Development and use of a gnotobiotic murine model for beef cattle to evaluate competitive exclusion of *Escherichia coli* O157:H7 using commensal *Escherichia coli* strains, and to ascertain the impact of physiological stress on the host-bacteria interaction

by

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Abstract

Enterohemorrhagic *Escherichia coli* (EHEC) serotype O157:H7 is an important foodborne pathogen, and cattle are considered the primary reservoir of this bacterium. Research was undertaken to ascertain factors that regulate competitive exclusion of *E. coli* O157:H7. A gnotobiotic (GB) murine model for cattle was used to study host-microbiota interactions. For years, isolators have been used to rear germ-free (GF) and GB mice however, these can be costly and the segregation of treatments within the same isolator is problematic. Recently, methodologies for housing GF mice in specially designed individually ventilated cages (IVCs) operated under barrier mode (outward directional airflow) have been developed; however this equipment can be expensive and their operation in barrier mode for research involving GF mice and pathogens is not permissible under modern biosafety and biosecurity regulations. Methods were developed to house GF mice in a commercially available conventional IVC system operated under containment mode (inward directional airflow). Moreover, the methods developed ensured that the GF or GB status of mice was maintained for at least 4 weeks with weekly handling. The use of a common IVC infrastructure with the application of operational procedures could be used to study *E. coli* O157:H7 in defined microbiota mice with each IVC treated as an experimental unit.

Currently there are no proven and effective methods of eliminating EHEC from cattle reservoirs and the impact of colonization resistance on EHEC in cattle is poorly understood. Using GB mice, a representative cattle EHEC infection model was developed to elucidate key aspects of the host-pathogen-microbiota interaction, and investigate competitive colonization between 20 phylogenetically-distinct commensal *E. coli* (EC) strains isolated from cattle and EHEC. Commensal strains were grown together or separately. Stress has been suggested as an important factor in intestinal tract colonization by EHEC in cattle, but this has not been experimentally investigated. To induce a physiological stress response, mice were administered the stress hormone corticosterone (CORT) in drinking water.

The EHEC strain FRIK 2001 was selected to colonize the intestinal tract of GB mice to mimic colonization of EHEC within the bovine gut. FRIK 2001 effectively colonized the gut with good bacterial densities and neither symptoms of disease nor metabolomic differences in kidney at 5 days post treatment were observed when compared to the other EHEC strains. Twenty bovine commensal strains of EC decreased EHEC densities in the cecum, proximal colon, and distal colon. These EC were equally effective at reducing growth of EHEC when grown before administration to GF mice individually or in combination. Moreover, histopathologic changes and expression of the pro-inflammatory cytokines, tumor necrosis factor alpha (*Tnf*α) and Keratinocyte-derived chemokine (*Kc)* were reduced in the distal colon of mice inoculated with commensal EC strains. A difference in mice behavior between the CORT– and CORT+ treatments was observed in the open field test for mean velocity and total distance travelled. Stress induced by CORT treatment, however, did not enhance FRIK 2001 colonization nor influence the efficacy of competitive exclusion of the bacterium.

Colonization of the intestinal tract of GF mice by a bovine isolated EHEC shared similarities with colonization of EHEC in cattle. The presence of commensal EC strains effectively reduced intestinal colonization and ameliorated disease, particularly within the distal colon, a key intestinal tract colonization site of EHEC in cattle. Notably, physiological stress did not potentiate enteric colonization nor intestinal disease in mice incited by EHEC.

Preface

This thesis is original work by Maximo E. Lange. The research project, of which this thesis is a part, received research ethics approval from the Agriculture and Agri-Food Canada Lethbridge Research and Development Centre Animal Care Committee (ACC) for Animal Use Protocol (AUP) #1623 ("Determining colonization mechanisms of *Escherichia coli O*157:H7 using a germ-free murine immune stress model"). The three separate experiments conducted under AUP #1623 are presented in chapters 2 and 3. In addition, ACC AUP #1931 ("Acquisition of sheep blood for use in microbiology research") was approved for the research (i.e. acquisition of blood for inclusion in microbiological media).

Chapter 2 of this thesis has been published as "Housing gnotobiotic mice in conventional animals facilities" in Current Protocols on Mouse Biology. The fluorescent *in situ* hybridization within the manuscript was selected as the cover image (March 2019 Volume 9). Chapter 3 is currently being submitted to the journal, Gut Pathogens.

I was responsible for the work conducted in this thesis, with the exception of metabolomics analyses presented in Chapter 3, which were completed by Catherine Brown, Tony Montina, Stephanie Sheppard, and Ben Wright (University of Lethbridge), and the corticosterone enzyme linked immunosorbent assays presented in Chapter 3, which was completed by Dr. Sandra Clarke (University of Alberta). Identification of strain specific markers in the DNA sequence of the five *Escherichia coli* O157:H7 strains evaluated was completed by Dr. Chad Laing (Canadian Food Inspection Agency).

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List of Abbreviations

A/E	Attaching effacing
ACTH	Adreno-corticotropic hormone
AI	Autoinducer
BSC	Biosafety cabinet
CORT	Corticosterone
CR	Colonization resistance
CRH	Corticotropin releasing hormone
DAPI	4',6-diamidino-2-phenylindole
EC	Escherichia coli
EHEC	Enterohemorrhagic Escherichia coli
Esp	Escherichia coli secreted protein
EtoH	Ethanol
FISH	Fluorescence in situ hybridization
GalNac	N-acetylgalactosamine
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase (GAPDH);
GB	Gnotobiotic
Gb3	Globotriaosylceramide
GF	Germ-free
GR	Glucocorticoid receptor
GRE	Glucocorticoid response elements
GUSβ	Beta-glucuronidase
HC	Hemorrhagic colitis
HPA	Hypothalamic-pituitary-adrenal axis
HPRT	Hypoxanthine-guanine phosphoribosyltransferase (GUSβ)
HUS	Hemolytic uremic syndrome
IACUC	Institutional Animal Care and Use Committee
IFN	Interferon
Ig	Immunoglobuline
IL	Interleukin
IVC	Individually ventilated cage
KC	Keratinocyte-derived chemokine
LB	Luria Bertani
LEE	Locus of enterocyte effacement
LPS	Lipopolyshaccharide
MR	Mineralocorticoid receptor
NAG	N-acetylglucosamine
NANA	N-acetylneuraminic acid
NBF	Neutral buffered formalin
NE	Norepinephrine
NF-κβ	Nuclear factor-κβ

OFE	Open field exploration
PAI	Pathogenicity island
PBS	Phosphate buffered saline
PFGE	Pulse field gel electrophoresis
Qse	Quorum sensing Escherichia coli
RG	Risk group
STEC	Shiga toxin Escherichia coli
Stx	Shiga toxin
TGF	Transforming growth factor
Th	T helper
Tir	Transmembrane intimin receptor
TLR	Toll-like receptor
TNF	Tumor necrosis factor
TTSS	Type three secretion system
VP	Vasopressin
VTEC	Vero cytotoxin Escherichia coli

Chapter 1: Literature Review

1.1 Introduction

Escherichia coli (*E. coli*) are bacteria commonly found in the intestinal tract of mammals, and are the most abundant facultative anaerobe within the human gut (Nataro *et al.*, 1998; Robinson *et al.*, 2006). Certain serotypes of *E. coli* can develop a mutualistic relationship with the host, while other serotypes are pathogens that incite acute intestinal and extraintestinal disease. The serotypes of *E. coli* are defined by the combination of their surface O (somatic), H (flagellar), and sometimes K (capsular) antigens (Nataro *et al.*, 1998). In many countries, *E. coli* O157:H7 is of particular public health interest, as a consequence of its pathogenicity in human beings, where it can incite non-bloody diarrhoea, haemorrhagic colitis (HC), and potentially, haemolytic uremic syndrome (HUS) (Kaper *et al.*, 2004). *E. coli* O157:H7 is considered to be a zoonotic pathogen, and its main reservoirs are healthy domesticated ruminants; predominantly cattle, and to a lesser extent sheep and goats (Ferens *et al.*, 2011).

1.2 *Escherichia coli* O157:H7 pathogenesis in human beings

Escherichia coli O157:H7 is an enterohemorrhagic *E. coli* (EHEC) belonging to a group of bacterial strains that are capable of expressing Shiga toxin (Stx), that cause hemorraghic colitis (HC) and hemolytic uremic syndrome (HUS), characterized by developing attaching/effacing (A/E) lesions on epithelial cells (Nataro *et al.*, 1998). This group of EHEC is included in a larger cohort of *E. coli* bacteria*,* known as Shiga toxin *E. coli* (STEC) or verocytotoxin *E. coli* (VTEC), all of which have the distinguishable ability of producing shiga toxin. All EHEC are believed to be pathogens, whereas not all STEC or VTEC bacteria are pathogenic (Nataro *et al.*, 1998).

Escherichia coli O157:H7 was first recognized as an incitant of enteric disease in human beings in 1982 (Lim *et al.*, 2010). Since then, the bacterium has been linked to diverse foodborne disease outbreaks, generally associated with the consumption of undercooked ground beef, but also with the ingestion of contaminated sausages, unpasteurized milk, lettuce, cantaloupe melon, apple juice, and radish sprouts, among other foods (Kaper *et al.*, 2004). Notably, relatively low infectious numbers of *E. coli* O157:H7 are required to induce disease, and it has been shown that ingestion of 100 EHEC cells can cause infection (Kaper *et al.*, 2004).

Although EHEC cellular mechanisms for inducing disease in humans are highly complex, for the scope of this thesis, a brief description will be provided as background information. The attachment between the bacterium and intestinal epithelial cells occurs by means of A/E lesions. Genes with the capacity of encoding for proteins required for A/E are found in the Locus of Enterocyte Effacement (LEE)

pathogenicity island (PAI) (Garmendia *et al.*, 2005). The LEE region is organized into five major operons composed of 41 total genes (LEE 1-5). Operon LEE 1 acts as a master regulator of LEE operons by codifying the protein *Ler* (LEE-encoded regulator) which activates transcription of LEE operons 2-5 (Garmendia *et al.*, 2005). The remaining LEE operons encode for proteins involved in a Type-III Secretion System (TTSS); multiple effector proteins required for binding of the bacterium to epithelial cells, formation of A/E lesions, and disruption of epithelial cell function. The TTSS is required to insert various effector proteins into the host cell. It is an apparatus that will form in the inner and outer membranes of bacteria ultimately forming a "needle" that extends from the bacteria and contacts the host cell (Moreira *et al.* 2010). Initially, upon LEE activation the proteins EscC (*E. coli* protein) and EscV establish themselves in the inner and outer membranes of the bacterium, respectively, forming an annular complex. EscJ a structural lipoprotein locates within the periplasmic space between EscC and EscV, and together these proteins provide a corridor on the bacterial membranes for effector proteins to be released. In order for effector proteins to travel from the bacterial membrane into the host cell a needle-like structure is extended from the annular complex of the bacteria to contact with the host cell membrane. This needle-like structure is comprised of proteins, EscF and EspA (*E. coli* secreted protein). EspA forms bonds with EscF, and polymerizes into the hollow needle-like structure that extends out and allows the contact with the host cells of the intestinal epithelium (Garmendia *et al.*, 2005). A pore is formed within epithelial cells via proteins EspB and EspD in combination with EspA. Through this mechanism it is believed EHEC introduces effector proteins into the host cell, and of particular importance the insertion of Transmembrane intimin receptor (Tir) protein. Transmembrane intimin receptor localizes within the host epithelial cell membrane and acts as the receptor for an adhesin on the membrane of EHEC. This adhesin protein is intimin, and the Tir/intimin connection enables the attachment of EHEC to host cells (Garmendia *et al.*, 2005). Tir also binds to the host cytoskeleton and induces polymerization of actin with the final formation of actin rich pedestals under the bacterium. Interestingly, these pedestals are not static structures, and the bacterium utilizes them to move across the cell surface (Garmendia *et al.*, 2005). Following formation of the pedestal, the epithelial microvilli are effaced inducing cell injury and intestinal inflammation within the distal gut (Nataro *et al.*, 1998, Garmendia *et al.*, 2005)

Another prominent virulence factor of EHEC is Stx, and this protein is the causative agent for inducing the often fatal HUS in human beings. Shiga toxin is phage encoded in the bacterial chromosome, and it is only released in the presence of disturbances in the bacterial DNA, membrane or

protein synthesis, which is the primary reason why antibiotic therapy to treat EHEC in people is controversial (Croxen *et al.*, 2010). This toxin is comprised of two immunologically non-cross reactive serotypes (i.e. Stx1 and Stx2); and these virulence factors can be expressed individually or together. Genetic sequence variations occur in Stx2, but both Stx1 and Stx2 express an identical A-B subunit complex. Notably the A-B subunit of the toxin is composed of a B pentameric and an A monomeric structure (Nataro *et al.*, 1998). The B subunit binds to the receptor glycolipid globotriaosylceramide (Gb3) on the cell surface, while the A subunit is introduced into the cell where it acts on the 60S subunit of the ribosome cleaving a single adenine from the 28S rRNA, and thus inhibiting cellular protein synthesis and subsequently leading to host cell death (Nataro at al., 1998). Human cells such as Paneth cells, endothelial cells, and kidney epithelial cells possessing the Gb3 receptor are susceptible to Stx binding and cell injury. In contrast, cattle lack the expression of Gb3 receptors in kidney glomeruli, and its believed this is why bovids are protected from developing HUS (Boyer *et al.*, 2011). HUS is characterized by acute renal failure, thrombocytopenia, and microangiopathic hemolytic anemia following Stx translocation into the systemic circulation. Necrosis of glomerular endothelial cells, in conjunction with renal inflammation and thrombosis of capillary lumen lowers glomerular filtration leading to acute renal failure (Nataro *et al.*, 1998).

Finally, *Escherichia coli* O157:H7 also contains a 60-MDa plasmid encoding enterohemolysin responsible for lysis of erythrocytes, as well as a pO157 plasmid that encodes the expression of fimbria. Both gene products are considered to further contribute to the pathogenesis of *Escherichia coli* O157:H7 (Nataro *et al.*, 1998).

1.3 *Escherichia coli* in ruminants

The intestinal tract of bovine species, and to a lesser extent small ruminants such as sheep and goats, is the main reservoir for human infectious *E. coli* O157:H7. The mechanism involved in the anatomical localization, intestinal colonization, shedding patterns, and low prevalence of *E. coli* O157:H7 disease in ruminants are not fully understood. Importantly, the elucidation of these mechanisms may facilitate the development of effective on farm mitigation strategies to reduce the transmission of *E. coli* O157:H7 from livestock to people. The carriage and shedding of *E. coli* O157:H7 in cattle can be highly complex. *E. coli* O157:H7 carriage can be sporadic and of short duration (Ferens *et al.*, 2011). Shedding of the bacterium in feces can be intermittent, the duration of shedding events can be short lived, and the quantity of cells released into the environment vary from 10 to 10^9 CFU/g of feces (Munns *et al.*, 2015). Typically, cattle shed EHEC at high densities for short periods of time, followed by prolonged

periods in which no fecal shedding occurs or the bacterium is shed at low cell densities (Stevens, 2002). Moreover, shedding of EHEC in feces tends to be higher during the summer and autumn, and lower during the winter months (Laven *et al.*, 2003). Cattle that shed the bacterium at densities greater than 10^4 CFU/g in feces are considered super shedders (Munns *et al.*, 2015). Omisakin *et al.*, (2003), found that 9% of cattle shedding at higher than 10^4 CFU/g in feces corresponded with more than 96% of the total *E. coli* O157:H7 present in the cattle tested. This supports the possibility that only a small group of animals account for the majority of the *E. coli* O157:H7 released in the farm environment. The duration of the super shedding period still remains unknown. Furthermore, not all fecal material collected from shedding cattle are positive for *E. coli* O157:H7; supporting the non-continuous intermittent shedding of the bacteria (Munns *et al.*, 2015).

1.3.1 Intestinal location and colonization in cattle

The intestinal location and conditions for colonization of EHEC in cattle is currently a subject of scientific debate. In cattle, *E. coli* O157:H7 is mainly identified with the large intestine, including the cecum, and cranial and distal colon and can be closely associated with the mucosal epithelium of the rectum (Grauke *et al.*, 2002; Naylor *et al.*, 2003). After inoculation in ruminants with *E. coli* O157:H7, the bacterium does not tend to persist in the rumen, upper or mid small intestine but will colonize in both the distal ileum, cecum and colon; with the greatest quantities of bacteria isolated within the large intestine (Grauke *et al.*, 2002). The highest densities of cells are isolated from feces, even after the bacterium is no longer isolated from intestinal tissues (Laven *et al.*, 2003). It is noteworthy that densities of *E. coli* 0157:H7 on the surface of feces are higher than within the fecal core, suggesting that the bacterium is prevalent in the distal parts of the large intestine (Naylor *et al.*, 2003). Several studies have indicated that the terminal rectum, a region rich in lymphoid tissue is the primary site of colonization for *E. coli* 0157:H7 (Naylor *et al.*, 2003), as the bacterium is most commonly isolated from this region of the gut. It is believed that *E. coli* 0157:H7 establishes intestinal adherence at the distal rectum. However, it is important to note that this research has primarily examined cattle that have been inoculated with *E. coli* O157:H7. Collectively, this information indicates that the niches and mechanisms required for *E. coli* 0157:H7 colonization and survival within the intestinal tract of cattle have yet to be fully determined.

Ruminants, particularly mature cattle, act as an asymptomatic reservoir for EHEC bacteria; however, it has been demonstrated that newborn calves (less than 36-hours-old) can present clinical manifestation and tissue injury due to infection with *E. coli* O157:H7, this includes watery diarrhea, neutrophilic infiltration, sloughing of epithelial cells and A/E lesions in large and small intestines (Dean-

Nystrom *et al.*, 1997). Moreover, there is also a higher prevalence of EHEC in cattle following: long distance transportation, changes in diet, and antibiotic therapy, possibly linked to immunocompetence of the animals and/or to disturbances to the structure of the intestinal tract microbiota (Stevens, 2002).

1.3.2 Bovine immune response to EHEC enteric colonization

Although cattle are the main reservoir of EHEC, infected adults typically do not present overt clinical symptoms of disease. Yet, EHEC is an established commensal organism of cattle and could be potentially pathogenic. There is convincing evidence of an immune response mounted by the bovine host in response to EHEC colonization (Vande Walle *et al.*, 2013). In this regard, EHEC have been found to form attaching effacing lesions on the intestinal epithelium however, in order to establish this close attachment with the host mucosa, the bacterium must first contact the epithelium. Flagella H7 contacts with enterocytes, which leads to the initiation of the TTSS (Vande Walle *et al.*, 2013). The flagellum is recognized by TLR-5 of the host that results in the activation of NF-κβ, and subsequently activation of IL-1B, IL-8, and TNF-α (Vande Walle *et al.*, 2013). The presence of EHEC lipopolyshaccharide (LPS) also activates TLR4, which stimulates a similar pro-inflammatory response (Vande Walle *et al.*, 2013). Once TTSS is activated, the bacterium attaches to the enterocyte and this process is associated with mild granulocytic mucosal infiltration accompanied by modest exfoliation of the epithelium at sites of colonization (Nart *et al.*, 2008). In neonatal calves challenged with *E. coli* O157:H7, granulocytic infiltration extends within the large intestine accompanied by substantive tissue congestion, edema and epithelial degeneration (Dean-Nystrom *et al.*, 1997). Disease severity is reduced in calves greater than 3-weeks-of-age, suggesting that the pathogenicity of *E. coli* O157:H7 in cattle is age dependant (Dean-Nystrom *et al.*, 1997).

Corbishley *et al.*, (2014) studied the gene expression of the rectal mucosa in 12-week-old calves inoculated with *E. coli* O157:H7. Cytokine profiles directed towards a Th1 response were observed, with an increase in the expression of IFNγ and T-bet as compared to control animals. There was also a reduction in TGFβ expression in inoculated animals while maintaining elevated IFNγ levels providing further evidence of an ongoing pro-inflammatory Th1 response (Corbishley *et al.*, 2014). Additionally, there were no observed changes in cytokines related with a Th2 response.

The presence of neutralizing antibodies against virulence factors of *E. coli* O157:H7 in naturally infected cattle have been observed. More specifically, antibodies generated against Stx1 and Stx2, LPS, TTSS proteins intimin, tir, EspA and Esp B and H7 flagellin (Vande Walle *et al.*, 2013). Moreover, evidence suggested that Stx can supress immune cell activity in cattle. In this regard, peripheral blood

mononuclear cells isolated from calves previously administered Stx2-positive *E. coli* O157:H7 failed to generate proliferative responses following a challenge with heat killed stx2-positive *E. coli* O157:H7 *in vitro*. This differed from peripheral blood mononuclear cells isolated form animals which were administered stx-negative *E. coli* O157:H7 bacteria and developed a robust response when re-challenged with heat killed stx2-positive *E. coli* O157:H7 *in vitro* (Hoffman *et al.*, 2006). Studies examining the transcriptome of the rectoanal junction in naturally infected super shedders compared to non-shedders, showed a downregulation of multiple immune factors in super shedders. This reduction was mainly related with function and chemoattraction of B cells and migration of neutrophils, macrophages and dendritic cells (Wang *et al.*, 2016). This suggests a potential decrease in numbers of granulocytes to areas of colonization of *E. coli* O157:H7; aiding to the establishment of the bacteria within the gut. It is still unclear if the reduced protective immune responses are associated with the colonization ability of the individual bacteria strains or a differing intrinsic property of super shedding hosts.

1.3.3 Control of EHEC in cattle

Multiple approaches have been implemented to reduce or eliminate the presence of EHEC in the cattle or within cattle processing plants. Methods to reduce the amounts of shedding and presence of the bacteria within the gut before the animal arrives at the processing plant are known as pre-harvest measures. Pre-harvest prevention was implemented to address high levels of bacterial contamination of cattle hides, particularly during the summer months, as elevated EHEC cell densities can overwhelm the sanitary measures utilized in plants to control EHEC levels. Many strategies to reduce bacterial load target farm production practices that are thought to facilitate the enteric proliferation of EHEC in cattle. These strategies target changes in concentrations of grain within the diet, the addition of prophylactic and therapeutic amounts of antimicrobial drugs to feed, changes in intensity and density of cattle production, and methods of manure disposal (Besser *et al.*, 2014). Many of these adjustments, however, are either partially effective or completely ineffective (Besser *et al.*, 2014). As a result, the development of alternate strategies to reduce the level of EHEC carriage and shedding are active areas of investigation.

Many pre-harvest control methods aim to limit exposure of the animals to EHEC by reducing animal density, exposure to wildlife potentially carrying the bacteria, and enhancing feed hygiene; however, most often these practices are impractical for Canadian and international cattle production systems (LeJeune *et al.*, 2007). Other mitigation strategies focus on reducing the amount of pathogen within the gut by utilizing feed that can alter short chain fatty acid concentrations, reducing pH and altering the

composition of resident intestinal bacteria (LeJeune *et al.*, 2007). Furthermore, probiotics such as *Lactobacillus acidophilus* are commercially available, and have been reported to help reduce the shedding of EHEC in feces (Sargeant *et al.*, 2007). Finally, strategies that directly target EHEC like hide washing, administration of antibacterial agents, such as sodium chlorate to feed and water, the use of bacteriophages (phages to eliminate EHEC have been successfully used *in vitro* and in murine models, but further research is needed for use in cattle) and anti-EHEC vaccines have been evaluated with variable results (LeJeune *et al.*, 2007). Several vaccines against *E. coli* O157:H7 have been developed and there are currently two commercial vaccines available. The first vaccine is directed at enhancing mucosal immunity against the TTSS, thereby reducing or preventing bacterial adherence (Besser *et al.*, 2014). The second vaccine stimulates the generation of antibodies against a siderophore receptor. This receptor is needed to sequester iron and antibodies binding to the receptor prevents *E. coli* O157:H7 from up-taking iron, an essential function for bacterial survival (Besser *et al.*, 2014). Vaccination has proven only partially effective in reducing prevalence of the bacteria within cattle, and has been unable to eliminate the bacteria from entire cattle herd. Although some of the currently available pre-harvest controls are partially effective, it is plausible that combination of multiple methods may increase their efficacy. However, no definite practical strategy that is economically feasible to reduce EHEC has been developed as yet. Thus, unfortunately, incidence of human infection with *E. coli* O157:H7 has remained fairly steady globally and throughout the years (Besser *et al.*, 2014).

1.3.4 Stress and the effects of glucocorticoid in cattle

Periods of stress are inevitable during livestock production. Social mixing, animal restraint and handling, introduction of cattle to new environments, transportation, weaning and processing (castration, vaccination, dehorning and branding) are examples of cattle production activities that induce stress.

Stress has been studied extensively and it is defined herein as the sum of all biologic reactions to physical, emotional, or mental stimuli that disturb homeostasis (Carroll *et al.*, 2007). Other authors define stress as the biological response elicited when an animal perceives a threat to its homeostasis (Moberg *et al.*, 2000). This threat is referred to as the stressor. Stress is not necessarily a negative and harmful process, since animals undergo and adapt to multiple stressful events throughout their lifetime. However, when the organisms' adaptation to the stressor is detrimental to the animal well-being, then the stress becomes distress. During periods of distress the biological processes of the animal attempt to restore the homeostatic balance, even after the stressor has ceased. Moberg *et al.*, (2000) presented a

model of animal stress in which they divided the stress response into the following three stages: recognition of a stressor; biological defense against the stressor; and lastly, consequences of the stress response. The last stage defines whether the animal is suffering from distress or was able to adapt without any adverse effects on its health.

Recognition of a threat is not perceived in equal manner by all animals. Inter-animal differences based on experience, genetics, age, physiological state, and season can influence how a stressor is perceived (Moberg *et al.*, 2000). The biological process to defend against stressors begins once functions of the central nervous system have been 'activated' in response to the threat. This can lead to a combination of the following four responses: behavioral; autonomic nervous system; neuroendocrine; and the immune (Moberg *et al.*, 2000). The most rudimentary response is a behavioral response, in which an animal simply attempts to avoid the stressor. Secondly, the autonomic nervous system triggered in the context of "fight or flight" response. This is a short-term response, characterized by elevated levels of circulating catecholamines epinephrine and norepinephrine (NE) which affects cardiac, respiratory, muscular, metabolic and other physiological function of the host (Moberg *et al.*, 2000). The third response is the activation of the hypothalamic-pituitary-adrenal axis (HPA axis), and is associated with the elevation of circulating glucocorticoids, steroid derivatives that can have prolonged and substantive effects on the long-term health of the host. Lastly the immune system can be activated with the innate and adaptive responses. Importantly, both glucocorticoids and catecholamines can influence an immune response, albeit temporally distinct from one another, following challenge to a stressor (Moberg *et al.*, 2000).

As a consequence of the stress response, the animal can enter a pre-pathological or pathophysiological state. It is in both these stages that energy requirements used to maintain a specific homeostatic function are shifted toward the physiological response associated with exposure to a stressor. This situation can substantively alter homoeostatic biological function and cause the induction (pre-pathological stage) or progression (pathological stage) of disease (Moberg *et al.*, 2000). As a consequence, this has a detrimental effect on the livestock producer, as energy reserves are redirected from animal performance to coping with the stressor induced event.

In livestock production, stressors are mainly grouped into the following three categories: psychologic stress; physical stress; and physiologic stress (Carroll *et al.*, 2007). Importantly, these three categories of stress are not mutually exclusive and often occur in tandem. Psychologic stress is associated with fear, and can be presented during periods of social mixing, introduction to new

environments, exposure to loud noises, unfamiliar restraints and equipment. Physical stress is that associated with animal injury and disease, extreme environmental temperatures, and periods of hunger, thirst, and fatigue (Carroll *et al.*, 2007). Physiological stress in cattle can be associated with loss of normal endocrine or neuroendocrine function caused by various conditions; including feed restriction, and endocrine disorders (Carroll *et al.*, 2007). Following challenge with an inducer of stress, the host develops a relatively uniform biological process that counteracts the stressful event, in order to return to physiological homeostasis. Briefly, a stressor will stimulate neuroendocrine systems including the HPA axis and the sympathetic nervous system (Carroll *et al.*, 2007). In the context of the HPA axis, diverse stimuli will trigger the secretion of corticotrophin-releasing hormone (CRH) and vasopressin (VP) from the hypothalamus and both of these hormones then stimulate the secretion of adrenocorticotropic hormone (ACTH) from corticotroph cells in the anterior pituitary gland. There, ACTH is released into circulation and induces secretion of glucocorticoids from the zona fasciculata and zona reticularis of the adrenal gland (Carroll *et al.*, 2007). An increase in glucocorticoid levels in blood will trigger a constellation of physiological responses. These include, activating gluconeogenesis process within the liver using different macromolecules including amino acids and lipids, stimulating synthesis and secretion of catecholamines and modulation of immune system function (Carroll *et al.*, 2007).

Glucocorticoids are comprised of different steroid products. Cortisol is the main glucocorticoid produced in the adrenal cortex of ruminants, while in other species, such as mice, the main glucocorticoid produced by the adrenal gland is corticosterone. Glucocorticoids are lipophilic and can penetrate cells through the lipid plasma membrane. There are two main intracellular glucocorticoid receptors; mineralocorticoid receptor (MR) and glucocorticoid receptor (GR). Corticosteroids have higher affinity for MR than GR; as such, at low basal physiological levels glucocorticoids primarily bind to MR. In contrast, following a stressful event, glucocorticoids can circulate in high quantities which enables binding to GR. Immune cells such as macrophages and T lymphocytes express GR as a primary receptor. It is suggested that this receptor is responsible for immunological changes that occur in the presence of high levels of glucocorticoids (Padgett *et al.*, 2003). The exact cellular mechanisms on how glucocorticoids alter the immune response are not entirely clear at present. The GR receptor remains inactive within the cytoplasm, but following the binding of glucocorticoids, the receptor translocates into the nucleus and binds to glucocorticoid response elements (GRE) (Padgett *et al.*, 2003). It is in the nucleus where transcription of immune elements can be modulated via a number of proposed mechanisms. Firstly, one model suggests that GR recognize a putative hormone response element in the

sequence of diverse cytokines and this enhances or represses transcription of various genes. This mechanism does not appear to be involved in the expression of important cytokines associated with immune function given that not all cytokines posses this response element (Padgett *et al.*, 2003). Another and the most accepted mechanism, is the down regulation of NF-κβ following GR translocation to the nucleus. It is believed that GR can activate the transcription of an NF-κβ inhibitor (IKb alpha), that will sequester NF-κβ in the cytoplasm and prevent it from entering the nucleus. Another proposed mechanism is the direct binding of GR to NF-κβ causing its inhibition (Padgett *et al.*, 2003). Regardless of the mechanism, glucocorticoids are potent immunomodulators able to reduce expression of important pro-inflammatory cytokines; TNF-α, IL-1, IL-12, or inhibit NF-κβ and IL-6, as well as decrease T and B lymphocytes numbers in host (Bartolomucci, 2007). Thus, it is plausible that during periods of prolonged stress, the immune function is altered in cattle, which subsequently affects the microbiota in the intestinal tract thereby opening niches or possibilities for invading bacteria that are not able to survive or colonize under normal circumstances.

As indicated previously, cortisol is the main glucocorticoid secreted in cattle, and its concentration within plasma is used as a measure of activation of the HPA axis (Chen *et al.*, 2015). Cortisol can also be measured in urine, saliva, milk, and feces (Mormede *et al.*, 2007). It is secreted in a pulsatile manner and as such the secretion of cortisol follows a diurnal cycle synchronized with the exposure to light, with higher concentrations in the morning, and lowest concentrations during the evening and night (Mormede *et al.*, 2007). Cattle show increases in cortisol release in response to acute periods of stress that include; dehorning (Sylvester, 1998), restraint, or mixing with unknown animals (Mormede *et al.*, 2007). The release of cortisol is a relatively slow process, requiring a few minutes after the stressful event for the hormone to reach peak levels in blood. Basal levels of cortisol in cattle are usually less than 15 nmol/L but can increase to 60-200 nmol/L in response to a stressor (Mormede *et al.*, 2007). The impact of different individual stressors on concentrations of cortisol is not well defined in cattle (Mormede *et al.*, 2007). During periods of chronic stress, the levels of cortisol are lower than levels associated with acute responses. Even if cortisol is at basal levels under chronic stress, the activation of the HPA system can still be observed.

Similar to cortisol, under situations of stress, the adrenal medulla composed primarily of chromaffin cells will also produce catecholamines: epinephrine, NE, and dopamine (Moreira *et al.*, 2010). These hormones are first synthesized from L-dopa into dopamine, and later into NE and epinephrine. NE and dopamine are located in sympathetic terminal nerve endings throughout the nervous system, including

the enteric nervous system (Moreira *et al.*, 2010). Epinephrine is secreted in the central nervous system and the adrenal gland, and can reach the intestinal tract through the systemic circulation. Catecholamines prepare the body for an attack or flight response, and can increase the heart rate, constrict blood vessels, dilate bronchioles, and increase metabolism (Carroll *et al.*, 2007). When secreted over prolonged periods catecholamines can also alter immune function. Immune cells, such as macrophages and T lymphocytes, express β2 adrenergic receptors which can modulate immune responses (Padgett *et al.*, 2003).

1.4 Quorum sensing and impacts of host stress on EHEC

The impact of stress on colonization of bacteria in the gut and the induction of EHEC associated disease has not been fully investigated. Studies examining inter-bacterial signaling and bacterial-host signaling have been conducted, and provide interesting information on the interaction of stress hormones and intestinal bacteria (Bansal *et al.*, 2007; Sperandio *et al.*, 2003; Vlisidou *et al.*, 2004). Bacterial interspecies communication can take place via a cell-to-cell signaling by a mechanism called quorum sensing. *E. coli* are able to produce molecules that bind to surface receptors of other *E. coli* bacteria, thereby stimulating or inhibiting a response. As an example, *E. coli* O157:H7 produces the autoinducer-3 (AI-3), a molecule that binds to a histidine kinase membrane receptor leading to the activation of virulence inducing transcription factors. This quorum sensing system is composed of quorum sensing regulators, named Quorum sensing *E. coli* (Qse), that will either act as histidine kinase membrane receptors or transcription factors that can regulate expression of virulence factors (Moreira *et al.*, 2010; Hughes *et al.*, 2008). EHEC has the histidine kinase membrane receptor QseC that will specifically recognize quorum sensing molecule AI-3. Once activated QseC will phosphorylate QseB, a response regulator that promotes the activation of LEE genes encoding for the TTSS as well as motility virulence genes activating the flagella (Moreira *et al.*, 2010). Furthermore, QseB will activate production of a second receptor QseE; another quorum sensing membrane receptor promoting A/E lesion formation (Moreira *et al.*, 2010; Hughes *et al.*, 2008). By signaling with commensal *E. coli* and other enteric bacteria, *E. coli* O157:H7 can activate genes responsible for colonization of the intestinal tract (Sperandio *et al.*, 2003). Importantly, this communication system can alert EHEC when it has reached the large intestine, given that commensal bacteria such as *E. coli*, *Enterococcus*, *Clostridium,* and *Bacteroides* spp. also produce AI molecules as part of their communication system (Sperandio *et al.*, 2003). Communication between these commensal bacteria and EHEC will help direct EHEC to its location in the intestinal tract as well as induce activation of its virulence factors (Sperandio *et al.*, 2003).

Histidine kinase receptors in EHEC, such as QseC, have been found to have a key role in communication between the mammalian host and the bacteria. Such interaction is known as inter-kingdom signalling. As such, the host catecholamines, epinephrine and NE act as agonists on QseC, the same receptor used by quorum sensing molecule AI-3 (Hughes *et al.*, 2008). In this manner, host stress molecules can stimulate the activation of EHEC virulence factors such as TTSS and flagella (Sperandio *et al.*, 2003). In essence, AI-3 cross talks with epinephrine and NE. Furthermore, by sensing catecholamines EHEC can recognize an altered physiologic and immunologic function in the host (Hughes *et al.*, 2008). As examples, recognition of catecholamines by QseC of EHEC results in the transcription of flagella genes and TTSS to facilitate colonization. In presence of epinephrine and NE, attachment of EHEC to HeLa cells was increased, as was its motility and ability to form biofilms (Bansal *et al.*, 2007). Furthermore, injections of NE into bovine ligated ileal loops showed increased epithelial cell adherence and induction of enteritis by EHEC (Vlisidou *et al.*, 2004). There were increased neutrophilic infiltrates within the lamina propia, submucosa, and intestinal lumen. In addition, extensive A/E lesions were found in bovine ileal loops inoculated with EHEC in the presence of NA (Vlisidou *et al.*, 2004). No EHEC incited lesions were found in the intestinal loops only inoculated with a diluent (Vlisidou *et al.*, 2004). Collectively, the observations suggest that stress in cattle can influence the colonization of EHEC within the intestinal tract through inter-kingdom quorum signalling, thereby promoting its virulence to facilitate survival.

1.5 A nutritional basis for EHEC and commensal *Escherichia coli* intestinal tract colonization

Freter *et al.*, (1983) highlighted that to colonize and survive within the gut an organism must successfully use at least one limiting nutrient more efficiently than other competing bacteria. In this manner, an organism is defined by its ability to occupy a nutrient defined ecological niche that differs from the other species present. The population size of a particular bacterium can be defined by the amount and availability of the nutrients required to survive. Based on these principles, many studies have tried to determine the complex processes of catabolic mechanisms needed to metabolize nutrients from intestinal tract mucus by EHEC. The nutrients accessed from intestinal tract mucus by EHEC and its commensal counterpart have been studied in both cattle and mice (Bertin *et al.*, 2013; Fabich *et al.*, 2008; Maltby *et al.*, 2013; Miranda *et al.*, 2004).

Mucus is an important component of the mucosal barrier and assists in protecting the host from pathogen invasion. Mucus can also be used as a substrate for bacterial growth and is a good source of energy from carbohydrate metabolism. Fabich *et al.*, (2008) compared *in vitro* EHEC and commensal *E. coli* metabolism of intestinal carbohydrates normally present in mucus of mice. Mucus is formed by

glycoproteins that in the murine intestinal tract are comprised of polysaccharides containing 13 different monosaccharides which are potentially available to EHEC via degradation of these polysaccharides by anaerobic enteric bacteria (Fabich *et al.*, 2008). Notably, EHEC is unable to hydrolase these polysaccharides as it does not possess the necessary hydrolases (Fabich *et al.*, 2008). EHEC grown in mucus expresses catabolic pathways for the utilization of 7 of the 13 monosaccharides. These carbohydrate metabolic mechanisms have been confirmed in a human isolate of EHEC (EDL933), but differed from that of a commensal human *E. coli* isolate (MG1655) (Fabich *et al.*, 2008). The differences observed in carbohydrate utilization suggest that both pathogenic and non-pathogenic *E. coli* can coexist in the intestine by occupying unique niches, and that more than one commensal strain is necessary to cover the broad range of carbohydrate metabolic pathways that EHEC can exploit (Fabich *et al.*, 2008). In this regard, multiple *E. coli* commensal strains that covered the full spectrum of carbohydrates used by EHEC were needed to competitively exclude EHEC (Maltby *et al.*, 2013). This is supported by the observation that multiple commensal *E. coli* organisms can co-colonize and coexist in the intestine of streptomycin-treated mice based on the difference in the types of carbohydrates that these bacteria need for colonization (Maltby *et al.*, 2013).

In cattle, the main fermentable carbohydrates from mucus in the small intestine are galactose, NAG (N-acetylglucosamine), GalNAc (N-acetylgalactosamine), fucose, mannose, and *N*-acetyl neuraminic acid (Bertin *et al.*, 2013). The human EHEC isolate EDL933 is able to utilize all six sugars, and competition assays suggest that the capacity of EHEC to metabolize mannose, NAG, GalNAc, and galactose is critical to achieve maximum growth of the bacterium within the bovine intestine. The genes required to utilize the six sugars are expressed at maximal levels during the exponential growth phase, except for the gene required to degrade mannose, which has the highest expression during stationary phase (Bertin *et al.*, 2013). Bertin *et al.*, (2013) established that mannose and NAG catabolism provides EHEC with the greatest competitive growth advantage in cattle, and this differs from the sugars needed for colonization of the mouse intestine.

Although the nutritional environment is only one factor involved in colonization resistance (i.e. the mechanisms by which the autochthonous microbiota regulates pathogens), the determination of each bacteria nutritional requirements is important to understand the mechanisms of competitive exclusion between non-pathogenic and pathogenic *E. coli* strains. It is noteworthy, that *E. coli* O157:H7 and commensal *E. coli* can metabolize multiple different carbohydrates in order to survive in the intestinal

tract, rendering the understanding of mechanisms involved in the competition for nutrients even more challenging.

1.6 Virulence gene expression and influence of the metabolic landscape on EHEC

Commensal bacteria can alter the nutritional environment of EHEC as well as influence the metabolic landscape of the bacterium in the gut. *E. coli* O157:H7 can react to subtle changes in the environment to either activate or suppress the expression of virulence factors (Curtis *et al.*, 2014; Njoroge *et al.*, 2012). Changes in carbohydrate concentrations can have an impact on gene expression in EHEC. For example, growth under glycolytic conditions (environment rich in glucose concentrations, such as the doudenum and jejunum) can inhibit the expression of the transcription factor *Ler*, a LEE-1 encoded regulator that controls the transcription of LEE operons (Njoroge *et al.*, 2012). Conversely, growth in a gluconeogenic environment (environment with low glucose concentrations, such as the distal colon) can activate the expression of LEE operons and consequently the virulence of EHEC (Njoroge *et al.*, 2012). This demonstrates that *E. coli* O157:H7 can recognize and follow a gradient of nutrient concentration, repressing expression of factors involved in colonization during unfavorable conditions (i.e. small intestine) and activating these factors under the favorable nutritional conditions (i.e. large intestine) (Pifer *et al.*, 2014). *E. coli* O157:H7 transcription factor *Cra* can also sense fluctuations in carbohydrate concentrations within the gut and this activates expression of LEE genes. The carbohydrate content present within this environment can be altered by the presence of other bacteria and in this manner stimulate or inhibit the production of virulence factors in EHEC, thus having an impact on its intestinal colonization and survival (Curtis *et al.*, 2014). *Bacteroides thetaiotaomicron* (*B. theta*) has been found to increase the expression of 20% of the *E. coli* genome (Curtis *et al.*, 2014). The presence of *B. theta* augmented the expression of LEE genes, *Stx2a*, and the *StcE* gene, a gene that encodes a mucinase. Furthermore, *B. theta* also increased expression of *Ler* and TTSS structural proteins such as *EspA*, TTSS receptor Tir (Curtis *et al.*, 2014). In summary, modification of the local gut environment by other bacteria can influence the expression of virulence factors in EHEC, and thereby modulate growth and colonization of EHEC within the intestinal tract.

1.7 Mouse models to evaluate EHEC intestinal colonization

The predominant animal model used to study pathogenesis of *E. coli* O157:H7 in humans is the mouse. I propose to use a murine model to elucidate mechanisms of competition between commensal and pathogenic *E. coli* from a cattle perspective. The main murine models utilized are conventional mice treated with streptomycin to induce intestinal dysbiosis or germ-free (GF) mice (Mohawk *et al.*, 2011).

GF mice are a mouse model devoid of microorganisms (Yi *et al.*, 2012). In contrast, mice with a fully established and known microbiota are considered a gnotobiotic mouse (GB). Thus, GF mice colonized with known strains of bacteria will become gnotobiotic mice (Wymore Brand *et al.*, 2015). Conventional mice (mice with normal flora in which the bacterial flora is undetermined) have also been used as *E. coli* O157:H7 colonization models. The fidelity of this model, however, can be compromised by the presence of resident commensal *E. coli* and other enteric bacteria that potentially confound EHEC colonization studies (Mundy *et al.*, 2006; Nagano *et al.*, 2003). Long-term fecal shedding was achieved in only one of the conventional mice tested, and intestinal colonization rates of *E. coli* O157:H7 were low (Mundy *et al.*, 2006; Nagano *et al.*, 2003). As such, other models have proven better to study EHEC induced intestinal disease. Streptomycin-treated mice have been used extensively as a human model to study EHEC infection, colonization and competitive exclusion (Fabich *et al.*, 2008; Gamage *et al.*, 2006; Leatham *et al.*, 2009; Maltby *et al.*, 2013; Miranda *et al.*, 2004; Wadolkowski *et al.*, 1990). Treatment with streptomycin induces intestinal dysbiosis by inhibiting the growth of commensal facultative anaerobic bacteria. More specifically, bacterial densities of enterococci, streptococci, lactobacilli, anaerobe lactobacilli, and bifidobacteria are reduced, while *Bacteroides* and *Eubacterium* species remain unaffected by antibiotic treatment (Leatham *et al.*, 2009). This allows an opportunity for EHEC and other commensal *E. coli* to successfully colonize and persist within the mouse intestinal tract. Another model used to study EHEC colonization and infection is the GF murine model. No competition occurs between EHEC and resident bacteria as the gut lacks microorganisms. Several studies have employed this model. Takahashi *et al.*,(2004) observed robust colonization of a hypertoxigenic EHEC strain (10^8-10^9 CFU/g of feces) by day 6 post inoculation, and increased death in mice mono-associated with EHEC on day 7 post treatment. Taguchi *et al.*,(2002) also observed high colonization rates EHEC in the intestinal tract of GF mice with corresponding manifestation of colonic injury and inflammation. Other studies have also observed successful EHEC colonization in GF mice, with indication of disease that include animals with marked neutrophilic necrotic enteritis that on occasion succumbed to disease (Isogai *et al.*, 1998). Moreover, Eaton *et al.*, (2008) tested ten different EHEC serotypes in Swiss-Webster GF mice, and showed colonization of all bacterial species within the gut. Colonization was unaffected by the dose or time interval of the oral inoculation, and bacterial shedding was robust at 10^9 to 10^{12} CFU/g of feces. This demonstrates that regardless of the inoculation dose, EHEC will colonize at a similar final density. Eaton *et al.*, (2008) claimed that GF mice are exquisitely susceptible to colonization by EHEC and that inoculation with numbers as low as 100 cells can increase growth to a persistent intestinal bacterial

density of 10^9 CFU/g or more within a single day. They also observed that EHEC colonizing the intestinal tract of GF mice caused clinical symptoms of disease, including lethargy, dehydration, polyuria, and polydipsia, with death of challenged mice occurring 4 to 7 days post inoculation. Interestingly, inoculated mice did not develop diarrhea, but cecum edema was observed (Eaton *et al.*, 2008). As well, when inoculated with ten different EHEC strains only seven strains caused disease in mice and it was speculated that the most significant causes of disease was renal injury; similar to HUS in people (Eaton *et al.*, 2008).

The Swiss Webster GF mouse model inoculated with 10^6 CFU of EHEC has been used in a number of studies (Goswami *et al.*, 2015; Tyler *et al.*, 2013). Mice inoculated with strain EDL933 became moribund and exhibited lower body weights, renal tubular necrosis, and renal failure 3 weeks post inoculation. The same bacterial strain, but lacking the ability to produce shiga toxin, did not develop disease and mice exhibited normal renal morphology (Tyler *et al.*, 2013).

In summary, GF mice are a valuable model to study colonization of the intestinal tract by EHEC. Studies can be directed at elucidating the competition of inoculated bacteria for the same niche as there is no intestinal microbiota to confound analyses. The streptomycin induced dysbiosis model allowed colonization of the murine intestinal tract by *E. coli* O157:H7, however, resident bacterial flora is present, thereby potentially limiting elucidation of specific bacterial colonization mechanisms.

1.8 *Escherichia coli* O157:H7 location in the intestinal tract of mice

Commensal *E. coli* (HS, MG1655 and Nissle 1917 strains) can be isolated from mucus of the entire intestinal tract of streptomycin treated mice with the highest densities collected from cecal and colonic mucus (Leatham *et al.*, 2009). Moreover, in absence of these commensal *E. coli* bacteria, *E. coli* O157:H7 effectively colonized along the entire gut (Leatham *et al.*, 2009). Similar to cattle, the highest densities of commensal *E. coli* were present in feces of mice as compared to bacteria isolated directly from intestinal samples (Leatham *et al.*, 2009). In another study, similar observations determined that densities of commensal *E. coli* and EHEC were 10 fold higher in cecal and colonic mucus compared to the rest of the intestinal tract (Miranda *et al.*, 2004). Although both strains (commensal and pathogenic) were found in mucus along the entire intestinal tract, higher densities of cells were observed in the large intestine. Using fluorescence *in situ* hybridization (FISH), *E. coli* O157:H7 (EDL933) was associated with the epithelium and mucus along the intestinal tract, with yet again, numbers ten-fold higher in the large intestine (Miranda *et al.*, 2004). Surprisingly, EHEC failed to grow in cecal luminal content and contrary to EHEC, commensal *E. coli* were not associated with the epithelium (Miranda *et al.*, 2004). Finally, in GF

mice challenged with EHEC, Eaton *et al.*, (2008) observed EHEC colonized the entire intestinal tract, with the highest densities of the bacteria in digesta from not only the cecum, and colon, but ileum as well. High levels of bacterial adherence to the epithelium were also observed in the ileum and cecum, with lower levels of adherence were observed in the colon. Furthermore, densities of bacteria within digesta were higher thorough out the intestinal tract than in association with the epithelium (Eaton *et al.*, 2008).

1.9 Mechanisms of competitive exclusion in the intestinal tract

In a healthy gut, a mutually beneficial relationship exists between the microbiota and the host. The host provides 'commensal' bacteria with a stable growth environment and nutrient supply, and in return, the commensal microbiota help develop and modulate the immune system, provides nutrients, and assists with both the prevention of colonization and elimination of pathogens from the gut (Stecher *et al.*, 2008). The mechanisms by which the commensal bacteria inhibit pathogen colonization within the intestinal tract is known as colonization resistance (CR) (Stecher *et al.*, 2008; Sassone-Corsi *et al.* 2015). There are several mechanisms that lead to successful CR of pathogens within the gut. These mechanisms include; direct inhibition of pathogens, nutrient depletion in specific intestinal locations, and modulation of intestinal and extra-intestinal immune responses (Stecher *et al.*, 2011; Sassone-Corsi *et al.* 2015). The production of antimicrobial peptides, such as bacteriocins, the release of inhibitory metabolites (e.g. butyrate or acetate), and competition for binding sites or stimulation of mucus secretion are processes involved in direct inhibition (Stecher *et al.*, 2011; Sassone-Corsi *et al.* 2015).

Many nutrients required for bacterial growth are limited within the gut, and the ability of bacteria to access and assimilate nutrients is vital for their growth. The high diversity of the microbiota within the intestinal tract of mammals results in vigorous competition for limited nutrients between all microorganisms (Stecher *et al.*, 2011; Sicard *et al.* 2017). In this regard, if a bacterial pathogen is unsuccessful at accessing required nutrients (e.g. as a result of competition by autochthonous bacteria) it is unable to successfully colonize the intestinal tract at densities needed to infect the host and subsequently incite disease. In some situations, pathogens can benefit from the presence of inflamed tissue within the intestine by possessing adaptive systems that preferentially overcome acute or chronic inflammation while other commensal organisms are eliminated by the inflammatory processes (Stecher *et al.*, 2007; Sassone-Corsi *et al.* 2015). Some pathogens have even evolved mechanisms that stimulate a pro-inflammatory immune response that reduce the diversity of commensal bacteria at the site of inflammation; allowing the pathogen to occupy niches that would have been previously unavailable (Stecher *et al.*, 2007). Moreover, pathogenic microorganisms have acquired the ability to differentially

exploit niches within the intestinal tract. For example, many pathogenic bacteria such as EHEC, possess specific pathogenicity factors such as adhesins or invasins that aid in epithelial attachment and enable the pathogen to successfully colonize the gut. Other bacteria can breach the mucus barrier, including the tightly adherent mucus layer, avoiding entrapment of the bacteria within the mucus (Sansonetti, 2004, Tyrrell, 2007). Finally, certain *Bacteroides* spp. possess a modified LPS that is less immunogenic as compared to the highly immunogenic LPS of EHEC thus reducing host recognition of the bacteria (Sansonetti, 2004).

1.9.1 Competitive exclusion in cattle

Several observational competitive exclusion studies in cattle using probiotic *E. coli* as a strategy to eliminate EHEC from the gut have been explored (Schamberger *et al.*, 2004; T. Zhao, *et al.* , 1998; T. Zhao *et al.*, 2003). In some instances, shedding of *E. coli* 0157:H7 in adult cattle and calves was reduced following challenge with the microorganism. Notably, colicin E7-secreting *E. coli* reduced EHEC numbers in cattle (Schamberger *et al.*, 2004). Colicins are antimicrobial proteins produced by some *E. coli* strains that can eliminate other bacteria by inhibiting peptidoclycan synthesis, forming membrane pores or cleaving DNA (Schamberger *et al.*, 2004). However, given the presence of a microbial community in the intestinal tract it is hard to conclude if the reduction of EHEC was associated with colicin E7 or due to other mechanisms. Conducting experiments directly in cattle that investigate mechanisms of competitive exclusion presents a number of salient limitations. These limitations can be related to their animal husbandry practises, cost, traction and size of the animals, and genetic, physiological, and microbial heterogeneity.

1.9.2 Competitive exclusion in mice

To date, EHEC competitive exclusion studies performed in mice have mainly focused on utilizing the mouse as a model of human intestinal competition. These studies have been conducted with human *E. coli* 0157:H7 isolates and have used human isolates of commensal *E. coli* as competitive strains (Fabich *et al.*, 2008; Gamage *et al.*, 2006; Leatham *et al.*, 2009; Miranda *et al.*, 2004). Competition between bovine isolated *E. coli* 0157:H7 and bovine commensal *E. coli* isolates have not been utilized in a mouse model. In this manner, the mouse model has not been used to study these factors in bovids, where the progression of intestinal inflammation mimics intestinal changes within the bovine host (i.e. a colonization model of chronic inflammation) without development of kidney injury and renal failure.

Competitive exclusion studies conducted in mice have provided valuable information on potentially excluding EHEC from the intestine. The specific mechanisms involved in these competitions, however,

are complex and are not fully elucidated. Presently, mechanistic studies conducted have mainly focused on the competition for limiting nutrients between commensal *E. coli* and EHEC, and in particular, researchers have selected non-pathogenic *E. coli* which metabolize all nutrients needed by EHEC to achieve colonization resistance (Maltby *et al.*, 2013). Moreover, researchers analyzing different carbohydrates metabolized by both commensal *E. coli* (MG1655) and EHEC (EDL933) strains concluded that both bacteria can coexist and colonize in the gut of mice. In addition, despite requiring some of the same carbohydrates to survive, both bacteria were able to co-colonize based on their ability to metabolize different sugars (Fabich *et al.*, 2008). The diverse metabolic tools that *E. coli* O157:H7 possesses and the ability of different *E. coli* strains to co-exist suggests that a single commensal *E. coli* strain will likely be insufficient to outcompete EHEC for colonization within the intestine. Leatham *et al.*, (2009) showed that individually, MG 1655, HS and Nissle 1917 *E. coli* strains were incapable of outcompeting EDL 933 EHEC strain for gut colonization in streptomycin-treated mice. However, when co-administered, these three commensal bacteria, having different nutrient requirements, were able to accomplish a 4 fold reduction in the number of EHEC collected in feces. Importantly, these commensal bacteria were introduced to the mice 10 days prior to the challenge with EHEC, allowing for successful colonization of the commensal microbes and thus preventing EHEC growth in the gut. Bacteria previously established in the intestinal tract (i.e. occupying specific niches) have a competitive advantage over the bacteria that are newly introduced and require the same niche. The specific mechanisms for exclusion of EHEC by the three commensal *E. coli* were not determined. Nutrition, innate immunity, or the indigenous microbiota (i.e. since the mice used were administered streptomycin to incite a dysbiosis, an undefined microbiota remained) could all have been involved in the competition. Contrary to this study, Gamage *et al.*, (2006) competed EHEC with a single commensal *E. coli* isolate in streptomycin-treated mice and showed reduced EHEC concentrations in feces at 4 days post-inoculation. The mechanisms behind this reduction were undetermined.

1.10 Germ-free mice as an animal model

Germ-free mice are considered 'free of demonstrable viable microbial associates', and they are a valuable model for studying inter-bacterial interactions and host-bacterial interactions *in vivo* (Gordon, 1960). The administration of different bacteria into the intestinal tract of GF mice allows researchers to specifically focus on the introduced bacteria without the confounding effects of the enteric commensal bacterial community. However, it must be considered that this lack of commensal microbiota can also affect the utility of GF mouse model. In this regard, the small intestine, and extra-intestinal tissue such

as lymph nodes and on occasion the liver, have reduced weights in comparison to conventional mice. In contrast, tissues that are naturally not in contact with microorganisms, such as extra-intestinal organs and the nervous system are equivalent in weight and size to conventional mice (Gordon, 1960). Moreover, IgA and IgG immunoglobulins are produced in smaller quantities following antigenic stimulation in GF mice. The structure of the intestinal tract in GF mice also differs substantially from conventional mice and it has been shown that bacteria will affect normal development (Thompson *et al.*, 1971). The small intestine is thinner and hypocellular with fewer numbers of lamina propria macrophages and lymphocytes. The villus crypts are shallower, with lower germinative cell mitotic activity and Peyers patches are reduced in size (Thompson *et al.*, 1971). Notably, the intestinal lymphoid tissue in GF mice is still functional and capable of mounting a response to antigenic stimulation (Thompson *et al.*, 1971). One prominent feature of the intestinal tract of GF mice is the enlarged cecum, which can weight up to 10 times more and contain 6 times more cecal content as compared to conventional mice. The cecal content also has a more liquid consistency and has a hypotonic osmolarity. Finally, motility and peristaltic waves of the intestinal tract are significantly reduced as compared to mice with an established intestinal microbiota. (Gordon, 1960; Thompson *et al.*, 1971).

Despite all of its advantages for studying bacterial colonization, competitive exclusion and the mechanism of pathogen induced tissue injury, conducting research using GF mice has many challenges; the most salient being prevention of bacterial contamination and retention of its GF status. Specialized equipment such as isolators are required in the main animal housing units (Arvidsson *et al.*, 2012). These isolators are fitted with HEPA filters and require a constant positive pressure airflow to prevent bacterial contamination of the mice. The implementation of strict operational procedures is also imperative. Currently, many experiments using GF mice are conducted within isolators; however multiple isolator units are often needed if studies involve inoculation of mice with different microorganisms. Furthermore, within individual isolators it is not possible to separate mice by treatments, necessitating the need to use multiple isolators for experimentation. The cost of establishing and maintaining GF mice infrastructure can be prohibitive, and many animal facilities do not have the resources for such equipment. Protocols have been developed for housing GF mice in individually ventilated cages (IVCs) in order to conduct experiments within the IVCs as opposed to the isolators (Hecht *et al.*, 2014; Paik *et al.*, 2015). Importantly, IVCs allow easy separation of experimental treatment groups, and can be operated in containment mode (negative pressure airflow), which is requirement when working with level 2 risk group (RG 2) pathogens, such as EHEC. The methods currently utilized for housing GF mice in IVCs are

developed for Isocage P cages, which are very expensive and specifically designed for facilities housing GF mice (Hecht *et al.*, 2014; Paik *et al.*, 2015). Thus, Isocage P cages are not commonly available for use in many animal facilities. Notably, the possibility of using conventional IVCs operated in containment mode to study RG2 pathogenic microorganisms in GF mice has not been determined.

1.11 Mice as animal stress models

As indicated previously, a stressful event can trigger the activation of the HPA axis elevating of glucocorticoids in blood and thereby inducing metabolic changes within the host. Mice have been used as animal models to study stress, depression, and anxiety. These models can be achieved by either directly administering exogenous corticosterone to mice or placing the mouse under stressful conditions and stimuli, such as physical restraint or forced periods of swimming ultimately elevating endogenous corticosterone blood levels (Demuyser, Deneyer, *et al.*, 2016).

The administration of glucocorticoids such as corticosterone to induce physiological changes representing chronic stress has been employed through various means, including; administration in drinking water, subcutaneous injections, oral gavage, and the implantation of slow-release subcutaneous pellets (Demuyser, Deneyer, *et al.*, 2016). Corticosterone in drinking water is the most commonly used method although it can be difficult to standardize dosage due to different amounts of water ingested by the animals. However, this method is preferred and often used given its ease of administration, especially when using GF mice, as this method of administration reduces the potential of an accidental bacterial contamination of the mice (Ardayfio *et al.*, 2006; Karatsoreos *et al.*, 2010; Kinlein *et al.*, 2015; Shahanoor *et al.*, 2017). Another advantage of providing corticosterone in water is the reduction in stress associated with animal handling and drug administration (Ardayfio *et al.*, 2006). Indeed, injections of corticosterone or subcutaneous surgeries required to implant corticosterone pellets can be particularly traumatic for mice, potentially generating unwanted stress and spiking endogenous corticosterone levels in control animals. Although dosage standardization is superior with corticosterone pellets or injections, inadvertent stress and the risk of bacterial contamination of GF mice are paramount considerations, and both are reduced by administering corticosterone *per os* in drinking water.

Multiple studies have examined the impact of different dosages of corticosterone on the induction of stress responses in mice. In this regard, a concentration of 25 µg/ml corticosterone in a 1% ethanol (EtOH) solution (i.e. low dose) will induce modest changes in the physiology and behavior while a high dose of 100 µg/ml corticosterone in a 1% EtOH vehicle causes more prominent effects (Karatsoreos *et*

al., 2010; Kinlein *et al.*, 2015). Importantly, it has been shown that corticosterone-induced physiological stress in mice also corresponds to behavioral changes (Demuyser *et al.*, 2016). Examples of behavioral modifications include increased levels of depression-like and anxiety-like behaviors in corticosterone treated mice. Behavioral changes are evaluated using different tests and some of these include; open field exploration (OFE) test, forced swim, elevated plus maze challenge (tests the anxiety-like behavior of mice by observing the movement, entries and exits of mice between an area protected by walls and an open unprotected area), tail suspension, and others (Demuyser *et al.*, 2016).

1.11.1 Open field exploration test

The open field exploration is a behavioral test that consists of an empty arena or enclosure (typically 30X30 cm or larger) of circular or square shape surrounded by walls to avoid escape of the mice. The mouse is individually placed in the enclosure and the behavior of the animal is observed for a defined period of time (Gould, 2009). This test measures exploratory behaviour of the mouse where qualitative and quantitative measurements are made. General locomotion activity, responses to a novel environment, and the induction of anxiety-related behaviour can be assessed (Bailey *et al.*, 2009; Gould, 2009). Although OFE measures various stress induced traits, this test has traditionally been used to study anxiety-like behavior such as fear in rodents. There are two main factors that influence anxiety-like behavior in an open field analysis; isolation of the individual animal, and stress induced by positioning the animal in a brightly lit unprotected novel environment (Bailey *et al.*, 2009).

The duration of the OFE test is typically between 2 and 10 minutes. This timeframe excludes the possibility of observing baseline activity or habituation to an environment, since the animal must be familiarized with the environment to observe such behaviors. Generally, baseline activity is observed after 30 minutes of exposure to the testing environment (Gould, 2009). The aim of conducting short tests is to study anxiety-like behavior when the animal is forced to experience a new environment. The traditional parameters measured in this test are distance travelled, time spent moving, time spent in the center of the open field arena, vertical activity, and number of fecal pellets excreted (i.e. good indicator of the activity of the autonomous nervous system). Typically, an animal introduced into an open field testing arena will spend most time near the walls of the enclosure, a behaviour known as thigmotaxis (Bailey *et al.*, 2009; Gould, 2009). This natural behaviour is observed because mice feel more protected close to the walls of the enclosure as opposed to the open and illuminated center of the arena. Mice that frequently explore the center of the enclosure are considered to exhibit less anxious behaviour, an observation in mice treated with anxiolytics (Bailey *et al.*, 2009). This type of testing has been used to

effectively measure exogenous corticosterone responses in mice. As an example, Sturm *et al.*,(2015) found that the mice strains, C57BL/6J and C57BL/6N treated with subcutaneous corticosterone pellets had reduced locomotor activity compared to placebo animals. Furthermore, C57BL/6N mice exhibited less frequent visits to the center of the arena as compared to placebo and treated C57BL/6J animals; showing that mice with different genetic backgrounds are affected differently by the presence of exogenous glucocorticoids. Demuyser *et al.*, (2016), also studied the impact of exogenous corticosterone in the behavior of mice. The researchers observed a significantly lower time spent in the center of the enclosure during 5 and 30 minute tests in mice that were treated with subcutaneous corticosterone pellets relative to untreated mice.

Finally, a recent study examining neonatal stress observed that GF mice separated from the dam did not demonstrate any significant differences in time spent in the center of the field relative to control mice. The neonatal mice, however, defecated higher quantities of feces during the test and it was suggested that this was a stress-induced increase in colonic motility (De Palma *et al.*, 2015). A limitation of the OFE testing in GF mice is the potential for bacterial contamination and loss of GF mouse status. Thus, repetitive OFE testing is not recommended and as such OFE is often the final test in the study before the experimental endpoint.

1.11.2 Nest building test

A non-invasive method to evaluate the behavior of mice is the assessment of nest building activities. Nests are essential structures for rodents. These are not only needed for protection and reproductive purposes, but also to aid in thermoregulation reducing heat loss (Deacon, 2006). The impact of stress on the nest building ability of mice has not been extensively studied. The non-invasive nature of nest building may be a valuable method in GF mice to assess behavioral responses associated with stress. Importantly, nest building behavior can be assessed in IVCs with minimal contact and handling, thus diminishing the potential for breach in containment and inadvertently introducing microorganisms to the mice.

1.12 Knowledge Gaps

Although *E. coli* O157:H7 has been studied extensively, there are many aspects of the host-pathogen interactions that still remain unknown. Current methods to control *E. coli* O157:H7 in cattle with the goal of preventing transmission of the pathogen to people have largely been unsuccessful. Commensal bacteria have been found to influence the course of EHEC's colonization (Curtis *et al.*, 2014), but to date, competitive exclusion studies utilizing mice have mainly focused on excluding EHEC from the

perspective of human medicine, and have thus used human commensal *E. coli* to study competition, colonization, pathogenesis and disease (Fabich *et al.*, 2008; Gamage *et al.*, 2006; Leatham *et al.*, 2009; Miranda *et al.*, 2004). To my knowledge, the ability of bovine commensal *E. coli* isolates to exclude EHEC has not be examined, nor have mice models been used for EHEC colonization that simulate the bacterial interactions encountered in cattle. Moreover, the mechanisms involved in the interaction mentioned above are currently unknown, and the acquisition of such information could be key toward the development of innovations to mitigate this important zoonotic pathogen on the farm.

Different methods of growing commensal bacteria have been previously compared to establish their influence in competition with a pathogen *in vivo*. In this regard, commensal bacteria grown individually and then mixed prior to inoculation provided less protection against *Salmonella* in chicken than the same commensal bacteria co-cultured for equal amounts of time and administered to the host (Stavric, 1992; Stavric *et al.*, 1985). In both scenarios the pathogen was grown separately from the commensal bacteria. The reason of the competitive advantage provided by communal growth (co-culture) of commensal bacteria prior to inoculation is unknown, and whether this phenomenon will apply to competitive exclusion of EHEC by commensal *E. coli* strains is currently unexplored. As such, this area of investigation would help further elucidate mechanism involved in competitive exclusion.

The immune response generated by cattle and mice colonized by *E. coli* O157:H7 over prolonged periods, and the impact of the host immune system on the bacteria are not fully understood. Moreover, the presence of the enteric microbiota makes assessing changes to colonization even more challenging. As such, I propose to use gnotobiotic mice colonized with an *E. coli* O157:H7 isolated from cattle to investigate the colonization of this important pathogen, and how it may be competitively excluded by bovine isolated commensal *E. coli* (i.e. that occupy the same niche). Most studies have focused on determining the effects of Shiga toxin on the host, mainly from a perspective of studying the pathogenesis of HUS, but relatively few studies have examined interactions between *E. coli* O157:H7 and commensal *E. coli* within the intestinal tract.

It has been proposed that EHEC resides in the lymphoid follicle rich mucosa of the terminal rectum of cattle (Naylor *et al.*, 2003); however, knowledge on mechanisms of colonization and EHEC interaction with the host are still broadly undefined and elucidation could potentially provide valuable information towards comprehending EHEC's behaviour in the gut. Furthermore, it is not fully resolved why EHEC colonization of the intestinal tract of adolescent and adult cattle does not incite clinical disease. This characteristic suggests ruminants act as a silent reservoir creating a complex scenario for both detecting

and reducing EHEC in cattle operations. Moreover, EHEC environmental shedding is not fully understood. Shedding patterns have shown to be variable throughout the seasons of the year presenting short peaks of shedding followed by prolonged periods of intermittent shedding of low numbers of bacteria or none at all (Stevens, 2002,Laven *et al.*, 2003). Additionally, the immune response mounted towards *E. coli* O157:H7 in the large intestine needs further exploration in order to enhance potential mitigation strategies. Little is know on the cellular and humoral responses in the intestinal tract when *E. coli* O157:H7 is forced to compete for colonization niches with commensal *E. coli* strains.

The impact of stress on *E. coli* O157:H7 intestinal tract colonization and disease has received relatively limited attention (Sperandio *et al.*, 2003). The influence of stress molecules, such as catecholamines, on *E. coli* O157:H7 has been studied in cell cultures and ileal loops; however, there is little knowledge on the impact of stress on *E. coli* O157:H7 colonization *in vivo.* Furthermore, corticosterone, a hormone elevated for prolonged periods during chronic stress, has not been studied as an inducer of stress in an *in vivo* model of *E. coli* O157:H7 colonization. Additionally, and to the best of my knowledge, there is little information on the impact of stress on EHEC intestinal colonization, intestinal immune function and competitive exclusion between commensal of *E. coli* and *E. coli* O157:H7 in a GB mouse model.

1.13 Objectives and hypotheses

The literature review demonstrates the need for development of mitigation strategies towards *E. coli* O157:H7 in its natural bovine reservoir. Further information of *E. coli* O157:H7 colonization mechanisms and competition with commensal *E. coli* bacteria is needed. The experiments conducted in this thesis attempt to target some of these questions. The objectives of this study have been divided into three experiments. Experiment 1, (a) establish a method to successfully transfer GF mice from an isolator to a conventional IVC and (b) establish a method to avoid contamination of GF mice housed in IVCs while conducting experimental research. Experiment 2, (a) establish an *E. coli* O157:H7 GF murine model of colonization, (b) ascertain mechanisms of colonization and host responses to different *E. coli* O157:H7 strains in GF mice and (c) select an appropriate *E. coli* O157:H7 strain for utilization in experiment 3. Experiment 3, (a) establish a corticosterone stress model in GF mice, (b) elucidate *E. coli* O157:H7 colonization mechanisms and in stressed versus non-stressed GF mice, (c) obtain a collection of characterized commensal *E. coli* strains, and select appropriate strains for evaluation, (d) use a GF murine model to elucidate mechanisms by which non-pathogenic *E. coli* inhibit *E. coli* O157:H7 disease incitement and/or colonization in the intestinal tract, (e) assess the influence of stress upon *E. coli*

O157:H7 competition with non-pathogenic *E. coli* and (f) asses the effectiveness of competitive exclusion of *E. coli* O157:H7 by non-pathogenic bovine *E. coli* strains grown communally as compared to strains grown separately.

The hypothesis tested in this thesis were:

a) Mice can be used as an intestinal tract colonization model for EHEC that mimics cattle.
b) Stress will enhance *E. coli* O157:H7 competitive advantage and alter the host response, thereby facilitating the colonization success of *E. coli* O157:H7 in the intestine relative to non-pathogenic *E. coli*.
c) Specific non-pathogenic bovine *E. coli* strains can competitively exclude *E. coli* O157:H7 by altering its virulence gene expression and the metabolic landscape in the intestine.
d) Non-pathogenic bovine *E. coli* strains grown communally will be more effective at competitive excluding *E. coli* O157:H7 than the same strains grown separately.

1.14 References

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Chapter 2: Housing gnotobiotic mice in conventional animal facilities[1]

2.1 Introduction

Germ-free (GF) animals are devoid of microorganisms (Yi *et al.*, 2012). Gnotobiotic (GB) animals possess a microbiota in which all microorganisms associated with the animal are known (e.g., Altered Schaedler Flora mice) (Dewhirst *et al.*, 1999; Wymore Brand *et al.*, 2015). *Sensu stricto* GF animals are GB, as the status of their microbiota is known. The characteristics of these mice make them an ideal mammalian model (Jiminez *et al.*, 2015), and GB mice have emerged as a powerful model to elucidate various aspects of the host-microbiota interaction (De Palma *et al.*, 2015; Eaton *et al.*, 2008; Gordon, 1960). Conducting logistically feasible experimentation without the introduction of contaminating microorganisms is a salient challenge facing researchers utilizing GB mice. Colonies of GF and GB mice are generally reared and kept in isolators with strict procedures to prevent contamination of the animals (Arvidsson *et al.*, 2012). Isolators have been used for decades as the sole housing option of GF and GB mice, and they are still a first choice for maintaining colonies (Al-Asmakh *et al.*, 2015; Arvidsson *et al.*, 2012; Reyniers, 1959). However, isolators possess many limitations for conducting experiments with GF and GB mice. For example, isolators are expensive to maintain; individual isolators do not allow physical segregation of treatments (e.g., when contamination of an isolator occurs, all animals within the isolator become contaminated); and most facilities do not possess isolators that can be operated under containment mode, which is required for experiments involving pathogenic microorganisms. Recently, equipment and techniques have been developed for GF and GB mice experimentation that utilize individually ventilated cage (IVC) systems (Hecht *et al.*, 2014; Paik *et al.*, 2015). Although any infrastructure and equipment needed for experimenting with GF mice is relatively expensive, IVCs are more cost- and space-effective than isolators; each IVC acts as a separate experimental unit, allowing colonization experiments with different microorganisms and different mice strains to be conducted; and IVCs facilitate the establishment of a specific environment in each cage, which is important for both experimental and ethical reasons. Previous investigations for conducting experiments with GF and GB mice have utilized specially designed Isocage P IVCs (Tecniplast) (Hecht *et al.*, 2014; Paik *et al.*, 2015). It is important to stress that these specialized IVC systems are not available at many institutes and the initial investment for obtaining them can be expensive. Thus, I present techniques for safely moving GF

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mice from an isolator into a commonly available conventional IVC system (Tecniplast) operated under containment mode, and for cage changing and sampling in this system without compromising the sterile status of the GF mouse model. Cages operated under containment mode can be used to maintain the GF status of mice for future experimentation involving GB mice with or without pathogenic microorganisms. I also describe the techniques that are employed in our lab to test the GF status of mice reared in the IVCs.

Basic Protocol 1 explains the sterile method for transporting mice from the colony in the isolator to the IVC cages placed on a rack. Basic Protocol 2 addresses the steps for weekly sampling and sterile cage changing of mice in the IVCs. In additions, four strategies are described to assess the sterile status of samples obtained from the GF mice: (1) detection of bacteria by aerobic and anaerobic culture (Support Protocol 1); (2) detection of bacteria using endpoint PCR targeting the 16S rRNA gene (Support Protocol 2); (3) visualization of bacteria within a filtered matrix suspension using fluorophore staining (Support Protocol 3); and (4) fluorescent *in situ* hybridization (FISH) of intestinal tissues using a bacterial probe (Support Protocol 4). The decision to apply one or more of these methods will depend on the experimental situation; however, application of more than one method is recommended. The protocols described herein possess high risks of contamination of the animal model if the steps described are not strictly followed.

Note: All protocols involving live animals must be reviewed and approved by an Institutional Animal Care and Use Committee (IACUC) and must conform to government regulations for the care and use of laboratory animals.

2.2 Materials and Methods

2.2.1 Basic Protocol 1

2.2.1.1 Transport of mice into individually ventilated cages

This protocol describes the procedures to transport GF mice from a GF isolator unit into IVCs and maintain microbiological sterility. Prior to commencement, the IVC HEPA filtered air-handling unit (Smart Flow, Tecniplast) should be set to containment mode (negative air pressure) to allow work with Risk Group 2 (RG2) pathogens in mice as a mammalian model. The air handling unit provides HEPA-filtered air into and exiting the IVCs, in this way avoiding any cross-contamination among cages. The efficacy of the HEPA filter unit is confirmed by a certified technician on an annual basis. Animal rooms are also set to operate in containment mode (inward directional airflow). The transport protocol employs the use of a TransDisk (27.7-cm diameter; Class Biologically Clean), which contains a HEPA filter

to allow breathing and prevent contamination. Mice are transferred under sterile conditions first from the isolator to the TransDisk and then from the TransDisk to the IVCs.

Two workers are required to perform the protocol. One individual serves as an assistant and is responsible for sanitizing instruments and cages with Clidox, and handing materials to the primary individual, who actively works inside an operating and certified Class 2A biosafety cabinet (BSC). The primary individual must follow procedures to ensure that the gloves and gown donned remain sterile. The BSC serves as sterile work station in which the GF mouse model is handled within the animal room; therefore, any items entering the BSC must be autoclaved or appropriately sanitized to ensure the sterile integrity of the work station.

This protocol is described here using our strain of choice, C57BL/6J (parental origins from University of North Carolina, Chapel Hill), at 5-weeks-of-age, the standard age at which we start most of the experiments in our research facility. However, it is applicable to any strain of mouse at any age, and can be conducted with any isolator capable of housing GF mice.

The mouse feed utilized must be autoclavable, because it must be sterile when provided to the mice. Our diet of choice is Prolab RMH 3500, Autoclavable 5P04 (LabDiet), although any high standard autoclavable diet should suffice. When autoclaved, it is important that feed be distributed in a relatively thin layer (≈2.5 cm) to ensure heat penetration. Although in the lab we routinely use a temperature of 121°C in concert with a quality assurance procedure, increasing the temperature of the autoclave step (e.g., to 132°C) is an option, although this may increase degradation of heat-labile ingredients in the diet.

2.2.1.2 In advance set up

1. Set up IVCs (Sterile Green Line GM500 Sealsafe Plus IVCs, Tecniplast) with bedding, a house, nest material, enrichment items, and food (autoclavable mouse feed).

2. Double-wrap IVCs, dissection forceps, mouse-handling forceps, beaker, tube rack, and paper towels in surgical wrap (Sontara disposable surgical drapes, Jorgesen Labs) and autoclave before use.

All autoclavable items should be sterilized at 121°C and 105 kPa. For cages containing feed, autoclave conditions (i.e., prevacuum cycle) are: 1 min purge; 60 min sterilization; and 15 min drying. For wrapped items, autoclave conditions (i.e., gravity cycle) are: 1 min purge, four pulses; 30 min sterilization; and 15 min drying. For liquids, autoclave conditions (i.e., liquid cycle) are: 1 min purge and 60 min sterilization. To ensure efficacy, include a test cage containing a biological indicator (i.e., *G.*

stearothermophilus, Attest Biological Indicator, VWR International) in each autoclave run and follow the manufacturer's instructions to determine viability of endospores.

Note: On a monthly basis, a DART (Bowie-Dick) test should be run to confirm the adequacy of air removal within the autoclave, and a leak test to measure the integrity of the sealed pressure vessel and the associated piping. For materials that cannot be autoclaved, sanitize with Clidox (1:3:1 [v/v/v] base/water/activator; Pharmacal Research Laboratories) as described below. Although an effective sanitizer with sporicidal properties (i.e., effective against bacterial endospores), Clidox is highly corrosive. Thus, do not surface sanitize surgical instruments with Clidox, and ensure residual Clidox is removed from metal objects after it is applied (e.g. rinsing with sterile water and removing liquid with sterile paper towel).

2.2.1.3 Day 1

Sanitize and run biosafety cabinet

3. Turn on the certified Class 2A BSC and let run for a minimum of 30 min.

4. Spray the surface of a spray bottle containing Clidox with Clidox, ensuring that the entire surface of the bottle is wetted, and let the bottle sit for ≈20 min. Re-spray the bottle with Clidox before introducing it into the BSC.

Important: This procedure must be applied to any instrument that can be sanitized with Clidox and will be placed in the BSC. Clidox will be active 15 min after preparation and will stay active at room temperature for 8 hr.

5. Using proper sterile surgical technique, don a sterile gown, respirator (Fit-tested P95 respirator with protection against nuisance acid gas, 3M Particulate Respirator), surgical cap, and surgical gloves to access the BSC. Thoroughly spray the walls and tray of the operating BSC, ensuring coverage of all surfaces. Keep the BSC running overnight.

2.2.1.4 Day 2

Set up biosafety cabinet

6. Re-sanitize the surfaces of the operating BSC with Clidox as in step 5 and allow 20 min of contact before use.

7. Two individuals are needed to introduce sterile materials into the operating BSC. Before beginning, both individuals don a sterile gown, a fit-tested P95 respirator, a surgical cap or hair net, and sterile surgical gloves using proper surgical technique (Figs. 2.1 and 2.2).

8. Introduce the following items into the BSC: dissection forceps, mouse-handling forceps, beaker, tube rack, and paper towels (double-wrapped in surgical wrap and autoclaved). Also, bottles containing sterile water and 70% ethanol, and containers with 2.0-ml screwcap tubes (sanitized with Clidox as in step 4). The primary individual handles items within the BSC, while the assistant sprays materials with Clidox a second time and hands materials to the primary individual.
Note: Exercise care to ensure that the number and size of the items within the BSC are kept to a minimum, and that items are positioned in a manner that minimizes disruption to airflow patterns within the BSC.

9. Primary individual: Within the operating BSC, fill beaker with 70% ethanol for sanitizing mouse-handling forceps.

2.2.1.5 Transfer germ free mice from germ free isolator to TransDisk

10. Prior to use, tape (3M Vinyl Tape 471) the TransDisk lid to the TransDisk body (Fig. 2.1 A,B) and autoclave at 121°C and 105 kPa for ≈30 min. After the autoclave cycle is complete, cover the breathing holes with tape (Fig. 2.1B).
Note: Do not tape the breathing holes in the lid prior to autoclaving, as they must remain open to allow effective killing of microorganisms within the TransDisk.

11. Remove the outer port cover of the GF isolator Flexible Film, Germ-Free Isolator (Class Biologically Clean), thoroughly spray the port and TransDisk with Clidox, and place the TransDisk within the port. Spray the interior surface of the outer port cover with Clidox and then replace the outer port cover.

12. Sanitize the interior of the outer port through the port nipple using an atomizer (Spraying Systems) containing Clidox (Fig. 2.1C). Allow ≈40 min contact time with Clidox.

13. Within the GF isolator, remove the inner port cover and transfer the TransDisk into the isolator using the GF integral gloves. Remove the tape sealing the TransDisk lid and remove the lid.

14. Individually place mice in the TransDisk using mouse-handling forceps, then carefully re-seal the lid of the TransDisk with tape.

15. Place the TransDisk back in the port and replace the inner port cover. Then remove the outer port cover and remove the TransDisk containing mice from the port.

16. Re-spray the port and port cover with Clidox, replace the outer port cover, and use the atomizer to sterilize the port interior.

2.2.1.6 Transfer mice from TransDisk to individually ventilated cages

17. Transport the TransDisk to the animal room containing the IVCs and operating BSC (Fig. 2.2A). Spray the surface of the TransDisk with Clidox, then remove the tape covering the breathing ports to ensure sufficient air exchange. Allow a Clidox contact time of 20 min, then cover the breathing ports with tape and re-spray the TransDisk with Clidox.

18. Two individuals, attired as in step 5, are needed to introduce the TransDisk into the operating BSC. The primary individual handles items and mice within the BSC, while the assistant sprays materials with Clidox a second time, hands materials to the primary individual, and places IVCs in the IVC rack. The primary individual must take care not to contact any item that is not sterile.

19. Primary individual: Sanitize the base tray of the BSC using Clidox from the spray bottle. Then, place the TransDisk (sanitized as in step 17 and handed by the assistant) in the BSC.

20. Assistant: Partially remove the outer wrap from the first IVC and hand the IVC to the primary individual, taking care not to make contact with the inner wrap (Fig. 2.2C).

21. Primary individual: Grasp the inner wrap enclosing the IVC handed from the assistant, being careful not to make contact with the outer wrap (Fig. 2.2C). Within the operating BSC, remove the IVC from the inner wrap and place it on the BSC base tray adjacent to the TransDisk.

22. Primary individual: Unclasp the IVC lid and use mouse-handling forceps to gently grasp the tail of a GF mouse within the TransDisk and transfer it to the IVC (i.e., with the IVC lid lifted by the other hand) (Fig. 2.1D). Re-clasp the IVC lid. Place forceps in the beaker with 70% ethanol. Fill the IVC water bottle with sterile water and situate the bottle in the IVC lid, ensuring the integrity of the seal. Hand the IVC containing the mouse to the assistant, being sure not to make contact with the assistant's hands.

23. Primary individual: With sterile dissecting forceps, transfer one to three fecal pellets excreted within the TransDisk into a sterile 2-ml screwcap tube.
Note: The fecal samples are used to confirm the GF status of the mouse at the time of removal from the TransDisk.

24. Assistant: Label the IVC and place it in the IVC rack, ensuring continuity with the HEPA air-handling unit operated in containment mode.

25. Repeat until all mice are transferred to IVCs and situated in the IVC rack.
Note: Depending on the experimental design, individual or multiple mice maybe placed in an IVC. The number of mice transferred to each IVC reflects the Animal Care Guidelines of the institute. All research conducted with GF and non-GF mice in the current study was reviewed by the Lethbridge Research and Development Centre Animal Care Committee and approved in advance of research commencement (i.e., Animal Use Protocols #1623 and #1631).

26. The primary individual transfers all fecal samples from the BSC to the assistant for subsequent processing according to Support Protocol 1. Apply the 1:3 pellet/PBS ratio to the number of pellets obtained.

2.2.2 Basic Protocol 2

2.2.2.1 Individually ventilated cage change and sampling protocol

Here I describe a method to successfully change cages and collect samples without compromising the GF status of mice. Detailed below is the cage change protocol coupled with fecal and feed sampling steps. Fecal and feed sampling and testing are conducted at 7-day intervals. The GF status of mice is evaluated for a period of up to 4 weeks in the current protocol, after which mice are humanely euthanized and intestinal samples collected to test the sterility of the animal model. It is noteworthy that 4 weeks is a typical endpoint for experiments involving assessment of pathogens in mice. Mice are sampled in week 0 (when moved from the isolator to the IVC cages) and subsequently in week 1-4 in each cage change and at the experimental endpoint.
Important: Two individuals are needed to transfer mice between IVCs and to obtain samples for analysis. Both should be properly attired and work together as described in Basic Protocol 1 and below.

1. Prepare and sterilize the BSC and all materials required, and introduce materials into the BSC using sterile technique as described in Basic Protocol 1 (see steps 1-9). In addition to the items described there, include autoclaved or sprayed scalpel handle, scalpel blades, and weigh boats.

2. Assistant: Remove an IVC containing a mouse/mice from the IVC rack and place on a surface sanitized with Clidox. Cover the filter on the IVC lid with a sterile paper towel to prevent damage from liquid during the spraying process, then spray the surfaces of the IVC with Clidox (Fig. 2.2B). Remove the paper towel and wipe the filter with a sterile paper towel moistened with Clidox. Return the IVC to the IVC

rack and allow ≈20 min Clidox contact time.

Note: Until this time is complete, the outer surface of cage should be considered contaminated; thus, it is returned to the IVC rack to allow sufficient contact time to kill any organisms on the cage. During the 20-min contact period, the cage remains wet with Clidox, precluding interim contamination. Importantly, the cage must not be placed in the BSC before the 20-min contact time is complete.

3. Assistant: After the 20-min Clidox contact time, detach the IVC from the IVC rack, repeat the Clidox surface sanitization process, and hand the re-sanitized IVC containing the mouse to the primary individual, who places it on the base plate of the operating BSC.

4. Both individuals: Following the protocol described in Basic Protocol 1 (see steps 20-21), transfer a sterilized and double-wrapped IVC to the operating BSC, remove it from the inner wrap, and place it on the BSC base plate adjacent to the IVC containing the mouse/mice.

Note: The sterile IVC contains bedding, a house, nest material, enrichment items, and food.

5. Primary individual: Unclasp the lid of the IVC containing the GF mouse/mice, remove the lid, and carefully place it with the outer surface of the lid against the wall of the BSC, so as to avoid contact between the BSC and the potentially contaminated interior surface of the lid. Gently grasp the tail of the mouse with sterile forceps, and lift the mouse from the IVC. Unclasp and open the lid of the adjacent sterile IVC, place the mouse in the IVC, and close and re-clasp the lid. Place the forceps in the beaker with 70% ethanol. Fill the IVC water bottle with sterile water and situate the bottle in the IVC lid, ensuring the integrity of the seal. Hand the IVC containing the transferred mouse/mice to the assistant, being sure not to make contact with the assistant’s hands.

6. Assistant: Label the IVC and place it in the IVC rack, ensuring continuity with the HEPA air-handling unit operated in containment mode.

7. Primary individual: Within the BSC, aseptically remove fecal and feed samples from the remaining IVC with sterile forceps (Fig. 2.2D). Place the samples into pre-weighed and labeled sterile 2-ml screwcap tubes. For feed pellets, arbitrarily select two individual pellets, hold each pellet with sterile forceps, and scrape off the outer layer of the pellet into a sterile weight boat using a sterile scalpel blade. Transfer the scrapings into a sterile tube.

Where possible, collect freshly defecated fecal pellets into three tubes: two tubes containing six pellets each and a third tube containing four to six pellets (for PCR analysis). To stimulate defecation the

mouse can be "scruff" restrained; however, making any contact with the mouse poses a risk of compromising its GF status. Replace the lid on the IVC, taking care to avoid contact with the inner surface of the lid, and hand the IVC to the assistant. Sanitize the base plate and walls of the BSC with Clidox.

8. Assistant: Wrap the IVC from which the mouse/mice was/were removed in an autoclavable plastic bag for subsequent autoclave decontamination.

9. Repeat until all mice are transferred into new IVCs and situated in the IVC rack and samples are obtained.

10. The primary individual transfers feed and fecal samples from the BSC to the assistant, who processes them as follows: set aside the feed tube and one fecal tube (containing six pellets) for detection of bacteria by culture (see Support Protocol 1) then, add 1 ml of 4% Neutral buffered formalin (NBF) to the second fecal tube (containing six pellets) for fluorophore staining (Support Protocol 3) and store a third fecal tube containing 180-220 mg feces (four to six pellets) at−80°C for PCR analysis (Support Protocol 2).

2.2.3 Support Protocol 1

2.2.3.1 Detection of bacteria by aerobic and anaerobic culture

This strategy is employed to test the status of mice by plating fecal and feed samples on Columbia agar with 5% sheep blood with an incubation period of 1 week at 37°C in aerobic and anaerobic atmospheres. Ingesta obtained from Support Protocol 4 can also be used in this protocol by following the same steps used to process fecal samples. In the lab we use blood obtained from sheep in the Lethbridge Research and Development Centre Flock (Animal Use Protocol #1631), but blood can also be purchased.

1. Within 30 min of collection, transfer subsamples of feces and feed from GF and non-GF mice (≈0.5 g) into pre-weighed 2-ml sterile screwcap tubes, weigh samples and tubes, and determine the weight of each sample. Suspend samples in sterile PBS at a 1:3 ratio (w/v) and vortex at a high setting for 20 sec. Note: In some instances, it may be necessary to use a sterile micropestle (Polypropylene micropestle, Fisher Scientific) to initially break up fecal subsamples. For fecal samples to be evaluated for viable anaerobic bacteria, samples should be placed in reduced PBS (PBS maintained in an anaerobic atmosphere to eliminate oxygen). Culturing can be completed as described in steps 2 and 3 within an

anaerobic chamber containing an anoxic atmosphere, or in ambient conditions with subsequent placement of the cultures in an anaerobic atmosphere within anaerobic jars (e.g., 2.5-liter anaerobic jars (Oxoid AnaeroJar, Thermo Scientific)).

2. Pipette 25 µl suspension from each sample onto the center of four CBA plates. Add 9-10 sterile glass beads (we utilize sterile 5-mm-diameter glass beads (Sigma-Millipore), aliquoted at 9-10 beads per 1.5-ml snap-cap tube) and shake in a back-and-forth manner to distribute the liquid over the surface of the medium. Remove beads by tilting the dish and allowing gravity to discharge the beads into a 200-ml beaker containing 70% ethanol.

3. Incubate two cultures (duplicates) per sample at 37°C in an anaerobic atmosphere (7-15% CO_2, <0.1% O_2 within anaerobic jars) and two cultures per sample at 37°C in an aerobic atmosphere.

4. After 7 days, examine anaerobic and aerobic cultures for microbial growth.

An alternative to direct plating is enrichment culture, which may increase the sensitivity of detection (e.g., to better detect bacteria present at very low densities and/or those that are damaged or in a quiescent state). To generate enrichment media, resazurin sodium salt (25 µg/ml) is added to each medium to verify that the medium is reduced, and the medium is then autoclaved for 5 min at 121°C. Once autoclaved, the warm medium is placed in a N_2 atmosphere chamber and vigorously agitated to displace oxygen. When it has cooled, cysteine (0.5 g/liter) is added to the medium to remove any residual oxygen. The liquid medium (10 ml) is transferred to 15-ml tubes, each tube is sealed with a screw cap fitted with a black butyl rubber stopper, and tubes are removed from the chamber and autoclaved for 30 min at 121°C. Media prepared for a CO_2 atmosphere chamber (90:10 CO_2/H_2) are prepared in the same fashion; however, sodium carbonate needs to be added before the 30 min autoclave step to prevent acidification (≈40 ml/liter of an 8% solution of sodium carbonate). Any number of enrichment media can be used. In the lab we routinely use Columbia broth.

2.2.4 Support Protocol 2

2.2.4.1 Detection of bacteria by endpoint PCR

The following protocol is employed to confirm the GF status of mice by testing for the presence of bacteria in feces and ingesta (ingested materials within the lumen of the intestine) by endpoint PCR targeting the 16S ribosomal RNA gene with bacteria-specific degenerate primers. The absence of DNA amplification correlates with the absence of bacterial cells in the tested sample. Testing the ingesta

collected from the intestine of mice during necropsy (see Support Protocol 4, step 8) is not a mandatory step to validate that the mice are devoid of bacteria. I have applied this step to validate the murine procedures applied in my study. An important goal of rearing GF mice in containment is to study the impact of pathogenic microorganisms on mice or to elucidate the impacts of an individual or consortium of 'commensal' bacteria on the host or the pathogen without the confounding effects of the indigenous enteric microbiota. For gnotobiotic mice (e.g., monoxenic mice), in the lab we routinely use quantitative taxon-specific primers to monitor the presence, as well as the spatial and temporal distribution, of the bacterium within the mouse. GF control mice are often included in such experiments.

1. Thaw tubes with fecal samples (see Basic protocol 2) on ice and extract DNA using the QIAamp Fast DNA Stool Mini Kit (Qiagen) according to manufacturer's recommendations.
Note: Genomic DNA is extracted from the bacterial cells along with DNA associated with the samples themselves (e.g., mouse genomic DNA within sloughed mucosa). QIAamp Fast DNA Stool Mini Kit first employs a lysis buffer to lyse and separate impurities from stool samples, subsequently utilizing a spin column through several steps to purify the DNA, with long-term storage capabilities at −80°C. Other DNA extraction kits may be used, but should be designed to remove PCR inhibitors present in ingesta and feces. Furthermore, an extraction protocol that is designed for both Gram-negative and Gram-positive bacteria is recommended. The QIAamp stool kit is also appropriate for ingesta samples.

2. Set up 20-µl PCR reactions consisting of: 2.0 µl 10× PCR buffer (Qiagen), 0.4 µl 10 mM dNTPs (Biobasic), 0.4 µl 25 mMMgCl2 (Qiagen), 2.0 µl 1 mg/ml Bovine serum albumin (Promega), 1.0 µl 10 µM primer UNI27F (5'-AGAGTTTGATCCTGGCTCAG-3') (Integrated DNA technologies), 1.0 µl 10 µM primer UNI1492R (5'-TACGG(C/T)TACCTTGTTACGACT-3') (Integrated DNA technologies), 0.1 µl HotStar *Taq* polymerase (Qiagen), 2.0 µl DNA template, 11.1 µl nuclease-free water (Qiagen). Use 2.0 µl DNA extracted from *E. coli* (20-50 µg/ml) as a positive PCR control, and 2.0 µl nuclease-free water as a negative PCR control.
Note: The indicated primers amplify variable regions V1 to V9 of the 16S rRNA gene (Kim *et al.*, 2012). Any bacterial genomic DNA can be utilized as a positive control. Genomic DNA from *E. coli* (American Type Culture Collection, ATCC 25922) possesses seven copies of the 16S rRNA gene. The use of an internal amplification control is recommended to guard against false negative results, e.g., due to PCR inhibition (Webb *et al.*, 2016).

3. Conduct target amplification in a Thermocycler (e.g., Eppendorf Mastercycler) as follows: 1 activation cycle at 95°C for 15 min, 35 cycles at 94°C for 30 sec (denaturation), 56°C for 90 sec (annealing), and 72°C for 2 min (extension), 1 final extension at 72°C for 10 min and hold at 4°C.

4. Electrophorese 5 µl of each PCR product and a 100-bp ladder on a 1% agarose Tris-acetate-EDTA gel. Run at a voltage and time adequate to the gel size. Visualize DNA bands with ethidium bromide (10 mg/ml ethidium bromide, Fisher Scientific) under UV light.

2.2.5 Support Protocol 3

2.2.5.1 Vital fluorescent DNA staining of fecal samples

The vital DNA staining method involves nonspecific binding of a dye to DNA with the goal of identifying the presence of microorganisms, including prokaryotes and eukaryotes. This protocol is aimed at testing mouse status by vacuum filtering fecal samples stored in formalin through a 0.2-µm polycarbonate membrane and later staining the DNA of cells trapped in the membrane with Fluoroshield containing 4',6-diamidino-2-phenylindole (DAPI). DAPI binds strongly to AT-rich regions of DNA, providing an option to test against unculturable bacteria as well as yeast or fungi. This protocol presents a fast and easy way of superficially testing the sterility of the mice.

1. Vortex tubes containing feces in 4% NBF (see Basic Protocol 2) vigorously at a high speed for 30 sec and fix for a minimum of 4 hr.
Note: In some instances, it may be necessary to use a sterile micropestle to initially break up fecal subsamples.

2. Filter suspension under vacuum through a 0.20-µmpolycarbonate membrane (Whatman Nuclepore Track-Etched Membranes; Sigma-Millipore) placed in a glass filter holder (Glass vacuum filter holder (Sigma-Millipore).

3. Mount the membrane on a microscope slide (25 × 75 × 1–mm Superfrost Plus Gold microscope slides, Fisher Scientific) and stain with a drop of Fluoroshield containing DAPI (Fluoroshield containing 4',6-diamidino-2-phenylindole (DAPI), Sigma-Millipore) according to manufacturer's instructions.
Note: Lower the lights of the laboratory when using DAPI and keep slides in a dark container, as DAPI is sensitive to light.

4. Apply a cover slip and observe slide using an epifluorescence microscope (e.g. Zeiss Axioscope). Record the presence or absence of bacteria.

2.2.6 Support Protocol 4

2.2.6.1 Fluorescent *in situ* hybridization of bacterial cells on intestinal histological slides

The following protocol describes the necessary steps to conduct a mouse euthanasia and necropsy to sample intestinal tissues and intestinal ingesta. Furthermore, I describe the necessary steps to process histology slides and stain bacterial cells with specific fluorescent probes.

The euthanization and necropsy procedures are conducted within an operating BSC using sterilized equipment/materials to ensure GF status of the samples collected. Tissues can be dehydrated manually, and any embedding center and microtome model will suffice.

Important: Two individuals are needed to introduce IVCs containing mice and all materials to the BSC and to perform necropsy. Both should be properly attired and work together as described in Basic Protocols 1 and 2.

2.2.6.2 Perform necropsy

1. Prepare and sterilize the BSC and all required necropsy materials, and introduce sterile materials into the BSC as described in Basic Protocol 1 (see steps 2-9). Include petri dishes, gauze, a syringe with isoflurane (Fresenius Kabi, Canada), empty 1-liter Nalgene container, forceps, cervical dislocation tool, solidified paraffin bed, scissors, sterile paper towels, pins, tissue cassettes, 1-liter Nalgene container with 10% NBF, 2-ml tubes and rack, mouse disposal bags (Bulldog Bag).

2. Introduce an IVC containing a mouse into the BSC as described in Basic Protocol 2 (see steps 2-3).

3. Within the BSC, place sterile gauze inside a Petri dish with a 0.2-ml aliquot of isoflurane. Place the dish inside a 1-liter Nalgene container and close the container. Open the lid of the IVC, lift the mouse by its tail with the mouse-handling forceps, and place the mouse inside the container.

4. Once the mouse is anesthetized, remove it from the container, place it on a bed of sterile towels, and humanely euthanize the animal by cervical dislocation, ensuring that the animal is dead (Cartner *et al.*, 2007).

5. Place sterile paper towel on a solidified paraffin bed, place the mouse on its back on the paraffin, and secure its legs to the paraffin using sterile pins.

Note: A solidified paraffin bed can be prepared beforehand by placing liquid paraffin in a plastic container (20 × 11.5 × 3 cm) and allowing the paraffin to solidify.

6. Using sterile iris scissors and forceps, conduct a midline laparotomy to exteriorize the intestine (Jiminez *et al.*, 2016).

7. Remove a ≈2-cm section of the cecum, proximal colon, and distal colon, immediately place in a labeled tissue cassette, and submerge the cassette in a container with 10% NBF. Fix for a minimum of 4 hr at room temperature.

Note: The containers with 10% NBF and samples can be removed from the BSC once the necropsies are finished without the need to follow sterile technique. Samples in formalin can be stored for long periods of time and processed when convenient.

8. Optional: Remove ingesta from the intestinal segments and place in 2-ml screwcap tubes for culture-based detection (Support Protocol 1) or endpoint PCR (see Basic Protocol 2). Store at –80°C.

9. Place the body in a water-impervious paper bag and dispose of according to institutional requirements (e.g., incineration).

2.2.6.3 Process tissue

10. Dehydrate samples manually or using an automated tissue processor (e.g. Leica TP1020 Benchtop Tissue Processor, Leica Biosystems) as follows: fresh 10% NBF for 5 min, alcoholic formalin for 1 hr, 80% ethanol for 45 min, 95% ethanol for 3× for 1 hr each, 100% ethanol for 2× for 1 hr each, Clearene 2× for 1.5 hr each, molten paraffin for 1 hr and molten paraffin for 1 hr under vacuum.

11. Embed samples in paraffin using an embedding center (Shandon Histocentre 3 embedding center, Thermo Scientific).

12. Prepare ≈5-µm-thick tissue sections using a microtome and place sections on microscope (Slides Finesse 325 manual rotary microtome, Thermo Scientific).

13. Wash and rehydrate sections as follows, taking care to ensure that sections are covered by each solution: xylenes 2× for 5 min each, 100% ethanol for 3 min, 90% ethanol for 3 min, 80% ethanol for 3 min and 70% ethanol for 3 min.

14. Circle the sections using an ImmEdge pen (Vector Laboratories) to contain probe in the area of the tissue segment.

15. Dilute bacterial probe EUB388 tagged with Alexa 555 (Bacterial probe EUB338 (5’-GCTGCCTCCCGTAGGAGT-3’; Kong, He, McAlister, Seviour, & Forster, 2010) tagged with Alexa 555,

Integrated DNA technologies) to a 2.5 ng/ml concentration in hybridization buffer and add to the tissue, ensuring coverage of the sections (≈10 µl per tissue sample). Incubate overnight at 37°C in the dark.

16. Without exposing the sections to light, wash at room temperature as follows: hybridization buffer for 15 min, wash buffer for 15 min and water for 5 min.

17. Apply a drop of Fluoroshield containing DAPI on each tissue section, cover with a cover slip, and seal with nail polish.
Note: DAPI acts as a counterstain by staining all nuclei of the mouse intestinal cells.

18. Observe using an epifluorescence microscope and record the presence or absence of bacteria.

2.2.7 Reagents and solutions

2.2.7.1 Alcoholic formalin

Combine 265 ml of 10% NBF and 735 mml of 95% ethanol to a 1-liter volume. Store up to 6 months at room temperature.

2.2.7.2 Hybridization buffer, pH 7.4

Heat 52.6 g sodium chloride (Sigma-Aldrich), 12.1 g Trizma base (Sigma-Aldrich), and 1 g sodium dodecyl sulfate (SDS, Sigma-Aldrich) in ≈300 ml sterile water to initially dissolve SDS. Add 300 ml formamide (Sigma-Aldrich) and sterile water to a final volume of 1 liter, ensuring that the pH of the solution is ≈7.4. Store up to ≈3 months at 4°C.

2.2.7.3 Phosphate-buffered saline (PBS), pH 7.4

Combine 800 g sodium chloride (Sigma-Aldrich), 20 g potassium chloride (Sigma-Aldrich), 144 g sodium phosphate dibasic (Sigma-Aldrich) and 24 g potassium phosphate monobasic (Sigma-Aldrich) in deionized sterile water and adjust volume to 8 liters with water to generate a 10× stock. Dilute to 1× with sterile deionized water, ensuring that the pH of the solution is ≈7.4. Store up to ≈2 years at room temperature.

2.2.7.4 Wash buffer, pH 7.4

Combine 52.6 g sodium chloride (Sigma-Aldrich) and 12.1 g Trizma base (Sigma-Aldrich), and adjust volume to 1 liter with sterile water, ensuring that the pH of the solution is ≈7.4. Store up to ≈2 years at room temperature.

2.3 Results

Maintaining GF status in mice is critical to the success of this novel protocol for housing GF mice in conventional animal facilities. As such assessing mice for microorganism contamination is required. Assays that measured the presence of bacteria content within fecal material and tissue were used and these include detection by culture (support protocol 1), PCR (support protocol 2), fluorophore staining (support protocol 3), and fluorescent *in situ* hybridization (support protocol 4).

2.3.1 Detection of bacteria by culture

No bacteria were isolated from fecal samples obtained from GF mice transported from the isolator to the IVC's (i.e. week 0) and no bacteria were isolated from feces obtained from GF mice maintained aerobically or anaerobically throughout the 4 week duration of the study (n=75) (Fig. 2.3A). In contrast, luxuriant bacterial growth emanated from feces collected from conventional control mice (n=15) (Fig. 2.3B). All the autoclaved feed pellets sampled throughout the observation period were sterile (data not shown). In contrast, bacteria were associated with 13.3% of the autoclaved feed sampled from cages of conventional mice.

2.3.2 Detection of bacteria by PCR

No amplicons were observed from endpoint PCR targeting the 16S rRNA gene from DNA extracted from fecal samples obtained from GF mice throughout a 4-week study period, nor from DNA extracted from ingesta samples throughout the intestinal tract of GF mice (n=90) (Fig. 2.4). In contrast, 16S rRNA gene amplicons were observed in all fecal and ingesta samples collected from conventional control mice during a 4 week study period (n=18) (Fig. 2.4). Amplicons of ≈1500 bp should be observed from samples obtained from/associated with conventional mice and the *E. coli* positive control.

2.3.3 Fluorophore staining

No fluorescing bacterial cells from fecal samples (n=75) obtained from GF mice were observed on the polycarbonate membrane (Fig. 2.5A). The absence of fluorescing bacterial cells confirms the GF status of the mouse. In contrast, abundant bacterial cells from fecal samples from conventional mice were observed on membranes (n=15) (Fig. 2.5B,C). When present, bacteria appear as a strong blue fluorescence on a blue background (i.e., the membrane).

2.3.4 Fluorescent *in situ* hybridization

No bacterial cells were observed in the lumen of the cecum, proximal and distal colon from GF mice by FISH (n=45) (Fig. 2.6A,B). When the orange fluorescence is reduced to the ingesta and bacterial structures cannot be observed, this confirms the GF status of the mouse. Conspicuous quantities of

bacteria were observed in the cecal and colonic lumen of conventional mice (n=9). When bacteria are present, they appear as a strong orange fluorescence in the lumen of the intestine (Fig. 2.6C,D). Intestinal epithelial cells can be observed by blue fluorescence.

2.4 Discussion

2.4.1 Background Information

In this research (published article) I present a protocol in which a Tecniplast Green Line Sealsafe Plus IVC infrastructure maintained in containment mode and used in concert with operational procedures can be successfully employed to maintain the axenic status of GF mice for a period of 4 weeks (I have confirmed GF status for up to 8 weeks). Importantly, these instructions allow investigators to conduct research with non-specialized IVCs acting as separate environmental units for colonization experiments with different microorganisms in GF and GB mice. Furthermore, they enable work with RG2 pathogens in GF and GB mice using conventional IVC equipment in a non-HEPA-filtered intake air small animal facility and meet the requisite biosafety and biosecurity standards of their respective institutes.

In recent years, specialized equipment has been developed to house and handle GB and GF mice, such as IVCs with isolator functions or completely sealed biosafety cabinet stations. However, this specialized equipment is expensive and many animal facilities do not possess such infrastructure. As conventional IVCs are the standard in many small animal facilities, I developed and evaluated a methodology to house and maintain the GF status of mice using a standard IVC system operated under containment mode. I determined that GF mice maintained in these IVCs with weekly handling retained their axenic status for the duration of the experiment (4 weeks), which meets or in many cases exceeds endpoints for challenge studies with pathogens (Brown *et al.*, 2016; Hertz *et al.*, 2018; Sevrin *et al.*, 2018; Taguchi *et al.*, 2002; Tyler *et al.*, 2013). I designed and implemented a strict operational plan to preclude contamination of GF mice. Sample collecting is necessary in the course of most experiments, and maintaining the GF or GB status of mice necessitates the implementation of effective operating procedures, which are conducted in concert with IVCs and ancillary equipment (Hecht *et al.*, 2014; Paik *et al.*, 2015). Previous studies have housed GB mice in IVCs under clean but not strict sterile procedures (Lundberg *et al.*, 2017). When studies demand the need for a precise GF or GB status, much more rigid protocols are imperative.

Although it is often necessary to collect samples for temporal analyses (e.g., densities of pathogens shed in feces), the impacts of this procedure on the physiology and behavior of mice should not be overlooked (Balcombe *et al.*, 2004; Reeb-Whitaker *et al.*, 2001). For example, weekly changes in static

isolator cages can adversely affect pup survival (Reeb-Whitaker *et al.*, 2001). In addition, handling of mice during cage changes can increase corticosterone levels and alter anxiety-like behaviors (Rasmussen *et al.*, 2011), and levels of aggression between male mice can be heightened after the cage cleaning process (Gray *et al.*, 1995; Van Loo *et al.*, 2000). A salient advantage of housing GF mice in IVCs is that the time between cage changes can be extended as a result of higher rates of ammonia and carbon dioxide elimination and moderation of ambient humidity, thereby reducing the confounding effects of animal handling (Memarzadeh *et al.*, 2004; Reeb-Whitaker *et al.*, 2001).

GB mice have emerged as an invaluable model to elucidate key aspects of the host-microbiota interaction in mammals (Diaz Heijtz *et al.*, 2011; McVey Neufeld *et al.*, 2015; Quach *et al.*, 2018; Sjogren *et al.*, 2012). This model has also proven itself to be of considerable value for studying pathogen-host interactions (Brown *et al.*, 2017; Fei *et al.*, 2013; Goswami *et al.*, 2015; Nascimento *et al.*, 2017; Soavelomandroso *et al.*, 2017). All scientific activities conducted within signatory countries that involve pathogens must adhere to United Nations conventions on biosafety and biosecurity. The small animal facility at Lethbridge Research and Development Centre is designed for research with RG2 pathogens, and the presence of containment zones is a critical component of the containment facility design to ensure the safety of staff and nonhuman animals within the facility, and people and non-human animals exterior to the facility. In many containment facilities, IVC systems are mandated as a primary containment device, and the animal room in which the IVCs are situated serves as a secondary containment (i.e., rooms operated in containment mode with indirectional airflow). Currently, IVC systems can be operated under positive pressure (barrier mode or bioexclusion) or negative pressure (containment or biocontainment mode). Positive pressure ventilation (Arvidsson *et al.*, 2012; Paik *et al.*, 2015) is applied to create a barrier that impedes microorganisms from entering the IVC. This is the same principle that applies to isolator ventilation and is the standard when working to prevent contamination of GF animals. However, when conducting research with pathogens in many small animal facilities, it is necessary to work with IVCs operated under containment conditions (i.e., negative pressure) to ensure that the pathogens are not released into the animal room. In addition to their containment characteristics, IVCs are also highly desirable from an animal care perspective, as air changes within the IVC greatly exceed the minimum number of air changes of (i.e., 15 to 20) specified by the Canadian Council on Animal Care (Canadian Council on Animal Care, 2003), thereby ensuring a high quality atmosphere for animals maintained in IVCs.

Although not specifically designed for work with GF or GB mice under containment operation, this protocol demonstrates that a conventional IVC equipment infrastructure available in most small animal facilities is suitable for maintaining the axenic status of mice and, in combination with appropriate operational procedures, allows researchers to conduct research with GF and/or GB mice inoculated with pathogens.

2.4.2 Critical Parameters

The protocols introduced do not present any technical difficulties; however, specific consideration must be placed on respecting the strict operational procedures when conducting the steps within the protocol. Sample collection and mouse manipulation are critical points for introduction of contaminant microorganisms, leading to loss of model integrity as well as compromise of the experiment and generation of inaccurate data.

2.4.3 Troubleshooting

Instances where elements are introduced or removed from the isolator as well as when mice are handled are high risk contamination points. Respecting the strict operational procedures is key to avoiding contamination in these instances. Autoclave cycles must be tested by placing an extra water bottle or cage containing a biological indicator (*G. stearothermophilus* endospores) situated within materials such as bedding (i.e., "spore tested"). Furthermore, care must be taken when working with samples, as external contamination of the samples can render a false positive result (i.e., due to ancillary contamination of the sample).

When contamination of mice within the GF isolator or IVCs is suspected, fecal sampling to test the isolator and cages is necessary to corroborate the status of the animals. Contamination of a single cage does not guarantee contamination of the totality of the cages. In contrast, a positive result from a sample obtained from an isolator signifies a contamination of the entire colony, which necessitates that the existing colony be euthanized, the isolator effectively sterilized, and the colony re-established. Samples can also be externally contaminated, particularly those utilized for endpoint PCR, which is a very sensitive detection method. Contamination of DNA reagents with bacteria is a possibility (Velasquez-Mejia *et al.*, 2018), and steps should be implemented to guard against false positive results. In this regard, a negative control (i.e., reagents without template) should be included in all PCR runs. In the event of a positive sample result (with a negative no-template sample), cross-checking must be done with the other detection tests to assess if there was a contamination for that particular sample or if the

animal was truly compromised. Duplication of samples to reduce false positives, as well as the inclusion of positive and negative controls, is essential.

In case of an unexpected contamination test result, the sterility of reagents and materials must be checked by conducting the same detection test with new reagents or instruments. A step-by-step process of individually testing and discarding reagents must be conducted until the compromised reagent/material has been identified.

2.4.4 Anticipated Results

Several tests can be employed to test the GF status of mice. I employ the four tests described here: detection of bacteria in ingesta and feces by anaerobic and aerobic culture; detection of bacterial DNA in ingesta and feces using endpoint PCR targeting the 16S rRNA gene; fluorophore staining of filtered fecal suspensions; and FISH analysis of intestinal tissues using a universal bacterial probe.

Mice that are GF will be negative for bacterial growth on Columbia agar containing 5% sheep blood after prolonged incubation in both aerobic and anaerobic atmospheres at 37°C (Fig. 2.3A), whereas growth will be present in cultures from non-GF animals (Fig. 2.3B). As many enteric bacteria are aerosensitive, we also use non-culture based methods to detect bacteria. For endpoint PCR, no amplicons are observed for DNA extracted from samples obtained from GF mice or the negative PCR control, whereas a amplicon of ≈1500 bp is observed for DNA extracted from samples obtained from non-GF mice or the positive PCR control (Fig. 2.4). Although endpoint PCR is very sensitive, a minimum threshold of bacteria is necessary to return a positive PCR result, due to dilution during the extraction procedure (i.e.,≈102 to 103 bacterial cells/g). For this reason, I also apply additional culture-independent methods. The first additional method is filtration of ingesta and/or fecal samples followed by staining with Fluoroshield containing DAPI and examination of the membrane using an epifluorescence microscope. Samples from GF mice exhibit no fluorescing bacterial cells on the membrane (Fig. 2.5A), whereas samples from non-GF mice exhibit conspicuous bacterial cell fluorescence on a dark blue background (Fig. 2.5B,C). An augment to membrane filtration is FISH for bacteria associated with intestinal tissues or ingesta within the intestinal lumen. Using this method, bacteria associated with mucosal surfaces or within ingesta in non-GF mice appear as a bright orange florescence (Fig. 2.6C,D). When bacteria are absent in GF mice, such bright orange fluorescence is not observed (Fig. 2.6A,B). In both, intestinal tissues fluoresce blue. Each of these methods possesses inherent advantages as well as disadvantages, and no method is foolproof on its own. Thus, it is important to apply all four methods in concert to ensure that mice are indeed GF (i.e., not contaminated at low levels) and avoid inaccurate

experimental findings and conclusions (i.e., by using mice that are assumed to be GF but contain microorganisms).

2.4.5 Time Considerations

The duration of Basic Protocols 1 and 2 is 2 days. The first day is utilized for sanitizing the BSC and the second is for conducting the protocol (i.e., transporting mice from isolator to IVCs or changing cages). Analysis of samples takes 2 to 7 days. Samples obtained for fluorophore staining and FISH do not have to be processed immediately, as they are stored in formalin. Ideally, these samples should be processed within a 6-month period. Samples for endpoint PCR also do not have to be processed immediately, as they are frozen, but should be processed within a 6 month period to ensure DNA integrity. Fecal and feed samples collected for culture-based detection should be processed on the same day that the sample was obtained.

2.5 Tables and figures

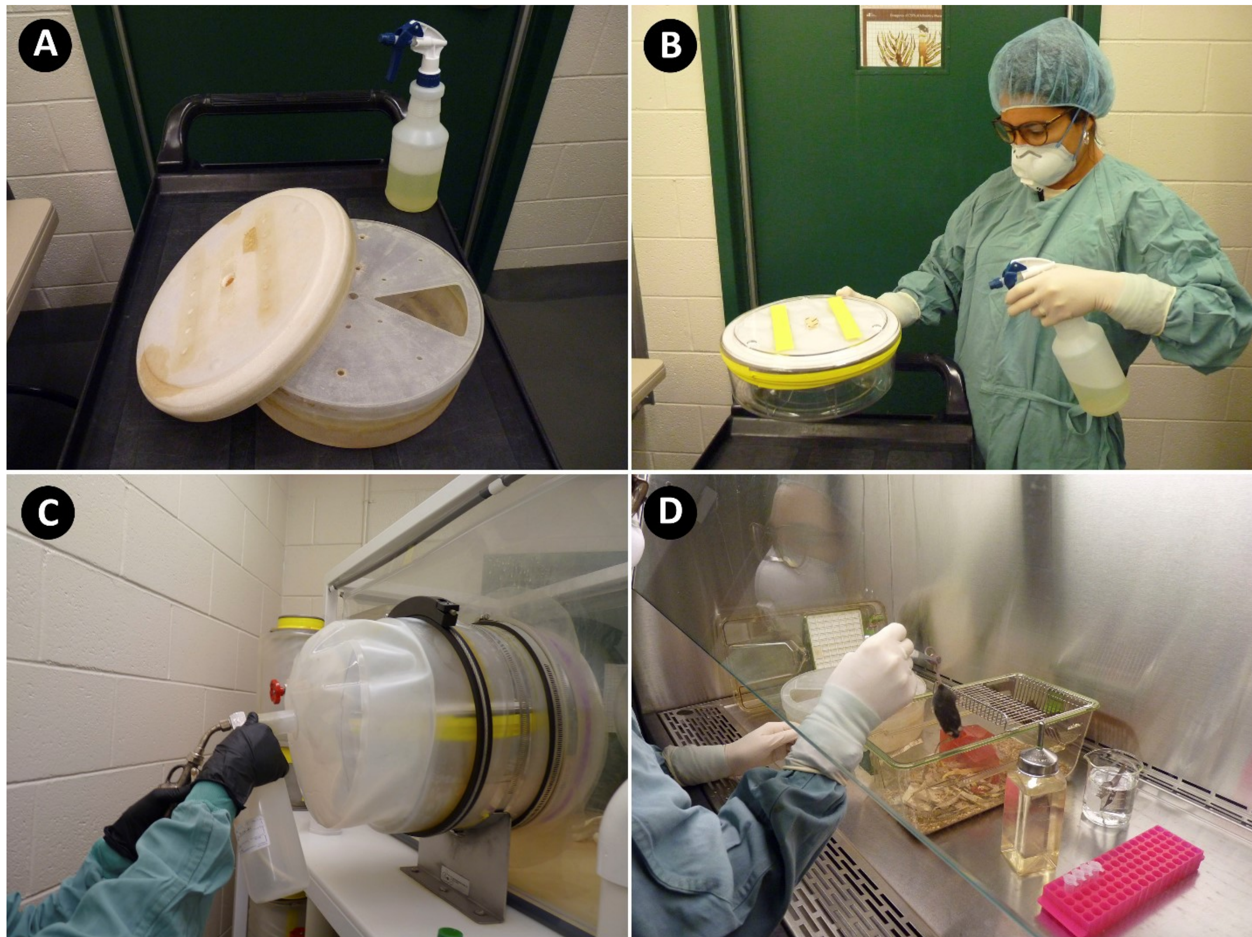

Figure 2.1 Transportation of mice from germ-free isolator into individually ventilated cages protocol. (A) The TransDisk shown with the open lid and no tape (i.e. before sterilization). (B) The assistant spraying the TransDisk prior to transfer to an operating BSC (note that the lid of the TransDisk is taped shut with the body and the air holes are covered with tape as well). (C) A technician spraying Clidox® into the isolator port containing the autoclaved TansDisk. (D) The primary individual transferring a mouse from the TransDisk into a Tecniplast® Green Line Sealsafe Plus individually ventilated cage.

Figure 2.2 Cage changing and sampling protocol. (A) Animal room with a Class 2A biological safety cabinet (BSC), Tecniplast® Green Line Sealsafe Plus individually ventilated cages (IVCs) in an IVC rack attached to a high efficiency particulate air handling unit operated in containment mode. (B) The assistant spraying Clidox® onto the IVC surface while covering the cage filter with a sterile towel. (C) The assistant aseptically handing the cage to the primary individual for placement of the IVC within the BSC (the assistant does not handle the cage by its inner sterile wrap, and both individuals wear sterile gloves and surgical gowns). (D) The primary individual collecting samples from the cage after the GF-mouse has been transferred to a replacement sterile cage and it has been returned to the IVC rack by the assistant.

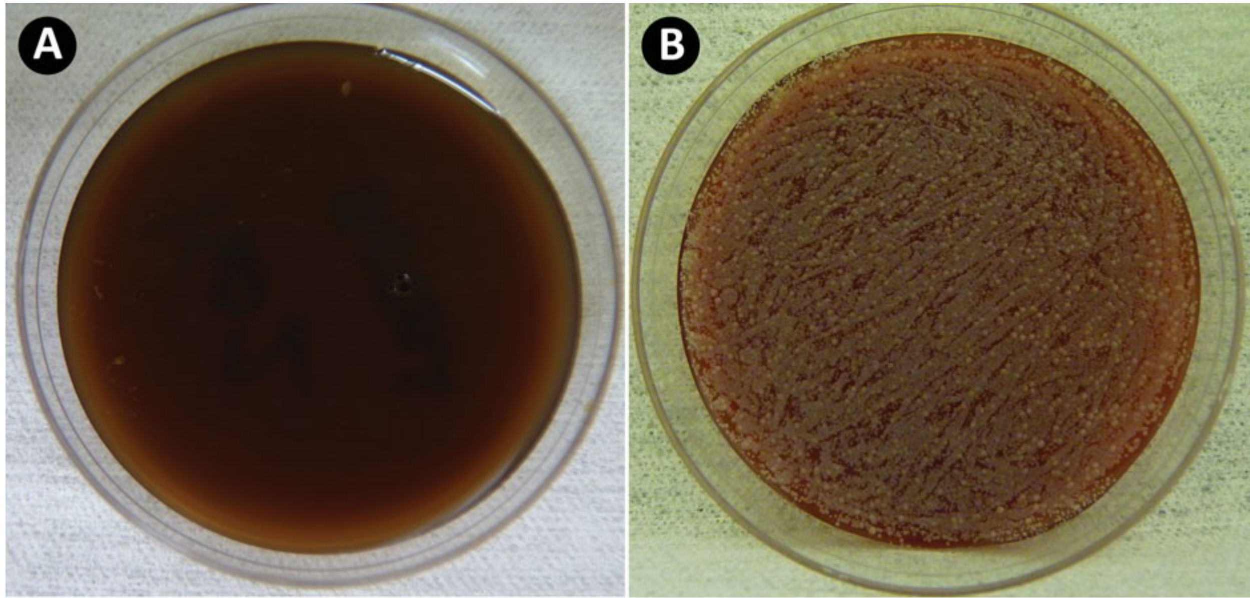

Figure 2.3 Suspensions generated from fecal samples from germ-free (GF) mice and non-GF control mice housed in Tecniplast® Green Line Sealsafe Plus individually ventilated cages attached to a high efficiency particulate air handling unit operated in containment mode for 4 weeks were spread on Columbia agar with 5% sheep blood and incubated for 7 days at 37ºC in an anaerobic atmosphere. (A) No growth from feces collected from a GF mouse. (B) Luxuriant bacterial growth from feces collected from a non-GF mouse.

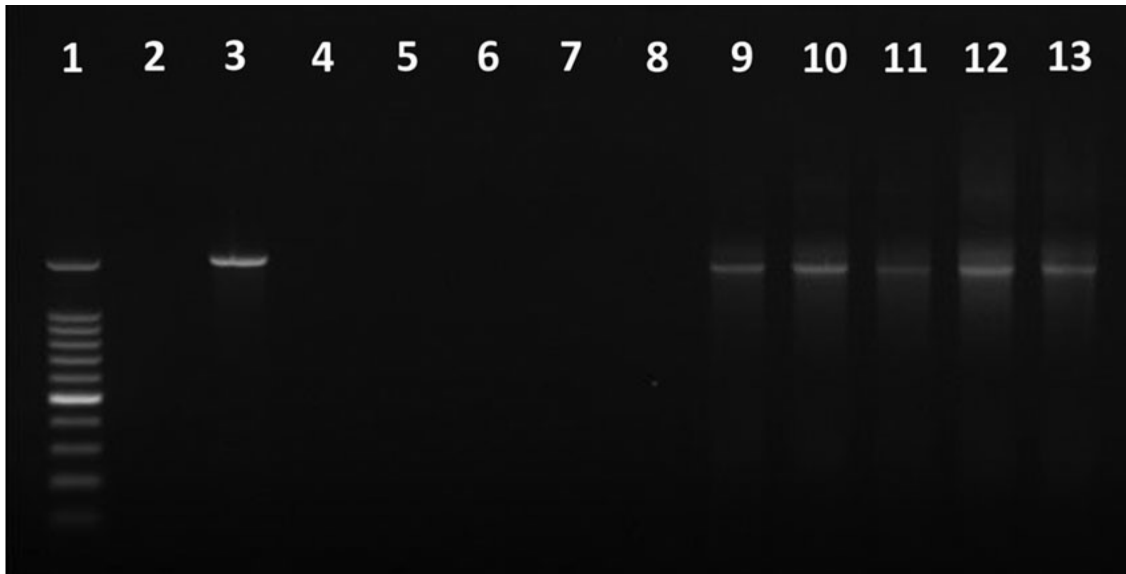

Figure 2.4 Endpoint PCR targeting the bacterial 16S rRNA gene in genomic DNA extracted from mouse feces comparing two representative germ-free (GF) mice from week 0 to week 4 housed in Tecniplast® Green Line Sealsafe Plus individually ventilated cages attached to a high efficiency particulate air handling unit operated in containment mode. Lane 1, 100 bp ladder; lane 2, negative PCR control; lane 3, positive PCR control; lanes 4-7 GF mice fecal samples week 1-4 in chronological order; lane 8, GF mice colonic ingesta; and lanes 9-12, non-GF control mice fecal samples week 1-4 in chronological order; lane 13 non-GF control mice colonic ingesta.

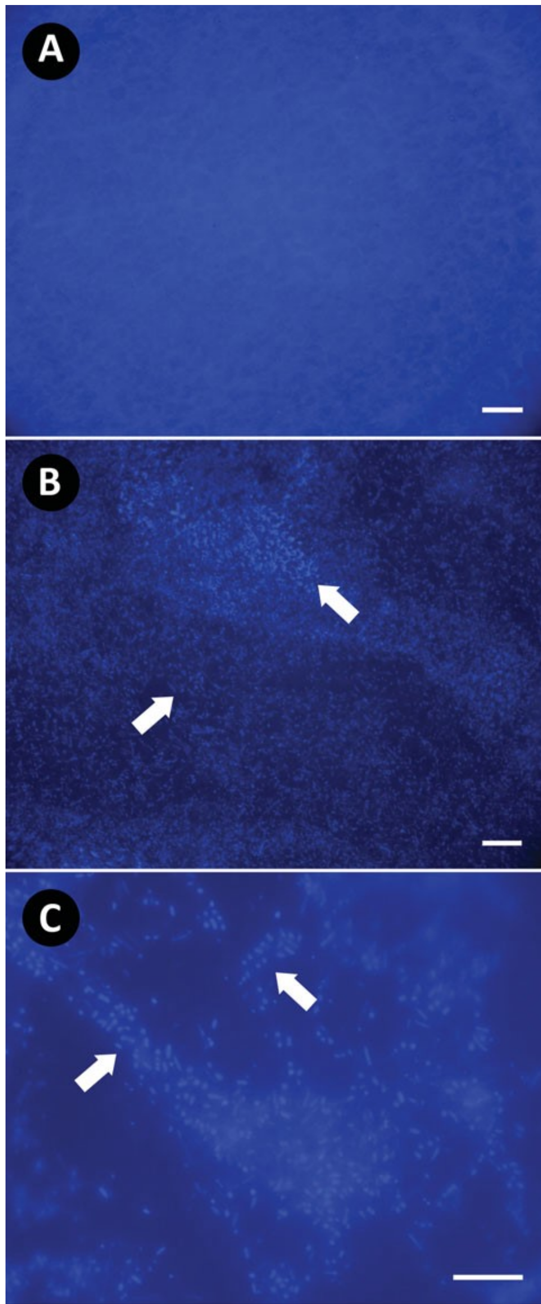

Figure 2.5 Fluorescent micrographs from representative formalin fixed fecal samples obtained from germ-free (GF) and non-GF control mice maintained in Tecniplast® Green Line Sealsafe Plus individually ventilated cages operated in containment mode for 4 weeks. Fecal suspensions were filtered through a 0.2 µm filter and stained with 4',6-diamidino-2-phenylindole (i.e. DAPI). (A) Feces from a respresentative GF mouse showing no fluorescing bacterial cells. (B-C) Feces from representative non-GF control mice showing fluorescing bacterial cells (arrows). Horizontal white bars are 100 µm.

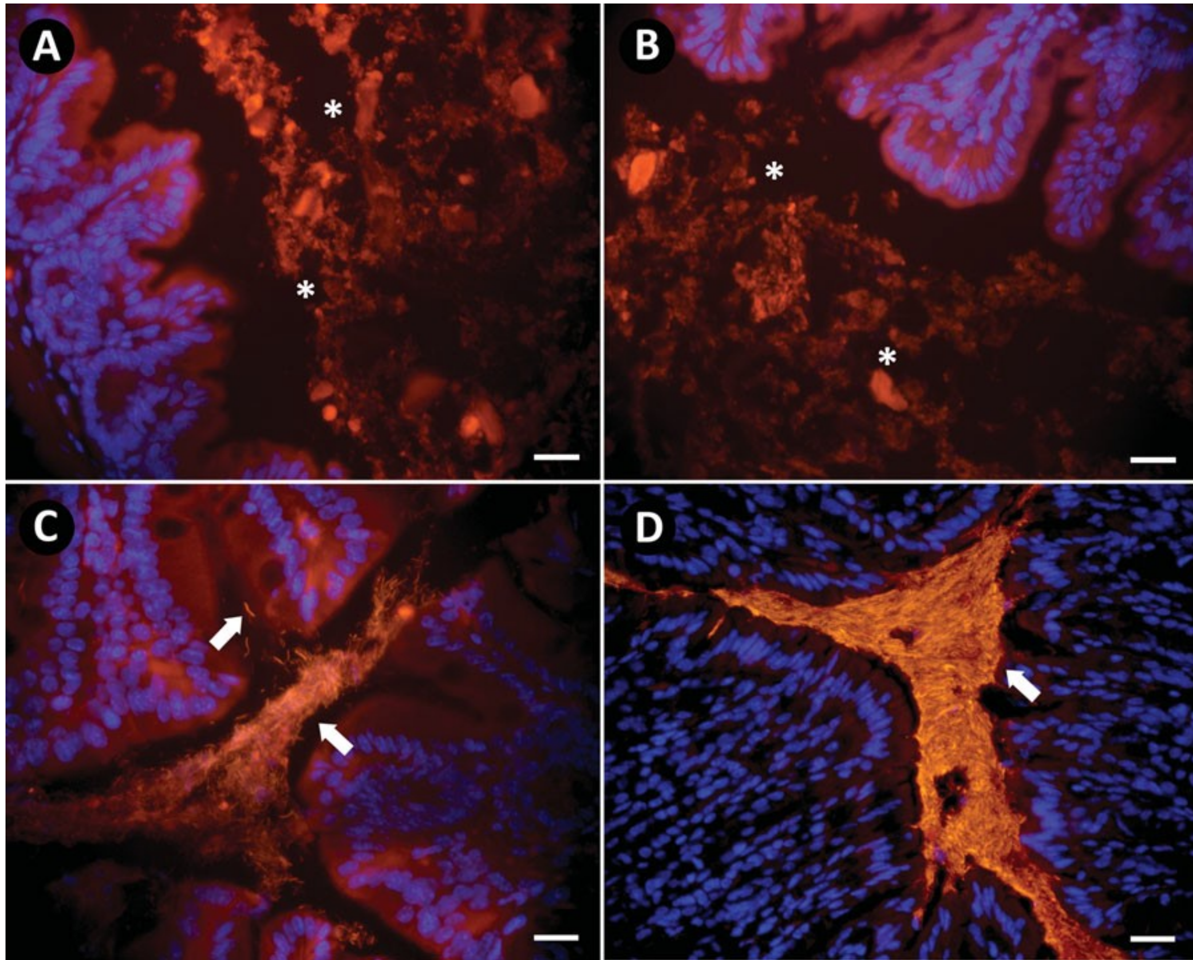

Figure 2.6 Cecal and colonic fluorescent micrographs from representative germ-free (GF) and non-GF control mice in a Tecniplast® Green Line Sealsafe Plus individually ventilated cage operated in containment mode for 4 weeks. Colon enterocytes were stained with 4',6-diamidino-2-phenylindole (i.e. DAPI), and bacterial cells were stained with the bacterial probe, EUB338 tagged with an orange fluorescent dye (Alexa 555). (A) Cecum from a representative GF mouse showing intestinal digesta within the lumen (asterisks), but no observable bacterial cells. (B) Proximal colon from a representative GF mouse showing intestinal digesta within the lumen (asterisks), but no observable bacterial cells. (C) Cecum from a representative non-GF control mouse showing abundant bacterial cells within the cecal lumen (arrows). (D) Proximal colon from a representative non-GF control mouse showing abundant bacterial cells within the colonic lumen. Horizontal white bars are 200 µm.

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Chapter 3: Competitive interaction between bovine enterohemorrhagic and commensal *Escherichia coli* and the impact of physiological stress on the host-bacteria interaction in a gnotobiotic murine model

3.1 Introduction

Escherichia coli O157:H7 is a human foodborne pathogen in which cattle are considered to be the main reservoir (Saeedi *et al.*, 2017). Patients infected with enterohaemorraghic *Escherichia coli* O157:H7 (EHEC) can present non-bloody diarrhea, bloody diarrhea, and potentially develop Hemolytic Uremic Syndrome (HUS) (Kaper *et al.*, 2004). Shiga toxin-producing *E. coli* have been estimated to cause 2,801,000 acute illnesses, 3,890 cases of HUS, 270 cases of permanent end stage renal disease, and 230 deaths per year worldwide (Majowicz *et al.*, 2014). It is not entirely clear how EHEC can successfully colonize and survive in the intestine of cattle whilst not causing symptoms of disease. Furthermore, the immune response of bovine hosts, colonization location and shedding patterns of EHEC are poorly understood (Munns *et al.*, 2015; Williams *et al.*, 2014). There are currently no definitive prevention strategies for eliminating EHEC in its natural bovine reservoir.

Cattle are exposed to multiple stressors during production; including weaning, vaccination, dietary changes, transportation, confinement, and others. Different handling and management procedures have been shown to increase cortisol levels in cattle (Agnes *et al.*, 1990; Fazio *et al.*, 2005; Locatelli *et al.*, 1989). Stress has been linked as a potential risk factor responsible for enhancing prevalence of EHEC fecal shedding, particularly in calves early in the feeding period (Bach *et al.*, 2004; Chase-Topping *et al.*, 2007; Garber *et al.*, 1995). It is well established that stress can have a variety of effects on the immune system altering the immune function (Cain *et al.*, 2017; Dhabhar, 2009), and this may potentially benefit EHEC colonization and persistence in cattle (Bach *et al.*, 2004). Furthermore, stress hormones such as catecholamines have been demonstrated to enhance the expression of virulence genes in EHEC that directly benefit colonization (Carlson-Banning *et al.*, 2018; Moreira *et al.*, 2010). At present, the degree and mechanisms by which physiological stress can potentially influence the intestinal colonization by EHEC is poorly understood and needs further investigation.

Elucidating the cardinal factors controlling intestinal colonization in cattle is challenging. The complexity of the intestinal microbiota makes it difficult to study EHEC colonization, including host bacteria interactions, and competition amongst bacteria *in vivo*. Additionally, husbandry practices and the costs involved in utilizing cattle in experiments can be a limiting factor for studies in some laboratories. Consequently, specific-pathogen-free and streptomycin-dysbiosis murine models have

been used in multiple occasions to investigate EHEC colonization, but the background noise generated by the intestinal microbiota can limit the ability of researchers to make definitive conclusions on interactions between bacteria and the host (Leatham *et al.*, 2009; Mundy *et al.*, 2006; Nagano *et al.*, 2003; Wadolkowski, Burris, *et al.*, 1990). Since a highly representative cattle model to elucidate key aspects of the host-pathogen-microbiota interaction is lacking, this study aimed to utilize a gnotobiotic (GB) C57BL/6 murine model to investigate the impacts of stress and a community of commensal *E. coli* strains on host and microbial responses, including competitive colonization by bovine EHEC. From a human health perspective, the interaction between EHEC, the host and other bacteria has been previously studied in GB mice and other murine models (Eaton *et al.*, 2008; Goswami *et al.*, 2015; Taguchi *et al.*, 2002; Takahashi *et al.*, 2004). However, to my knowledge, these interactions with the added effects of physiological stress have not been examined. In this regard, I introduced stress as a factor that can potentially benefit EHEC colonization when competing with other commensal *E. coli* bacteria. Additionally, immunological responses and colonization impacts of a bovine EHEC in a murine model of stress have not been previously studied.

In an initial experiment, five different EHEC strains were tested in a non-stress GB murine model to assess intestinal colonization patterns and virulence of the bacterial strains. Secondly, I examined the degree to which a bovine isolated EHEC could outcompete 20 commensal *E. coli* strains in GB mice with or without exogenous corticosterone as an incitant of physiological stress . I hypothesized that stress predisposes the host by altering the immune response and directly influencing the bacterial virulence factors, thereby providing EHEC with a competitive advantage over the commensal strains of *E. coli*. My overarching goal was to use a gnotobiotic murine model to elucidate the impact that physiological stress has on EHEC colonization, a potential key factor regulating the EHEC-host-microbiota interaction in cattle.

3.2 Materials and Methods

3.2.1 Ethics statement

All experiments involving mice were carried out in strict accordance with the recommendations specified in the Canadian Council on Animal Care Guidelines. The project was approved by the Agriculture and Agri-Food Canada Lethbridge Research and Development Centre (AAFC LeRDC) Animal Care Committee (Animal User Protocol #1623), and LeRDC Biosafety and Biosecurity Committee before commencement of the research.

3.2.2 Mice

All mice were produced from a breeding colony of GF C57BL/6 mice maintained at AAFC LeRDC, which was generated from adult breeding pairs obtained from the National Gnotobiotic Rodent Resource Center (NGRRC) at the University of North Carolina. Methods for housing and handling of GF mice in IVCs are described in chapter 2. Briefly, GF C57BL/6 mice were reared and maintained in a Flexible Film Germ Free isolator (Class Biologically Clean Ltd., Madison, WI, USA), and they were transferred to and housed in Tecniplast® Green Line GM500 Sealsafe Plus Individually Ventilated Cages (IVC) (Tecniplast, Toronto, ON) situated in a single-sided Sealsafe Plus rack (Tecniplast) attached to a Smart Flow (Tecniplast) HEPA filtered air handling unit operated in containment mode (i.e. negative air pressure circulation) accordingly to the manufacturer's recommendations (Lange *et al.*, 2019). The IVCs were situated within a vivarium in animal rooms operated in containment mode (inward directional airflow), with unfiltered ambient air entering and exiting the room. Mice were maintained on a 12 hr light/dark cycle, and were allowed to drink and eat *ad libitum*.

To confirm the GF status of mice, feces from non-inoculated GF mice were weighed, suspended in sterile 1 mL PBS and vortexed (high setting) for 20 sec. Aliquots of the suspension (25 µL) were spread in quadruplicate onto Columbia agar (Difco; Becton Dickinson Canada Inc., Mississauga, ON) containing 5% sheep blood. Half of the cultures were incubated in an anoxic atmosphere (9-13% CO_2, with less than 0.1% O_2) at 37°C in 2.5 L anaerobic jars (Oxoid™ AnaeroJar™ 2.5L, Thermo Scientific™, Ottawa, ON), and the other cultures were incubated in an aerobic atmosphere at 37°C. After 7 days, cultures were examined for microbial growth.

3.2.3 *Escherichia coli* strains

Five EHEC strains representing different phylogenetic groups were evaluated in the study: (1) EDL933 (first recognized human outbreak); (2) FRIK 2001 (bovine isolate); (3) TW14359 (hyper-virulent human isolated strain); (4) ECI-1375 (bovine isolate); and (5) ECI-1911 (bovine isolate). Details on Shiga toxin production of each strain are in Table 3.1.

Twenty commensal *E. coli* strains recovered from beef cattle in Nova Scotia and Alberta were also evaluated. These isolates, confirmed not to carry shiga toxin, were selected based on their ability to competitively exclude *E. coli* O157:H7 in a chemostat (unpublished data). To determine that the commensal *E. coli* isolates represented unique subtypes, they were genotyped by pulsed field gel electrophoresis (PFGE) using the protocol specified by the Centers for Disease Control and Prevention (CDC) (CDC, 2013). Briefly, the enzyme *Xba*I (New England Biolabs) was used for restriction

endonuclease digestion, and electrophoresis was executed with a CHEF-DR® III Pulsed Field Electrophoresis System (Bio-Rad Laboratories Inc., Hercules, CA) using a 1% agarose gel. Electrophoresis conditions were as follows: initial switch time 2.2 sec, final switch time 54.2 sec, voltage 6V, included angle 120°, flow rate 1 L/min, run time 19 hr at 14°. Gel images were captured utilizing an Alphaimager 2200 (Alpha Innotech) and analyzed with BioNumerics 6.6. A reference *E. coli* commensal strain (LCMB-18-J) was used as a standard.

3.2.4 Design and validation of primers to detect and quantify EHEC strains

Regions unique to the genome of each of the five EHEC strains were identified utilizing Panseq (using default settings). Specific primers were designed utilizing Geneious 5.3.6 (Table 3.2) targeting the putative unique sequences of each strain's genome (i.e. relative to each other). The specificity of all designed primers was determined by end point PCR using genomic DNA extracted from cells of each strain in late log stage of growth using a thermocycler (Eppendorf) according to the manufacturer's recommendations. Reaction mixtures consisted of a total volume of 20 µL containing: 2 µL of genomic DNA; 2 µL of reaction buffer (Qiagen Inc.), 0.4 µL of deoxynucleoside triphosphates (0.2mM), 0.4 µL of $MgCl_2$ (2mM), 1 µL of each primer (0.5 µM; Integrated DNA Technologies, Coralville, IA), 0.1 µL of HotStar Taq polymerase (10 units/400 µL; Qiagen Inc.) and 13.1 µL of nuclease-free water. PCR cycle conditions were: one activation cycle at 95°C for 5 min; 35 cycles at 94°C for 15 sec, 62°C for 30 sec, and 72°C for 30 sec; and a final extension at 72°C for 5 min. The primers were also designed for quantitative PCR (qPCR). The following reagents were used: 2.0 µl of DNA, 10 µl of 2X Quantitect® SYBR® Green Master Mix (Qiagen Inc.), 1.0 µl of the forward and reverse primer (10 µM; Integrated DNA Technologies), and 6.0 µl of nuclease-free water (Qiagen Inc.). PCR conditions were: one activation cycle at 95°C for 15 min; 40 cycles at 94°C for 15 sec, 62°C for 30 sec, and a final cycle 95°C for 1 min, 55°C for 30 sec and 95°C for 30s. Furthermore, a melt curve analysis was conducted at the end of amplification. A Mx3005p Real Time PCR instrument (Agilent Technologies Canada Inc.) was used for qPCR. To calculate a density value, threshold cycles (Ct values) for each sample were compared to a standard curve generated from known quantities of DNA extracted from each EHEC strain. One primer set that was determined to be specific for each EHEC strain was selected.

By endpoint and qPCR, specificity of the EHEC primers was confirmed to not produce an amplicon against the 20 commensal *E. coli* strains isolated from beef cattle.

3.2.5 Virulence of EHEC strains

The colonization characteristics and virulence of the five different EHEC strains were determined in GF mice. Thirty six mice (4- to 6-week-old male GF mice) were transferred from the GF isolators into IVCs and acclimated for a period of 1 week at which point they were inoculated with EHEC strains. The experiment was designed as a two (time point) by six (*E. coli* treatments) factorial with three replicates conducted on separate occasions (twelve mice per replicate). Mice were administered the following EHEC strains/treatments: (1) EDL933; (2) FRIK 2001; (3) TW14359; (4) ECI-1375; (5) ECI-1911; and (6) PBS alone (i.e. no bacteria control treatment). Mice were inoculated with bacteria or PBS alone on day 0, and were humanely euthanized on 5 and 10 days post-inoculation (p.i.).

3.2.6 Competitive colonization by EHEC in mice under physiological stress

The ability of a bovine strain of EHEC (i.e. FRIK 2001) to competitively colonize mice under conditions of physiological stress was determined. The experiment was conducted as a two (± corticosterone) by six (*E. coli* treatment) factorial. Three replicates were conducted on separate occasions (twelve mice per replicate). For the corticosterone treatment, 4- to 8-week-old male GF mice were arbitrarily assigned to one of two groups; one group of mice was administered corticosterone in water to induce physiological stress (CORT+), and the second group was provided drinking water free of the glucocorticoid (CORT-). Corticosterone is a key hormone released in stress situations and has been used in stress models of mice or measured as a response to tests inciting stress (Ardayfio *et al.*, 2006; Cain *et al.*, 2017; Kim *et al.*, 2013; Murray *et al.*, 2008; Sturm *et al.*, 2015). Mice were transferred from GF isolators into IVCs and permitted to acclimate for a period of 1 week before commencement of corticosterone administration. The *E. coli* treatments consisted of: (1) GF mice inoculated with *E. coli* O157:H7 (EHEC); (2) GF mice inoculated *E. coli* O157:H7 and 20 commensal bovine *E. coli* grown communally (EHEC+CC); (3) GF mice inoculated with *E. coli* O157:H7 and 20 commensal bovine *E. coli* grown separately (EHEC+CS); (4) GF mice inoculated with 20 commensal *E. coli* grown communally (CC); (5) GF mice inoculated with 20 commensal *E. coli* grown separately (CS); and (6) PBS alone (PBS). On day 0, corticosterone treatment was initiated and was continued for 9 days. On day 6 of the 9 day corticosterone treatment regime, *E. coli* were administered, and mice were humanely euthanized on day 9 (i.e. 3 days p.i. with bacteria). The idea of including a comparison between communal and individual production of the commensal inoculum is based on previous studies, in which selected mixtures of bacteria grown communally were more effective than the same bacteria propagated

separately for competitively excluding *S. enterica* Typhimurium from the intestinal tract of chickens (Stavric *et al.*, 1985).

3.2.7 **Propagation of *Escherichia coli***

All *E. coli* O157:H7 strains were grown aerobically in 20 mL of Luria-Bertani (LB) broth at 37°C while shaking at 115 rpm to a mid-logarithmic phase growth as determined by optical density at 600 nm. The cultures were centrifuged at 12000 × *g* for 5 min, the supernatant removed, and bacterial cells re-suspended in phosphate-buffered saline (PBS) to a final concentration of 1 × 10^6 cells/mL. The density of cells was confirmed by diluting the cell suspension in a 10-fold dilution series, spreading 100 µL onto LB agar in duplicate, and counting colonies at the dilution yielding 30 to 300 colonies.

Commensal *E. coli* were grown separately or communally. For commensal *E. coli* strains grown separately, each isolate was grown in 20 mL of LB broth, the medium was removed by centrifugation, and cells were re-suspended in PBS as above. The *E. coli* strains were combined by pooling 1 mL of each culture in PBS together to achieve a final concentration of 1 × 10^6 CFU/mL. For commensal *E. coli* strains grown communally, one colony of each strain was placed into a common tube with 35 mL of LB broth. After 5 hr of growth at 37°C, 5 mL of culture were removed, centrifuged, and supernatant removed. The pellet was re-suspended in 45 mL of PBS to achieve a final concentration of 1 × 10^6 CFU/mL, which was confirmed using the dilution spread-plate method. To determine the relative abundance of commensal strains grown communally in LB both, pulsed field gel electrophoresis (PFGE) was used as follows. On the day of cell harvest for inoculation of mice, the culture broth was diluted in a ten-fold series, and 100 µL of each dilution was spread onto LB agar. After 24 hr growth at 37°C, biomass from 100 randomly-selected colonies were collected, isolates were propagated in LB broth, and biomass was stored in LB with 30% glycerol at -80°C. The *E. coli* isolates were grown from frozen stocks on Sorbitol MacConkey agar, and their PFGE fingerprints were obtained by PFGE, and using BioNumerics 6.6. was assigned to a strain using the PFGE fingerprints obtained from pure cultures previously.

3.2.8 Inoculation of mice

Escherichia coli were administered to mice in sterile raspberry Jell-O (Kraft-Heinz Canada, Don Mills, ON). An 100 µl suspension of bacterial cells (1 × 10^6 cells/mL) or PBS alone (100 µl) was uniformly mixed into 7 mL of sterile raspberry Jell-O placed in sterile 60-mm-diameter Petri dishes. In instances where commensals and EHEC strains were inoculated together, cells suspensions of both were mixed into the Jell-O at the same time. All mice consumed the Jell-O within 1-2 hr. This method of inoculation was selected to reduce handling and risk of compromising the GF status of the mice.

3.2.9 Corticosterone administration

To induce stress, mice were administered corticosterone (100 µg/mL; Sigma Aldrich, Oakville, ON) in sterile drinking water (Ardayfio *et al.*, 2006; Karatsoreos *et al.*, 2010). The corticosterone was dissolved in absolute ethanol before addition to water (1% final ethanol concentration v/v) placed in conventional water dispensers. Control animals were administered water containing ethanol (1%) alone. Mice were allowed to drink *ad libitum*.

3.2.10 Health assessments

Following administration of corticosterone and inoculation with EHEC, mice were scored each morning at the same time (i.e. 9:00 am) for activity (0-4), haircoat and appearance (0-3), and vocalization (0-1). The score of each category was added to create the total score. Details of the scoring system are in Table 3.3.

3.2.11 Behavioral assessments

On day 3 of the competitive colonization experiment, enrichment items were removed from the cages and mice were provided with 3.0 g of a sterile cotton nestlet. The nestlet was supplied 1 hr before the dark phase. Nest building quality was assessed the next morning following a 1 to 5 rating scale (Deacon, 2006). On day 9 (i.e. immediately prior to euthanization), an open field test was performed. A 30 × 30 cm Phenotyper cage (Noldus Information Technology Inc., Leesburg, VA) was utilized to record exploratory behaviour. Each mouse was placed in the center of the cage, and the behaviour was recorded for 10 min. Personnel remained behind curtains during the video recording to minimize environmental distractions. All videos were analyzed with Ethovision XT10 (Noldus Information Technology Inc.), and measurements of center zone frequency, cumulative time in center, latency to first in center, total distance moved, and velocity of movement were quantified (Gould, 2009).

3.2.12 Sample collection

At experimental endpoints, mice were anaesthetized with isoflurane, and blood was collected by cardiac puncture. Blood for serum separation was collected into BD Microtainer® SST tubes (BD, Franklin Lake, NJ, USA), and serum for quantification of corticosterone was stored at -80°C until analyzed. During anaesthesia, mice were then humanely euthanized by cervical dislocation. A ventral mid-line laparotomy was completed with sterile tools to exteriorize the intestine. Sections from kidney, ileum, cecum, and proximal and distal colon were removed. Within ca. 5 min of death, samples for gene expression were placed in RNALater® (Qiagen Inc.), and stored at -80°C. Tissue samples for quantitation metabolomics were snap frozen in liquid nitrogen, and subsequently stored at -80°C. Samples for histopathologic

examination were placed in 10% neutral buffered formalin at room temperature. In addition, intestinal biopsies (4-mm diameter) were obtained from the cecum, proximal colon, and distal colon, and stored at -80°C for enumeration of *E. coli* O157:H7 by quantitative PCR (qPCR). Subsamples of digesta (180-220 mg) from the ileum and cecum were also collected, and where possible from the proximal colon and distal colon, and stored at -80°C for enumeration of *E. coli* O157:H7 by qPCR, and to quantify corticosterone.

3.2.13 Histology

Sections of caecum, proximal colon, and distal colon fixed in 10% neutral buffered formalin were dehydrated using a Leica tissue processor (Leica TP1020 Benchtop Tissue Processor, Leica Biosystems, Concord, ON). Following dehydration, tissues were embedded in paraffin using a Shandon Histocentre 3 Embedding Center (Thermo Scientific, Ottawa, ON), sectioned (≈5 µm) using a Finesse 325 Manual Rotary Microtome (Thermo Scientific), and the sections placed on 25 × 75 × 1–mm Superfrost Plus Gold microscope slides (Fisher Scientific) and deparaffinized. The slides were then stained with hematoxylin and eosin, and sections (mucosa and submucosa) were examined using a Zeiss Axioskop Plus microscope (Carl Zeiss Canada Ltd, North York, ON). Total histopathological score for cell infiltrate severity (1-4) and extent (1-3), epithelial hyperplasia (1-5), epithelial injury (1-4), cryptitis (2-3), crypt abscess (4-5), goblet cell loss (1-4), granulation tissue (4-5), crypt loss (4-5), apoptosis (0-3), occluding thrombi (0-3), mucosal hemorrhage (0-3), irregular crypts (4-5), villus blunting (1-5), and ulceration (3-5) as described previously (Eaton *et al.*, 2017; Erben *et al.*, 2014; Koelink *et al.*, 2018). A total score of 62 was calculated by combining the scores for all metrics. Samples were scored by a board-certified pathologist (V.F.B.) who was blinded to treatment.

3.2.14 EHEC quantification

Densities of *E. coli* O157:H7 associated with mucosa and within digesta were determined by qPCR (following the settings developed on section 3.2.4). Genomic DNA was extracted from 80-120 mg of thawed ingesta samples using the QIAamp® Fast DNA stool extraction kit (Qiagen Inc.) according to the manufacturer's recommendations. Also, genomic DNA from mucosal biopsies was extracted using the DNAeasy blood and tissue extraction kit (Qiagen Inc.). Quantitative PCR was conducted as described above.

3.2.15 Quantification of inflammation gene mRNA

To quantify mRNA of targets of interest, RNA was extracted from ≈0.5 × 0.5 cm sections of distal colon using a RNeasy mini kit (Qiagen Inc.) with a DNase step added to eliminate residual genomic DNA.

RNA quantity and quality were determined using Bioanalyzer 2100 (Agilent Technologies Canada Inc., Mississauga, ON). RNA (1000 ng) was transcribed into cDNA using a Quantitec reverse transcription kit (Qiagen inc.). Expression of mRNA for interferon-gamma (*Ifnγ*)*,* interleukin (Il) 4 (*Il4*)*, Il22,* keratinocyte-derived cytokine (*Kc*)*,* transforming growth factor beta (*Tgfβ*)*,* toll-like receptor-4 (*Tlr4*), and tumour necrosis factor-alpha (*Tnfα*) were standardized against hypoxanthine-guanine phosphoribosyltransferase (*Hprt*), beta-glucuronidase (*Gusβ*) and glyceraldehyde 3-phosphate dehydrogenase (*Gapdh*); these reference genes were selected due to the low variation between samples. Primer sequences are in Table 3.4.

3.2.16 Corticosterone quantification

Serum and fecal corticosterone extractions were carried out according the manufacturer's instructions (Cayman Chemical, Ann Arbor, MI, USA). The optical densities of all corticosterone ELISAs (wavelength of 412 nm) were measured using a Synergy HT multi-detection microplate reader (BioTek Instruments Inc., Winooski, VT, USA) with Gen5 analysis software (BioTek Instruments Inc., Winooski, VT, USA).

3.2.17 Tissue metabolomics

Kidney tissue were homogenized in 4 mL/g methanol and 1.6 mL/g deionized water. Tissues were homogenized with 6-mm-diameter steel bead for 5 min intervals using Qiagen Tissue Lyser at 50 Hz followed by 1 min of vortexing. This step was repeated twice more to ensure complete tissue homogenization. To each sample, 2 mL/g chloroform was added and vortexed thoroughly. Next, 2 mL/g chloroform and 4 mL/g deionized water were added to each sample and vortexed until thoroughly mixed. Samples were then incubated at 4°C for 15 min followed by centrifugation at 1000 x g for 15 min at 4°C. Next, 600 µl of the supernatant was removed and left until evaporated. Samples were rehydrated in 480 µl of metabolomics buffer (0.125 M KH_2PO_4, 0.5 M K_2HPO_4, 0.00375 M NaN_3, and 0.375 M KF; pH 7.4). A 120 µl aliquot of deuterium oxide containing 0.05% v/v trimethylsilylpropanoic acid (TMSP) was added to each sample (final total volume of 600 µl); TMPS was used as a chemical shift reference for 1H-NMR spectroscopy. A 550 µl aliquot was then loaded into a 5 mm NMR tube and run on a 700 MHz Bruker Avance III HD spectrometer (Bruker, ON, Canada) for spectral collection. Data acquisition and processing were followed as previously described (Paxman *et al.*, 2018).

3.2.18 Analyses

The majority of the statistical analysis were performed using Statistical Analysis Software (SAS Institute Inc. Cary, NC). Least square means test was used to compare treatments within factors in

bacterial densities, gene expression, and cytokine concentrations. The open field test behavioral results were compared utilizing an Student's t test. Fisher's exact test was utilized to analyze categorical data (histopathological scoring and health assessment data). For metabolomics data, NMR spectra were exported to MATLAB (Math Works, MA, USA) where they underwent spectral peak alignment and binning using Recursive Segment Wise Peak Alignment (Veselkov *et al.*, 2008) and Dynamic Adaptive Binning (Anderson *et al.*, 2011), respectively. After these analyses the dataset was normalized to the total metabolome, excluding the region containing the water peak, and pareto scaled. MetaboanalystR was used to perform univariate and multivariate statistics including calculation of fold changes of specific metabolites, heat map creation, and hierarchical clustering analysis (Chong *et al.* 2019). These tests were carried out using the bins identified as significant by univariate tests in order to observe group separation. Univariate measures include the t-test and the Mann-Whitney U test. Both tests determine if there is a significant difference between the means of the two groups; however, the t-test and the Mann-Whitney U test are applied in instances where the data is normally distributed (parametric) or not, respectively. The test for data normality was carried out using a decision tree algorithm as described by Goodpaster *et al.* (2010). All p-values obtained from analysis were Bonferroni-Holm corrected for multiple comparisons. Metabolites were then identified using Chenomx 8.2 NMR Suite (Chenomx Inc., AB, Canada).

3.3 Results

3.3.1 Differences were detected in the virulence of and intestinal colonization by EHEC strains

At day 5 p.i., four of the EHEC strains (EDL933, TW14359, ECI-1375, and ECI-1911) impacted ($P<0.001$) the health of mice (Fig. 3.1A). In contrast, EHEC strain FRIK 2001 did not affect the activity or appearance of mice. The impacts of EHEC strains EDL933, TW14359, ECI-1375 and ECI-1911 on mice required that they were all humanely euthanized before or at 5 day p.i. endpoint. Only EHEC negative control mice and those inoculated with FRIK 2001 were able to be analyzed at both endpoints; 5 day p.i. and 10 day p.i. At day 10 p.i., FRIK 2001 strain resulted in an average health score of 2.0 ± 0.5, which was higher ($P<0.022$) than mice not inoculated with EHEC (0.0) (Fig. 3.1B).

All five EHEC strains colonized the mucosa in the ileum, cecum, proximal colon, and distal colon of mice at 5 days p.i. with no differences ($P\geq0.200$) in bacterial densities between intestinal sites (Fig. 3.2A-D). The degree of colonization was similar among the five EHEC strains in mice at 5 day p.i., with the exception of FRIK 2001 and ECI-1375 in the ileum and distal colon where these were detected at a lower density ($P<0.05$) than other strains. The mucosal colonization densities of FRIK 2001 between day 5 and

day 10 p.i. in all the intestinal sections analyzed remained the same ($P \geq 0.1$). As the goal was to generate a bovine model of EHEC colonization and strain FRIK 2001 was the only one evaluated that did not incite acute disease within 5 days p.i., this EHEC strain was selected for further evaluation.

3.3.2 EHEC strains generated histopathological changes in the intestine

Analysis of histopathological slides revealed leukocyte infiltration, loss of goblet cells, epithelial hyperplasia and apoptosis in all treatments and all tissues with exception of the control treatments. Specifically, the distal colon had elevated scores, particularly strains EDL933 and ECI-1911, which presented significant differences with FRIK 2001 ($P=0.001$ and $P=0.01$) at 5 days p.i. (Fig. 3.3). Interestingly histopathological scores tended to decrease in FRIK 2001 at 10 days p.i. in the distal colon ($p=0.07$). Bacterial strains other than FRIK 2001 were not analyzed beyond 5 days p.i.

3.3.3 Differences were detected in the metabolite profiles in kidney samples of strain FRIK 2001

Analysis of the treatment groups included 472 total spectral bins from the kidney. The metabolome of mice inoculated with EHEC strains EDL-933, TW14359, ECI-1375, and ECI-1911 was substantially changed ($P<0.001$) relative to non-treated mice at 5 days p.i. In contrast, minimal changes in the metabolome of mice inoculated with FRIK 2001 were observed relative to un-inoculated mice at day 5 p.i. (Fig. 3.4A), whereas more substantive differences were observed in the metabolome of kidney (193 altered bins) as compared to control mice at 10 days p.i. (Fig. 3.4B). Consistent with this observation, unsupervised hierarchical clustering indicated that strain FRIK 2001 causes a large number of differences in the metabolome of the kidney (77 altered bins) of mice inoculated with the pathogen at 10 days p.i. relative to 5 days p.i. (Fig. 3.4C). A number of specific metabolites were differentially present at 10 days p.i. in the kidney of mice inoculated with FRIK 2001 and control mice. Of importance, kynurenine ($P=0.01$) and carnitine ($P=0.05$) were decreased compared to control mice.

Health assessment scores, intestinal colonization, histopathologic changes and metabolomic profiles, at day 5 and 10 p.i. provided evidence that EHEC strain FRIK 2001 mimics colonization in the cattle host.

3.3.4 Stress affected mouse behavior

Mice that were not administered corticosterone travelled at a faster rate during the exploration of the arena ($P=0.012$) than mice administered corticosterone (i.e. stressed mice) (Fig. 3.5A). In addition, the total distance travelled by corticosterone negative mice was greater ($P=0.020$) than by stressed mice (Fig. 3.5B). There was no effect of EHEC administration on behavior of mice. No differences ($P>0.68$) in nest building behavior were observed for either the stress or bacterial treatments.

3.3.5 Corticosterone concentrations were higher in stressed mice

Concentrations of corticosterone were higher in the serum ($P=0.006$) and feces ($P<0.001$) of stressed compared to non-stressed mice when measured on day 9 (Fig. 3.6A-B). All bacterial treatments were combined when comparing corticosterone concentrations.

3.3.6 Communal growth affected the population structure of commensal *Escherichia coli* inoculum

Seventeen of the 20 commensal *E. coli* strains grown communally in LB for 5 hr were isolated; the prevalence of strain recovery ranged from 1.1% to 13.9% (Table 3.5).

3.3.7 EHEC was reduced in mice administered commensal *Escherichia coli*

At day 3 p.i., bacterial densities of FRIK 2001 associated with mucosa in the cecum were lower in mice inoculated with commensal strains produced together (CC; $P=0.017$) and separately (CS; $P<0.001$). The same response was observed in the proximal colon (EHEC+CC $P=0.015$; EHEC+CS $P=0.01$) and distal colon (EHEC+CC $P=0.05$; EHEC+CS $P=0.009$) (Fig. 3.7). Furthermore, the administration of commensal strains reduced densities of FRIK 2001 in digesta within the caecum (EHEC+CC $P=0.001$;EHEC+CS $P<0.001$), proximal colon (EHEC+CC $P=0.002$; EHEC+CS $P<0.001$), and distal colon (EHEC+CC $P=0.004$;EHEC+CS $P=0.003$) (Fig. 3.8). In no instance was FRIK 2001 detected in mice not inoculated with the pathogen. Stress did not alter ($P\geq0.118$) the densities of FRIK 2001 in digesta or associated with mucosa. Communal or individual growth of the commensal inoculum did not affect ($P=0.196$) the densities of FRIK 2001.

3.3.8 Histopathologic changes associated with EHEC were greatest in the distal colon

More extensive histopathologic changes incited by FRIK 2001 were observed in the distal colon relative to the caecum ($P<0.001$) and proximal colon ($P<0.001$) (Fig. 3.9A). Stress, alone or in conjunction with EHEC did not affect tissue morphology or histopathologic scoring.

3.3.9 Histopathologic changes in the distal colon were reduced in mice administered commensal *Escherichia coli*

Both commensal *E. coli* treatments (i.e. CC and CS) reduced ($P\leq0.003$) histopathologic changes in the distal colon of mice infected with EHEC FRIK 2001 (Fig. 3.9B). No adverse effects ($P\geq0.100$) were observed in mice not infected with FRIK 2001 (CC, CS, and PBS). There was no effect ($P=0.100$) of stress induction on histopathologic scores.

3.3.10 Expression of inflammatory marker genes in the distal colon were reduced in mice administered commensal *Escherichia coli*

Gene expression of *Tnfα* and neutrophil attractant cytokine *Kc* mRNA were reduced ($P<0.001$) in mice administered EHEC FRIK2001 with commensal *E. coli* (Fig. 3.10A-B). Administration of commensal *E. coli* did not affect ($P \geq 0.1238$) quantities of *Il4, Il22, Tlr4, or Ifnγ* mRNA. The corticosterone stress treatment affected ($P=0.018$) quantities of *Tnfα* in mice administered EHEC alone (Fig. 3.10A). Moreover, corticosterone treatment reduced ($P=0.018$) *Tgfβ* mRNA independent of *E. coli* administration (Fig. 3.10C).

3.4 Discussion

3.4.1 Development of a bovine EHEC model

Due to the complex nature of the microbiota present in the gastrointestinal tract of individual cattle, it is challenging to elucidate interactions among bacteria, and their impacts on the host. Colonization mechanisms in the intestine, shedding patterns, and lack of *E. coli* O157:H7 disease in cattle are factors that are still not entirely understood, and further information is needed to enable the development of on farm mitigation strategies. A simpler and more prescribed model devoid of intestinal microbiota can prove to be a valuable resource for understanding bacterial competition and bacteria-host interaction. Understanding of mechanisms in such a model can later be applied to formulate hypotheses with validation in cattle experiments. The development and use of a GB murine model of enteric bovine *E. coli* O157:H7 colonization could provide key information on the pathogen-host-microbiota interaction. An objective of this study was to develop a GB colonization model of *E. coli* O157:H7 with reduced kidney damage characteristic of cattle colonized by the pathogen. *Escherichia coli* O157:H7 mouse models have mainly been used as a human model to study HUS (Eaton *et al.*, 2008; Taguchi *et al.*, 2002; Wadolkowski, Sung, *et al.*, 1990). In this regard, both GB and streptomycin-treated murine models have been utilized due to their susceptibility to Stx with subsequent development of renal injury and death (Eaton *et al.*, 2017; Eaton *et al.*, 2008; Taguchi *et al.*, 2002). Initially, I examined the colonization and health status of mice inoculated with five different EHEC strains, representing different phylogenic lineages. All five strains successfully colonized mucosa in the large intestine (cecum, proximal colon and distal colon) at 5 days p.i. with no differences in bacterial densities between the intestinal sites. However, FRIK 2001 was the only strain that did not present elevated health scores before day 5 p.i. In contrast, mice inoculated with either EDL933, TW14359, ECI-1375, or ECI-1911 presented high scores in the health assessments by day 5 p.i. *Escherichia coli* O157:H7 FRIK 2001 is a lineage II EHEC from bovine,

and mice inoculated with this strain displayed mild reduction in activity and modest changes in grooming and hair coat appearance at or after 8 days p.i. Therefore, FRIK 2001 could colonize the intestinal tract of GF mice with limited presentation of symptoms similar to cattle. In GB mice infected with EHEC, there is typically a rapid progression from colitis to renal injury and failure, where Stx produced by EHEC causes acute tubular necrosis that can lead to death (Eaton *et al.*, 2017; Eaton *et al.*, 2008). Therefore, the manifestations of disease observed in animals inoculated with either EDL933, TW14359, ECI-1375 or ECI-1911, could be related to kidney injury and failure. It is noteworthy that in contrast to FRIK 2001, EHEC strains EDL933, TW14359, ECI-1375, and ECI-1911 belong to lineages I or I/II.

Mice infected with FRIK 2001 did not develop diarrhea; however, the bacterium did trigger mucosal leukocyte infiltration, goblet cell loss, epithelial hyperplasia, and apoptosis of epithelial cells. The highest histopathologic scores were observed in the distal colon of mice, similar to cattle where the distal colon is the primary location where attaching effacing lesions develop (Naylor *et al.*, 2003). Other strains such as EDL933 and ECI-1911 had significantly higher histopathologic scores at day 5 p.i. than FRIK 2001. Notably, inflammation in mice infected with FRIK 2001 at day 5 p.i. was categorized as mild, and was reduced in the distal colon by day 10 p.i. Previous studies utilizing strain EDL933 in GF mice have categorized the colonic changes as a necrotizing colitis accompanied with a few attaching effacing lesions (Eaton *et al.*, 2017). Much like my results they found EHEC necrotizing colitis peaked at 1-4 days p.i., and was gradually reduced thereafter (Eaton *et al.*, 2017). Although I did not observe conspicuous histopathologic changes, significant metabolomic changes were observed in the kidneys of mice infected with *E. coli* O157:H7 strains and mirrored other health metrics. Renal metabolomic profiles of mice infected with FRIK 2001 followed a similar progression to the health assessment scores. In this regard, there were significantly altered metabolite bins in infected mice at day 5 p.i. relative to 10 p.i. Moreover, no differences were observed between mice infected with FRIK 2001 and un-inoculated control mice at day 5 p.i., and significantly altered metabolite bins were only observed in the kidneys of mice inoculated with FRIK 2001 and un-inoculated control mice at day 10 p.i. The lack of metabolomic differences between FRIK 2001 inoculated mice and control mice at 5 days p.i. supports my conclusion that early intestinal colonization by this EHEC strain potentially occurs in absence of kidney pathology (ca. ≤5 days p.i.). However, by 10 days p.i. evidence of kidney pathology was observed. Carnitine, a biological compound, was found to have a 25% decrease in mice infected with FRIK 2001 10 days p.i. as compared to control mice. Carnitine functions transporting free fatty acids into the mitochondria for β-

oxidation (Reuter *et al.*, 2012). The presence of Stx2a plus TNFα reduced carnitine species in human renal glomerular endothelial cells (HRGEC) (Betzen *et al.*, 2016). Low levels of carnitine show a perturbation in the normal use of free fatty acids (Betzen *et al.*, 2016). This suggests that an alteration in normal metabolism could be taking place due to kidney disruption at 10 days p.i. The metabolite kynurenine was also significantly reduced in mice infected with FRIK 2001 at 10 days p.i. Elevated levels of kynurenine in HRGEC have been associated with the combined administration of Stx2a and TNFα (Betzen *et al.*, 2016). However, the role of kynurenine is complex since elevated levels of kynurenic acid (a metabolite of kynurenine) act as an early mediator of leukocyte recruitment (Barth *et al.*, 2009). Nonetheless, kynurenic acid is also capable of reducing LPS stimulated secretion of IFNγ and TNFα (J. Wang *et al.*, 2006). This indicates a downregulating role of some factors of the immune response. Given the lower amounts of kynurenine found in kidney samples, it is possible that an alteration in the regulation of certain aspects of the immune response was taking place at 10 days p.i. Intestinal colonization, histopathologic changes, metabolomic profiles, and health assessment scores at day 5 and 10 p.i. indicated that EHEC strain FRIK 2001 mimics colonization in bovine. Notably lineage II strains exhibit unique host ecology relative to lineage I and I/II EHEC strains (Sadiq *et al.*, 2014; Zhang *et al.*, 2010), and are more commonly isolated from bovine hosts, whereas lineage I strains are more frequently isolated from human beings (Sadiq *et al.*, 2014).

3.4.2 Impact of physiological stress on EHEC intestinal colonization and pathology

The GB murine model allowed me to examine the impact of physiological stress on bacterial interactions within the host and among bacteria. To generate a representative physiological condition of stress in mice, I administered the glucocorticoid corticosterone in drinking water. To evaluate the impact of stress, the behavior of mice was assessed with the open field test and the nest building ability. The open field test is utilized as a test to observe mice exploratory drive (curiosity) as well as anxiety-like behavior (fear), which can be directly affected under stressful scenarios (Gould *et al.*, 2009). Similar to others, I observed that mice administered corticosterone presented a reduction in total locomotion as well as a slower travelling rate than non-stressed mice. This indicates that stressed mice exhibit reduced exploratory behavior when encountering a new open and unprotected environment (Kim *et al.*, 2013; Sturm *et al.*, 2015). Although others have reported that corticosterone treated mice exhibit a significant reduction in the time spent in the center of the open field (David *et al.*, 2009), I did not observe this behavior. It has been suggested that GF mice show reduced anxiety-like behavior (anxiolytic) as compared to normal flora mice when placed in the elevated plus maze, indicating that the microbiota

plays a role in the development of anxiety-like behavior in a mouse (Neufeld *et al.*, 2011). The mice in this study were GB, and thus the differences I observed in anxiety-like behavior could be attributed directly to physiological stress induced by corticosterone. This was supported by the higher levels of corticosterone in serum of stress treatment mice. In contrast, I did not observe that corticosterone administration influenced nest building ability of the mice. This could be due to mice only being stressed for 3 days prior to introducing the nestlets into the cages. It is noteworthy that maintaining the GF and GB status of mice represents challenges in conducting behavioral analyses, which necessitated that I limit the behavioral analysis to in-cage assessments (e.g. nest building), and to more classical behavioral assessments at the end of the experimental period (i.e. immediately prior to euthanasia).

Previous studies have suggested that stress may be a predisposing factor benefiting colonization of the intestine of cattle by *E. coli* O157:H7 (Vlisidou *et al.*, 2004). In the model presented in this study, I did not find that a general state of stress benefitted intestinal colonization by EHEC FRIK 2001. Previous observations have shown that stress hormones, such as catecholamines affect the expression of virulence factors by *E. coli* O157:H7 (Lyte *et al.*, 2011; Sperandio *et al.*, 2003; Vlisidou *et al.*, 2004). These molecules were found to enhance the expression of type three secretion system, a complex specifically targeted towards binding with epithelial cells and forming attaching effacing lesions (Lyte *et al.*, 2011; Sperandio *et al.*, 2003; Vlisidou *et al.*, 2004). In the current study, stress did not enhance the densities or intestinal injury of EHEC FRIK 2001. It is noteworthy that mice underwent physiological stress with exogenous corticosterone, a known systemic glucocorticoid hormone released during stressful conditions (Moberg *et al.*, 2000). Glucocorticoids have also been shown to stimulate production of catecholamines (Sharara-Chami *et al.*, 2010). I am not aware if corticosterone stimulated the secretion of catecholamines in the intestine of the mice; the influence of catecholamine on *E. coli* O157:H7 is difficult to replicate *in vivo*, and has only previously been observed *in vitro* or in ligated intestinal loops (Sperandio *et al.*, 2003; Vlisidou *et al.*, 2004).

I observed an impact of stress in the expression of *Tnfα* and *Tgfβ* in the distal colon. Stressed mice presented lower levels of *Tgfβ*. Regulatory molecules such as TGFβ have the important function of controlling the inflammatory response avoiding collateral damage to host tissue (Letterio *et al.*, 1998). The reduction in expression of this cytokine in the distal colon could lead to an unbalanced inflammatory response and possibly benefit *E. coli* O157:H7 colonization. Moreover, *E. coli* O157:H7 infection has been shown to cause reduced protein expression of tight junctions and barrier dysfunction (Howe *et al.*, 2005). TGFβ can prevent epithelial barrier dysfunction generated by *E. coli* O157:H7 and

potentially reducing the penetration of Shiga toxin past the epithelium (Howe *et al.*, 2005). The observed reduction of *Tgfβ* expression in the distal colon in stressed animals could possibly play a pivotal role in the colonization of FRIK 2001. Stressed mice mono-colonized with FRIK 2001 presented significantly elevated levels of expression of *Tnf*α in the distal colon as compared to non-stressed FRIK 2001 treatments. TNFα stimulates a downstream cascade with the consequent arrival of neutrophils to the lamina propria of the intestine (Li *et al.*, 2002; Pearson *et al.*, 2014). Neutrophils represent a first line of defense in response to *E. coli* O157:H7, and therefore are of importance in the ability to eliminate bacterial pathogens (Amulic *et al.*, 2012). However, TNFα can possibly benefit EHEC, since administration of a TNFα inhibitor reduced pathological symptoms in mice infected with EHEC (Isogai *et al.*, 1998).

3.4.3 Impact of commensal *Escherichia coli* strains on EHEC colonization and pathology

In the current study, a combination of 20 *E. coli* commensal strains of bovine origin significantly reduced densities of EHEC FRIK 2001 in the intestinal tract of mice, particularly in the distal colon. Previous studies have utilized murine models to ascertain the impact of human commensal *E. coli* strains on *E. coli* O157:H7 with the goal of eliminating EHEC from the intestine of afflicted people (Leatham *et al.*, 2009; Maltby *et al.*, 2013; Takahashi *et al.*, 2004). In these studies, the commensal *E. coli* strains were effective at reducing EHEC colonization (Leatham *et al.*, 2009); however, a dysbiosis was first achieved with streptomycin, and mice were pre-colonized with the commensal strains 10 days prior to inoculation with EHEC. It is noteworthy that adhesion of strains to the epithelium can provide an advantage for strains pre-colonizing the intestine (Freter *et al.*, 1983). In contrast to human focused studies, I inoculated GF mice with FRIK 2001 and commensal *E. coli* strains at the same time with the idea of precluding a pre-colonization advantage to the commensal competitors. Freter *et al.*, (1983) proposed that the competition for colonization niches could be, in part, based on competition for nutrients. Different strains of *E. coli* have different nutrient requirements (Maltby *et al.*, 2013), and the inability to utilize a nutrient at a higher rate than other bacteria can be a limiting factor for surviving within the intestinal environment. It is clear from previous studies that a single strain of commensal *E. coli* cannot outcompete *E. coli* O157:H7 (Leatham *et al.*, 2009), and it is plausible that in my study one or all of the 20 commensal *E. coli* strains administered were able to impede FRIK 2001 from accessing key nutrients, thus reducing its survival.

Histopathological changes such as inflammatory infiltrate in the mucosa, apoptosis of enteric cells, epithelial hyperplasia, cryptitis, and goblet cell loss were highest in the distal colon. I was particularly

interested in looking at the intestinal histopathology in the distal colon as this area of the intestine, specifically the recto anal junction, is considered the main site of *E. coli* O157:H7 colonization in cattle (Naylor *et al.*, 2003). In this regard, the highest concentrations of *E. coli* O157:H7 have been isolated from this region of the bovine intestine (Naylor *et al.*, 2003). The presence of competing *E. coli* significantly reduced the histopathological score in the distal colon, suggesting that the 20 selected strains can interfere with the proper colonization and epithelial damage generated by FRIK 2001. The histological changes that I observed in mice mono-colonized with EHEC FRIK 2001 correspond with histopathological changes previously described in cattle (Dean-Nystrom *et al.*, 1997; Nart *et al.*, 2008; Zhao *et al.*, 2003). Calves colonized with *E. coli* O157:H7 develop a mild neutrophilic inflammation in the mucosa of the large intestine (Nart *et al.*, 2008), an alteration that I observed in the mucosa layer of the distal colon of mice.

Relative expression of *Tnfα* and *Kc* in the distal colon were significantly reduced in mice colonized by commensal *E. coli* strains, indicating that they ameliorated the inflammatory impact of EHEC. Kc is a chemokine that shares functional properties with the human IL8, causing strong neutrophil attraction to the site of inflammation (Roche *et al.*, 2007). Kc has been linked with the functional role of inducing neutrophil accumulation in the glomeruli of the kidney of mice when stimulated with *E. coli* LPS or Stx2 (Roche *et al.*, 2007). The expression of this chemotactic molecule is of particular importance as accumulation of neutrophils are observed in the colonic mucosa of calves infected by EHEC (Dean-Nystrom *et al.*, 1997). This suggests that neutrophils are important in the clearance of *E. coli* O157:H7 from the intestinal tract of cattle. The increase in *Kc* expression observed in the distal colon is consistent with the neutrophilic infiltration I observed in mice infected with EHEC. However, other studies found a reduction in the expression of factors involved in immune function in the rectoanal junction of super shedding cattle; suggesting this decrease in response as a possible component in *E. coli* O157:H7 colonizing the rectoanal area (O. Wang *et al.*, 2016). *Tnfα* was also reduced in mice colonized by commensal *E. coli* strains. TNFα is a pro-inflammatory cytokine known to trigger the activation of several elements of the immune response including the transcription factor, NF-κβ with the consequent activation of inflammatory signaling pathways and pro-inflammatory cytokines such as IL8 (Li *et al.*, 2002; Naude *et al.*, 2011). Inhibition of TNFα in mice infected with *E. coli* O157:H7 was associated with reduced pathology and animal lethality (Isogai *et al.*, 1998). Furthermore, the colonization of *E. coli* O157:H7 induces the secretion of NF-κβ and consequently IL8 (Bellmeyer *et al.*, 2009). It is noteworthy that infection by *E. coli* O157:H7 Stx positive strains induce greater NF-κβ expression than infection by *E.*

coli O157:H7 Stx negative strains (Bellmeyer *et al.*, 2009). Although *E. coli* O157:H7 infection elevates the secretion of TNFα, infection by EHEC can also reduce the activation of NF-κβ; the mechanism, however, is not fully understood (Xue *et al.*, 2014). It is plausible that in my study, competition with commensal *E. coli* disrupted the ability of EHEC FRIK 2001 to interact with intestinal mucosa, thereby reducing host recognition and subsequent the expression of inflammatory markers. Notably, the reduced expression of *Tnfα* in mice inoculated with commensal *E. coli* correlated with the reduced intestinal damage. In contrast to mice however, adolescent cattle inoculated with EHEC do not exhibit elevated expression of *Tnfα* (Corbishley *et al.*, 2014). Overall, bacterial competition reduced FRIK 2001 densities, lessened histopathological lesions and decreased pro-inflammatory markers. It is plausible that commensal *E. coli* denied access to nutrients, thereby reducing densities of FRIK 2001. Furthermore, by interfering with direct access to the epithelium it would be expected to reduce recognition of the pathogen by the host, thus lowering activation of pro-inflammatory markers and ensuing damage to the hosts epithelium.

3.4.4 Communal growth of commensal *Escherichia coli* strains

There was no observable difference on the efficacy of EHEC exclusion between commensal *E. coli* strains grown individual or communally. Previous studies reported success in reducing infection of chickens by *Salmonella enterica* Typhimurium via incubating mixtures of cultures of commensal bacteria before competition with the pathogen (Stavric, 1992; Stavric *et al.*, 1985). The mechanism for the enhanced efficacy of cultures grown communally is currently unknown. A possible explanation is that competition amongst commensal bacteria enhances glycocalyx formation, providing a survival advantage later in the intestine (Stavric, 1992). Another possibility is that communal incubation triggers quorum sensing communication between bacteria, where signals can be used to synchronize behaviors of the population and prompt bacteria to act as multicellular organisms (Waters *et al.*, 2005). Bacteria can also quorum quench, where they inactivate signals from other bacteria to interfere with communal communication (Waters *et al.*, 2005). At high bacterial numbers sufficient molecules are produced for communal detection, however quorum sensing has not been studied under this specific condition. It is noteworthy that communal incubations of bacteria for use against *S. enterica* Typhimurium were conducted for 7, 24, and 48 hr, and the latter two times were found to be the most effective (Gleeson *et al.*, 1989; Stavric *et al.*, 1985). Longer incubation periods (i.e. beyond log phase) may enhance the competitive efficacy of *E. coli* strains grown communally.

3.4.5 Conclusions

In the current study, I was able to achieve a GB mouse model to study host-pathogen interactions in a manner that mimics cattle. The advantage of this model is the absence of enteric microbiota that can confound the elucidation of mechanisms involved in bacterial competition and colonization. Contrary to my hypothesis, the administration of exogenous corticosterone, as a model of general physiological stress did not provide an advantage in *E. coli* O157:H7 colonization. However, commensal *E. coli* strains administered at the same time as EHEC effectively reduced densities of the pathogen and injury to the host. Importantly, the murine model developed in the current study can be used to elucidate mechanisms of pathogenesis and colonization resistance leading towards the development of effective on farm mitigation of EHEC in cattle.

3.5 Tables and figures

Table 3.1 Presence of Shiga toxin gene in the five analyzed strains

Strain	Stx1	Stx2
TW-14359	0	1
ECI-1375	1	1
ECI-1911	0	1
FRIK-2001	0	1
EDL-933	1	1

Table 3.2 Detail of forward and reverse primers targeting specific DNA sequences unique to each EHEC strain.

Strain	Forward primer	Reverse primer
EDL-933	TGACCGCTTACGCAGTTCGC	AATGGTTGCTGCCACGGCTC
FRIK2001	GGATGACGGTGCCTGTGCTG	GGCTGGCGGGAGCTACCTAA
TW14359	ATCGGTGGCTGGAATGGGCT	AAGTGCAACTGGTGCGTGCT
ECI-1375	CGCTCGCCCTAAGATGGGGA	TTCGACGGCTTTCGCTGACG
ECI-1911	CACACTGTCCGGTGATGCCG	CGCGCTGTTCAGCATGAGGT

Table 3.3 Health assessment scoring system

Category	Score	Definition
Activity	0	Bright and alert
	1	Slow to move, stays in nest area, restless/agitated or separated
	2	Reluctant to move, not bright or alert
	3	Reluctant to move even if prodded gently and depressed, hunched, eyes partly closed
	4	Moribund
Hair coat and appearance	0	Fur coat shiny and smooth, regular grooming behavior
	1	Reduced grooming behavior, fur coat dry, beginning to huddle
	2	Huddled, fur coat rough, dry, and stands up
	3	Huddled, ungroomed, severe piloerection observed
Vocalization	0	No vocalization
	1	Presence of vocalization

Table 3.4 Sequences for primers used for gene expression

Target gene	**Forward**	**Reverse**
Ifnγ	ACGGCACAGTCATTGAAAGC	TCTGGCTCTGCAGGATTTTCA
Il4	CAGCAACGAAGAACACCACAG	GGCATCGAAAAGCCCGAAAG
Il22	TGACACTTGTGCGATCTCTGA	CTTGCACCGGGTGTTGACG
Kc	AACCGAAGTCATAGCCACAC	CGTTACTTGGGGACACCTTT
Tgfβ	GTCCAAACTAAGGCTCGCCA	CATAGTAGTCCGCTTCGGGC
Tlr4	GGCAACTTGGACCTGAGGAG	TTCCTTCTGCCCGGTAAGGT
Tnfα	GATCGGTCCCCAAAGGGATG	GCTCCTCCACTTGGTGGTTT
Hprt	ACAGGCCAGACTTTGTTGGAT	ACTTGCGCTCATCTTAGGCT
Gusβ	GCTCATCTGGAATTTCGCCG	CGGTTTCGTTGGCAATCCTC
Gapdh	TACACTGAGGACCAGGTTGT	CCAGGAAATGAGCTTGACGA

Table 3.5 Frequency of presence of colonies isolated from the collectively grown inoculum of commensal strains. There were three strains that were absent when conducting the PFGE analysis.

Strain	**Frequency (expressed in %)**
1	9.9
2	8.8
3	9.9
4	4.4
5	4.4
6	2.2
7	1.1
8	5.5
9	2.2
10	13.2
11	1.1
12	9.9
13	3.3
14	5.5
15	2.2
16	7.7
17	8.8
18	0
19	0
20	0

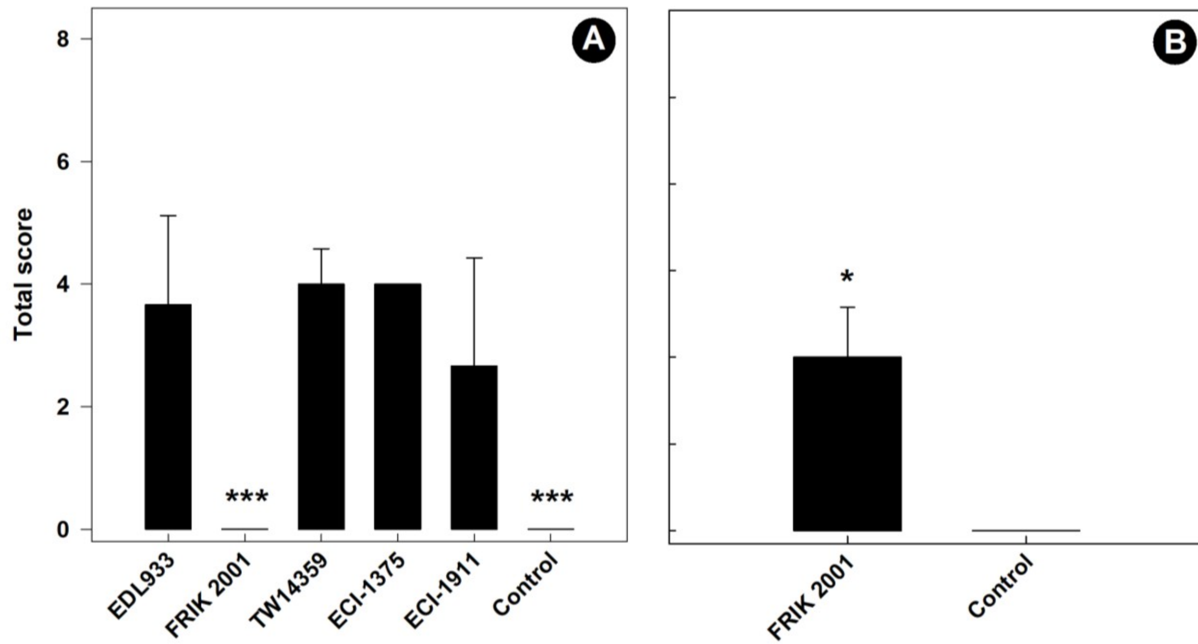

Figure 3.1 Health assessment scores between five EHEC strains (A) Health assessment score comparisons between all treatments at day 5 (B) Health assessment score comparison between control treatment (PBS) and FRIK 2001 at day 10. Histogram bars with asterisks indicate differences (*P<0.050, **P<0.010, ***P<0.001) between treatments.

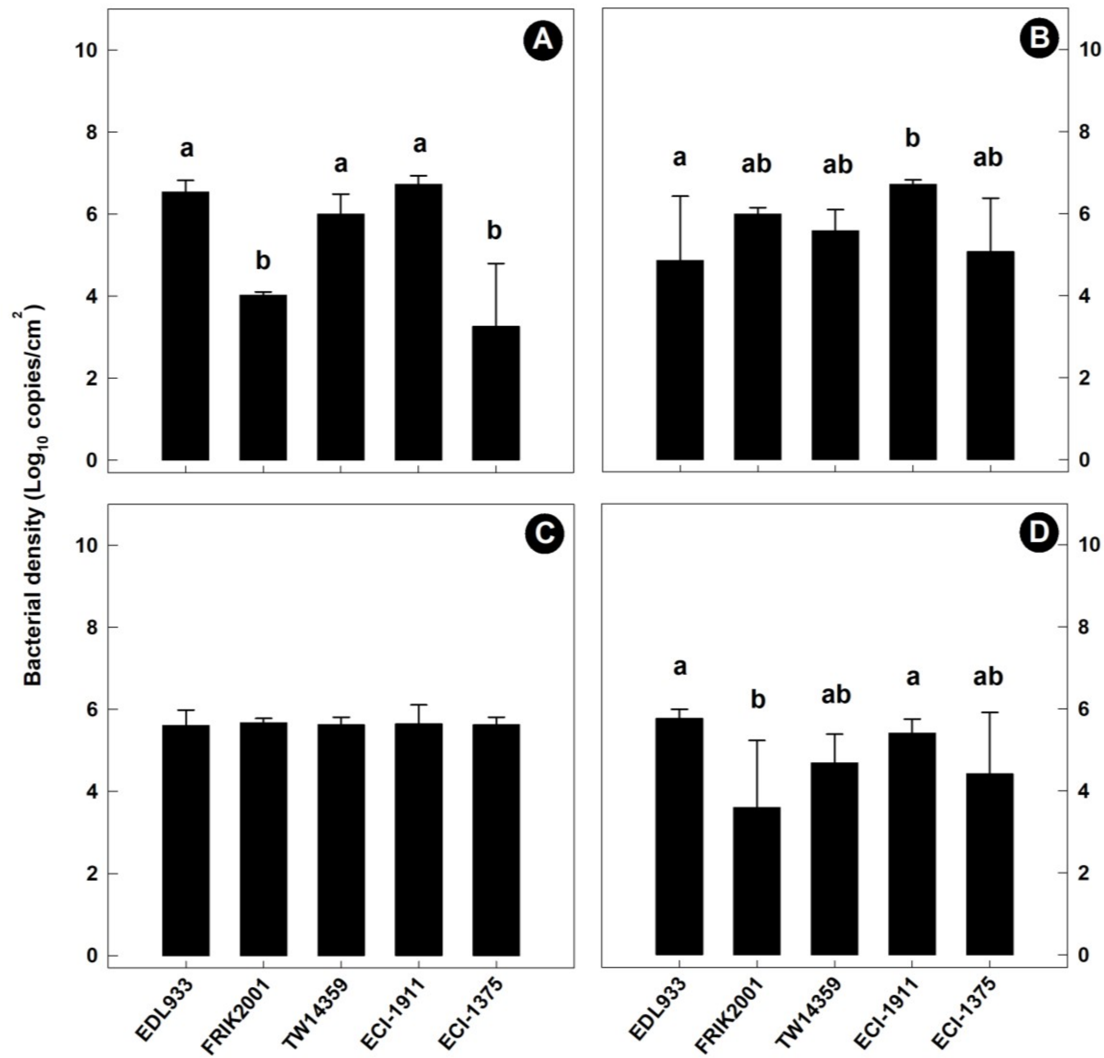

Figure 3.2 Mucosal bacterial densities associated with mucosa (cm^2) at day 5 (A) Ileum (B) Cecum (C) Proximal colon (D) Distal colon. Each strain presented in this graph was individually inoculated into mice. Histogram bars not indicated with the same letter differ in densities ($P \leq 0.050$). Control mice (not shown) presented no bacterial densities associated with the mucosa.

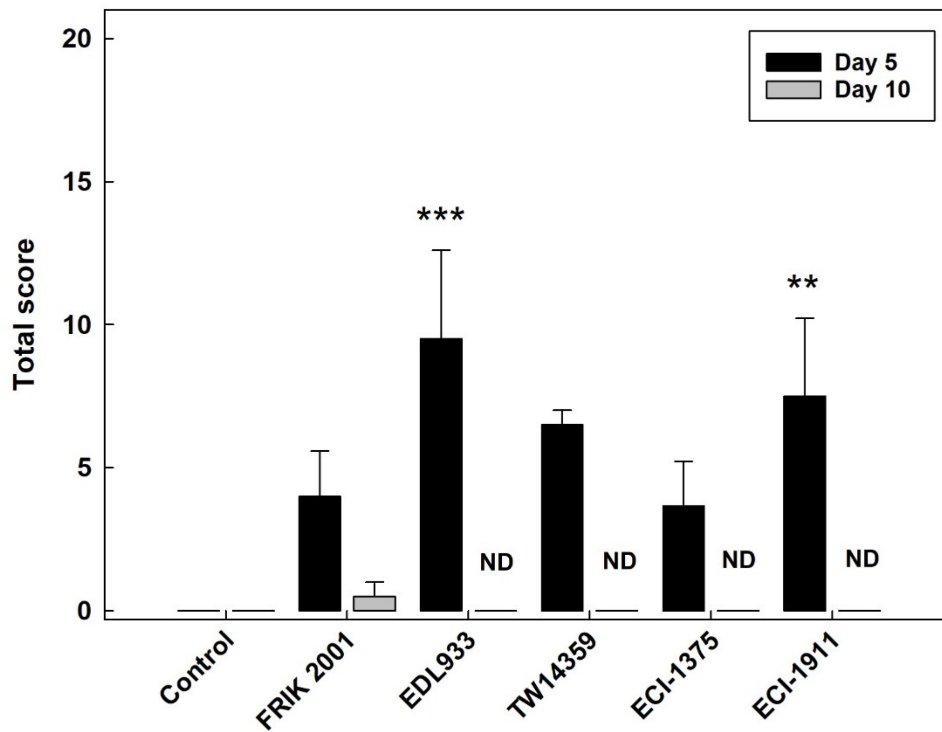

Figure 3.3 Total histopathological score comparison in the distal colon at day 5 and 10 p.i. EDL933 and ECI-1911 exhibited higher total score than FRIK 2001 (P=0.001 and P=0.01). Scores of mice inoculated with FRIK 2001 at day 10 were lower than at day 5, no statistical difference was found, but a trend was observed (p=0.07). The remaining four strains could not be analyzed at day 10 p.i. Histogram bars with asterisks indicate differences (*P<0.050, **P<0.010, ***P<0.001) between treatments.

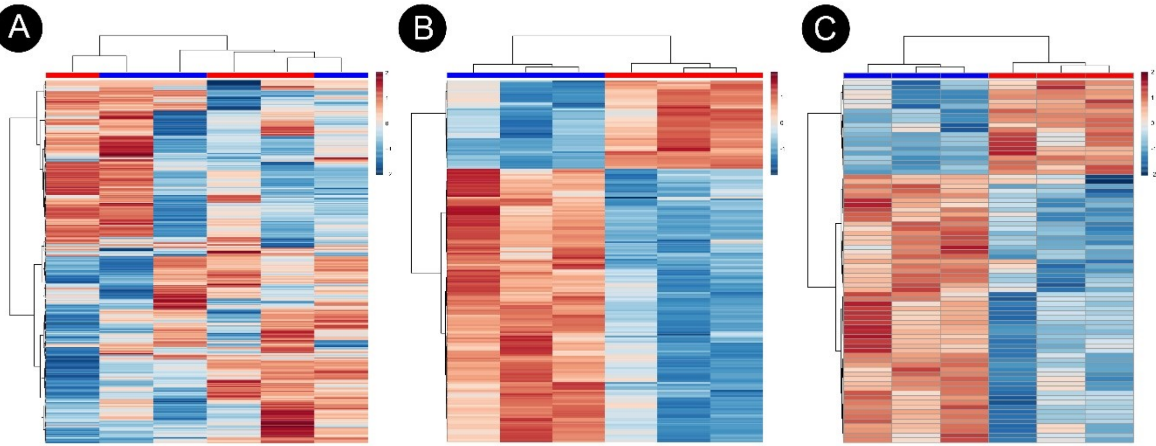

Figure 3.4 Heat maps for (A) Strain FRIK 2001 (red) vs control (blue) at 5 days p.i. in kidney, (B) Strain FRIK 2001 (blue) vs control (red) at 10 days p.i in kidney and (C) Strain FRIK 2001 at 5 days p.i. (red) vs 10 days p.i. (blue) in kidney. The heat maps visually indicate whether a bin was up- or down- regulated in each group. The dendrogram at the top of each heat map illustrates the results of the unsupervised hierarchical clustering analysis.

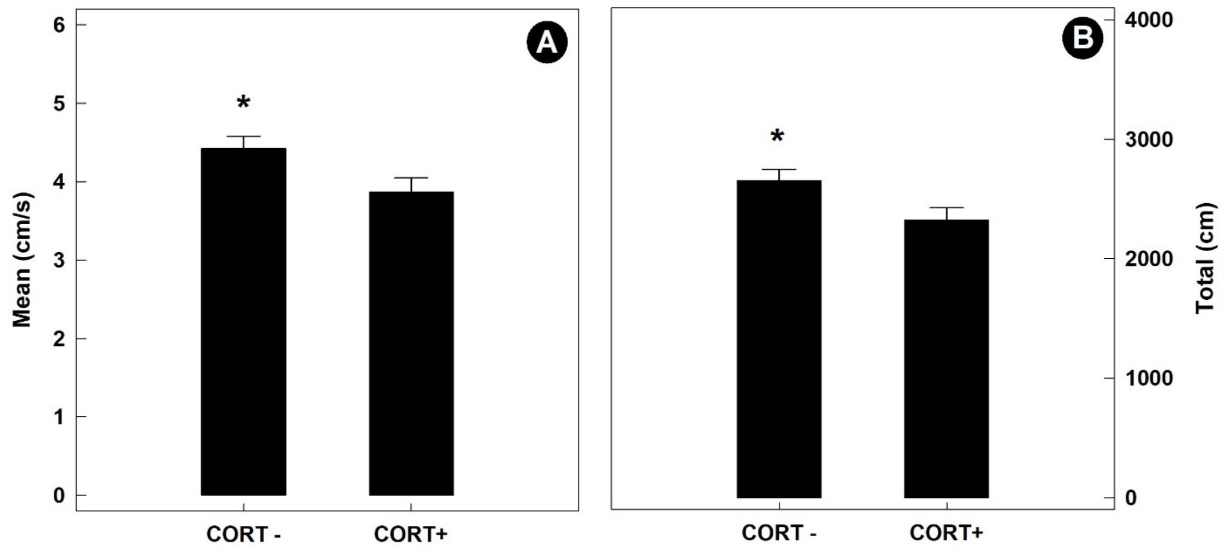

Figure 3.5 Results of a 10 min open field test after a 9-day CORT + or CORT - treatment in drinking water. (A) Mean velocity of mice during the entire 10 min open field test (*p*=0.012) (B) Total distance travelled in a 10 min open field test (*p*=0.02). Histogram bars with asterisks indicate differences (*P<0.050, **P<0.010, ***P<0.001) between treatments.

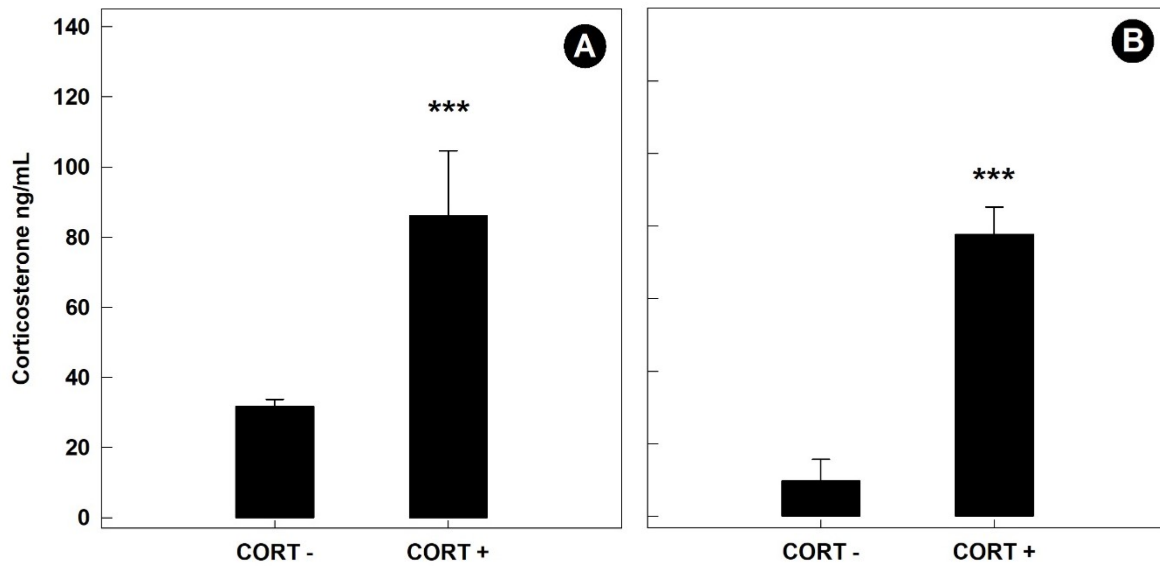

Figure 3.6 ELISA corticosterone concentration measurements following a 9-day CORT + or CORT - treatment in drinking water. Bacterial treatments were combined into CORT + or CORT - (A) Serum (B) Feces. Histogram bars with asterisks indicate differences (*P<0.050, **P<0.010, ***P<0.001) between treatments.

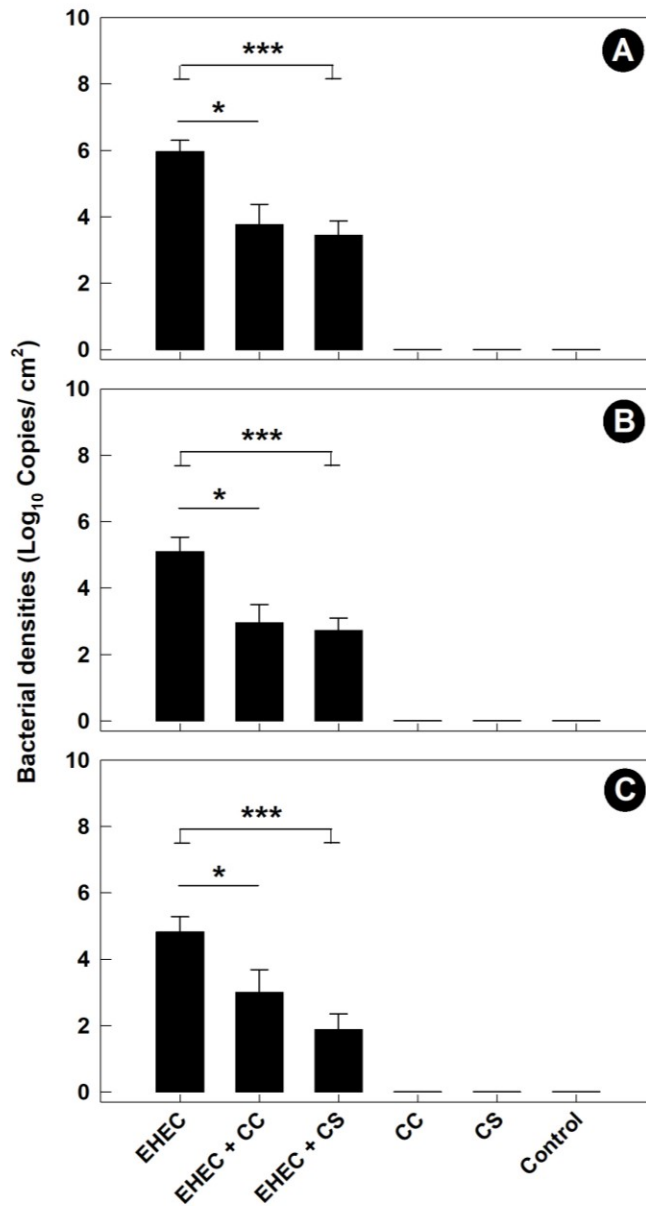

Figure 3.7 EHEC mucosal densities in (A) caecum, (B) proximal colon and (C) distal colon quantified with Real Time qPCR with specific primers for EHEC (FRIK 2001) at day 3 p.i. Significant differences were observed in all three locations. (A) p=0.017; p<0.01 (B) p=0.015; p<0.01 (C) p=0.05; p<0.01. The graph depicts combined CORT treatments since no CORT difference was found. As expected no presence of EHEC was found in CC, CS and control mice. Histogram bars with asterisks indicate differences (*P<0.050, **P<0.010, ***P<0.001) between treatments.

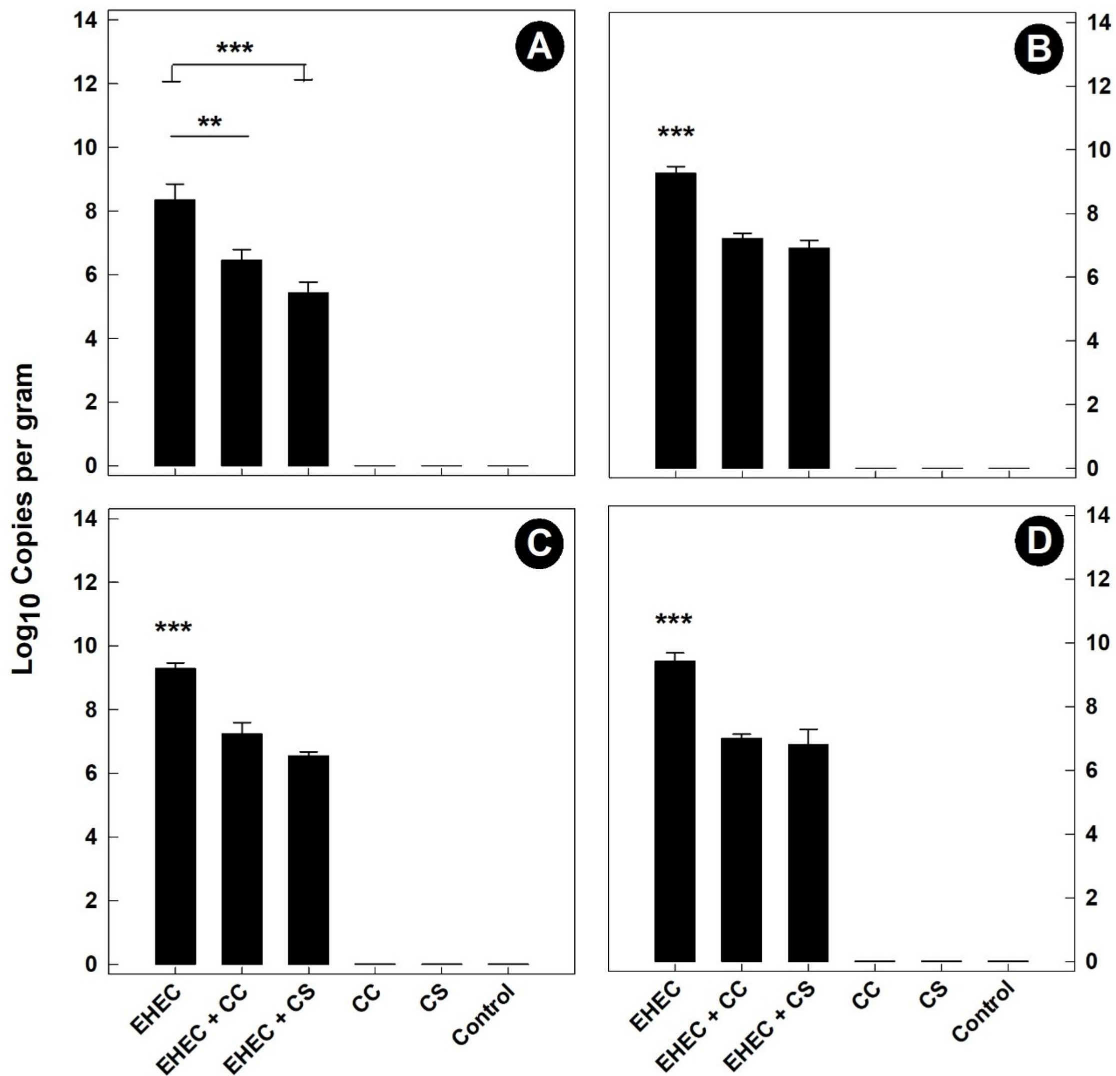

Figure 3.8 EHEC ingesta densities in (A) Ileum, (B) caecum, (C) proximal colon and (D) distal colon quantified with Real Time qPCR with specific primers for EHEC (FRIK 2001). Significant differences were observed in all four locations. The graph depicts only bacterial treatments since there was no difference in CORT treatment. As expected no presence of EHEC was found in treatment CC, CS and control mice. Histogram bars with asterisks indicate differences (*P<0.050, **P<0.010, ***P<0.001) between treatments.

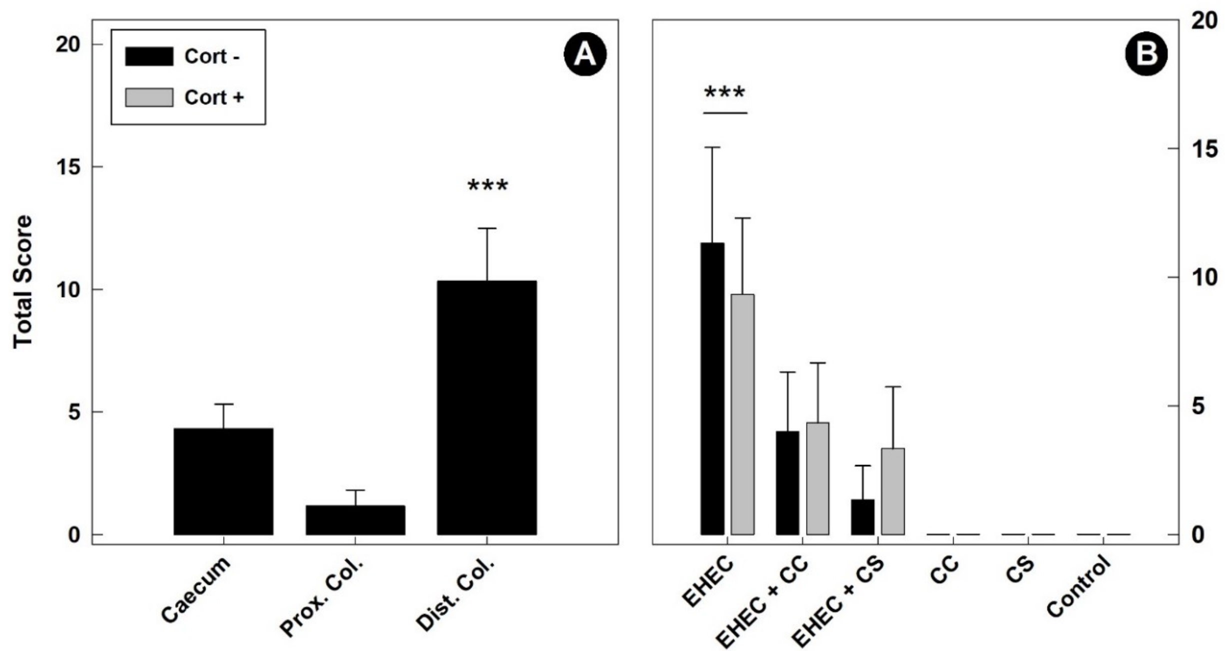

Figure 3.9 (A) Total histopathological score comparison in large intestine at day 3 (B) total histopathological score comparison between treatments in the distal colon at day 3. EHEC exhibited higher total score than EHEC+CC and EHEC+CS (*p*≤0.003). No differences were found between corticosterone treatments. Histogram bars with asterisks indicate differences (*P<0.050, **P<0.010, ***P<0.001) between treatments.

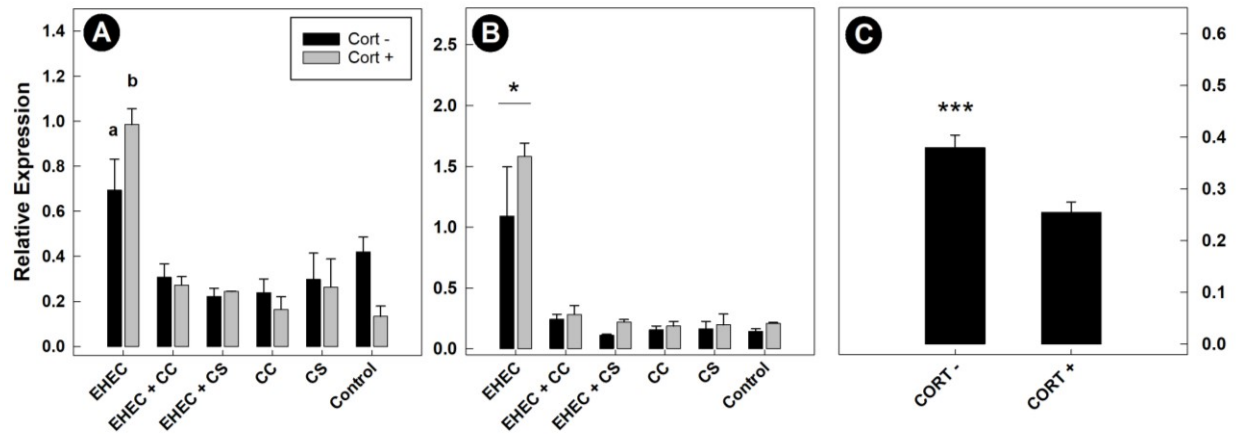

Figure 3.10 Relative mRNA gene expression in distal colon tissue of GB mice (A) *Tnfα* expression (B) *Kc* expression (C) *Tgfβ* expression comparing CORT – with CORT + treatments.

3.6 References

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Chapter 4: General conclusions and future research

4.1.1 General conclusions

Escherichia coli O157:H7 was first recognized as a human pathogen in 1982 (Kaper *et al.*, 2014). It is estimated that shiga toxin-producing *E. coli* is responsible for 2,801,000 acute illnesses, 3,890 cases of HUS, 270 cases of permanent end stage renal disease, and 230 deaths per year worldwide (Majowicz *et al.*, 2014). Its ability to cause bloody diarrhea and HUS in humans makes this bacterium a major public health concern and currently, there are no effective treatments of HUS nor proven methods to eliminate the pathogen within its primary reservoir, cattle (Besser *et al.*, 2014). Mice have been extensively used as a model to study *E. coli* O157:H7 pathogenesis, particularly the development and impact of HUS on the host (Eaton *et al.*, 2008; Goswami *et al.*, 2015; Taguchi *et al.*, 2002; Tyler *et al.*, 2013; Wadolkowski *et al.*, 1990). Many of these studies have been conducted using a GB mouse model mainly due to the susceptibility of GB mice to the effects of Stx on the kidney (Eaton *et al.*, 2008). Furthermore, the use of GF and GB mice provides the major advantage of allowing researchers to elucidate key aspects of the microbiota-host-pathogen interactions (Al-Asmakh *et al.*, 2015). However, conducting research with GF mice in conventional facilities without introduction of bacterial contamination is challenging. Housing GF and GB mice requires specialized equipment, is very labor intensive and costly. In general, experiments using GF and GB mice are conducted within specialized isolators. However, physical separation of treatment groups is often not possible (i.e. multiple isolators are required), nor is maintaining the isolators in containment mode (inward airflow). Most, facilities are not equipped with multiple isolators or specialized GF IVC units. Thus, the development of a methodology that allows research with use of pathogens in conventional IVC equipment, yet maintaining the GF and GB status of mice would facilitate the use of this model to elucidate key aspects of the host-pathogen interaction toward development of effective mitigation strategies.

Multiple aspects of *E. coli* O157:H7 colonization in cattle remain unknown. The ability to colonize the intestine of ruminants without causing symptoms of illness is puzzling. Moreover, the precise anatomical location of colonization, the immune response generated by the host, and factors that regulate shedding of the bacterium are not fully understood (Munns *et al.*, 2015; Williams *et al.*, 2014). In addition, presently, there are no definitive prevention strategies for eliminating EHEC in its natural bovine reservoir. Mice have been utilized as models to study *E. coli* O157:H7 infection and the induction of disease within the host. Mice are not only a cost effective alternative to cattle, the genetic status and physiological processes of the animals, as well as the composition of the enteric microbiota can be

controlled. Thus, the use of murine models are ideal for elucidating mechanisms involved in disease and colonization. However, the vast majority of research conducted with mice and *E. coli* O157:H7 to date targets the pathophysiology of disease in humans (Eaton *et al.*, 2008; Goswami *et al.*, 2015; Nagano *et al.*, 2003; Taguchi *et al.*, 2002; Tyler *et al.*, 2013; Wadolkowski *et al.*, 1990). Very little research has been performed in mice with an understanding of the role of cattle as a reservoir of *E. coli* O157:H7. A model that can simulate a *E. coli* O157:H7 intestinal colonization in GB mice without the early development of HUS is still lacking. One of the aims of my research was to study the colonization of GF mice with different *E. coli* O157:H7 strains; and analyze bacterial densities, and its impacts on the host. This included examining histopathological and metabolomic changes in the host to select the appropriate strain. Indeed, the advantages of using mice as a model for *E. coli* O157:H7 in cattle may give further understanding of disease process and host carriage of the bacteria.

Competitive exclusion with commensal bacteria has been previously attempted in cattle to reduce intestinal colonization by *E. coli* O157:H7 (Schamberger *et al.*, 2004; Wilson *et al.*, 2016; T. Zhao *et al.*, 1998; T. Zhao, *et al.*, 2003). However, assessing the impact of *E. coli* O157:H7 with competing strains in the cattle is challenging on a number of fronts; including the extreme complexity of the interactions with the bacterial community present in the intestine. A simpler model devoid of microbial interference, such as a GB mouse, can be of great value when investigating mechanisms of pathogen-host interactions. Additionally, previous studies have suggested a competitive advantage when bacteria are incubated together before inoculation and competition *in vivo* (Stavric, 1992; Stavric *et al.*, 1985). The validity of this claim as well as the mechanisms involved in the competition are currently unknown. Furthermore, stress has been proposed as a possible factor that benefits *E. coli* O157:H7 colonization in the intestinal tract (Lyte *et al.*, 2011). Stress related hormones such as catecholamines can induce bacterial adherence to the mucosa (*in vitro* and *ex vivo)* and these have been found to promote activation of the TTSS consequently forming attaching effacing lesions in the host (Lyte *et al.*, 2011; Moreira *et al.*, 2010; Vlisidou *et al.*, 2004). In contrast, little is known about the effect of an another important stress hormone, glucocorticoids, on *E. coli* O157:H7 colonization and mechanisms involved in enteric disease.

The current study used a defined microbiota model to elucidate the impacts of physiological stress, experimentally induced by administering corticosterone in drinking water, and the ability of commensal *E. coli* strains to outcompete an *E. coli O157:H7* strain. Salient outcomes of this research include:

a) The establishment of a successful method to maintain GF and GB mice in conventional IVCs under containment mode (negative airflow pressure) to logistically facilitate experimentation with risk group 2 pathogens in defined microbiota models
b) Development of strain specific primers, and determination of colonization and host impacts of five *E. coli* O157:H7 strains, representing different phylogenetic lineages
c) Identification of an *E. coli* O157:H7 strain (i.e. FRIK 2001) that successfully colonizes the intestinal tract of GB and incites a mild inflammation with limited symptomatology similarly to the interaction between *E. coli* O157:H7 and beef cattle
d) Application of metabolomics to characterize the effects of *E. coli* O157:H7 on kidneys of mice, which showed that the pathologic impacts of FRIK 2001 corresponded with other health metrics
e) Establishment of a physiological stress model using GF and GB mice, and the application of behavior (e.g. open field test) and host (e.g. corticosterone concentration) metrics of stress
f) Identification of commensal *E. coli* strains of bovine origin that are able to significantly reduce *E. coli* O157:H7 densities in the intestinal tract of GB mice
g) Determination that competition between commensal *E. coli* and *E. coli* O157:H7 significantly reduced histologic changes and pro-inflammatory cytokines and chemokines in the distal colon
h) Physiological stress induction did not provide a colonization advantage to *E. coli* O157:H7
i) Determination that growing commensal *E. coli* strains together did not enhance efficacy over growing strains individually

The use of IVCs provides many advantages over germ-free isolators, including the ability to conduct studies in separate unique environments as well as allow segregation of treatments. Previous research developed techniques for managing and handling GF mice in IVC units; however, these methods utilized specialized Isocage P units specifically designed for such purposes (Hecht *et al.*, 2014; Paik *et al.*, 2015). Furthermore, the units were operated under positive airflow, opposed to negative airflow required for containment. The methodology that I developed enables researchers to safely use a conventional IVC infrastructure to conduct research with RG2 pathogens using GF and GB mice models. Furthermore, the utilization of IVCs enables longer periods between cage changes, reducing stress in the animals (Rasmussen *et al.*, 2011) and workload for researchers.

A primary goal of the current study funded by Agriculture and Agri-Food Canada was to establish and utilize a GB murine model of enteric bovine *E. coli* O157:H7 colonization. I proposed that such a model would allow the acquisition of key information on the pathogen-host-microbiota interaction

toward development of rationale-based mitigation strategies. *Escherichia coli* O157:H7 strain FRIK 2001 successfully colonized the intestinal tract of mice with no health symptoms or evidence of kidney damage at day 5 p.i. This is characteristic of *E. coli* O157:H7 colonization in cattle (Boyer *et al.*, 2011), and contrasted with other strains of *E. coli* O157:H7, which incited severe disease soon after inoculation of mice. Importantly, FRIK 2001 successfully colonized the intestinal tract at similar densities to the other *E. coli* O157:H7 strains. This is expected and in line with evidence that when devoid of the normal microbiota EHEC strains are able to colonize the intestinal tract of mice within the first day following their introduction (Eaton *et al.*, 2008). I observed that enteric inflammation incited by the FRIK 2001 strain of E. coli O157:H7 was mild, whereas other completed studies that used mice as a human model of disease reported necrotizing colitis (Eaton *et al.*, 2017). The specific cause of inflammation that was observed in mice is speculative. It has previously been shown that Stx2 is not essential to generate histological changes in the intestinal epithelium, suggesting that there are other factors involved in pathogenesis (Eaton *et al.*, 2017). It is plausible that H7 flagellin, *E. coli* pili, or other mechanisms were responsible for triggering inflammation (Eaton *et al.*, 2017). It is noteworthy that *E. coli* O157:H7 FRIK 2001 is a lineage II EHEC which differed from the lineages of the other EHEC strains examined. Lineage I and I/II strains are highly pathogenic, particularly in human beings (Zhang *et al.*, 2010). That EHEC lineage II was able to effectively colonize the intestinal tract of GB mice, but exhibit a significantly lower rate of disease development suggests that this lineage of *E. coli* O157:H7 is appropriate for bovine models.

I did not obtain any evidence to indicate that a general state of physiological stress benefitted colonization and disease in GB mice infected with *E. coli* O157:H7. It is known that stress hormones can trigger the TTSS activation and bacterial attachment to the mucosa (Chen *et al.*, 2003; Moreira *et al.*, 2010; Vlisidou *et al.*, 2004). I did not determine if corticosterone administration stimulated the production of catecholamines. Nonetheless, corticosterone is known to affect the immune system (Cain *et al.*, 2017), and such shifts in the immune response could prove to be beneficial for *E. coli* O157:H7 when attempting to colonize the intestinal epithelium. Although I observed changes in the expression of immune markers in stressed mice, I did not obtain evidence of an increase in colonization indicative that stress conferred a competition advantage in GB mice. Mice administered corticosterone did however exhibit altered behavior in an open field test as well as metabolomics changes in the kidney and liver (information not shown).

Effective mitigation strategies to reduce *E. coli* O157:H7 colonization in bovine hosts do not currently exist. Using a GB model, I observed that *E. coli* O157:H7 densities were reduced by 20 commensal strains of *E. coli.* Moreover, *E. coli* commensal strains decreased histopathologic changes caused by *E. coli* O157:H7 in the distal colon, and reduced expression of pro-inflammatory immune markers (*TNFα* and *Kc*). This suggests that the commensal *E. coli* strains reduced the ability of *E. coli* O157:H7 to interact with the intestinal epithelium, thereby reducing recognition of the pathogen-associated molecular patterns (PAMPs) resulting in decreased tissue injury and expression of inflammation. In this regard, inhibition of TNFα in mice colonized with *E. coli* O157:H7 reduced pathology and mortality (Isogai *et al.*, 1998). Furthermore, decreased expression of *Kc,* a homolog of IL-8, provides evidence for reduced recruitment of leukocytes to the intestinal mucosa. Notably, KC is linked with inducing neutrophil accumulation in the glomeruli of the kidney of mice when stimulated with *E. coli* LPS or Stx2 (Roche *et al.*, 2007).

4.1.2 Future research

The study that I completed provides insights into the direction that future research could take. In this regard, the GB model I have developed could be used to conduct studies to elucidate mechanisms towards reducing the threat posed by *E. coli* O157:H7. Work still needs to be conducted in the attempt to eliminate *E. coli* O157:H7 from the main reservoir. A combination of the knowledge of studies in cattle with that obtained in murine models can provide useful information on the colonization, survival and competition mechanisms of *E. coli* O157:H7 in its natural host.

Future work following the line of research conducted here could focus on techniques aimed at finding the specific intestinal location of FRIK 2001 in the distal colon. Specific probes for fluorescence *in situ* hybridization (FISH) can help identify attachment of the bacterium to the epithelium. Previous studies have utilized this technique to locate the pathogen's location in the intestine (Nagano *et al.*, 2003; Poulsen *et al.*, 1994); however, this has not been explored in the scenario of a bovine model of colonization or relative to competition with other strains. Also, immunofluorescence assays can be helpful to locate *E. coli* O157:H7 in the intestinal tract (Mundy *et al.*, 2006). Conducting FISH analyses is challenging, as was my experience in the current study, and transforming fluorescence into FRIK 2001 (e.g. to express green fluorescent proteins) is another option that could be applied to facilitate visualization of the bacterium *in vivo*. Additionally, the analysis of virulence genes expressed by FRIK 2001 during competition with other commensal bacteria could be of great value, specifically those related with the TTSS and flagella (Garmendia *et al.*, 2005). Contrary to my hypothesis, I did not observe

that stress induced by corticosterone was advantageous to colonization of the intestinal tract or induction of disease by FRIK 2001. Catecholamines act on the receptor of the quorum sensing molecule Autoinducer 3 to stimulate activation of virulence factors in *E. coli* O157:H7. However, the dosage of epinephrine and NE needed to stimulate these factors is currently unknown (Vlisidou *et al.*, 2004). Furthermore, measuring catecholamines in the intestine of stressed mice may provide insight on mechanisms. Also, measuring the expression of Autoinducer 3 receptor in *E. coli* O157:H7 bacteria during competition with commensal *E. coli* under stressful scenarios could shed light into the advantage conferred by stress hormones on *E. coli* O157:H7 colonization. Other methods of inducing stress on the animals can be utilized, including stressors that mimic production settings (e.g. constraint, transport). My strategy was to achieve defined and consistent physiological stress via administration of the stress hormone, corticosterone. Moreover, I chose this approach for logistical reasons; administering corticosterone in drinking water was the safest approach to not compromise the GF or GB status of the mice. Although, mimicking stress that beef cattle experience in confined feed operations is one possibility, another option would be to expose mice to behavioral stress that have been used extensively in rodent research. For example, daily forced swimming or forced restraint have been shown to induce stress in a measurable and reproducible manner (Bowers *et al.*, 2008). This being said, inducing stress in GB mice without compromising the integrity of the model is very challenging.

I evaluated and observed that twenty commensal *E. coli* strains isolated from cattle were able to reduce the densities of FRIK 2001 in the intestine, but the role that individual strains played in the competition is unknown. It is noteworthy that the commensal strains were selected based on their ability to outcompete EHEC strains in a chemostat model (Kalmokoff pers. comm.), but their efficacy *in vivo* was unknown. Moreover, I contrasted the efficacy of the commensal *E. coli* strains grown separately and grown together based on a previous observation of increased efficacy against *S. enterica* for the bacteria grown communally (Stavric *et al.*, 1985). For strains grown communally, I subtyped isolates to ascertain the degree to which competition occurred among strains during communal growth, and observed that the majority of strains were conserved in the inoculum a prevalence of strain recovery ranging from 1.1% to 13.9%. A limitation of my research is that neither the location nor abundance of individual commensal strains was measured. Developing specific genetic markers for each strain to measure densities and target specific locations would be beneficial (e.g. by qPCR and FISH). In this regard, samples for quantifying/visualizing commensal strains were collected, and archived. The genomes of all 20 commensal *E. coli* strains are in the process of being sequenced at the National

Microbiology Laboratory in Winnipeg, and the sequence data will be subjected to comparative whole genome sequence analysis to identify strain-specific markers (i.e. using the same bioinformatics methods applied to EHEC strains in the current study). Additionally, the development and use of strain specific markers for commensals and FRIK 2001 will allow me to ascertain locations co-colonized toward determining whether commensals and EHEC compete for the same ecological niche. This could be augmented with *in vivo* models and metrics, such as artificial intestine model with metabolomics to gain information on niches for these bacteria. These models and the information obtained would be expected to facilitate the rationale-based selection of highly competitive commensal strains.

A potential criticism of my research may be that mice are not directly representative of cattle. As indicated previously in my thesis, mice possess many advantages over cattle to elucidate mechanisms toward the identification of mitigation strategies, which would then be evaluated in young ruminants. Significantly, the lack of a normal microbiota in the GB mice provides a strong advantage to study host-microbiota-pathogen interactions and to elucidate key mechanisms, and the use of this model has been extensively used as a model for human beings. I believe that the GB mouse model is equally useful to elucidate mechanisms that are applicable to cattle, and the use of this model will expedite the development of innovations for the sector. Extensive research has been placed in the competition of *E. coli* O157:H7 with other bacteria in mice, claiming that the competitive advantage of the bacteria relies on a nutritional advantage, the production of bacteriocins or short chain fatty acids (Gamage *et al.*, 2006; Leatham *et al.*, 2009; Maltby *et al.*, 2013; Miranda *et al.*, 2004; Takahashi *et al.*, 2004). Yet, the exact mechanisms of interaction taking place during these specific competitions are not entirely clear and further exploration is needed. The GB model used herein possesses characteristics that facilitate the elucidation of mechanisms of competitive colonization and exclusion. In this regard, these mice can be used to develop a defined enteric microbiota model. For example, human flora mice models have been established (Gaboriau-Routhiau *et al.*, 2003) and it would be possible to establish the bovine colonic microbiota in GB mice to emulate the distal colon environment of cattle. Competition between FRIK 2001 and autochthonous commensal bacteria could be studied in this model in a cost effective manner. Multiple studies have utilized streptomycin-treated normal flora mouse models to study *E. coli* O157:H7 colonization and the interaction with the host microbiota from a human health perspective (Fabich *et al.*, 2008; Maltby *et al.*, 2013; Miranda *et al.*, 2004; Wadolkowski *et al.*, 1990). However, this is a dysbiosis microbiota model that is not representative of a normal enteric microbiota (i.e. a eubiosis

situation). Thus, the use of a bovine flora mice model would be more representative than the streptomycin dysbiosis model facilitating the study EHEC-bovine-microbiota interactions.

I believe that the model developed and the research presented in this thesis on the competitive exclusion of *E. coli* O157:H7 is the first step toward developing effective mitigation of this important pathogen for the beef cattle sector in Alberta and elsewhere. In this regard, my research used a GB mouse model to ascertain mechanisms. The model I developed could be used to further explore stress on the host-pathogen interaction. Research could then use a bovine flora mouse model to validate and expand upon my findings (e.g. identification of bacteria that colonize enteric niches currently occupied by EHEC), with subsequent validation in ruminant models (e.g. sheep), and eventually in cattle. Similarly, host immune metrics identified in my research (e.g. relevant targets and markers) will advance research in more complex models, such as cattle.

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