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The Influence of the Bovine Fecal Microbiota on the Shedding of Shiga Toxin-Producing *Escherichia coli* (STEC) by Beef Cattle

Nirosh D. Aluthge
University of Nebraska-Lincoln, niroshaluthge@gmail.com

THE INFLUENCE OF THE BOVINE FECAL MICROBIOTA ON THE SHEDDING OF SHIGA TOXIN-PRODUCING *ESCHERICHIA COLI* (STEC) BY BEEF CATTLE

by

Nirosh D. Aluthge

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Nirosh Dilshan Aluthge, M. S.

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Adviser: Samodha Fernando

During the past three decades, Shiga toxin-producing *E.coli* (STEC) have emerged as an important food safety concern. Although initially *E. coli* O157 was the main focus, recent outbreaks and resulting investigations have shown that certain non-O157 STEC are as much a threat to food safety as their O157 counterparts. To the beef industry, STEC have been of particular concern due to the frequent association of beef and beef products as vehicles of STEC infection. As a result, along with *E. coli* O157, six non-O157 STEC serogroups (known as the 'big six') are now regulated as adulterants in certain raw beef products in the United States. Compared to STEC O157, relatively little is known about the prevalence and pathogenicity of the non-O157 STEC in beef production systems. Fecal shedding of STEC by cattle is considered the main route of entry of these pathogens to the environment.

The main objective of this study was to investigate if differences existed in the fecal bacterial composition of beef cattle based on their level of STEC shedding. In addition, this study also investigated the fecal prevalence of virulent strains of STEC O157 and the 'big six' non-O157 STEC (EHEC-7) within a beef cattle population to assess if the fecal

microbiota had an influence on the shedding of these virulent STEC strains. A total of 328 cross-bred beef steers from two separate years were fecal sampled and the fecal bacterial composition assessed using high-throughput DNA sequencing. NeoSEEK^{TM} STEC assay was used to determine the prevalence of EHEC-7. No higher order differences were detected that suggests that STEC shedding was associated with changes in fecal bacterial composition. However, some genera and OTUs were associated with a given shedding category. Only 4.08% of the fecal samples yielded a member of the EHEC-7. The low number of samples positive for EHEC-7 prevented an analysis being done to determine the influence of the fecal microbiota on their shedding.

DEDICATION

I dedicate this work to my loving parents, brother, and everyone who helped with this project.

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Chapter 1

LITERATURE REVIEW

1.1 Introduction

Shiga toxin-producing *Escherichia coli* (STEC) are strains of *E. coli* which possess at least one member of a class of cytotoxins known as 'Shiga toxins' (Gyles, 2007). This group of bacteria, whose routes of transmission include food and water, are now recognized as an important cause of gastrointestinal disease in humans, particularly since such infections may result in life-threatening consequences such as hemolytic-uremic syndrome (HUS) and thrombotic thrombocytopenic purpura (Paton & Paton, 1998).

1.2 Shiga toxin-producing *E. coli* (STEC) as foodborne pathogens

The first published report on Shiga toxin- producing *E. coli* appeared in 1977, when Konowalchuk et al. (1977) described a novel cytotoxin produced by certain strains of *E. coli* (mostly isolated from children with diarrhea), which had a profound and irreversible cytopathic effect on Vero (African Green Monkey Kidney) cells (Paton and Paton, 1998). Thus, the toxin was called 'verocytotoxin' (or simply verotoxin) and the *E. coli* strains producing these toxins came to be known as verotoxin-producing *E. coli* (VTEC). Subsequently, the cytotoxin produced by one of the isolates in the above mentioned study was purified and characterized by O'Brien et al. (1983). They found that this verotoxin had a strikingly similar structure and biological activity to Shiga toxin (Stx) produced by *Shigella dysenteriae* type-1, and also that it could be neutralized by anti-Stx, resulting in the new nomenclature of Shiga-like toxin (SLT) being attributed to this toxin. As a result

of these findings, the term ‘Shiga toxin-producing *E. coli* (STEC)’ was introduced, and has become the more popular term to describe these *E. coli* strains in the United States while the earlier ‘VTEC’ nomenclature is more commonly used in Europe (Bolton, 2011).

STEC were first implicated as etiologic agents in foodborne disease in 1982, when Riley et al. (1983) investigated two outbreaks of an unusual gastrointestinal illness that involved over 40 people in the states of Oregon and Michigan, from February through March, and May through June 1982. The authors described how they isolated the then ‘rare’ *E. coli* serotype O157:H7 from stool samples of patients as well as from a beef patty from a suspected lot of meat in Michigan. At this time, the only previous known isolation of *E. coli* O157:H7 was from a sporadic case of hemorrhagic colitis in 1975 (Riley *et al*., 1983). The report by Riley et al. described a clinically distinctive gastrointestinal illness associated with *E. coli* O157:H7, apparently transmitted by undercooked meat. The first reports of sporadic HUS due to an STEC serotype that was not O157:H7 (non-O157 STEC) appeared in 1975 in France, when *E. coli* O103 was isolated from some patients in a hospital (Karmali *et al*., 1985) while the first outbreak caused by a non-O157 STEC (*E. coli* O145:H-) occurred in Japan in 1984, although the vehicle of infection was not determined in this instance (Johnson *et al*., 1996).

Shiga toxin-producing *E. coli* have been a major public health concern in recent times because of their association with foodborne and waterborne disease outbreaks. According to published data, it is estimated that over 63,000 human disease cases due to O157 STEC strains and around 112,000 cases due to non-O157 STEC strains occur annually in

the United States (Scallan *et al*, 2011.). Diseases due to STEC can range from mild, self-limiting diarrhea to hemorrhagic colitis and HUS, and have gained widespread media attention due to the life-threatening nature of some of these diseases. In addition to the consequences on human health, STEC outbreaks have resulted in costly product recalls for the food industry and has damaged consumer confidence when it comes to the safety of the food supply.

1.2.1 Classification of STEC

STEC are commonly classified into serotypes based on their O- and H- antigens. The O (Ohne) antigen is determined by the polysaccharide portion of the cell wall lipopolysaccharide layer (LPS) while the H (Hauch) antigen is based on the flagella protein (Gyles, 2007). The serogroup is determined by the O-antigen; the serotype is determined by both the O- and H-antigens (Campos *et al*., 2004).

There are many hundreds of different serotypes of STEC based on O- and H- antigen classification; however, only a small number of these serotypes have been associated with human illness (Farrokh *et al*., 2013). Based on the association of these serotypes with disease of varying severity in humans, and with sporadic disease or outbreaks, a grouping of STEC into 5 seropathotypes (from A to E) has been proposed (Karmali et al., 2003; Gyles, 2007). The most virulent are categorized under Seropathotype A, and consists of the serotypes O157:H7 and O157:NM (non-motile). Seropathotype B consists of O26:H11, O103:H2, O111:NM, O121:H19, and O145:NM. These serotypes can also cause severe disease and outbreaks, but occur at a lower frequency than the O157 serotypes (Gyles, 2007). Seropathotype C includes STEC serotypes, such as O91:H21

and O113:H21, which are infrequently implicated in sporadic HUS but are not associated with outbreaks. A vast majority of STEC fall under seropathotypes D and E, which consists of serotypes which are either rarely associated with or have never been implicated in human illness (Gyles, 2007).

Because of their differences in virulence, association with human disease outbreaks and certain biochemical characteristics, STEC are commonly divided in two major groups: the O157 STEC and the non-O157 STEC.

1.2.2 O157 STEC

In recognition of their importance as etiological agents of potentially fatal human illness, O157 STEC strains have historically gained a lot of attention from the scientific community, regulators, and the public in general. The major serotype of public health significance within this group is *E. coli* O157:H7 (STEC O157) and much effort has been expended to understand the prevalence, transmission, and disease causing traits of this organism. Since the first recording of *E. coli* O157:H7 as a foodborne pathogen in 1982 in the United States, infections have been reported in over 50 countries covering all continents except Antarctica (Chase-Topping *et al*, 2008). The highest annual incidences of human infection have been reported from Scotland, in parts of Canada, the United States, and Japan (Chase-Topping *et al*., 2008).

Early outbreaks caused by STEC O157 in the 1980's was mainly through contaminated beef products and unpasteurized milk (Griffin and Tauxe, 1991). Since then, it has been shown that outbreaks are associated with a wide range of food products, including unpasteurized apple juice, spinach, and salami (Chase-Topping *et al*, 2008). The source

of contamination for most foods is thought to be through contact with animal feces; either directly in the field or indirectly through runoff water from farms (Fairbrother and Nadeau, 2006; Chase-Topping *et al*, 2008), further highlighting the importance of food animals, especially cattle, as major reservoirs for STEC O157. Although mainly identified as a foodborne pathogen, environmental exposure can also lead to human infection by STEC O157 (Chase-Topping *et al*, 2008).

1.2.3 Non-O157 STEC

Although the O157 STEC group has received much of the attention of the scientific community and regulatory authorities, over 200 non-O157 STEC serotypes have also been isolated from outbreaks and sporadic cases of HUS and severe diarrhea in the US (Kaspar et al., 2010). In certain parts of the world, such as continental Europe, Australia and Argentina, infections with non-O157 STEC serotypes are actually more common than infections with O157 STEC (Caprioli *et al*., 1998; Blanco *et al*., 2004; Johnson *et al*., 2006). However, non-O157 STEC are increasingly recognized as contributing significantly to the STEC disease burden (Gould et al., 2013). In fact, recent estimates suggest that in the US as well non-O157 STEC may cause more cases of disease than STEC O157 (Hale et al. (2012) estimated that STEC O157 caused 40.3% of domestically acquired STEC infections, whereas the non-O157 STEC were responsible for 59.7% of these illnesses).

Although many different non-O157 STEC strains have been isolated from patients, only a handful of serogroups and serotypes account for a majority of human non-O157 STEC illnesses. According to published reports from 1984 – 2009, the most common non-O157

STEC serogroups identified worldwide were O26 (37%), O111 (31%), O103 (6%), O121 (5%), O145 (5%), and O45 (1%) (Kaspar et al., 2010; Kalchayanand et al., 2011). In the United States from 1983 – 2002, the breakdown in proportions of STEC serogroups isolated from patients with illness was O26 (22%), O111 (16%), O103 (12%), O121 (8%), O45 (7%), and O145 (5%) (Brooks et al., 2005). Thus, these six major non-O157 STEC serogroups (known as the 'big six' non-O157 STEC) are said to account for 71% of non-O157 STEC disease cases in the US (Brooks et al., 2005). Within these six serogroups, the most common serotypes associated with illness are O26:H11 or non-motile (NM); O45:H2 or NM; O103:H2, H11, H25, or NM; O111:H8 or NM; O121:H19 or H7; and O145:NM (Brooks et al., 2005; Kalchayanand et al., 2011).

Similar to O157 STEC, non-O157 STEC serotypes are often associated with cattle and other ruminants (Kaspar et al., 2010; Kalchayanand et al., 2011). As a result of this ecology, meat, milk, water and fresh produce have been implicated in non-O157 STEC transmission as well (Kaspar et al., 2010).

1.2.4 Enterohemorrhagic *Escherichia coli* (EHEC)

Enterohemorrhagic *E. coli* are the sub group of STEC that is more often associated with hemorrhagic colitis and HUS (Gyles, 2007), and as such, are considered to be the more virulent members of the STEC group. The most common serotypes of EHEC associated with severe disease are O157:H7, O26:H11: H^-, O111:H8: H^-, and O103:H2: H^- (Venturini et al., 2010). EHEC members have a common set of virulence factors which account for their enhanced pathogenicity in humans. These include the Shiga toxins 1 and 2, several effector proteins encoded by the LEE, and EHEC-hemolysin (Kim et al., 1999)

1.2.4.1 Evolution of EHEC

Pioneering work on the evolution of *E. coli* O157:H7 by Whittam et al. (1993) using a method based on allelic variation among 20 enzyme-coded genes detected by multilocus enzyme electrophoresis, revealed that *E. coli* O157:H7 formed a separate clonal population only distantly related to other STEC and that it probably evolved from an O55:H7-like enteropathogenic *E. coli* (EPEC) progenitor cell that already had acquired the LEE island. According to the proposed stepwise evolutionary model, this EPEC O55:H7 ancestor was lysogenized by Shiga toxin-converting phages, followed by a serotype switch via the acquisition of genes within the *gnd* region and subsequent acquisition of the large pO157 plasmid leading to the emergence of *E.coli* O157:H7 (Feng et al., 1998).

Based on population genetic studies, extant EHEC strains are believed to have derived from two distinct lineages (Whittam et al., 1993). The EHEC 1 lineage is composed of only closely related strains of the serotype O157:H7 whereas the EHEC 2 lineage, which is only distantly related to the EHEC 1 lineage, is much more diverse, both serotypically and genotypically (Whittam et al., 1993; Boerlin et al., 1998; Feng et al., 1998). The EHEC 2 lineage is primarily composed of the serotypes O111:H8, O111:H-, O26:H11, and O26:H-even though strains with many different O:H combinations, including some nontypable strains, fall into this group (Donnenberg and Whittam, 2001). The emergence of the EHEC 2 lineage is hypothesized to have begun with the acquisition of a LEE island located at the *pheU* site (in contrast, members of EHEC I have the LEE near the *selC* gene) (Donnenberg and Whittam, 2001). The subsequent evolution process is believed to have involved multiple gains and losses of Shiga toxin genes and pathogenicity islands.

An ancestral O26:H11 strain is thought to have acquired a *stx1* phage and an EHEC plasmid, giving rise to the EHEC O26:H11 clone. The same O26:H11 ancestor is also posited to have experienced an antigenic shift to O111, resulting in the EHEC O111 clone (Donnenberg and Whittam, 2001).

1.2.4.2 EHEC virulence

Although composed of different *E. coli* serotypes, members of EHEC 1 and EHEC 2 lineages have similar virulence factors (Ogura et al., 2009) and, as a result, similar pathogenic potential. All EHECs have much larger genomes (5.5 – 5.9 Mb) than nonpathogenic *E. coli* and contain unusually large numbers of prophages and integrative elements (Ogura et al., 2009). Based on their comparison of the genomes of EHEC O157:H7 and three non-O157 EHECs (O26, O111, and O103), Ogura et al. (2009) found that many lambdoid phages, integrative elements, and virulence plasmids carried similar virulence genes among these EHECs, but that they had distinct evolutionary histories. This suggested independent acquisition of these mobile genetic elements, leading to the parallel evolution of virulence among O157 and non-O157 EHEC strains (Ogura *et al*, 2009).

1.3 STEC Pathogenicity and Virulence Factors

1.3.1 Shiga toxins

The principal virulence factor associated with STEC pathogenesis is Shiga toxin (Stx) (Ritchie et al., 2003). There are two major types of Stx known as Stx 1 and Stx 2 (carried by lysogenic bacteriophages), with each having several antigenic variants (Gyles, 2007). Stx is composed of five identical B subunits that are responsible for binding the holotoxin

to the glycolipid globotriaosylceramide (Gb3) receptors and a single A subunit that cleaves ribosomal RNA (rRNA), which results in inhibition of protein synthesis (Melton-Celsa and O'Brien, 1998). Stx produced in the human colon can travel via the blood stream to the kidney, where it damages renal endothelial cells and occludes the microvasculature, resulting in renal inflammation (Kaper et al., 2004). This damage can lead to development of the hemolytic uremic syndrome (HUS), especially in children < 5 years old and in the elderly (Fuller et al., 2011).

1.3.2 Locus of enterocyte effacement (LEE)

In addition to Stx, STEC strains associated with the more severe forms of STEC disease, such as HUS, tend to possess accessory virulence factors.Among these, the pathogenicity island known as the locus of enterocyte effacement (LEE) is one of the most prominent. The LEE contains genes which encode for a type III secretion system and effector proteins that enables intimate adherence of the bacterial cells to colonic epithelial cells (Kaper et al., 2004). The tight adherence is mainly due to the adhesin called intimin, encoded by the *eae* (*E. coli* attaching and effacing) gene (Goosney et al., 1999). There are 19 variants of this gene and this variation may result in specificity for different host tissues (Bolton, 2011). The receptor for intimin is known as the translocated intimin receptor (TIR), and both intimin and TIR are encoded by the LEE pathogenicity island (Perna et al., 1998). The LEE-encoded factors induce profound structural modifications in underlying epithelial cells, resulting in the formation of attaching and effacing (A/E) lesions. A/E lesions involve ultrastructural changes, including loss of enterocyte microvilli ('effacing') and intimate attachment of the bacterium to the cell surface.

Beneath the adherent bacteria, accumulation of cytoskeletal components occurs, leading to the formation of characteristic ‘pedestals’ (Paton & Paton, 1998).

1.3.3 O Island 122 (OI-122)

O island 122 is a 23,092-bp genomic island composed of 26 open reading frames (ORFs), including those showing significant homology to virulence genes such as *Salmonella enterica* serovar Typhimurium *pagC*, *Shigella flexneri* enterotoxin 2 and the EHEC factor for adherence (*efa1*), which is also referred to as lymphocyte inhibition factor (*lifA*) (Karmali *et al*., 2003). This pathogenicity island is present in *E. coli* O157:H7 and in many non-O157 STEC strains that are associated with outbreaks and HUS (Wickham et al., 2006).

1.3.4 Virulence plasmids

Most pathogenic STEC also possess a highly conserved plasmid such as pO157, pSFO157, and pO113 (Grant et al., 2011). Initially identified in *E. coli* O157:H7, pO157 is a 92-kb F-like plasmid composed of segments of putative virulence genes (Burland et al., 1998). These potential virulence genes include those encoding a potential adhesin (ToxB), Enterohemorrhagic *E. coli* (EHEC)-hemolysin, and a serine protease (EspP) (Grant et al., 2011). ToxB is thought to contribute to the adherence of EHEC to epithelial cells through promoting the production and/or secretion of type III effector proteins (Tatsuno et al., 2001). EspP may be involved in downregulation of complement and influence EHEC colonization of the human gut (Orth et al., 2010). The EHEC-hemolysin is related to α-hemolysin, and its toxicity is due to the disruption of permeability of cytoplasmic membranes of target mammalian cells (Grant et al., 2011). The pO113 mega

plasmid is known to carry genes such as *saa* and the operon *lpf* which encode the putative adhesins Saa (STEC agglutinating adhesin) and long polar fimbriae (LPF), respectively (Bolton, 2011). HUS-causing STEC strains which lack the LEE pathogenicity island are believed to colonize the human gut by making use of these putative adhesins encoded on pO113 (Paton et al., 2001; Vidal et al., 2008; Bolton, 2011).

1.3.5 Non-LEE-encoded effectors

The LEE was initially assumed to represent a self-contained unit, containing not only the genes for the type III secretion system (TTSS), but also all of the effectors that might be secreted through the system (Tobe et al., 2006). However, in a proteomic analysis of proteins secreted by the LEE-encoded TTSS, Gruenheid et al. (2004) identified a novel protein which was encoded in a prophage-associated pathogenicity island at a site distinct from the LEE but translocated through the TTSS. As a result, this protein was named non-LEE-encoded effector A (NleA). Subsequent studies have found >20 putative or proven non-LEE effector proteins. Tobe et al. (2006) noted that the majority of functional effector genes were encoded by exchangeable effector loci that lie within lambdoid prophages. The closely related enteropathogenic *E. coli* (EPEC) also code for non-LEE encoded effectors, although the effector repertoire is smaller than that of STEC (Dean and Kenny, 2009). NleA has been shown to be essential for virulence in the EHEC-related pathogen *Citrobacter rodentium* in a mouse model (Gruenheid et al., 2004) as has NleB (Wickham et al., 2006) while other non-LEE encoded effectors such as NleH seem to have ‘accessory’ functions indirectly related to virulence such as blocking apoptosis of infected cells (Hemrajani et al., 2010).

1.3.6 Markers of increased risk to humans

The pathogenicity of an STEC strain depends on production of key virulence factors. Although the precise set of virulence factors necessary to cause STEC-related disease in humans has not been strictly defined, associations between carriage of certain genes and the ability to cause severe disease in humans have been made (Arthur *et al*., 2002). Several studies have indicated that STEC strains carrying *stx2* alone were more likely to cause severe disease compared to STEC strains carrying *stx1* or both *stx1* and *stx2* (Boerlin *et al*., 1999; Ostroff *et al*., 1989). However, it is not known whether the association of Stx2 with HUS is due to the action of Stx2 itself or whether it's simply a marker for increased disease severity, although it has been shown that Stx2 is about 1,000× more toxic to renal microvascular endothelial cells than is Stx1 (Gyles, 2007; Louise et al., 1995). In addition to *stx2*, the LEE-associated *eae* (codes for intimin) and EHEC *hlyA* (EHEC hemolysin) have also been found in a high proportion of STEC strains causing human disease (Acheson, 2000; Beutin *et al*., 1998; Bonnet *et al*., 1998; Eklund *et al*., 2001; Gyles *et al*., 1998; Schmidt *et al*., 1995; Boerlin *et al*., 1999; Ethelberg et al., 2004). Thus, the carriage of the combination of *stx2*, *eae*, and *hlyA* is considered a good indicator of the pathogenic potential of STEC strains (Meng et al., 1998). However, neither *eae* nor *hlyA* appear to be essential for pathogenicity as clinical isolates lacking these factors have been reported (Paton et al., 1999; Ritchie et al., 2003).

Wickham et al. (2006) carried out a study to determine the genetic determinants of non-O157 STEC associated with HUS and outbreaks. The main targets of this study were the genes that were part of O island 122 (OI-122). The OI-122 genes *pagC, Z4322, ent, nleB, nleE* and *efa1/lifA* were more prevalent in HUS-associated non-O157 STEC strains while

Z4323, ent, nleB, nleE and *efa1/lifA* were each more prevalent in non-O157 STEC strains associated with outbreaks. These virulence determinants were also encountered in all the *E. coli* O157:H7 strains investgated in this study. The authors further posited that the additive effect of a variable repertoire of virulence determinants in a particular STEC strain governed its disease-causing potential (Wickham et al., 2006).

In a molecular risk assessment aimed at identifying non-O157 STEC virulence factors associated with public health risk, Coombes et al. (2008) identified three genomic islands encoding non-LEE effector genes and 14 individual *nle* genes that correlated independently with outbreak and HUS potential in humans. The same authors also suggested that pathogenicity islands as well as non-LEE effectors may contribute additively to non-O157 STEC virulence (Wickham et al., 2006).

1.4 Routes of Infection

Direct contact: Both O157 and non-O157 STEC are known to have caused infections in humans as a result of direct contact with animals or their environment. *E. coli* O157:H7 in ruminant feces may be directly ingested by persons working or interacting with animals (Doyle et al., 2006). Several non-O157 STEC outbreaks among children who visited farms or petting zoos have also been reported (Akiba et al., 2005; Hanna et al., 2007; Stephan et al., 2008; Kaspar et al., 2010). Inadequate hand washing following contact with animals and/or their surroundings was the major cause for these illnesses. Person-to-person spread of STEC has been the primary mode of infection in outbreaks involving day-cares, schools, senior-care facilities and hospitals, especially where there have been lapses in hygiene (Doyle et al., 2006; Anon, 2008; Anon, 2009; Brooks et al.,

2005; Combs et al., 2003; Belongia et al., 1993; Pennington, 2000; Reida et al., 1994; Kaspar et al., 2010). Contact with domestic animals, such as cats, has also been a route of STEC infection (Busch et al., 2007).

Contaminated Food: Meats such as beef, lamb, and mutton can be contaminated during slaughter and processing by exposure to feces or hides containing STEC. Similarly, milk from dairy cows, sheep, and goats can be contaminated with STEC, although these bacteria are destroyed during the pasteurization process (Kaspar et al., 2010). Thus, milk-related outbreaks of STEC are due to consumption of unpasteurized milk (Allerberger et al., 2003; Ammon, 1997; Deschênes et al., 1996) or post-pasteurization contamination (Moore et al., 1995). Manure and irrigation water contaminated with STEC can contaminate fruits and vegetables (Islam et al., 2005). This presents a risk when consuming those fruits and vegetables that are not normally cooked before eating. In addition, experiments done with *E. coli* O157:H7 has demonstrated the survival and growth of these bacteria in shredded lettuce, carrots, and cucumbers under the modified atmosphere conditions used in commercial packaging (Abdul-Raouf et al., 1993; Doyle et al., 2006).

Contaminated water: Water used for drinking or recreation has been reported as the source of several STEC outbreaks (Kaspar et al., 2010). Infected persons are likely the source of bacteria for the cases involving recreational water. Unchlorinated drinking water was implicated in a large O157 outbreak in Missouri (Swerdlow et al., 1992). Fecal material contaminated with STEC from domestic and/or wild ruminant animals may also have played a part in some of these water related outbreaks (Doyle et al., 2006).

1.5 Reservoirs of STEC

Ruminants are the major reservoir for STEC O157 and may be an important reservoir for non-O157 STEC as well (Kaspar et al., 2010; Smith et al., 2014). Among ruminants, cattle are thought to be the most important reservoir (Doyle et al., 2006), although STEC have also been isolated from other ruminants such as sheep, goats and deer (Doyle et al., 2006; Kaspar et al., 2010). Sheep have been shown to harbor a diverse number of STEC serotypes (Kaspar et al., 2010). However, *E. coli* O157:H7 appears to be infrequently isolated and is probably a minor component of the total STEC load in sheep (Kaspar et al., 2010).

Non-ruminant animals such as swine and horses are also known to carry STEC. In swine, the STEC strains usually isolated are associated with edema disease of those animals and the strains are usually specific for pigs (Gannon et al., 1988; Fratamico et al., 2004). Thus, although virulent strains of STEC, including *E. coli* O157:H7, have occasionally been isolated from swine, these animals are not considered important in the transmission of human virulent STEC (Desrosiers et al., 2001). STEC are rarely isolated from poultry, although there have been occasions where poultry have tested positive for *E. coli* O157:H7 (Doyle et al., 2006).

STEC are also occasionally isolated from other wild and domestic animals but it is believed that these animals are transient hosts of these bacteria rather than true hosts (Kaspar et al., 2010). These animals may have acquired STEC from foods or water contaminated with fecal material from ruminants (Kaspar et al., 2010).

1.6 STEC, the beef industry, and federal regulation

Because of the well-known association of *E. coli* O157 with beef cattle and their products and the occurrence of non-O157 STEC in these animals, STEC have become an important food safety challenge to the beef industry as well as a concern for federal food safety regulators. According to the U. S. Centers for Disease Control and Prevention (CDC)'s Foodborne Outbreak Online Database (FOOD, wwwn.cdc.gov/foodborneoutbreaks/), between 1998 and 2012, 28.6% (123/430) of outbreaks associated with STEC were related to beef. Interestingly, only one of these beef-related outbreaks involved a non-O157 STEC serogroup (*E. coli* O26 outbreak originating from ground beef in June 2010).

The association of STEC with beef has invariably had a negative economic impact on the beef industry as well. The beef industry had an estimated $2.7 billion cost due to *E. coli* O157:H7 from 1993-2003 (Kay, 2003). Of this total expense, approximately 60% was thought to be due to loss in demand for beef due to consumer concerns over the safety of ground beef (Smith, 2014; Kay, 2003). Additional expenses due to implementation of strategies to prevent beef contamination by STEC and costs related to defending lawsuits has further added to the economic burden of the beef industry due to these pathogens (Smith, 2014; Kay, 2003).

E. coli O157:H7 was declared an adulterant in raw ground beef in August 1994 by the U. S. Department of Agriculture's Food Safety and Inspection Service (USDA, n. d.). According to this policy, raw chopped or ground beef products that contained *E. coli* O157:H7 required further processing to destroy these pathogens. In September 2011, the

FSIS announced that raw, non-intact beef products or raw, intact beef products that are intended for use in raw non-intact product that are contaminated with the 'Big Six' non-O157 STEC serogroups (O111, O26, O45, O145, O121, and O103) were also considered adulterated (USDA, 2011).

In response to the continued involvement of beef and related products in the transmission of STEC and in order to abide by regulatory requirements, the beef industry has adopted several intervention strategies to reduce STEC contamination of beef. Most of these control strategies have been targeted and validated for O157 STEC, although non-O157 STEC strains are also thought to exhibit similar susceptibility to these interventions (Kalchayanand et al., 2011).

Pre-harvest intervention strategies which have been tested include: feeding direct-fed microbials to cattle to competitively exclude colonization by STEC of these animals, (e. g. feeding *Lactobacillus acidophilus* NP51, Peterson et al., 2007), use of bacteriophages and vaccines to control these pathogens in live animals (Kalchayanand et al., 2011; Potter et al., 2004), and washing the hides of animals with water or other chemicals to reduce bacterial levels on hides before hide removal (Arthur et al., 2007; Bosilevac et al., 2005; Kalchayanand et al., 2011). Post-slaughter interventions have included the use of a sequence of treatments implemented at various processing steps. These treatments include hide-washing, steam-vacuuming, trimming, carcass washing, and subprimal treatment with various compounds (Kalchayanand et al., 2011). Effective carcass decontamination strategies have included the use of hot water, lactic acid, bromine compound washes, and steam (Koohmaraie et al., 2005; Kalchayanand et al., 2009). In

addition to their effect on *E. coli* O157:H7, some of these interventions have been shown to be effective against non-O157 STEC serotypes such as O26:H11 and O111:H8 as well (Cutter and Rivera-Betancourt, 2000; Kalchayanand et al., 2011). Novel technologies such as high hydrostatic pressure processing, pulsed electric field, electrolyzed water treatment, and irradiation have also been explored as intervention strategies (Kalchayanand *et al*., 2011).

1.7 Cattle as reservoirs of STEC

In North America, beef and dairy cattle are the most significant reservoir of STEC (Gyles, 2007) and based on published literature, more than 400 different serotypes of STEC have been recovered from cattle (Beutin et al., 1993; Blanco et al., 2004). Cattle are considered to be asymptomatic carriers of STEC since these animals lack the Stx receptor globotriaosylceramide (Gb_3) in their gastrointestinal tracts, and are thus protected from the effects of these toxins (Pruimboom-Breese et al., 2000). Understanding the prevalence and ecology of STEC among cattle and the factors which lead to the colonization of these animals by STEC can potentially lead to the development of on-farm intervention strategies to reduce STEC contamination of the food supply.

1.7.1 Prevalence of STEC among cattle

Prevalence rates of both O157 STEC and non-O157 STEC in cattle have been determined by various investigators, most of them involving the examination of individual or pooled bovine fecal samples of cattle at slaughter or on the farm (Gyles, 2007). Researchers have used multiple isolation and detection procedures in different studies due to a lack of a

standard efficacious procedure. This makes it difficult to compare different studies since the methodologies used in determining prevalence are not homogeneous. In addition, It has been shown that STEC O157 are excreted at higher frequency in warmer (summer) months and at lower frequency during the colder (winter) months (Chapman et al., 1997; Jenkins et al., 2002; Dunn et al., 2004). It has also been observed for some time that prevalence of STEC O157 is higher in younger animals and in animals subject to transit, feed changes, and antimicrobial therapy (Hancock et al., 1998; Stevens et al., 2002b). Thus these factors also need to be factored in when comparing different STEC prevalence studies.

For STEC O157:H7, the reported prevalences have ranged from 0.3-19.7% in feedlots and from 0.7% to 27.3% for cattle on pasture (Hussein, 2007). Less work has been done with regard to determining the non-O157 STEC prevalence in cattle, mainly due to limitations in detection and enumeration techniques. Nonetheless, reported non-O157 STEC prevalence rates have ranged from 4.7 to 44.8% in grazing cattle and 4.6 to 55.9% in feedlot cattle (Hussein and Bolinger, 2005; Kalchayanand et al., 2011).

A more recent study by Cernicchiaro et al. (2013) used two detection protocols to determine the prevalence of O157 STEC and the 'big six' non-O157 STEC in feces of commercial feedlot cattle. The first protocol involved performing an 11-gene multiplex PCR assay (which detects the O157 and the 6 major non-O157 serogroups as well as four virulence genes including *Stx*1 and *Stx*2) using purified total fecal DNA ('direct PCR' method) while the other protocol involved the use of immunomagentic separation using Dynabeads specific for serogroups O26, O103, and O111 followed by selective plating

on MacConkey agar ("culture-based method"). The direct PCR method results showed that serogroup O157 was the most prevalent with a prevalence rate of 48.2%. Among the non-O157 serogroups, O26 (23.4%), O121 (16.4%), and O103 (11.8%) were the most prevalent. However, these cannot be considered estimates for 'Shiga toxin-producing' members of these serogroups since it cannot be established whether the Shiga toxin genes also originated from the same serogroups. The culture-based method showed 30.5% prevalence for O26 and 29.7% and 10.1% prevalence for serogroups O103 and O111, respectively. Thus, more O26, O103, and O111 positive samples were detected by culturing than by direct PCR. Importantly, the authors reported that a large number of samples positive for the major O serogroups, by both culture-based and direct PCR methods, did not possess Shiga toxin genes, indicating that cattle harbor Shiga toxin–negative *E. coli* belonging to these seven major O serogroups (Cernicchiaro *et al*., 2013).

Studies have been conducted which have compared the prevalence of STEC among different cattle production types. Cobbold et al. (2004b) sampled cattle for STEC from 3 different cattle production systems: dairy, feedlot, and range cow-calf operations. The prevalence of both *stx* and STEC in fecal/environmental samples from feedlots was significantly lower than those from dairy and range operations (Cobbold et al., 2004b). In a comparison of the prevalence of STEC O157 and O26 among beef and dairy cattle in Japan, Sasaki et al. (2013) reported that the prevalence of STEC O157 was higher in beef cattle than in dairy cattle. The low isolation rate of STEC O26 from both types of animals precluded the researchers from carrying out statistically valid comparisons regarding the prevalence of this serogroup (Sasaki et al., 2013).

1.7.2 Factors affecting prevalence and levels of STEC in the farm environment

Several biological, environmental, and management factors have been identified that affect the incidence of *E. coli* O157 in cattle and in the production environment (Berry and Wells, 2010). These same factors may play a role in the prevalence and persistence of non-O157 STEC in these environments as well.

1.7.2.1 Seasonal variability of STEC

Season has been the one environmental factor which has consistently been shown to influence shedding of *E. coli* O157:H7 (Berry and Wells, 2010). Studies conducted on feedlot cattle in North America have shown that the greatest rate of STEC O157 carriage occurs during the warmer summer months while the lowest carriage rates typically occur in colder winter months (Smith et al., 2005; Renter et al., 2008; Van Donkersgoed et al., 2001). However, it has been reported that the prevalence of non-O157 STEC on hides was lower in winter, spring and summer and highest in fall (Barkocy-Gallagher et al., 2003). Research done in Scotland has shown a higher incidence of *E. coli* O157 among cattle during the winter months, although this is thought to be due to the practice of housing cattle during this period which may bring animals closer together thus increasing the chances of transmission (Ogden et al, 2004; Synge et al., 2003).

The precise reason(s) for an increase in prevalence of *E. coli* O157 during the warmer months is still not clear. The more favorable growth temperatures during summer were thought to influence the ability of these bacteria to replicate in environmental reservoirs such as feed or water (Hancock et al., 2001). However, studies have shown that cooler temperatures can enhance the persistence of *E. coli* O157 in water as well as in manures

and soils (Berry and Wells, 2010). Cattle heat stress has also been considered a potential cause of increased prevalence of O157 during the summer months, although clear evidence for this has not been presented (Berry and Wells, 2010). Seasonal variation in shedding has also been hypothesized to be due to physiological responses of the animal in response to changing day length (Edrington et al., 2006). Flies in the farm environment are known to be involved in the transmission of O157, and the warmer seasons result in an increase in the fly populations (*Ahmad* et al., 2007). However, any influence of flies on seasonal prevalence of *E. coli* O157 has not been demonstrated (Berry and Wells, 2010).

1.7.2.2 Age of cattle

Shedding of O157 STEC and some non-O157 STEC appear to be related to weaning and age of bovine animals (Gyles, 2007). Lowest rates have been shown to occur in calves before weaning, with highest rates in calves post-weaning and intermediate rates in adult cattle (Mechie et al., 1997; Shinagawa et al., 2000; Nielsen et al., 2002).

1.7.2.3 Impact of the environmental habitat

Based on studies done with STEC O157, several factors related to the farm environment appear to be related to the prevalence of STEC. In a study of cattle from 29 pens of 5 Midwestern feedlots, Smith et al. (2001) reported a higher prevalence of *E. coli* O157:H7 among cattle from ‘muddy’ pens compared to cattle from ‘normal’ pens. In other studies involving feedlot cattle, fecal prevalence was associated with the condition of the floor surface and with the presence of STEC O157 in other environmental samples such as fresh fecal pats, drinking water, etc. (Smith, 2014; Smith et al., 2005; Renter et al., 2008).

1.7.2.4 Impact of diet on STEC prevalence

Much of the work on the impact of diet on STEC shedding has been concentrated on STEC O157. Though there are many studies in the literature implicating various diets affecting O157 shedding, the results of these studies have often been conflicting or not repeatable (Jacob et al., 2009). The difference in prevalence observed between different diets has often been thought to be due to changes in hindgut ecology, particularly in pH and VFA concentrations (Jacob et al., 2009). The pH and VFA concentrations throughout the rumen and intestine are believed to be directly related to feed composition (Jacob et al., 2009).

Several studies have positively associated barley grain with *E. coli* O157 shedding in both experimental and observational settings (Jacob et al., 2009; Dargatz et al., 1997; Buchko et al., 2000; Berg et al., 2004). Berg et al. (2004) reported that cattle fed a barley grain diet shed higher concentrations of *E. coli* O157 and had a higher fecal pH when compared with animals fed a corn-based diet. The specific mechanism for the observed increase in shedding is not known, although changes in hindgut ecology is suspected (Jacob et al., 2009). Generally, a large percentage of starch (80-95%) is fermented in the rumen, and a significant proportion of the remaining starch undergoes digestion in the small intestine (Huntington, 1997). Starch that escapes ruminal and small intestinal degradation can undergo secondary fermentation in the large intestine, similar to ruminal fermentation (Ørskov et al., 1970). Barley has a lower concentration of starch than most other cereal grains (Huntington, 1997) and as a result is rapidly and efficiently digested in the rumen (Ørskov, 1986), leaving little starch available for secondary fermentation in the large intestine. Thus, cattle fed barley grain-based diets have an increased pH and

decreased volatile fatty acids (VFA) in the hindgut (Jacob *et al*., 2009) which may create a more conducive environment for O157 growth.

Garber et al. (1995) reported a negative correlation between whole cottonseed diets and fecal shedding of *E. coli* O157 in heifers. Other studies have shown no relationship between the two factors (Dargatz et al., 1997; Buchko et al., 2000).

Grain-processing method has also been reported to affect *E. coli* O157 prevalence in cattle (Fox et al., 2007). Heifers fed steam-flaked grains were reported to have higher O157 prevalence than heifers fed dry-rolled grain diets on most occasions. Depenbusch et al. (2008) also reported higher O157 prevalence in cattle fed steam-flaked grain diets compared with cattle fed dry-rolled grain diets for 30 days. However, Dewell et al. (2005) found no significant effect of grain processing on *E. coli* O157 prevalence in cattle.

Studies done with experimentally-inoculated cattle (and sheep) have shown that animals fed forage diets shed *E. coli* O157 in the feces for a longer duration than animals consuming grain-based diets (Kudva et al., 1997; Van Baale et al., 2004; Jacob et al., 2009). The general hypothesis for this observation is an increased ruminal and/or hindgut pH and decreased VFA content associated with forage diets (Jacob et al., 2009). In contrast, Diez-Gonzalez et al. (1998) reported significantly higher total *E. coli* concentrations in feces of cattle fed concentrate diets compared to cattle fed forage diets, although the relationship between generic *E. coli* and *E. coli* O157 populations is not known (Jacob et al., 2009). Diez-Gonzalez (1998) also observed that increased

concentrations of acid-resistant *E. coli* were found in cattle fed diets with grain than in cattle fed diets with no grain.

Several studies have reported an association between feeding distillers or brewers grains (ethanol co-products) and increased *E. coli* O157 prevalence in cattle (Synge et al., 2003; Dewell et al., 2005). Jacob et al. (2008) reported that cattle fed dried distillers grains with solubles (DDGS) at 25% of the final diet had a twofold higher prevalence of *E. coli* O157:H7 than cattle not fed DDGS. According to a recent review by Wells et al. (2014), cumulative data indicates that high levels of distillers grain (i. e., fed at 40% or greater, dry matter basis) in the finishing diet of feedlot cattle appear to increase fecal and hide loads for *E. coli* O157:H7. However, it has been noted that although potential associations between dietary distillers grains and *E. coli* O157 prevalence and/or persistence in cattle have been well described, statistically significant associations have not always been found (Jacob et al., 2009).

The exact mechanism responsible for increased *E. coli* O157 shedding when distillers grains are fed to cattle is unclear. Two proposed possibilities are: (1) distillers grains may alter the hindgut ecology of cattle resulting in a more suitable environment for *E. coli* O157, or (2) a component of distillers grains stimulates *E. coli* O157 growth (Jacob et al., 2008). The high ruminal escape property of protein in dried distillers grain diets described by Klopfenstein et al. (2008) could provide more protein to the hindgut environment. Also, since the starch content of corn has been removed in distillers grains, this may result in less rumen fermentation compared to corn-based diets (Jacob *et al*., 2009).

1.7.3 The ecology of STEC in cattle

It seems the probability for cattle to carry STEC depends on both gastrointestinal tract (GIT)-associated conditions and environmental conditions which are regularly changing over time (Smith, 2014). All *E. coli* have two main habitats: a primary habitat in the lower GIT of warm-blooded animals and a secondary habitat in the outside environment (i. e., water, sediment, and soil; Smith, 2014). Factors such as cattle diet, immunological state, physiological state and interactions with other microorganisms in the cattle GIT can be expected to influence the suitability of the cattle primary environment for STEC colonization (Smith, 2014). The lower GIT of cattle is uniformly warm with an approximate temperature of 37 ^{0}C and is also rich in nutrients, which enable active growth of STEC, which then exit by bulk transfer to the secondary habitat (Smith, 2014).

1.7.4 STEC colonization of cattle

STEC O157:H7 has been shown to occur at the beginning (oral cavity) and the end (feces, rectoanal mucosa) of the bovine GIT. In studies done with experimentally challenged weaned calves, Brown et al. (1997) recovered *E. coli* O157:H7 from almost all sites sampled with the highest numbers being recovered from the fore stomach. Similarly, Cray and Moon (1995) demonstrated a ubiquitous STEC O157 distribution with the highest recovery rate in large intestinal sites. Contradicting these observations of a wide distribution of *E. coli* O157:H7 in the bovine GIT, Grauke et al. (2002) reported that these bacteria could not be recovered from rumen and duodenal cannulae samples after 16 days, even though some of these animals had STEC O157-positive fecal samples for up to 34 days. This seemed to suggest a large intestinal sight of colonization. Subsequently, Naylor et al. (2003) provided evidence of tropism of *E. coli* O157:H7 to

the mucosal epithelium within a defined region extending up to 5 cm proximally from the recto-anal junction (RAJ) of experimentally infected calves. The RAJ colonization by EHEC O157:H7 was accompanied by the formation of characteristic attaching and effacing (A/E) lesions. However, in a later study involving naturally STEC shedding cattle, Keen et al. (2010) managed to isolate *E. coli* O157:H7 from throughout the bovine GIT, including the tonsils, reticulum, rumen, omasum, abomasum, duodenum, jejunum, cecum, spiral colon, rectum, and even the liver, suggesting STEC O157 is broadly adapted to many cattle GI microhabitats. An early study looking into the rumen as a potential source of *E. coli* O157:H7 contamination at harvest had noted the growth inhibition of these bacteria in well-fed animals (Rasmussen et al., 1993). Subsequent research has also indicated that the rumen is not a likely reservoir for *E. coli* O157:H7 (Berry and Wells, 2010).

Extensive bacterial adherence to the colonic epithelium of calves by the non-O157 STEC serogroups O5, O26, and O111 has been observed (Hall et al., 1985; Pearson et al., 1999; Stevens et al., 2002c). Studies carried out using bovine tissue explants of calves have shown that *E. coli* O26 and O111 are also capable of binding at the RAJ (Girard et al., 2007). Van Diemen et al. (2005) showed that *E. coli* O26 strains had the capacity to colonize the spiral colon of 4-day old calves. In a previous study, Cobbold and Desmarchelier (2004) had developed a quantitative colonization assay to comparatively measure attachment of STEC to bovine mucosal tissues maintained *in vitro*. No significant differences were noted in the numbers of STEC colonizing tissues from weaning or adult cattle, or from cattle fed either forage or grain-based diets. However, of the STEC serogroups used in the study, the counts for STEC O157 were greater than

those for O26 and O111. The authors also looked at the impact of the volatile fatty acids (VFA) acetate, propionate and butyrate on STEC colonization. The presence of high concentrations of VFA (120 mM) resulted in a reduction in STEC colonization, regardless of VFA composition. Based on this observation, the authors suggested that under conditions where large amounts of VFA are being produced, there may be a reduction in STEC adherence to the gut wall, and therefore a potential reduction in STEC carriage (Cobbold and Desmarchelier, 2004).

1.7.4.1 Factors affecting STEC colonization of cattle

The bacterial factors of the locus of enterocyte effacement (LEE) pathogenicity island (such as intimin and Tir) of EHEC and their contribution to the formation of attaching and effacing lesions have been demonstrated to play an important role in the persistent colonization of the bovine distal gut (Naylor et al., 2005). Intriguingly, different intimin subtypes are able to confer a tropism for different intestinal sites (Phillips and Frankel, 2000). However, the LEE has not been found in all STEC which have been isolated from diarrheagenic calves and healthy cattle, suggesting the involvement of other factors in the colonization process (Stevens et al., 2002a; Wieler et al., 1996; Sandhu et al., 1996).

The EHEC factor for adherence (*efa1*) gene has been identified as mediating the colonization of the bovine intestine by non-O157 STEC (Stevens et al., 2002c). Mutation of this gene in STEC O5 and O111 was shown to significantly reduce fecal shedding and adherence to the colonic epithelium in experimentally infected calves. Almost all non-O157 STEC tested seem to possess the *efa1* gene (Nicholls et al., 2000) while STEC O157 appear to possess a truncated version of this gene (Stevens et al., 2002c). These

observations have led to the suggestion that O157 and non-O157 STEC may potentially use different strategies to colonize the ruminant host (Stevens et al., 2002b).

1.7.4.2 Host animal responses to STEC infections

Colonization of cattle by STEC is believed to result in asymptomatic infection in adult cattle (Pruimboom-Brees et al., 2000). However, studies based on STEC O157 have shown that following STEC infection, inflammation and innate and adaptive immune responses occur in cattle of all ages (Moxley and Smith, 2010; Smith, 2014). In calves, STEC are actually considered to be pathogens as infection tends to result in diarrheagenic conditions in these animals (Moxley and Smith, 2010). Natural and artificial infection of susceptible calves with bovine virulent STEC strains has been shown to produce diarrhea, villous atrophy, epithelial cell damage, and infiltration of neutrophils into the lamina propria among other clinical manifestations (Stevens et al., 2002b). Dean-Nystrom et al. (1997) also showed that infection of neonatal colostrum-deprived calves with STEC O157 results in diarrhea and colonic oedema (Dean-Nystrom et al., 1997). Generally, the duration of infection is short-lived, about a month, and reinfection is common in the field environment (Khaitsa et al., 2003).

1.8 Human health risk of STEC isolated from cattle

Most of the STEC serotypes that have been isolated from cattle or beef appear to be of minimal or insignificant health risk to humans (Kalchayanand et al., 2011). As noted previously, the presence of the combination of *stx2*, *eae*, and *hlyA* in an STEC isolate is considered a good indicator of its pathogenic potential in humans (Meng et al., 1998). In a survey of 361 non-O157 STEC isolates from beef carcasses, Arthur et al. (2002)

reported that 40 (11%) of the isolates possessed the above mentioned combination of virulence genes indicating potential human pathogenicity.

In a review of the published literature spanning a 25 year period (1982 – 2006), Hussein (2007) revealed that out of 373 serotypes isolated from beef cattle, 65 had previously been isolated from HUS patients and a further 62 were known to cause human illnesses.

Research done over the past one-and-a-half decades has shown that STEC O157:H7 strains are non-randomly distributed among human and cattle isolates. Using an octamer-based genome scanning (OBGS) approach, Kim et al. (1999) were able to reveal the presence of two distinct lineages of *E.coli* O157:H7 which were disseminated among cattle in the United States and also that human and bovine isolates were distributed non-randomly among these two lineages. Based on OBGS analysis of human isolates from 9 states and dairy cattle isolates from 16 different states, it was shown that the isolates constituted a monophyletic lineage that has diverged into two distinct populations, one comprising the majority of human isolates (lineage 1) and the other containing most of the cattle isolates (lineage 2).The authors have suggested that this nonrandom distribution of isolates among the two lineages may reflect differences in human virulence or efficiency of transmission to humans from bovine sources (*Kim* et al., *1999*) .

Evidence has also been presented for differences in Shiga toxin (Stx) production between HUS-associated and bovine-associated STEC strains. In a study involving multiple STEC serotypes, Ritchie et al. (2003) observed that basal Stx production by HUS-associated STEC exceeded that of bovine-associated STEC. In addition, the authors also observed that the induction of both Stx 1 (low-iron induced) and Stx 2 (mitomycin C induced)

production was more marked for HUS-associated STEC than for bovine-associated STEC (Ritchie et al., 2003).

In an interesting study by Bono et al. (2007), polymorphisms in the LEE-encoded genes *tir* and *eae* from STEC O157:H7 isolates from clinically ill humans and healthy cattle were identified and these identified polymorphisms were tested for association with human (vs bovine) isolate source. Out of 5 polymorphisms identified in a segment of *tir*, alleles of polymorphisms *tir* 255 T>A and repeat region I –repeat unit 3 (RRI –RU3, presence or absence) were observed to have dissimilar distributions among human and bovine isolates. Remarkably, more than 99% of 108 human isolates possessed the *tir* 255 T>A T allele and lacked RR1-RU3 (Bono et al., 2007). In contrast, only 55% of 77 bovine isolates had the *tir* 255 T>A T allele. This provides evidence for the potential use of the *tir* 255 T>A T allele as a marker for identifying human virulent strains of STEC O157:H7 (Bono et al., 2007).

1.9 The bovine gut microbiota

The microbial populations inhabiting the GI tract of cattle play an important role in ensuring the health and well-being of these animals, and much work has been done regarding the microbes and their contribution to digestion in the pregastric compartments of the reticulorumen (Russell and Rychlik, 2001). However, much less is known about the microbiota of other compartments of the bovine gastrointestinal tract, such as the large intestine (Wells et al., 2014).

The early studies which examined the cattle microbiota were based on traditional microbiological culture methods (Dowd et al., 2008). However, these culture-dependent

methods are limited since only a small percentage of the microbial community of a given environment is able to grow in laboratory growth media (Spiegelman et al., 2005). Culture-independent methods, such as 16S rRNA gene-based deep sequencing, are capable of identifying community members that are recalcitrant to culture, thus enabling a broader understanding of the microbial communities inhabiting the bovine GIT (Durso et al., 2010).

Several studies in the recent literature have taken a sequencing-based, culture-independent approach to the characterization of microbial communities of the cattle GIT. In a full-length 16S rRNA gene-based Sanger sequencing survey of the fecal microbiota[1] of beef feedlot cattle, Durso et al. (2010) identified the bacterial phylum Firmicutes as being the most abundant, with Bacteroidetes and Proteobacteria being the other abundant phyla. At the genus level, *Prevotella* was the most common. This study further identified a 'core' set of bovine GIT bacterial taxa, composed of the Bacteroidetes members *Prevotella* and *Bacteroides*; the Firmicutes *Faecalibacterium*, *Ruminococcus*, *Roseburia*, and *Clostridium*; and the Proteobacterium *Succinivibrio*. Based on comparisons with published work on the microbial community composition of dairy cattle, the authors suggested that although beef and dairy cattle seemed to share many of the same major bacterial groups, the relative abundances of these groups were different among the two types of cattle. In addition, animal-to-animal variation in fecal microbial communities was observed which cannot be attributed to breed, gender, diet, age, or weather (Durso et al., 2010). Sanger sequencing of 16S rRNA clone libraries has also been used to

[1] Although the 'microbiota' includes different types of microorganisms including Archae, viruses, fungi, etc., for the purpose of this thesis, only the bacterial component of the fecal microbiota is considered.

investigate the effects of feeding dietary monensin on the bacterial population structure of dairy cattle colonic contents (McGarvey et al., 2010).

Few studies have used next generation sequencing to evaluate the bovine fecal microbiota. Using 16S rDNA bacterial tag-encoded FLX amplicon pyrosequencing (bTEFAP) to characterize the fecal microbiota of 20 commercial, lactating dairy cows, Dowd et al. (2008) reported that the most common genera identified were *Clostridium, Bacteroides, Porphyromonas, Ruminococcus, Alistipes, Prevotella, Lachnospira, Enterococcus, Oscillospira, Cytophaga, Anaerotruncus,* and *Acidaminococcus*. Callaway et al. (2010) used bTEFAP to study the change in ruminal and fecal microbial populations in cattle fed diets containing 0, 25, or 50% dried distillers grain (DDGS). Members of the genus *Prevotella* accounted for 18.2% of the total ruminal population while the genus *Clostridium* predominated the fecal microbial population (19.7% of total population). Some genera such as *Megasphaera*, *Butyrivibrio*, *Ruminobacter*, *Cytophaga*, *Roseburia,* and *Selenomonas* were detected exclusively in the rumen samples. Across all 3 diets, more than 400 different bacterial species belonging to 56 genera were detected in the rumen samples. For the fecal samples, over 540 different bacterial species corresponding to 94 genera were observed. Compared to the diet without DDGS, cattle fed 50% DDGS had a reduced level of *Succinivibrio* (not statistically significant) and an increased population of *Bacteroides* which reached statistical significance. In the fecal samples, only levels of *Acinetobacter* showed a statistically significant increase in response to DDGS feeding (Callaway et al., 2010). The 454 GS FLX pyrosequencing platform was used by Shanks et al. (2011) in a study which looked into the influence of animal management practices on the fecal microbiota of cattle from 6 different feeding

operations. The six different cattle populations came from four different geographic locations and were organized into three management groups: forage group, processed-grain group, and unprocessed-grain group. A total of 633,877 high-quality sequences, covering the V6 hypervariable region of the bacterial 16S rRNA gene, were obtained from 30 beef cattle fecal samples, with 5 animals representing each cattle feeding operation. Similar to other studies, the most abundant members of the fecal microbiota were those of the phyla Firmicutes and Bacteroidetes, while Tenericutes and Proteobacteria were the next most abundant phyla. This study revealed that the bacterial community composition correlated significantly with fecal starch concentrations, which was largely reflected in changes in the Bacteroidetes, Proteobacteria, and Firmicutes populations. The Firmicutes decreased in abundance across a starch concentration gradient whereas the Bacteroidetes increased across the gradient. It was also noted that, in contrast to some other studies which noted significant animal-to-animal variation in terms of bacterial community structure, animals from a given management grouping shared a highly similar fecal microbiota. In conclusion, it was deemed that bovine fecal bacterial communities can be dramatically different in different animal feeding operations, and that the feeding operation is a more important determinant of the cattle microbiome than is the geographic location of the feedlot (Shanks et al., 2011). Barcoded DNA pyrosequencing was also used in a later study which compared the fecal microbiota of beef steers fed different levels of wet distillers grains (Rice et al., 2012). A total of 24 bacterial phyla were observed distributed across all animals on all diets, revealing a considerable amount of animal-to-animal variation. Six phyla were observed in all animals regardless of dietary treatment and were considered as core phyla. These

phyla were Firmicutes, Bacteroidetes, Proteobacteria, Tenericutes, Nitrospirae, and Fusobacteria (Rice et al., 2012).

A recent study by Kim et al. (2014) investigated the fecal bacterial diversity of cattle fed different diets (high grain, moderate grain and silage/forage) using the 454 GS FLX Titanium pyrosequencing platform. Firmicutes and Bacteroidetes were the dominant phyla observed in all fecal samples. It was reported that about 6% of the cleaned sequences could not be classified into known phyla. Members of the genera *Oscillobacter, Turicibacter, Roseburia, Faecalibacterium, Coprococcus, Clostridium, Prevotella,* and *Succinivibrio* were the most commonly observed, with *Prevotella* being the most dominant genus, representing 6.99% of all sequences. The greatest bacterial diversity was observed for the moderate grain diet while the lowest diversity was observed for the high grain diet. Out of a total of 176,692 OTUs only 2,359 (1.3%) were shared across all three diets. The authors concluded that bacterial communities in cattle feces were dramatically affected by diet, particularly between forage- and concentrate-based diets (Kim et al., 2014).

Based on the studies mentioned above, it appears that Firmicutes, Bacteroidetes and, to a lesser extent, Proteobacteria are the predominant bacterial phyla of the bovine gut microbiota, regardless of cattle types and diets. This implies that these core taxa are involved in performing fundamental metabolic functions essential to the collective cattle microbiota (Shanks et al., 2011).

1.10 The bovine gut microbiota and STEC shedding

The bovine gut consists of complex microbial communities which are constantly competing with each other for colonization space and nutrients. This raises the question as to whether other autochthonous microbes play a role in the colonization of the cattle gut by STEC. Some *in-vitro* studies have shown that intestinal microbial communities can negatively impact the growth of STEC, although these studies were not done in the context of the bovine gut microbiota (Poole et al., 2003; Kim and Jiang, 2010; Momose et al., 2008). Nutritional competition between indigenous microbial communities and STEC has been suggested as a possible mechanism for the observed growth inhibition (Momose et al., 2008).

Few studies have looked at the influence of the gut microbiota on fecal shedding of STEC *in vivo* in cattle. Using denaturing gradient gel electrophoresis (DGGE) coupled to polymerase chain reaction (PCR), Zhao et al. (2013) assessed the effects of the fecal microbiota on total STEC shedding in young calves and their dams. The results showed that bacterial diversity increased as cattle age increased, which corresponded with lower STEC shedding levels and prevalence. This led to the inference that a high-diversity bacterial community might be a factor that influences STEC survival, attachment, and shedding in the bovine intestine. A negative correlation was observed between the butyrate-producing bacterium *Anaerostipes butyraticus* and STEC shedding, with a high abundance of this bacterium found in low level STEC-shedding animals. A similar negative correlation was also observed between the expression of genes related to butyrate synthesis by the microbial community and STEC shedding. This led the authors

to suggest that a high concentration of butyrate-producing bacteria might play a role in controlling STEC shedding by bovine animals (Zhao et al., 2013).

1.11 Fecal shedding patterns of STEC by cattle

Fecal shedding of STEC by cattle is probably the most important means through which these bacteria contaminate the farm environment. Fecal contamination of the farm or feedlot environment causes cyclic colonization of ruminant animals and aids in persistence of these pathogens in these environments (Kalchayanand et al., 2011). Most of what is known about STEC shedding patterns in cattle is based on what is known through studies focused on *E. coli* O157:H7.

1.11.1 Super-shedders

Research conducted with cattle has shown that, within a herd, some animals tend to excrete *E. coli* O157 at levels as high as $> 4 \times 10^7$ CFU/g of feces whereas in a majority of animals the concentrations are less than 10 – 100 CFU/g (Fegan et al., 2004; Widiasih et al., 2004). The term "super-shedder" has been used to describe the subset of animals which transiently shed O157:H7 STEC at levels $> 1 \times 10^4$ CFU/g of feces (Chase-Topping et al., 2008; Arthur et al., 2010). However, there is a lack of a formal definition for a super-shedder: reports in the literature have used cut-offs of $\geq 10^3$ or $\geq 10^4$ CFU/g of feces (Omisakin et al., 2003; Low et al., 2005; Robinson et al., 2004b; Ogden et al., 2004) and some have simply used outlying counts in their definitions of super-shedders (Bach et al., 2005). However, it is thought that an 'ideal' definition of a super-shedder should encompass both the concentration as well as the duration of shedding (Chase-Topping et al., 2008). This type of definition was used in a longitudinal study by Davis et

al. (2006) when they defined a super-shedding animal based on a mean fecal concentration of $\geq 10^4$ CFU/g as well as having at least 4 consecutive STEC O157:H7 positive recto-anal mucosal swabs.

Naylor et al. (2003) demonstrated that bovine animals colonized at the recto-anal junction of the terminal rectum shed high concentrations of *E. coli* O157:H7 in their feces for several weeks and that these animals contributed disproportionately to contamination of beef and the environment with these organisms. Subsequent studies have shown similar correlations between colonization at the RAJ and persistent shedding of *E. coli* O157:H7 by bovine animals (Rice et al., 2003). Based on these observations, Chase-Topping et al. (2008) hypothesized that super-shedders were the subset of animals that were colonized at the terminal rectum by *E. coli* O157:H7 and that, in contrast, in other animals which shed low levels of this organism, the bacteria were amplified in the feces during transient passage through the animal or colonized at sites other than the terminal rectum within the cattle gastrointestinal tract.

1.11.2 Non-O157 STEC super-shedders

As regards the non-O157 STEC, currently it is not known whether a 'super-shedder' phenomenon is associated with these serotypes as well. Menrath et al. (2010) published a report claiming to show the occurrence of non-O157 STEC 'super-shedders' in a 12-month study involving 133 dairy cows. However, the definition of a 'super-shedder' in this study was purely based on the duration of non-O157 STEC fecal shedding and not on the quantitative threshold ($> 1 \times 10^4$ CFU/g feces) commonly used to identify STEC O157 super-shedders. Thus, the 'non-O157 super-shedder' status of these dairy animals

is debatable. This study nevertheless demonstrated that some animals within the herd shed non-O157 STEC more persistently than others (Menrath et al., 2010).

High-levels of fecal shedding of STEC leads to an increased risk of beef carcass contamination by these pathogens and also results in an increased STEC load in the farm environment. Since run-off water from cattle farms may come into contact with vegetable crops and cattle manure is used as fertilizer, increased fecal shedding of STEC may impact the safety of produce as well. Therefore, understanding the factors which lead to the emergence of super-shedders and implementing strategies to minimize STEC fecal shedding by these animals will likely lead to increased safety of beef and other food products.

1.11.3 Factors leading to the emergence of super-shedders

Limited research has focused on the exact risk factors which lead to the emergence of a super-shedder (Chase-Topping et al., 2008; Xu et al., 2014). Potential factors include (i) the phylogenetic lineage or strain-specific characteristics of the strains being shed, (ii) the microbiota community composition at the RAJ, and (iii) the genotype and phenotype of the host animals, including innate and adaptive immune responses, as well as (iv) environmental factors such as route of transmission or exposure dose (Arthur et al., 2013; Chase-Topping et al., 2008).

1.11.3.1 Strain specific characteristics of E. coli O157:H7

In a study examining the risk factors associated with the emergence of super-shedders in Scottish farms, Chase-Topping et al. (2007) found an association between the *E. coli* O157 phage type (PT) 21/28 and super-shedders. It has been suggested that altered

regulation of the type III secretion system (T3SS) of PT 21/28 strains compared to other PT strains may enable these bacteria to better colonize and be excreted at higher levels (Chase-Topping et al. 2008). However, in a study by Arthur et al. (2013) which characterized *E. coli* O157:H7 strains from super-shedding cattle, PT 21/28 strains were not found among the 19 different phage types isolated, suggesting that this PT was not a common source of super-shedding in the United States. The authors further concluded that no exclusive *E. coli* O157:H7 genotype could be identified that was common to all super-shedder isolates (Arthur et al., 2013).

1.11.3.2 Super-shedding and the bovine gut microbiota

A recent study conducted by Xu et al. (2014) compared the fecal bacterial communities of 11 *E. coli* O157:H7 super-shedder and 11 non-shedder feedlot steers using 454 pyrosequencing. The data was analyzed using five different clustering methods to minimize the introduction of potential biases. The authors reported that super-shedders exhibited higher bacterial richness and diversity than non-shedders. Based on clustering of samples on Nonmetric Multidimensional Scaling (*NMDS*) plots and on analysis of similarity (ANOSIM) it was claimed that the super-shedders and non-shedders harbored different fecal bacterial communities. Furthermore, 72 operational taxonomic units (OTUs) were identified as differentially abundant between the two shedding phenotypes. Of these, 17 OTUs were enriched in the non-shedders while 55 were more abundant in the super-shedders. The authors posited that the particular microbial community in super-shedders may be capable of differentially degrading organic matter leading to a nutritional environment that is more favorable for the growth and proliferation of *E. coli* O157:H7 (Xu et al., 2014). However, an important limitation of this study was that it

sampled only 22 animals (11 super-shedders and 11 non-shedders) out of a total of 400 animals.

Although not directly related to STEC super-shedding in cattle, the involvement of the gut microbiota in the generation of super-shedders of *Salmonella enterica* Typhimurium (*S*. Typhimurium) has been demonstrated in a mouse model (Lawley et al., 2008). In this model, 129X1/SvJ mice provide a natural model of *Salmonella enterica* Typhimurium transmission. According to the model only the super-shedders shed high levels of *S*. Typhimurium (> 10^8 CFU/g) in their feces and, as a result, rapidly transmit infection. The development of the super-shedder phenotype was related, at the level of the bacterium, to the possession of the virulence factors *Salmonella* pathogenicity islands (SPIs) 1 and 2, as well as to the intestinal microbiota. The researchers demonstrated that treatment of mice with the antibiotics streptomycin and neomycin, which altered the indigenous intestinal microbiota, rapidly induced the super-shedder phenomenon in infected mice and predisposed uninfected mice to the super-shedder phenotype for several days (Lawley et al., 2008).

1.11.4 Importance of super-shedders in STEC O157 transmission

The importance of super-shedders stems from their perceived role in the increased transmission of STEC in cattle production systems. This may be through greater incidence or persistence of infection, excretion of greater concentrations of *E. coli* O157:H7, or a combination of these factors (Cobbold et al., 2007). One study showed that 9% of animals shedding *E. coli* O157:H7 at harvest contributed to over 96% of the total *E. coli* O157:H7 fecal load for the group (Omisakin et al., 2003). Studies done with

feedlot cattle have shown that cattle that did not shed *E. coli* O157:H7 over a study period were five-times more likely to have been housed in a pen that did not have a super-shedder in it (Cobbold et al., 2007). Similarly, in a study done by Arthur et al. (2009), 95% of feedlot pens containing at least one super-shedder were shown to have STEC O157 hide prevalence rates >80%. Stephens et al. (2009) showed that pens with animals carrying fecal pats inoculated with STEC O157 to simulate the presence of a super-shedder increased the likelihood of previously culture-negative cattle to transiently shed STEC O157. In a cross-sectional study of cattle groups from 474 cattle farms in Scotland, Matthews et al. (2006b) determined by relating *E. coli* O157 bacterial counts to infectiousness and fitting dynamic epidemiological models to prevalence data that approximately 80% of the transmission arises from the 20% most infectious individuals. However, the aforementioned study by Stephens et al. (2009) did not support this mathematical model-based finding that suggested super-shedders contribute the majority of the *E. coli* O157 load at the pen level (Stephens et al., 2009).

The presence of a super-shedder in a truckload of cattle on their way to harvest has been shown to increase the chances of carcass contamination with *E. coli* O157 in animals originating from that truckload (Fox et al., 2008).

Although the work presented above perceive super-shedders as important agents of STEC O157 transmission within cattle in the farm environment, other studies have shown conflicting results, questioning the importance of super-shedders in this capacity. Munns et al. (2014) identified *E. coli* O157:H7 super-shedders among a group of feedlot steers in a commercial feedlot, and transported these super-shedding animals to a research feedlot.

Freshly voided fecal pats from these animals were then enumerated for *E. coli* O157:H7 in the morning and evening for the first seven days and, subsequently, once a day for a further 19 days. Of the 11 super-shedders initially identified at the commercial feedlot, only five were confirmed as super-shedders after their arrival at the research feedlot, and none of the animals shed *E. coli* O157:H7 at super-shedder levels after 2–days at the research feedlot. Moreover, super-shedding was not consistent in fecal pats collected from the *same* individual at different times of the day. Based on the lack of consistency of super-shedding and the short duration of shedding observed in this study, the authors concluded that super-shedding cattle may not play as great a role in transmission and contamination of the feedlot environment by *E. coli* O157:H7 as has been previously proposed. The authors further suggested that super-shedding may be more a function of the time a sample is collected, rather than it being a function of the characteristics of the *E. coli* O157:H7 subtype shed or the host animal. Smith (2014) also noted the inconsistency of STEC O157 super-shedding and also pointed out that it is not yet understood whether super-shedding is a characteristic of certain cattle or merely a stage of pathogenesis that cattle transition through following infection.

1.12 Detection and enumeration methods for STEC

To study STEC to better understand their biological characteristics, it is essential to have robust methods by which these bacteria can be isolated, characterized, and enumerated from foods, host animals, and other sources. Many culture-based, immunological and molecular techniques are available for the detection and isolation of O157 STEC, which is in part due to its historical importance as a human pathogen but also because *E.coli* O157:H7 is a single, specific serotype. In contrast, as noted earlier, the non-O157 STEC

have generated attention relatively recently and are composed of many different serotypes with different biological characteristics. Thus, developing assays for the detection and enumeration of non-O157 STEC has been much more challenging, particularly as there are similar *E.coli* strains that are non-pathogenic (Grant et al., 2011).

1.12.1 Methods for detecting STEC in bovine feces

The common procedure used to detect STEC from cattle feces involves enrichment, direct plating of the enriched sample on to selective agar, followed by confirmation via polymerase chain reaction (Moxley, 2003).

The enrichment step is necessary especially if the target bacteria are present in low concentrations in the fecal samples. Both selective and non-selective enrichment media have been used for this step. Buffered peptone water and trypticase soy broth have been used as non-selective media (Pearce et al., 2004; Shaw et al., 2004). Selective enrichment broths, for example, those used for isolating STEC O157, contain antibiotics such as vancomycin, cefixime, and cefsulodin which repress the growth of the background bacteria (Moxley, 2003).

After enrichment, fecal samples may be tested for selected virulence genes and STEC O-serogroups as a means of screening samples in order to establish which fecal samples merit further isolation and testing (Paddock, 2013). Commonly, DNA is extracted and purified from a sub sample of the enrichment using commercially available kits and subsequently used as template DNA for PCR reactions. Multiplex PCR can be used to screen for several genes at the same time (e. g., *stx* genes and O-serogroup genes). However, since this is ‘total’ fecal DNA and not DNA from a pure culture of a single

bacterial species, it is not possible to say that genes detected by PCR originate from the same bacterium (Paddock, 2013).

Following enrichment, immunomagnetic separation can be used to isolate specific serogroups of STEC. Magnetic beads for O157 and the 'big six' non-O157 STEC are commercially available (Abraxis Inc., Warminster, PA). The final IMS preparation is then plated onto a selective medium such as sorbitol MacConkey agar for STEC O157 or Rainbow agar (Biolog Inc., Hayward, CA) and CHROMagar STEC (CHROMagar, Paris, France) for non-O157 STEC (Paddock, 2013). Incubation temperatures in the range of 37^0 C to 42^0 C have been used, with the optimal growth temperature for STEC O157 reported as 40^0 C (Nauta et al., 1999; Gonthier et al., 2001). Better detection limits for non-O157 STEC have been reported when incubated at 41^0 C (Gonthier et al., 2001).

After isolated colonies are obtained on the selective media following incubation, they still need to be confirmed as STEC colonies and may also need to be tested for the presence of virulence genes. Colony hybridization, which involves 'replica plating' onto a nitrocellulose/nylon membrane followed by hybridization with specific DNA oligo-nucleotides (Paton and Paton, 1998) is the most comprehensive way of testing and confirming all colonies growing on a plate. However, this method is time-consuming and is difficult to perform when a large number of samples are being screened (Paddock, 2013). Thus, in most studies a small number of colonies are sub-cultured and subsequently tested for STEC serogroup and virulence factors using multiplex PCR reactions such as those described by Bai et al. (2012).

1.12.2 *E. coli* O157 enumeration

In the past, enumeration of STEC O157 was carried out by using the most probable number (MPN) technique which provides an indirect estimate of the number of bacteria present in a sample (Barkocy-Gallagher et al., 2003). Major drawbacks of the MPN method are its time-consuming and labor-intensive nature which makes this technique less amenable for high-throughput processes (Brichta-Harhay et al., 2007). In contrast, direct plating methods are faster and provide an estimate of viable bacterial counts without the need for an enrichment step. The hydrophobic grid membrane filter method (HGMF) and the spiral plate count method (SPCM) have both been used to enumerate STEC O157 load in bovine fecal samples (Brichta-Harhay et al., 2007).

1.12.2.1 Spiral plate count method (SPCM)

This method is particularly suitable for the enumeration of STEC O157 from feces since it can be used with samples which have a high background microbial load (Brichta-Harhay et al., 2007). The homogenized sample is dispensed in a logarithmic spiral pattern on to the surface of a rotating agar plate with a larger amount of the inoculum in the center of the plate and a decreasing amount towards the edge of the plate, typically resulting in a 1000-fold dilution from the center to the outer edge of the spiral (Robinson et al., 2004b; Brichta-Harhay et al., 2007). Selective culture media such as Sorbitol MacConkey agar supplemented with cefixime and tellurite (CT-SMAC) and ntCHROM-O157 agar containing novobiocin and potassium tellurite have been used as the plating media (Omisakin et al., 2003; Brichta-Harhay et al., 2007).

In a study involving *E. coli* O157 spiked bovine fecal samples, Robinson et al. (2004b) reported a lower detection limit for the SPCM of 10^2 CFU/g of feces for direct plating. The count data was deemed most repeatable and accurate when over the range of 1.0 X 10^2 – 1.0 X 10^8 CFU/g feces. In a similar study in which the SPCM technique was used, Brichta-Harhay et al. (2007) also observed a lower detection limit of 2.0 X 10^2 CFU/g for *E. coli* O157 from cattle fecal samples with the counts being most reliable when the inoculum levels were $\geq$ 1.0 X 10^3 CFU/g.

1.12.3 Enumeration of total STEC

A recent publication by Zhao et al. (2013) used a direct plating method to enumerate total STEC (both O157 and non-O157 STEC) from dam and calf fecal samples using CHROMagar™ STEC medium (CHROMagar Microbiology, Paris, France). While the composition of this medium has not been made publicly available (Gouali et al., 2013) the selective mechanism of this chromogenic medium is not based on sorbitol fermentation but partly involves tellurite resistance (Hirvonen et al., 2012; Zhao et al., 2013). This medium had previously been evaluated for its performance characteristics in isolation of STEC from human fecal samples (Hirvonen et al., 2012; Wylie et al., 2013; Gouali et al, 2013).

Hirvonen et al. (2012) used a collection of STEC, enteropathogenic *E. coli* (EPEC), enterotoxigenic *E. coli* (ETEC), and enteroaggregative *E. coli* (EAEC) strains, representing 49 different serotypes, to study the ability of CHROMagar STEC to support the growth of STEC and other diarrheagenic *E. coli* strains. The researchers also employed a collection of non-STEC strains and other microbes to investigate the

specificity of the medium. A high specificity of 98.9% was observed for the medium with only 3 non-toxin-producing isolates out of 186 *E. coli* strains growing as mauve color colonies. Other microbes were inhibited or grew as colorless or blue colonies. A low sensitivity was observed, however, for STEC strains which were *stx*-positive but *eae*-negative as only one-fifth of such isolates grew on the medium. In addition, only 49% of the different STEC serotypes used in this study actually showed characteristic growth. Interestingly, the authors observed that 97.4%of the non-O157 isolates grown on CHROMagar STEC formed fluorescent colonies when observed under UV light, whilst all the O157 colonies were non-fluorescent (Hirvonen et al., 2012).

Wylie et al. (2013) reported sensitivity and specificity values for CHROMagar STEC of 85.7% and 95.8% repectively, while the corresponding values in a study by Gouali et al. (2013) were 89.1% and 83.7%. Gouali et al. also noted that isolates that grew on CHROMagar STEC medium belonged to the most prevalent EHEC serogroups, including O157, O26, and O103, as well as to less common serogroups such as O118, O148, and O121. However, the authors also noted that certain non–O157 STEC serotypes (e. g., O148:H8 and O80:H2) as well as sorbitol-fermenting O157:H7 did not grow on this medium.

1.12.4 Detection of major virulent STEC serogroups using genetic markers

Recently, Neogen (Neogen Corp., Lansing, MI) introduced a novel assay for detecting pathogenic strains of the seven major STEC serogroups (O157, O145, O121, O111, O103, O45, and O26). Known as 'NeoSEEK^{TM} STEC Confirmation', this test is based on the Sequenom platform that the company currently uses for high throughput single

nucleotide polymorphism (SNP) genotyping (Hosking and Petrik, unpublished). This method relies on matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) spectrometry-based multiplexing (Hosking and Petrik, unpublished). The test is performed by looking for the presence/absence pattern of a particular set of target genes which include O-group, Stx 1 and 2, Eae, fliC, and other virulence associated genes. A total of 70 independent targets are assayed (Hosking and Petrik, unpublished). The number and types of targets assayed are able to provide enough evidence to make an identification of O-serogroup (if present) and whether the O-group(s) detected are associated with pathogenic strains (without the need for colony isolation) (Hosking and Petrik, unpublished).

In the current published literature, there is only a single study which compares the fecal bacterial communities of *E. coli* O157:H7 super-shedder and non-shedder beef cattle using a next generation sequencing approach (Xu et al., 2014). This study only focused on STEC O157 shedding and had the major limitation of having a very small sample size (only 22 animals in total). Furthermore, all the animals were fed a single diet which is not reflective of the 'real-world' situation where different types of finishing diets are used. This study by Xu et al. (2014) identified certain bacterial OTUs as being significantly different in abundance between super-shedders and non-shedders; However, since diet has a known influence on structuring bacterial communities in cattle (Kim et al., 2014), whether these findings can be extrapolated to animals fed a different diet(s) is unknown.

To address these gaps in knowledge, the current study investigated the fecal bacterial communities of over 300 beef steers from two separate sampling years to identify any

relationship of fecal bactrerial community structure and shedding of STEC (both O157 and non-O157 STEC). Because the lower gastrointestinal tracts of cattle, where STEC are believed to colonize, harbor complex resident bacterial communities which potentially interact with STEC, the hypothesis of this study was that there was an association between the fecal bacterial community composition of feedlot steers and shedding of STEC.

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Chapter 2

Impact of fecal bacterial communities on shedding of Shiga toxin-producing *Escherichia coli* (STEC) by beef steers

2.1 Introduction

Shiga toxin-producing *Escherichia coli* (STEC) are important zoonotic human pathogens which have a natural reservoir in ruminant animals, especially cattle (Gyles, 2007). In humans, complications due to STEC infections can range from mild self-limiting diarrhea to more serious conditions such as hemorrhagic colitis, hemolytic uremic syndrome (HUS) and even death (Paton and Paton, 1998). STEC are commonly divided into two major subgroups, the O157 STEC (e. g., *E. coli* O157:H7) and the non-O157 STEC, owing to differences in certain biochemical properties (e. g., ability to ferment sorbitol) and frequency of association with sporadic cases and disease outbreaks. In the United States, it is estimated that over 63,000 human disease cases due to O157 STEC and around 112,000 cases due to non-O157 STEC occur annually (Scallan et al., 2011).

E. coli O157:H7 (O157 STEC) is the best known member of the STEC group and was first implicated in foodborne disease in the early 1980's (Riley et al., 1983). Since then, several non-O157 STEC serotypes (e. g. *E. coli* O26:H11, O111:H8) have also been associated with human disease and have been recognized for their pathogenic potential which can rival that of the O157 STEC (Johnson et al., 2006). Chief among these are 6 major non-O157 STEC serogroups (O111, O26, O103, O45, O121, and O145), known as

the ‘big six’, which are said to account for >70% of non-O157 STEC isolates recovered from human cases in the United States (Brooks et al., 2005).

Cattle are a major reservoir for O157 STEC in the United States (Doyle et al., 2006) and are a known reservoir for non-O157 STEC as well (Smith et al., 2014). Fecal shedding of STEC by cattle is thought to be the main route through which these bacteria enter the environment (Callaway et al., 2013). In cattle, the colonization of STEC results in asymptomatic infection (Cray and Moon 1995). This is due to the fact that cattle lack vascular expression of the Shiga toxin receptor globotriaosylceramide-3 (Gb3) (Pruimboom-Brees et al., 2000). In contrast, humans express Gb3 on the vascular endothelium, which promotes much of the pathophysiology associated with Shiga toxin. Thus, the insensitivity to Shiga toxin enables cattle to be more tolerant hosts for STEC and may contribute to persistence and transmission of these human pathogens in the bovine reservoir (Pruimboom-Brees et al., 2000; Nguyen and Sperandio, 2012).

To the beef industry, STEC have been of particular concern due to the frequent association of beef and beef products as vehicles of STEC infection (CDC, 2014). As a result, along with *E. coli* O157, the ‘big six’ non-O157 STEC serogroups are now regulated as adulterants in certain raw beef products in the United States (USDA n. d., USDA, 2011). Compared to STEC O157, relatively little is known about the prevalence and pathogenicity of the non-O157 STEC in beef production systems.

Research conducted over the last decade regarding shedding of *E. coli* O157:H7 has revealed the heterogeneous nature of shedding by individual animals (Matthews et al. 2006). Certain animals within a herd, known as ‘super-shedders’, transiently shed *E. coli*

O157:H7 at levels >10^4 colony-forming units/g of feces (Arthur et al., 2010; Chase-Topping et al., 2008) and contribute disproportionately to the transmission of this pathogen among animals in cattle production and lairage environments, resulting in increased hide and subsequent carcass contamination (Arthur et al., 2010). It is currently unknown whether the super-shedder phenomenon extends to non-O157 STEC as well, although differences in persistence of shedding of certain non-O157 serotypes among dairy cattle has been observed (Menrath et al., 2010).

More than 400 STEC serotypes have been isolated from cattle (Gyles, 2007) and not all of them are equally pathogenic to humans. Although the precise combination of virulence factors necessary to cause STEC-related disease has not been strictly defined, associations between carriage of certain genes and the ability to cause severe disease in humans have been made (Arthur et al., 2002). These virulence factors are commonly found in the subgroup of STEC known as the enteohaemorrhagic *E. coli* (EHEC). Several studies have indicated that STEC strains carrying *stx2* alone were more likely to cause severe disease compared to STEC strains carrying *stx1* or both *stx1* and *stx2* (Boerlin et al., 1999; Ostroff et al., 1989). In addition to *stx2*, the LEE-associated *eae* (intimin) and the plasmid-encoded EHEC *hlyA* (hemolysin) have also been found in a high proportion of STEC strains causing human disease (Acheson, 2000; Beutin et al., 1998; Eklund at al., 2001; Gyles et al., 1998; Schmidt et al., 1995).

Since STEC inhabit the gastrointestinal tracts of healthy cattle (Sandhu and Gyles, 2002), competition with other members of the bovine gut microbiota for nutrients and colonization space is essential. The composition of the gut microbiota varies considerably

between individual animals and these differences cannot be solely attributed to such factors as differences in diet, age, weather conditions, etc. (Durso et al., 2010). This raises the question whether the gut microbiota composition of a given animal has a role to play in determining the animal's propensity to shed STEC at high levels. Such a scenario is plausible if a given gut microbiota, due to its metabolic activities or through some other mechanism, can create an environmental milieu in the bovine gut which is either favorable or hostile for STEC colonization and proliferation. Another interesting question is whether the gut microbiota has a role to play in the ability of more virulent STEC to colonize and persist in the bovine gastrointestinal tract, especially since some of these virulence factors (e. g., intimin) are involved in the attachment of bacterial cells to the bovine gut epithelium (Naylor et al., 2005).

The advent of 'culture-independent' techniques, such as next-generation DNA sequencing technologies, and their use in microbial ecology studies has enabled researchers to study gastrointestinal microbial communities of both humans and animals in much greater detail. However, thus far, only a few studies have used these culture-independent approaches to study the gut microbiota composition of cattle and its relationship to STEC shedding (Xu et al. 2014; Zhao et al. 2013). This study investigates the influence of the bovine fecal bacterial community structure on the level of STEC shedding. In addition, the fecal prevalence of potentially human pathogenic strains of the 7 major STEC serogroups (O157 and the 'big six') is assessed using the molecular approach of the NeoSEEK^{TM} STEC assay.

2.2 Materials and methods

2.2.1 Animals and diets

Cross-bred yearling beef steers from two different sampling years – 2011 and 2013 - were involved in this study. Fecal samples were collected in July 2011 and August-October 2013. In 2011, fecal samples were collected from a herd of 170 animals (body weight (BW) = 383 ± 19 lb) from three sampling time points. However, based on availability, quality, and quantity, only fecal samples from 103 animals covering two consecutive sampling time points (one week apart from each other) were selected for the current study. These animals were fed three different diets which included: wet distillers grains with solubles (WDGS), dried distillers grains with solubles (DDGS), and a corn-based control diet (CON) **(**see Table 1 for diet compositions**)**. There were 31 animals on the CON diet, while there were 36 each in DDGS and WDGS. The 2013 samples were collected from 225 animals (BW = 347 ± 27 lb) at four sampling time points. The first three samplings were performed at 3-week time intervals whereas the fourth sampling was done just 2 weeks after the third sampling. Fourty-five animals were shipped out of the feedlot at the end of the third sampling time point so the fourth sampling involved only 180 animals. Enumeration of STEC was done only for the fecal samples from time points three and four. The animals from 2013 were fed five finishing diets which included: 15% corn silage and 20% modified distillers grains with solubles (15Sil:20MDGS), 45% corn silage and 20% MDGS (45Sil:20MDGS), 45% corn silage and 40% MDGS (45Sil:40MDGS), 15% corn silage and 40% MDGS (15Sil:40MDGS), and Control (5% corn stalks and 40% MDGS) **(**see Table 2 for diet compositions**)**. There were 45 animals on each diet. In both sampling years and all sampling time points, fecal

samples were collected as rectal grabs from cattle restrained in a chute, using a separate sterile sleeve for each animal. Once a fecal sample was collected, the sleeve was inverted, labeled for identification, carefully tied and placed inside an ice container for transport to the laboratory. The samples were transported to the laboratory within 2-3 hours of collection on each sampling day.

2.2.2 Microbiological culture for enumeration of STEC

Five grams of fecal grab sample were mixed and homogenized in 45 ml of phosphate buffered saline (1*x*) using a homogenizer set at a paddle speed of 2400 rpm for 1 minute. A 50 µl volume of the homogenate was spread on an agar plate containing CHROMagar STEC^{TM} medium (CHROmagar, Paris, France) using an Eddy Jet spiral plater (IUL instruments, Barcelona, Spain). Each sample was plated in duplicate. The plates were incubated at 42^0 C for 24 hours and enumerated according to the guidelines provided in the spiral plater documentation. The average colony-forming unit (cfu) count/g of feces of presumptive STEC was calculated for each sample. Based on this enumeration, the following criteria were established to categorize fecal samples into three shedding categories: fecal samples with > 4.0 logs CFU/g of feces as 'High-shedder', 4.0 – 3.0 log CFU/g of feces as 'Medium-shedder', and < 3.0 logs CFU/g of feces as 'Low-shedder'. The high-shedder threshold of >4.0 logs CFU/g was selected based on the STEC O157 working definition for a super-shedder (Arthur et al., 2010); the remaining two thresholds were selected arbitrarily.

2.2.3 DNA extraction and PCR amplification

DNA extraction from fecal grab samples was carried out using the PowerMag™ Soil DNA Isolation Kit (MO BIO Laboratories, Carlsbad, CA) according to the manufacturer's protocol with the following modification: bead-beating was performed in a Tissue Lyser (QIAGEN Inc., Valencia, CA) at full speed (30 beats/s) for 10 minutes, twice, with incubation of the samples in a heated water bath at 95^0 C for 10 mins between the two bead-beating steps. The rest of the steps were carried out according to the manufacturer's protocol. The extracted DNA was used to PCR amplify the V3 hypervariable region of the bacterial 16S rRNA gene using 341F and 518R (barcoded) primers with adapters. The forward primer (P1-341F) was 5-ccactacgcctccgctttcctctctatgggcagtcggtgatCCTACGGGAGGCAGCAG-3 with the P1-adaptor sequence shown in lower case letters. The reverse primer (A-518R) had the sequence 5-ccatctcatccctgcgtgtctccgactcagNNNNNNNNNNNNATTACCGCGGCT

GCTGG-3 where the A-adaptor is represented in lower case letters and the sample-specific unique barcode is represented by a string of N's. The PCR reactions were performed in 25 µl volumes containing 4 µl (10-30 ng/µl conc.) of template DNA, 0.50 µl of 341F primer (final concentration 0.5 µM), 1.00 µl of 518R primer (0.4 µM) (Integrated DNA Technologies, Coralville, IA), 0.25 µl of bovine serum albumin (New England Biolabs, Ipswich, MA) (10 mg/ml; final conc. 1.5 µM), 0.5 µl of deoxynucleoside triphosphates (0.2 µM), 0.25 µl of Terra PCR Direct Polymerase Mix (0.625 units) (Clontech Laboratories Inc., Mountain View, CA), 12.5 µl of 2×Terra PCR Direct Buffer (Clontech Laboratories Inc., Mountain View, CA) and 6 µl of nuclease-free water (Hoefer Inc., Holliston, MA). The amplifications were performed on a Veriti 96-

well thermocycler (Life Technologies, Carlsbad, CA). The PCR reaction conditions were 3 mins at 98^0 C followed by 25 cycles of 30s at 98^0 C, 30s at 52^0 C, and 40s at 68^0 C, with a final elongation step of 4 mins at 68^0 C.

2.2.4 Preparation of amplicon libraries and DNA sequencing

Eight microliter volumes of the 16S rRNA gene amplicons were resolved in a 2% agarose gel and quantified using the GeneTools software package (Syngene, Frederick, MD). Based on these intensities, amplicons from up to 96 samples were 'pooled' together using an epMotion M5073 liquid handler (Eppendorf AG, Hamburg, Germany) to ensure equal representation of amplicon DNA from each sample. Each pooled library was size selected for the target amplicons using a 2% E-Gel® SizeSelect™ gel (Life Technologies, Carlsbad, CA). The size-selected fragments were quantified using an Agilent Bioanalyzer 2100 high sensitivity chip (Agilent Technologies, Santa Clara, CA) and subsequently subjected to sequencing on an Ion Torrent™ Personal Genome Machine (Life Technologies, Carlsbad, CA) using 316 chips. The sequencing was done in the 518R to 341F direction. Emulsion PCR, enrichment, bead deposition, and sequencing was performed according to the manufacturer's protocol.

2.2.5 Determining the presence of pathogenic strains of the 7 major EHEC serogroups (O157, O111, O26, O45, O145, O121, and O103)

The fecal DNA extracted from all bovine fecal samples were sent to the GeneSeek section of Neogen Corp. (Lincoln, NE) where the NeoSEEK™ STEC confirmation assays were carried out to detect the presence of potentially pathogenic strains of the 7 major EHEC serogroups.

2.2.6 Bioinformatics pipeline

2.2.6.1 Quality filtering, OTU picking, and generation of OTU table

The quality-trimmed FASTAQ file obtained from the Ion Torrent™ Personal Genome Machine was converted to a FASTA file and the sequences in the resulting file were then de-multiplexed into their respective samples using the open-source bioinformatics platform Quantitative Insights Into Microbial Ecology (QIIME) (Caporaso et al., 2010). Sequences that contained more than one mismatch to the primer or barcode, reads with an average quality score of less than 15 along a 30 bp sliding window (starting from 3' end), and homopolymer runs over 6 bp were removed. In addition, sequences were trimmed at the first ambiguous base (N) character. The forward primer, adapters, and barcodes were also removed from the reads. The fasta files thus generated from each sequencing run were then concatenated to form a single large file containing all the data (i. e., sequence data from both sampling years). To remove the reverse primer from these sequences, the QIIME command **truncate_reverse_primer.py** was used. Subsequently, the quality-filtered sequences were run through a perl script (min_max_length.pl; see Appendix II for code) to trim all sequences (from the 3'end) to a uniform length of 130 bp (the actual complete amplicon was 160 bp in size). Sequences shorter than 130 bp were removed from further analyses. The trimmed sequences were reverse complemented using the command **reverse.seqs** in MOTHUR (Schloss et al., 2009). Subsequently, chimera detection and filtering, sequence clustering, and OTU-picking (97% sequence similarity) was performed using the UPARSE pipeline (Edgar, 2013) using the **usearch_batch_master.pbs** batch script (Appendix II**)**. Taxonomic classification was assigned within QIIME to the resulting OTU table using the greengenes database (Wang

et al., 2007) version gg_13_5 (May 2013). The representative OTU sequences generated from the UPARSE pipeline were aligned using the RDP Aligner tool (https://pyro.cme.msu.edu/aligner**)** and any OTUs which didn't align within the target region of the 16S rRNA gene were removed. In addition, OTUs classified as Cyanobacteria were also removed. Rarefaction curves for samples from each year were generated within QIIME according to the steps described by Kuczynski et al. (2011) using the observed_species rarefaction measure.

2.2.6.2 Comparing the distribution of core taxa between each year

For each year, a core measurable microbiota (CMM) was defined by retaining only the bacterial taxa that were present in at least 75% of the samples. For taxa, only the 'Family' and 'Genus' levels were considered (for commands used to derive cores, please refer Appendix I). Beta diversity estimates were compared between the fecal samples of the two years using Bray-Curtis distance matrices within QIIME.

2.2.6.3 Analysis for influence of fecal microbiota on STEC shedding

The data analysis was performed within each year. Thus the complete OTU table was split into two OTU tables corresponding to each year and subsequent analyses were performed using each 'year-specific' OTU table. Only fecal samples which had shedding information were considered for this analysis (201 fecal samples from 2011 and 358 from 2013 resulting in a total of 559 samples).

2.2.6.4 Comparing alpha diversity among shedding categories

To see whether there was a difference in alpha-diversity between the three shedding categories, Shannon diversity Indices were generated within QIIME using the

alpha_diversity.py command. The alpha diversity values for each metric was plotted as box-and-whisker plots using the ggplot2 package (Wickham, 2009) in R studio (R Core Team, 2014). The Mann-Whitney test was used to compare the alpha-diversity indices of the fecal bacterial communities among the different shedding categories.

2.2.6.5 Comparison of the CMM between the different shedding categories

For each shedding category (i. e., High-shedder, Medium-shedder, and Low-shedder), a separate CMM was defined by retaining only taxa and OTUs that were present in at least 75% of the respective fecal samples. Beta diversity estimates were calculated for these CMMs at the family and genus levels as well as at the OTU level. The command **beta_diversity_through_plots.py** was implemented in QIIME to compare beta diversity. This command also generated principal coordinate analysis (PCoA) plots to observe clustering of samples. Bray-Curtis distance matrices were used for the beta diversity analyses.

2.2.6.6 Selecting features associated with shedding categories and shedding levels

To select features (taxa/OTUs) that were significantly different in abundance between the shedding categories within each year, the bioinformatics tool Linear Discriminate Analysis Effect Size (LEfse) (Segata et al., 2011) was used with default parameters. This comparison was done only between the high-shedder and low-shedder samples as these were the shedding categories of most interest. LEfse uses the non-parametric Kruskal-Wallis rank-sum test to identify features (taxa/OTUs) with significant differential abundance with respect to the classes of interest (shedding category). Subsequently, this tool uses Linear Discriminate Analysis (LDA) to estimate the effect size of each

differentially abundant feature (Segata et al., 2011). LEfSe was implemented through the Galaxy server of the Huttenhower research group available online (http://huttenhower.sph.harvard.edu/galaxy/). Features identified by LEFse were further evaluated for their influence on shedding as described below.

2.2.7 Statistical analysis

Permutational multivariate analysis of variance (PERMANOVA) was used to examine the influence of shedding category on fecal bacterial community structure at the phylum, class, family, genus, and OTU levels (both core and total OTUs were considered). The commands were run using the R statistical software environment (version 3.1.3) (R Core Team, 2014). Bray-Curtis distance matrices were used for statistical analyses, which were generated using input files containing relative abundances of each taxon or OTU in corresponding samples (OTU relative abundances were calculated using the perl script normalize_otu_table.pl; Appendix II). These input files were generated within QIIME using the command **summarize_taxa.py** and subsequently imported into R. The PERMANOVA commands were run in R package vegan (Oksanen et al., 2015) via the 'Adonis' function. In the statistical model, shedding category, diet, and time point were considered as fixed effects, while animal was considered as a random effect. The distance matrix was the response variable. P-values <0.05 were considered to be statistically significant. The PERMANOVA test was also used to assess the influence of sampling year on fecal bacterial communities (see section 2.2.6.3 above). Year, time point, and diet were considered as fixed effects, while animal was considered a random effect.

The differentially abundant taxa/OTUs identified by LEfse were further tested for their influence on shedding by way of a multi-factor ANOVA. This was done using the linear models function (lm) in R. For the LEfse-selected OTUs, only the top 15 OTUs with the highest LDA scores were considered for each shedding category. The statistical model accounted for shedding category, diet, time point (fixed effects) and animal (random effect). The relative abundance of the taxa/OTU of interest (these were the 'features' identified by LEfse) across samples was the response variable. P-values <0.05 were considered to be statistically significant. Taxa/OTUs that showed a significant association with shedding were further examined using Box-and-whisker plots and correlation plots. The box-and-whisker plots were generated using the ggplot2 package in R. To compare the relative abundance of target taxa/OTUs among the shedding categories, the Mann-Whitney test was used. Correlations between STEC shedding level and relative abundances of these target taxa/OTUs were assessed using GraphPad Prism version 6.0 (GraphPad Software, San Diego, CA). Relative abundances (number of sequences of taxon/total number of reads in sample) were logarithm with base 10 (log 10) transformed in an attempt to achieve normality prior to correlation analysis. For samples which had read counts of zero for the relevant taxa/OTU, prior to log10 transformation the zeros were replaced with the following formula: (0.5/total number of reads in that sample). Normality of the data was tested using the Kolmogorov-Smirnov test. For correlation analysis, Pearson's correlation (normally distributed data) or Spearman's test (non-normally distributed data) were used.

2.2.8 Comparison of fecal bacterial communities between fecal samples based on the prevalence of EHEC of the 7 major O-serogroups (EHEC-7)

There were only 42 fecal samples out of a total of 1030 (includes samples from both 2011 and 2013) in which a member of the EHEC-7 was detected. Because the number of samples in which EHEC-7 was not detected far out-numbered the number of samples which were positive for EHEC-7, it was deemed that statistically valid conclusions would be difficult to obtain and, as a result, this analysis was not performed.

2.3 Results

2.3.1 Prevalence of EHEC-7 in fecal samples

The number of fecal samples in which any of the 7 major EHEC serogroups were detected in samples is graphically represented in figure 2.1. Of the 1030 fecal samples which were subjected to NeoSEEK^{TM} STEC confirmation assays, only 42 samples were positive for the presence of a major EHEC (Appendix IV). The serogroups detected were O103, O111, O45, O145, and O157. All of the detected serogroups carried *eae* along with *stx* (Appendix IV). The overall fecal prevalence in EHEC and non-EHEC of the 7 target O-serogroups is depicted in figure 2.2.

For 2011, 20 fecal samples were positive for EHEC. Among the serogroups detected were O103, O111, O45, and O157. EHEC O103 was the most frequently encountered EHEC; EHEC O157 was detected in only a single sample. Fecal samples from both sampling time points were positive for EHEC O103 for 3 animals. Two fecal samples had more than one EHEC serogroup being detected; one had both O103 and O45 while the other had O103 and O157 (Appendix IV).

Major EHEC serogroups were detected in 22 fecal samples from 2013. O103, O145, O45, and O157 were the EHEC serogroups detected. EHEC O103 along with EHEC O157 were the most frequently detected serogroups. Two samples were positive for more than one EHEC serogroup; both O103 and O157 were detected in one sample, while the other sample was positive for both O157 and O45. None of the animals had more than one sampling time point from which an EHEC was detected.

2.3.2 Multiplex 16S rRNA gene-based sequencing of bovine fecal samples

Sequencing of 16S rRNA tags using the Ion Torrent™ Personal Genome Machine resulted in a total of 26,351,825 sequences after de-multiplexing and removal of adapters and barcodes. After removal of short sequences (<130 bp) and quality filtering, the data set retained 16,025,749 sequence reads. Following further quality filtering, chimera detection and cropping to a fixed size of 130 bp (see methods section), the number of sequences used for OTU clustering was 14,622,764 with a mean of 14,336 sequences per sample. Any sample with <3,000 sequences were removed from further analysis. This resulted in 201 samples for 2011 and 804 samples for 2013 with >3,000 sequences which were used for subsequent analyses. The depth of sequencing for each sample with respect to OTUs discovered is represented as rarefaction curves in figure 2.3.

2.3.3 Alpha diversity estimates among shedding categories

For 2011, there were 81 high-shedder, 81 medium-shedder, and 39 low-shedder fecal samples, while for 2013 the corresponding numbers were 127, 191, and 40 respectively. The alpha diversity estimates for the shedding categories for each year are represented in

figure 2.4. No significant differences in the alpha diversity between shedding categories were detected by the Shannon diversity index for either sampling year.

2.3.4 Comparison of the core taxa distribution between fecal samples from the two years (2011 and 2013)

The distribution of the core taxa among the two sampling years was compared using beta diversity estimates in QIIME. This analysis revealed separate clustering of samples based on sampling year, as is evident by the principal coordinate analysis (PCoA) plots depicted in figure 2.5 which are based on Bray-Curtis distances. Multivariate statistical analysis using PERMANOVA also confirmed that 'year' had a significant influence on structuring of the microbial communities at both the 'Family' and 'Genus' levels (p=0.001, R^2=0.10848 and p=0.001, R^2=0.09661 respectively, Appendix V). A comparison of the major phyla (>1% relative abundance) revealed that the fecal samples from both years were composed of the same predominant phyla (Figure 2.6). However, there were significant differences in the relative abundances of these phyla between the two years (Figure 2.7).

2.3.5 Influence of fecal bacterial community on shedding category

The influence of shedding category on phylum, class, family, genus and OTU level changes in the fecal bacterial communities was assessed using PERMANOVA. For the family, genus, and OTU levels, core measurable microbiotas (CMMs) were compared (in addition, total OTUs were also compared) as described in section 2.2.6.6 of methods section. For 2011, there were 23 core families which accounted for 99.3% of the total number of sequences that could be classified at the family level. Similarly, the genus

level CMM consisted of 31 core genera and made up 98.7% of the total genus level sequences. For 2013, the family level core consisted of 25 families covering 99.3% of all sequences classified at the family level and the genus level core consisted of 32 genera accounting for 98% of all sequences that were classified at the genus level. At the OTU level, for 2011, there were 318 core OTUs in the CMM which made up 41.25% of total sequences of the 2011 data set. Likewise, the 2013 CMM consisted of 385 OTUs, accounting for 44.38% of sequences from fecal samples with shedding information.

PERMANOVA test results are summarized in table 2.3 (see Appendix V for actual outputs). Based on these results, phylum, class, or family level changes in fecal bacterial community were not significantly influenced by shedding category (this is the case for samples from both years). However, for fecal samples from 2011, shedding category appears to contribute to changes at the genus level (p=0.003, R^2=0.01971). For 2013 samples, the influence of shedding category on genus level taxa approached significance (p =0.055, R^2=0.00523). At the core OTU level, for both years, shedding category was significantly associated with fecal bacterial community structure (p=0.001, R^2=0.01961 and p=0.028, R^2=0.00505 for 2011 and 2013, respectively). Similar to core OTUs, total OTUs also had a significant association with shedding category (see Appendix V). Principal coordinate analysis (PCoA) plots for the core genera and core OTUs generated using Bray-Curtis distance matrices are shown in figures 2.8 and 2.9 respectively.

2.3.6 LEfse results for genera/OTUs with significant differential abundance between shedding categories

The LEfse outputs for genera within each year with significant differential abundance between the high-shedder and low-shedder categories are shown in figure 2.10. For 2011, there were 8 genera which were significantly more abundant among high-shedder samples, while 7 genera were significantly more abundant in low-shedders. Four genera were significantly more abundant in low-shedder samples, while only a single genus (*Prevotella*) was significantly more abundant among high-shedders for 2013. Since the LEfse test didn't take into account all the factors - such as diet and time point - which may have influenced these genera to be significantly more abundant in a given shedding category, each of these taxa were further tested using a multi-factor ANOVA (see 2.2.7 in methods section) to establish whether they were significantly influenced by shedding. The results of these analyses (Table 2.4) revealed that, among genera that were more abundant in high-shedders, only *Butyrivibrio* (p=0.017) and CF231 (p=0.003) were significantly affected by shedding among the 2011 genera, while among the 2013 genera, only *Prevotella* (p=0.044) was significantly influenced by shedding. It is interesting to note that CF231 and *Prevotella* were also the genera with the highest LDA scores for the high-shedder category for 2011 and 2013, respectively (Figure 2.10). None of the genera which were significantly more abundant among low-shedders appeared to be significantly influenced by STEC shedding. Most of the other genera that were picked up by LEfse as being more abundant in either shedding category appeared to be influenced more by diet. The box-and-whisker plots and the correlation analysis plots (see below) for *Butyrivibrio* and CF231 further demonstrated their association with high-shedding.

At the OTU level, several OTUs were detected by LEfse as having significantly greater relative abundance in high-shedder or low-shedder fecal samples (Figures 2.11 – 2.13). Similar to the genus level, these OTUs were also further tested using multi-factor ANOVA to assess their effect on shedding (Table 2.5). For 2011, only OTU 15828 (abundant in high-shedders) was significantly affected by shedding (p=0.004). In contrast, eleven OTUs were significantly associated with shedding category from the 2013 data set. All of these 2013 OTUs were more abundant in the low-shedders according to the LEFse results.

2.3.7 Box-and-whisker plots and correlation analysis

The genera/OTUs which were significantly influenced by STEC shedding based on the multi-factor ANOVA were further analyzed using box-and-whisker plots and correlation analysis. The genus level results of these analyses are presented in figure 2.14, while the results at the OTU level are presented infigure 2.15. OTU 15828, which was more abundant in high-shedders according to LEfse and was also significantly associated with shedding based on the multi-factor ANOVA for the 2011 data, was confirmed to be significantly more abundant in the high-shedders compared to the medium- and low-shedders based on the box-and-whisker plots (Figure 2.15(l)). The Spearman test also showed a positive correlation between the relative abundance of this OTU in fecal samples and STEC shedding level (Figure 2.15(l), r=0.2772, p=0.0001). For 2013, 11 OTUs that were shown by LEfse to be significantly more abundant in low-shedders were also significantly associated with shedding according to the multi-factor ANOVA. Nine of these 11 OTUs were significantly more abundant in the low-shedders compared to both medium- and high-shedders (Figure 2.15(a-k)). The remaining two OTUs (OTU 118

and OTU 10480) were significantly more abundant in low-shedders only compared to the high-shedders; there was no significant difference in relative abundance when medium-shedders were compared to low-shedders. The correlation analyses confirmed a significant negative correlation between the relative abundance of the OTU and level of STEC shedding for all these OTUs except OTU 118 and OTU 16990 (Figure 2.15). A summary of the OTUs from both 2011 and 2013 which were significantly associated with a given shedding caregory based on LEfse, multi-factor ANOVA, as well as box-and-whisker plots and correlation analysis is presented in table 2.6.

OTU 15828 was identified by LEfse as more abundant in high-shedder fecal samples in both 2011 and 2013 (Figures 2.11 and 2.13). The box-and-whisker plots and correlation analyses for this OTU in both years are shown in figure 2.16.

2.4 Discussion

To understand the role of the fecal microbiota on level of Shiga toxin-producing *E. coli* (STEC) shedding in beef cattle, this study characterized the fecal bacterial community composition of >300 beef steers (from two separate sampling years) at multiple time points using a culture-independent approach at varying levels of STEC shedding. Fecal prevalence of the 7 major EHEC serogroups that are regulated as adulterants in beef was also determined within this cohort of animals.

2.4.1 Fecal prevalence of EHEC-7

Several studies in the last few decades have investigated the prevalence of STEC O157 in cattle (Chapman et al., 1997; Omisakin et al., 2003; Dunn et al., 2004). However, relatively few studies have been carried out to determine the prevalence of the non-O157

STEC in the bovine reservoir, and even fewer studies have investigated the prevalence of potentially human pathogenic STEC/EHEC in these animals.

One of the reasons for this scarcity of studies investigating the prevalence of non-O157 STEC is related to challenges associated with detecting these serogroups in foods and food-related animals (Barlow and Mellor, 2010). In contrast to *E. coli* O157:H7, phenotypic characteristics (e.g., sorbitol fermentation) that distinguish non-O157 STEC from generic *E. coli* are lacking (Wang et al., 2013). Thus, it is not easy to readily culture non-O157 STEC. In addition, non-O157 STEC are a diverse group of bacteria encompassing over 170 O-serogroups with different characteristics, whereas *E. coli* O157:H7 is a single serotype.

Several recent studies have used multiplex PCR as a means of detecting STEC (both O157 and non-O157) in cattle feces (Paddock et al., 2012; Cernicchiaro et al., 2013). These multiplex PCR tests usually screen DNA extracted from fecal samples in enriched broths for the presence of virulence genes commonly associated with pathogenic STEC (such as *stx*, *eae*, and *hly*), and any samples positive for these virulence factors are further tested using primers specific for O-serogroups of interest such as O157 and the 'big six' non-O157 STEC serogroups. An important limitation of this approach is that it is difficult to confirm whether signals associated with the virulence genes and the serogroup-specific genes originate from individual cells or from different cells in the population (Barlow and Mellor, 2010). Thus, plating and isolation of pure cultures from suspected fecal samples has to be performed in order to verify that virulence factors originate from specific colonies belonging to target EHEC serogroups. The ability to isolate these EHEC

serogroups harboring known virulence markers from a given sample is often a difficult undertaking (Barlow and Mellor, 2010).

The NeoSEEK™ STEC confirmation assay uses PCR coupled with mass spectrometry to generate genetic profiles (genetic 'fingerprint'), based on more than 70 single nucleotide polymorphisms (SNPs), of the bacteria present in a given sample (Neogen Corp, n. d.; Quality Assurance Magazine, 2012). These profiles are subsequently compared to the genetic fingerprint of known pathogenic EHEC strains in order to determine whether target EHEC strains belonging to the 7 major O-serogroups are present in a given sample (Neogen Corp, n. d.; Quality Assurance Magazine, 2012). Thus, the NeoSEEK™ STEC confirmation assay has the capacity to differentiate between non-pathogenic and potentially pathogenic strains of EHEC O157 and the 'big six' non-O157 EHEC. The ability to distinguish between pathogenic STEC and non-pathogenic generic *E. coli* is important, as reports have suggested a high prevalence of non-STEC *E. coli* belonging to the 7 major O-serogroups in bovine feces (Cernicchiaro et al., 2013).

Out of the 1030 bovine fecal DNA samples (from both 2011 and 2013) which were screened using the NeoSEEK™ STEC assay, only 42 samples (4.08%) yielded a positive result for potentially virulent strains of the 7 major EHEC serogroups. This low prevalence of EHEC in bovine feces was also noted by Barlow and Mellor (2010) in a study involving post-evisceration beef cattle fecal samples from Australian abattoirs. These investigators screened 300 cattle fecal samples (from 25 abattoirs) for virulence markers *stx1,stx2,* and *eae* using real-time PCR followed by isolation of target EHEC O-serogroups using immunomagnetic separation and colony-hybridization (Barlow and

Mellor, 2010). Seventy-eight of the 300 samples (26%) tested positive for *stx* and *eae*, and 30 of these also tested positive for at least one of the target EHEC serogroups. However, isolation of these EHEC serogroups and subsequent testing for virulence markers identified only 1 *E. coli* O91, 1 *E. coli* O26, and 5 *E. coli* O157 as possessing the requisite virulence genes. This led to the conclusion that the overall prevalence of EHEC in Australian beef cattle was very low (Barlow and Mellor, 2010). The authors further remarked that testing for the presence of virulence determinants and serogroup-specific genes alone overestimated the presence of pathogenic STEC and that strain isolation and confirmation of the presence of virulence determinants in those strains should be an essential part of any test protocol (Barlow and Mellor, 2010).

During this entire study, covering both sampling years, 5 out of the 7 major EHEC serogroups were detected. Serogroups O103, O157, and O45 were detected in fecal samples from both years, whereas O111 and O145 were only detected in 2011 and 2013 samples, respectively. EHEC O26 and EHEC O121 were not detected during the entire study. Overall, the most prevalent EHEC serogroup detected was O103, which was detected in 26 samples (2.5%) (figure 2.2 (a)). Serogroup O157 was the second most frequently detected with an overall prevalence of 0.97%. Interestingly, EHEC O157 was detected in only 1 fecal sample in 2011, while it was detected in 9 samples from 2013. This may simply have been a result of the greater number of samples (829) screened for the 2013 sampling year compared to 2011 (201). Serogroups O45, O145, and O111 were encountered the least, with prevalence values of 0.48, 0.29, and 0.19% respectively (Figure 2.12). It was interesting to note that, apart from 3 animals, none of the other animals had more than one sampling time point in which a given pathogenic EHEC was

detected. This might reflect natural variation owing to infrequent colonization of cattle by these EHEC strains, or it might have been due to other factors such as unequal distribution of these organisms in fecal samples or their occurrence at very low levels below the detection limit of the assay (~10^3 CFU/ml – S. Hinkley, Neogen Corp., personal communication).

Many fecal samples were positive for *E. coli* strains that belonged to one of the 7-major EHEC O-serogroups but did not match the known virulence profiles of target reference pathogenic EHEC strains used in the NeoSEEK^{TM} STEC assay. The prevalence of these *E. coli* serogroups in fecal samples was as follows: O103 (29.4%), O26 (19.3%), O45 (9.9%), O157 (5.3%), and O111 (0.097%) (figure 2.2 (b)). Based on recent research, it appears that, regardless of detection method or Shiga toxin-production, *E. coli* belonging to serogroups O103 and O26 are the most commonly encountered of the 7 major serogroups in cattle feces (Miller et al., 2014; Noll et al., 2014; Shridhar et al., 2014; Cernicchiaro et al., 2013; Joris et al., 2011). Nonetheless, the prevalence of 31.9 % (when both EHEC and non-EHEC are considered) for O103 in fecal samples is much lower than the fecal prevalence rates of 41.1% (Miller et al., 2014), 56.6% and 60.2% (Noll et al., 2014), and 80.5% (Shridhar et al., 2014) obtained by other workers for this serogroup. This may be due to differences in the detection methodologies employed in the current study compared to those used in the other studies cited above (for example, this study did not use an enrichment step whereas the other studies did).

2.4.2 STEC shedding and bovine fecal microbiota

In terms of public health, currently, only STEC O157 and the 'big six' non-O157 STEC are regulated. However, there is the possibility that STEC of other serogroups which are present in cattle can also pose a threat to human health. Since important STEC virulence factors are carried on mobile elements (e. g., bacteriophages and virulence plasmids), it is feasible that exchange of these virulence determinants in cattle environments would result in the emergence of new strains of human virulent STEC. Therefore, the possibility that any STEC can acquire additional virulence factors and become pathogenic led the current study to evaluate the influence of the fecal microbiota composition on 'all' STEC shedding instead of selected serogroups/serotypes.

Due to differences in diets, period of sampling, and animal factors, it was not surprising to observe that there was a significant difference in the composition of the fecal microbiota when comparing the fecal bacterial community from one year's samples with that of the others (Figure 2.5). The bacterial phyla Bacteroidetes, Firmicutes, Proteobacteria, and Tenericutes, were dominant in fecal samples from both years (Figure 2.6), and is in agreement with previous reports (Shanks et al., 2011; Rice et al., 2012) which identified these phyla as 'core' bovine taxa, regardless of differences in diets and cattle management practices. Nonetheless, the relative abundances of these phyla were different between the two years; Bacteroidetes and Proteobacteria were significantly more abundant in 2011 compared to 2013 ($p < 0.0001$), while the phyla Firmicutes and Tenericutes were significantly more abundant ($p < 0.0001$) in 2013 compared to 2011 (figure 2.7). In addition, a notable difference was observed in the relative abundance of the phylum Spirochaetes between the two years ($p < 0.0001$), where the percentage of

reads attributed to this phylum increased significantly from 0.4% in 2011 to 5.6% in 2013 (figure 2.7). These changes are likely due to differences in the availability of substrates for microbial growth as a result of the different diets the animals were fed in the two separate years.

The shedding level of STEC among the animals of both years was heterogeneous, similar to observations made for the shedding of *E. coli* O157 among cattle (Matthews et al., 2006). For certain animals, the level of STEC shedding varied considerably between the two sampling time points; as a result, the shedding phenotype of these animals changed from one time point to the other (Appendix III). For 2011, there were only 30 animals (out of 103) which shed high-shedder levels on both sampling time points. Similarly, 25 and 10 animals, respectively, shed medium- and low-shedder levels of STEC consistently during both sampling time points. The number of animals from 2013 which consistently shed high-, medium-, and low-shedder levels of STEC in their feces was 23, 59, and 16 respectively. Thus, instead of categorizing individual animals, their fecal samples were categorized as high-, medium-, or low-shedder according to the level of STEC detected. This variability in fecal shedding level of STEC for an individual animal at different sampling time points was also observed previously by Munns et al. (2014) for *E. coli* O157:H7 in feedlot steers.

When comparing fecal microbial communities based on shedding category, no significant differences were observed for alpha diversity based on Shannon diversity index for either year (Figure 2.4). This is in contrast to the observations made by Zhao et al. (2013) who, using the same diversity index, reported an increased diversity in the

fecal microbiota of cattle shedding low levels of STEC compared to those shedding high levels. Conversely, Xu et al. (2014) noted that *E. coli* O157:H7 super-shedders had a higher diversity fecal bacterial community compared to non-shedders. However, many factors such as animal age, sex, breed, diets fed, etc., can influence bacterial diversity. The current study and previous studies do not use similar diets or similar animals, and therefore it is hard to compare bacterial diversity among studies.

No significant differences were observed at the taxonomic levels of phylum, class, and family of the CMM between fecal samples belonging to different shedding categories. However, at the genus and OTU levels, the PERMANOVA analysis revealed significant differences between the shedding categories. Specifically, in year 2011 both OTU and genus levels were significantly associated with shedding category, while in 2013 only the OTU level showed a significant association with shedding category. However, for 2013 the genus level comparisons were approaching significance (p=0.055). The observation that no large scale (i. e., phylum level or class level) differences existed in the fecal bacterial communities between the shedding categores is not surprising as in a biological context, STEC shedding may only influence a few bacterial species that would occupy the same niche within the ecosystem. Within the complex bacterial community (10^{10} – 10^{11} bacteria per gram of feces (Dowd et al., 2008)), Even super-shedders only account for $\geq 10^{4}$ CFU/g feces. Therefore, the abundance of STEC is still small compared to the total bacterial population found in bovine feces, which would only lead to changes in a few closely associated species. Furthermore, adult cattle shedding different levels of STEC are asymptomatic, suggesting that STEC shedding probably has little impact on the overall health and well-being of cattle. Therefore, for reasons described above, it is

unlikely that differences in the levels of STEC shedding would result in global changes in fecal bacterial community structure and are more likely to have positive or negative effects on a few bacterial genera, or more likely, a few species or strains. Certain bacterial species may have a preference for the same ecological niche as STEC (for example the recto-anal junction mucosa for STEC O157) and can compete for colonization space and nutrients, thus influencing the ability of STEC to grow and proliferate within the bovine gastrointestinal tract. Alternatively, some members of the bovine fecal bacterial community might produce compounds to promote or inhibit the growth and proliferation of STEC and thereby influence the colonization and shedding of these pathogens.

To identify which genera or OTUs potentially influence STEC colonization and shedding, LEfse was implemented. Several genera were identified by LEfse that discriminated between high-shedder and low-shedder fecal samples. However, when the discriminative genera were compared between the two sampling years, certain genera that were associated with high-shedder samples in 2011 appeared to be associated with low-shedder samples in 2013 and vice versa (for example, *Butyrivibrio* was related to high-shedder samples in 2011 and to low-shedder samples in 2013; figure 2.10). Similar observations were made at the OTU level as well. This suggested that some of these apparently discriminative taxa/OTUs may actually be related to other factors, such as diet or time point, rather than STEC shedding itself as previous studies have shown that, in the context of STEC O157, diet influences shedding (Jacob et al., 2008; Wells et al., 2014). Thus, the taxa/OTUs identified by LEfse were further tested using multi-factor ANOVA to identify taxa/OTUs influencing STEC shedding while accounting for the

other confounding factors. This analysis demonstrated that only very few taxa/OTUs identified by LEfse were significantly influenced by STEC shedding category. Box-and-whisker plots comparing the relative abundance of these identified taxa/OTUs among the different shedding categories and the correlation analyses further confirmed the association of these taxa/OTUs with STEC shedding.

Interestingly, none of the genera and OTUs that were shown to be significantly associated with either high- or low-shedding categories (based on LEfse, multi-factor ANOVA, and the other statistical analyses) in one sampling year were detected as having a significant relationship with STEC shedding in the other year. Two possible explanations as to why OTUs that are significant for shedding in one year are not significant or not even identified as associated with shedding in the other year may be: (1) they are part of the CMM in only one of the two years so are not identified in the other year or (2) although they are part of the CMMs of both years, their abundance in one year is much lower compared to the other year that they have a minimal impact on STEC shedding. To investigate these possibilities, the distribution of core OTUs that were significantly associated with shedding in one year were compared to their distribution in the CMM of the other year (Figure 2.17). Indeed, OTUs 26, 42, 63, 316, 580, and 677, which were associated with low-shedding in 2013 (Table 2.6) were absent from the CMM of 2011. OTUs 10480 and 10659, which were also associated with low-shedders in 2013 were significantly less abundant in 2011, while OTU 15828 (significantly associated with high-shedders samples in 2011) had a significantly lower abundance in 2013 (Figure 2.17). These OTUs may not have been identified by the multi-factor ANOVA as significantly associated with STEC shedding due to their low abundance. As diet is

known to have a significant effect on microbial community composition (Kim et al., 2014), the differences in the diets used within the two years (differences in nutrient availability to microbes) may have influenced this observation. Thus, these results would suggest that there is no genus or OTU which can consistently be linked to either high- or low-shedding regardless of dietary and temporal differences. Thus when comparing bacterial shifts based on STEC shedding, it is critical to account for diet and therefore the bacterial populations that are identified to influence STEC shedding appear to be limited to the diets tested.

It was noteworthy that OTU 15828 was significantly more abundant in high-shedders in both years, and the correlation analyses also showed a significant positive correlation of this OTU with STEC shedding level (Figure 2.16). Of note was that even though this OTU was significantly lesser in abundance in 2013 compared to 2011 (Figure 2.16), it was still associated with high-shedder samples. OTU 15828 was classified as a member of the genus CF231 in the family Paraprevotellaceae (Table 2.6) which are known to inhabit the rumen (McCann et al., 2014). No information was found in the literature regarding any relationship between STEC shedding and either CF231 or Paraprevotellaceae.

Although, as mentioned previously, the PERMANOVA results at the genus and OTU levels showed that shedding category had a statistically significant influence ($p<0.05$) on microbial community, the effect size given by the R^2 values (which indicates the percentage of variance explained by each factor in the model) are quite low (table 2.3 (a) and (b)). The PCoA plots in figures 2.8 and 2.9 also indicate that there isn't clear

clustering of the fecal samples based on shedding category. This may suggest that STEC shedding category has only a minor influence on fecal bacterial community. Indeed, the observation of 'significant' *p*-values along with low R^2 values might indicate a statistical scenario referred to as "the *p*-value problem" (Lin et al., 2013). This is a situation where even minuscule/weak effects may become significant as a result of large sample sizes used in a study (there were 201 samples for 2011 and 358 samples for 2013). This might also explain why even though PERMANOVA results suggest significant differences exist at the genus and OTU levels between different shedding categories, the 'post hoc' tests (LEfse followed by multi-factor ANOVA, box-and-whisker plots, and correlation analysis) identify very few genera/OTUs that appear to be associated with STEC shedding.

Similarly, the significance associated with the correlation analyses of target OTUs which have an apparent relationship with STEC shedding levels, also needs to be interpreted with caution, as these parameters are also influenced by large sample sizes (Taylor, 1990). Even OTU 15828, which had a highly significant *p*-value based on correlation analysis (p=0.0001, Figure 2.15(l)) had a relatively low Spearman r value (0.2772). Furthermore, if the coefficient of determination (r^2) is calculated, this amounts to only 0.077. The coefficient of determination is defined as the percent of variation in the values of the dependent variable that can be used to explain variations in the value of the independent variable (Taylor, 1990). Thus, the variation in the relative abundance of OTU 15828 only explains 7.7%, of the total variation observed in STEC shedding levels. These percentages are even lower for the remaining genera and OTUs that appear to have a potential influence on STEC shedding. Nonetheless, it is still worth noting that, in spite

of the differences in diet, animals, and time of sampling between the two sampling years, OTU 15828 was significantly more abundant in high-shedder fecal samples compared to low-shedder fecal samples in both sampling years as mentioned before.

2.4.3 Conclusions

The main aims of this study were to answer the following questions: (1) are there significant differences in the bovine fecal bacterial community composition based on STEC shedding? (2) are there differences in the fecal bacterial community structure between fecal samples which harbor virulent EHEC strains and those in which these pathogens are not detected? and (3) what is the fecal prevalence of human pathogenic EHEC of the 7 major serogroups in feedlot steers?

No conclusive evidence was found during this study to suggest that STEC shedding had a major influence on fecal bacterial community composition. In fact, based on the results of this study, it seems that large scale changes in fecal bacterial communities doesn't occur as a result of varying levels of STEC shedding by feedlot steers. However, certain genera and OTUs that were significantly associated with high- or low-shedder categories were detected.

Since very few fecal samples were positive for pathogenic members of the 7 major EHEC serogroups, it was not possible to perform statistically valid analyses to compare fecal bacterial community structures between fecal samples which harbored virulent EHEC and those which did not.

Lastly, the NeoSEEK^{TM} STEC assay results revealed a low fecal prevalence rate for pathogenic strains of the 7 major EHEC serogroups in the two herds of feedlot steers sampled in this 2-year study.

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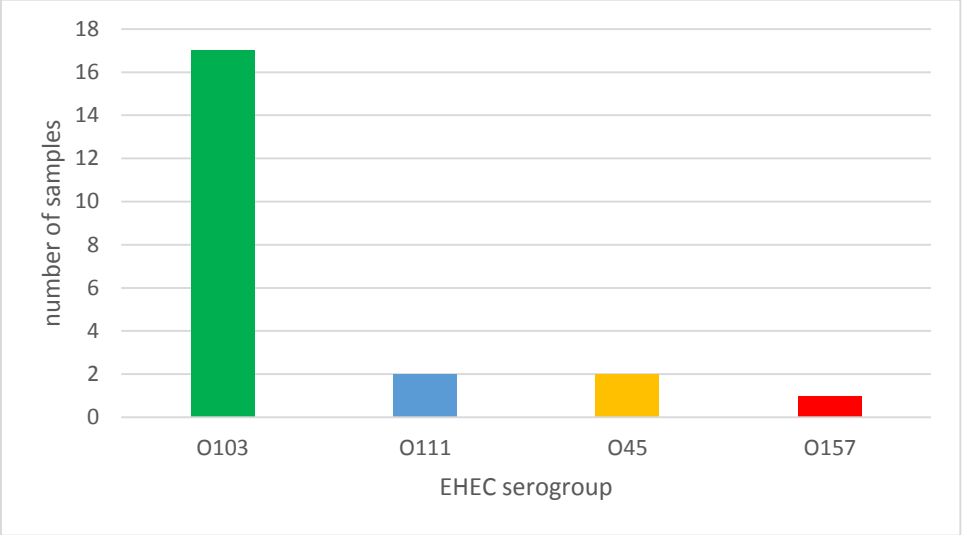

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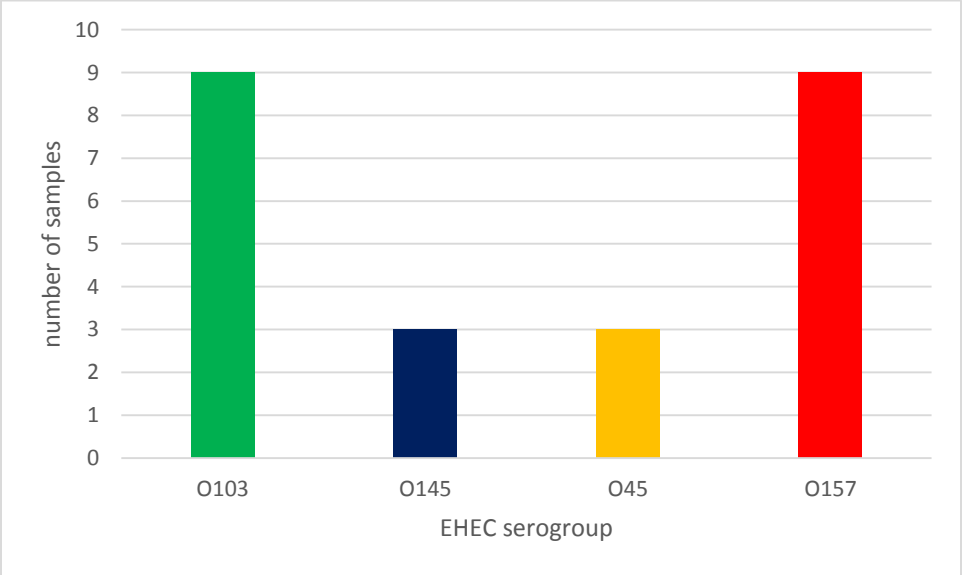
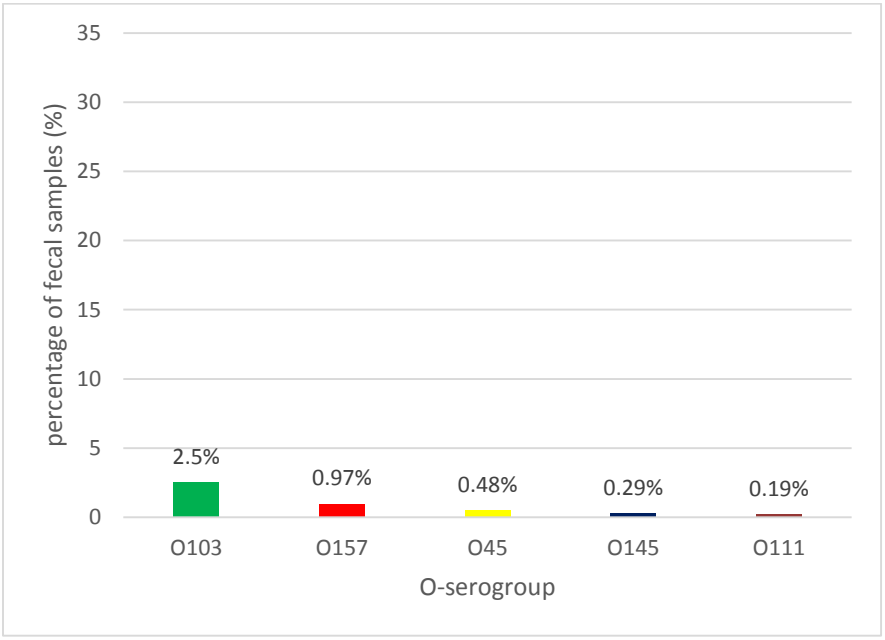


(b)

Figure 2.1: The prevalence of the major EHEC serogroups among bovine fecal samples as determined by the NeoSEEK^{TM} STEC confirmation assay. (a) 2011 samples (b) 2013 samples.



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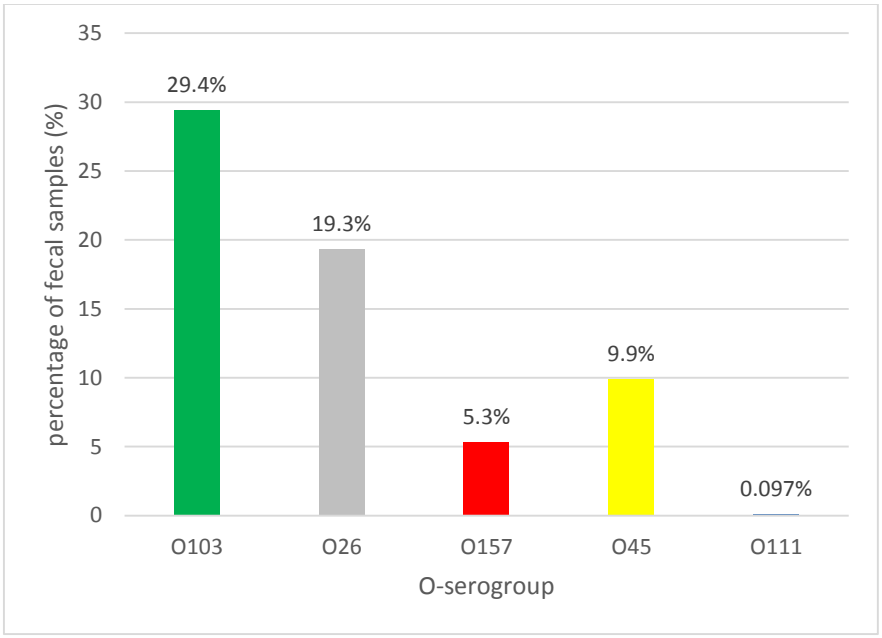
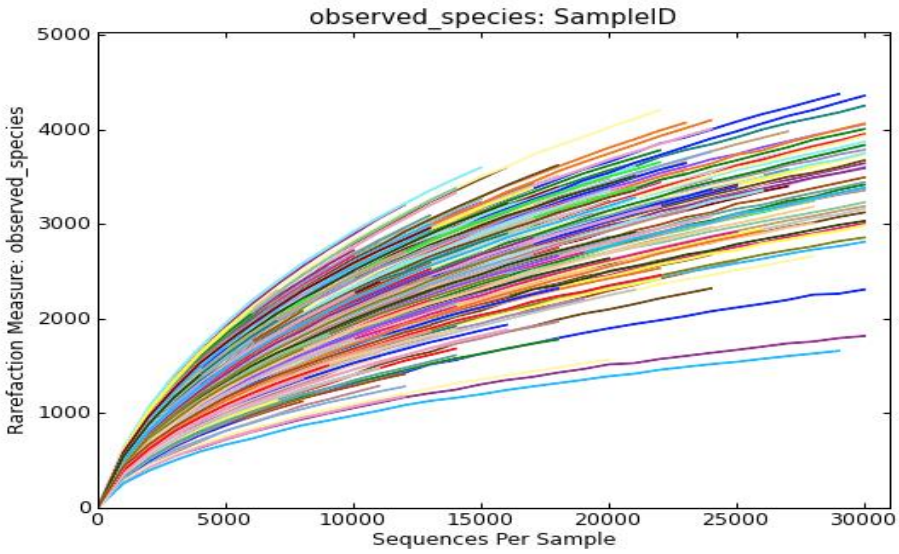
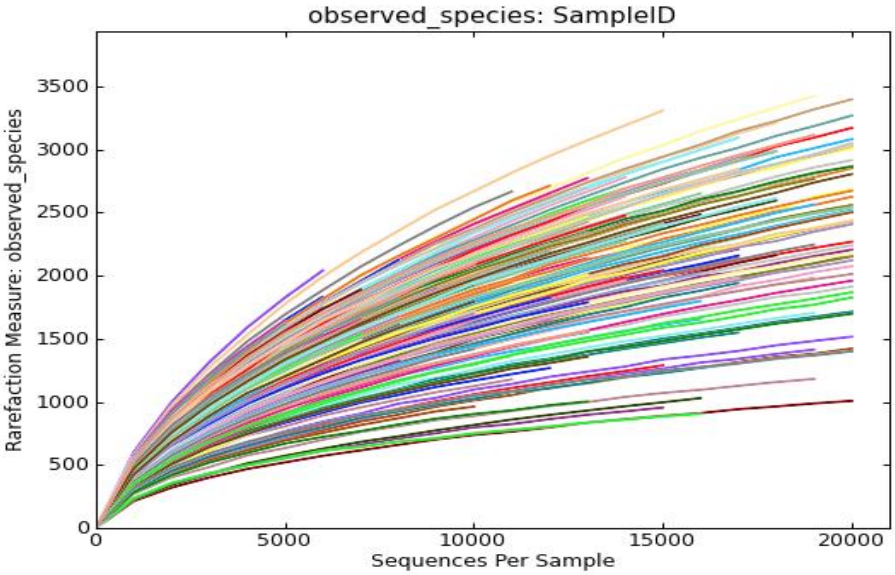


(b)

Figure 2.2: Overall fecal prevalence of (a) EHEC and (b) non-EHEC of the 7-major serogroups regulated in beef

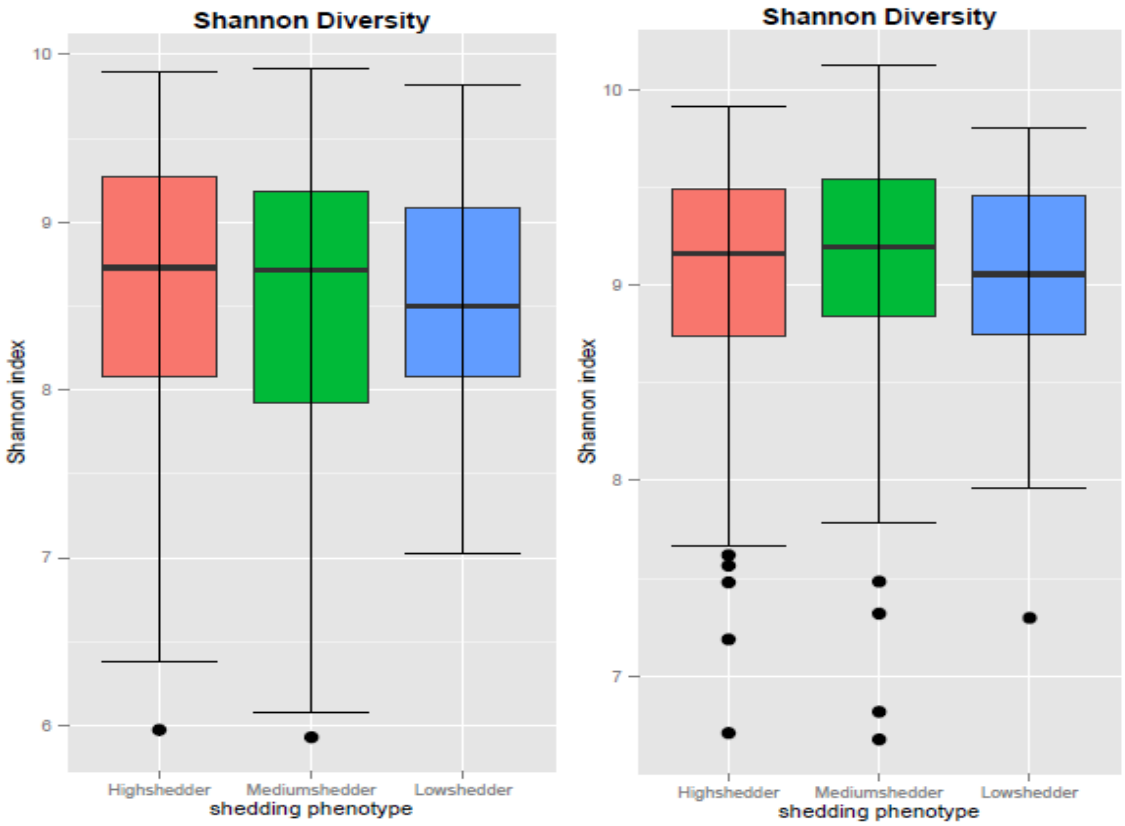


(a)



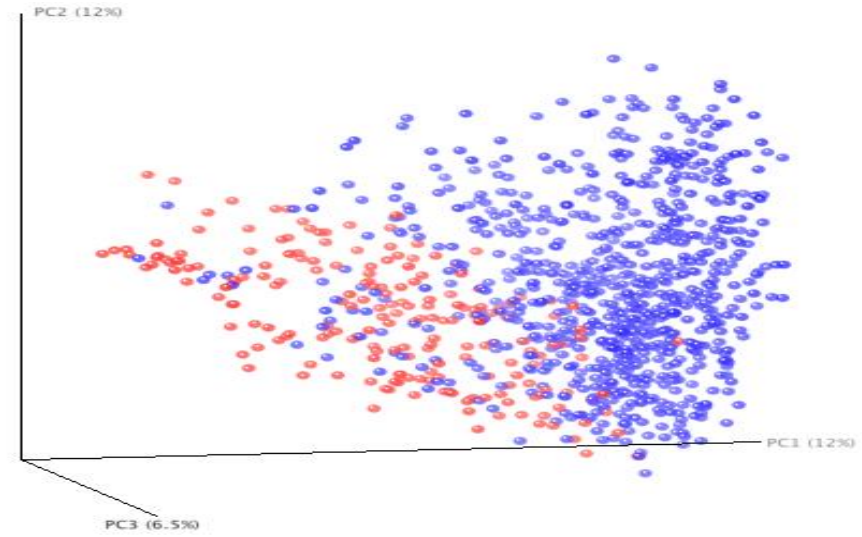
(b)

Figure 2.3: Rarefaction curves based on observed_species rarefaction measure. (a) 2011 samples (b) 2013 samples.

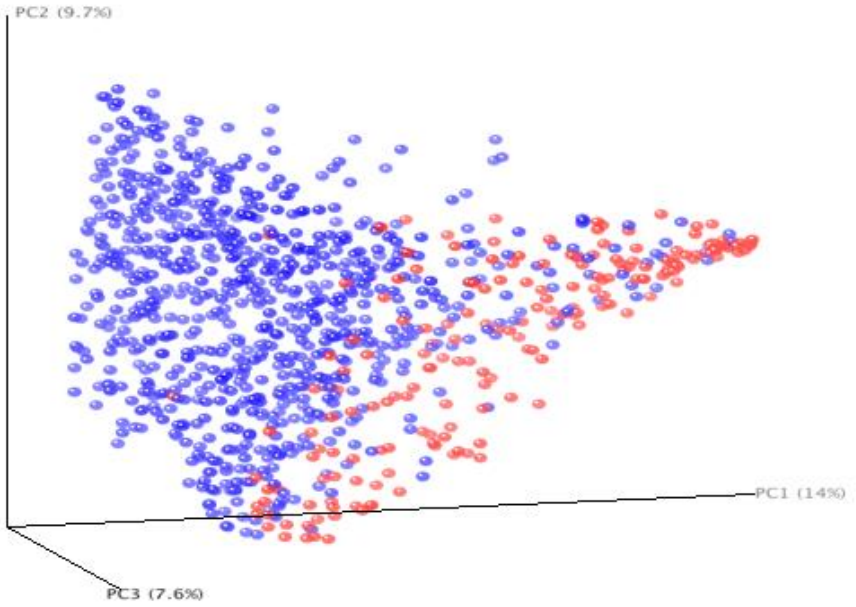


(a) (b)

Figure 2.4: Alpha diversity based on Shannon diversity index for fecal samples from each shedding category within each sampling year. (a) 2011 samples (b) 2013 samples. ● – outliers



(a)



(b)

Figure 2.5: Principal coordinate analysis (PCoA) plots for fecal samples from the two sampling years. The distances were calculated based on Bray-Curtis distance matrices. (a) Family level (b) Genus level. ●2011 samples ●2013 samples

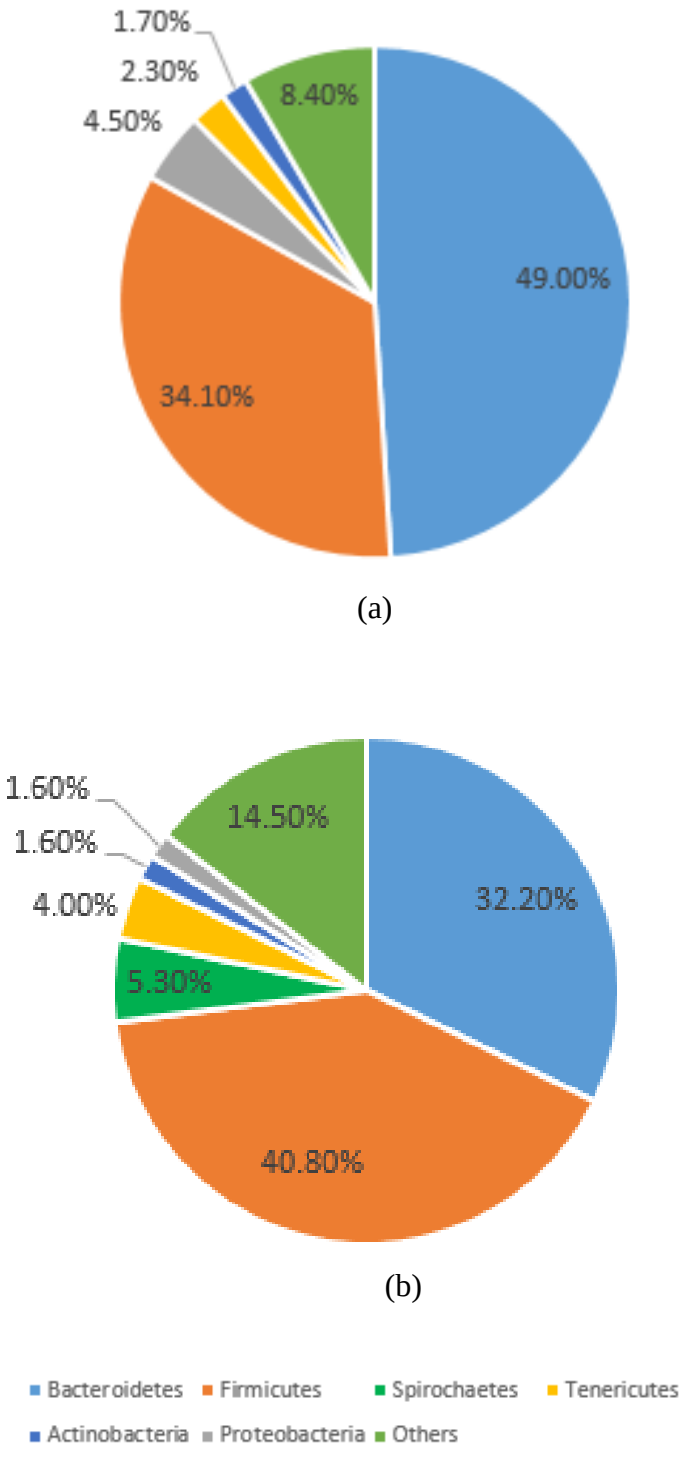


Figure 2.6: Composition of the predominant bacterial phyla in bovine fecal samples (a) 2011 (b) 2013.

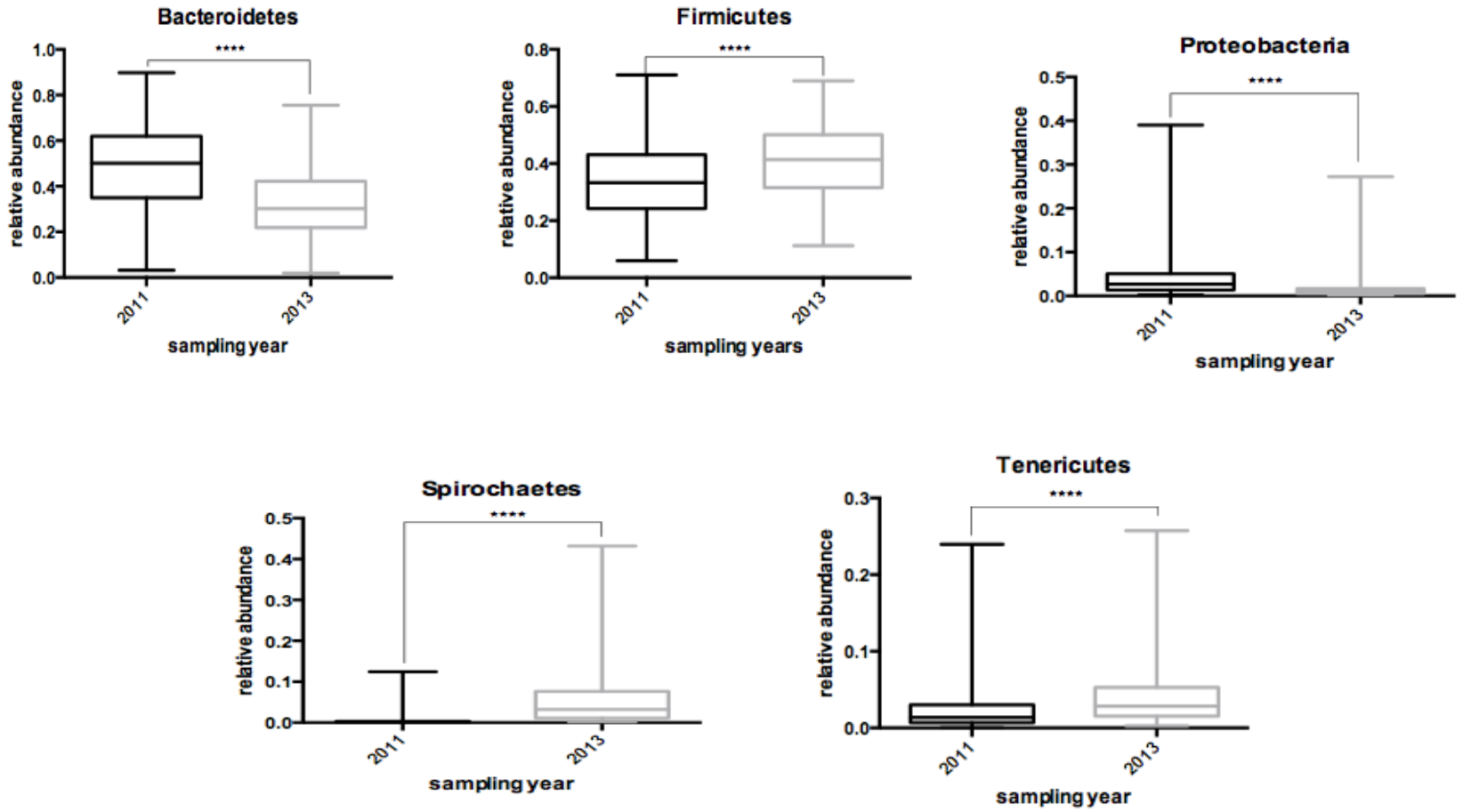


Figure 2.7: Comparison of the relative abundances of major bacterial phyla between the two sampling years (2011 and 2013). **** signifies *p*-value < 0.0001 (Mann-Whitney test).

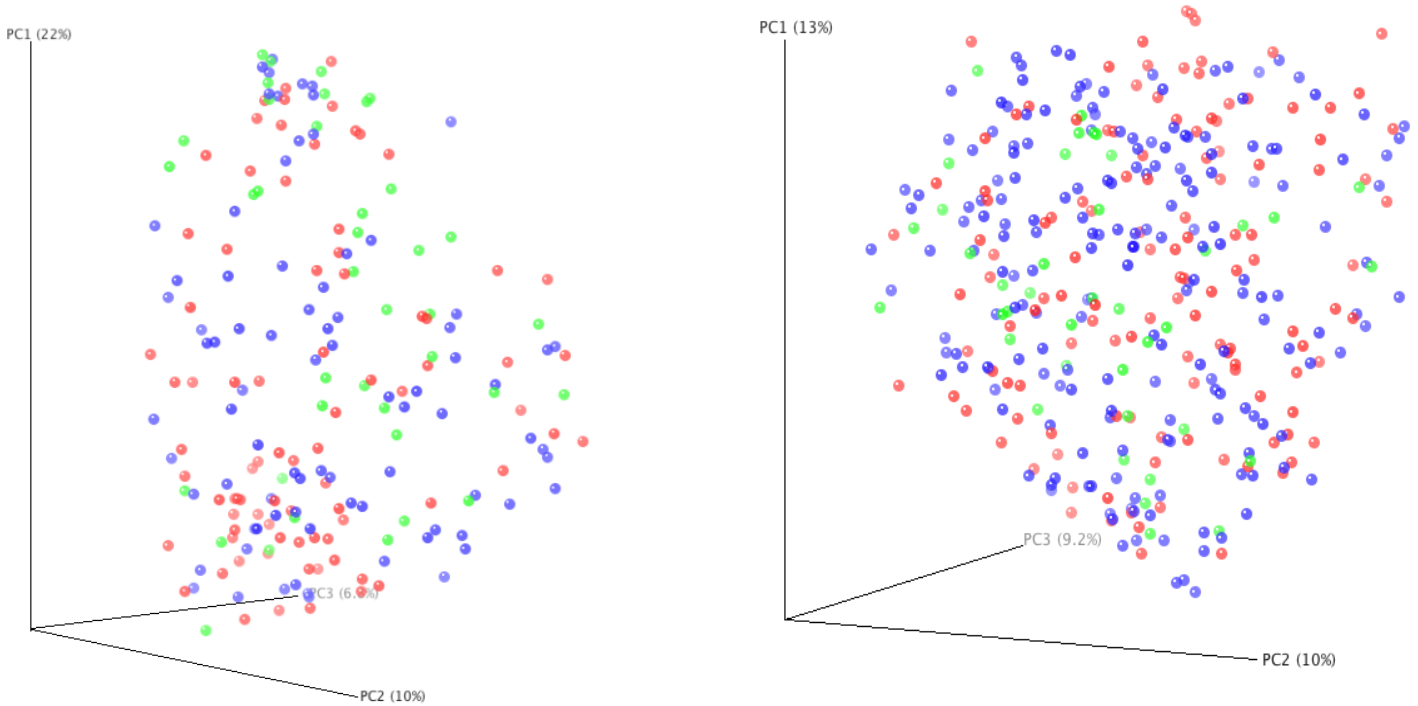
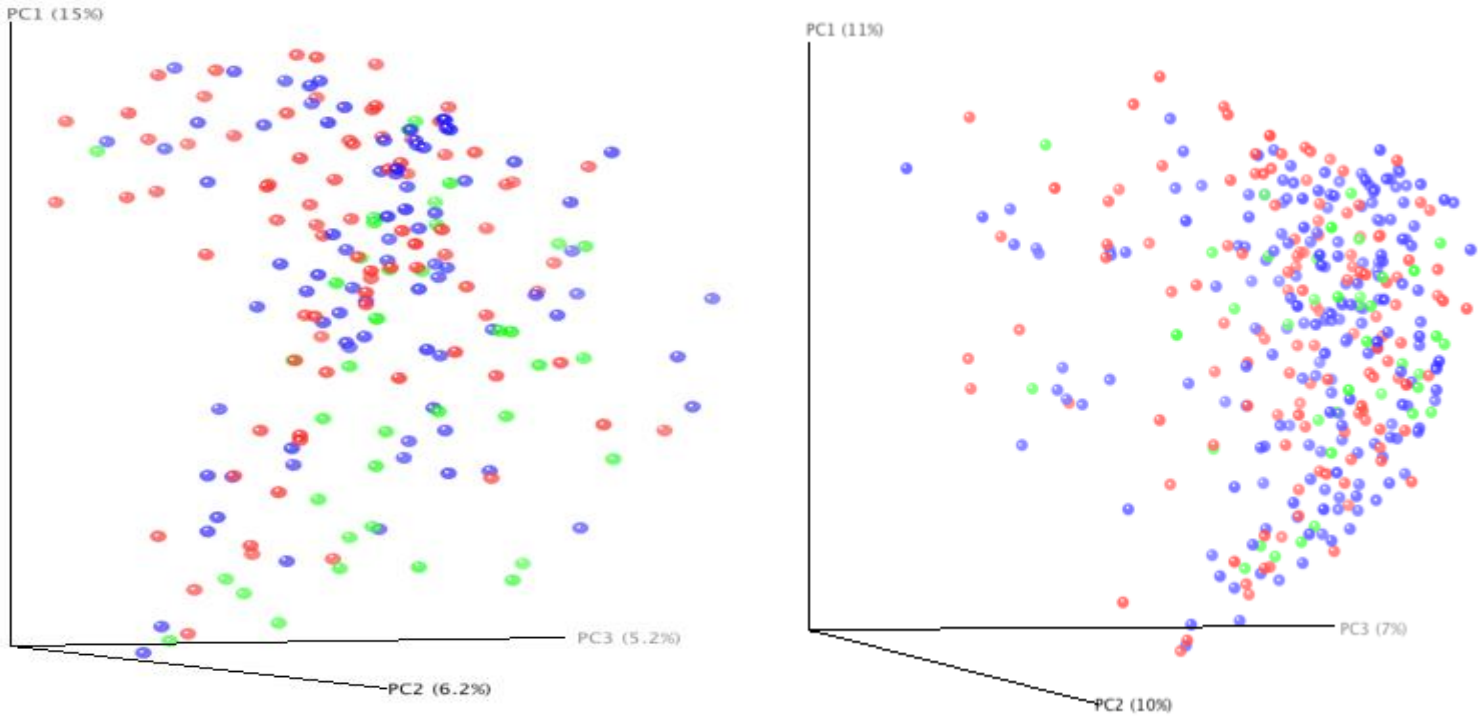
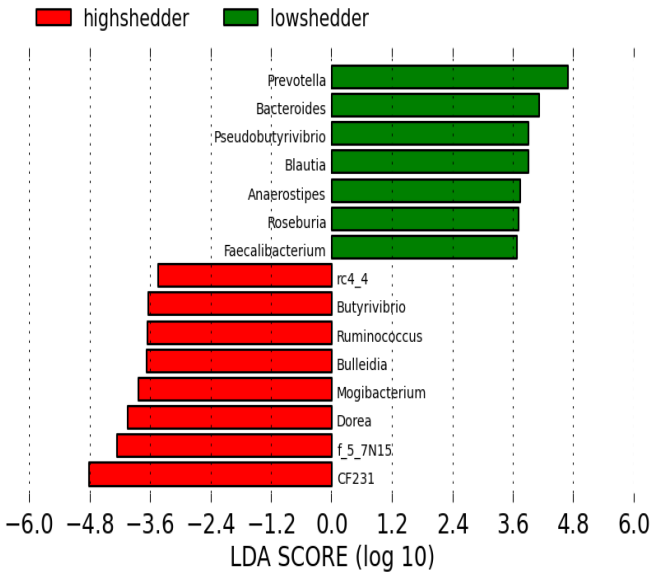


Figure 2.8: Principal coordinate analysis (PCoA) plots for core genera in fecal samples based on Bray-Curtis distances. (a) 2011 (b) 2013. ● High-shedder ● Medium-shedder ● Low-shedder

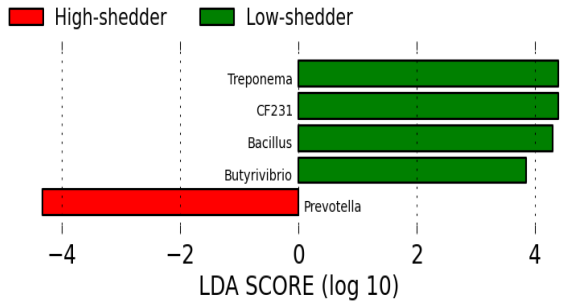


(a) (b)

Figure 2.9: Principal coordinate analysis (PCoA) plots for core OTUs in fecal samples based on Bray-Curtis distances. (a) 2011 (b) 2013. ● High-shedder ● Medium-shedder ● Low-shedder



(a)



(b)

Figure 2.10: LEfse outputs for genera which were differentially abundant between high-shedder and low-shedder fecal samples. (a) 2011 samples (b) 2013 samples. An LDA score of 2.0 was used as the threshold.

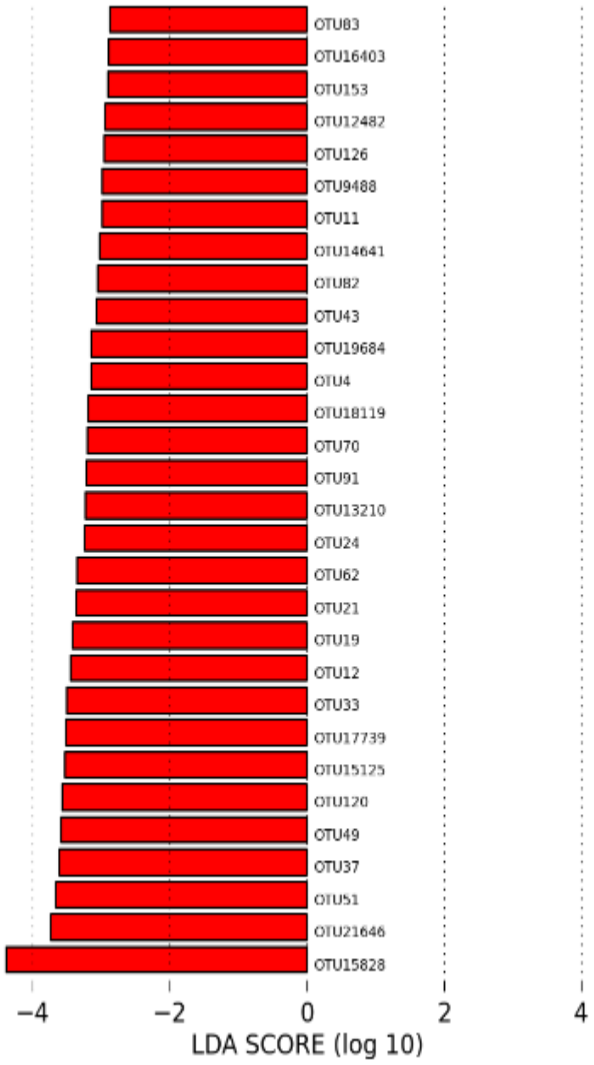


Figure 2.11: LEfse results for OTUs which were significantly more abundant in high-shedders for 2011 samples. Only the top 30 OTUs with the highest LDA scores are shown.

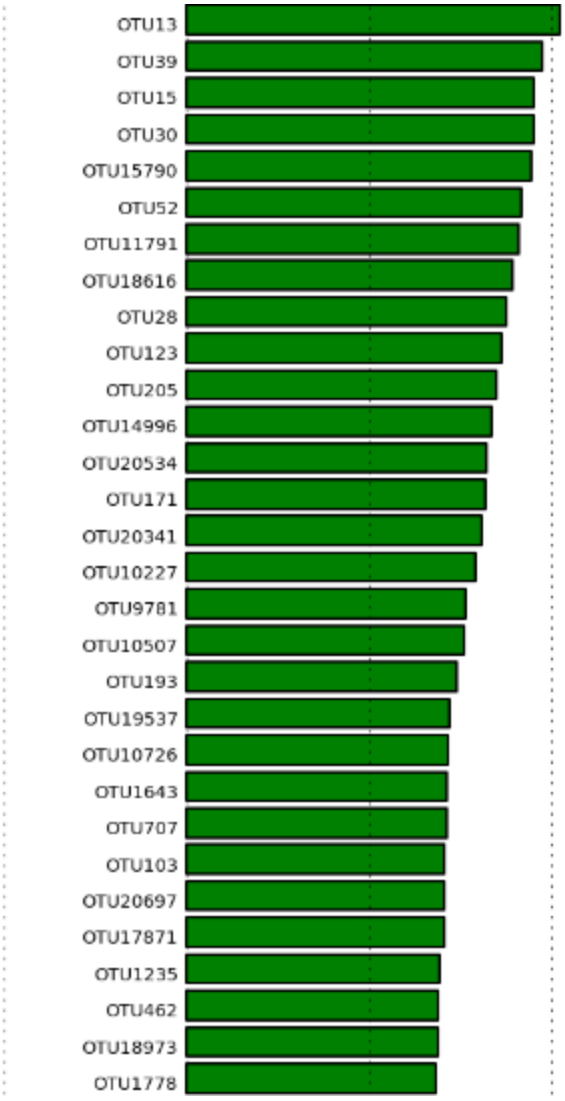


Figure 2.12: LEfse results for OTUs which were significantly more abundant in low-shedders for 2011 samples. Only the top 30 OTUs with the highest LDA scores are shown.

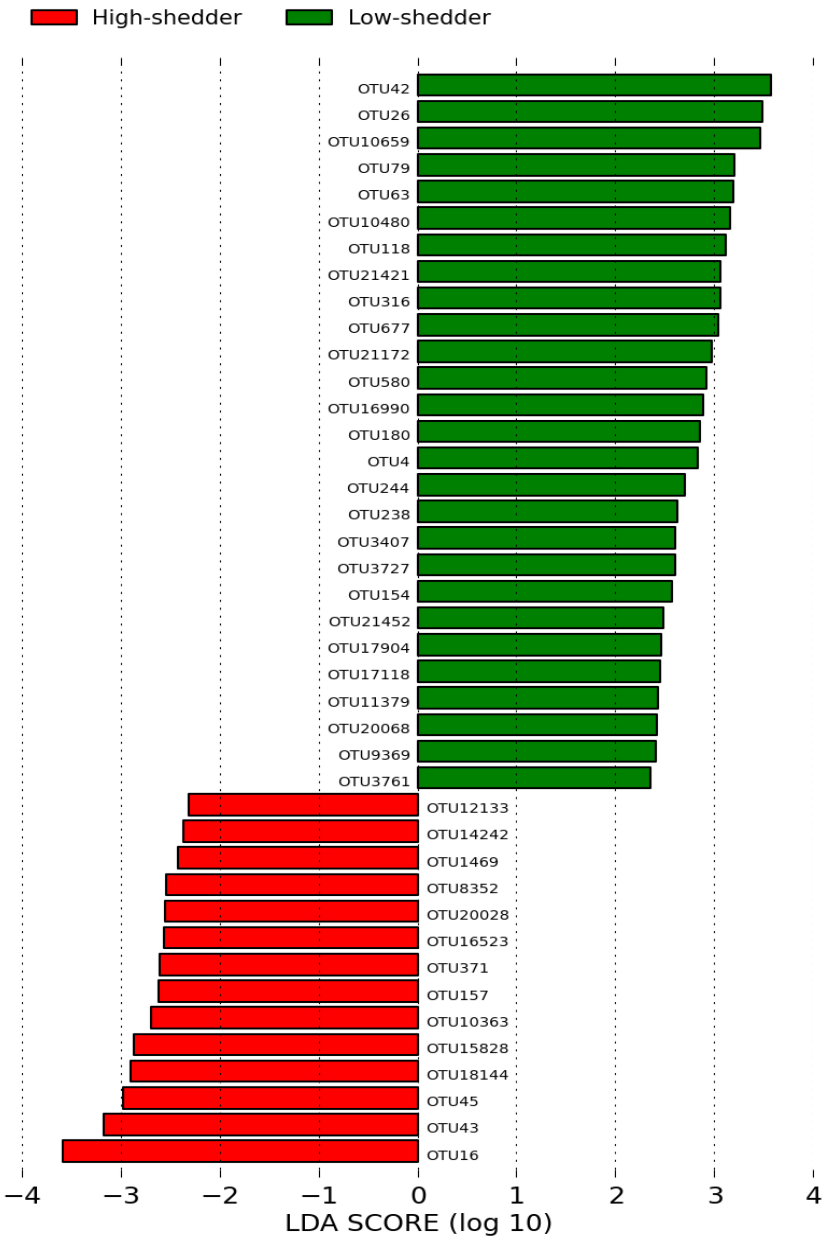


Figure 2.13: LEfse results for OTUs which were discriminative of low-shedders and high-shedders for 2013 samples.

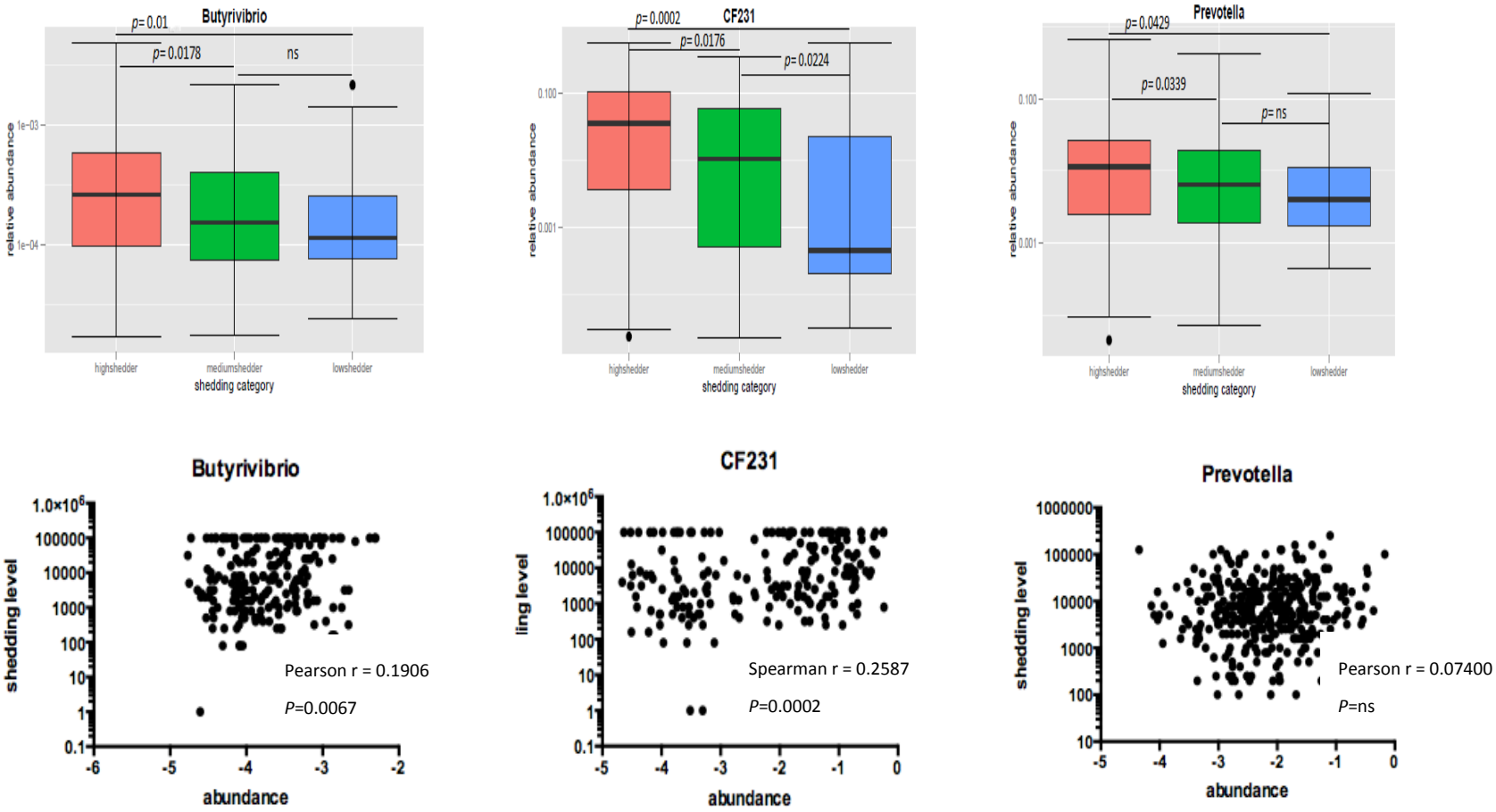
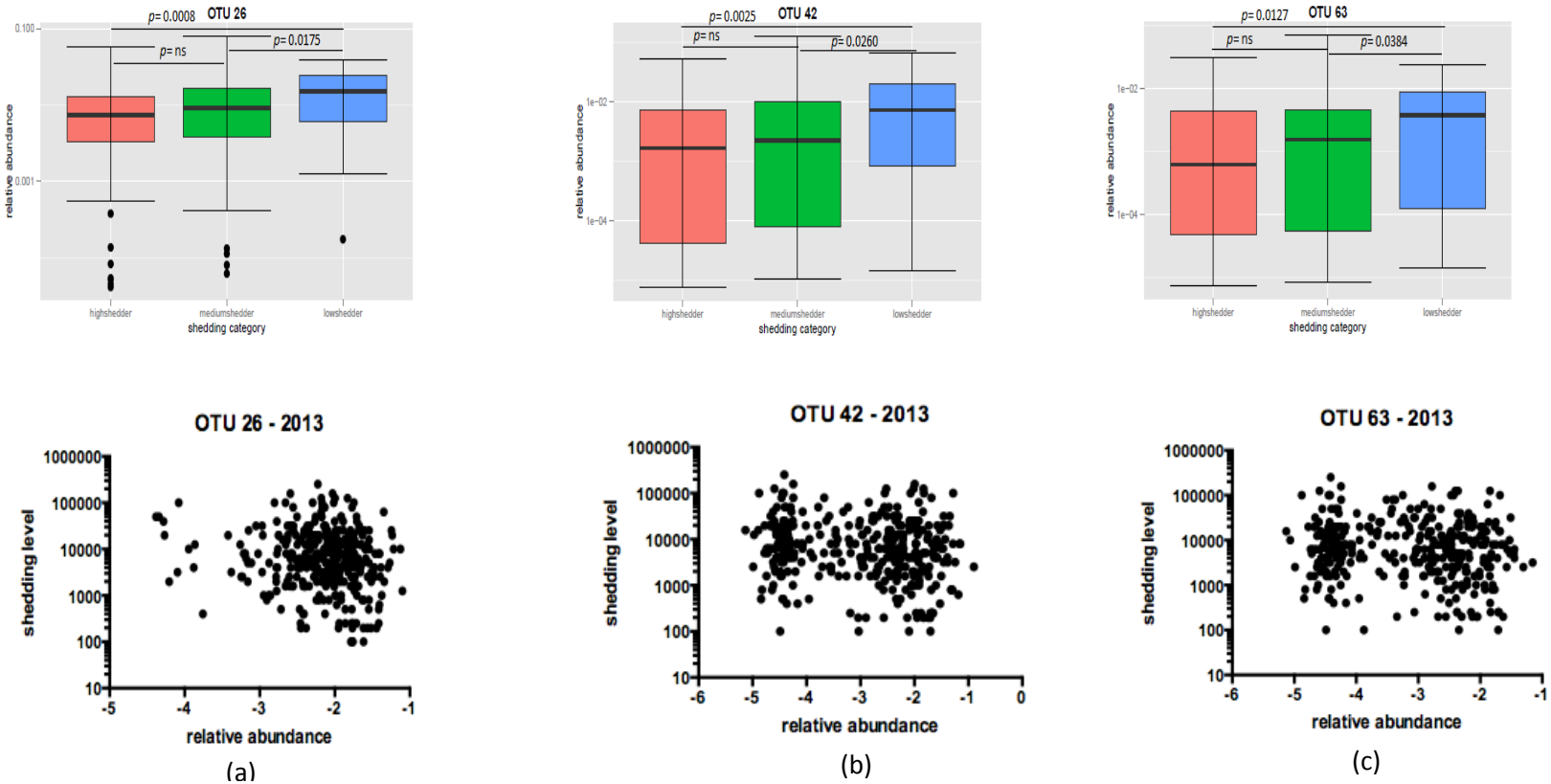


Figure 2.14: Box-and-whisker plots and correlation analysis results for genera that were significantly associated with shedding based on multi-factor ANOVA. *Butyrivibrio* and CF231 were from 2011; *Prevotella* was from 2013. ● - outliers in box-and-whisker plots. Statistical comparisons for box-and-whisker plots were performed using the Mann-Whitney test.

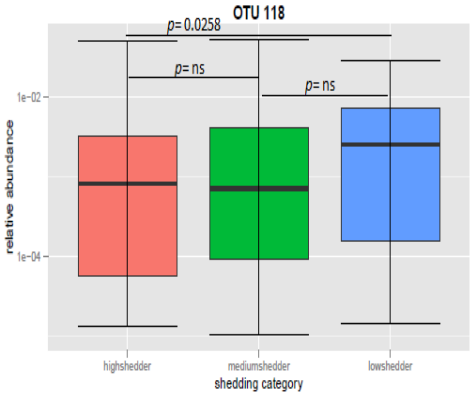


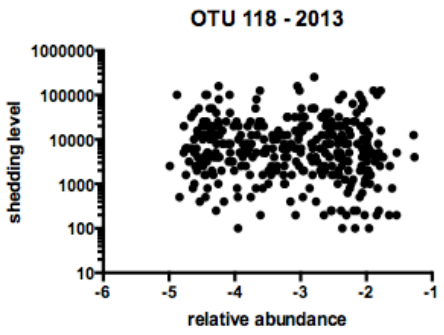
Spearman r = -0.1350, P=0.0106

Spearman r = -0.1485, *P*=0.0049

Spearman r = -0.1085, *P*=0.0401

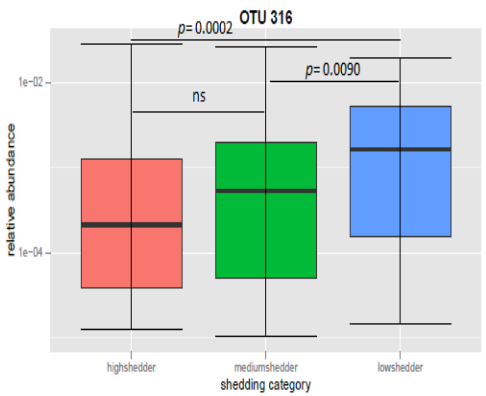
Figure 2.15: Box-and-whisker plots and correlation analysis results for core OTUs that were significantly associated with shedding based on multi-factor ANOVA. ● - outliers in box-and-whisker plots. Statistical comparisons for box-and-whisker plots were performed using the Mann-Whitney test. (a) – (k) OTUs significantly associated with shedding in 2013; (l) OTU significantly associated with shedding in 2011.

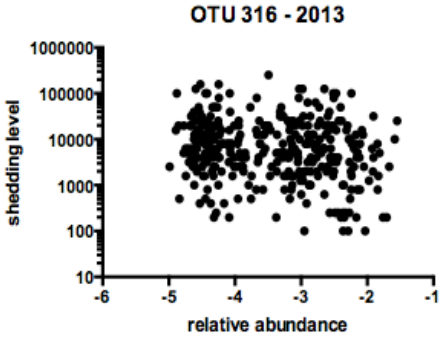




(d)

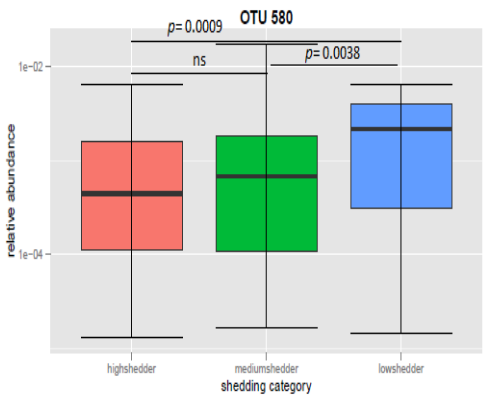
Spearman r = -0.1026, *P*=ns

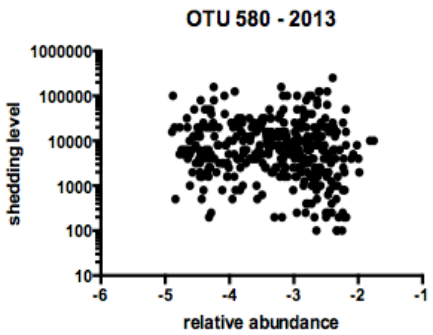




(e)

Spearman r = -0.1899, *P*=0.0003

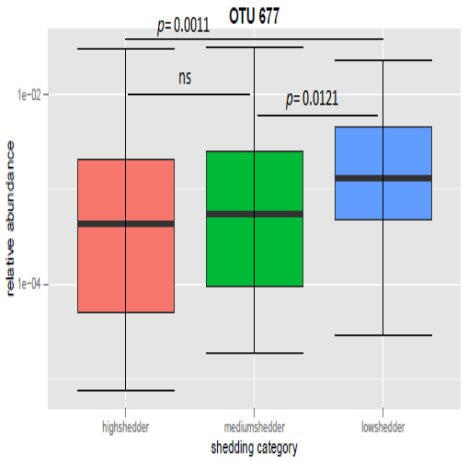


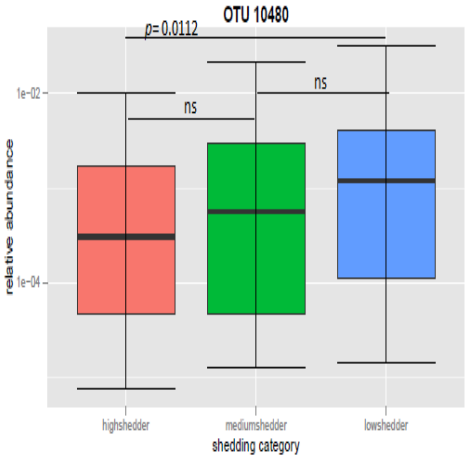


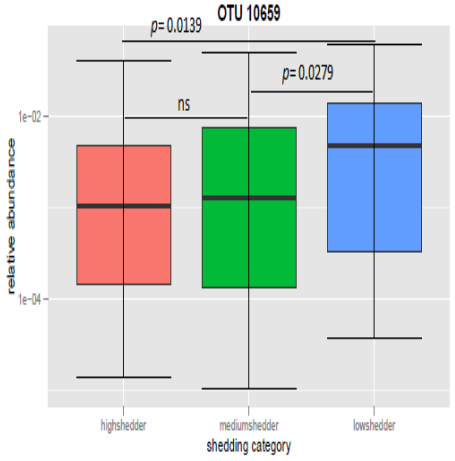
(f)

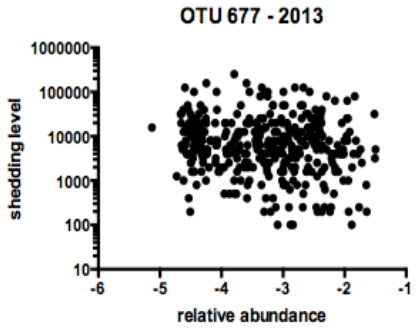
Spearman r = -0.1388, *P*=0.0085

Figure 2.15 continued…….



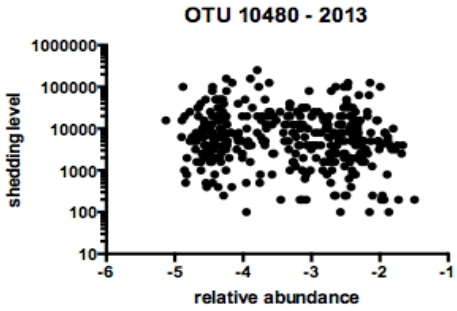






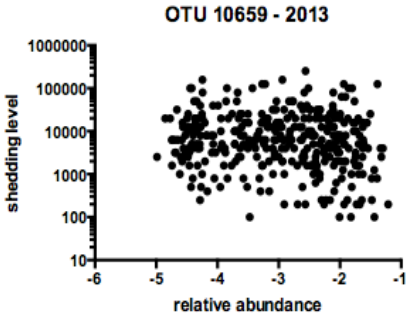
(g)

Spearman r = -0.1254, *P*=0.0176



(h)

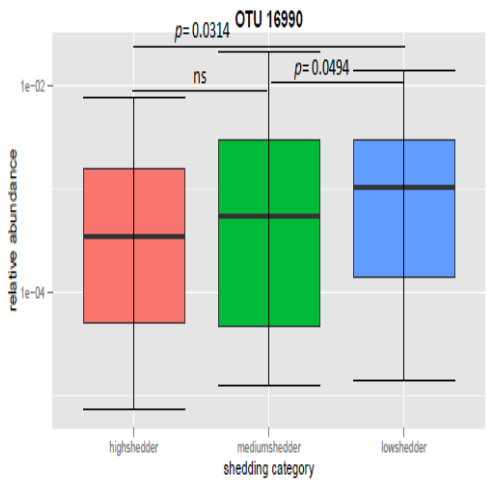
Spearman r = -0.1229, *P*=0.0200

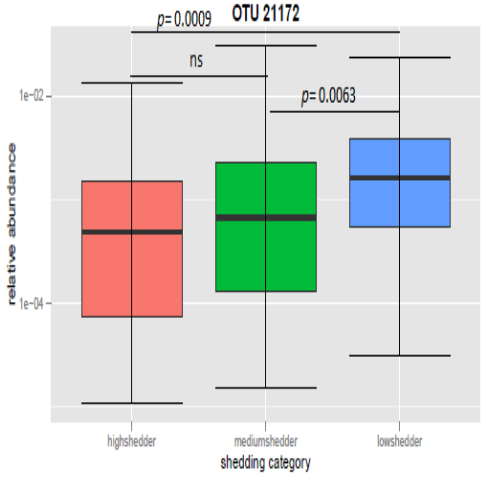


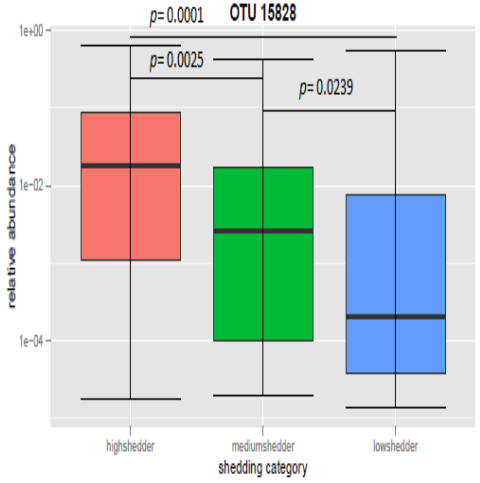
(i)

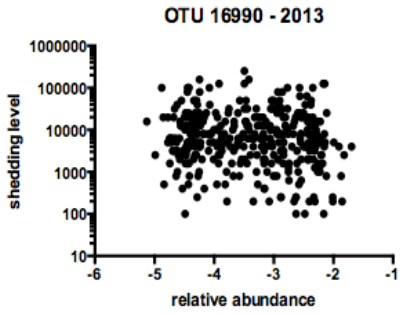
Spearman r = -0.1191, *P*=0.0243

Figure 2.15 continued…….



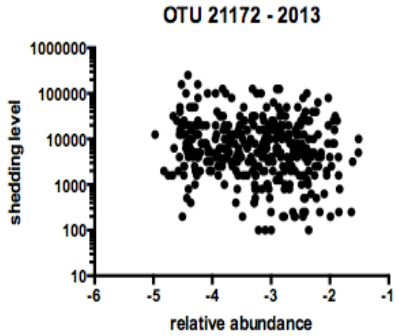






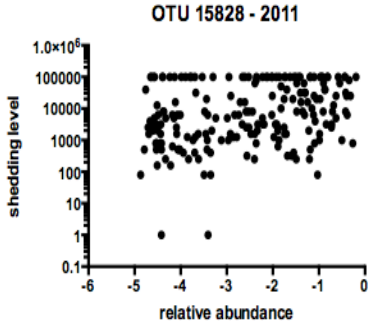
(j)

Spearman r = -0.08485, *P*=ns



(k)

Spearman r = -0.1376, P=0.0092



(l)

Spearman r = 0.2772, *P*=0.0001

Figure 2.15 continued…….

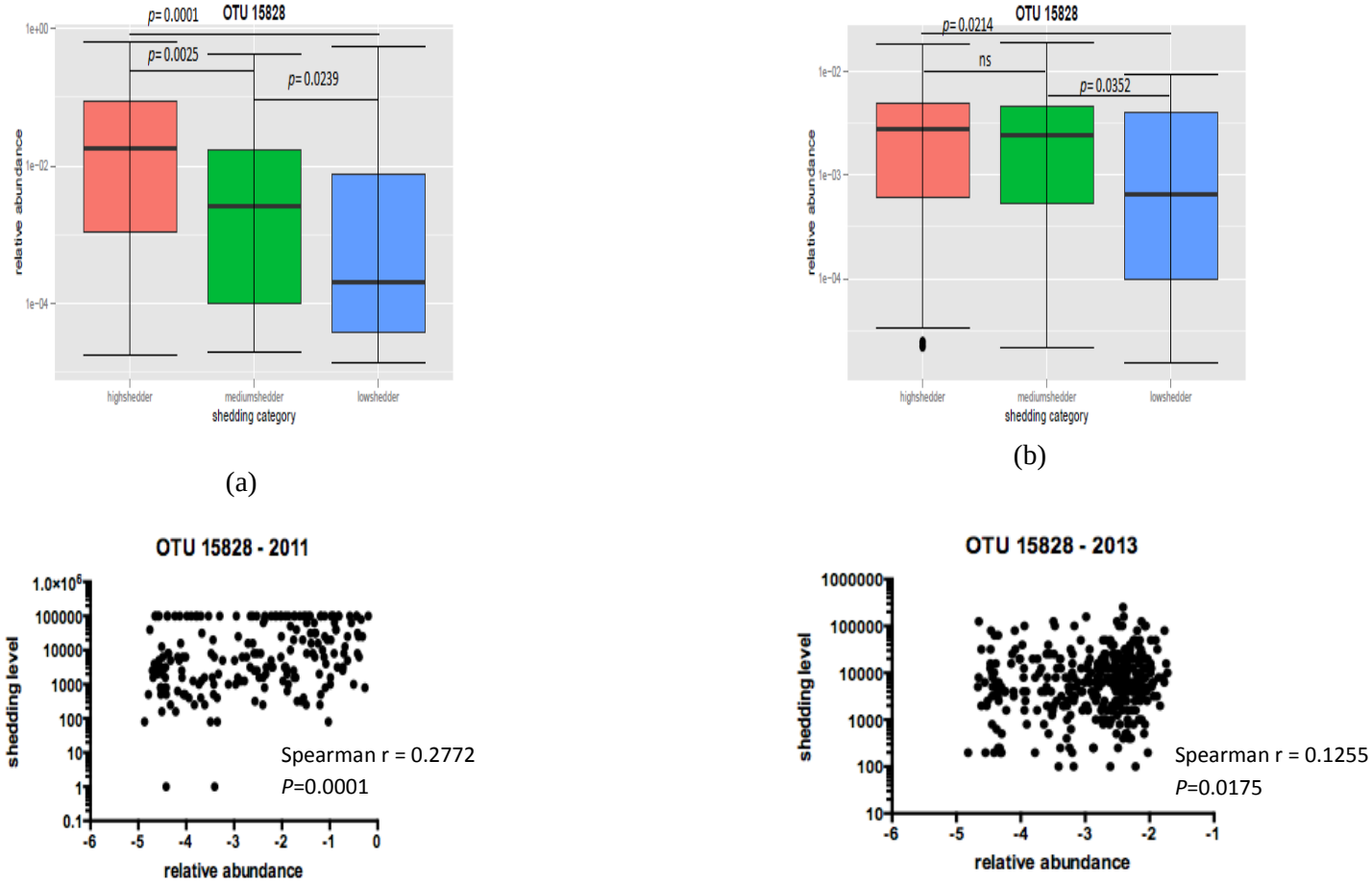


Figure 2.16: Box-and-whisker plots and correlation analysis results for OTU 15828 in (a) 2011 and (b) 2013. ● - outliers in box-and-whisker plots. Statistical comparisons for box-and-whisker plots were performed using the Mann-Whitney test.

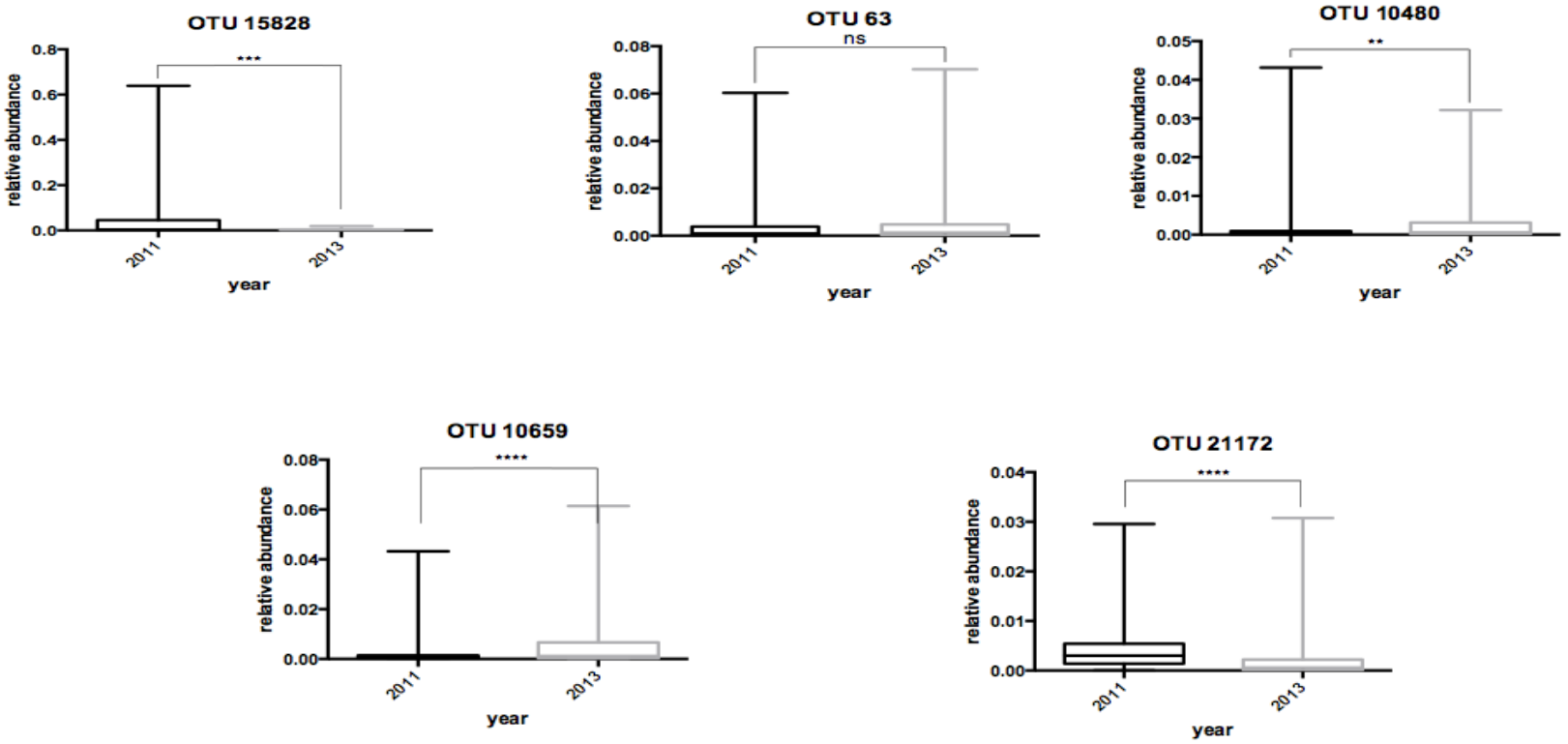


Figure 2.17: Comparison of the relative abundance of OTUs associated with STEC shedding between the two sampling years .

**** $p < 0.0001$; *** $p < 0.001$; ** $p < 0.01$; ns – not significant ($p > 0.05$); Mann-Whitney test.

Table 2.1: Compositions of diets fed to beef steers in 2011

Diet	Component	Amount (kg)
(1) Control Diet (CON)	HMC	48
	DRC	32
	Corn Silage	15
	Supp BN _1112	5
	Sum	100
(2) Dried Distillers Grains with Solubles (DDGS)	DDGS	40
	HMC	24
	DRC	16
	Corn Silage	15
	Supp BN _1112	5
	Sum	100
(3) Wet Distillers Grains with Solubles (WDGS)	WDGS	40
	HMC	24
	DRC	16
	Corn Silage	15
	Supp BN _1112	5
	Sum	100

Table 2.2: Compositions of diets fed to beef steers in 2013

Diet	Component	Amount (kg)
(1) Control Diet	Alfalfa	0
	Stalks	5
	HMC	25.5
	DRC	25.5
	MDGS	40
	Supp BN _1326	4
	Sum	100
	Roughage	5
	Corn	53.5
(2) 15Sil:20MDGS Diet	Alfalfa	0
	Silage	15
	HMC	30.5
	DRC	30.5
	MDGS	20
	Supp BN _1326	4
	Sum	100
	Roughage	7.5
	Corn	68.5
(3) 15Sil:40MDGS Diet	Alfalfa	0
	Silage	15
	HMC	20.5
	DRC	20.5

	MDGS	40
	Supp BN _1326	4
	Sum	100
	Roughage	7.5
	Corn	48.5
(4) 45Sil:20MDGS Diet	Alfalfa	0
	Silage	45
	HMC	15.5
	DRC	15.5
	MDGS	20
	Supp BN _1326	4
	Sum	100
	Roughage	22.5
	Corn	53.5
(5) 45Sil:40MDGS Diet	Alfalfa	0
	Silage	45
	HMC	5.5
	DRC	5.5
	MDGS	40
	Supp BN_1326	4
	Sum	100
	Roughage	22.5
	Corn	33.5

Table 2.3: PERMANOVA results at the (a) core genus (b) core OTU levels.

	Year			
	2011		**2013**	
Factor	**R^2**	**Pr(>F)**	**R^2**	**Pr(>F)**
Shedding categoty	0.01971	0.003	0.00523	0.055
Diet	0.06607	0.001	0.00635	0.019
Time point	0.00895	0.078	0.02089	0.001
Animal	0.00811	0.106	0.00206	0.658

(a)

	Year			
	2011		**2013**	
Factor	**R^2**	**Pr(>F)**	**R^2**	**Pr(>F)**
Shedding category	0.01961	0.001	0.00505	0.028
Diet	0.05852	0.001	0.01005	0.001
Time point	0.01183	0.004	0.02497	0.001
Animal	0.00626	0.177	0.00173	0.901

(b)

Table 2.4: Multi-factor ANOVA results summary showing the significance of each factor on the relative abundance of target genera. (a) 2011 results (b) 2013 results. *P*-values <0.05 are in bold phase.

	Factors		
Genus	**Shedding category**	**Diet**	**Time point**
	***P*-value**	***P*-value**	***P*-value**
Prevotella	0.938	**<0.001**	0.537
Bacteroides	0.141	**<0.001**	0.746
Pseudobutyrivibrio	0.803	**0.024**	0.850
Blautia	0.836	0.611	**0.009**
Anaerostipes	0.918	0.370	0.560
Roseburia	0.697	**0.007**	**0.018**
Faecalibacterium	0.560	0.1832	**0.007**
rc4_4	0.532	**<0.001**	0.087
Butyrivibrio	**0.017**	0.218	0.658
Ruminococcus	0.595	**<0.001**	0.994
Bulleidia	0.366	**0.015**	0.2515
Mogibacterium	0.865	**<0.001**	0.012
Doria	0.624	**<0.001**	0.016
f_5_7N15	0.967	0.098	0.860
CF231	**0.002**	**<0.001**	0.470

(a)

Table 2.4 continued……

Genus	Factors		
	Shedding category	**Diet**	**Time point**
	***P*-value**	***P*-value**	***P*-value**
Treponema	0.998	0.331	0.137
CF231	0.432	0.528	**<0.001**
Bacillus	0.125	0.120	**0.043**
Butyrivibrio	0.751	**0.002**	0.431
Prevotella	**0.044**	0.114	0.479

(b)

Table 2.5: Multi-factor ANOVA results summary showing the significance of each factor on the relative abundance of target core OTUs. (a) 2011 results (b) 2013 results. *P*-values <0.05 are in bold phase (see Appendix VI for actual ANOVA outputs for OTUs which showed significance for shedding category).

	Factors		
OTU number	**Shedding category**	**Diet**	**Time point**
	***P*-value**	***P*-value**	***P*-value**
OTU13	0.076	**0.018**	0.816
OTU39	0.267	**0.005**	**0.043**
OTU15	0.149	0.570	0.698
OTU30	0.378	**<0.001**	0.227
OTU28	0.908	0.786	0.123
OTU52	0.615	**<0.001**	0.752
OTU123	0.875	**0.002**	0.226
OTU171	0.786	**<0.001**	**0.034**
OTU205	0.181	**<0.001**	0.833
OTU11791	0.201	**<0.001**	0.076
OTU14996	0.892	**<0.001**	0.138
OTU15790	0.315	**<0.001**	0.195
OTU18616	0.309	**<0.001**	0.013
OTU20341	0.165	**0.002**	0.802
OTU20534	0.159	0.533	0.028

OTU12	0.653	**0.027**	0.908
OTU19	0.250	0.381	0.171
OTU21	0.490	**0.013**	0.858
OTU24	0.851	**<0.001**	0.769
OTU33	0.816	0.912	0.022
OTU37	0.688	0.064	0.578
OTU49	0.580	0.326	0.003
OTU51	0.482	**0.011**	0.178
OTU62	0.486	0.843	0.006
OTU120	0.821	0.954	0.055
OTU13210	0.388	**0.012**	0.202
OTU15125	0.365	0.200	0.422
OTU15828	**0.004**	**<0.001**	0.898
OTU17739	0.795	**0.027**	0.619
OTU21646	0.127	**<0.001**	<0.001

(a)

Table 2.5 continued……

OTU number	Factors		
	Shedding category	**Diet**	**Time point**
	***P*-value**	***P*-value**	***P*-value**
OTU4	0.121	0.240	**<0.001**
OTU26	**0.002**	**0.006**	0.289
OTU42	**0.004**	0.715	**<0.001**
OTU63	**0.020**	**0.040**	0.041
OTU79	0.231	0.850	0.394
OTU118	**0.031**	**0.043**	0.743
OTU180	0.876	0.490	0.265
OTU316	**<0.001**	**0.015**	0.061
OTU580	**<0.001**	0.161	**<0.001**
OTU677	**0.014**	**0.015**	0.249
OTU10480	**<0.001**	0.146	0.365
OTU10659	**0.001**	0.246	0.022
OTU16990	**0.006**	0.074	0.309
OTU21172	**0.011**	0.288	0.228
OTU21421	0.355	0.504	**<0.001**
OTU16	0.083	**0.001**	**0.037**
OTU43	0.246	**0.043**	0.498
OTU45	0.117	0.322	**<0.001**

OTU157	0.074	0.115	0.172
OTU371	0.138	0.238	**<0.001**
OTU1469	0.099	0.1929	0.685
OTU8352	0.109	0.723	**<0.001**
OTU10363	0.549	0.224	0.127
OTU12133	0.507	0.225	0.141
OTU14242	0.117	0.396	0.333
OTU15828	0.099	**0.015**	0.248
OTU16523	0.137	**0.020**	**0.012**
OTU18144	0.249	0.356	**0.012**
OTU20028	0.479	**0.004**	0.248

(b)

Table 2.6: OTUs which were significantly associated with shedding based on multi-factor ANOVA, box-and-whisker plots, and correlation analysis

OTU	Classification[2]	Associated shedding category	Year
OTU15828	Genus CF231(Family Paraprevotellaceae)	High-shedders	2011
OTU26	Genus CF231 (Family Paraprevotellaceae)	Low-shedders	2013
OTU42	Family Ruminococcaceae	Low-shedders	2013
OTU63	Kingdom Bacteria	Low-shedders	2013
OTU316	Genus *Oscillispira*	Low-shedders	2013
OTU580	Kingdom Bacteria	Low-shedders	2013
OTU677	Class Clostridia	Low-shedders	2013
OTU10480	Family Clostridiaceae	Low-shedders	2013
OTU10659	Kingdom Bacteria	Low-shedders	2013
OTU21172	Family Lachnospiraceae	Low-shedders	2013

[2] Represents the lowest taxonomic level to which an OTU could be classified with a confidence threshold of at least 80% based on the greengenes database version gg_13_5 (May 2013).

Chapter 3

Concluding remarks and future directions

As *Escherichia coli* O157 and non-157 STEC continue to be an important concern to the beef industry, identifying and implementing measures to reduce the entry of these pathogens to the food supply are becoming critically important. It has been well-established that cattle are the major natural reservoir for STEC O157 and these animals are also known to harbor non-O157 STEC strains as well (Smith et al., 2014). Therefore, pre-harvest interventions to reduce fecal shedding of STEC by cattle can potentially minimize the entry of these pathogens to the environment and subsequent contamination of the food supply.

As was mentioned previously, most of the information regarding the ecology of STEC in bovine animals, including heterogeneity of shedding and the 'super-shedder'phenomenon, is heavily biased towards STEC O157. Comparatively little is known about non-O157 STEC in cattle, including whether these bacteria are also shed at super-shedder levels or if non-O157 STEC have a preferred site of colonization within the bovine gastrointestinal tract (GIT), such as the RAJ for *E. coli* O157:H7 (Naylor et al., 2003). As this information becomes available in the future, it may be more appropriate to study shedding levels of particular STEC serogroups, preferably in relation to the microbiota composition of their major site(s) of colonization in the bovine GIT. Additionally, as STEC constitute a diverse group of *E. coli* strains which likely possess different characteristics (e. g., preferred site of colonization within the bovine GIT), it is unlikely that a single specific mechanism (e. g., bacteriocin production) by a member of

the commensal microbiota can inhibit/affect the growth and survival of all STEC in their bovine hosts. However, it can be speculated that a more general mechanism, such as acid production or production of volatile fatty acids (VFAs) by members of the microbiota might have a general inhibitory effect on STEC. Since VFAs are produced in large amounts in the rumen by ruminal microbial species (Dijkstra, 1994), and also because STEC need to transit through the rumen in order to reach the lower gastrointestinal tract (Burow et al., 2005), these factors may also influence STEC colonization, and it might be worth studying the rumen microbial communities between different shedding phenotypes.

This study used the threshold of $>10^4$ CFU/g of feces to define 'high-shedders' of 'total STEC', based on this widely used concentration to define STEC O157 'super-shedders' (Chase-Topping et al., 2008; Arthur et al., 2010). However, STEC O157 is a single serotype while total STEC would constitute a greater number of different serotypes. Therefore, the thresholds used in this study to define the different shedding categories may not have been the most appropriate (medium- and low-shedders were defined using arbitrary thresholds). However, one can argue that if all different STEC within a sample are pathogenic like O157 and behave the same way, the threshold of $>10^4$ CFU/g of feces may be adequate to define a high-shedder, although the necessary research is yet to be performed to make this assumption.

An important observation was that for some animals the shedding phenotype changed between the two sampling time points. This shows the dynamic nature of the shedding phenotype and complicates the process of defining individual animals based on their shedding phenotype. Thus, a robust way of defining shedding phenotypes needs to be established and future studies should use more stringent definitions to identify 'true'

high-shedders, medium-shedders, and low-shedders. Ideally, cattle should be sampled and enumerated for STEC shedding levels during multiple time points and only those animals which consistently shed a given level of STEC should be categorized into different shedding phenotypes and subsequently included in the analysis.

A major confounding factor in this study was the use of different diets in the two sampling years, making it very difficult to compare findings from one year with those of the other year. If both years had the same diets, it would have been interesting to observe if the same OTUs consistently showed a significant association with a given shedding category. In the current study, several OTUs which showed significant differential abundance between shedding categories in one year were under-represented in the other year, most likely due to dietary effects. Therefore, a follow-up study using the same diets as those used in one of the years of this study might yield interesting results. However, using different diets in the two years suggest that dietary factors influence fecal bacterial communities and the correlations of shedding and community composition are diet-specific.

The lowest taxonomic level at which the bioinformatics analysis could be done in this study was the OTU level, which when defined at 3% dissimilarity, has an often cited (albeit controversial) operational definition of a bacterial ‘species’ (Schloss and Westcott, 2011). If there were differences in the fecal microbiota between shedding categories at lower taxonomic levels, such as subspecies or strain, then these differences would not have been detected in this study. Future studies looking at differences in microbial communities at finer taxonomic levels than species might yield new insights on this topic.

With the declaration of the ‘big six’ non-O157 STEC as adulterants in certain raw beef products, there has been renewed interest among the scientific community in better understanding the ecology and prevalence of these bacteria, along with STEC O157, in bovine populations. The results of this study suggest that the fecal prevalence of pathogenic strains of STEC O157 and the ‘big six’ non-O157 STEC among the two herds of feedlot steers surveyed was very low. The finding that there was a high fecal prevalence of non-EHEC *E. coli* belonging to these 7 STEC serogroups emphasizes the need for detection methodologies which not only detect the O-serogroups of concern, but also possess the capacity to indicate their virulence potential.

As mentioned previously, the difficulty of culturing and enumerating non-O157 STEC relative to their O157 counterparts has resulted in a deficiency of information regarding the ecology and prevalence of these pathogens in beef production systems. Initiatives such as the United States Department of Agriculture-National Institute of Food and Agriculture (USDA-NIFA) Coordinated Agricultural Project (CAP) grant on STEC (STEC CAP Team, n. d.), which aims to mitigate risk associated with the 7 major STEC serogroups along the beef production chain, are trying to bridge this gap in knowledge by (in addition to other measures) developing methodologies to better detect and enumerate both O157 and non-O157 STEC. Such initiatives will enable a better understanding of STEC dynamics in beef production environments which can potentially lead to science-based interventions to reduce the disease burden of these pathogens associated with beef and beef products.

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APPENDIX I

Bioinformatics Pipeline

Commands used for initial quality filtering

- Converting the FASTQ file to a FASTA file : convert_fastaqual_fastq.py -c fastq_to_fastaqual –f STEC_plate.fastq –o fastaqual
- split_libraries.py command eg: split_libraries.py –m STEC_2014_Mapping.txt -b variable_length -l 0 -L 1000 –x –o split_library/ -f STEC_plate.fna
- truncate_reverse_primer.py -f STEC_all_plates_seqs.fna -m STEC_rev_prim_mapping.txt -z truncate_only -M 2 -o reverse_primer_removed_truncate_only/
- ./min_max_length.pl –min=130 –max=130 –fasta=STEC_plates_rev_primer_truncated.fna
- mothur > summary.seqs(fasta=STEC_plates_trimmed.fasta)

The resulting reverse complemented file was used as the input file for the batch script 'usearch_batch_master.pbs' (Appendix II) where it was initially converted to a form compatible with USEARCH using the perl script qiime_to_userach.pl (Appendix II).

QIIME commands for generating OTU table

The 'test.otus2.fa' file resulting from the command **fasta_number.py** (see above) was used as the input file for the QIIME command **assign_taxonomy.py**. This command assigns the taxonomy information for the representative OTUs in the 'test.otus2.fa' file.

assign_taxonomy.py -i test.otus2.fa –t /Users/samodhafernando/nirosh_BI/gg_13_5_otus/taxonomy/97_otu_taxonomy.txt –r /Users/samodhafernando/nirosh_BI/gg_13_5_otus/rep_set/97_otus.fasta –o assign_gg_taxonomy/

The taxonomy information thus generated was annotated to the corresponding OTUs in the 'test.otu_table.txt'. This OTU table was then converted to the 'biom' format using the following QIIME command:

convert_biom.py –i test.otu_table.txt –o test.otu_table.biom --biom_table_type="otu table" –process_obs_metadata taxonomy

Singleton OTUs (2) as well as OTUs with Cyanobacterial assignments (183 OTUs) were filtered out from the OTU table using the QIIME command **filter_otus_from_otu_table.py**. Two samples which had <3,000 sequences were also removed from the OTU table using the QIIME command **filter_samples_from_otu_table.py**. Any OTU not aligning within the target region of the 16S rRNA gene (see below) was also removed. The resulting complete OTU table contained 1005 samples with a total of 20633 OTUs comprising 14343659 sequences.

Removing non-aligning OTUs

The output aligned file from RDP contained the summary file 'alignment_summary.txt'. This file contains information regarding the starting and ending alignment positions within the 16S rRNA gene for each representative OTU. Based on this data, 984 OTUS which aligned outside of the target region of the 16S rRNA gene were removed from the OTU table. This was done by only retaining the properly aligning OTUs:

filter_otus_from_otu_table.py –i test.otu_table.biom –o test.otu_table_aligning.biom –negate_ids_to_exclude –e properly_aligning_otus.txt

Shedding-based data analysis

Since the data analysis was to be done separately for each year, the 'total' otu table was split into two separate otu tables corresponding to each year:

MacQIIME pcp166836pcs:shedding_analysis_130 $ **split_otu_table.py** -i STEC_2014_130_correct.otu_table_sorted.biom -o year_based_split -m STEC_master_mapping.txt -f Year

For the 2013 samples, only the samples which had shedding information were retained while the rest were filtered out

MacQIIME pcp166836pcs:2013 $ **filter_samples_from_otu_table.py** -i STEC_2014_130_correct.otu_table_sorted_2013.biom -o STEC_onlyshedders_2013.biom --sample_id_fp only_shedders.txt

Determining alpha diversity estimates for the shedding phenotypes

MacQIIME pcp166836pcs:2011 $ **alpha_diversity.py** -i STEC_2014_130_correct.otu_table_shedding_sorted_2011.biom -m chao1,observed_species,shannon -o alpha_div_results_2011.txt

MacQIIME pcp166836pcs:2013 $ **alpha_diversity.py** -i STEC_onlyshedders_2013_sorted.biom -m chao1,observed_species,shannon -o alpha_div_results_2013.txt

Establishing core measurable microbiomes (CMMs)

To look at each 'shedding core' separately, the OTU table was split based on shedding phenotype:

MacQIIME pcp166836pcs:2011 $ **split_otu_table.py** -i STEC_2014_130_correct.otu_table_shedding_sorted_2011.biom -o shedding_split -m STEC_2014_shedders_mapping.txt -f Sheddingphenotype

Within each shedding phenotype, a 'family' and 'genus' level core (only taxa or OTUs present in at least 75% of the samples) was established as follows;

For High-shedders:

MacQIIME pcp166836pcs:HS $ **summarize_taxa.py** -i STEC_2014_130_correct.otu_table_shedding_sorted_2011_High-shedder.biom -L 2,3,4,5,6,7 -o total_analysis/total.summarize_taxa_bioms

For Medium-shedders:

MacQIIME pcp166836pcs:HS $ **summarize_taxa.py** -i STEC_2014_130_correct.otu_table_shedding_sorted_2011_Medium-shedder.biom -L 2,3,4,5,6,7 -o total_analysis/total.summarize_taxa_bioms

For Low-shedders:

MacQIIME pcp166836pcs:HS $ **summarize_taxa.py** -i STEC_2014_130_correct.otu_table_shedding_sorted_2011_Low-shedder.biom -L 2,3,4,5,6,7 -o total_analysis/total.summarize_taxa_bioms

The perl script 'parse_taxa_table.py' (Appendix II) was used to determine the core taxa at the family and genus levels.

the 'shedding cores' were merged to make a single OTU table. First, the taxa cores needed to be converted from the .txt format to the .biom format:

MacQIIME pcp166836pcs:taxa_based_cores_75 **$ convert_biom.py** -i STEC_2011_HS_family_core75.txt -o STEC_2011_HS_family_core75.biom --biom_table_type="otu table"

MacQIIME pcp166836pcs:taxa_based_cores_75 **$ convert_biom.py** -i STEC_2011_MS_family_core75.txt -o STEC_2011_MS_family_core75.biom --biom_table_type="otu table"

MacQIIME pcp166836pcs:taxa_based_cores_75 **$ convert_biom.py** -i STEC_2011_LS_family_core75.txt -o STEC_2011_LS_family_core75.biom --biom_table_type="otu table"

MacQIIME pcp166836pcs:taxa_based_cores_75 $ **merge_otu_tables.py** -i

STEC_2011_HS_family_core75.biom,STEC_2011_MS_family_core75.biom,STEC_2011_LS_family_core75.biom -o merged_family_core.biom

MacQIIME pcp166836pcs:taxa_based_cores_75 $ **convert_biom.py** -i merged_family_core.biom -o merged_family_core.txt -b

#from this merged otu table, can get the 'list' of core families (core_families.txt) so that they can be filtered out of the main 'family' taxa table

#To do that need to have a 'total family' otu table for 2011:

MacQIIME pcp166836pcs:2011 $ **summarize_taxa.py** -i STEC_2014_130_correct.otu_table_shedding_sorted_2011.biom -L 2,3,4,5,6,7 -o total_analysis/total.summarize_taxa_bioms

#Now from the output file 'total.summarize_taxa_bioms/ STEC_2014_130_correct.otu_table_shedding_sorted_2011_L5.biom' can filter out the core:

MacQIIME pcp166836pcs:2011 $ **filter_otus_from_otu_table.py** -i STEC_2014_130_correct.otu_table_shedding_sorted_2011_L5.biom -o STEC_2011_overall_core_families.biom --negate_ids_to_exclude -e 2011_core_families.txt

The same was done with the 2013 data set as well

#The OTU-based core was also determined for each shedding phenotype

2011 data set

#The core OTUs for each shedding phenotype were determined by only retaining those OTUs which were present in at least 75% of the relevant fecal samples. This was done using the 'filter_otus_from_otu_table.py' command.

MacQIIME pcp166836pcs:shedding_split $ **filter_otus_from_otu_table.py** -i STEC_2014_130_correct.otu_table_shedding_sorted_2011_High-shedder.biom -o High-shedder_core.biom -s 61

MacQIIME pcp166836pcs:shedding_split $ **filter_otus_from_otu_table.py** -i STEC_2014_130_correct.otu_table_shedding_sorted_2011_Medium-shedder.biom -o Medium-shedder_core.biom -s 61

MacQIIME pcp166836pcs:shedding_split $ **filter_otus_from_otu_table.py** -i STEC_2014_130_correct.otu_table_shedding_sorted_2011_Low-shedder.biom -o Low-shedder_core.biom -s 30

MacQIIME pcp166836pcs:shedding_split $ **merge_otu_tables.py** -i High-shedder_core.biom,Medium-shedder_core.biom,Low-shedder_core.biom -o merged_cores.biom

#From this merged_cores.biom OTU table a list of the 'overall core' OTUs was obtained. This was subsequently used to make the overall core OTU table:

MacQIIME pcp166836pcs:2011 $ **filter_otus_from_otu_table.py** -i STEC_2014_130_correct.otu_table_shedding_sorted_2011.biom -o STEC_2011_overall_core.otu_table.biom --negate_ids_to_exclude -e 2011_core_otus.txt

#The same was done with the 2013 data set as well

Determining beta diversity estimates for shedding phenotypes

2011 data set

At the level of core taxa

MacQIIME pcp166836pcs:2011 $ **beta_diversity_through_plots.py** -i STEC_2011_overall_core_families_raw_reads.biom -o core_families_beta_diversity -e 1645 -m STEC_2014_shedders_mapping.txt -p qiime_parameters_working-1.txt -c Sheddingphenotype

Similarly, beta diversity was determined at the genus level for the core genera among the shedding phenotypes

At the level of core OTUs

MacQIIME pcp166836pcs:2011 $ **beta_diversity_through_plots.py** -i STEC_2011_overall_core.otu_table.biom -o core_beta_diversity -e 1474 -m STEC_2014_shedders_mapping.txt -p qiime_parameters_working-1.txt -t aligned_STEC_130_select_repset.phylip.tre -c Sheddingphenotype

2013 data set

At the level of core taxa

MacQIIME pcp166836pcs:2013 $ **beta_diversity_through_plots.py** -i STEC_2013_overall_core_family_raw_reads.biom -o core_families_beta_diversity -e 1236 -m STEC_2014_shedders_mapping.txt -p qiime_parameters_working-1.txt -c Sheddingphenotype

MacQIIME pcp166836pcs:2013 $ **beta_diversity_through_plots.py** -i STEC_2013_overall_core_genus_raw_reads.biom -o core_genera_beta_diversity -e 473 -m STEC_2014_shedders_mapping.txt -p qiime_parameters_working-1.txt -c Sheddingphenotype

At the level of core OTUs

MacQIIME pcp166836pcs:2013 $ **beta_diversity_through_plots.py** -i STEC_2013_overall_core.otu_table.biom -o core_beta_diversity -e 1005 -m STEC_2014_shedders_mapping.txt -p qiime_parameters_working-1.txt -t aligned_STEC_130_select_repset.phylip.tre -c Sheddingphenotype

Comparing overall core microbiotas between the two years

#had to make new cores for both years as this analysis wasn't based on shedding

MacQIIME pcp166836pcs:2011 $ **summarize_taxa.py** -i STEC_2014_130_correct.otu_table_sorted_2011.biom -L 2,3,4,5,6,7 -o overall_total_analysis/total.summarize_taxa_raw_reads –a

MacQIIME pcp166836pcs:2013 $ **summarize_taxa.py** -i STEC_2014_130_correct.otu_table_sorted_2013.biom -L 2,3,4,5,6,7 -o all_animals_total_analysis/total.summarize_taxa_raw_reads -a

The perl script 'parse_taxa_table.py' (Appendix II) was used to determine the core taxa at the family and genus levels.

APPENDIX II

Scripts used for data analysis

(1) Perl script **min_max_length.pl**

```
#!/usr/bin/perl -w

use strict;
use Getopt::Long;

#Command line parameters:
my $fasta = "";
my $min = "";
my $max = "";

#Setup the command line options using Getopt:Long
my $commandline = GetOptions("fasta:s", \$fasta,"min:s", \$min,"max:s", \$max);

if (!$commandline || $fasta eq "" || $min eq "" || $max eq "") {
        print STDERR "Usage: $0 -fasta -min -max \n";
        print STDERR "example: ./header_lines_sff_split.pl -fasta=16S.fasta -min=100 -
max=467 \n\n";
        exit;
}

open (my $FASTA_FILE, "$fasta") or die "Can't open FASTA file!";
open (my $NEW_FASTA, ">trimmed.fasta") or die "Can't open output FASTA file";

my $input_read_count = 0;
my $output_read_count = 0;
my $header;
my $index_max = ($max - 1);
my $sequence;

while (my $line = readline($FASTA_FILE)) {
        chomp $line;
        my $check_line = substr ($line, 0, 1);

        if ($check_line eq ">") {
                $header = $line;
                $input_read_count ++;
```

```
                next;
        }

        my $seq_length = length ($line);
   #print "$seq_length\n" ;

        if ($seq_length < $min) {
                next;
        }

        if ($seq_length > $max) {
                my $max_line = substr ($line, 0, $index_max);
                if ($output_read_count == 0) {
                        print $NEW_FASTA "$header\n";
                        print $NEW_FASTA "$max_line";
                }
                print $NEW_FASTA "\n$header\n";
                print $NEW_FASTA "$max_line";
                $output_read_count ++;
        } else {
                if ($output_read_count == 0) {
                        print $NEW_FASTA "$header\n";
                        print $NEW_FASTA "$line";
                }
                print $NEW_FASTA "\n$header\n";
                print $NEW_FASTA "$line";
                $output_read_count ++;
        }
}

print "\nInput read count: $input_read_count \n";
print "Output read count: $output_read_count \n\n";

close ($FASTA_FILE) or die "Can’t close the FASTA file!";
close ($NEW_FASTA) or die "Can’t close the NEW_FASTA file!";
```

(2) Perl script **qiime_to_usearch.pl** (Author: Chris Anderson/Fernando Lab UNL ANSC)

```
#!/usr/bin/perl -w

use strict;

use Getopt::Long;


#Command line parameters:
```

```
my $fasta = "";
my $prefix = "";
#Setup the command line options using Getopt:Long
my $commandline = GetOptions("fasta:s", \$fasta,
                             prefix:s, \$prefix);
if (!$commandline || $fasta eq "" || $prefix eq "" ) {
        print STDERR "Example: ./qiime_ \n";
        exit;
}
my $output = 0;
my @split_line;
my @split_number;
my @split_id;

open (my $FASTA_FILE, "$fasta") or die "Can't open the input FASTA file";
open (my $FORMAT_FILE, ">format.fasta") or die "Can't open the otuput FASTA file!";

while (my $line = readline($FASTA_FILE)) {
        chomp $line;
        my $check_line = substr ($line, 0, 1);
        if ($check_line eq ">") {
                @split_line = split /_/, $line;
                @split_number = split /\s/, $split_line[1];
                @split_id = split />/, $split_line[0];
                print $FORMAT_FILE ">$prefix$split_number[0];barcode=$split_id[1]\n";
        } else {
                print $FORMAT_FILE "$line\n";
                $output ++;
        }
```

```
}


print "Output Sequences: $output\n";


close ($FASTA_FILE) or die "Can't close input FASTA file! \n";

close ($FORMAT_FILE) or die "Can't close output FASTA file! \n";
```

(3) **Batch script for performing UPARSE pipeline commands** (This batch script was run on the 'Tusker' server at the UNL Holland Computing Center) - (Author: Chris Anderson/Fernando Lab UNL ANSC)

```
#!/bin/sh
#SBATCH --ntasks=10
#SBATCH --time=6:00:00
#SBATCH --mem-per-cpu=5000
#SBATCH --output=usearch.%J.stdout
#SBATCH --error=usearch.%J.stderr

/home/samodha/shared/Programs/./qiime_to_usearch.pl -fasta=test.trim.rc.fasta -prefix=test

/home/samodha/shared/Programs/./usearch7.0.10 -derep_fulllength format.fasta -sizeout -output
test.derep.fa

/home/samodha/shared/Programs/./usearch7.0.10 -sortbysize test.derep.fa -minsize 2 -output
test.derep.sort.fa

/home/samodha/shared/Programs/./usearch7.0.10 -cluster_otus test.derep.sort.fa -otus
test.otus1.fa

/home/samodha/shared/Programs/./usearch7.0.10 -uchime_ref test.otus1.fa -db
/home/samodha/shared/Programs/gold.fasta -strand plus -nonchimeras test.otus1.nonchimera.fa

python /home/samodha/shared/Programs/usearch_python_scripts/fasta_number.py
test.otus1.nonchimera.fa > test.otus2.fa

/home/samodha/shared/Programs/./usearch7.0.10 -usearch_global format.fasta -db test.otus2.fa -
strand plus -id 0.97 -uc test.otu_map.uc

python /home/samodha/shared/Programs/usearch_python_scripts/uc2otutab.py test.otu_map.uc >
test.otu_table.txt
```

(4) Perl script **parse_taxa_table.pl** (Author: Nirosh Aluthge/Fernando Lab UNL ANSC)

```
#!/arch/bin/perl -w

#PURPOSE: to look at the core taxa among samples in an otu table

#INPUT: Tab-delimited OTU table which has been transposed with taxa as rows and samples as columns. A good example would be an output file from the QIIME commmand summarize_taxa.py.This file is already transposed in the required way. However, the headers need to be removed ('Taxon' and sample IDs).

#OUTPUT: core_taxa_otu_table

use strict;

my $ele ;
my $sample_presence = 0 ;
my @cols ;
my $total_input = 0 ;
my $core_output = 0 ;
my $not_core_output = 0 ;

open (my $INPUT, "/home/perlcourse/2012/nirosh/course/STEC_project/STEC_2014_130_correct.otu_table_sorted_2013_L6.txt") or die "Can't open input file" ;

open (my $OUTPUT1, "+>/home/perlcourse/2012/nirosh/course/STEC_project/STEC_2013_overall_genus_core75_raw_reads.txt") or die "Can't open output file1" ;

open (my $OUTPUT2, "+>/home/perlcourse/2012/nirosh/course/STEC_project/LS_notcore50_family.txt") or die "Can't open output file2" ;

while (my $line = readline ($INPUT)) {
  chomp $line;
  if ($line =~ /;g__\w/) {# to check if the taxonomy assigned goes down to the required level. in this example, it is looking for phylum level taxa hence the checking for ';p_'
    $total_input ++ ;
    @cols = split/\t/, $line ;
    foreach $ele (@cols) {
      if ($ele !~ /k__/) {# leaving out the taxon field since it doesn't have a numerical value
```

```
            if ($ele != 0) { # if a value is not equal to zero, it means that that taxon is present in
that sample
              $sample_presence++ ; # keeps count of the number of samples in which taxon is
present
            }
     }
   }
   #print "$sample_presence\n" ;
   if ($sample_presence >=603) { # compares the number of samples in which taxon was detected
to check whether it's greater than or equal to the number of samples in which it should be present
in order to be considered as part of the core

     print $OUTPUT1 "$line\n" ;# if requirements are satisfied to be considered part of the core,
that taxon info will be written to the output file
     $core_output++ ;
   } else {
     print $OUTPUT2 "$line\n" ;
     $not_core_output++
   }
  }
  undef @cols ; # preparing variables to loop through the next taxon
  $sample_presence = 0 ;
}

print "2013 total genera: $total_input\n" ;
print "2013 core genera: $core_output\n" ;
print "2013 not core genera: $not_core_output\n" ;

close ($INPUT) or die "Can't close input file!" ;
close ($OUTPUT1) or die "Can't close output file1!" ;
close ($OUTPUT2) or die "Can't close output file2!" ;
```

(5) Perl script **normalize_otu_table.pl** (Author: Nirosh Aluthge/Fernando Lab UNL ANSC)

```
#!/arch/bin/perl -w

#PURPOSE: To convert a 'raw numbers' OTU table to a normalized OTU table

#INPUT: Tab-delimited OTU table which has been transposed and the headers removed

#OUTPUT: normalized_otu_table

use strict;
```

```
my $ele1 ;
my $ele2 ;
my @norm_val_array ;
my @cols2 ;
my $tot_count = 0 ;
my $norm_val = 0 ;
my $INPUT2 ;
#my @tot_count_array ;

open (my $INPUT1, "/home/perlcourse/2012/nirosh/course/STEC_project/STEC_2013_eae.txt")
or die "Can't open input file" ;

open (my $OUTPUT,
"+>/home/perlcourse/2012/nirosh/course/STEC_project/normalized_STEC_2013_eae.txt") or die
"Can't open output file" ;

while (my $line1 = readline ($INPUT1) ) {
  chomp $line1 ;
  my @cols1 = split/\t/, $line1 ;
  foreach $ele1 (@cols1) {
    $tot_count = $tot_count + $ele1 ; #getting the total sequence count for that sample
  }
  @cols2 = split/\t/, $line1 ;
  foreach $ele2 (@cols2) {
    $norm_val = $ele2/$tot_count ; #each of the values for the sample are divided by the total
count
    my $rounded_norm_val = sprintf ("%.6f" , $norm_val);#round value to 4 decimal places
    push (@norm_val_array, $rounded_norm_val) ;#all the normalized values of the sample are
pushed into an array in order
  }
  $" = "\t";
  print $OUTPUT "@norm_val_array\n" ;
  undef @norm_val_array ; #preparing variables for the next line
  undef @cols2 ;
  $tot_count = 0 ;
}

close ($INPUT1) or die "Can't close input file!" ;
close ($OUTPUT) or die "Can't close output file!" ;
```

APPENDIX III

Level of presumptive STEC enumerated in bovine fecal samples

(a) 2011 sampling data

Animal_ID	Level of presumptive STEC (log cfu/g feces)	
	Time_point 1	Time_point 2
3149	4.5	4.1
3190	5.0	5.0
3191	2.9	1.9
3214	2.9	2.5
3221	4.3	3.8
3230	5.0	4.9
3233	2.4	3.5
3240	3.9	3.1
3241	2.6	3.1
3242	4.6	3.0
3249	2.7	3.3
3258	4.6	3.8
3260	3.1	3.1
3261	5.0	4.4
3282	3.8	2.7
3284	2.4	1.9
3290	2.5	2.9
3295	4.8	5.0
3471	3.8	4.4
3820	5.0	5.0
4090	3.4	5.0
4208	3.5	3.8
4228	3.5	3.1
4232	4.2	2.7
4240	5.0	4.2
4288	4.3	4.4
4304	4.4	3.5
4307	2.7	2.7
4415	2.6	5.0

4525	4.2	3.0
4534	3.9	2.4
4538	4.2	3.4
4539	2.8	1.9
4547	3.2	5.0
4593	5.0	4.9
4610	3.9	3.8
4631	2.6	2.4
4635	3.7	2.9
4649	3.5	3.8
4670	2.7	1.9
4683	3.4	3.0
4693	5.0	3.9
4695	3.9	3.5
4696	3.5	2.9
4705	5.0	5.0
4712	3.7	3.3
4723	3.3	2.7
4724	3.5	3.2
4726	3.2	5.0
4728	3.2	3.1
4733	3.8	3.3
4736	3.9	4.5
4739	5.0	3.6
4749	3.9	3.8
4752	5.0	5.0
4761	2.9	4.8
4766	4.3	3.0
4767	3.5	3.5
4768	2.9	5.0
4770	5.0	5.0
4793	4.8	4.5
4797	3.8	3.2
4798	4.7	4.6
4799	3.4	3.2
4806	5.0	5.0
4811	2.9	2.2
4814	3.0	2.5
4821	4.4	4.6

4830	5.0	5.0
4834	3.0	2.4
4856	3.4	3.2
4859	3.9	3.2
4868	3.2	2.2
4871	3.5	4.3
4873	3.3	2.4
4880	5.0	5.0
4883	5.0	4.8
4885	5.0	5.0
4888	3.7	3.7
4891	3.7	2.6
4894	4.3	3.3
4899	3.9	3.5
4903	4.0	2.9
4908	4.1	5.0
4914	3.6	3.4
4923	3.7	3.0
4925	5.0	5.0
4927	5.0	4.8
4936	5.0	5.0
4943	5.0	5.0
4945	5.0	5.0
4948	3.4	3.8
4951	5.0	4.8
4956	3.9	4.5
4958	1.9	ND
4959	3.4	3.9
4961	3.7	5.0
4972	2.4	ND
4981	5.0	5.0
4982	5.0	4.4
4996	4.0	4.2
5001	3.2	3.2
5133	2.7	2.8

(b) 2013 sampling data

Animal_ID	Level of presumptive STEC (log cfu/g feces)	
	Time_point 1	Time_point 2
1030	4.3	ND
1529	4.8	ND
1540	2.9	ND
1541	3.6	ND
1544	3.4	4.3
1556	4.5	ND
1557	3.6	2.9
1563	4	3.6
1565	3.7	ND
1574	3.7	ND
1576	4.7	ND
1582	3.5	ND
1583	4.6	3.9
1591	3.7	3.5
1593	4.3	3.4
1597	3.2	3
1601	4.3	ND
1608	ND	3.2
1609	4.9	3.6
1617	2.3	3.2
1626	ND	4.7
1635	3.5	3.4
1639	3.8	4
1643	3.8	ND
1651	3.9	3.3
1652	3.9	2.9
1657	4.5	4.1
1664	4.2	5.2
1680	ND	ND
1682	3.8	ND
1690	4.6	4.7
1725	3.7	2.6
1727	5.1	ND
1735	4.4	4.3
1835	3.4	ND
1851	3.6	3.8
1853	3.9	3.6

1856	3.8	4.4
1890	3.3	3.6
1924	3.2	3.6
1941	3.2	2.4
1945	3.6	3.6
1953	2.7	4
1959	4.2	2.4
1960	3.7	3.3
1961	3.8	4.6
1962	ND	3
1973	ND	4.5
1977	4.9	3.3
2125	4.3	ND
2129	3.7	3.2
2136	3.5	3.9
2143	5.1	4.3
2173	2.9	ND
2197	4.4	4.2
2211	3.9	ND
2222	ND	4.3
2237	4.4	2.9
2249	3.9	4.1
2278	3.8	3.6
2293	2.3	ND
2310	4.9	3.7
2313	3.6	5.4
2320	4.1	3
2322	3.7	4.1
2323	ND	4.3
2324	4.9	3.6
2329	4.1	4
2344	3.8	2.9
2345	3.9	2.9
2348	4.4	4.4
2361	3.5	3.3
2368	3.4	2.3
2373	4.1	4
2380	ND	3
2389	3.3	3.8

2390	4.4	3.2
2391	3.4	3.7
8810	4.4	3.6
8818	4.3	2
8820	3.4	4.1
8827	3.7	3.9
8852	2.8	2
8877	4.7	ND
8884	3.5	3.8
8887	3.3	ND
8888	3.7	2.7
8890	ND	4.3
8892	3.9	4.2
8933	4.5	ND
8936	4.1	ND
8944	3.8	ND
8948	2.7	ND
8954	3.5	ND
8956	3.6	3.2
8959	5.2	4
8960	3.6	4.1
8986	3.7	ND
8992	3.8	ND
8994	5	ND
8999	4.1	4.1
9000	4.3	ND
9002	5	4.2
9008	4.1	3.7
9010	3.8	3.9
9012	3.9	2.6
9016	4.3	ND
9018	4	4.1
9019	3.5	2.3
9021	ND	ND
9022	3.2	4
9024	3.6	4.1
9025	4.2	4.1
9026	ND	4.7
9027	4.2	3.3

9028	4.2	3.1
9029	4.7	4.4
9032	3.3	4.1
9035	4.1	ND
9036	3.4	4.1
9037	4.6	4.5
9039	ND	4.4
9041	4.6	3.4
9046	4.4	4.3
9048	2.3	ND
9049	3.6	ND
9052	4.5	4.5
9054	4.5	ND
9058	3.5	ND
9064	3.7	ND
9071	3.7	4.5
9072	3.6	3.8
9073	3.7	4.6
9074	4	ND
9079	3.4	4.2
9080	3.9	ND
9106	4.1	3.9
9111	4	4
9112	4	4.3
9114	4.4	3.4
9115	ND	2
9116	4.6	3.8
9117	2.6	3
9118	2.7	ND
9119	2.6	2.7
9122	3.9	4.2
9126	3.9	3.2
9129	3.7	2
9134	4	ND
9135	3.5	3.6
9139	4.3	3.9
9141	4.1	2.4
9142	4	ND
9143	3.9	3.7

9144	ND	ND
9145	4	3.6
9148	3.6	3.2
9150	3.7	3.7
9151	2.3	ND
9152	4.3	4
9154	3.8	ND
9156	3.4	3.8
9157	3.3	5.1
9158	4.5	4.5
9159	3.6	3.3
9160	ND	ND
9162	4.1	ND
9164	4.2	3
9166	2.8	ND
9167	3.4	3.8
9170	3.5	ND
9175	ND	3.8
9176	3.3	ND
9177	3.6	ND
9178	4.3	3.2
9179	ND	4.7
9180	4.2	2.9
9181	3.3	3.6
9182	3.3	ND
9185	3.1	4
9187	4.2	4.2
9188	3.5	ND
9191	3.4	2.3
9192	5.1	5
9193	2.4	3.3
9195	5	4.2
9197	4.8	3.7
9200	3.7	3.5
9201	5	5
9204	3.6	3.2
9354	2.7	ND
9530	2.9	3.9
9693	3.9	ND

9698	4.7	4.4
9707	3.4	3.2
9711	4.1	ND
9714	4.7	4.2
9719	4	3.8
9721	4.6	3.9
9722	3.6	ND
9747	3	2.4
9749	4.3	3.8
9759	3.2	ND
9760	3.7	2.3
9761	4.3	3.4
9764	4.3	ND
9766	5	2.4
9770	ND	3.6
9774	3.7	ND
9777	3.1	3
9780	3.8	3.9
9784	4.1	3.5
9785	3.9	3.4
9791	4.4	4.3
9806	3.3	2.4
9814	3	4.4
9829	3.7	3.3
9830	4.1	3.2
9833	4.3	3.9
9838	ND	3.6
9844	4.3	4.8
9871	3.5	4.3
9874	4.2	3.7
9878	4.3	2.4
9952	3.6	ND

ND – No Data

APPENDIX IV

Fecal samples in which EHEC-7 were detected by NeoSEEK™ STEC assay

2011 Fecal sampling

Sample_ID	Animal_ID	EHEC_presence	EHEC_serogroup	Eae	Stx	Time_point	Year
428	4736	yes	O103	yes	yes	2	2011
519	4959	yes	O103	yes	yes	2	2011
287	4945	yes	O111	yes	yes	1	2011
472	4610	yes	O103	yes	yes	2	2011
526	4798	yes	O103	yes	yes	2	2011
364	4798	yes	O103	yes	yes	1	2011
238	4959	yes	O103	yes	yes	1	2011
338	4873	yes	O103	yes	yes	1	2011
306	4693	yes	O103	yes	yes	1	2011
215	3242	yes	O103	yes	yes	1	2011
285	4090	yes	O111	yes	yes	1	2011
258	4981	yes	O103	yes	yes	1	2011
209	4228	yes	O103.O45	yes	yes	1	2011
247	3190	yes	O45	yes	yes	1	2011
518	4635	yes	O103	yes	yes	2	2011
315	4733	yes	O103	yes	yes	1	2011
432	4726	yes	O103.O157	yes	yes	2	2011

339	4925	yes	O103	yes	yes	1	2011
514	4797	Yes	O103	yes	yes	2	2011
224	4610	Yes	O103	yes	yes	1	2011

2013 Fecal Samples

Sample_ID	Animal_ID	EHEC_presence	EHEC_serogroup	Eae	Stx	Time_point	Year
1639.1	1639	yes	O103	yes	yes	1	2013
9719.1	9719	yes	O157	yes	yes	1	2013
8936.1	8936	yes	O103, O157	yes	yes	1	2013
9162.1	9162	yes	O157, O45	yes	yes	1	2013
9530.1	9530	yes	O103	yes	yes	1	2013
2389.1	2389	yes	O157	yes	yes	1	2013
9195.1	9195	yes	O157	yes	yes	1	2013
1727.1	1727	yes	O103	yes	yes	1	2013
2197.1	2197	yes	O103	yes	yes	1	2013
1977.1	1977	yes	O103	yes	yes	1	2013
9022.1	9022	yes	O145	yes	yes	1	2013
1583.1	1583	yes	O103	yes	yes	1	2013
9000.1	9000	yes	O157	yes	yes	1	2013
9151.2	9151	yes	O145	yes	yes	2	2013
9115.2	9115	yes	O157	yes	yes	2	2013
8992.2	8992	yes	O45	yes	yes	2	2013
9126.2	9126	yes	O157	yes	yes	2	2013

9191.2	9191	yes	O157	yes	yes	2	2013
9770.3	9770	yes	O103	yes	yes	3	2013
9029.3	9029	yes	O145	yes	yes	3	2013
9722.3	9722	yes	O45	yes	yes	3	2013
9814.1	9814	Yes	O103	yes	yes	1	2013

APPENDIX V

PERMANOVA outputs

Comparison of fecal bacterial community between sampling years

(a Family level

	Df	SumsOfSqs	MeanSqs	F.Model	R2	Pr(>F)	
year	1	13.490	13.4895	123.700	0.10848	0.001	***
diet	1	0.795	0.7951	7.291	0.00639	0.001	***
time_point	1	0.861	0.8608	7.894	0.00692	0.001	***
animal	1	0.158	0.1582	1.451	0.00127	0.180	
Residuals	1000	109.051	0.1091		0.87693		
Total	1004	124.354			1.00000		

(b Genus level

	Df	SumsOfSqs	MeanSqs	F.Model	R2	Pr(>F)	
year	1	19.906	19.9059	108.883	0.09661	0.001	***
diet	1	1.545	1.5448	8.450	0.00750	0.001	***
time_point	1	1.466	1.4657	8.017	0.00711	0.001	***
animal	1	0.314	0.3143	1.719	0.00153	0.108	
Residuals	1000	182.819	0.1828		0.88726		
Total	1004	206.049			1.00000		

Shedding phenotype and fecal bacterial community

For 2011 data set

(a Phylum level

	Df	SumsOfSqs	MeanSqs	F.Model	R2	Pr(>F)	
sheddingphenotype	1	0.0299	0.02989	0.7728	0.00343	0.421	
diet	1	1.0250	1.02497	26.5005	0.11758	0.001	***

timepoint	1	0.0698	0.06982	1.8051	0.00801	0.179
animal_ID	1	0.0121	0.01212	0.3134	0.00139	0.745
Residuals	196	7.5808	0.03868		0.86960	
Total	200	8.7176			1.00000	

(b Class level

	Df	SumsOfSqs	MeanSqs	F.Model	R2	Pr(>F)
sheddingphenotype	1	0.0278	0.02782	0.6721	0.00300	0.477
diet	1	1.0202	1.02025	24.6498	0.11020	0.001 ***
timepoint	1	0.0765	0.07654	1.8492	0.00827	0.141
animal_ID	1	0.0211	0.02112	0.5104	0.00228	0.575
Residuals	196	8.1124	0.04139		0.87625	
Total	200	9.2581			1.00000	

(c Core family level

	Df	SumsOfSqs	MeanSqs	F.Model	R2	Pr(>F)
sheddingphenotype	1	0.1786	0.17861	1.5374	0.00721	0.170
diet	1	1.4063	1.40625	12.1040	0.05677	0.001 ***
timepoint	1	0.2810	0.28097	2.4184	0.01134	0.030 *
animal_ID	1	0.1353	0.13525	1.1641	0.00546	0.297
Residuals	196	22.7715	0.11618		0.91922	
Total	200	24.7725			1.00000	

(d Core genus level

	Df	SumsOfSqs	MeanSqs	F.Model	R2	Pr(>F)
sheddingphenotype	1	0.811	0.81137	4.3069	0.01971	0.003 **
diet	1	2.719	2.71933	14.4349	0.06607	0.001 ***
timepoint	1	0.368	0.36824	1.9547	0.00895	0.078 .
animal_ID	1	0.334	0.33382	1.7720	0.00811	0.106
Residuals	196	36.924	0.18839		0.89715	
Total	200	41.156			1.00000	

(e) Core OTUs

	Df	SumsOfSqs	MeanSqs	F.Model	R2	Pr(>F)

sheddingphenotype	1	0.882	0.88172	4.2538	0.01961	0.001 ***
diet	1	2.631	2.63070	12.6917	0.05852	0.001 ***
timepoint	1	0.532	0.53181	2.5657	0.01183	0.004 **
animal_ID	1	0.281	0.28143	1.3577	0.00626	0.117
Residuals	196	40.626	0.20728		0.90377	
Total	200	44.952			1.00000	

(f) Total OTUs

	Df	SumsOfSqs	MeanSqs	F.Model	R2	Pr(>F)
sheddingphenotype	1	1.048	1.04753	3.4676	0.01631	0.001 ***
diet	1	2.495	2.49455	8.2576	0.03884	0.001 ***
timepoint	1	1.030	1.03045	3.4111	0.01604	0.001 ***
animal_ID	1	0.452	0.45234	1.4974	0.00704	0.024 *
Residuals	196	59.210	0.30209		0.92177	
Total	200	64.234			1.00000	

For 2013 data set

(a Phylum level

	Df	SumsOfSqs	MeanSqs	F.Model	R2	Pr(>F)
sheddingphenotype	1	0.0107	0.01068	0.2655	0.00070	0.845
diet	1	0.0860	0.08599	2.1375	0.00564	0.102
timepoint	1	0.9108	0.91077	22.6409	0.05979	0.001 ***
animal	1	0.0251	0.02509	0.6238	0.00165	0.562
Residuals	353	14.2001	0.04023		0.93222	
Total	357	15.2326			1.00000	

(b Class level

	Df	SumsOfSqs	MeanSqs	F.Model	R2	Pr(>F)
sheddingphenotype	1	0.0123	0.01231	0.2604	0.00069	0.883
diet	1	0.1031	0.10306	2.1810	0.00582	0.094 .
timepoint	1	0.8894	0.88941	18.8230	0.05020	0.001 ***
animal	1	0.0313	0.03134	0.6632	0.00177	0.549
Residuals	353	16.6797	0.04725		0.94151	
Total	357	17.7158			1.00000	

(c Family level

	Df	SumsOfSqs	MeanSqs	F.Model	R2	Pr(>F)	
sheddingphenotype	1	0.176	0.17569	1.7562	0.00480	0.097	.
diet	1	0.147	0.14733	1.4728	0.00402	0.168	
timepoint	1	0.892	0.89235	8.9200	0.02437	0.001	***
animal	1	0.083	0.08250	0.8247	0.00225	0.579	
Residuals	353	35.314	0.10004		0.96455		
Total	357	36.612			1.00000		

(c Genus level

	Df	SumsOfSqs	MeanSqs	F.Model	R2	Pr(>F)	
sheddingphenotype	1	0.327	0.32725	1.9128	0.00523	0.055	.
diet	1	0.397	0.39745	2.3230	0.00635	0.019	*
timepoint	1	1.306	1.30649	7.6363	0.02089	0.001	***
animal	1	0.129	0.12862	0.7518	0.00206	0.658	
Residuals	353	60.395	0.17109		0.96547		
Total	357	62.555			1.00000		

e) Core OTUs

	Df	SumsOfSqs	MeanSqs	F.Model	R2	Pr(>F)	
sheddingphenotype	1	0.317	0.31652	1.8601	0.00505	0.028	*
diet	1	0.630	0.62991	3.7019	0.01005	0.001	***
timepoint	1	1.565	1.56536	9.1995	0.02497	0.001	***
animal	1	0.109	0.10870	0.6388	0.00173	0.901	
Residuals	353	60.066	0.17016		0.95820		
Total	357	62.686			1.00000		

f) Total OTUs

	Df	SumsOfSqs	MeanSqs	F.Model	R2	Pr(>F)	
sheddingphenotype	1	0.473	0.47346	1.6781	0.00461	0.010	**
diet	1	0.748	0.74823	2.6519	0.00729	0.001	***
timepoint	1	1.669	1.66877	5.9146	0.01625	0.001	***
animal	1	0.206	0.20552	0.7284	0.00200	0.949	
Residuals	353	99.597	0.28214		0.96985		
Total	357	102.693			1.00000		

```
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1
```

APPENDIX VI

Multi-factor ANOVA results

2011 Genus level

Butyrvibrio

```
Analysis of Variance Table

Response: X14
                  Df     Sum Sq    Mean Sq F value  Pr(>F)
sheddingcategory   1 2.6810e-06 2.6811e-06  5.7663 0.01727 *
diet               1 7.1100e-07 7.1132e-07  1.5299 0.21761
timepoint          1 9.1000e-08 9.1450e-08  0.1967 0.65789
Residuals        197 9.1598e-05 4.6496e-07
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1
```

CF231

```
Analysis of Variance Table

Response: X7
                  Df  Sum Sq  Mean Sq F value    Pr(>F)
sheddingcategory   1 0.11010 0.110101  9.2397 0.0026899 **
diet               1 0.13947 0.139466 11.7040 0.0007582 ***
timepoint          1 0.00625 0.006246  0.5242 0.4699195
Residuals        197 2.34747 0.011916
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1
```

2011 Genus level

Prevotella

```
Analysis of Variance Table

Response: X6
                  Df  Sum Sq   Mean Sq F value  Pr(>F)
sheddingcategory   1 0.01659 0.0165920  4.0689 0.04443 *
diet               1 0.01023 0.0102349  2.5100 0.11402
timepoint          1 0.00205 0.0020453  0.5016 0.47928
Residuals        354 1.44352 0.0040777
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1
```

2011 OTU level

OTU15828

```
Analysis of Variance Table

Response: X231
                  Df  Sum Sq  Mean Sq F value    Pr(>F)
sheddingcategory   1 0.09013 0.090129  8.6269  0.003707 **
diet               1 0.17334 0.173342 16.5920 6.713e-05 ***
timepoint          1 0.00017 0.000172  0.0165  0.897927
Residuals        197 2.05813 0.010447
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1
```

2013 OTU level

OTU26

```
Analysis of Variance Table

Response: X16
                  Df   Sum Sq    Mean Sq F value   Pr(>F)
sheddingcategory   1 0.001247 0.00124736  9.3445 0.002407 **
diet               1 0.001016 0.00101641  7.6144 0.006091 **
timepoint          1 0.000150 0.00015047  1.1272 0.289096
Residuals        354 0.047254 0.00013349
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1
```

OTU42

```
Analysis of Variance Table

Response: X21
                  Df   Sum Sq    Mean Sq F value    Pr(>F)
sheddingcategory   1 0.001459 0.00145895  8.4581 0.0038636 **
diet               1 0.000023 0.00002297  0.1332 0.7153661
timepoint          1 0.002334 0.00233446 13.5337 0.0002707 ***
Residuals        354 0.061062 0.00017249
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1
```

OTU63

```
Analysis of Variance Table

Response: X31
                  Df    Sum Sq    Mean Sq F value  Pr(>F)
sheddingcategory   1 0.0003208 0.00032076  5.4517 0.02011 *
diet               1 0.0002479 0.00024788  4.2129 0.04085 *
```

```
timepoint           1 0.0002465 0.00024653  4.1899 0.04140 *
Residuals         354 0.0208286 0.00005884
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1
```

OTU118

```
Analysis of Variance Table

Response: X53
                   Df    Sum Sq    Mean Sq F value  Pr(>F)
sheddingcategory    1 0.0001706 1.7057e-04  4.6991 0.03084 *
diet                1 0.0001495 1.4953e-04  4.1193 0.04314 *
timepoint           1 0.0000039 3.9160e-06  0.1079 0.74278
Residuals         354 0.0128497 3.6299e-05
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1
```

OTU316

```
Analysis of Variance Table

Response: X87
                   Df    Sum Sq    Mean Sq F value    Pr(>F)
sheddingcategory    1 0.0001480 1.4800e-04 11.4664 0.0007882 ***
diet                1 0.0000770 7.7041e-05  5.9688 0.0150492 *
timepoint           1 0.0000456 4.5627e-05  3.5350 0.0609081 .
Residuals         354 0.0045692 1.2907e-05
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1
```

OTU580

```
Analysis of Variance Table

Response: X96
                   Df     Sum Sq    Mean Sq F value    Pr(>F)
sheddingcategory    1 0.00005131 5.1313e-05 11.9411 0.0006158 ***
diet                1 0.00000846 8.4610e-06  1.9689 0.1614427
timepoint           1 0.00005769 5.7691e-05 13.4255 0.0002862 ***
Residuals         354 0.00152119 4.2970e-06
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1
```

OTU677

```
Analysis of Variance Table

Response: X333
                   Df    Sum Sq    Mean Sq F value  Pr(>F)
sheddingcategory    1 0.0001228 1.2275e-04  6.1510 0.01360 *
diet                1 0.0001202 1.2024e-04  6.0248 0.01459 *
timepoint           1 0.0000266 2.6586e-05  1.3322 0.24920
Residuals         354 0.0070647 1.9957e-05
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1
```

OTU10480

```
Analysis of Variance Table

Response: X353
                  Df    Sum Sq    Mean Sq F value    Pr(>F)
sheddingcategory   1 0.0002300 2.3003e-04 15.4928 9.972e-05 ***
diet               1 0.0000315 3.1514e-05  2.1225    0.1460
timepoint          1 0.0000122 1.2194e-05  0.8213    0.3654
Residuals        354 0.0052561 1.4848e-05
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1
```

OTU10659

```
Analysis of Variance Table

Response: X152
                  Df    Sum Sq    Mean Sq F value   Pr(>F)
sheddingcategory   1 0.0008345 0.00083455 10.7580 0.001141 **
diet               1 0.0001046 0.00010462  1.3487 0.246293
timepoint          1 0.0004087 0.00040873  5.2689 0.022294 *
Residuals        354 0.0274613 0.00007757
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1
```

OTU16990

```
Analysis of Variance Table

Response: X198
                  Df     Sum Sq    Mean Sq F value   Pr(>F)
sheddingcategory   1 0.00004969 4.9692e-05  7.7554 0.005643 **
diet               1 0.00002053 2.0535e-05  3.2048 0.074275 .
timepoint          1 0.00000665 6.6490e-06  1.0378 0.309041
Residuals        354 0.00226823 6.4070e-06
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1
```

OTU21172

```
Analysis of Variance Table

Response: X250
                  Df    Sum Sq    Mean Sq F value  Pr(>F)
sheddingcategory   1 0.0000950 9.4968e-05  6.4821 0.01132 *
diet               1 0.0000166 1.6583e-05  1.1319 0.28810
timepoint          1 0.0000213 2.1343e-05  1.4568 0.22825
Residuals        354 0.0051864 1.4651e-05
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1
```