numerator and/or denominators for prevalence, unspecified study population, and a wide array of sample collection and laboratory methodologies employed. Firstly, there is no clear and consistent case definition for STEC and/or EHEC reported in the literature. Therefore, outcome classifications were categorized by reviewers based on non-O157 O gene and virulence gene profiles leading to our outcome classifications for serogroup, STEC, and EHEC. Articles retrieved in the search included combined estimates of "STEC" or "non-O157 STEC" which included O groups not of interest and/or did not allow for data to be extracted by O gene; as a result, these articles did not meet the risk of bias assessment criteria and were excluded. Excluding these articles may have biased our overall non-O157 STEC estimates obtained; however, our objective was to obtain estimates of the most prevalent O groups (or top 6), rather than other non-O157 groups. Conversely, in some articles, when researchers reported serotypes, data were extracted for serogroup, STEC, and EHEC by O gene rather than serotype.

During the risk of bias assessment, many articles were excluded because a numerator and/or denominator was not reported (criterion 5 in the risk of bias assessment) and crude prevalence could not be calculated. In some instances, fecal samples from cattle of different ages were combined into one estimate and we could not identify a numerator and denominator for our population age group of interest (i.e., adult cattle). Additionally, in some cases, fecal samples from multiple ruminant species were combined and the numerator and denominator for each species could not be determined, thus values could not be extracted. Although these articles contain information that may be relevant to our research question, we could not distinguish and accurately attribute it to our target population.

There are several methodologies utilized to sample, isolate, and quantify STEC in cattle feces, hides, and carcasses. Sample collection methods and actual sample specimens collected varied between studies, especially for fecal sampling. The types of fecal specimen data extracted in this review included pen-floor, rectal grab, rectal swab, cecal, and unreported. For the hide and

carcass matrices, samples were typically collected with sponges, however, the surface area swabbed, stage of harvest, and media used were not consistent among studies. Many laboratory detection methods for isolation and quantification of STEC in these matrices exist. In this review, we chose to exclude articles published before the year 2000 in an attempt to minimize the variability in laboratory methods and their corresponding sensitivity of detection.

Specifically, we wanted to incorporate studies that employed an IMS step, as this procedure has improved the sensitivity of culture-based methods (Chapman *et al.,* 1994; Cernicchiaro *et al.,* 2013). However, the majority of articles relied on culture and/or molecular testing, and only 25 of the 57 articles included in the worldwide fecal prevalence meta-analysis reported using IMS. Additionally, whereas IMS has demonstrated an increased sensitivity, available culture methods are not equivalent in terms of detection, hence broadly categorizing laboratory methods as done in this study may contribute to the heterogeneity observed (Stromberg *et al.,* 2016a). Nevertheless, publication year did not necessarily reflect study year as some of the studies published in early 2000 were conducted in mid or late 1990s, and as such, some of their diagnostic protocols are not comparable to the ones currently used. We did not correct for these anomalies, but we did categorize laboratory methodology to account for the different methods employed the best way we could while still attempting to deduce any methodological differences. The variability in methodology for sample collection and laboratory testing creates challenges when trying to compare prevalence estimates retrieved from studies worldwide. For example, in this review, articles where researchers reported the utilization of detection methods considered standard (e.g., culture, IMS, and PCR), the top 6 EHEC prevalence on peri-harvest cattle hides ranged from undetected to 5.0% (Thomas *et al.,* 2012; Stromberg *et al.,* 2015, 2016b). Articles reporting the use of more recent technology, such as the BAX® System (DuPont Qualicon, Wilmington, DE) or NeoSeek™ STEC detection and identification test (Neogen, Lansing, MI), reported a wider range (undetected to 57.6%) of top 6 EHEC peri-harvest cattle hide prevalence (Chaves *et al.,* 2013; Schneider *et al.,* 2018b; Stromberg *et al.,* 2015, 2016b). In this review, top 6 EHEC hide prevalence estimates seem to be highly variable and numerically higher compared to the other outcome classifications (i.e., serogroup and STEC), which may be due to the laboratory methodologies used to obtain these estimates. For example, in two articles (Stromberg *et al.,* 2015, Stromberg *et al.,* 2016b) where researchers

compared two laboratory methodologies —'Other (NeoSeek™)' and 'culture + IMS'— prevalence estimates for the top 6 EHEC ranged from undetected to 5.0% and 0.5 to 49.0% for 'culture + IMS' and 'Other (NeoSeek™)', respectively. Whereas data were reported descriptively for hide prevalence, the variability between these two methodologies (i.e., 'culture + IMS' and 'Other (NeoSeek™) is clear as they yield very different prevalence estimates when testing the same samples. Due to the numerous detection methods reported in the 70 articles retrieved, at the sacrifice of losing methodological details that may explain the variability in prevalence estimates and between-study heterogeneity observed, laboratory methodologies employed had to be broadly categorized for analysis. Therefore, when trying to evaluate laboratory methodologies employed as a potential variable contributing to between-study heterogeneity, categorization of these methods into wider categories such as: culture, culture + IMS, PCR only, or other, likely oversimplified the complexities of the laboratory methodology employed. In this review, we found that the type of laboratory methods significantly explained some of the between-study heterogeneity in uni-variable and multi-variable meta-regression models for the top 6 fecal EHEC. Since apparent prevalence estimates are directly impacted by the accuracy of the detection protocols used, the estimates of the present analysis may be biased, however, given the diversity of detection protocols employed and their different accuracy, it will be difficult to predict the directionality of the potential bias. Sources of between-study heterogeneity were not evaluated for hide and carcass prevalence data.

Limitations of the review

Key limitations of this study include: only peer-reviewed literature was considered, limited data used to populate some analyses may not yield reliable estimates, unexplained heterogeneity remains in our models, and several forms of bias are plausible. Non-primary research (literature reviews, short communications, abstract-only, conference proceedings), non-peer reviewed, and grey literature were not included in this systematic review. In addition to electronic databases, we hand searched reference lists of peer-reviewed papers and 17 articles were identified in the hand search that were not found in the electronic search. By limiting this review to only consider peer-reviewed literature, we were not able to include data that were not yet published at the time of our search (March 2019) but were pertinent to our research question such as Cernicchiaro *et al.* (2020) and other studies discussed internally and/or at conferences but not yet published.

While this limited our sample size of eligible articles, the articles that were included in this review underwent a rigorous peer-review process and are more likely representative of final, accurate estimates, which may or may not be the case for preliminary data shared at conferences. Additional concerns with the inclusion of non-primary research, non-peer reviewed, and grey literature would be the possibility of including redundant estimates from research that was presented at a conference and later published. If our hypothesis is accurate that inclusion of grey literature leads to overrepresentation/repetition of certain data, our model estimates likely would not change, but the measures of variability (e.g., standard errors, confidence intervals) may be smaller, however, these would be artifactual (given by a larger number of studies being represented in the data).

In total, 70 articles were retrieved worldwide, however when considering articles by outcome classification, across three matrices, and by region, data were especially limited for some subgroup analyses. Due to the small number of studies included in some of the subgroup analyses and meta-regression models, estimates should be interpreted with caution (Higgins and Thompson 2004; Higgins *et al.,* 2019). Similarly, very few articles reported model-adjusted prevalence estimates after accounting for the hierarchical structure of the data or the study design features. Except those cases, the precision of the estimates may be underestimated. To avoid such methodological differences, only raw data were extracted as well as sole information from the respective organizational level (e.g., sample-level). Additionally, given the structure of our dataset there is also a hierarchical structure to consider: we have extracted data from studies nested within articles, and articles within region for each matrix and outcome. Whereas a multi-level (three-level meta-analysis) model would likely not have a large impact on the coefficients we obtained, the standard errors associated with the estimates would be smaller as the hierarchy and correlation of studies within articles would be accounted for. As region, considered an important source of variability, was accounted for in subgroup analysis and given the scarcity of articles retrieved for analysis, we chose to fit a simpler, more parsimonious model acknowledging our standard errors may be underestimated for the fecal prevalence estimates obtained.

The most significant and novel aspect of this review was the exploration of sources of between-study heterogeneity of serogroup, STEC, and EHEC fecal prevalence estimates on a global scale. For the worldwide meta-analysis, between-study heterogeneity was evaluated for key variables of interest. Cattle type, specimen type, and region were all significant variables in multi-variable meta-regressions for serogroup, STEC, and EHEC outcome classifications for global fecal prevalence ($P \leq 0.05$). It is likely that the differences in animal and farm management and production systems among different regions contributed to the between-study heterogeneity. Although the exact management/production systems were not directly reported and extracted, we classified the study population by type (beef or dairy) to attempt to measure these differences. Eight articles presented estimates for beef and dairy combined, whereas in another eight articles we could not determine if they were beef and/or dairy cattle. This lack of separation between cattle production type for sixteen articles may have limited the ability to detect potential management and/or production system differences, if present, for beef and dairy cattle in this review. In addition, we grouped the extracted fecal prevalence data into geographical regions to minimize variability and account for regional differences in production systems. Whereas reasons for combining estimates within region are intuitive, analyses of North America demonstrated significant differences in observed STEC and EHEC fecal prevalence estimates between the USA and Canada. In addition to North America, four other regions were included in the worldwide analysis, with the following countries within each region: Asia (Bangladesh, India, Japan, Korea, South Korea), Australia/Oceania (Australia, New Zealand), Europe (Belgium, France, Germany, Ireland, Italy, Scotland, Spain, Switzerland), and South America (Argentina, and Brazil). Therefore, by combining estimates within region we may have masked some local differences, of unknown sources, that are present in the real-world. Although North America was the only region further explored, it is plausible countries within other regions in this review could also be significantly different in terms of apparent prevalence.

Variables, in addition to region, such as specimen type, laboratory method, and time of harvest, explained some of the between-study heterogeneity observed in global and North American fecal prevalence meta-analyses. However, additional factors and their potential interactions may further explain the observed variability among studies and prevalence estimates. Season, age, and diet are factors that are known to influence *E. coli* O157 fecal shedding in cattle and have

been well-established in peer-reviewed literature (Barkocy-Gallagher *et al.,* 2003; Edrington *et al.,* 2006; Callaway *et al.,* 2009; Ekiri *et al.,* 2014). Whereas the seasonality of the top 6 has been recently evaluated (Dewsbury *et al.,* 2015; Ekiri *et al.,* 2014; Schneider *et al.,* 2018a), the limited number of studies precluded us from evaluating season in this review. Recently, our group (Cernicchiaro *et al.,* 2020) published a study evaluating associations between season, processing plants, and hide cleanliness scores with prevalence and concentration on beef cattle hides in the USA for non-O157 STEC. This research demonstrated the seasonality of non-O157 STEC, by O group, as well as differences observed between plants and with quantification data on cattle hides presented. Unfortunately, this study was published after our search was conducted and therefore was not eligible to be included in this review. Though these newly published data are extremely valuable, data remains limited to comprehensively assess all potential pertinent risk factors and potential underlying complex interactions for shedding as well as synthesizing estimates for hide and carcass prevalence and concentration.

Overall, heterogeneity in this study could not be attributed to a particular source of bias. In addition to publication bias, many other sources of selection bias such as those associated with geographic region could be present, along with differences in study quality and design, true heterogeneity, and chance (Chan *et al.,* 2004; Egger *et al.,* 1997; Higgins and Green, 2011; O'Connor *et al.,* 2014; Sterne *et al.,* 2000, 2011). It is possible that empirical data produced in certain geographical locations are published in local reporting systems or journals in the native language rather than in international journals. Since articles written in languages other than English were excluded, there is potential for language bias as valuable data available in other languages would have been missed. In summary, we attempted to control for internal and external validity factors that could have biased our estimates during the risk of bias assessment step and acknowledge other limitations previously discussed which could potentially lead to bias.

Conclusions

This study, the first of its kind, gathered and synthesized estimates of prevalence and concentration of top 6 non-O157 serogroup, STEC, and EHEC in fecal, hide, and carcass samples from pre- and peri-harvest cattle from countries across the globe. Furthermore, this study identified important knowledge gaps in published literature for hide and carcass prevalence

data, in addition to concentration data for all matrices. Peri-harvest hide and carcass prevalence and concentration data—arguably the most important data for mitigating beef adulteration—were the most limited. In addition to summarizing measures of pathogen frequency and concentration, this study identified some of the factors responsible for between-study heterogeneity, such as region, cattle type, and specimen type, for cattle fecal prevalence worldwide. Although this review summarizes all relevant data currently available, future research is needed to obtain additional hide and carcass prevalence data as well as quantification of these pathogens in all matrices of interest. The synthesized estimates of prevalence from this review could be integrated into a quantitative microbial risk assessment model to assess the potential risks attributable to non-O157 STEC in the beef chain. Similarly, this evidence is highly valued in expert panels such as the ones convened by the Food and Agriculture Organization of the United Nations, the World Health Organization, as well as the Codex Alimentarius Commission when developing guidelines on various food safety topics (e.g., Microbiological Risk Assessment Series).

With robust estimates of frequency and quantity of these foodborne pathogens in these cattle matrices, we could better identify primary targets for pre- and peri-harvest intervention methods to optimize STEC mitigation strategies to reduce adulteration of beef products worldwide.

Other information

Protocol

The initial study protocol for this project is not publicly accessible.

Availability of data, code, and other supplementary materials

The supplementary material containing the causal diagram can be found in Appendix A. The supplementary material containing the methodology and results for the outlier and influential diagnostics performed for this study can be found in Appendix B with the attached annotated R code and datafile.

Author contributions

DD was the graduate student responsible for the study with direct oversight by NC, conducted all steps of the research under her guidance, performed the data analysis, and drafted the manuscript. NC was responsible for obtaining funding, identifying the research team, providing a protocol, training graduate student (DD), directly involved in all methodology, reviewed the data analysis, assisted with the interpretation of results, and manuscript preparation. MS contributed intellectual input throughout the systematic review process, directly involved with the risk of bias assessment and the data extraction processes, and provided input on manuscript drafts. AD contributed extensively to R coding, data analysis, and manuscript preparation. PE contributed to the risk of bias assessment, data extraction template, and provided input on manuscript drafts. All co-authors have read and approve the final manuscript draft.

Support

This project was supported by the Agriculture and Food Research Initiative Grant No. 2012-68003-30155 from the USDA National Institute of Food and Agriculture, Prevention, Detection and Control of Shiga Toxin-Producing *Escherichia coli* (STEC) from Pre-Harvest Through Consumption of Beef Products Program–A4101, and the College of Veterinary Medicine, at Kansas State University.

Competing interests

No competing financial interests or conflicts exist for any of the authors.

Acknowledgements

The authors wish to thank Monica Anderson for help with retrieving full-text articles.

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Table 5-1. Inclusion and exclusion criteria for eligibility (relevance screening) of articles for the present systematic review of the literature.

Criteria	Inclusion	Exclusion
Language	English	Languages other than English
Publication year	2000-2019	Prior to 2000
Population	Healthy, adult cattle (8 months and older) pre- and peri-harvest Pre-harvest: cattle before transport to the harvest facility Peri-harvest: the time after cattle leave the farm until after stunning and hide removal prior to any carcass interventions Any breed	Calves (< 8 months) Species other than cattle Diseased cattle
Sample type	Fecal: pen-floor, rectal swab, rectal grab/intestinal contents, cecal content (sampled pre- or post-harvest) Hide and carcass: samples (e.g., sponge, swab, etc.) prior to any in-plant intervention	Hide and carcass: samples post in-plant interventions (e.g., hide wash, carcass wash).
Study type	Observational studies (cross-sectional, cohort, case-control) Laboratory trials (using field samples)	Experimental studies In vitro (laboratory) experiments Non-primary research (e.g., literature reviews)
Outcomes	*Escherichia coli* O26, O45, O103, O111, O121, and O145 Virulence genes: *stx*$_1$, *stx*$_2$, *eae*	Bacterial species other than *Escherichia coli* All other *Escherichia coli* O serogroups All other virulence genes
Outcome measures	Prevalence (or proportion positive), concentration	Outcomes other than prevalence and concentration
Region	North America (United States of America, Mexico, and Canada)	See below*

*Initially, the search was restricted to articles produced in North America; however, given the low number of articles, we expanded the search to include articles available in English from peer-reviewed literature and cattle populations worldwide.

Table 5-2. Risk of bias assessment criteria.

		Data Extracted	Data Not Extracted**
Criteria	**Outcome**	**No. Articles**	**No. Articles**
1. Was the sample size justified?	No / unknown / not reported	58	74
	Yes	12	9
***2. Was the study population properly described?**	No / unknown	0	10
	Yes (cattle; beef and/or dairy cattle)	70	73
***3. Were the animals housed or grouped in a way that is representative of field/ commercial conditions?**	No / unknown / not reported	0	9
	In part - closed system; research farms	5	6
	Yes - typical of commercial operations	65	68
4. Study catchment area	Single-site (one operation / farm / processing plant)	26	24
	Multi-site (multiple operation / farms / processing plants / multiple states)	44	59
***5. Were the numerator and denominator for the prevalence provided?**	No numerator and/or denominator (can't calculate prevalence)	0	60
	Provided both numerator and denominator (or prevalence and numerator/denominator; can calculate prevalence)	70	23
6. Was time/duration (month, season) of study reported?	No / unknown / multiple seasons but cumulative prevalence	50	68
	Less than 3 months	6	5
	Three months or more (full season)	14	10
***7. Can clearly identify at least one non-O157 STEC serogroup (O26, O45, O103, O111, O121, or O145)**	No	0	28
	Yes	70	55

*Articles that did not meet criteria 2, 3, 5 or 7 were excluded and were not considered for data extraction.

**There were 168 articles deemed relevant for the risk of bias assessment. In total, 70 articles met the risk of bias assessment criteria and data were extracted, 83 articles failed the risk of bias assessment and data were not extracted. Additionally, upon further reviewing the full-text articles that were eligible for the risk of bias assessment, the did not meet inclusion criteria (e.g., study type) and were excluded.

Table 5-3. List of the articles included in the worldwide meta-analysis of fecal prevalence across all outcome classifications by key study variables.

Variable	No. articles	References
Region		
Asia	11	Das *et al.*, 2005; Islam *et al.*, 2008; Jeon *et al.*, 2006; Kang *et al.*, 2014; Khan *et al.*, 2002; Kijima-Tanaka *et al.*, 2005; Kobayashi *et al.*, 2001; Sasaki *et al.*, 2011, 2013a, 2013b, Shinagawa *et al.*, 2000
Australia/Oceania	6	Barlow and Mellor 2010; Cobbold and Desmarchelier, 2000; Hornitzky *et al.*, 2002; Jaros *et al.*, 2016; Mellor *et al.*, 2016; Midgley and Desmarchelier, 2001
Europe	17	Bibbal *et al.*, 2015; Bolton *et al.*, 2014; Bonardi *et al.*, 2005, 2007; Joris *et al.*, 2011, 2013; Lynch *et al.*, 2012; Monaghan *et al.*, 2011; Murphy *et al.*, 2016; Orden *et al.*, 2002; Pearce *et al.*, 2004, 2006; Pradel *et al.*, 2000; Shaw *et al.*, 2004; Thomas *et al.*, 2012; Zschöck *et al.*, 2000; Zweifel *et al.*, 2005
North America	18	Agga *et al.*, 2017; Bai *et al.*, 2012; Baltasar *et al.*, 2014; Cull *et al.*, 2017; Dargatz *et al.*, 2013; Dewsbury *et al.*, 2015; Ekiri *et al.*, 2014; Hallewell *et al.*, 2016; Karama *et al.*, 2008; Paddock *et al.*, 2012; Renter *et al.*, 2007; Schneider *et al.*, 2018a; Schurman *et al.*, 2000; Shridhar *et al.*, 2017; Singh *et al.*, 2015; Stanford *et al.*, 2016; Stromberg *et al.*, 2016b; Thran *et al.*, 2001
South America	5	Farah *et al.*, 2007; Fernández *et al.*, 2010; Meichtri *et al.*, 2004; Padola *et al.*, 2004; Timm *et al.*, 2007
Time of Harvest		
Pre-harvest	37	Bai *et al.*, 2012; Baltasar *et al.*, 2014; Bolton *et al.*, 2014; Cobbold and Desmarchelier, 2000; Cull *et al.*, 2017; Dargatz *et al.*, 2013; Das *et al.*, 2005; Dewsbury *et al.*, 2015; Ekiri *et al.*, 2014; Fernández *et al.*, 2010; Hallewell *et al.*, 2016; Hornitzky *et al.*, 2002; Jeon *et al.*, 2006; Joris *et al.*, 2013; Kang *et al.*, 2014; Khan *et al.*, 2002; Kijima-Tanaka *et al.*, 2005; Kobayashi *et al.*, 2001; Lynch *et al.*, 2012; Midgley and Desmarchelier, 2001*; Monaghan *et al.*, 2011; Murphy *et al.*, 2016; Orden *et al.*, 2002; Paddock *et al.*, 2012; Padola *et al.*, 2004; Pearce *et al.*, 2004, 2006; Renter *et al.*, 2007; Sasaki *et al.*, 2011, 2013a, 2013b; Schneider *et al.*, 2018a; Shaw *et al.*, 2004; Shridhar *et al.*, 2017; Singh *et al.*, 2015; Thran *et al.*, 2001; Zschöck *et al.*, 2000
Peri-harvest	22	Adamu *et al.*, 2018; Agga *et al.*, 2017; Barlow *et al.*, 2010; Bibbal *et al.*, 2015; Bonardi *et al.*, 2005, 2007; Farah *et al.*, 2007; Islam *et al.*, 2008; Jaros *et al.*, 2016; Joris *et al.*, 2011; Karama *et al.*, 2008; Meichtri *et al.*, 2004; Mellor *et al.*, 2016; Midgley and Desmarchelier, 2001*; Pradel *et al.*, 2000; Schurman *et al.*, 2000; Shinagawa *et al.*, 2000; Stanford *et al.*, 2016; Stromberg *et al.*, 2016b; Thomas *et al.*, 2012; Timm *et al.*, 2007; Zweifel *et al.*, 2005

Cattle Type		
Beef	33	Agga *et al.*, 2017; Bai *et al.*, 2012; Baltasar *et al.*, 2014; Barlow and Mellor 2010; Bibbal *et al.*, 2015*; Cull *et al.*, 2017; Dargatz *et al.*, 2013; Dewsbury *et al.*, 2015; Ekiri *et al.*, 2014; Farah *et al.*, 2007; Hallewell *et al.*, 2016; Hornitzky *et al.*, 2002*; Jaros *et al.*, 2016; Joris *et al.*, 2011, 2013; Kang *et al.*, 2014*; Karama *et al.*, 2008; Kijima-Tanaka *et al.*, 2005; Meichtri *et al.*, 2004; Mellor *et al.*, 2016*; Midgley and Desmarchelier, 2001; Paddock *et al.*, 2012*; Padola *et al.*, 2004; Pearce *et al.*, 2004; Renter *et al.*, 2007; Sasaki *et al.*, 2011, 2013a*; Schneider *et al.*, 2018a; Schurman *et al.*, 2000; Shaw *et al.*, 2004; Shridhar *et al.*, 2017; Thomas *et al.*, 2012; Timm *et al.*, 2007
Beef and Dairy	8	Bonardi *et al.*, 2005, 2007; Hornitzky *et al.*, 2002*; Jeon *et al.*, 2006; Monaghan *et al.*, 2011; Paddock *et al.*, 2012*; Sasaki *et al.*, 2013a*; Shinagawa *et al.*, 2000
Dairy	16	Bibbal *et al.*, 2015*; Cobbold and Desmarchelier 2000; Das *et al.*, 2005; Fernández *et al.*, 2010; Kang *et al.*, 2014*; Kobayashi *et al.*, 2001; Lynch *et al.*, 2012; Mellor *et al.*, 2016*; Murphy *et al.*, 2016; Paddock *et al.*, 2012*; Sasaki *et al.*, 2013a*, 2013b; Singh *et al.*, 2015; Stromberg *et al.*, 2016b; Thran *et al.*, 2001; Zschöck *et al.*, 2000
Unknown	8	Bolton *et al.*, 2014; Islam *et al.*, 2008; Khan *et al.*, 2002; Orden *et al.*, 2002; Pearce *et al.*, 2006; Pradel *et al.*, 2000; Stanford *et al.*, 2016; Zweifel *et al.*, 2005
Laboratory Methods		
Culture	29	Bai *et al.*, 2012*; Baltasar *et al.*, 2014; Bolton *et al.*, 2014; Cobbold and Desmarchelier, 2000; Das *et al.*, 2005; Ekiri *et al.*, 2014*; Farah *et al.*, 2007; Fernández *et al.*, 2010; Hornitzky *et al.*, 2002; Islam *et al.*, 2008; Kang *et al.*, 2014; Khan *et al.*, 2002; Kijima-Tanaka *et al.*, 2005; Kobayashi *et al.*, 2001; Lynch *et al.*, 2012*; Meichtri *et al.*, 2004; Monaghan *et al.*, 2011; Orden *et al.*, 2002; Paddock *et al.*, 2012; Padola *et al.*, 2004; Pradel *et al.*, 2000; Renter *et al.*, 2007; Schurman *et al.*, 2000; Shaw *et al.*, 2004; Singh *et al.*, 2015; Thran *et al.*, 2001; Timm *et al.*, 2007; Zschöck *et al.*, 2000; Zweifel *et al.*, 2005
Culture + IMS	25	Bai *et al.*, 2012*; Barlow and Mellor 2010; Bibbal *et al.*, 2015; Bonardi *et al.*, 2005, 2007; Cull *et al.*, 2017; Dewsbury *et al.*, 2015; Ekiri *et al.*, 2014*; Hallewell *et al.*, 2016; Jaros *et al.*, 2016, Jeon *et al.*, 2006; Joris *et al.*, 2011, 2013; Lynch *et al.*, 2012*; Mellor *et al.*, 2016; Murphy *et al.*, 2016; Pearce *et al.*, 2004, 2006; Sasaki *et al.*, 2011, 2013a, 2013b; Shinagawa *et al.*, 2000; Shridhar *et al.*, 2017, Stromberg *et al.*, 2016b*; Thomas *et al.*, 2012
PCR only	3	Dargatz *et al.*, 2013; Karama *et al.*, 2008; Stanford *et al.*, 2016
Other	4	Agga *et al.*, 2017; Midgley and Desmarchelier, 2001; Schneider *et al.*, 2018a; Stromberg *et al.*, 2016b*

Specimen Type		
Pen-floor	11	Bolton *et al.*, 2014; Cull *et al.*, 2017; Dewsbury *et al.*, 2015; Midgley and Desmarchelier, 2001; Monaghan *et al.*, 2011; Paddock *et al.*, 2012; Pearce *et al.*, 2006; Renter *et al.*, 2007; Schneider *et al.*, 2018a; Shridhar *et al.*, 2017; Stanford *et al.*, 2016
Rectal grab	20	Agga *et al.*, 2017*; Baltasar *et al.*, 2014; Barlow and Mellor 2010; Das *et al.*, 2005; Ekiri *et al.*, 2014; Hallewell *et al.*, 2016; Islam *et al.*, 2008; Joris *et al.*, 2013; Karama *et al.*, 2008; Kobayashi *et al.*, 2001; Mellor *et al.*, 2016; Orden *et al.*, 2002; Sasaki *et al.*, 2011, 2013a, 2013b; Shaw *et al.*, 2004; Singh *et al*., 2015; Stromberg *et al.*, 2016b, Thomas *et al.*, 2012; Thran *et al.*, 2001
Rectal swab	16	Agga *et al.*, 2017*; Cobbold and Desmarchelier, 2000; Dargatz *et al.*, 2013; Farah *et al.*, 2007; Fernández *et al.*, 2010; Jaros *et al.*, 2016; Joris *et al.*,2011; Kang *et al.*, 2014; Lynch *et al.*, 2012; Meichtri *et al.*, 2004; Murphy *et al.*, 2016; Padola *et al.*, 2004; Pearce *et al.*, 2004; Schurman *et al.*, 2000; Timm *et al.*, 2007; Zschöck *et al.*, 2000
Cecal	2	Bonardi *et al.*, 2005, 2007
Unknown	9	Bai *et al.*, 2012; Bibbal *et al.*, 2015; Hornitzky *et al.*, 2002; Jeon *et al.*, 2006; Khan *et al.*, 2002; Kijima-Tanaka *et al.*, 2005; Pradel *et al.*, 2000; Shinagawa *et al.*, 2000; Zweifel *et al.*, 2005

*Indicates article is present in more than one category within variable (e.g., time of harvest, cattle type, etc.).

Table 5-4. Hide prevalence data extracted with key study characteristics.

Author, year	Cattle Type	Region (country)	Sample Description	Laboratory Method*	Outcome Classification	Prevalence, % (# positives/total)
Chaves *et al.*, 2013	Beef	Central America (Honduras[δ])	swab from foreshank prior to skinning; area not stated	PCR only (BAX® System (DuPont Qualicon)	EHEC O26	36.0 (11/30)
					EHEC O45	31.0 (9/30)
					EHEC O103	3.0 (1/30)
					EHEC O111	0.0 (0/30)
					EHEC O121	24.0 (7/30)
					EHEC O145	6.0 (2/30)
		Central America (Nicaragua[δ])			EHEC O26	47.0 (24/50)
					EHEC O45	4.0 (2/50)
					EHEC O103	1.0 (1/50)
					EHEC O111	0.0 (0/50)
					EHEC O121	46.0 (23/50)
					EHEC O145	1.0 (1/50)
Midgley and Desmarchelier, 2001	Beef	Australia	25 cm^2 area swabbed at the brisket prior to hide removal	Other (Colony hybridization, Microbact 12E (Oxoid) kit, PFGE, latex agglutination kits)	EHEC O26	4.0 (2/50)
					EHEC O111	0.0 (0/50)
Monaghan *et al.*, 2012	Beef	Europe	100 cm^2 area swabbed at the rump immediately prior to hide removal	Culture	serogroup O26	0.0 (0/450)
					STEC O26	0.0 (0/450)
					EHEC O26	0.0 (0/450)
Schneider *et al.*, 2018b	Beef, dairy	North America[‡] (Northern USA)	1,000 cm^2 behind shoulder (approximately 15 cm from midline) after	Other (NeoSeek™)	EHEC O26	13.4 (49/365)
					EHEC O45	53.2 (194/365)
					EHEC O103	34.8 (127/365)
					EHEC O111	13.7 (50/365)
					EHEC O121	17.3 (63/365)

		North America‡ (Southern USA)	exsanguination prior to hide removal		EHEC O145	17.5 (64/365)
					EHEC O26	20.3 (77/379)
					EHEC O45	57.5 (218/379)
					EHEC O103	35.9 (136/379)
					EHEC O111	29.3 (111/379)
					EHEC O121	18.2 (69/379)
					EHEC O145	22.4 (85/379)
Stromberg *et al.*, 2015	Beef	North America (Central USA)	1,000 cm^2 behind shoulder (approximately 15cm from midline) after exsanguination, immediately prior to hide wash and removal (*bacteriophage for *E. coli* O157 was applied in holding pens, cattle were rinsed with H_2O just after stunning per routine plant protocol)	Other (NeoSeek™)	EHEC O26	0.5 (3/576)
					EHEC O45	39.4 (227/576)
					EHEC O103	18.6 (107/576)
					EHEC O111	2.3 (13/576)
					EHEC O121	2.4 (14/576)
					EHEC O145	49.0 (282/576)
				Culture + IMS	EHEC O26	0.4 (2/476†)
					EHEC O45	0.0 (0/476†)
					EHEC O103	0.0 (0/476†)
					EHEC O111	0.0 (0/476†)
					EHEC O121	0.0 (0/476†)
					EHEC O145	0.2 (1/476†)
Stromberg *et al.*, 2016b	Dairy	North America (Western USA)	1,000 cm^2 behind shoulder (approximately 15 cm from midline) after exsanguination, immediately prior to hide wash and removal	Other (NeoSeek™)	EHEC O26	7.0 (7/100)
					EHEC O45	36.0 (36/100)
					EHEC O103	10.0 (10/100)
					EHEC O111	15.0 (15/100)
					EHEC O121	3.0 (3/100)
					EHEC O145	23.0 (23/100)
				Culture + IMS	EHEC O26	5.0 (5/100)

			(*cattle were rinsed with H_2O just after stunning per routine plant protocol)		EHEC O45	0.0 (0/100)
					EHEC O103	0.0 (0/100)
					EHEC O111	1.0 (1/100)
					EHEC O121	1.0 (1/100)
					EHEC O145	0.0 (0/100)
Svoboda *et al.*, 2013	Beef	North America (USA)	100 cm^2 area swabbed at each the flank, brisket, and rump	Culture + IMS	STEC O26	0.0 (0/27)
					STEC O45	0.0 (0/27)
					STEC O103	0.0 (0/27)
					STEC O111	0.0 (0/27)
					STEC O121	0.0 (0/27)
					STEC O145	0.0 (0/27)
Thomas *et al.*, 2012	Beef	Europe (Ireland)	100 cm^2 area swabbed at the brisket prior to hide removal	Culture + IMS	serogroup O26	6.0 (24/402)
					serogroup O111	0.0 (0/402)
					serogroup O103	27.1 (109/402)
					serogroup O145	2.5 (10/402)
					STEC O26	0.3 (1/402)
					STEC O111	0.0 (0/402)
					STEC O103	0.3 (1/402)
					STEC O145	0.0 (0/402)
					EHEC O26	0.3 (1/402)
					EHEC O111	0.0 (0/402)
					EHEC O103	0.0 (0/402)
					EHEC O145	0.0 (0/402)

*Laboratory methods presented in this table are how authors extracted and categorized data for analysis; for full laboratory method protocols used refer to the original manuscript referenced. If category was "Other", method of detection was stated in parenthesis.

[δ]Sample numerators and prevalence estimates were estimated from Figure 1 (Chaves *et al.*, 2013) to report estimates by region.

[‡]Schneider *et al.*, 2018b data are also presented by season, for presentation purposes authors chose to present by region.

[†]The sample denominator for Stromberg *et al.*, 2015 differed between the Other (NeoSeek™) method and Culture + IMS methods extracted by 100, due to inadequate DNA for 100 samples collected.

Table 5-5. Carcass prevalence data extracted with key study characteristics.

Author, year	Cattle Type	Region (country)	Laboratory Method	Laboratory Method*	Outcome Classification	Prevalence, % (# positives/total)
Stromberg *et al.*, 2015	Beef	North America (Central USA)	Sponge 1: 1,000 cm^2 brisket-short plate region sponge Sponge 2: 3,000 cm^2 lateral hock and round rump regions area prior to the first carcass wash (both sponges were combined for each animal)	Other (NeoSeek™)	EHEC O26	0.4 (2/576)
					EHEC O45	1.4 (8/576)
					EHEC O103	1.7 (10/576)
					EHEC O111	0.0 (0/576)
					EHEC O121	0.0 (0/576)
					EHEC O145	1.9 (11/576)
Stromberg *et al.*, 2016b	Dairy	North America (Western USA)	Sponge 1: 1,000 cm^2 brisket-short plate region sponge Sponge 2: 3,000 cm^2 lateral hock and round rump regions area prior to the first carcass wash (both sponges were combined for each animal)	Other (NeoSeek™)	EHEC O26	0.0 (0/100)
					EHEC O45	0.0 (0/100)
					EHEC O103	0.0 (0/100)
					EHEC O111	0.0 (0/100)
					EHEC O121	0.0 (0/100)
					EHEC O145	0.0 (0/100)
				Culture + IMS	EHEC O26	3.0 (3/100)
					EHEC O45	0.0 (0/100)
					EHEC O103	4.0 (4/100)
					EHEC O111	0.0 (0/100)
					EHEC O121	0.0 (0/100)
					EHEC O145	2.0 (2/100)
Svoboda *et al.*, 2013	Beef	North America (USA)	100 cm^2 area swabbed at each the flank, brisket, and rump, prior to	Culture + IMS	serogroup O26	4.9 (10/203)
					serogroup O45	13.8 (28/203)
					serogroup O103	11.8 (24/203)

			interventions immediately following surface trimming		serogroup O111	0.0 (0/203)
					serogroup O121	10.8 (22/203)
					serogroup O145	1.5 (3/203)
Thomas *et al.*, 2012	Beef	Europe (Ireland)	100 cm^2 area swabbed on the right brisket prior to evisceration	Culture + IMS	serogroup O26	0.5 (2/402)
					serogroup O111	0.0 (0/402)
					serogroup O103	5.5 (22/402)
					serogroup O145	0.5 (2/402)
					STEC O26	0.0 (0/402)
					STEC O111	0.0 (0/402)
					STEC O103	0.0 (0/402)
					STEC O145	0.0 (0/402)
					EHEC O26	0.0 (0/402)
					EHEC O111	0.0 (0/402)
					EHEC O103	0.0 (0/402)
					EHEC O145	0.0 (0/402)

*Laboratory methods presented in this table are how authors extracted and categorized data for analysis; for full laboratory method protocols used refer to the original manuscript referenced. If category was "Other", method of detection was stated in parenthesis.

Table 5-6. Pooled serogroup, STEC, and EHEC fecal prevalence estimates by region obtained from random-effects meta-analysis models.

Outcome	Region	No. articles	No. studies	Prevalence (95% CI), %	Cochrane's chi-square statistic (*Q*)	*P*-value*	I^2,%
Serogroup	Asia	2	49	5.2 (3.9-6.8)	160.90	≤0.01	70.2
	Australia/Oceania	2	13	1.7 (1.0-2.9)	51.57	≤0.01	76.7
	Europe	6	30	1.9 (0.9-4.3)	1548.88	≤0.01	98.1
	North America	8	73	6.4 (3.7-10.8)	14311.99	≤0.01	99.5
	South America[δ]	1	-	-	-	-	-
	Worldwide	18	165	4.7 (3.4-6.3)	16116.40	≤0.01	99.0
STEC	Asia	9	26	0.8 (0.5-1.1)	38.69	0.04	35.4
	Australia/Oceania	2	14	1.3 (0.7-2.5)	11.42	0.58	0.0
	Europe	9	42	0.4 (0.3-0.6)	157.83	≤0.01	74.0
	North America	8	79	1.1 (0.8-1.5)	321.09	≤0.01	75.7
	South America	5	30	0.3 (0.2-0.4)	31.17	0.36	7.0
	Worldwide	33	191	0.7 (0.5-0.8)	675.82	≤0.01	71.9
EHEC	Asia	7	18	1.0 (0.6-1.5)	16.57	0.48	0.0
	Australia/Oceania	3	17	0.3 (0.2-0.4)	9.94	0.87	0.0
	Europe	16	140	1.3 (1.0-1.7)	362.17	≤0.01	61.6
	North America	10	170	1.2 (0.9-1.5)	1909.67	≤0.01	91.2
	South America	4	24	0.2 (0.1-0.4)	30.74	0.13	25.2
	Worldwide	40	369	1.0 (0.8-1.1)	3142.84	≤0.01	88.3

*The *P* value presented demonstrates the statistical significance of heterogeneity using the Cochrane's *Q* statistic method. The null hypothesis is there is 'no heterogeneity' with a Chi-square distribution and n - 1 degrees of freedom, where n is number of studies (Dohoo *et al.*, 2009).

[δ] Only one article retrieved presented data at the serogroup level for South America; therefore, South America was excluded from the serogroup meta-analyses and meta-regression analyses.

Table 5-7. Pooled serogroup, STEC, and EHEC cattle fecal prevalence estimates in North America by O gene obtained from random-effects meta-analysis models.

Outcome	O gene	No. articles	No. studies	Prevalence (95% CI), %	Cochrane's chi-square statistic (*Q*)	*P*-value*	I^2, %
Serogroup	O26	7	12	15.1 (4.1-42.7)	1973.34	≤0.01	99.4
	O45	7	12	10.2 (3.9-23.9)	881.71	≤0.01	98.8
	O103	8	13	19.6 (5.6-50.2)	4480.4	≤0.01	99.7
	O111	7	12	1.0 (0.2-5.8)	642.76	≤0.01	98.3
	O121	7	12	3.7 (1.1-11.6)	773.13	≤0.01	98.6
	O145	7	12	1.0 (0.3-4.2)	620.38	≤0.01	98.2
	Top 6	8	73	6.4 (3.7-10.8)	14311.99	≤0.01	99.5
STEC	O26	6	14	1.1 (0.5-2.3)	31.50	≤0.01	58.7
	O45	4	12	0.7 (0.4-1.5)	8.42	0.67	0.0
	O103	7	15	1.6 (0.7-3.7)	62.82	≤0.01	77.7
	O111	4	12	0.6 (0.3-1.3)	7.27	0.78	0.0
	O121	6	14	1.3 (0.5-3.7)	99.61	≤0.01	87.0
	O145	4	12	1.4 (0.5-3.5)	51.64	≤0.01	78.7
	Top 6	8	79	1.1 (0.8-1.5)	321.09	≤0.01	75.7
EHEC	O26	7	28	0.8 (0.5-1.4)	311.23	≤0.01	91.3
	O45	7	28	1.7 (0.8-3.7)	447.01	≤0.01	94.0
	O103	9	27	2.8 (1.6-4.9)	194.76	≤0.01	86.7
	O111	8	29	1.4 (0.7-2.7)	218.38	≤0.01	87.2
	O121	8	29	0.5 (0.3-0.8)	123.33	≤0.01	77.3
	O145	8	29	1.0 (0.6-1.7)	254.52	≤0.01	89.0
	Top 6	10	170	1.2 (0.9-1.5)	1909.67	≤0.01	91.2

*The *P*-value presented demonstrates the statistical significance of heterogeneity using the Cochrane's *Q* statistic method. The null hypothesis states that there is 'no heterogeneity' with a Chi-square distribution and n - 1 degrees of freedom, where n is number of studies (Dohoo *et al.*, 2009).

Table 5-8. Uni-variable and multi-variable meta-regression models for non-O157 *serogroup* fecal prevalence in cattle worldwide.

Variables	No. articles	No. studies	Uni-variable		Multi-variable	
			Prevalence (95% CI), %	*P*-value	Prevalence (95% CI), %	*P*-value
Region*				**≤0.01**		**≤0.01**
Asia	2	49	4.4 (2.5-7.8)		Referent	
Australia/Oceania	2	13	1.5 (0.2-9.0)		4.4 (0.1-80.9)	
Europe	6	30	1.7 (0.4-7.4)		3.3 (0.0-79.1)	
North America	8	73	5.5 (1.5-18.4)		69.4 (2.2-99.6)	
Time of Harvest[δ]				**≤0.01**		
Pre-harvest	12	132	5.3 (3.8-7.3)			
Peri-harvest	6	33	1.0 (0.3-3.0)			
Cattle Type[‡]				**≤0.01**		**0.02**
Beef and Dairy	1	48	4.6 (2.6-8.0)		Referent	
Beef	12	86	3.7 (1.0-12.6)		0.0 (0.0-1.8)	
Dairy	3	15	14.5 (2.9-48.7)		0.0 (0.0-2.7)	
Unknown	3	16	0.6 (0.1-3.4)		0.1 (0.0-11.6)	
Laboratory Methods[†]				**≤0.01**		**≤0.01**
PCR only	2	24	0.9 (0.4-1.8)		Referent	
Culture	5	33	9.6 (1.8-38.2)		99.2 (25.6-99.8)	
Culture + IMS	12	108	4.2 (0.9-17.6)		77.8 (9.7-99.1)	
Specimen Type				**0.02**		**≤0.01**
Rectal swab	5	35	1.7 (0.9-3.3)		Referent	
Pen-floor	5	40	6.9 (1.5-26.4)		0.3 (0.0-7.7)	
Rectal grab	5	24	4.5 (0.8-20.9)		1.8 (0.1-26.8)	
Unknown	3	66	3.6 (0.8-14.6)		0.0 (0.0-1.8)	

*South America was not included in these analyses as only one article presented data.

[δ] Time of harvest was not significant (*P*-value < 0.05) in the multi-variable model.

[‡] Beef and dairy cattle fecal prevalence were estimated and reported separately for each cattle type (Paddock *et al.*, 2012).

[†] Bai *et al.*, reported fecal prevalence data using two methodologies categorized as Culture and Culture + IMS (Bai *et al.*, 2012).

Table 5-9. Uni-variable and multi-variable meta-regression models for non-O157 *STEC* fecal prevalence in cattle worldwide.

Variables	No. articles	No. studies	Uni-variable		Multi-variable	
			Prevalence (95% CI), %	*P*-value	Prevalence (95% CI), %	*P*-value
Region				**≤0.01**		**≤0.01**
Asia	7	26	0.6 (0.4-1.0)		Referent	
Australia/Oceania	3	14	1.3 (0.3-5.1)		1.2 (0.2-7.5)	
Europe	16	42	0.4 (0.1-1.3)		0.3 (0.1-1.7)	
North America	10	79	1.2 (0.4-3.5)		0.4 (0.1-2.3)	
South America	4	30	0.2 (0.1-0.8)		0.1 (0.0-0.9)	
Time of Harvest*				**0.05**		
Pre-harvest	23	149	0.8 (0.6-1.0)			
Peri-harvest	10	42	0.5 (0.2-1.0)			
Cattle Type[δ]				**≤0.01**		**≤0.01**
Beef and Dairy	3	9	0.3 (0.1-0.8)		Referent	
Beef	17	126	1.0 (0.2-6.0)		0.7 (0.1-5.2)	
Dairy	10	36	0.4 (0.1-2.5)		0.3 (0.0-2.3)	
Unknown	5	20	0.3 (0.0-2.0)		0.3 (0.0-2.3)	
Laboratory Methods*[‡]				**0.10**		
PCR only	1	2	0.3 (0.1-1.8)			
Culture	23	125	0.6 (0.0-18.0)			
Culture + IMS	10	64	0.9 (0.0-25.4)			
Specimen Type				**≤0.01**		**≤0.01**
Rectal swab	12	68	0.4 (0.3-0.5)		Referent	
Pen-floor	3	22	0.2 (0.1-0.6)		0.2 (0.0-1.2)	
Rectal grab	13	81	1.6 (0.7-3.2)		0.9 (0.2-4.7)	
Unknown	5	20	0.6 (0.2-1.6)		0.4 (0.1-2.6)	

*Time of harvest and laboratory method variables were not significant (*P* value < 0.05) in the multi-variable model.

[δ] Two articles presented data for more than one cattle type. Kang *et al.*, reported data on beef and dairy cattle separately (Kang *et al.*, 2014) and Hornitzky *et al.*, presented data for beef cattle and a combination of dairy and beef cattle (Hornitzky *et al.*, 2002).

[‡] Ekiri *et al.*, 2014 reports data using two separate methods, categorized as Culture and Culture + IMS.

Table 5-10. Uni-variable and multi-variable meta-regression models for non-O157 *EHEC* fecal prevalence in cattle worldwide.

Variables	No. articles	No. studies	Uni-variable		Multi-variable	
			Prevalence (95% CI), %	*P*-value	Prevalence (95% CI), %	*P*-value
Region				**≤0.01**		**≤0.01**
Asia	7	18	0.7 (0.3-1.4)		Referent	
Australia/Oceania	3	17	0.2 (0.0-1.5)		0.0 (0.0-0.4)	
Europe	16	140	1.3 (0.2-5.6)		0.2 (0.0-4.1)	
North America	10	170	1.3 (0.3-5.8)		0.1 (0.0-1.5)	
South America	4	24	0.2 (0.0-1.0)		0.0 (0.0-1.2)	
Time of Harvest*				**≤0.01**		
Pre-harvest	26	283	1.2 (1.0-1.4)			
Peri-harvest	15	86	0.7 (0.4-1.2)			
Cattle Type [δ]				**≤0.01**		**≤0.01**
Beef and Dairy	5	17	0.4 (0.2-0.8)		Referent	
Beef	21	267	1.4 (0.3-6.7)		0.19 (0.0-5.5)	
Dairy	12	59	0.8 (0.1-4.2)		0.11 (0.0-3.5)	
Unknown	6	26	0.2 (0.0-1.2)		0.05 (0.0-1.4)	
Laboratory Methods[‡]				**≤0.01**		**≤0.01**
PCR only	1	2	0.2 (0.0-1.4)		Referent	
Culture	19	91	0.3 (0.0-21.8)		0.8 (0.0-37.4)	
Culture + IMS	17	161	1.1 (0.0-48.2)		1.4 (0.0-53.2)	
Other	4	115	2.5 (0.0-68.7)		9.3 (0.1-88.8)	
Specimen Type[†]				**≤0.01**		**≤0.01**
Rectal swab	11	63	0.6 (0.4-1.0)		Referent	
Cecal	2	8	0.4 (0.1-2.3)		0.1 (0.0-2.2)	
Pen-floor	9	141	1.0 (0.4-2.4)		0.1 (0.0-1.8)	
Rectal grab	15	136	1.8 (0.7-4.3)		0.3 (0.0-5.7)	
Unknown	4	21	0.3 (0.1-1.0)		0.1 (0.0-2.0)	

*Time of harvest was not significant (*P*-value < 0.05) in the multi-variable model and Midgley and Desmarchelier present EHEC fecal prevalence data for both pre-harvest and peri-harvest times of harvest (Midgley and Desmarchelier, 2001).

[δ] Three articles presented data for more than one cattle type category, two articles presented dairy for beef and dairy separately (Bibbal *et al.,* 2015; Mellor *et al.,* 2016) and one article presented data for beef and dairy in combination, and for dairy and beef cattle types separately.

[‡] Stromberg *et al.,* presented prevalence estimates from two different methodologies, categorized as Culture and Culture + IMS (Stromberg *et al.,* 2016b).

[†]Two specimen types, Rectal swab and Rectal grab, were collected and prevalence estimates reported separately in Agga *et al.,* 2017.

Table 5-11. Uni-variable and multi-variable meta-regression models for non-O157 *serogroup* cattle fecal prevalence in North America.

Variables	No. articles	No. studies	Uni-variable		Multi-variable	
			Prevalence (95% CI), %	*P*-value	Prevalence (95% CI), %	*P*-value
Time of Harvest*				**0.18**		
Pre-harvest	7	67	5.9 (3.3-10.3)			
Peri-harvest	1	6	1.5 (0.1-18.0)			
Cattle Type*[δ]				**0.08**		
Unknown	1	6	1.5 (0.2-9.5)			
Beef	6	60	4.9 (0.1-73.5)			
Dairy	2	7	23.3 (0.3-96.8)			
Laboratory Methods[‡]				**≤0.01**		**≤0.01**
PCR only	2	24	0.9 (0.4-1.9)		Referent	
Culture	3	19	22.4 (3.4-70.3)		14.1 (1.1-71.5)	
Culture + IMS	4	30	9.1 (1.4-41.8)		3.5 (0.3-33.8)	
Specimen Type				**≤0.01**		**≤0.01**
Rectal swab	1	18	0.7 (0.3-1.8)		Referent	
Pen-floor	4	36	8.6 (1.0-46.0)		1.5 (0.1-18.1)	
Rectal grab	2	7	46.4 (4.7-93.8)		12.1 (0.5-79.5)	
Unknown	1	12	5.6 (0.4-45.0)		0.6 (0.0-11.6)	

*All variables were subjected to a uni-variable screen and significant variables ($P < 0.1$) were evaluated in a backward stepwise multi-variable model. Variables not significant at $P < 0.05$ were removed from the multi-variable model.

[δ] Data for two cattle types, beef and dairy, were extracted independently for one article (Paddock *et al.*, 2012).

[‡] Bai *et al.*, presented prevalence estimates from two different methodologies, categorized as Culture and Culture + IMS (Bai *et al.*, 2012).

Table 5-12. Uni-variable and multi-variable meta-regression models for non-O157 *STEC* cattle fecal prevalence in North America.

Variables	No. articles	No. studies	Uni-variable		Multi-variable	
			Prevalence (95% CI), %	*P*-value	Prevalence (95% CI), %	*P*-value
Time of Harvest				**≤0.01**		**≤0.01**
Pre-harvest	6	74	1.4 (1.0-1.9)		Referent	
Peri-harvest	2	5	0.2 (0.0-0.9)		0.0 (0.0-0.3)	
Cattle Type				**0.07**		**0.02**
Beef and Dairy	1	6	0.3 (0.1-1.0)		Referent	
Beef	5	71	1.3 (0.1-16.3)		1.6 (0.1-17.7)	
Dairy	2	2	0.8 (0.0-27.6)		0.8 (0.0-23.8)	
Laboratory Methods*[δ]				**0.14**		
PCR only	1	2	0.3 (0.0-1.9)			
Culture	6	41	1.0 (0.0-31.7)			
Culture + IMS	2	36	1.5 (0.0-42.6)			
Specimen Type*				**≤0.01**		
Rectal swab	1	3	0.1 (0.0-0.5)			
Pen-floor	2	18	0.3 (0.0-4.3)			
Rectal grab	5	58	2.2 (0.2-23.1)			

*All variables were subjected to a uni-variable screen and significant variables ($P < 0.1$) were evaluated in a backward stepwise multi-variable model. Variables not significant at $P < 0.05$ were removed from the multi-variable model.

[δ] Bai *et al.,* presented prevalence estimates from two different methodologies, categorized as Culture and Culture + IMS (Bai *et al.*, 2012).

Table 5-13. Uni-variable and multi-variable meta-regression models for non-O157 *EHEC* cattle fecal prevalence in North America.

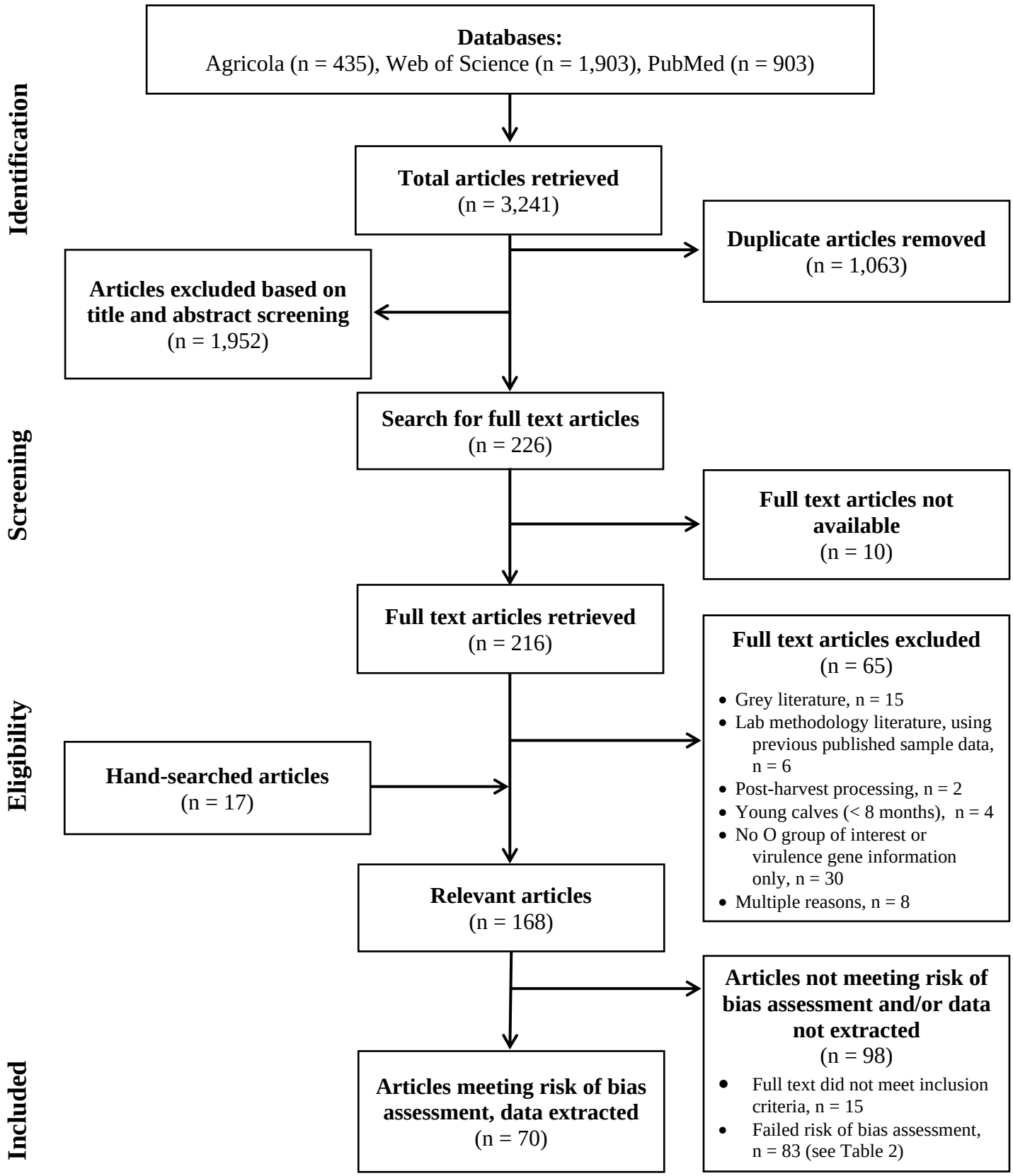
Variables	No. articles	No. studies	Uni-variable		Multi-variable	
			Prevalence (95% CI), %	*P*-value	Prevalence (95% CI), %	*P*-value
Time of Harvest				**0.04**		**0.03**
Pre-harvest	7	144	1.1 (0.8-1.5)		Referent	
Peri-harvest	3	26	2.3 (0.9-5.7)		0.2 (0.0-2.5)	
Cattle Type*				**0.06**		
Beef and Dairy	1	6	0.2 (0.1-1.0)			
Beef	7	151	1.3 (0.1-21.3)			
Dairy	2	13	1.9 (0.1-33.4)			
Laboratory Methods[δ]				**≤0.01**		**≤0.01**
PCR only	1	2	0.2 (0.0-1.3)		Referent	
Culture	4	27	0.4 (0.0-24.5)		0.4 (0.0-26.8)	
Culture + IMS	3	30	0.4 (0.0-23.0)		0.3 (0.0-23.0)	
Other	3	111	2.6 (0.0-67.6)		2.3 (0.0-67.0)	
Specimen Type*[‡]				**0.22**		
Rectal swab	1	6	3.7 (1.1-12.3)			
Pen-floor	5	125	1.2 (0.1-14.2)			
Rectal grab	5	39	1.2 (0.1-15.1)			

*All variables were subjected to a uni-variable screen and significant variables ($P < 0.1$) were evaluated in a backward stepwise multi-variable model. Variables not significant at $P < 0.05$ were removed from the multi-variable model.

[δ] Stromberg *et al.*, presented prevalence estimates from two different methodologies, categorized as Culture and Culture + IMS (Stromberg *et al.*, 2016b).

[‡] Two specimen types, Rectal swab and Rectal grab, were collected and prevalence estimates reported separately in Agga *et al.*, 2017.

Figure 5-1. Flow chart of study selection for meta-analysis eligibility.



Appendix A: Supplementary material

Causal diagram for fecal prevalence meta-analysis and meta-regression models constructed *a priori.*

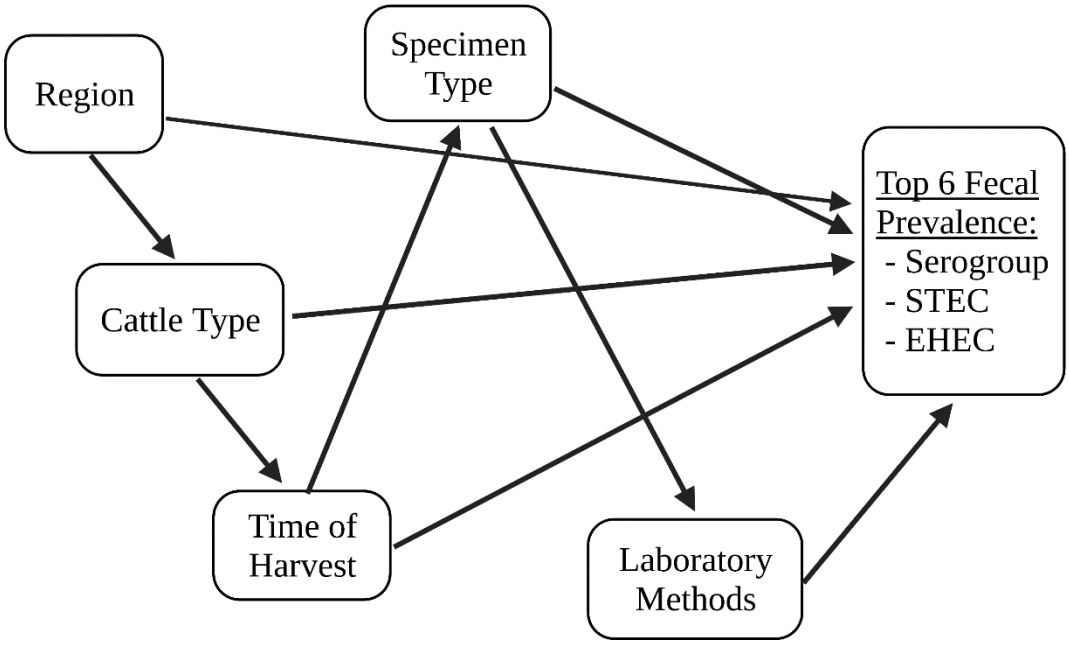


Diagram created with BioRender.com

Appendix B: Supplementary material

Evaluation of outliers and influence diagnostics for worldwide and North America fecal prevalence meta-analyses

Methods

In an effort to evaluate the robustness and validity of our meta-analyses and multi-variable meta-regression analyses, outliers and influential statistics were evaluated utilizing the "leave-one-out method". All data were analyzed using R version 3.6.1 (R Core Team, 2019) using the meta package (version 4.9-9; Balduzzi et al., 2019). Meta-analysis models for the worldwide by region and North American dataset by O group models were evaluated using the 'metainf' function. Specific outlier and influence statistics and cut-offs were not explored for the meta-analyses, but their overall impact on the pooled prevalence estimates were observed and documented descriptively.

Studies identified as potential outliers or influential for each multi-variable meta-regression model were evaluated following procedures outlined by Viechtbauer and Cheung (2010b) using the function 'influence.rma.uni' in the *metafor* package (version 2.1-0). Studies were considered potential outliers if the externally studentized, or studentized deleted, residuals exceeded a value ± 1.96. The following diagnostics were used to evaluate influence: a) DFITS, b) Cook's Distance, c) hat values, d) DFBETAS, and e) covariance ratios. A study was considered potentially influential based on the following cut-off criteria according to Viechtbauer (2010a) unless otherwise stated:

a) DFITS value greater than $3\sqrt{p/(k-p)}$ where p is the number of model coefficients and k is the number of observations,

b) Cook's distance $\geq 4/k$ (Dohoo *et al.*, 2009),

c) hat value > 3 $(p/(k)$,

d) DFBETAS value > 1, and

e) a covariance ratio < 1 indicated removing the i^{th} study would yield more precise estimates of model coefficients.

All formulas for detailed calculations of outlier and influence statistics can be found in Viechtbauer and Cheung (2010b) in addition to cut-off recommendations (Viechtbauer, 2010a). The objective of this outlier and influence diagnostic evaluation was not to remove potential

outliers or influential studies, but rather to assess the robustness and validity of the models. Findings were summarized descriptively below.

Results

Exploration of outliers by the leave-one-out method of our worldwide and North America fecal prevalence meta-analyses for serogroup, STEC, and EHEC identified minor changes in the variability around the mean prevalence estimates. Worldwide mean fecal prevalence (minimum and maximum mean prevalence using the leave-one-out method; range) for serogroup, STEC, and EHEC was 4.7 (range = 4.3 – 4.9%), 0.7 (range = 0.6 – 0.7%), and 1.0% (range = 0.9 – 1.0%), respectively. Similarly, North American cattle fecal prevalence (range) estimates were 6.4 (range = 6.0 – 6.9%), 1.1 (range = 1.0 – 1.1%), and 1.2% (1.1 – 1.2%) for serogroup, STEC, and EHEC, respectively. Utilizing the leave-one-out method, overall mean prevalence estimates were not significantly impacted, indicating our meta-analyses models yielded fairly robust estimates.

Considering the worldwide dataset, 34.5 (57/165), 22.0 (42/191) and 25.2% (93/369) of studies were classified as a potential outlier and/or potentially influential study for serogroup, STEC, and EHEC outcome classifications, respectively. Worldwide, 83.3 (15/18), 57.6 (19/33), and 70.0% (28/40) of articles retrieved for the serogroup, STEC, and EHEC outcome classifications contained at least one study that was considered a potential outlier and/or influential study. Whereas, in the North America outlier and influential analyses, we classified 34.2 (25/73), 38.0 (30/79), and 22.9% (39/170) of studies as potential outliers and/or influential studies for serogroup, STEC, and EHEC outcome classifications, respectively. The potential outliers/influential studies were represented by 100.0 (8/8), 100.0 (8/8), and 80.0% (8/10) of articles retrieved for serogroup, STEC, and EHEC cattle fecal prevalence in North America, respectively.

For the worldwide and North America outlier and influence analyses, the majority of articles contained potential outliers/influential studies. Considering there could be up to six different O groups extracted from each article, in addition to different outcome classifications, methods, regions, sample type, seasons, feedlots, and/or processing plants represented in the same article, we are not surprised that there are so many studies deemed potential outliers/influential studies.

While our analyses indicate there are many potential outliers/influential studies, the overall mean estimates obtained in our models did not substantially differ when evaluating models using the leave-one-out method demonstrating the robustness of our models despite these potential outliers/influential studies.

In summary, studies deemed as potential outliers and/or influential reported either extremely low or very high prevalence estimates. For serogroup and STEC outcome classifications, we did not identify any common variables of interest among the potential outlier and influential studies. Within EHEC studies, worldwide and in North America, studies identified as outliers or influential reported having used laboratory methods categorized as "other". Methods in the "other" category include a flow cytometry-based method, such as NeoSeek™ which had notably produced higher prevalence estimates than standard cultural or molecular methods of detection (Agga *et al.*, 2017; Schneider *et al.*, 2018a; Stromberg *et al.*, 2016). No trends in potential outlier and/or influential studies were observed for key variables of interest (e.g., cattle type) across outcome classifications. Supplementary materials include values for all evaluated outlier and influence statistics for worldwide and North America analyses.

Chapter 6 - Conclusions

Outcomes research has been a formal discipline for over two decades, and more formally implemented in the animal health industry in recent years. The discipline of outcomes research was reviewed in Chapter 1 with a lens specifically focusing on the animal health industry by reviewing key outcomes, associated value metrics, common design methods, potential applications and overall impact, while making parallels to its human medicine counterpart. Following the introduction, four research chapters were presented. Chapters 2 through 5 were wide-ranging in objective, outcomes of interest, value, stakeholder, study population, design approach, and impact. The research presented in this dissertation utilized several different experimental study designs including: a completely randomized design (Chapter 2), a 2 x 2 complete cross-over design (Chapter 3), and a randomized complete block design (Chapter 4), in addition to formal systematic review and meta-analysis research synthesis methods (Chapter 5). The primary objectives of Chapters 2, 3, 4, and 5 were to evaluate clinical efficacy, acceptability, field effectiveness, and to summarize and synthesize estimates of prevalence and concentration from published literature, respectively. In this dissertation, the value and application of the research presented herein to the scientific community have been demonstrated, especially in the areas of veterinary product evaluation, food safety, and One Health. In addition to scientific contributions, the work described offers real-world data to key stakeholders, including veterinarians, pet-owners, food safety professionals, livestock producers, government agencies such as the United States Department of Agriculture (USDA), and United States Food and Drug Administration (FDA), Food and Agriculture Organization of the United Nations (FAO), World Health Organization (WHO), and World Organisation for Animal Health (OIE) to help guide decision-making. The overall conclusions of this dissertation work follow.

The foundation of outcomes research is comprised of two elements, studying outcomes and evaluating value, which were thoroughly reviewed in the context of the animal health industry in Chapter 1. This added component of value, is what sets the discipline of outcomes research apart from other traditional research approaches. Value can be measured by many characteristics that satisfy four primary needs: emotional, functional, societal, and life-changing. Ultimately the perceived value is determined by the stakeholder. The most important needs in determining value differ depending on the stakeholder, considering the different perspectives of a veterinarian, pet-owner, and livestock producer in the animal health industry. As a discipline,

outcomes research principles used in human healthcare can be easily translated to animal health. However, human health outcomes research continues to pave the way through shaping outcomes research based on current trends, and through the development and implementation of novel methodologies. While the animal health industry has formal methodology and guidelines for reporting, work is still needed in translating novel technologies utilized in human healthcare, such as long-term cost-effectiveness and decision analytical models, to the animal health industry. Additionally, the use of Real-World Data (RWD) and Real-World Evidence (RWE) to aid and/or supplement in the product licensure process in animal health could transform veterinary product development. Unique to the animal health industry, outcomes research impacts a multitude of species and has many applications in One Health in comparative medicine, food safety and security, as well as in zoonotic and vector-borne diseases. Outcomes research is also utilized, and provides an area for growth in the future, in veterinary product development. While underrepresented in the literature during development, due to confidentiality and intellectual property, outcomes research plays a role in licensure of veterinary products during the development phase. In addition, outcomes research is utilized and findings are more frequently published post-licensure as the focus of marketing veterinary products is through comparative efficacy and value proposition, the focus of the following two research chapters.

Chapters 2 and 3, focused on the comparison between two licensed veterinary products to evaluate outcomes with an emphasis on value proposition. While licensed veterinary products have been formally evaluated for safety and efficacy, they are not traditionally appraised for value. All licensed veterinary products are successful in preventing, reducing, managing a disease or condition per labeled use, as approved by the appropriate regulatory agency. However, the underlying focus of these two research chapters was to assess the added value or competitive advantage of a product compared to a competitor (Chapter 2) or pioneer product (Chapter 3). In Chapter 2, the study objective was to compare the efficacy of two licensed antimicrobials, Zactran® (gamithromycin; Boehrinnger Ingelheim; Duluth, Georgia) and Excede® (ceftiofur crystalline free-acid; Zoetis; Kalamazoo, Michigan), commonly administered for bovine respiratory disease (BRD) metaphylaxis, in backgrounded stocker calves. A completely randomized design, with pasture serving as the experimental unit, was utilized to evaluate health, production, and economic outcomes associated with naturally occurring BRD. In the United States, BRD is the most economically devastating disease facing the beef industry. The overall

conclusion of this study was that steers administered Zactran® had lower morbidity, increased weight gain, and increased net revenue compared to steers administered Excede® as metaphylaxis. This study focused on key health, production, and economic outcomes of importance, the scientific contributions from this research trial were subsequently relevant to veterinarians and cattle producers in the beef industry when deciding on an antimicrobial for BRD metaphylaxis, a common practice in the commercial production environment. While fundamentally, both Zactran® and Excede® have been proven effective in reducing BRD, through outcomes research we demonstrated that Zactran® was overall more valuable in terms of disease reduction, increased productivity, and increased economic revenue—all key outcomes and elements of value relevant to veterinarians and ultimately producers. However, it is worth noting that lower costs and higher net return for cattle producers is paramount to the sustainability and longevity of beef production while maintaining affordable beef prices impacting food security. Additionally, with a direct comparison to a competitor, this study provides an excellent reference for veterinarians and producers when communicating the effects and uses of these two drugs while contributing to the body of literature available for beef production practices.

As previously mentioned, Chapter 3 was also an experimental research study designed to compare two licensed veterinary products, in this study—Rimadyl® (Zoetis; Kalamazoo, Michigan) and Carprieve® (Norbrook Laboratories Limited; Newry, Northern Ireland). The objective of this study was to assess canine acceptance of these two bioequivalent carprofen liver-flavored chewable tablets. Carprofen is a common non-steroidal anti-inflammatory drug (NSAID) used to manage signs of clinical canine osteoarthritis (OA). Canine OA symptoms include pain, discomfort, and lameness subsequently leading to disability and affects nearly 20% of all canines older than one year of age. This study was designed as a 2 x 2 complete cross-over, with the individual canine serving as the experimental unit. Acceptability tests used were consistent with industry evaluation of oral palatability of pet foods. Canine acceptability was measured through voluntary prehension of the chewable tablet, this voluntary prehension represents an element of functional value through ease of administration. Comparative acceptability of orally administered veterinary products is fundamental in pet owner compliance based on ease of administration. Additionally, generic products offer a more affordable alternative, and with the costs of veterinary care oftentimes a financial stress, this is also crucial

for pet owner compliance. The results of this study demonstrated that canine acceptability did not significantly differ between Rimadyl® and Carprieve® chewable tablets. While there are limitations inherent in all experimental research studies, especially in regard to external validity, the key finding of canine acceptability between the two products is a contribution to the body of knowledge regarding canine NSAIDs for use by veterinarians and pet-owners. This experimental study also provides a direct comparison between Rimadyl® and Carprieve®, which as demonstrated in Chapter 2, could provide a competitive edge through marketing. With these two products serving as bioequivalent formulations licensed by the FDA, the generic product, Carprieve®, offers an alternative to the pioneer product at a more affordable price for pet-owners with comparable acceptability by canines. Comparable in acceptability and formulation, paired with a reduction in cost, Carprieve® increases the potential for pet owners to comply with veterinarian prescribed treatment as it is voluntarily prehended similar to Rimadyl® thus resulting in greater treatment adherence by the pet owner and subsequently a better quality of life for the pet through proper pain management, especially for chronic conditions such as OA.

The final two research chapters (Chapters 4 and 5) fall under the One Health umbrella and more specifically—food safety. Pre-harvest food safety research has been at the forefront of beef safety research for decades, specifically for *E. coli* O157:H7, in the efforts to reduce these foodborne pathogens in cattle prior to harvest. Whereas the effectiveness of pre-harvest interventions to mitigate these foodborne pathogens in commercial settings is not consistent across published studies, direct-fed microbials (DFM) have shown promise in reducing *E. coli* O157:H7 in commercial feedlot cattle. As *E. coli* O157:H7 is the serotype most commonly implicated in human disease, this was the targeted organism to evaluate the effectiveness of the inclusion of a DFM product, BactaShield™ (Legacy Animal Nutrition, LLC; Wamego, Kansas), which contains *Lactobacillus acidophilus* and *Lactobacillus casei,* in the finishing diet of commercial feedlot cattle in Kansas and Nebraska—presented in Chapter 4. This multi-site experimental study utilized a randomized complete block design with pen serving as the experimental unit. Within feedlot, cattle were grouped in blocks of two based on allocation date, and within block randomly assigned to BactaShield™ treatment or control. Data were analyzed for each feedlot independently. In this study, the inclusion of BactaShield™ in the commercial finishing diet did not show a significant reduction of *E. coli* O157:H7 fecal prevalence or super-shedding ($\geq 10^4$ CFU/gram of feces) prevalence in feedlot pens at either commercial feedlot in

Kansas or Nebraska. While this study did not demonstrate this DFM product as a potential intervention to reduce *E. coli* O157:H7 in feedlot cattle, it did reveal the inherent challenges of conducting DFM research in the commercial production environment, which is supported by prior research in this area. While exact reasons for lack of effectiveness in this field trial are unclear, plausible reasons for lack of effectiveness in this study included study limitations, environmental conditions, and/or simply a real lack of field effectiveness of this DFM product. Future research is needed on this and other pre-harvest interventions to decrease bacterial load of these foodborne pathogens in cattle. With more stringent use of antimicrobials in veterinary medicine and production agriculture, alternatives such as inclusion of DFMs in finishing diets offer a desirable pre-harvest intervention in beef production and sustainability.

While *E. coli* O157:H7 has been widely researched for over three decades, the Top 6 non-O157 serogroups (O26, O45, O103, O111, O121, O145) have been more recently declared as adulterants in non-intact beef products, such as ground beef and trim in the United States. Thus, these six serogroups have not been as thoroughly researched as *E. coli* O157:H7 in the cattle reservoir which warranted the application of research synthesis methods to gather, integrate, and interpret data on the prevalence and concentration of these bacteria and their associated virulence genes in fecal, hide, and carcass samples of pre- and peri-harvest cattle worldwide (Chapter 5). Methods including a formal systematic review, followed by meta-analyses and meta-regression models were implemented. The three matrices of interest—fecal, pre-intervention hide, and pre-intervention carcass—provide estimates along the continuum of beef production and offer estimates of the foodborne pathogens of interest at the key sources of contamination of beef products. This study was the first conducted for these non-O157 pathogens of interest in cattle globally and regionally in the United States. We demonstrated that the Top 6 are prevalent in cattle and are widespread globally. Existing knowledge gaps were highlighted acknowledging the lack of prevalence data in hide and carcass matrices as well as limited concentration data for all matrices. However, despite limited information for some matrices, the data synthesized for fecal prevalence estimates greatly contribute to quantitative microbial risk assessments and is heavily sought after by expert panels such as those assembled by the FAO , WHO, and OIE. Furthermore, this research provides robust estimates of the Top 6 pathogens regionally and globally at the serogroup, STEC, and EHEC levels in cattle and may be utilized in developing standards for food safety on behalf of efforts such as those of the Codex Alimentarius

Commission. The research efforts of this work are valuable to stakeholders globally. Through One Health efforts in food safety, data was contributed to better understand and mitigate the risk of these global foodborne pathogens in cattle.

The future of outcomes research, especially in the animal health industry, could benefit from investing resources in scientific communication efforts to increase awareness and transparency to the animal health industry as a whole, and especially to production animal agriculture. Additional efforts should be given to build good-will and overall trust with the general public in addition to understanding the complexities of what factors impact perception around common hot topics such as antimicrobial resistance, antibiotic use in animals, common production practices, as well as research using animals. In conclusion, the application of outcomes research in the context of animal health is relatively novel, however, real-world impacts of research described herein have been demonstrated. The research presented in this dissertation has been valuable to companion animal and large animal veterinarians, beef producers, and pet-owners, in addition to impacting global One Health initiatives through food safety research. Future efforts of outcomes research in the animal health industry should focus on bridging the gap between novel methodologies and technologies utilized in human health and translating their efforts into the context of animal health.