# Investigation of host mechanisms associated with *Escherichia coli* O157:H7 super-shedding in beef steers

by

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## Abstract

Cattle shedding > $10^4$ CFU *E. coli* O157/gram of feces are defined as super-shedder (SS) which are a major disseminator of this foodborne pathogen into the environment. The recto-anal junction (RAJ) has been identified as the primary colonization site of *E. coli* O157within the gastrointestinal tract. To date, the mechanisms of *E. coli* O157 super-shedding in beef cattle remain unidentified. This thesis aimed to identify host mechanisms that are potentially associated with super-shedding through performing four studies on host gene expression throughout the intestinal tract and associated regulation mechanisms. Study 1 performed comparative transcriptomic analysis of rectal tissue collected from 5 SS and 4 cattle free from fecal *E. coli* O157 (non-shedders, NS) using RNA-Seq, and identified 58 differentially expressed (DE) genes including 11 that were up-regulated and 47 that were down-regulated in SS compared to NS. Functional analysis of these DE genes revealed that 31 down-regulated genes were potentially associated with reduced innate and adaptive immunity including decreased quantity and migration of immune cells such as lymphocytes, neutrophils and dendritic cells in SS. This suggests that the RAJ of SS may be less effective in terms of immune protection. Study 2 performed RNA-Seq to characterize and compare the transcriptomes of the whole intestinal tissues of SS and NS to understand whether other gut regions could also be associated with super shedding. In total, 351 DE genes were identified throughout the gastrointestinal tract between SS and NS, with 101 being up-regulated and 250 down-regulated in SS. Functional analysis of these DE genes suggested increased T-cell responses and cholesterol absorption in the distal jejunum and descending colon, and decreased B-cell maturation in the distal jejunum of SS. Further, single nucleotide polymorphism analysis of these DE genes identified association between 33 SNPs in 7 DE genes and *E. coli* O157 shedding. The functions of these 7 genes were associated

with leukocyte activation and cholesterol transportation, suggesting that host genetic variation may influence gene expression leading to altered immune functions and cholesterol transportation in SS. Study 3 analyzed miRNAomes of the whole intestinal tract of SS and NS, aiming to identify the possible post transcriptional regulatory mechanisms that altered gene expression observed in studies 1 and 2. In total, the number of DE miRNAs ranged from 1 (in descending colon) to 8 (in distal jejunum) and 7 miRNAs were up-regulated and 7 were down-regulated throughout the gut of SS. Functional analysis indicated that the DE miRNAs potentially regulate genes involved in host immune function including hematological system development and immune cell trafficking. These findings suggest that the alternation of miRNA expression may be one of the regulatory mechanisms responsible for the altered expression of genes associated with immune function in the gut of SS. In study 4, bacterial 16S rRNA gene amplicon profiling was performed to characterize the RAJ mucosa associated microbiota and their functions (using PICRUSt). Differential abundance analysis of operational taxonomic units (OTUs) identified 2 OTUs unique to SS which potentially represented members *Bacteroides* and *Clostridium*, and 7 OTUs unique to NS which potentially represented members of *Coprococcus*, *Prevotella*, *Clostridium* and *Paludibacter*. The relative abundance of microbial taxa and their function were correlated with the DE genes of RAJ reported in study 2, with results suggesting that *Clostridium, Coprococcus* and *Paludibacter* may alter the host host epithelium in a manner that deters the colonization of the RAJ by *E. coli* O157. In summary, this thesis provides knowledge of the host gut gene expression, miRNA regulation, and host-microbial interaction in beef cattle that differ in their *E. coli* O157 shedding capacity. Our findings suggest that *E. coli* O157 shedding in cattle could be systematically attributed to differences in host immune responses, genetic variation, miRNA regulation, and the nature of the gut microbiota.

## Preface

This thesis is an original work by Ou Wang, and it is a research project conducted by Ou Wang and supervised by Dr. Leluo Guan. This research project was funded by Alberta Innovates Bio Solutions (Project Number: FSC-12-017). The animal trial for Chapters 2 to 5 was approved by the Animal Care Committee of the Lethbridge Research Centre, Agriculture Agri-food Canada (Animal Care Committee protocol number: 1120), and followed Canadian Council of Animal Care Guidelines.

The identification of *E. coli* O157 super-shedder cattle were performed by Dr. Tim A. McAllister's group at Agriculture and Agri-Food Canada and Dr. Kim Stanford's group at Alberta Agriculture and Forestry. The animal tissue sampling was completed by Dr. Brent Selinger's group at the University of Lethbridge.

Chapter 2 has been published as Wang O, Liang G, McAllister TA, Plastow G, Stanford K, Selinger B, Guan LL (2016) Comparative Transcriptomic Analysis of Rectal Tissue from Beef Steers Revealed Reduced Host Immunity in *Escherichia coli* O157:H7 Super-Shedders. PLOS ONE 11(3): e0151284. Under Dr. Leluo Guan's supervision, I was responsible for RNA-Seq data generation and analysis, as well as drafting and editing the manuscript. All co-authors contributed to editing and composing the manuscript.

**Dedication**

I dedicate this work to my family, friends, and everyone who helped with this thesis!

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## List of Abbreviations

APOA1: Apolipoprotein A1;

BATF2: Basic Leucine Zipper ATF-Like Transcription Factor 2;

BLNK: B-Cell Linker;

CCL21: Chemokine (C-C Motif) Ligand;

DAVID: Database for Annotation, Visualization and Integrated Discovery;

CD19: CD19 Molecule;

CD79A: CD79a Molecule, immunoglobulin-associated alpha;

CD79B: CD79b Molecule, Immunoglobulin-associated Beta;

CFU: Colony Forming Unit;

CNR2: Cannabinoid Receptor 2;

CT-SMAC: Cefixime and potassium tellurite sorbitol MacConkey agar;

CXCL13: Chemokine (C-X-C Motif) Ligand 13;

cpm: Counts Per Million;

DE: Differential expressed;

DSC1: Desmocollin 1;

eaeA: Intimine;

F3: Coagulation Factor III;

fliC: H7 flagellin;

FPKM: Fragments Per Kilobase of transcript per Million mapped reads;

GP2: Glycoprotein 2; Glycerol-3-Phosphate Acyltransferase, Mitochondrial;

GPR132: G Protein-Coupled Receptor 132;

HBEGF: Heparin Binding EGF Like Growth Factor;

HC: Hemorrhagic colitis;

HUS: Hemolytic uramic symdrome;

IL2RA: Interleukin 2 Receptor, Alpha;

IL18R1: Interleukin 18 Receptor 1;

ILFs: Isolated lymphoid follicles;

LTB: lymphotoxin beta;

IPA: Ingenuity Pathway Analysis®;

ITK: IL2 Inducible T-Cell Kinase;

KLHL6: Kelch-Like Family Member 6;

LTB: Lymphotoxin Beta;

MS4A1: 4-Domains, Subfamily A, Member 1;

NS: non-shedder;

OTUs: Operational Taxonomic Units;

PICRUSt: Phylogenetic Investigation of Communities by Reconstruction of Unobserved States;

POU2AF1: POU Class 2 Associating Factor 1;

QIIME: Quantitative Insights into Microbial Ecology;

RALB: RAS Like Proto-Oncogene B;

RAN-Seq: RNA Sequencing;

RAJ: Recto-Anal Junction;

RHOH: Ras Homolog Family Member H;

S100A8: S100 Calcium Binding Protein A8;

S100A9: S100 Calcium Binding Protein A9;

S1PR2: Sphingosine-1-Phosphate Receptor 2;

SASH3: SAM and SH3 Domain Containing 3;

SCARB1: Scavenger Receptor Class B Member 1;

SCFA: Short Chain Fatty Acid;

SH2D1A: SH2 Domain Protein 1A;

SIT1: Signaling Threshold Regulating Transmembrane Adaptor 1;

SS: super-shedder;

SYK: Spleen Associated Tyrosine Kinase;

THEMIS: Thymocyte Selection Associated;

VT: Verotoxin

# Chapter 1 Literature Review

## 1.1 Introduction

*E. coli* O157 contamination can lead to human illness, such as bloody diarrhea, hemolytic uremic syndrome (HUS) and even death. From the XL Foods Inc. recall in 2012 to the very recent ground beef products recall by Intercity Packer in November, 2016, this bacterium has caused many recalls of beef product in Canadian history (CFIA, 2017). Contamination of *E. coli* O157 is costing the beef industry enormous economic losses annually. For instance, the XL Foods Inc. recall, which was the largest beef recall in Canadian history, caused at least $16 million in losses in the for treatment of the disease, food recalls and disposal, inspections, and delay of production (Charlebois et al., 2015). A consequential impact of this recall was a decrease in the consumers' confidence of the safety of Canadian beef products.

*E. coli* O157 infections are frequently reported in the U.S., Canada, Japan, Australia and the European Union (Chase-Topping et al., 2008), among which the U.S. and Canada exhibit a particularly high prevalence (Griffin and Tauxe, 1991; Boyce et al., 1995). In Alberta, Canada, the mean annual rate of *E. coli* O157 infection was estimated to be 12.6 per 100,000 persons, and in every 14 cases there is one case of HUS (Waters et al., 1994). In the U.S. from 1983 to 2012, a total of 740 outbreaks were reported, which involved 13,515 cases including 2,765 hospitalizations, and 73 deaths (Rangel et al., 2005; Heiman et al., 2015). An estimation based on the epidemiological data from 1983-2002 suggested that *E. coli* O157 may cause 73,480 illnesses, 2,168 hospitalizations, and 61 deaths in the U.S. each year (Rangel et al., 2005), resulting in an annual loss of $ US 405 million (Frenzen et al., 2005). In addition, according to data collected on *E. coli* O157 outbreaks in the United States over a 20 (1983-2002) and 10 year

(2003-2012) period identified food (increased from 52% to 65%), person to person (increased from 9% to 10%), animal contact (increased from 3% to 10%) and water (decreased from 9% to 4%) as the major routes of transmission of this pathogen (Rangel et al., 2005; Heiman et al., 2015).

The largest outbreak of *E. coli* O157 in Canada occurred in Walkerton, Ontario, which was caused by contamination of a well by surface water containing *E. coli* O157 from a farm. The contamination led to adulteration of town's water supply with *E. coli* O157 which caused infection in more than 5,000 people and resulted in 7 deaths (Goss et al., 2002; Livernois, 2002). The economic loss of this Walkerton outbreak was estimated to be more than C$ 60 million, showing that the outbreak not only caused a health issue, but also resulted in significant economic losses (Goss et al., 2002; Livernois, 2002). The most notorious outbreak in the U.S. occurred during 1992 to 1993, which was caused by *E. coli* O157 contaminated hamburgers provided by the Jack in the Box fast food chain restaurant. The outbreak eventually led to more than 700 illnesses and the deaths of 4 children, and resulted in more than one hundred individuals suffering permeant kidney damage (Powell, 2000). The cause of the outbreak was undercooked hamburger due to the failure of the restaurant to maintain the standard cooking temperature that is necessary to kill *E. coli* O157.

The concept "from farm to fork" clearly stated that the control of food safety is more effective if it begins at the farm instead of only focusing on control measures within processing plants and consumers' kitchens. Ruminants, such as cattle, are suggested as the primary carriers for *E. coli* O157, and the possible source of *E. coli* O157 infection in humans (Pa et al., 1993). *E. coli* O157 cross contamination can occur on farm and at slaughter, and any on-farm control could be helpful in mitigating risks. Although non-O157 *E. coli* have recently received attention,

human illness due to *E. coli* O157 in Canada far exceeds illness caused by non-O157 serogroups (Gill and Gill, 2010). Consequently, a focus on defining the mechanisms of shedding of *E. coli* O157 in ruminant, such as cattle, is justified as this serogroup remains the leading threat to human health.

### 1.2 Foodborne pathogenic *E. coli*

*E. coli* is a bacterial species that belong to the family *Enterobateriaceae*, which have the characteristics of being rod-shaped, Gram-negative and facultative anaerobic. These bacteria are normally found in the intestine of human and animals, and account for about 0.1% of the total bacterial flora within the human gut (Eckburg et al., 2005). Most *E. coli* strains are beneficial to their hosts as they can exclude pathogenic bacteria such as *Salmonella typhimurium* (Hudault et al., 2001; Nuotio et al., 2013), and provide vitamins to the host (e.g., $K_2$, Newton et al., 1971). *E. coli* strains initiate colonization of the new born's intestine within hours after birth (Adlerberth et al., 1991). However, in immunocompromised individuals, pathogenic *E. coli* can cause disease, including urinary tract infections, meningitis and diarrhea (Nataro and Kaper, 1998). Most pathogenic *E. coli* can be classified into the following eight pathotypes: enterotoxigenic *E. coli* (ETEC), enteropathogenic *E. coli* (EPEC), enteroaggregative *E. coli* (EAEC), enteroinvasive *E. coli* (EIEC), diffusely adherent *E. coli* (DAEC), uropathogenic *E. coli* (UPEC), meningitis/sepsis-associated *E. coli* (MNEC), and enterohemorrhagic *E. coli* (EHEC) (Nataro and Kaper, 1998). Of these, EHEC strains cause hemorrhagic disease in humans, including HUS, bloody diarrhea and even death (Croxen et al., 2013), clinical outcomes that arise from the Shiga-like toxins that they produce.

### 1.2.1 Non-O157 Shiga-toxin producing *E. coli*

More than 200 serotypes of non-O157 Shiga-toxin producing *E. coli* (STEC) have been isolated from hemorrhagic colitis (HC) and HUS patients in previous investigations (Brooks et al., 2005). By 2002, it was reported that the most common non-O157 *E. coli* serogroups that cause human infections were O26 (22%), O111 (16%), O103 (12%), O121 (8%), O45 (7%), and O145 (5%), which were referred as "THE Big Six" (Yoon et al., 2013), which account for 70% of non-O157 STEC infections as interpreted from data collected from 1983-2002 (Brooks et al., 2005). The "Big Six" STEC were defined as food adulterants by the Food Safety and Inspection Service (FSIS) of the U.S. Department of Agriculture (USDA) in 2011, and the raw meat contaminated by these EHEC serotypes cannot be sold (Yoon et al., 2013). However, in Canada the EHEC that cause the most human infection is *E. coli* O157 (Gill and Gill, 2010), and thus current study focused on defining the mechanisms of shedding of *E. coli* O157 in cattle.

### 1.2.2 *E. coli* O157:H7 and *E. coli* O157:NM

*E. coli* O157:H7 and O157:NM (both referred to *E. coli* O157 forthward) are the major safety concern in the food industry in Canada and the United States (Gill and Gill, 2010), as it is the serotype most commonly isolated from infected patients (Su, 1995). The O indicates the O-polysaccharide, which is a part of the lipopolysaccharide on the cell membrane of Gram-negative bacteria, and the H factor indicates the composition of the flagellar antigens (Whitfield and Roberts, 1999). A combination of O and H types defines the serotype of the isolated *E. coli*.

Two key investigations led to the recognition and identification of *E. coli* O157:H7 as an important pathogen. The first was the investigation of more than 30 cases of hemorrhagic colitis (HC) in the United States in 1982, which led to the isolation of *E. coli* O157:H7 from the stools of patients with watery diarrhea and bloody diarrhea (Karmali et al., 1983). The second outbreak

investigation was conducted on 47 cases of HC in Oregon and Michigan in 1982 by Riley et al. (1983) who isolated *E. coli* O157:H7 from stool samples collected from patients. The cytotoxins produced by *E. coli* O157 were identified as the causative agent of hemorrhagic colitis, and were named verotoxins because of their toxic effects on vero-cells (Karmali et al., 1983). They were also described as Shiga-like toxins as they shared properties with a similar toxin produced by *Shigella dysenteriae* type 1 (Faruque et al., 2003).

### 1.3 Pathogenesis of *E. coli* O157 infection

Shiga-toxins (Stx) are the main causative agents of HC and hemolytic uremic syndrome (HUS), and belong to a family of cytotoxins consisting of two groups of toxins: Stx1 and Stx2 (Friedman and Court, 2001). It was reported that the toxicity of Stx2 is higher than Stx1, and EHEC strains producing Stx2 are more likely to cause HC and HUS in humans (Cimolai et al., 1994). The Stx consists of one A subunit and five identical B subunits, forming a $AB_5$ toxin molecule (Croxen and Finlay, 2010). The B subunits are responsible for binding to the glycolipid globotriaosylceramide (Gb3) receptor on the surface of Paneth cells and lead to endocytosis of the toxin, whereas the A subunit cleaves ribosomal RNA and stops protein synthesis (Shaikh and Tarr, 2003; Schüller et al., 2007). In the human colon, bloody diarrhea and HC are results of tissue damage caused by Stx (Nataro and Kaper, 1998). Although produced in the intestinal tract, Stx can be absorbed across the intestinal epithelium into the bloodstream and travel to the kidney where it causes HUS as a result of kidney damage (Andreoli et al., 2002).

The virulence genes of *E. coli* O157 encode the Shiga-toxins and the translocated effector proteins that enable *E. coli* O157 to cause A/E lesions. The Shiga-toxin genes have been proposed to be acquired as result of the integration of lambdoid bacteriophages into the *E. coli* O157 genome (Shaikh and Tarr, 2003). Many effector proteins that function in actin recruitment

and pedestal formation in host cells are encoded by genes located on a > 35.6 kb pathogenicity island which is commonly known as the locus of enterocyte effacement (LEE) (Nataro and Kaper, 1998). The virulence genes on the LEE include the two of the most important proteins for attachment of *E. coli* O157 to the epithelium, intimin and Tir, other proteins that form T3SS (*secretion of E. coli proteins* (SEP)*;* and *chaperone of E. coli secretion* (ESC), and effector proteins that are translocated into the host cell (Reiland et al., 2014). In addition, although numerous non-LEE virulent genes have been reported, only a few of them have been well characterized. For example, the gene encoding NleD was reported to be associated with persistent shedding of *E. coli* O157 in a calf (Dziva et al., 2004), and *TccP* (*Tir-cytoskeleton coupling protein*) encodes for a translocated protein that induces actin polymerization and pedestal formation during EHEC colonization (Croxen and Finlay, 2010).

Invasion of pathogenic *E. coli* occurs four steps: (1) colonization; (2) avoidance of the host immune response; (3) reproduction; and (4) causing host tissue damage (Nataro and Kaper, 1998). For *E. coli* O157, the flagella, the *E. coli* common pilus, and the hemorrhagic coli pilus have been suggested to initiate attachment to the intestinal epithelium of the host (Xicohtencatl-Cortes et al., 2009; Walle et al., 2013). The attachment is enhanced through interaction between intimin, a receptor on the cell membranes of *E. coli* O157 and the nucleolin of host cells, as well as by Tir (Translocated intimin receptor), a receptor encoded by *E. coli* O157 and translocated into host cells (Xicohtencatl-Cortes et al., 2009; Croxen and Finlay, 2010; Walle et al., 2013). Upon attachment, *E. coli* O157 injects various effector proteins into the host cell by a type III secretion system (T3SS) that alter the physiology of the intestinal cell. It has been reported that the genome of *E. coli* O157 Sakei strain contains more than 62 genes that may encode for 3SS effector proteins (Wong et al., 2011). In addition to enhancing attachment by Tir translocation,

an important function of T3SS proteins is to cause dysregulation to actin polymerization within host cells, leading to the formation of an actin-rich pedestal structure that allows *E. coli* O157 to adhere to the intestinal epithelium of the host (Campellone et al., 2004). Formation of actin-rich pedestal and attachment of *E. coli* O157 results in the removal of microvilli on the epithelial cell surface, an alteration referred to as the formation of attaching and effacing lesions (A/E lesion) (Campellone et al., 2004). In addition, the effector proteins have anti-phagocytosis functions that deter host immune responses. For example, EspB (*Escherichia* secretion protein B) has been reported to interact with actin binding domains of myosin proteins to inhibit phagocytosis (Iizumi et al., 2007). The EspH (*Escherichia* secretion protein H) component has been proposed to block phagocytosis by interfering with pathways involving FCγ (Fc-gamma) and complement (CR3) receptors, which both mediate phagocytosis (Dong et al., 2010). The effector proteins, NleE, NleB, NleC, NleD and NleH (non-Lee effectors E, B, C, D and H) have been reported to inhibit the activation of NF-κB pathways in the host, Which can potentially lead to repressed innate and adaptive immune responses in the host (Nadler et al., 2010; Newton et al., 2010; Yen et al., 2010; Baruch et al., 2011; Mühlen et al., 2011; Pearson et al., 2011).

### 1.4 Methods for *E. coli* O157 detection

Many methods have been reported and are available to detect *E. coli* O157 in feces and other matrices. Culture based methods are most commonly used for the numeration and isolation of *E. coli* O157 with sorbitol MacConkey agar being the most widely used medium (Farmer and Davis, 1985). As *E. coli* O157 strains do not ferment sorbitol, they utilize the peptone for growth, resulting in raised pH value in sorbitol MacConkey medium. The non-pathogenic *E. coli* can ferment sorbitol and produce acid to reduce pH. A pH indicator contained in sorbitol MacConkey agar will change to colors, allowing differentiation of *E. coli* O157 strains from

other *E. coli* strains. Both cefixime and tellurite are often added o sorbitol MacConkey medium (CT-SMAC agar) to inhibit the growth of other non-pathogenic *E. coli* strains (Zadik et al., 1993). Two chromogenic media were also widely used in *E. coli* O157 isolation: CHROMagar O157 agar and Rainbow O157 agar (Bettelheim, 2005). The CHROMagar O157 agar is frequently used for *E. coli* O157 detection (forms pink or purple colonies), and has been reported to have better sensitivity and similar specificity compared with sorbitol MacConkey medium (Church et al., 2007). The *E. coli* O157 strains that grow on Rainbow O157 agar are typically black colonies, while some other EHEC strains, such as O113, could be mauve, red or pink (Bettelheim, 1998).

Immunoassays are non-culture based methods for *E. coli* O157 detection, which usually target the O and H antigens of STEC, and many ELISA (enzyme-linked immunosorbent assay) or latex agglutination test based kits are commercially available for this purpose. A study comparing the ELISA and sorbitol-MacConkey agar suggested that ELISA has better sensitivity and consume less times, but it was recommended that positive samples be confirmed using PCR based methods (Park et al., 1996). If the test purpose is to measure the population of all STEC strains, immunoassays targeting Shiga-toxins can also be used (Kehl et al., 1997). Immunomagnetic separation (IMS) is particularly useful when the initial population of *E. coli* O157 is low. With a short period of enrichment (4 h), IMS can greatly improve the sensitivity of *E. coli* O157 detection (Karch et al., 1996), even when compared to PCR based methods (Chapman and Siddons, 1996; Cubbon et al., 1996; Nataro and Kaper, 1998). A limitation of IMS is that it may not work well for *E. coli* O157 enumeration as enrichment if often required.

PCR procedures are rapid detection methods for *E. coli* O157, the primers usually are designed to target Shiga-toxins encoding genes, *stx1* and *stx2* (Brown et al., 1989); the intimin

gene, *eae;* and the flaglin gene, *fliC* (Gannon et al., 1997). The PCR based method can be used for various samples, such as food, stool, water and soil samples (Paton and Paton, 1998; Holland et al., 2000; Campbell et al., 2001). A study reported that the sensitivity could be as low as $10^3$ CFU of stx1-producing STEC cells per g of feces, in a mix containing $10^8$ CFU of stx2-producing STEC per gram of feces (Ramotar et al., 1995), showing high specificity and sensitivity of PCR based methods. Another report by Karch et al. (1996) suggested that the PCR could show positive results when the population of *E. coli* O157 is higher than $10^5$ CFU per g of feces. In addition, with pre-enrichment, qPCR methods detected as little as 0.5 to 1 CFU of STEC per g of ground beef (Witham et al., 1996).

### 1.5 Cattle as the primary reservoir of *E. coli* O157

Ruminants, especially cattle are reported to be the primary carrier of *E. coli* O157 (Chase-Topping et al., 2008), and the majority of outbreaks of *E. coli* O157 have been linked to cattle, as indicated by the survey data from 1982-2012 that showed ground beef to be responsible for > 20% of *E. coli* O157 outbreaks in the U.S. (Rangel et al., 2005; Heiman et al., 2015). *E. coli* O157 can colonize the intestinal tract of cattle without causing hemorrhagic disease as Gb3 receptors are not present in bovine blood vessels (Pruimboom-Brees et al., 2000), although bovine epithelial cells express this receptor (Hoey et al., 2002). The transmission of *E. coli* O157 among cattle occurs through animal-animal contact, feces, water, feed, birds, insects, and rodents (Van Donkersgoed et al., 2001). *E. coli* O157 can be ingested and survive within the gastrointestinal tract of cattle (Keen et al., 2010), and although it can pass through the rumen it does not appear to colonize the epithelial tissue within this region of the gastrointestinal tract (Grauke et al., 2002). The production of high concentrations of volatile fatty acids (VFA) by the rumen microbiota may explain why colonization does not take place at this site (Wallace et al.,

1989). *E. coli* O157 can endure the low pH environment in the abomasum (approximately pH 1-2) of cattle because of its acid resistance systems, including an arginine- and glutamate-dependent systems (Lin et al., 1996). Compared to the rumen and abomasum, the lower gastrointestinal tract (cecum, colon, rectum) present are more suitable environment for *E. coli* O157 to grow and proliferate (Grauke et al., 2002). A study on the colonization of *E. coli* O157 in the gut of calves reported A/E lesions in the gut of calves < 3 weeks old (Dean-Nystrom et al., 1997). One possible reason for it would be that the underdeveloped immune system in neonatal calves enables this bacterium to colonize these regions of the gastrointestinal tract. Studies have suggested that the bovine terminal rectum (recto-anal junction, RAJ) is the primary colonization site of *E. coli* O157, and colonization at RAJ of cattle may be associated with a high level of shedding of this bacterium (Naylor et al., 2003; Rice et al., 2003; Greenquist et al., 2005; Naylor et al., 2005; Davis et al., 2006; Lim et al., 2007).

As mentioned previously, the Gb3 receptors can be expressed by intestinal epithelial cells of cattle (Hoey et al., 2002), but not by vascular vessels (Pruimboom-Brees et al., 2000), suggesting that *E. coli* O157 still has potential to cause damage to the epithelium of cattle. Indeed, challenging neonatal calves with *E. coli* O157 has been shown to cause diarrhea (Dean-Nystrom et al., 1997), and A/E lesions in the ileum and rectum, and the A/E lesions in RAJ of cattle that are naturally shed *E. coli* O157 were also observed (Phillips et al., 2000; Naylor et al., 2005). In *E. coli* O157 challenged yearling steers, two types of tissue damage to the jejunum, ileum and colon of persistent *E. coli* O157 shedders, focal petechiae and mucosal hemorrhagesin, were detected (Baines et al., 2008b). However, it was reported that shedding of *E. coli* O157 did not affect the performance of cattle (Brashears et al., 2003).

Upon attachment by *E. coli* O157 via T3SS, the bovine intestinal epithelial cells recognize the *E. coli* O157:H7 flagellum with toll-like receptor 5 (TLR5) (Mahajan et al., 2009; Walle et al., 2013), leading to activation of NF-κB pathways. Based on evidence of altered gene expression, lipopolysaccharide (LPS) from *E. coli* O157:H7 have also been suggested to be recognized by macrophages and bovine colonic cells (Chitko-McKown et al., 2004). Furthermore, innate immune responses as demonstrated by neutrophil infiltration, can also be detected in the RAJ of cattle colonized by *E. coli* O157 (Nart et al., 2008b). Adaptive immune protection can also be mounted in the gut of cattle against *E. coli* O157:H7, including IgG and IgA antibodies against Stx, LPS, intimin, and the outer membrane porin C protein (OmpC), as well as translocated effector proteins (Nart et al., 2008a).

### 1.6 *E. coli* O157 super-shedder cattle

The densities of fecal *E. coli* O157 can vary greatly among cattle, ranging from 10 to $10^7$ CFU per gram of feces (Chase-Topping et al., 2007; Stephens et al., 2009). The frequency of *E. coli O157* among cattle also varies greatly according to different studies: Hancock et al (1997b) investigated 11,881 fecal samples collected from cattle of 100 feedlots in 13 states (U.S.), and found 210 (1.8%) were positive for *E. coli* O157. In another study, Lejeune et al. (2004) studied twenty feedlots of beef cattle, and reported that 13% of 4,790 collected fecal samples were positive for *E. coli* O157 while Smith et al. (2001) investigated 29 feedlots in Midwestern U.S. and 719 of 3,162 cattle (23%) were positive for *E. coli* O157.

Cattle that shed fecal *E. coli* O157 > $10^4$ CFU per gram of feces are often defined as super-shedders (SS) (Chase-Topping et al., 2008). Super-shedding has been reported in numerous studies, with SS usually accounting for less than 10% of cattle in the herd, but more than 90% of the *E. coli* O157 that enters the farm environment (Omisakin et al., 2003; Chase-

Topping et al., 2007; Stephens et al., 2009; Munns et al., 2015). Shedding of *E. coli* O157 is not a continuous process, rather it is intermittent and shedding levels are inconsistent. Stanford et. al (2005) reported that after inoculation with $10^{10}$ CFU of *E. coli* O157, yearling steers shed *E. coli* O157 for more than 12 weeks, and although the shedding level fluctuated it did decrease over time. Munns et al. (2014) monitored the fecal O157 of 11 SS purchased from a feedlot for 34 days, and reported that after 6 days, none of the steers tested for SS were shedding > $10^4$ CFU *E. coli* O157. In fact, only two previous SS were positive for *E. coli* O157 after 30 days. Baines et al. (2008b) suggested that the duration of fecal shedding of *E. coli* O157 varies greatly among cattle which could be classified into three types, including non-persistent, moderately persistent, and persistent SS (Baines et al., 2008b). Non-persistent SS were defined as cattle that shed *E. coli* O157 for less than 14 days; moderately persistent SS were defined as cattle shedding *E. coli* O157 for about 30 days; and persistent shedders continued to shed *E. coli* O157 for several months (Baines et al., 2008b). Targeting the persistent shedders from the herd is critical, as they can continuously spread *E. coli* O157 into the farm environment, and the hygiene of the farm environment was suggested to influence the prevalence of *E. coli* O157 shedders (Herriott et al., 1998: 157; Keen et al., 2006). Also, such shedding patterns suggest a potential problem in many studies on SS: the animals with non-detectable fecal *E. coli* O157 may be previously colonized by *E. coli* O157. Therefore, for future studies on super-shedding phenomenon, to monitor the cattle for a long-period, such as months, may ensure the reliability of negative control selection.

*E. coli* O157 strains associated with SS have been reported to cause disease in humans. Halliday et al. (2006) and Pearce et al. (2009) both reported that the *E. coli* O157 phage types 21/28, which were prevalent in SS, were associated with human infections in Scotland. Arthur (2013) reported that the *E. coli* O157 phage type 4 was associated with SS identified in U.S.

farms, and it was also the most common strain associated with human infections in the United States. Munns et al. (2016) reported that the phage type 14a was associated with SS identified in an Alberta feedlot, and that it was also one of the most prevalent phage types responsible for human infections in Canada.

### 1.6.1 Factors influencing *E. coli* O157 shedding in cattle

#### 1.6.1.1 Genetics of super-shedder associated *E. coli* O157 strains

As mentioned previously, *E. coli* O157 phage types 21/28 have been frequently isolated from SS cattle housed at Scottish farms. The authors suggested that the genetic variation among genes encoding T3SS might lead to differential regulation and expression of T3SS system between phage types 21/28 and other phage types isolated from low-shedder cattle (Chase-Topping et al., 2008). Such differential regulation of expression in genes may enhance the ability of *E. coli* O157 phage types 21/28 to colonize the intestinal epithelium of cattle (Chase-Topping et al., 2008). Bono et al. (2007) identified a genetic variation at the 255th nucleotide of *Tir* between isolates obtained from SS vs low shedders. Subsequently, Arthur et al. (2013) and Munns et al. (2016) confirmed the results of Bono et al. 2007 with further genomic analysis of SS isolates. These authors suggested such genetic variation may lead to improved potential of SS associated *E. coli* O157 strains to colonize the bovine intestine. Moreover, increased Stx2 expression has been proposed to be associated with increased adherence of *E. coli* O157 to the intestinal epithelium of cattle (Baines et al., 2008a). This suggests that *E. coli* O157 strains that produce higher levels of Stx2 may play an important role in the etiology of SS cattle.

#### 1.6.1.2 Host intestinal microbiota

Bovine gut microbiota may also play a role in influencing the shedding of *E. coli* O157, as they share the host's gut with the other microbial species. The size of gut metagenome can be

more than a hundred times larger than the host genome (Qin et al., 2010), and the microbiota has been proposed to play a role in regulating the host immune system, nutrient metabolism, and liver, muscle and brain function (Nicholson et al., 2012). Healthy gut microbiota has been suggested to inhibit the growth of pathogenic microbes through direct effects, such as releasing antimicrobials and competitive exclusion, as well as indirect effects, such as activation of host immunity (Buffie and Pamer, 2013). Zhao et al. (1998) examined 1,200 bovine gut microbes, and isolated 18 bacteria that competitively excluded *E. coli* O157. The authors found that 17 of the screened *E. coli* isolates were non-pathogenic, with the production of colicin potentially responsible for their anti- *E. coli* O157 activity (Schamberger et al., 2004). Judging from the complexity of the gut microbiota, the potential of gut microbiota to regulate *E. coli* O157 shedding is unlikely to be limited to competitive exclusion and production of antimicrobials. Indeed, beneficial strains such as *Lactobacillus* and *Bifidobacterium* have been reported to reduce *E. coli* O157 shedding in cattle (Peterson et al., 2007a; Fukuda et al., 2011), probably due to their ability to stimulate the development of host immune system, and lower intestinal pH through the production of lactic acid (Cross, 2002). Niu et al. (2014) reported that the T1-like gut bacteriophages of a *Siphoviridae* family can lyse common *E. coli* O157 strains, suggesting that the microbiota of intestinal tract of SS, low-shedders, and cattle negative for fecal *E. coli* O157 (non-shedders, NS) may harbor different bacteriophages. In addition, a previous fecal microbiome study reported that there was a difference in the diversity of fecal microbiota between SS and NS, which also suggested a link between gut microbiota and *E. coli* O157 shedding (Xu et al., 2014).

#### 1.6.1.3 Host immune functions and immunity

Host immune responses are known to be induced by both oral challenge and rectal inoculation with *E. coli* O157. Woodward et al. (1999) reported that inflammatory responses, mainly eosinophils response, were induced in the intestinal tract of gnotobiotic calves orally challenged with *E. coli* O157. Dean-Nystrom et al. (1997) reported that *E. coli* O157 challenge via stomach tube can cause neutrophil infiltration in colon, ileum and rectum of neonatal calves. Stoffregen et al. (2004) observed infiltration of neutrophils and eosinophils of the mucosa of the gallbladder in calves challenged with *E. coli* O157. Nart el al. (2008b) detected neutrophil responses and antibody responses against *E. coli* O157 in the mucosa of RAJ in challenged calves. Corbishley et al. (2014) suggested that T-cell responses were involved in *E. coli* O157 colonization in challenged cattle via measurement of *IFN-γ* and *T-bet* expressed in the RAJ of dairy calves. The findings of these studies suggest that the host and *E. coli* O157 interactions involve both innate and adaptive immune responses.

As described previously, *E. coli* O157 can colonize the intestinal tract of the bovine via T3SS, which translocates effector proteins encoded within the LEE and non-LEE genomic regions. It is reasonable to assume cattle with immunity against antigens from *E. coli* O157 may differ in their responses to *E. coli* O157 colonization as compared to cattle that lack an immune response. Various vaccines have been developed in an effort to prevent intestinal colonization and fecal shedding of *E. coli* O157, through immunization of the host against specific bacterial antigens associated with colonization (Walle et al., 2013). Several studies have reported vaccines developed based on using T3SS proteins to immunize cattle have shown variable efficacy at reducing *E. coli* O157 colonization and lowering the prevalence of this pathogen in the herd (Van Donkersgoed et al., 2005; Peterson et al., 2007b; Smith et al., 2008; Moxley et al., 2009;

Smith et al., 2009). McNeilly et al. (2010) reported a vaccine based on H7 antigen, intimin and EspA, exhibited protection against colonization of the intestinal tract of cattle by *E. coli* O157. Furthermore, a vaccine based on siderophore receptor and porin proteins (SRP) (proteins involved in *E. coli* O157 iron transportation system) also resulted in a reduction in the shedding and prevalence of *E. coli* O157 in cattle (Fox et al., 2009: 157; Thornton et al., 2009). These vaccination studies suggest that host immunity may also play a role in *E. coli* O157 colonization and shedding in cattle.

#### 1.6.1.4 Dietary factors

Diet is known to influence ruminal and intestinal microbiota in mammals, and has been suggested that it influences *E. coli* O157 shedding in cattle (Tkalcic et al., 2000; Tajima et al., 2001; Walker et al., 2011). Feeding forage 48 h after fasting has been reported to increase *E. coli* O157 numbers in the intestinal tract and shedding duration, possibly resulting in greater colonization and prevalence of *E. coli* O157 in cattle (Buchko et al., 2000a). One possible reason for this is that fasting decreases VFA concentrations in rumen and intestinal contents, thus reducing the extent to which they inhibit the growth of *E. coli* O157 (Callaway et al., 2009). Barley is more rapidly fermented in the rumen than corn, reducing the amount of starch that reaches the lower intestinal tract. This can lead to higher pH values and lower VFA concentration in digesta within the lower intestinal of barley-fed as compared to corn-fed cattle (Buchko et al., 2000a; Berg et al., 2004; Bach et al., 2005). Thus, barley-fed cattle have been reported to be associated with increased shedding of *E. coli* O157 (Dargatz et al., 1997; Buchko et al., 2000b). Differences in feed processing methods can also result in differences in the shedding of *E. coli* O157. Fox et al (2007) reported that dry-rolled corn reduced *E. coli* O157 shedding as compared to steam-flaked corn, possibly because steam-flaked corn is more rapidly

fermented in the rumen, leading to less starch reaches to intestine. The composition of the diet may also influence *E. coli* O157 shedding. Tkalcic et al. (2000) reported that grain fed cattle shed higher levels of *E. coli* O157 than forage-fed cattle; while others have reported that forage-fed cattle shed more *E. coli* O157 for a longer duration (Van Baale et al., 2004). Inconsistencies in the impact of shifting diets from forage to grain and from grain to forage on the shedding of *E. coli* O157 in cattle have also been reported and Callaway et al. (2009) suggested that this variation may arise from difference in the types and ratio of forages and grain investigated. More importantly, Callaway et al. (2009) also suggested intrinsic factors of forages, including the forage quality and the presence of antimicrobials, such as tannins, vanillin and other phenolic acids, may also contribute to the variability observed.

#### 1.6.1.5 Environmental factors

It has been suggested that the shedding of *E. coli* O157 is seasonal. In most studies, it has been reported that the prevalence of *E. coli* O157 among cattle is higher during the warm than the cold season (Hancock et al., 1997a; Bonardi et al., 1999; McEvoy et al., 2003; Ogden et al., 2004; Ferens and Hovde, 2011). However, Ogden et al. (2004) reported that in Scottish farms, the prevalence of *E. coli* O157 was higher in winter than summer possibly due to intensive housing increasing animal to animal transmission, while the shedding level of *E. coli* O157 was indeed higher in summer. It was also suggested that the husbandry and management practices and hygienic practices on farm could influence *E. coli* O157 shedding in beef and dairy cattle (Herriott et al., 1998: 157; Keen et al., 2006).

### 1.6.2 Preharvest interventions

The sustainable agriculture production concept of "from farm gate to food plate" makes food safety an important component for the whole production chain. Although controlling *E. coli*

O157 at food processing plants can ensure the safety of food products, the risk of contamination of irrigation and recreation water cannot be reduced without on-farm control strategies.

#### 1.6.2.1 Probiotics

Beneficial bacteria, such as *Lactobacillus* spp., have been reported to reduce the prevalence of *E. coli* O157 in cattle, possibly due to their ability to produce lactic acids and to stimulate the development of host immune system (Malin et al., 2004; Younts-Dahl et al., 2004). Direct-fed microbials (DFM) that contain *Lactobacillus* spp. have shown potential as a preharvest intervention strategy. Lema et al. (2001) reported that a mixed inoculant containing *L. acidophilus*, *L. casei*, *L. fermentum* and *L. plantarum* reduced *E. coli* O157 shedding in lambs. Younts-Dahl et al. (2004) reported that feeding high doses ($10^9$ CFU/ml per animal) of *L. acidophilus* strains to beef steers reduced fecal *E. coli* O157. Brashears et al. (2003) also reported that a *L. acidophilus* based DFM reduced *E. coli* O157 density on the hide and in feces from feedlot cattle. Some of these DFM products are commercially available and are being used by beef producers (Callaway et al., 2004). However, none of the DFM products have been reported to completely eliminate shedding of *E. coli* O157, as how the host and gut microbial interaction can influence shedding of *E. coli* O157 was not understood, which impedes the development of novel DFM products. Also, *E. coli* O157 was known to resist acidic environment, which may also explain why probiotics treatment cannot completely prevent from *E. coli* O157 shedding in cattle (Leyer et al., 1995).

#### 1.6.2.2 Colicin-producing *E. coli*

Colicins are antimicrobials produced by certain strains of *E. coli* to kill other strains including *E. coli* O157, and thus such *E. coli* strains (as DFM) were proposed to be used as a preharvest intervention to control EHEC in cattle (Schamberger et al., 2004). Schamberger and

Diez-Gonzalez (2002) identified 24 colicin-producing *E. coli* from humans and animals which showed an ability to inhibit *E. coli* O157 *in vitro*. Zhao et al. (1998) isolated 17 *E. coli* strains from the cattle intestinal tract and fecal samples which inhibited growth *E. coli* O157. Inoculation of these isolates into calves resulted in a reduction in the carriage of *E. coli* O157 within the gastrointestinal tract. However, using of colicins to reduce *E. coli* O157 shedding is still at experimental stage, and there are problems associated with of applying colicins-producing *E. coli*, which require further research. For example, some colicin-producing *E. coli* may produce endotoxins, and treatment with *E. coli* may induce host immune responses, and also the potential strains need to survive the digestive tract of the host (Murinda et al., 1996: 7).

#### 1.6.2.3 Vaccine

It is challenging to develop a vaccine for cattle against *E. coli* O157 because cattle do not always exhibit clinical symptoms as a result of *E. coli* O157 colonization. As mentioned previously, vaccination was reported to reduce *E. coli* O157 prevalence and fecal shedding in cattle, but inconsistent results were reported in different studies (Sargeant et al., 2007). In Canada, Econiche™ is a commercially available vaccine based on T3SS proteins. This vaccine was reported to effectively reduce the shedding of *E. coli* O157 in cattle, but 3 vaccinations are needed to elicit an adequate immune response and even with this it does not completely prevent shedding (Rich et al., 2010). Another vaccine, EpitopixTM SRP, has been granted a conditional license from the USDA and although it did reduce the fecal shedding of *E. coli* O157 in cattle, responses were marginal (Thornton et al. 2009). However, none of the vaccines have been adopted by the farm in U.S. and Canada. Potential reason is that application of these vaccines requires serval times of vaccinations, and none of the vaccines can effectively eliminate the

shedding of *E. coli* O157. Also, the cost of applying these vaccines may also impedes the adoption of *E. coli* O157 vaccines to cattle production.

#### 1.6.2.4 Sodium chlorate

Sodium chlorate kills *E. coli* O157 because it is reduced to chlorite by an enzyme within the cytoplasm, and chlorite is toxic to the bacteria (Stewart, 1988). Callaway et al. (2002) supplied cattle with chlorate through drinking water, and within 24 h a 2 log and 3 log, decrease in *E. coli* O157 was noted in the rumen and feces, respectively. Administration of chlorate to sheep also resulted in a 2 to 4 log reduction of fecal *E. coli* O157 within 24 h (Callaway et al., 2003; Edrington et al., 2003). These studies suggest that sodium chlorate shows promise as an approach to reducing *E. coli* O157 in ruminants prior to slaughter, pending its regulatory approval.

#### 1.6.2.5 Hide cleaning

The hide of cattle is regularly washed to remove physical contaminates prior slaughter, a practice that greatly reduces foodborne pathogens on the hide, including *E. coli* O157 (Arthur et al., 2007). Using organic acid additives may increase the efficiency by which washing reduces *E. coli* O157 on the hide, as these compounds are routinely used for carcass decontamination (Berry and Cutter, 2000). Also, Coffey et al. (2011) tested the performance of bacteriophages e11/2 and e4/1c on reducing the hide *E. coli* O157, suggesting that after 1 hour of bacteriophages spray onto the hide, the population of *E. coli* O157 decreased 1.5 log. Although the bacteriophage treatment can reduce the density of *E. coli* O157 in beef, it does not prevent contamination after the treatment (Carter et al., 2012). Also, Sheng et al. (2006) reported that the application of bacteriophage to the recto-anal junction of cattle can reduce average *E. coli* O157 shedding, but not eliminate this bacterium.

### 1.7 Understanding the regulatory mechanisms of super-shedding at the gene level

Gene expression analysis studies the transcripts that are expressed in certain types of cell or tissue in an effort to gain an understanding of physiological processes, such as immune responses, pathogenesis and regulation of gene expression at molecular level. If the sequences of interested transcripts are known, there is a possibility to investigate genes of interest. Researchers have been using gene expression analysis to further understand *E. coli* O157 shedding in cattle. For example, Li and Hovde (2007) studied t gene expression at the RAJ using microarrays and suggested that host immune response, signal transduction and metabolism were involved in *E. coli* O157 colonization at this site. Corbishley et al. (2014) reported increased expression of IFN-γ and T-bet genes in the RAJ of *E. coli* O157 challenged cattle and suggested T-cell responses might occur upon *E. coli* O157 colonization. Many methods can be used for gene expression analysis, including real-time quantitative PCR, microarrays, and RNA-sequencing (RNA-seq).

#### 1.7.1 Studying host gene expression using real-time quantitative PCR (RT-qPCR)

RT-qPCR is a method based on polymerase chain reaction and it measures the accumulation of amplificons for every cycle of the reaction. For gene expression analysis, reverse transcription is employed prior to RT-qPCR measurement. RT-qPCR is a reliable, relatively simple, sensitive, and specific method to measure gene expression in a rapid manner (Bustin et al., 2009). It is commonly regarded as the gold standard for gene expression measurement (Bustin et al., 2005). However, selected primers need to be specific to target sequences for reliable results, and optimization of reaction conditions can be time consuming. SYBR Green (Bulcke et al., 2010) is an economical DNA-binding dye and commonly used principally for RT-qPCR. However, SYBR green can interact with all double stranded DNA,

including non-specific amplicons which could result in errors. Using a fluorescent reporter probe, such as Taqman (Ponchel et al., 2003) increases specificity and accuracy, as the fluorescence can only be detected from amplicons that contain sequences that are complementary to the reporter probe. When simultaneous detection of a large number of genes is required or novel as of yet undefined transcripts are targeted, RT-qPCR is no longer a suitable method.

### 1.7.2 Microarrays

Microarrays are also known as DNA-chips, which allow for simultaneous detection and quantification of thousands of DNA sequences. Transcripts are often reverse transcribed into cDNA for gene expression analysis using microarrays. The surface of the microarray is consists of an array of probe DNA sequences (Bumgarner, 2013) which hybrid with labeled (commonly fluoresce) DNA/cDNA (targets) probes with the quantity of the target measured on the basis of the fluorescence signal. Although high throughput, microarrays do not allow detection of novel transcripts and absolute quantitation of gene expression. In addition, as the technologies of high throughput sequencing have advanced substantially in recent years, and the cost of parallel sequencing has declined significantly, next generation sequencing methods have gained preference over microarrays.

### 1.7.3 Next-generation sequencing

The Human Genome Project was completed in 2003, which took more than 10 years and cost more than $4 billion (USD), but a decade later the next generation sequencing technologies (Illumina NovaSeq® series sequencers) is aiming to lower the cost of sequencing human genome to $100 (Kamps et al., 2017) within several days. Next-generation sequencing involves three principal steps, library construction, sequencing, and bioinformatic data analysis (Kamps et al., 2017). The advantages of next-generation sequencing over traditional Sanger sequencing are

high throughput, high sensitivity and specificity (Chin et al., 2013), as well as the ability to detect novel nucleic acid sequences.

RNA-Seq is the term used to refer to studies on gene expression profiles using next-generation sequencing technologies. It is revolutionary as it allows the investigation genome wide transcriptome, which includes all the genes expressed within a biological system. By increasing sequencing depth, novel and rare transcripts can be detected, with paired end sequencing ensuring a high level of mapping accuracy (Wang et al., 2009). The challenges for RNA-Seq are that (1) it may not be able to quantify absolute gene expression (2) data analysis requires sophisticated bioinformatics tools, and (3) well annotated genomes are needed to acquire accurate information from RNA-Seq data.

Transcriptome profiling has been used in many areas of research, including in the characterization of diseases, such as cancer, diabetes, obesity, and host-microbial interactions (Eizirik et al., 2012; Eizirik et al., 2012; Kommadath et al., 2014; Cloney, 2017). Sun et al. (2012) conducted RNA-Seq using RNA isolated from the gill of catfish challenged by *Flavobacterium columnare*, and identified potentially altered inflammatory response pathways, including the NF-κB pathway and interferon signaling. Kommadath et al. (2014) performed transcriptomic analysis on the blood of pigs challenged by *Salmonella*, and identified co-expressed gene networks that were potentially associated with *Salmonella* shedding in swine. Transcriptome studies have been used to investigate *Mycobacterium* infections in cattle, which causes Johne's disease. McLoughlin et al. (2014) performed RNA-Seq and microarray to profile the transcriptome of peripheral blood leukocytes from cattle infected with *Mycobacterium bovis*, and they suggested that immunomodulation by *M. bovis* led to repressed innate immune responses and signaling, which impeded adaptive immune responses, allowing the progression of

*Bovine tuberculosis*. Casey et al. (2015) investigated the transcriptome of bovine macrophages infected by *Mycobacterium avium* subspecies *paratuberculosis*, and identified altered signaling pathways in infected macrophage, including CD40, interferon, and IL-15 signaling. Based on transcriptome profiling of bovine peripheral blood mononuclear cells, Bhuju et al. (2012) reported that vaccination of cattle with Bacillus Calmette–Guérin against *M. bovis* resulted in upregulation of INF-γ which in turn induced T-helper cell responses. The authors also proposed that *IL-22* could be used as an indicator for successful vaccination of cattle, based on its expression pattern after vaccination. These studies suggest that RNA-Seq based transcriptomic analysis has considerable potential to help in understanding the mechanisms of host and pathogen interaction, and thus could be used to investigate the mechanisms that lead to colonization and high levels of shedding of *E. col* O157 in cattle.

### 1.7.4 miRNAomes

Small non-coding RNAs, such as microRNAs (miRNAs) are important components of the transcriptome; however, miRNAs are not translated into proteins, but rather they play in regulating transcription. MiRNAs are about 22 nucleotides in length (Bartel, 2009), and can be expressed from miRNA encoding genes which are located in intergenic or intragenic regions of the genome (Du and Zamore, 2005). Within the nucleus, an miRNA genes encode for a primary miRNA, which is processed by a complex of Drosha (RNase III enzyme) and DGCR8 (DiGeorge syndrome critical region in gene 8) into a precursor miRNA which in turn is processed by a RNase, Dicer, into miRNAs in the cytoplasm (Bernstein et al., 2001; Lee et al., 2003). The mature miRNAs bind to an RNA-induced silencing complex (RISC) which then binds to an Argonaute (AGO) protein to form a complex that mediates mRNA expression (Huntzinger and Izaurralde, 2011). The regulation of miRNA is achieved through recognition of complementary sequences on transcripts, commonly in the 3'-UTR region (Fang and Rajewsky,

2011), which are usually 6-8 nucleotides long and referred to as seed regions (Bartel, 2009). Such miRNA recognition mechanisms have been widely used in computer algorithms for miRNA target prediction (Thomas et al., 2010). Upon target transcript recognition, three mechanisms of silencing of transcripts by miRNA have been reported: cleavage of mRNAs; destabilization of mRNA; and reducing translation efficiency (Huntzinger and Izaurralde, 2011). It has been reported that miRNA transfection or inhibition its expression could cause perturbations in the expression of hundreds of genes (Grimson et al., 2007; Frankel et al., 2008), and that 30%-40% of genes contain complementary sequences with miRNA seed regions (Krützfeldt et al., 2005; Frankel et al., 2008).

miRNAs can be expressed within plant and animal cells, and they are involved in various functions, including tissue development, metabolism, immune function and disease development (Wahid et al., 2010). In addition, miRNAs have been detected in extracellular spaces, such as milk, serum, urine and feces (Weber et al., 2010; Liu et al., 2016), suggesting that miRNAs may also be associated with regulating extracellular functions associated with immunity, cell communication and the development of the gut microbiota (Turchinovich et al., 2012; Liu et al., 2016).

Many studies have reported that miRNA expression was associated with the host-microbial interactions. Bao et al. (2015) studied the whole blood miRNAomes of *Salmonella* challenged pigs, suggesting miR-214 and miR-331-3p may regulate host immune responses that arise as a result of the persistent shedding of *Salmonella*. The miRNA-155 has been reported to control *Helicobacter pylori* infections by mediating T-cell responses in mice (Oertli et al., 2011). The presence of *Mycobacterium tuberculosis* has also been reported to interfere with the expression of miR-125b and miR-155 so as to block the expression of Tumor Necrosis Factor

(TNF) responses in macrophages (Rajaram et al., 2011). Ma et al. (2011) reported that miR-29 mediated natural killer cells and T helper responses against *Listeria monocytogenes* and *M. bovis* infection via regulation of IFN-γ. These authors also identified that lowered expression of miR-29 upon pathogen infection led to enhanced host resistance to *L. monocytogenes*. Moreover, a recent study revealed that the expression of miRNAs involved in B-cell function in the small intestine were associated with beneficial bacteria (*Lactobacilli* and *Bifidobacterium*) present in the gut of young calves (Liang et al., 2014). Therefore, miRNAs appear to play an important role in host-microbial interactions, making it logical to investigate miRNA expression in the gut of cattle as a mean of shedding light on the mechanisms of colonization that result in super-shedding of *E. coli* O157 in cattle.

### 1.8 Studying of gut microbiome and its role in *E. coli* O157 super shedding

The gut microbiota consists of all the microorganisms that inhabit the intestinal tract of a host. As mentioned previously, the nature of the gut microbiota may be one of factors that influences *E. coli* O157 shedding in cattle, and it is already known to play a role in various important host physiological processes (Nicholson et al., 2012; Xu et al., 2014). For example, the microbiota can influence the development of the mucosal associated lymphoid system. Hamada et al. (2002) reported that isolated lymphoid follicles were under-developed in germ germ-free mice; and Rakoff-Nahoum et al. (2004) suggested that the toll-like receptor signaling induced by LPS of commensal bacteria was necessary for the maintenance of epithelial homeostasis. Also, *Clostridium* spp., which produce butyrate (Pryde et al., 2002), have been suggested to provide energy to the gut and to enhance the epithelial barrier through improved tight junction assembly (Peng et al., 2009; Hudcovic et al., 2012) although not all species of *Clostridium* are beneficial bacteria.

Due to the large number of species that comprise the gut microbiota and the fact that many of these species are unculturable, traditional culturing methods are not ideal for studying the whole microbial community in the gut (Amann et al., 1995). One common method to investigate the members of the gut microbiome is 16S rRNA gene amplicon sequencing, as 16S rRNA gene is present in all prokaryotic cells, and contains hypervariable regions which are ideal for taxonomic analysis (Woese, 1987; Yang et al., 2016). The limitation of 16S rRNA gene amplicon sequencing is that it does not provide information on the function of the genes, although methods are available for prediction of the gene families associated with predicted taxa (Langille et al., 2013). Therefore, following the sequencing based taxonomic analysis, metagenomic, proteomic and metabolomic analysis can be used to further understand the actual function of members of the microbiome.

### 1.9 Hypothesis and key objectives

Identification of super-shedding status on farm could be an important step in allowing both beef and dairy producers to apply preharvest interventions, such as dietary management, feed additives, or hide washes prior to slaughter. Current identification methods still rely on plate culturing, which is inefficient and time consuming. Identification of biomarkers, such as genes, miRNAs or gut microbes may facilitate targeting of potential super-shedders within herds. However, a limited understanding of the mechanisms of super-shedding has greatly impeded the development of effective biomarkers that could be used to target SS with management strategies aimed at reducing overall *E. coli* O157 shedding.

I hypothesize that differences in the physiological environment between the small and the large intestine may contribute to the tropism of *E. coli* O157 colonization, and that gene/miRNA expression profiles of the whole gastrointestinal tissue differ between NS and naturally colonized

*E. coli* O157 SS, especially at the RAJ. In addition, the differences in gene expression profiles could be due to genetic variation within the host. Secondly, I hypothesize that the mucosa associated microbiota of NS is different from that of SS, and such differences could cause distinct microbe-to-microbe and host-microbial interactions, and gene expression at RAJ of NS and SS.

The specific objectives of this thesis were: (1) to investigate the transcriptome of RAJ, and to identify genes that are potentially associated with super-shedding and colonization of *E. coli* O157 using RNA-Seq (Chapter 2); (2) to investigate the host mechanisms associated with *E. coli* O157 shedding in cattle by studying the transcriptome of the whole intestinal tract using RNA-Seq (Chapter 3); (3) to investigate the miRNA expression and miRNA regulation in the whole intestinal tract of cattle, and to identify miRNAs potentially associated with *E. coli* O157 shedding using miRNA-Seq (Chapter 4); (4) to investigate the RAJ mucosa associated bacterial community and its predictive microbial function, as well as potential host-microbial interactions that may influence host gene expression and *E. coli* O157 shedding (Chapter 5). The long-term goal of this project is to further understand *E. coli* O157 super-shedding in cattle from a perspective of host genomics and the gut microbiome, as well as to provide information to develop potential strategies that can rapidly identify SS based on differences in genes/miRNAs and gut microbes between SS and NS.

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# Chapter 2. Comparative transcriptomic analysis of rectal tissue from beef steers revealed reduced host immunity in *Escherichia coli* O157:H7 super-shedders[1]

## 2.1 Introduction

*Escherichia coli* O157:H7 is a foodborne pathogen that can cause disease in humans, including abdominal cramps, bloody diarrhea and hemolytic uremic syndrome. It is estimated that this bacterium causes more than 60,000 illnesses in the U.S. annually (Scallan et al., 2011). Cattle are the main reservoir of *E. coli* O157:H7, and many *E. coli* O157:H7 outbreaks have been traced to contaminated meat, fruits or vegetables that have direct contact with cattle feces or contaminated water (Sodha et al., 2015). Cattle shedding more than $10^4$ CFU *E. coli* O157:H7 per gram of feces are defined as "super-shedders" (SS) (Chase-Topping et al., 2008). It is speculated that these individuals are the source of the majority of *E. coli* O157:H7 cells that enter the environment and/or food processing chain (Omisakin et al., 2003). Three types of super-shedders have been described, which are non-persistent shedders (shedding lasts ~14 days after *E. coli* O157:H7 challenge), moderately persistent shedders (lasts ~30 days), and persistent shedders (lasts several months) (Baines et al., 2008b). Many studies have reported that the recto-anal junction (RAJ) of cattle is the primary colonization site of *E. coli* O157:H7, with the formation of biofilms at this location playing an integral role in super-shedding (Munns et al., 2015). Although the mechanism of the super-shedding phenomenon is large unknown, many factors such as diet, seasonality, environment, cattle host, and interactions between the

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microbiome and the intestinal epithelium as well as interactions within the microbiome have been considered to affect this phenomenon (Munns et al., 2015).

The interactions between *E. coli* O157:H7 and the immune system of cattle have been previously reviewed (Walle et al., 2013). In general, the flagellum of *E. coli* O157:H7 has been suggested to initiate the colonization of the bovine epithelium, and upon colonization the type III secretion system injects effector proteins, including intimin receptor (Tir), and *Escherichia* secretion proteins (EspA, EspB and EspD) into epithelial cells. In response, the bovine intestinal epithelial cells recognize the *E. coli* O157:H7 flagellum with toll-like receptor 5 (TLR5) activating NF-κB pathways. Lipopolysaccharide (LPS) from *E. coli* O157:H7 can also be recognized by macrophages and bovine colonic cells, based on the evidence of altered gene expression in cultured cells. Adaptive immune protection can be mounted in cattle against *E. coli* O157:H7, including IgG and IgA antibodies against Stx1 (Shiga-like toxin), Stx2, LPS and intimin, as well as *E. coli* O157:H7 secretive proteins. Previous gene expression measurement for rectal tissue of cattle shedding *E. coli* O157 indicated that the changes in gene expression related to immune responses may be sustained while the number of fecal *E. coli* O157 decreases, although the maximum changes in expression may occur when the number of *E. coli* O157 peaks (Corbishley et al., 2014). Interestingly, a study challenging calves and peripheral blood mononuclear cells with *E. coli* O157 revealed that the *Stx* produced by *E. coli* O157:H7 is able to inhibit the proliferation of lymphocytes (Hoffman et al., 2006).

To date, few studies have examined the contributions of host mechanisms at the RAJ to the growth of *E. coli* O157:H7 and the super-shedding phenomenon. Antibody secretion and immune cell infiltration and changes in gene expression at this site have been observed in cattle challenged with *E. coli* O157:H7 (Walle et al., 2013). However, such changes at the RAJ have

not been examined in cattle that are natural super-shedders. In this study, we hypothesized that the gene expression profiles of RAJ tissue differ between SS and non-shedding cattle (NS). Comparative transcriptome analysis of RAJ tissues collected from cattle previously shown to be SS and NS (Munns et al., 2014) was performed using high throughput RNA-Seq.

## 2.2 Materials and methods

### 2.2.1 Identification of SS cattle and rectum tissue collection

Fecal samples were collected from 400 British x Continental feedlot steers (452 kg ± 23 kg) that were fed a barley-grain based finishing diet and cared for in accordance with the guidelines of the Canadian Council of Animal Care (Animal Care Committee protocol number: 1120) (Munns et al., 2014). A fecal sample (50 g) from each steer was collected from the rectum, transferred to a sterile container, placed on ice and immediately transported to the laboratory for enumeration using culture-based methods as outlined previously (Munns et al., 2014). Briefly, individually collected samples were plated for microbial analysis within 4 h after sample collection. The fecal sample (1 g) was serially diluted with 9 mL of phosphate buffered saline (PBS) from $10^{-1}$ to $10^{-4}$. The dilutions were plated onto sorbitol MacConkey agar with 2.5 mg/L potassium tellurite and 0.05 mg/L cefixime (CT-SMAC; Dalynn Biologicals, Calgary, AB), and incubated at 37°C for 18 to 24 h. A colony counter (Reichert, Depew, NY) was used for bacterial enumeration (colony forming unit, CFU), and steers identified with $\geq 10^4$ CFU of *E. coli* O157:H7 per gram of feces were defined as SS. The CT-SMAC selected isolates were then examined using an *E. coli* O157 Latex Test kit (Oxoid Ltd, Basingstoke, Hampshire, UK) following the manufacturer's instruction, and the positive agglutination isolates were further verified using a multiplex PCR assay targeting genes encoding virulent factors including

verotoxin (*vt*), intimine (*eaeA)*, H7 flagellin (*fliC*)) as described previously (Gannon et al., 1997) to define the *E. coli* O157:H7 serotype. Based on this evaluation, 5 SS and 4 control pen-mates which tested negative for *E. coli* O157:H7 (NS) were selected for slaughter. Prior to slaughter, the fecal samples of SS were collected twice daily for 4-10 days, and the fecal *E. coli* O157:H7 was monitored using the culture based method as described above and subsequently an immunomagnetic separation assay using anti-*E. coli* O157 Dynabeads (Invitrogen, Carlsbad, CA) following manufacturer's instructions as outlined in a previous study (Munns et al., 2014). When animals were slaughtered, two 2 $cm^2$ biopsies 3-5 cm proximal to the RAJ were collected and immediately flash frozen in liquid nitrogen within 10 min after death of the animal. Samples were stored at -80 °C until RNA was extracted.

### 2.2.2 RNA extraction

Before RNA extraction, frozen RAJ tissue samples from 5 SS and 4 NS were each ground to a fine powder in liquid nitrogen using a pestle and mortar. Total RNA was extracted from about 100 mg of powdered tissue using a mirVana total RNA Isolation Kit (Ambion, Carlsbad, CA, USA) following the manufacturer's instructions. The Agilent 2200 TapeStation (Agilent Technologies, Santa Clara, CA, USA) and Qubit 2.0 Fluorometer (Invitrogen, Carlsbad, CA, USA) were used to measure RNA quality and quantity respectively. RNA with an integrity number (RIN) greater than 7.0 (and ratio of 28S/18S ranging from 1.7 to 2.4) was used for RNA-Seq library construction.

### 2.2.3 RNA-Seq library preparation and sequencing

Extracted total RNA (1 μg) was used for library preparation using a Truseq Stranded Total RNA Sample Preparation kit (Illumina, San Diego, CA, USA) following the manufacturer's instructions. Firstly, the rRNAs were depleted using biotinylated, target-specific

oligos combined rRNA removal beads, and the remaining RNA was fragmented followed by first strand cDNA synthesis with reverse transcriptase and random primers. For the second strand cDNA synthesis, DNA polymerase I and RNase H were used, followed by ligation of the indexed-adapters and PCR enrichment (98°C for 30 sec, followed by 15 cycles of: 98°C for 10 sec, 60°C for 30 sec, 72°C for 30 sec, and 72°C for 5 min; the final products were held at 4°C). To ensure that the size of the products was around 250-270 bp and the concentration was adequate for sequencing, libraries were measured using Agilent 2200 TapeStation and a Qubit 2.0 Fluorometer, respectively. RNA sequencing was performed using a HiSeq 2000 sequencing system (Illumina, San Diego, CA, USA), with paired-end (100 bp) sequencing at Genome Quebec Innovation Centre, Montreal, Quebec, Canada.

**2.2.4 mRNA profiling and differential expression analysis**

Sequencing reads were first mapped against the reference bovine genome UMD3.1 (DNA source was Hereford cattle) assembly (Zimin et al., 2009) using Tophat2 (Kim et al., 2013). The Tophat2 splice aligner was used to address the two most challenging issues in the mapping of RNA-Seq reads: (1) reads spanning multiple exons, and (2) reads more readily mapping to pseudogenes than to their real positions (Kim et al., 2013). To study gene expression profiles of RAJ tissues, the read counts mapped to the genome were normalized into FPKM (fragments per kilobase of exon per million fragments mapped) using Cufflinks (Trapnell et al., 2012).

The Cufflinks pipeline (Trapnell et al., 2012) was used for differentially expressed **(**DE) gene identification. Cufflinks takes the raw-reads alignment output from Tophat2 and assembles the mapped reads into transcripts to construct a transcriptome for each sample. The reads were assembled into transcripts by Cufflinks, and the transcripts were annotated using Ensembl umd3.1.GTF file (ftp://ftp.ensembl.org/pub/release-80/gtf/bos_taurus). After transcriptome

assembly, Cuffmerge was used to merge the transcriptomes into a uniform basis for expression level calculation. The DE analysis were then performed using Cuffdiff with FPKM ≥ 0.3, FDR < 0.05 and log2-fold-change > 1 or < -1 as cut-off, which have been applied in other RNA-Seq based studies (Bottomly et al., 2011; Toung et al., 2011). Only the DE genes expressed in at least 50% of NS (at least 2 NS) and SS (at least 3 SS), were retained and subjected to functional analysis.

### 2.2.5 Functional analysis of rectal transcriptome and differentially expressed genes

Functional analysis of the transcriptome of RAJ tissue and DE genes were performed using Ingenuity Pathway Analysis® (IPA, IPA®, QIAGEN Redwood City, www.qiagen.com/ingenuity) with the Ingenuity Knowledge Base (gene only) used as a reference set for our analysis. The function of the core transcriptome of RAJ tissue was based on the top 8,000 genes with the greatest FPKM values (normalized abundance) as IPA does not support analyses with more than 8000 molecules. This software package performs a downstream effect analysis for the biological influence of the input gene set (the transcriptome or DE genes), which first identifies biological function, and then predicts if it is increased or decreased based on up- or down-regulation of genes. A z-score generated from IPA analysis is used to indicate the direction of change of a certain function, with z-scores ≥ 2.0 or ≤ -2.0 indicating the significant increase or decrease in a function, respectively. DAVID Bioinformatics (Huang et al., 2009) and KEGG_PATHWAY (pathways were enriched by DAVID bioinformatics) (Kanehisa and Goto, 2000) were used for enrichment of biological pathways, and pathway analysis, respectively.

### 2.2.6 Validation of gene expression using quantitative real time PCR (qPCR)

The main purpose to perform qPCR on gene expression is to validate the reliability of differential expression identified from RNA-Seq data. Ten identified DE genes related to

immune function from RNA-Seq analysis were selected for qPCR validation, including chemokine (*C-C motif*) *ligand 21* (*CCL21*), *chemokine* (*C-C motif*) *receptor 7* (*CCR7*), *CD22 molecule* (*CD22*), *chemokine* (*C-X-C motif*) *ligand 13* (*CXCL13*), *lymphotoxin beta* (*LTB*), *CD19 molecule* (*CD19*), *interleukin 2 receptor, alpha* (*IL2RA*), *SH2 domain protein 1A* (*SH2D1A*), *POU class 2 associating factor 1 (POU2AF1)* and *4-domains, subfamily A, member 1* (*MS4A1*). The primers (Table 2.1) were designed using NCBI primer blast, and the primer pair specificity was verified by sequencing the PCR products (Table 2.1) before qPCR analysis.

Total RNA (1 μg) was first reverse transcribed to single stranded cDNA using Oligo $(dT)_{12\text{-}18}$ (Life Technologies, Carlsbad, CA, USA) and SuperScript™ II RT (Life Technologies, Carlsbad, CA, USA). Quantitative real time PCR was performed using a StepOnePlus™ Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) using SYBR® Green (Life Technologies, Carlsbad, CA, USA) chemistry and the following program: 95 °C for 20 sec, followed by 40 cycles of denaturation at 95 °C for 3 sec, and annealing/extension for 30 sec. The annealing temperature varied for each gene (Table 2.1). Four commonly used reference genes, bovine *GAPDH*, *18S rRNA*, *RPLP0* and *β-actin* (Rekawiecki et al., 2012), were used for qPCR analysis. Of the genes examined, *β-actin* had the most consistent Cq across all samples, and therefore it was used as the standard reference gene for comparison purposes. ΔCq value was used to indicate the relative quantity of target genes, which was calculated with the equation: $\Delta Cq = Cq_{\text{target genes}} - Cq_{\text{referece gene}}$. Statistical analysis was performed using two-sample t-test with SAS (version 9.2; SAS Institute Inc., Cary, NC) to compare the ΔCq of NS and SS.

### 2.2.7 Availability of sequencing data

The RNA-Seq data are available at NCBI Gene Expression Omnibus (GEO) database under accession number GSE67099.

## 2.3 Results and Discussion

### 2.3.1 Identification of super-shedders

Eleven SS were identified from 400 animals (2.75% of sampled cattle) and the 5 individuals that exhibited the highest shedding levels were slaughtered (Table 2.2). Although these five individuals were not all shedding > $10^4$ CFU/g feces at the time of slaughter, they were confirmed as SS within the previous ten days. Cattle in the present study meet the definition of a non-persistent super-shedder as outlined previously (Baines et al., 2008b). In addition, although the tissue sampling was performed after SS identification and the number of fecal *E. coli* O157 decreased afterwards in the SS, it does not undermine the validity of using collected tissue samples for super-shedding phenomenon investigation. The changes in gene expression potentially induced by *E. coli* O157 can last even after the *E. coli* O157 shedding drops under super-shedding level (Corbishley et al., 2014). The tissue sampling was managed to be accomplished after cattle were purchased from the commercial feedlot, within 4-10 days after the animals were identified as super-shedders, and all the super-shedders were at least positive for *E. coli* O157 before sampling. Therefore, the detected changes in gene expression may be still potentially related to *E. coli* O157 colonization.

### 2.3.2 Transcriptome profiling of bovine rectal tissue

The whole tissue of the RAJ was used for transcriptome profiling using RNA-Seq as the lymphoid structures underlying epithelial tissues might also influence super-shedding (Naylor et al., 2003). In total, the number of paired-end reads generated from parallel sequencing of RAJ tissues ranged from 25.5 M to 38.2 M (Table 2.3), with 17,859 ± 354 genes identified (at least

one read mapped to the gene in at least one animal). The core transcriptome of RAJ tissue included 11,773 genes (with FPKM ≥ 0.03 in all animals).

The top physiological functions associated with the core transcriptome (analyzed from the most abundant 8,000 genes) of RAJ tissue is shown in Figure 2.1, and the identified function was plotted in $-\log_{10}$ (p-value) scale, with the p-value measuring if the association between a set of uploaded genes and a given function was due to random chance (Figure 2.1). The identified basic physiological functions fit this tissue since the rectum contains muscles, blood vessels (arteries, veins), lymphatics, and nerve fibers (Barleben and Mills, 2010). The main physiological function of the RAJ is to store and eliminate feces through peristalsis under the control of the nervous system (Brading and Ramalingam, 2006). As shown in Figure 2.1, genes associated with the development of the muscular (involved 275 genes) and nervous system (involved 562 genes) are included in the core transcriptome of the rectal tissue. Considering that connective tissue is one of main components of the mucosa, submucosa, muscularis and adventitia of the RAJ, it is not surprising that "connective tissue development and function" was one of the top physiological processes (Figure 2.1). Other identified prominent physiological functions included hematological system development (1,145 genes), lymphoid tissue structure development (526 genes), and immune cell trafficking (580 genes). These observations correspond to the highly vascularized nature (Barleben and Mills, 2010) of the RAJ and the presence of lymphoid follicles (ILFs) and lamina propria lymphocytes at this site as identified in cattle (Naylor et al., 2003).

### 2.3.3 Differentially expressed genes in RAJ between super-shedders and non-shedders and qPCR validation

Based on DE analysis, a total of 58 DE genes were identified between SS and NS, with 11 up-regulated and 47 down-regulated genes in SS, with log2-fold-change ranging from -5.5 to 4.2. Among ten genes selected for qPCR, *CCR7, CD22, CXCL13, LTB, IL2RA, CD19,* and *SH2D1A* showed significantly lower expression in SS compared with NS, similar to the RNA-Seq results(Figure 2.2). While expressions of *MS4A1, POU2AF1* and *CCL21* were not significantly different between SS and NS, their ΔCq in SS was greater than in NS, indicating a trend for lower expression in SS. The consistency between qPCR and RNA-Seq is in agreement with previous studies that gene expression levels detected by these two methods are strongly correlated, although not completely consistent (Nagalakshmi et al., 2008; Griffith et al., 2010).

### 2.3.4 Differentially expressed genes that are associated with host adaptive immunity

When the functions of all DE genes were analyzed, the top enriched GO (Gene Ontology) term was "immune system process" based on DAVID bioinformatics functional annotation. IPA functional analysis also showed that 31 down regulated DE genes were related to immune functions, and several of the gene products were immune cell membrane receptors and cytokines (Table 2.4).

According to IPA functional analysis, the identified DE genes were involved in 56 physiological processes (IPA z-score $\leq$ -2.0 or $\geq$ 2.0), which belonged to 10 categories (Figure 2.3). The "hypoplasia of lymphatic system" was the only function predicted to be increased in SS, and five down-regulated genes were involved in this function, including *Kelch-Like Family Member 6* (*KLHL6*), *POU2AF1*, *Ras Homolog Family Member H* (*RHOH*), *SAM and SH3 Domain Containing 3* (*SASH3*), and *Sirtuin 1* (*SIT1*) (Table 2.4). The transcription factor

encoded by *POU2AF1* is involved in development of B-cells, and formation of germinal centers in peripheral lymphoid organs (Nielsen et al., 1996), while *RHOH* and *SITI* have been reported to be involved in regulation of maturation of T-cells (Simeoni et al., 2005; Dorn et al., 2007). Moreover, *SASH3* is involved in B- and T-cell proliferation (Beer et al., 2005), and *KLHL6* is suggested to play a role in B-cell maturation (Kroll et al., 2005). Down-regulation of these genes suggests possible disrupted maturation and proliferation of B- and T-cells in RAJ tissues.

Eleven down-regulated DE genes in SS were associated with decreased functions in "quantity of lymphocytes", "development of lymphocytes", "activation of lymphocytes" and "lymphocytes migration". These genes include *KLHL6*, *POU2AF1, SASH3*, *MS4A1*, *CD22*, *CD19*, *CD79a molecule, immunoglobulin-associated alpha (CD79A)*, *CD79b Molecule, Immunoglobulin-Associated Beta* (*CD79B)*, *CXCL13*, *CCL19*, and *CCL21* (Table 2.5). *KLHL6* is associated with B-cell activation and proliferation, and insufficient expression of this gene causes a reduction in B-cell subsets in mice (Kroll et al., 2005). The expression of *POU2AF1* is essential for B-cells activation and germinal center development (Nielsen et al., 1996), while *SASH3* is a signaling adapter gene expressed in lymphocytes, and decrease in its expression is associated with a decrease in activation and production of B-cells (Beer et al., 2005). The *MS4A1* is important for B-cell proliferation, decrease in the number of B-cells was observed with insufficient expression of this gene (Uchida et al., 2004). The *CD22*, *CD79a*, *CD79b* and *CD19* encode B-cell specific membrane proteins, and down-regulation of these genes indicate a potential decrease in B-cell number (Kraus et al., 2004; Poe et al., 2004). The CD19 was also reported to promote the activation of B-cells, and its down-regulation may contribute to decreased activation of B-cells (Karnell et al., 2014) in SS. The *CXCL13* gene encodes a chemoattractant of B-cells, and expression of this gene is required for migration of B-cells to gut

associated lymphoid tissues and formation of lymphoid follicle (Bowman et al., 2000). The CCL19 and CCL21 are chemoattractants for B-cells, and CCL19 is associated with migration of B-cells from bone marrow to peripheral lymphoid tissues (Bowman et al., 2000), while CCL21 regulates B-cells movement in secondary lymphoid tissues (Nagira et al., 1998). Down-regulation of these genes suggests possible reduction in B-cell quantity, development and migration in SS, which may lead to impairment in mucosal IgA production. As a result, the susceptibility to colonization by *E. coli* O157:H7 at RAJ of SS may be higher than NS.

The isolated lymphoid follicles (ILFs) in the bovine rectum were suggested to be the source of secretory antibodies in the gut, serving as the first line of defense in the gastrointestinal (GI) tract (Tsuji et al., 2008). Lymphotoxin is necessary for the initiation of ILF development (McDonald et al., 2005), and the down-regulation of *lymphotoxin beta* (*LTB*) indicated probable decrease in production of lymphotoxin (Table 2.4). Impaired ILF was reported to cause a 10-fold increase of segmented filamentous bacteria (Suzuki et al., 2004), and a 100-fold increase of anaerobic bacteria in the small intestine of mice (Fagarasan et al., 2002). Others have observed that impairment in ILF function resulted in a 10 to 100-fold increase of *Enterobacteriaceae* colonization in the ileum of mice (Bouskra et al., 2008). These observations suggest that ILFs play an important role in regulating the density of bacterial populations attached to the intestinal epithelium of the host. Down-regulation of *LTB* indicated a possibility that the RAJ of SS contains less well-developed ILF than NS, and thus we speculate that a higher diversity of microbes can be harbored by RAJ of SS. This hypothesis is supported by the observation of greater diversity of fecal microbiota in SS as compared to NS from the same animals (Xu et al., 2014), and a tissue colonized microbiota study is required to further confirm our speculation.

In addition to ILFs, functions on the "movement of T lymphocytes", "quantity of T lymphocytes" and "T-cell development" decreased in the RAJ of SS as compared to NS (Table 2.5). This suggests that the cell-mediated immune response could also be related to super-shedding. T-cells are distributed among the epithelium (intraepithelial T-cells, IET) and the lamina propria, with IET providing rapid T-cell responses to numerous antigens and lamina propria T-cells undertaking regulatory functions (Sheridan and Lefrançois, 2010). The decreased function in the movement, development and quantity of T-cells may lead to the reduced accumulation of IET and lamina propria T-cells in SS, enabling *E. coli* O157:H7 biofilms to more readily form on the RAJ epithelium.

### 2.3.5 Differentially expressed genes that are associated with host innate immunity

The DE analysis of genes also revealed a decrease in the functions of "cell movement of phagocytes", "chemotaxis of neutrophils" and "migration of dendritic cells", and "activation of natural killer cells" (Table 2.5), suggesting less active innate immune protection in SS than NS.

Intestinal phagocytes, especially macrophage, can rapidly eliminate bacteria without eliciting a strong inflammatory response (Smythies et al., 2005). In SS, down-regulation of six cytokine and cytokine receptor genes including *CCL13*, *CCL21*, *CXCL13*, *SPP1*, *LTB* and *CCR7* (Table 2.5) suggest a reduction in the function of the movement of phagocytes, including macrophage, neutrophils and dendritic cells (Saeki et al., 2000; Steitz et al., 2002; Comerford and Nibbs, 2005; Wolf et al., 2010). Down-regulation of two S100 family genes, *S100 Calcium Binding Protein A8* (*S100A8*) and *S100 Calcium Binding Protein A9* (*S100A9*), involved in movement of neutrophils may also be an indication of decreased chemotaxis of neutrophils (Ryckman et al., 2003) in SS. Also, the function of "migration of dendritic cells" was predicted to be decreased in SS as a result of down-regulation of *CCL19*, *CCL21, CCR7, CXCL13* and

*SPP1* (Table 2.5). All these genes encode cytokines/cytokines receptors which positively regulate chemotaxis of dendritic cells (Sozzani et al., 1998; Saeki et al., 2000; Jang et al., 2006; Shao et al., 2014), which play an important role in mucosal immune functions, such as antigen presenting and activation of B- and T-cells. The decreased function of migration of dendritic cells suggests reduced innate immune protection through fewer activated effector B- and T-cells distributed in the epithelium and lamina propria at the RAJ of SS. In addition, down-regulation of *CD69, S100A9, SH2D1A* and *SPP1* (Table 2.4) in SS is suggested to be associated with the decreased function in "activation of natural killer cells (NK cells)" (Table 2.5). The *CD69* gene encodes a cell membrane protein of activated NK cells (Borrego et al., 1993) and the *S100A9*, *SH2D1A* and *SPP1* gene products have been reported to positively regulate activation of NK cells (Zhang et al., 2010; Dong et al., 2012; Arnold et al., 2013), and therefore, their down-regulation suggest a decreased NK cell activation at the RAJ of SS.

### 2.3.6 Pathway enrichment analysis of differentially expressed genes

To further define the potential functional outcomes of the 58 DE genes, KEGG_PATHWAY analysis was performed. Four pathways were enriched by DAVID Bioinformatics including the hematopoietic cell lineage (KEGG PATHWAY entry: bta04640), cytokine-cytokine receptor interaction (KEGG PATHWAY entry: bta04060), the chemokine signaling pathway (KEGG PATHWAY entry: bta04062), and the B-cell receptor signaling pathway (KEGG PATHWAY entry: bta04662). Four down-regulated DE genes including *IL2RA*, *CD19*, *CD22* and *MS4A1*were found to be associated with the hematopoietic cell lineage pathway in SS. The down-regulation of *IL2RA* suggests the decrease in T-cell differentiation, and the down-regulation of *CD19*, *CD22*, *IL2RA* and *MS4A1* suggests the decrease in B-cell differentiation. Six down-regulated genes involved in cytokine-cytokine receptor interaction and

chemokine signaling pathways were also identified, including *CCL19*, *CCL21*, *CCR7*, *CXCL13*, *IL2RA* and *LTB*. The down-regulation of these genes may be associated with a reduction in the chemotaxis of leukocytes in SS. Four down-regulated genes, *CD19*, *CD22*, *CD79a* and *CD79b* involved in the B-cell receptor signaling pathway also indicate a potential reduction in antibody production at the RAJ of SS. The pathway analysis of DE genes further suggested the potentially reduced innate and adaptive immune functions at RAJ of SS as identified above in the functional analysis.

### 2.3.7 Other factors possibly contributing to decrease in host immune functions in SS

Environmental and bacterial factors could also contribute to the decrease in the immune function of SS. Since the cattle were from the same feedlot, it would be reasonable to assume that the environmental factors were similar for each of the sampled individuals. Colonization of the RAJ itself by *E. coli* O157:H7 could impair host immune responses as several EHEC secretive proteins, such as NleB (non-LEE-encoded type III effector B), NleC, NleE and NleH have been shown to impair host innate immunity by disrupting NF-κB pathways (Walle et al., 2013). Furthermore, Shiga-toxins produced by EHEC could also suppress cellular immune responses, as shown by the down-regulation of membrane surface proteins on macrophages (Menge et al., 2015) and a reduction in the activation and proliferation of B- and T-cells (Menge et al., 2003). Therefore, *E. coli* O157:H7 mediating mechanisms may also contribute to the observation that the rectal tissue of SS exhibited less effective innate and adaptive immune protection.

Based on above detected changes in rectal transcriptomes, it is difficult to conclude if the potentially reduced immune functions in SS are an inherent property of these animals, or those immune functions were repressed by *E. coli* O157 mediated activities. It is worthy to mention

that although the current study is the first to clearly present a connection between host gene expression and the super-shedding phenomenon, there are limitations in terms of discrepancy of sampling time between tissue collection and feces *E. coli* O157 enumeration. To define whether such connection can be biologically meaningful and to gain fundamental understanding of super-shedding, a complicated biological phenomenon, as well as to investigate immunomodulation of *E. coli* O157, further research including (1) synchronized tissue and fecal sampling from live SS and (2) longitudinal monitoring of both host gene expression and *E. coli* O157 shedding levels is required.

**2.4 Conclusion**

Transcriptomic analysis of rectal tissues from NS and SS suggested a potentially decreased function in the number and movement of phagocytes and lymphocytes in the rectum of SS. Because the ILFs, epithelium and lamina propria contain the majority of mucosal immune cells at the RAJ, our findings reflect potential impairment of these rectal lymphoid structures in SS. Based on the transcriptomic analysis, the rectal mucous membrane ILF of SS may contain less lymphocytes, macrophage and dendritic cells than NS. The decreased expression of immune related genes also indicates a failure to mount adequate immune protection in the RAJ of SS which can result in enhanced colonization by *E. coli* O157:H7, and/or that colonization by the bacterium itself suppresses immune function. Our study showed contradictory results to previous studies on *E. coli* O157:H7 experimentally challenged cattle which showed innate and adaptive immune responses could be promoted at the RAJ, including increased neutrophil infiltration and IgA production (Nart et al., 2008c) and increased release of cytokine and proliferation of T-cells and NK cells (Corbishley et al., 2014). Unlike the previous studies, we focused on cattle naturally-colonized with *E. coli* O157:H7, which may have different response mechanisms

compared to the experimentally challenged animals. In addition, sampling from a commercial feedlot limited our sampling approaches, rendering a necessity of further verification for current findings. However, current findings not only justify further research, but also raised an important question: is the repressed immune protection a result of *E. coli* O157 activity or an inherent property of SS? Improved sampling technique that allow synchronized rectal tissue and feces sampling from SS would potentially answer this question. To advance our understanding of the mechanisms of *E. coli* O157:H7 colonization at the RAJ of cattle, and the mechanisms resulting in the super-shedding phenotype as well as to verify our findings, immunological and proteomic studies are also required. The transcriptomes of other regions of the bovine gut should also be investigated to define if there are any specific genes expressed at RAJ that make it the preferred site for colonization by *E. coli* O157:H7 in the gastrointestinal tract. From our results, the transcriptome of non-persistent super-shedders appears to differ from those of non-shedders, largely due to factors linked to immune function. This is an important preliminary step in helping to identify pre-disposing factors for super-shedding, although additional information is required to define the actual mammalian and bacterial cellular response that lead to super-shedding.

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## 2.6 Tables and figures

**Table 2.1 Primer sequences, amplicon sizes and annealing temperatures for qPCR assays**

| Genes | Oligo sequence (5' to 3') | Amplicon size, bp | Access. No.** | Annealing temp, °C |
|---|---|---|---|---|
| ***CCL21*** | F: GCTATCCTGTTCTCGCCTCG<br>R: ACTGGGCTATGGCCCTTTTG | 222 | NM_001038076.2 | 60 |
| ***CCR7*** | F: ACCCTCGCTAGCTACCTCAA<br>R: CGGTCTCTTGTCTTGGGGAC | 293 | NM_001024930.3 | 64 |
| ***CD22*** | F: ACCTCAGTTTCCAGCCCAAG<br>R: CCTCATGGTCACAGACTCGC | 188 | XM_003587236.2 | 64 |
| ***CXCL13*** | F: AACCCTCAAGCCAAATGGACA<br>R: AACCCGGAGCAGGAATGTTG | 154 | NM_001015576.2 | 60 |
| ***LTB*** | F: TGGGAAGAGGAGGTCAGTCC<br>R: TAGCTTGCCATAAGTCGGGC | 215 | XM_002697371.2 | 62 |
| ***MS4A1*** | F: GCGGAGAAGAACTCCACACA<br>R: GGGTTAGCTCGCTCACAGTT | 206 | NM_001077854.2 | 64 |
| ***IL2RA*** | F: GCACGGTCAGGCTTCAGAT<br>R: TTCTTGACTTCTTCTGGCCTTG | 288 | NM_174358.2 | 64 |
| ***CD19*** | F: CTCCCATACCTCCCTGGTCA<br>R: GCCCATGACCCACATCTCTC | 127 | NM_001245998.1 | 64 |
| ***POU2AF1*** | GAGACCATGGTGACTGGTGG<br>AATACGGCCATTGTGGGGAG | 246 | NM_001075915.1 | 62 |
| ***SH2D1A*** | CAGCACCGGGGGTACATAAA<br>TCCTGTAGCACCTTGTGTACTT | 146 | NM_001034733.2 | 62 |
| ***β-actin**** | F: CTAGGCACCAGGGCGTAATG<br>R: CCACACGGAGCTCGTTGTAG | 177 | AF191490.1 | 60 |

* Primer sequence from (Malmuthuge et al., 2012b)

**NCBI accession number

**Table 2.2 *Escherichia coli* O157:H7 numbers ($log_{10}$ CFU/g feces)***

| Steer ID | *E. coli* O157:H7 numbers at the day of sampling, log CFU/g feces | Length of time between the first sampling and the day of slaughter, days | *E. coli* O157:H7 numbers day of/prior to slaughter, log CFU/g feces |
|---|---|---|---|
| **274_SS** | 6.7 | 8 | +** (a day prior to slaughter) |
| **287_SS** | 5.4 | 8 | + (a day prior to slaughter) |
| **294_SS** | 5.8 | 10 | + (a day prior to slaughter) |
| **299_SS** | 7.8 | 10 | + (a day prior to slaughter) |
| **310_SS** | 7.5 | 4 | 5.8 (day of slaughter) |

**E. coli* O157:H7 number in fecal samples of steers identified as super-shedders at the day of sampling and day of/prior to slaughter.

***+, positive for *E. coli* O157:H7 via immunomagnetic separation assay.

**Table 2.3 Sequencing results and percentage of reads mapped to reference genome by Tophat2**

| Library name (Steer ID) | No. of Reads generated | Average Quality score | No. of reads mapped to reference | % of mapped reads |
|---|---|---|---|---|
| **108_NS** | 38.2M | 35 | 30.3M | 79.2 |
| **152_NS** | 25.6M | 35 | 20.1M | 78.5 |
| **165_NS** | 27.6M | 35 | 22.9M | 82.9 |
| **242_NS** | 30.1M | 35 | 23.5M | 78.2 |
| **274_SS** | 27.0M | 35 | 16.5M | 61.1 |
| **287_SS** | 28.3M | 35 | 24.1M | 85.3 |
| **294_SS** | 28.7M | 36 | 21.7M | 75.5 |
| **299_SS** | 27.2M | 36 | 21.0M | 77.2 |
| **310_SS** | 35.0M | 35 | 27.7M | 79.2 |

**Table 2.4 Functions of 31 down-regulated DE genes in super-shedders that are associated with immune functions predicted by IPA**

| Symbol | Entrez Gene Name | Location | Type (s) | log2-fold-change |
|---|---|---|---|---|
| ***CD69*** | *CD69 molecule* | Plasma Membrane | transmembrane receptor | -1.8 |
| ***CD180*** | *CD180 molecule* | Plasma Membrane | other | -1.9 |
| ***TIMD4*** | *T-cell immunoglobulin and mucin domain containing 4* | Plasma Membrane | other | -2.0 |
| ***RHOH*** | *ras homolog family member H* | Plasma Membrane | enzyme | -2.0 |
| ***SIT1*** | *signaling threshold regulating transmembrane adaptor 1* | Plasma Membrane | other | -2.3 |
| ***CD79A*** | *CD79a molecule, immunoglobulin-associated alpha* | Plasma Membrane | transmembrane receptor | -2.4 |
| ***FAIM3*** | *Fas apoptotic inhibitory molecule 3* | Plasma Membrane | other | -2.6 |
| ***CD19*** | *CD19 molecule* | Plasma Membrane | transmembrane receptor | -2.6 |
| ***IL2RA*** | *interleukin 2 receptor, alpha* | Plasma Membrane | transmembrane receptor | -2.7 |
| ***CD22*** | *CD22 molecule* | Plasma Membrane | transmembrane receptor | -2.8 |
| ***MS4A1*** | *membrane-spanning 4-domains, subfamily A, member 1* | Plasma Membrane | other | -2.9 |
| ***CD79B*** | *CD79b molecule, immunoglobulin-associated beta* | Plasma Membrane | transmembrane receptor | -3.3 |
| ***CCR7*** | *chemokine (C-C motif) receptor 7* | Plasma Membrane | G-protein coupled receptor | -3.6 |
| ***KLHL6*** | *kelch-like family member 6* | Other | other | -2.0 |
| ***POU2AF1*** | *POU class 2 associating factor 1* | Nucleus | transcription regulator | -2.0 |

| | | | | |
|---|---|---|---|---|
| ***TCF7*** | *transcription factor 7 (T-cell specific, HMG-box)* | Nucleus | transcription regulator | -2.0 |
| ***LEF1*** | *lymphoid enhancer-binding factor 1* | Nucleus | transcription regulator | -3.1 |
| ***CCL19*** | *chemokine (C-C motif) ligand 19* | Extracellular Space | cytokine | -2.2 |
| ***SPP1*** | *secreted phosphoprotein 1* | Extracellular Space | cytokine | -2.9 |
| ***LTB*** | *lymphotoxin beta (TNF superfamily, member 3)* | Extracellular Space | cytokine | -2.9 |
| ***CCL21*** | *chemokine (C-C motif) ligand 21* | Extracellular Space | cytokine | -3.4 |
| ***CXCL13*** | *chemokine (C-X-C motif) ligand 13* | Extracellular Space | cytokine | -4.3 |
| ***SASH3*** | *SAM and SH3 domain containing 3* | Cytoplasm | other | -1.7 |
| ***GIMAP5*** | *GTPase, IMAP family member 5* | Cytoplasm | other | -2.0 |
| ***FAM65B*** | *family with sequence similarity 65, member B* | Cytoplasm | other | -2.1 |
| ***THEMIS*** | *thymocyte selection associated* | Cytoplasm | other | -2.2 |
| ***SH2D1A*** | *SH2 domain containing 1A* | Cytoplasm | other | -2.2 |
| ***PLA2G2A*** | *phospholipase A2, group IIA (platelets, synovial fluid)* | Cytoplasm | enzyme | -3.6 |
| ***S100A12*** | *S100 calcium binding protein A12* | Cytoplasm | other | -3.8 |
| ***S100A9*** | *S100 calcium binding protein A9* | Cytoplasm | other | -5.0 |
| ***S100A8*** | *S100 calcium binding protein A8* | Cytoplasm | other | -5.5 |

* log2-fold-change is log ratio of gene expression level in super-shedders to non-shedders.

**Table 2.5 Decreased immune functions in super-shedders identified by the DE genes using IPA**

| Associated immune cells | Functions Annotation | z-score | Associated molecules |
|---|---|---|---|
| **T-cells** | cell movement of T lymphocytes | -2.4 | *CCL19, CCL21, CCR7, CD69, CXCL13, IL2RA, LTB, SPP1* |
| | T cell homeostasis | -2.4 | *CD79A, IL2RA, LEF1, LTB, RHOH, SH2D1A, SPP1, TCF7, THEMIS* |
| | T cell development | -2.4 | *CD79A, IL2RA, LEF1, LTB, RHOH, SH2D1A, SPP1, TCF7, THEMIS* |
| | quantity of T lymphocytes | -2.3 | *CCL19, CCL21, CCR7, CD79A, IL2RA, LTB, POU2AF1, RHOH, SASH3, SH2D1A, SIT1, THEMIS* |
| | quantity of CD4+ T-lymphocytes | -2.2 | *CD69, RHOH, SH2D1A, SIT1, THEMIS* |
| | quantity of regulatory T lymphocytes | -2.2 | *CCL19, CCL21, IL2RA, LTB, THEMIS* |
| **T-, B-cell** | quantity of lymphocytes | -2.6 | *CCL19, CCL21, CCR7, CD19, CD22, CD79A, CD79B, CXCL13, IL2RA, KLHL6, LTB, MS4A1, POU2AF1, RHOH, SASH3, SH2D1A, SIT1, THEMIS* |
| | development of lymphocytes | -2.5 | *CD19, CD79A, GIMAP5, IL2RA, LEF1, LTB, RHOH, SH2D1A, SPP1, TCF7, THEMIS* |
| | binding of lymphocytes | -2.2 | *CCL19, CCL21, CD22, CXCL13, SH2D1A* |
| | infiltration by lymphocytes | -2.2 | *CCL19, CCL21, CCR7, IL2RA, LTB* |
| | activation of lymphocytes | -2.0 | *CD19, CD69, PLA2G2A, POU2AF1, S100A9, SASH3, SH2D1A, SPP1* |
| | maturation of lymphocytes | -2.0 | *CD19, LEF1, RHOH, TCF7* |
| | cell viability of lymphocytes | -2.4 | *CD19, CD22, CD79A, GIMAP5, LEF1, TCF7* |

| | | | |
|---|---|---|---|
| | Lymphocyte migration | -2.1 | *CCL19, CCL21, CCR7, CD69, CXCL13, IL2RA, LTB, SH2D1A, SPP1* |
| **Phagocyte, macrophage, dendritic, neutrophils** | cell movement of phagocytes | -2.7 | *CCL19, CCL21, CCR7, CXCL13, LTB, S100A8, S100A9, SPP1* |
| **Neutrophils** | chemotaxis of neutrophils | -2.6 | *CCL19, CCL21, CCR7, CD69, S100A8, S100A9, SPP1* |
| **Dendritic cells** | migration of dendritic cells | -2.2 | *CCL19, CCL21, CCR7, CXCL13, SPP1* |
| **NK cells*** | activation of natural killer cells | -2.0 | *CD69, S100A9, SH2D1A, SPP1* |

***** NK cells: natural killer cells

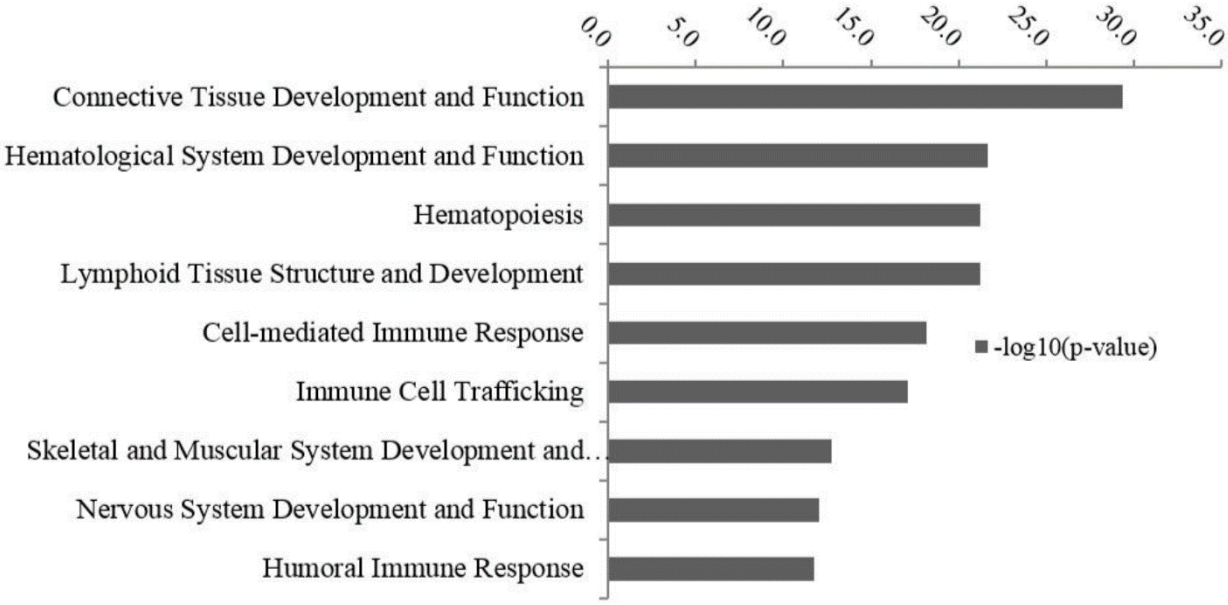


**Figure 2.1 Top enriched functional terms (physiological process) by IPA for the core transcriptome of rectal anal junction (RAJ).** The y-axis is on $-\log_{10}$ (P-value) scale: the greater the $-\log_{10}$ (P-value), the more likely that function is associated with the core transcriptome of the RAJ.

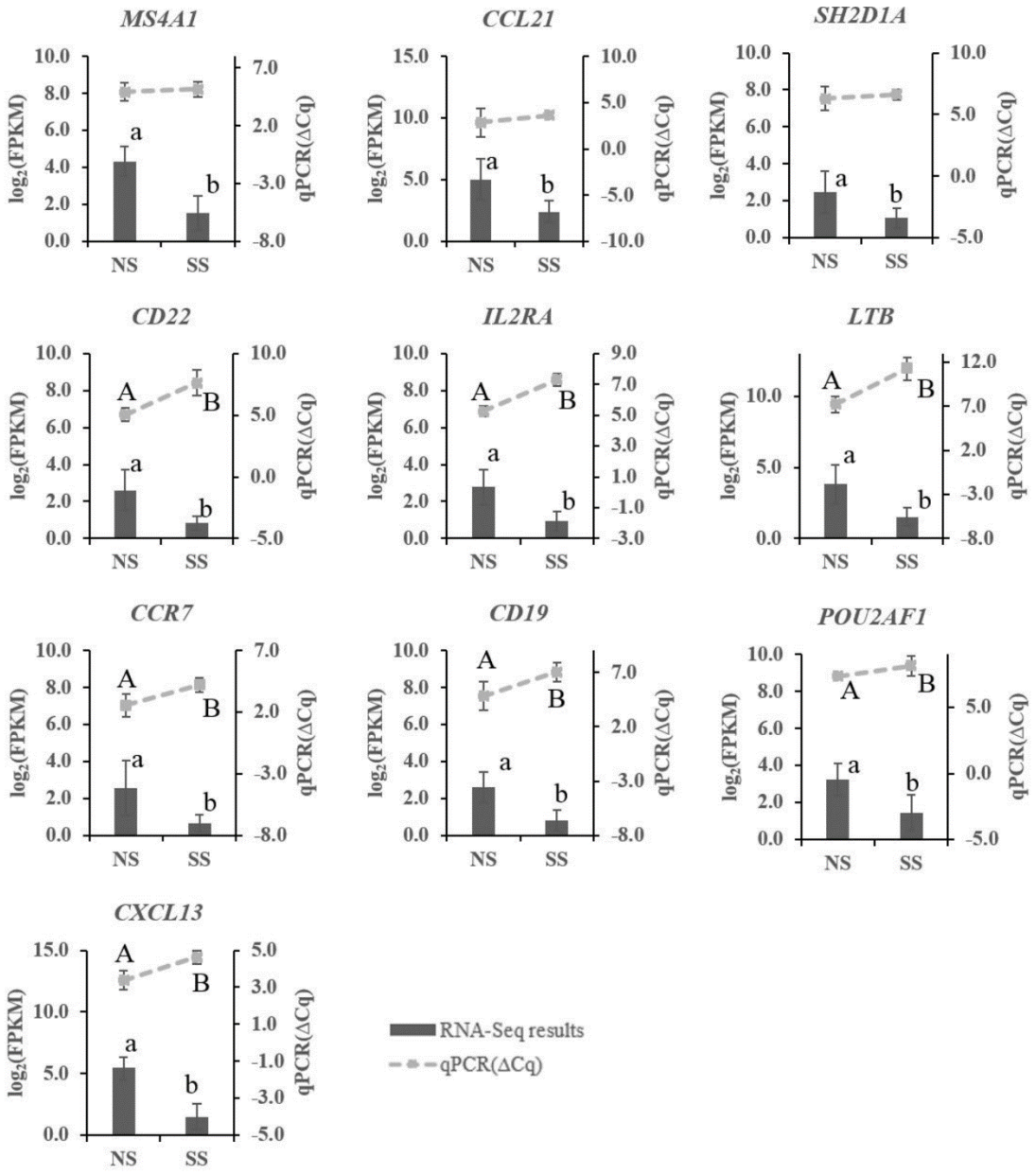


**Figure 2.2 Expression of selected differentially expressed (DE) genes as detected by qRT-PCR and RNA-Sequencing.** Differentially expressed genes as measured by qPCR are shown by lines on the top and values are indicated by the right Y-axis as relative expression level (ΔCq). Lower ΔCt values represent higher gene expression levels and *vice versa*. Gene expression as measured by RNA-Seq are shown by bar graphs on the bottom and values are indicated by the left Y-axis as log2 (FPKM). A, B indicate a significant difference in the relative expression detected by qPCR (P-value < 0.05); a, b indicate a significant difference in expression of genes detected by RNA-Seq (FDR < 0.05). Data are presented as mean ± standard deviation. NS and SS represent non-shedders and super-shedders, respectively.

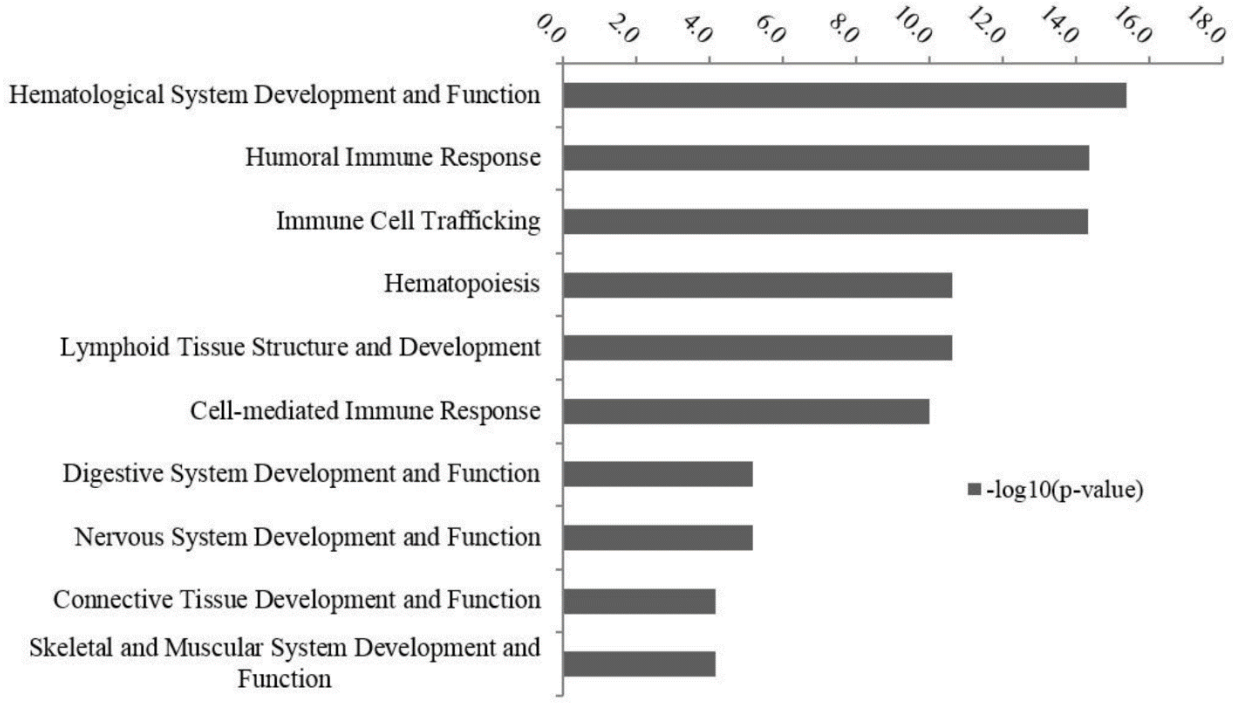


**Figure 2.3 Top enriched functional categories for the differentially-expressed genes by IPA.** The y-axis is on $-\log_{10}$ (P-value) scale: the greater the $-\log_{10}$ (P-value), the more likely that function is associated with the differentially expressed genes.

# Chapter 3. Host mechanisms involved in cattle *Escherichia coli* O157 shedding: a fundamental understanding for reducing foodborne pathogen in food animal production[2]

## 3.1 Introduction

The sustainable agriculture production concept of "from farm gate to food plate" makes food safety an important component through the production chain. *Escherichia coli* O157:H7 (*E. coli* O157) is a zoonotic pathogen which produces Shiga toxins that can cause severe disease in humans including bloody diarrhea, hemolytic uremic syndrome and in some circumstances even death (Scallan et al., 2011). From 2000-2008, It was estimated that this pathogen is linked to more than sixty thousands cases of illness in humans and caused more than one hundred million dollars losses in United States annually (Scallan et al., 2011). Cattle are the primary reservoir for *E. coli* O157, and individuals that shed greater than $10^4$ CFU/g feces are defined as super-shedders (SS) (Chase-Topping et al., 2008). *E. coli* O157 originated from cattle could spread into the farms and processing environments where it contaminates vegetables and beef products through the food production chain (Ferens and Hovde, 2011). *E. coli* O157 can be shed by cattle for days, weeks or even months (Baines et al., 2008), with shedding patterns differing substantially among individuals (Chase-Topping et al., 2008).

Environmental, host, and microbial factors have been proposed to affect super-shedding (Munns et al., 2015). Among these factors, the environmental factors including diet and seasonality have been reported to be associated with super-shedding (LeJeune and Wetzel, 2007;

[2] Chapter 3 is a part of a manuscript submitted to Scientific Reports, and currently under revision.

Ferens and Hovde, 2011). With regard to the microbial factor, *in vitro* studies have revealed that several *E. coli* O157 non-LEE (locus for enterocyte effacement)-encoded type III effector proteins may impair host innate immunity and that Shiga toxins can repress lymphocytes responses (Menge et al., 2003; Walle et al., 2013), which could lead to potential colonization of this pathogen *in vivo*. It has been reported that a high dose challenge of *E. coli* O157 to yearling steers was able to cause damage to epithelium of both the small and large intestine (Baines et al., 2008). Also, *E. coli* O157 colonization of the intestinal epithelium of challenged cattle could activate both innate and adaptive immune responses initiated by bovine macrophage and enterocytes through the recognition of the flagellum and lipopolysaccharide (LPS) of *E. coli* O157 (Walle et al., 2013). Recent study has also revealed distinct faecal microbiota between SS and cattle that are negative for *E. coli* O157 (non-shedders, NS) (Xu et al., 2014). However, the host mechanisms involved in this foodborne pathogen shedding are largely unknown. Studies of the transcriptome of recto-anal junction (RAJ) of SS, the primary site of *E. coli* O157 colonization (Cobbold et al., 2007), revealed lower expression of genes involved in humoral and cell-mediated immune responses (Wang et al., 2016). Based on the findings to date, super-shedding is a complicated process which may involve multi-biological processes and the mode of action regulating of this process is unclear. Moreover, most attempts on understanding super-shedding were based on *E. coli* O157 challenged cattle (Walle et al., 2013), and it is unknown whether the mechanisms of *E. coli* O157 shedding are the same between challenged and naturally-occurring SS.

It has been widely reported that less than 10% of the animal in a herd are SS (Omisakin et al., 2003; Matthews et al., 2006b; Matthews et al., 2006a; Arthur et al., 2009). However, it is difficult to explain such phenomena by only considering microbial (pathogen) and environmental

factors, as all the animals that are fed with the same diet and raised in the same environment should have equal possibility to ingest *E. coli* O157. In addition, the colonization tropism by *E. coli* O157 towards the RAJ (Naylor et al., 2003; Rice et al., 2003; Greenquist et al., 2005; Naylor et al., 2005; Davis et al., 2006; Lim et al., 2007) of cattle is difficult to be well explained solely by microbial effects. Therefore, we hypothesize that host gene expression throughout the whole gastrointestinal tract, as one of host related mechanisms, is different between SS and NS, and that the altered gene expression in SS, can be partially influenced by genetic variations. We also hypothesis that the difference in physiological functions determined by the transcriptome between small and the large intestine may contribute to the tropism of *E. coli* O157 colonization in cattle. Therefore, we compared the transcriptome profiles of tissues through the whole gastrointestinal tract including duodenum, proximal jejunum, distal jejunum, cecum, spiral colon and descending colon between SS and NS using RNA-Seq and performed SNP discovery on the identified differentially expressed genes.

### 3.2 Materials and Methods

The study was approved by the Animal Care Committee of the Lethbridge Research and Development Centre, Agriculture Agri-Food Canada (Animal Care Committee protocol number: 1120), and the steers were managed according to the Canadian Council of Animal Care Guidelines.

#### 3.2.1 Super-shedder identification and intestinal tissues collection

Super-shedder identification and the tissue sampling procedure was outlined in previous studies[11,22]. Briefly, 50 g of faecal samples were collected from 400 yearling steers with similar body weights (452 kg ± 23 kg, mean ± standard deviations) that were on a finishing diet and

were used for *E. coli* O157 enumeration using CT-SMAC agar (Dalynn Biologicals, Calgary, AB. Canada). Confirmation of O157 serogroup was performed using an *E. coli* O157 Latex Test kit (Oxoid Ltd, Basingstoke, Hampshire, UK). Isolates were further confirmed to be *E. coli* O157:H7 using a multiplex PCR assay targeting *VT*, *eaeA*, *fliC*[23]. Steers identified with ≥ $10^4$ CFU of *E. coli* O157/g feces were defined as SS. Of the 400 steers, 5 SS (out of 11 identified SS) and 5 NS pen-mates were transferred to the Lethbridge Research Centre research feedlot. Steers were slaughtered within 4-11 days after purchase, and all the SS were positive for *E. coli* O157 prior to tissue sampling although not all were shedding at > $10^4$ CFU/g feces (Wang et al., 2016). Tissues of duodenum, proximal jejunum, distal jejunum, cecum, spiral colon and descending colon were collected immediately after slaughter and snap frozen in liquid nitrogen.

### 3.2.2 RNA-extraction, RNA-Seq library preparation and RNA sequencing

RNA extraction and library preparation procedures have been previously outlined by Wang et al (2016). Briefly, total RNA was immediately extracted from about 100 mg of ground powdered tissue using a mirVana total RNA Isolation Kit (Ambion, Carlsbad, CA, USA). One micro gram of RNA was used for library preparation using a Truseq Stranded Total RNA Sample Preparation kit (Illumina, San Diego, CA, USA) following the manufacturer's instructions. The cDNAs were confirmed to meet quality control standards and sequenced (paired-end, 2 ×100 bp) using a HiSeq 2000 sequencing system (Illumina, San Diego, CA, USA) at Genome Quebec Innovation Centre, Montreal, Quebec, Canada.

### 3.2.3 Identification of transcriptomes and differentially expressed genes

The RNA-Seq data analysis was performed using the pipeline previously reported (Wang et al., 2016). Briefly, adapter sequence removal and quality filtering of sequencing reads were performed by fastq-mcf (Aronesty, 2013), and the remaining reads were mapped against the

reference bovine genome UMD3.1 assembly (Zimin et al., 2009) using the splice junction mapper, Tophat2 (Kim et al., 2013). HtSeq-count (Anders et al., 2015) was used to quantify the mapped reads to each known bovine gene. The number of mapped reads was then normalized into counts per million (cpm) to eliminate variation introduced by sequencing depth, using the formula: cpm = (Number of reads mapped to a gene) ÷ (total number of reads mapped to all annotated genes) × $10^6$. Any gene with a cpm higher than 1 was considered as being expressed (Wilkinson et al., 2016), and the genes expressed in more than 2 of animals in each group were further subjected to differential expression and functional analysis. Bioconductor package edgeR was used for differentially expressed (DE) gene identification between SS and NS in each intestinal tissue (Robinson et al., 2010), using negative binomially distributed RNA-Seq counts. A false discovery rate (FDR) of 0.05 was used as the cut-off to define DE genes. Log2 fold change of each DE gene was calculated using the equation: log2 fold change = log2(average cpm of SS / average cpm of NS). Log2 fold change threshold for DE genes were set to ≤ -1 and ≥ 1 with negative values indicating down-regulated genes in SS and positive values indicating up-regulated in SS.

### 3.2.4 Functional analysis of the transcriptome and differentially expressed genes

Functional analysis of RNA-Seq data was performed using several bioinformatics tools. The PANTHER (Protein Analysis Through Evolutionary Relationships) classification system (Mi et al., 2016) was used for gene ontology (GO) terms annotation and enrichment, with p-value 0.05 used as cut-off. Ingenuity Pathway Analysis® (IPA, QIAGEN, Redwood City, CA, United States www.qiagen.com/ingenuity) was used for functional term enrichment and canonical pathways analysis for core transcriptomes and DE genes for each tissue, and a downstream effects analysis was performed to identify the biological influence of the DE genes.

As the maximal number of genes that IPA can analyze is 8,000, the 8,000 genes in the core transcriptome with the highest cpm values were used for functional analysis for core transcriptome of each tissue. Based on the fold change of DE genes, the IPA also predicted if a certain enriched biological function was increased or decreased, and a z-score was used to indicate whether a predicted function was increased (z-score > 2.0) or decreased (z-score < 2.0). A similar z-score algorithm was used by IPA to predict if a canonical pathway was inhibited (z-score < -2.0) or activated (z-score > 2.0).

### 3.2.5 RNA-Seq based SNP discovery

To identify potential causal effects of DE genes, single nucleotide polymorphism (SNP) analysis was performed using RNA-Seq reads for these DE genes. To increase read coverage for SNP analysis, RNA-Seq reads of all tissues were combined for each animal and SNP calling was performed by VarScan2 (Koboldt et al., 2012), which is a mutation caller that uses a heuristic algorithm for sequence variant detection. For SNP calling, the minimum base quality of reads was 15, minimum read depth at a position to call a SNP was 8, minimal read number that supports an allele was 2, and the minimum variant allele frequency threshold was 0.1. The SNPs were kept for association analysis by the Fisher's exact test if the genotypes for all 10 animals were detected by VarScan2. Shedding of *E. coli* O157 was considered as a phenotype of SS, and the Fisher's exact test was used to analyze for associations between alleles and super-shedding with significance at 0.05.

### 3.2.6 Cholesterol quantitation

To quantify the cholesterol concentration in distal jejunum and descending colon, a Cholesterol Quantitation Kit (Catalog Number: MAK043, Sigma-Aldrich, St. Luis, MO, USA) was used for lipid extraction and quantitation by fluorometric detection following manufacturer's

instruction. Prior to cholesterol extraction, adipose tissues were removed manually using a scalpel, as the study of lipid storage was not the objective of this study, and the rest of the tissues were ground and mixed. Approximately 100 mg of tissue was used for cholesterol extraction and measurement according to the kit instructions, and the fluorescence intensity was detected at: $\lambda_{ex}$ = 535 and $\lambda_{em}$ = 587 nm. Two tailed sample t.test (significance level: 0.05) was performed to determine whether cholesterol content of the distal jejunum and descending colon differed between SS and NS.

### 3.2.7 Availability of sequencing data

The RNA-Seq data are available at NCBI Gene Expression Omnibus (GEO) database under accession number GSE85277.

## 3.3 Results

### 3.3.1 Transcriptome profiling of bovine intestinal tissues

The number of paired-end sequence reads for each tissue sample ranged from 185.0 million in the descending colon to 203.7 million in the cecum, with the number of genes detected in each tissue ranging from 16,846 ± 639 (mean ± standard deviation) in the cecum to 18,137 ± 696 (mean ± standard deviation) in the distal jejunum. For each tissue, transcripts with counts per million higher than 1 (cpm ≥ 1) in all 10 animals were defined as the core transcriptome for that tissue. The core transcriptomes consisted of 12,813 expressed genes in the duodenum, 12,552 expressed genes in the proximal jejunum, 12,905 expressed genes in the distal jejunum, 12,627 expressed genes in the cecum, 12,404 expressed genes in the spiral colon, and 12,216 expressed genes in the descending colon. The hierarchical cluster analysis of core transcriptomes revealed no clear separation between SS and NS in most of the gut regions, except for

descending colon where SS (with the exception of SS 310) and NS were clustered into separate groups (Figure 3.1). Principal component analysis (PCA) plots based on the whole transcriptome revealed that 4 of NS were clustered closely for duodenum, cecum and spiral colon, 3 of NS were clustered closely for distal jejunum, and NS and SS were separated on the direction of first principal component (except for SS 310) for descending colon (Figure 3.2). For SS animals, they were not closely grouped, indicating a large variation among SS. Certain SS were more closely related to NS in several tissues. For example, the duodenum transcriptome of SS 274 was similar to that of NS, while the spiral colon transcriptome of SS 287 and descending colon transcriptome of SS 310 were similar to those of NS.

### 3.3.2 Functional analysis of core transcriptomes of bovine gastrointestinal tissues

Functional analysis of the core transcriptome using biological process GO terms (Gene Ontology) enrichment showed consistent enriched GO terms for all intestinal tissues, including metabolic processes, cellular processes, biological regulation, localization and development processes (based on p-values). Further functional analysis using IPA revealed 16 enriched biological process categories (Figure 3.3A). Although similar functional categories were identified for all intestinal tissues, the likelihood of association (indicated by Fisher's exact test p-value calculated by IPA) between functional categories and the transcriptome of a tissue varied, rendering the tissues of small and large intestines clustered separately (Fig.3A). Immune functions such as cell mediated immune responses, hematological system development and function, humoral immune responses and lymphoid tissue structure and development were more associated with the core transcriptome of small intestinal tissues than with large intestinal tissues, particularly the distal jejunum (Figure 3.3A). Functional analysis by the Panther Classification System also showed that more transcripts (t.test, p-value $< 0.05$) in the core transcriptome of

small intestinal tissues were associated with immune system processes: 562 transcripts in the duodenum, 574 transcripts in the proximal jejunum, 579 transcripts in the distal jejunum, 545 transcripts in the cecum, 497 genes in the spiral colon, and 514 transcripts in the descending colon.

### 3.3.3 Identification of tissue dependent differentially expressed genes between super-shedders and non-shedders through comparative transcriptomic analysis

The number of DE genes ranged from 1 (in the proximal jejunum) to 248 in the distal jejunum (Figure 3.4A) between SS and NS, and the Log2 (fold change) of DE genes ranged from 8.9 for *APOB* in the distal colon to -7.9 for *CPS1* in the spiral colon of SS. In total, 101 genes were up-regulated and 250 were down-regulated when the transcriptomes of gut tissues of SS to NS were compared (Figure 3.4A and B).

### 3.3.4 Functional analysis of differentially expressed genes

Functional analysis revealed that the GO terms were enriched for distal jejunum and descending colon DE genes, but not for the duodenum, proximal jejunum, cecum or spiral colon DE genes (p-value > 0.05). Similarly, the IPA functional terms were enriched for DE genes identified from distal jejunum, cecum and descending colon, but not for those from duodenum, proximal jejunum, or the spiral colon.

For 67 up-regulated DE genes in distal jejunum, 33 enriched GO terms were identified within 11 functional classes based on the hierarchical structure of GO term. The most specific GO term in each enriched class includes “regulation of interferon-gamma production”, “positive regulation of inflammatory response” and “immune response-activating cell surface receptor signalling pathway” (Table 3.1). Among these 11 GO terms, 10 of them were associated with immune functions (Table 3.1), which involved 22 DE genes including five genes encoding

cytokines (*CCL2, CXCL10, CXCL9, IL1A* and *TNFSF10*) and three genes encoding cytokine receptors (*IL18R1*, *IL1RL1* and *CCR9*) (Table 3.2). According to IPA functional analysis of DE genes of distal jejunum, five up-regulated genes (*F3*, *CCL2, CCR9, GPR132, S1PR2*) were associated with increased leukocyte migration (z-score ≥ 2.0) (Figure 3.3B and Table 3.3), while five down-regulated DE genes (*BCL6, BLNK, CD79A, CD79B, EBF1*) were involved in B-cell signalling pathway (z-score ≤ -2.0). For DE genes in descending colon, IPA analysis showed four up-regulated genes (*APOA1, CD36, GP2* and *GPAM*) were associated with increased proliferation of T-cell (z-score ≥ 2.0) in SS (Figure 3.3B and Table 3.3).

In addition to immune functions, IPA pathway analysis of DE genes in distal jejunum showed inhibited LXR/RXR activation pathway (z-score ≤ -2.0) which is associated with increased cholesterol absorption in SS. Six genes were associated with this pathway, and two genes (*APOE, ARG2)* were down-regulated, while four genes (*CCL2*, *IL1A, IL1RL1, PTGS2*) were up-regulated (Table 3.3). Similarly, for the DE genes in the descending colon, five enriched GO terms were also associated with cholesterol transportation, including "cholesterol import", "sterol import" and "cholesterol transport" (Table 3.1). Four DE genes (*APOB*, *APOA1*, *CD36* and *AGPAT9*) associated with these GO terms were up-regulated (Table 3.1).

### 3.3.5 SNP identification of DE genes

In total, 33 DE genes associated with immune functions and cholesterol transportation were selected for SNP identification analysis. In total, 1,575 SNPs were identified based on RNA-Seq dataset of these DE genes, and 1,477 of them have been previously reported in the dbSNP database (Sherry et al., 2001). Among those SNPs, 67 were three prime UTR variants, 9 were five prime UTR variants, 1,283 were intronic variants, 41 were missense variants, 9 were splice region variants (3 missenses variants), and 71 were synonymous variants. The association

analysis (Fisher's exact test) showed that 33 SNPs (Table 3.4) in seven genes (p-value < 0.05) including *BATF2*, *THEMIS*, *ITK*, *BLNK*, *IL18R1, EBF1,* and *APOA1* were associated with super-shedding (Figure 3.5). As indicated by GO term enrichment and IPA pathway analysis, *BATF2*, *THEMIS*, *ITK*, *BLNK*, *IL18R1* and *EBF1* were related to leukocyte activation and *APOA1* was associated with cholesterol transportation. Of these 33 SNPs, rs42084078 in *BLNK* and rs109755291 in *IL18R1* were missense variants and rs384985356 in *APOA1* was a synonymous variant, while the others were intronic variants. Furthermore, the rs42084078 (A/G) causes an amino acid difference (A/V) at position 101 of BLNK (Accession number: NP_001039519.1), and rs109755291 (C/T) also causes an A/V difference at position 531 of IL18R1 (Accession number: XP_005212515.1).

### 3.3.6 Cholesterol quantitation in distal jejunum and descending colon

To further verify the identified potential cholesterol metabolism difference in distal jejunum and in descending colon between SS and NS, the cholesterol concentrations in these two tissues were evaluated. The cholesterol content ranged from 0.57 to 1.15 mg/g in the distal jejunum, and 0.26 to 0.68 mg/g in the descending colon. The cholesterol content was higher in small intestinal tissue than in large intestinal tissue (t-test, p-value < 0.05); however, there was no statistical difference between SS and NS in both tissues (p-values were 0.89 and 0.48 for descending colon and distal jejunum, respectively) (Figure 3.6).

## 3.4 Discussion

To our knowledge, this study is the first to investigate gene expression (transcriptome) throughout the whole gastrointestinal tract of beef cattle with different *E. coli* O157 shedding phenotypes. Although the rectal-anal junction has been suggested to be the primary *E. coli* O157

colonization site, this pathogen has also been isolated from other regions of the digestive tract (Keen et al., 2010), suggesting that the gut environment throughout the gastrointestinal tract could impact on the colonization of this pathogen which can attribute to the shedding capacity of the animals. Therefore, the information obtained from the transcriptome can help us understand gut physiology and biology of cattle intestinal tract at molecular level and how it differs between SS and SS, which may partially attribute to the super-shedding phenomena. To better assess such information, the whole intestinal tissue (epithelium and tissues underneath) for each region was used for transcriptome profiling as the tissues underlying the epithelium (such as the lymphoid structures) may also play a role in super-shedding phenomena (Naylor et al., 2003).

Similar numbers of expressed genes were detected for all gut tissues, which was not surprising due to their physiological nature as digestive tract. When the whole transcriptomes were compared, most of tissues (especially small intestinal tissues) showed no segregation between NS and SS, suggesting that the variation in *E. coli* O157 shedding status was not the key factor to influence the overall host gut transcriptomes. Indeed, these animals were healthy and no significant difference was observed in terms of their growth. The association of immune functions with small intestine regions, especially for distal jejunum, was in agreement with a previous report that jejunum also plays a role in mucosal immunity because Peyer's patches (PP) are present in this region (Mutwiri et al., 1999), and ileal PP extend from the distal jejunum to ileocecal valve (Mutwiri et al., 1999). Higher activity of immune functions in the small intestine than large intestine in beef steers suggests potential lower chance for *E. coli* O157 to colonize in small intestine. Thus, the colonization of *E. coli* O157 in large intestine of cattle, especially the RAJ (Naylor et al., 2003) may be a result from lower host immunity of these regions. Further validation is needed to measure whether lymphoid structures in small intestine can contribute to

the tropism of *E. coli* O157. Separation of transcriptome of NS and SS in PCA analysis (in cecum and descending colon) suggest that difference in gut physiological environment regulated by gene expression profiles may contribute to the difference in *E. coli* O157 shedding. Such findings were also in agreement with previous report that the colonization of *E. coli* O157 was prevalent in lower digestive tracts of challenged yearling cattle, including cecum and colon (Grauke et al., 2002). It is possible that the environment of hindgut of SS is more favorable for *E. coli* O157 to survive, proliferate and colonize. Future studies to quantify the *E. coli* O157 population and measure the physiological parameters as well as immunological features in each region of the gut is necessary to identify alternation in which host functions through the gut can regulate *E. coli* O157 colonization and the high level fecal shedding of *E. coli* O157.

Although there were no significant differences in core transcriptomes among the small intestinal regions (indicated by PCA analysis), DE genes were detected for each gut region between SS and NS. In the small intestinal region, lower number of DE genes were identified in duodenum and proximal jejunum compared with distal jejunum, suggesting that the gut environment in anterior part of small intestine is similar between SS and NS. The highest number of DE genes (248, including 181 down-regulated and 67 up-regulated) was detected in distal jejunum with the up-regulated DE genes *F3*, *GPR132*, *CCR9*, *CXCL9* and *CXCL10* involved in several GO terms including "cytokine-mediated signaling pathway", "positive regulation of intracellular signal transduction", "defense response". The *F3* (also called TF, log2 fold change: 1.3) product was reported to play a regulatory role in recruiting leukocytes in intestine of mice (Anthoni et al., 2007); the *GPR132* (also called *G2A*, log2 fold change: 1.3) product was reported to enhance chemotaxis of T-cells *in vitro* (Radu et al., 2004); the requirement of *CCR9* (log2 fold change: 1.2) for migration of T helper 17 cells (Th17) to the small intestine was

demonstrated using mice (Wang et al., 2010); CXCL9 (log2 fold change: 3.2) and CXCL10 (log2 fold change: 2.5) can induce chemotaxis of T-helper cells (Kagnoff, 2014). Up-regulation of these genes in distal jejunum of SS may suggest increased T-cell migration. In addition, the cytokine *IL1A* and two cell-membrane receptor genes, *IL18R1* and *IL1RL1* were upregulated and were involved in GO terms including "cytokine-mediated signaling pathway" and "leukocyte activation". *IL1A* (log2 fold change: 1.6) product plays a key role in the differentiation of Th17 cells (Chung et al., 2009); IL18R1 (log2 fold change: 1.3) was suggested to promote cell-mediated responses (Sims and Smith, 2010); and IL1RL1 (log2 fold change: 1.4) is involved in activation of T-helper cells (Schmitz et al., 2005; Sims and Smith, 2010). Up-regulation of these cytokine/receptor genes suggests a potential of increased T-cell differentiation and proliferation in distal jejunum of SS (Figure 3.6A).

For descending colon, the functional analysis of the up-regulated DE genes also suggests a trend of increased proliferation of T-cell (z-score ≥ 2.0) in SS. The up-regulation of *CD36* (log2 fold change: 4.1) was reported to increase T-cell proliferation i*n vitro* (Vallejo et al., 2000); *GPAM*, also known as *GPAT-1,* (log2 fold change: 2.4) was reported to increase T-cell proliferation in mice (Collison et al., 2008). These findings are in agreement with a previous report that increased expression of genes involved in the proliferation of T-cells in the rectum of *E. coli* O157 challenged cattle (Corbishley et al., 2014). However, different from our previous transcriptomic analysis of rectal tissues which suggested a potential decrease in both humoral and cell-mediated immune functions in SS (Wang et al., 2016), current transcriptome analysis indicate potentially enhanced T-cell migration and proliferation at the distal jejunum and descending colon of SS. In gut associated lymphoid system, T-cells need to migrate from thymus to the gut epithelium to form intraepithelial T-cells (IETs), which are mature and activated cells

serve as front line to protect the intestinal epithelium (Cheroutre et al., 2011). Compared with NS, the gut transcriptome of SS may have a higher level of T-cell migration in locations anterior to RAJ, while lower T-cell migration and quantity at RAJ (Wang et al., 2016), suggesting a potential dysregulation of migration of T-cells in SS cattle. Such potential dysregulation may result in reduced IETs accumulation at RAJ, leaving it more vulnerable to *E. coli* O157 colonization.

In addition, the functional analysis of down-regulated DE genes in distal jejunum suggested association between genes, *CD79A*, *CD79B*, *BLNK*, *BCL6* and *EBF1* and decreased B-cell signaling. CD79A (log2 fold change: -2.2) and CD79B (log2 fold change: -4.3) are components of B-cell receptor (BCR) that initiates the B-cell signalling pathway (Liu et al., 2010). Upon BCR interaction with antigens, BLNK (log2 fold change: -2.5) can be phosphorylated, leading to downstream signal transduction (Minegishi et al., 1999), such as the mediation of transcription by BCL6 and EBF1, two transcription factors essential to B-cell maturation (Crotty et al., 2010; Nechanitzky et al., 2013). *E. coli* O157 mediated activities, such as Shiga-toxin production, were reported to suppress lymphocyte responses in cattle (Menge et al., 2004), which may partially explain why the transcriptome analysis suggested inhibited B-cell signaling pathway in distal jejunum and the trend of reduction in lymphocyte activation and migration in cecum.

Besides the potential involvement of immune functions, the pathway analysis and GO term enrichment of DE revealed inhibited LXR/RXR pathway and altered cholesterol transportation in distal jejunum and descending colon of SS. It has been reported that activation of LXR/RXR pathway in intestinal tract leads to decreased cholesterol absorption/synthesis and increased excretion of cholesterol in faeces (Zhao and Dahlman-Wright, 2010). Thus, inhibition

of LXR/RXR pathway could lead to increased cholesterol absorption in distal jejunum of SS. In addition, the up-regulated DE genes in descending colon of SS, including *APOB* (log2 fold change: 8.9), *APOA1* (log2 fold change: 6.0), *CD36* (log2 fold change: 4.1) and *AGPAT9* (log2 fold change: 3.2) also suggest the altered cholesterol transportation in SS. The *APOB* encodes a key component of chylomicrons and low density lipoproteins which are responsible for cholesterol transportation between luminal content and intestinal tissues (Iqbal et al., 2003). The APOA1 was reported to regulate cholesterol transportation between cell membrane (Danielsen et al., 2012) and CD36 can facilitate cholesterol absorption in the intestinal tract (Nauli et al., 2006). Moreover, AGPAT9 was reported to be involved in cholesterol metabolism, because the deficiency of AGPAT9 (in mice) led to dysregulation in cholesterol metabolism with higher free cholesterol and cholesteryl esters in plasma and liver (Cao et al., 2014). The observations of differences in the expression of genes involved in cholesterol transportation and immune functions in the gut between SS and NS are not likely due to coincidence, because lipid metabolism plays a key role in immune system (Wolowczuk et al., 2008). The LXR pathway was reported to interact with both the innate and adaptive immune systems, especially with macrophage functions (Castrillo et al., 2003; Bensinger et al., 2008). In addition, Bensinger *et al.* (2008) reported that increased T-cell activation in mice was accompanied by inhibition of LXR pathway and promotion of cholesterol synthesis. A possible mechanism is that cholesterol is required for cell membrane construction and cell proliferation, and thus increased sterol absorption/synthesis may act as a stimulus to lymphocytes proliferation (Bensinger et al., 2008). Similar to the report by Bensinger *et al.* (2008), current findings also indicated potentially increased T-cell migration and proliferation accompanied by a trend of increased lipid absorption in the distal jejunum and descending colon of SS, suggesting that both alternations of host

immune functions and lipid metabolism could influence *E. coli* O157 shedding. However, no difference was observed for cholesterol concentrations in distal jejunum and descending colon in both tissues. One possibility could be that the absorption/synthesis of cholesterol may have increased in the epithelial tissues, but it may have been utilized during host cell proliferation, including T-cells (Bensinger et al., 2008). In addition, cholesterol may be transported to liver via mesenteric lymph upon absorption. Therefore, although the gene expression suggested increased cholesterol absorption, the cholesterol level may have remained unchanged due to the immediate transportation.

It is noticeable that highly upregulated expression of *FOLH1* (log2 change fold: 4.5 in RNA-Seq results; for qPCR measurement, the ΔCq was -1.04 in NS and -1.97 in SS, ΔCq was calculated by $Cq_{FOLH1}$ - $Cq_{\beta\text{-actin}}$, lower ΔCq indicated higher expression) occurred in descending colon of SS, suggesting that the expression level of *FOLH1* was up-regulated in SS. Up-regulation of *FOLH1* was observed in intestine of rat in a folate-deficient situation (Said et al., 2000). Because the environmental and dietary factors are the same in the NS and SS, it is possible that the potential folate-deficiency in descending colon was due to lower density of folic acid synthesizing commensal bacteria, including beneficial microorganisms such as *Bifidobacterium* species (Pompei et al., 2007). To identify how host-commensal interactions can influence *E. coli* O157 shedding requires further research, such as 16S rDNA amplicon sequencing for tissue attached microbes and metagenomics studies.

Further SNPs discovery analysis identified 33 SNPs in seven DE genes associated with immune functions and cholesterol transportation. Among those SNPs, rs42084078 (in *BLNK*) and rs109755291 (in *IL18R1*) are non-synonymous, and allele A of rs42084078 and allele C of rs109755291 were only seen in SS. Thirty of the SNPs showing association with super-shedding

were in the intron of genes *BATF2*, *BLNK*, *IL18R1*, *ITK*, *THEMIS* and *EBF1*. Although SNPs in the intron do not alter protein sequences, they are still important since the introns are known to regulate gene expression, and intronic variation was reported to be associated with phenotypes (Sturm et al., 2008). For example, the DE gene *EBF1* is a transcription factor essential for B-cell development from innate lymphoid cells (Nechanitzky et al., 2013), and intronic SNPs in *EBF1* was reported to be associated with autoimmune disease in human (Nordmark et al., 2011). Therefore, we speculate that the detected genetic variations may influence their expression and lead to difference in B-cell signaling, T-cell responses and cholesterol absorption in the gastrointestinal tract between SS and NS. Future validation study is needed for the identified SNPs, and current results can be used as a reference for selection of candidate gene/SNPs for testing using larger population as well as with more defined shedding status through a long-term monitoring approach.

It is known that shedding levels vary greatly in SS, ranging from $10^4$ to $10^9$ CFU/g of feces (Munns et al., 2015), with three types of SS being observed: non-persistent shedders (shedding < 14 days), moderately persistent shedders (shedding ~30 days), and persistent shedders (shedding last several months) (Baines et al., 2008). As described by Munns (2014), the SS used in this study had different fecal *E. coli* O157 counts before and upon their slaughter. For example, SS 310 was slaughtered on the 5th day of shedding and the fecal *E. coli* O157 in this animal upon slaughter was still greater than $10^4$ CFU/g of feces. Similarly, SS 274/SS 287 and SS 294/SS 299 were slaughtered on the 8th day and 11th day, respectively, and their shedding level was below $10^4$ CFU/g of feces at the slaughter. Different shedding patterns among these five SS may be the reason underlying the variation observed in gene expression through the gut of SS in PCA plots. However, since they were monitored for only 11 days at most, it is difficult

to define the which types of shedder they belonged to. Therefore, future studies using the samples collected from long term monitored SS are needed to determine how the gut transcriptomes differ among different types of SS.

### 3.5 Conclusion

Our results suggest that (1) the bovine small intestine may have more active immune system than the large intestine, making the distal colon towards rectum more prone to colonization with *E. coli* O157; (2) the distal jejunum and descending colon of SS showed potentially higher level of T-cell migration and proliferation, and cholesterol absorption, as well as inhibited B-cell maturation (Figure 3.6A); (3) host genetic variation in the genes involved in immune function and cholesterol absorption may play a role in *E. coli* O157 shedding in SS, but genetic association analysis with larger number of animals is required to validate such speculation. Based on the transcriptome data, we speculate that host mechanisms, including genetic variation, alternation in immune functions and cholesterol metabolism, may be critical to super-shedding (Figure 3.6B). Although this study is stronger at revealing the alternation of gene expression during high level shedding of *E. coli* O157 in cattle, the potential host functions that influence SS shedding were also identified. Further research is needed focusing on the associations among host mechanisms (such as genetics, immunity and cholesterol metabolism, as well as host-microbial interaction) and the *E. coli* O157 immunomodulatory effects, with larger population of cattle and/or using a longitude approach. In addition, long-term monitoring fecal *E. coli* O157 for animals used in future studies to define NS as negative control and the type of SS (temporary, intermediate, and persistent) is critical to investigate more specific host effects that are associated with super-shedding.

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### 3.7 Tables and figures

**Table 3.1 Gene ontology (GO) term enrichment (biological process, p-value < 0.05) for DE genes between non-shedders and super-shedders in distal jejunum and descending colon, no functional terms were enriched for other tissues (p-value ≥ 0.05).**

| GO Terms | Fold enrichment*** | Genes |
|---|---|---|
| **Up-regulated genes in DJ*** | | |
| regulation of interferon-gamma production | 22.6 | *LOC100848575, IL1RL1, PD-L1, KLRK1, IL18R1* |
| positive regulation of inflammatory response | 22.6 | *IL1RL1, CD6, IDO1, CCL2, GBP5* |
| immune response-activating cell surface receptor signaling pathway | 22.5 | *LOC100848575, PTPN22, IKT, THEMIS, ICP2, KLRK1* |
| negative regulation of homotypic cell-cell adhesion | 22.0 | *PTPN22, ADAMTS18, ALOX12, PD-L1, IDO1* |
| positive regulation of cytokine production | 12.2 | *LOC100848575, PTPN22, IL1RL1, IL1A, PD-L1, CD6, KLRK1, IL18R1, IDO1, GBP5* |
| negative regulation of immune system process | 9.5 | *PTPN22, IL1RL1, GPR171, PD-L1, KLRK1, IDO1, CCL2, GPR55* |
| cytokine-mediated signaling pathway | 8.8 | *CXCL10, IL1RL1, IL1A, CXCL9, CCR9, IL18R1, F3, CCL2* |
| leukocyte activation | 8.4 | *LOC100848575, PTPN22, BATF2, LCP2, ITK, CD6, KLRK1, IL18R1, THEMIS* |
| immune response | 8.1 | *BOLA-NC1, CLNK, CXCL10, TNFSF10, IL1A, LCP2, ITK, PD-L1, CD6, CXCL9, CCR9, CD1D, IL18R1, THEMIS, CCL2, LOC407111, GBP5, F1N3I2* |
| positive regulation of intracellular signal transduction | 5.4 | *CXCL10, TNFSF10, PTPN22, IL1A, CXCL9, IL8R1, F3, NRG1, CCL2, PPR55, LOC407111* |
| defense response | 5.0 | *CXCL10, BATF2, IL1A, ITK, CD6, CXCL9, KLRK1, F3, IDO1, CCL2, GBP5, IFI47* |
| **Down-regulated genes in DJ** | | |
| regulation of nuclease activity | 33.8 | *HMGB1, NEIL1, PCNA, LOC618297* |
| base-excision repair | 21.1 | *HMGB1, NEIL1, NEIL3, MUTYH, UNG, LIG1* |

| | | |
|---|---|---|
| nucleosome assembly | 7.5 | *H2B1, HIST1H2BJ, HMGB1, HIST1H2BI, HIST1H1C, DCK, HIST1H1A, HIST1H1E, HIST1H1D* |
| **Up-regulated genes in DC**** | | |
| Cholesterol import | > 100 | *APOA1, CD36* |
| Cholesterol transport | 95.5 | *APOB, APOA1, CD36* |
| Sterol transport | 90.9 | *APOB, APOA1, CD36* |
| Sterol import | > 100 | *APOA1, CD36* |
| Triglyceride metabolic process | 93.2 | *APOB, APOA1, AGPAT9* |

*Distal jejunum

**Descending colon

***Number of genes observed in the uploaded list over the expected number genes involved in that term for *Bos taurus* (which is determined by the Gene Ontology database)

**Table 3.2 Differentially expressed genes in distal jejunum enriched for GO terms that are associated with immune functions.**

| Gene | log2-fold-change | Location | Type(s) |
|---|---|---|---|
| ***CCL2*** | 1.1 | Extracellular Space | cytokine |
| ***CXCL10*** | 2.5 | Extracellular Space | cytokine |
| ***CXCL9*** | 3.2 | Extracellular Space | cytokine |
| ***FASLG*** | 1.4 | Extracellular Space | cytokine |
| ***IL1A*** | 1.6 | Extracellular Space | cytokine |
| ***TNFSF10*** | 1.2 | Extracellular Space | cytokine |
| ***CD274*** | 2.1 | Plasma Membrane | enzyme |
| ***GBP5*** | 2.3 | Plasma Membrane | enzyme |
| ***IDO1*** | 2.9 | Cytoplasm | enzyme |
| ***CCR9*** | 1.2 | Plasma Membrane | G-protein coupled receptor |
| ***GPR171*** | 1 | Plasma Membrane | G-protein coupled receptor |
| ***GPR55*** | 1.3 | Plasma Membrane | G-protein coupled receptor |
| ***ITK*** | 1.1 | Cytoplasm | kinase |
| ***BATF2*** | 2 | Other | other |
| ***CLNK*** | 1.4 | Cytoplasm | other |
| ***LCP2*** | 1.1 | Cytoplasm | other |
| ***THEMIS*** | 1.4 | Cytoplasm | other |
| ***PTPN22*** | 1.2 | Cytoplasm | phosphatase |
| ***CD6*** | 1.1 | Plasma Membrane | transmembrane receptor |
| ***IL18R1*** | 1.3 | Plasma Membrane | transmembrane receptor |
| ***IL1RL1*** | 1.4 | Plasma Membrane | transmembrane receptor |
| ***KLRK1*** | 1.3 | Plasma Membrane | transmembrane receptor |

**Table 3.3 IPA downstream functional analysis and pathways analysis for DE genes between non-shedders and super-shedders in distal jejunum, cecum, and descending colon, no functional terms or pathways with $|z| \geq 2$ were enriched for the other tissues.**

| Tissues | Functional terms/pathways | z-score | Involved genes |
|---|---|---|---|
| **Distal jejunum** | leukocyte migration | 2.2 | *CCL2, CCR9, F3, GPR132, S1PR2* |
| **Distal jejunum** | LXR/RXR activation | -2.4 | *APOE, ARG2, CCL2, IL1A, IL1RL1, PTGS2* |
| **Distal jejunum** | B-cell receptor signaling | -2.0 | *BCL6, BLNK, CD79A, CD79B, EBF1* |
| **Descending colon** | proliferation of T-cells | 2.0 | *APOA1, CD36, GP2, GPAM* |

**Table 3.4 SNPs identified in DE genes which showed association with super-shedding phenomena.**

| Gene | Variant.ID | Location | Alleles | Freq_NS* | Freq_SS** | Consequence | p-value |
|---|---|---|---|---|---|---|---|
| ***APOA1****** | rs384985356 | 15:27932563 | A/C | A, 50% | A, 100% | Synonymous variant | 0.033 |
| ***BATF2****** | rs42191303 | 29:43828428 | C/T | C, 100% | C, 20% | Intron variant | 0.001 |
| ***BLNK****** | rs42088770 | 26:17382354 | A/T | A, 100% | A, 50% | Intron variant | 0.033 |
| ***BLNK*** | rs110491800 | 26:17395942 | A/G | A, 50% | A, 100% | Intron variant | 0.033 |
| ***BLNK*** | rs110241837 | 26:17398172 | G/T | G, 0% | G, 70% | Intron variant | 0.003 |
| ***BLNK*** | rs109825977 | 26:17398543 | A/G | A, 0% | A, 60% | Intron variant | 0.011 |
| ***BLNK*** | rs209836657 | 26:17398630 | G/C | G, 0% | G, 70% | Intron variant | 0.003 |
| ***BLNK*** | rs42088798 | 26:17399306 | G/C | G, 0% | G, 70% | Intron variant | 0.003 |
| ***BLNK*** | rs385916156 | 26:17399758 | T/A | T, 0% | T, 60% | Intron variant | 0.011 |
| ***BLNK*** | rs211039227 | 26:17401401 | T/A | T, 0% | T, 60% | Intron variant | 0.011 |
| ***BLNK*** | rs42088814 | 26:17401969 | A/G | A, 100% | A, 40% | Intron variant | 0.011 |
| ***BLNK*** | rs42088815 | 26:17402059 | T/C | T, 100% | T, 50% | Intron variant | 0.033 |
| ***BLNK*** | rs42084078 | 26:17419883 | A/G | A, 0% | A, 50% | Missense variant | 0.033 |
| ***BLNK*** | rs135453434 | 26:17427529 | C/T | C, 100% | C, 50% | Intron variant | 0.033 |
| ***BLNK*** | rs42084065 | 26:17428014 | T/C | T, 100% | T, 50% | Intron variant | 0.033 |
| ***BLNK*** | rs132877528 | 26:17431964 | C/T | C, 0% | C, 50% | Intron variant | 0.033 |
| ***BLNK*** | rs134560601 | 26:17432163 | A/G | A, 0% | A, 60% | Intron variant | 0.011 |
| ***IL18R1****** | rs109078612 | 11:7153863 | T/C | T, 0% | T, 60% | Intron variant | 0.011 |
| ***IL18R1*** | rs109700098 | 11:7169952 | T/C | T, 0% | T, 60% | Intron variant | 0.011 |
| ***IL18R1*** | rs109755291 | 11:7179544 | C/T | C, 0% | C, 60% | Missense variant | 0.011 |
| ***ITK****** | rs109137099 | 7:70923893 | T/C | T, 80% | T, 20% | Intron variant | 0.023 |
| ***THEMIS****** | rs135858596 | 9:66782892 | C/A | C, 30% | C, 90% | Intron variant | 0.020 |
| ***THEMIS*** | rs207702093 | 9:66756813 | C/T | C, 100% | C, 50% | Intron variant | 0.033 |
| ***EBF1****** | rs207972417 | 7:72475725 | C/T | C, 100% | C, 40% | Intron variant | 0.011 |
| ***EBF1*** | rs207622846 | 7:72477936 | T/C | T, 100% | T, 50% | Intron variant | 0.033 |
| ***EBF1*** | rs209147903 | 7:72478125 | G/A | G, 100% | G, 40% | Intron variant | 0.011 |
| ***EBF1*** | rs211282271 | 7:72478236 | T/C | T, 100% | T, 50% | Intron variant | 0.033 |
| ***EBF1*** | rs384133035 | 7:72481066 | A/G | A, 100% | A, 50% | Intron variant | 0.033 |
| ***EBF1*** | rs41656033 | 7:72582112 | G/C | G, 100% | G, 30% | Intron variant | 0.003 |
| ***EBF1*** | rs132998336 | 7:72588676 | G/A | G, 90% | G, 30% | Intron variant | 0.020 |
| ***EBF1*** | rs135578882 | 7:72616057 | C/T | C, 100% | C, 40% | Intron variant | 0.011 |
| ***EBF1*** | rs43524161 | 7:72701165 | C/G | C, 50% | C, 0% | Intron variant | 0.033 |
| ***EBF1*** | rs29027619 | 7:72710782 | G/C | G, 70% | G, 10% | Intron variant | 0.020 |

***Allele frequency in non-shedders, total frequency of two alleles are 100%**

****Allele frequency in super-shedders, total frequency of two alleles are 100%**

*****APOA1, Apolipoprotein A1; BATF2, Basic Leucine Zipper ATF-Like Transcription Factor 2; *BLNK, B-Cell Linker; IL18R1, Interleukin 18 Receptor 1; THEMIS, Thymocyte Selection Associated; EBF1, Early B-Cell Factor 1.***

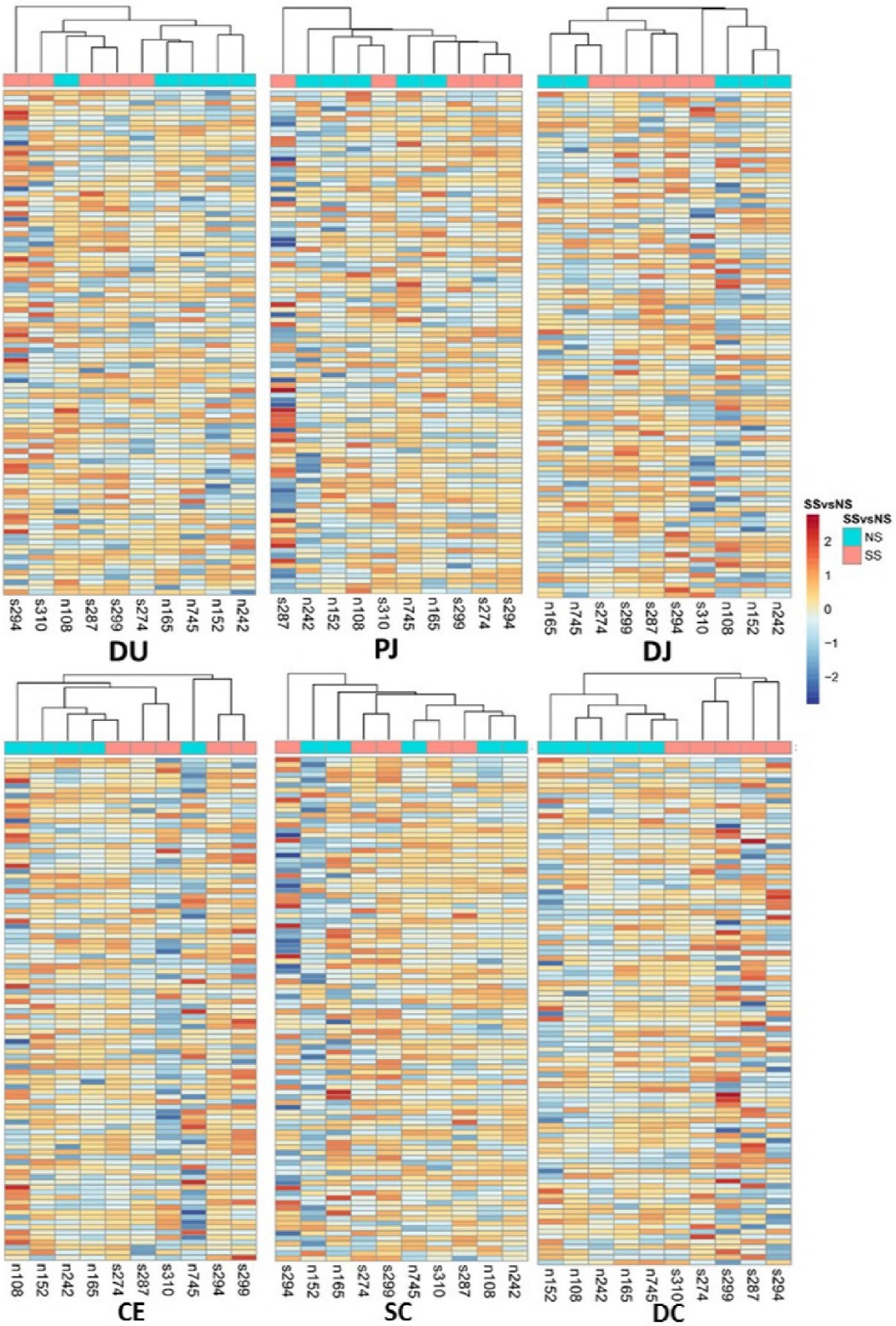
DU
s294 s310 n108 s287 s299 s274 n165 n745 n152 n242
PJ
s287 n242 n152 n108 s310 n745 n165 s299 s274 s294
DJ
n165 n745 s274 s299 s287 s294 s310 n108 n152 n242
SSvsNS
SSvsNS
NS
SS
2
1
0
−1
−2
CE
n108 n152 n242 n165 s274 s287 s310 n745 s294 s299
SC
s294 n152 n165 s274 s299 n745 s310 s287 n108 n242
DC
n152 n108 n242 n165 n745 s310 s274 s299 s287 s294

**Figure 3.1 Heatmaps for core transcriptomes of gastrointestinal tract of beer steers.** The genes in core transcriptomes were first aggregated into 100 clusters using k-means clustering. DU, duodenum; PJ, proximal jejunum; DJ, distal jejunum; CE, cecum; SC, spiral colon; DC, descending colon.

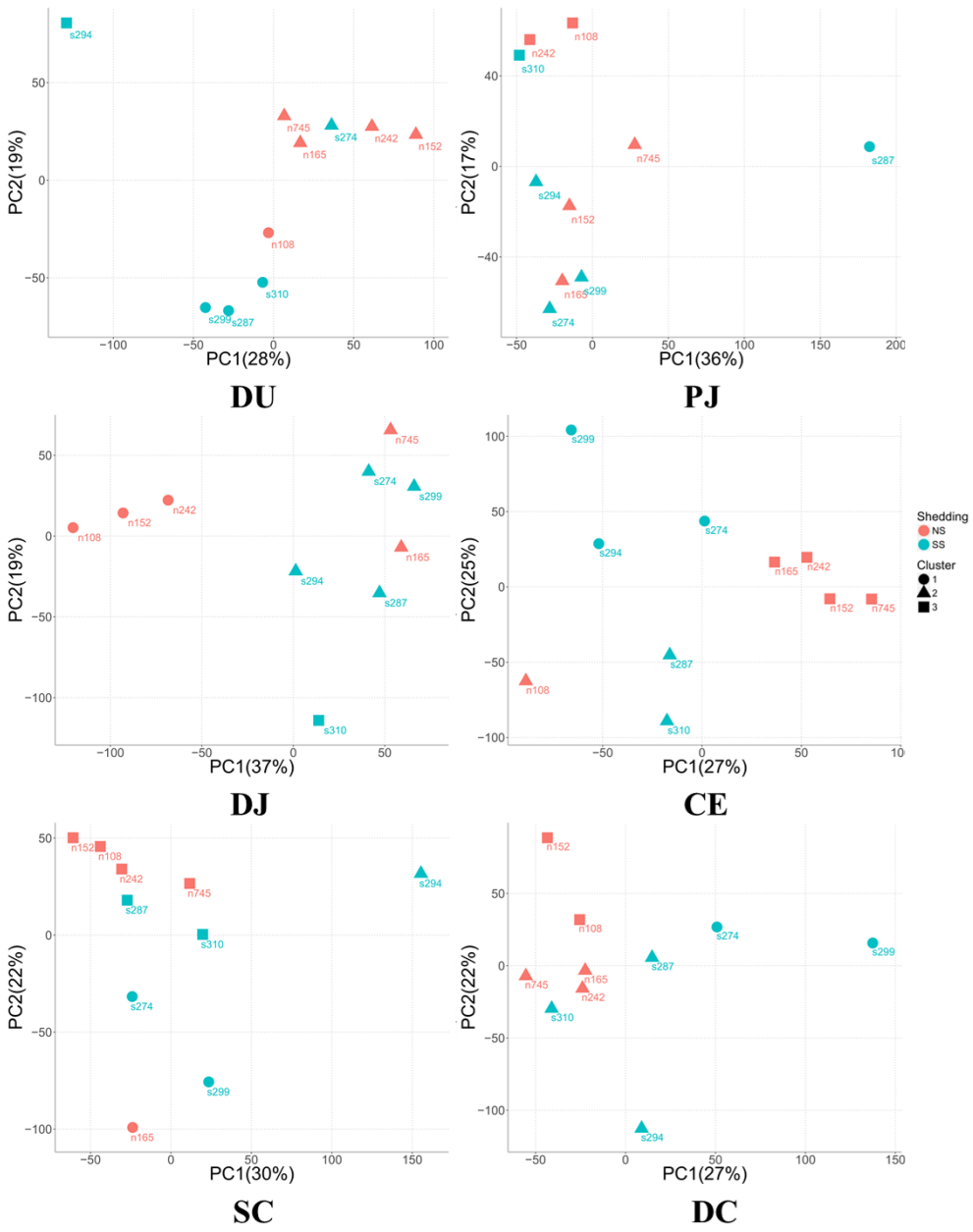


**Figure 3.2 Principal component analysis of the core transcriptome of all tissues.** The red circles indicate non-shedders that are clustered together based on their gene expression profiles. The expression values of each gene were scaled by subtracting mean expression value then dividing by standard deviation. The cluster analysis was performed using k-mean algorithm. DU, duodenum; PJ, proximal jejunum; DJ, distal jejunum; CE, cecum; SC, spiral colon; DC, descending colon.

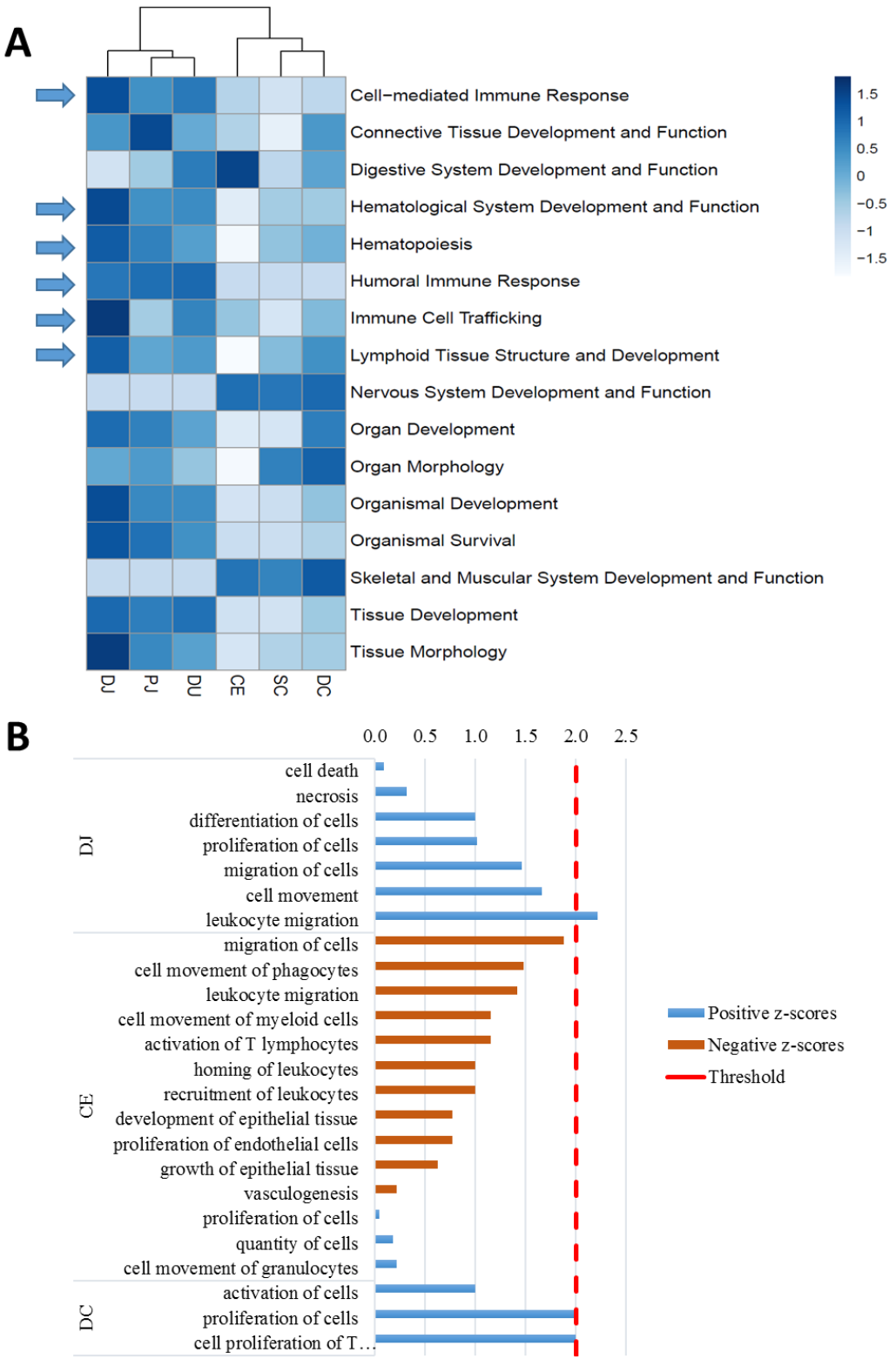
A
Cell−mediated Immune Response
Connective Tissue Development and Function
Digestive System Development and Function
Hematological System Development and Function
Hematopoiesis
Humoral Immune Response
Immune Cell Trafficking
Lymphoid Tissue Structure and Development
Nervous System Development and Function
Organ Development
Organ Morphology
Organismal Development
Organismal Survival
Skeletal and Muscular System Development and Function
Tissue Development
Tissue Morphology
1.5
1
0.5
0
−0.5
−1
−1.5
DJ
PJ
DU
CE
SC
DC
B
0.0
0.5
1.0
1.5
2.0
2.5
DJ
cell death
necrosis
differentiation of cells
proliferation of cells
migration of cells
cell movement
leukocyte migration
CE
migration of cells
cell movement of phagocytes
leukocyte migration
cell movement of myeloid cells
activation of T lymphocytes
homing of leukocytes
recruitment of leukocytes
development of epithelial tissue
proliferation of endothelial cells
growth of epithelial tissue
vasculogenesis
proliferation of cells
quantity of cells
cell movement of granulocytes
DC
activation of cells
proliferation of cells
cell proliferation of T…
Positive z-scores
Negative z-scores
Threshold

**Figure 3.3 Function analysis for the core transcriptome of cattle intestinal tissues.** (A) IPA (Ingenuity Pathway Analysis) functional analysis of the core transcriptome of intestinal tissues. The heatmap shows scaled values of $-\log_{10}$(p-value) indicated with blue colours: the darker the blue, the more likelihood that a function is associated with the core transcriptome of a tissue. (B) Functional analysis for differentially expressed (DE) genes. Results obtained from IPA downstream analysis for DE genes of each tissue. Positive z-scores were indicated with blue bars and negative z-scores with brown bars. The absolute values of z-scores were plotted, and those with a threshold greater than 2 were considered to indicate a difference in functionality between super-shedders and non-shedders. A positive z-score indicates enhancement, while a negative z-score indicates a reduction in function. DJ, distal jejunum; CE, cecum; SC, spiral colon; DC, descending colon; for differentially expressed genes identified in the other tissues, no IPA functional terms were enriched.

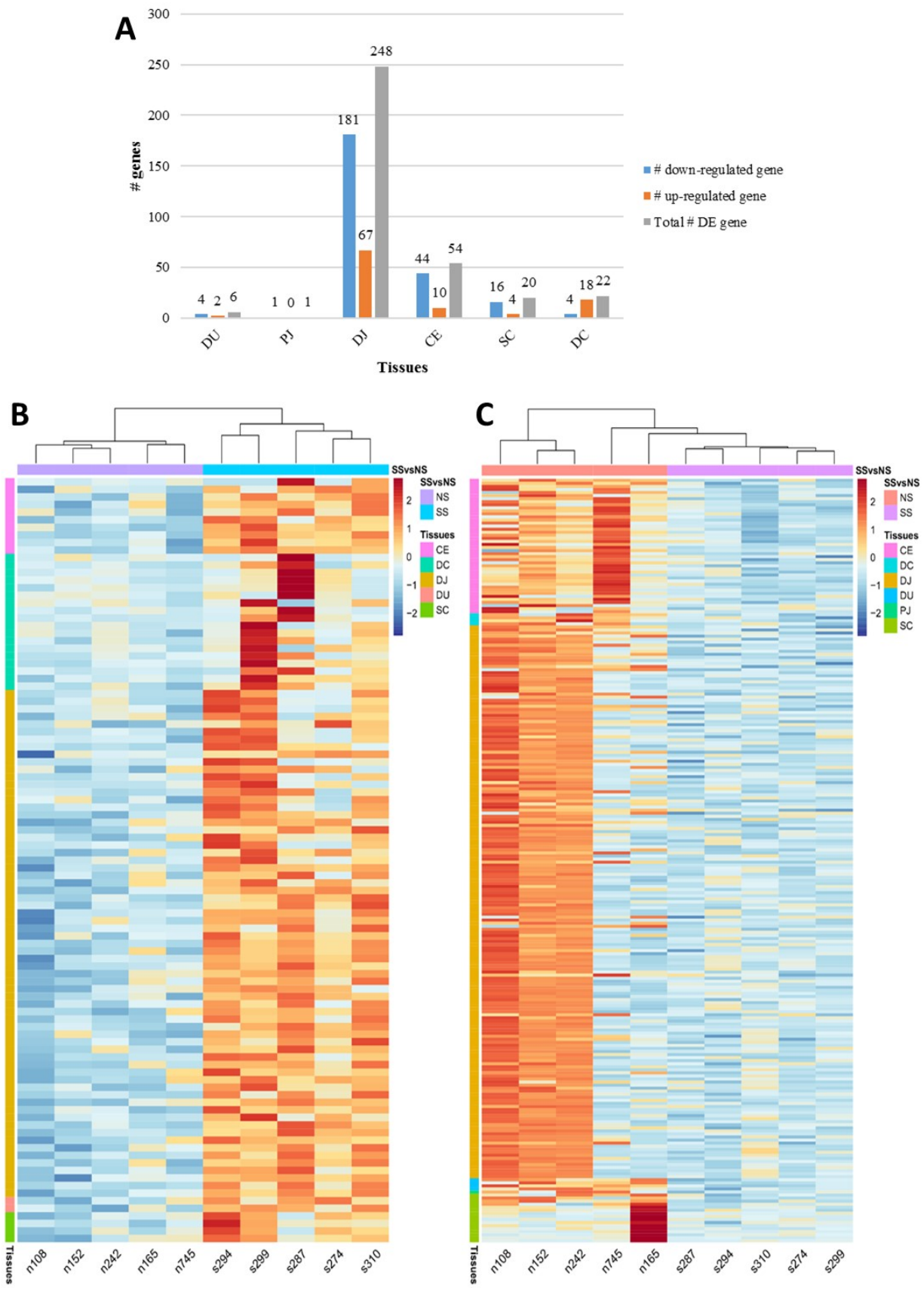
A
300
250
200
150
100
50
0
# genes
248
181
67
44
54
10
16
4
20
4
18
22
4 2 6
1 0 1
DU
PJ
DJ
CE
SC
DC
Tissues
# down-regulated gene
# up-regulated gene
Total # DE gene
B
SSvsNS
NS
SS
Tissues
CE
DC
DJ
DU
SC
2
1
0
−1
−2
n108
n152
n242
n165
n745
s294
s299
s287
s274
s310
C
SSvsNS
NS
SS
Tissues
CE
DC
DJ
DU
PJ
SC
n108
n152
n242
n745
n165
s287
s294
s310
s274
s299

**Figure 3.4 Differentially expressed genes between super-shedders and non-shedders.** (A) Number of differentially expressed (DE) genes identified in intestinal regions. DE genes: FDR < 0.05, log2-fold-change < -1 or > 1, cpm ≥ 1 in at least 50% of SS and NS. (B) and (C) are Heatmaps for $log_2$(counts per million) of differentially expressed genes in all tissues: (B) up-regulated differentially expressed genes in super-shedders; (C) down-regulated differentially expressed genes in super-shedders. Scaled log2(counts per million) was indicated by red and blue colors, and red indicates higher expression level, while blue colour indicates lower expression level. The column labels indicate super-shedders and non-shedders: NS, non-shedders; SS, super-shedders. The row labels indicate the tissue where differentially expressed genes were identified. DU, duodenum; PJ, proximal jejunum; DJ, distal jejunum; CE, cecum; SC, spiral colon; DC, descending colon.

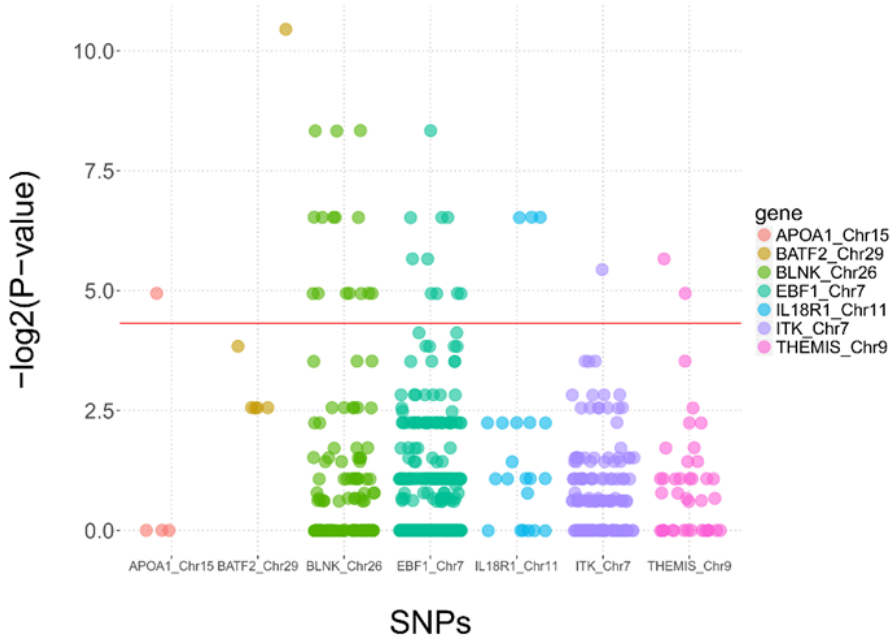


**Figure 3.5 SNPs detected in DE genes based on RNA-Seq data.** The x-axis shows the location of a SNP on the genes, and y-axis shows the –log2(p-values). The SNPs that shows association with super-shedding phenomena are presented above the red line (p-value < 0.05).

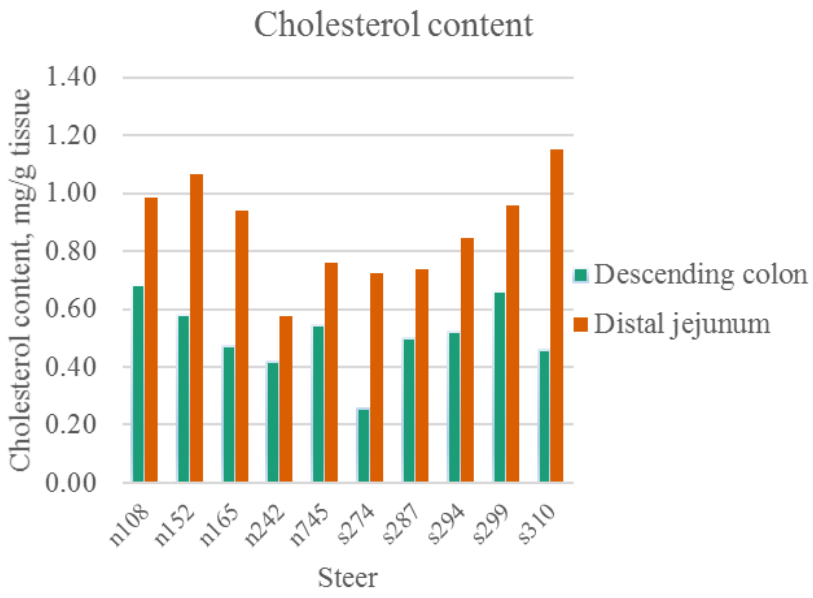


**Figure 3.6 Cholesterol quantification results of distal jejunum and descending colon** (P-values of each SNP).

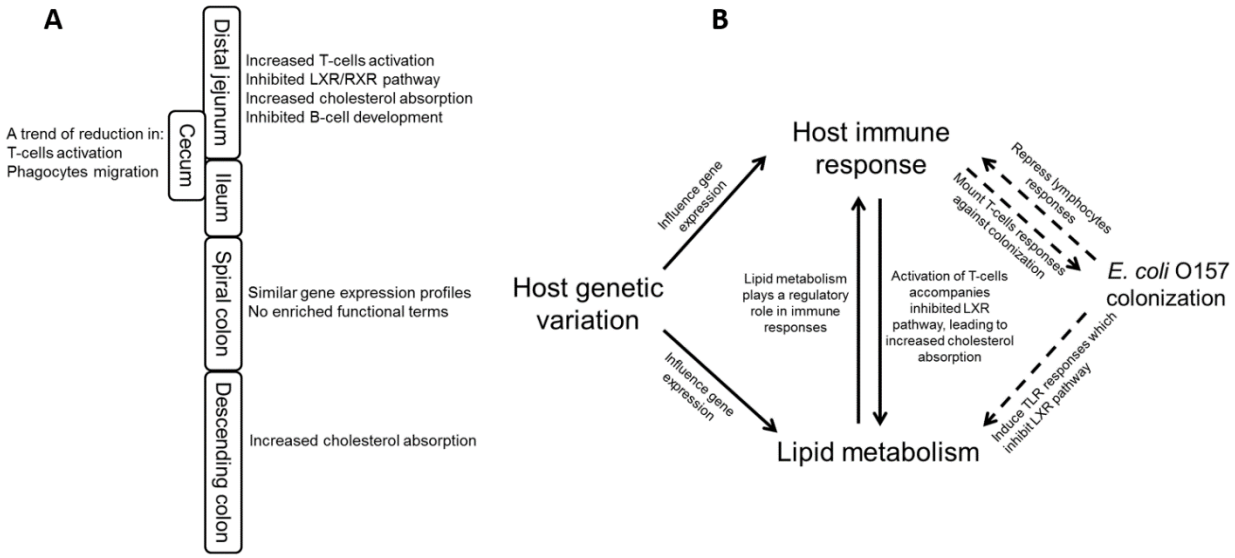


**Figure 3.6 Possible host and *E. coli* O157 interactions in super-shedder cattle that may affect gene expression in the gut of super-shedders.** (A) Changes in functions of different intestinal regions of super-shedders. Functions were predicted based on expression of DE genes of each gut region. (B) Possible association among lipid metabolism, host immune responses and *E. coli* O157 colonization.

# Chapter 4. MicroRNAomes of cattle intestinal tissues revealed possible miRNA regulated mechanisms involved in *Escherichia coli* O157 fecal shedding

## 4.1 Introduction

*Escherichia coli* (*E. coli*) O157 is a foodborne pathogen that causes severe human disease, such as hemolytic uremic syndrome, bloody diarrhea, and even death (Pennington, 2010). The *E. coli* O157 in the feces of cattle shed into the farm environment can survive in soil and water for extended periods of time (Maule, 2000) and can cause contamination of vegetables and fruits during planting and irrigation. Furthermore, fecal contamination in the food processing and preparation chain can also lead the adulteration of meat (Ferens and Hovde, 2011). Cattle shedding > $10^4$ CFU *E. coli* O157 per gram of feces are often referred to as super-shedders (SS), which have been reported to be responsible for most of the *E. coli* O157 spread into the farm environment (Chase-Topping et al., 2008). However, only a small portion (< 10%) of the cattle in a herd have been reported to be SS in a number of studies (Chase-Topping et al., 2008; Munns et al., 2014), suggesting certain individuals have a propensity to become SS. The recto-anal junction (RAJ) of cattle has been suggested to be the primary colonization site of *E. coli* O157 (Naylor et al., 2003), and our recent study revealed that expression of genes involved in immune functions at the RAJs differed between natural SS and non-shedders (NS, cattle negative for *E. coli* O157) (O. Wang et al., 2016). However, the regulatory mechanisms responsible for this differential gene expression in the RAJ of SS vs NS remains unclear.

One possibility is that the non-LEE-encoded type III secretive proteins and Shiga toxins produced by *E. coli* O157 may suppress host lymphocyte responses, as suggested by both *in vitro* and *in vivo* studies (Menge et al., 2003; Hoffman et al., 2006; Walle et al., 2013). Another possibility is that the regulatory mechanisms of the transcriptome that lead to immune responses in the gut may differ between SS and NS. One form of transcriptome regulation occurs through non-coding RNAs, especially microRNAs (miRNAs), which have been reported to regulate gene expression in many biological processes (Wahid et al., 2010). In addition, detection of miRNAs in extracellular spaces, such as milk, serum, urine and feces (Weber et al., 2010; Liu et al., 2016), suggests that the functions of miRNAs are not restricted to within cells, but also with extracellular functions associated with including immune functions, cell communication and even shaping the gut microbiota (Turchinovich et al., 2012; Liu et al., 2016).

Gene expression is regulated by miRNA through recognition of complementary sequences on transcripts, commonly through binding to the 3'-UTR region (Bartel, 2009; Fang and Rajewsky, 2011). Many studies have reported that miRNA expression was associated with host-microbial interaction. Bao et al. (2015) studied whole blood miRNAomes of *Salmonella* challenged pigs, and suggested that miR-214 and miR-331-3p may regulate host immune responses associated with persistent shedding of *Salmonella*. Others have reported that miRNA-155 influences the presence of *Helicobacter pylori* in mouse gastrointestinal tract by mediating T-cell responses (Oertli et al., 2011). *Mycobacterium tuberculosis* has been reported to interfere with the expression of miR-125b and miR-155 blocking the expression of Tumor Necrosis Factor (TNF) responses in macrophages (Rajaram et al., 2011). Moreover, a recent effort revealed that the expression of miRNAs involved in B-cell functions in the small intestine were associated with beneficial bacterial (*Lactobacilli* and *Bifidobacterium*) populations in the gut of

young calves (Liang et al., 2014). Therefore, we hypothesize that the expression of gut miRNAs differs between SS and NS, and that it is one of the key regulatory mechanisms of the reported differential gene expression through the gut in response to the *E. coli* O157 colonization and shedding in cattle (O. Wang et al., 2016). We performed miRNA expression profiling on the whole gut of cattle, including the duodenum, proximal jejunum, distal jejunum, cecum, spiral colon and descending colon using miRNA sequencing to exam whether miRNAs may play a regulatory role in host immune functions that influence the presence of *E. coli* O157 within the digestive tract.

## 4.2 Materials and Methods

The steers used in this experiment followed the Canadian Council of Animal Care Guidelines, and the protocol was reviewed and approved by the Animal Care Committee of Lethbridge Research Centre, Agriculture Agri-food Canada (Animal Care Committee protocol number: 1120).

### 4.2.1 Super-shedder cattle identification and intestinal tissue collection

The protocol for identification of SS and tissue collection were as detailed previously (Zaheer et al., 2017). Briefly, fecal samples (50 g each) were collected from RAJ of 400 British x Continental feedlot steers for *E. coli* O157 identification and quantification using plate culturing methods on sorbitol MacConkey agar with 2.5 mg/L potassium tellurite and 0.05 mg/L cefixime. In total, 11 SS were identified with fecal *E. coli* O157 $\geq 10^4$ CFU per gram of feces. The *E. coli* O157 isolates from CT-SMAC were confirmed using an *E. coli* O157 Latex Test kit (Oxoid Ltd, Basingstoke, Hampshire, UK) and the serotype (*E. coli* O157:H7) was further confirmed with multiplex PCR targeting *verotoxin* (*vt*), *intimine* (*eaeA*) and *H7 flagellin* (*fliC*) following protocols described previously (Gannon et al., 1997). Based on fecal *E. coli* O157 numbers, 5 SS

(out of 11 identified) and 5 control pen-mates (NS, negative for fecal *E. coli* O157) were used for sampling of intestinal tissue. The fecal *E. coli* O157 of SS were monitored for 4 to 10 days prior to slaughter, and all SS were positive for fecal *E. coli* O157 before slaughter (Munns et al., 2014), although they were not necessarily shedding at SS levels. From the slaughtered NS and SS, two 2 $cm^2$ of tissues were collected from duodenum, proximal jejunum, distal jejunum, cecum, spiral colon, descending colon and RAJ (one RAJ sample from a NS was lost during storage), and immediately snap-frozen in liquid nitrogen and stored at -80°C.

### 4.2.2 RNA extraction and miRNA sequencing

The tissue samples were ground into fine powders in liquid nitrogen prior to RNA extraction. Approximately 100 mg of tissue were used for RNA extraction using a mirVana total RNA Isolation Kit (Ambion, Carlsbad, CA, USA) per manufacturer's instructions. The RNA integrity was measured using an Agilent 2200 TapeStation (Agilent Technologies, Santa Clara, CA, USA), and the RNA concentration was measured using a Qubit 2.0 Fluorometer (Invitrogen, Carlsbad, CA, USA). The RNA samples with an integrity number (RIN) ≥ 7.0 and ratio of 28S/18S ≥ 1.7 were used for miRNA-Seq library construction. For each sample, 1 μg of total RNA was used for library preparation using a TruSeq Small RNA sample preparation kit (Illumina, San Diego, CA) following manufacturer's instruction. RNA sequencing was performed using an Illumina HiSeq 2000 (illumina, San Diego, CA, USA) sequencing platform (single end, 1 × 50 bp) at the Genome Quebec Innovation Centre (Montreal, PQ, Canada).

### 4.2.3 miRNA sequencing data processing, functional analysis, differential analysis

All the data were presented as mean ± standard deviation. The miRNA sequencing reads were trimmed, quantity filtered and processed using a web based analysis tool, "sRNAtoolbox" (using default parameters) (Rueda et al., 2015). The read number for detected miRNAs were

normalized as counts per million (cpm), and the miRNAs with cpm > 1 were defined as expressed miRNAs. The miRNA family conservation was defined according to miRNA family information from TargetScan (release 7.0, 2016) (Agarwal et al.). The miRNA target prediction was performed using PITA (Probability of Interaction by Target Accessibility) (Kertesz et al., 2007) and miRanda (Betel et al., 2008). The targets predicted by both miRanda (matching score > 145, free energy < -10) and PITA (free energy < -10) were used for functional analysis for selected miRNA. The Ingenuity Pathway Analysis® (IPA, IPA®, QIAGEN Redwood City, www.qiagen.com/ingenuity) was used for function enrichment, and the Fisher's exact text implemented by internal functions of IPA was used for the association analysis for miRNA targets and functions, with significance level 5% (Fisher's exact test p-value 0.05) for selection of enriched functions. The Bioconductor edgeR (Robinson et al., 2010), which fits the reads counts to a binomial distribution, and estimate the dispersion and determine differential expression by exact test, was used to identify differentially expressed (DE) miRNA. Only miRNAs expressed in > 2 of either NS or SS group were used for differential expression analysis, and FDR (false discovery rate) of 10% was used as a cut-off for DE miRNA identification.

### 4.2.4 Identification of potential miRNA regulation on target mRNA expression

To assess the potential for interactions between identified DE miRNAs and predicted target genes, a Spearman's rank-order correlation analysis between expression of DE miRNAs and all genes that encode predicted target transcripts (gene expression levels are reported in Chapter 3). The correlation analysis was performed using R (version 3.3.2), and only miRNA/transcripts with cpm > 1 in at least 5 animals were used for correlation analysis. The

correlation coefficient, ρ (rho)-value < -0.6, and p-value < 0.05 were considered as significant negative correlation.

## 4.3 Results

### 4.3.1 miRNAome profiling of intestinal tissues collected from beef steers

The sequencing reads were generated from 69 miRNA libraries prepared from intestinal tissues collected at 7 different locations, including duodenum, proximal jejunum, distal jejunum, cecum, spiral colon, descending colon, and RAJ. The number of sequencing reads ranged from 30 to 75 million for each region (Table 4.1), and the reads number for each tissue ranged from 0.9 to 22 million (Figure 4.1A). The number of reads mapped to miRNA ranged from 0.5 to 19 million per sample (Figure 4.1B), and the number of miRNAs detected in each region ranged from 390 ± 13 (duodenum) to 413 ± 49 (descending colon) (Table 4.1 and Figure 4.1C). In total, 464 miRNAs were shared by all the intestinal regions (7 regions), while 39 miRNAs were only detected in 1 region (Figure 4.1D). Among the identified miRNAs in each region, the number of highly conserved miRNAs ranged from 151 (spiral colon) to 157 (descending colon), and the number of conserved miRNAs ranged from 83 (duodenum and RAJ) to 88 (cecum and descending colon), while 280 (RAJ) to 307 (descending colon) of detected miRNAs were poorly conserved (defined by TargetScan 7.0 which claims that certain poorly conserved miRNAs may also be sequences misannotated as miRNAs) (Figure 4.2).

The bta-miR-143 (48.8%), bta-miR-192 (10.8%) and bta-miR-148a (3.29%) were the three most abundant miRNAs expressed in duodenum, and bta-miR-143 (44.3%), bta-miR-192 (14.1%) and bta-miR-10a (2.7%) were the three most abundant miRNAs expressed in proximal jejunum. In distal jejunum, cecum, spiral colon, descending colon and RAJ, the bta-miR-143 (16.2% in cecum to 59.3% in descending colon), bta-miR-192 (5.4% in spiral colon to 12.5% in

cecum) and bta-miR-10b (4.7% in descending colon to 14.6% in cecum) are the three most abundant miRNAs. The relative abundance of bta-miR-143 and bta-miR-192 miRNA accounted for more than 50% of the miRNAs detected in each intestinal region, with the abundance of top 15 miRNAs of each region accounted for > 83% of total expressed miRNAs. Among the top 15 most abundant expressed miRNAs, 10 were shared by all 7 regions, including bta-miR-143, bta-miR-192, bta-miR-10b, bta-miR-27b, bta-miR-26a, bta-miR-26c, bta-miR-21-5p, bta-miR-148a, bta-miR-22-3p, and bta-let-7f. Functional enrichment by IPA for PITA and miRanda, predicted that these miRNAs most frequently regulated the vascular system functions (cardiovascular system development and function, p-values ranged from 4.2E-07 to 3.3E-05) and organismal survival (p-value ranged from 8.9E-07 to 1.6E-04) (Table 4.2). Also, bta-miR-10b was potentially associated with digestive system development and function (p-value: 1.4E-04), with the bta-miR-148a, bta-miR-192 and bta-miR-22-3p being associated with nervous system development and function (p-value ranged from 2.9E-06 to 3.4E-5) (Table 4.2). In addition, Euclidean distance based hierarchical clustering and PCA analysis indicated that the miRNA expression profiles were not distinct for each of the intestinal regions (data not shown).

### 4.3.2 Differential expressed miRNAs between non-shedders and super-shedders in each intestinal tract

Comparing SS and NS for each region, the number of differentially expressed miRNAs ranged from 1 (in descending colon) to 8 (in distal jejunum) in the gut tissues, and the log2-fold-change ranged from -4.5 to 4.5 (Table 4.3). For duodenum (3 DE miRNAs), cecum (3 DE miRNAs), spiral colon (4 DE miRNAs) and descending colon (1 DE miRNAs), all identified DE miRNAs were down-regulated in SS. For proximal jejunum (2 DE miRNAs) and distal jejunum (8 DE miRNAs), equal numbers of DE miRNAs were up-regulated and down-regulated in SS.

For DE miRNAs identified in RAJ, 4 were down-regulated and 3 were up-regulated. Four DE miRNAs were shared by more than one intestinal regions (Table 4.3): the bta-miR-378b was down-regulated in all regions (log2-fold-change ranged from -3.3 to -4.5); bta-miR-2284j was down-regulated (log2-fold-change ranged from -2.9 to -4.1) in including duodenum, distal jejunum, cecum, spiral colon and RAJ; bta-miR-2284d (log2-fold-change ranged from -2.7 to -2.9) was down-regulated in duodenum, distal jejunum, spiral colon and RAJ; and bta-miR-99a-5p was up-regulated in proximal and distal jejunum (log2-fold-change were 3.3 and 2.7, respectively) (Table 4.3).

For the family conservation of detected DE miRNAs, 5 miRNAs were defined as poorly conserved (or misannotated as miRNAs), 1 was conserved, and 7 were highly conserved (Table 4.4). Ten of the DE miRNAs were in intergenic regions of the genome, and 4 of the DE miRNAs were in the intragenic regions (introns) (Table 4.4). Two intronic miRNAs including bta-miR-2284j and bta-miR-2284d, which were likely bovine specific miRNAs, were present in the introns of *Glutamate Ionotropic Receptor Kainate Type Subunit 4* (*GRIK4*) and *SWAP Switching B-Cell Complex 70kDa Subunit* (*SWAP70*), respectively (Table 4.4). The other two intronic miRNAs were highly conserved including bta-miR-1271 and bta-miR-211, which were associated with *ADP Ribosylation Factor Like GTPase 10* (*ARL10*) and *Transient Receptor Potential Cation Channel Subfamily M Member 1* (*TRRM1*), respectively (Table 4.4).

### 4.3.3 Target gene prediction and functional enrichment for differential expressed miRNAs

The number of predicted targets of DE miRNAs ranged from 37 (bta-miR-99b) to 803 (bta-miR-211) (Table 4.5 and 4.6). For the up-regulated miRNAs, functions of tissue development (p-value ranged from 5.73E-06 to 1.83E-5), organismal development (p-value ranged from 4.42E-07 to 4.64E-06) and nervous system development and functions (p-value

ranged from 5.73E-06 to 9.01E-04) were the most frequently enriched (Table 4.5). For down-regulated miRNAs, functions of organismal development (p-value ranged from 4.87E-07 to 1.12E-04) and cardiovascular system development (p-value ranged from 1.73E-06 to 2.0E-05) were the most frequently enriched (Table 4.6). In addition, the down-regulated bta-miR-451(log2-fold-change: -1.77 in spiral colon) and bta-miR-2284j (log2-fold-change ranged from -2.9 to -4.1), as well as up-regulated bta-miR-211 (log2-fold-change: 4.5 in RAJ) were associated with immune functions, such as development of the hematological system (p-value ranged from 1.8E-05 to 1.8E-05) (Table 4.5 and 4.6).

### 4.3.4 miRNA and mRNA correlation and functional analyses

For each intestinal region, the correlation analysis between expression of DE miRNAs and expression of all the computationally predicted target genes indicated that only a small number of predicted target genes were negatively correlated with the miRNAs: 1 (bta-miR-99a-5p, bta-miR-100 and bta-miR-99b in distal jejunum) to 74 (bta-miR-29d-3p in RAJ) of predicted target genes showed correlation with miRNAs (Spearman's $\rho < -0.6$, and p-value $< 0.05$) (Table 4.7). The functional enrichment for negatively correlated target transcripts of each miRNA indicated association between the DE miRNAs and immune functions. As shown in Table 4.7, with bta-miR-378b, the number of negatively correlated transcripts ranged from 2 (in cecum) to 38 (in spiral colon), and in duodenum, proximal jejunum and distal jejunum, the top enriched functions (smallest p-value) were associated with immune functions, including cell-mediated immune responses and hematological system development (p-values $< 0.05$). In RAJ, two transcripts were negatively correlated with bta-miR-378b, *PRDM1* ($\rho = -0.68$, p-value $< 0.05$) and *CYLD* ($\rho = -0.81$, p-value $< 0.05$), which were also associated with humoral and cell-mediated immune responses (enriched by IPA). For the bta-miR-2284d in distal jejunum, the top

function enriched (smallest p-value) for its negatively correlated target transcripts was hematological system development and function (p-value < 0.05). Also in duodenum, two target transcripts negatively correlated with bta-miR-2284d, *MYC* (ρ = -0.78, p-value < 0.05) and *NLK* (ρ = -0.85, p-value < 0.05), were associated with lymphoid tissue structure and development. For the bta-miR-211 (log2-fold-change: 4.51) and bta-miR-29d-3p (log2-fold-change: 2.23) which were up-regulated in the RAJ of SS, the top enriched functions of their negatively correlated genes were hematological system development and function (p-value < 0.05), immune cell trafficking (p-value < 0.05) and hematopoiesis (p-value < 0.05) (Table 4.7). The bta-miR-18a (log2-fold-change: -1.58) in the distal jejunum was negatively correlated with *ITCH*, *GJA1*, *IL4R*, *F3* and *CD7* (ρ-values < -0.6, p-values < 0.05), which were associated with immune cell trafficking and humoral immune response (p-values < 0.05). In the cecum, the negatively correlated target transcripts of bta-miR-2094 (log2-fold-change: -2.72), *SYK* and *CD22* (ρ-values < -0.6, p-values < 0.05), were associated with humoral and cell-mediated immune response (p-values < 0.05). In RAJ, the bta-miR-1271 (log2-fold-change: 2.59) was negatively correlated with *SCARB1*, *CNR2*, *RALB*, *HBEGF*, *DSC1* and *SIT1* (ρ-values < -0.6, p-values < 0.05), which were associated with humoral immune response and lymphoid tissue structure and development (p-values < 0.05).

In the distal jejunum, several negatively correlated transcripts with bta-miR-18a, bta-miR-378b and bta-miR-2284d were also identified as DE genes between NS and SS, including the *F3* (log2-fold-change: 2.23) with bta-miR-18a (ρ = -0.67, p-value < 0.05) and with bta-miR-2284d (ρ = -0.85, p-value < 0.05); the *PTGS2* (log2-fold-change: 1.3) with bta-miR-2284d (ρ = -0.78, p-value < 0.05); and *THEMIS* (log2-fold-change: 1.4), *ITK* (log2-fold-change: 1.1) and *TIFA* (log2-fold-change: 1.2) with bta-miR-378b (ρ-values were -0.73, -0.71 and -0.78

respectively, and p-values < 0.05). Also, in RAJ, the DE genes *SIT1* (log2-fold-change, -2.3) and *RGS13* (log2-fold-change, -2.2) were predicted as targets of bta-miR-1271 and bta-miR-29d-3p, respectively, with expression being negatively correlated with these miRNAs (ρ-values were -0.67 and -0.83 respectively, and p-values < 0.05).

**4.4 Discussion**

This is the first study to investigate the potential role of miRNA in shedding of *E. coli* O157 in cattle through comparison of miRNAomes of the whole intestinal tract of SS and NS beef steers. Our findings indicated that different regions of the gut tended to share the same expressed miRNAs and the hierarchical cluster and PCA analyses suggested similar expression patterns throughout the regions of the bovine intestinal tract. Similar results have previously been reported for miRNA expression patterns in duodenum and jejunum of dairy cattle (D. Wang et al., 2016). The observation of high abundance of bta-miR-143 and bta-miRNA-192 was also observed in previous miRNA profiling studies of the gut tissues of calves (Liang et al., 2014; Liang et al., 2016), and dairy cattle (D. Wang et al., 2016). The bta-miR-143, which had the highest abundance in all the intestinal regions, belongs to miR-143 family, and miR-143 has been reported to be highly expressed in the gut of humans (Gaulke et al., 2014) and mice (Singh et al., 2011). This likely reflects the its important function that bta-miR-143 plays in maintaining smooth muscle cells, vascular homeostasis, epithelium regeneration and epithelial tumor repression (Slaby et al., 2007; Elia et al., 2009; Chivukula et al., 2014). Functional enrichment for the most abundant miRNAs showed associations with development of the nervous system, vascular system and digestive system, suggesting the critical role of abundantly expressed miRNAs in the bovine gut.

Although both environmental and host related factors could influence on *E. coli* O157 shedding in cattle, since both NS and SS were raised in the same environment, we speculate that the identified DE miRNAs between SS and NS animals are host related mechanisms which regulate gut gene expression and alter gut environment in a manner that contribute to the shedding of *E. coli* O157. The distal jejunum and RAJ were the regions where most DE miRNAs were identified, and thus it is reasonable to speculate that certain miRNAs expressed in the these may be involved in *E. coli* O157 super-shedding. For example, the miRNAs, bta-miR-2284j, bta-miR-378b and bta-miR-2284d are simultaneously down-regulated in the distal jejunum and RAJ of SS. In addition, the intestinal mucosal immune surveillance components, Peyer's patches (PP) and isolated lymphoid follicles (ILFs) are known to be present in distal jejunum (Mutwiri et al., 1999) and RAJ (Naylor et al., 2003), respectively. Also, he attaching and effacing lesion caused by *E. coli* O157 was reported in the jejunum of challenged neonatal calves (Dean-Nystrom et al., 1997), and both innate and adaptive immune responses were detected in the RAJ of challenged cattle (Nart et al., 2008). Therefore, the miRNAs, bta-miR-2887 (down-regulated in RAJ of SS) and bta-miR-211 (up-regulated in RAJ of SS), which potentially target mRNAs involved in host immune functions, may also play a role in *E. coli* O157 shedding in SS cattle.

Down-regulation of bta-miR-378b in all the intestinal regions of SS indicates its potentially important role in *E. coli* O157 super-shedding. According to miRbase (Release 21) (Griffiths-Jones et al., 2006), bta-miR-378b is encoded by miR-378 gene family, and miRNAs of this family were suggested to regulate lipogenesis in adipose tissues (Gerin et al., 2010). Also, miR-378 was detected in monocytes and T-cells (Allantaz et al., 2012), and has been reported to regulate cytotoxicity of natural killer cells (Wang et al., 2012), indicating a potential role of miR-378 in mediating innate and adaptive immune functions. Indeed, in all the intestinal regions, the

immune functions were enriched for the negatively correlated target transcripts of bta-miR-378b. The *PRDM1* and *CYLD* were two predicted target transcripts of bta-miR-378b that were negatively correlated with bta-miR-378b in RAJ, the known primary colonization site of *E. coli* O157 in cattle (Nart et al., 2008). The *PRDM1* encodes a Blimp1 protein, which was known to positively regulate the differentiation of B-cells into long-lived plasma cells, and it is required for the release of antibodies by these cells (Martins and Calame, 2008). Lipopolysaccharide (LPS), a byproduct of Gram-negative bacteria, was reported to cause increase in expression of *PRDM1* (Savitsky and Calame, 2006). *CYLD* was confirmed to be a NF-κB repressor to prevent inflammatory diseases, and also, with the LPS being one of the factors that induce an increase in its expression (Courtois, 2008). The functions of these two transcripts in humoral and cell-mediated immune responses suggest their potential role in host and gut microbial interactions. Down-regulation of bta-miR-378b suggested a trend of increased expression of both genes in RAJ of SS, which may be influenced by translocation of Gram-negative bacteria (as indicated above LPS may lead to altered expression of these genes), which is commonly observed when the host intestinal mucosal barrier is disrupted or immune defenses are deficient (Berg, 1999). Indeed, our previous RNA-Seq based study of the rectal tissues of SS suggested possible immunodeficiency in RAJ of SS (O. Wang et al., 2016), and the capability of *E. coli* O157 to disrupt and invade cattle intestinal epithelium has also been demonstrated (Sheng et al., 2011). Future studies to measure LPS and to evaluate the abundance of Gram-negative bacteria in SS vs NS, as well as to examine the integrity of host intestinal mucosal barriers of SS are needed to validate the above speculation.

The bovine specific bta-miR-2284d was down-regulated in both the small and large intestine of SS, including in the RAJ. Although its expression level was low in all the tissues

(average cpm = 2.2), it may still play a critical role in bovine physiology, as the number of its predicted targets is higher than that of other DE miRNAs except for bta-miR-211. Bta-miR-2284d has been reported in several bovine miRNA studies (Liu et al., 2014; Zhao et al., 2016), however, information about its function is limited. The correlation analysis suggests that this miRNA may play a regulatory function with regard to immunity the bovine distal jejunum. Among the transcripts showing negative correlation with bta-miR-2284d in distal jejunum, the DE gene *F3* (also called *TF*) was reported to be involved in recruiting leukocytes in the intestine of mice (Anthoni et al., 2007), and the *PTGS2* (also called *COX2*) was suggested to promote humoral immune responses (Ryan et al., 2006). However, data of bacterial translocation, host mucosal integrity as well as the state of host immune defenses in the intestinal regions studied are not available, and the interaction between miR-378b/bta-miR-2284d and their target transcripts requires further validation. Nevertheless, these findings highlight the importance of further research on the role of miRNA regulations of immune functions including the intestinal mucosal barrier and immune defenses of SS cattle using histological and immunological methods, to identify miRNAs associated with host responses against *E. coli* O157. Also, to better understand the role of bta-miR-378b in super-shedding phenomena, it is worth further investigating whether it directly targets the genes associated with the immune system, or regulates immune functions through regulating lipid metabolism, as lipids are important components for cell signaling and cell proliferation involved in immune responses (Bensinger et al., 2008). In addition, to validate the interaction between bta-miR-2284d and potential targets, especially the targets involved in immune functions will help understand the functions of this bovine specific miRNA, and how its regulation may influence *E. coli* O157 shedding.

Extracellular miRNAs were discovered in feces and body fluids, such as plasma, saliva, tears and urine (Weber et al., 2010; Liu et al., 2016). Their functions were suggested to be involved in immune response regulation, cell communication and shaping gut microbiota (Turchinovich et al., 2012; Liu et al., 2016). There is limited information about bovine fecal miRNA expression pattern and their functions, but it was suggested that the small RNA species in the extracellular exosome were similar to the fecal small RNA species identified in humans (Liu et al., 2016). In the current study, the identified DE miRNAs expressed in gut tissue were not only secretive, but also showed great difference in abundance between the gut tissues and the serum/seral exosome as reported by Zhao et al. (2016), implying that the miRNAs showing higher abundance in the serum (including bta-miR-451, bta-miR-18a, bta-29d-3p and bta-miR-2904, with log2-fold-change ranged from 2.1 to 8.1), may serve important functions in the extracellular spaces, including in the luminal space of the intestinal tract. Indeed, the bta-miR-451 was reported to be abundantly present in various bovine biological fluids, including colostrum (Sun et al., 2013), milk (Oh et al., 2015) and serum (Zhao et al., 2016). Current data indicated that these miRNAs were significantly different within the gut tissues of SS and NS, which could lead to differential abundance in extracellular luminal content. It has been suggested that fecal miRNAs were capable of maintaining normal gut microbiota (Liu et al., 2016) by entering bacteria cells including *Fusobacterium nucleatum* (*F. nucleatum*) and *E. coli*, to influence bacterial gene expression possibly through interaction with bacterial DNA/RNA (Liu et al., 2016). It was reported that miRNAs (hsa-miR-515-5p and hsa-miR-1226-5p) interactions with bacterial 16S rRNA and ribozyme RNaseP genes, enhanced growth *F. nucleatum* and *E. coli* (Liu et al., 2016). It is possible that the extracellular miRNAs, including bta-miR-451, bta-miR-18a, bta-29d-3p and bta-miR-2904, may potentially interact with microbial genomes,

including *E. coli* O157. However, the capability for those miRNAs entering bacterial cells including *E. coli* O157 requires further study, and their effects on the growth of *E. coli* O157 and other gut microbes also need further examination. Even if these miRNAs can enter bacterial cells, they may not directly act against/enhance the growth of *E. coli* O157. Instead, they may shape the microbiota by interacting with other gut microorganisms of SS, leading to a microbial composition and gut environment that are more conducive to growth and colonization by *E. coli* O157.

### 4.5 Conclusion

This study investigated the miRNA expression in the whole intestine of *E. coli* O157 SS and NS beef steers. The miRNA profiling results indicated that the majority of expressed miRNAs were common within the regions of the intestinal tract that were investigated. Comparative expression analysis of miRNAs revealed that the distal jejunum and RAJ may play an important role in host responses against *E. coli* O157, as most DE miRNAs were identified in these regions. Down-regulation of bta-miR378b and bta-miR-2284d in multiple intestinal regions of SS suggested that these two miRNAs may differentially alter lipid metabolism and immune functions in the intestinal tract of in the SS vs NS. The DE miRNAs, bta-miR-451, bta-miR-18a, bta-29d-3p and bta-miR-2904, may function in extracellular spaces, and in the intestinal tracts of SS these miRNAs may have potential to interact with the genome of *E. coli* O157 or affect composition of gut microbiota, eventually leading to growth, proliferation and shedding of *E. coli* O157.

## 4.6 References

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## 4.7 Tables and figures

**Table 4.1 Number of reads generated by Illumina HiSeq 2000 system for miRNA sequencing and average number of miRNA detected in each intestinal region of beef steers.**

| Tissue | Number of reads, Million (M) | # identified miRNA |
|---|---|---|
| **Duodenum** | 30.0 | 390 ± 13 |
| **Proximal jejunum** | 41.1 | 393 ± 20 |
| **Distal jejunum** | 37.7 | 395 ± 19 |
| **Cecum** | 39.9 | 411 ± 16 |
| **Spiral colon** | 38.2 | 409 ± 15 |
| **Descending colon** | 75.4 | 413 ± 49 |
| **Rectum**[1] | 33.5 | 398 ± 15 |

[1]Data of rectal tissue were also reported in previous publication (O. Wang et al., 2016).

**Table 4.2 Functions enriched for predicted target genes of top 10 abundant miRNAs that are shared by all the intestinal regions.** (Only the two functions with smallest p-values were shown).

| miRNA | Function categories[1] | p-value[2] |
|---|---|---|
| **bta-let-7f** | organismal development | 7.89E-6 |
| | tissue development | 7.89E-6 |
| **bta-miR-10b** | cardiovascular system development and function | 1.45E-4 |
| | digestive system development and function | 1.45E-4 |
| **bta-miR-143** | organ development | 1.28E-5 |
| | organismal survival | 1.01E-5 |
| **bta-miR-148a** | cardiovascular system development and function | 3.31E-5 |
| | nervous system development and function | 4.44E-6 |
| **bta-miR-192** | nervous system development and function | 3.45E-5 |
| | organismal development | 1.61E-4 |
| **bta-miR-21-5p** | cardiovascular system development and function | 4.24E-7 |
| | organismal survival | 2.59E-9 |
| **bta-miR-22-3p** | nervous system development and function | 2.91E-6 |
| | organismal development | 2.22E-6 |
| **bta-miR-26a** | cardiovascular system development and function | 8.93E-7 |
| | organismal development | 8.93E-7 |
| **bta-miR-26c** | cardiovascular system development and function | 1.58E-4 |
| | organismal development | 1.58E-4 |
| **bta-miR-27b** | organismal survival | 1.96E-9 |
| | tissue development | 2.54E-8 |

[1]Functions enriched for the predicted mRNA genes using Ingenuity Pathway Analysis.

[2]The Fisher's exact test p-value calculated by Ingenuity Pathway Analysis, and the top 3 functions with the smallest p-values were shown.

**Table 4.3 Differentially expressed miRNAs detected in each region of the intestinal tract of beef steers** (10 tissue samples for each region, including 5 collected from non-shedders and 5 from super-shedders, but only 4 rectal tissues from non-shedders).

| Tissue | Differentially expressed miRNA | Log2-fold-change | FDR |
|---|---|---|---|
| **Duodenum** | bta-miR-378b | -4.37 | < 0.01 |
| | bta-miR-2284j | -3.28 | 0.02 |
| | bta-miR-2284d | -2.90 | 0.02 |
| **Proximal jejunum** | bta-miR-378b | -4.50 | < 0.01 |
| | bta-miR-99a-5p | 3.30 | 0.04 |
| **Distal jejunum** | bta-miR-378b | -3.74 | < 0.01 |
| | bta-miR-2284j | -4.11 | < 0.01 |
| | bta-miR-100 | 3.77 | 0.01 |
| | bta-miR-2284d | -2.92 | 0.04 |
| | bta-miR-99b | 2.53 | 0.09 |
| | bta-miR-409a | 2.51 | 0.09 |
| | bta-miR-99a-5p | 2.69 | 0.09 |
| | bta-miR-18a | -1.58 | 0.09 |
| **Cecum** | bta-miR-378b | -3.92 | < 0.01 |
| | bta-miR-2284j | -3.36 | 0.02 |
| | bta-miR-2904 | -2.72 | 0.02 |
| **Spiral colon** | bta-miR-378b | -3.95 | < 0.01 |
| | bta-miR-451 | -1.77 | 0.01 |
| | bta-miR-2284j | -2.92 | 0.01 |
| | bta-miR-2284d | -2.95 | 0.01 |
| **Descending colon** | bta-miR-378b | -3.30 | 0.09 |
| | bta-miR-2284j | -3.76 | 0.01 |
| | bta-miR-1271 | 2.59 | 0.02 |
| | bta-miR-378b | -3.42 | 0.02 |

| | | | |
|---|---|---|---|
| **Rectum** | bta-miR-2887 | -3.20 | 0.05 |
| | bta-miR-211 | 4.51 | 0.05 |
| | bta-miR-2284d | -2.72 | 0.07 |
| | bta-miR-29d-3p | 2.23 | 0.09 |

**Table 4.4 Differentially expressed miRNA, family conservation, and number of predicted targets.**

| miRNAs | Conservation[1] | Chromosome | Location |
|---|---|---|---|
| **bta-miR-29d-3p** | 2 | Chr16 | intergenic |
| **bta-miR-99a-5p** | 2 | Chr1 | intergenic |
| **bta-miR-100** | 2 | Chr15 | intergenic |
| **bta-miR-99b** | 2 | Chr18 | intergenic |
| **bta-miR-18a** | 2 | Chr12 | intergenic |
| **bta-miR-451** | 2 | Chr19 | intergenic |
| **bta-miR-211** | 2 | Chr21 | TRPM1[2], intron 7 |
| **bta-miR-1271** | 2 | Chr7 | ARL10[3], intron 2 |
| **bta-miR-409a** | 1 | Chr21 | intergenic |
| **bta-miR-378b** | -1 | Chr15 | intergenic |
| **bta-miR-2904** | -1 | Chr2, Chr3 | intergenic |
| **bta-miR-2887** | -1 | Chr2, Chr3 | intergenic |
| **bta-miR-2284j** | -1 | Chr15 | GRIK4[4], intron 3 |
| **bta-miR-2284d** | -1 | Chr15 | SWAP70[5], intron 2 |

[1]Defined by TargetScan (release 7, 2016). 2 = highly conserved across human, mouse, rat, dog, and chicken; 1 = conserved across human, mouse, rat and dog; -1 = poorly conserved and possibly misannotated as a miRNA.

[2]Transient receptor potential cation channel subfamily M member 1.

[3]ADP ribosylation factor like GTPase 10.

[4]Glutamate ionotropic receptor kainate type subunit 4.

[5]SWAP switching B-cell complex 70kDa subunit.

**Table 4.5 Up-regulated miRNAs in super-shedders and the functions enriched for their predicted target genes.**

| miRNA | #Target[1] | Regions[2] | Enriched functions[3] | P-value[4] |
|---|---|---|---|---|
| **bta-miR-99a-5p** | 40 | Pj, Dj | organ development | 3.92E-04 |
| | | | organismal development | 3.32E-06 |
| | | | tissue development | 3.32E-06 |
| **bta-miR-100** | 47 | Dj | nervous system development and function | 9.01E-04 |
| | | | organismal development | 4.64E-06 |
| | | | tissue development | 4.64E-06 |
| **bta-miR-409a** | 175 | Dj | cardiovascular system development and function | 4.35E-06 |
| | | | organismal development | 4.35E-06 |
| | | | tissue development | 1.83E-05 |
| **bta-miR-99b** | 37 | Dj | organismal development | 2.67E-06 |
| | | | tissue development | 2.67E-06 |
| | | | tissue morphology | 2.63E-05 |
| **bta-miR-1271** | 443 | Re | nervous system development and function | 5.73E-06 |
| | | | skeletal and muscular system development and function | 5.07E-06 |
| | | | tissue development | 5.73E-06 |
| **bta-miR-211** | 803 | Re | hematological system development and function | 1.78E-05 |
| | | | nervous system development and function | 3.73E-06 |
| | | | tissue development | 3.73E-06 |
| **bta-miR-29d-3p** | 521 | Re | organ morphology | 2.17E-05 |
| | | | organismal development | 4.42E-07 |
| | | | organismal survival | 8.16E-07 |

[1]Genes encodes target mRNAs predicted by PITA and miRanda.

[2]Intestinal regions where the differentially expressed miRNAs were identified. Pj, proximal jejunum; Dj, middle jejunum; Re, rectum.

[3]Functions enriched for the predicted mRNA genes using Ingenuity Pathway Analysis.

[4]The Fisher's exact test p-value calculated by Ingenuity Pathway Analysis, and the top 3 functions with the smallest p-values were shown.

**Table 4.6 Down-regulated miRNAs in super-shedders, and the functions enriched for their predicted target genes.**

| miRNA | #Target[1] | Tissues[2] | Enriched functions[3] | P-value[4] |
|---|---|---|---|---|
| **bta-miR-378b** | 514 | Du, Pj, Dj, Ce, Sc, Dc, Re | cardiovascular system development and function | 1.73E-06 |
| | | | connective tissue development and function | 5.47E-06 |
| | | | tissue morphology | 1.73E-06 |
| **bta-miR-2284j** | 486 | Du, Dj, Ce, Sc, Re | cardiovascular system development and function | 2.00E-05 |
| | | | organismal development | 1.12E-04 |
| | | | tissue development | 3.07E-05 |
| **bta-miR-2284d** | 696 | Du, Dj, Sc, Re | cardiovascular system development and function | 4.87E-07 |
| | | | organ morphology | 4.87E-07 |
| | | | organismal development | 4.87E-07 |
| **bta-miR-18a** | 559 | Dj | cardiovascular system development and function | 1.33E-06 |
| | | | organ morphology | 4.83E-06 |
| | | | organismal survival | 1.54E-07 |
| **bta-miR-2904** | 678 | Ce | digestive system development and function | 4.12E-05 |
| | | | hematological system development and function | 4.31E-05 |
| | | | organismal development | 3.48E-06 |
| **bta-miR-451** | 120 | Sc | nervous system development and function | 3.75E-06 |
| | | | skeletal and muscular system development and function | 2.64E-05 |
| | | | tissue development | 3.75E-06 |

| | | | | |
|---|---|---|---|---|
| **bta-miR-2887** | 226 | Re | hematological system development and function | 1.76E-04 |
| | | | nervous system development and function | 6.50E-05 |
| | | | organismal development | 2.28E-05 |

[1]Genes encodes target mRNAs predicted by PITA and miRanda.

[2]Intestinal regions where the differentially expressed miRNAs were identified. Du, duodenum; Pj, proximal jejunum; Dj, middle jejunum; Ce, cecum; Sc, spiral colon; Dc, Descending colon; Re, rectum.

[3]Functions enriched for the predicted mRNA genes using Ingenuity Pathway Analysis.

[4]The Fisher's exact test p-value calculated by Ingenuity Pathway Analysis, and the top 3 functions with the smallest p-values were shown.

**Table 4.7 Differentially expressed miRNAs and number of targets negatively correlated with miRNA expression in different intestinal regions.**

| miRNA | # predicted targets[1] | # negatively correlated target genes | Enriched functions[2] |
|---|---|---|---|
| **bta-miR-378b** | 514 | Duodenum:23<br>Proximal jejunum:8<br>Distal jejunum:36<br>Cecum:2<br>Spiral colon:38<br>Descending colon:3<br>Rectum:5 | hematological system development and function; hematopoiesis<br>cell-mediated immune response; hematological system development and function<br>hematological system development and function; immune cell trafficking<br>nervous system development and function: tissue morphology<br>connective tissue development and function; organismal development<br>tissue morphology; connective tissue development and function<br>organismal development; tissue morphology |
| **bta-miR-2284j** | 486 | Duodenum: N/A[3]<br>Distal jejunum: N/A<br>Cecum: N/A<br>Spiral colon: N/A<br>Rectum: N/A | n/a<br>n/a<br>n/a<br>n/a<br>n/a |
| **bta-miR-2284d** | 696 | Duodenum:21<br>Distal jejunum:65<br>Spiral colon: N/A<br>Rectum: N/A | tissue morphology; cardiovascular system development and function<br>hematological system development and function; tissue morphology<br>n/a<br>n/a |
| **bta-miR-99a-5p** | 40 | Proximal jejunum:1<br>Distal jejunum:1 | not enriched<br>not enriched |
| **bta-miR-100** | 47 | Distal jejunum:1 | not enriched |
| **bta-miR-99b** | 37 | Distal jejunum:1 | not enriched |
| **bta-miR-409a** | 175 | Distal jejunum:5 | organismal development; digestive system development and function |
| **bta-miR-18a** | 559 | Distal jejunum:16 | cardiovascular system development and function; skeletal and muscular system development and function |
| **bta-miR-2904** | 678 | Cecum:7 | hematopoiesis; humoral immune response |
| **bta-miR-451** | 120 | Spiral colon:3 | cardiovascular system development and function; tissue development |
| **bta-miR-1271** | 443 | Rectum:8 | cardiovascular system development and function; connective tissue development and function |
| **bta-miR-2887** | 226 | Rectum: N/A | n/a |
| **bta-miR-211** | 803 | Rectum:69 | hematological system development and function; immune cell trafficking |
| **bta-miR-29d-3p** | 521 | Rectum:74 | hematological system development and function; hematopoiesis |

[1]Number of genes encoding miRNA target transcripts, predicted by miRanda and PITA.

[2]The Fisher's exact test p-value < 0.05 calculated by Ingenuity Pathway Analysis, and the top 2 functions with the smallest p-values were shown. Function enrichment was not performed if the negatively correlated miRNA-mRNA pairs were less than 2.

[3]The correlation analysis not performed because the miRNA expressed (cpm > 1) in less than 5 animals.

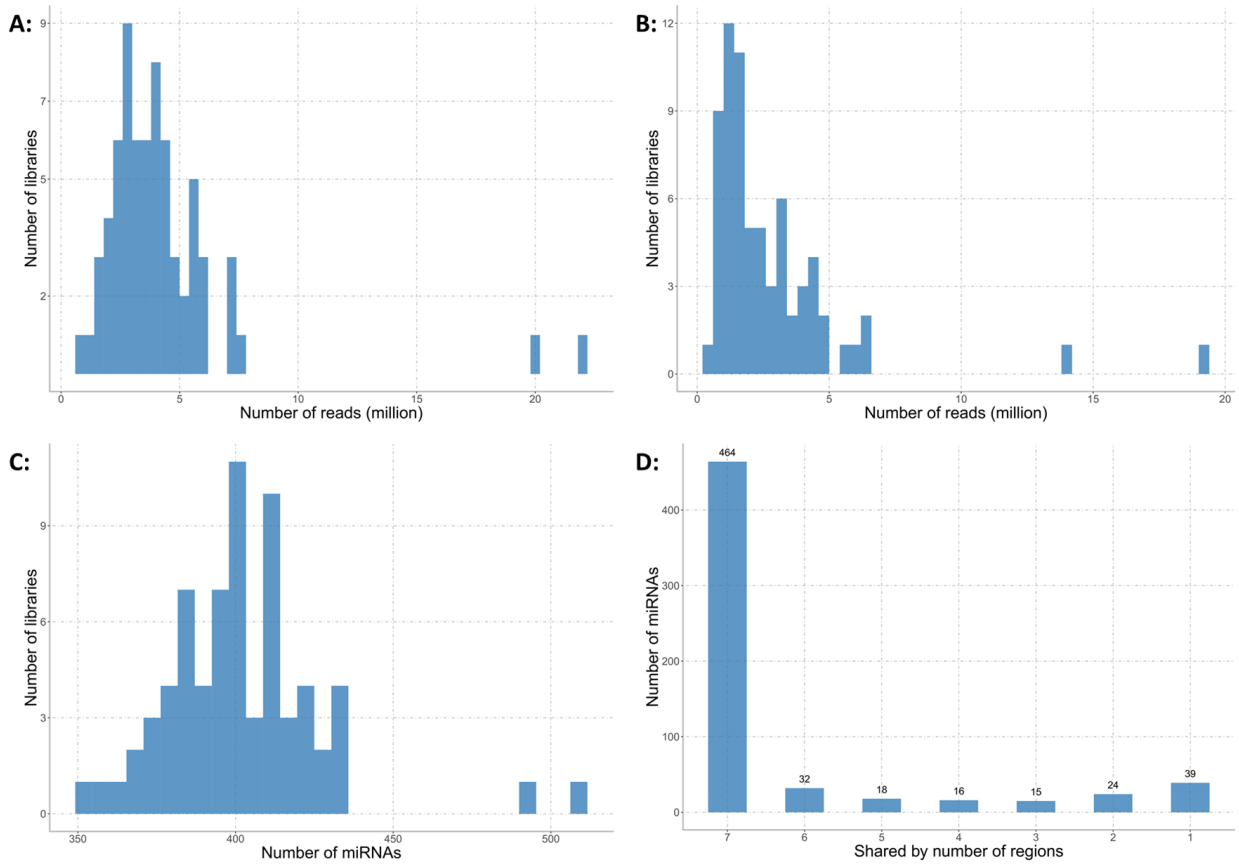


**Figure 4.1 General sequencing results and miRNA mapping results.** (A) Distribution of numbers of reads generated from HiSeq 2000 sequencing platform. (B) Distribution of numbers of reads mapped to known bovine miRNA after quality filtering. (C) Distribution of numbers of detected miRNA from the intestinal regions. (D) Number of miRNA shared by the intestinal regions, the number on x-axis represents the number of intestinal regions a miRNA is shared by, 7, all intestinal regions; 6, 6 out of 7 regions, 5, 5 out of 7 regions, 4, 4 out of 7 regions; 3, 3 out of 7 regions; 2, 2 out of 7 regions; 1, only detected in one of 7 regions.

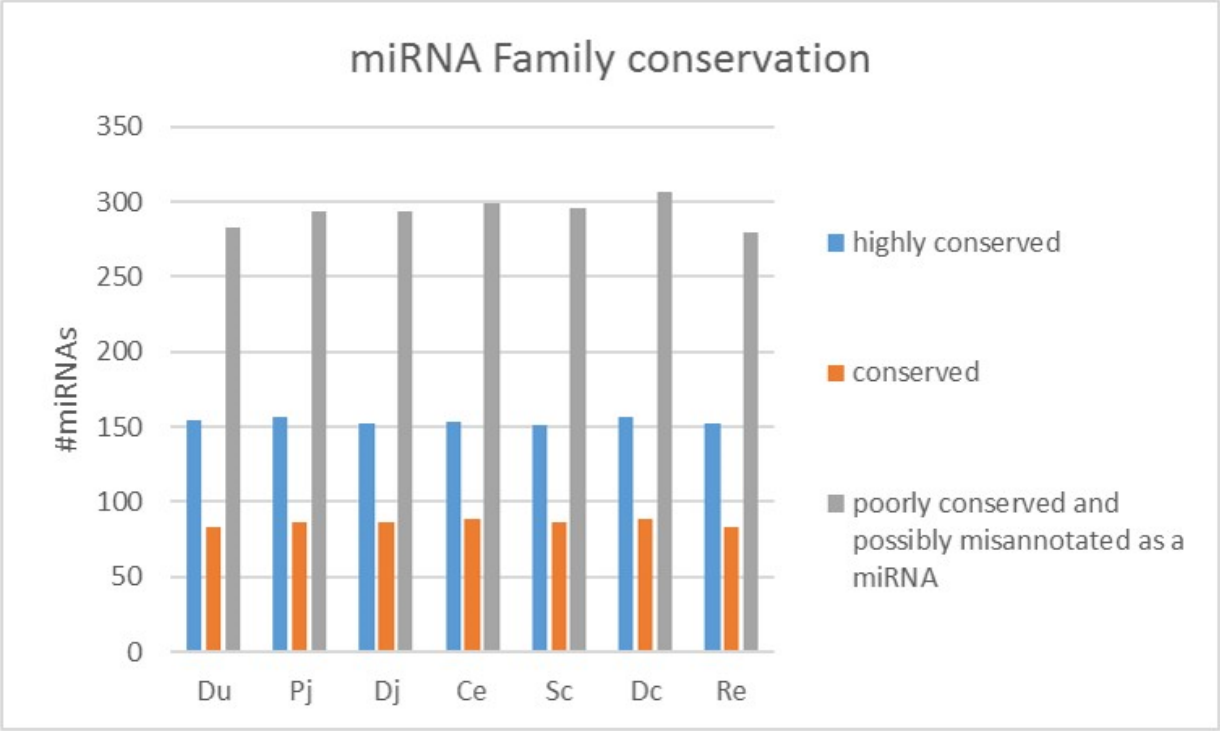


**Figure 4.2 Family conservation of detected bovine miRNAs in each region.** Du, duodenum; pj, proximal jejunum; Dj, distal jejunum; Ce, cecum; Sc, spiral colon; Dc, descending colon; Re, rectum.

# Chapter 5. Assessment of recto-anal junction mucosa associated microbiome reveals potential microbial factors associated with *Escherichia coli* O157 shedding in beef steers

## 5.1 Introduction

The metagenome of gut microbiota can be more than a hundred times greater than the genome of its host (Qin et al., 2010), and gut microbiota has been suggested to regulate the host immune system, nutrient metabolism, energy harvest, liver, muscle and even brain functions (Nicholson et al., 2012). It has been suggested that gut microbiota can inhibit pathogenic microbes through direct effects, such as releasing antimicrobials and competitive exclusion, as well as indirect effects, such as activation of host immune protection (Buffie and Pamer, 2013). Thus, investigating the composition and the function of gut microbiota can benefit our understanding of the interaction between the host and gut commensal/pathogenic microorganisms.

The foodborne pathogen, *E. coli* O157, is known to inhabit the intestinal tract of cattle without causing symptoms of illness. Howerver, it has been suggested that it can occasionally cause pathogenesis in cattle evidenced by the formation of attaching and effacing lesions at the rectum of calves and damage to intestinal epithelium of yearling cattle (Naylor et al., 2005; Baines et al., 2008). Cattle shedding > $10^4$ CFU of *E. coli* O157 per gram of feces have been defined as super-shedders (SS) (Chase-Topping et al., 2008). These cattle usually account for less than 10 % of the animals in a feedlot, but are the source of most of *E. coli* O157 that is shed into the farm environment. Furthermore, those *E. coli* O157 strains that are most commonly isolated from SS have also been reported to be associated with human infection (Chase-Topping

et al., 2008). The capability of this bacterium to cause severe human diseases, including hemolytic uremic syndrome and even death, creates an urgency to better understand super-shedding phenomena to develop control strategies for this pathogen. Studies on SS cattle have been conducted for more than a decade, but the mechanisms of super-shedding remain unclear, and few studies investigated this problem from the perspective of gut microbiota. A previous fecal microbiome study reported that the diversity of fecal microbiota between SS and cattle with negative fecal *E. coli* O157 (NS) differed, suggesting a link between gut microbiota and *E. coli* O157 shedding (Xu et al., 2014). However, whether such a link was through direct or indirect interaction was unknown. In addition, since it has been shown that mucosal and luminal microbiota differ in composition (Malmuthuge et al., 2012), RAJ mucosal associated microbiota is worthy of investigation, as *E. coli* O157 shedding has been linked to colonization at the epithelium of RAJ.

It is possible that gut microbiota play a role in regulating shedding of *E. coli* O157, as this bacterium cohabitates the host's gut with other microbial species. However, judging from the complexity of the gut microbiota, the potential of gut microbiota to regulate *E. coli* O157 shedding may not be limited to direct effects, such as competitive exclusion and byproducts from beneficial bacteria. Indeed, our previous study (Wang et al., 2016) suggested that host gene expression profiles differed between SS and NS, and the differentially expressed (DE) genes were associated with immune function, but whether such a difference is caused by host or microbial factors is unknown. In this study, we hypothesize that the RAJ mucosa-associated microbiota of NS is different from that of SS, and such difference could cause distinct microbe-to-microbe and host-microbial interactions, leading to better host intestinal barriers at RAJ of NS and a less favorable gut environment for growth of *E. coli* O157. Taxonomic profiling was

performed to characterize the mucosa associated microbiota by 16S rDNA amplicon sequencing, and the functions of the metagenome were also predicted using PICRUSt based on the identified bacterial taxa. Correlation analysis was conducted to identify potential bacterial groups and bacterial functions involved in super-shedding.

## 5.2 Materials and Methods

### 5.2.1 Animal tissue sample collection and sample preparation for sequencing

The details of the animal trial have been reported previously (Munns et al., 2014). It followed the Canadian Council of Animal Care Guidelines (Animal Care Committee protocol number: 1120) and was reviewed and approved by the Animal Care Committee of Lethbridge Research and Development Centre, Agriculture Agri-food Canada. Animal tissue sample collection and SS identification protocols have also been described in previous studies (Munns et al., 2014; Wang et al., 2016). Briefly, the fecal samples (50g) were collected from 400 beef steers in an Alberta feedlot. Plate counting was used to enumerate *E. coli* O157 using CT-SMAC agar (Dalynn Biologicals, Calgary, AB. Canada). The positive culture isolates were then confirmed with an *E. coli* O157 Latex Test kit (Oxoid Ltd, Basingstoke, Hampshire, UK), followed by further verification using a multiplex PCR targeting *VT*, *eaeA*, and *fliC*. Five of selected SS (out of 11 identified SS) were slaughtered within 11 days of being purchased, and the fecal shedding of *E. coli* O157 was monitored daily until slaughter. All the SS steers were fecal positive for *E. coli* O157 before slaughter. The RAJ tissue samples were collected and snap frozen immediately following slaughter.

The RAJ tissue samples were ground into fine powder prior to DNA extraction. DNA extraction was performed using about 100 mg of tissue and QIAamp DNA Stool Mini Kit (QIAGEN, Germantown, MD, USA). The amplicon of partial bacterial 16S rRNA gene was

generated using a pair of primers 27f-AGAGTTTGATCMTGGCTCAG, and 519r-GWATTACCGCGGCKGCTG, targeting V1-V3 hypervariable region of 16S rRNA gene (St-Pierre and Wright, 2014). The amplicon sequencing (paired end, 2 × 300) was carried out by the Genome Quebec Innovation Centre (Montreal, Quebec, Canada) using the MiSeq platform.

### 5.2.2 Sequence data processing and microbial community analysis

The removal of adapter sequences and quality control for raw sequencing reads were performed using fastq-mcf (Aronesty, 2013). The pairs of reads were joined using QIIME (quantitative insights into microbial ecology) (Caporaso et al., 2010), and the joined reads were filtered with the following criteria: the minimal length, 400; maximum length of homopolymer, 8; and reverse primers removal was enabled and the default setting was used for the rest of the parameters. Chimera detection and removal was performed using usearch61 (Edgar, 2010). The operational taxonomic units (OTUs) picking followed the de novo protocol using usearch61 at 97% identity, followed by taxonomic assignment. To evaluate the adequacy of the sequencing depth for detecting the microbes present in the sequenced samples, the good's coverage index was calculated using the formula: good's coverage = 1-S/N, where S is the number of singleton OTUs, and N is the total number of OTUs in a sample. The alpha-diversity (within sample diversity) was evaluated using Chao1 and Shannon indices. The Chao1 index estimates the number of species present in a sample, which was calculated with the formula: Chao1 = $S_{obs}$ + $N_1^2/2N_2$, where $S_{obs}$ is the total number of species detected in a sample, $N_1$ is the number of species observed once and $N_2$ is the number of species observed twice. The Shannon index estimates both the richness and evenness of a microbial community, which was calculated with the formula: Shannon = $-\Sigma P_i * \ln(P_i)$, where $P_i$ is the proportion of i-th OTU to the total number of OTUs in a sample. To evaluate beta-diversity (between samples diversity), a principal

coordinate analysis was performed based on a weighted unifrac distance. All the indices and beta-diversity were calculated using the script implemented in QIIME. For the taxonomy analysis, only taxa with a relative abundance > 0.1% in at least one sample were defined as detectable. The phylogenetic tree was built using FastTree algorithm which was implemented by QIIME.

### 5.2.3 Microbial function prediction

The PICRUSt was used to predict the functional composition of the metagenome using KEGG (Kyoto Encyclopedia of Genes and Genomes) Orthology classification schemes at the second and third KEGG Pathway hierarchy levels (Langille et al., 2013). KEGG Pathway is a collection of hierarchically classified pathway maps divided into four classification levels with the higher levels being more specific, and the third and fourth levels corresponding to individual pathway maps and KEGG orthology entries, respectively. For PICRUSt analysis, pathways belonging to human diseases, organismal systems, drug development and plant functions were filtered out, as they do not reflect microbial functions.

### 5.2.4 Differential abundance analysis and correlation analysis

All the data were presented as mean ± standard deviation unless otherwise indicated. The differential abundance analysis was performed using the statistical analysis package edgeR (Robinson et al., 2010). The edgeR modeled the read counts as a negative binomial distribution, and normalized the read counts according to sequencing depth and RNA composition by calculating the scaling factors computed by internal functions of edgeR. After data fitting to a negative binomial distribution and dispersion estimation, the differential abundance was determined by an exact test implemented in edgeR. The differential abundance analysis was performed at OTU level for taxonomic data, and the second and third KEGG pathway

hierarchical level for functional comparison (Kanehisa and Goto, 2000). Only OTUs/microbial functions with a relative abundance > 0.1% in > 50% of either group (NS or SS) were subjected to differential abundance analysis using edgeR. A p-value less than 0.1 was considered as showing statistically significant different between NS and SS. The correlation was performed using Spearman's rank correlation, and the Spearman's correlation coefficient rho-value > 0.8, and p-value < 0.01 as cutoff to select significantly correlated pairs.

## 5.3 Results

### 5.3.1 Taxonomic assessment of the microbial community of the rectum of beef steers

For joined paired-end MiSeq sequencing reads of the 9 RAJ samples, 94,512 reads passed quality filtering (Table 5.1), with a length of 478 ± 14 bases. In total, 1,278 OTUs were identified from all the samples, and the number of OTUs identified in each sample ranged from 223 (SS 294) to 523 (SS 310) (Table 5.1). In all the RAJ samples, > 99% reads were classified into 14 phyla with *Firmicutes* (61.5 ± 7.5%), *Bacteroidetes* (27.9 ± 6.4%), *Proteobacteria* (5.5 ± 2.1%) and *Spirochaetes* (2.9 ± 3.3%) being the predominant phyla (relative abundance > 1%). Only four phyla were identified in all the steers, the *Firmicutes*, *Bacteroidetes*, *Proteobacteria* and *Tenericutes.* At the family level, 66 families were detected in all the steers, with 56 classified and 10 unclassified. *Ruminococcaceae* (28.9 ± 6.8%) and *Lachnospiraceae* (9.9 ± 4.4%) from the *Firmicutes* phylum, and *Bacteroidaceae* (8.2 ± 3.4%) from the *Bacteroidetes* phylum were the most abundant families, and 13 families were identified in all steers. At the genus level, 101 genera were detected in all animals including 45 unclassified and 57 classified, with genera *5-7N15* (6.9 ± 2.4%, belonging to family of *Bacteroidaceae*), *Prevotella* (4.7 ± 4.1%), and *Ruminococcus* (3.8 ± 6.1%) being the most abundant classified genera. Seven genera were detected in all steers, including *5-7N15*, *Prevotella*, *Ruminococcus*, *CF231* (belonging to family

of *Paraprevotellaceae*), *Dorea*, *Clostridium* and *Oscillospira*. The most abundantly detected genera (36 genera) belonged to *Firmicutes*. These included 27 classified and 9 unclassified, and *Ruminococcus*, *Dorea*, and *Clostridium* were the most abundant (relative abundance ranged from 1% to 7%). Twenty-three detected genera belonged to *Bacteroidetes*, including 11 classified and 12 unclassified. Of these, *5-7N15*, *Prevotella*, and *CF231* were the most abundant (relative abundance ranged from 2% to 6%). Twenty-three genera belonged to *Proteobacteria*, including 11 classified and 12 unclassified genera, and *Pseudomonas*, *Succinivibrio*, and *Sutterella* were the most abundant (average abundance ranged from 0.5% to 0.9%).

### 5.3.2 Comparison of rectal mucosal microbiota between super-shedders and non-shedders

The good's coverage index was above 99% in all samples indicating adequate sequencing depth to represent the RAJ attached bacterial community. The Chao1 was 321.1 ± 71.4 for NS and 368.8 ± 130.6 for SS, and Shannon was 7.2 ± 0.2 for NS and 7.2 ± 0.5 for SS. Neither of the alpha diversity indices, Chao1 and Shannon, showed statistical significance (t-test, p-value > 0.05) between SS and NS, indicating no statistical difference in microbial diversity at the rectum. Animal SS 310 had the highest Chao1 value (539.2), while SS 299 had the lowest Chao1 value (241.3), indicating that mucosal bacterial populations exhibited great individual variation within SS. According to beta-diversity analysis, the SS animals formed two clusters, one cluster included SS 310, 274 and 287, and another cluster included SS 299 and 294 (Figure 5.1).

Although alpha indices were similar between NS and SS, there were differences in composition of RAJ mucosa-associated microbiota. At the phylum level, 9 phyla were detected in both NS and SS, while 4 phyla including *Elusimicrobia*, *Fusobacteria*, *Lentisphaerae*, and *OD1* (Figure 2A) were only detected in NS steers (Figure 5.2A). At the genera level, 11 genera were specific to NS, 10 were specific to SS (Table 5.2), and 36 were shared by NS and SS

(Figure 5.2B). Differential abundance analysis showed difference in 9 OTUs (P-value < 0.1), including 7 unique to NS and 2 unique to SS (Figure 5.3). However, based on the Greengene database, 7 OTUs could not be classified to genus, with only OTU108 and OTU260 mapped to *Coprococcus* and *Prevotella*, respectively. The phylogenic analysis indicated that OTU56 was closely related to *Bacteroides*; OTU121 and OTU66 were closely related to *Clostridium*; OTU45 and OTU180 were closely related to *Paludibacter*; and the OTU228 and OTU336 were clustered together, and shared common ancestors with microbes within *Proteobacteria* (Figure 5.4).

### 5.3.3 Functional prediction of mucosal attached microbiota using PICRUSt

KEGG pathways predicted by PICRUSt belonged to 23 categories (abundance > 0.1%), with the 10 most abundant categories shown in Figure 5.5A. Among these categories, six were related to metabolism, including metabolism of amino acid, carbohydrate, energy, lipid, cofactors and vitamins, as well as nucleotides; three were related to genetic information processing, including replication and repair, translation and transcription; one was membrane transport. Comparing SS and NS, two pathways had lower abundance (p-value < 0.1) in SS including signal transduction ($log_2$-fold-change: -0.15) and cell motility ($log_2$-fold-change: -0.25), whereas replication and repair ($log_2$-fold-change: 0.07) showed higher abundance in SS.

In total, 120 individual pathway maps were predicted to be associated with the RAJ microbiota (abundance > 0.1%,), and 10 the most abundant pathways are shown in Figure 5.5B. These pathways included 3 associated with environmental information processes, including ABC transporters, transporters, and two-component system; 4 associated with genetic information processes, including chromosome, DNA repair and recombination proteins, transcription factors, ribosome; and 3 associated with metabolism, including peptidases, purine metabolism, and pyrimidine metabolism. When predicted pathways were further compared between SS and NS,

12 of them had lower abundance ($P < 0.1$), while 3 of them had higher abundance ($P < 0.1$) in SS (Table 5.3). Three low abundant pathways in SS were related to cell motilities, including flagellar assembly ($log_2$-fold-change: -0.36), bacterial motility proteins ($log_2$-fold-change: -0.33), and bacterial chemotaxis ($log_2$-fold-change: -0.32). Nutrient metabolism was also predicted to differ between RAJ microbiota of SS vs NS. For example, pyruvate metabolism ($log_2$-fold-change: 0.10) showed a higher abundance in SS, while pathways associated with glycan biosysnthesis and metabolism ($log_2$-fold-change -0.29 to -0.28), tryptophan metabolism ($log_2$-fold-change: 0.17) and lipid metabolism pathways ($log_2$-fold-change: -0.27 to -0.21) showed lower abundance in SS (Table 5.3). The cellular signaling showed differences between microbiota of NS and SS as the function of two-component system was predicted to be lower ($log_2$-fold-change: -0.20) in SS (Table 5.3). In addition, the biosynthesis of ansamycins ($log_2$-fold-change: 0.21) exhibited a higher abundance in SS (Table 5.3).

### 5.3.4 Relationship between host gene expression with microbial abundance and predicted microbial functions

To further understand the association between altered RAJ mucosal associated microbiota and host functions, the expression of 58 DE genes identified between SS and NS (reported in Chapter 2) were subjected to correlation analysis with the relative abundance of microbial taxa and predicted function identified above. The relative abundance of *Succinivibrionaceae* family was negatively correlated with four DE genes, including *LOXL1*(rho-value: -0.80, p-value: 0.009), *MMP16* (rho-value: -0.88, p-value: 0.001), *MXRA8* (rho-value: -0.92, p-value < 0.001) and *HSPB6* (rho-value: -0.88, p-value: 0.002) (Figure 5.6A). Three S100 proteins genes showed correlation with several bacterial families, including *S100A8* with *Pseudomonadaceae* (rho-value: 0.80, p-value: 0.009), *S100A9* with *Ruminococcaceae* (rho-value: -0.85, p-value: 0.003)

and *Paraprevotellaceae* (rho-value: 0.87, p-value: 0.002), and *S100A12* with *Lachnospiraceae* (rho-value: 0.85, p-value: 0.004) (Figure 5.6A). For the correlation between bacterial genera and DE genes, 3 S100 proteins showed correlation with 6 genera, including *S100A8* with *Pseudomonas* (rho-value: 0.82, p-value: 0.007) and *Clostridium* (rho-value: 0.92, p-value < 0.001), *S100A9* with (rho-value: 0.82, p-value: 0.007) *Parabacteroides* (rho-value: 0.80, p-value: 0.009) and *Clostridium* (rho-value: 0.83, p-value: 0.005), and *S100A12* with *YRC22* (rho-value: 0.82, p-value: 0.007), *Dorea* (rho-value: 0.80, p-value: 0.009) and *Blautia* (rho-value: 0.80, p-value: 0.009) (Figure 5.6B).

Moreover, the expression of DE genes was correlated with the abundance of predicted microbial function. In total, 19 DE genes showed correlation with at least one bacterial function. The expression of *S100A8* and *S100A9* were both negatively correlated with the relative abundance of 5 microbial functions, including chromosome, DNA replication proteins, homologous recombination, mismatch repair and RNA polymerase (rho-values < -0.8, p-values < 0.01) (Figure 5.7A). The expression of *FCN2* was negatively correlated with relative abundance of metabolic pathways, including amino acid related enzymes, one carbon pool by folate, terpenoid backbone biosynthesis, and thiamine metabolism (rho-values < -0.8, p-value < 0.01); and positively correlated with glyoxylate and dicarboxylate metabolism, and base excision repair (rho-values > 0.8, p-value < 0.01) (Figure 5.7B). The expression of *SFRP2* was positively correlated with relative abundance of microbial amino acid metabolism pathways (i.e., valine, leucine and isoleucine, tryptophan, and lysine metabolism; rho-values > 0.8, p-values < 0.01) (Figure 5.7C). The expression of cytokine genes, *CCL19* and *CXCL13*, were positively correlated with microbial glycosyltransferases (rho-values > 0.8, p-values < 0.01), the enzymes involved in glycan biosynthesis (Figure 5.7D). The relative abundance of bacterial pathway,

butanoate metabolism, was positively correlated with the expression of *GIMAP5*, *CD69* and *THEMIS* (rho-value < -0.8, p-values < 0.01) (Figure 5.7D).

**5.4 Discussion**

This study characterized the RAJ mucosa associated bacterial community of NS and SS yearling beef steers, with the aim to identify whether the composition of gut microbiota could be associated with the *E. coli* O157 shedding. The findings indicated that members of *Firmicutes*, *Bacteroidetes*, and *Proteobacteria* were the core microbes of RAJ mucosa associated microbiota, which is consistent with previous studies of in cattle (Shanks et al., 2011; Mao et al., 2015; Kaevska et al., 2016). In fact, studies on human and mice also indicated that these microbial groups were the predominant phyla in the gut, suggesting they play an important role in mammals (Ley et al., 2005). The current study and that of Mao et al (2015) showed consistent findings with predominance of *Bacteroidetes* and *Firmicutes* genera at the rectum of cattle. For example, the *Prevotella* and unclassified genera of *Prevotellaceae* and *Rikenellaceae* were the predominant genera belonging to *Bacteroidetes*, although the relative abundance of *Prevotella* was > 5% in current results but it was < 2% in study by Mao et al (2015). Also, in Mao et al.'s (2015) study, the unclassified genera of *Ruminococcaceae* and *Lachnospiraceae* were the most abundant *Firmicutes*, which was in agreement with our findings. However, for *Proteobacteria*, there was more variation. Mao et al. (2015) reported that *Campylobacter*, *Desulfobulbus*, and *Acinetobacter* were the predominant *Proteobacteria* genera, while in this study *Pseudomonas*, *Succinivibrio*, and *Sutterella* were more predominant. Because the study by Mao et al. (2015) focused on dairy cattle while this study focused on beef cattle, these results suggest that beef and dairy cattle may have similar gut microbial groups, but the relative abundance of them differs. The higher abundance of *Prevotella* in rectal content of beef cattle compared with dairy cattle

was also reported by Durso et al. (2010). Xu et al. (2014) investigated the rectal fecal microbiota of the same beef steers, suggesting *Firmicutes*, *Bacteroidetes*, and *Proteobacteria* are the most predominant phyla, similar to our results for mucosa associated microbiota in beef cattle. Nevertheless, the relative abundance of *Firmicutes, Bacteroidetes,* and *Proteobacteria* were 53.9%, 35.6% and 1.7% in fecal samples (Xu et al., 2014), respectively, while they were 61.5%, 27.9% and 5.5% in mucosa, respectively, suggesting higher numbers of *Firmicutes* and *Proteobacteria*, and lower numbers of *Bacteroidetes* associated with the mucosa of RAJ.

For the RAJ mucosa associated microbiota, large animal to animal variations were indicated by the taxonomic and beta-diversity analysis. Even for the predominant taxa shared by all the animals, the relative abundance varied from individual to individual. Such individual variation in fecal microbiota was previously reported in cattle by Durso et al. (2010) who suggested that individual animal variation cannot be simply attributed to breed, gender, diet, age or environmental factors. As several of these variables were controlled in this study, these findings further support the position of Durso et al. (2010).

When comparing NS and SS, the diversity indices did not statistically differ, suggesting that the estimated richness and evenness of identified species at RAJ of NS and SS were comparable. Although the richness and evenness of microbes were not statistically significant, it is still possible that different microbial groups made up the RAJ microbial community in SS and NS, and that the unique microbes associated with NS may have reduced the colonization of *E. coli* O157. The OTU108, which is unique to NS, which has been previously documented in the feces of cattle (Kim and Wells, 2016). *Coprococcus* spp. produces butyric acid and can lower the pH of the media when cultured *in vitro* (Holdeman and Morre, 1974), which may lead to an gut environment unfavorable to *E. coli* O157. The OTU45 and OTU180 were also unique to NS and

were closely related to *Paludibacter*, a genus previously detected in colonic mucosa of cattle (Reti et al., 2013) and reported to produce propionate (Ueki et al., 2006). The short chain fatty acids, including butyric and propionic acid, have been suggested to decrease the shedding of *E. coli* O157 in cattle (Jacob et al., 2009). Thus, the *Coprococcus* and *Paludibacter* that are corresponding to OTU108, OTU45 and OTU180 may play a protective role against colonization of *E. coli* O157 at RAJ of NS. Indeed, the volatile fatty acid production by gut microbiota was suggested by many studies as an important factor influencing the shedding of *E. coli* O157 in cattle (Kudva et al., 1997; Cobbold and Desmarchelier, 2004; Bach et al., 2005). Low levels or absence of these VFA producing microbes at the RAJ of SS may be a factor that enables *E. coli* O157 to proliferate in SS. Further research, such as *in vitro* tests are needed to confirm the capability of these microbes to inhibit the growth of *E. coli* O157.

The functional predictions revealed that the most abundant microbial functions of RAJ mucosa microbiota included metabolism, genetic information processing and cellular processes and signaling. Several microbial pathways with differential abundance between SS and NS may be potentially associated with *E. coli* O157 shedding, including lipopolysaccharide biosynthesis, biosynthesis of ansamycins, and carbohydrate metabolism. The gene families associated with lipopolysaccharide (LPS) biosynthesis had lower abundance for the RAJ microbiota of SS, which may suggest lower abundance of Gram-negative bacterial strains at the RAJ of SS. On one hand, lower LPS biosynthesis could be beneficial to the host because it is an endotoxin that induces release of proinflammatory cytokines (Cani et al., 2007). However, less Gram-negative bacteria at the RAJ of SS may not create an environment that competitively excludes *E. coli* O157, as Zhao et al. (1998) reported that 18 fecal bacteria (screened from 1200 isolates from gut tissues and fecal samples of non-shedder cattle) capable of inhibiting the growth of *E. coli* O157

were Gram-negative. More interestingly, 17 out of the 18 isolates screened were non-pathogenic *E. coli* strains (Zhao et al., 1998), possibly due to production of colicin by certain *E. coli* strains (Schamberger et al., 2004). The higher abundance of microbial gene families associated with biosynthesis of ansamycins was shown in SS, and ansamycins were reported to have antimicrobial activity towards many Gram-positive bacteria and bacteriophages (Price et al., 1977; Wehrli, 1977). However, certain Gram-positive bacteria, such as *Lactobacillus* (Peterson et al., 2007) and *Bifidobacterium* (Fukuda et al., 2011), and T1-like bacteriophages (Niu et al., 2014), were reported to have inhibitory effect on the growth and shedding of *E. coli* O157 in cattle. Thus, a higher potential of RAJ mucosal microbiota of SS to produce ansamycins may put beneficial Gram-positive bacteria and bacteriophage at a disadvantage, contributing to the growth of *E. coli* O157 and super-shedding. It is also interesting that the gene families associated with carbohydrate, lipid and amino acid metabolism showed differential abundance between RAJ mucosal microbiota of SS and NS. This may indicate that microbial nutrient metabolism by RAJ mucosal microbiota is an important factor associated with shedding of *E. coli* O157. The products of such metabolism, such as short chain/branched-chain fatty acids and biogenic amines can cause changes in intestinal environment, such as pH which may mediate the growth of *E. coli* O157 (Neis et al., 2015). These bacterial metabolites can also impact the physiology of the host, by providing energy to epithelial cells and modulating the development of the mucosal immune system and intestinal barriers, eventually influencing the colonization of *E. coli* O157 (Tremaroli and Bäckhed, 2012). Further studies are required to investigate the rectal microbial functional composition using metagenomics and metabolomics methods, as well as using larger numbers of animals for comparisons of population of *E. coli* O157 and potential beneficial bacteria.

The gut microbiota can influence host gut physiologies, such as energy metabolism, mucosal immune system development, gut epithelial cell proliferation and gene expression (Nicholson et al., 2012). Thus, it is possible that certain microbial groups and microbial functions may influence RAJ physiology of SS by affecting host gene expression, leading to super-shedding. Because the DE genes used for correlation analysis showed significant different expression level between SS and NS (reported in Chapter 2), it is reasonable to speculate that the abundance of correlated microbial groups/functions may also be potentially different. It is worth noting that the genes encoding S100 family of proteins, including *S100A8*, *S100A9*, and *S100A12* were correlated with abundance of several microbial genera, such as *Clostridium* and *Pseudomonas*. The members of S100 protein family are associated with variety of functions, including protein phosphorylation, cell trafficking, cell proliferation and differentiation (Donato, 2001). *Clostridium* was suggested as one of essential butyrate producers in the human gut microbiota (Pryde et al., 2002), and butyrate-producing *Clostridium* spp. specifically colonized the mucins in an *in vitro* gut model (Van den Abbeele et al., 2013). Microbial butyrate was suggested to enhance the tight junctions of the epithelium, and act as a chemoattractant to neutrophils and provide energy to the epithelial cells (Lopetuso et al., 2013). The neutrophils and epithelial cells are the two major type of host cells that express S100A8 and S100A9 (Donato, 2001). Thus, butyrate production may be a potential mechanism of the observed positive correlation between *S100A8*/*S100A9* and *Clostridium.* More importantly, although the expression of S100A8/S100A9 by leukocytes was suggested to be associated with inflammation (Gebhardt et al., 2006), the expression and release of S100 proteins by epithelial cells were suggested to serve as antimicrobials to maintain intestinal mucosal homeostasis (Cua and Tato, 2010). It is possible that the butyrate-producing *Clostridium* strains had higher abundance (as it was

positively correlated with *S100A8*/*S100A9* which had higher expression in NS) at the RAJ of NS, leading to the development of stronger intestinal barrier against colonization by pathogens, including *E. coli* O157 (Peng et al., 2009). However, the quantification of *Clostridium* using qPCR measurement did not show a statistical difference in the rectal populations of SS and NS (measured using qPCR, compared using t-test, p-value > 0.05, data not shown), which is also in agreement with sequencing data (compared using Mann-Whitney U test, P > 0.05). Nevertheless, OTU121 and OTU66, which were closely related to *Clostridium*, were unique to SS and NS, respectively, suggesting different composition of *Clostridium* spp. between SS and NS. It is possible that although the total number of *Clostridium* is comparable between SS and NS, different *Clostridium* spp. were harbored by SS and NS, leading to differential abundance of butyric acid from *Clostridium*. Further characterization of *Clostridium* genus at RAJ of SS and NS is needed to identify the role of *Clostridium* in super-shedding phenomena.

The pathways associated with bacterial DNA replication and RNA expression were negatively correlated with expression of *S100A8* and *S100A9*, indicating that bacterial proliferation and gene expression may influence host gene expression. Also, the predicted microbial pathways, which were associated with metabolism of amino acids, vitamin B and butyrate, were correlated with expression of host genes, suggesting that bacterial metabolism plays a regulatory role in host cell gene expression and physiology. The growth of gut microbiota has long been suggested to be associated with host innate (Chu and Mazmanian, 2013) and adaptive immune system development (Brestoff and Artis, 2013). Microbial nutrient metabolism and vitamin synthesis, as well as butyrate production were also reported to influence host immune system development, energy harvest, epithelial cell proliferation, and even the development of disease (Brestoff and Artis, 2013). The findings here suggest that the difference

in the functions of gut microbiota between SS and NS may contribute to the differential expression of genes in RAJ, especially those associated with microbial functions, such as cell proliferation, nutrient, vitamin and butyrate metabolism.

Our previous speculation was that the host genetic variation might lead to the differential expression of host genes (Wang et al., 2016), and current results suggested that the gut microbiota and their functions are also an important factor. It is interesting that studies on using probiotics and dietary modification to reduce *E. coli* O157 shedding in ruminants reported inconsistent results, as reviewed by Sargeant el al. (2007), only 16 out of 27 probiotics additives decreased *E. coli* O157 shedding through probiotic inoculation or diet changes. It is possible that probiotic and dietary changes may not directly affect the growth/colonization of *E. coli* O157, but rather change the composition of the gut microbiota, which in turn reduces shedding through modifying gut environment and/or enhanced host intestinal barriers. Thus, the studies which suggested little effect of using probiotics and dietary modification may have performed their experiment on older cattle with gut microbiota more resistant to probiotic treatment and diet modification. Nevertheless, our findings still support the validity of using microbial treatment and dietary modification to control *E. coli* O157 shedding, but we suggest to also focus on their efficacy on modifying the gut microbiota to a composition less favorable to the survival of *E. coli* O157, besides their potential to eliminate *E. coli* O157 directly.

**5.5 Conclusion**

In conclusion, this study characterized the rectal mucosa associated microbiota of beef steers. The results indicated that the members of *Firmicutes*, *Bacteroidetes*, and *Proteobacteria* represent the majority population of RAJ microbiota, and metabolism, genetic information processing and cellular signaling were the most abundant microbial functions. The findings

indicated that the mucosa microbiota of RAJ differed between SS and NS, possibly leading to different abundance of gene families that constitute the metagenome. The microbiota may influence the growth and colonization of *E. coli* O157 at RAJ through two mechanisms. First, certain commensal bacteria may enhance host immune system and intestinal barriers to reduce *E. coli* O157 growth and colonization, such as the short chain fatty acids producing *Coprococcus* and *Paludibacter*. Secondly, the microbial metabolites, such as short chain fatty acids and antimicrobials, may shape the gut environment of cattle to inhibit/enhance the growth of *E. coli* O157. Although studies on using probiotic treatment against *E. coli* O157 shedding showed inconsistent results, our findings support the validity of using such strategies to control *E. coli* O157 shedding, but we suggest to also focus on their efficacy on modifying the gut microbiota, besides their potential to directly eliminate *E. coli* O157.

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## 5.7 Tables and figures

**Table 5.1 Sequencing results and alpha diversity indices for all steers.**

| Animal_id | #Reads* | #OTU | Good's coverage | Chao1 | Shannon |
|---|---|---|---|---|---|
| **NS108_NS** | 10,199 | 407 | > 99% | 435.5 | 7.6 |
| **NS152_NS** | 7,795 | 239 | > 99% | 260.0 | 7.2 |
| **NS165_NS** | 10,175 | 275 | > 99% | 285.5 | 7.3 |
| **NS242_NS** | 10,515 | 296 | > 99% | 310.0 | 7.0 |
| **SS274_SS** | 9,919 | 372 | > 99% | 391.3 | 7.6 |
| **SS287_SS** | 12,468 | 409 | > 99% | 431.7 | 7.1 |
| **SS294_SS** | 10,308 | 223 | > 99% | 255.7 | 6.8 |
| **SS299_SS** | 11,339 | 225 | > 99% | 241.3 | 6.8 |
| **SS310_SS** | 11,794 | 523 | > 99% | 539.2 | 7.7 |

*Number of reads passed quality filtering

**Table 5.2 Non-shedders and super-shedders specific genera, and the microbial families and phyla that these genera belonged to.**

| **NS specific microbe** | | |
|---|---|---|
| **Phylum** | **Family** | **Genus** |
| *Actinobacteria* | *Intrasporangiaceae* | *Janibacter* |
| *Bacteroidetes* | *Paraprevotellaceae* | *Paraprevotella* |
| *Bacteroidetes* | *Sphingobacteriaceae* | *Pedobacter* |
| *Firmicutes* | *Lachnospiraceae* | *Epulopiscium* |
| *Firmicutes* | *Veillonellaceae* | *Mitsuokella* |
| *Firmicutes* | *Ruminococcaceae* | *Faecalibacterium* |
| *Fusobacteria* | *Fusobacteriaceae* | *u114* |
| *Proteobacteria* | *Bradyrhizobiaceae* | *Bradyrhizobium* |
| *Proteobacteria* | *Alcaligenaceae* | *Pelistega* |
| *Proteobacteria* | *Bdellovibrionaceae* | *Bdellovibrio* |
| *Proteobacteria* | *Shewanellaceae* | *Shewanella* |
| **SS specific microbes** | | |
| **Phylum** | **Family** | **Genus** |
| *Actinobacteria* | *Corynebacteriaceae* | *Corynebacterium* |
| *Actinobacteria* | *Gordoniaceae* | *Gordonia* |
| *Actinobacteria* | *Propionibacteriaceae* | *Luteococcus* |
| *Firmicutes* | *Lactobacillaceae* | *Lactobacillus* |
| *Firmicutes* | *Erysipelotrichaceae* | *Coprobacillus* |
| *Firmicutes* | *Staphylococcaceae* | *Jeotgalicoccus* |
| *Firmicutes* | *Veillonellaceae* | *Acidaminococcus* |
| *Firmicutes* | *Mogibacteriaceae* | *Anaerovorax* |
| *Proteobacteria* | *Moraxellaceae* | *Acinetobacter* |
| *Proteobacteria* | *Rhodobacterales* | *Rhodobacter* |

**Table 5.3 KEGG pathways that showed differential abundance between the rectal microbiota of SS and NS.**

| Level 2 | Level 3 | Abundance* | logFC** | P-Value |
|---|---|---|---|---|
| **Amino Acid Metabolism** | Tryptophan metabolism | 0.17 | -0.32 | 0.09 |
| **Carbohydrate Metabolism** | Pyruvate metabolism | 1.07 | 0.10 | 0.09 |
| **Cell Motility** | Bacterial chemotaxis | 0.62 | -0.32 | 0.05 |
| | Bacterial motility proteins | 1.27 | -0.33 | 0.04 |
| | Flagellar assembly | 0.55 | -0.36 | 0.06 |
| **Glycan Biosynthesis and Metabolism** | Lipopolysaccharide biosynthesis | 0.21 | -0.28 | 0.07 |
| | Lipopolysaccharide biosynthesis proteins | 0.30 | -0.29 | 0.02 |
| **Lipid Metabolism** | Biosynthesis of unsaturated fatty acids | 0.14 | -0.27 | 0.07 |
| | Sphingolipid metabolism | 0.21 | -0.21 | 0.06 |
| **Membrane Transport** | Secretion system | 1.35 | -0.14 | 0.07 |
| **Metabolism of Other Amino Acids** | Glutathione metabolism | 0.18 | -0.25 | 0.04 |
| **Metabolism of Terpenoids and Polyketides** | Biosynthesis of ansamycins | 0.12 | 0.21 | 0.07 |
| **Signal Transduction** | Two-component system | 1.58 | -0.20 | 0.02 |
| **Xenobiotics Biodegradation and Metabolism** | Aminobenzoate degradation | 0.13 | -0.26 | 0.06 |
| | Naphthalene degradation | 0.16 | 0.23 | 0.07 |

*Average abundance among all the steers

** log2-fold-change

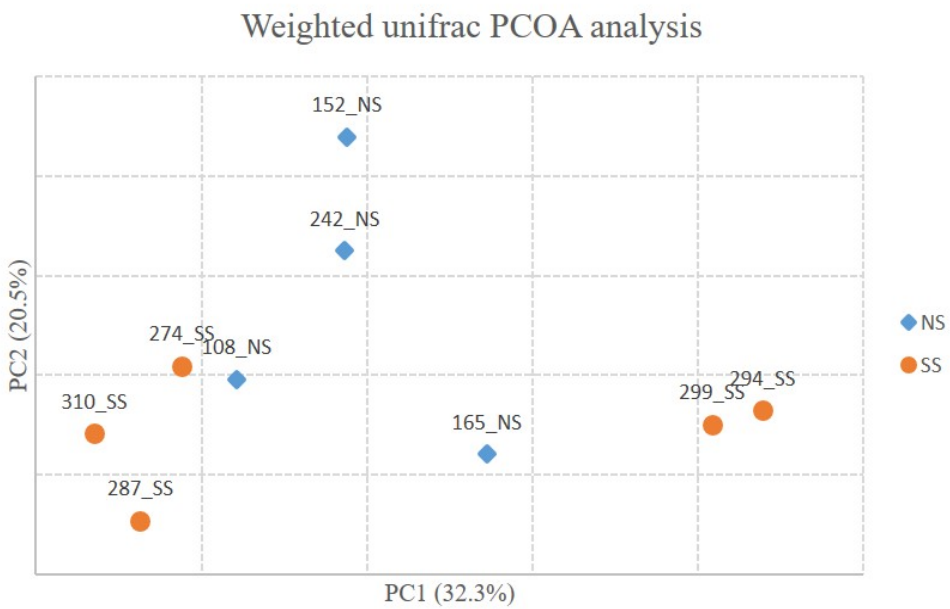


**Figure 5.1 Principal coordinator analysis based on unifrac distance for OTUs identified for recto-anal junction tissue.** NS: non-shedders; SS: super-shedders.

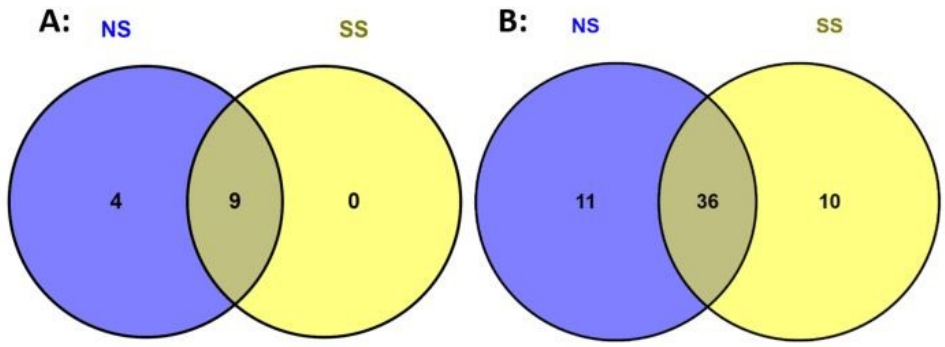


**Figure 5.2 Taxonomic analysis of recto-anal junction tissue collected from super-shedders and non-shedders.** (A) Phyla detected in non-shedders and super-shedders. Detected phyla: relative abundance > 0.1% in at least one non-shedder or super-shedder. (B) Genera detected in non-shedders and super-shedders. Detected genera: relative abundance > 0.1% in at least one non-shedder or super-shedder. NS: non-shedders; SS: super-shedders.

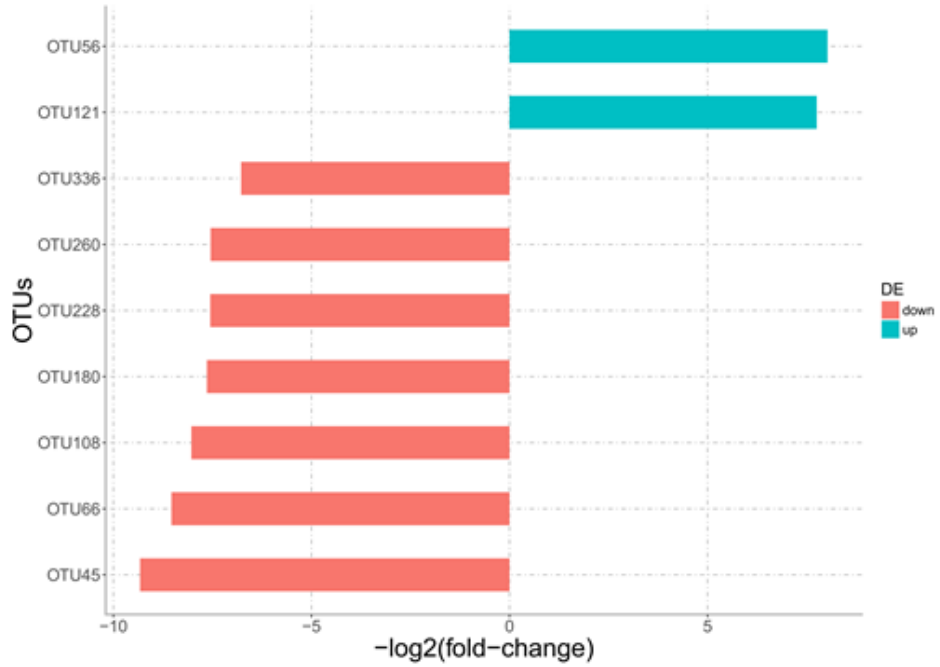


**Figure 5.3 Differentially abundant OTUs between non-shedders and super-shedders.** The blue bars indicate that the OTUs were more abundant in super-shedders, and red bars indicate OTUs were less abundant in super-shedders.

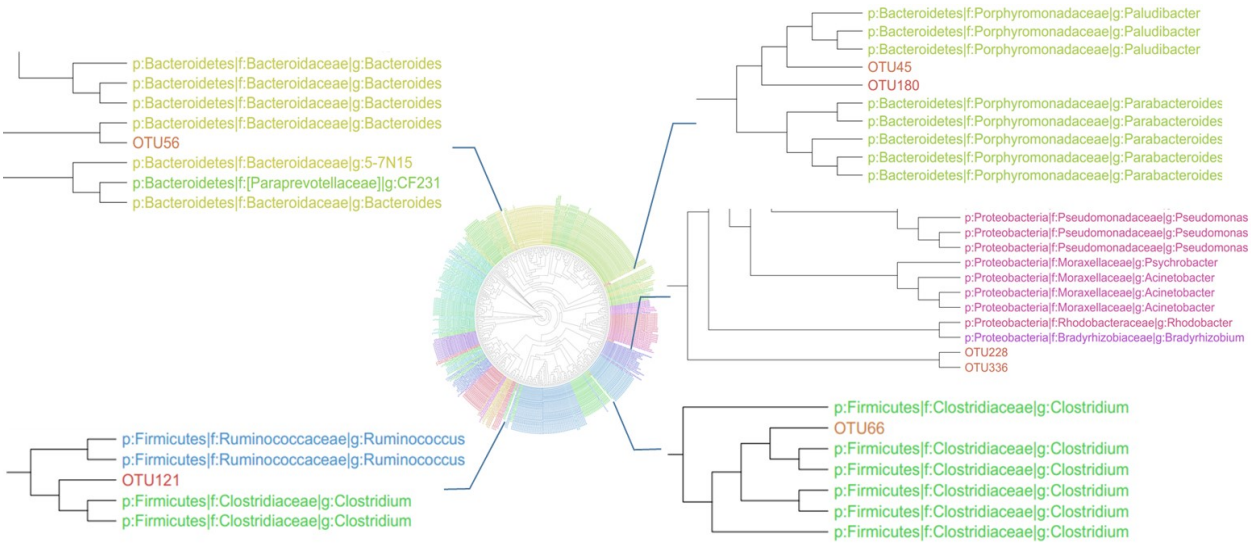


Figure 5.4 Phylogenetic tree built based on sequence of OTUs assigned to known bacterial genera.

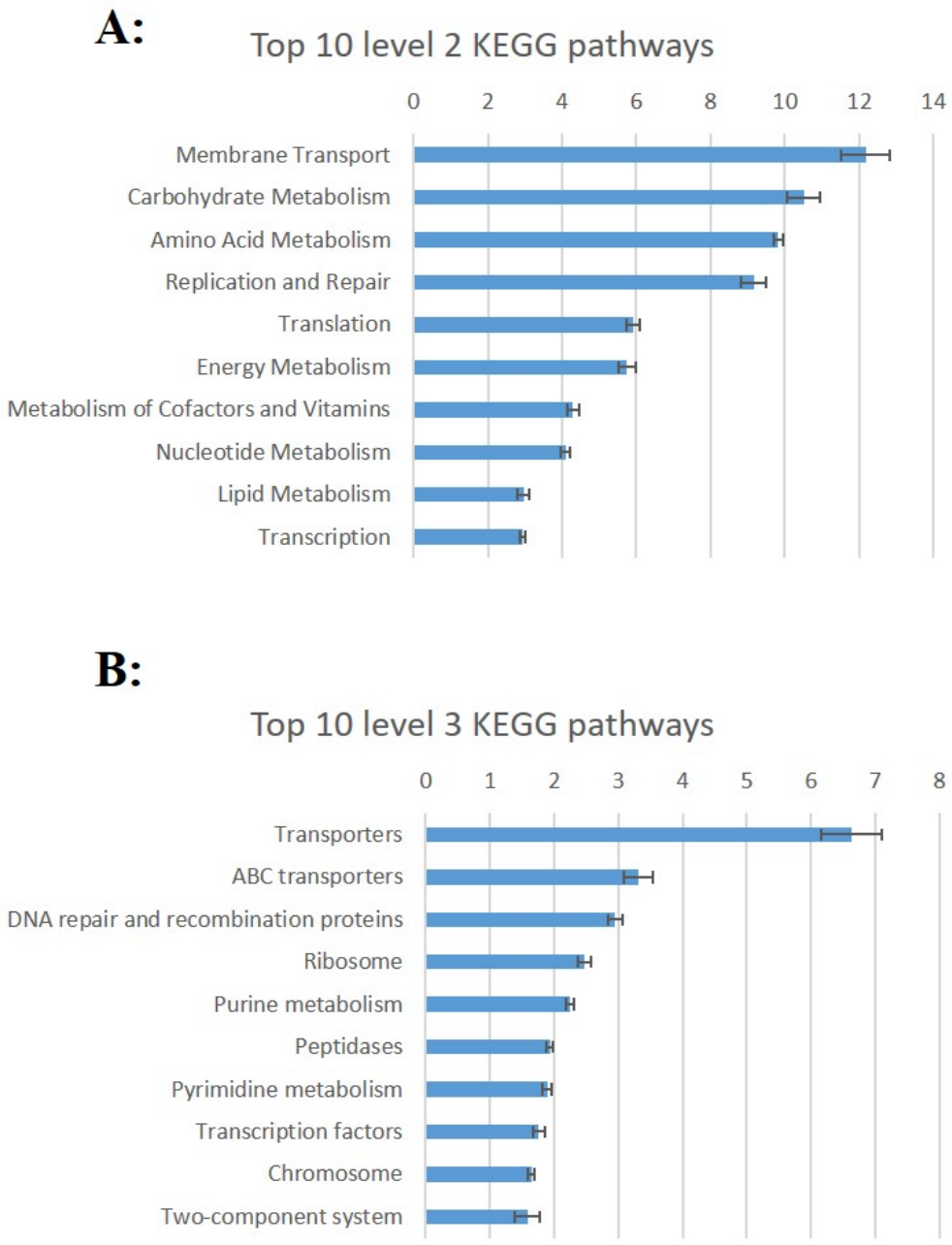


Figure 5.5 (A) The top ten predicted metagenomic functions at level 2 of the KEGG Pathway.

(B) The top ten predicted metagenomic functions at level 3 of KEGG Pathway.

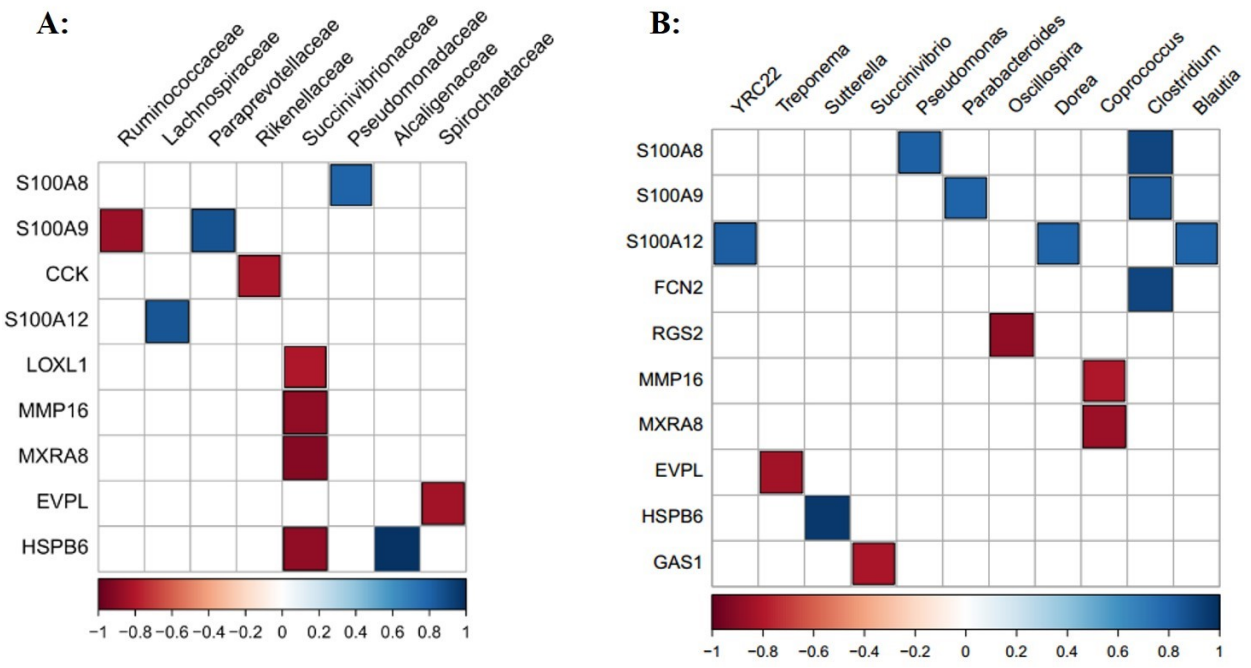


**Figure 5.6 (A) Correlation analysis results of differentially expressed genes identified in rectal-anal junction samples and microbial abundance at the family level. (B) Correlation analysis results of differentially expressed genes identified in rectal-anal junction samples and microbial abundance at the genus level.** Color bars indicate Spearman correlation coefficient rho-value, and only correlation with rho-value > 0.8 and p-value < 0.01 were indicated by blue/red colors. Genes/microbial families that did not show significant correlation are not shown.

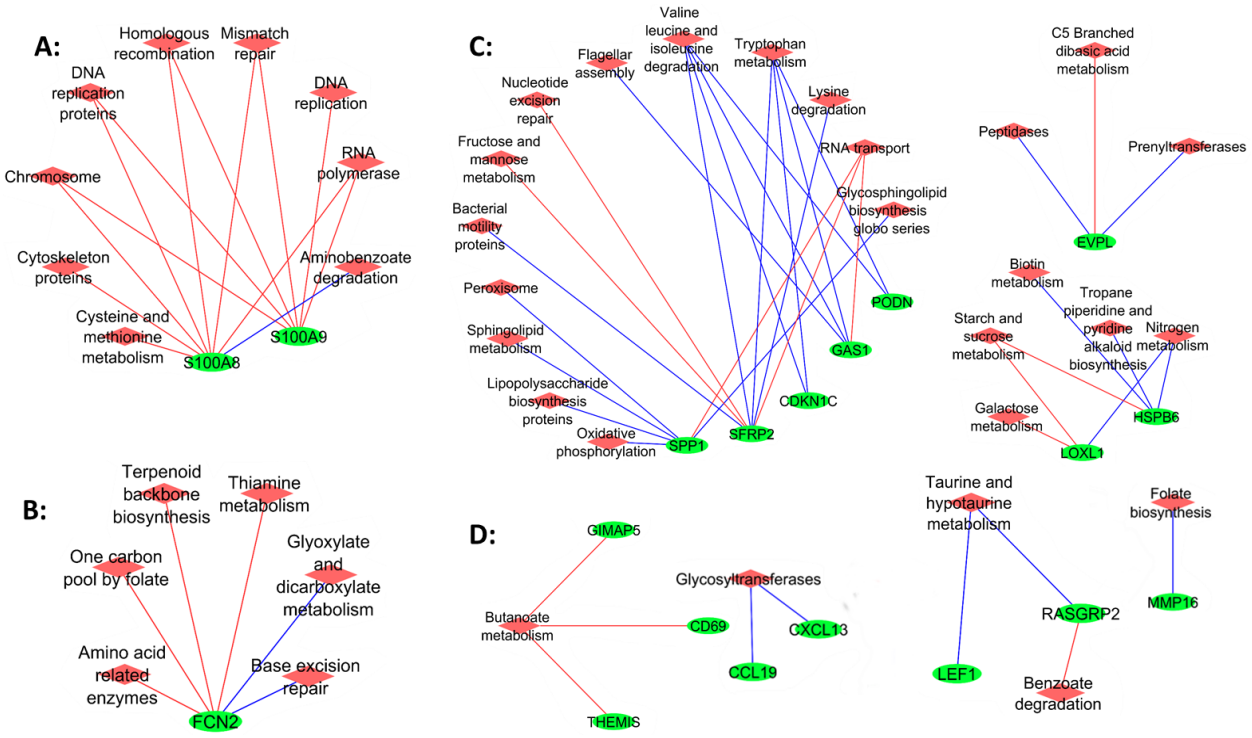


**Figure 5.7 Correlation networks for differentially expressed genes identified in rectal-anal junction samples and predicted metagenomic functions at KEGG pathway hierarchical level 3.** The green circles were differentially expressed genes, and the red diamonds are the predicted pathway for the metagenome. The blue lines indicat positive correlation and red lines indicates negative correlation. Only correlation with rho-value > 0.8 and p-value < 0.01 are shown in these figures. Genes/functions that did not show significant correlation are not shown.

# Chapter 6. General discussion

*E. coli* O157 shedding in cattle is a complicated biological process, which is also the origin of the transmission of this foodborne pathogen that has been directly associated with many past outbreaks. *E. coli* O157 contamination is a serious issue threatening food safety, but the host mechanisms associated with super-shedding are unclear. Identifying the host mechanisms that may regulate this process is vital to provide the fundamental knowledge for future on-farm intervention strategies. In this thesis, I performed studies to elucidate the host mechanisms through studying host gene expression and miRNA driven posttranscriptional regulation in the whole gastrointestinal tract, as well as host-microbial interactions at the recto-anal junction (RAJ), a major site of *E. coli* O157 colonization. The super-shedder (SS) used in this study were naturally colonized by *E. coli* O157 and were selected from 400 beef cattle fed a barley grain-based diet in a southern Alberta feedlot. It has been reported that SS represent the minor population (less than 10%) of the herd (Omisakin et al., 2003; Chase-Topping et al., 2007; Stephens et al., 2009; Munns et al., 2015), suggesting that certain individual cattle have a higher propensity to become SS. Thus, the intrinsic mechanisms of the host are associated with the propensity to shed high levels of *E. coli* O157. The transcriptome and miRNAome profiling data obtained from this thesis suggest that the host immune systems and cholesterol metabolism in the gastrointestinal tract may be associated with super-shedding of *E. coli* O157 in cattle. In addition, the RAJ mucosal tissue associated microbiome and its predicted functions also revealed the potential role of bacteria to contribute to host defense against *E. coli* O157. The RAJ mucosal microbiota could influence host immune functions through affecting host gene expression to impact the colonization of *E. coli* O157, or it may influence *E. coli* O157 growth and shedding through competitive exclusion or modifying the gut environment (Figure 6.1).

### 6.1 Altered gene expression in the gut of super-shedder compared with non-shedder cattle

As described in Chapter 2, gene expression of the RAJ tissue from SS and NS was compared, as RAJ has been suggested to be the primary colonization site of *E. coli* O157 (Greenquist et al., 2005; Naylor et al., 2005; Davis et al., 2006; Lim et al., 2007). The findings in Chapter 2 indicate that genes involved in humoral and cell-mediated immune functions decreased in the RAJ of SS, suggesting that SS may fail to mount adequate immune responses against *E. coli* O157 colonization. However, investigation only of the RAJ cannot generate a complete understanding of the mechanisms involved in the apparent tropism of *E. coli* O157 colonization towards RAJ. It has been reported that attaching and effacing (A/E) lesions have been detected in the ileum and rectum of *E. coli* O157 challenged cattle (Phillips et al., 2000; Naylor et al., 2005). Also, epithelium damage caused by *E. coli* O157 in the jejunum, ileum and colon of yearling steers challenged with this bacterium have also been reported (Baines et al., 2008). Therefore, as described in Chapter 3, transcriptomic analysis of the whole intestinal tract was undertaken, and the results suggest that in regions anterior (the distal jejunum and descending colon) to the rectum, the functions involved in cell-mediated responses may be increased in SS. In Chapter 3, results revealed that most of the differentially expressed (DE) genes between SS and NS were identified in the distal jejunum, suggesting that in addition to RAJ, the functions of distal jejunum may also affect *E. coli* O157 super-shedding. A possible reason is that the distal jejunum is close to the ileum, and the mucosal immune surveillance components, Peyer's patches (PP), which sense pathogens and induce immune responses against them (Jung et al., 2010), are present in these regions (Mutwiri et al., 1999). Although it is important to investigate the ileum to study the effects of PP in super-shedding phenomena, due to SRM (specified risk material) regulations in Canada, the ileum of SS cannot be transported to

our research facility. Further research is needed to study ileum of SS to better our understanding of the role of PP in *E. coli* O157 colonization and shedding in cattle. In addition, a potential for increased T-cell responses in the descending colon of SS was also identified by DE genes, suggesting that immune responses may also be induced by *E. coli* O157 in the colon of SS. Such differences between RAJ and other intestinal regions suggest a possible mechanism of *E. coli* O157 colonization tropism: in SS, the RAJ mounted less-effective immune protection than the small intestine and other regions of the large intestine, rendering the RAJ of SS a niche for *E. coli* O157 to survive, colonize and proliferate.

Many other studies on SS have focused on immune responses (Hoffman et al., 2006; Nart et al., 2008b; Nart et al., 2008a; Corbishley et al., 2014), and our findings in Chapter 3 suggest that in addition to immune function, host cholesterol metabolism may also play a role in shedding of *E. coli* O157 in SS. The DE genes identified in the distal jejunum indicate the probability of inhibited LXR/RXR activation pathway, and the DE genes in the descending colon suggest increased cholesterol transportation in SS. A possible mechanism is that cholesterol is required for cell membrane construction and cell proliferation, and thus increased sterol absorption/synthesis may act as a stimulus for lymphocyte proliferation (Bensinger et al., 2008). Also, the microvilli of epithelial cells are rich in cholesterol (Koichi et al., 1974), and as the A/E lesions caused by *E. coli* O157 colonization disrupt the microvilli, and so the renewal of epithelial cells may lead to increased cholesterol absorption. However, no difference in cholesterol concentration was detected in these tissues (distal jejunum and descending colon) between SS and NS, one possibility could be that the absorption/synthesis of cholesterol may have increased in the epithelial tissues of SS, but it may have been utilized during the host immune responses (Bensinger et al., 2008) or transported to the liver via mesenteric lymph upon

absorption. Therefore, understanding the liver function can provide further evidence on altered cholesterol pathway, RNA-Seq was performed for liver tissues collected from SS and NS, but the functional analysis was not included in the thesis. Future studies to measure cholesterol in liver or blood may also provide insights into how cholesterol absorption could impact *E. coli* O157 shedding. In addition, it was uncertain whether *E. coli* O157 colonized the distal jejunum and descending colon in the SS that were used in this study, further work is needed to validate the potential association between cholesterol metabolism and *E. coli* O157 colonization and shedding.

Overall, findings on altered gene expression in SS compared with NS as described in Chapters 2 and 3 suggest that biological processes (e.g. immune functions, lipid metabolisms) may potentially differ between SS and NS in the gut. Some researchers have suggested that super-shedding may be the result of microbial effects, as the *E. coli* O157 possess the non-LEE-encoded type III secretive proteins and Shiga toxins which are reported to suppress host lymphocyte responses (Menge et al., 2003; Hoffman et al., 2006; Walle et al., 2013). However, if the suppression of immune functions in SS is only caused by such microbial effects, it is difficult to explain why only less than 10% of the steers (11 out of 400 in my study) are SS, as all the steers were fed with the same diet and raised in the same farm, and they should have equal risk of ingesting *E. coli* O157. Therefore, we speculated that host genetics are involved in the observed differential gene expression between SS and NS, in addition to the microbial effects. Indeed, in Chapter 3, we identified the association between SNPs (single nucleotide polymorphisms) in the DE genes and the SS status. Although we cannot conclude that the identified SNPs are responsible for the differences in expression of genes due to the small sample size, our findings justify further research on host genetic variation and the association

with *E. coli* O157 shedding. This is the first study to pinpoint some evidence that host genetic variation and gene expression are associated with *E. coli* O157 shedding, and such findings provide information about candidate SNPs for further validation studies using larger populations.

### 6.2 Altered miRNA expression in the gut of super-shedder compared with non-shedder cattle

As described above, microbial immunomodulatory effects by *E. coli* O157 cannot fully explain why only a small number of individual cattle become SS. In addition to genetic variation (Chapter 3) and altered gene expression (Chapters 2 and 3), the findings in Chapter 4 indicate that miRNA regulation can contribute to differential gene expression between SS and NS. Firstly, the comparative analysis for the miRNA expression profiles between NS and SS indicate that the distal jejunum and RAJ were the regions where most DE miRNAs were identified. This is consistent with the altered gene expression patterns identified in Chapters 2 and 3 where most of the DE genes identified. Secondly, some of the identified DE genes were the putative targets of these DE miRNAs including bta-miR-18a, bta-miR-378b and bta-miR-2284d based on computational prediction and correlation analysis between miRNAs and predicted target mRNAs. In the distal jejunum, the DE gene, *F3* was potentially targeted by bta-miR-18a and bta-miR-2284d; the *PTGS2* was potentially targeted by bta-miR-2284d; *THEMIS* and *ITK* were potential targets of bta-miR-378b. In the RAJ, *SIT1* and *RGS13* were putative targets of bta-miR-1271 and bta-miR-29d-3p, respectively. These DE genes were associated with potentially altered immune functions and LXR/RXR pathway reported in Chapters 2 and 3. In addition, the DE miRNAs, bta-miR-378b, bta-miR-2284j, and bta-miR-2284d were simultaneously down-regulated in multiple regions including the distal jejunum and RAJ. Functional analysis of their putative targets indicates that these miRNAs potentially regulate genes involved in immune

function, including immune cell trafficking and hematological system development. Indeed, these functions were also enriched for the DE genes identified in the distal jejunum and RAJ as shown in Chapters 2 and 3. As miRNAs regulate gene expression at the posttranscriptional level, such regulation could lead to repression of translation and/or degradation of transcripts (Cai et al., 2009). Thus, expression of proteins can also be useful to study the regulatory mechanism of miRNAs, and the proteomics study of the host intestinal tissues may also reveal the effects of regulation by identified DE miRNAs. The validation experiment using cell model, such as bovine intestinal epithelial cell line (Miyazawa et al., 2010), to overexpress or to knockdown the DE miRNAs followed by measurement of expression of putative target transcripts could facilitate understanding the functions of those miRNAs in cattle.

### 6.3 Role of gut microbiota in altered gene expression in the gut of super-shedder compared with non-shedder cattle

In addition to host genetics and miRNA regulation, microbiota is also an important factor that influence host gene expression in the gut, because disturbance to the gut microbiota can cause changes of physiological functions in the host (Holmes et al., 2012; Nicholson et al., 2012). Indeed, the relative abundance of microbial taxa and predicted functions (described in Chapter 5) were correlated with the expression of DE genes in RAJ (as reported in Chapter 2), suggesting the potential role of microbiota in influencing host gene expression. The correlation between *Clostridium* and DE genes, *S100A8* and *S100A9*, suggests that *Clostridium* spp. may play a role in *E. coli* O157 shedding via influence the functions of the intestinal epithelium, as these two S100 proteins were reported to be highly expressed in epithelial (Cua and Tato, 2010). A recent study on the digesta and fecal microbiota of SS and NS revealed that the gut microbiota of steers showed greater diversity in the colon, suggesting that the microbiota of hindgut gut may

impact *E. coli* O157 super-shedding in cattle (Zaheer et al., 2017). In addition, our findings indicated distinct RAJ mucosal microbiota between SS and NS, suggesting that the mucosal associated microbiota may play an important role in *E. coli* O157 super-shedding, possibly due to their ability to interact with the RAJ epithelium and affect host gene expression.

In addition, *Clostridium* spp. belongs to a group of bacteria producing butyrate (Van den Abbeele et al., 2013), a short chain fatty acid that has been suggested to enhance the tight junction, and thereby improve the epithelium barrier against colonization of pathogens (Peng et al., 2009). *E. coli* O157 colonization has been shown to disrupt the tight junction (Tomson et al., 2004), and butyrate producing *Clostridium* spp. has been reported to lower the shedding of *E. coli* O157 in mice, and to increase the survival of *E. coli* O157 infected in mice (Takahashi et al., 2004). This suggests that *Clostridium* spp. associated with RAJ mucosa may impact *E. coli* O157 shedding. Also, the correlation between DE genes, *S100A8*/*S100A9*, with bacterial DNA replication and gene expression pathways suggest that bacterial proliferation may influence host epithelium function. Moreover, the findings in Chapter 5 suggest that the microbiota may mediate *E. coli* O157 colonization through modifying gut environment and/or enhancing host intestinal barriers. Recent studies have proposed to apply probiotic based approaches to reducing *E. coli* O157 shedding in cattle, including single strain *Lactobacillus acidophilus*, or mixed probiotics strains of *Lactobacillus. casei, Lactobacillus fermentum, Lactobacillus plantarum* and *Streptococcus faecium* (Sargeant et al., 2007). However inconsistent efficacy of probiotic treatments has been reported (Sargeant et al., 2007). The lack of understanding of the variation in host microbiota among individual animals could prevent such application, as those studies may have been performed on cattle with gut microbiota that were less conducive to responding to probiotics.

Furthermore, studies have revealed the association between gut microbiota and obesity in human, as gut microbiota is one of the mechanisms that influence host energy and lipid metabolism (Azorín-Ortuño et al., 2009; Caesar et al., 2010; Velagapudi et al., 2010). Alteration of gut microbiota can result in different composition of lipids in serum, liver, and adipose tissue (Velagapudi et al., 2010). In addition, links between lipid metabolism and the immune system have been demonstrated in many studies. For example, the activation of liver X receptor (LXR) by fatty acids and sterols can lead to inhibition of inflammatory responses induced by NF-κB (Seo et al., 2004a; Seo et al., 2004b). Responses in TLR can lead to inhibited LXR activation, causing increased absorption of cholesterol (Castrillo et al., 2003). Most of these studies focused on association between fecal samples or luminal content with diseases, especially cancer (Pereira et al., 2013). Our study is the first to link the RAJ tissue mucosal associated microbiota and host immune function with *E. coli* O157 shedding in cattle. A potential mechanism could include differences in lipid metabolism leading to variation in host immune function and increased susceptibility among cattle to *E. coli* O157 colonization (Figure 6.1).

**6.4 Future direction**

This is the first study that applied transcriptomic, miRNAome and microbiome approaches to study host mechanism involved in *E. coli* O157 shedding in cattle. Although this study sheds some light on the potential roles of host gene expression, posttranscriptional regulation of gene expression and host microbial interactions in the super-shedding, there are limitations in the current studies.

Firstly, the types of SS were not determined in this study, due to challenges in continuously monitoring fecal *E. coli* O157 density for months. As reported in a previous study that monitored *E. coli* O157 shedding patterns of beef steers for 77 days, three shedding patters

were observed, including non-persistent (last ~7 days), moderately persistent (last ~30 days), and persistent shedding (last > 30 days) (Baines et al., 2008). The SS used in my thesis may belong to different types of SS, and the segregation in transcriptome profiles within the SS group may indicate the different shedding types. As persistent shedders post the greatest threat to the farm environment, future studies using persistent SS are needed to investigate the mechanisms that lead to long-term fecal shedding of *E. coli* O157. Targeting persistent SS could not only facilitate identifying potential mechanisms that lead to persistent shedding, but also mitigating *E. coli* O157 contamination of the environment. Secondly, the extent of immunomodulatory effects by *E. coli* O157 were not examined, and thus it is difficult to determine which down-regulated genes or inhibited immune functions were due to microbial effects or host genetic variation and/or regulation by miRNAs. Future studies by synchronized monitoring the changes of *E. coli* O157 shedding and gene expression are needed to determine whether the observed difference in gene expression is due to host genetic mechanisms or microbial immunomodulatory effects or both. Thirdly, although genetic variation was identified between SS and NS located in the DE genes associated with immune functions, and the association analysis indicated potential SNPs that may play a role in the observed differential expression of genes between SS and NS, it requires further validation with larger populations of SS and NS. Fourthly, the miRNA functional analysis was based on the putative target mRNAs that were predicted by computational algorithms and correlation analysis. To further understand the role of miRNA regulation, their putative targets need to be validated using experimental methods, such as luciferase reporters assays (Jin et al., 2013) or overexpression/knock down of miRNAs in bovine epithelial cell model (Miyazawa et al., 2010). A follow up study through long term monitoring of fecal *E. coli* O157 and expression of candidate gene/miRNA in the RAJ should be undertaken.

This could be accomplished using the rectal biopsy procedure and fecal samples collected daily from cattle. By performing such experiments, the type of SS, and the microbial effects on host gene/miRNA expression could be determined. Finally, because the microbiota functions were based on prediction, a metagenomic, or/and metatranscriptomic analysis can be performed to better investigate the microbial gene families that are present in the intestinal mucosa samples. Also, proteomic and metabolomic analysis should be performed to further define microbial functions, and to study how such functions can influence host physiological processes, and eventually affect fecal shedding of *E. coli* O157. Bovine intestinal epithelial cell model (Miyazawa et al., 2010), or germ-free animal models may also facilitate understanding of how microbes such as *Clostridium, Coprococcus*, and *Paludibacter* can interact with enterocytes and the gut mucosal associated immune system in cattle, and how they may influence the presence of *E. coli* O157 in the intestinal tract of cattle. In addition, as shown in Chapter 5, there is large individual variation among steers in terms of gut mucosal associated microbiota. To minimize such individual variation, an *in vivo* intestinal loop model (Vlisidou et al., 2004) could be adopted to examine the effects of *Clostridium, Coprococcus*, and *Paludibacter* on the growth and colonization of *E. coli* O157 in the intestinal tract of cattle.

**6.5 Implications**

The goals of this thesis were to reveal the mechanisms that are associated with *E. coli* O157 persistent shedding in cattle, and to develop a diagnostic tool that could rapidly detect SS based on identified candidate genes/miRNAs and microbes. Learning the shedding mechanisms could facilitate development of more effective preharvest interventions, such as probiotics and antimicrobial treatments. Also, diagnostic tools that allow early identification of SS could allow real time treatments to reduce *E. coli* O157 colonization and fecal shedding prior to slaughter.

Measurement of expression of candidate genes/miRNAs may be useful to diagnose whether a super-shedder belongs to non-persistent or persistent categories, allowing farms to isolate persistent shedders from the herd so as to reduce animal-to-animal contamination. Such information could also be useful in developing methods to immunize cattle against *E. coli* O157. The identified SNPs associated with *E. coli* O157 shedding may benefit the breeding industry to selectively breed cattle with less genetic propensity to shed this foodborne pathogen. By investigating cattle-*E. coli* O157 interaction, our ultimate goal is to develop methods that can effectively reduce fecal *E. coli* O157, eventually leading to reduced risk of beef recalls and human illness.

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## 6.7 Figures

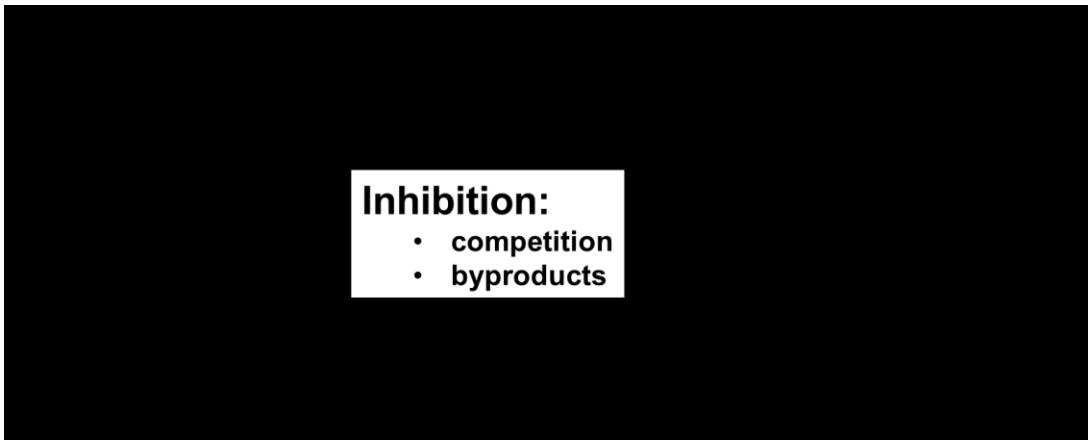


**Figure 6.1 Potential host-microbial interactions that may influence shedding of *E. coli* O157 in beef steers**

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