DISSERTATION

THE EPIDEMIOLOGY AND ECOLOGY OF ESCHERICHIA COLI O157 ON U.S. DAIRIES

Submitted by

Chloe Marie Stenkamp-Strahm

Department of Clinical Sciences

In partial fulfillment of the requirements

For the Degree of Doctor of Philosophy

Colorado State University

Fort Collins, Colorado

Summer 2018

Doctoral Committee:

Advisor: Stephen Reynolds
Co-Advisor: Craig McConnel

Sheryl Magzamen
Jason Lombard
Zaid Abdo

ABSTRACT

THE EPIDEMIOLOGY AND ECOLOGY OF ESCHERICHIA COLI O157 ON U.S. DAIRIES

Escherichia coli O157 (O157) is a bacterium that causes human foodborne disease outbreaks worldwide. Beef and dairy cattle are reservoirs for O157, as they harbor the bacteria in their lower gastrointestinal (GI) tracts and shed it in feces without clinical illness. Humans become infected with O157 after contacting cows or manure, or ingesting the bacteria on dairy, meat or produce products. Dairy cattle are a central part of the U.S food supply, providing milk for a multitude of dairy products, and 15-20% of the beef produced. Transmission of O157 from dairy cattle to humans is reduced by techniques that limit bacterial survival after food is harvested (i.e. post-harvest). However, O157 outbreaks occur after post-harvest dairy pasteurization and slaughter laws are applied across the U.S food chain. Due to these outbreaks, an emphasis has been placed on developing methods that reduce O157 presence prior to harvest (i.e pre-harvest) at the dairy farm. An understanding of dairy cow O157 prevalence and magnitude of shedding, and animal-level correlates for shedding, may aid in the development of pre-harvest O157 strategies.

We hypothesized that life history features (parity, history of disease, others) would be associated with O157 shedding by adult cows on Colorado dairies, and that shedding in early lactation would be correlated with shedding detected during the pre-weaning period of these dams' calves. Although overall prevalence was low (3.0%) and only one individual shed O157 at a high magnitude (>10^3 CFU/g feces), a higher number of adult cows shed O157 between June and October. Dams were at increased risk of shedding if they were a lower parity, earlier days in milk, or had a history of antibiotic use. Calf shedding was not detected on the Colorado dairies studied; no correlation between dam and calf O157 shedding was present. We hypothesized that the lack of calf shedding was due to the sampling time-frame, calf management, and the geographic region of

study. Using fecal samples collected by the National Animal Health Monitoring System (NAHMS) from dairy calves across the U.S, we estimated the prevalence of O157 shedding and managerial, environmental, and calf-level variables associated with pathogen presence. U.S calf shedding of O157 was low (2.5 %) and not influenced by geographic region. Calves were at increased risk to shed if they received colostrum from their own dam, which suggests that increased time spent with the dam is associated with shedding. Results indicated that the passive transfer status of calves also influenced shedding, but was affected by the temperature and humidity index (THI) calves were exposed to during pre-weaning. Calves experiencing thermoneutral or heat-stress THIs were more likely to shed O157 if they had poor or moderate passive transfer. Calves were unlikely to shed if they had excellent passive transfer, regardless of THI.

Herds of cattle likely have uniform levels of O157 exposure, but only some individuals shed the bacteria. We hypothesized that the GI microbial community influenced which cows become colonized with O157 post-ingestion. After measuring microbial communities in naturally infected cows on Colorado dairies, lower microbial richness (i.e. total number of unique species) was associated with intermittent or multi-day shedding of O157. The species *Bacillus coagulans* was lower in abundance in fecal samples that contained O157, while the genus *Moryella spp* was higher in abundance.

The results of this dissertation highlight factors associated with O157 shedding by dairy cows and calves. This information may be used when developing techniques that reduce transmission between dairy cows, or dissemination of O157 beyond the dairy. Because O157 does not adversely affect cows, the future adoption of O157 mitigation strategies relies on whether or not these approaches benefit the dairy operation. Based on our results, we hypothesize that ill health and cow stress is associated with shedding, but is difficult to measure and monetarily quantify. At the current time, development of reduction strategies should focus on methods that reduce O157 while simultaneously improving cow health and production (e. g reducing stress

during cow transition periods, improving passive transfer and limiting dam exposure of calves, feeding probiotics that improve GI health, creating multi-pathogen vaccines). We propose that future studies should also focus on determining whether O157 augments milk production and cow fertility.

ACKNOWLEDGEMENTS

"*To whom much is given, much is required*" - JFK, 1961

When invited to join the DVM/PhD program at Colorado State University, I received one of the greatest opportunities. The DVM/PhD community of students and professors is small, close-knit, and composed of intelligent individuals. To Ed Hoover, Sue VandeWoude and Justin Lee; thank you for your unwavering support over the last five years. I am also grateful to past and present DVM/PhD students. They have given me sage advice, friendship, and provided a mutual understanding regarding the challenges of doing research and vet school simultaneously.

Much of the success of this dissertation is also owed to my committee. Zaid: thank you for guiding me through many of my analyses, and offering invaluable insight when it was needed. Steve: thank you for continually offering critical thoughts, and methods to translate our research to the bigger 'food safety' picture. Sheryl: thank you for always questioning my assumptions, and making astute observations about data. You helped me narrow or widen my focus when I needed to, and constantly reinforced the tenets of good epidemiologic research. As the only female on my committee, you are not simply a mentor but one of my greatest role models. Jason: thank you for providing opportunities to work with APHIS, and always listening critically to my thoughts and ideas. Your encouragement to consider all aspects of the information gathered, and the stakeholders that may be impacted, was invaluable guidance when answering our one-health research questions. And may you continue to run faster than me up the hills along Horsetooth reservoir. Craig: thank you for being both my mentor and confidante, from the very beginning. You helped me identify when I needed to think more (or less) deeply, and always emboldened me to reason as both a clinician and a researcher. We joke about it, but you have also reinforced how important humility is. I hope this is something I never lose sight of. And thank you to my lab-mates

Dr. Lyndsey Linke and Roberta Magnuson. You never hesitated to talk about lab issues and offer helpful advice. We also laughed about manure. A lot.

And thank you to those mentors from an earlier time. To Onesmo Balemba, whose intellect and passion for veterinary research helped me decide to pursue a DVM/PhD. To all the veterinarians I had the pleasure of working with at clinics across Washington state: Drs. Ross, McAuliffe, Choker, Demarco, O'Dea, Deaver and Brown. Your hard work, dedication to the veterinary craft, and good humor, drove my desire to be a part of the DVM community.

I also must thank others that have both enriched and made my Fort Collins life enjoyable. To my girls in the CSU DVM Class of 2018; you are comrades in the truest sense. Each and every one of you inspires me in different ways, both in and outside of school. To my crossfit-mates and running pals, thank you for pulling me into a world beyond academia, and being an incredible source of support and friendship.

To my brother Beau: thank you for being one of my best friends and biggest champions. You remind me each day how important it is to be passionate, and show kindness to others. And to Ben: Thank you for your continued love, support, and distraction.

DEDICATION

They say it takes a village to raise a child. In terms of this PhD, it took the mentorship of multiple intelligent people, from slightly different fields, to form me into a veterinary physician scientist. I humbly dedicate this dissertation to my entire PhD committee. They allowed me to answer the questions that I wanted to answer. No matter how thinly they were spread, they answered my messages and were willing to meet with me. I truly believe this was paramount my progress, and never went unnoticed. I will continue to learn and develop as a researcher, but so much of my current scientific acumen is owed to these wonderful individuals.

TABLE OF CONTENTS

INTRODUCTION

A Story

On the evening of Friday September 15, 2006, Idaho residents Robyn and Jeff Allgood noticed that their two-year old son, Kyle, was showing signs of the flu. Expecting infection with a seasonal virus, they tended to their son before realizing a sharp decline in his condition at the end of the weekend. The couple took him to a medical center near their home that Monday. At the hospital, they were informed by doctors that Kyle had developed hemolytic uremic syndrome (HUS). HUS is a type of kidney failure caused by strains of pathogenic *Escherichia coli* bacteria carrying shiga-toxins (stx) (164). After a lack of improvement in his condition, Kyle was flown to Primary Children's Hospital in Salt Lake City, UT. Unfortunately, Kyle passed away the night of Wednesday, September 20, 2006, only five days after his parents noticed the first sign of illness.

Kyle's infection was acquired after eating bagged spinach contaminated with large amounts of *Escherichia coli* O157:H7 (O157). The work of this dissertation focuses on these pathogenic bacteria. O157 was first discovered as a human pathogen in 1983, and gained notoriety after an outbreak associated with contaminated Jack-In-The-Box fast-food hamburgers in 1992 (11, 33). Even though regulations for food processing were implemented in meat, dairy, and produce sectors after the initial outbreak (23, 75), O157 stubbornly persists as an agent of food-related disease throughout the world (99). In the U.S alone, these bacteria still causes up to 149,000 domestically acquired annual infections (176). These O157 illnesses are mostly associated with ingesting contaminated food, but can also be caused by drinking contaminated water, having direct or indirect contact with animals, or by person-to-person transmission (67). Of outbreaks linked to specific food vehicles, the top three contaminated products are beef, leafy vegetables (like the outbreak spinach), and unpasteurized dairy products.

After an incubation period that averages 3-4 days, O157 infections can cause a range of symptoms in humans, starting with abdominal pain, non-bloody diarrhea, and nausea. At times,

symptoms progress to bloody diarrhea, HUS, shock, and death. This range of symptoms earned stx-carrying O157 a designation of 'enterohemorrhagic *E. coli*' (EHEC) (55). In Kyle's case, the stx created by O157 caused advanced stages of infection. Once absorbed into his bloodstream, stx bound receptors on his kidney cells and induced cellular apoptosis, leading to acute renal failure (164). Specific treatment for humans with severe O157 infections are limited, and the administration of antibiotics is contraindicated due to a potential for increased absorption of stx and advanced disease (59, 222). In hospitalized patients like Kyle, only supportive treatment is given. Importantly, genes encoding stx are found on bacteriophage DNA, which may be horizontally transmitted among bacterial strains (55). Several non-O157 stx bacterial strains (O26, O45, O103, O111, O121, O145) have been implicated in disease outbreaks in recent years (137). Although these other strains exist, a disproportionately higher number (36%) of all stx-related diseases are traced to strains of O157, and a larger number of infections from O157 result in hospitalization (176). Of note, O157 itself can vary in level of virulence due to the type of stx (e.g stx1, stx2, etc) carried. Compared to stx1 containing strains, stx2 containing variants (like the one on the contaminated spinach) more commonly cause advanced kidney disease (78, 117), and non-stx containing O157 strains (atypical enteropathogenic *E. coli* or aEPEC) still cause significant GI upset and diarrhea during infection (178, 158, 52).

The spinach implicated in the multi-state outbreak causing Kyle's death was traced to Salinas Valley, CA. Here, a 50-acre parcel of row-crop land had been leased from a local cattle ranch to a spinach-growing produce company (34). Cattle serve as principal reservoirs for O157, as the bacteria can sub-clinically colonize the bovine lower gastrointestinal (GI) tract to be passed (shed) in feces (73). Bovine colonization and shedding tends to be intermittent within different types of cattle, but the bacterium is propagated among herd individuals via the fecal-oral route (Figure 1; Herd Transmission). During the 2006 outbreak, O157 strains found on contaminated spinach were also found in feces from the nearby ranch cattle (159). O157 prevalence can be highly variable

within and between cattle farms, but rates of cattle shedding tend to uniformly increase in warm months (late summer, early fall), comparable to those of the outbreak (184). These increases have been correlated with temporal fluctuations of other O157 outbreaks (92) and proximity to cattle has also been correlated with a high risk of non-outbreak related infections (103).

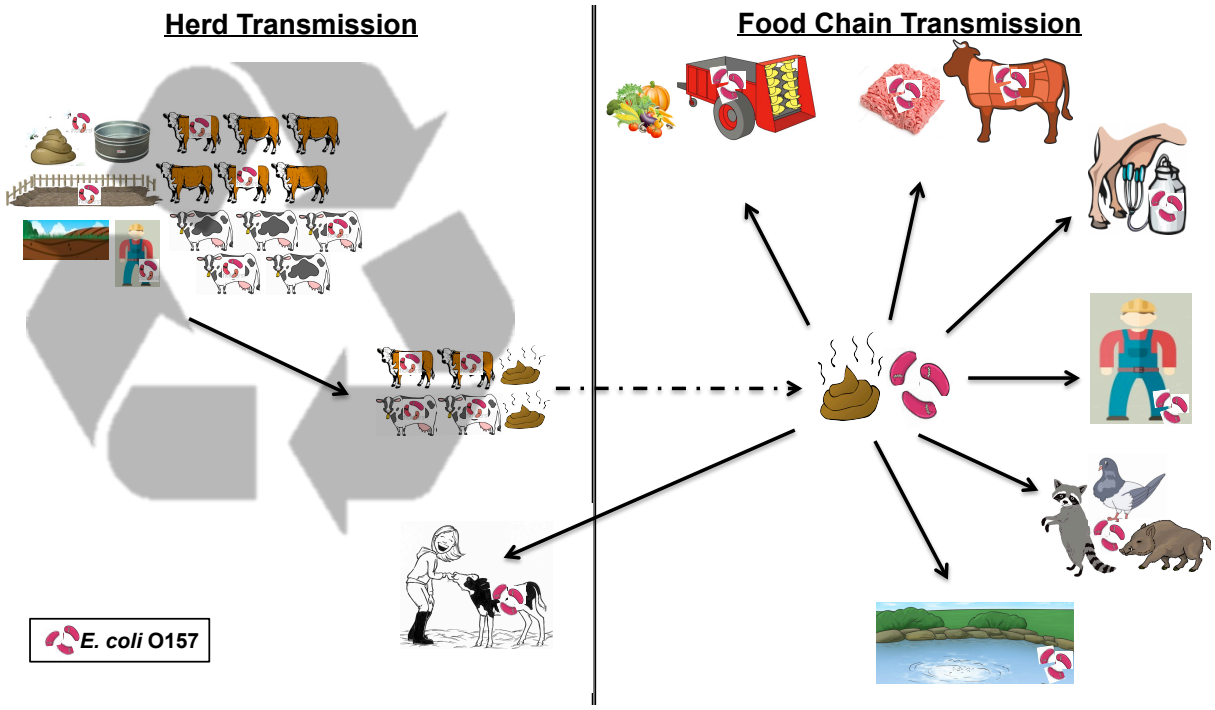

Figure 1: Transmission of *E. coli* O157 through cattle herds and potential dissemination routes to humans and the food chain.

In the Salinas Valley, O157 shedding cattle were separated from spinach fields by fencing. An investigation by the Food and Drug Administration (FDA) and California Department of Health Services showed that outbreak O157 strains could be isolated from river water and wild pig feces found within one mile of the farm field. Proximity of irrigation wells to surface waterways accessible by cattle may have caused both spinach and swine to be exposed to cattle manure containing O157 (109). Although other animal hosts experience natural O157 shedding to a lesser degree than cattle, shedding by livestock and wildlife (such as feral hogs) has been described and linked to human outbreaks (15, 18, 114, 169). In this outbreak, swine manure may have

contaminated the spinach, because there was evidence of wild pig tracks, wild pig feces, and pig rooting marks in crop fields.

Once outside of bovine or other animal hosts, O157 has a unique ability to survive in diverse environments for varying amounts of time. O157 isolates matching the infectious strain in the spinach outbreak were recovered from the surrounding farm environment for up to three months (109). Because of the survivability and the ability of O157 to relocate via animal transmission, irrigation, manure fertilization, climatic, and other natural processes (Figure 1; Food Chain Transmission), cattle are commonly implicated in outbreaks linked to produce, not unlike the Salinas Valley spinach (13).

Control of O157 and other food contaminants during food collection, storage, distribution, and live animal slaughter ("post-harvest" control), involves the use of quality control check-points. For example, Hazard Analysis and Critical Control Point (HACCP) testing involves the enumeration of indicator organisms at different points during meat processing, and is widely accepted as a management technique that allows delivery of microbiologically safe beef (23). Guidelines to limit microbial contamination specifically in the produce chain have been outlined by the FDA (75). Check-point procedures are widely implemented in the U.S food chain for post-harvest pathogen control, but their strict adherence still does not completely prevent food safety issues associated with O157 (120). These control measures are overwhelmed when food production is at its peak, and the seasonal variation in shedding increases O157 infection pressure on cattle (14, 127). The 2006 outbreak investigation revealed that the produce company did not adhere to FDA techniques that limit bacterial contamination during vegetable harvest. As a result, contaminated greens went on to be packaged, and shipped throughout the U.S and Canada.

At the time of Kyle's death, individuals in multiple U.S states were experiencing symptoms of *E. coli* infection after eating the bagged spinach. By the time the Salinas Valley outbreak was over, humans in 26 states and Canada had been affected. More than 200 illnesses, 31 cases of HUS, and 3

deaths had been reported, making it one of the largest O157 outbreaks in U.S history (34). It rivaled the initial hamburger outbreak in 1993, where over 700 individuals in 4 states became infected, and 4 children with HUS died (36, 36). Total diseases attributed to O157 have remained stable in recent years (14, 92). CDC data collected between 2000 and 2008 shows that there were 63,158 cases of infection, 2,138 hospitalizations, and 20 deaths in the U.S still attributed to the pathogen (176). Infections and related illnesses associated with O157, more than 20 years after the pathogen's initial discovery, are estimated to cost more than $405 million annually, which includes medical care, lost productivity, and the cost of premature death (77).

This 2006 Salinas Valley spinach outbreak highlights three important details regarding pathogenic *E. coli* O157 in the U.S: 1) O157 infection remains a public health threat due to disease incidence, and lack of effective treatment in humans 2) Control strategies that reduce post-harvest food contamination with O157 exist, but can be overwhelmed 3) O157 transmission to the human food chain is multifactorial and complex, but cows serve as a principal source for pathogen amplification and dissemination.

To aid in the reduction of human O157 infection, the ensuing chapters of this dissertation aim to better understand O157 and its ecology on U.S dairies. Additional motivations for this focus are highlighted below.

Epidemiology of Food Safety

Classic epidemiology is the study of a disease host, an agent of disease, and the environment that encourages their interaction. By understanding the dynamics between these three factors, one can appreciate the acquisition and distribution of disease across a population of hosts. Epidemiologists commonly utilize data regarding the presence or absence of disease in light of environmental and host factors. After determining associations between disease outcomes and

these other factors, researchers are able to discern characteristics or patterns that may increase or decrease the risk of disease occurrence (risk factors).

The epidemiology of food safety usually involves humans, an infectious agent of disease found on or in food products, and the growing, processing and transportation environment that enabled contamination and delivered the food to the consumer. Agents involved in foodborne disease include viral (e.g. Norovirus, Rotavirus, Hepatitis A Virus, etc) and bacterial (e.g. *Salmonella enterica spp*., *Campylobacter sp*., *Clostridium botulinum*, *Escherichia coli spp*., etc.) pathogens. Similar to the classic epidemiology framework, the study of foodborne disease involves the establishment of risk factors that enabled the contamination of products and movement of these infectious agents to humans. This provides a structure for investigating foodborne disease outbreaks.

Two avenues exist for maintaining non-contaminated food (produce, meat, dairy products), and reducing infectious foodborne disease in humans. These include pre-harvest and post-harvest strategies (Figure 2). Post-harvest strategies are those implemented after food products have been gathered for further processing and transport to consumers. These are the strategies most readily employed during food production in the U.S. Post-harvest approaches might include scrubbing produce and rinsing in chlorinated water, or regularly disinfecting equipment used during beef grinding and tenderizing. These control strategies may also include consumer behaviors that lessen viability of foodborne disease agents, such as cooking meat products to an internal temperature of $\geq$ 160°F (11). An additional strategy, and also a means to measure effectiveness of post-harvest intervention, includes the use of check-point inspections mentioned previously. The volume of food harvested in the U.S on a daily basis makes a scrutiny of pathogen-reduction measures outside of check-point approaches relatively impossible. Evidenced by a consistency in U.S foodborne disease outbreaks (127), existing check-point inspection strategies do not fully avoid post-harvest contamination of food with disease agents. Pre-harvest strategies are those that are implemented prior to food being gathered for processing. These might include treatments that

reduce the amount of bacteria or viruses found in farm soil, or teat sanitation procedures that reduce milk contamination with pathogenic organisms originally found on dairy cow teats during milking. Compared to post-harvest, pre-harvest strategies that control foodborne disease agents are less commonly used in U.S production systems.

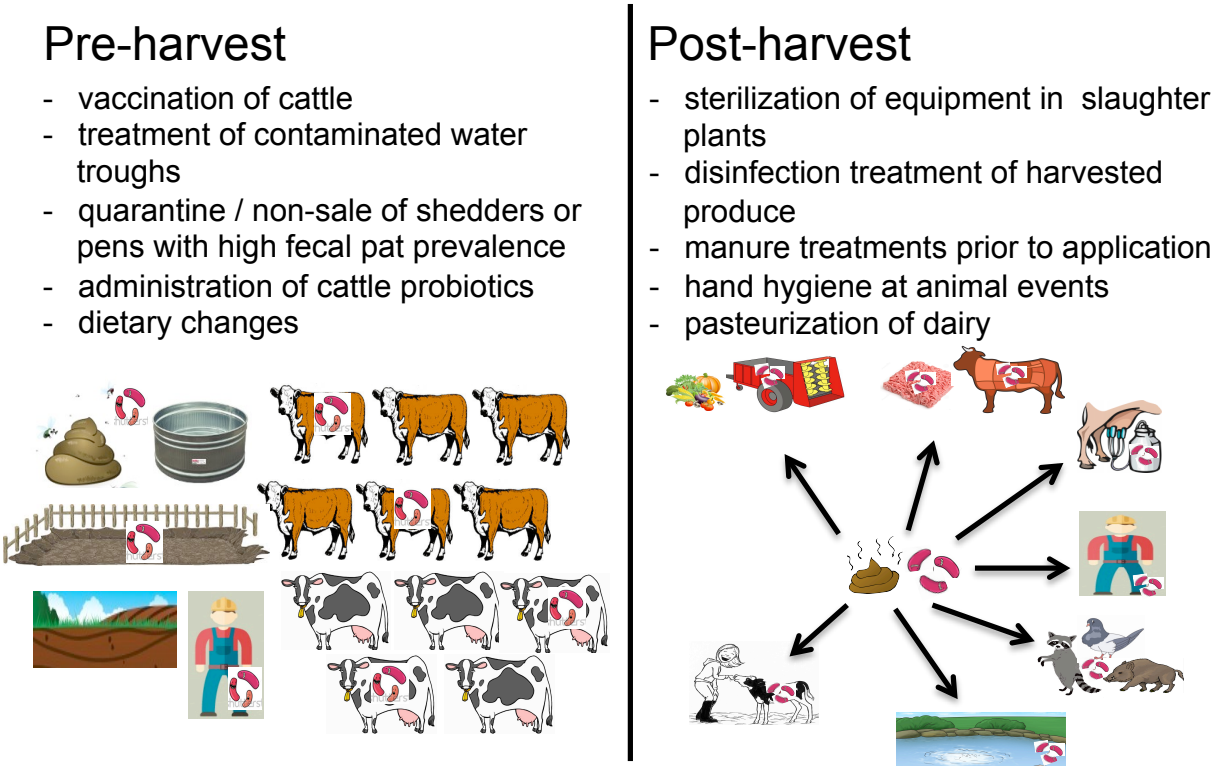

Figure 2: Areas within the transmission chain of O157 to implement specific food safety control strategies, with general examples of each type.

Because foodborne disease caused by O157 is a persistent problem, attempts are made to control the pathogen post-harvest. As inefficiencies in post-harvest strategies are evident, recent research has more prominently focused on understanding pre-harvest transmission and control (Figure 2). Since pre-harvest strategies are primarily focused at the farm level, control of O157 from this direction may not only reduce the occurrence of disease caused by contaminated food, but via direct contact with animals. This is important for O157 in particular, as HUS rates tend to be higher for transmission by animal contact (92). Risk factor establishment in this realm of study concerns

the carriage of O157 in cows, versus O157 disease in humans. The ensuing chapters of this dissertation concentrate on this point, specifically focusing on dairy cows and calves.

Food Safety in the Dairy Chain

According to the National Agricultural Statistics Service (NASS), 9.4 million dairy cows were milked in the U.S in 2017 (207). These cows produced over 215 billion pounds of milk, creating multiple dairy products including fluid milk, butter, cheese, and frozen products (206). Although the primary income of U.S dairy farms comes through the sale of milk, the national dairy herd also supplies feeder cattle and cull cows to fill U.S beef demand. To account for fluctuating beef inventory, the dairy sector supplies an average of 15-20% of beef in the U.S annually (17). In 2016 alone dairy cows contributed 5.7 billion pounds of beef (22.7%) to the supply chain (206).

Dairy cattle are important reservoirs of human foodborne and other zoonotic pathogens. Pathogens carried by dairy cattle include bacterial (*Escherichia coli spp*, *Salmonella enteric spp*., *Listeria monocytogenes*, *Leptospira interrogans*), viral (Pseudocowpox) and parasitic (*Cryptosporidium* spp, *Giardia* spp) agents (168). Dairy cattle that shed[1] pathogens in feces and other body fluids have an ability to directly contaminate beef and milk products. Dairy products contaminated with these bacterial and viral agents accounted for 13.8% of all foodborne disease illnesses in the U.S between 1998 and 2008 (156). Beyond ingesting contaminated beef and dairy products, multiple avenues for dairy cow pathogen transmission exist. Environmental contamination with manure is important, because soil and surface waters may eventually affect

[1] A note on terminology: 'shed', 'shedding', and 'colonized' are used interchangeably throughout this dissertation, and are used to describe cows passing detectable quantities of O157 in their feces. Non-colonized or non-shedding cows are those that ingest quantities of O157 from their environment and have it pass through their GI system without adhering and replicating. There is some disagreement in the literature as to whether or not cattle with O157 positive feces are actually 'colonized' with the bacteria, versus passively ingesting it. Because gold-standard detection methods are unable to identify < 10^2 CFU/g of O157 in feces, the general consensus is that 'shedding' animals are, in fact, colonized. Further, early work showed that high levels of O157 were consistently found at specific sites of the bovine lower GI (recto-anal mucosal junction or RAJ), which suggests colonization.

non-bovine food products, or enable transmission to humans via outdoor recreation (6, 41). Human infections with these agents can also occur through occupational or direct cow contact, or via direct contact with contaminated manure (35, 177, 58).

One of the most important pathogens carried by dairy cattle is O157, which silently colonizes their lower GI tracts. U.S dairy cows are frequently exposed to O157, as prior work with national herds suggests that the pathogen is ubiquitous across farm environments (216, 202). Although pasteurization kills O157 and laws for pasteurization exist in the U.S (Milk Pasteurization Ordinance), dairy O157 contamination occurs regularly and may be increasing. When comparing U.S O157 outbreaks from 2003-2007 versus 2008-2012, the median number of outbreaks did not change, but those attributed to dairy food vehicles more than doubled (92). In a study that assessed outbreaks between 1982 and 2006 in the UK, Ireland, Denmark, Norway, Finland, Canada, Japan, and the U.S, dairy products were the source of transmission in 12.2% of cases (189). Ingestion of unpasteurized products may be playing a role in these trends. Regardless of its ability to contain pathogens, raw milk products continue to be consumed by pro-raw milk advocates because of perceived health benefits (130). Outbreaks of O157 have specifically been associated with drinking raw milk (88, 63), or unpasteurized cheeses (83, 97). When looking at dairy attributed O157 infections as a whole, a large proportion are attributed to contacting animals, in addition to ingesting raw products (Figure 3). Importantly, both of these transmission routes are not affected by currently employed U.S. post-harvest food safety procedures.

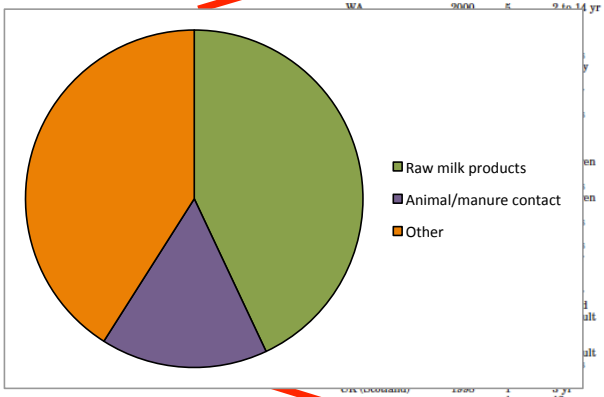

Table 1. Human infections with *Escherichia coli* O157[1] from dairy cattle.

Country	Year	Cases (no.)	Age	Illness (no.)	Infection route	Reference
US						
WI	1986	2	<13 mo	HUS[2]	Raw milk	Martin et al., 1986
WA	1986	37	Adults	HC[3] (all), HUS (1), and TTP[4] (3)	Ground beef	Ostroff et al., 1990
	1990	2	Adults	HUS	Raw milk	Wells et al., 1991
OR	1993	14	Adults	Diarrhea	Raw milk	Keene et al., 1997
WI	1998	55	15 mo to 90 yr	Abdominal pain/bloody diarrhea	Cheese	CDC, 2000
PA	2000	47	<10 yr	Abdominal pain/bloody diarrhea (all) and HUS (8)	Visiting a dairy farm	Crump et al., 2002
		4	Adults	Abdominal pain/bloody diarrhea		
WA	2000	5	2 to 14 yr	Abdominal pain/bloody diarrhea (all) and HUS (1)	Visiting a dairy farm	Georgia Division of Public Health, 2002
				Abdominal pain/diarrhea (all) and HUS (3)	Raw milk	Borczyk et al., 1987
				Abdominal pain/diarrhea		
				Abdominal pain/bloody diarrhea	Ground beef	Todd et al., 1993
				Diarrhea or HC (all) and HUS (5)	Yogurt	Morgan et al., 1993
				Diarrhea or HC		
				No signs of illness	Raw milk or animal/manure contact	Wilson et al., 1996
				Abdominal pain (all) and HUS (3)	Raw milk	Wall et al., 1996[5]
				Abdominal pain		
				Abdominal pain/bloody diarrhea (2) and HUS (1)	Cheese	Reid, 2001[5]
				Abdominal pain/bloody diarrhea		
				Abdominal pain/diarrhea	Raw milk	CDSC, 1996[5]
				Bloody diarrhea (all) and HUS (2)	Raw milk or animal/manure contact	Lahti et al., 2002
				Diarrhea		
				No signs of illness		
				No signs of illness		
				Bloody diarrhea and HUS	Visiting a dairy farm	Bielaszewska et al., 2000[6]
				Bloody diarrhea	Raw milk	Lahti et al., 2002
				Abdominal pain/diarrhea (all) and HUS (4)	Cream	CDSC, 1998[5]
				Abdominal pain/diarrhea	Raw milk	Reid, 2001
		1	12 yr	HUS	Cheese	
		1	An adult	Abdominal pain/diarrhea	Butter	
		3	Adults	Abdominal pain/diarrhea (all) and HUS/death (1)	Cheese	
UK (England)	1996	60	Adults	Abdominal pain/diarrhea	Raw milk	CDSC, 1999a[5]
	1999	1	Adults	Abdominal pain/diarrhea	Cheese	CDSC, 1999b[5]
	2000	1	Child	HUS	Raw milk	International Association of Milk, Food, and Environmental Sanitarians, 2000[5]
		3	Adults	Bloody diarrhea		
Austria	2001	1	6 yr	Bloody diarrhea and HUS	Raw milk	Allerberger et al., 2001[6]

[1]All isolates were *E. coli* O157:H7 unless indicated otherwise.
[2]HUS = Hemolytic uremic syndrome.
[3]HC = Hemorrhagic colitis.
[4]TTP = Thrombotic thrombocytopenic purpura.
[5]The *E. coli* O157 isolates were not typed for the H antigen.
[6]A nonmotile *E. coli* O157 isolate.

From: Hussein and Sakuma, *J. Dairy Science*, 2005

Figure 3: Infections resulting from dairy-linked O157 (adapted from Hussein and Sakuma, 2005 [99], with proportions of broad infection routes quantified. A significant amount of dairy O157 infections result from ingestion of raw products, and either contact with cows or cow manure.

To understand dynamics of O157 and foodborne pathogen transmission within dairies and to the greater food chain, dairy operation management and production strategies must be appreciated. Although the U.S. dairy industry is growing, over the last several years it has consolidated. The industry is now comprised of a fewer number of operations, each with a larger number of high producing milk cows. A majority of operations are conventional (non-grazing), and the U.S rolling herd average for milk production (19,932 lb/cow) has gone up 12% over the last 10 years (205, 206, 207). These industry trends are based on an economic advantage; an increase in farm size and milk pounds per cow minimizes fixed costs and maintains variable costs. Economic influence is a key driver for how specific management choices are made on each individual operation.

The success of a dairy is predicated on maintaining healthy cows that regularly produce calves. The importance of regular calving is not just related to the continued production of milk, but also maintenance of the herd population. Conventional U.S dairies remove calves from dams within a few hours of calving, so milk from the cows can be sold for human consumption. To lessen the occurrence of certain diseases and monitor growth, calves are commonly reared individually (in hutches or pens) in locations geographically separate from other herd members. These orphaned calves (referred to as 'preweaned') are fed milk replacer or waste milk until weaning. Removal of cows from a farm at any time is influenced by the number of replacement heifers available to maintain herd numbers. The gestation of a cow is around 9 months, and heifers experience their first heat cycle at around 15 months. Most U.S dairy farms have year-round calving schedules to ensure an adequate number of replacement heifers is available when needed. This requires that cows become pregnant while they are still lactating (i.e 'in milk'). A goal of farms may be to maintain a 12 month individual cow calving interval: birth on day 1, lactation through day 105, conception, lactation while pregnant through day 305, a non-lactating 'dry-off' period through day 365, new birth on day 1 (171). Dairy cattle have been bred historically to create high volumes of milk, which may have had negative influences on herd fecundity (84, 146, 82). Although 12 month calving intervals may be a goal, fertility rates often do not allow for this level of efficiency, and low fertility is a common reason for cow removal from U.S herds (44, 1).

Beyond their regular production of calves, the health of cows on an operation is also crucial for farm productivity. Most diseases in dairy cattle cause a decrease in milk production, and changes in milk yield can often be used to identify health issues before a cow shows clinical signs of disease. Because cattle experience stresses related to their stage of the production cycle, specific diseases are often correlated to age group, lactation stage, or lactation cycle (parity). For instance, cows that have recently calved and are early in their lactation cycle commonly experience negative energy balance and ketosis (171). Cows at later parity, producing more milk compared to their

younger counterparts, are more likely to experience hypocalcemia or 'milk fever' during the early stage of lactation (171). Pre-weaned calves that have poor passive transfer (a low amount of immunoglobulins [IgG] in the bloodstream) are at increased risk for mortality or morbidity due to a multitude of factors (168). Screening a few individuals for common diseases in certain production or age groups is a low-cost method that often identifies health issues across the population. An example would be collecting blood to examine biomarkers of ketosis in cows that are early days in milk (DIM), or measuring serum levels of IgG in calves that are under one week old. These are management techniques that can be employed by both farm operators and veterinarians, to ensure the health of the dairy herd

When these points are taken together, the production success of U.S dairy farms is predicated on a management structure that allows for short calving intervals of high producing, healthy dairy cattle. Any managerial change that is implemented within a dairy must be justified through its impact on production. Unless a change is cost free, the outcome of its implementation needs to improve production, increase revenue, or decrease costs. Examples of such changes may include the implementation of a new treatment to reduce disease burden, modification of pen size to reduce cow stress, or augmenting rations fed at different lactation stages to improve cow macronutrient parameters.

When attempting to make managerial changes that reduce the amount of zoonotic pathogens present on a dairy, the same production tenets apply. The implementation of pre-harvest O157 control strategies may rely on deciphering currently unknown or misunderstood detrimental consequences associated with cattle carriage. The research work of this dissertation attempts to incorporate animal health and production measures, when possible, during the study of dairy O157 ecology.

Controlling Dairy O157; What we Do and Do Not Know

An overarching goal of this dissertation is to collect information that guides development and implementation of pre-harvest strategies that lessen dissemination of O157 on dairies. If we are to accomplish this goal, a foundation of knowledge regarding transmission and physiology of the pathogen within dairy cattle must be established. To date, there are many gaps in this understanding factors associated with O157 colonization. Therefore, the specific aims of this dissertation address *1) The prevalence and risk factors for O157 shedding in different age cohorts of dairy cattle on U.S operations and 2) the composition and structure of GI microbial communities found in naturally shedding and non-shedding dairy cattle.*

Although O157 prevalence and transmission within the beef cattle setting has been studied, less is known about the prevalence and dynamics of transmission in dairy cows. Studies to date have shown that dairy cows experience site-specific colonization with O157 at the recto-anal mucosal junction (RAJ), similar to what is appreciated in beef cattle (125). Carriage in dairy cows is intermittent, and documented prevalence is highly variable across dairy operations internationally (0.2% - 48.8%)(99, 173). Some studies have indicated that levels of shedding by dairy cows, especially calves, may be higher than those seen in feedlot cattle (48, 74). The occurrence of 'super-shedding' (individual cows shedding over 10^3 - 10^4 CFU / g feces) has been established in beef herds, and correlated with an increase in herd transmission and an increased risk for food chain contamination (49, 154). Although dairy heifers have been detected to be super-shedders, whether or not this is a common occurrence has not been established (218, 219). General prevalence has not been established on large conventional U.S dairy farms, even though this may be crucial to developing O157 control measures in the U.S. For beef cows, 20% pen fecal pat prevalence has been suggested as a functional threshold to target for O157 reduction. This value can be used as a marker to predict when groups of beef cattle headed to slaughter are at increased risk of hide contamination with O157 (14). It's difficult to ascertain whether this same level should be a target

when implementing pre-harvest dairy interventions, due to gaps in knowledge concerning prevalence. A focus of the second chapter of this dissertation was to establish prevalence levels of shedding and super-shedding in cows on conventional Colorado dairies. Chapters 3 and 4 aimed to establish the prevalence of calves shedding, both on conventional Colorado dairies and dairy operations across the U.S.

The gold standard for detection of O157 from cattle feces involves enrichment, culture, immunomagnetic separation, bacterial plating, latex agglutination, de-novo culture, and PCR (134, 160). This requires several days of lab-based experiments before a final determination of whether or not the cow was shedding. These laborious detection techniques, coupled with a lack of clinical signs, make it difficult to identify cows colonized with the O157 pathogen. Defining epidemiologic risk factors that may influence cow colonization with O157 is key, because targeting certain groups for treatment may be the best way to curtail shedding on very large operations. Small studies on variably managed dairies have suggested that multiple factors influence O157 colonization. Carriage may fluctuate due to age, and be influenced by cow-calf contact, ventilation management systems, and methods of manure storage and removal (39, 74, 48, 173, 50, 81). Certain environmental factors (high temperature, high humidity, increased rainfall) have been positively associated with shedding, while others (increased solar exposure) have been negatively associated with shedding (219). Risk factors related to individual cow production including number of lactation days, lactation number, somatic cell count, and milk protein have also been associated with the presence of O157 (142). Lactation was also shown as a risk factor for shedding in beef herds (122). Along with a focus on prevalence, chapters 2 and 3 attempted to define variables that may be risk factors for shedding in early lactation cows and calves on conventional Colorado dairies. Taken together, these chapters also help discern age-related changes in O157 shedding, and the potential for O157 transmission between groups (dams and calves).

Most studies that examine O157 on dairies have been relatively small and focused in discrete geographic areas. At a national scale, there may be specific geographical or managerial characteristics between operations that change the shedding prevalence of one U.S farm compared to others. If this is understood, we may be able to alter dairy management to mitigate O157 shedding through policy design. Chapter 4 of this dissertation used information collected from shedding and non-shedding calves from operations across the U.S. Managerial, environmental and animal health information was then used to discern which factors influence shedding in this national cohort of animals.

During early lactation, cows experience high levels of hormonal, nutritional and metabolic stress, causing them to potentially be a high-risk shedding group. These individuals are also at increased risk for diseases like ketosis, subclinical laminitis, displaced abomasa, udder edema, and mastitis (135). To date, correlates between these diseases and shedding have not been made in the literature. It is therefore difficult to augment management procedures on single or multiple operations, because it is unknown if cattle experience negative health or production consequences during pathogen colonization. Chapter 2 looks at associations between O157 and historical disease episodes and treatments given to early lactation cattle. In addition, chapter 4 looks at associations between growth parameters and disease outcomes in O157 shedding calves.

There are current treatment and management techniques that aim to reduce or mitigate pre-harvest shedding of O157 in cattle. For example, cattle vaccines for siderophore receptor proteins (SRP) or type 3 secretion system proteins have been developed and shown to reduce colonization (94). Future work may also result in pathogen neutralization vaccines that target stx2 (3). Other pre-harvest techniques include treating cows with bacterial agents such as *Lactobacillus acidophilus* N51 and *Propionibacterium freudenreichii* (165, 194, 175), or with bacterial component products containing bacteriocins and bacteriophages (66). Studies looking at dietary change for shedding mitigation have had conflicting outcomes, but forage-based rations with ionophore

supplementation may cause a reduction in O157 shedding also (208, 26). Although these pre-harvest reduction strategies exist, none of them has been shown to sufficiently or completely quell O157 shedding in cows.

Dairy cattle that live in the same environment likely have similar environmental exposures to the pathogen. Some individuals will become colonized with O157 after ingestion and shed detectable quantities, while other individuals do not shed detectable levels. The proteins that O157 uses during colonization of the bovine GI have been elucidated, as well as the genes that underlie these proteins, but a better understanding of the GI physiology when colonization occurs would be advantageous. In uniformly exposed individual cows, variable microbial communities thrive throughout the GI tract, and may influence whether or not the O157 pathogen is able to adhere and replicate post-ingestion. These community members are important to consider, as previous laboratory studies have shown certain commensal bacteria to inhibit O157 growth in vitro, or be advantageous for O157 adherence and continued survival (20, 87, 144, 157). An overview of these bacteria and their impact on pathogenic *E. coli* growth can be found in Table 1.

Table 1: Literature evidence of probiotic and other bacterial strains reducing viability of pathogenic *Escherichia coli*

Study Citation	Bacteria name(s) and strain(s)	In Vitro / In Vivo	Methods Used
Gopal et al., 2001	*Lactobacillus rhamnosus* DR20 *Lactobacillus acidophilus* HN017 *Bifidobacterium lactis* DR10	In Vitro	Pre-treatment of O157 with culture supernatants of each bacteria reduced culturable numbers of O157 on TSB[1] plates and reduced the invasiveness and cell association characteristics of O157 (using intestinal cell lines
Lodemann et al., 2015	*Enterococcus faecium* NCIMB 10415	In Vitro	When co-incubated with ETEC, E. faecium ameliorated the transepithelial electrical resistance reduction usually caused by the pathogen. Experiments were done

			using human epithelial cell lines
Michail and Abernathy, 2002	*Lactobacillus plantarum* 299v	In Vitro	Preincubation with *L. plantarum* reduced the attachment of EPEC to epithelial cells, and reduced the ability of EPEC to increase the short circuit current across cells
Boudeau et al 2003	*Escherichia coli* Nissle 1917	In Vitro	Reduced the adherence and invasion of of human epithelial cells by adherent-invasive E. coli strains
Mack et al., 1999	*Lactobacillus plantarum* 299v *Lactobacillus rhamnosus* GG	In Vitro	Inhibited adherence of attaching and effacing (both EPEC and EHEC) *E. coli* to intestinal epithelial cells and increased epithelial cell expression of MUC2 and MUC3 intestinal mucins
Parassol et al., 2005	*Lactobacillus casei* DN 114-001	In Vitro	Inhibited the paracellular permeability increase caused by EPEC in human colon cells, but did not reduce pathogen cellular adherence
Brashears et al, 2003	*Lactobacillus acidophilus* NPC 747	In Vivo	When given as a direct fed microbial, L. acidophilus NPC 747 reduced the individual animal prevalence of O157 shedding and decreased the number of hide samples positive at harvest
Tkalcic et al., 2003	Probiotic 3-strain *E. coli* 'competitive exclusion' mixture	In Vivo	Calves experimentally infected with O157 and then given probiotics had a reduction in pathogen shedding compared to untreated calves. At necropsy O157 was isolated from fewer calves treated with probiotics

Zhao et al., 1998	Probiotic *E. coli* mixture and *Proteus mirabilis*	In Vivo	18 strains of bacteria isolated from cows were shown to inhibit O157 growth in vitro. 17 of these were E. coli strains and one was Proteus Mirabilis. Calves fed the probiotic cocktail did not develop clinical signs of disease. Calves infected with O157 prior to cocktail administration exhibited shedding for a variable number of days
Asahara et al., 2004	*Bifidobacterium breve* strain Yukault	In Vivo	In a fatal mouse stx infection model, *B. breve* feeding inhibited imouse body weight loss and death, and also inhibted stx production by O157
Peterson et al., 2007	Lactobacillus acidophilus strain NP51	In Vivo	Over a two year study period, L. acidophilus treated steers were 35% less likely to shed O157 than untreated steers
Tabe et al., 2008	*Lactobacillus acidophilus* 51 and *Propionibacterium freudenreichii 24*	In Vivo	Steers placed on the dual probiotic supplement were almost three times less likely to shed O157 than non-supplemented control seers
Takahashi et al., 2004	*Clostridium butyricum* MIYAIRI strain 588	In Vitro / In Vivo	Growth of O157 and production of stx was inhibited in broth cultures where the pathogen was co-incubated with C. butyricum. Gnotobiotic mice prophylactically treated with C. butyricum survived O157 infection challenge

[1] TSB = tryptic soy broth

Next-generation sequencing methods that target the bacterial 16s rRNA gene have enabled researchers to characterize taxa present in microbial communities across multiple systems (30, 118). Such approaches also enable evaluation of the diversity and relative abundance of different members within a given community. Studying the cow GI microbiota during or between shedding

events could reveal new taxa to target when developing pre-harvest O157 probiotics, or reveal indicator species that signify a cow's likelihood to be actively shedding, or risk to shed in the near future. Chapter 5 of this dissertation focused on defining changes in the dairy cow GI microbiota before, after, and during O157 shedding events.

Queries of Dissertation Research

To fit within the broad goal and specific aims above, the work of the ensuing chapters of this dissertation attempted to answer the following questions:

Chapter 2: What level of cow O157 prevalence exists on representative conventional dairies in Colorado? What are the risk factors for O157 colonization of cattle in these environments?

Chapter 3: What levels of pre-weaned dairy calf O157 prevalence exist on representative conventional dairies in Colorado? Can colonization in calves be correlated with that of their dams, or with O157 in the surrounding environment?

Chapter 4: What temporal, managerial, environmental, and animal health parameters are associated with pre-weaned calf O157 shedding on dairies across the U.S?

Chapter 5: Is the cow GI microbiota (taxa seen, diversity of community) associated with O157 shedding events?

CHAPTER 1: CLIMATE, LACTATION, AND TREATMENT FACTORS INFLUENCE FAECAL SHEDDING OF ESCHERICHIA COLI O157 PATHOTYPES IN DAIRY COWS

Summary

Among pathogens shed by cattle, Escherichia coli O157 ranks highest in those causing human illness. To date, prevalence and risk factors for O157 shedding have been assessed in feedlot, but not dairy cattle. The study aimed to determine prevalence levels and risk factors for O157 atypical enteropathogenic E. coli (aEPEC) and enterohaemorrhagic E. coli (EHEC) shedding in dairy cattle. Dairy cattle (n = 899) within the first 21 days of lactation were sampled monthly over the course of 1 year, on three dry lot dairies surrounding Fort Collins, CO. During visits multiple factors were measured (disease history, pharmaceutical use, climate measures, etc.), and cattle faeces were collected and assessed for presence of O157 and virulence genes. Logistic regression analysis was performed using O157 outcomes and measured factors. Prevalence of O157 aEPEC was 3·7%, while EHEC was 3·0%. Many potential risk factors were highly correlated, and used to build separate multivariable models. An increase in humidity was positively associated with aEPEC, while fluid faeces and history of disease showed a negative association. Meanwhile, an increase in temperature and antibiotic treatment was positively associated with EHEC, while more days in milk, higher hygiene score and cow contact were negatively associated. These results may guide mitigation strategies that reduce O157 shedding, and contamination of the human food chain.

Background

Escherichia coli (E. coli) O157:H7 infection continues to be one of the most common food-animal borne human diseases. National Centers for Disease Control surveillance estimates that upwards of 96,000 human cases occur annually, causing symptoms of bloody diarrhea, abdominal cramps, and hemolytic uremic syndrome (38, 150). Historically, outbreaks have occurred after the

pathogen is introduced to the human food chain through bovine products or contaminated produce, or through direct human contact with cows (55). Understanding and reducing the presence and dissemination of *E.coli* O157:H7 (O157) from bovine hosts remains paramount to ultimately mitigating human infection with this organism.

The pathogenicity of O157 strains shed by cattle is defined by the presence of virulence genes retained on mobile genetic elements (89). These genes include *stx1* and *stx2*, which produce shiga toxins and exist on phage DNA (55). The virulence gene *eae* lies in a pathogenicity island and encodes the protein intimin, which allows bacterial adherence to epithelial cells and subsequent attaching and effacing lesions in humans and cattle (53). Because of the mobile nature of these elements, serotyping alone is an inferior method to use when determining the pathogenicity of *E.coli* strains (121). Indeed, cattle have been shown to harbor O157 pathotypes with severe human infectivity, containing both *stx* and *eae* genes (enterohemorrhagic *E.coli*; EHEC), yet they may also harbor the O157 pathotype containing only *eae*, or *eae* with a bundle forming pilus (*bfp*) gene (atypical enteropathogenic *E.coli;* aEPEC, and enterepathogenic *E.coli*) (16). O157 aEPEC is an *E.coli* variant from which EHEC likely descended, and although less studied may have a high prevalence on dairy farms and an ability to cause serious diarrheic infection and calf diarrheal disease (72, 145). Not all studies to date have characterized the nature of the O157 pathogen beyond serotype, yet this is a critical step in truly understanding bovine transmission dynamics and human health consequences (39, 218).

Dairy cattle are unique in their ability to contaminate diverse food products including milk, meat, produce, and manure-fertilized crops. Even though O157 is ubiquitous in dairy herds across the US, risk factors for colonization and shedding dynamics have more commonly been studied in beef herds (13, 143). Dairy studies are especially relevant due to the increasing popularity of drinking raw milk, and the fact that high prevalence levels of O157 have been noted in the few published studies (48, 74, 105, 218). Previous work has shown several environmental risk factors

for dairy O157 shedding, including season, temperature, humidity, rainfall and solar exposure (219). Herd level variables of importance appear to be dairy production methods, the size of herd, use of total mixed ration or pasture growth, methods for manure storage and ventilation, and number of birds present (39, 74). To our knowledge, O157 shedding studies that include dairy cow health and treatment data have yet to be done, but one study that assessed lactation characteristics determined that the parity, number of days in milk, somatic cell count, and milk content of individuals were linked with shedding status (142). Given the factors that may influence shedding, early lactation cows exposed to nutritional, environmental and social changes, along with high levels of metabolic and hormonal stress due to calving and lactation, are thought to present a high risk group (106).

In the US, the intermittent nature and daily variation of bovine colonization with O157 indicates that implementation of a longitudinal sampling scheme is necessary to fully appreciate and define shedding dynamics (142, 173, 218). Importantly, those animals shedding high quantities of the pathogen ('high-shedders;' $>10^3$-10^4 CFU/g feces) may account for the majority of O157 contamination within a herd, increasing animal-to-animal transmission and risk of food chain contamination (142, 49, 154). When classifying cows based on O157 shedding magnitude versus positive shedding alone, outcomes for risk factor analyses can become altered (219).

Determining the risk factors for individual dairy cow O157 shedding may provide a framework within which to develop targeted prevention or treatment strategies to quell shedding and the subsequent introduction of pathogenic O157 to the human food chain. In an attempt to gain a comprehensive picture of dairy O157 shedding dynamics, the present study aimed to determine O157 shedding status, magnitude, and virulence in early lactation dairy cattle from three northern Colorado dairy herds sampled monthly over the course of one year. Concurrently, environmental, individual animal, and herd level data were collected for inclusion in multivariable risk factor analyses.

Methods

Samples and Data Collection

Three dry-lot dairies located within a 20-mile radius of Fort Collins, CO and representing a combined population of 2,750 lactating cattle were utilized for the study. Herds were sampled monthly (every 2-4 weeks) for a period of one year (July 2013 – June 2014, n= 939, representing 899 individuals). Although few cows were sampled in duplicate, each sample was considered independent due to the daily variation in shedding, change of season between sample acquisition, and variation in lactation characteristics (days in milk, disease and treatment status, contact with other cows, etc) between collections. Herd 3 could not be sampled during the month of December due to barn construction. During sampling, 10g feces were collected from cows within the first 21 days of lactation via rectal palpation. As the herds studied calve on a year-round basis, cattle sampled tended to be evenly dispersed within this 21-day lactation window. At collection the rectal temperature, fecal consistency, hygiene and body condition scores for each animal were recorded, as well as pen stocking number (cow contact). Management of the herds studied included removal of calves from the dam immediately post-partum. As youngstock are reared in geographically separated individual hutches, it is of note that the variable 'cow contact' measures only the influence of other adult cows. The level of precipitation, ambient average temperature and humidity were determined each week sampling was completed using National Oceanic and Atmospheric Administration values (www.noaa.gov). Animal life history features including lactation number, calving ease, days in milk, prior disease, and disease treatments were obtained from on-farm computer record systems (Dairy Comp 305™, Valley Agricultural Software, Tulare, CA). Diseases and injuries catalogued in Dairy Comp and collected for analysis included history of bloat, diarrhea, dystocia, hemorrhagic bowel syndrome, Johne's disease, ketosis, hoof lameness, lameness due to injury, mastitis, metritis, pneumonia, retained placenta, udder injury, metabolic symptoms, edema, abomasal ulceration, displaced abomasum, and fever. All recorded treatments

were analyzed, and included ceftiofur, ampicillin, oxytetracycline, pirlimycin, cephapirin, flunixin meglumine, dexamethasone, and aspirin administration. Disease and treatment data were only collected for the given lactation cycle. Specific definitions for variable measures can be found in Table 16 (in supplement).

Given the data collected, treatment types were further collapsed and assessed as broad categories by type (antibiotic treatment, other drug treatment, never treated) and administration and type (intramammary antibiotic, systemic antibiotic, systemic anti-inflammatory, never treated). Categories were also created for cows receiving multiple treatment types (Table 16). The covariate 'disease' was also assessed using a binary (disease/no disease) method. Since many cows were listed as having a disease but not being treated, a binary variable of 'disease or treatment' versus 'no disease or treatment' was created. While over-assessment of recorded risk factors may result in an ultimate loss of model information, all classifications herein were evaluated in an attempt to better understand if disease or treatment may have influenced shedding status, as this is a novelty of the present study. Other variables were collapsed based on biological plausibility and historical relevance. For example, parity comparisons in the dairy setting are often reduced to compare first to second, or third and greater lactation cycles, as cattle commonly remain in the system only to lactation cycles 2-5. Fecal consistency was broken down to compare a level of 3 (normal consistency) to levels 1 + 2 (more liquid) and levels 4 +5 (more solid). All data collected for use as variables in regression modeling are described in Table 2, and collapsed variables are further described within Tables 3-6 of the results section.

Table 2: Variables used in regression analysis

Variable name	Type	Levels
Dairy farm	Categorical (GEE for clustering)	Herd 1, Herd 2, Herd 3
Fecal score	Categorical	<=2, 3 (ref), >= 4
Hygiene score	Categorical	<=2 (ref), >2
Body condition score	Categorical	<2, 3 (ref), >3
Cow contact	Continuous	NA

Parity	Categorical	1, 2, >=3
Calving ease	Categorical	<=2 (ref), >2
Milk production	Difference in mean value between outcomes	NA
Days in milk	Continuous	NA
Vaccine use (SRP, HBS)	Dichotomous	0, 1
Ionophore use	Dichotomous	0, 1
Disease	Categorical based on disease type; Dichotomous	1-18 vs 0; 0, 1
Treatment type	Categorical based on treatment type; Categorical based on treatment class	1-8 vs 0; Antibiotic treatment, Other treatment, vs Never treated
Any Disease or Treatment	Dichotomous	0, 1
Average temperature (°F)	Continuous	NA
Average humidity (%)	Continuous	NA
Amount of precipitation (inches)	Continuous	NA

GEE, Generalized estimating equations; SRP, *Salmonella* siderophore receptor and porin; HBS, haemorrhagic bowel syndrome; n.a., not applicable

Isolation and characterization of O157 pathotypes

O157 isolation was performed following selective enrichment and detection 'gold standard' procedures. O157 presence and enumeration was assessed initially through direct plating. Samples were mixed 1:10 in buffered peptone water (BPW) for both enrichment and direct plating. One hundred µl was spread-plated on sorbitol MacConkey agar with BCIG (Oxoid Diagnostic Reagents, Basingstoke, Hampshire England) containing 1.25mg potassium tellurite and 0.025mg cefixime (CT-SMAC-BCIG; HiMedia Laboratories, Mumbai India). Plates were incubated at 37°C for 24h (134). As pathogenic O157 has been known to adapt a sorbitol fermenting phenotype within 24 hours, 'suspect' O157 colonies seen on plates throughout experiments were deemed as those with straw, gray, pink-grey, or too small/difficult to characterize colony coloration (7, 178).

After direct plate incubation, plates containing ≥100 suspect colonies were chosen for latex agglutination. Three to 15 colonies per plate, depending on how many colonies were determined 'suspect', were tested for O157 by agglutination using an *E.coli* O157 latex kit, following

manufacturer's instructions (Oxoid Diagnostic Reagents, Basingstoke, Hampshire England). Positive colonies were enriched in BPW for 6h and stored at -80°C in 10% sterile glycerol. For PCR experiments, 10µl of thawed isolates were centrifuged at 5,000 x g for 5 minutes and re-suspended in 30µl molecular grade water. Once re-suspended, 5µl culture template was placed into Qiagen Multiplex PCR Plus Kit reaction master mixes, according to manufacturer instructions (Qiagen, Venlo Netherlands). Briefly, each 25 µl PCR reaction consisted of 12.5 µl master mix, 2.5 µl primer mix containing 0.2 µM each primer, 5 µl molecular grade water, and 5 µl of culture template. The thermal cycling conditions consisted of an initial incubation at 95°C for 5 min to activate the polymerase, followed by 40 cycles of amplification with denaturation at 95°C for 30 s, annealing at 57°C for 1 min and 30 s, and extension at 72°C for 30 s, ending with a final extension at 68°C for 10 min. Thermocycling was performed using a Bio-Rad S1000 Thermal Cycler (Bio-Rad, Sydney, Australia). PCR products were analyzed by agarose gel electrophoresis using a 2% agarose gel (Lonza Group Ltd., Basel Switzerland).

The fecal dilution remaining after direct plating was enriched for 6 hours at 37°C, and stored overnight at 4°C. Enriched samples not confirmed as O157 positive through direct plating (those not 'high-shedding'; having either none or less than 10^3-10^4 CFU/g feces) were subjected to immunomagnetic separation (IMS) using Dynabeads anti-*E.coli* O157 and a BeadRetriever System (Life Technologies, Oslo Norway). IMS samples were subsequently plated onto CT-SMAC-BCIG and incubated for 24 hours at 37°C. Suspect colonies were confirmed by latex agglutination and PCR targeting O157 *rfb* gene (213). All *rfb* (and thus O157) positive isolates were subsequently PCR tested for *stx1*, *stx2*, and *eaeA* (a variant of the *eae* intimin gene) using the same PCR protocol outlined above (160).

In addition to isolates that contained *rfb*, *eaeA*, and any *stx* genes (EHEC), or *rfb* and *eaeA* genes alone (aEPEC), many were seen to only harbor the O157 *rfb* gene. To further characterize these allegedly non-virulent isolates, complete typing (O, H, toxin and fimbriae) was performed on a

subset of samples. Briefly, an '*rfb* only' isolate was selected from each herd, at three staggered time points throughout the year-long sample period (9 isolates total). Typing was performed by the AAVLD accredited *E.coli* reference center at The Pennsylvania State University, in the College of Agricultural Sciences.

Statistical analyses

Monthly and annual prevalence of pathogenic variants of O157 were estimated within and among herds. Confidence intervals of shedding prevalence were calculated using the Wilson procedure with a correction for continuity. Shedding outcomes with respect to presence of 'O157 pathotype' were evaluated for association with potential risk factors. Pathotypes were assessed in separate models because of their different indications for human disease and food safety, and were either those bacteria with the presence of both *rfb* and *eaeA* genes (low pathogenicity aEPEC), or those with presence of *rfb*, *eaeA*, and any *stx* genes (high pathogenicity EHEC). Potential risk factors associated with pathotype outcome were pre-selected using a threshold of $p<0.25$ in a univariate logistic regression analysis. As a main project goal was to identify the specific factors most significantly associated with the risk of shedding, factors that showed significant correlations or associations with one another were then included in separate multivariable models. This strategy was also chosen because the adoption of future cattle management methods, aimed at reducing O157 in the food chain, may ultimately be more conceivable through targeting one significant collinear factor, and not the other. For each model, a backward step-wise selection process was implemented using a p-to-retain criteria of 0.1 at each step. Final covariate selection was done using a p-value of < 0.05. Interaction terms, between all significant variables, were evaluated for significance at $p < 0.05$. All multivariable analyses were adjusted for the effect of 'dairy herd' using a generalized estimating equations (GEE) approach. Models created for the same outcome were assessed for biological plausibility and compared using QIC (quasi likelihood independent model

criterion) values based on GEE, to determine models with the best working correlation structure for the data and to identify the most robust shedding predictors based on model fit (57). All statistical analyses were performed using SAS v9.4 (SAS Institute Inc., Cary NC).

Results

Only one sample was confirmed as O157 positive after direct plating, and deemed to originate from a 'high-shedder' (>10^3-10^4 CFU/g feces when collected). The sample was collected mid-September from Herd 2, and contained all tested virulence genes.

Univariate and multivariable statistical analysis was not performed using stratified shedding outcomes of 'high' or 'low' given the lack of high shedder detection. Samples were simply classified as positive ($\geq 10^2$ CFU/g feces) or negative for O157 pathotypes after IMS, latex agglutination, and PCR. Level of detection is based on IMS manufacturer indication, which claims the ability to detect as low as 100 CFU/g feces in enriched samples, assuming the presence of high amounts of background flora.

Prevalence of E.coli O157

During the year-long study period, 939 fecal samples from early lactation cows were collected during 38 separate visits. One hundred and eighty-nine (21%; 95% CI = 17.6% - 22.9%) isolates contained the *rfb* gene alone, 35 (3.9%; 95% CI = 2.7% - 5.2%) contained both *rfb* and *eaeA* (aEPEC), 28 (3.1%; 95% CI = 2.0% - 4.3%) contained *rfb, eaeA*, and any or both *stx* genes (EHEC), and 5 (0.55%; 95% CI = 0.19% – 1.3%) contained *rfb* and any *stx* genes. For EHEC isolates, a total of 22 contained the *stx2* gene (79%; 95% CI = 59.0% - 91.0%), 26 contained *stx1* (93%; 95% CI = 75.1% – 98.8%), and 20 contained both *stx1* and *stx2* (71%; 95% CI = 51.1% - 86.1%).
The number of aEPEC and EHEC isolates varied by dairy herd and season, with a majority of O157 pathotypes being isolated between the months of June and October (Figure 4). Herd 1 had the

greatest proportion of aEPEC isolates (20/35) and a low proportion of EHEC (5/28). Herd 2 had the greatest proportion of EHEC isolates (23/28) and a moderate number of aEPEC (8/35). In comparison, Herd 3 showed relatively few isolates of aEPEC (7/35) and no EHEC (Figure 4).

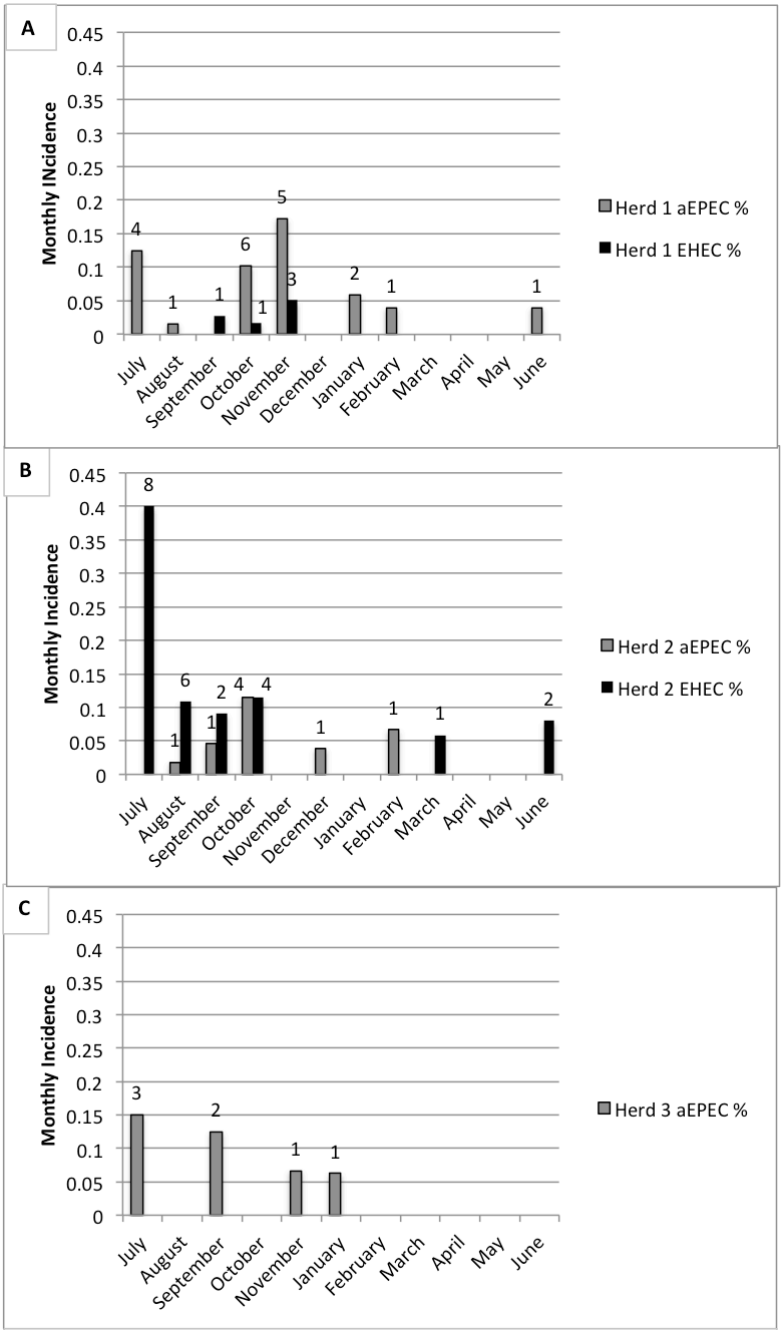

Figure 4: Monthly prevalence of atypical enteropathogenic *E. coli* (aEPEC) and enterohaemorrhagic *E. coli* (EHEC) measured for Herd 1 (A), Herd 2 (B), and Herd 3 (C). No isolates from Herd 3 were positive for EHEC, respectively.

A subset of isolates possessing only the *rfb* gene were typed by the Pennsylvania State *E.coli* reference center, where all were confirmed to contain the *rfb* gene and lacked the *stx1*, *stx2*, *eae*, STa, cnf1, cnf2, CS31A, F41, and K99 virulence genes. Isolates were all sorbitol fermenting and H+, being either O157:H12, O157:H45 or O157:H+(untypeable).

Significant factors

Risk factor categories were collapsed based on the power of study data and biological plausibility for comparing measured levels (Table 2). For aEPEC shedding, several variables univariately influenced status (Table 3). Many pre-selected aEPEC parameters were significantly ($p < 0.05$) associated with one another (Table 4). Each set of variables without correlations was included in a multivariable model with 'dairy herd' as a clustering constraint. Backward step-wise regression was used to determine multiple significant models for aEPEC (Table 17 in supplement).

Table 3: Herd, climate and individual cow parameters pre-selected (P<0.25) for inclusion in aEPEC modeling

Parameter	***Level***	***n value***	***OR[1]***	***95% CI***		***P value***
Fecal score	(1,2)	3	0.937	0.218	4.035	0.931
	(4,5)	2	0.466	0.140	1.545	0.212
	3 [2]	30				
Hygiene score	>2	7	0.609	0.263	1.412	0.248
	1 or 2[2]	28				
Cow Contact	Each unit increase	NA	1.010	1.000	1.020	0.080
Any Disease or Treatment	Any	9	0.550	0.190	1.570	0.260
	None listed[2]	26				
Average weekly humidity (%)	Each unit increase	NA	1.067	1.024	1.111	0.002
Weekly precipitation (inches)	Each unit increase	NA	0.740	0.520	1.070	0.113

aEPEC, atypical enteropathogenic *E. coli*; OR, Odds ratio; CI, confidence interval; n.a., not applicable

[1] *Comparison Odds of shedding aEPEC*

[2] *Reference category*

Table 4: Pre-selected parameters for aEPEC modeling found to be correlated at p<0.05

Parameter	*Avg weekly humidity (%)*	*Cow Contact*	*Weekly precipitation (inches)*	*Any Disease or Treatment*	*Fecal Score*	*Hygiene Score*
Average weekly humidity (%)			x	x		x
Cow contact			x	x	x	
Weekly precipitation (inches)	x	x				x
Any Disease or Treatment	x	x				
Fecal score		x				
Hygiene score	x		x			

aEPEC, atypical enteropathogenic *E. coli*

Many factors were also univariately pre-selected ($p \leq 0.25$) for association with EHEC shedding (Table 5). Significant associations ($p < 0.05$) between univariately significant EHEC parameters can be found in Table 6. All EHEC multivariable models determined using combinations of non-collinear factors can be found in Table 17 in supplement.

Table 5: Herd, climate and individual cow parameters pre-selected (P<0.25) for inclusion in EHEC modeling

Parameter	***Level***	***n value***	***OR[1]***	***95% CI***		***P value***
Hygiene score	>2	5	0.53	0.199	1.409	0.203
	1 or 2[2]	23				
Cow Contact	Each unit increase	NA	0.971	0.958	0.984	<0.0001
Parity	>=3	7	0.512	0.206	1.272	0.149
	2	6	0.572	0.219	1.495	0.255
	1 [2]	15				
Days in milk	Each day increase	NA	0.884	0.819	0.953	0.0014

Any Disase or Treatment	Any	10	1.758	0.761	4.06	0.187
	None listed[2]	18				
Antibiotic or Other Treatment	Ever antibiotic	8	1.66	0.709	3.886	0.243
	Ever other	2	0.467	0.107	2.035	0.311
	None listed[2]	18				
Average weekly temperature (°F)	Each unit increase	NA	1.06	1.027	1.093	0.0002
Average weekly humidity (%)	Each unit increase	NA	1.031	0.987	1.076	0.167
Weekly precipitation (inches)	Each unit increase	NA	1.19	0.91	1.54	0.21

EHEC, enterohaemorrhagic *E. coli*; OR, Odds ratio; CI, confidence interval; n.a., not applicable

[1] *Comparison Odds of shedding aEPEC*

[2] *Reference category*

Table 6: Pre-selected parameters for EHEC modeling found to be correlated at p<0.05

Parameter	*Hygiene Score*	*Avg weekly humidity (%)*	*Cow Contact*	*Parity*	*Days in milk*	*Avg weekly temp (°F)*	*Any Tx*	*Antibiotic or Other Treatment*	*Weekly Precip (inches)*
Hygiene Score		x		x	x	x			x
Avg weekly humidity (%)	x				x		x	x	x
Cow contact				x	x	x	x		x
Parity	x		x			x	x	x	x
Days in milk	x	x	x				x	x	x
Avg weekly temp (°F)	x		x	x				x	x
Any Disease or Treatment		x	x	x	x				
Antibiotic or Other Treatment		x		x	x	x			x
Weekly Precip (inches)	x	x	x	x	x	x		x	

EHEC,enterohaemorrhagic *E. coli*

Biological significance and QIC correlation structure values were used to determine two final 'best' multivariate models for aEPEC and EHEC (Table 7). In a final model, history of any disease or treatment, as a single factor, significantly reduced the odds of shedding aEPEC. In a second model, an increase in the average weekly humidity significantly increased, and a more watery fecal score significantly decreased, the odds of shedding aEPEC. For EHEC, an increase in hygiene score and cow contact were shown to reduce the odds of shedding, while recent treatment with any antibiotic significantly increased the odds. In a second model, an increase in the average weekly temperature increased the odds of EHEC shedding, while an increase in the number of days in milk reduced the odds of shedding.

Table 7: Final multivariable models determined for aEPEC and EHEC shedding, using 'dairy herd' as a clustering constraint

Final Models aEPEC	***Parameter***	***Comparison***	***OR[1]***	***95% CI***	***P-value***	***QIC***	***QICu***
1)	Any Disease or Treatment	Any vs None[2]	0.55	0.33-0.90	0.018	297.9	299.9
2)	Average weekly humidity (%)	Each unit increase	1.067	1.034-1.10	<0.0001	290.3	294.6
	Fecal Score	(1,2) vs 3[2]	0.45	0.21-0.95	0.037		
		(4,5) vs 3[2]	0.94	0.28-3.19	0.92		
Final Models EHEC							
1)	Hygiene score	>2 vs (1,2)[2]	0.4	0.25-0.65	0.0002	237.9	235.3
	Cow contact	Each unit increase	0.97	0.96-0.99	0.0002		
	Antibiotic or Other Treatment	Ever antibiotic vs None listed[2]	1.32	1.017-1.72	0.0373		
		Ever other vs None listed[2]	0.49	0.17-1.44	0.196		
2)	Average weekly temperature (°F)	Each unit increase	1.06	1.02-1.10	0.003	238.9	229.9
	Days in milk	Each day increase	0.88	0.79-0.99	0.028		

aEPEC, atypical enteropathogenic *E. coli*; EHEC, enterohaemorrhagic E. coli; OR, Odds ratio; CI, confidence interval; n.a., not applicable

QIC, quasi-likelihood independent model criterion

[1] *Comparison Odds of shedding*

[2] *Reference category*

Discussion

E.coli O157 has been shown to adapt a sorbitol fermenting phenotype within 24 hours, and sorbitol fermenting strains have historically caused outbreaks and harbored a high number of virulence genes (116). Many O157 strains were isolated in the current study in an attempt to detect all bacteria, sorbitol fermenting and non-sorbitol fermenting, of public health importance. Some studies to date have not PCR confirmed IMS-isolated O157 strains, resulting in unknown 0157 pathogenicity. The multitude of non-virulent O157 isolates found in the current study further validates that PCR confirmation of virulence genes during O157 studies is an absolute necessity.

Strains not confirmed as having *eaeA* or *stx* genes in the current study were confirmed to have O157 and H12 or H45 flagellar antigens. H12 subtypes have previously been isolated from watersheds and cattle at slaughter, and non-pathogenic strains have been used as control strains in EHEC studies (4, 102). Although H12 strains can become infected with similar phages to O157:H7, it is unknown whether strains isolated in the current study retain an ability to become pathogenic. When isolated, O157:H45 strains commonly have contained EPEC virulence genes (*bfp, eae, tir*) (91, 147). These strains have been detected in human prevalence and gastroenteritis outbreak studies (158, 52). Strains in the current study appeared avirulent based on AAVLD typing, and future studies are needed to determine if they have capacity to acquire mobile virulence elements.

On the dairies studied, shedding prevalence of aEPEC and EHEC were both relatively low (3.7% and 3%, respectively). Although studies to date have not assessed aEPEC prevalence, other studies of *stx* and O157:H7 dairy prevalence have shown both higher (72.7%; 11.1% - 32.3 %) (74, 142) and lower (1%) levels (39). Importantly, high shedding cattle have been hypothesized to influence herd EHEC propagation, and only one was observed in the current study (49, 154). To date, this is the first assessment of EHEC prevalence on dairies in the High Plains or Rocky Mountain biome, so it is possible that lower shedding prevalence and outcomes may be inherent to the particular environment studied.

aEPEC was isolated more commonly than EHEC on farms studied. To our knowledge this shedding outcome has not been modeled previously, but may pose a public health risk. Although not associated with hemolytic uremic syndrome like EHEC, the intimin gene conserved between aEPEC and EHEC strains imparts the ability for aEPEC to cause attaching and effacing lesions, watery diarrhea, and pediatric infection (158). A high infective dose is required for human EPEC infection (16), which is likely owing to its lower virulence as a human pathogen. However, strong evidence exists for EHEC strain evolution from EPEC, and a close homology among these strains (52). This homology and the mobile nature of phage transmitted *stx* genes indicates that modeling aEPEC may be advantageous for both the prediction of future food-borne EPEC infections, and potentially understanding the emergence of new *stx* strains.

Final multivariable models for aEPEC revealed positive associations between shedding and increased humidity, while watery feces and a recent history of any disease or treatment showed reduced odds of shedding. In prior studies climatic factors and fecal consistencies have been shown to influence virulent O157 shedding in dairy cattle (219). The negative association of disease and treatment with aEPEC shedding is difficult to interpret. The stress that accompanies disease processes and administration of treatments may influence intrinsic immune or physiological bovine processes that permit less ideal colonization conditions of the lower gastrointestinal tract to ingested aEPEC strains. Future studies are required to confirm this assumption, however.

Multivariable models of EHEC revealed a negative association between shedding and a high hygiene score, greater cow contact, and a higher number of days in milk. An increased hygiene score and greater cow contact appear counterintuitive with historical factors thought to influence shedding. Although a subjective measure, hygiene scores are meant to reflect animal cleanliness, and cleanliness of the farm environment. Dirtier cows may reflect a combination of changes in environmental parameters (rainfall, humidity) more discretely than those parameters do when measured directly and individually. The exact mechanism by which this influences shedding

remains to be determined. Cows housed with a greater number of other cows would presumably have an increased risk of shedding pathogenic O157, both through an increase in exposure and stress. The current study revealed an opposite trend, and this may be due to the daily employee modifications of pen size depending on cow number, which was especially evident based on the management of Herd 2 (having the highest prevalence of EHEC). A more meaningful measure of animal contact would have taken into account both the number of cows in a pen and the size of the enclosure, which was not possible due to daily fluctuations. With changes in pen size it is possible that increasing animal numbers actually reflected a less confined environment for the animals studied. Previous studies have shown that cow age, a higher parity, and greater number of days in milk may be associated with reduced EHEC shedding (48, 142). The current study showed that both a higher parity and number of days in milk were associated with reduced shedding, with a significant interaction of these factors (Table 17 in supplement and Table 7). Cattle were studied within the first 21 days of milk, so it is of note that the findings associated with this initial time from calving may become more or less apparent, should studies of shedding be carried further into the lactation period. Regardless, these results continue to support a notion that early lactation heifers, experiencing their initial calving and metabolic challenge of milk production, are a group more likely to shed EHEC. These individuals may therefore be targeted during future EHEC intervention studies.

Recent treatment with an antibiotic and a higher environmental temperature were significantly associated with an increase in EHEC shedding. Temperature has been previously implicated in O157 shedding in both beef and dairy settings, especially as it pertains to season (219, 142). To date, this is the first study that has assessed medical treatments given during the current lactation, and their influence on the risk of a cow to shed EHEC. Results showed that cows treated with any antibiotic (both systemic and intramammary administration) had an increased risk of shedding compared to those individuals receiving no treatments. This indicates that antibiotic

administration may modulate the bovine flora in a manner that facilitates future colonization with EHEC. Although prevalence levels had low power to detect bovine disease parameters that influenced EHEC, the collapsed variable that investigated cows with any disease or treatment history indicated that these animals are at increased risk of shedding, compared to non-diseased and non-treated cows, respectively (Table 17 in supplement). Taken together, these EHEC results support an idea that early lactation heifers with a reported status of disease, and history of receiving any antibiotic during the current lactation, may be the individuals most likely to contribute to EHEC shedding and contamination of the farm.

The differences in aEPEC and EHEC shedding prevalence between farms are likely due to variation in management factors, specifically those related to nutrition and modulation of the GI tract. These factors may include feeding ionophores, and using hemorrhagic bowel syndrome (HBS) and SRP vaccines. Herd 1 and Herd 3 both fed ionophores during the study period. Herd 1 also employed use of a *Salmonella* Newport SRP vaccine (Epitopix, Willmar MN) while Herd 2 utilized an autogenous *Clostridium perfringens* type A, HBS vaccine. Parallels between management variability and O157 pathotype prevalence indicates a need to look at GI bacterial communities under the influence of these managerial factors, and determine the specific intestinal environment that facilitates or impedes O157 growth. To truly understand the ecology of O157 shedding and farm prevalence, future studies should include an assessment of calves and cattle beyond the early lactation period, and a comparison of cows in ill-health with treatment history (specifically antibiotic use for EHEC) to those with no prior disease or history of treatment administration.

Given it's ubiquity in a multitude of unprocessed food products and ability to cause outbreaks and isolated incidents of human illness worldwide, *E.coli* O157 remains a pertinent public health concern. Many strains and pathotypes of the O157 pathogen exist, having been isolated and characterized during instances of disease. Both the current and previous studies have shown that shedding of this pathogen from bovine hosts is complex, and may vary due to season,

environment, specific host factors, and bacterial pathotype. The current study aimed to comprehensively model the shedding of O157 pathotypic variants from reservoir dairy hosts. Our findings related to risk factors and shedding dynamics of pathogenic strains can guide future studies aimed to detect dairy hosts colonized with O157, and influence the development and assessment of prevention or treatment techniques directed at reducing O157 shedding and food chain contamination.

CHAPTER 2: PREVALENCE OF ESCHERICHIA COLI O157 SHEDDING IN PREWEANED CALVES ON COLORADO DAIRIES

Summary

To gain insight into a potential age-related predisposition for Escherichia coli pathogen shedding on dairies, this pilot study measured the prevalence of E. coli O157 (ECO157) in the feces of preweaned dairy calves. An aim of this study was to link these outcomes with the concurrent environmental presence of ECO157 and dam ECO157 shedding elucidated in a parallel study. Recto-anal mucosal swabs and a subset of fecal grab samples were collected from calves (2 to 8 weeks of age; n = 399) monthly between December 2013 and June 2014 on three dairies in northern Colorado. A subset of calf dams (n = 111) were also sampled via fecal grab. Concurrently, environmental samples were collected from locations within the vicinity of the calves: farm tractor tires, steering wheels, hutches, buckets, and gloves from the research technicians and the employees involved in calf rearing. The presence of ECO157 and virulence genes was measured in the samples and confirmed via PCR. Of the calves, only 1 (0.25%) of 399 individuals shed during the time period, and the ECO157 strain detected carried no measured virulence genes (eaeA, stx1, and stx2). No difference was seen in detection between the recto-anal mucosal swabs and the fecal grab technique. In contrast, 32% (35 of 111) of the dams shed ECO157, with 1.8% (2 of 111) of the shed isolates containing virulence genes. No ECO157 was detected in the environmental samples. These outcomes demonstrate a disparity between dam and calf ECO157 shedding and indicate that preweaned calves, managed similarly to those of this study, probably have a minor influence on dairy contamination and the transmission of ECO157.

Background

Escherichia coli O157 (ECO157) is a gram-negative bacterium capable of causing human infection when ingested at a very low dose (38). It is well understood that cattle are a primary ECO157 reservoir, and human infections can result from the ingestion of dairy, meat, and produce products originally contaminated by these ruminants (89). Recently, several studies (211, 218, 219) have contributed to a better understanding of ECO157 shedding and transmission dynamics in dairy cattle. These include a study (190) that was conducted alongside the current study: a year-long risk factor analysis of ECO157 shedding in adult cattle from three dry-lot dairies in northern Colorado. Although the concurrent study elucidated the risk factors for shedding in an early-lactation cohort of adult cows, it did not address the importance of the calves recently born to these dams.

ECO157 shedding in dairy calves may be of particular importance to the farm transmission of the ECO157 pathogen. Previous studies (50, 54) have shown a greater magnitude of shedding in calves (1 to 14 weeks of age) than in adults, a longer period of shedding postinoculation, and likelihood that weaning animals will shed. The type of housing and management of calves used has been shown to modulate their rates of ECO157 shedding (200, 46). Although not specifically measured, the survival of ECO157 in the environmental vicinity of these calves probably plays a role in their increased shedding rates because ECO157 persists for varying periods in diverse environments (209). On large conventional dairies, milk-fed heifer calves (preweaned calves) are removed from their dams immediately postpartum, fed a milk replacer, and often housed individually until weaning. To our knowledge, no previous analysis of the shedding similarities between naturally infected calves and dams postseparation has been performed, and the impact of parturition and the immediate postpartum period on ECO157 transmission is unknown.

The goal of the current pilot study was to determine the prevalence of ECO157 shedding in preweaned dairy calves and to link the results to maternal shedding and the distribution of ECO157

in the surrounding dairy calf environments. Information of this nature may improve our understanding of ECO157 dairy ecology by designating the routes of ECO157 survival and infection within farm environments and herd animals.

Methods

Preweaned calves (2 to 8 weeks of age; n = 399) and dairy environmental locations (n = 129) were sampled every 2 to 6 weeks (approximately once per month) between December 2013 and July 2014. A cohort of dams within 21 days of calving (n = 111) was also sampled during the study period as part of a larger study (190) of ECO157 risk factors in early-lactation cows. Calves and dams that met the specified criteria were convenience sampled during each sampling period in an effort to obtain samples from as many eligible pairs as possible. Each individual was sampled only once during the study. The three freestall and dry-lot dairies sampled were contracted with Colorado State University herd health management, were within a 20-mi (32.2-km) radius of Fort Collins, Colorado, and represented a combined population of 2,750 lactating cattle. Herds 1 and 3 were sampled nine times during the study period, and herd 2 was sampled eight times. The calves were reared individually in hutches and bedded at the beginning of the preweaning period with either fresh straw or shavings. Additional bedding was provided on an as-needed basis but was not changed prior to weaning. The individual calves did not suckle but were removed from their dams post-calving and given 4 L of either previously frozen or fresh unpasteurized individual colostrum via orogastric intubation in the first 12 h of life. Each day, the calves were fed 6 to 8 L of milk replacer, pasteurized or unpasteurized whole milk, along with free access to either a mixed grain or grain and pellet calf starter. Although calf feeding strategies were generically similar among the herds, there were characteristic differences. The managers of herd 3 supplemented the calves' milk replacer with *Lactobacillus* (Superior Milk Products, Keenesburg, CO), those of herd 2 added trimethoprim-sulfadiazine (Uniprim, 3.5 g per calf; Neogen, Lansing, MI) to the milk for 12 days,

and those of herd 1 supplemented the milk replacer with decoquinate (Decoxx, 0.5 mg/kg; Zoetis, Parsipanny, NJ) and used a medicated dairy calf grain containing 50 g per U.S. ton (907 kg) of chlortetracycline and 50 g per U.S. ton of lasolocid (Ranch Way Feeds, Fort Collins, CO).

At each visit, a foam-tipped recto-anal mucosal swab (RAMS; foam-tipped applicators, VWR International, Radnor, PA) was used to sample the recto-anal-mucosal junction of each calf using light pressure. Swiffer (Proctor and Gamble, Cincinnati, OH) swabs were used separately to sample the inside of the calf hutches, the inside of the calf feed buckets, the steering wheel and tires of tractors used for calf feeding, and the boots of personnel via wiping with moderate pressure. The gloves (VWR International) worn by the research technicians and employees involved in calf rearing were also collected on the day of sampling. The farm employees were not made aware of the purpose or goals of this study. The dams were sampled by obtaining more than 10 g of feces via rectal palpation. To verify that the detection results were not affected by the sensitivity of the RAMS collection technique, 10% (n = 39) of study calves were convenience sampled via fecal collection (following digital stimulation) during the last two sampling periods.

ECO157 isolation was performed following blind standard laboratory procedures, as previously described (190). Briefly, the Swiffer swabs and the gloves were placed in 90 mL of buffered peptone water (HiMedia Laboratories, Mumbai, India) the RAMS were placed in 5 mL of buffered peptone water, and the fecal samples were diluted (1:10) in buffered peptone water for enrichment. We direct plated 100 µL of the solutions onto sorbitol MacConkey agar with 5-bromo-4-chloro-3-indolyl-b-D-glucuronide (Oxoid Diagnostic Reagents, Basingstoke, Hampshire, England) containing 1.25 mg of potassium tellurite and 0.025 mg of cefixime (HiMedia Laboratories). The fecal enrichment solution remaining after direct plating was incubated for 6 h at 37°C, followed by storage overnight at 48°C. The plates were incubated at 37°C for 24 h, and those containing >100 suspect colonies were chosen for ECO157 latex agglutination, following manufacturer's instructions (Oxoid Diagnostic Reagents). Latex positive colonies were grown in tryptic soy broth (BD, Sparks,

MD) for 6 h and stored at -80°C in 10% sterile glycerol (Sigma Aldrich, St. Louis, MO). The enriched samples stored at 48°C and not confirmed as ECO157 positive through direct plating were subjected to immunomagnetic separation using Dynabeads anti-*E. coli* O157 and a BeadRetriever System (Life Technologies, Oslo, Norway). The immunomagnetic separation sample extracts were plated onto 5-bromo-4-chloro-3-indolyl-b-D-glucuronide containing 1.25 mg of potassium tellurite and 0.025 mg of cefixime and incubated for 24 h at 37°C. Suspect colonies were confirmed using latex agglutination and archived as previously described.

PCR targeting the ECO157 rfb gene was conducted on all latex-positive isolates, and all rfb-positive (and thus ECO157 positive) isolates were subsequently PCR tested for the enterohemorrhagic *E. coli* virulence genes stx1, stx2, and eaeA using the PCR protocol outlined next. For PCR, 10 µL of thawed tryptic soy broth was centrifuged at 5,000g for 5 min and re-suspended in 30 µL of molecular-grade water. Once it was resuspended, 5 µL of the culture template was placed in Qiagen Multiplex PCR Plus Kit reaction master mixes (Qiagen, Venlo, Limburg, The Netherlands), according to the manufacturer instructions, with minor modifications. Briefly, each 25 µL PCR reaction consisted of 12.5 µL of master mix, 2.5 µL of primer mix containing 0.2 µM each primer, 5 µL of molecular-grade water, and 5 µL of culture template. The thermal cycling conditions consisted of an initial incubation at 95°C for 5 min, followed by 40 cycles of amplification with denaturation at 95°C for 30 s, annealing at 57°C for 1.5 min, and extension at 72°C for 30 s, ending with a final extension at 68°C for 10 min. Thermocycling was performed using an MJ Research PTC-100 thermal cycler (Bio-Rad, Hercules, CA). The PCR products were analyzed visually using agarose gel electrophoresis with a 2% agarose gel (Lonza Group Ltd., Basel, Switzerland).

Results

During the period of the study, ECO157 was detected in one calf sample (1 of 399; 0.25%) from herd 3 (Table 8). The sample was collected via RAMS in December, contained the rfb gene, and

lacked all the measured virulence genes (stx1, stx2 , and eaeA). Although every potential *E. coli* virulence gene was not assessed, this isolate was probably non-virulent owing to the lack of major virulence genes found during in-depth characterization of isolates containing only the rfb gene from the early-lactation cows (190). The collection and enrichment of the calf feces collected via digital stimulation showed no difference in ECO157 outcome from the sampling using RAMS. ECO157 was not detected in any samples collected from the calves' environment (gloves, boots, tractor tires, tractor steering wheel, hutches, and feed buckets).

Table 8: *Total calf and dam samples tested and ECO157 isolates detected, by herd*

Dairy herd	Total Calves Sampled, *n*	Calves with isolates containing only *rfb*[a], *n* (%)	Total Dams Sampled, *n*	Dams with isolates containing only *rfb*[a], *n* (%)	Dams with isolates containing *rfb* and *stx(2)*[b], *n* (%)	Dams with isolates containing *rfb/eaeA*[c], *n* (%)
1	179	0	53	11 (20.8%)	1 (1.8%)	0
2	70	0	15	2 (2.8%)	0	0
3	150	1 (0.7%)	43	22 (51.2%)	0	1 (2.3%)
Total	399	1 (0.3%)	111	35 (31.5%)	1 (0.9%)	1 (0.9%)

[a]*E. coli* O157; Stx(2)

[b]*E. coli* Shiga toxin gene 2

[c]*E. coli* attaching and effacing gene

Of the dams, ECO157 isolates with only the rfb gene were detected in 35 samples (32%). In addition, one dam sample (0.9%) from herd 3 contained an ECO157 isolate with the rfb and eaeA genes, and one (0.9%) from herd 1 contained an isolate with the rfb and stx2 genes (Table 8). The dam of the calf that produced the non-virulent ECO157 isolate did not shed any variant of ECO157 when sampled. Additional statistical analyses were not performed on the gathered data because of the lack of positive outcomes. The larger study (190) performed in early-lactation cattle showed that dams shed ECO157 at low rates between the months of August and November, prior to the December onset of this calf study. Because this pilot project did not sample the calves during these

fall and late summer months, it is unknown whether the prevalence of calf shedding would have been different during these seasons.

Discussion

Similar to the results of this study, a low prevalence of ECO157 shedding has been reported (151, 155) in calves younger than 2 months. In the current literature (50, 95, 141, 151, 155, 200), there seems to be a consensus that calves at weaning, as opposed to prior to weaning, are an age group more at risk for both shedding and shedding higher levels of ECO157. Studies (46, 200) that looked at factors associated with shedding demonstrated that group housing and the nonuse of the coccidiostat decoquinate increased the risk of Shiga toxin bacterial shedding in preweaned calves. In the current study, decoquinate was used only in herd 1 during the study period. However, changes in nutrition and nutritional additives did not seem to increase or decrease the shedding rates among the groups because ECO157 was detected in only a single calf sample. The management strategy on all three farms was to house the calves in individual hutches during the preweaning phase and to move them to group housing only after the weaning period. Not initiating the group housing of calves until postweaning is a common strategy employed on dairies, and this may help explain both the higher levels of ECO157 excretion seen in previous studies of weaned calves and the low level of ECO157 excretion seen in the preweaned calves of this study.

In the seasons of our study, ECO157 was detected at a higher frequency in the dams than in their calves. Pearce et al. (162) saw no association between Shiga toxin E. coli shedding in beef calves and their dams within 1 week of birth. In contrast, Cobbold and Desmarchelier (50) showed that dairy calves were twice as likely to shed Shiga toxin *E. coli* when their dams shed Shiga toxin *E. coli*. As previously mentioned, the housing management and immediate removal of the calves from their dams on the farms in the current study probably played a role in the lack of shedding association, and this seems to be an advantageous managerial approach for low-pathogen calf

rearing. It is important to note that these pilot results represent observations from naturally infected dairy herds in a defined location; controlled studies over a longer time frame need to be conducted to confirm that the differences in ECO157 prevalence in calves are due to specific managerial and farm level factors.

The two most common methods of sample collection for ECO157 detection are RAMS and collection of feces. RAMS have been cited previously as being a more sensitive detection method, especially for colonized animals, because the recto-anal-mucosal junction tends to contain ECO157 to a greater extent than the rest of the gastrointestinal tract (49, 125, 129, 149). However, conflicting evidence from other studies (115, 152) showed that the rectoanal- mucosal junction plays a small role in an animal's shedding status; these studies determined that collecting fecal material is a more sensitive method for detecting ECO157 shedding. When it became apparent during the current study that we were not detecting ECO157 via RAMS in the calves, yet were detecting it via fecal retrieval in their dams, we questioned the detection sensitivity of RAMS and chose to use both methods in parallel on a subset of the calves. However, the same ECO157 outcome was achieved regardless of the method we used. A previous study (218) that used both methods in parallel to sample heifers reported that fecal sampling showed a slight increase in sensitivity compared with RAMS. In the current study, not all the calves were sampled using both methods, so it is unknown whether our results would change if we had a greater number of fecal collections. Given low prevalence of shedding (1 of 399), there was not enough power in the current study to detect a difference in sensitivity between the two methods.

Although these results may be specific to geographical location, season, and the type of dairies (dry-lot and freestall), the current study supports the notion that preweaned calves that have been immediately removed from their dams and reared in individual hutches have a small impact on dairy contamination and the dissemination of ECO157. Nevertheless, this does not mean that management strategies to reduce ECO157 in dairies should ignore the peripartum period.

Previous studies show a high variability in calf shedding rates from farm to farm, probably because of the different management strategies employed for calf rearing.

CHAPTER 3: FACTORS ASSOCIATED WITH ESCHERICHIA COLI O157 PRESENCE IN PREWEANED DAIRY HEIFERS ON U.S. OPERATIONS

Summary

Dairy calves shed pathogenic *E. coli* O157 (O157) in feces and are a potential route of exposure for human infections. As part of the National Animal Health Monitoring System's (NAHMS) Dairy 2014 study, we evaluated farm, animal, and environmental factors associated with O157 presence in dairy heifer calves. For this O157 study, calves were enrolled from 100 dairy operations in 13 states. Each operation collected data from calves from birth to weaning, over an 18-month period. A single fecal sample was collected from 487 calves in western states and 871 calves in eastern states (n = 1,358 total). O157 was detected in 2.5% (n = 34) of fecal samples. Descriptive statistics and univariable screening were used to determine which farm practices, environmental factors, and calf health measures were associated with O157 detection. Mixed logistic models, controlling for dairy operation, were created using backward elimination of screened variables. The final main effects model included variables for source of colostrum, temperature and humidity index (THI), and serum IgG concentration. Higher serum IgG was associated with lower odds of O157 shedding, while calves fed colostrum from their own dam had higher odds of O157 shedding compared with calves fed colostrum from pooled sources. Interaction models showed that THI level modified the effect of colostrum source on O157 shedding; calves living at a heat stress THI had a significantly increased presence of O157 when fed colostrum from a first lactation dam. THI level also modified the effects of serum IgG. Calves assigned thermoneutral or heat stress THI values had increased presence of O157 with poor (<10 g/L) or adequate (10–15 g/L), compared with excellent (≥15 g/L), serum IgG levels. These results highlight factors that influence O157 presence in preweaned dairy heifer calves and may be used to guide practices that mitigate shedding through improved animal husbandry.

Background

Escherichia coli O157:H7 (O157) is a gram negative, pathogenic bacterium capable of causing bloody diarrhea, abdominal cramps, and hemolytic uremic syndrome in humans (113, 150, 55). It is estimated that up to 149,000 domestically acquired human O157 infections occur annually in the United States, associated with ingestion of contaminated bovine products and other food sources (176). Cattle play a central role in contamination of the food chain, as they can shed O157 in their feces. Once shed from bovine reservoirs, the O157 bacterium can survive under a multitude of conditions and travel far from sites of deposition by weather and processes such as irrigation and manure fertilization (6, 41, 209). Attempts to control O157 after it is disseminated in feces (Food Safety and Inspection Service standard operating procedures for slaughter, USDA codes for food preparation, Pasteurized Milk Ordinance) have had limited success, evidenced by persistent human outbreaks (42, 132). To reduce the future burden of human disease, understanding the dynamics of cattle carriage with O157 and developing strategies that reduce shedding at the animal level are necessary.

After infection via the fecal-oral route, dairy cows that shed O157 have the ability to directly contaminate diverse food products, including milk, meat, and manure-fertilized crops (143). This is particularly important, as national studies have indicated that O157 is frequently present within the environment of US dairy herds (216, 202). Previous studies have revealed that dairy cow shedding patterns are intermittent and complex, but can be influenced by environmental, farm management, host health, and lactation factors (185, 142, 219, 211, 190). Although risk factors in lactating cows have been measured, less is known about O157 shedding in preweaned calves. Calves may play a pivotal role in both maintaining O157 within the farm environment and directly spreading the pathogen to humans. Calves may shed a higher magnitude of O157 for prolonged periods (62, 50), and both on- and off-farm human contact with calves tends to be greater than with adult cows (56, 188). Shedding of O157 in calves cannot be directly compared with shedding in

adult cows because preweaned calves are monogastrics, and their diet, housing, and overall management on a dairy operation are different from cows. Further, O157 may cause diarrhea in calves, whereas adults do not experience clinical signs of disease during carriage (54, 168).

Previous studies have shown a low prevalence of shedding in calves (80, 174, 191), but this finding is not consistent across the literature (141m 50, 104, 108). When attempting to define factors that influence carriage of O157 by calves, studies have indicated that dietary shifts, the presence of multiple GI pathogens, and administration of anti-coccidial agents may play a role (54, 188, 46). Operation-level factors, including calving strategy, colostrum administration, housing, and amount of contact between adult cows and calves, may also impact preweaning O157 prevalence (201, 174, 39). In the United States, dairy operations are located across the country, and are diverse in their calf-rearing approaches and overall methods of farm management (205). Although some factors for calf shedding have been elucidated, results from studies to date have lacked generalizability. This is due to an absence of comprehensive managerial and calf health data, and characterization of samples from geographically discrete areas, at few seasonal time points. To develop strategies that reduce the prevalence of O157 on-farm, the dynamics of shedding in calves should be analyzed on a national scale that incorporates information for geography, management, and animal health. The objective of the current study was to examine managerial, environmental, and animal health parameters for associations with the presence of O157 in preweaned heifer calves, using USDA's National Animal Health Monitoring System (NAHMS) Dairy 2014 Calf Component study data. Understanding these parameters may then be used to guide the development of management strategies that reduce O157 shedding in calves.

Methods

National surveys of US livestock and poultry industries are conducted annually by NAHMS. In 2014, NAHMS researchers collected information on the health, management, and productivity of

the US dairy industry (204). This study also included a longitudinal study that collected data regarding dairy heifer calf health (NAHMS Dairy 2014 Calf Component).

The NAHMS Dairy 2014 Calf Component study was conducted over 18 months from March 2014 to September 2015. A convenience sample of 104 dairy operations was enrolled from 13 states in two regions: West (California, Colorado, and Washington) and East (Iowa, Michigan, Minnesota, Missouri, New York, Ohio, Pennsylvania, Vermont, Virginia, and Wisconsin). Enrolled operations were categorized based on the number of mature cows: small (30–99 cows), medium (100–499 cows) and large (≥500 cows).

At the study outset, each operation was instructed to enroll 24 heifer calves (roughly 2/mo) for a 1-yr period. Due to lower-than-anticipated participation and a lag in calf enrollment, the target number for participating operations was increased to 48 calves, over an 18-mo period.

Calf Health Card and Sampling of Biologics

Prior to enrollment, calves were tested for bovine viral diarrhea virus (BVDV) and excluded if positive. For on-farm testing, the IDEXX SNAP BVDV Antigen Test (IDEXX, Westbrook, ME) was used, with ear notch samples collected via V-cut ear notchers. BVDV calves were excluded so as not to confound the goals of the Calf Component study: to assess preweaned calf health and growth.

For each enrolled heifer, a Heifer Calf Health Card (Calf Card, available at https://www.aphis.usda.gov/animal_health/nahms/dairy/downloads/dairy14ques/CalfHealth.pdf) was filled out by the producer, the calf handler, a veterinary medical officer, veterinary extension personnel, or a combination of these individuals The Calf Card was written in both English and Spanish, and had sections describing birth data (e.g., birth date, birth weight, calving ease of dam), colostrum feeding data (timing, amount, method of feeding), preweaning housing and procedures (e.g., type of housing, ventilation, bedding, dehorning, and navel disinfection), milk feeding (including type of liquid diet, additives, method of feeding) and milk consumption record (volume

and frequency of feedings). The Calf Card also contained a biweekly growth record (hip height, heart girth), biologic sampling record (serum collection and fecal sampling dates), vaccination history, disease incidence and treatment, weaning data (weaning date, primary weaning criteria), and any additional notes. Starter feed and milk replacer labels were also collected, if applicable.

For each calf enrolled in the study, colostrum from the initial feeding (40–50 mL) was collected in conical screw-cap tubes and frozen prior to shipping. Similarly, blood (5 mL) was collected from each calf between 1 and 7 d of age and placed in serum separator tubes, which were centrifuged when possible prior to shipping. Both colostrum and serum were shipped together on ice to the USDA's National Veterinary Service Laboratories (NVSL; Ames, IA). At the NVSL, serum was separated and serum and colostrum samples accumulated and were then shipped to Saskatoon Colostrum Company (SCC; Saskatoon, Saskatchewan) where they were tested for IgG concentration using radial immunodiffusion (RID).

For RID, 24 mL agarose plates were punched with 42 wells of 6 µL volume. Wells 1–4 and 39–42 were for 2 replicates each of 4 calibrators used to generate a standard curve, wells 5–9 and 10–14 were for each of the 2 reference standards that were used to qualify the plate, and wells 15–38 were used for test samples. Serial 2 – fold dilutions (1:4, 1:8, 1:16, 1:32) of the bovine IgG standard (Bovine Serum calibrator cat. #4055, Midland BioProducts Corp., Boone, IA), reference bovine serum (CVB bovine IgG species standard; working stock diluted 1:4), and reference colostrum (diluted 1:15) were prepared in PBS. A 1:4 dilution of test serum and a 1:15 dilution of test colostrum were prepared using PBS. Two replicates of 4 µL of each dilution of bovine IgG standard were dispensed for the standard curve. Two replicates of 4 µL of the diluted reference colostrum and reference serum and 4 µL of the diluted test samples were also dispensed on the plate. Plates prepared in house using commercially available ingredients and reagents were incubated at 20°–25ºC for 18–19 h in a humidified chamber. A plate reader was used to measure and record the ring diameters of precipitin rings surrounding wells (Digital RID reader product

code AD400, The Binding Site Inc., San Diego, CA). Using the ring diameters obtained for each of the 2-fold dilutions of bovine IgG standard, a regression line was generated for each plate for the variables R (ring diameter) versus $\log_{10}$ (concentration). The plate was considered acceptable if the R^2 was greater than 0.97 for the standard curve and the mean values for the reference colostrum and reference serum were the expected values ±10%. IgG concentration (g/L) for test samples were determined using the regression line of bovine IgG standards obtained for each plate. To calculate the IgG consumed during the first colostrum feeding for each calf, the concentration of IgG in colostral samples was multiplied by the volume fed, which was recorded on the Calf Card.

Field personnel were instructed to collect a single fecal sample directly from the rectum of calves between 2 and 4 wk of age. Feces were placed in cylindrical screw-top containers and shipped on ice to the USDA's Agricultural Research Service's Environmental Microbial Food Safety Laboratory (EMFSL; Beltsville, MD) for enteric parasite detection. Fecal samples were tested by immunofluorescence microscopy for *Cryptosporidium* and *Giardia,* as described previously (71). Briefly, 15 g of feces were mixed with 35 mL of distilled water. Each fecal suspension was passed through a sieve with 45 µm pores. Distilled water was added to the filtrate up to a 50-mL volume. Samples were then centrifuged for 15 min at 1800 x g. Pellets were re-suspended in a solution of 25 mL distilled water with 25 mL of 1.4 g/L CsCl solution. Samples were again centrifuged at 300 g for 20 min and the supernatant was aspirated. Each pellet was washed with distilled water, and 2 µL of pellet suspension was transferred to an 11-mm diameter well of a glass microscope slide. 2 µl of Merifluor reagent (Meridian Diagnostics, Cincinnati, OH) was added to each well. A Zeiss Axioscop microscope (Jena, Germany) with a 400x objective, epifluorescence, and FITC-Texas Red™ dual wavelength filter, was used to examine each well in its entirety and count oocysts/cysts.

After testing at EMFSL, leftover feces were stored frozen before being shipped on dry ice to the Colorado State University Animal Population Health Institute Laboratory (APHI; Fort Collins, CO) for detection of enteric bacteria. Once all study samples were received at APHI, they were

thawed and diluted 1:10 in buffered peptone water (BPW; Oxoid Ltd, Cheshire, England). Samples were then incubated for 6 h at 37°C and enrichments were stored at -80°C in 10% sterile glycerol. Enrichments were thawed, boiled for 10 min, and centrifuged at 9300 x g for 5 min. The DNA in culture supernatants was purified using a GeneClean Turbo Kit based on manufacturer instructions (MP Biomedicals, Solon, OH).

Each extracted DNA sample was subjected to real-time multiplex PCR reactions (qPCR), using a BioRad iQ5 machine (BioRad, Hercules, CA). The multiplex combination described below was previously validated to have bacterial detection sensitivity of <18 CFU/10 g in artificially contaminated ground beef, and minimum interference by an internal amplification control (IAC) (193). Reactions targeted the *invA* (*Salmonella enterica; Salmonella spp*), *rfbE* (*Escherichia coli* O157; O157) genes, and human adenovirus IAC DNA, as previously described (Suo et al., 2010). For the IAC, a 79-bp region of the human adenovirus was used, based on exclusivity of the viral sequence to bacterial genomes. The DNA was originally PCR amplified from a long oligonucleotide (5'-TGGAAGCAATGCCAAATGTGTATGTGGTGGCATTGTCTTCTCCCGTTGTAACTATCCACTGAGATGTGTTAGGCGCGCC - 3'), using the forward and reverse primers described in Table 9, and purified with a QIAquick gel extraction kit (Qiagen, Valencia, CA). IAC DNA was prepared and validated at the USDA Molecular Characterization of Foodborne Pathogens Research Unit in Wyndmoor, PA, and shipped to the APHI Laboratory on ice.

Table 9: Forward and reverse primers and TaqMan probes used in bacterial detection qPCR

Organism	Gene	Primer/Probe	Sequence (5' - 3')	Amplicon Size	Probe 5' Reporter / 3' Quencher
Escherichia coli O157	*rfbE*	Forward	TGTTCCAACACTGACATATATAGCATCA	93	HEX / BHQ 2
		Reverse	TGCCAAGTTTCATTATCTGAATCAA		
		Probe	ATGCTATAAAATACACAGGAGCCACCCCCA		
Salmonella enterica	*invA*	Forward	GTTGAGGATGTTATTCGCAAAGG	75	6-FAM / BHQ 1

		Reverse	GGAGGCTTCCGGGTCAAG		
		Probe	CCGTCAGACCTCTGGCAGTACC TTCCTC		
Human adenovirus (control)	IAC	Forward	GGCGCGCCTAACACATCT	79	Texas Red/ BHQ 2
		Reverse	TGGAAGCAATGCCAAATGTGTA		
		Probe	TTACAACGGGAGAAGACAATGC CACCA		

The following amplification procedure was used for all real-time reactions: 95°C x 10 minutes, 40 cycles of 95°C x 15 seconds and 60°C x 1 minute. Initially, amplification curves of serially diluted IAC (10^6–10^1 copies/µL), *Escherichia coli* O157:H7 ATCC 35150, and *Salmonella enterica* ssp Typhimurium DNA were performed. Results showed that IAC concentrations of 1.4 x 10^3 copies had low impedance of bacterial amplification at both low and high concentrations of bacterial template, similar to that seen by Suo and colleagues (193). After running dilution curves of individual and multiplexed positive controls, amplification efficiencies for both *rfbE* and *invA* targets were determined as ≥94%.

Forward and reverse primers and TaqMan probe sequences can be found in Table 9. All of the primers and probes were obtained from Eurofins Genomics (Louisville, KY). The following was added to each 20-µl reaction: 1x PerfeCTa qPCR ToughMix (Quanta Biosciences, Beverly, MA), 200 nm each of forward, reverse, and probe oligonucleotides (3 sets), 1.4 x 10^3 copies of IAC, and 2 µL of control or test DNA. Each test DNA sample was run in triplicate.

Growth Measurements

Height and weight measures of calves were gathered approximately every 2 wk during the preweaning period. Height/weight tapes from Coburn® (Nasco, Fort Atkinson, WI) were provided for making measurements. Birth weights (kg) were estimated using a scale, hoof circumference, or heart girth circumference. When measured, hip height and heart girth circumference were recorded in centimeters. Heart girth (cm) was converted to kilograms using the following equation from Heinrichs et al., (93):

(eq 1) weight (kg) = ((0.02655*(cm^2))+(-2.876*cm)+102.71)

Average daily gain (ADG) was calculated by subtracting the birth weight from the final weight and dividing this quantity by the number of days between the two measurements (approximately equivalent to the duration of the preweaning period). Final weights did not always occur at the same time as weaning, though only calves with final weight measurements within 14 d (standard deviation = 8.9 d) of weaning were included in analyses.

Meteorological Measurements

County measures of the temperature and humidity index (THI) were obtained using data from the National Oceanic and Atmospheric Administration (www.noaa.org). THI provides an index that accounts for the effects of temperature and humidity, using dry bulb temperature (T, °F) and the relative humidity (RH) (25, 51). The equation for THI used in this analysis was:

(eq 2) THI = T – (0.55 – (0.55*RH/100) * (T – 58))

Values from the month of fecal sample collection were obtained, and each calf was assigned a single THI value. THI values were then categorized based on thermoneutral zones (180), since external temperature likely influences the shedding of O157 (219). The following categories were created: cold stress (THI < 50), thermoneutral (THI ≤ 50 and < 70), heat stress (THI ≥ 70).

Because calves with fecal samples taken toward the beginning of the month may have been assigned THI values that did not truly reflect the time immediately prior to O157 detection, a sensitivity analysis was performed. Data were categorized based on whether or not calves had a THI value recorded for their birth month. Since these were more likely to reflect calves with THI values conducive to the time period prior to shedding, mixed regression models (see below) were

run using both data sets (birth month matched or non-birth month matched), in turn, after randomly subsampling to the same number of observations.

Data Management and Statistical Analysis

At calf weaning, each Calf Card was mailed to USDA NAHMS. Each Calf Card was validated by NAHMS personnel when it arrived. Once all Calf Cards were entered, information was again validated by NAHMS employees and merged with results from colostrum and serum testing at SCC, fecal testing at EMFSL and APHI, and THI data. Variables assessed for association with O157 presence were selected based on their biologic or historically relevant plausibility to impact colonization of the pathogen. Broadly, these variables were related to calf growth parameters, immune status, housing and maintenance, and presence of GI microbes and parasites. These variables can be found in Table 10 and included: days of age at fecal, birth weight, weight at weaning, days of age at weaning, average daily gain, IgG consumed in colostrum, serum IgG, colostrum source, hours until colostrum given, type of liquid diet, milk pasteurization, milk delivery, calf bedding type, size of herd, calf breed, housing ventilation, calving ease, dam lactation, temperature and humidity index, any disease, coccidiostat administration, dehorning, housing type, presence of Giardia, presence of Cryptosporidium, presence of Salmonella enterica spp, region of farm, navel disinfection, administration of direct-fed agents, administration of any *E. coli* preventative.

Continuous variables were tested for linearity. Hours to colostrum administration was dichotomized based on this variable's non-linear nature. Due to low values in several categories, the incidence of disease as indicated on Calf Cards (cough, runny nose, runny eyes, breathing difficulty, head tilt, listlessness, droopy ears, off of feed, dull, diarrhea) was collapsed to a binary outcome of "any disease" or "no disease." In addition, the variable for calf bedding initially included options for straw/hay, shavings, sand, manure bio-solids, none, or other/combination. This variable was

collapsed to reflect the most prominent categories, while still discerning bedding types shown previously to impact O157 growth: straw/hay, shavings/woodchips, and other/combination (124, 70). Direct fed agents administered to calves in this study, as indicated on calf cards, included CONVERT™ Day One Calf Gel, NuLife® ReBOUND™, First Arrival®, Last Stand® Calf Paste, Vita Charge®, and probiotics. This variable was dichotomized into "any direct-fed agent" or "no direct-fed agent." Calves of the current study were also given many vaccines. To reflect vaccinations that might impact colonization or shedding of the O157 pathogen, vaccines indicated on Calf Cards were categorized based on their efficacy against gastrointestinal microbes. Four vaccines were selected and further investigated for their association with O157 shedding. These vaccines were categorized as "*E. coli* preventatives," and identified on Calf Cards as Bovi-Sera, Piliguard® E.coli-1, First Defense®, and Ecolizer™. The variable indicating "hours to colostrum feeding" was dichotomized (≤ 4 h or > 4 h) based on industry recommendations. Variables that may temporally be associated with O157 (disease incidence, dehorning, administration of *E. coli* preventative) were classified based on their occurrence prior to fecal sampling.

Distributions of calf variables by presence of O157 were measured and tested for differences using Fisher's exact test for categorical variables, and t-tests for continuous variables (Table 11). Due to a low number of calves with O157, variables with $p < 0.20$ were selected and only calves with all variables in Table 11 were included in regression analyses.

Mixed regression models were implemented to determine factors significantly associated with the presence of O157. Screened variables were initially assessed via univariable mixed logistic regression models, with dairy operation as a random variable. Variables with $p < 0.20$ in univariable screening were assessed for collinearity and included in multivariable models. Initial multivariable models were stable. Step-wise backward elimination model selection was then performed, screening for confounding at each step. Variables with $p < 0.05$ were retained in a finalmodel of main effects. An interaction analysis was then performed. Multiplicative interactions

between main effects were assessed by introducing a product term for all final model covariate pairs, in turn, in mixed regression statements. For interaction variables that met a $p < 0.05$ threshold for significance, stratified odds ratios were determined for ifferent combinations of each variable level.

Initial statistical analysis, screening of variables, and interaction assessments were performed in R version 3.3.2 or later. Mixed logistic regression analysis was performed in SAS (version 9.4; SAS Institute Inc., Cary, NC) using the GLIMMIX procedure.

Results

For the study, fecal samples were obtained from 2,249 calves. Of these samples, 1,358 had a large enough quantity for laboratory analysis of all parasitic and bacterial pathogens. These calves were from 100 different operations in western (n = 487; CA, CO, WA) and eastern (n = 968; IA, MI, MN, MO, NY, OH, PA, VT, VA, WI) states (Figure 5). For fecal level measures, 31% (n = 421) of calf samples contained *Giardia*, while 44% (n = 593) of samples contained *Cryptosporidium.* Relatively few samples (n = 12, 0.8%) contained *Salmonella spp.* detected via qPCR, and a higher number (n = 34, 2.5%) were found to contain O157.

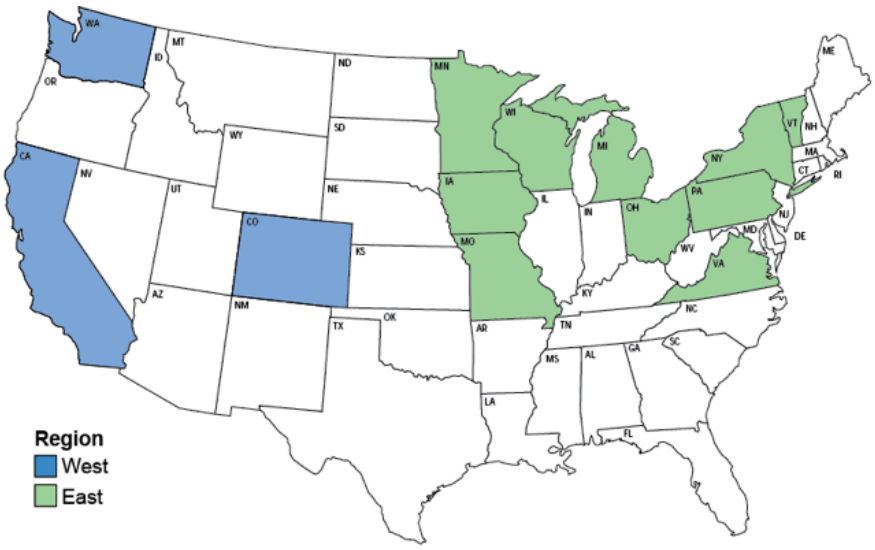

Figure 5: Map of states participating in the calf component of the dairy 2014 study. Regions were defined as west (blue states) and east (green states).

We quantified farm practices, environmental factors, and calf health measures by O157 status (Table 10). Thirteen variables with differences ($p < 0.2$) in O157 presence were retained for evaluation in univariable mixed models (Table 11). These variables included days of age at fecal collection, days of age at weaning, average daily gain (kg), serum IgG, calf bedding, size of herd, hours until colostrum feeding, colostrum source, temperature and humidity index (THI), *Giardia*, *Cryptosporidium*, *Salmonella*, and any *E. coli* preventative. Calves with missing values for any of the retained variables were omitted from further analysis. The regression dataset included information from 1,106 calves (omitting 252), with 2.5% (n = 28) of calf samples containing O157.

Table 10: Herd and calf level descriptors by *E.coli* O157 presence in preweaned dairy calves

	No O157 (n = 1324 max)	O157 (n = 34 max)	*P* - value
Days of Age at Fecal (SD)[1]	21.8 (6.2)	23.6 (7.5)	0.18
Birth Weight, kg (SD)[1]	42.8 (5.5)	43.0 (5.5)	0.88

Weight at Weaning, kg (SD)[1]			
	88.4 (20.8)	86.5 (20.7)	0.62
Days of Age at Weaning (SD)[1]			
	63.8 (17.4)	69.3 (17.2)	0.08
Average Daily Gain, kg (SD)[1]			
	0.7 (0.2)	0.6 (0.2)	0.05
IgG (g) consumed in Colostrum (SD)[1]			
	220.9 (115.9)	197.8 (119.9)	0.31
Serum IgG (g/L) (SD)[1]			
	22.2 (10.6)	15.7 (8.9)	<0.01
Colostrum Source (%)[2]			
First lactation dam	228 (18%)	8 (23%)	0.021
Second lactation dam	171 (13%)	8 (23%)	
Third or higher lactation dam	236 (18%)	10 (30%)	
Unknown Lactation dam / not calf's dam	377 (29%)	7 (21%)	
Pooled	262 (20%)	1 (3%)	
Commercial replacer	20 (2%)	0 (0%)	
Type of Liquid Diet (%)[2]			
Milk replacer	454 (34%)	10 (29%)	0.76
Whole/waste milk	508 (38%)	13 (38%)	
Both milk replacer and waste milk	362 (27%)	11 (32%)	
Milk Pasteurization (%)[2]			
Pasteurized	428 (32%)	11 (32%)	0.80
Not Pasteurized	442 (33%)	13 (38%)	
Milk Replacer only	454 (34%)	10 (29%)	
Milk Delivery (%)[2]			
Bottle	348 (26%)	8 (24%)	0.35
Bucket	230 (17%)	2 (6%)	
Milk Bar	18 (1%)	0 (0%)	
Robot	17 (1%)	0 (0%)	
Bottle + Bucket	639 (48%)	23 (68%)	
Other Combinations	72 (5%)	1 (3%)	
Calf Bedding (%)[2]			
Straw/hay	732 (55%)	15 (44%)	0.13
Shavings/woodchips	299 (23%)	13 (38%)	

	Other/Combo	293 (22%)	6 (18%)	
Size of Herd (%)[2]				
	Small (30-99 cows)	188 (14%)	8 (24%)	0.15
	Medium (100-499 cows)	353 (27%)	11 (35%)	
	Large (500 or more cows)	783 (59%)	15 (44%)	
Breed (%)[2]				
	Holstein	1182 (89%)	31 (91%)	0.68
	Jersey	57 (4%)	2 (6%)	
	Other	82 (6%)	1 (3%)	
Housing Ventilation (%)[2]				
	Outside/Natural	1073 (81%)	31 (91%)	0.38
	Positive Pressure Tubes	130 (10%)	1 (3%)	
	Other / Cross Ventilation	121 (9%)	2 (6%)	
Calving Ease (%)[2]				
	Unassisted	954 (78%)	27 (79%)	1.00
	Easy / 1 person help	205 (17%)	6 (18%)	
	Difficult / >= 2 people help	58 (5%)	1 (3%)	
	Meachanical / Surgical Extraction	7 (1%)	0 (0%)	
Dam Lactation (%)[2]				
	1	467 (39%)	14 (41%)	0.98
	2	317 (27%)	9 (26%)	
	>= 3	406 (34%)	11 (32%)	
Temperature and Humidity Index (THI) (%)[2]				
	< 50, cold stress	594 (45%)	3 (9%)	<0.01
	50 - 69, thermoneutral	576 (44%)	18 (53%)	
	>= 70, heat stress	154 (12%)	13 (38%)	
Any Disease (%)[2]				
	Yes	335 (25%)	5 (15%)	0.23
	No	989 (75%)	29 (85%)	
Coccidiostats Added to Milk (%)[2]				
	Yes	516 (40%)	14 (41%)	0.86
	No	789 (60%)	20 (59%)	
Dehorned (%)[2]				
	Yes	286 (24%)	7 (23%)	1.00
	No	925 (76%)	23 (77%)	

Calf Housing (%)[2]				
	Pen / Hutch	1173 (89%)	31 (91%)	1.00
	Group	151 (11%)	3 (9%)	
Giardia isolated (%)[2]				
	Yes	407 (31%)	14 (41%)	0.18
	No	917 (69%)	20 (59%)	
Cryptosporidium isolated (%)[2]				
	Yes	574 (43%)	19 (56%)	0.16
	No	750 (57%)	15 (44%)	
Salmonella enterica spp isolated (%)[2]				
	Yes	9 (1%)	3 (9%)	<0.01
	No	1315 (99%)	31 (91%)	
Region (%)[2, 3]				
	West	475 (36%)	12 (35%)	1.00
	East	849 (64%)	22 (65%)	
Navel Disinfection (%)[2]				
	Yes	981 (76%)	25 (74%)	0.84
	No	315 (24%)	9 (26%)	
Any Direct-Fed Agent (%)[2]				
	Yes	15 (1%)	1 (3%)	0.34
	No	1309 (99%)	33 (97%)	
Any *E.coli* Preventative (%)[2]				
	Yes	166 (13%)	0 (0%)	0.02
	No	1158 (87%)	34 (100%)	
Hours until Colostrum Given (%)[2]				
	< = 4	1016 (82%)	20 (61%)	<0.01
	> 4.0	201 (18%)	13 (39%)	

[1]Continous variables assessed with students t-test

[2]Categorical/Dichotomous variables analyzed with Fisher's exact test

[3]Western states included California, Colorado and Washington, Eastern states included Iowa, Michigan, Minnesota, Missouri, New York, Ohio, Pennsylvania, Vermont, Virginia, Wisconsin

Associations with O157 were measured for each variable individually, using mixed logistic regression with operation as a random variable (Table 11; Crude OR). Those with individual $p < 0.2$

significance (all variables but *Giardia* and *Cryptosporidium*) were used in an initial mixed multivariable model, with operation as a random variable (Table 11; Adjusted OR). After backward step-wise elimination, three main effects variables were significantly ($p < 0.05$) associated with O157 presence in calves. These included colostrum source, THI, and serum IgG (Table 12). Compared to pooled sources, colostrum from the calf's dam (lactation 1, 2, or 3+) was associated with an increased odds of O157 presence (example: OR from 2nd lactation calf dam = 1.045, 95% CI = 1.011–1.081). When comparing to a thermoneutral THI, a heat stress THI showed a significant association with increased O157 presence (OR = 1.057, 95% CI = 1.023–1.089) and a cold stress THI showed significant association with decreased O157 presence (OR = 0.974, 95% CI = 0.955–0.933). Values for serum IgG showed that each 1-gram increase was significantly associated with reduced odds of O157 presence (OR = 0.998, 95% CI = 0.997–0.999).

Table 11: Results of crude (univariable) and adjusted (multivariable) mixed logistic regression modeling of O157 presence in preweaned dairy calves

Variable	Unit Increase	Crude OR[1]	Crude 95% CI	*P* - value	Adjusted OR[2]	Adjusted 95% CI	*P* - value
Days of Age at Fecal	1 day	1.001	0.999 - 1.003	0.235	1.001	0.999-1.003	0.235
Days of Age at Weaning	1 day	1.000	0.999 - 1.001	0.135	1.000	1.000 - 1.001	0.551
Average Daily Gain, kg	1 kg	0.956	0.916 - 0.996	0.033	0.973	0.931 - 1.016	0.215
Serum IgG (g / L)	1 g / L	0.998	0.9975 - 0.9993	<0.001	0.998	0.998 - 0.999	0.0008
Calf Bedding							
Shavings / Woodchips		1.249	1.216 - 1.282	0.183	1.014	0.987 - 1.041	0.326
Other / Combo		1.001	0.974 - 1.028	0.966	0.993	0.964 - 1.022	0.636
Straw/Hay		REFERENCE			REFERENCE		
Size of Herd							
Small (30-99 cows)		1.030	0.997 - 1.064	0.080	0.997	0.960 - 1.036	0.888
Medium (100-499 cows)		REFERNCE			REFERENCE		
Large (500 or more cows)		1.013	0.989 - 1.036	0.300	1.007	0.979 - 1.035	0.648

Hours until Colostrum Given						
> 4 hours	1.035	1.010 - 1.060	0.006	1.017	0.990 - 1.045	0.228
< = 4 hours	REFERENCE			REFERENCE		
Colostrum Source						
1st lactation dam	1.029	1.000 - 1.063	0.0854	1.031	0.996 - 1.068	0.0876
2nd lactation dam	1.046	1.010 - 1.084	0.0113	1.044	1.006 - 1.083	0.0236
3+ lactation dam	1.044	1.011 - 1.079	0.0088	1.043	1.006 - 1.081	0.0211
Unknown lactation dam / Not calf's dam	1.011	0.981 - 1.042	0.476	1.007	0.976 - 1.039	0.666
Colostrum Replacer	0.99	0.916 - 1.069	0.784	0.975	0.902 - 1.054	0.525
Pooled Colostrum	REFERENCE			REFERENCE		
Temperature and Humidity Index (THI)						
>70, heat stress	1.056	1.025 - 1.087	<0.001	1.056	1.024 - 1.089	0.0005
50 - 69, thermoneutral	REFERENCE			REFERENCE		
< 50, cold stress	0.975	0.955 - 0.994	<0.001	0.978	0.968 - 0.988	0.0445
Giardia isolated						
Yes	1.011	0.991 - 1.032	0.286	NA	NA	NA
No	REFERENCE					
Cryptosporidium isolated						
Yes	1.011	0.992 - 1.030	0.277	NA	NA	NA
No	REFERENCE					
Salmonella isolated						
Yes	1.078	0.978 - 1.188	0.133	1.064	0.967 - 1.172	0.2044
No	REFERENCE			REFERENCE		
Any *E.coli* Preventative						
Yes	0.968	0.938 - 0.998	0.037	0.976	0.944 - 1.008	0.135
No	REFERENCE			REFERENCE		

[1]Odds of O157 presence, calculated from univariable mixed logistic regression using operation as random variable
[2]Odds of O157 presence, calculated from multivariable mixed logistic regression using operation as random variable. Multivariable terms were selected based on a Crude OR $P < 0.2$

Table 12: Main effects multivariable mixed logistic regression model of O157 presence in preweaned dairy calves

Variable	Unit Increase	OR[1]	Standard Error	95% Confidence Interval	*P*-value
Serum IgG (g / L)	1 g / L	0.998	0.0005	0.997 - 0.999	0.0002

Temperature and humidity for stress (THI)				
>70, heat stress	1.057	0.0149	1.023 - 1.089	0.0002
50 - 69, thermoneutral	REFERENCE			
< 50, cold stress	0.974	0.0100	0.955 - 0.993	0.0085
Colostrum source				
1st lactation dam	1.033	0.0158	1.002 - 1.066	0.0396
2nd lactation dam	1.045	0.0171	1.011 - 1.081	0.0101
3+ lactation dam	1.042	0.0157	1.010 - 1.075	0.0091
Unknown lactation dam / Not calf's dam	1.012	0.0145	0.983 - 1.041	0.4270
Colostrum Replacer	0.977	0.0388	0.906 - 1.055	0.5550
Pooled Colostrum	REFERENCE			

[1]Odds of O157 presence, calculated from mixed logistic regression using operation as random variable

After interaction models were created, THI was found to significantly modify the effect of colostrum source and serum IgG (Table 13; p = <0.001 for THI and colostrum source and p = 0.0007 for THI and serum IgG). To measure the relationship between these variables, serum IgG was categorized and samples were stratified based on standard industry cut-off measures (<10 g / L, 10.1–14.9 g/L, ≥ 15 g/L) (USDA, 2010). Mixed regression modeling was performed using multiplicative interaction terms, andO157 logit values were plotted for each level of serum IgG (Figure 6) and colostrum source (Figure 7) using the lowest risk categories from the main effect model (low THI, high serum IgG, pooled colostrum). The odds of O157 presence increased as THI level increased, and as serum IgG level decreased (Table 13). Specifically, compared with calves exposed to a cold stress THI with high (≥15 g/L) serum IgG values, calves exposed to thermoneutral or heat stress THI, with low or moderate values of serum IgG, were at increased risk of O157 presence (thermoneutral and low IgG OR: 1.076, 95% CI = 1.008–1.149; heat stress and low IgG OR: 1.203, 95% CI = 1.103–1.311). Compared to calves exposed to a cold stress THI and fed pooled colostrum, calves fed colostrum from their own first lactation dam and exposed to a heat stress THI were at increased risk of O157 presence (OR: 1.238, 95% CI = 1.128 – 1.360). Calves fed colostrum

from their own second lactation dam and exposed to a thermoneutral THI were also at increased risk of O157 presence compared to the reference group (OR: 1.076, 95% CI = 1.007 – 1.150).

Table 13: Interaction multivariable mixed logistic regression model of O157 presence in preweaned dairy calves

Variable	N O157(+) / O157 (-)	OR[1]	Standard Error	95% Confidence Interval	*P*-value
Categorized Serum IgG (g / L)					
< 10	10 / 117 (7.9%)	0.992	0.025	0.944 - 1.04	0.732
10 - 14.9	6 / 150 (3.8%)	0.993	0.019	0.957 - 1.03	0.704
> 15	12 / 811 (1.5%)	REFERENCE			
Temperature and humidity for stress (THI)					
>70, heat stress	12 / 131 (8.4%)	0.988	0.031	0.930 - 1.050	0.697
50 - 69, thermoneutral	14 / 457 (3.0%)	0.9970	0.022	0.956 - 1.041	0.905
< 50, cold stress	2 / 490 (0.4%)	REFERENCE			
Colostrum source					
1st lactation dam	6 / 190 (3.0%)	1.001	0.022	0.959 - 1.046	0.956
2nd lactation dam	7 / 138 (4.8%)	1.003	0.025	0.955 - 1.053	0.920
3+ lactation dam	9 / 191 (4.5%)	1.025	0.023	0.980 - 1.072	0.274
Unknown lactation dam / Not calf's dam	5 / 300 (1.6%)	1.000	0.021	0.960 - 1.042	0.986
Colostrum Replacer	1 / 17 (5.6%)	1.001	0.055	0.899 - 1.115	0.984
Pooled Colostrum	1 / 262 (0.4%)	REFERENCE			
Categorized Serum IgG x THI					
<10 g / L, heat stress	5 / 19 (20.8%)	1.203	0.044	1.103 - 1.311	<0.0001
10 - 14.9 g / L, heat stress	3 / 17 (15.0%)	1.108	0.042	1.02 - 1.202	0.014
<10 g / L, thermoneutral	5 / 53 (8.6%)	1.076	0.033	1.008 - 1.149	0.028
10 - 14.9 g / L, thermoneutral	3 / 54 (5.3%)	1.047	0.029	0.989 - 1.107	0.114
Any serum IgG, cold stress	2 / 490 (0.4%)	REFERENCE			
>15 g / L, any THI	12 / 811 (1.5%)	REFERENCE			
Colostrum source x THI					
1st lactation dam, thermoneutral	1 / 79 (1.4%)	1.004	0.031	0.944 - 1.067	0.908
2nd lactation dam, thermoneutral	6 / 60 (9.0%)	1.076	0.034	1.007 - 1.150	0.031
3+ lactation dam, thermoneutral	5 / 83 (5.7%)	1.020	0.031	0.959 - 1.084	0.532
Uknown lactation / other dam, thermoneutral	1 / 127 (0.8%)	0.999	0.028	0.945 - 1.057	0.983
Colostrum replacer, thermoneutral	0 / 9 (0.0%)	0.949	0.079	0.813 - 1.107	0.506
1st lactation dam, heat stress	5 / 16 (24.0%)	1.238	0.048	1.128 - 1.360	<0.0001
2nd lactation dam, heat stress	1 / 16 (5.9%)	1.000	0.053	0.902 - 1.110	0.996
3+ lactation dam, heat stress	2 / 23 (7.7%)	1.020	0.046	0.932 - 1.117	0.662

Uknown lactation / other dam, heat stress	4 / 42 (8.7%)	1.038	0.041	0.957 - 1.125	0.370
Colostrum replacer, heat stress	1 / 0 (100%)	0.999	0.162	0.727 - 1.374	0.998
Any THI and pooled colostrum	1 / 262 (0.4%)	REFERENCE			
Cold THI and any colostrum source	2 / 490 (0.4%)	REFERENCE			

[1]Odds of O157 presence, calculated from mixed logistic regression using operation as random variable

We performed a sensitivity analysis using THI values from birth month matched or non-birth month matched calves. The change in model fit (AIC value) was negligible between data sets (data not shown).

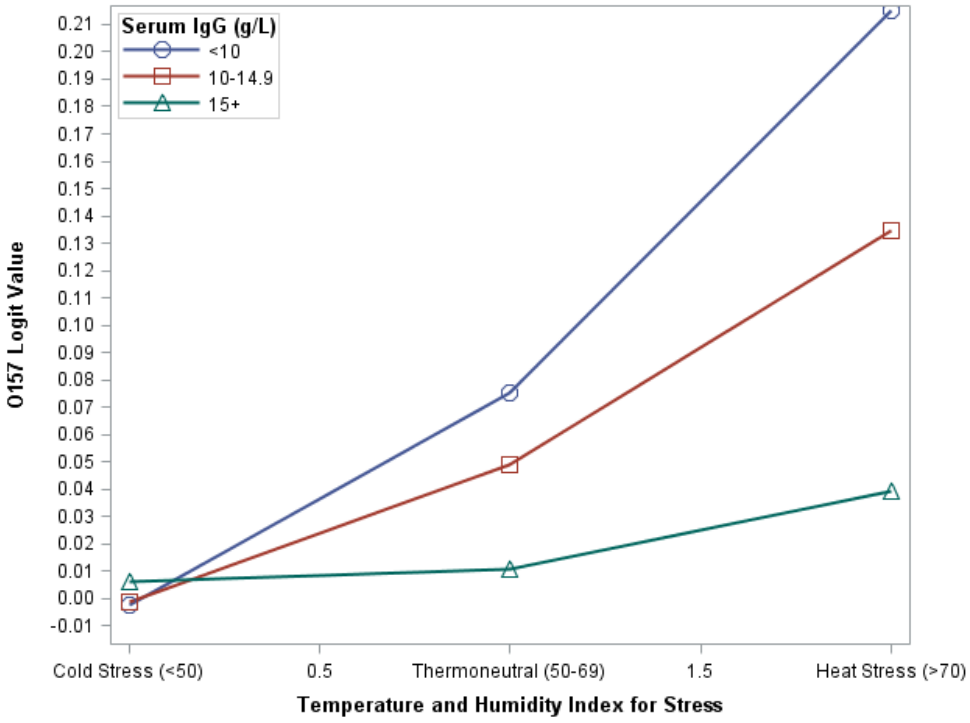

Figure 6: Interaction of categorized serum IgG and temperature and humidity index for stress on O157 shedding status of calf fecal samples. Logit values were obtained via mixed logistic regression, using calf operation as a random variable.

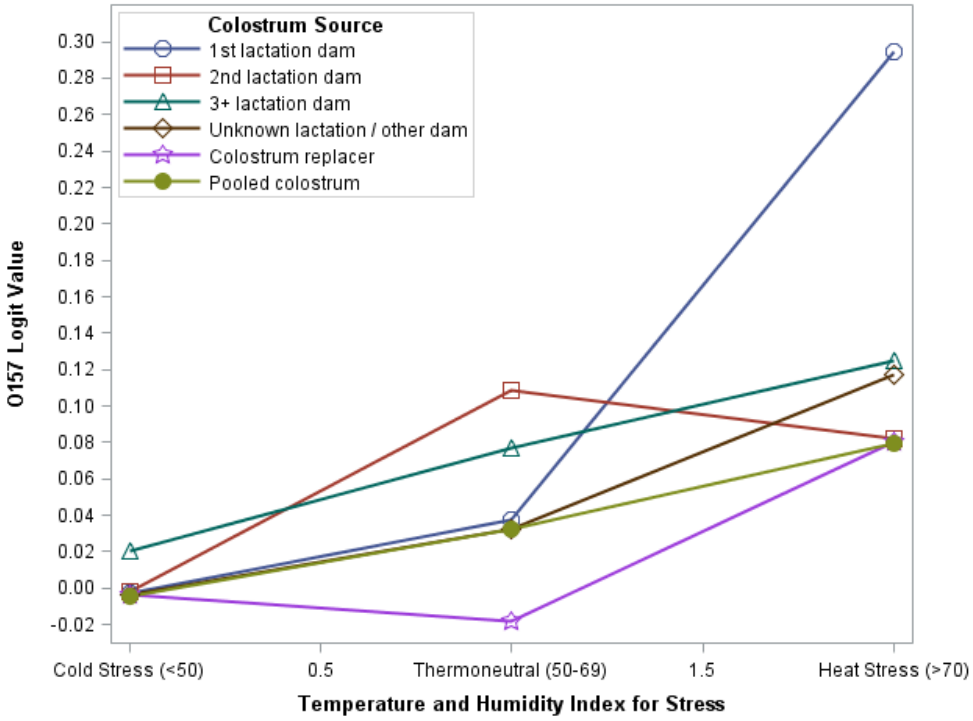

Figure 7: Interaction of colostrum source and temperature and humidity index for stress on O157 shedding status of calf fecal samples. Logit values were obtained via mixed logistic regression, using calf operation as a random variable.

Discussion

In the current study, we detected O157 in 2.5% of tested NAHMS calf fecal samples. The calves studied were raised on diverse operations across the United States. By comparison, during the NAHMS 1991–92 National Dairy Heifer Evaluation Project (NDHEP), approximately 7,000 calf samples from over 1,000 US operations were tested for O157, and the prevalence was found to be 0.36% (201). Calves positive for O157 from both the current study and NDHEP showed no regional clustering across the United States. Another report that included case and control herds from the original NDHEP showed calf O157 prevalence to be 1.9% (225). These results support a systematically low prevalence of O157 in preweaned calves, with little variation across the country.

Operations commonly house young calves in individual pens or hutches to reduce exposure and spread of enteric and respiratory pathogens. During the NDHEP, a significant managerial factor

that influenced shedding was whether or not preweaned calves were group housed. Calves grouped before weaning were 9 times more likely to test positive for O157 than those that were not (201). In the current study, a low percentage (11%) of operations group housed their animals. Although this left little power for detection, the distribution of operations that group housed did not explain the presence of O157, as presence did not differ by housing status. Conducting studies that attempt to specifically look at animal contact as it relates to O157 shedding is necessary to understand if group housing truly influences shedding. .

In these analyses, calves given colostrum from their own dam, compared to pooled sources, had a higher risk of O157 presence. Previous studies have shown that calves left to nurse colostrum from their own dams may take longer to ingest colostrum, ingest less colostrum overall, and ultimately experience poor passive transfer (203). We postulate that calves fed colostrum from their own dams likely spent a greater period of time with their dam after birth, compared to calves fed colostrum from another dam or a pooled source. Although NAHMS did not capture the amount of time calves spent with their dam, the average time to colostrum feeding was significantly greater for calves fed colostrum from their own dam compared with other sources (data not shown), which supports this hypothesis. Although total colostral IgG given was not associated with O157, our interaction model showed that calves fed colostrum from their own first lactation dam were at increased risk of shedding O157, when also experiencing heat stress temperature and humidity levels. Previous studies have shown that first lactation dams experience a higher prevalence of O157 shedding, which tends to increase earlier in their lactation cycle and during warms months of the year (142, 211, 190). Coliforms have also been shown to exist in high quantities in raw colostrum (179). In summary, our data suggest that calves that spend a longer time with their dam before separation may have a greater exposure to O157 in the pen environment, and also within the colostrum they are administered. . Very few operations enrolled in the NAHMS Dairy 2014 study pasteurized their individual cow colostrum (2.6%), and it was not possible to correlate

pasteurization with a potential reduction in O157 presence in the current study. To understand whether preweaned calf O157 exposure occurs during colostrum feeding and/or during time periods spent with their dam, future studies that measure O157 in colostrum and the calving pen environment are warranted.

Calves that experienced high THI levels representative of heat stress were more likely to have O157 positive fecal samples. Previous studies have shown that O157 prevalence in dairy cows fluctuates during the year. A study by Williams et al. (219) showed that higher temperature and humidity was positively associated with increased shedding in dairy heifers on pasture. Other studies that looked at annual dairy farm prevalence of O157 showed levels to be highest during the warm late summer and early autumn months (204, 142, 190).

Sheng and colleagues (184) conducted a study that indicated that a change in seasonal exposure, rather than intrinsic animal change, caused warm-month increases in cattle shedding. Although O157 is capable of surviving in a multitude of environments, survival in manure is lower when temperatures are fluctuating (182). It is known that very high temperatures can stress O157 microbes, but protection within manure and lack of temperature variation during the warm months may equate to enhanced O157 survival. This may increase oral exposure in calves and lead to the increase in presence seen during heat stress. Our results also showed a slight reduction of O157 presence in calves experiencing cold stress, as only 2 individuals had O157 at THI values <50. Although O157 can survive in cold or frozen manure, exposure of O157 to calves may be reduced because of reduced environmental movement by moisture (119).

Changes in ambient temperature and humidity can impact not just environmental, but also calf physiological factors. Our results showed an interaction between THI and serum IgG value. Calves with a high IgG had lower odds of shedding during thermoneutral and heat stress THI, compared with calves with moderate or low IgG in the same groups. This suggests that the immune health of the calf is important to reduce the presence of the O157 pathogen, particularly at times

when calves may be physiologically predisposed to shedding due to high seasonal exposure. Because of this, strategies that mitigate the impact of seasonal increases in temperature (e.g modifying ventilation, providing reflective roofing, placing hutches in shaded areas) may be advantageous to reduce shedding in calves with poor passive transfer. It is also possible that heat stress of calves in-utero, or of dams during dry-off, impairs IgG absorption in the calf, resulting in lower levels of serum IgG. To avoid calves with poor passive transfer, ensuring the timely administration of sufficient amounts of high quality colostrum especially during times of heat stress may also be an advantageous strategy (186). More work is required to better understand if the association between THI value and O157 presence is due to environmental influence, animal health factors, or both.

During this study, serum IgG values were inversely associated with the presence of O157 in calves. Immune status may be important for O157 presence in calves because of their potential to have a clinical response to colonization. In contrast to adults, calves colonized with O157 can develop diarrhea and attaching and effacing lesions along the GI villous border (61, 62). However, there was no association between a history of any disease (including diarrheal disease) and O157 presence in the current study. Compared with O157 alone, literature suggests that loose stools are more commonly a result of other calf pathogens or other pathogens in concert with O157 (112, 10, 168). As incubation periods may influence when clinical disease is detected, a sensitivity analysis was performed that looked at any disease within 4 d of fecal sample collection (data not shown). We found no significant association between disease and the presence of O157.

It is difficult to directly tie serum IgG levels to O157 colonization in the absence of disease, as evidence indicates dams are not passing anti-O157 antibodies to calves in colostrum. Previous studies have supported this notion. In one study that looked at 1-wk-old calves at slaughter, serum IgG values were not associated with O157 carriage (104). Other results showed that colostrum-fed experimentally infected calves did not have greatly different clinical outcomes than colostrum-

deprived experimentally infected calves (61). In another study, newborn calves were fed hyperimmune colostrum from dams immunized with the O157 antigens γ -intimin C280 and EspB. These specific antigens were selected because they allow O157 to bind eukaryotic host cells (γ-intimin) and create pores in these cells post-binding (EspB), and adult cattle also respond serologically to these antigens during experimental infection (22). Although calves showed increased mucosal antibodies to both of these antigens at sites of O157 colonization (the recto-anal junction and ileum), fecal shedding of O157 was not reduced and enterocyte erosion still occurred after infection challenge at 10 wk of age (170). This body of evidence indicates that cattle do not pass anti-O157 antibodies in colostrum, and colostrum quantity and quality may not affect clinical or shedding outcomes in O157 infected calves. Interpreting changes in serum IgG alone should be done cautiously, because a strong and significant interaction was found with this variable and THI in the current models. However, it is plausible that the detected association between O157 presence and serum IgG is due to broadly enhanced health parameters related to improved immunity. This correlates with previous research showing that O157 is associated with the presence of other GI microbes (188). The immunity and GI health of calves is undoubtedly influenced by their GI microbiota. Work has shown that the presence of other gram-negative pathogens, reductions in microbial diversity, and the absence of key taxa may influence whether or not O157 is able to colonize cows post ingestion (223, 47, 192). Pathogen variables analyzed in the current study (i.e., *Salmonella* spp, *Cryptosporidium*, *Giardia*) were associated with O157 presence in initial screening but did not remain in the final multivariable model. This supports an idea that immunity and overall health of the calf GI tract are important factors that may influence the fecal presence of O157.Although the health of the GI tract cannot be understated, serum IgG values also represent systemic health parameters of calves. In the NAHMS Dairy 2014 Calf Component study, an increase in serum IgG was associated with both decreased calf morbidity and mortality (199). Taken

together, these outcomes suggest that adequate passive transfer is important to reduce the overall prevalence of disease and death in US dairy calves, but also may reduce the fecal presence of O157.

The current study had several limitations. The low prevalence of shedding in calves left little power to detect associations between O157 and several variables (housing ventilation, calving ease, milk delivery method, direct fed agents, history of disease and calf breed). Although consistency of weight measurements was a goal, the variable for average daily gain (initially associated with O157 shedding) may have been confounded by individual calves who had final weight measurements taken in days prior to, or after, the pre-weaning period. Further, studies to date have not assessed if O157 colonization in calves may occur during a specific time post-birth, similar to what is seen for K99 enterotoxigenic *E. coli* and other GI pathogens. The collection of only a single fecal sample from these calves between 2-4 weeks of age therefore may have limited the overall detection of O157 shedding.

Our results show that certain environmental, managerial, and calf health factors may impact the occurrence of preweaned calf O157 shedding. Compared to pooled sources, feeding colostrum from the calf's dam increased the likelihood of calf O157 presence. This was impacted by the level of THI, becausecalves that drank colostrum from their own first lactation dam during heat stress THI had an even greater risk of shedding. Additionally, a decrease in serum IgG concentration increased the likelihood of O157 presence at different THI levels. Specifically, calves with poor or moderate serum IgG had increased odds of shedding during thermoneutral or heat stress THI, compared to calves with excellent serum IgG. These outcomes highlight factors that influence the odds of O157 presence in preweaned calves, and may be used to guide future managerial strategies aimed at reducing O157 on dairy operations across the United States. On-farm management strategies that mitigate shedding in calves may just modestly reduce environmental contamination with the pathogen, however, because the prevalence of preweaned calves shedding O157 at any one

time is low. Regardless, the potential to limit the number of human infections due to direct animal and manure contact makes pursuing these strategies valuable.

CHAPTER 4: ASSOCIATIONS BETWEEN ESCHERICHIA COLI O157 SHEDDING AND THE FAECAL MICROBIOTA OF DAIRY COWS

Summary

Dairy cattle shed pathogenic *Escherichia coli* O157 (O157) in faeces, playing a role in human exposure. We aimed to measure faecal microbial communities in early lactation dairy cattle, and model outcomes with O157 shedding metrics. Daily faecal samples were collected from 40 cattle on two Colorado dairies for five consecutive days, and characterized for O157. 16S rRNA gene sequencing was used to measure sample-level microbial communities. Alpha-diversity metrics were associated with O157 outcomes via regression modeling, adjusting for confounders. Differential abundance of taxa were identified between O157(+) and O157(-) samples and between shedding days of individuals, using matched Wilcoxon rank-sum tests, zero-inflated Gaussian (ZIG) regression and negative binomial regression. After removing an outlier, multi-day and intermittently shedding cows had lower average richness compared to those that never shed. ZIG modeling revealed *Bacillus coagulans* to be more abundant in O157(-) samples, while *Moryella spp* were more abundant in O157(+) samples. Negative binomial models and Wilcoxon tests revealed no differentially abundant taxa between O157(+) vs O157(-) samples, or between shedding days of individuals. Microbial diversity and some taxa may be influenced by or affect O157 shedding by dairy cattle. If future work corroborates these findings, dairy cow microbial community changes may be used to guide on farm strategies that mitigate O157 dissemination, protecting the human food chain.

Background

Pathogenic *Escherichia coli* O157 (O157) is a food safety pathogen that causes an estimated 2,801,000 cases of human disease each year, some of which lead to haemolytic uraemic syndrome,

end-stage renal disease and death (132). Asymptomatically shedding ruminants are linked to a majority of these illness events (150). Results from national studies suggest that O157 is ubiquitous in the environment of US dairy farms, implicating dairy cattle as principle O157 reservoirs (216, 202). Despite current control measures (e.g. Food Safety and Inspection Service standard operating procedures for slaughter, laws for dairy pasteurization, United States Department of Agriculture codes for food preparation) O157 from dairies still regularly enters the human food supply (55). As the human food chain is vast and complicated, controlling O157 at the dairy cattle source is a logical strategy to reduce the burden of human disease.

In the last decade, there has been much interest in characterizing the pattern in which cows shed O157 in their faeces (185, 173). Although cows in US dairies are exposed to O157 in the surrounding environment, only a portion of animals will presumably become colonized with the bacteria after ingestion, and intermittently shed detectable faecal quantities (49). Cattle do not mount an immune response to O157, and lack formal clinical signs during carriage. This equates to an inability to discern shedding from non-shedding cattle without laboratory based bacterial enrichment and characterization of faecal samples (160, 213). These difficulties and the intermittent and variable nature of shedding have required strategic design for risk factor studies when looking at naturally colonized herds. Regardless, previous evaluation of O157 risk factors has revealed that environment and host-level factors, including humidity, temperature, parity, lactation days, prior treatment, disease status and the specific operation, influence a given dairy cow's risk of having an O157 shedding event (48, 142, 219, 211, 190). Given these findings, there remains a need to identify interventions and preventive strategies that may be implemented to reduce the number of shedding events in at-risk individuals.

Recently, next-generation sequencing of the 16S rRNA gene has led to an increase in the number of microbial community surveys (MCS) measuring gastrointestinal (GI) microbes, and their influence on pathogen and disease presence (45). Most of these studies define changes in microbial

community (beta diversity) among study environments via ordination methods, and tie these conclusions to environmental metadata. Studies of this nature in dairy cows have defined faecal communities and those within the rumen, focusing on community structure and its relation to animal growth, development and milk production (107, 110, 133, 65). Animal-level characteristics identified as risk factors for O157 shedding have been shown through diverse mammalian MCS studies to be correlated with changes in the GI microbiota (181, 24, 32, 43). Although the GI microbiota in dairy cows has been studied previously, it has not been thoroughly evaluated relative to O157 colonization and the cow life history features previously shown to impact shedding events.

Identifying specific taxa that are associated with shedding might discern whether the microbiota itself should be defined as a risk factor, or be considered as an O157 treatment target. In many MCS studies, including those of cows, relative changes in abundance of operational taxonomic units (OTUs) are measured after sequence counts are rarefied to the level of the lowest sample, commonly omitting large percentages of sequence data (140). With the progression of microbiome research has also come the development of analytic approaches that incorporate epidemiologic measures and metadata relating these communities to disease outcomes. It is also possible to measure sample-specific differential abundance of taxa without omitting sequence data, using robust normalization and modeling of data distributions (197). These different analytic approaches are advantageous when using MCS data to understand the associations between dairy cow microbial communities and the presence of O157. Given the considerations above, we postulated that the dairy cow GI microbiota would be associated with O157 colonization and shedding events. Specifically, we hypothesized that GI microbial diversity would be different between O157 shedding and non-shedding individuals, and that the presence of certain taxa would be associated with the presence of O157.

Methods

Study Population

Two commercial, freestall dairies (Farm 1, Farm 2) in northern Colorado and representing a combined population of 2,350 lactating cattle participated in this pilot study. Both dairies consisted of predominantly Holstein Friesian cows fed total mixed rations (TMR) formulated by the same nutritionist and with nutrient contents similar to one another. Forage was predominantly corn silage and alfalfa hay with additional carbohydrates provided in the form of hominy, brewer's grain, distiller's grain and wheat middlings. Each TMR was supplemented with vitamin and trace minerals designed for high-producing cows and Diamond V original line XPC supplement (Cedar Rapids, IA). Early lactation cows were targeted for the study, as they are a cohort at higher risk of O157 shedding, and this enabled our detection of both shedding and non-shedding individuals (141, 211).

Our sampling design was employed to assess the change in microbial communities between all O157-positive and -negative samples, and between shedding events and shedding patterns of individual cows. On sample day 1, all cows within the first 21 days postpartum on each dairy (n = 74) were sampled by obtaining >10 g faeces via rectal palpation. Samples were kept on ice prior to laboratory characterization. On-farm record systems were used to gather animal life-history features: lactation number (parity), days in milk (DIM), disease during current lactation and disease treatments during current lactation (Dairy Comp 305TM, Valley Agricultural Software, Tulare, CA; DHI-Plus, DHI Computing Service Inc., Provo, UT). Diseases recorded included retained placenta, mastitis, metritis, fever of unknown origin, pneumonia, enteric disease, dystocia (including severity), ketosis and lameness. Recorded treatments included penicillin, oxytetracycline, ceftiofur, flunixin meglumine, drench (oral electrolytes) and propylene glycol.

Laboratory enrichment and latex agglutination procedures described below were used to identify bacterial isolates containing the rfb (O157) gene in day 1 faecal samples. Based on those

preliminary results, 10 cows that shed O157 on day 1, and 10 cows that did not, were selected per farm and tested in the same fashion for five consecutive days (n = 40 cows, n = 200 samples).

Laboratory Characterization of O157

O157 isolation was performed via selective enrichment and detection 'gold standard' procedures with slight modification, as described previously (190, 191). Briefly, samples were mixed 1:10 in buffered peptone water (BPW) for both enrichment and initial direct plating. One hundred microlitre was spread plated on sorbitol MacConkey agar with BCIG (Oxoid Diagnostic Reagents, Basingstoke, Hampshire, UK) containing 1.25 mg potassium tellurite and 0.025 mg cefixime (CTSMAC- BCIG; HiMedia Laboratories, Mumbai, India). These direct plates were incubated at 37°C for 24 h (134). As pathogenic O157 has been known to adapt a sorbitol fermenting phenotype within 24 h, 'suspect' O157 colonies seen on plates throughout experiments were deemed as those with straw, grey, pink– grey or too small/difficult to characterize colony coloration (178, 7).

Direct plates containing ≥100 suspect colonies after incubation were chosen for latex agglutination. Three to 15 colonies per plate were tested for O157 by agglutination using an *E. coli* O157 latex kit, following manufacturer's instructions (Oxoid Diagnostic Reagents). Positive colonies were enriched in BPW for 6 h and stored at -80°C in 10% sterile glycerol. For PCR experiments, 10 L of thawed isolates were centrifuged at 5000 g for 5 min and re-suspended in 30 µL molecular grade water. A volume of 5 µL re-suspended culture template was placed into Qiagen Multiplex PCR Plus Kit reactions, according to the manufacturer's instructions (Qiagen, Venlo, the Netherlands). In brief, each 25 µL PCR reaction consisted of 12.5 µL master mix, 2.5 µL primer mixcontaining 0.2 $umol^{-1}$ each primer, 5 µL molecular grade water and 5 µL culture template. The thermal cycling conditions consisted of an initial incubation at 95°C for 5 min to activate the polymerase, followed by 40 cycles of amplification with denaturation at 95°C for 30 s, annealing at 57°C for 1 min and 30

s and extension at 72°C for 30 s, ending with a final extension at 68°C for 10 min. Thermocycling was performed using a Bio-Rad S1000 Thermal Cycler (Bio-Rad, Sydney, Australia). PCR products were analysed by agarose gel electrophoresis using a 2% agarose gel (Lonza Group Ltd., Basel, Switzerland).

The faecal dilution remaining after direct plating was enriched for 6 h at 37°C. Enriched samples not confirmed as O157 positive through direct plating were subjected to immunomagnetic separation (IMS) using Dynabeads anti-*E. coli* O157 and a BeadRetriever System (Life Technologies, Oslo, Norway). IMS samples were subsequently plated onto CT-SMAC-BCIG and incubated for 24 h at 37°C. Suspect colonies were confirmed by latex agglutination and PCR targeting O157 rfb (O-antigen) gene (213). All rfb-positive preliminary isolates were subsequently PCR tested for stx1, stx2 (shiga toxin genes) and eaeA (a variant of the eae intimin gene) using the same PCR protocol outlined above (primers; 160). O157 were not enumerated after IMS detection and PCR confirmation.

Samples were deemed to be enterohaemorrhagic *E. coli* (EHEC) O157 positive when isolates contained rfb, eaeA and any stx genes. Samples were deemed to be atypical enteropathogenic *E. coli* (aEPEC) O157 positive when isolates contained rfb and eaeA genes. For subsequent statistical analysis, all pathogenic strains (EHEC and aEPEC) were considered together and are hereafter referred to as 'O157'.

Library Preparation and 16s Sequencing

DNA library preparation and 16S rRNA sequencing were performed at the Argonne National Laboratory and followed Earth Microbiome Project suggested protocols (85, www.earthmicrobiome.org, accessed August 2015). Briefly, genomic DNA was extracted from faecal samples stored at -80°C using the PowerSoil DNA Isolation Kit (MoBio/Qiagen, Carlsbad, CA). To support pooling of all collected samples during a paired-end 2 x 150-base pair Illumina sequencing

run, the amplification primer set contained nine extra bases in the adapter region of the forward primer and a 12-base Golay barcode sequence in the reverse amplification primer (29). For amplification of the V4 hypervariable region of the 16S rRNA gene, 515F (5'-GTGYCAGCMGCCGCGGTAA-3') and 806R (5'-GGACTACNVGGGTWTCTAAT-3') primers with the defined barcodes and Illumina flow cell adapter sequences were used for amplification. Each 25 μL PCR reaction contained 12 μL of certified DNA-free water (MoBio/Qiagen), 10 μL of 5-Prime HotMasterMix (1x, Quanta Biosciences, Beverly, MA), 1 μL of forward primer (5 umol l^{-1} concentration, 200 pmol l^{-1} final), 1 uL Golay Barcode Tagged Reverse Primer (5 umol l^{-1} concentration, 200 pmol l^{-1} final) and 1 μL of genomic DNA. The PCR conditions were as follows: 94°C for 3 min, 35 cycles of 94°C for 45 s, 50°C for 60 s, 72°C for 90 s and a final single extension of 10 min at 72°C. Following PCR, amplicons were quantified using a Quant-iTTM PicoGreen_ dsDNA Assay Kit (Invitrogen, Waltham, MA). Based on different quantification values, volumes of each sample were pooled to achieve equal representation. Pools were cleaned using the UltraClean PCR Clean-Up Kit (MoBio/Qiagen) and quantified de novo. Pool molarity was determined, and the pool was then diluted to 2 nmol l^{-1}, before denaturing with NaOH. The sample was then repeat diluted to a final concentration of 2 pmol l^{-1}. A 30% PhiX spike was added, prior to loading on an Illumina HiSeq sequencer. Samples were sequenced using a 300-cycle V2 reagent cartridge (Illumina, San Diego, CA).

Sequence Pre-processing and OTU Selection

Sequence read quality was analysed via FastQC. Raw fastq files were demultiplexed with a maximum barcode error of 0 using default methods in QIIME (28). Reads not assigned via barcode were removed. QIIME was then used to preprocess reads to OTUs. During this approach, reads were clustered using USEARCH, and chimeras were removed using the UCHIME algorithm (ver. 9.0; 68). Reads were aligned to the Greengenes core alignment with 97% sequence identity using PyNAST

(64, 28). Taxonomy was assigned using the Ribosomal Database Project 2.2 classifier and the Greengenes 13.8 taxonomy reference (214, 138). A maximum likelihood approximated tree was built using FastTree 2.1.9 (166).

During this preprocessing, an open reference approach was utilized with the packages listed above (172). The open reference algorithm allowed for an initial clustering and closed reference sequence alignment, followed by de novo clustering of reads that failed to align. During the second clustering, representative sequences were created using the centroid of those that failed to align, and these were used during a closed reference picking process. OTUs that were only represented by a single read were discarded.

Nonmetric multi-dimensional scaling with a Bray–Curtis distance was used to ordinate OTUs from O157-positive and -negative samples in space. An unweighted unifrac distance was used to perform a principal coordinate analysis of OTUs from O157-positive and -negative samples. The relative abundance of taxa within each sample was measured using the R package Phyloseq (139).

Statistical Analysis

O157 categories were used to classify the presence of O157 at either the sample or the cow level. These categories are listed in Table 14 and include for the sample level: pathotype, day prior vs day of and day of vs day after. 'Pathotype' classified samples as containing aEPEC/ EHEC, or no pathogenic O157. For 'day prior vs day of' and 'day of vs day after' samples were paired to compare either the day prior to shedding or the day after shedding to the day of the shedding event in that individual. At the cow level, categories included pattern and ever vs never. 'Pattern' was a category that defined cows as multi- day (≥2 days shed), intermittent (1 day shed) or never shedders (0 days shed). 'Ever vs never' classified cows as having shed or not shed pathogenic O157 at least once during the study period. For 'pattern' and 'ever vs never', each cow was classified the same across days.

Table 14: Definition of O157 categories used to classify cows and samples for subsequent statistical analyses

Category Name	*Measure*	*Levels of variable*	*Analysis*
Pathotype	Sample	No O157 O157	Epidemiologic modeling, Differential abundance testing
Ever vs Never (Binary)	Cow	Never shed O157 Shed O157 at least 1 time	Epidemiologic modeling
Pattern (Polytomous)	Cow	Never shed O157 Shed O157 1 time (intermittent) Shed O157 > 2 times (multi-day)	Epidemiologic modeling
Day prior versus Day of (matched)	Sample	Day prior to a shedding day Shedding day	Differential abundance testing
Day of versus Day after (matched)	Sample	Shedding day Day after a shedding day	Differential abundance testing

Using these samples and cow defined categories, two different statistical approaches were used to analyse data from microbial count tables: epidemiologic modeling of microbial alpha diversity and measurement of taxa differential abundance. These approaches are described, in turn, below. As taxa within microbial communities are defined at the sample level, for cow-level categories (pattern, ever vs never) only the epidemiologic modeling of diversity measures was used. For matched samples (day of vs day after, day prior vs day of), only measuring differential abundance of taxa was used.

Relative abundance of OTUs aggregated to different taxa levels were quantified using Phyloseq. Alpha-diversity metrics were quantified using Phyloseq and Vegan, including observed richness (R), Shannon's index (S) and Pielou's measure of species evenness ((S)/Log(R)) (139, 153). Previous studies have shown that rarefying 16S rRNA count data prior to analysis is necessary to control for sequencing depth, but can negatively influence results (140). For analyses, data were normalized using cumulative sum scaling (CSS) in metagenomeSeq to correct for this bias (161).

Random effects, logistic and multinomial modeling were completed to measure the association between O157 categories and diversity measures. In brief, for the category 'pathotype',

random effects regression was used with sample-level diversity measures, and a random effect for cow. For the category 'ever vs never', a logistic regression model was used with diversity measures averaged across days for each individual cow. For the 'pattern' analysis, multinomial models were used with diversity measures averaged across days for each individual cow. Separate animal-level covariates that modify the risk of shedding in dairy cows have been reported previously (142, 211, 190). As these variables may cause, confound or mediate the association between alpha diversity and O157 shedding status, a directed acyclic graph was used to evaluate the role of these factors in the association between O157 categories and diversity (Figure 13 of supplement). The distribution of the variables parity, days in milk, disease status, farm and treatment were measured across O157 categories. These variables were assessed individually for associations with diversity, and those that met the screening criteria ($P \leq 0.2$) were included in the O157 models of diversity. Odds ratios and confidence intervals were calculated from model coefficients and standard errors using the interquartile range (IQR) of diversity values.

Using metagenomeSeq, taxa were aggregated to the family, genus and species levels (161). Aggregated tables were filtered to reflect taxa present in at least 25–30% of samples, and differential abundance at each taxonomic level was measured in turn. The ideal methodology used to test for differential abundance between non-paired 16S rRNA count tables is not agreed upon in the literature (140, 197, 215); therefore, two modeling approaches were used to assess changes in abundance for the 'pathotype' metric. In metagenomesSeq, zero-inflated Gaussian (ZIG) regression was used to evaluate changes between CSS normalized count tables (161). In DESeq2, negative binomial regression was used to evaluate the same changes in count tables internally normalized by calculating geometric means and median count ratios (128). For matched samples ('day prior vs day of' and 'day of vs day after'), aggregated count tables were normalized with CSS and compared using a Wilcoxon rank-sum test on table differences. After adjusting for multiple testing using the

Benjamini and Hochberg (12) correction, a P-value cutoff of <0.1 was used to detect differences. This P-value was selected due to the preliminary nature of the project, and relatively low power.

All procedures were approved by Colorado State University's Institutional Animal Care and Use Committee. All statistical analyses were performed in R ver. 3.3.2 or later.

Results

O157 Isolates and Initial Abundance Data

The study population consisted of 40 cows (20 per farm) that were sampled for five consecutive days. Of these individuals, 14 (35%) shed EHEC, 4 (10%) shed aEPEC and 31 (78%) shed rfb isolates without virulence genes. Samples taken on day 1 of the study were preliminarily assessed for rfb (38% shed rfb+ strains, 62% did not), prior to continued sampling of O157-positive and -negative cows on days 2–5. PCR characterization of isolates revealed many fewer cows harbouring O157 with virulence genes (EHEC and aEPEC) than those identified as rfb positive on day 1. Of the 200 study samples, 20 (10%) contained EHEC isolates, 4 (2%) contained aEPEC isolates and 67 (34%) contained rfb isolates that did not have virulence genes.

During 16S library preparation of faecal sample DNA, four samples either did not amplify well during PCR or did not sequence adequately, and were omitted from further analysis. Of these samples, three were from Farm 1 and one was from Farm 2. None of the omitted samples were identified as having aEPEC or EHEC. After sequencing and prior to OTU analysis in QIIME, all Illumina DNA sequence reads were seen to have an average quality PHRED score ≥30 via FASTQC. The data set revealed a total count of 12,225,598 reads, and 159,354 unique OTUs. Mean sequence read count per sample was 39,059.

Bacterial communities and member taxa were strikingly similar among cow samples, and did not cluster in ordination space based on O157 presence or absence (Figure 8). Measures related to animal life-history characteristics and other shedding categories also did not show clustering of

samples in space. Relative abundances of taxa at the family and phylum levels further revealed that communities with and without O157 were similar (Figure 8 a, b). Differences, where noted, were only slight. O157-positive and -negative bacterial communities contained 62 and 56% Firmicutes, 35 and 31% Bacteroidetes and 2.2 and 2.4% Tenericutes respectively. Actinobacteria was slightly higher in non- O157 samples (2.5 vs 1.9% in O157-positive samples) as were Spirochaetes (1.6 vs 1.0% in O157-positive samples). Proteobacterial percentages were 4.2% in non-O157 samples and 4.6% in O157 containing samples.

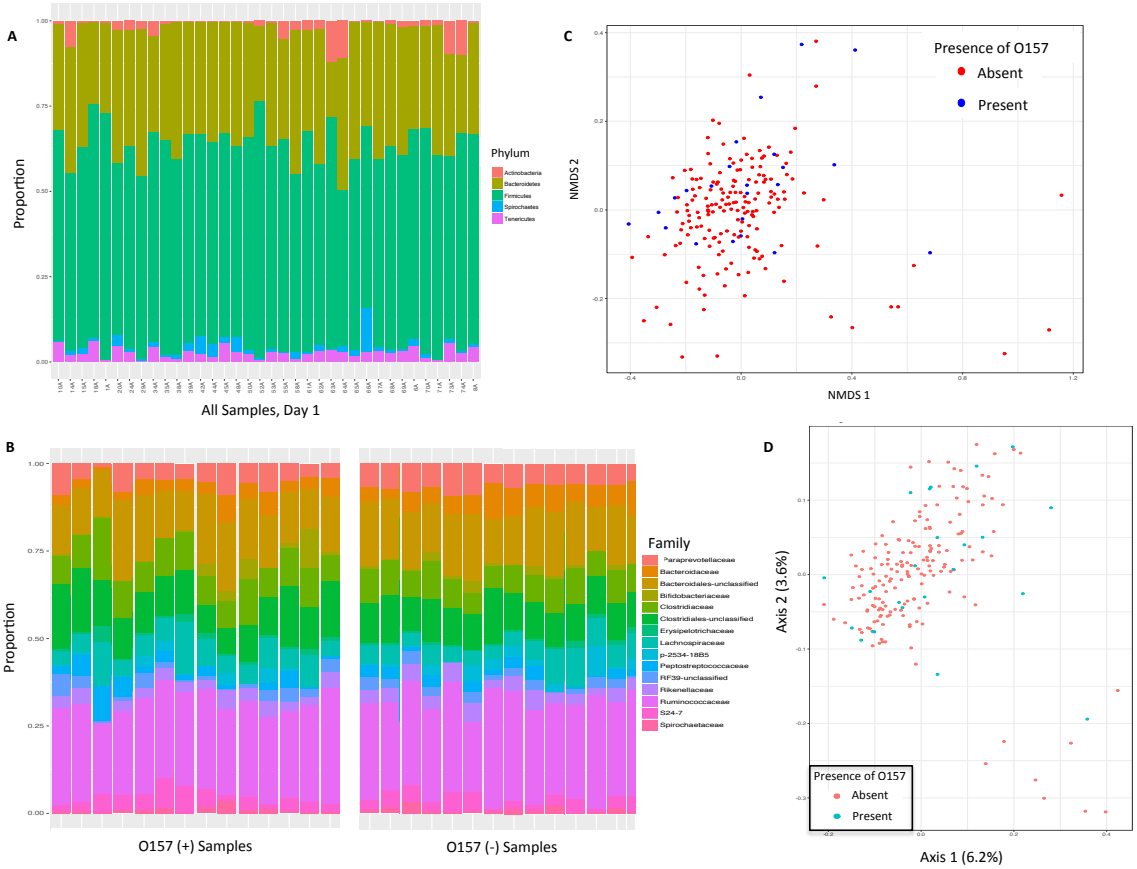

Figure 8: Microbial communities of dairy cow faecal samples were mostly uniform. Proportions of microbial community members at the phylum level among all cow samples taken on the initial day of the study (a) showed low variability. Proportions of community members at the family level, in a subset of O157-positive and -negative samples taken throughout the study (b; one sample per cow), additionally showed that taxa were not dependent on O157 pathogen status. Looking at communities via ordination with NMDS and Bray–Curtis (c) or PCoA with un-weighted Unifrac (d) revealed a lack of clustering in samples, and a spatial resolution that was not related to the presence or absence of O157

When looking at community composition at the family level, several taxa were seen to be slightly higher in abundance in non-O157 samples compared to O157 containing samples: Paraprevotellaceae (5.4 vs 4.7% in O157 positive), Bacteroidaceae (4.7 vs 3.6% in O157 positive), Bifidobacteraceae (2.4 vs 1.7% in O157 positive) and Spirochaetaceae (1.5 vs 1.0% in O157 positive). Meanwhile, Lachnospiraceae (6.0% in non-O157 vs 7.1% in O157 positive), Erysipelotrichaceae (0.9% in non-O157 vs 1.4% in O157 positive) and Peptostreptococcaceae (2.2% in non-O157 vs 2.8% in O157 positive) were slightly higher in O157-positive samples.

Epidemiologic Modeling of Microbial Diversity

Five different variables described previously to influence a dairy cow's risk of shedding pathogenic O157 (farm, DIM, parity, disease status and treatment status) may confound or mediate the association between microbial diversity and O157. Cows in the current study experienced ketosis (n = 5), metritis (n = 4), mastitis (n = 2), retained placenta (n = 2), lameness (n = 2), enteric disease (n = 2), fever of unknown origin (n = 1) and pneumonia (n = 1). The variable 'disease' was collapsed into a binary yes/no category due to sparseness of most disease types (no disease: n = 21; disease: n = 19). Treatments given to cows in the current study included penicillin (n = 3), oxytetracycline (n = 5), ceftiofur (n = 7), flunixin meglumine (n = 2), drench (n = 3) and propylene glycol (n = 4). Due to sparseness of treatment types, the variable 'treatment' was collapsed into a categorical variable with three levels. These levels represented cows that were never treated (n = 23), cows that were ever treated with any antibiotic (penicillin, oxytetracycline, ceftiofur; n = 11) and cows that were not treated with an antibiotic but were treated with a non-antibiotic agent (flunixin meglumine, propylene glycol, drench; n = 6). Enrolled cattle were parity 1 (n = 19), parity 2 (n = 7), parity 3 (n = 8), parity 4 (n = 3), parity 5 (n = 1) and parity 6 (n = 2). The variable parity was also collapsed into three categories due to sparseness of cells and biological reasoning (parity 1, n = 19; parity 2, n = 7; parity ≥3, n = 14). All of the variables known to influence shedding of O157

were grouped by O157 categories, described in Table 15. Of these variables, farm, parity and treatment differed by O157 'pathotype' status (Table 15; P ≤ 0.08). Treatment and parity had variable distributions across 'pattern' categories (P = 0.03 and P = 0.33 respectively) and treatment varied between categories of 'ever vs never' (P = 0.13).

Table 15: Herd and cow level descriptors by O157 pathotype and shedding status

		Pathotype, n = Sample (196)		*P-value*
		No O157	*aEPEC/EHEC*	
Farm*				
	1	83 (84%)	16 (16%)	
	2	90 (93%)	7 (7%)	0.08
DIM (median (IQR))§				
		9 (7.0)	8 (7.5)	0.752
Parityt				
	1	83(88%)	11 (12%)	
	2	27 (77%)	8 (23%)	
	≥ 3	63 (94%)	4 (6%)	0.047
Disease*				
	Absent	92 (90%)	10 (10%)	
	Present	81 (86%)	13 (14%)	0.51
Treatmentt				
	None	100 (90%)	11 (10%)	
	Antibiotic	53 (96%)	2 (4%)	
	Other	20 (66%)	10 (33%)	0.0004
		Ever/Never, n = Cow (40)		
		Never Shed	*Shed ≥ 1 time*	
Farm*				
	1	11 (55%)	9 (45%)	
	2	13 (65%)	7 (35%)	0.75
DIM (SD)₮				
		8.04 (4.54)	7.06 (4.54)	0.51
Parityt				
	1	12 (63%)	7 (37%)	
	2	3 (37%)	5 (63%	
	≥ 3	9 (69%)	4 (31%)	0.4
Disease*				
	Absent	12 (57%)	9 (43%)	
	Present	12 (63%)	7 (37%)	0.95
Treatmentt				

	None	13 (57%)	10 (43%)		
	Antibiotic	9 (82%)	2 (18%)		
	Other	2 (33%)	4 (66%)		0.13
		Pattern, n = Cow (40)			
		Never shed	*Intermittent*	*Multi-day*	
Farm[t]					
	1	11 (58%)	5 (26%)	4 (21%)	
	2	13 (65%)	7 (35%)	0 (0%)	0.18
DIM (SD)[¶]					
		8.04 (4.54)	6.33 (4.45)	9.25 (4.65)	0.437
Parity[t]					
	1	12 (63%)	4 (21%)	3 (16%)	
	2	3 (37%)	4 (50%)	1 (13%)	
	≥ 3	9 (69%)	4 (31%)	0 (0%)	0.33
Disease[t]					
	Absent	12 (57%)	8 (38%)	1 (5%)	
	Present	12 (63%)	4 (21%)	3 (16%)	0.36
Treatment[t]					
	None	13 (57%)	9 (39%)	1 (4%)	
	Antibiotic	9 (82%)	2 (18%)	0 (0%)	
	Other	2 (33%)	1 (17%)	3 (50%)	0.03

[*] Variables analyzed with chi-square test

[t] Variables analyzed with fisher's exact test

[Ŧ] Continuous variables assessed with student's t-test

[§] Continuous variables assessed with wilcoxon rank test

[¶] Continuous variables assessed with one-way anova

Richness, evenness and Shannon's diversity were computed for all samples using normalized 16S rRNA read counts. The IQR of these values by O157 categories are presented in Figure 9. In multinomial models, cows that were classified as multi-day shedders were seen to have lower average richness (Fig. 10; OR = 0.51; 95% CI: 0.41–0.64) compared to cows that never shed during the study period. Odds ratios and confidence intervals were non-significant for other diversity models comparing O157 categories.

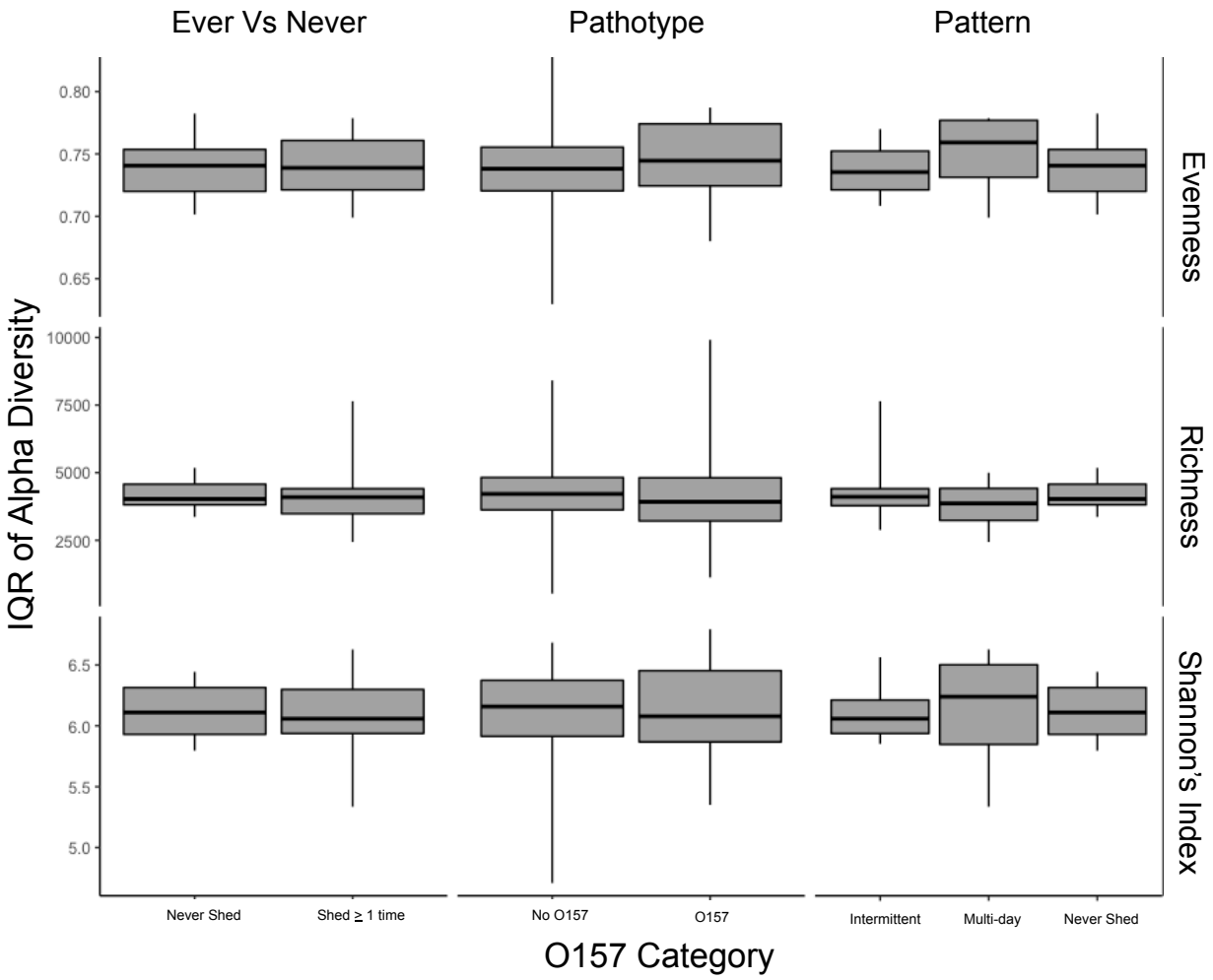

Figure 9: Interquartile range of normalized alpha-diversity measures (Shannon's, richness, evenness) defined by sample or cow O157 category. Boxes define values between the second and third quartiles, with the median value defined by a horizontal line. Whiskers define maximum and minimum diversity values respectively. For cow-level categories (ever vs never, pattern), each individual's diversity values were averaged.

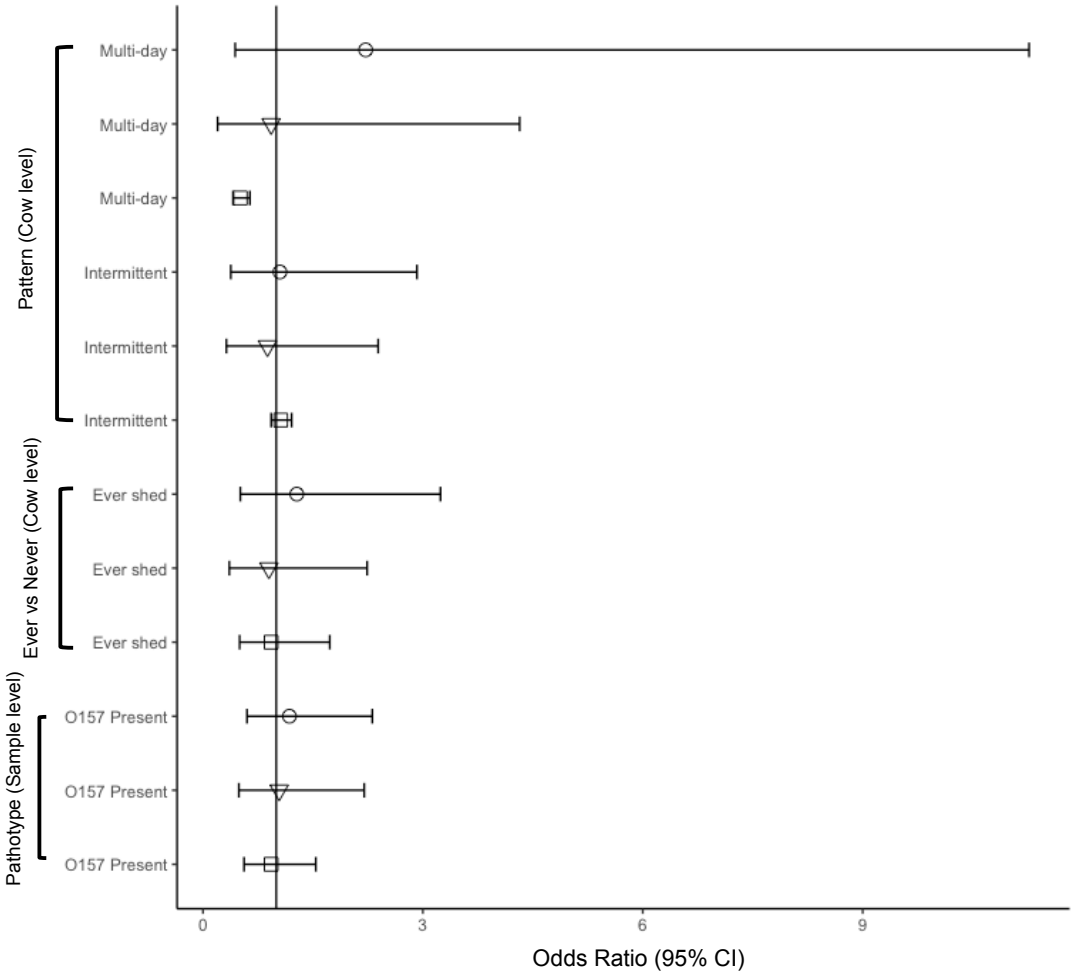

Figure 10: Associations between O157 metrics and evenness (O), Shannon's index (Δ) and richness ( ) measures using regression modelling. Odds ratios and confidence intervals were calculated from model coefficients using SE and interquartile range. For pattern and ever vs never, multinomial and logistic models were used to compare values to cows that never shed, with diversity measures averaged by cow. For pathotype, mixed logistic models with 'cow' as a random effect were used to compare O157-positive to -negative samples.

Variables that may confound or mediate the association between O157 categories and diversity measures (Figure 18 of supplement, Table 15) were assessed for individual associations with richness, Shannon's and evenness values (Table 19 of supplement). Variables with $P < 0.2$ model results were considered further in influencing the regression outcomes between O157 category and diversity seen in Figure 11. These variables included parity, farm and treatment, and

were the same variables seen to be different when distributed by O157 shedding metrics (Table 15).

These three variables were included in models of O157 and alpha diversity. Each variable was first assessed by itself with an O157 metric, and then in turn with other variables. For most models, the non-significant associations seen in the original crude values were not altered with adjustment (Table 20 of supplement). Cows with a multi-day shedding pattern still had lower average richness compared to never shedding cows, regardless of adjusting with any or all variables. However, when the variable parity was added to pattern models with any combination of farm, treatment or no other confounders, cows with an intermittent shedding pattern were seen to have significantly higher average richness compared to never shedding cows (Table 20 of supplement; parity added alone: OR = 1.23; 95% CI: 1.07–1.40). Due to the discrepant nature of these conclusions, raw values of richness for cows of all parity levels were evaluated. A single intermittently shedding, parity 1 cow had much higher richness values than nearly all other cows in the study, including on the day she shed O157. The average richness value for this cow on days 1–5 was 7644, compared to average values that fell in the range of 3000– 5000 for other cows. Given the relatively small sample size in this pilot project, it was hypothesized that this individual was driving the increase in average richness seen between intermittently and never shedding cows, when controlling for parity. A sensitivity analysis was performed removing this outlier (Figure 11). When re-running models of richness and O157 pattern adjusting for confounders, intermittently shedding cows were seen to have significantly lower average richness (OR = 0.568; 95% CI: 0.49–0.66) compared to never shedding cows. Similar to results when the single high-richness cow was included, multi-day shedding cows still had significantly lower average richness (OR = 0.415; 95% CI: 0.33–0.52) compared to never shedding cows.

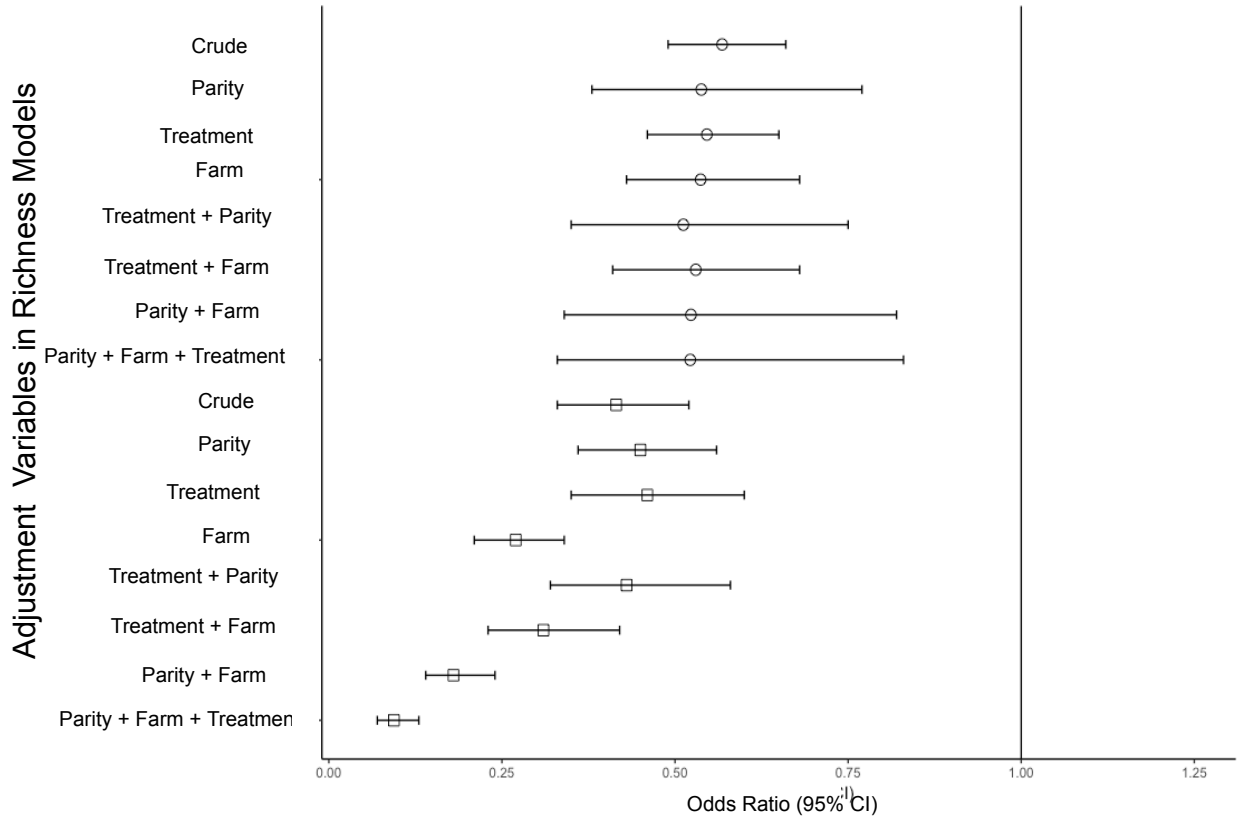

Figure 11: Crude and confounder adjusted estimates of association between O157 intermittent (O) and multi-day ( ) shedding pattern and richness, omitting a single high-richness cow. Odds ratios and confidence intervals were calculated from model coefficients using SE and interquartile range. Multinomial models were used to compare values of cows that were intermittent or multi-day shedders to cows that never shed O157.

Differential Abundance Testing

To examine taxa that may be driving associations seen during diversity modeling, differential abundance testing was performed using sample-level O157 metrics. Both ZIG and negative binomial regression were used to evaluate differences in taxa defined at the species, genus and family levels. When aggregating taxa to these different taxonomic levels and filtering to those seen in 25% of samples, there were 195 unique species, 590 unique genus and 290 unique family level taxa for analysis. Comparing samples with O157 to those without O157 and controlling for 'cow', one genus (*Moryella*; P = 0.04) was seen to be higher in abundance and one species (*Bacillus coagulans*; P = 0.09) was seen to be lower in abundance using ZIG models (Figure 12 A). Negative binomial models did not show similar conclusions; there were no family-, genus- or species-level

taxa seen to have differential abundance between O157-positive and -negative samples (Figure 12 B).

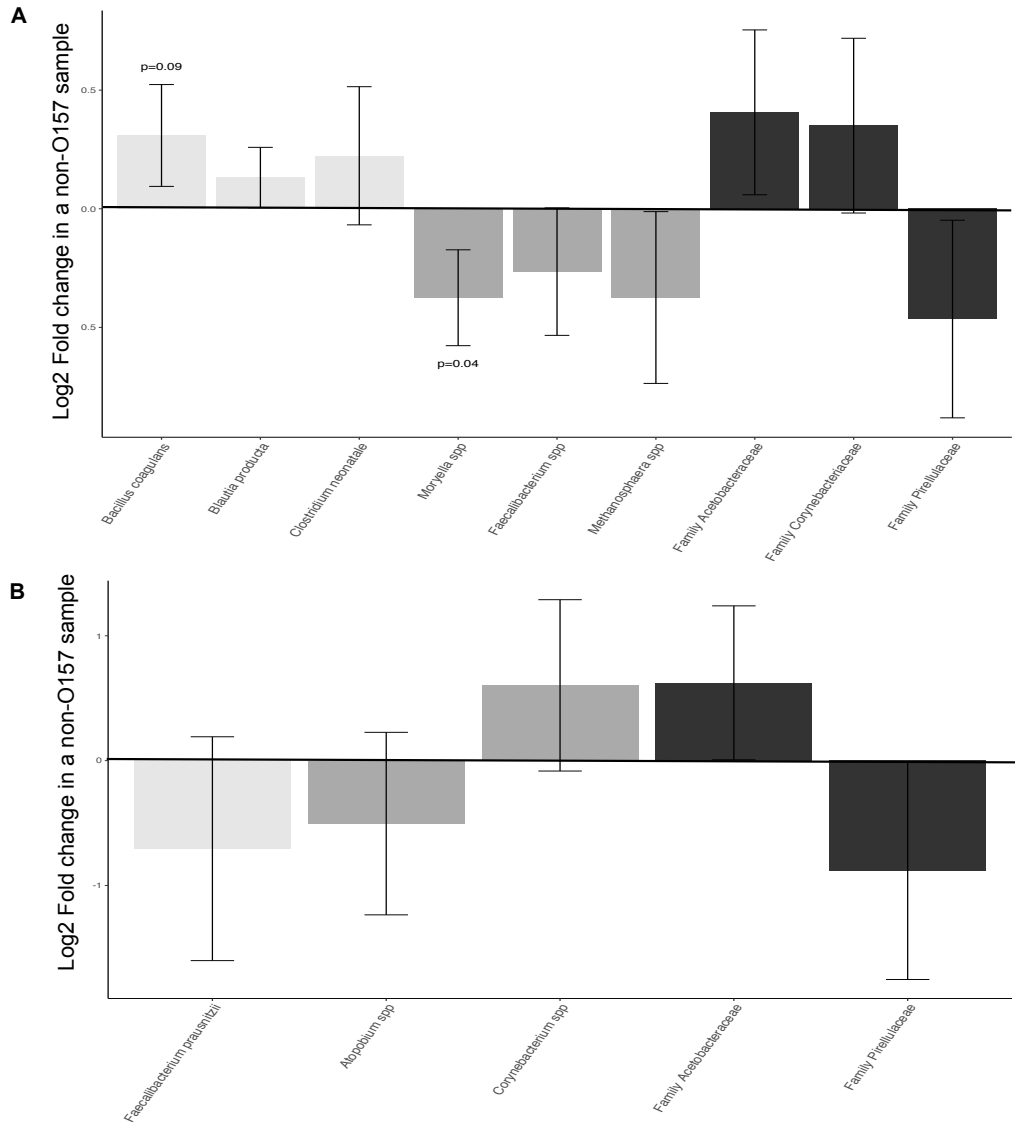

Figure 12: Log2 fold changes and 95% confidence intervals of selected species (light grey), genus (medium grey) and family (black)-level bacterial taxa in faecal samples that do not harbour pathogenic O157 compared to faecal samples that do. Using a zero-inflated Gaussian model (A), two taxa were seen to be significantly different from O157 samples after FDR correction at $P < 0.1$; *Bacillus coagulans* ($P = 0.09$) and *Moryella* ($P = 0.04$). When using a negative binomial model (B), no taxa were seen to be significantly different from O157-negative samples after FDR correction.

We compared day prior vs shedding day (n = 12 pairs), as well as shedding day vs day after shedding (n = 10 pairs), by running Wilcoxon rank-sum tests on the differences in family, genus and

species taxa abundance. After correcting for multiple testing, no specific taxa were observed to differ in days prior or after O157 shedding, when compared to the O157 shedding day.

Discussion

Faecal bacterial communities influence the health and disease status of animals, and have been studied specifically in cows in terms of animal growth and development. Although the roles these communities play in pathogen presence has been investigated in mouse and other comparative models, less is known about their influence on the risk of O157 shedding in dairy cows. The current pilot study aimed to elucidate bacterial community differences associated with O157 presence and with the shedding pattern cows display by tracking communities in a cohort of shedding and nonshedding early lactation cows over the course of 5 days.

Reported shedding prevalence of O157 in dairy cows has been highly variable (1–72.7%) (142, 39, 74). We estimated a prevalence of 10% EHEC-positive samples and 2% aEPEC-positive samples during our 5-day study period. Previous work on the farms under study showed annual early lactation prevalence of 3.7 and 3% for EHEC and aEPEC respectively (190). It is difficult to compare shedding rates between the current study and previous ones, as current samples were only obtained during the month of July and repeatedly from the same individuals. However, 35% of the early lactation cows shed EHEC at some point during a 5-day period. A study by Williams et al. (218) sampled nulliparous dairy heifers at pasture for two periods over 8 days, and each individual was seen to shed O157 at least once during this time frame. The current project sampled primiparous and multiparous cows, and demonstrated that high rates of O157 shedding may take place during the early stages of lactation.

In the current work, sorbitol and non-sorbitol fermenting strains were initially isolated, as O157 with both characteristics can result in bovine-associated human outbreaks (116). After PCR confirmation, non-eaeA+ strains were not considered further, as previous identification of similar

rfb+ strains from these herds showed them to be H12 or H45 (190). Although virulence of these strains is possible, they were not the focus of the study. The authors chose to look at both aEPEC and EHEC O157 strains together during downstream analyses. This was due in part to the low number of samples with each specific pathotype, which impeded individual (bacteria level) analyses. Furthermore, there is evidence for an evolutionary relationship between aEPEC and EHEC O157 and high sequence homology (52). Given this information, and the fact that stx genes reside on mobile genetic elements and harbour an ability to be 'shared' among genetically similar bacteria, the authors hypothesized that the strains likely share a niche in the bovine GI microbiota.

A few phylum-level community members slightly less abundant in O157-positive compared to -negative samples (Actinobacteria, Bacteroidetes, Spirochaetes) were likely offset by a higher proportion of Firmicutes that defined these pathogen-harbouring communities. Regardless, the early lactation cows of this study showed similarities in beta diversity when ordinating OTU counts, and in the proportions of different taxa present. This was regardless of life-history features and farm sampled. Contrary to this finding, a study by Chopyk et al. (47) showed that EHEC positive cattle hide communities clustered separately from those that did not contain EHEC. That study subtracted the OTUs found in faeces by those measured on hides, and is not directly comparable to results of this analysis. Faecal microbial communities in cows have been seen previously to become more similar as animals age, with calf communities being highly variable compared to those of adults or calf dams (223, 65). Other cow studies have shown that the feeding operation and type of ration may be more important than the history of the cow itself for causing rumen and faecal microbiota changes (183). The farms in the current study fed similar rations to their early lactation cattle. We propose that the lack of separation based on beta diversity and only slight taxa proportion differences seen by O157 status of these faecal samples is likely attributable to herd nutritional and managerial uniformity. These outcomes presented an ideal platform for measuring

O157-related changes in abundance of specific taxa within the samples, and modeling diversity measures with O157 shedding metrics.

When modeling bacterial diversity measures, cows that were classified as intermittent or multi-day O157 shedders were seen to have lower average richness than cows that never shed during the study period. These findings remained when controlling for life-history features of cows shown to influence shedding and faecal-level alpha diversity. Research has shown that the presence of different commensal microbes can reduce the proliferation of invading pathogens through competitive exclusion for space, nutrient depletion and creation of antimicrobial compounds. Results from studies looking at cattle environments suggest that O157 tends to thrive where there is less microbial diversity. For instance, O157 was seen to survive better in manure-amended soil that was autoclaved vs manure-amended soil that was not autoclaved (111). A study that used denaturing gradient gel electrophoresis to classify beef microbial communities containing STEC showed that Shannon's diversity was lower in STEC-positive compared to -negative samples (223). Likewise, bacterial diversity was lower in cattle hide samples that contained STEC compared to those that did not (47). Contrary to these results, a study by Xu and colleagues (221) revealed that 'super-shedding' ($\geq 10^4$ CFU per gram of faeces) feedlot steers had significantly higher richness compared to their O157-negative pen-mates. Changes in outcomes between that and the current study likely have to do with no cattle in the current study 'super shedding', sequencing depth for analysis, differences in study animals and sequencing approaches.

Shannon's diversity index takes into account both the richness of samples and the relative abundance of taxa. We speculate that Shannon's index was not associated with O157 categories, while richness was, because the proportions of taxa within samples were very similar. Furthermore, we were unable to establish temporality between O157 presence and richness due to the nature of microbial community data collection, which took place only during a 5-day window. This is perhaps a reason why analysis at the sample level (pathotype) did not reveal associations

between O157 presence and richness, while those at the cow level (pattern of shedding) did demonstrate these associations. Perhaps it is not the richness of the community during the shedding event, but the community immediately preceding, or preceding for a span of days, that influences eventual O157 colonization. Based on our conclusions, we can hypothesize that O157 is either able to gradually occupy an empty niche in less diverse bacterial communities, or acts to outcompete other bacteria and reduce or change the community diversity by its presence alone, prior to colonization. It remains possible that diversity outcomes are dependent on faecal quantities or exposure dose of the O157 pathogen, especially given the single outlier cow that shed while having a GI community with very high richness. More research is required to know if this is the case.

There is an interest in identifying specific faecal community members that are associated with the presence or absence of O157. Although previous microbiome studies have identified OTUs in communities that harbour STEC (221, 47), this study is the first of its kind to measure changes in community members aggregated to specific taxa levels (family, genus, species) via robust modeling of normalized sequence counts. Perhaps the most important change in this approach is that no sequencing data have been omitted, as is commonly done in studies that rarefy counts to the lowest sample.

Using ZIG modeling of counts normalized with CSS, we identified one genus, *Moryella*, more abundant in O157-positive samples. Currently, the genus *Moryella* contains only one defined species: *Moryella indoligenes* (31). This is an indole-producing, strictly anaerobic bacterium isolated from clinical abscesses in humans, presumably originating from within the human GI tract (31). Other information on this species and genus remain scant, although the bacteria seem to be closely related to species of the genus *Clostridium*, and are weakly saccharolytic. The production of indole by *Moryella spp*. may be a reason why it exists in concert with O157. Indole is produced by Gram-positive and -negative bacteria, including *E. coli* and many pathogens, and may act as an

interspecies signaling molecule (123). Other diverse activities of the molecule include mediating virulence genes of EPEC, modifying EHEC genetic activation to enhance attaching and effacing lesion formation and stabilizing *E. coli* plasmids to maintain high copy numbers (40, 96). In vitro the molecule can also decrease surface colonization and motility, and down-regulate genes that cause EHEC biofilm formation (9). The exact mechanism by which *Moryella spp.* associated indole may influence O157 presence in our study requires further research.

The ZIG model also showed that *B. coagulans* was more abundant in non-O157 containing communities. Ba*cillus coagulans* is a Gram-positive mobile facultative anaerobe that produces lactic acid and the bacteriocin-like inhibitory substance coagulin (101). This species is currently marketed as a beneficial probiotic for humans, dogs, pigs, horses and cows. The bacterium is used as an add-in to supplements that contain electrolytes or vitamins and minerals, and is advertised to support calf health and growth, promote optimal digestion and ease the transition to lactation in dairy cows. The herds of this study were not supplementing their stock with any product that may have contained *B. coagulans*, or other probiotics. Our differential abundance result is supported by a recent study that showed *B. coagulans* supplementation reducing the total faecal coliform count in rats (90). It is possible that the lactic acid producing properties of this species form the basis for its negative correlation with O157. Lactic acid, produced by strains like *B. coagulans* and also *Lactobacillus* species, has been shown to permeabilize the Gram-negative membrane of pathogenic bacteria like *Salmonella*, *E. coli* and *Pseudomonas aeruginosa* (2). Strains in the genus *Bifidobacterium* and *Lactobacillus* were seen to be antagonistic against pyelonephritic *E. coli* in vitro (100). In another study, the background flora in ground beef products, which contained a large percentage of lactic acid-producing bacteria, inhibited both the aerobic and anaerobic growth of O157 (212). *Lactobacillus spp*. supplementation has also been studied specifically in light of O157 shedding in cows. Strains in this genus were seen to reduce, but not eliminate, faecal shedding of O157 by beef feedlot steers in two separate studies (21, 165). The exact mechanism by which

bacteria like *Lactobacillus spp.* and *B. coagulans* are antagonistic towards O157 in the GI communities of cattle remains to be determined, but merits further investigation.

Because these changes in taxa were seen with the shedding event itself, we could not determine if they were prior to or a result of O157 presence. We sought to assess the differences in microbial community taxa of shedding animals on days preceding and following their shedding event, in an effort to establish temporality. Due to the pilot nature of the project and small number of matched sample pairs, the statistical rank test did not reveal significant changes in any taxa. Day-to-day changes in GI communities of animals with unchanged diet or environment are likely minimal. True assessment of differences in O157 between days would require a high-powered match test, so these results cannot conclusively determine if taxa differences exist before and after shedding events. When using a differential abundance modeling strategy with a negative binomial distribution, the taxa changes seen via ZIG modeling were not upheld. Although many of the same taxa were close to significance in both models, we cannot overlook the implication of this outcome. There is a lack of consensus in the research community regarding the best approaches to measure differential microbial abundance using 16S rRNA data (140, 197, 215). Both modeling approaches utilized in the current study presented different strengths. ZIG models have been shown to have higher false-positive rates, but concurrently higher detection sensitivity, compared to others. Given this pilot study comparison between relatively few (n = 40) individual cows, and similarities in overall community due to diet and environment, the authors chose to use the high-sensitivity ZIG model in concert with a more conservative negative binomial. Changes in outcome between models may also be due to differences in the normalization strategies; ZIG in metagenomeSeq utilizes a cumulative sum-scaling approach, while DeSeq2 uses an approach common to RNASeq analyses involving internal normalization with geometric means. To validate the O157-associated changes of taxa seen in this study future work should employ in vitro experiments, as well as additional MCS measure of shedding cows (beef and dairy) that are managed in different ways.

Mitigating shedding in cattle is one approach to reducing outbreaks of human O157 disease. Historically, understanding the dynamics of cow O157 colonization has proven difficult, and blocked the progression towards developing cow management or treatment strategies that reduce shedding. Understanding the cow microbiota and changes surrounding shedding events can theoretically lead to a better understanding of shedding dynamics, while also revealing potential treatment strategies. Results of the current work showed that regardless of life-history features known to influence shedding, lower average bacterial richness was associated with an intermittent or multi-day shedding pattern in dairy cows. The species *B. coagulans* was negatively associated with the presence of O157 in faecal samples, while the genus *Moryella* was positively associated with O157. These species may be used as targets for therapeutic agents (*Moryella*), or as therapeutic agents (*B. coagulans*). In vitro work is required to confirm and validate these taxa findings first, however, and investigate the mechanisms by which these bacteria influence O157. Taken together, results of this study suggest that the cow microbiome plays a role in O157 pathogen presence. Modulating microbial communities may be a useful approach to reduce O157 shedding in cows.

CONCLUSIONS AND NEXT STEPS

The two major aims of this dissertation were to define: 1) the prevalence and risk factors for O157 shedding in different age cohorts of dairy cattle on U.S operations and 2) the composition and structure of GI microbial communities found in naturally shedding and non-shedding cattle exposed to O157. By tying together results from the previous chapters, this chapter will first present a discussion of outcomes regarding the two major aims. This section will then reveal options for next steps to continue the experimental and analytical work that may support, or address the limitations of, those conclusions.

Finally, it will discuss findings of the dissertation work within the context of the broad goal: to collect information that aids in the development and implementation of pre-harvest O157 reduction procedures on dairy operations. This includes discussion of which O157 reduction strategies may be used, with a specific nod to their plausibility for being adopted within the U.S industry.

Aim 1:

Define prevalence and risk factors for O157 shedding in different age cohorts of dairy cattle on U.S operations

Aim 1 Summary of Findings

When longitudinally sampled over one year, early lactation cows on Colorado dairies were seen to shed not only EHEC, but also aEPEC O157. At the current time, few studies report on the prevalence of non-stx O157 shedding in cows, even though these strains cause clinical disease in humans and may acquire stx genes via phage transfer (55, 178). For both strains, however, prevalence of shedding was low (< 4 %) compared to many previous studies (99). These values add

to the current body of O157 knowledge, as studies to assess O157 prevalence on large conventional U.S. operations had not been completed prior to this work. Although there may be variability due to the operations and sampling, these outcomes suggest that conventional dry-lot dairy cows living in the High Plains or Rocky Mountain biome may only modestly contribute to O157 amplification. Risk factors for shedding in these cows were slightly different for the different strains of bacteria. Focusing on EHEC, a majority of O157 was shed from cows during summer and fall months (between June and November). This coincides with and supports prior work; seasonal prevalence increases have been seen in other dairy studies, and studies of beef cows. Increased risk for shedding was seen in cows that had been given antibiotics prior to sampling, and were generally earlier in their lactation cycle (i.e. fewer DIM). Risks associated with antibiotic use suggest that GI microbial health and diversity plays a role in O157 colonization. Because Chapter 2 focused on risk factors for cows within three weeks of calving, the significance that earlier DIM resulted in increased risk suggests that the immediately post-partum period may be a critical time for pathogen colonization. This is supported by the work of Chapter 4, where 35% of early lactation dairy cows shed O157 at least one time during a brief, 5-day window. Cows at earlier parity (heifers) were also seen as more likely to shed. The first lactation transition a dairy cow goes through represents a high-stress time period potentially exacerbated by negative energy balance, which is commonly associated with sub-clinical and clinical disease. Evidence of prior disease was not found to be a significant risk factor in this study. However, the power to detect disease associations was low, due to the low prevalence of O157 shedding and relatively low occurrence of post-partum disease in cows on these dairies. When treatment and disease were assessed as a single variable (e.g. classifying cows as having any treatment or disease during the lactation sampled), a significant association with O157 shedding was seen. This work has revealed, for the first time, which risk factors for O157 shedding exist in adult cows on large conventional U.S. operations.

When the calves of a select group of dams from the Colorado study were sampled during the pre-weaning period, pathogenic O157 was not found in fecal samples. Two different sampling techniques (fecal grab and recto-anal mucosal swab) were used in these calves to ensure a lack of O157 recovery was not due to experimental procedure. Recovery was the same across sampling techniques; no calf samples were ever positive for pathogenic O157. This remained true even when dams of these calves were positive during early lactation. This finding suggests that although calves may be exposed to O157 in the calving pen, other factors may contribute to continued shedding during the preweaning period

While this particular study did not detect shedding of preweaned calves, other studies have suggested that young calves are a high-risk cohort (62, 50). U.S. dairies recruited during the NAHMS 2014 study enrolled heifer calves on a monthly basis for the preweaned period, and single fecal samples taken from these individuals were characterized for O157. Calf shedding across U.S operations was low (2.5 %). To probe why differences were seen in national herds versus those in Colorado, risk factor analyses looked at managerial, environmental, and calf health and production variables collected by NAHMS. Results showed that U.S calves fed colostrum from their own dam were at increased risk to shed O157. Heat treatment of colostrum will kill any viable O157 that may be present, but the pathogen has been found in unpasteurized colostrum samples (86, 37). Time to colostrum administration was greater in calves given colostrum from their own dams, which suggests that time spent with the dam is associated with shedding. Study results further indicated that the passive transfer status of calves influenced shedding, but was affected by the temperature and humidity index (THI) calves experienced during the preweaning period. Calves at thermoneutral or heat stress THI were seen to shed O157, and were more likely to do so if they had poor or moderate passive transfer. They were unlikely to shed if passive transfer levels were considered excellent, regardless of THI. The calves from Colorado dairies studied in Chapter 3 were only assessed in months with commonly low THI (December - June), which may have played a role

in their O157 results. These outcomes have indicated for the first time that the passive immunity of young calves may impact their shedding of O157, and is likely influenced by the season of study.

Putting outcomes of adults and calves together, first parity and very early lactation cows seem more likely to shed O157. If these individuals aid in transmission of the pathogen to calves, a route of exposure may be via colostrum, and environmental peri-parturient contact (i.e. exposure to feces or hide in the calving pen). Successful transmission via colostrum route may be due to levels of the pathogen in colostrum versus the environment, or the route of exposure (more GI direct in the case of suckling calves). The work of chapters 2-4 also suggests that O157 shedding and exposures vary seasonally in early lactation cows and their calves, with important months spanning June to November. It seems likely that **season affects levels of O157 in the farm environment, and fecal-oral availability of the pathogen to herd members.** This hypothesis has been supported by results of many cattle O157 studies. Ecological modeling of O157 populations has shown that water trough, feedbunk and pen floor habitats can maintain viable populations of the bacteria, without their net growth in the cow GI tract (8). These results cause some researchers to argue that the 'true' reservoir for O157 exists outside of the cow GI, and merits further identification so it can be targeted for farm pathogen reduction (14). Finally, we postulate that adult cow and calf health parameters play a role in their shedding of O157. Risk factor outcomes suggest that a correlation between adult cow stress and shedding exists, even though 'history of disease' alone was not associated with O157. First parity and very early lactation cows experience nutritional, metabolic and other stress related to recent calving, the onset of milking activities, production of milk, and movement between pens (135). Parameters of stress that correlate with first parity and early lactation may represent a variable that is not easily quantifiable. Higher risk was seen in cows that had any disease or treatment listed for the current lactation, or a specific history of antibiotic use, which suggests that other unmeasured indicators of poor health may be evident in these shedding animals. In calves, GI parasites and pathogenic bacteria (*Giardia*,

Cryptosporidium, *Salmonella spp*.) were associated with O157 presence during univariable analyses. Poor passive transfer was also associated with shedding in calves. It is evident from other studies that the immunoglobulins transferred to calves in colostrum are not specific for the O157 bacteria, and poor passive transfer in general is associated with increased morbidity and mortality in these individuals. The significance of these variables suggests that the GI and overall immune status of calves is related to O157 colonization.

Collectively**,** these results reveal for the first time that **changes in cow stress level, immune status, and GI health may modulate the risk of shedding O157**. The influence of these factors may be subtle within an individual (adult cow or calf), but the overall impact on O157 amplification may be quite large when viewed across thousands of dairy herd members.

Aim 1 Next Steps

A) Use a daily sampling scheme to perform a longitudinal risk factor study of early lactation cows, on varied U.S operations.

The conclusions of this work have led to a hypothesis that cow stress and ill-health underlie the occurrence of O157 shedding. A cause-effect relationship has not been made, however, as models shown are simply associative. Further, sampling an individual cow one time may estimate herd trends for O157 shedding, but not shedding dynamics specific to an individual cow. To investigate whether O157 precedes or results from cow health events, a future study should sample and characterize cows for O157 shedding immediately post-calving, and on a daily basis through the first three weeks of lactation. Data regarding disease and treatment events should concurrently be gathered, so temporal relationships may be built between O157 and these outcomes. The work of chapter 4 revealed that findings on Colorado dairies regarding calf shedding were not similar across dairies within the U.S. The risk factor findings in early lactation cows shown in chapter 2 may also vary by operation at the national level. Therefore, it would be advantageous for the daily

sampling scheme proposed to occur on operations in Colorado and other geographically distinct locations. Conclusions of this proposed work may then support the risk factor outcomes from Colorado cows, and externally validate these findings on farms across the U.S. Outcomes of these studies may also elucidate whether factors of stress that vary by operation (i.e. pen density of cows, water soaking/misting and summer shade management) influence O157 shedding.

B) Quantify environmental sources of dairy O157 across seasons.

Multiple studies have shown a seasonal (warm month) increase in O157 shedding in cattle. Bacterial proliferation in environmental reservoirs may occur during these times, or reservoirs may increase in type and shift in terms of proximity to cattle pens. It is important to study the levels of O157 in these areas, as many varied locations (e.g. cattle bedding, water troughs, floors of common alleyways) may work in concert to ensure continued herd O157 exposure. Once identified, environmental reservoirs may also be the easiest to manage for reducing exposure of dairy cattle to O157 (67).

C) Investigate sources of O157 that allow transmission to pre-weaned calves

When taken together, risk factor results from adult cows in chapter 2 and calves in chapter 4 suggest that calves may be exposed to O157 by prolonged dam contact, or through colostrum. When tying shedding results from dams and calves to the actual occurrence of transmission, there is disconnect in the timing of the studies done in this dissertation. Measuring O157 exposures in the calving pen or during colostrum feeding, and then connecting with shedding later in the preweaning period is only appropriate if it can be shown that fecal-oral exposures in the surrounding environment are ongoing, and/or calves remain colonized with the bacteria for an extended period of time. Future work should study the length of shedding in calves, and their environmental and colostrum exposures to O157. Environmental quantification of O157 should

start with sampling the calving pen and then continue to sample calves, their food, and areas within hutches and pens throughout the preweaning time period.

D) Implement O157 reduction strategies in high-risk shedders

Although we cannot adequately predict shedding, targeting high-risk individuals for O157 reduction treatments may reduce individual cow amplification of the bacteria, overall herd transmission, and lessen levels of O157 in the dairy environment. Vaccination of cows was determined to be a cost-effective approach to prevent O157 illness in humans, and continues to be suggested as a viable approach for pre-harvest O157 control (220, 210). A vaccine that mitigates O157 shedding in an individual cow is the SRP subunit vaccine, which affects O157's ability to acquire iron (66, 94). In different trials with steers, this vaccine has been seen to reduce the prevalence of O157 shedding, the number of days cattle test culture positive for O157, and the overall concentration of O157 in fecal samples (196, 76). Different stressors related to lactation and variable GI microbiotas may influence SRP vaccine efficacy in early lactation dairy cows. A vaccine trial to determine anti-O157 SRP effects in these animals would include vaccinating or sham vaccinating two groups of early lactation heifers, and collecting fecal and environmental samples 2-3 times per week over a two-month period. Results would compare the number of positive fecal samples from vaccinated versus control animals, and the relative quantity of O157 (i.e IMS positive versus direct plating positive and 'super-shedding') seen within the samples of each group. Relative quantities of O157 in environmental samples should also be compared between vaccinated and control pens.

E) Perform a longitudinal study that attempts to associate O157 with changes in milk production

Because changes come with a cost, dairy owners are unlikely to alter farm management or begin cow treatments that limit O157 shedding unless the production of the farm or health of cows

will benefit. The work of this dissertation aimed to associate O157 shedding with cow factors (overall health, disease) that may influence these choices. Future studies that define whether or not production parameters are associated with O157 are still warranted, however. Studies would ideally measure milk yield, somatic cell counts, and fertility of shedding and non-shedding cows within naturally infected dairy herds. These production parameters would then be modeled with O157 outcomes similar to the approaches used in chapter 2.

Aim 2:

Define the composition and structure of GI microbial communities found in naturally shedding and non-shedding cattle exposed to O157

Aim 2 Summary of Findings

After measuring microbial communities in naturally infected shedding and non-shedding dairy cattle, we realized an association between low diversity and O157 presence. To the best of our knowledge, this is the first time GI microbial diversity has been measured in relation to the presence of the O157 pathogen in dairy cows. Because factors associated with O157 seen in Chapter 2 (DIM, parity, history of treatment) may be instigating this change in diversity rather than pathogen presence alone, these confounders were controlled for when modeling shedding outcomes. Specifically, richness (the total number of unique species present) was lower in cows shedding intermittently or for multiple days during the study period. Because antibiotics may reduce the overall diversity of gut flora, this outcome of low richness agrees with the Chapter 2 finding that antibiotic use was associated with EHEC O157. Pathogens found in NAHMS calves that were initially associated with shedding (*Cryptosporidium*, *Giardia*, *Salmonella*) may also be more likely to thrive in lower diversity microbial communities (60, 69). These findings suggest that **a**

healthy and diverse bovine GI microbiota may be advantageous to reduce O157 pathogen colonization.

Owing to common exposures, the overall structure of communities was similar between shedding and non-shedding herd members. However, our results suggest that specific bacteria are associated with the presence and absence of O157. *Moryella* spp., while not commonly discussed in the literature, may be a genus that prefers co-habitation with O157. It's possible that these bacteria may be used as an O157 detection target in the future. Meanwhile, *Bacillus coagulans* was seen in higher abundance in non-shedding animals. *B. coagulans* produces lactic acid, which may provide a basis for it's antagonism toward O157. Similar lactic acid producing strains (*Lactobacillus* spp., *Bifidobacteria* spp.) may disrupt O157 cell membranes, and have been used as probiotic treatments in shedding steers (2, 21, 165). These outcomes have associated specific taxa with the presence of the O157 pathogen for the first time. Taken as a whole, the work of this dissertation suggests that **the GI microbiota of dairy cows can be a focus when developing treatments that may prevent or reduce O157 shedding.** This focus may surround enriching diversity, or modulating microbial composition in a way that causes antagonism toward the O157 pathogen.

Aim 2 Next Steps

A) Validate and characterize the microbial physiology behind specific bacterial findings, using bench-top detection and co-habitation studies

After defining and modeling microbial taxa in shedding animals, *B. coagulans* and *Moryella* spp. were negatively and positively associated with O157, respectively. These bioinformatic findings should be corroborated by the detection of these specific bacterial species in the fecal samples used for sequencing. Bench-top culture, enrichment, and isolation strategies can be employed to accomplish this. If the findings from this bench-top work agree with those of 16s sequencing, in-vitro studies should be performed. Bovine cell lines can be grown with O157 and the

supernatants of *Moryella indoligenes* and *B. coagulans* cultures. Metabolomics may be employed to detect specific molecules in culture supernatants that potentiate results (e.g. changes to O157 colonization efficiency, O157 cell death, etc.). These same cell lines can also be colonized with O157 first, before placing whole cell cultures of *M. indoligenes* and *B. coagulans* into flasks. Growth parameters of O157 with and without these other bacterial agents can then be compared.

B) Repeat 16s rRNA studies with a larger (non-pilot) cohort of naturally infected dairy cattle and beef steers

Because the microbiota projects of this dissertation were pilot in nature (n = 40 individuals) outcomes should be corroborated in studies that sample a larger number of naturally shedding cows. As the microbiota of naturally shedding beef steers is likely different than that of dairy cows, these projects should include a scrutiny of the GI microbiota in both beef and dairy cohorts.

C) Measure changes in the microbiota of cows that shed stx-containing *E. coli* O26, O45, O103, O111, O121, and O145 (e.g 'the big 6')

The pathogenesis of human disease caused by non-O157 *E. coli* that contain stx genes is similar to that for stx-containing O157. Cows can be colonized with and shed these other 'big 6' stx-serotypes. It is plausible that these bacteria and O157 have a similar niche in the bovine microbiota. If this is true, microbiota outcomes in non-O157 stx shedding cows would be similar to those seen in this dissertation. Future probiotic prevention measures or treatments to reduce O157 shedding may then be used to reduce shedding of these other stx *E. coli*. Performing a study similar to that of Chapter 5, but assessing herds infected with non-O157 stx, would reveal these outcomes.

Overarching Goal:

Collect information that aids in the development and implementation of pre-harvest O157 reduction procedures on dairy operations.

Potential pre-harvest strategies

On conventional dairies, this dissertation work identified early lactation, first parity heifers as being at high-risk for shedding O157. These individuals may contribute more to environmental maintenance of the pathogen, and transmission to other herd members. To reduce O157 transmission, these individuals may be kept in pens separate from other herd members during their early DIM. Pen separation of this nature would be most feasible on operations that experience a high prevalence of O157 shedding. Strategies that aim to kill environmental O157 have been previously discussed in the literature, and can be used in this high-risk shedding pen. Environmental procedures to use when targeting this pen may include using acidic agents to regularly treat and flush water troughs (226), using sand bedding rather than bedding of sawdust and other materials (124), omitting flush-type manure removal systems and using scraping or other methods (81), and allowing intermittent pen-drying or 'resting' under sun exposure (187). These environmental treatments would also reduce worker risk of exposure to the pathogen. If transmission risk of O157 from a particular farm to humans intensifies (e.g. recent outbreaks have occurred in the same geographic area as the farm) animals in the high-risk shedding pen may be managed differently if sold for slaughter. For instance, animals may be sampled for O157 detection before sale, or hides may be treated with agents like cetylpyridinium chloride (19) prior to shipping. Previous work has shown that cattle trucks not sufficiently cleaned prior to picking up new shipments of animals may aid in O157 transmission (136). Designing a policy that ensures slaughter truck disinfection, especially after movement of high-risk shedding cattle from dairies, is another avenue that may be explored.

This dissertation also identified pre-weaned calves with poor passive transfer, exposed to temperature and humidity levels indicative of heat stress, as being more likely to shed O157. Improving passive transfer in calves may be advantageous to reduce O157 shedding. Testing levels of serum IgG a few hours post-colostrum ingestion and supplementing calves with low values may be advisable, but perhaps impossible in a dairy herd setting with multiple individuals. As an alternative approach, farmers currently not doing so can measure levels of IgG in colostrum prior to calf administration, using an inexpensive Brix refractometer (167). If the Brix percentage of a sample is low, workers may supplement the calf with either more of the colostrum (if deemed high enough quality) or a different aliquot of colostrum. If heat stress truly influences colonization, calves identified as having poor passive transfer may be moved to hutches with greater amounts of shade in the late summer and early autumn months. Operators may choose to rear these calves in hutches with different structure that can be positioned in a way that maximizes air movement based on wind patterns, or allows for minimal sun exposure in the late afternoon. If future studies corroborate the finding that these calves are exposed to O157 via colostrum or dam contact, dairies may also choose to begin colostrum pasteurization and suckling avoidance procedures. These strategies could target all post-partum dams and hand-fed colostrum aliquots, or just focus on instances where pathogen transmission is most likely (i.e. when a calf may suckle colostrum from first lactation dams). Although we still cannot predict calf O157 shedding, we can aim to reduce transmission to humans if farms develop policies that limit interaction between high-risk calves and individuals who may be visiting. These particular calves should also not be donated for use at farm animal expos or petting zoo events.

To limit disease transmission, worker movement from older to younger animals is currently not recommended on dairy operations. These policies may be further developed, with regard for reducing transmission of O157 from high-risk shedders to other herd members, or to dairy workers. Workers that interact with these animals may choose to wear specific personal protective

equipment (PPE; masks, coveralls that cinch at the wrist, gloves) to limit their own exposure when working with high-risk shedders. To reduce worker ability to transmit from one group to another, owners may choose to limit worker milking procedures during a shift that includes high-risk cohort interaction, or require a full clothing change before performing tasks with other age and lactation cohorts. These strategies would only be useful in cases where similar on-farm biosecurity practices are not currently used.

A project of this dissertation showed that *B. coagulans* is a possible anti-O157 probiotic agent. Probiotic supplementation is a mitigation avenue worthy of further exploration, as other probiotic treatments have proven successful at reducing O157 shedding by cows (194, 165, 198, 21). While the prospect of the *B. coagulans* target is exciting, more work must be done before developing this bacteria as a therapeutic agent, or even planning a clinical trial around its use. The finding of higher amounts of *B. coagulans* in non-shedding cows does not discern whether *B. coagulans* antagonizes O157, or O157 antagonizes *B. coagulans*. After this is established (Aim 2 Next Steps A), there are basic tenets that any probiotic agent must satisfy in order to be efficacious and safe (79). A specific strain of the bacteria must be isolated and validated to not contain known antimicrobial resistance (AMR) genes, or virulence factors seen in other pathogens. If the strain creates bacteriocins or regularly carries phages, these components should be screened for their influence on other commensal bovine bacteria. The strain must also be screened for both acid and bile resistance, to ensure it can reach the bovine lower GI tract and still remain viable. Before cows may be enrolled in a clinical trial with a screened strain of *B. coagulans*, the bacteria must be proven in efficacy studies to not affect growth or health of animals supplemented, and a modest duration of strain presence (shedding) must be shown after feasible quantities are fed (i.e amounts must not exceed what can be plausibly added within conventional rations). Although much continuing work is necessary, the utility of *B. coagulans* as a therapeutic agent should continue to be explored.

Although not the focus of this dissertation, vaccination to reduce O157 shedding has been explored in the literature, and commercially produced vaccines are now available (217, 3, 76, 148, 175, 127). Because cows do not mount an immune response to the normal components of O157, a vaccine engineered to create a bovine antibody response to specific O157 iron-acquisition proteins (siderophore receptor and porin proteins; SRP) has been manufactured (227). The SRP vaccine contains large amounts of both receptor and porin proteins isolated from bacterial culture, coupled with an adjuvant. When cattle mount an immune response to these O157 proteins, the bacteria is unable to sequester iron and will fail to thrive. At the current time, dairy operators vaccinate cows prior to calving to prevent post-partum diseases, and transfer antibodies for different pathogens to newborn calves via colostrum (163). Such vaccines include those that prevent *E. coli* mastitis (e.g. Enviracor™ *E. coli* J5), and others diseases associated with *Leptospira* spp and *Clostridia* spp (e.g. Leptoshield®, Ultrachoice™ 7-way *Clostridium*). Studies that assess the efficacy and side-effects of the SRP vaccine in peri-parturient dairy cows still need to be performed (Aim 1 Next Steps D above), but it's likely that a reduction in shedding would be achieved in these cohorts similar to what has been seen in beef herds (196, 76). If this outcome is supported, producers may choose to administer the O157 SRP vaccine during the same pre-partum window as the other vaccinations, to reduce post-partum cow shedding of O157.

Feasibility of implementation

Some O157 mitigation strategies, based on the outcomes of this dissertation, have now been proposed. A viable question remains regarding the feasibility of implementing these measures. For a farm operator, the benefit of any mitigation strategy must outweigh the costs of incorporating it. Based on our risk factor outcomes, we proposed that subclinical parameters of metabolic and hormonal stress are likely tied to shedding. These notions are still hypotheses, however, and subclinical variables of stress are difficult to both measure and place a monetary value on. At the

current time it is difficult to argue that mitigating O157 is explicitly advantageous to cows and farmers, because measurable changes in cow health and production have not been specifically linked with O157 shedding.

Some arguments can be made regarding the farm benefits of these measures, however. Separating first parity, early lactation heifers may ease operator management of health issues specifically seen in this cohort of animals. Heifers going through their first lactational transition can experience ketosis, complications from dystocia, retained fetal membranes, udder edema and subclinical laminitis. If animals at risk for these outcomes are already separated from herd members, screening for these diseases, and treating and monitoring recovery, may be easily facilitated. In calves separated for stress reduction and cooling, the same argument can be made for managing illness related to poor passive transfer. Historically, calves with poor passive transfer are at higher risk for death and most diseases. Keeping these cohorts in a specific area where they are easily monitored may ultimately be advantageous. O157 may be shed in concert with other GI pathogens, so separating these individuals may further reduce harmful bacterial and parasitic exposures to other preweaned calves in the herd. The average daily gain of calves was also slightly lower in shedding compared to non-shedding calves, so separating shedders may allow for easy nutritional supplementation, when warranted.

Moving forward

A focus for the future may be to recognize strategies that reduce O157 shedding, while conferring independent benefits to the farm. For instance, the use of probiotic agents that are antagonistic for O157 may result in measurable health and production changes (i. e increase in milk yield, reduction of GI disease incidence) in cows. Their use in that event would benefit both food safety and farm production. Regarding vaccination, it may be possible to manufacture a product that simultaneously creates anti-O157 antibodies and antibodies for other disease agents. Many

Clostridial vaccines given pre-partum contain both bacterins (attenuated pathogenic bacteria) and toxoids (disease agents secreted by pathogens). It may be possible to include purified O157 SRP proteins in these products, so immunized cattle mount immune responses against both *Clostridial* and *E. coli* O157 bacteria. Although not as commonly used, vaccines that target *Salmonella enterica* SRP proteins also exist, and may be similarly modified with O157 SRP. Administration of these modified vaccines would not greatly augment farm management if such products are already administered, and be cost effective if new vaccines are similar in price to those originally used.

A global approach to reduce O157 shedding may be to reduce animal stress (heat induced or otherwise) and improve cow and calf health. This is already a main goal for many dairy producers, and could be supported by future studies that measure cause and effect relationships between O157 and ill-health (Aim 1 Next Steps A). Reducing the number of disease events and treatments given, especially to cows in the calve-to-milk transition period, derives a production benefit to the operator. This benefit comes in the form of an overall reduction in the cost of managing disease, an increase in animal longevity, and higher milk production. This also applies for improving the immune status and health of calves, which would reduce overall morbidity and mortality during the pre-weaning period. Conclusively, approaches for future O157 mitigation may ultimately rely on increasing what is currently considered the 'standard' of good cow health and management on dairy operations.

As the work of this dissertation mainly focused on the health outcomes of shedding cows, O157 studies in the future should specifically aim to measure the association between O157 colonization and milk production parameters. Studies should also focus on changes in cow fertility, because low fertility remains a top reason for cow removal from conventional dairy herds in the U.S. If an association were made between O157 and fertility issues or low production, implementation of O157 mitigation strategies would become a priority for farmers.

Before successfully implementing O157 mitigation strategies on dairies, more information should be gathered. Regardless, the work of this dissertation has moved us toward one day realizing such activities, which would lessen dissemination of O157 to the human food chain and the occurrence of O157 infection in humans.

REFERENCES

1. Adamczyk K, Makulska J, Jagusiak W, Weglarz A (2017) Associations between strain, herd size, age at first calving, culling reason and lifetime performance characteristics in Holstein-Friesian cows. Animal 11: 327-334. doi: 10.1017/s1751731116001348

2. Alakomi HL, Skytta E, Saarela M, Mattila-Sandholm T, Latva-Kala K, Helander IM (2000) Lactic acid permeabilizes gram-negative bacteria by disrupting the outer membrane. Appl Environ Microbiol 66: 2001-2005.

3. Albanese A, Sacerdoti F, Seyahian EA, Amaral MM, Fiorentino G, Fernandez Brando R, Vilte DA, Mercado EC, Palermo MS, Cataldi A, Zotta E, Ibarra C (2018) Immunization of pregnant cows with Shiga toxin-2 induces high levels of specific colostral antibodies and lactoferrin able to neutralize E. coli O157:H7 pathogenicity. Vaccine 36: 1728-1735. doi: 10.1016/j.vaccine.2018.02.060

4. Alonso S, Mora A, Blanco M, Blanco JE, Dahbi G, Ferreiro MT, Lopez C, Alberghini L, Albonetti S, Echeita A, Trevisani M, Blanco J (2007) Fecal carriage of Escherichia coli O157:H7 and carcass contamination in cattle at slaughter in northern Italy. Int Microbiol 10: 109-116.

5. Asahara T, Shimizu K, Nomoto K, Hamabata T, Ozawa A, Takeda Y (2004) Probiotic bifidobacteria protect mice from lethal infection with Shiga toxin-producing Escherichia coli O157:H7. Infect Immun 72: 2240-2247.

6. Avery LM, Williams AP, Killham K, Jones DL (2008) Survival of Escherichia coli O157:H7 in waters from lakes, rivers, puddles and animal-drinking troughs. Sci Total Environ 389: 378-385. doi: 10.1016/j.scitotenv.2007.08.049

7. Ayaz ND, Gencay YE, Erol I (2014) Prevalence and molecular characterization of sorbitol fermenting and non-fermenting Escherichia coli O157:H7(+)/H7(-) isolated from cattle at slaughterhouse and slaughterhouse wastewater. Int J Food Microbiol 174: 31-38. doi: 10.1016/j.ijfoodmicro.2014.01.002

8. Ayscue P, Lanzas C, Ivanek R, Grohn YT (2009) Modeling on-farm Escherichia coli O157:H7 population dynamics. Foodborne Pathog Dis 6: 461-470. doi: 10.1089/fpd.2008.0235

9. Bansal T, Englert D, Lee J, Hegde M, Wood TK, Jayaraman A (2007) Differential effects of epinephrine, norepinephrine, and indole on Escherichia coli O157:H7 chemotaxis, colonization, and gene expression. Infect Immun 75: 4597-4607. doi: 10.1128/iai.00630-07

10. Bartels CJ, Holzhauer M, Jorritsma R, Swart WA, Lam TJ (2010) Prevalence, prediction and risk factors of enteropathogens in normal and non-normal faeces of young Dutch dairy calvesPrev Vet Med. 2009 Elsevier B.V, Netherlands, pp. 162-169

11. Benedict J (2011) *Poisoned: The True Story of the Deadly E. coli Outbreak That Changed the Way Americans Eat*. Inspire Books, Buena Vista, VA

12. Benjamini Y, Hochberg Y (1995) Controlling the False Discovery Rate: a practical and powerful approach to multiple testing. Journal of the Royal Statistical Society Series B (Methodological) 57: 289-300.

13. Berry ED, Wells JE (2010) Escherichia coli O157:H7: recent advances in research on occurrence, transmission, and control in cattle and the production environment. Adv Food Nutr Res 60: 67-117. doi: 10.1016/s1043-4526(10)60004-6

14. Besser TE, Schmidt CE, Shah DH, Shringi S (2014) "Preharvest" Food Safety for Escherichia coli O157 and Other Pathogenic Shiga Toxin-Producing Strains. Microbiol Spectr 2. doi: 10.1128/microbiolspec.EHEC-0021-2013

15. Best A, Clifford D, Crudgington B, Cooley WA, Nunez A, Carter B, Weyer U, Woodward MJ, La Ragione RM (2009) Intermittent Escherichia coli O157:H7 colonisation at the terminal rectum mucosa of conventionally-reared lambs. Vet Res 40: 9. doi: 10.1051/vetres:2008047

16. Bieber D, Ramer SW, Wu CY, Murray WJ, Tobe T, Fernandez R, Schoolnik GK (1998) Type IV pili, transient bacterial aggregates, and virulence of enteropathogenic Escherichia coli. Science 280: 2114-2118.

17. Boetel B (2017) In the cattle markets: dairy cattle impact on beef supplies. Dairy Herd Management, University of Wisconsin, River Falls.

18. Booher SL, Cornick NA, Moon HW (2002) Persistence of Escherichia coli O157:H7 in experimentally infected swine. Vet Microbiol 89: 69-81.

19. Bosilevac JM, Arthur TM, Wheeler TL, Shackelford SD, Rossman M, Reagan JO, Koohmaraie M (2004) Prevalence of Escherichia coli O157 and levels of aerobic bacteria and Enterobacteriaceae are reduced when hides are washed and treated with cetylpyridinium chloride at a commercial beef processing plant. J Food Prot 67: 646-650.

20. Boudeau J, Glasser AL, Julien S, Colombel JF, Darfeuille-Michaud A (2003) Inhibitory effect of probiotic Escherichia coli strain Nissle 1917 on adhesion to and invasion of intestinal epithelial cells by adherent-invasive E. coli strains isolated from patients with Crohn's disease. Aliment Pharmacol Ther 18: 45-56.

21. Brashears MM, Galyean ML, Loneragan GH, Mann JE, Killinger-Mann K (2003) Prevalence of Escherichia coli O157:H7 and performance by beef feedlot cattle given Lactobacillus direct-fed microbials. J Food Prot 66: 748-754.

22. Bretschneider G, Berberov EM, Moxley RA (2007) Isotype-specific antibody responses against Escherichia coli O157:H7 locus of enterocyte effacement proteins in adult beef cattle following experimental infection. Vet Immunol Immunopathol 118: 229-238. doi: 10.1016/j.vetimm.2007.06.005

23. Brown MH, Gill CO, Hollingsworth J, Nickelson R, 2nd, Seward S, Sheridan JJ, Stevenson T, Sumner JL, Theno DM, Usborne WR, Zink D (2000) The role of microbiological testing in systems for assuring the safety of beef. Int J Food Microbiol 62: 7-16.

24. Buffie CG, Pamer EG (2013) Microbiota-mediated colonization resistance against intestinal pathogens. Nat Rev Immunol 13: 790-801. doi: 10.1038/nri3535

25. Buffington CS, Collazo-Arocho A, Canton GH (1977) Black globe humidity index (BGHI) as a comfort equation for dairy cows. Am Soc Agric Eng, St. Joseph, pp. 19.

26. Callaway TR, Anderson RC, Edrington TS, Genovese KJ, Bischoff KM, Poole TL, Jung YS, Harvey RB, Nisbet DJ (2004) What are we doing about Escherichia coli O157:H7 in cattle? J Anim Sci 82 E-Suppl: E93-99. doi: 10.2527/2004.8213_supplE93x

27. Caporaso JG, Bittinger K, Bushman FD, DeSantis TZ, Andersen GL, Knight R (2010) PyNAST: a flexible tool for aligning sequences to a template alignment. Bioinformatics 26: 266-267. doi: 10.1093/bioinformatics/btp636

28. Caporaso JG, Kuczynski J, Stombaugh J, Bittinger K, Bushman FD, Costello EK, Fierer N, Peña AG, Goodrich JK, Gordon JI, Huttley GA, Kelley ST, Knights D, Koenig JE, Ley RE, Lozupone CA, McDonald D, Muegge BD, Pirrung M, Reeder J, Sevinsky JR, Turnbaugh PJ, Walters WA, Widmann J, Yatsunenko T, Zaneveld J, Knight R (2010) QIIME allows analysis of high-throughput community sequencing data. Nat Methods 7. doi: 10.1038/nmeth.f.303

29. Caporaso JG, Lauber CL, Walters WA, Berg-Lyons D, Huntley J, Fierer N, Owens SM, Betley J, Fraser L, Bauer M, Gormley N, Gilbert JA, Smith G, Knight R (2012) Ultra-high-throughput microbial community analysis on the Illumina HiSeq and MiSeq platforms. Isme j 6: 1621-1624. doi: 10.1038/ismej.2012.8

30. Caporaso JG, Lauber CL, Walters WA, Berg-Lyons D, Lozupone CA, Turnbaugh PJ, Fierer N, Knight R (2011) Global patterns of 16S rRNA diversity at a depth of millions of sequences per sample. Proc Natl Acad Sci U S A 108 Suppl 1: 4516-4522. doi: 10.1073/pnas.1000080107

31. Carlier JP, K'Ouas G, Han XY (2007) Moryella indoligenes gen. nov., sp. nov., an anaerobic bacterium isolated from clinical specimens. Int J Syst Evol Microbiol 57: 725-729. doi: 10.1099/ijs.0.64705-0

32. Carrothers JM, York MA, Brooker SL, Lackey KA, Williams JE, Shafii B, Price WJ, Settles ML, McGuire MA, McGuire MK (2015) Fecal Microbial Community Structure Is Stable over Time and Related to Variation in Macronutrient and Micronutrient Intakes in Lactating Women. J Nutr 145: 2379-2388. doi: 10.3945/jn.115.211110

33. CDC (1997) Isolation of E. coli O157:H7 from sporadic cases of hemorrhagic colitis--United States. 1982. MMWR Morb Mortal Wkly Rep 46: 700-704.

34. CDC (2006) Ongoing multistate outbreak of Escherichia coli serotype O157:H7 infections associated with consumption of fresh spinach--United States, September 2006. MMWR Morb Mortal Wkly Rep 55: 1045-1046.

35. CDC (2012) Outbreak of Shiga toxin-producing Escherichia coli O111 infections associated with a correctional facility dairy - Colorado, 2010. MMWR Morb Mortal Wkly Rep 61: 149-152.

36. CDC (2017) Notice to Readers: Special Podcast: "Defining Moments in MMWR History - E. coli O157:H7". MMWR Morb Mortal Wkly Rep 66: 566. doi: 10.15585/mmwr.mm6621a5

37. CDC (2008) Escherichia coli 0157:H7 infections in children associated with raw milk and raw colostrum from cows--California, 2006. MMWR Morb Mortal Wkly Rep 57: 625-628.

38. CDC (2012) National Shiga toxin-producing Escherichia coli (STEC) Surveillance Overview. In: Services, DoHaH (ed.). CDC, Atlanta, Georgia, pp. 4.

39. Cernicchiaro N, Pearl DL, McEwen SA, Harpster L, Homan HJ, Linz GM, Lejeune JT (2012) Association of wild bird density and farm management factors with the prevalence of E. coli O157 in dairy herds in Ohio (2007-2009). Zoonoses Public Health 59: 320-329. doi: 10.1111/j.1863-2378.2012.01457.x

40. Chant EL, Summers DK (2007) Indole signalling contributes to the stable maintenance of Escherichia coli multicopy plasmids. Mol Microbiol 63: 35-43. doi: 10.1111/j.1365-2958.2006.05481.x

41. Chauret C (2011) Survival and control of Escherichia coli O157:H7 in foods, beverages, soil and water. Virulence 2: 593-601. doi: 10.4161/viru.2.6.18423

42. Chekabab SM, Paquin-Veillette J, Dozois CM, Harel J (2013) The ecological habitat and transmission of Escherichia coli O157:H7. FEMS Microbiol Lett 341: 1-12. doi: 10.1111/1574-6968.12078

43. Chevalier C, Stojanovic O, Colin DJ, Suarez-Zamorano N, Tarallo V, Veyrat-Durebex C, Rigo D, Fabbiano S, Stevanovic A, Hagemann S, Montet X, Seimbille Y, Zamboni N, Hapfelmeier S, Trajkovski M (2015) Gut Microbiota Orchestrates Energy Homeostasis during Cold. Cell 163: 1360-1374. doi: 10.1016/j.cell.2015.11.004

44. Chiumia D, Chagunda MG, Macrae AI, Roberts DJ (2013) Predisposing factors for involuntary culling in Holstein-Friesian dairy cows. J Dairy Res 80: 45-50. doi: 10.1017/s002202991200060x

45. Cho I, Blaser MJ (2012) The Human Microbiome: at the interface of health and disease. Nat Rev Genet 13: 260-270. doi: 10.1038/nrg3182

46. Cho S, Fossler CP, Diez-Gonzalez F, Wells SJ, Hedberg CW, Kaneene JB, Ruegg PL, Warnick LD, Bender JB (2013) Herd-level risk factors associated with fecal shedding of Shiga toxin-encoding bacteria on dairy farms in Minnesota, USA. Can Vet J 54: 693-697.

47. Chopyk J, Moore RM, DiSpirito Z, Stromberg ZR, Lewis GL, Renter DG, Cernicchiaro N, Moxley RA, Wommack KE (2016) Presence of pathogenic Escherichia coli is correlated with bacterial community diversity and composition on pre-harvest cattle hides. Microbiome 4: 9. doi: 10.1186/s40168-016-0155-4

48. Cobbaut K, Berkvens D, Houf K, De Deken R, De Zutter L (2009) Escherichia coli O157 prevalence in different cattle farm types and identification of potential risk factors. J Food Prot 72: 1848-1853.

49. Cobbold RN, Hancock DD, Rice DH, Berg J, Stilborn R, Hovde CJ, Besser TE (2007) Rectoanal junction colonization of feedlot cattle by Escherichia coli O157:H7 and its association with supershedders and excretion dynamics. Appl Environ Microbiol 73: 1563-1568. doi: 10.1128/aem.01742-06

50. Cobbold R, Desmarchelier P (2000) A longitudinal study of Shiga-toxigenic Escherichia coli (STEC) prevalence in three Australian diary herds. Vet Microbiol 71: 125-137.

51. Collier RJ, Zimbelman RB, Rhoads RP, Rhoads ML, Baumgard LH (2011) A re-evaluation of the impact of temperature humidity index (THI) and black globe humidity index (BGHI) on milk production in high producing dairy cows. Western Dairy Management Conference, Reno, NV. USA, pp. 113 - 125.

52. Cookson AL, Cao M, Bennett J, Nicol C, Thomson-Carter F, Attwood GT (2010) Relationship between virulence gene profiles of atypical enteropathogenic Escherichia coli and Shiga toxin-producing E. coli isolates from cattle and sheep in New Zealand. Appl Environ Microbiol 76: 3744-3747. doi: 10.1128/aem.02528-09

53. Cornick NA, Booher SL, Moon HW (2002) Intimin facilitates colonization by Escherichia coli O157:H7 in adult ruminants. Infect Immun 70: 2704-2707.

54. Cray WC, Jr., Moon HW (1995) Experimental infection of calves and adult cattle with Escherichia coli O157:H7. Appl Environ Microbiol 61: 1586-1590.

55. Croxen MA, Law RJ, Scholz R, Keeney KM, Wlodarska M, Finlay BB (2013) Recent advances in understanding enteric pathogenic Escherichia coli. Clin Microbiol Rev 26: 822-880. doi: 10.1128/cmr.00022-13

56. Crump JA, Sulka AC, Langer AJ, Schaben C, Crielly AS, Gage R, Baysinger M, Moll M, Withers G, Toney DM, Hunter SB, Hoekstra RM, Wong SK, Griffin PM, Van Gilder TJ (2002) An outbreak of Escherichia coli O157:H7 infections among visitors to a dairy farm. N Engl J Med 347: 555-560. doi: 10.1056/NEJMoa020524

57. Cui J (2007) QIC program and model selection in GEE analyses. Stata Journal 7: 209-220.

58. Curran K, Heiman KE, Singh T, Doobovsky Z, Hensley J, Melius B, Burnworth L, Williams I, Nichols M (2015) Outbreak of Escherichia coli O157:H7 Infections Associated with Dairy Education Event Attendance - Whatcom County, Washington, 2015. MMWR Morb Mortal Wkly Rep 64: 1202-1203. doi: 10.15585/mmwr.mm6442a5

59. Davis TK, McKee R, Schnadower D, Tarr PI (2013) Treatment of Shiga toxin-producing Escherichia coli infections. Infect Dis Clin North Am 27: 577-597. doi: 10.1016/j.idc.2013.05.010

60. de la Fuente R, Luzon M, Ruiz-Santa-Quiteria JA, Garcia A, Cid D, Orden JA, Garcia S, Sanz R, Gomez-Bautista M (1999) Cryptosporidium and concurrent infections with other major enterophatogens in 1 to 30-day-old diarrheic dairy calves in central Spain. Vet Parasitol 80: 179-185.

61. Dean-Nystrom EA, Bosworth BT, Cray WC, Jr., Moon HW (1997) Pathogenicity of Escherichia coli O157:H7 in the intestines of neonatal calves. Infect Immun 65: 1842-1848.

62. Dean-Nystrom EA, Bosworth BT, Moon HW (1997) Pathogenesis of O157:H7 Escherichia coli infection in neonatal calves. Adv Exp Med Biol 412: 47-51.

63. Denny J, Bhat M, Eckmann K (2008) Outbreak of Escherichia coli O157:H7 associated with raw milk consumption in the Pacific Northwest. Foodborne Pathog Dis 5: 321-328. doi: 10.1089/fpd.2007.0072

64. DeSantis TZ, Hugenholtz P, Larsen N, Rojas M, Brodie EL, Keller K, Huber T, Dalevi D, Hu P, Andersen GL (2006) Greengenes, a chimera-checked 16S rRNA gene database and workbench compatible with ARB. Appl Environ Microbiol 72: 5069-5072. doi: 10.1128/aem.03006-05

65. Dill-McFarland, A. K, Breaker JD, Suen G (2017) Microbial succession in the gastrointestinal tract of dairy cows from 2 weeks to first lactation. Scientific Reports 7: 40864.

66. Doyle MP, Erickson MC (2012) Opportunities for mitigating pathogen contamination during on-farm food production. Int J Food Microbiol 152: 54-74. doi: 10.1016/j.ijfoodmicro.2011.02.037

67. Duffy G, McCabe E (2014) Veterinary Public Health Approach to Managing Pathogenic Verocytotoxigenic Escherichia coli in the Agri-Food Chain. Microbiol Spectr 2. doi: 10.1128/microbiolspec.EHEC-0023-2013

68. Edgar RC (2010) Search and clustering orders of magnitude faster than BLAST. Bioinformatics 26: 2460-2461. doi: 10.1093/bioinformatics/btq461

69. Edrington TS, Dowd SE, Farrow RF, Hagevoort GR, Callaway TR, Anderson RC, Nisbet DJ (2012) Development of colonic microflora as assessed by pyrosequencing in dairy calves fed waste milk. J Dairy Sci 95: 4519-4525. doi: 10.3168/jds.2011-5119

70. Ellis-Iversen J, Smith RP, Van Winden S, Paiba GA, Watson E, Snow LC, Cook AJ (2008) Farm practices to control E. coli O157 in young cattle--a randomised controlled trial. Vet Res 39: 3. doi: 10.1051/vetres:2007041

71. Fayer R, Trout JM, Graczyk TK, Lewis EJ (2000) Prevalence of Cryptosporidium, Giardia and Eimeria infections in post-weaned and adult cattle on three Maryland farms. Vet Parasitol 93: 103-112.

72. Feng PC, Keys C, Lacher DW, Beutin L, Bentancor A, Heuvelink A, Afset JE, Rumi V, Monday S (2012) Clonal relations of atypical enteropathogenic Escherichia coli O157:H16 strains isolated from various sources from several countries. FEMS Microbiol Lett 337: 126-131. doi: 10.1111/1574-6968.12017

73. Ferens WA, Hovde CJ (2011) Escherichia coli O157:H7: animal reservoir and sources of human infection. Foodborne Pathog Dis 8: 465-487. doi: 10.1089/fpd.2010.0673

74. Ferreira MR, Freitas Filho EG, Pinto JF, Dias M, Moreira CN (2014) Isolation, prevalence, and risk factors for infection by shiga toxin-producing Escherichia coli (STEC) in dairy cattle. Trop Anim Health Prod 46: 635-639. doi: 10.1007/s11250-014-0541-5

75. FDA (1998) Guide to Minimize Microbial Food Safety Hazards for Fresh Fruits and Vegetables. In: Services, USDoHaH (ed.). Center for Food Safety and Applied Nutrition, Washington, DC.

76. Fox JT, Thomson DU, Drouillard JS, Thornton AB, Burkhardt DT, Emery DA, Nagaraja TG (2009) Efficacy of Escherichia coli O157:H7 siderophore receptor/porin proteins-based vaccine in feedlot cattle naturally shedding E. coli O157. Foodborne Pathog Dis 6: 893-899. doi: 10.1089/fpd.2009.0336

77. Frenzen PD, Drake A, Angulo FJ (2005) Economic cost of illness due to Escherichia coli O157 infections in the United States. J Food Prot 68: 2623-2630.

78. Fuller CA, Pellino CA, Flagler MJ, Strasser JE, Weiss AA (2011) Shiga toxin subtypes display dramatic differences in potency. Infect Immun 79: 1329-1337. doi: 10.1128/iai.01182-10

79. Gaggia F, Mattarelli P, Biavati B (2010) Probiotics and prebiotics in animal feeding for safe food production. Int J Food Microbiol 141 Suppl 1: S15-28. doi: 10.1016/j.ijfoodmicro.2010.02.031

80. Garber LP, Wells SJ, Hancock DD, Doyle MP, Tuttle J, Shere JA, Zhao T (1995) Risk factors for fecal shedding of Escherichia coli O157:H7 in dairy calves. J Am Vet Med Assoc 207: 46-49.

81. Garber L, Wells S, Schroeder-Tucker L, Ferris K (1999) Factors associated with fecal shedding of verotoxin-producing Escherichia coli O157 on dairy farms. J Food Prot 62: 307-312.

82. Garcia-Ruiz A, Cole JB, VanRaden PM, Wiggans GR, Ruiz-Lopez FJ, Van Tassell CP (2016) Changes in genetic selection differentials and generation intervals in US Holstein dairy cattle as a result of genomic selection. Proc Natl Acad Sci U S A 113: E3995-4004. doi: 10.1073/pnas.1519061113

83. Gaulin C, Levac E, Ramsay D, Dion R, Ismail J, Gingras S, Lacroix C (2012) Escherichia coli O157:H7 outbreak linked to raw milk cheese in Quebec, Canada: use of exact probability calculation and casecase study approaches to foodborne outbreak investigation. J Food Prot 75: 812-818. doi: 10.4315/0362-028x.jfp-11-385

84. Gernand E, Konig S (2017) Genetic relationships among female fertility disorders, female fertility traits and productivity of Holstein dairy cows in the early lactation period. J Anim Breed Genet 134: 353-363. doi: 10.1111/jbg.12274

85. Gilbert JA, Jansson JK, Knight R (2014) The Earth Microbiome project: successes and aspirations. BMC Biol 12: 69. doi: 10.1186/s12915-014-0069-1

86. Godden S, McMartin S, Feirtag J, Stabel J, Bey R, Goyal S, Metzger L, Fetrow J, Wells S, Chester-Jones H (2006) Heat-treatment of bovine colostrum. II: effects of heating duration on pathogen viability and immunoglobulin G. J Dairy Sci 89: 3476-3483. doi: 10.3168/jds.S0022-0302(06)72386-4

87. Gopal PK, Prasad J, Smart J, Gill HS (2001) In vitro adherence properties of Lactobacillus rhamnosus DR20 and Bifidobacterium lactis DR10 strains and their antagonistic activity against an enterotoxigenic Escherichia coli. Int J Food Microbiol 67: 207-216.

88. Guh A, Phan Q, Nelson R, Purviance K, Milardo E, Kinney S, Mshar P, Kasacek W, Cartter M (2010) Outbreak of Escherichia coli O157 associated with raw milk, Connecticut, 2008. Clin Infect Dis 51: 1411-1417. doi: 10.1086/657304

89. Gyles CL (2007) Shiga toxin-producing Escherichia coli: an overview. J Anim Sci 85: E45-62. doi: 10.2527/jas.2006-508

90. Haldar L, Gandhi DN (2016) Effect of oral administration of Bacillus coagulans B37 and Bacillus pumilus B9 strains on fecal coliforms, Lactobacillus and Bacillus spp. in rat animal model. Vet World 9: 766-772. doi: 10.14202/vetworld.2016.766-772

91. Hazen TH, Sahl JW, Fraser CM, Donnenberg MS, Scheutz F, Rasko DA (2013) Draft Genome Sequences of Three O157 Enteropathogenic Escherichia coli Isolates. Genome Announc 1. doi: 10.1128/genomeA.00516-13

92. Heiman KE, Mody RK, Johnson SD, Griffin PM, Gould LH (2015) Escherichia coli O157 Outbreaks in the United States, 2003-2012. Emerg Infect Dis 21: 1293-1301. doi: 10.3201/eid2108.141364

93. Heinrichs AJ, Rogers GW, Cooper JB (1992) Predicting body weight and wither height in Holstein heifers using body measurements. J Dairy Sci 75: 3576-3581. doi: 10.3168/jds.S0022-0302(92)78134-X

94. Hermesch DR, Thomson DU, Loneragan GH, Renter DR, White BJ (2008) Effects of a commercially available vaccine against Salmonella enterica serotype Newport on milk production, somatic cell count, and shedding of Salmonella organisms in female dairy cattle with no clinical signs of salmonellosis. Am J Vet Res 69: 1229-1234. doi: 10.2460/ajvr.69.9.1229

95. Heuvelink AE, van den Biggelaar FL, Zwartkruis-Nahuis J, Herbes RG, Huyben R, Nagelkerke N, Melchers WJ, Monnens LA, de Boer E (1998) Occurrence of verocytotoxin-producing Escherichia coli O157 on Dutch dairy farms. J Clin Microbiol 36: 3480-3487.

96. Hirakawa H, Kodama T, Takumi-Kobayashi A, Honda T, Yamaguchi A (2009) Secreted indole serves as a signal for expression of type III secretion system translocators in enterohaemorrhagic Escherichia coli O157:H7. Microbiology 155: 541-550. doi: 10.1099/mic.0.020420-0

97. Honish L, Predy G, Hislop N, Chui L, Kowalewska-Grochowska K, Trottier L, Kreplin C, Zazulak I (2005) An outbreak of E. coli O157:H7 hemorrhagic colitis associated with unpasteurized gouda cheese. Can J Public Health 96: 182-184.

98. Hosmer DW, Lemeshow S (1992) Confidence interval estimation of interaction. Epidemiology 3: 452-456.

99. Hussein HS, Sakuma T (2005) Prevalence of shiga toxin-producing Escherichia coli in dairy cattle and their products. J Dairy Sci 88: 450-465.

100. Hutt P, Shchepetova J, Loivukene K, Kullisaar T, Mikelsaar M (2006) Antagonistic activity of probiotic lactobacilli and bifidobacteria against entero- and uropathogens. J Appl Microbiol 100: 1324-1332. doi: 10.1111/j.1365-2672.2006.02857.x

101. Hyronimus B, Le Marrec C, Urdaci MC (1998) Coagulin, a bacteriocin-like inhibitory substance produced by Bacillus coagulans I4. J Appl Microbiol 85: 42-50.

102. Ingram DT, Callahan MT, Ferguson S, Hoover DG, Chiu PC, Shelton DR, Millner PD, Camp MJ, Patel JR, Kniel KE, Sharma M (2012) Use of zero-valent iron biosand filters to reduce Escherichia coli O157:H12 in irrigation water applied to spinach plants in a field setting. J Appl Microbiol 112: 551-560. doi: 10.1111/j.1365-2672.2011.05217.x

103. Innocent GT, Mellor DJ, McEwen SA, Reilly WJ, Smallwood J, Locking ME, Shaw DJ, Michel P, Taylor DJ, Steele WB, Gunn GJ, Ternent HE, Woolhouse ME, Reid SW (2005) Spatial and temporal epidemiology of sporadic human cases of Escherichia coli O157 in Scotland, 1996-1999. Epidemiol Infect 133: 1033-1041. doi: 10.1017/s0950268805003687

104. Irshad H, Cookson AL, Hotter G, Besser TE, On SL, French NP (2012) Epidemiology of Shiga toxin-producing Escherichia coli O157 in very young calves in the North Island of New Zealand. N Z Vet J 60: 21-26. doi: 10.1080/00480169.2011.627063

105. Jackson EE, Erten ES, Maddi N, Graham TE, Larkin JW, Blodgett RJ, Schlesser JE, Reddy RM (2012) Detection and enumeration of four foodborne pathogens in raw commingled silo milk in the United States. J Food Prot 75: 1382-1393. doi: 10.4315/0362-028x.jfp-11-548

106. Jacob ME, Callaway TR, Nagaraja TG (2009) Dietary interactions and interventions affecting Escherichia coli O157 colonization and shedding in cattle. Foodborne Pathog Dis 6: 785-792. doi: 10.1089/fpd.2009.0306

107. Jami E, Mizrahi I (2012) Similarity of the ruminal bacteria across individual lactating cows. Anaerobe 18: 338-343. doi: 10.1016/j.anaerobe.2012.04.003

108. Jaros P, Cookson AL, Reynolds A, Prattley DJ, Campbell DM, Hathaway S, French NP (2016) Nationwide prevalence and risk factors for faecal carriage of Escherichia coli O157 and O26 in very young calves and adult cattle at slaughter in New Zealand. Epidemiol Infect 144: 1736-1747. doi: 10.1017/s0950268815003209

109. Jay MT, Cooley M, Carychao D, Wiscomb GW, Sweitzer RA, Crawford-Miksza L, Farrar JA, Lau DK, O'Connell J, Millington A, Asmundson RV, Atwill ER, Mandrell RE (2007) Escherichia coli O157:H7 in feral swine near spinach fields and cattle, central California coast. Emerg Infect Dis 13: 1908-1911. doi: 10.3201/eid1312.070763

110. Jewell KA, McCormick CA, Odt CL, Weimer PJ, Suen G (2015) Ruminal Bacterial Community Composition in Dairy Cows Is Dynamic over the Course of Two Lactations and Correlates with Feed Efficiency. Appl Environ Microbiol 81: 4697-4710. doi: 10.1128/aem.00720-15

111. Jiang X, Morgan J, Doyle MP (2002) Fate of Escherichia coli O157:H7 in manure-amended soil. Appl Environ Microbiol 68: 2605-2609.

112. Kang SJ, Ryu SJ, Chae JS, Eo SK, Woo GJ, Lee JH (2004) Occurrence and characteristics of enterohemorrhagic Escherichia coli O157 in calves associated with diarrhoea. Vet Microbiol 98: 323-328. doi: 10.1016/j.vetmic.2003.11.004

113. Karmali MA, Gannon V, Sargeant JM (2010) Verocytotoxin-producing Escherichia coli (VTEC). Vet Microbiol 140: 360-370. doi: 10.1016/j.vetmic.2009.04.011

114. Keene WE, Sazie E, Kok J, Rice DH, Hancock DD, Balan VK, Zhao T, Doyle MP (1997) An outbreak of Escherichia coli O157:H7 infections traced to jerky made from deer meat. Jama 277: 1229-1231.

115. Khaitsa ML, Bauer ML, Gibbs PS, Lardy GP, Doetkott D, Kegode RB (2005) Comparison of two sampling methods for Escherichia coli O157:H7 detection in feedlot cattle. J Food Prot 68: 1724-1728.

116. King LA, Loukiadis E, Mariani-Kurkdjian P, Haeghebaert S, Weill FX, Baliere C, Ganet S, Gouali M, Vaillant V, Pihier N, Callon H, Novo R, Gaillot O, Thevenot-Sergentet D, Bingen E, Chaud P, de Valk H (2014) Foodborne transmission of sorbitol-fermenting Escherichia coli O157:[H7] via ground beef: an outbreak in northern France, 2011. Clin Microbiol Infect 20: O1136-1144. doi: 10.1111/1469-0691.12736

117. Kruger A, Lucchesi PM (2015) Shiga toxins and stx phages: highly diverse entities. Microbiology 161: 451-462. doi: 10.1099/mic.0.000003

118. Kuczynski J, Stombaugh J, Walters WA, Gonzalez A, Caporaso JG, Knight R (2011) Using QIIME to analyze 16S rRNA gene sequences from microbial communities. Curr Protoc Bioinformatics Chapter 10: Unit 10.17. doi: 10.1002/0471250953.bi1007s36

119. Kudva IT, Blanch K, Hovde CJ (1998) Analysis of Escherichia coli O157:H7 Survival in Ovine or Bovine Manure and Manure Slurry. Appl Environ Microbiol 64: 3166-3174.

120. Kumar D, Thakur S (2018) Molecular Tools To Study Preharvest Food Safety Challenges. Microbiol Spectr 6. doi: 10.1128/microbiolspec.PFS-0019-2017

121. Lambertini E, Karns JS, Van Kessel JA, Cao H, Schukken YH, Wolfgang DR, Smith JM, Pradhan AK (2015) Dynamics of Escherichia coli virulence factors in dairy herds and farm environments from a longitudinal study in the United States. Appl Environ Microbiol. doi: 10.1128/aem.00465-15

122. Lammers GA, Mc CC, Jordan D, Ayton MS, Morris S, Patterson EI, Ward MP, Heller J (2015) Synchronization of E. coli O157 shedding in a grass-fed beef herd: a longitudinal study. Epidemiol Infect 143: 3244-3255. doi: 10.1017/s0950268815000588

123. Lee JH, Lee J (2010) Indole as an intercellular signal in microbial communities. FEMS Microbiol Rev 34: 426-444. doi: 10.1111/j.1574-6976.2009.00204.x

124. Lejeune JT, Kauffman MD (2005) Effect of sand and sawdust bedding materials on the fecal prevalence of Escherichia coli O157:H7 in dairy cows. Appl Environ Microbiol 71: 326-330. doi: 10.1128/aem.71.1.326-330.2005

125. Lim JY, Li J, Sheng H, Besser TE, Potter K, Hovde CJ (2007) Escherichia coli O157:H7 colonization at the rectoanal junction of long-duration culture-positive cattle. Appl Environ Microbiol 73: 1380-1382. doi: 10.1128/aem.02242-06

126. Lodemann U, Strahlendorf J, Schierack P, Klingspor S, Aschenbach JR, Martens H (2015) Effects of the Probiotic Enterococcus faecium and Pathogenic Escherichia coli Strains in a Pig and Human Epithelial Intestinal Cell Model. Scientifica (Cairo) 2015: 235184. doi: 10.1155/2015/235184

127. Loneragan GH, Brashears MM (2005) Pre-harvest interventions to reduce carriage of E. coli O157 by harvest-ready feedlot cattle. Meat Sci 71: 72-78. doi: 10.1016/j.meatsci.2005.04.005

128. Love MI, Huber W, Anders S (2014) Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. Genome Biol 15: 550. doi: 10.1186/s13059-014-0550-8

129. Low JC, McKendrick IJ, McKechnie C, Fenlon D, Naylor SW, Currie C, Smith DG, Allison L, Gally DL (2005) Rectal carriage of enterohemorrhagic Escherichia coli O157 in slaughtered cattle. Appl Environ Microbiol 71: 93-97. doi: 10.1128/aem.71.1.93-97.2005

130. Macdonald LE, Brett J, Kelton D, Majowicz SE, Snedeker K, Sargeant JM (2011) A systematic review and meta-analysis of the effects of pasteurization on milk vitamins, and evidence for raw milk consumption and other health-related outcomes. J Food Prot 74: 1814-1832. doi: 10.4315/0362-028x.jfp-10-269

131. Mack DR, Michail S, Wei S, McDougall L, Hollingsworth MA (1999) Probiotics inhibit enteropathogenic E. coli adherence in vitro by inducing intestinal mucin gene expression. Am J Physiol 276: G941-950.

132. Majowicz SE, Scallan E, Jones-Bitton A, Sargeant JM, Stapleton J, Angulo FJ, Yeung DH, Kirk MD (2014) Global Incidence of Human Shiga Toxin–Producing Escherichia coli Infections and Deaths: A Systematic Review and Knowledge Synthesis. Foodborne Pathog Dis 11: 447-455. doi: 10.1089/fpd.2013.1704

133. Mao S, Zhang M, Liu J, Zhu W (2015) Characterising the bacterial microbiota across the gastrointestinal tracts of dairy cattle: membership and potential function. Sci Rep 5: 16116. doi: 10.1038/srep16116

134. March SB, Ratnam S (1986) Sorbitol-MacConkey medium for detection of Escherichia coli O157:H7 associated with hemorrhagic colitis. J Clin Microbiol 23: 869-872.

135. Marek RE (2011) Dairy Cows: Nutrition, Fertility and Milk Production. Nova Science Publishers, Inc., New York

136. Mather AE, Reid SW, McEwen SA, Ternent HE, Reid-Smith RJ, Boerlin P, Taylor DJ, Steele WB, Gunn GJ, Mellor DJ (2008) Factors associated with cross-contamination of hides of Scottish cattle by Escherichia coli O157. Appl Environ Microbiol 74: 6313-6319. doi: 10.1128/aem.00770-08

137. Mathusa EC, Chen Y, Enache E, Hontz L (2010) Non-O157 Shiga toxin-producing Escherichia coli in foods. J Food Prot 73: 1721-1736.

138. McDonald D, Price MN, Goodrich J, Nawrocki EP, DeSantis TZ, Probst A, Andersen GL, Knight R, Hugenholtz P (2012) An improved Greengenes taxonomy with explicit ranks for ecological and evolutionary analyses of bacteria and archaea. ISME J 6. doi: 10.1038/ismej.2011.139

139. McMurdie PJ, Holmes S (2013) phyloseq: an R package for reproducible interactive analysis and graphics of microbiome census data. PLoS One 8: e61217. doi: 10.1371/journal.pone.0061217

140. McMurdie PJ, Holmes S (2014) Waste not, want not: why rarefying microbiome data is inadmissible. PLoS Comput Biol 10: e1003531. doi: 10.1371/journal.pcbi.1003531

141. Mechie SC, Chapman PA, Siddons CA (1997) A fifteen month study of Escherichia coli O157:H7 in a dairy herd. Epidemiol Infect 118: 17-25.

142. Menrath A, Wieler LH, Heidemanns K, Semmler T, Fruth A, Kemper N (2010) Shiga toxin producing Escherichia coli: identification of non-O157:H7-Super-Shedding cows and related risk factorsGut Pathog, England, pp. 7

143. Meyer-Broseta S, Bastian SN, Arne PD, Cerf O, Sanaa M (2001) Review of epidemiological surveys on the prevalence of contamination of healthy cattle with Escherichia coli serogroup O157:H7. Int J Hyg Environ Health 203: 347-361. doi: 10.1078/1438-4639-4410041

144. Michail S, Abernathy F (2002) Lactobacillus plantarum reduces the in vitro secretory response of intestinal epithelial cells to enteropathogenic Escherichia coli infection. J Pediatr Gastroenterol Nutr 35: 350-355.

145. Monaghan A, Byrne B, Fanning S, Sweeney T, McDowell D, Bolton DJ (2013) Serotypes and virulence profiles of atypical enteropathogenic Escherichia coli (EPEC) isolated from bovine farms and abattoirs. J Appl Microbiol 114: 595-603. doi: 10.1111/jam.12064

146. Montaldo HH, Castillo-Juarez H, Valencia-Posadas M, Cienfuegos-Rivas EG, Ruiz-Lopez FJ (2010) Genetic and environmental parameters for milk production, udder health, and fertility traits in Mexican Holstein cows. J Dairy Sci 93: 2168-2175. doi: 10.3168/jds.2009-2050

147. Mora A, Blanco M, Yamamoto D, Dahbi G, Blanco JE, Lopez C, Alonso MP, Vieira MA, Hernandes RT, Abe CM, Piazza RM, Lacher DW, Elias WP, Gomes TA, Blanco J (2009) HeLa-cell adherence patterns and actin aggregation of enteropathogenic Escherichia coli (EPEC) and Shiga-toxin-producing E. coli (STEC) strains carrying different eae and tir alleles. Int Microbiol 12: 243-251.

148. Moxley RA, Smith DR, Luebbe M, Erickson GE, Klopfenstein TJ, Rogan D (2009) Escherichia coli O157:H7 vaccine dose-effect in feedlot cattle. Foodborne Pathog Dis 6: 879-884. doi: 10.1089/fpd.2009.0297

149. Naylor SW, Low JC, Besser TE, Mahajan A, Gunn GJ, Pearce MC, McKendrick IJ, Smith DG, Gally DL (2003) Lymphoid follicle-dense mucosa at the terminal rectum is the principal site of colonization of enterohemorrhagic Escherichia coli O157:H7 in the bovine host. Infect Immun 71: 1505-1512.

150. Nguyen Y, Sperandio V (2012) Enterohemorrhagic E. coli (EHEC) pathogenesis. Front Cell Infect Microbiol 2. doi: 10.3389/fcimb.2012.00090

151. Nielsen EM, Tegtmeier C, Andersen HJ, Gronbaek C, Andersen JS (2002) Influence of age, sex and herd characteristics on the occurrence of Verocytotoxin-producing Escherichia coli O157 in Danish dairy farms. Vet Microbiol 88: 245-257.

152. Niu YD, Xu Y, McAllister TA, Rozema EA, Stephens TP, Bach SJ, Johnson RP, Stanford K (2008) Comparison of fecal versus rectoanal mucosal swab sampling for detecting Escherichia coli O157:H7 in experimentally inoculated cattle used in assessing bacteriophage as a mitigation strategy. J Food Prot 71: 691-698.

153. Oksanen J, Blanchet FG, Friendly M, Kindt R, Legendre P, McGlinn D, Minhcin PR, O'Hara RB, Simpson GL, Solymos P, Stevens MHH, Szoecs E, Wagner H (2017) vegan: Community Ecology Package. R package Version 2.4-3., https://CRAN.R-project.org/package=vegan.

154. Omisakin F, MacRae M, Ogden ID, Strachan NJ (2003) Concentration and prevalence of Escherichia coli O157 in cattle feces at slaughter. Appl Environ Microbiol 69: 2444-2447.

155. Paiba GA, Wilesmith JW, Evans SJ, Pascoe SJ, Smith RP, Kidd SA, Ryan JB, McLaren IM, Chappell SA, Willshaw GA, Cheasty T, French NP, Jones TW, Buchanan HF, Challoner DJ, Colloff AD, Cranwell MP, Daniel RG, Davies IH, Duff JP, Hogg RA, Kirby FD, Millar MF, Monies RJ, Nicholls MJ, Payne JH (2003) Prevalence of faecal excretion of verocytotoxigenic Escherichia coli O157 in cattle in England and Wales. Vet Rec 153: 347-353.

156. Painter JA, Hoekstra RM, Ayers T, Tauxe RV, Braden CR, Angulo FJ, Griffin PM (2013) Attribution of foodborne illnesses, hospitalizations, and deaths to food commodities by using outbreak data, United States, 1998-2008. Emerg Infect Dis 19: 407-415. doi: 10.3201/eid1903.111866

157. Parassol N, Freitas M, Thoreux K, Dalmasso G, Bourdet-Sicard R, Rampal P (2005) Lactobacillus casei DN-114 001 inhibits the increase in paracellular permeability of enteropathogenic Escherichia coli-infected T84 cells. Res Microbiol 156: 256-262. doi: 10.1016/j.resmic.2004.09.013

158. Park JH, Oh SS, Oh KH, Shin J, Jang EJ, Jun BY, Youn SK, Cho SH (2014) Diarrheal outbreak caused by atypical enteropathogenic Escherichia coli O157:H45 in South Korea. Foodborne Pathog Dis 11: 775-781. doi: 10.1089/fpd.2014.1754

159. Parker CT, Kyle JL, Huynh S, Carter MQ, Brandl MT, Mandrell RE (2012) Distinct transcriptional profiles and phenotypes exhibited by Escherichia coli O157:H7 isolates related to the 2006 spinach-associated outbreak. Appl Environ Microbiol 78: 455-463. doi: 10.1128/aem.06251-11

160. Paton AW, Paton JC (1998) Detection and characterization of Shiga toxigenic Escherichia coli by using multiplex PCR assays for stx1, stx2, eaeA, enterohemorrhagic E. coli hlyA, rfbO111, and rfbO157. J Clin Microbiol 36: 598-602.

161. Paulson JN, Stine OC, Bravo HC, Pop M (2013) Differential abundance analysis for microbial marker-gene surveys. Nat Methods 10: 1200-1202. doi: 10.1038/nmeth.2658

162. Pearce MC, Jenkins C, Vali L, Smith AW, Knight HI, Cheasty T, Smith HR, Gunn GJ, Woolhouse ME, Amyes SG, Frankel G (2004) Temporal shedding patterns and virulence factors of Escherichia coli serogroups O26, O103, O111, O145, and O157 in a cohort of beef calves and their dams. Appl Environ Microbiol 70: 1708-1716.

163. Penn Vet Learning Center (2018) Diary Field Service; Vaccination Protocols. Field Service, New Bolton Center.

164. Pennington H (2010) Escherichia coli O157. Lancet 376: 1428-1435. doi: 10.1016/s0140-6736(10)60963-4

165. Peterson RE, Klopfenstein TJ, Erickson GE, Folmer J, Hinkley S, Moxley RA, Smith DR (2007) Effect of Lactobacillus acidophilus strain NP51 on Escherichia coil O157:H7 fecal shedding and finishing performance in beef feedlot cattle. J Food Prot 70: 287-291.

166. Price MN, Dehal PS, Arkin AP (2010) FastTree 2--approximately maximum-likelihood trees for large alignments. PLoS One 5: e9490. doi: 10.1371/journal.pone.0009490

167. Quigley JD, Lago A, Chapman C, Erickson P, Polo J (2013) Evaluation of the Brix refractometer to estimate immunoglobulin G concentration in bovine colostrum. J Dairy Sci 96: 1148-1155. doi: 10.3168/jds.2012-5823

168. Quinn PJ, Markey BK, Leonard FC, Hartigan P, Fanning S, Fitzpatrick ES (2015) Veterinary Microbiology and Microbial Disease 2nd Edition. Wiley-Blackwell (STMS), Chichester, West Sussex, UK

169. Rabatsky-Ehr T, Dingman D, Marcus R, Howard R, Kinney A, Mshar P (2002) Deer meat as the source for a sporadic case of Escherichia coli O157:H7 infection, Connecticut. Emerg Infect Dis 8: 525-527. doi: 10.3201/eid0805.010373

170. Rabinovitz BC, Vilte DA, Larzabal M, Abdala A, Galarza R, Zotta E, Ibarra C, Mercado EC, Cataldi A (2014) Physiopathological effects of Escherichia coli O157:H7 inoculation in weaned calves fed with colostrum containing antibodies to EspB and Intimin. Vaccine 32: 3823-3829. doi: 10.1016/j.vaccine.2014.04.073

171. Radostits OM (2001) Herd Health Food Animal Production Medicine. Saunders

172. Rideout JR, He Y, Navas-Molina JA, Walters WA, Ursell LK, Gibbons SM, Chase J, McDonald D, Gonzalez A, Robbins-Pianka A, Clemente JC, Gilbert JA, Huse SM, Zhou HW, Knight R, Caporaso JG (2014) Subsampled open-reference clustering creates consistent, comprehensive OTU definitions and scales to billions of sequences. PeerJ 2: e545. doi: 10.7717/peerj.545

173. Robinson SE, Wright EJ, Hart CA, Bennett M, French NP (2004) Intermittent and persistent shedding of Escherichia coli O157 in cohorts of naturally infected calves. J Appl Microbiol 97: 1045-1053. doi: 10.1111/j.1365-2672.2004.02390.x

174. Rugbjerg H, Nielsen EM, Andersen JS (2003) Risk factors associated with faecal shedding of verocytotoxin-producing Escherichia coli O157 in eight known-infected Danish dairy herds. Prev Vet Med 58: 101-113.

175. Sargeant JM, Amezcua MR, Rajic A, Waddell L (2007) Pre-harvest interventions to reduce the shedding of E. coli O157 in the faeces of weaned domestic ruminants: a systematic review. Zoonoses Public Health 54: 260-277. doi: 10.1111/j.1863-2378.2007.01059.x

176. Scallan E, Hoekstra R, Angulo F, Tauxe R, Widdowson M, Roy S (2011) Foodborne illness acquired in the United States -- major pathogens. Emerging Infectious Disease 17: 7 - 15.

177. Schatzmayr HG, Costa RV, Goncalves MC, D'Andrea PS, Barth OM (2011) Human and animal infections by vaccinia-like viruses in the state of Rio de Janeiro: a novel expanding zoonosis. Vaccine 29 Suppl 4: D65-69. doi: 10.1016/j.vaccine.2011.09.105

178. Schmidt H, Scheef J, Huppertz HI, Frosch M, Karch H (1999) Escherichia coli O157:H7 and O157:H(-) strains that do not produce Shiga toxin: phenotypic and genetic characterization of isolates associated with diarrhea and hemolytic-uremic syndrome. J Clin Microbiol 37: 3491-3496.

179. Schneider J, Mohle-Boetani J, Vugia D (2008) *Escherichia coli* 0157:H7 Infections in Children Associated with Raw Milk and Raw Colostrum From Cows --- California, 2006. In: Services, DoHaH (ed.), vol. 57. Morbidity and Mortality Weekly Report, Centers for Disease Control and Prevention, pp. 625 - 628.

180. Scibilia LS, Muller LD, Kensinger RS, Sweeney TF, Shellenberger PR (1987) Effect of environmental temperature and dietary fat on growth and physiological responses of newborn calves. J Dairy Sci 70: 1426-1433. doi: 10.3168/jds.S0022-0302(87)80165-0

181. Sekirov I, Tam NM, Jogova M, Robertson ML, Li Y, Lupp C, Finlay BB (2008) Antibiotic-induced perturbations of the intestinal microbiota alter host susceptibility to enteric infection. Infect Immun 76: 4726-4736. doi: 10.1128/iai.00319-08

182. Semenov AV, van Bruggen AH, van Overbeek L, Termorshuizen AJ, Semenov AM (2007) Influence of temperature fluctuations on Escherichia coli O157:H7 and Salmonella enterica serovar Typhimurium in cow manure. FEMS Microbiol Ecol 60: 419-428. doi: 10.1111/j.1574-6941.2007.00306.x

183. Shanks OC, Kelty CA, Archibeque S, Jenkins M, Newton RJ, McLellan SL, Huse SM, Sogin ML (2011) Community structures of fecal bacteria in cattle from different animal feeding operations. Appl Environ Microbiol 77: 2992-3001. doi: 10.1128/aem.02988-10

184. Sheng H, Shringi S, Baker KN, Minnich SA, Hovde CJ, Besser TE (2015) Standardized Escherichia coli O157:H7 Exposure Studies in Cattle Provide Evidence that Bovine Factors Do Not Drive Increased Summertime Colonization. Appl Environ Microbiol 82: 964-971. doi: 10.1128/aem.02839-15

185. Shere JA, Bartlett KJ, Kaspar CW (1998) Longitudinal study of Escherichia coli O157:H7 dissemination on four dairy farms in Wisconsin. Appl Environ Microbiol 64: 1390-1399.

186. Shivley C, Urie N, Lombard J, Haines D, Sargent R, Kopral C, Earleywine T, Olson J, Garry F (2018) Factors associated with colostrum quality and passive transfer status of dairy heifer calves on U.S dairy operations. Journal of Dairy Science Accepted January 2018.

187. Smith D, Blackford M, Younts S, Moxley R, Gray J, Hungerford L, Milton T, Klopfenstein T (2001) Ecological relationships between the prevalence of cattle shedding Escherichia coli O157:H7 and characteristics of the cattle or conditions of the feedlot pen. J Food Prot 64: 1899-1903.

188. Smith KE, Stenzel SA, Bender JB, Wagstrom E, Soderlund D, Leano FT, Taylor CM, Belle-Isle PA, Danila R (2004) Outbreaks of enteric infections caused by multiple pathogens associated with calves at a farm day camp. Pediatr Infect Dis J 23: 1098-1104.

189. Snedeker KG, Shaw DJ, Locking ME, Prescott RJ (2009) Primary and secondary cases in Escherichia coli O157 outbreaks: a statistical analysis. BMC Infect Dis 9: 144. doi: 10.1186/1471-2334-9-144

190. Stenkamp-Strahm C, Mc CC, Rao S, Magnuson R, Hyatt DR, Linke L (2017) Climate, lactation, and treatment factors influence faecal shedding of Escherichia coli O157 pathotypes in dairy cows. Epidemiol Infect 145: 115-125. doi: 10.1017/s0950268816001928

191. Stenkamp-Strahm C, McConnel C, Hyatt DR, Magnuson R, Tenneson P, Linke L (2017) Prevalence of Escherichia coli O157 Shedding in Preweaned Calves on Colorado Dairies. J Food Prot 80: 990-993. doi: 10.4315/0362-028x.jfp-16-531

192. Stenkamp-Strahm C, McConnel C, Magzamen S, Abdo Z, Reynolds S (2018) Associations between Escherichia coli O157 shedding and the faecal microbiota of dairy cows. J Appl Microbiol 124: 881-898. doi: 10.1111/jam.13679

193. Suo B, He Y, Tu SI, Shi X (2010) A multiplex real-time polymerase chain reaction for simultaneous detection of Salmonella spp., Escherichia coli O157, and Listeria monocytogenes in meat products. Foodborne Pathog Dis 7: 619-628. doi: 10.1089/fpd.2009.0430

194. Tabe ES, Oloya J, Doetkott DK, Bauer ML, Gibbs PS, Khaitsa ML (2008) Comparative effect of direct-fed microbials on fecal shedding of Escherichia coli O157:H7 and Salmonella in naturally infected feedlot cattle. J Food Prot 71: 539-544.

195. Takahashi M, Taguchi H, Yamaguchi H, Osaki T, Komatsu A, Kamiya S (2004) The effect of probiotic treatment with Clostridium butyricum on enterohemorrhagic Escherichia coli O157:H7 infection in mice. FEMS Immunol Med Microbiol 41: 219-226. doi: 10.1016/j.femsim.2004.03.010

196. Thomson DU, Loneragan GH, Thornton AB, Lechtenberg KF, Emery DA, Burkhardt DT, Nagaraja TG (2009) Use of a siderophore receptor and porin proteins-based vaccine to control the burden of Escherichia coli O157:H7 in feedlot cattle. Foodborne Pathog Dis 6: 871-877. doi: 10.1089/fpd.2009.0290

197. Thorsen J, Brejnrod A, Mortensen M, Rasmussen MA, Stokholm J, Al-Soud WA, Sorensen S, Bisgaard H, Waage J (2016) Large-scale benchmarking reveals false discoveries and count transformation sensitivity in 16S rRNA gene amplicon data analysis methods used in microbiome studies. Microbiome 4: 62. doi: 10.1186/s40168-016-0208-8

198. Tkalcic S, Zhao T, Harmon BG, Doyle MP, Brown CA, Zhao P (2003) Fecal shedding of enterohemorrhagic Escherichia coli in weaned calves following treatment with probiotic Escherichia coli. J Food Prot 66: 1184-1189.

199. Urie N, Lombard J, Shivley C, Kopral C, Adams A, Earleywine T, Olson J, Garry F (2018) A descriptive overview of morbidity and mortality and factors associated with morbidity and mortality in preweaned dairy heifer calves on U.S. dairy operations. Journal of Dairy Science Accepted January 2018.

200. USDA (1994) *Escherichia coli* O157:H7 in U.S Dairy Calves. In: National Animal Health Monitoring System, VS, Centers for Epidemiology and Animal Health (ed.). United States Department of Agriculture, Animal and Plant Health Inspection Service, Veterinary SErvices, Fort Collins, Colorado.

201. USDA (1996) Dairy 1996: National dairy health evaluation project. Escherichia coli O157:H7 in U.S. Dairy Calves. United States Department of Agriculture, National Health Monitoring System, Fort Collins, CO.

202. USDA (2003) Escherichia coli O157 on US Dairy Operations. United States Department of Agriculture, National Animal Health Monitoring System, Fort Collins, CO.

203. USDA (2010) Passive transfer status of heifer calves on US dairies, 1991-2007. United States Department of Agriculture, National Animal Health Monitoring System, Fort Collins, Colorado.

204. USDA (2013) NAHMS 2014 Dairy Study: Launch. United States Department of Agriculture, National Health Monitoring System, Fort Collins, CO.

205. USDA (2016) Dairy Cattle Management Practices in the United States, Part 1. United States Department of Agriculture, National Health Monitoring System, Fort Collins, CO.

206. USDA (2017) Livestock, Dairy and Poultry Outlook. In: Service, ER (ed.), Ithaca, NY.

207. USDA N (2018) National Agricultural Statistics Service, Milk Production Per Year, U.S. Online.

208. Van Baale MJ, Sargeant JM, Gnad DP, DeBey BM, Lechtenberg KF, Nagaraja TG (2004) Effect of forage or grain diets with or without monensin on ruminal persistence and fecal Escherichia coli O157:H7 in cattle. Appl Environ Microbiol 70: 5336-5342. doi: 10.1128/aem.70.9.5336-5342.2004

209. van Elsas JD, Semenov AV, Costa R, Trevors JT (2011) Survival of Escherichia coli in the environment: fundamental and public health aspects. Isme j 5: 173-183. doi: 10.1038/ismej.2010.80

210. Varela NP, Dick P, Wilson J (2013) Assessing the existing information on the efficacy of bovine vaccination against Escherichia coli O157:H7--a systematic review and meta-analysis. Zoonoses Public Health 60: 253-268. doi: 10.1111/j.1863-2378.2012.01523.x

211. Venegas-Vargas C, Henderson S, Khare A, Mosci RE, Lehnert JD, Singh P, Ouellette LM, Norby B, Funk JA, Rust S, Bartlett PC, Grooms D, Manning SD (2016) Factors Associated with Shiga Toxin-Producing Escherichia coli Shedding by Dairy and Beef Cattle. Appl Environ Microbiol 82: 5049-5056. doi: 10.1128/aem.00829-16

212. Vold L, Holck A, Wasteson Y, Nissen H (2000) High levels of background flora inhibits growth of Escherichia coli O157:H7 in ground beef. Int J Food Microbiol 56: 219-225.

213. Wang G, Clark CG, Rodgers FG (2002) Detection in Escherichia coli of the genes encoding the major virulence factors, the genes defining the O157:H7 serotype, and components of the type 2 Shiga toxin family by multiplex PCR. J Clin Microbiol 40: 3613-3619.

214. Wang Q, Garrity GM, Tiedje JM, Cole JR (2007) Naive Bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy. Appl Environ Microbiol 73: 5261-5267. doi: 10.1128/aem.00062-07

215. Weiss S, Xu ZZ, Peddada S, Amir A, Bittinger K, Gonzalez A, Lozupone C, Zaneveld JR, Vazquez-Baeza Y, Birmingham A, Hyde ER, Knight R (2017) Normalization and microbial differential abundance strategies depend upon data characteristics. Microbiome 5: 27. doi: 10.1186/s40168-017-0237-y

216. Wells S, Fedorka-Cray P, Besser T, McDonough P, Smith B (1998) E.coli O157 and Salmonella-Status on US Dairy Operations. In: VS, U-A- (ed.). CEAH, Fort Collins, CO.

217. Wileman BW, Thomson DU, Olson KC, Jaeger JR, Pacheco LA, Bolte J, Burkhardt DT, Emery DA, Straub D (2011) Escherichia coli O157:H7 shedding in vaccinated beef calves born to cows vaccinated prepartum with Escherichia coli O157:H7 SRP vaccine. J Food Prot 74: 1599-1604. doi: 10.4315/0362-028x.jfp-11-034

218. Williams KJ, Ward MP, Dhungyel OP (2015) Daily variations in Escherichia coli O157 shedding patterns in a cohort of dairy heifers at pastureEpidemiol Infect, England, pp. 1388-1397

219. Williams KJ, Ward MP, Dhungyel OP, Hall EJ (2015) Risk factors for Escherichia coli O157 shedding and super-shedding by dairy heifers at pasture. Epidemiol Infect 143: 1004-1015. doi: 10.1017/s0950268814001630

220. Withee J, Williams M, Disney T, Schlosser W, Bauer N, Ebel E (2009) Streamlined analysis for evaluating the use of preharvest interventions intended to prevent Escherichia coli O157:H7 illness in humans. Foodborne Pathog Dis 6: 817-825. doi: 10.1089/fpd.2008.0255

221. Xu Y, Dugat-Bony E, Zaheer R, Selinger L, Barbieri R, Munns K, McAllister TA, Selinger LB (2014) Escherichia coli O157:H7 super-shedder and non-shedder feedlot steers harbour distinct fecal bacterial communities. PLoS One 9: e98115. doi: 10.1371/journal.pone.0098115

222. Zangari T, Melton-Celsa AR, Panda A, Smith MA, Tatarov I, De Tolla L, O'Brien AD (2014) Enhanced virulence of the Escherichia coli O157:H7 spinach-associated outbreak strain in two animal models is associated with higher levels of Stx2 production after induction with ciprofloxacin. Infect Immun 82: 4968-4977. doi: 10.1128/iai.02361-14

223. Zhao L, Tyler PJ, Starnes J, Bratcher CL, Rankins D, McCaskey TA, Wang L (2013) Correlation analysis of Shiga toxin-producing Escherichia coli shedding and faecal bacterial composition in beef cattle. J Appl Microbiol 115: 591-603. doi: 10.1111/jam.12250

224. Zhao T, Doyle MP, Harmon BG, Brown CA, Mueller PO, Parks AH (1998) Reduction of carriage of enterohemorrhagic Escherichia coli O157:H7 in cattle by inoculation with probiotic bacteria. J Clin Microbiol 36: 641-647.

225. Zhao T, Doyle MP, Shere J, Garber L (1995) Prevalence of enterohemorrhagic Escherichia coli O157:H7 in a survey of dairy herds. Appl Environ Microbiol 61: 1290-1293.

226. Zhao T, Zhao P, West JW, Bernard JK, Cross HG, Doyle MP (2006) Inactivation of enterohemorrhagic Escherichia coli in rumen content- or feces-contaminated drinking water for cattle. Appl Environ Microbiol 72: 3268-3273. doi: 10.1128/aem.72.5.3268-3273.2006

227. Zoetis (2018) E. coli O157 SRP Technology. Zoetis Animal Health.

APPENDIX 1: SUPPLEMENTARY FIGURES AND TABLES FOR CHAPTER 1

Table 16: Definition of select variables used in regression analysis

Variable name	Levels	Definition
Fecal score	<=2, 3, >= 4	1= very liquid with no rings or dimples in pats 2= runny and does not pile, sits less than 1" high, appearance of rings in pats 3= more thickened consistency, stands 1.5" high and has rings or dimples 4= thick, does not stick to shoes and shows no rings or dimples 5= manure is firm balls that stack 2-4" high
Hygiene score	<=2, >2	1= little to no manure above the coronary band 2= minor splashing of manure above the coronary band and near the teat/upper leg/flank 3= distinct plaques of manure above the coronary band and on teats, upper leg and flank, with hair still visible 4= solid plaques of manure extending high up on the leg, on and around the teats, upper legs, and flank
Body condition score	<2, 3, >3	1= deep cavity around tailhead, pelvis and short ribs sharp and easily felt 2= shallow cavity around tailhead, some fat covering pin bones, pelvis easily felt, ends of short ribs feel rounded, loin depression visible 3= fatty tissue filling cavity around tailhead, pelvis felt with slight pressure, short ribs felt with pressure, slight loin depression 4= folds of fat around tailhead and pin bones, pelvis felt with firm pressure, short ribs are not palpable, no loin depression 5= fatty tissue covers tailhead, pelvic bones non-palpable, short ribs covered by thick layer of fat
Calving ease	<=2, >=3	1= no assistance 2= minimal assistance from a single person 3= significant manipulation and relatively hard pull from single person 4= manipulation and hard pulls from two people 5= cesarian section or fetotomy
Disease	1 through 18, Ever vs Never	1= bloat 2= diarrhea 3= down, metabolic 4= dystocia 5= hemorrhagic bowel syndrome 6= Johne's disease 7= ketosis 8= lame foot 9= lame from injury 10= mastitis 11= metritis

		12= pneumonia 13= retained placenta 14= udder injury 15= edema 16= displaced abomasum 17= fever 18= ulcer
Treatment*	1 through 8, Categorized: Ever antibiotic, ever other treatment, vs never treatment	1= sick with none or non-listed treatment 2= systemic drug only 3= intramammary drug only 4= antiinflammatory drug only 5= systemic and intramammary drugs 6= systemic and antiinflammatory drugs 7= intramammary and antiinflammatory drugs 8= systemic, antiinflammatory and intramammary drugs
Any Disease or Treatment	Ever vs Never	1= any disease or treatment listed at least once 2= never disease or treatment listed

*Systemic drugs include: Ceftiofur, Ampicillin, Oxytetracycline

*Intramammary drugs include: Ceftiofur, Pirlimycin, Cephapirin

*Antiinflammatory drugs include: Flunixin meglumine, Dexamethasone, Aspirin

Table 17: Multivariate models determined for aEPEC shedding, using 'dairy' as a clustering variable

	Comparison	*OR[1]*	*95% CI*	*P-value*	*QIC*	*QICu*
Model 1						
Hygiene score	>2 vs (1,2)	1.523	0.473-4.898	0.481	300.8	303
Fecal score	(1,2) vs 3	0.497	0.222-1.111	0.089		
	(4,5) vs 3	0.973	0.284-3.331	0.965		
Any Disease or Treatment	Any vs None	0.558	0.337-0.924	0.024		
Final Model 1						
Any Disease or Treatment	Any vs None	0.545	0.330-0.899	0.0175	297.9	299.9
Model 2						
Hygiene score	>2 vs (1,2)	1.623	0.501-5.255	0.419	300.4	300.4
Cow contact	Each unit increase	1.01	1.001-1.019	0.0245		
Final Model 2						
Cow contact	Each unit increase	1.01	1.0017-1.0184	0.0175	298.1	299.8
Model 3						
Fecal score	(1,2) vs 3	0.489	0.205-1.168	0.107	304.1	306

						.6
	(4,5) vs 3	0.982	0.327-2.951	0.975		
Any Disease or Treatment	Any vs None	0.544	0.329-0.899	0.0175		
Weekly precipitation (inches)	Each unit increase	0.735	0.467-1.157	0.184		
Final Model 3						
Any Disease or Treatment	Any vs None listed	0.545	0.330-0.899	0.0175	297.9	299.9
Model 4						
Crowding	Each unit increase	1.012	0.997-1.026	0.1115	288.9	291.2
Average weekly humidity (%)	Each unit increase	1.067	1.040-1.095	<0.0001		
Final Model 4						
Average weekly humidity (%)	Each unit increase	1.07	1.032-1.101	0.0001	290.2	292.6
Model 5 and Final Model 5						
Average weekly humidity (%)	Each unit increase	1.067	1.034-1.101	<0.0001	290.3	294.6
Fecal score	(1,2) vs 3	0.451	0.213-0.954	0.0373		
	(4,5) vs 3	0.938	0.276-3.187	0.919		

[1] *Odds of shedding aEPEC*

Table 18: Multivariate models determined for EHEC shedding, using 'dairy' as a clustering variable

	Comparison	***OR[1]***	***95% CI***	***P-value***	***QIC***	***QICu***
Model 1 and Final Model 1						
Hygiene score	>2 vs (1,2)	0.401	0.246-0.651	0.0002	237.9	235.3
Cow contact	Each unit increase	0.973	0.959-0.987	0.0002		
Antibiotic or Other Treatment	Ever antibiotic vs None listed	1.322	1.017-1.719	0.0373		
	Ever other vs None listed	0.495	0.171-1.437	0.196		
Model 2 and Final Model 2						
Average weekly humidity (%)	Each unit increase	1.049	1.000-1.098	0.044	244.2	231.9
Cow contact	Each unit increase	0.968	0.966-0.971	<0.0001		
Model 3 and Final Model 3						
Any Disease or Treatment	Any vs None listed	1.613	0.913-2.847	0.0995	255.6	239.9
Average weekly temperature (°F)	Each unit increase	1.058	1.010-1.108	0.0173		

Model 4 and Final Model 4						
Hygiene score	>2 vs (1,2)	0.52	0.37-0.72	<0.0001	266.1	254.5
Any Disease or Treatment	Any vs None listed	1.81	1.24-2.66	0.0023		
Hygiene score * Any Disease or Treatment	>2 and Any vs (1,2) and None	0.43	0.241-0.766	0.0042		
Model 5 and Final Model 5						
Hygiene Score	>2 vs (1,2)	0.507	0.362-0.710	<0.0001	266.4	254.6
Antibiotic or Other Treatment	Ever antibiotic vs None listed	1.715	1.082-2.717	0.0217		
	Ever other vs None listed	0.459	0.115-1.830	0.269		
Model 6 and Final Model 6						
Cow Contact	Each unit increase	0.971	0.962-0.981	<0.0001	242.9	236.9
Antibiotic or Other Treatment	Ever antibiotic vs None listed	1.271	0.986-1.64	0.0641		
	Ever other vs None listed	0.519	0.168-0.159	0.253		
Model 7						
Parity	2 vs 1	0.565	0.326-0.979	0.042	274.2	255.4
	>=3 vs 1	0.527	0.486-0.559	<0.0001		
Average weekly humidity (%)	Each unit increase	1.03	0.981-1.082	0.237		
Final Model 7						
Parity	2 vs 1	0.572	0.324-1.009	0.054	271.4	255.2
	>=3 vs 1	0.512	0.457-0.573	<0.0001		
Model 8						
Weekly precipitation (inches)	Each unit increase	1.185	0.914-1.537	0.2002	270.1	253.6
Any Disease or Treatment	Any vs None listed	1.804	1.219-2.672	0.0032		
Final Model 8						
Any Disease or Treatment	Any vs None listed	1.758	1.199-2.58		268.6	253
Model 9						
Average weekly humidity (%)	Each unit increase	1.03	0.97-1.10	0.341	263.6	241.9
Average weekly temperature (°F)	Each unit increase	1.06	1.01-1.11	0.0225		
Final Model 9						
Average weekly temperature (°F)	Each unit increase	1.06	1.012-1.111	0.0131	258.8	239.9
Model 10 and Final Model 10						
Parity	2 vs 1	1.383	0.988-1.935	0.059	250	246.9

	>=3 vs 1	0.68	0.545-0.849	0.0006		
Days in milk	Each day increase	0.915	0.842-0.995	0.037		
Days in milk * Parity	Increase DIM and Parity 2 vs Baseline DIM and Parity 1	0.868	0.74-1.02	0.083		
	Increase DIM and Parity >=3 vs Baseline DIM and Parity 1	0.95	0.908-0.996	0.033		
	Increase DIM and Parity >=3 vs Baseline DIM and Parity 2	1.01	0.892-1.34	0.387		
Model 11 and Final Model 11						
Average weekly temperature (°F)	Each unit increase	1.06	1.02-1.10	0.003	238.9	229.9
Days in milk	Each day increase	0.88	0.79-0.99	0.028		

[1] *Odds of shedding EHEC*

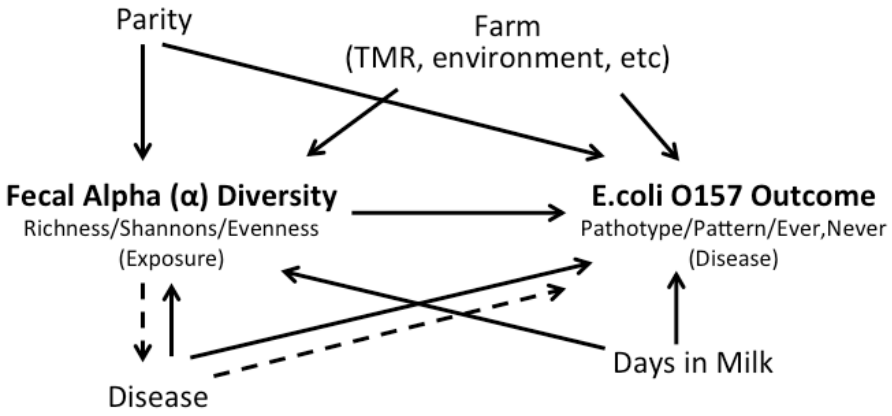

Figure 13: Directed acyclic graph of factors hypothesized to confound or mediate the association between alpha diversity and O157 outcome. Solid lines delineate factors that may be confounders, while dashed lines represent those that may be mediators.

Table 19: Associations between cow covariates and alpha diversity measures using regression modeling

Covariate	Alpha Diversity	Levels	OR *	Lower CI*	Upper CI*
[t] DIM (continuous)	Richness		0.91	0.67	1.24
	Shannons		0.91	0.57	1.46
	Evenness		1.05	0.69	1.60
[Ŧ] Parity (1, 2, 3)	Richness	Parity 1	1.00		
		Parity 2	0.48	0.41	0.57
		Parity ≥ 3	0.99	0.87	1.13
	Shannons	Parity 1	1.00		
		Parity 2	0.28	0.06	1.23
		Parity ≥ 3	1.64	0.52	5.17
	Evenness	Parity 1	1.00		
		Parity 2	0.26	0.06	1.16
		Parity ≥ 3	1.57	0.53	4.69
[§] Disease (0, 1)	Richness	Absent	1.00		
		Present	0.96	0.53	1.75
	Shannons	Absent	1.00		
		Present	1.05	0.43	2.58

	Evenness	Absent	1.00		
		Present	1.22	0.49	3.03
§Farm (1, 2)	Richness	Farm 1	1.00		
		Farm 2	0.35	0.14	0.92
	Shannons	Farm 1	1.00		
		Farm 2	0.37	0.13	1.06
	Evenness	Farm 1	1.00		
		Farm 2	0.52	0.20	1.35
ᵀTreatment (0, 1, 2)	Richness	No Treatment	1.00		
		Antibiotic	0.90	0.81	1.00
		Other	0.88	0.74	1.04
	Shannons	No Treatment	1.00		
		Antibiotic	0.83	0.29	2.36
		Other	0.68	0.19	2.47
	Evenness	No Treatment	1.00		
		Antibiotic	0.82	0.29	2.37
		Other	1.00	0.27	3.70

* Odds ratios and confidence intervals were calculated from model coefficients and SE using interquartile range

ᵗMixed linear regression model with 'cow' as a random effect. P values reflect models comparison to null

ᵀMultinomial model with diversity measure averaged by cow

§Logistic model with diversity measure averaged by cow

Table 20: Crude and confounder adjusted estimates of associations between O157 category and alpha diversity

O157 Category (levels)	Alpha Diversity	Measurement	OR* (CI)
ᵗPathotype	Richness	Crude	0.931 (0.56 - 1.54)
(aEPEC vs EHEC)		Adjusted Parity	0.985 (0.59 - 1.65)
		Adjusted Treatment	0.964 (0.60 - 1.56)
		Adjusted Farm	0.874 (0.53 - 1.44)
		Adjusted Treatment + Parity	1.02 (0.62 - 1.69)
		Adjusted Treatment + Farm	0.95 (0.58 - 1.57)
		Adjusted Parity + Farm	0.915 (0.55 - 1.52)
		Adjusted Parity + Farm + Treatment	1.00 (0.60 - 1.68)
	Shannons	Crude	1.042 (0.49 - 2.20)
		Adjusted Parity	1.240 (0.57 - 2.69)
		Adjusted Treatment	1.09 (0.58 - 2.05)
		Adjusted Farm	0.929 (0.45 - 1.90)
		Adjusted Treatment + Parity	1.29 (0.67 - 2.51)
		Adjusted Treatment + Farm	1.08 (0.56 - 2.07)

		Adjusted Parity + Farm	1.106 (0.53 - 2.29)
		Adjusted Parity + Farm + Treatment	1.27 (0.64 - 2.50)
	Evenness	Crude	1.176 (0.60 - 2.31)
		Adjusted Parity	1.412 (0.72 - 2.75)
		Adjusted Treatment	1.18 (0.66 - 2.11)
		Adjusted Farm	1.083 (0.56 - 2.10)
		Adjusted Treatment + Parity	1.44 (0.80 - 2.59)
		Adjusted Treatment + Farm	1.17 (0.64 - 2.12)
		Adjusted Parity + Farm	1.339 (0.70 - 2.56)
		Adjusted Parity + Farm + Treatment	1.43 (0.79 - 2.58)
ŦEver/Never (0,1) (Shed vs Never Shed)	Richness	Crude	0.927 (0.50 - 1.73)
		Adjusted Parity	1.026 (0.54 - 1.94)
		Adjusted Treatment	0.92 (0.48 - 1.74)
		Adjusted Farm	1.187 (0.60 - 2.33)
		Adjusted Treatment + Parity	1.02 (0.54 - 1.93)
		Adjusted Treatment + Farm	0.91 (0.45 - 1.83)
		Adjusted Parity + Farm	0.930 (0.47 - 1.84)
		Adjusted Parity + Farm + Treatment	0.99 (0.50 - 2.00)
	Shannons	Crude	0.896 (0.36 - 2.24)
		Adjusted Parity	1.205 (0.42 - 3.39)
		Adjusted Treatment	0.90 (0.34 - 2.38)
		Adjusted Farm	0.800 (0.30 - 2.11)
		Adjusted Treatment + Parity	1.25 (0.44 - 3.58)
		Adjusted Treatment + Farm	0.89 (0.31 - 2.59)
		Adjusted Parity + Farm	1.078 (0.37 - 3.13)
		Adjusted Parity + Farm + Treatment	1.24 (0.40 - 3.85)
	Evenness	Crude	1.281 (0.51 - 3.24)
		Adjusted Parity	1.894 (0.65 - 5.54)
		Adjusted Treatment	1.25 (0.47 - 3.36)
		Adjusted Farm	1.211 (0.47 - 3.13)
		Adjusted Treatment + Parity	2.06 (0.63 - 6.77)
		Adjusted Treatment + Farm	1.29 (0.46 - 3.58)
		Adjusted Parity + Farm	1.795 (0.61 - 5.33)
		Adjusted Parity + Farm + Treatment	2.09 (0.61 - 7.12)
§Pattern (0,1,2) (Intermittent[1] or Multiday[2] vs Never Shed)	Richness	Crude[1]	1.060 (0.93 - 1.21)
		Adjusted Parity[1]	1.227 (1.07 - 1.40)
		Adjusted Treatment[1]	1.03 (0.91 - 1.18)

	Adjusted Farm[1]	1.112 (0.90 - 1.37)
	Adjusted Treatment + Parity[1]	1.22 (1.06 - 1.40)
	Adjusted Treatment + Farm[1]	1.12 (0.91 - 1.38)
	Adjusted Parity + Farm[1]	1.465 (1.23 - 1.74)
	Adjusted Parity + Farm + Treatment[1]	1.39 (1.12 - 1.71)
	Crude[2]	0.515 (0.413 - 0.641)
	Adjusted Parity[2]	0.599 (0.48 - 0.75)
	Adjusted Treatment[2]	0.51 (0.39 - 0.66)
	Adjusted Farm[2]	0.326 (0.26 - 0.41)
	Adjusted Treatment + Parity[2]	0.62 (0.47 - 0.83)
	Adjusted Treatment + Farm[2]	0.43 (0.33 - 0.56)
	Adjusted Parity + Farm[1]	0.251 (0.19 - 0.33)
	Adjusted Parity + Farm + Treatment[2]	0.36 (0.26 - 0.49)
Shannons	Crude[1]	0.876 (0.32 - 2.39)
	Adjusted Parity[1]	1.171 (0.36 - 3.82)
	Adjusted Treatment[1]	0.82 (0.27 - 2.47)
	Adjusted Farm[1]	0.900 (0.29 - 2.75)
	Adjusted Treatment + Parity[1]	1.27 (0.33 - 4.88)
	Adjusted Treatment + Farm[1]	0.87 (0.25 - 2.98)
	Adjusted Parity + Farm[1]	1.401 (0.33 - 5.93)
	Adjusted Parity + Farm + Treatment[1]	1.65 (0.31 - 8.65)
	Crude[2]	0.931 (0.20 - 4.32)
	Adjusted Parity[2]	1.285 (0.25 - 6.52)
	Adjusted Treatment[2]	1.07 (0.27 - 4.27)
	Adjusted Farm[2]	0.640 (0.16 - 2.54)
	Adjusted Treatment + Parity[2]	1.65 (0.34 - 8.12)
	Adjusted Treatment + Farm[2]	0.91 (0.22 - 3.76)
	Adjusted Parity + Farm[1]	0.941 (0.24 - 3.63)
	Adjusted Parity + Farm + Treatment[2]	1.29 (0.27 - 6.10)
Evenness	Crude[1]	1.052 (0.38 - 2.92)
	Adjusted Parity[1]	1.498 (0.45 - 4.98)
	Adjusted Treatment[1]	1.03 (0.35 - 3.05)
	Adjusted Farm[1]	1.097 (0.39 - 3.09)
	Adjusted Treatment + Parity[1]	1.76 (0.46 - 6.72)
	Adjusted Treatment + Farm[1]	1.10 (0.36 - 3.37)
	Adjusted Parity + Farm[1]	1.587 (0.46 - 5.42)
	Adjusted Parity + Farm +	1.95 (0.47 - 8.01)

Treatment[1]	
Crude[2]	2.219 (0.44 - 11.27)
Adjusted Parity[2]	3.248 (0.57 - 18.36)
Adjusted Treatment[2]	2.27 (0.40 - 12.81)
Adjusted Farm[2]	1.703 (0.29 - 9.92)
Adjusted Treatment + Parity[2]	3.22 (0.47 - 21.88)
Adjusted Treatment + Farm[2]	2.00 (0.33 - 12.23)
Adjusted Parity + Farm[1]	3.422 (0.49 - 23.79)
Adjusted Parity + Farm + Treatment[2]	3.08 (0.41 - 23.04)

* Odds ratios and confidence intervals were calculated from model coefficients and SE using interquartile range

[t] Mixed logistic regression model with 'cow' as a random effect

[T] Logistic regression model with diversity measure averaged by cow

[§] Multinomial model with diversity measure averaged by cow