MECHANISMS OF COLONIZATION RESISTANCE TO *SHIGELLA FLEXNERI*

by

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABSTRACT

Shigella flexneri is an enteric bacterial pathogen responsible for shigellosis. Components of the commensal microbiota promote colonization resistance in mice and antibiotic treatment is required for susceptibility to *S. flexneri* infection. The mechanisms of resistance to colonization by *S. flexneri* have not been defined in the streptomycin-treated mouse model of infection. We hypothesize that a consortium of anaerobes cultured from healthy mice and serially passaged to select for streptomycin-resistant bacteria can confer protection against *S. flexneri* infection in streptomycin-treated mice. A close relative of *Shigella, E. coli,* uses nitrogen respiration to overcome colonization resistance in the streptomycin-treated mouse. We hypothesize that *S. flexneri* similarly uses nitrogen respiration to overcome colonization resistance and that a mutant for nitrate reductase activity is impaired in the ability to colonize streptomycin-treated mice. Intracellular carbon metabolism through the phosphotransacetylase-acetate kinase pathway has been demonstrated by *S. flexneri* during *in vitro* infection of cells in tissue culture. We hypothesize that this form of intracellular carbon metabolism also contributes to the ability of *S. flexneri* to colonize.

Consortia of commensal bacteria were cultured from healthy mice under selective conditions and characterized by 16S rRNA sequencing. Proton Nuclear Magnetic Resonance Spectroscopy was used to demonstrate the production of a short-chain fatty acid by the cultured bacteria that has previously been implicated in counteracting *Shigella*-mediated virulence. The cultured consortia of bacteria did not confer protection in the murine model of *S. flexneri* infection, but the selective culturing method repeatedly enriched for specific genera of bacteria and yielded 16S rRNA profiles distinct from the gut of the healthy mouse.

Nitrogen respiration and carbon metabolism pathways were investigated as potential mechanisms used by *S. flexneri* to promote colonization. The importance of the phosphotransacetylase-acetate kinase carbon metabolism pathway in intracellular proliferation of *S. flexneri* was confirmed *in vitro*, while nitrate reductase activity was dispensable for colonization of streptomycin-treated mice. By addressing the role of commensal bacteria, as well as nitrogen respiration and intracellular carbon metabolism, this work advances our understanding of the factors allowing colonization by *S. flexneri* and sets the stage for future study.

LIST OF ABBREVIATIONS USED

^{1}H NMR	Proton Nuclear Magnetic Resonance Spectroscopy
aceE	Pyruvate Dehydrogenase
Acetyl-CoA	Acetyl Coenzyme A
ACKA	Acetate Kinase
AcP	Acetyl Phosphate
ADP	Adenosine Diphosphate
AfaE	Afimbrial Adhesin E
AMPs	Antimicrobial Peptides
ANOVA	Analysis of Variance
ATP	Adenosine Triphosphate
BLAST	Basic Local Alignment Search Tool
bp	Base Pairs
C. acidotolerans	*Clostridium acidotolerans*
C. nitrophenolicum	*Clostridium nitrophenolicum*
$CaCl_2$	Calcium Chloride
CFU	Colony Forming Units
CGEB	Comparative Genomics and Evolutionary Bioinformatics
CI	Competitive Index
CO_2	Carbon Dioxide
D_2O	Deuterium Oxide
DH5α	Doug Hanahan Strain 5α
DMEM	Dulbecco's Modified Eagle Medium
DNA	Deoxyribonucleic Acid
DSS	Dextran Sodium Sulfate
E. coli	*Escherichia coli*
ED	Enter-Doudoroff
EDTA	Ethylenediaminetetraacetic acid
EG	Eggerth-Gagnon
EHEC	Enterohaemorrhagic *E. coli*
EMP	Embden-Meyerhof-Parnas
FBS	Fetal Bovine Serum
FLP	Flippase Recombinase
FRT	FLP Recognition Target
GI	Gastrointestinal
GLP-1	Glucagon-like Peptide 1
GPCR	G-protein-coupled Receptor
hBD-1	Human β-defensin-1
hCAP	Human Cationic Antimicrobial Protein
HDAC	Histone Deacetylase
IBD	Inflammatory Bowel Disease
IgA	Immunoglobulin A
IgE	Immunoglobulin E
IMR	Integrated Microbiome Resource
iNOS	Inducible Nitric Oxide Synthase

IpaH	Invasion Plasmid Antigen H
KH_2PO_4	Potassium Dihydrogen Phosphate
LB	Luria Bertani
MCT-1	Monocarboxylate Transporter 1
MET-1	Microbial Ecosystem Therapeutic-1
mg	milligram
$MgSO_4$	Magnesium sulfate
MHz	megaherz
mL	milliliter
mM	millimolar
MOI	Multiplicity of Infection
MOPS	3-morpholinopropane-1-sulfonic Acid
Na_2HPO_4	Sodim Phosphate Dibasic
NaCl	Sodium Chloride
NADH	Nicotinamide Adenine Dinucleotide Hydrogen
NaOH	Sodium hydroxide
NCBI	National Center for Biotechnology
NH_4Cl	Ammonium chloride
NK-κB	Nuclear Factor-kappa B
NOD	Nucleotide Oligomerization Domain
NOS	Nitric Oxide Synthase
NOS1	Neuronal Nitric Oxide Synthase
NOS2	Nitric Oxide Synthase Type 2
NOS3	Endothelial Nitric Oxide Synthase
ORF	Open Reading Frame
OTU	Operational Taxonomic Unit
PAMPS	Pathogen Associated Molecular Patterns
PBS	Phosphate Buffered Saline
PCA	Principal Component Analysis
PCR	Polymerase Chain Reaction
PICRUST	Phylogenetic Investigation of Communities by Reconstruction of Unobserved States
PP	Pentose Phosphate
ppm	Parts Per Million
PTA	Phosphotransacetylase
PYY	Peptide YY
RNA	Ribonucleic Acid
RNI	Reactive Nitrogen Intermediates
RNS	Reactive Nitrogen Species
ROI	Reactive Oxygen Intermediates
ROS	Reactive Oxygen Species
rRNA	Ribosomal Ribonucleic Acid
S. boydii	*Shigella boydii*
S. dysenteriae	*Shigella dysenteriae*
S. flexneri	*Shigella flexneri*
S. sonnei	*Shigella sonnei*

SCFA	Short-chain Fatty Acid
SD	Standard Deviation
sIgA	Secretory Immunoglobulin A
SMCT-1	Sodium-Coupled Monocarboxylate Transporter-1
spp	Species
T3SS	Type III Secretion System
TCA	Tricarboxylic Acid
T_H1	Type 1 helper cells
T_H2	Type 2 helper cells
TLR	Toll-like Receptor
T_{reg}	Regulatory T Cells
TSB	Trypticase Soy Broth
UI	Uninoculated
UV	Ultra Violet
V. cholera	*Vibrio cholerae*
VRE	Vancomycin Resistant *Enterococcus*
WHO	World Health organization
WT	Wild type
µF	microfarad
µg	microgram
µL	microliter
µM	micromolar

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Chapter 1: Introduction

1.1: *Shigella* spp.

1.1.1: Epidemiology

Diarrheal diseases caused by enteric infection continue to place a strenuous burden on global health. One of the leading contributors to diarrheal disease is *Shigella* spp., a Gram-negative bacterium that accounts for approximately 188 million cases and 64,993 deaths annually, according to a recent estimate by the World Health Organization (WHO) (Pires *et al*., 2015). *Shigella* spp. cause a severe diarrheal disease known as shigellosis. Children under five in developing countries are most susceptible to shigellosis (Kotloff *et al*., 1999; Schroeder and Hilbi, 2008). Despite considerable efforts, there is no licensed vaccine for shigellosis and the development of a suitable candidate antigen is complicated by the diversity of antigens across the multiple serotypes of bacteria (Livio *et al*., 2014).

Shigella serotypes are divided into four species including *S. dysenteriae, S. boydii, S. sonnei, and S. flexneri. S. dysenteriae* produces Shiga toxin and is responsible for multiple pandemics spanning Central America, South Asia, and Central and East Africa from the 1970s to the 1990s (Levine *et al*., 2007). *S. boydii* is endemic in certain developing countries but is generally restricted to Southern Asia (Levine *et al*., 2007; von Seidlein *et al*., 2006). Presently, *S. dysenteriae* and *S. boydii* are less frequently isolated, while the remaining two subgroups show continued prevalence on a global scale (Kotloff *et al*., 1999; Thompson *et al*., 2015). The Shiga toxin-producing *S. dysenteriae* is generally associated with large outbreaks, (for example in refugee camps) (Kerneis *et al*., 2009). Traditionally, *S. sonnei* infection correlated with high-income regions of the world

but recent evidence demonstrates its emergence in developing countries where *S. flexneri* is most prevalent (Thompson *et al*., 2015). *S. flexneri* is the most commonly used laboratory strain and is the focus of this thesis.

In recent decades, *Shigella* has acquired plasmid-encoded resistance to the antibiotics previously constituting the first-line of treatment for shigellosis, including sulfonamides, tetracycline, ampicillin, and trimethoprim-sulfamethoxazole (Kotloff *et al*., 1999). More recent reports document resistance to second-line therapies including the quinolones and third generation cephalosporin (Gu *et al*., 2012; Vinh *et al*., 2009). In 2006, the WHO recommended Ciprofloxacin, a fluoroquinolone, as the new first-line of treatment against *Shigella* infection but Ciprofloxacin resistant isolates were detected not long after (von Seidlein *et al*., 2006). Continuous emergence of antimicrobial resistance among the *Shigella* spp. represents an ongoing challenge and continues to drive the need for research in the areas of vaccine development and pathogenesis to address remaining knowledge gaps that may inform prevention efforts.

1.1.2: Pathogenesis

Shigella is a non-motile bacillus of the *Enterobacteriacea* family that has evolved as a human specific pathogen, although certain primates are also known to be reservoirs (WHO, 2005). In susceptible hosts, *Shigella* causes a disease known as bacillary dysentery, or shigellosis, that is characterized by abdominal cramps, fever, and mucopurulent stool with or without the presence of blood (Levine *et al*., 2007; Phalipon and Sansonetti, 2007; WHO, 2005). Spread by the fecal-oral route, *Shigella* causes disease by transmission of as few as 10 organisms in contaminated food or water (Schroeder and Hilbi, 2008). In the gastrointestinal (GI) tract, *Shigella* uses transcytosis

through specialized epithelial cells known as M cells to bypass the epithelial cell barrier and access the sub-epithelial layer (Mounier *et al*., 1992; Sansonetti and Phalipon, 1999). Once the mucosal barrier is breached, *Shigella* induces programmed cell death in both dendritic cells and macrophages in the sub-epithelial space and invades epithelial cells through the basolateral membrane (Edgeworth *et al*., 2002; Fink and Cookson, 2005; Phalipon and Sansonetti, 2007). Throughout these processes, *Shigella* triggers the release of many proinflammatory factors including interleukin-1β and interleukin-18 from macrophages (Schroeder and Hilbi, 2008), as well as interleukin-8 and tumor necrosis factor α from epithelial cells. These proinflammatory factors result in inflammation and recruitment of neutrophils to the site of infection. While neutrophils may act to resolve infection by trapping and killing bacteria, they can also further the inflammatory process through the release of cytokines and chemokines, proteases, reactive oxidants, as well as prostaglandins and leukotrienes (Sansonetti and Phalipon, 1999; Wright *et al*., 2010). Granule structures within neutrophils store and release lytic enzymes and bactericidal substances such as peroxidase, lysozyme, and hydrolytic enzymes that are active in bacterial killing but can also damage host tissue (Borregaard and Cowland, 1997; Wright *et al*., 2010). These events culminate in the destabilization of and extensive damage to the epithelial cell barrier, facilitating entry for additional infective *Shigella* and prolonging the inflammatory environment characteristic of *Shigella* colonization (Phalipon and Sansonetti, 2007).

Following entry into epithelial cells, *Shigella* ruptures the phagocytic vacuole, replicates in the host cytoplasm, and uses actin-based motility to spread to adjacent cells in the mucosal membrane (Bernardini *et al*., 1989; Schroeder and Hilbi, 2008). For

successful pathogenesis, *Shigella* relies on specific factors with specialized functions encoded on its large virulence plasmid (Sansonetti *et al*., 1982). The 200 kb plasmid is among the main features distinguishing *Shigella* from closely related *Escherichia coli* (Schroeder and Hilbi, 2008). Approximately 100 genes are encoded on the virulence plasmid including translocators and substrates of *Shigella'*s Type III Secretion System (T3SS), regulators, chaperones, and the T3SS machinery itself (Schroeder and Hilbi, 2008). The design of the T3SS allows for elegant delivery of protein effectors into the host cell cytoplasm in a tightly regulated fashion (Buchrieser *et al*., 2000; Le Gall *et al*., 2005; Mavris *et al*., 2002).

The first step in regulation of *Shigella'*s T3SS occurs in a temperature-dependent manner. At 37° C, assembly of the T3SS occurs but the system remains inactive with transcription of the first wave of effectors occurring to produce secretion-competent forms of proteins in complex with their respective chaperones (Parsot, 2009). Upon contact with host cells, the T3SS is activated, a first "wave" of effectors are released that mediate entry of the bacterium, and the transcription of a second wave of effectors is induced (Parsot, 2009; Schroeder and Hilbi, 2008). *Shigella* genes that encode second wave effectors have a 17 bp motif in their promoters that is recognized by MxiE, an AraC-class transcriptional activator (Le Gall *et al*., 2005). During secretion conditions, translocators known as IpaB and IpaC are secreted from the cytoplasm to liberate their chaperone, IpgC. The IpgC chaperone then acts as a co-activator to turn on MxiE activity through a mechanism that is not fully understood (Le Gall *et al*., 2005). Following transcriptional activation by MxiE, second wave effectors mediate post-invasion virulence functions such as modulation of the host immune response (Schroeder and

Hilbi, 2008). While several genes have been well characterized in *Shigella* virulence, the role of many plasmid-encoded genes remains unknown.

1.1.3: MxiE-Mediated Downregulation of Host AMPs

MxiE-dependent effectors have been implicated in down regulating a critical component of host innate immunity. Antimicrobial peptides (AMPs) are small, generally cationic peptides conserved among most organisms that have direct and indirect roles in antimicrobial defense (Bahar and Ren, 2013; Wassing *et al*., 2015). AMPs are expressed at high levels at mucosal surfaces and are synthesized by epithelial cells as well as immune cells including phagocytes and lymphocytes (Bahar and Ren, 2013). Two families, the defensins and the cathelicidins, comprise the two main classes of AMPs in humans (Ganz, 2003; Zanetti, 2004). In biopsies from patients with shigellosis as well as in epithelial and monocyte tissue culture models, downregulation of the human AMPs human cationic antimicrobial protein (hCAP or LL-37) and human β-defensin-1 (hBD-1) has been documented (Islam *et al*., 2001). More recent work has attributed this phenomenon to transcriptional suppression of several cationic AMPs by the MxiE-dependent repertoire of effectors in virulent *Shigella* (Schroeder and Hilbi, 2008; Sperandio *et al*., 2008).

1.1.4: *Shigella* Effector IpaH9.8

The IpaHs (invasion plasmid antigen H) are a family of closely related effector proteins that interfere with host cell processes and whose expression is regulated by MxiE. *Shigella* possesses 12 IpaHs with some encoded on the virulence plasmid and others representing the only chromosomally encoded T3SS substrates (Schroeder and Hilbi, 2008). The IpaHs are E3 ubiquitin ligases with homologs among many plant and

animal pathogens (Rohde *et al*., 2007). The IpaHs contain a variable N-terminal domain harboring a series of leucine-rich repeats and a highly conserved C-terminal domain shared among all of the IpaHs (Tanner *et al*., 2015). Functional E3 ligase activity is dependent on a catalytic Cys residue within the C-terminal domain that mediates transfer of ubiquitin, a small protein with a central role in eukaryotic cell signaling, to substrate targets within the host cell (Rohde *et al*., 2007). Targets may be mono- or poly-ubiquitinated with the nature of the ubiquitin linkage dictating the fate of ubiquitinated proteins. Polyubiquitination with lysine 48-linked chains is associated with degradation of targets by the proteasome, while lysine 63-linked chains are involved in non-proteolytic processes including protein trafficking, DNA repair, and inflammation (Xu *et al*., 2009). Through interference with the host ubiquitin signaling system, the IpaHs are believed to target host factors that regulate inflammation (Tanner *et al*., 2015). While a handful of substrates have been identified for the IpaHs, the search for remaining substrates continues and ongoing research aims to understand the precise host cell pathways subverted.

Recent work by a previous Master's student in the Rohde Lab, Kaitlyn Tanner, identified the host cell factor ZKSCAN3, a negative regulator of autophagy, as a target of the *Shigella* effector IpaH9.8. Autophagy is a catabolic process of tightly regulated self-degradation of organelles or cytosolic components via formation of a double-membrane autophagosome (He and Klionsky, 2009). Following its formation, the autophagosome fuses with hydrolase-containing lysosomes or vacuoles and the enclosed material is degraded. Autophagy is commonly upregulated in response to cellular stresses such as starvation and serves to replenish the cell with nutrients including amino acids and other

small molecules (He and Klionsky, 2009). Kaitlyn's work proposes a mechanism whereby IpaH9.8 is secreted into the host cell cytoplasm by *Shigella's* T3SS, where it upregulates autophagy through negative regulation of ZKSCAN3 to acquire nutrients for intracellular growth (Tanner, 2014).

1.2: Animal Infection Models

The current body of knowledge surrounding *Shigella* pathogenesis has depended on decades of research using tissue culture models. While informative, many of the findings derived from *in vitro* studies remain to be validated *in vivo.* The development of an effective animal model for shigellosis has been confounded by the specificity of *Shigella* as a human pathogen and the differences in host response to infection between humans and animal models. First used in 1955, the Sereny test is the oldest animal model of shigellosis (Sereny, 1955). The sereny test is an assay that tests the ability of *Shigella* to invade mucosal epithelial cells in the keratoconjuctival sac surrounding the mouse or guinea-pig eye. While this assay allows avirulent mutant *Shigella* to be identified, it fails to distinguish between defects in epithelial cell invasion, cell-to-cell spread, or initiation of inflammation, due to a lack of specificity in the response (Philpott *et al*., 2000).

Another model that has been used to study *Shigella* is the rabbit ligated ileal loop model. In this model, a surgical incision is made in the abdominal wall of anaesthetized animals and sections of the ileum are ligated to preserve vasculature and introduce an ileal loop that is injected with a high inoculation of bacteria (Philpott *et al*., 2000). Upon euthanasia, the infected loop can be examined for bacterial invasion, inflammation, and fluid accumulation. While the rabbit ileal loop model has helped inform certain aspects of *Shigella* research (Wassef *et al*., 1989), its use has been restricted to select labs and has

not been widely taught or practiced. The high level of training required and the invasive nature of the model render it impractical for widespread use. Another major drawback of using rabbits in *Shigella* studies is the lack of dysenteric symptoms in response to oral or gastric inoculation. Since *Shigella* targets the distal colon in the natural human host, studies using the rabbit ileal loop model do not accurately reflect the tissue specificity during the host-pathogen interaction (Philpott *et al*., 2000).

The murine lung has also been used to model *Shigella* infection but similarly lacks relevance in terms of organ specificity of shigellosis (Mallett *et al*., 1993; van de Verg *et al*., 1995). Mice are inoculated via the intranasal route allowing bacteria to invade the bronchial and alveolar epithelia resulting in acute bronchiolitis and development of lethal pneumonia with pulmonary lesions resembling those observed in *Shigella* induced colitis (Philpott *et al*., 2000; van de Verg *et al*., 1995). Despite the inability to accurately mimic *Shigella'*s natural niche, this model has been used to study aspects of local and systemic immunity including antibody and cytokine responses to *Shigella* infection (Mallett *et al*., 1993; van de Verg *et al*., 1995).

Macaque monkeys are one of the only animals that develop dysentery-like disease in response to oral infection with *Shigella.* Macques have been used in studies evaluating vaccine prototypes using live attenuated strains (Sansonetti *et al*., 1991). While the ability to induce dysentery is an attractive quality in an animal model of *Shigella* infection, this is offset by the substantial ethical and financial challenges associated with this model as well as the high infectious dose required to induce dysentery, which is 10-100 million times higher than that required for human infection (Philpott *et al*., 2000).

Due to the drawbacks associated with each of the models discussed above, the search for an effective and robust small animal model to study *Shigella* pathogenesis continued until a recent advancement was made. In 2005, Martino *et al.* adapted a model first used to study another Gram-negative, intracellular pathogen, *Salmonella,* to *Shigella* research. As with *Shigella,* mice are intrinsically resistant to *Salmonella* infection. This phenomenon is known as colonization resistance and is discussed in more detail throughout this thesis. Due to the natural resistance to *Salmonella,* orally infected mice show little or no signs of intestinal inflammation. When mice are pretreated with streptomycin, however, oral infection with *Salmonella enterica* serovar Typhimurium results in colitis with many similarities to human pathology such as epithelial ulceration, edema, and extensive neutrophil infiltration (Barthel *et al*., 2003). Streptomycin is a broad-spectrum antibiotic that binds to the bacterial 30S ribosomal subunit to inhibit the initiation and elongation phases of protein synthesis (NIH, 2015). Pretreatment of mice with streptomycin similarly renders mice susceptible to infection by *Shigella* and is the basis for the first model of shigellosis in which orally administered *Shigella* interact with the colonic tissue representing the natural target of infection (Martino *et al*., 2005). Streptomycin pretreated, wild-type *Shigella* infected mice are burdened with high levels of bacteria in the colon, cecum, ileum, and liver, and express an abundance of inflammatory cytokines in the intestinal tissue (Martino *et al*., 2005). Aggregates of mononuclear cells and polymorphonuclear leukocytes at the site of infection are also detectable via histopathological analysis (Martino *et al*., 2005). In addition to characterizing the manifestations of disease during infection, this work was key in establishing optimal infection conditions. Although *Shigella* does not induce dysentery in

mice, the streptomycin-treated mouse model of infection represents a significant advancement for the study of *Shigella* pathogenesis. This model of infection has been established in the Rohde lab and was recently used to validate the role of the *Shigella* effector OspG (Pruneda *et al*., 2014).

1.3: Colonization Resistance

1.3.1: Introduction

Intestinal colonization resistance is defined as the resistance to colonization by ingested bacteria imposed by the normal flora occupying the intestinal tract. Commensal bacteria, which begin to colonize the body during and immediately after birth, have evolved to occupy distinct sites in the body and are in constant contact with the host. As a result, commensals are in tune with the physiological conditions of the host and have certain advantages over invading bacteria (Sun and O'Riordan, 2013). The high density of commensal microbiota residing in the intestine prevents colonization by ingested pathogens as well as the overgrowth of certain commensals, also known as pathobionts, that are normally present at low levels in the gut (Lawley and Walker, 2013). Pathobionts are opportunistic pathogens that can cause inflammation and disease in genetically susceptible hosts or when the balance of the normal biota in the gut is perturbed (Kaiko and Stappenbeck, 2014). Perturbation of the normal biota inhabiting the gut is also known as dysbiosis and has recently been associated with several diseases including autism, asthma, and inflammatory bowel diseases such as ulcerative colitis and Crohn's (Abrahamsson *et al*., 2014; Frank *et al*., 2007; Parracho *et al*., 2005). Antibiotic use is one of the major factors associated with alterations in the intestinal microbiota contributing to dysbiosis (Hawrelak and Myers, 2004).

The phenomenon of colonization resistance was first recognized in 1955 when Bohnhoff *et al.* determined that the dose of *Salmonella enterica* serovar Typhimurium required to infect mice is 100 000-fold lower when the normal intestinal flora of the mice is first subjected to antibiotic treatment (Bohnhoff *et al*., 1955). The established streptomycin-treated mouse model of *Shigella* infection, which requires pretreatment with streptomycin for intestinal colonization to occur, also demonstrates the colonization resistance barrier. The model is consistent with the role of the intestinal flora in preventing oral infections, and is similarly observed in other models of enteropathogen infection such as *Salmonella* (Barthel *et al*., 2003; Martino *et al*., 2005; Que *et al*., 1986).

1.3.2: Mechanisms of Colonization Resistance

The proposed mechanisms of colonization resistance suggest that resistance is a complex state involving direct microbe-microbe interactions between commensals within the host as well as indirect effects resulting from stimulation of the host mucosal immune system by the commensal microbiota (Lawley and Walker, 2013). One way that the commensal microbiota promotes colonization resistance is through competition with ingested pathogens or pathobionts for niches and nutrients within the host. This mechanism of colonization resistance has been shown to drive the elimination of *Clostridium difficile* from the infected mouse gut. Since it is less efficient than other members of the resident microbiota at competing for nutrients such as glucose, N-acetylglucosamine, and sialic acids derived from the colon, the opportunistic pathogen, *C. difficile*, is outcompeted by the mouse cecal microbiota (Wilson and Perini, 1988). Colonization by intestinal pathogens is also influenced by the ability of invading bacteria to traverse the outer mucin layer, an area heavily colonized by commensals, and to

compete with the normal biota for attachment to adhesion receptors on the gut epithelium (Juge, 2012).

Colonization resistance can also occur as a result of modifications to the intestinal environment by the resident microbiota. Metabolic processes carried out by anaerobic organisms residing in the gut reduce free oxygen to maintain a predominantly anaerobic atmosphere in the lumen of the GI tract. Anaerobiosis in the gut lumen can act as a barrier to pathogens requiring available oxygen for survival and can limit growth and virulence gene expression even in facultative anaerobes like *Shigella* whose secretion machinery is inhibited until a significant increase in oxygen is detected (Lawley and Walker, 2013; Marteyn *et al*., 2010).

Products of the intestinal microbiota can contribute further to inhospitable gut conditions in support of colonization resistance. Commensal microbes synthesize diverse antimicrobial compounds such as bacteriocins that have bacteriocidal activity against invading pathogens (Lawley and Walker, 2013). Bacteriocins are biologically active proteins with bacteriocidal activity against closely related bacterial targets. Killing mechanisms are diverse and can include pore formation in the cellular membrane of the target bacteria and nuclease activity against DNA, ribosomal RNA, or transfer RNA (Riley and Wertz, 2002).

Short-chain fatty acids (SCFA) are another product of the normal biota with a role in promoting colonization resistance. SCFAs comprise a subset of saturated fatty acids with six or less carbon atoms. SCFAs are carboxylic acids characterized by a carboxyl group attached to an aliphatic tail (Tan *et al*., 2014). They are synthesized when carbohydrates that are indigestible by the host are broken down by the intestinal

microbiota and released as byproducts of fermentation (Sun and O'Riordan, 2013). The predominant SCFAs in the colon are acetate followed by propionate and butyrate, respectively, with a combined concentration ranging from 70 to 120 mM (Hooper *et al*., 2002). The concentration and distribution of SCFAs in the colon varies and is dependent on local colonization patterns and abundance of the microbiota, with the large intestine being the more heavily colonized site (Sun and O'Riordan, 2013).

Increased rates of bacterial fermentation and corresponding SCFA production in this area contribute to local reductions in pH, particularly at the interface between the small and large intestine where undigested carbohydrates first become available for breakdown. In addition to SCFAs, other organic acids produced by the microbiota, such as lactate, also contribute to increased acidity. Localized reductions in pH result in a suboptimal environment for certain enteric pathogens, thus contributing to colonization resistance (Lawley and Walker, 2013). Studies demonstrate the suppression of replication rate as a consequence of reduced pH in certain species of *Salmonella* and *E. coli* (Cherrington *et al*., 1991; Duncan *et al*., 2009). In a murine model of colitis, increased acetic, propionic, and butyric acid profiles resulting from administration of certain commensals contained in a fermented milk product lowers inflammation and contributes to a nonpermissive environment for colitogenic Enterobacteriaceae (Veiga *et al*., 2010).

Butyrate has also been implicated in relieving aspects of pathology associated with *Shigella* infection. In a rabbit model of shigellosis, administration of butyrate improves clinical symptoms including inflammation, restores epithelial production of a main AMP targeted for downregulation by *Shigella,* and reduces intestinal bacterial load (Rabbani *et al*., 1999; Raqib *et al*., 2006; Sarker *et al*., 2011).

SCFAs can also directly influence virulence gene expression in enteric pathogens to support colonization resistance. Exposure to butyrate results in the down-regulation of genes encoding the T3SS required for invasion in *Salmonella enterica* and decreases invasion of chicken cecal epithelial cells (Gantois *et al*., 2006; Van Immerseel *et al*., 2004). Acetate produced by the mouse commensal *Bifidobacterium,* inhibits the growth of pathogenic *E. coli* and prevents its virulence by blocking translocation of the lethal Shiga toxin into the blood in a murine model of infection (Fukuda *et al*., 2011).

Stimulation of the host mucosal immune system by the commensal microbiota is another way that colonization resistance is encouraged. An important aspect of host innate defense against invading microbes is the system of specific receptors located in the cytoplasm, within certain organelles, and in the cellular membrane of target cells. Toll-like receptors (TLRs) and nucleotide-binding oligomerization domain-like receptors (NODs) are stimulated by various pattern associated molecular patterns (PAMPS) such as components of the pathogen membrane, nucleotides, lipoproteins, and proteins to initiate host defense. Interactions between TLRs and the commensal microbiota have been shown to help maintain intestinal epithelial homeostasis and protect from injury (Lawley and Walker, 2013; Rakoff-Nahoum *et al*., 2004). In addition, commensal –TLR interactions on the surface of Paneth intestinal epithelial cells contribute to the expression of antimicrobial factors and limit invasion of host tissues by pathogenic *Salmonella* (Vaishnava *et al*., 2008). Increasing evidence shows that the commensal microbiota also maintains gut integrity to promote colonization resistance by inducing the expansion of regulatory T cells and helping to maintain the T cell/ T helper type 17 balance in the gut (Lawley and Walker, 2013).

1.4: The Commensal Microbiota

1.4.1: Introduction

Advances in DNA sequencing technologies has made possible the study of the (largely unculturable) commensal microbiota. The eukaryotic, viral, archaeal, and bacterial microbes colonizing the body, collectively called the microbiota, are now considered a distinct organ impacting host physiology during both health and disease (Lawley and Walker, 2013). Little is known about the non-bacterial components, while bacteria have been the focus of studies on the microbiota. Approximately 10^{14} microbes colonize the body at adulthood and vastly outnumber human cells by a factor of 10^{3} (Lawley and Walker, 2013). The most densely populated region is the GI tract which increases in diversity and number of bacteria toward the colon (Sekirov *et al*., 2010).

The intestinal microbiota is dominated by strict anaerobes and four prominent phyla, however some facultative anaerobes and aerobes are present and over 50 bacterial phyla have been described (Sekirov *et al*., 2010). Dominant phyla include the Gram-positive Firmicutes and Actinobacteria and the Gram-negative Bacteroidetes and Proteobacteria (Kaiko and Stappenbeck, 2014). While recent studies show an association between features of the human microbiome and specific traits including diet, health status, age and geography (Greenblum *et al*., 2012; Wu *et al*., 2011; Yatsunenko *et al*., 2012), the study of the microbiota in health and disease is complicated by the inherent variability in microbiota between individuals (Franzosa *et al*., 2015; Lawley and Walker, 2013).

The microbiota starts to develop at birth and is shaped by a process of ecological succession involving commensal microbe-microbe interactions, host factors, and

environmental exposures. Nutrition, microenvironments within the host, and immune responses to pathogens and commensals all play a role in determining the mature microbial composition (Kaiko and Stappenbeck, 2014). Despite fluctuations that can occur during early development and disease, the gut microbiota is relatively resilient and maintains a general level of stability over time (Lozupone *et al*., 2012).

1.4.2: Role of the Commensal Microbiota

An important function of the intestinal microbiota is to prevent pathogen invasion and maintain gut homeostasis to avoid overgrowth of certain commensals, a function known as colonization resistance (Lawley and Walker, 2013). Beyond colonization resistance, the commensal microbiota and its products carry out many additional functions contributing to host health status and physiology.

One of the major roles of the commensal microbiota is reflected in its contribution to host cell metabolism. The microbiota metabolizes many potentially harmful substances involved in the digestive process including bile acids, bilirubin, and heterocyclic amines that might otherwise lead to toxic effects in the body (Hooper *et al*., 2002).

Carbohydrate metabolism is another host cell process heavily influenced by the commensal microbiota. While equipped with the metabolic machinery needed to absorb simple sugars, hydrolyze disaccharides, and breakdown starch into monosaccharides, the human genome does not encode the genes necessary to degrade most plant- and animal-derived dietary glycans (Koropatkin *et al*., 2012; Musso *et al*., 2011). In particular, is the lack of enzymes needed to hydrolyze many plant polysaccharides. In exchange for a nutrient-rich niche, the commensal microbiota colonizing the distal GI tract is responsible for breaking down undigested plant material including xylan, cellulose, and pectin, as

well as partially digested starch (Musso *et al*., 2011). The SCFAs resulting from these fermentation events contribute an estimated 5-10% of the total calorie requirement in humans (Lawley and Walker, 2013).

SCFAs produced by the commensal microbiota have a plethora of effects on host physiology and have become a popular area of study for their association with gut health. Abnormally low levels of SCFAs including propionate and butyrate have been documented in patients suffering from inflammatory bowel disease (IBD) compared to healthy controls (Huda-Faujan *et al*., 2010). Furthermore, butyrate has been implicated in suppressing colonic tumor growth (Gupta *et al*., 2006) and an inverse relationship between colonic levels of SCFAs and incidence of colorectal cancer has been demonstrated (Bingham *et al*., 2003).

Following their production, the majority of SCFAs are utilized in the gut. Butyrate in particular, represents the primary source of energy for epithelial cells in the colon. The other predominant SCFAs acetate and propionate are absorbed into the blood and make their way to the liver. In the liver, acetate and propionate are metabolized by hepatocytes or released systematically into the peripheral venous system (Pomare *et al*., 1985; Tan *et al*., 2014). The fate of the SCFAs that remain in the gut is influenced by the ionization state of the acid with unionized SCFAs freely crossing the epithelial barrier and ionized SCFAs relying on specialized transporters for uptake into cells. Two main receptors, monocarboxylate transporter 1 (MCT-1) and sodium-coupled monocarboxylate transporter 1 (SMCT-1) receptors, are expressed along the length of the GI tract including on colonocytes as well as in the small intestine and the cecum, and mediate the active transport of ionized SCFAs across the epithelial barrier (Tan *et al*., 2014). MCT-1 is also

expressed on immune cells including lymphocytes (Merezhinskaya *et al*., 2004) while SMCT-1 can also be found on kidney and thyroid cells (Ganapathy *et al*., 2008).

SCFAs are known to impact host biology through two major mechanisms, inhibition of histone deacetylase (HDAC) activity, and interaction with host G-protein-coupled receptors (Tan *et al*., 2014). Acetylation of lysine residues within histones induces gene expression by promoting the access of transcription factors to gene promoters. By catalyzing the removal of acetyl groups from histones, histone deacetylases reverse the effects of histone acetylation on gene expression (DesJarlais and Tummino, 2016). SCFAs regulate gene expression by suppressing histone deacetylation through inhibition of HDAC activity and contributing to increased levels of acetylation (Boffa *et al*., 1978; Soliman and Rosenberger, 2011). While the exact mechanism of HDAC inhibition by SCFAs remains elusive, the effects of SCFAs on HDAC activity have been associated with an anti-inflammatory phenotype. SCFAs contribute to decreased inflammation by interfering with HDAC activities such as HDAC-mediated effects on NF-kB and inflammatory cytokine expression (Tan *et al*., 2014; Usami *et al*., 2008).

SCFAs have also been recognized as ligands for G-protein-coupled receptors (GPCRs) including GPR41, GPR43, and GPR109A. GPR43 is expressed along the length of the GI tract and recognizes a range of SCFAs (Tan *et al*., 2014). GPR43 is highly expressed on specialized endocrine cells known as L-cells populating the distal colon and rectum. In response to SCFA stimulation of GPR43, L-cells release Peptide YY (PYY) and glucagon-like peptide 1 (GLP-1) (Gunawardene *et al*., 2011; Tolhurst *et al*., 2012). GLP-1 and PYY are anorectic gut hormones released in response to food intake that

regulate satiety (De Silva and Bloom, 2012). GLP-1 also has a central role in regulating insulin release (Meloni *et al*., 2013). Through indirect effects on PYY and GLP-1 secretion, it is possible that SCFAs contribute to body weight regulation and food intake (Tan *et al*., 2014). GPR43 is also expressed on several immune cells and the SCFA-GPR43 interaction has been linked to the resolution of inflammatory responses as demonstrated in a DSS-induced model of colitis (Maslowski *et al*., 2009).

SCFA-mediated stimulation of another GPCR, GPR41, has a regulatory role in the maintenance of metabolic homeostasis. In the colonic mucosa, GPR41 is expressed in enterocytes and enteroendocrine cells and is most potently stimulated by propionate followed by butyrate and acetate, respectively (Tazoe *et al*., 2009). GPR41 is also abundantly expressed in the sympathetic ganglia; an area of adrenergic neuron cell body aggregates that function in the sympathetic nervous system. Propionate functions adversely with the ketone body β-hydroxybutyrate on GPR41 to stimulate and antagonize this receptor, respectively, to promote or suppress sympathetic nervous system activity allowing control over energy expenditure and maintenance of metabolic homeostasis (Kimura *et al*., 2011). Additional functions mediated by SCFA through their interactions with GPCRs are reviewed in Tan *et al* (2014).

Intestinal colonization by the commensal microbiota also impacts the structure and function of the mucosal epithelial layer. In contrast to the small intestine, the large intestine is coated in a much thicker mucus layer and is divided into two distinct sub layers. The outer layer is thicker and loosely arranged allowing for heavy colonization by the commensal flora. The thin inner layer is largely free of microbes due to its close association with the underlying epithelial cells and densely organized mucin (Johansson

et al., 2011). Together, these features contribute to the barrier function and integrity of the intestinal epithelium. The microbiota inhabiting the outer mucus layer degrades glycans derived from shed epithelial cells and mucous secretions in the host, contributing to glycan turnover at the mucosal layer (Koropatkin *et al*., 2012).

While the complete mechanisms are unknown, the gut microbiota play a significant role in the development of the mammalian immune system, both locally and at a systemic level. The role of the commensal flora in inducing the development of secondary lymphoid tissues has been demonstrated. In mice, intestinal lymphoid tissue in the small intestine provides a structural platform for lymphoid cells to initiate immune responses (Hamada *et al*., 2002). In the absence of the commensal flora, axenic mice display underdeveloped intestinal lymphoid tissue, a phenotype restored by colonization with commensal bacteria (Pabst *et al*., 2006). In another study, axenic mice were found to be defective in intestinal immunoglobulin A (IgA) plasmocytes (Moreau *et al*., 1978). IgA plays a critical role in host defense and in maintaining homeostasis with the microbiota at the intestinal epithelial cell surface (Macpherson *et al*., 2000; Macpherson and Uhr, 2004). Regulatory T cells (T_{reg}) in the colon also contribute to intestinal and systemic immune homeostasis (Geuking *et al*., 2011). Similar to observations with IgA, axenic mice are deficient in T_{reg} cells and recent work demonstrates that members of the commensal biota including indigenous *Bacteroides* and *Clostridium* species restore these cell populations to levels observed in conventionally-raised mice (Atarashi *et al*., 2013; Atarashi *et al*., 2011; Round and Mazmanian, 2010).

At the systemic level, serum immunoglobulin E (IgE) levels are also influenced by the commensal microbiota. In axenic mice, the balance of Type 1 helper (T_H1) to Type

2 helper (T_H2) cells is disturbed leading to a dominant T_H2 response associated with elevated IgE levels (Lee and Hase, 2014). An immunomodulatory molecule, PSA, synthesized by the commensal bacteria *Bacteroides fragilis,* is associated with restoration of the systemic T_H2/ T_H1 balance in axenic mice (Mazmanian *et al*., 2005).

Despite the abundance of recent data implicating the microbiota in human health status and disease, many follow up studies are required and many aspects of this broad research area remain to be addressed. The influence of antibiotics, physical and psychological stress, diet, and other factors on the microbiota remain to be fully characterized and associations between specific diseases and microbe imbalances are lacking. The anaerobic commensals colonizing the mammalian gut have been particularly difficult to study, largely due to the un-culturable nature of many of these organisms. Culture-independent techniques such as next-generation sequencing, described below, represent a useful tool for this area of study.

1.4.3: Tools to Study the Microbiota

1.4.3.1: Next-generation Sequencing

In the 1980s, the foundation for culture-independent identification of bacteria was established with the discovery that phylogenetic relationships between bacteria could be identified by comparing stable oligonucleotide signatures within the genetic code (Clarridge, 2004). Currently, the gene coding for the 16S ribosomal RNA (16S rRNA) is the most commonly used bacterial marker. The 16S rRNA gene also allows for comparison with archaea, while the 18S rRNA gene is used when dealing with identification of eukaryotic samples (Clarridge, 2004). The 16S rRNA gene is highly conserved denoting its importance as an essential part of cell function (Pei *et al*., 2010).

Universal primers corresponding to conserved regions at the start and either 540-bp downstream or at the end of the 1550 bp sequence within the 16S rRNA gene are commonly used for amplification and the variable region in between conserved sequences is used for comparative taxonomy (Clarridge, 2004).

Paired with recent advances in sequencing technology, the 16S rRNA gene marker provides a powerful tool for the study of the microbiome. In recent decades, the field of genetics has been revolutionized by the speed, cost efficiency, and massive data production associated with modern sequencing (Mardis, 2008). Originally, sequencing was a tedious and largely non-automated process with the capability of determining only a few hundred nucleotides at a time. Gel electrophoresis was used to sort DNA fragments by molecular weight but was eventually replaced by capillary electrophoresis (Kircher and Kelso, 2010; Sanger *et al*., 1977). The 1990s saw the development of the first commercially available 96 capillary sequencers, which became known as the earliest form of high-throughput sequencing (Huang *et al*., 1992; Kircher and Kelso, 2010). Within the last decade however, sequencing technologies have continued to improve with daily throughput up to 1000 times that of traditional Sanger-sequencing technologies, causing the term high-throughput to be redefined as next-generation sequencing (Kircher and Kelso, 2010).

Five main next-generation sequencing platforms exist for massively parallel DNA sequencing read production. These include the Roche/454 FLX, the Illumina/Solexa Genome Analyzer, the Applied Biosystems SOLiD™ System, the Helicose Heliscope™, and the Pacific Biosciences SMRT (Mardis, 2008). In contrast to traditional methods of sequencing, next-generation sequencing technologies use adapter sequences that are

ligated to blunt-ended fragments of DNA generated from the source of interest. Adapter sequences allow the selective amplification of DNA molecules using PCR and alleviate the need for a bacterial cloning step to amplify DNA fragments (Mardis, 2008). Traditional methods using a bacterial intermediate encounter the challenges associated with cloning such as dealing with sequences of difficult base pair composition or length, and interactions that can occur with the bacterial host system (Kircher and Kelso, 2010). Depending on the platform used, the average run time for next-generation sequencing ranges from 8 hours to 10 days and can generate between hundreds of thousands and tens of millions of reads (Mardis, 2008).

In the present study, the Illumina Genome Analyzer platform was used and will therefore be the focus of the next-generation sequencing technologies discussed. The Illumina Genome Analyzer platform uses a "sequencing by synthesis" approach. In this method, the purified DNA source is randomly fragmented and adaptors are ligated to both ends of the fragments. Sequencing primers anneal to the adaptors and polymerases extend the sequencing primers by incorporating fluorescently labelled reversible chain terminator nucleotides. Due to the chain terminating nature of the first nucleotide, the incorporation stops and excess nucleotides are washed off. The fluorescent label of the incorporated nucleotides is then read through nucleotide-specific filters with lasers that excite the fluorophores. Finally, the blocked 3' terminus and associated fluorophore are removed from each newly incorporated base and the sequencing reaction is repeated for each of the three remaining bases (Kircher and Kelso, 2010; Mardis, 2008).

Next-generation sequencing has revolutionized the field of metagenomics due to the many advantages it holds over past sequencing techniques. Metagenomics is defined

as the study of the total genetic material directly obtained from an environmental sample. It is used for direct sampling of specific environmental niches and has diverse applications. In one study, metagenomics was applied to monitor the diversity, composition, and functional traits of bacterial communities in rapidly moving freshwater sites dispersed along southwestern Canada. Studies such as this have the potential to directly inform the development of biomarkers for water quality monitoring systems and contribute to a greater understanding of watershed ecosystem composition (Van Rossum *et al*., 2015).

In another study, metagenomic analysis was used to investigate the microbial ecology associated with red and white snow phenotypes in a Russian Arctic archipelago. This study highlights the adaptations of a pigment-producing unicellular snow algae associated with the red snow phenotype to increasing UV exposure and the related shift in microbial community composition supported by the *Chlamydomonas* algae species (Hisakawa *et al*., 2015). Studies like this deepen our understanding of the impact of warming temperatures on polar environments and the microbial communities residing there. In addition to these studies, metagenomics has been used to study the microbial community composition in sites such as soil, marine environments, and in urban settings heavily influenced by anthropogenic activity (Afshinnekoo *et al*., 2015; Mendes *et al*., 2011; Sunagawa *et al*., 2015). One of the most critical applications of metagenomics in terms of its contribution to human health is the study of the human microbiome. A variety of sites on and in the body are colonized and therefore represent targets for human microbiome studies. Human microbiome projects share the common goal of defining the roles that our microbial symbionts have in human health and disease (Ursell *et al*., 2012).

One of the most high profile human microbiome studies to date is the Human Microbiome Project. Launched in 2008 by the National Institutes of Health, the Human Microbiome Project was an initiative aimed at conducting culture-independent identification and molecular characterization of the microbes associated with healthy and diseased human states. In this study, a comprehensive data resource was generated for diverse body sites including the mouth, skin, intestine, and vagina (Kaiko and Stappenbeck, 2014). Samples were collected from over 200 volunteers and subjected to 16S rRNA and metagenomic DNA sequencing (Segata *et al*., 2012). Findings from the Human Microbiome Project revealed strong habitat specificity among body sites between individuals but showed substantial variation in diversity and abundance of the microbes present at each body site between healthy individuals. This study also suggested that diverse bacterial compositions have the same overall functional potential since strong functional conservation of bacterial genes was observed between individuals with distinct microbial profiles (Kaiko and Stappenbeck, 2014).

As demonstrated in the Human Microbiome Project, next-generation sequencing is a powerful tool for the investigation and characterization of the human microbiome and can be used to contribute to our understanding of the role of microbial symbionts in health and disease (Group *et al*., 2009). While these studies have already advanced our understanding, they open up additional questions and set the stage for future study. One such question is how the microbial communities colonizing the body are shaped by both diet and host genetics (Kaiko and Stappenbeck, 2014). Another emerging area of interest is the direct and indirect interactions between the commensal microbiota and invading pathogens and how these interactions culminate in colonization resistance.

1.4.3.2: Murine Models in Human Microbiome Studies

Germ-free or axenic, gnotobiotic, and specific-pathogen free mouse models have all been used extensively to study host-microbiome interactions and the role of the gut microbiota in mammalian physiology (Fritz *et al*., 2013; Kostic *et al*., 2013). While other models are available for host-microbiome research, mice are the most commonly used model organisms (Fritz *et al*., 2013). Despite their widespread use, these models do not accurately reflect all aspects of human host physiology and are associated with certain disadvantages. Discrepancies include immune system differences, differential environmental and diet-associated factors, distinct GI tract topologies, and differences in microbiota composition at the genus level between mice and humans (Fritz *et al*., 2013). Additional factors such as coprophagic behavior might also differentially influence the microbial communities colonizing the mouse (Kostic *et al*., 2013).

Despite these differences, other features of murine models make them beneficial to host-microbiome research. Easy manipulations such as the ability to control breeding strategy, littermates, housing conditions, diet, and light exposure lend well to the study of environmental factors on the microbiota. Research is also facilitated by availability of genetically altered mice including knockout, knock-in, and transgenic mutants (Kostic *et al*., 2013). Other advantages include the short breeding time, availability of fully sequenced genomes, and the conservation of organs between mice and humans, although organs are present in different proportions (Fritz *et al*., 2013). A major advantage of using murine models to investigate host-microbiota interactions is the level of similarity between the mouse and human microbiota at the phylum to family level (Fritz *et al*.,

2013; Kostic *et al*., 2013). For these reasons, host-microbiota research in murine models is a powerful tool with relevance to human biology.

Related to their use as models to investigate host-microbiota interactions, murine models have been helpful in revealing mechanisms of colonization resistance. These studies are facilitated by the murine characteristics described above, the range of available immunomodulatory reagents, and the diversity of select human pathogens to colonize the mouse and inflict morbidity (Lawley and Walker, 2013).

1.4.4: The Microbiota and Enteric Infection

The involvement of the commensal microbiota in altering the course of disease in several models of enteric infection has been described. Infection with the Gram-positive, extracellular bacteria, *Clostridium difficile,* can result in severe diarrhea and inflammation of the colon and is a leading cause of antibiotic-associated diarrhea (Bartlett, 2002). It is now understood that the disease phenotype in individuals with recurrent *C. difficile* infection, is associated with decreased diversity of the fecal microbiome (Chang *et al*., 2008). Furthermore, re-establishing the balance of commensal bacteria in the intestine through fecal bacteriotherapy has been shown as an effective treatment against recurrent *C. difficile* infection (Bakken, 2009; Petrof *et al*., 2013; Rohlke *et al*., 2010).

Another intestinal pathogen, the waterborne Gram-negative bacteria *Vibrio cholerae* is the causative agent of the secretory diarrheal disease known as cholera. *V. cholerae* colonizes the small bowel and releases a potent enterotoxin responsible for the massive depletion of electrolytes from target intestinal epithelial cells resulting in diarrhea (Finkelstein, 1996). Recently, Gordon *et al* (2014) identified bacterial taxa

associated with recovery from *V. cholerae* infection using next-generation sequencing on stool samples collected from adults living in an area with high disease burden. In a murine model, co-infection of *Vibrio* with certain isolates identified by next-generation sequencing significantly lowered the burden of *V. cholerae.* Specifically, authors demonstrate that one isolate, *Ruminococcus obeum,* interferes with the virulence of *V. cholerae* to restrict colonization (Hsiao *et al*., 2014).

Commensals in the gut have also been linked with altering the outcome of *Salmonella typhimurium* infection. Perturbation of the intestinal microbiota with antibiotic treatment increases the susceptibility of mice to *Salmonella* colonization and correlates with more severe intestinal pathology (Sekirov *et al*., 2008). In another study, the presence of a complex microbiota was found to act in concert with secretory IgA antibody (sIgA) to mediate pathogen clearance from the gut lumen in mice (Endt *et al*., 2010). More recently, the effects of a synthetic community of bacteria originally isolated from healthy human donor stool and used to treat recurrent *C. difficile* infections were evaluated in the context of a murine model of *S. typhimurium.* The synthetic community of commensals attenuated features of infection including components of the pro-inflammatory response and tight junction disruption at the intestinal epithelial barrier (Martz *et al*., 2015).

Enterococcus is a genus of Gram-positive bacteria ubiquitous in the human intestine as well as the environment. Two species, *Enterococcus faecalis* and *Enterococcus faecium,* cause the majority of human infections and are increasingly problematic in hospital settings due to high levels of intrinsic and acquired antibiotic resistance (Cetinkaya *et al*., 2000). Antibiotic induced changes in the composition of the

murine microbiota have been shown to promote efficient displacement of the microbiota by Vancomycin-resistant *Enterococcus* (VRE) in the intestinal environment (Ubeda *et al*., 2010). More recently, Ubeda *et al.* demonstrated that administration of an obligate anaerobic collection of commensal bacteria containing members of the *Barnesiella* genus eliminates VRE from the intestine of densely colonized mice (Ubeda *et al*., 2013).

These studies highlight the emerging theme of the microbiota acting as a crucial determinant in the onset and course of disease caused by enteric infection. A more thorough understanding of the mechanisms by which the microbiota impacts the health status of the host during infection and disease and whether microbiota-mediated effects occur at the species or community level remain to be addressed. The role of commensals in other enteropathogen infection models such as *Shigella* remains to be investigated and these efforts have therapeutic potential that has yet to be explored.

1.5: Colonization of the Gut by Enteropathogens

Enteropathogens face many challenges associated with overcoming the barrier of colonization resistance for successful infection to occur. In addition to having to outcompete and withstand the commensals inhabiting the gut, enteric pathogens are exposed to an array of other conditions that impact their ability to colonize. One major obstacle is the limited oxygen concentration in the gut. Oxygen levels in the gut follow a gradient with highest concentrations detected in the lamina propria, located under the intestinal epithelial layer, and decreasing levels toward the intestinal lumen (Albenberg *et al*., 2014). To adapt to the anaerobic conditions within the GI tract during the course of infection, *Shigella* differentially regulates hundreds of genes. Genes involved in carbon transport and metabolism, DNA topology and regulation, and host interaction and

survival within the GI tract are among the genes up-regulated, while genes involved in *Shigella*'s T3SS are down regulated under anaerobiosis (Vergara-Irigaray *et al*., 2014). Many other facultative anaerobes undergo similar adaptations in their gene expression patterns to acclimatize to anaerobiosis (Muller-Herbst *et al*., 2014; Salmon *et al*., 2003).

Additional obstacles to successful colonization include reactive oxygen and nitrogen species resulting from innate immune cell output and inflammatory stimuli. Reactive oxygen intermediates (ROI) are species that are produced as intermediates in the reduction of O_2 to water and include superoxide, hydrogen peroxide, and hydroxyl radical. Reactive nitrogen intermediates (RNI) are nitrogenous species derived from nitric oxide which is produced by nitric oxide synthases (NOS). RNI include among others dinitrogen trioxide, dinitrogen tetraoxide, peroxynitrate, dinitrosyl-iron complexes, and nitrate. ROI and RNI are antimicrobial products involved in host innate immunity and are produced by several phagocytic cells in response to the binding of PAMPs associated with invading microbes to host TLRs (Werling and Jungi, 2003). ROI and RNI function by damaging both DNA and substances involved in the propagation and protection of DNA such as iron-sulphur clusters and hemes. As well as their ability to directly damage pathogens, immunoregulatory signaling by ROI and RNI also contributes to host defense (Nathan and Shiloh, 2000).

While several enzymes produce ROI, nitric oxide, from which RNI are derived, is produced exclusively by NOS (nitric oxide synthase) enzymes. iNOS (inducible nitric oxide synthase) also known as NOS2 (nitric oxide synthase type 2) is the inducible NOS isoform activated in response to inflammatory stimuli, while neuronal NOS (NOS1) and endothelial NOS (NOS3) are constitutively expressed. Mice deficient in nitric oxide

production by iNOS experience increased disease severity during infection with many pathogens including *Salmonella typhimurium* and *Listeria monocytogenes* compared to wild type mice (Bogdan *et al*., 2000). Expression of NOS2 at high levels in human macrophages can kill *leishmania* and *mycobacteria in vitro* (Nathan and Shiloh, 2000). Consistent with this data, RNI demonstrate toxicity against many microorganisms in culture (Nathan and Shiloh, 2000). To evade ROI- and RNI-mediated killing during host invasion, pathogens must interrupt host cell production of these species, repair the damage they cause, or catabolize these products (Nathan and Shiloh, 2000).

1.6: Overcoming Colonization Resistance by Exploiting Pathogen-induced Niches

Relating to these challenges, an emerging area of study has begun to address how certain pathogens thrive under inflammatory conditions within the host and manipulate this environment to advance infection. The inflammatory environment stimulated by enteropathogenic infection has recently been associated with shifts in microbial homeostasis that allow enteric pathogens to overcome colonization resistance. Using the murine pathogen *Citrobacter rodentium* as a model for human disease caused by enteropathogenic and enterohemorrhagic *E. coli*, Lupp *et al.* reported significant alterations in the microbial community structure in response to inflammation and demonstrated that host-mediated inflammation supports Enterobacteriaceae growth. Furthermore, the total resident bacteria in the colon was reduced as a result of the combined effect of enteropathogen colonization and host-mediated inflammation (Lupp *et al*., 2007). In another study, host inflammatory responses triggered by infection with *S. enterica* serovar Typhimurium altered the composition of the host microbiota and effectively suppressed commensal growth, allowing colonization by *S. enterica* (Stecher

et al., 2007). The mechanisms involved in overcoming colonization resistance by *Shigella* remain unknown.

Although intracellular, *Shigella* is closely related to its extracellular relative *E. coli* (Wei *et al*., 2003). Recently, a mechanism by which *E. coli* overcomes colonization resistance in the inflamed host has been described in a murine model of infection and is dependent on host-derived reactive nitrogen species and the ability of *E. coli* to utilize the anaerobic nitrogen respiration pathway. *E. coli* and *Shigella*, both members of the Enterobacteriaceae family, encode three nitrate reductases on the *narGHJI*, *narZYWV*, and *napFDAGHBC* operons (Blasco *et al*., 1990; Choe and Reznikoff, 1993; Jin *et al*., 2002). Similar to the murine model of *Shigella* infection, colonization of the murine GI tract with *E. coli* requires streptomycin treatment. Spees *et al.* (2013) tested whether the ability of *E. coli* to overcome colonization resistance in streptomycin-treated mice was a result of the antibiotic-mediated elimination of microbe-microbe interactions or the result of antibiotic induced alterations in mucosal immune responses. Streptomycin treatment resulted in a mild inflammatory response in the cecal mucosa characterized by elevated iNOS expression. Nitrate species generated downstream of iNOS activity as a by-product during the inflammatory response correlated with enhanced growth of *E. coli* in the murine GI lumen (Spees *et al*., 2013). Similar results were obtained in a murine model of DSS-induced colitis and in mice manipulated for the development of spontaneous colitis as a result of a deficiency in T cell production of the anti-inflammatory cytokine IL-10 (Winter *et al*., 2013). These studies suggest that intestinal inflammation and the ability to respire nitrate permit *E. coli* to overcome colonization resistance.

1.7: Overcoming Colonization Resistance by Hijacking Eukaryotic Metabolic Pathways

Another challenge to colonization is the nutrient availability within the host during infection. The biochemical environment within the host cell cytosol has not been fully characterized, confounding our understanding of the nutritional content used to promote the growth and virulence of intracellular pathogens (Ray *et al*., 2009). Generally, the mammalian cell cytosol is characterized as having low concentrations of magnesium, sodium, and calcium ions, a high concentration of potassium, and limited iron bound to carrier molecules (Ray *et al*., 2009). To better understand host nutrient availability, studies have aimed to identify bacterial genes involved in intracellular growth and replication. Auxotrophic mutants defective for replication have been used to identify limiting nutrients for bacterial proliferation and intracellular survival. Through such studies, guanine, thymine, and *p*-aminobenzoic acid synthesis have been identified as requirements for intracellular *Shigella* replication (Cersini *et al*., 2003; Cersini *et al*., 1998; Noriega *et al*., 1996), while the synthesis of diaminopimelate, a component of the aspartate amino acid family, has been associated with survival inside the host cytosol (Cersini *et al*., 1998). Genes involved in the recycling of peptidoglycan, adaptation to oxygen tension, as well as iron, magnesium, and phosphate uptake are upregulated in response to the intracellular environment, however their role in intracellular growth remains to be described (Ray *et al*., 2009). Proteome analysis of intracellular *Shigella* has demonstrated that metabolic pathways undergo changes in response to the intracellular environment (Pieper *et al*., 2013), however, until recently, the carbon source utilized by intracellular *Shigella* was unknown.

Colonization and pathogenesis of enteric pathogens is reliant on the ability to successfully and efficiently break down carbon sources. Carbon catabolism yields energy available as reducing equivalents and ATP and provides the cell with several biosynthetic precursor requirements (Munoz-Elias and McKinney, 2006). Members of the *Enterobacteriaceae* family including *E. coli* and *Salmonella* rely on three major pathways for carbon metabolism: the Embden-Meyerhof-Parnas (EMP), the Entner-Doudoroff (ED), and the pentose phosphate (PP) pathways (Gotz *et al*., 2010). While *Shigella* encodes the genetic machinery for these common glycolytic pathways, recent work suggests that host-derived pyruvate fuels intracellular growth of *Shigella* following epithelial cell invasion, and that *Shigella* relies on a distinct metabolic pathway for carbon metabolism (Kentner *et al*., 2014; Waligora *et al*., 2014).

Upon invasion of host epithelial cells, *Shigella* undergoes rapid proliferation within the host cytosol. Using a HeLa epithelial cell model of infection, Kentner *et al.* investigated the basis of intracellular carbon metabolism responsible for fueling *Shigella*'s extensive proliferation. In the intracellular environment, *Shigella* captures the glycolytic output of the infected host cell in the form of pyruvate and uses a three-step pyruvate-to-acetate pathway (PTA-ACKA pathway) (Kentner *et al*., 2014), a pathway commonly used by *Enterobacteriaceae* under nutrient-rich conditions during rapid growth (el-Mansi and Holms, 1989; Wolfe, 2005). The key enzymes of this pathway, pyruvate dehydrogenase (aceE), phosphotransacetylase (PTA), and acetate kinase (ACKA) convert host-derived pyruvate to acetyl coenzyme A (acetyl-CoA), acetyl-CoA to acetyl phosphate, and acetyl phosphate and ADP to acetate and ATP, respectively. As a result of this pathway, infected cells excrete acetate rather than the lactate normally

excreted by uninfected HeLa cells (Kentner *et al*., 2014). For each molecule of pyruvate that enters the PTA-ACKA pathway, a single molecule of ATP is generated as well as some additional ATP that arises from oxidation of NADH. Compared to the TCA cycle which generates 14 molecules of ATP per molecule of pyruvate, the PTA-ACKA pathway is far less efficient, however its advantages include the low enzyme cost, minimal membrane space requirements, and fast rate of ATP generation (Kentner *et al*., 2014). Kentner *et al.* demonstrate the use of the PTA-ACKA pathway to fuel intracellular growth in a cell culture model of *Shigella* infection, however the use of this pathway remains to be confirmed *in vivo.*

1.8 Research Described in this Thesis

This thesis addresses three areas of research relating to our current understanding of colonization resistance to *Shigella* infection. The commensal microbiota and the metabolic pathways in bacteria for nitrogen respiration and carbon utilization have been implicated in either colonization resistance to infection by enteric pathogens or in promoting bacterial colonization, however little is known regarding colonization resistance to *Shigella*. We hypothesized that the commensal microbiota as well as the bacterial pathways for nitrogen and carbon metabolism are involved in colonization resistance to *Shigella* and the ability of *Shigella* to overcome colonization resistance respectively, and that by testing the involvement of these pathways and of the commensal bacteria in our murine model of *Shigella* infection, we can broaden our understanding of the mechanism(s) underlying colonization resistance to *Shigella*. The ability of *Shigella* to overcome colonization resistance was investigated through the study of two potential mechanisms used to promote bacterial colonization. Firstly, the role of the intracellular

PTA-ACKA carbon metabolism pathway was investigated. Mutants were constructed to interrupt the flow of carbon through the PTA-ACKA pathway and tested in a cell culture model to confirm the phenotypes previously described in the literature. The mutant strain created for the *pta* gene was further used to create additional mutants impaired in intercellular spread and E3 ubiquitin ligase activity by the IpaH9.8 effector. Using the respective mutant strains, the intracellular proliferation defect associated with the *pta* gene was confirmed in cell culture. Furthermore, the intracellular growth phenotype of the strain deleted for both the *ipaH9.8* and *pta* genes was tested to investigate the role of the IpaH9.8 effector in supporting nutrient acquisition in the host cytosol.

Another bacterial mechanism, nitrate respiration, was investigated for its potential role in the ability of *Shigella* to overcome colonization resistance during infection of the streptomycin-treated mouse. Three chromosomally encoded nitrate reductase genes were sequentially deleted to create a *S. flexneri* mutant strain defective in nitrogen respiration. The inability to respire nitrate was confirmed *in vitro* and complemented by expressing one of the deleted nitrate reductases on a plasmid. The nitrogen respiration defective mutant was tested *in vivo* using the murine model of *Shigella* infection and the competitive index was evaluated using a co-infection model with wild-type nitrate reductase expressing *Shigella.*

Thirdly, the involvement of the commensal microbiota in colonization resistance to *S. flexneri* infection in the murine intestine was investigated. The hypothesis that a streptomycin-resistant consortium of commensals cultured from the healthy mouse gut could confer protection against *Shigella* infection was tested. To test this idea directly, we developed a procedure to enrich for SCFA-producing bacteria that would be compatible

with our *Shigella* infection model. Bacteria were cultured anaerobically on minimal glucose media in the presence of increasing concentrations of streptomycin and butyrate from the feces of healthy BALB/c mice. The resulting consortium of butyrate associated, streptomycin resistant anaerobes was characterized using 16S ribosomal RNA (16S rRNA) sequencing. Proton Nuclear Magnetic Resonance Spectroscopy (^{1}H NMR) was applied to test the short-chain fatty acid profile of the consortium bacteria and test for the qualitative presence of butyrate. The consortium of commensal bacteria was introduced into the mouse model prior to *Shigella* infection and the clinical outcome of mice was evaluated. 16S rRNA sequencing was applied to characterize the intestinal bacteria before and after treatment with consortium and prior to and following *Shigella* infection.

To further investigate the role of commensals in colonization resistance, modified Eggerth-Gagnon selective media was used in an attempt to culture *Barnesiella* species from the feces of healthy AKR/J mice. *Barnesiella* was not successfully cultured, however sequencing with universal 16S rRNA primers was used to identify additional bacterial species isolated from the feces of healthy AKR/J mice. The short-chain fatty acid profiles of the bacterial isolates were characterized by ^{1}H NMR spectroscopy.

Chapter 2: Materials and Methods

2.1: Growth and Maintenance of Bacterial Strains

For the construction of all *Shigella* mutants used in this study, the streptomycin resistant parental strain *Shigella flexneri* M90T-Sm serotype 5a was used. Throughout the study, it is referred to as wild-type *S. flexneri* or by the name of the deleted gene(s) when mutants were generated and used. *Shigella* strains were grown overnight at 37° C in 30 mg/mL Trypticase Soy Broth (TSB) with shaking for liquid cultures or on TSB agar (20 mg/mL agar) containing 0.01% (w/v) Congo red. The *Escherichia coli* (*E. coli)* K-12 strain MG1655 containing the appropriate in-frame gene deletion in which a kanamycin cassette replaces the deleted gene was used as template for the PCR reactions to generate knock-out cassettes for *Shigella* mutants (Baba *et al*., 2006). *E. coli* strain DH5α was used as a cloning vector. *E. coli* S17-1λpir harboring an FLP C1857 containing plasmid was used for mating experiments to remove kanamycin or tetracycline cassettes during sequential gene deletions in *Shigella* mutants. *E. coli* strains were grown overnight at 37° C in Luria-Bertani (LB) broth containing tryptone (10 mg/mL), yeast extract (5 mg/mL), and sodium chloride (10 mg/mL) with shaking for liquid cultures and with the addition of 20 mg/mL agar when grown on solid media. Antibiotic selection was used when appropriate at concentrations listed in Table 1. The bacterial strains used in this study are listed in Table 2.

Table 1: Antibiotic Concentrations

Antibiotic	Final Concentration
Ampicillin	100 μg/mL
Chloramphenicol	25 μg/mL
Gentamicin	15 μg/mL
Kanamycin	25 μg/mL
Spectinomycin	100 μg/mL
Streptomycin	100 μg/mL
Tetracycline	5 μg/mL

Table 2: Bacterial Strains used in this Study

Strain	Genotype	Source
M90T-Sm	Wild-type *Shigella flexneri*	Institute Pasteur
ΔicsA	M90T-Sm with *icsA* replaced by a tetracycline resistance gene	Rohde Lab
ΔptaΔicsA	M90T-Sm with *icsA* replaced by a tetracycline resistance gene and *pta* replaced by a kanamycin resistance gene	Constructed in this study
ΔptaΔicsAΔipaH9.8	M90T-Sm with in-frame *icsA* and *pta* deletions and *ipaH9.8* replaced by a tetracycline resistance gene	Constructed in this study
ΔnarGΔnarZΔnapA	M90T-Sm with in-frame *narG* and *narZ* deletions and *napA* replaced by a kanamycin resistance gene	Constructed in this study
ΔtrbH	M90T-Sm with *trbH* replaced by a tetracycline resistance gene	Rohde Lab
Δpta	*Escherichia coli* MG1655 with *pta* replaced by a kanamycin resistance gene	Keio University
ΔnarG	*Escherichia coli* MG1655 with *narG* replaced by a kanamycin resistance gene	Keio University
ΔnarZ	*Escherichia coli* MG1655 with *narZ* replaced by a kanamycin resistance gene	Keio University
ΔnapA	*Escherichia coli* MG1655 with *napA* replaced by a kanamycin resistance gene	Keio University
S17-1λpir	*Escherichia coli* lab strain	Rohde Lab
DH5α	*Escherichia coli* lab strain	Rohde Lab

2.2: Oligonucleotides and Plasmids used in this Study

Oligonucleotides used for mutant construction to generate knock-out cassettes and confirm gene deletions, for cloning, and for sequence identification are listed in Table 3. Oligonucleotides were purchased from Sigma-Aldrich, diluted to 100 μM stock concentrations and 10 μM working stocks, and stored at -20 °C. Plasmids used in this study are listed in Table 4.

Table 3: Oligonucleotides

Name	Description	Sequence
ptaFW	Forward primer for construction of the *pta::kan* knock-out cassette	GGTACCCGTCCTGCGGTGGTTATCCC
ptaRV	Reverse primer for construction of the *pta::kan* knock-out cassette	CACCAACGTATCGGGCATTGCCC
ptaFWcheck	Forward primer upstream start of *pta* deletion to confirm knock-out cassette integration	CGGTGGTTCCGTTTCTGCTATCCGCAACGGT
narGFW	Forward primer for construction of the *narG::kan* knock-out cassette	ATGCGTCATTTAGTTACAACATAC
narGRV	Reverse primer for construction of the *narG::kan* knock-out cassette	GCGCGGTATGCAGGTTCTGATAGTC
narGFWcheck	Forward primer upstream start of *narG* deletion to confirm knock-out cassette integration	GTTCGCCAGTCGGCGCAATGAAG
narZFW	Forward primer for construction of the *narZ::kan* knock-out cassette	ATCTCAGCCATTGGCGCAGTG
narZRV	Reverse primer for construction of the *narZ::kan* knock-out cassette	TCGTAGTAATCATCAATCTGCGGCAC
narZFWcheck	Forward primer upstream start of *narZ* deletion to confirm knock-out cassette integration	AGTGGTTCTACTTTCCAGATGATCGCCGTC
napAFW	Forward primer for contruction of the *napA::kan* knock-out cassette	TCAGACATCAGCACCCAACTGA
napARV	Reverse primer for construction of the *napA::kan* knock-out cassette	CGTTGCGGTCAGTGTGTTCAG

Table 3: Oligonucleotides

Name	Description	Sequence
napAFWcheck	Forward primer upstream start of *napA* deletion to confirm knock-out cassette integration	CGCGGCGCGGGCGGCTATCCGAGCG
narZxhoIFW	Forward primer for cloning *narG* with *XhoI* restriction site	TATCTCGAGGATGAGTAAACTTTTGGATCGC
narZxbaIRV	Reverse primer for cloning *narG* with *XbaI* restriction site	TATTCTAGATCATTTTTTCGCCTCCTGTACC
narZ2fw1	Forward internal check primer to sequence *narZ* insert	GCTAACGTCTGGACCATC
narZ2fw2	Forward internal check primer to sequence *narZ* insert	CGATAATCCCAGCGACTAC
narZ2fw3	Forward internal check primer to sequence *narZ* insert	GGAAGATGAAAACAGCGCGAAAG
narZ2fw4	Forward internal check primer to sequence *narZ* insert	GTATGGGCTGGCTACCTTC
narZ2fw5	Forward internal check primer to sequence *narZ* insert	GAAATCTACAAAGGTATTGCC
narZ2fw6	Forward internal check primer to sequence *narZ* insert	GAGGACGAGAAGATTCGC
narZ2fw7	Forward internal check primer to sequence *narZ* insert	GACCATGATGTATCACGCC
ipaH9.8-ntermdel-for	Forward primer to amplify *ipaH9.8* knock-out cassette	GTAATTTCCTCACTGAGCTACCAGCATTTTCTGAGGGAAATAATCCCGTTATTCCGGGGATCCGTCGACC
ipaH9.8-C337del-rev	Reverse primer to amplify *ipaH9.8* knock-out cassette	ACCAGGAGGGTTTTCCGGAGATTGTTCCATGTGAGCGCGACACGGTCCTCTGTAGGCTGGAGCTGCTTCG
ipaH9.8prIF	Forward primer upstream *ipaH9.8* deletion to check knock-out cassette integration	GTATCGCTCGAGATATAAATGTCAGGCTAGGGTCA
icsALR1F	Forward primer to amplify *icsA* knock-out cassette	CGGAATCTTTTCAGGGGTTTATCAACCACTTACTGATAATATAGTGCATGATTCCGGGGATCCGTCGACC

Table 3: Oligonucleotides

Name	Description	Sequence
icsALR1R	Reverse primer to amplify *icsA* knock-out cassette	GGACATCAACACGCCCTGCATTTTTATTATCA GAAGGTATATTTCACACCTGTAGGCTGGAGCT GCTTCG
icsALR2F	Forward primer upstream *icsA* deletion to check integration of knock-out cassette	ATTTCTCCCGTTGCATTGATATA
P1	Forward primer to check integration of knock-out cassettes containing FRT sites	ATTCCGGGGATCCGTCGACC
P2	Reverse primer to check integration of knock-out cassettes containing FRT sites	TGTAGGCTGGAGCTGCTTCG
T7F	Forward primer flanking pBluescript multiple cloning site to check insert	TAATACGACTCACTATAGGG
T3R	Reverse primer flanking pBluescript multiple cloning site to check insert	AATTAACCCTCACTAAAGGG
V1V3univ8F	Forward primer to amplify V1V3 region of 16S rRNA gene for sequencing	AGAGAGTTTGATCCTGGCTCAG
534R	Reverse primer to amplify V1V3 region of 16S rRNA gene	ATTACCGCGGCTGCTGG

Table 4: Plasmids used in this Study

Plasmid name	Features	Marker	Source
p*afaE*	Encodes the *Escherichia coli* protein Afimbrial Adhesin E	spectinomycin	Sansonetti Lab
pRR008	λ Red expression plasmid	gentamicin	Rohde Lab
pRR003	Encodes flippase recombinase enzyme (FLP)	kanamycin	Rohde Lab
p*narZ*	Contains *narZ* cloned into pBluescript II KS^+ under lac promoter	ampicillin	Constructed in this study
pBluescript II KS^+	Empty vector	ampicillin	Thomas Lab
tetRA-pGEM	tetRA amplified with T3LR1R and T7LR1F primers and cloned into pGEM-TEasy	tetracycline	Rohde Lab

2.3: Construction of Mutants

Knock-out cassettes were generated using polymerase chain reaction (PCR) with the primers indicated in Table 2 to amplify linear fragments of DNA containing the kanamycin resistance gene flanked by 150-460 bp of homology sequence to the gene of interest using the *pta::kan, narG::kan*, *narZ::kan*, or *napA::kan* deletion mutant strains from the Keio collection as template (Baba *et al*., 2006). To prepare template, a colony from the appropriate Keio mutant strain was suspended in 100 µL of distilled water and 1µL was added to the 25 µL or 20 µL PCR reaction. For deletion of *ipaH9.8* and *icsA*, knock-out cassettes containing the tetracycline resistance gene flanked by Flippase Recognition Target (FRT) sequences and 50 bp of homology to the gene of interest were generated using *tetRA*-pGEM as template with primers listen in Table 2. The construction of *tetRA*-pGEM is described in Sidik *et al* (2014). Knock-out cassettes were purified using QIAGEN PCR purification kit (Toronto, ON, catalogue number 28104) and used for λ red-mediated recombination to create the mutants as described in Sidik *et al* (2014), but with kanamycin used in the recovery media where necessary (Sidik et al., 2014). Integration of the knock-out cassette at the desired location was confirmed by PCR with the reverse primer used to create the knock-out cassette and the indicated forward check primers upstream of the region deleted for each mutant. In some cases, the P1 or P2 primers (Table 2) containing homology to FRT sequences within the knock-out cassettes were also paired with check primers targeting sequences outside the knock-out cassettes and used to confirm integration at the desired location. The general strategy of mutant construction is depicted in Figure 1.

For the construction of *Shigella* strains with multiple gene deletions, genes were deleted sequentially. Following integration of each knock-out cassette, the kanamycin or tetracycline resistance gene was removed by a recombination event between FRT sites flanking the kanamycin or tetracycline gene in the knock-out cassette (Baba et al., 2006). Recombination was catalyzed by flippase machinery encoded on the plasmid pRR003 and mobilization of the plasmid into *Shigella* occurred by mating the *Shigella* mutant strains with the *E. coli* strain S17-1λ pir, as described in Sidik *et al.* (2014).

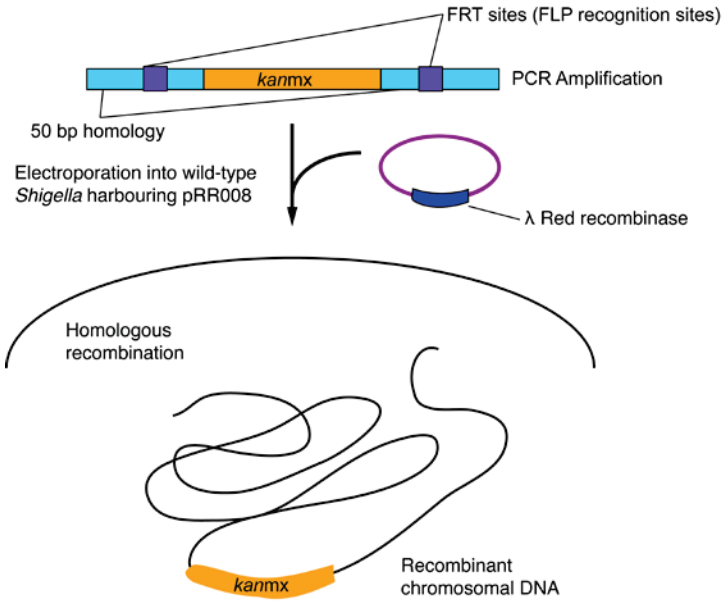


Figure 1: General strategy of mutant construction using λ red-mediated recombination. FRT = flippase recognition target, FLP = flippase recombinase, PCR = Polymerase Chain Reaction.

2.4: Cell Culture Maintenance

HeLa cells were grown in Dulbecco's Modified Eagle Medium (DMEM) (Sigma-Aldrich) supplemented with 10% fetal bovine serum (FBS) in twelve-well dishes or 50 mL, 25 cm^2 polystyrene tissue culture flasks. Cells were maintained at 37° in a humidified incubator at 5% CO_2.

2.5: Gentamicin Protection Assays

All strains used for analysis in the gentamicin protection assay were first transformed with an *afaE* expressing plasmid, as described in chapter 2.10. Afimbrial adhesin E (AfaE) is a structural adhesion protein from *E. coli* that mediates attachment to epithelial cells (Garcia *et al*., 1994). Twelve well culture plates were seeded with HeLa cells two days prior to infection and grown to 60-80% confluency. Prior to infection, cells were washed with phosphate buffered saline (PBS) and given fresh media. One well was trypsinized and the cell number was determined using a hemacytometer. HeLa cells were infected with bacteria grown to mid-log-phase at a multiplicity of infection (MOI) of 10 bacteria to 1 HeLa cell. Infected cells were incubated for one hour at 37 °C with 5% CO_2. Following incubation, the media was replaced with DMEM- FBS containing 50 µg/mL gentamicin and cells were returned to the incubator for one hour. Media was removed and cells were washed once with PBS then lysed with 500 µL/well NP-40 lysis buffer (0.1% NP-40, 50 mM Tris-HCl pH 7.5, 5 mM ethylenediaminetetraacetic acid (EDTA), 10% glycerol, 100mM sodium chloride). Lysate was used to make seven 1:10 serial dilutions in PBS and 20 µL volumes were plated on Congo red TSB agar and incubated at 37 °C overnight. Bacterial counts were obtained the following day by counting spots containing between 20-100 colonies.

2.6: Chemical Complementation

For chemical complementation of the *Shigella* mutants deleted for the *pta* gene in the gentamicin protection assay, lithium potassium acetyl phosphate (Sigma-Aldrich) was added to cell culture medium at a concentration of 100 mM. Acetyl phosphate supplemented medium was introduced to HeLa cells directly before infection and was maintained in the media used for both incubations with and without supplemental gentamicin. The gentamicin protection assay was conducted as described in Chapter 2.5.

2.7: *In Vitro* Nitrate Reduction Test

To test for inactivation of the nitrogen respiration pathway in *S. flexneri ΔnarGΔnarZΔnapA::kan* mutant strain, the nitrate reduction test kit (Sigma-Aldrich, product number 73426) was used following the manufacturer's instructions.

2.8: *In Vitro* Competitive-Growth Assay

Competitive growth assays were done as described by Spees *et al* (2013) but with 0.2% glucose added to the mucin medium. Briefly, bacteria were grown in 0.25% type II porcine mucin (Sigma-Aldrich), 40 mM MOPS (morpholinepropanesulfonic acid) buffer, trace elements (Price-Carter *et al*., 2001), magnesium sulfate heptahydrate (265 mg/L), and the added glucose with or without 40 mM sodium nitrate (Sigma-Aldrich). The mucin media was inoculated with a 1:1 ratio of *trbH::tet* and *ΔnarGΔnarZnapA::kan* for a total concentration of approximately 10^{10} CFU/mL and incubated anaerobically at 37 °C for sixteen hours. Seven 1:10 serial dilutions were made and bacteria were plated on two types of Congo red TSB agar, each containing the appropriate antibiotic for the selection of each strain. Bacterial counts were recorded after an overnight incubation at 37 °C and

the competitive index was calculated by dividing the number of *ΔtrbH* mutant by the number of *ΔnarGΔnarZnapA* mutant bacteria.

2.9: Cloning *narZ*

The *narZ* gene was amplified by PCR with primers listed in Table 2 and wild-type *S. flexneri* as template. To prepare template, a colony of bacteria was suspended in 100 µL of distilled water and 1 µL was added to the 25 µL PCR reaction. PCR product was then purified and digested with the restriction enzymes *XbaI* and *XhoI*, purified again, and ligated into digested pBluescript II KS^+. The ligated plasmid was then transformed into calcium-chloride competent DH5α as described in Chapter 2.8. The insert was sequenced using the primers used for initial amplification of *narZ* and internal check primers listed in Table 2 to check for the lack of mutations that would affect the encoded gene.

2.10 Generation and Transformation of Calcium Chloride Competent DH5α and Electrocompetent *S. flexneri*

To make calcium chloride ($CaCl_2$) competent DH5α, a 200 mL culture of bacteria was grown to an OD_{600} of 0.4-0.6 then pelleted at 5000 g for fifteen minutes at 4°C in four 50 mL Falcon tubes. The pellets were resuspended in 25 mL of ice cold 100 mM $CaCl_2$, pelleted, resuspended, and pelleted again. Pellets were resuspended in 500 µL/tube of ice cold 100 mM $CaCl_2$ with 15% (v/v) glycerol. Cells were aliquoted into 100 µL volumes and stored at -80 °C until further use. To transform $CaCl_2$ competent DH5α, a 100 µL aliquot of cells was thawed on ice and the entire plasmid ligation reaction (20 µL) was added. Cells were incubated on ice for thirty minutes then heat shocked at 42 °C for forty five seconds. Cells were then incubated on ice for a further two minutes, then 1

mL of LB broth was added and cells were allowed to recover at 37 °C with shaking for one to two hours. Cells were plated on LB with the appropriate antibiotic to select for the transformed plasmid.

To make electrocompetent *S. flexneri*, 30 mL cultures were grown to an OD_{600} of 0.4-0.6 and centrifuged at 5000 g for fifteen minutes at 4 °C. Cells were resuspended in ice cold water, pelleted, resuspended, and pelleted again. Pellets were resuspended in approximately 100 µL of residual water, mixed with 1 µL of plasmid mini prep for transformation, and transferred to electroporation cuvettes with 2 mm gaps. Bacteria were electroporated (2.5 kV) then inoculated into 1 mL of fresh TSB broth and allowed to recover at 37 °C with shaking at 200 RPM for one to two hours. Bacteria were plated on Congo red TSB agar plates with the appropriate antibiotics for selection of the transformed plasmid.

2.11: Mouse Infections

All animal experiments were conducted in the IWK Health Centre under the approval of the University Committee on Laboratory Animals at Dalhousie University. The murine model of *Shigella* infection was adapted from the procedure outlined in Martino *et al* (2005) and is described in Pruneda *et al* (2014) (Martino *et al*., 2005; Pruneda *et al*., 2014). For all experiments 6-8 week old female BALB/c mice from Charles River Laboratory (Saint-Constant, Quebec) were used. Two days prior to infection, mice were given streptomycin in their drinking water (5 g/L) and streptomycin treatment was maintained for the duration of the experiment. On the day of infection, food was removed from the cages for six hours leading up to infection. Overnight cultures of the appropriate strain of *Shigella* were used to inoculate 10 mL cultures of

TSB (1:10) with antibiotic selection and grown to mid-log phase with shaking at 37 °C. Bacteria were then pelleted (3,220 g, 10 minutes) and resuspended in 1mL PBS for every 10 mL of original culture. Mice were infected by oral gavage with 100 µL/mouse of the bacterial suspension (approximately 10^8 CFU of *Shigella*). Mice were monitored daily for three or six days following infection. Clinical scores were recorded based on appearance, posture, behavior, appetite, hydration, body temperature, and weight loss. Mice with clinical scores that reached 12 were placed on 2-hour watch and euthanized if scores reached 15 or when 20 % of the body weight was lost. To assess bacterial burden, fecal samples were obtained daily, weighed, diluted 1:10 in PBS, and used to make seven 1:10 serial dilutions. Dilutions were plated on MacConkey agar (5 g/L bile salts, 10 g/L lactose, 0.075 g/L neutral red, 20 g/L peptone, 5 g/L sodium chloride, 20 g/L agar) containing 100 µg/mL streptomycin unless otherwise indicated. Following overnight incubation at 37 °C, bacterial colonies were counted and used to calculate the number of CFU per gram of feces. On the third day post-infection, mice were put into surgical plane with isoflurane gas and euthanized by cardiac puncture and cervical dislocation. The liver, spleen, colon, and cecum were collected during dissection for further analysis. A portion of the liver and spleen were placed in PBS, homogenized, serially diluted, and plated on MacConkey agar with 100 µg/mL streptomycin to count bacterial burden and assess dissemination. The cecal content was also used for 1:10 serial dilutions and plated to assess bacterial burden. A longitudinal piece of colon was prepared using the "Swiss roll" method (Moolenbeek and Ruitenberg, 1981). The rolled colon along with the remaining portion of the spleen and the cecum were fixed in 10 % (v/v) formalin and

embedded in paraffin for histological analysis. Histology was performed by the IWK's Histology Department.

In murine infections in which the consortium of bacteria cultured from healthy BALB/c mice was introduced to test for a protective role against *Shigella*, the consortium bacteria were administered the day following the start of streptomycin treatment and one day prior to *Shigella* infection. Bacteria were grown as described in Chapter 3.2 then recovered from the agar plate and resuspended in 1 mL PBS using a sterilized loop. An approximate bacterial count was obtained using a Helber Counting Chamber (Hawksley, catalogue number Z30000). Prior to treatment with the cultured bacterial consortium, food was removed from the mice cages for a period of 6 hours. The suspension of bacteria was administered to mice (100µL/mouse) by oral gavage (approximately 10^7 bacteria). Infections were otherwise carried out as described above. Additional fecal samples were obtained from the healthy untreated BALB/c mouse, the streptomycin treated mouse, the consortium treated mouse pre-infection, the consortium treated mouse post *Shigella* infection, and the *Shigella* infected mouse in the absence of consortium treatment. Samples were used for genomic DNA purification as described in Chapter 3.5 and used for 16s rRNA Sequencing analysis as described in Chapter 3.6.

In co-infection experiments with the *ΔtrbH* and *ΔnarGΔnarZnapA Shigella* mutants, mice were inoculated with a 1:1 ratio of the strains for a total of approximately 10^8 CFU of *Shigella*. Bacteria were recovered from the mice by plating serial dilutions of the feces, cecum, or homogenized liver and spleen on both streptomycin (100µg/mL) MacConkey agar containing 100 µg/mL tetracycline, and streptomycin (100µg/mL) MacConkey agar containing 100 µg/mL kanamycin. The infection was otherwise

conducted as described above but histological analysis was not included. The competitive index was calculated by normalizing the final bacterial counts by the number of bacteria in the original inoculum for each strain and calculating the ratio of *ΔtrbH* to *ΔnarGΔnarZnapA* mutant bacteria. For all experiments, data from mice that reached clinical endpoints within 24 hours of treatment or infection by oral gavage were excluded from the study and death was attributed to gavage error.

2.12: Isolation of the Consortium of bacteria from healthy BALB/c mice

Fresh fecal pellets were obtained from healthy BALB/c mice. Fecal pellets were reuspended in PBS (2-3 pellets per mL), vortexed, and plated on M9 minimal media containing M9 salts (12.8 g/L Na_2HPO_4, 3 g/L KH_2PO_4, 0.5 g/L NaCl, 1 g/L NH_4Cl), $MgSO_4$ (0.24 g/L), 0.2% glucose, $CaCl_2$ (0.11 g/L), and agar (15 g/L). A fresh solution of filter sterilized sodium butyrate was made and added to the plates as an overlay at a final concentration of 50 mM. This concentration was increased to 100 mM on the second round of growth. Plates were incubated in an anaerobic jar using the GasPak™ EZ Anaerobic Pouch System (BD) at 37 °C for 2-3 days. After 2-3 days, a lawn of growth was visible on the plates and transferred with a sterile loop into 1 mL of fresh PBS. The suspension was plated on fresh M9 agar plates with a final concentration of 100 mM sodium butyrate and the addition of streptomycin (50 µg/mL). The anaerobic incubation of the plates was repeated and at the third round of growth, concentrations of butyrate were maintained at 100 mM and streptomycin was increased to 100 µg/mL for the third round of growth. Bacteria grown on M9 agar plates with 100 mM butyrate and 100 µg/mL streptomycin were resuspended in 1 mL of PBS and 500 µL of 50% glycerol and stored at -80 °C. For murine infections or ^1H NMR experiments, frozen stocks were

recovered by plating on M9 media with the final concentrations of butyrate and streptomycin as described above and grown anaerobically for 3 days as described. For the no butyrate control, a consortium of bacteria was cultured as described from the feces of healthy BALB/c mice but using M9 media without butyrate and containing 2% glucose.

2.13: Isolation of Commensal Anaerobes from the Feces of Healthy AKR/J Mice

Fecal pellets were obtained from healthy AKR/J mice from Jackson Laboratory (Bar Harbor, Maine). Pellets were resuspended in PBS (2-3 pellets per mL), vortexed, and plated on modified Eggerth-Gagnon (EG) agar plates containing peptone (10 g/L), $Na_2HPO_4 \times 2H_2O$ (4 g/L), porcine gastric mucin (2 g/L), sheep blood (50 mL), horse blood (50 mL), and agar (15 g). Plates were incubated for 2 days anaerobically at 37 °C. Universal primers targeting the V1-V3 region of the bacterial 16S rRNA gene were used for colony PCR to amplify DNA for sequence identification. For each isolate, a single colony was resuspended in 100 µL of distilled water and 1 µL of the suspension was used as template in a 20 µL PCR reaction with Taq DNA polymerase. Primers used for amplification and sequencing are listed in Table 3.

2.14: Detection of Bacterial Metabolites using ^{1}H NMR

Bacteria were grown as described in chapter 2.12 or 2.13 of the materials and methods. Bacteria were then washed with PBS (10 mL), resuspended in PBS supplemented with 0.2 % glucose (10 mL) and incubated at 37 °C anaerobically for 24 hours. Following incubation, suspensions were pelleted to remove the bacteria and the supernatants were collected. NaOH (1 M) was added to the supernatants until a pH of 9.5 was reached. Supernatants were frozen at -20 °C until further use. Supernatants were lyophilized for 24-48 hours and the residue was dissolved in deuterium oxide

(approximately 10-20 mg/0.8 mL). The standard sodium butyrate solution was made by dissolving 6 mg in 900 µL deuterium oxide. The standard glucose solution was made by dissolving 20 mg in 800 µL deuterium oxide and the solution was left at room temperature for one week to allow glucose to reach its equilibrium form. Spectra were acquired (typically 32 or 256 scans) using a Bruker AVANCE 300 MHz Spectrometer.

2.15: Genomic DNA Purification

Genomic DNA was purified using the DNeasy Blood and Tissue Kit (Qiagen) following the manufacturer's instructions for purifying genomic DNA from Gram-positives and Gram-negatives. Purified DNA obtained from following the Gram-negative and the Gram-positive purification protocol separately were eluted with equal volumes at the final step of purification and were pooled to use for 16S rRNA sequencing analysis. When the source of DNA was mouse fecal pellets, the PowerSoil DNA Isolation Kit from MO BIO (Carlsbad, California) was used following the manufacturer's instructions.

2.16: 16S rRNA Sequencing and Bioinformatics Analyses

16S rRNA sequencing was done at the Integrated Microbiome Resource (IMR) Facility at Dalhousie University. Using the DNA extracted from the protocols described in chapter 2.15, 16S fragments were amplified by PCR in duplicate with template dilutions of 1:1 and 1:10 with the high-fidelity Phusion DNA polymerase using specific fusion primers containing Illumina adaptors, indices, and specific regions that target 438 bp regions of the V6/V7/V8 16S rRNA gene and allow for multiplexing with runs of up to 384 samples. PCR products were confirmed on a high-throughput Invitrogen 96-well E-gel and any failed samples were re-amplified with optimized PCR conditions. PCR products were normalized using the high-throughout Invitrogen SequalPrep 96-well Plate

Kit and pooled to create a single library. The library was quantified and subjected to quality control prior to sequencing. Amplicon samples were run on the Illumina MiSeq with output of approximately 12400 reads per sample. Bioinformatics analysis was conducted using QIIME.

2.17: Statistical Methods

Statistical analyses were performed using GraphPad Prism. A two-way analysis of variance (ANOVA) was performed followed by a Tukey's multiple comparisons test on the bacterial levels recovered from mutant *S. flexneri* infected cells during the gentamicin protection assay. Two-way ANOVAs were performed on the clinical scores and bacterial burden recovered from wild-type and *ΔnarGΔnarZΔnapA* infected mice on days 1-6 post-infection. Bacterial burdens recovered form the spleen and cecum of infected mice on day 6 post-infection were analyzed using a student's t-test. The competitive index of bacteria recovered during the *in vitro* growth assay in the absence and presence of nitrate and from the feces during the murine co-infection experiment were analyzed using a one-way ANOVA with a Dunnett's multiple comparisons test to compare mean values to the reference value of 1. The *ΔtrbH* and *ΔnarGΔnarZΔnapA* mutant *S. flexneri* recovered from the cecal content of co-infected mice were analyzed using a student's t-test. For *Shigella* challenge experiments on consortium-treated and untreated mice, a two-way ANOVA was used to analyze the fecal burden and clinical scores of *Shigella* over the 3-day infection. To evaluate the cecal burden on day 3 post-infection, a student's t-test was used. Survival curves were analyzed using a logrank Mantel-Cox test.

Chapter 3: Results

3.1: A Phosphotransacetylase Mutant of *S. flexneri* is Impaired in Intracellular Growth

To investigate the role of the intracellular PTA-ACKA carbon metabolism pathway in colonization by *S. flexneri*, a mutant defective in a key enzyme in this pathway, phosphotransacetylase (Pta) was constructed using λ red-mediated recombination. Using the same method, the *icsA* gene was also deleted from this strain. Confirmation of knock-out cassette integration at the desired location for all gene deletions is shown in Figure 2. The *icsA* gene is found on the *Shigella* virulence plasmid and its gene product mediates cell to cell spread (Bernardini *et al*., 1989). By deleting *icsA,* intercellular spread is minimized to allow for more accurate measurement of intracellular growth. A gentamicin protection assay was used to assess intracellular growth of the *ΔptaΔicsA* mutant. Compared to the *ΔicsA* control, significantly lower numbers of *ΔptaΔicsA* mutant bacteria were recovered from infected HeLa cells (Figure 3), supporting the previously reported role of the intracellular PTA-ACKA pathway in fueling proliferation of *Shigella* within host cells (Kentner *et al*., 2014). A third deletion was made in the *ΔptaΔicsA* mutant to create a strain that is deleted for the middle third of the *ipaH9.8* gene (amino acids 183-337). Confirmation of the *ipaH9.8* knock-out cassette integration at the desired location is shown in Figure 2C. As discussed in Sidik *et al.* (2014), deletion of the entire *ipaH9.8* gene resulted in a polar mutation therefore the sequence encoding the middle third of *ipaH9.8* was deleted to create the nonpolar mutant. Kaitlyn Tanner, a former student in the Rohde Lab, identified a substrate of the IpaH9.8

E3 ubiquitin ligase as ZKSCAN3, a negative regulator of autophagy. Her work suggests that a putative virulence mechanism of *Shigella* is to induce autophagy to acquire nutrients in the mammalian cytosol through IpaH9.8 activity. To test this hypothesis, the *ΔptaΔicsAΔipaH9.8* mutant was also assessed in the gentamicin protection assay to test whether loss of IpaH9.8 activity in the *Δpta* background would further impair intracellular proliferation. Levels of *ΔptaΔicsAΔipaH9.8* mutant bacteria recovered from infected HeLa cells were also significantly reduced compared to the *ΔicsA control* but similar to levels recovered from *ΔptaΔicsA* infected cells (Figure 3).

In the PTA-ACKA pathway, Pta converts acetyl-CoA produced by the upstream aceE enzyme to acetyl phosphate. Levels of bacteria recovered from both *ΔptaΔicsA* and *ΔptaΔicsAΔipaH9.8* infected cells were restored to *ΔicsA* levels by supplementing the cell culture medium with 100 mM acetyl phosphate. (Figure 3). To ensure that the increase in bacteria recovered from *ΔptaΔicsA* and *ΔptaΔicsAΔipaH9.8* infected cells in the presence of exogenous acetyl phosphate was not a result of the compound interfering with gentamicin-mediated killing, wild type *S. flexneri* was grown in the presence and absence of gentamicin, and in the presence of both acetyl phosphate and gentamicin. In the presence of acetyl phosphate, gentamicin retained its ability to significantly inhibit the growth of wild type *S. flexneri* (data not shown).

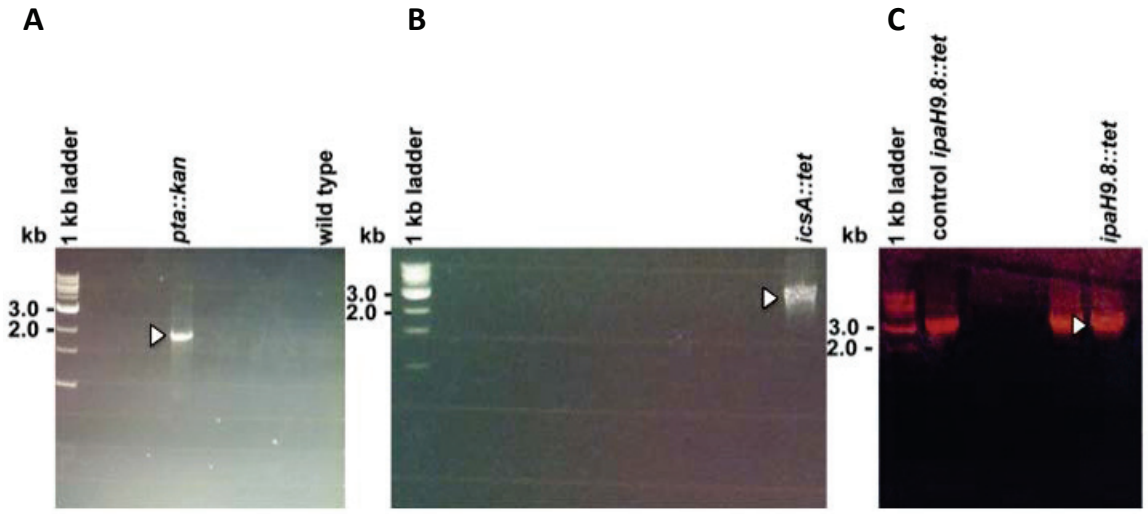


Figure 2: Confirmation of knock-out cassette integration for construction of the *ΔptaΔicsA* and *ΔptaΔicsAΔipaH9.8* mutants. DNA fragments were amplified by colony PCR with a reverse primer targeting the knock-out cassette and a forward primer targeting sequence outside of the region of gene deletion, indicated in Table 3. Wild type *S. flexneri* (wild type) or *ΔipaH9.8* mutant *S. flexneri* (control *ipaH9.8::tet*) was used as a negative or positive control, respectively, where indicated. PCR products were separated by ethidium bromide 1% agarose gel electrophoresis. White arrows indicate bands of the appropriate size confirming (A) deletion of the *pta* gene in the wild type *S. flexneri* background, expected size: 2.1 kb with the primers ptaFWcheck and P2, (B) deletion of the *icsA* gene in the *Δpta* mutant *S. flexneri* background, expected size: 2.2 kb with the primers icsALR1F and icsAR2F, (C) deletion of the middle third of the *ipaH9.8* gene in the *ΔptaΔicsA* background, expected size: 2.8 kb with the primers ipaH9.8prIF and ipaH9.8-C337del-rev.

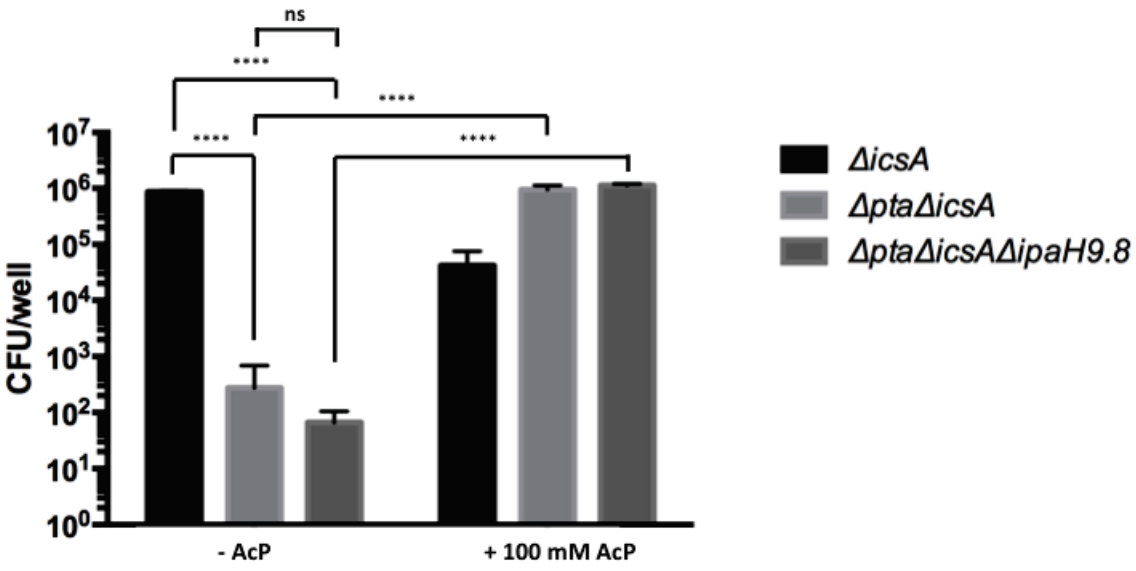


Figure 3: Gentamicin protection assays were performed using HeLa cells infected with *ΔicsA*, *ΔptaΔicsA*, and *ΔptaΔicsAΔipaH9.8* mutant *S. flexneri* transformed with the *afaE* expressing plasmid at an MOI of 10. The number of colony-forming units (CFU) recovered from each well of infected cells was determined in the absence and presence of exogenous acetyl phosphate (100mM). A two-way analysis of variance (ANOVA) was performed with a Tukey's multiple comparison post test. Error bars indicate standard deviation of three replicates. ****P < 0.0001.

3.2: Deletion of *narG, narZ,* and *napA* Genes Results in Loss of Nitrate Reductase Activity in *S. flexneri*

To investigate the role of nitrogen respiration in colonization by *S. flexneri*, three genes, each encoding a component of *Shigella's* nitrate reductase machinery, were sequentially deleted using λ red-mediated recombination. Confirmation of knock-out cassette integration at the desired location for each gene deletion is shown in Figure 4. Following deletion of the nitrate reductase genes to create the *ΔnarGΔnarZΔnapA* triple mutant, the mutant was tested for the ability to reduce nitrate using a qualitative assay. Wild type *S. flexneri* gave a positive test result indicated by the appearance of a red precipitate known as prontosil (Figure 5). By contrast, the *ΔnarGΔnarZΔnapA* mutant retained its original media colour and a red precipitate was not visible (Figure 5). To test for false negatives, zinc powder was added as a reducing agent to encourage the reduction of any unused nitrate in the tube inoculated with the *ΔnarGΔnarZΔnapA* mutant. Following the addition of zinc, a red precipitate was visible (Figure 5), indicating a true negative result for nitrate reductase activity. I conclude that the *ΔnarGΔnarZΔnapA* mutant is completely defective for nitrate reductase activity.

To confirm that the loss of nitrate reductase activity in the mutant strain was a result of the deletion of the *narG*, *narZ*, and *napA* genes, *narZ* was cloned into the pBluescript II KS+ expression vector to make p*narZ*. Mutant *ΔnarGΔnarZΔnapA Shigella* was transformed with either p*narZ* or pBluescript II KS+ empty vector and nitrate reductase assays were performed. We observed that a *ΔnarGΔnarZΔnapA* strain harboring p*narZ* could reduce nitrate while *ΔnarGΔnarZΔnapA* could not (Figure 5). These data indicate that the presence of *narZ* was sufficient to restore nitrate reductase

activity to the *ΔnarGΔnarZΔnapA* mutant strain. The addition of zinc powder to *ΔnarGΔnarZΔnapA* transformed with empty vector as well as to the uninoculated control resulted in the formation of a red precipitate, indicating a lack of nitrate reductase activity in these tubes (Figure 5).

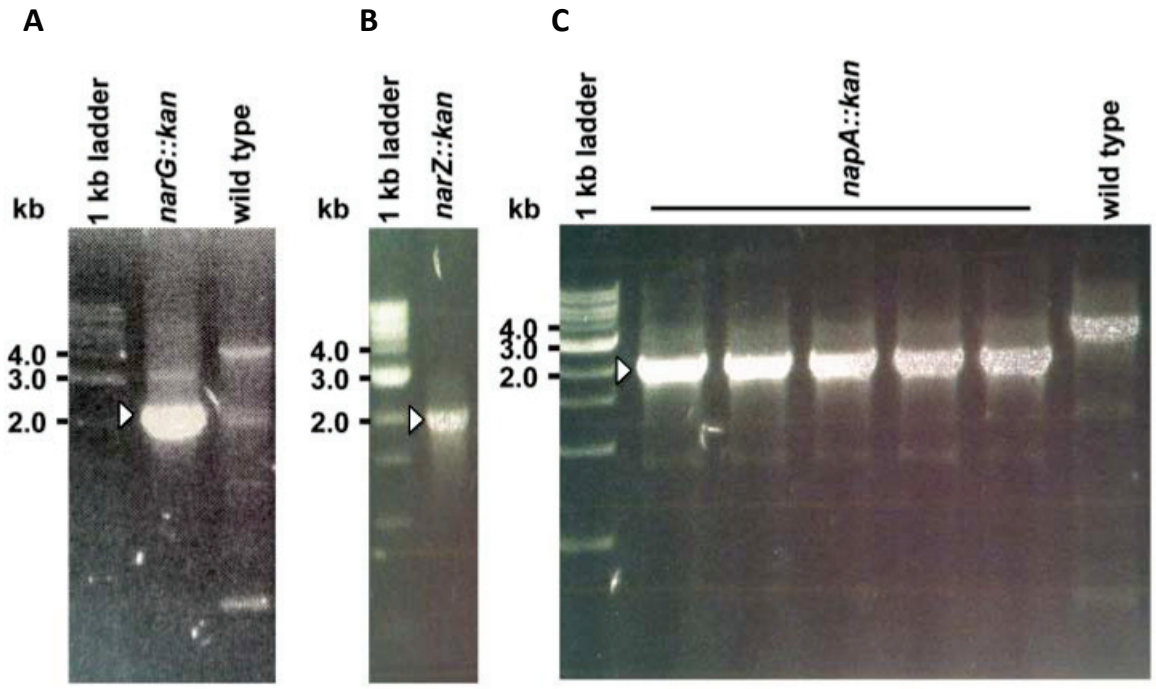


Figure 4: Confirmation of knock-out cassette integration for construction of the *ΔnarGΔnarZΔnapA::kan* mutant. DNA fragments were amplified by colony PCR with a reverse primer targeting the knock-out cassette and a forward primer targeting sequence outside of the region of gene deletion, indicated in Table 3. Wild type *S. flexneri* (wild type) was used as a negative control where indicated. PCR products were separated by ethidium bromide 1% agarose gel electrophoresis. White arrows indicate bands of the appropriate size confirming (A) deletion of the *narG* gene in the wild type *S. flexneri* background, expected size: 2.5 kb with the primers narGFWcheck and narGRV, (B) deletion of the *narZ* gene in the *ΔnarG* mutant *S. flexneri* background, expected size: 2.2 kb with the primers narZFWcheck and narZRV, (C) deletion of the *napA* gene in the *ΔnarGΔnarZ* background, expected size: 2.3 kb with the primers napAFWcheck and napARV.

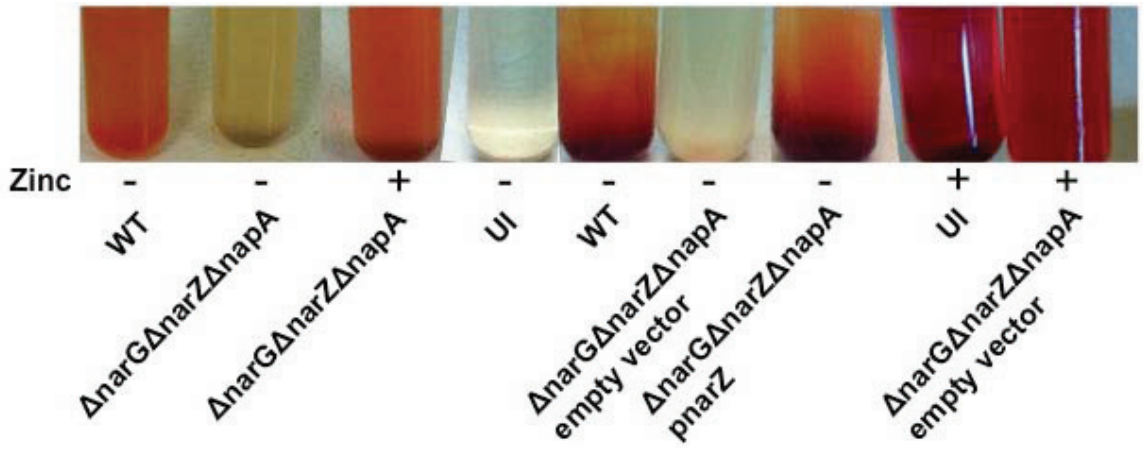


Figure 5: *S. flexneri* deleted for the *narG*, *narZ*, and *napA* genes encoding nitrate reductase components was tested for nitrate reductase activity using the Nitrate Reductase Test Kit (Sigma-Aldrich). Tubes containing nitrate broth were inoculated with wild type *S. flexneri* (WT), the *ΔnarGΔnarZΔnapA* mutant, *ΔnarGΔnarZΔnapA* transformed with empty vector, *ΔnarGΔnarZΔnapA* transformed with p*narZ*, or left uninoculated (UI). Bacteria were grown anaerobically at 37 °C for 48 hours and kit reagents were added according to the manufacturer's instructions.

3.2.1: *ΔnarGΔnarZΔnapA* and *ΔtrbH* Mutant *Shigella* Display Similar *in vitro* Growth in the Absence and Presence of Nitrate.

To test whether the ability to reduce nitrate confers a growth advantage to *S. flexneri* in nitrate medium *in vitro*, the competitive index was measured during anaerobic growth in mucin broth containing 40 mM sodium nitrate. To allow co-inoculation and subsequent recovery of single strains, a mutant for *trbH* was used to represent wild type *S. flexneri* and for comparison with the *ΔnarGΔnarZΔnapA* mutant. Nitrate reductase genes in the *trbH* mutant are uninterrupted and deletion of the small 720 bp *trbH* ORF encoded on *Shigella*'s virulence plasmid has no effect on plasmid stability (Radnedge *et al*., 1997). The role of the putative TrbH protein is unknown but the gene product has no homologues among proteins with available sequences to date. The *trbH* mutant was chosen since the deletion of *trbH* is likely inconsequential (Radnedge *et al*., 1997) and the mutant can be easily selected when grown with *ΔnarGΔnarZΔnapA* since these strains have tetracycline, and kanamycin resistance markers, respectively. The nitrate-containing medium was inoculated with a 1:1 ratio of *ΔtrbH* and *ΔnarGΔnarZΔnapA* and grown for sixteen hours anaerobically. Serial dilutions were made and the inoculated culture was plated on both selective Congo red agar media containing the appropriate antibiotics. Colonies were counted following overnight incubation at 37 °C and the competitive index was calculated. In the absence of nitrate, *ΔtrbH* and *ΔnarGΔnarZΔnapA* bacteria were recovered in equal amounts while in the presence of nitrate, the number of *ΔtrbH* mutant bacteria recovered was approximately 1 log higher than the number of *ΔnarGΔnarZΔnapA* bacteria recovered but the competitive index value was not significantly different from the reference value of 1 (Figure 6).

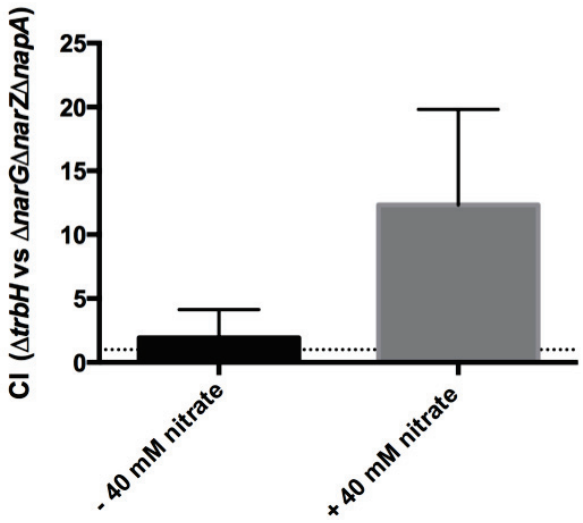


Figure 6: Competitive Index (CI) of *ΔtrbH* versus *ΔnarGΔnarZΔnapA* mutant *S. flexneri* following anaerobic growth in mucin broth in the absence (-) and presence (+) of 40 mM nitrate. Mucin broth was inoculated with a 1:1 ratio of the bacterial strains and incubated anaerobically at 37 °C for sixteen hours. Serial dilutions were plated and bacterial colonies were counted following overnight growth. The competitive index was calculated by dividing the number of *ΔtrbH* by the number of *ΔnarGΔnarZΔnapA* mutant bacteria recovered from growth in each condition. Means with standard deviations are shown. Data were analyzed using a one-way ANOVA with a Dunnett's multiple comparisons test to compare mean values to a reference value of 1 (indicated by the dotted line) and found to be non significant.

3.2.2: Mice Infected with Wild type and *ΔnarGΔnarZΔnapA* Mutant *S. flexneri* Show Similar Signs of Illness, and Have Similar Bacterial Burden in the Feces, Cecum, and Spleen.

To test the hypothesis that loss of nitrate reductase activity impairs the ability of *S. flexneri* to colonize *in vivo* using the murine model of *Shigella* infection, 6-8 week old female BALB/c mice were infected with wild type *S. flexneri* or *ΔnarGΔnarZΔnapA* mutant *S. flexneri*. A total of 10 mice, n=5 per group, were infected with each of the *S. flexneri* strains at a dose of approximately 10^8 CFU/mouse two days after streptomycin treatment was initiated. Over the course of the 6-day infection, clinical scores and bacterial burden in the feces were monitored daily and bacterial burden in the cecum, spleen, and liver was measured after euthanizing the mice on day 6. To measure bacterial burden, fecal, cecal, or organ samples obtained were weighed, diluted in PBS when appropriate, and homogenized when liver and spleen samples were used. Serial dilutions (1:10) were made and plated on MacConkey agar with streptomycin. Bacterial burden in the feces was similar between wild type-infected and *ΔnarGΔnarZΔnapA*-infected mice during the course of the 6 day infection. (Figure 7A). Although, the bacterial burden was slightly higher in *ΔnarGΔnarZΔnapA*-infected mice from day 3-6 post infection, differences were not significant.

Similar bacterial burden in the spleen and cecal content, sampled on day 6 post infection, was observed in both wild type-infected and *ΔnarGΔnarZΔnapA*-infected mice (Figure 7C and 7D, respectively). Bacterial dissemination to the liver during infection was not observed therefore these data are not shown. Clinical scores were similar between wild type- and *ΔnarGΔnarZΔnapA*-infected mice on day 1-5 post infection with

no significant differences between groups (Figure 7B). On day 6, *ΔnarGΔnarZΔnapA*-infected mice displayed significantly elevated clinical scores compared to the wild type-infected group (Figure 7B). Taken together, these data suggest that the ability to reduce nitrate does not play a role in colonization in our mouse model of *Shigella* infection.

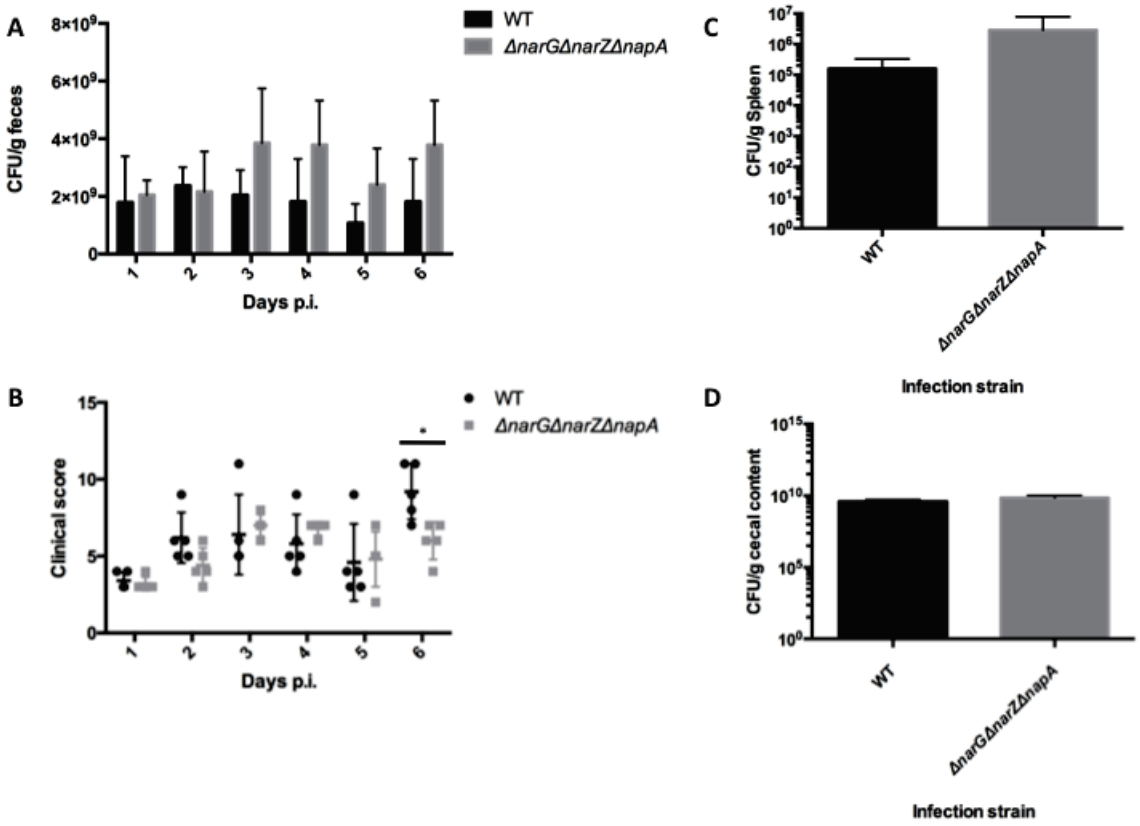


Figure 7: (A) Bacteria recovered in colony forming units (CFU) per gram (g) of fecal content from wild type-infected (n=5) and *ΔnarGΔnarZΔnapA* infected (n=5) mice on day 1-6 post infection (p.i.). Values of means with standard deviations are shown. Bacterial burden between strains was not significant at each day p.i. Statistical analysis was performed using a two-way ANOVA. (B) Clinical scores of wild type- and *ΔnarGΔnarZΔnapA*- infected mice from 1-6 days p.i. Clinical scores between infection groups were non significant except on day 6 p.i. A two-way ANOVA was performed. (C) Bacteria recovered in log (CFU/g) of spleen from wild type-infected and *ΔnarGΔnarZΔnapA*-infected mice on day 6 p.i. Bacterial burden between strains was not significant. Statistical analysis was performed using a t-test. (D) Bacteria recovered in log (CFU/g) of cecal content from wild type-infected and *ΔnarGΔnarZΔnapA*-infected mice on day 6 p.i. Bacterial burden between strains was not significant. Statistical analysis was performed using a t-test. In all panels, results shown are means ± SD. There were 5 mice in each group.

3.2.3: Nitrate Reductase Mutant *S. flexneri* are not Impaired for Colonization During Co-infection of the Streptomycin Treated Mouse.

To test whether nitrate reductase activity confers a competitive advantage to *S. flexneri* during colonization of the murine intestine, BALB/c mice were co-infected with a 1:1 ratio of *ΔtrbH* and *ΔnarGΔnarZΔnapA* mutant *S. flexneri*. Clinical scores were monitored and fecal samples were obtained daily, serially diluted, and plated on both MacConkey streptomycin agar containing tetracycline and MacConkey streptomycin agar with kanamycin to measure the bacterial burden of each strain. Mice were euthanized at day 6 post infection and the liver, spleen, and cecal content were harvested. The liver and spleen were first homogenized and then all samples were used for serial dilutions and plated to measure bacterial burden at these sites within the mouse. The *ΔtrbH* and *ΔnarGΔnarZΔnapA* mutant strains were recovered in relatively equal amounts from the feces over the six day infection with the average competitive index (CI) of *ΔtrbH to ΔnarGΔnarZΔnapA* mutant *S. flexneri* not exceeding values of 0.5 above or below the reference CI of 1 (Figure 8A). Similarly, the bacterial burden in the cecal content on day 6 post infection was not significantly different between *ΔtrbH* and *ΔnarGΔnarZΔnapA* mutant *S. flexneri* (Figure 8B). Dissemination of bacteria to the liver and spleen during infection was not detected.

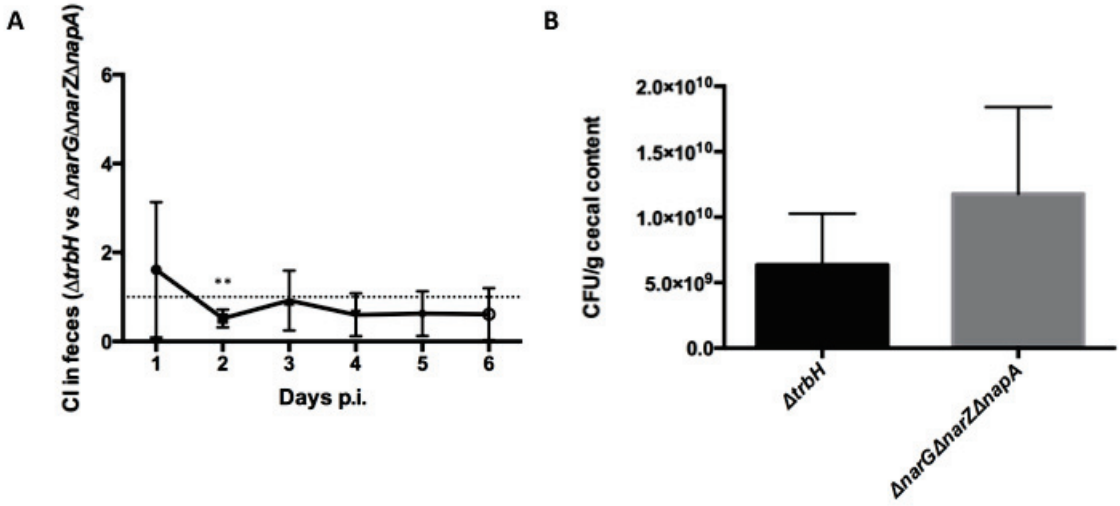


Figure 8: (A) Competitive Index (CI) of *ΔtrbH* versus *ΔnarGΔnarZΔnapA* mutant *S. flexneri* recovered from the feces of mice co-infected with a 1:1 ratio of the bacterial strains over 6 days post-infection. Means with standard deviations are shown. Statistical analysis was performed using a one-way ANOVA followed by a Dunnet's multiple comparisons test to compare mean values to a reference value of 1 (indicated by the dotted line). ** $P < 0.01$, otherwise non-significant. (B) Bacteria recovered in colony forming units per gram (CFU/g) of cecal content from mice co-infected with a 1:1 ratio of wild type and *ΔnarGΔnarZΔnapA* mutant *S. flexneri* on day 6 post-infection (p.i). Means with standard deviations are shown. Bacterial burden between strains was non significant. Statistical analysis was performed using a t-test.

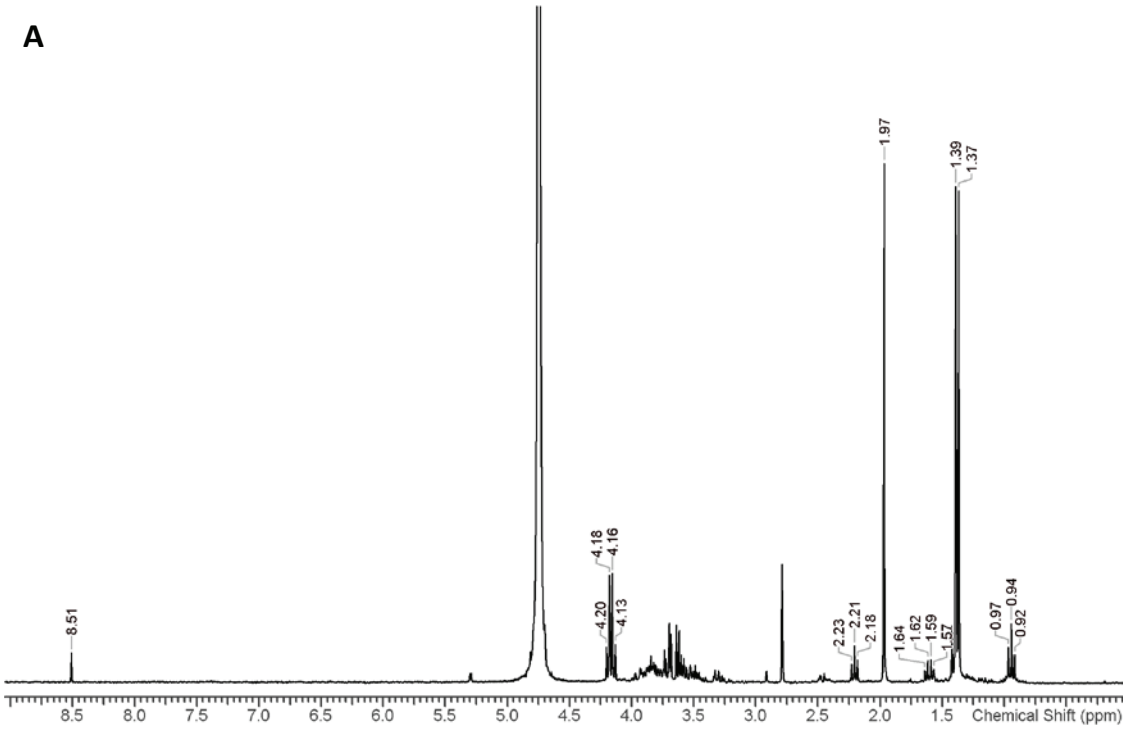
3.3: A Consortium of Streptomycin-Resistant, Butyrate-Associated Anaerobes Isolated from the Healthy Mouse Produces Butyrate *In Vitro*

Next, the role of the commensal bacteria in colonization resistance to *S. flexneri* in the murine intestine was investigated. Fecal pellets were obtained from healthy, specific pathogen free, 6-8 week old female BALB/c mice from Charles River Laboratories. Fecal pellets were used as the source from which anaerobic commensal bacteria were cultured. For bacteria to be compatible with the murine model of *Shigella* infection, streptomycin was included in the culture media to select for streptomycin resistance. Butyrate was also included in the culture media to enrich for butyrate-associated bacteria that we hypothesized might produce butyrate and contribute to colonization resistance against *S. flexneri*. The process of growing streptomycin-resistant, butyrate-associated anaerobes is described in detail in chapter 2.12 of the materials and methods. Cultured consortium 1 refers to the first experiment, while cultured consortium 2 and 3 were obtained from new mice each time and were used for ^{1}H NMR analysis. The control consortium grown in the absence of butyrate was cultured from the same mice and fecal matter used to culture the cultured consortium 3.

Following the enrichment process, the consortium bacteria were tested for butyrate production using ^{1}H NMR. Multiple SCFAs were detected in the cultured consortium 2 bacterial supernatant including formate, lactate, acetate, and butyrate (Figure 9A). Unmetabolized glucose was also detected (Figure 9A). When this experiment was repeated using the cultured consortium 3 bacteria, acetate and lactate were detected however butyrate and formate were not (Figure 9C). Unmetabolized glucose was also detected in the supernatant of the cultured consortium 3 bacteria as well

as in the supernatant from bacteria grown in the absence of butyrate (Figure 9C and D). No further metabolites were detected in the consortium grown in the absence of butyrate (Figure 9D). Standard solutions of sodium butyrate and glucose were also made as described in chapter 2.14 of the materials and methods and ^{1}H NMR spectra were obtained (Figure 9B and Figure 9E, respectively).

A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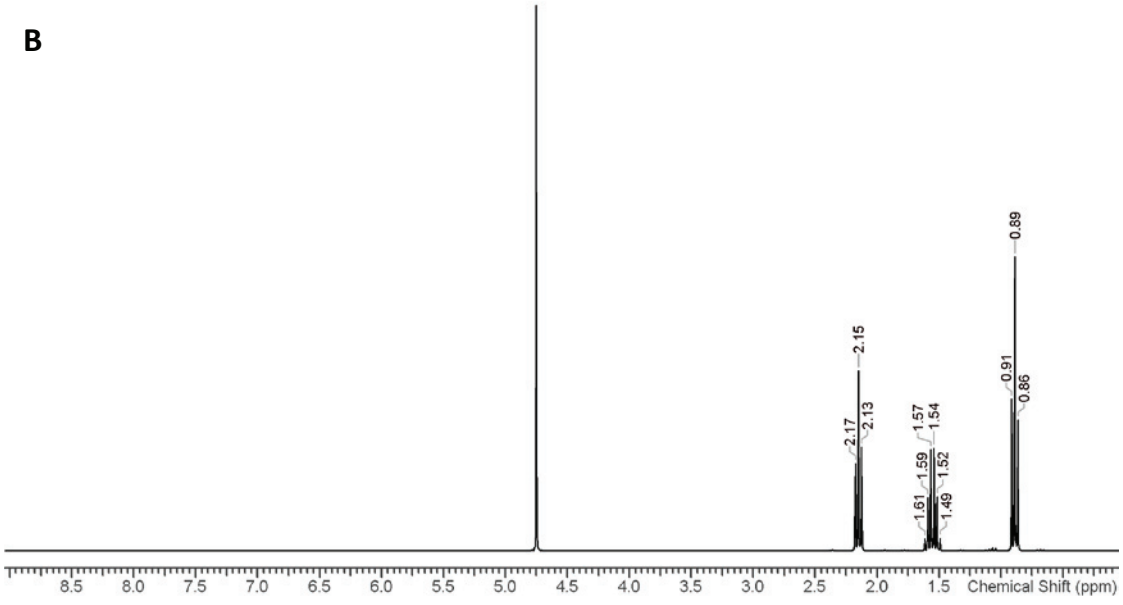
B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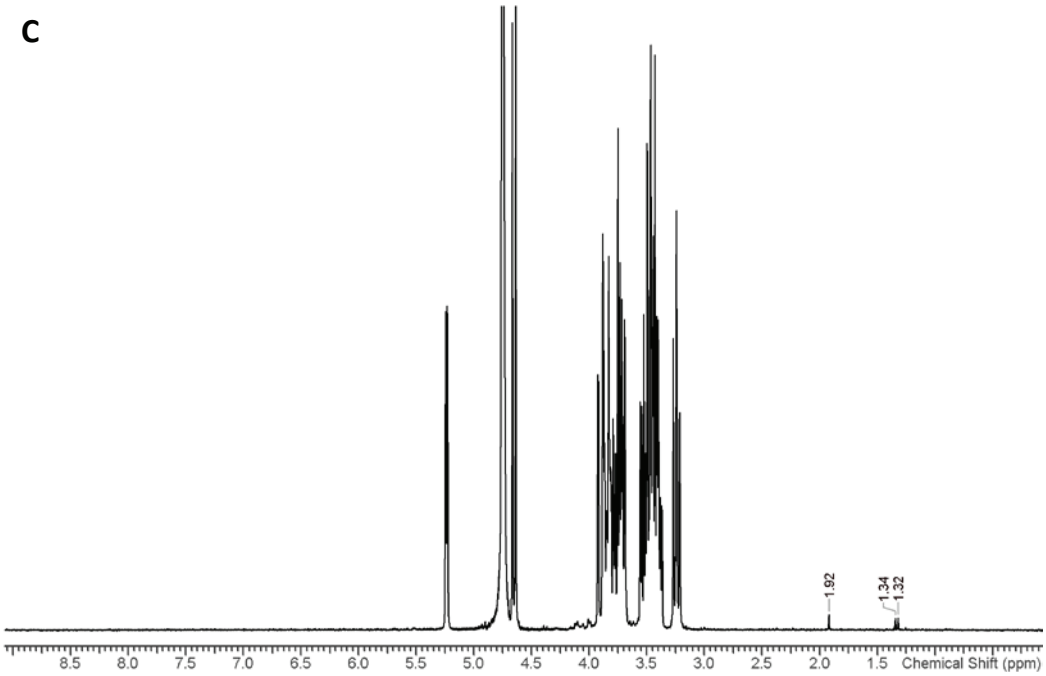
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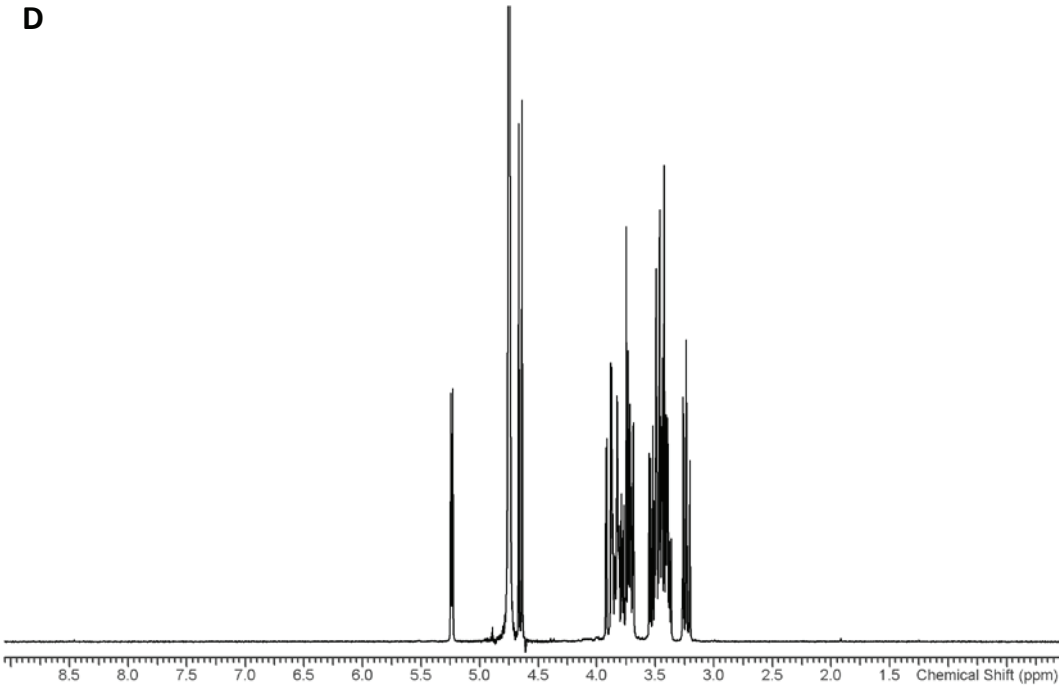
D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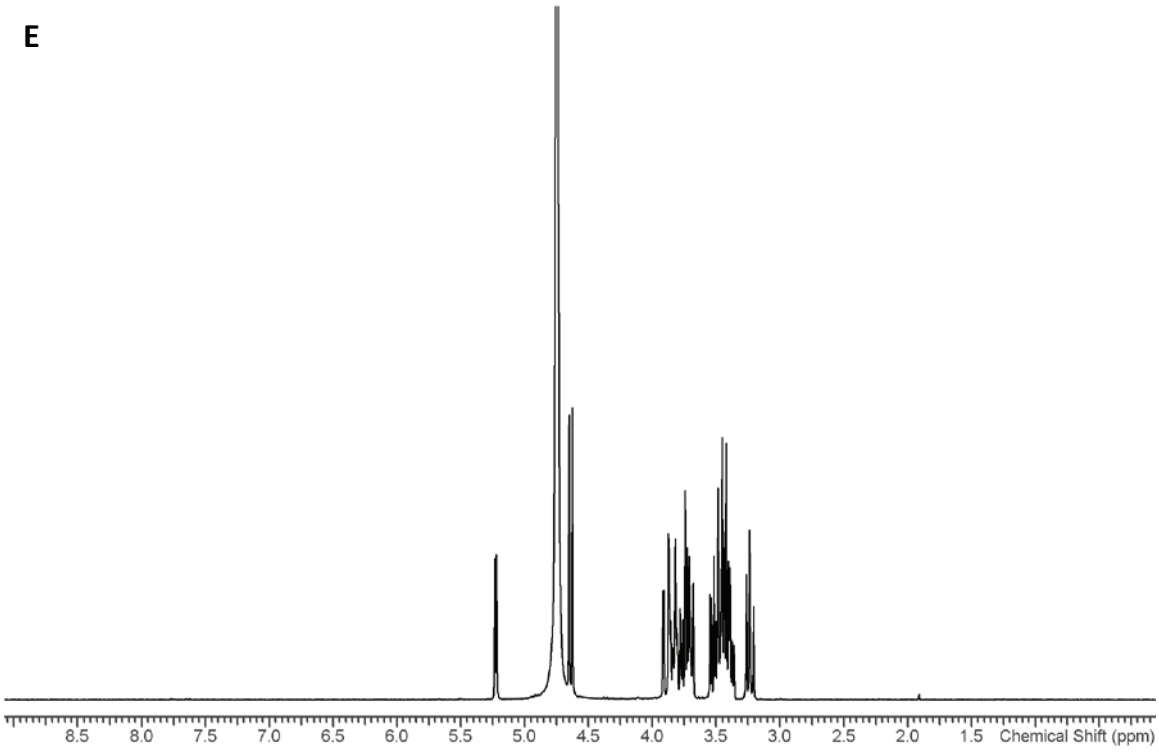
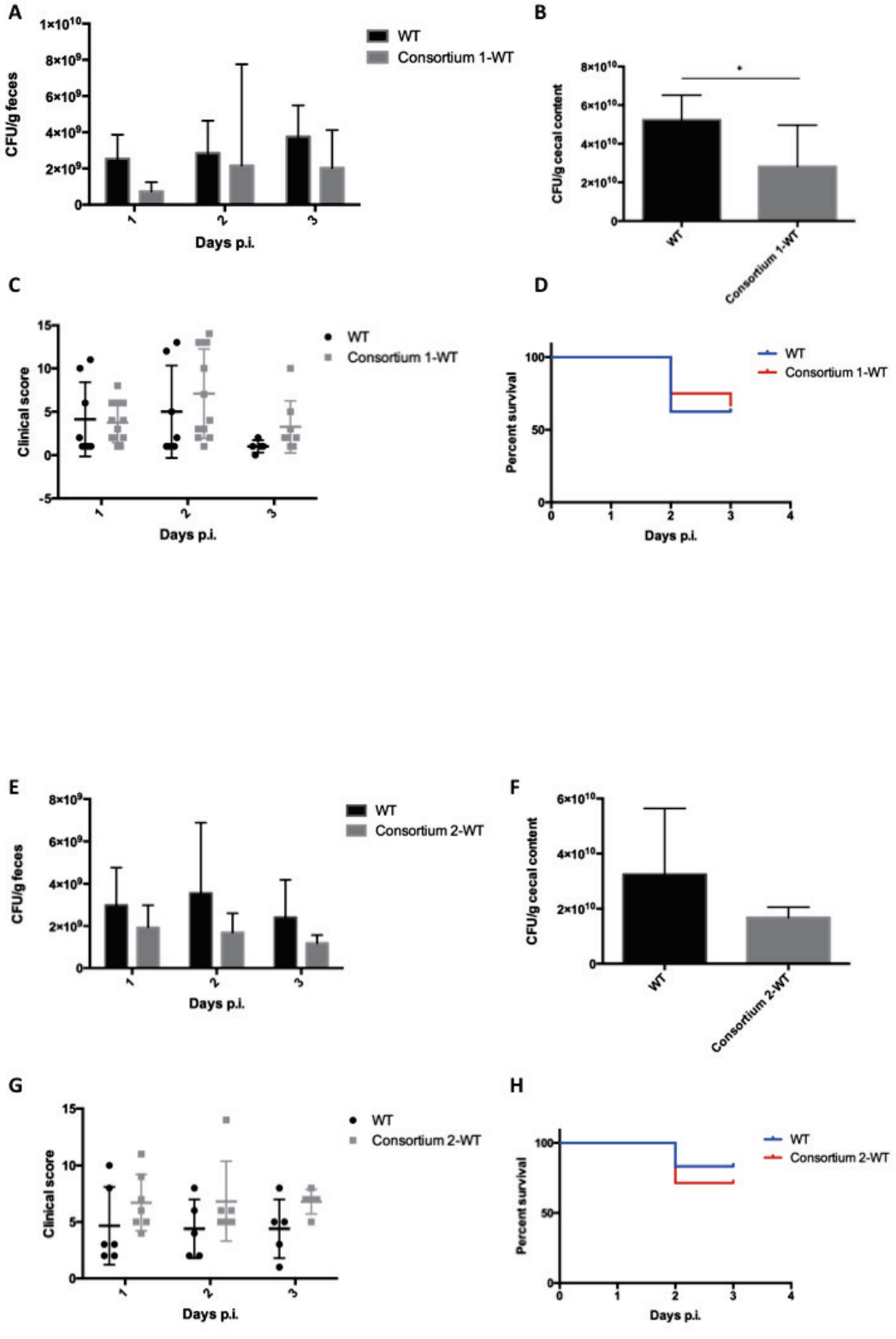


Figure 9: ^{1}H NMR spectra of the supernatant of cultured consortia of bacteria or standard solutions. Cultured consortium bacteria were incubated in PBS with 10 mM glucose for 24 hours anaerobically. After incubation, the supernatant was adjusted to pH 9.5 and lyophilized. A portion of the residue was dissolved in D_2O. Reference signals appear at approximately δ 4.75. (A) ^{1}H NMR spectrum of cultured consortium 2 with signals assigned to formate (δ 8.51), lactate (δ 1.37-1.39, 4.13-4.20), acetate (δ 1.97), and butyrate (δ 0.92-0.97, 1.57-1.64, 2.18-2.23). (B) ^{1}H NMR spectrum of a standard solution of sodium butyrate made by dissolving 6 mg in 900 μL D_2O. Signals appear at δ 0.86-0.91, 1.49-1.61, 2.13-2.17. (C) ^{1}H NMR spectrum of cultured consortium 3 with signals assigned to acetate (δ 1.92) and lactate (δ 1.32, 1.34). (D) ^{1}H NMR spectrum of control consortium grown in the absence of butyrate. (E) ^{1}H NMR spectrum of a standard solution of glucose made by dissolving 20 mg in 800 μL D_2O.

3.3.2 Administration of Streptomycin-resistant, Butyrate-associated Consortium Bacteria Prior to Infection with *S. flexneri* Does Not Alter *Shigella* Burden, Clinical Score, or Percent Survival.

To test the hypothesis that a streptomycin-resistant, butyrate-associated consortium of commensals cultured anaerobically from the healthy BALB/c mouse confers protection against colonization by *Shigella*, the consortium of cultured commensal bacteria was introduced into the murine model prior to challenge with *S. flexneri*. Cultured consortium 1 and cultured consortium 2 were each introduced into the mouse model in independent sets of experiments. Mice were given streptomycin 2 days prior to infection and the following day treated with a suspension of consortium bacteria by oral gavage, or left untreated. The next day, mice were orally infected with *S. flexneri* and monitored for 3 days post-infection. Clinical scores were measured and fecal samples were obtained daily to assess the burden of *S. flexneri*. Percent survival was similar between consortium-treated and untreated, wild type *S. flexneri* infected mice (Figure 10D and H). Clinical scores between the consortium-treated and untreated *S. flexneri* infected mice over the course of the 3-day infection were also similar (Figure 10C and G). The fecal burden of *S. flexneri* was also similar between consortium-treated and untreated *S. flexneri* infected mice on days 1-6 post-infection (Figure 10A and E). The burden of *S. flexneri* recovered from the cecal content of untreated *S. flexneri* infected mice was slightly higher than the levels recovered from infected mice pre-treated with the cultured consortium 1 bacteria (Figure 10B), however similar levels of *S. flexneri* were recovered from the cecal content between *S. flexneri* infected mice and infected mice pretreated with the cultured consortium 2 bacteria (Figure 10F).


A
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Clinical score
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D
Percent survival
WT
Consortium 1-WT
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E
CFU/g feces
WT
Consortium 2-WT
Days p.i.
F
CFU/g cecal content
WT
Consortium 2-WT
G
Clinical score
WT
Consortium 2-WT
Days p.i.
H
Percent survival
WT
Consortium 2-WT
Days p.i.

Figure 10: (A, E) *Shigella* recovered in colony forming units (CFU) per gram (g) of fecal content from untreated wild type *S. flexneri* infected (WT) (A: n=8 on day 1, 7 on day 2, 5 on day 3, E: n=6 on day 1, 5 on days 2 and 3) and consortium 1 or 2-treated wild type *S. flexneri* infected (Consortium 1-WT or Consortium 2-WT) (A: n=12 on day 1, 11 on day 2, 8 on day 3, E: n=6 on day 1, 5 on days 2 and 3) mice on day 1-3 post infection (p.i.). Values of means with standard deviations are shown. Bacterial burden between consortium-treated and untreated mice was non significant at each day p.i. Statistical analysis was performed using a two-way ANOVA. (B, F) *Shigella* recovered in colony forming units (CFU) per gram (g) of cecal content from untreated *S. flexneri* infected (WT) (B: n=5, F n=5) and consortium 1 or 2-treated *S. flexneri* infected (Consortium 1-WT or Consortium 2-WT) (B: n=8, F: n=6) mice on day 3 post-infection (p.i.). Values of means with standard deviations are shown. * $P < 0.05$. Statistical analysis was performed using a t-test. (C,G) Clinical scores of untreated wild type *S. flexneri* infected (WT) (C: n=8 on day 1, 7 on day 2, 5 on day 3, G: n=6 on day 1, 5 on days 2 and 3) and consortium-treated wild type *S. flexneri* infected (Consortium 1-WT or Consortium 2-WT) (C: n=12 on day 1, 12 on day 2, 8 on day 3, G: n=7 on day 1, 6 on day 2, 5 on day 3) mice from 1-3 days p.i. Values of means with standard deviations are shown. Statistical analysis was performed using a two-way ANOVA. Means were not significantly different between consortium-treated and untreated infected groups on days 1-3 p.i. (D, H) Percent survival of untreated wild type *S. flexneri* infected (WT) (D: n=8, H: n=6) and consortium 1 or 2-treated wild type *S. flexneri* infected (Consortium 1-WT or Consortium 2-WT) (D: n=12, H: n=7) mice from days 1-3 post infection (p.i.). Statistical analysis was performed using a logrank Mantel-Cox test.

3.3.1 Characterization of Consortium Bacteria by 16S rRNA Sequencing

To characterize the streptomycin-resistant, butyrate-associated consortium of bacteria cultured from the healthy BALB/c mouse, genomic DNA was purified from the cultured consortium 1, 2, and 3 bacteria as well as the control consortium of bacteria grown in the absence of butyrate and used for 16S rRNA sequencing. DNA was also extracted from the feces of of mice treated with streptomycin and orally gavaged with the cultured consortium 2 bacteria one day after consortium-treatment and prior to *S. flexneri* infection and also from streptomycin-treated mice orally gavaged with the cultured consortium 2 bacteria 1 day post *S. flexneri* infection. The two samples obtained from consortium 2-treated mice were taken from independent experiments. DNA samples extracted from the feces of healthy, streptomycin-treated, and streptomycin-treated *S. flexneri* infected BALB/c mice were also included in the analysis and were prepared by Angela Daurie, a fellow student in the Rohde Lab. 16S rRNA sequencing was conducted in collaboration with the Comparative Genomics and Evolutionary Bioinformatics (CGEB) group at the IMR Facility at Dalhousie University.

The variability in the sequenced samples is represented in the principal component analysis (PCA) in figure 11. The two samples representative of the healthy BALB/c mouse intestinal microbiota clustered in close proximity to one another as did the cultured consortium samples (1, 2, and 3) grown anaerobically in the presence of streptomycin and butyrate and a clear separation among these two groups is visible (Figure 11). The samples representative of the streptomycin treated, the streptomycin consortium 2-treated, and the streptomycin treated *S. flexneri* infected mice are clearly separated from the healthy mice samples and the cultured consortium 1, 2, and 3 samples

(Figure 11). The sample representative of the consortium cultured anaerobically in the absence of butyrate is in close proximity to the sample representative of the *S. flexneri* infected mouse gut (Figure 11). Further analysis showed that the sample representative of the control consortium cultured in the absence of butyrate was dominated by high levels (90%) of unclassified Enterobacteriaceae (data not shown).

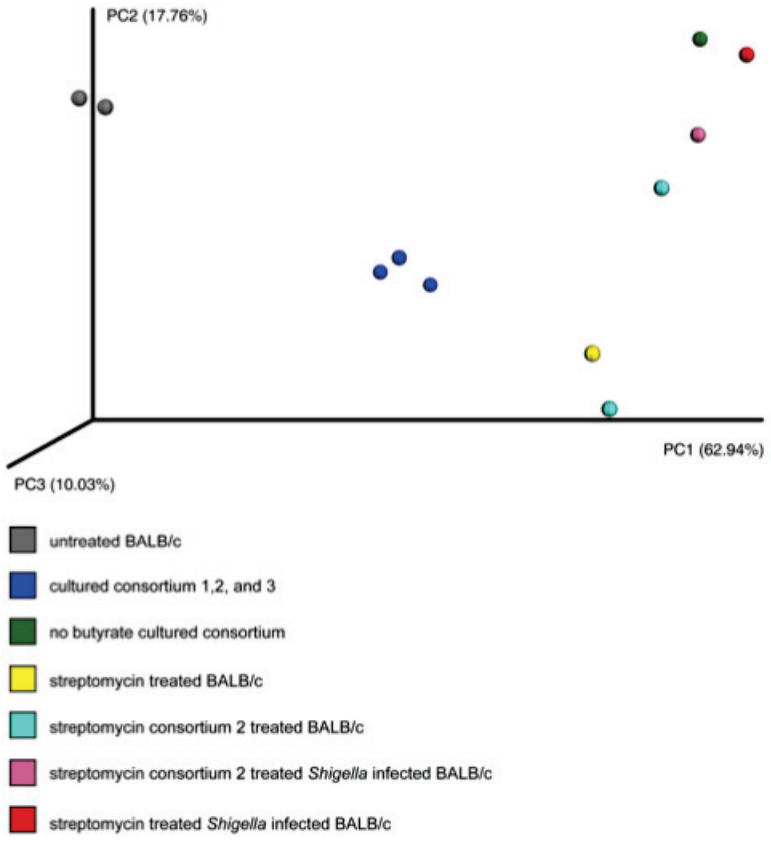


Figure 11: Principal component analysis (PCA) plot showing the first (x-axis) and second (y-axis) principal components of the weighted beta-diversity of 16S rRNA sequences derived from DNA samples extracted from the feces of BALB/c mice under the indicated treatment and/or infection conditions or from DNA samples extracted from the cultured consortium bacteria grown in the presence (dark blue) and absence (green) of butyrate.

Stacked bar graphs showing the microbial diversity in the samples representative of the healthy BALB/c mouse gut and the three consortiums of bacteria cultured independently in the presence of streptomycin and butyrate are shown in Figure 12. The predominant genera detected in the healthy BALB/c mouse were *Parabacteroides*, unclassified members of the order Clostridiales, *Bacteroides*, unclassified members of the Lachnospiraceae family, unclassified members of the Ruminococcaceae family and lower levels of genera including *Lactobacillus*, and unclassified members of the Peptococcaceae family (Figure 12). Species within the *Bacteroides*, *Parabacteroides,* *Paenibacillus,* and *Lactococcus* genera were reproducibly predominant in the consortia of bacteria cultured using the selective butyrate media (Figure 12). High proportions of *Lactobacillus* were common to the cultured consortium 2 and 3 samples, while higher proportions of members of the Clostridiales order were common between the cultured consortium 2 and 3. Unclassified members of the Clostridiaceae family were also reproducibly detected in the cultured consortium samples at varying levels (Figure 12).

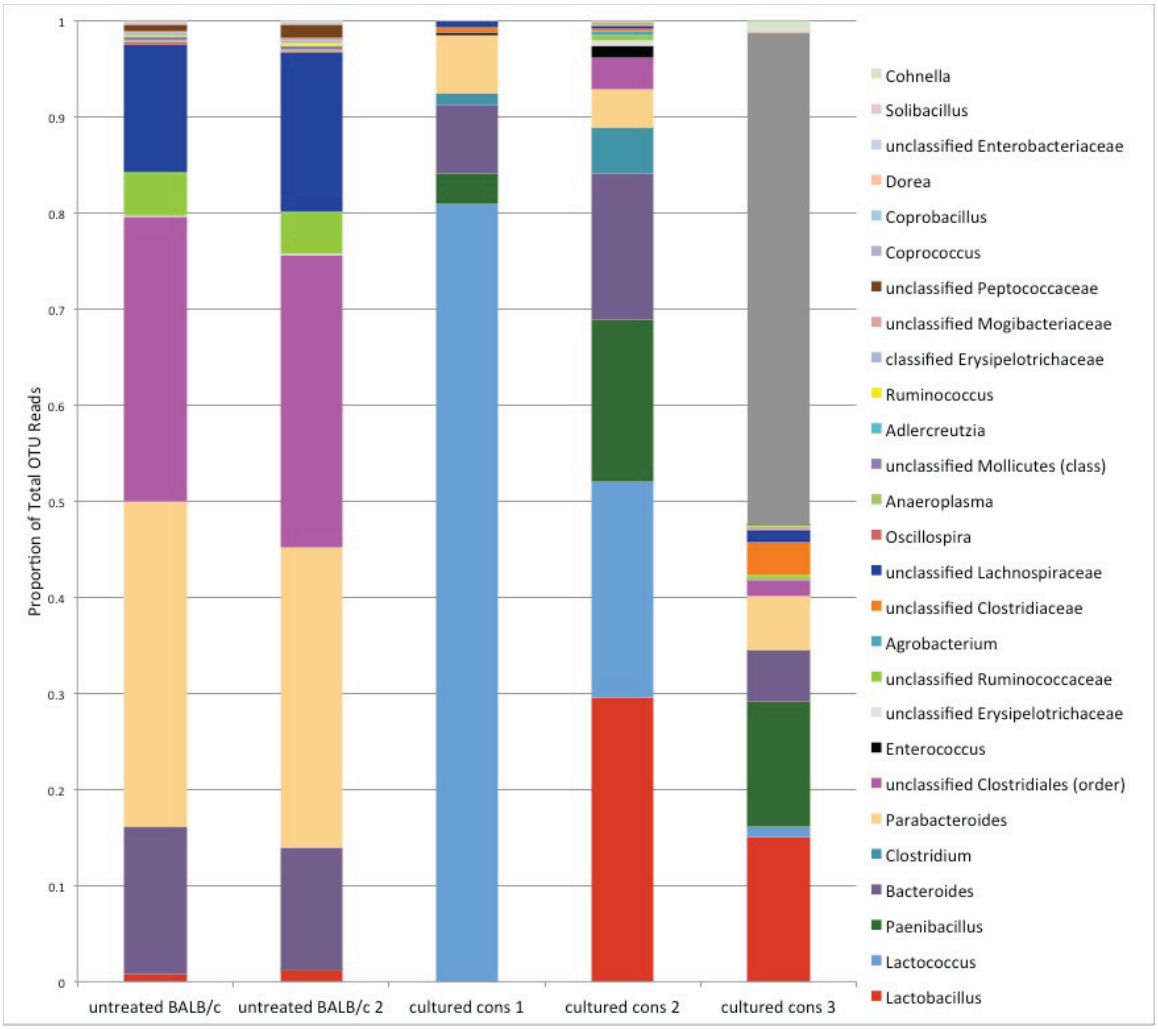


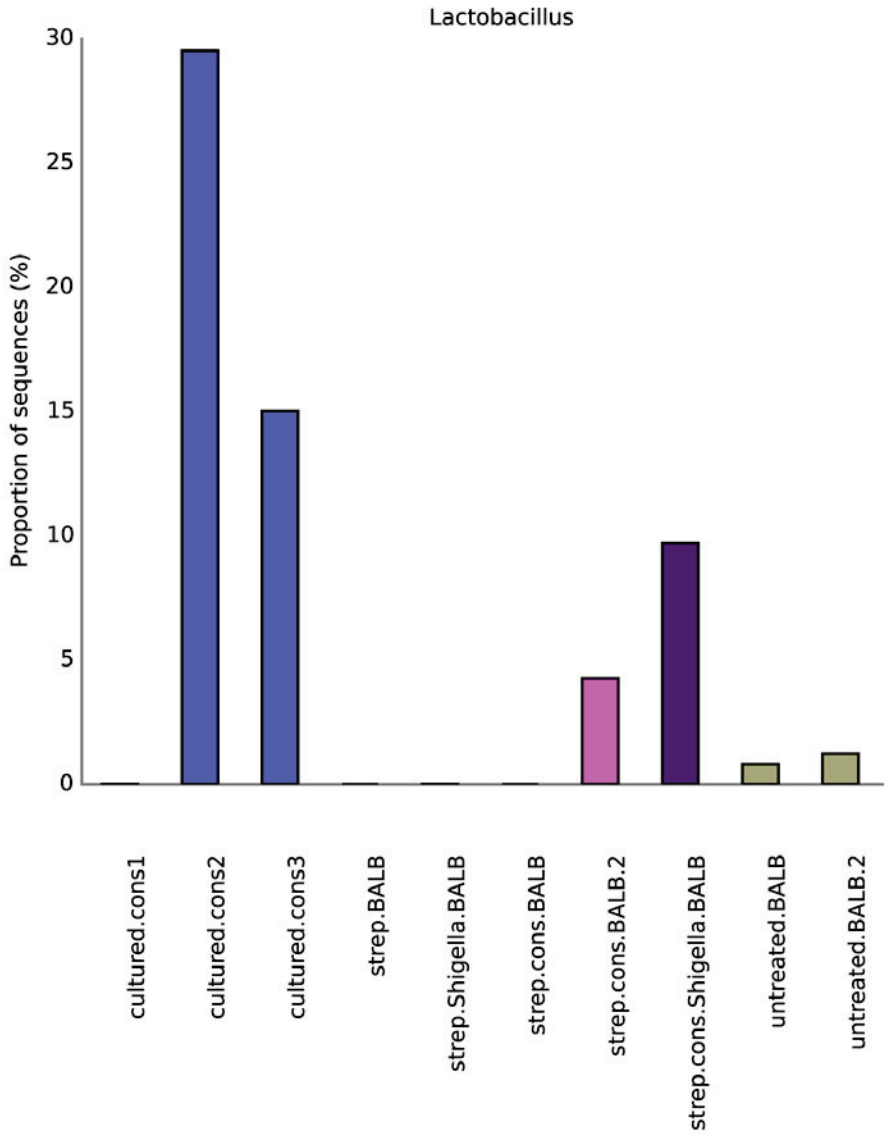
Figure 12: Stacked bar graph showing proportions of total operational taxonomic unit (OTU) reads detected by 16S rRNA sequencing in DNA samples extracted from the feces of healthy BALB/c mice in independent experiments (untreated BALB/c and untreated BALB/c 2) and from the cultured consortium 1, 2, and 3 bacteria grown on minimal media in the presence of butyrate and streptomycin. The Illumina MiSeq was used to obtain an output of approximately 12422 reads.

3.3.3 16S rRNA Characterization of the Mouse Gut Following Consortium Treatment Before and After Challenge with *S. flexneri.*

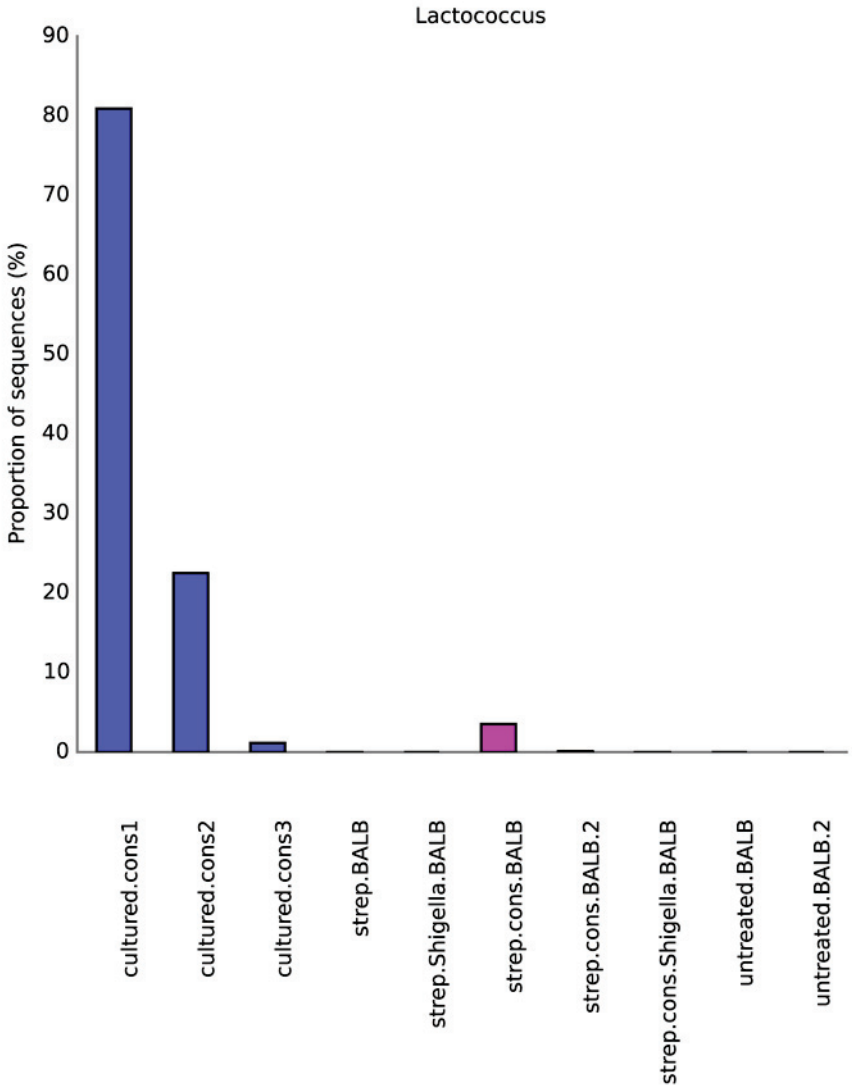
To test whether administration of the cultured consortium 2 bacteria resulted in colonization in treated mice and whether colonization persisted after *Shigella* infection, fecal samples were obtained from mice following treatment with the consortium of cultured commensal bacteria (cultured consortium 2) prior to and following *S. flexneri* infection. Mice were treated with streptomycin 2 days prior to *Shigella* infection and the cultured consortium 2 bacteria were administered one day prior to infection. Fecal samples from consortium-treated mice were obtained the following day prior to infection with *S. flexneri.* One day after *Shigella* infection, the fecal samples were obtained from consortium-treated *S. flexneri* infected mice and genomic DNA was extracted. DNA extracted from the feces of healthy, streptomycin treated, and streptomycin treated *S. flexneri* infected mice were also included in the analysis. For comparison, the corresponding levels of bacterial genera detected in the DNA extracted directly from the cultured consortium 1, 2, and 3 samples grown in the presence of streptomycin and butyrate are also shown (Figure 13A-F). *Lactobacillus* was detected at low levels in the untreated BALB/c mice but reached proportions of almost 30% in the cultured consortium 2 sample (Figure 13A). In one of the samples from mice treated with the cultured consortium 2, proportions were reduced to just under 5% but remained at levels slightly higher than in the untreated mice samples (Figure 13A). In samples obtained from mice treated with the cultured consortium 2 post infection with *Shigella*, the proportion of *Lactobacillus* persisted at levels reaching almost 10% (Figure 13A). Another genus that was enriched under anaerobic growth in the presence of butyrate,

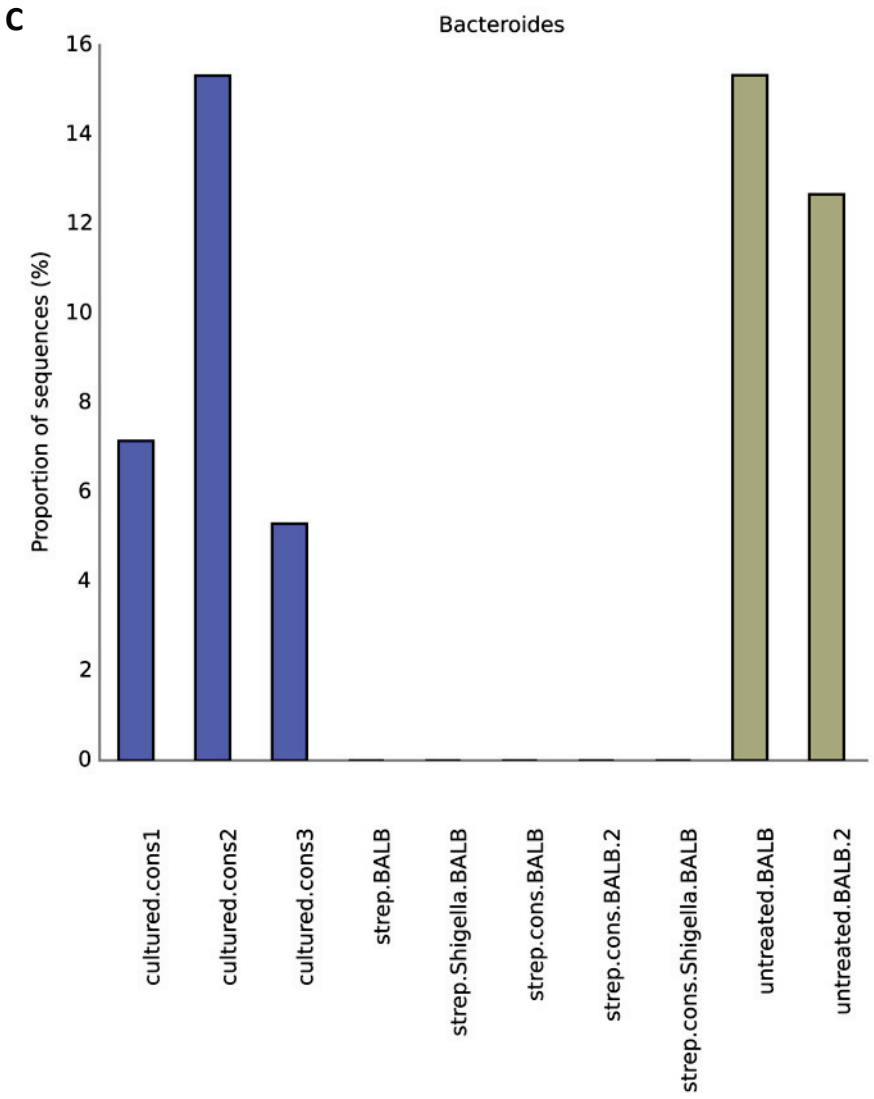
Lactococcus, was detected at low levels in the feces of streptomycin, consortium 2-treated mice, however decreased levels were observed in these mice following infection with *S. flexneri* (Figure 13B). A similar pattern was observed with the detection of *Clostridium* with high levels in the cultured consortium bacteria and in one of the two samples representative of streptomycin, cultured consortium 2-treated mice prior to *S. flexneri* infection (Figure 13E). Members of the *Bacteroides* and *Parabacteroides* genera were detected at high proportions in the feces of untreated mice and were repeatedly detected in the cultured consortium samples resulting from anaerobic growth in the presence of butyrate (Figure 13C and D). These genera were not detected to the same extent in the feces of the samples representative of the consortium-treated BALB/c mice, the consortium-treated *Shigella* infected mice, or the streptomycin treated mice (Figure 13C and D). Proportions of *Paenibacillus* were very low in samples representative of the untreated BALB/c mice but enriched to proportions of between 2 and 20% in the cultured consortium samples (Figure 13F). In the samples representative of cultured consortium 2-treated BALB/c mice, the proportions of *Paenibacillus* detected were between 35 and 60% (Figure 13F). In the sample representative of mice treated with the cultured consortium 2 and infected with *Shigella*, the proportion was approximately 25% (Figure 13F). In samples representative of untreated *Shigella* infected mice and streptomycin-treated mice, *Paenibacillus* proportions were between 2 and 7% (Figure 13F).

A

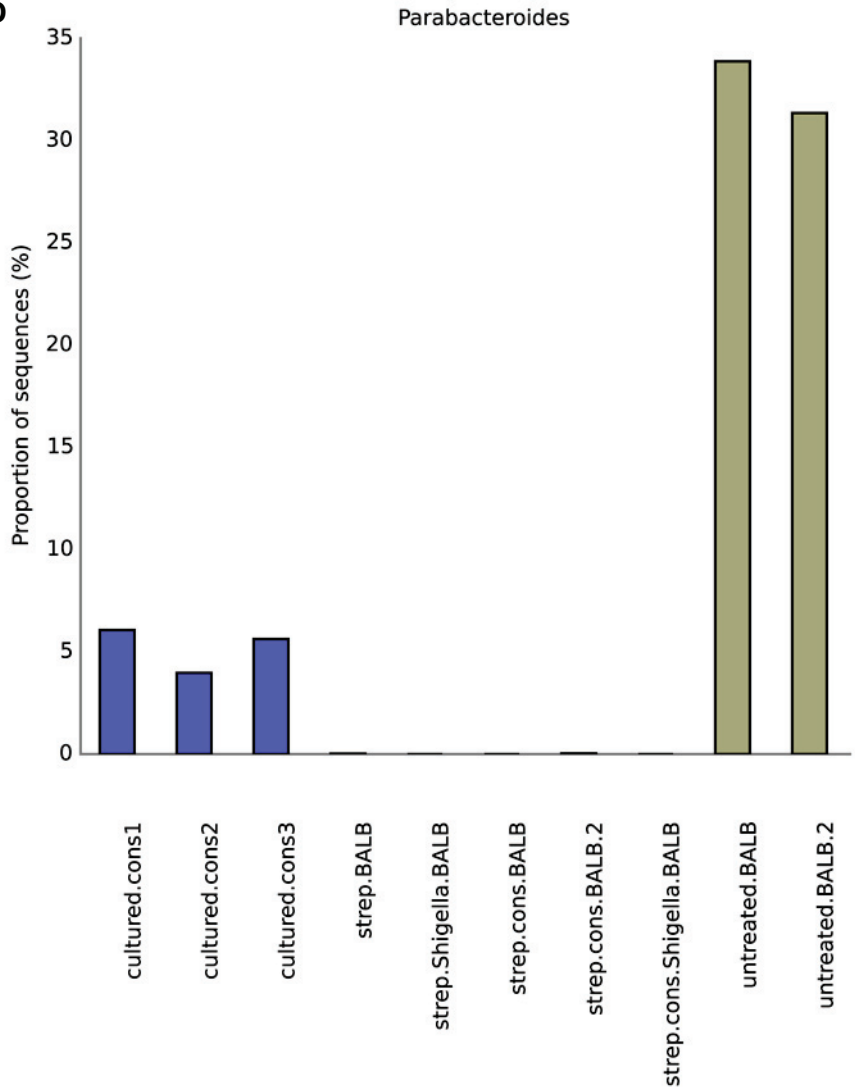

Lactobacillus
Proportion of sequences (%)
30
25
20
15
10
5
0
cultured.cons1
cultured.cons2
cultured.cons3
strep.BALB
strep.Shigella.BALB
strep.cons.BALB
strep.cons.BALB.2
strep.cons.Shigella.BALB
untreated.BALB
untreated.BALB.2

B

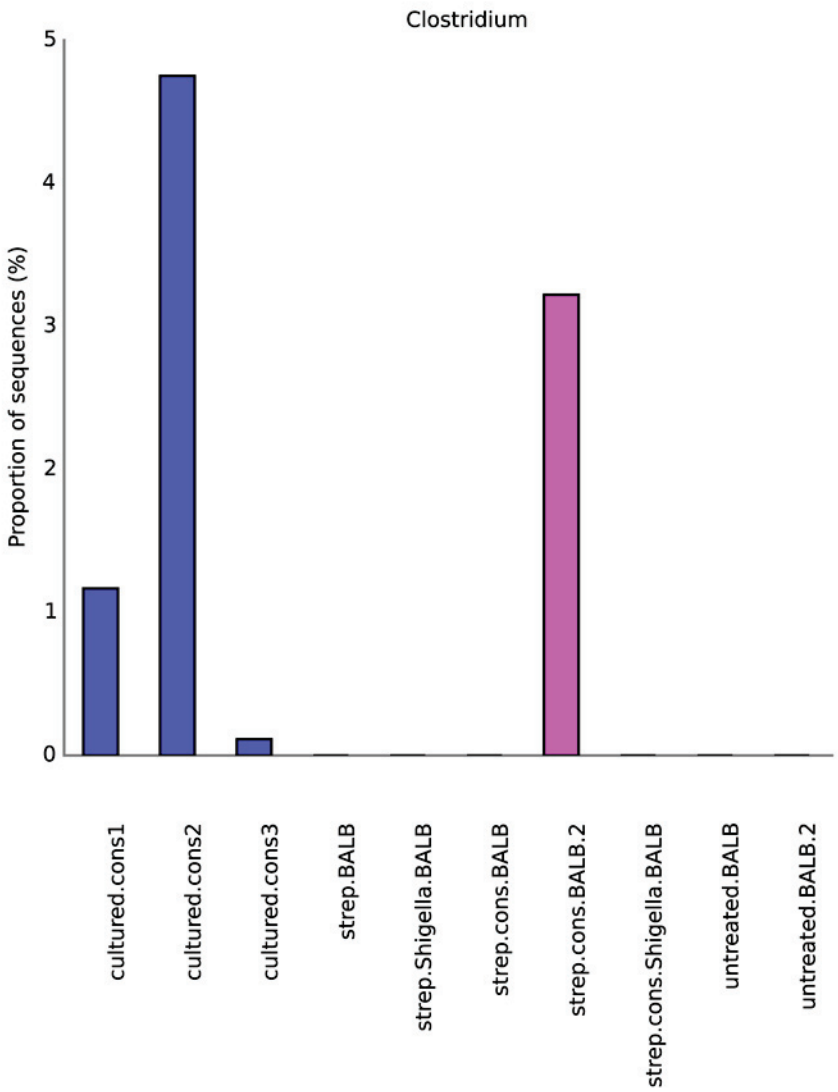

Lactococcus
Proportion of sequences (%)
90
80
70
60
50
40
30
20
10
0
cultured.cons1
cultured.cons2
cultured.cons3
strep.BALB
strep.Shigella.BALB
strep.cons.BALB
strep.cons.BALB.2
strep.cons.Shigella.BALB
untreated.BALB
untreated.BALB.2


C
Bacteroides
Proportion of sequences (%)
0
2
4
6
8
10
12
14
16
cultured.cons1
cultured.cons2
cultured.cons3
strep.BALB
strep.Shigella.BALB
strep.cons.BALB
strep.cons.BALB.2
strep.cons.Shigella.BALB
untreated.BALB
untreated.BALB.2

D


Parabacteroides
Proportion of sequences (%)
35
30
25
20
15
10
5
0
cultured.cons1
cultured.cons2
cultured.cons3
strep.BALB
strep.Shigella.BALB
strep.cons.BALB
strep.cons.BALB.2
strep.cons.Shigella.BALB
untreated.BALB
untreated.BALB.2

E


Clostridium
Proportion of sequences (%)
0
1
2
3
4
5
cultured.cons1
cultured.cons2
cultured.cons3
strep.BALB
strep.Shigella.BALB
strep.cons.BALB
strep.cons.BALB.2
strep.cons.Shigella.BALB
untreated.BALB
untreated.BALB.2

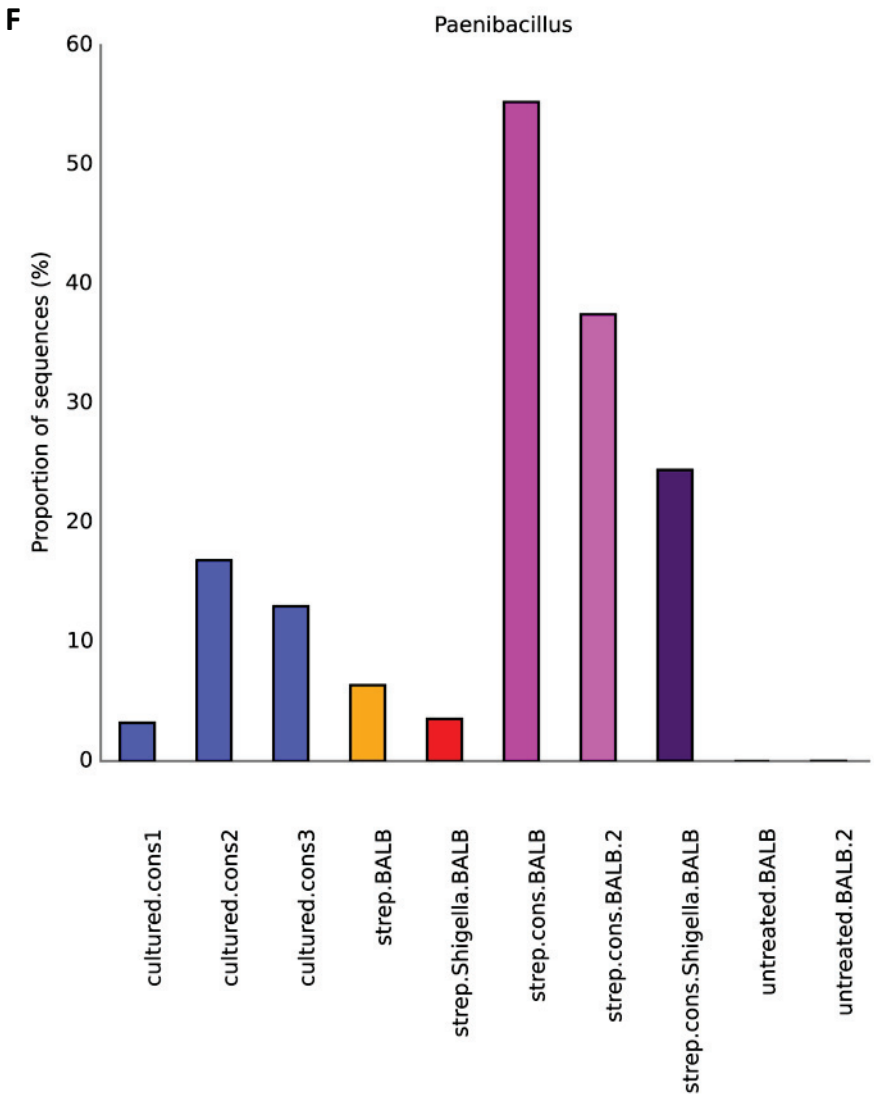
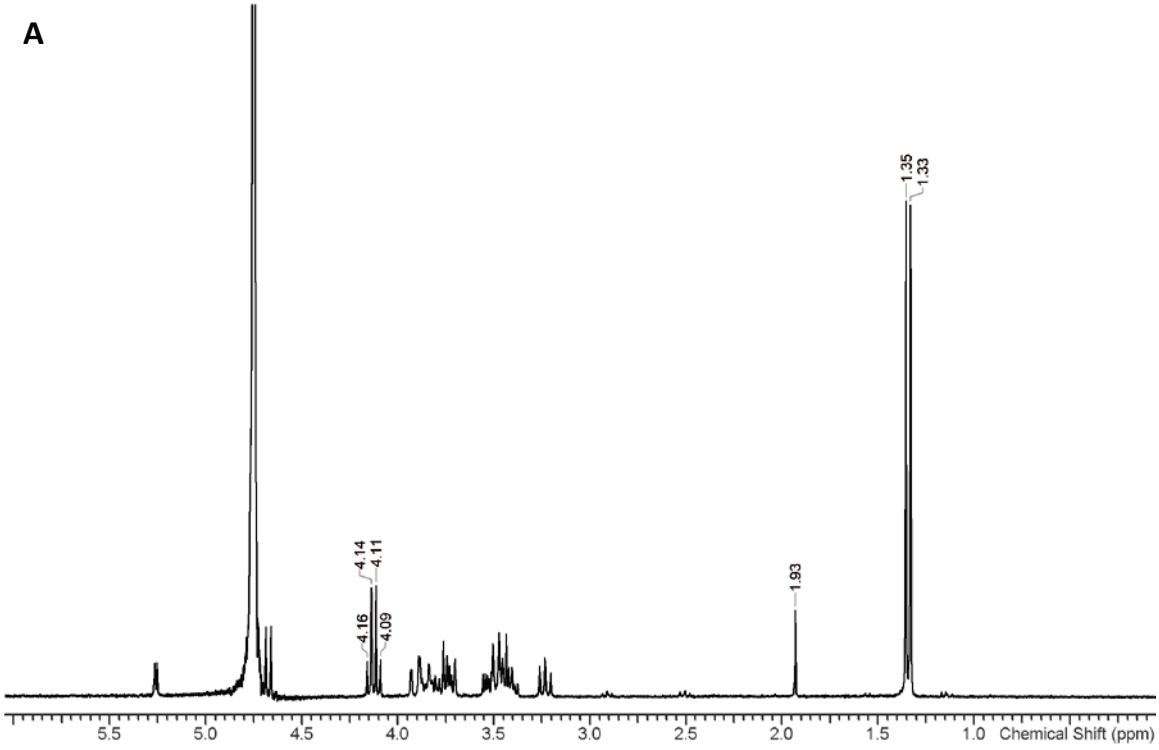
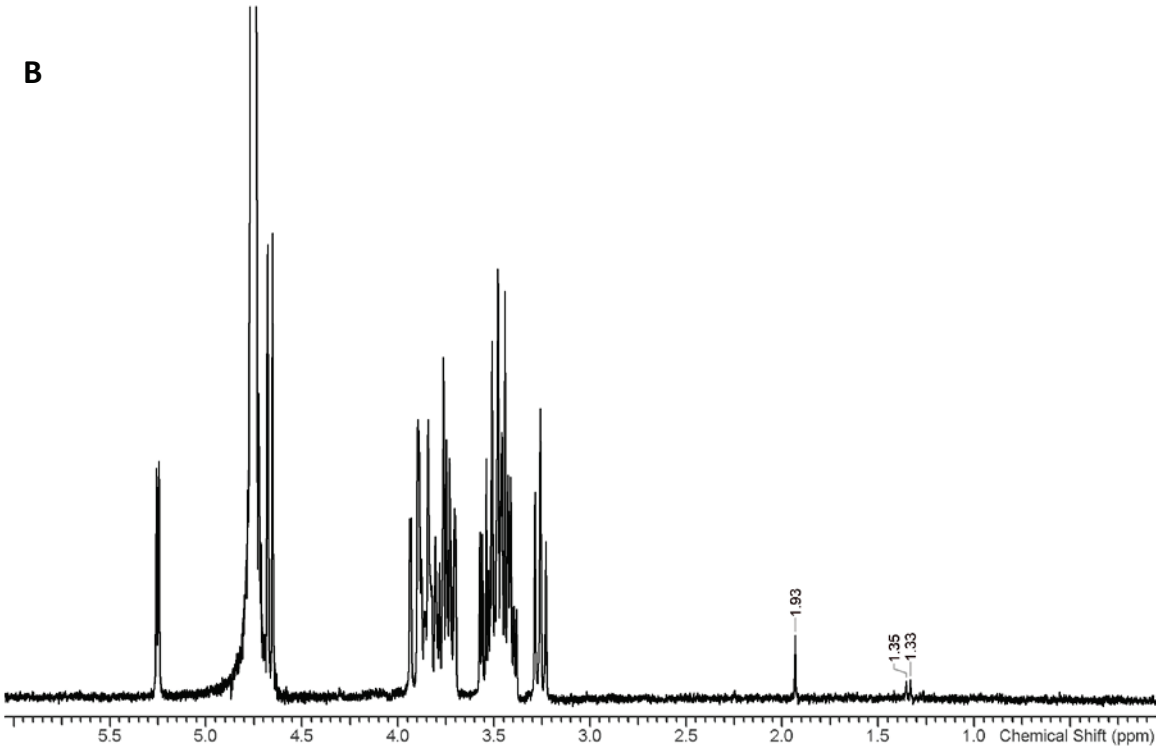


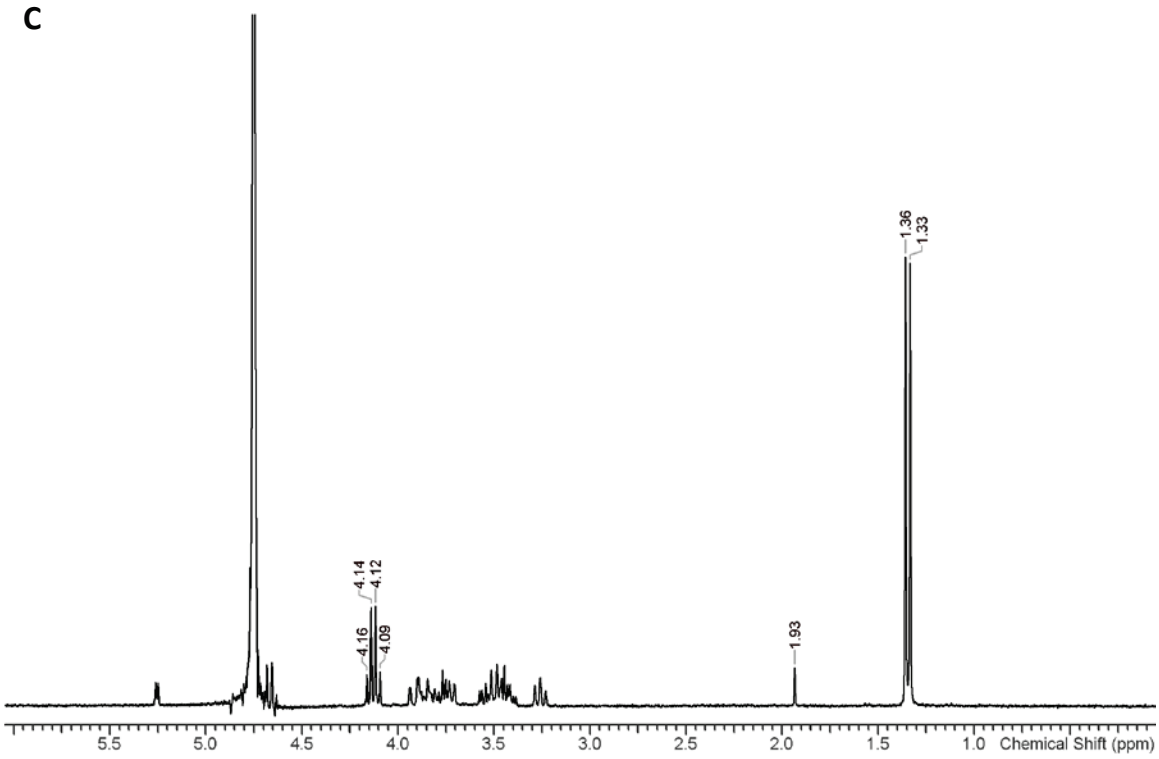
Figure 13: Bar graphs showing the proportion of sequences of approximately 12422 output reads detected by 16S rRNA sequencing on DNA extracted from the consortium of bacteria grown in the presence of streptomycin and butyrate (cultured consortium 1,2, and 3: cultured.cons1, cultured.cons2, and cultured.cons3, respectively), and from the feces of healthy mice (untreated.BALB and untreated.BALB2), streptomycin treated mice (strep.BALB), streptomycin treated *S. flexneri* infected mice (strep.Shigella.BALB), streptomycin consortium 2 treated mice (strep.cons.BALB and strep.cons.BALB2), and streptomycin consortium 2 treated *S. flexneri* infected mice (strep.cons.Shigella.BALB). (A) Proportions of *Lactobacillus* (B) Proportions of *Lactococcus* (C) Proportions of *Bacteroides* (D) Proportions of *Parabacteroides* (E) Proportions of *Clostridium,* and (F) Proportions of *Paenibacillus*.

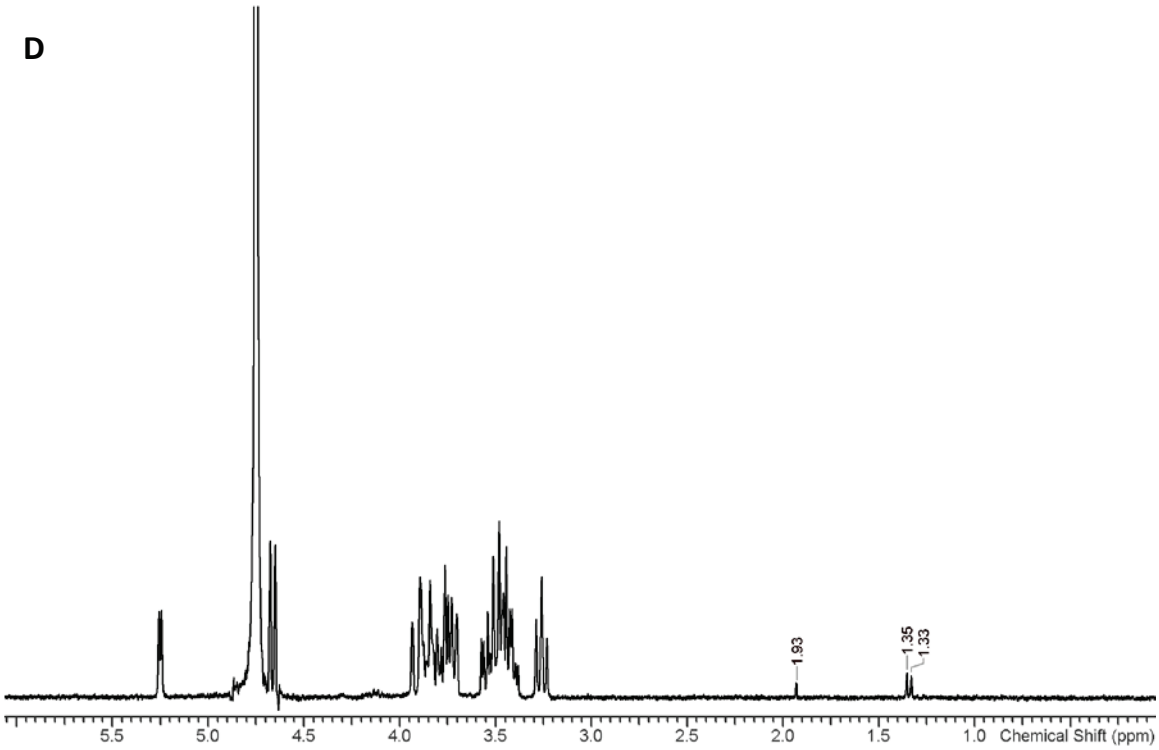
3.4: Isolation and ^{1}H NMR analysis of Commensals Cultured from the Healthy AKR/J Mouse.

To investigate the role of commensals in colonization resistance in an alternative mouse strain, AKR/J, attempts were made at culturing the commensal anaerobe, *Barnesiella*, from AKR/J mice on selective EG media. Multiple colony morphologies were observed following a 2-day period of anaerobic growth at 37 °C. After streak purification, the V1-V3 region of the 16S rRNA gene was amplified from each isolate by PCR using primers listed in Table 2 and sequenced through the Genewiz sequencing service. Sequence identification was made using the NCBI Basic Local Alignment Search Tool (BLAST) with the16S ribosomal RNA sequence database. Sequences derived from four isolates were identified as *Enterrococcus faecalis* (100% identity) (AKR/J isolate 1), *Enterrococcus gallinarum and Enterococcus casseliflavus* (99% identity with both) (AKR/J isolate 2), *Clostridium botulinum* and *Clostridium sporogenes* (98% identity with both) (AKR/J isolate 3), and *Clostridium nitrophenolicum* and *Clostridium aciditolerans* (99% identity with both) (AKR/J isolate 4), respectively. The metabolic profiles of the cultured bacteria were examined by ^{1}H NMR. Unmetabolized glucose, lactate, and acetate were detected in the supernatant of all four bacteria cultured from the feces of healthy AKR/J mice (Figure 14A-D). The spectrum obtained from the standard solution of glucose is also shown (Figure 14E).


A
1.35
1.33
4.14
4.11
4.16
4.09
1.93
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
Chemical Shift (ppm)


B
1.93
1.35
1.33
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
Chemical Shift (ppm)


C
1.36
1.33
4.14
4.12
4.16
4.09
1.93
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
Chemical Shift (ppm)


D
1.93
1.35
1.33
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
Chemical Shift (ppm)

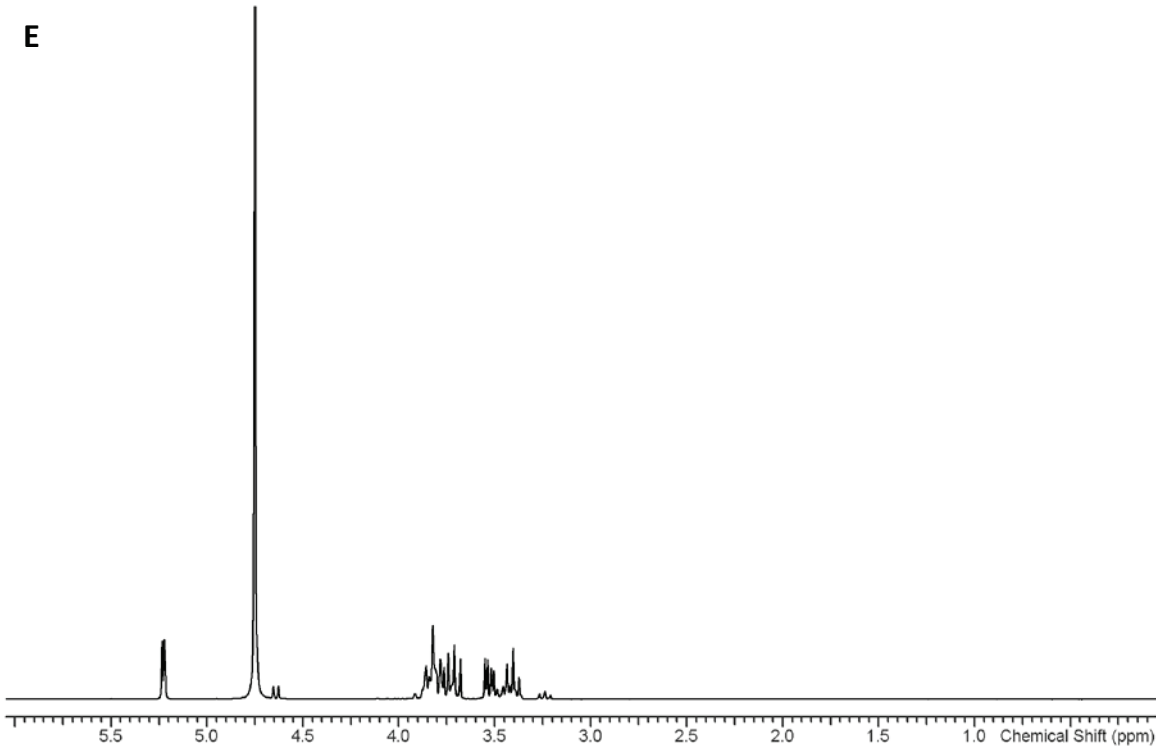


Figure 14: ^{1}H NMR spectra of the supernatant of four isolates of bacteria cultured on selective Eggerth Gagnon media from the feces of healthy AKR/J mice. Bacteria were incubated in PBS with 10 mM glucose for 24 hours anaerobically. After incubation, the supernatant was adjusted to pH 9.5 and lyophilized. A portion of the residue was dissolved in D_2O. Reference signals appear at approximately δ 4.75. (A) ^{1}H NMR spectrum of AKR/J isolate 1 with signals assigned to lactate (δ 1.33, 1.35, 4.09-4.16), and acetate (δ 1.93). (B) ^{1}H NMR spectrum of AKR/J isolate 2 with signals assigned to lactate (δ 1.33, 1.35), and acetate (δ 1.93). (C) ^{1}H NMR spectrum of AKR/J isolate 3 with signals assigned to lactate (δ 1.33, 1.36, 4.09-4.16), and acetate (δ 1.93). (D) ^{1}H NMR spectrum of AKR/J isolate 4 with signals assigned to lactate (δ 1.33, 1.35), and acetate (δ 1.93). (E) ^{1}H NMR spectrum of a standard solution of glucose made by dissolving 20 mg in 800 μL D_2O.

Chapter 4: Discussion

4.1: Involvement of the PTA-ACKA Pathway in Intracellular Growth of *S. flexneri*

Attempts to confirm the previously reported phenotype of impaired intracellular growth associated with a mutant for the phosphotransacetylase (*pta)* gene component of the PTA-ACKA pathway *in vitro* were successful. Kentner *et al.* (2014) observed a significant decrease in the rate of intracellular generation of a mutant for *pta* produced in the *S. flexneri* 2a 2457T *icsA* background. Using the parental strain studied in the Rohde Lab, *S. flexneri* M90T-Sm serotype 5a, to create deletions in the *pta* and *icsA* genes, a significant impairment in intracellular growth was similarly observed using the gentamicin protection assay. Kentner *et al.* (2014) were able to complement the defect in intracellular growth of the *pta* mutant by expressing the gene and native promoter on a medium copy number plasmid, however my attempts to do so were unsuccessful. Using an alternative method of complementation involving the addition of exogenous acetyl phosphate, the product of the Pta enzyme, to the cell culture medium during the intracellular growth assay, levels of *ΔptaΔicsA* mutant bacteria recovered were restored to levels recovered from *ΔicsA* infected cells. Since addition of the Pta enzyme product complemented the *ΔptaΔicsA* phenotype of impaired intracellular growth, Pta likely contributes to the growth of *Shigella* in host cells and the PTA-ACKA carbon metabolism pathway seems to support intracellular growth *in vitro*, as reported in Kentner *et al.* (2014).

By deleting the IpaH9.8 E3 ubiquitin ligase in the *ΔptaΔicsA* background and assessing this mutant for intracellular growth, I aimed to test the hypothesis that a mutant deleted for both *ipaH9.8* and *pta* (*ΔptaΔicsAΔipaH9.8*) would display a phenotype of

increasingly impaired intracellular growth compared to the *pta* mutant (*ΔptaΔicsA*). Lower levels of bacteria recovered from the *ΔptaΔicsAΔipaH9.8* mutant-infected HeLa cells in the gentamicin protection assay compared to levels recovered from *ΔptaΔicsA*-infected cells would support the hypothesis that IpaH9.8 activity contributes to nutrient acquisition in the *Shigella*-infected cell. This work builds off of previous findings in the Rohde Lab: a yeast two-hybrid screen and *in vitro* ubiquitination assays, demonstrating that IpaH9.8 targets the negative regulator of autophagy, ZKSCAN3, to induce autophagy. While slightly lower amounts of bacteria were recovered from the *ΔptaΔicsAΔipaH9.8* mutant-infected HeLa cells compared to cells infected with the *ΔptaΔicsA* mutant, the difference was not statistically significant. In the protocol used, HeLa cells were infected for a period of 1 hour before gentamicin was introduced to kill all extracellular bacteria. After an additional 1 hour incubation in the gentamicin containing media, cells were lysed and bacteria were recovered to quantify levels of intracellular growth. The generation time of wild type intracellular *Shigella* is approximately 37 minutes and it was previously reported that *ΔptaΔicsA* mutant *Shigella* had intracellular growth defects of 40-60% of the parental strain (Kentner *et al*., 2014). It is possible that quantification of intracellular growth using a longer infection time (3-5 hours) would show larger differences between the intracellular growth of *ΔptaΔicsA* and *ΔptaΔicsAΔipaH9.8* mutant *Shigella.*

The aim of my project was to investigate mechanisms of colonization by *S. flexneri in vivo* using our established murine infection model. While I did not reach the point of testing the *Δpta* mutant *in vivo* during my study, the construction of this mutant

is the first step toward this goal and this strain can be used as a future tool in the Rohde Lab to further the study of the PTA-ACKA pathway in *Shigella* colonization.

4.2: Investigation of the role of Nitrogen Respiration in Colonization by *S. flexneri*

A second aim of this study was to investigate the role of nitrogen respiration in colonization by *S. flexneri*. It has been recently demonstrated that the commensal strain of *E. coli,* Nissle 1917, uses nitrate reduction to utilize nitrate species generated during inflammation in the streptomycin treated mouse (Spees *et al*., 2013). Metabolism of nitrate produced by host iNOS confers an advantage allowing *E. coli* to overcome colonization resistance. By creating a mutant for genes encoding *Shigella'*s nitrate reductase machinery, the involvement of nitrate reduction in the ability of *S. flexneri* to colonize the streptomycin treated mouse was successfully tested. Similar to findings reported with *E. coli* (Spees *et al*., 2013), deletion of the *narG*, *narZ*, and *napA* genes was sufficient to abrogate nitrate reductase activity. The loss of nitrate reductase activity was confirmed using the *in vitro* test from Sigma-Aldrich. A red precipitate was visible in nitrate medium inoculated with wild type *S. flexneri* upon addition of the reagents according to the manufacturer's instructions. In contrast, the *ΔnarGΔnarZΔnapA* mutant remained colorless until the zinc catalyst was added. Upon the addition of zinc, a red precipitate formed in the *ΔnarGΔnarZΔnapA* inoculated tube indicating that unused nitrate was present and confirming that nitrate reductase activity was abrogated in the mutant strain. To confirm that the deleted genes were responsible for nitrate reductase activity, one of the genes, *narZ* was cloned into an expression vector and transformed into the *ΔnarGΔnarZΔnapA* mutant. Expression of *narZ* restored nitrate reductase activity in the *ΔnarGΔnarZΔnapA* mutant, indicated by the formation of a red precipitate

in the nitrate media upon addition of the appropriate reagents. These results demonstrate that *narG, narZ,* and *napA* are required for nitrate reduction during anaerobic growth *in vitro* in *S. flexneri*, as in *E. coli* (Spees *et al*., 2013).

The ability of nitrate reduction to confer an advantage to *S. flexneri* during growth in nitrate broth was tested. In *E. coli*, a mutant for nitrate reductase machinery was outcompeted by a strain with wild type nitrate reductase genes when grown in medium containing 40 mM nitrate (Spees *et al*., 2013). In contrast, both strains were recovered in equal ratios in the absence of nitrate (Spees *et al*., 2013). Under the same conditions, *ΔtrbH* mutant *S. flexneri* did not outcompete the *ΔnarGΔnarZΔnapA* strain in the presence of nitrate. While *ΔtrbH* mutant *S. flexneri* was recovered in slightly higher amounts compared to the *ΔnarGΔnarZΔnapA* mutant in the presence of nitrate, levels of recovered bacteria were not significantly different. These results suggest that *S. flexneri* may be less reliant on available nitrate during anaerobic *in vitro* growth in medium than *E. coli.*

The *in vitro* assay was conducted in mucin medium with or without nitrate supplementation, to mimic conditions within the host intestine. In addition to mucin, many other aspects of host physiology are likely required to more accurately represent infection conditions. BALB/c mice were infected with wild type *S. flexneri* or the *ΔnarGΔnarZΔnapA* mutant using the standard murine infection model used in the Rohde Lab to test whether nitrate reductase activity confers an advantage to *S. flexneri* during colonization of the streptomycin-treated mouse. Bacterial burden in the feces over the six-day infection as well as in the cecal content and spleen as assessed on day 6, was similar between wild type infected and *ΔnarGΔnarZΔnapA* infected mice. Clinical scores

were similar between the two groups of infected mice with the exception of day 6 post infection when wild type infected mice displayed elevated clinical scores compared to the group infected with nitrate reductase mutant *S. flexneri*. Clinical scores are monitored during infection of mice with *S. flexneri* as an indicator for disease associated with infection and to ensure that ethical endpoints put in place are being adhered to during infection experiments. Several parameters are measured including appearance, posture, behavior, appetite, hydration, weight, and body temperature, and contribute to the cumulative clinical score with a higher score correlating with increased severity of symptoms. While clinical scoring is a valuable tool in murine infection studies, caution should be exercised when evaluating phenotypes assigned by clinical scores. Other than body weight and temperature, measurements used to determine clinical scores are subjective and can therefore be prone to inconsistencies or other manifestations of human error. While wild type infected mice displayed elevated clinical scores on day 6 post infection compared to the *ΔnarGΔnarZΔnapA* mutant-infected mice, this result was not accompanied by differences in bacterial burden in the feces, cecum, or spleen, suggesting that genes for nitrate reductase activity in *S. flexneri* are dispensable during colonization *in vivo* of the streptomycin treated mouse.

Spees *et al*. (2013) observed that wild type *E. coli* outcompete a strain deleted for nitrate reductase activity in streptomycin treated mice using a co-infection model in which mice were inoculated with a mixture of each strain at a 1:1 ratio, rather than a model with two distinct groups infected in parallel with the wild type and mutant strain. Another group investigating the role of genes involved in sugar-phosphate uptake in the murine adapted enterohaemorrhagic *E. coli* (EHEC) strain *Citrobacter rodentium*

reported that bacteria deleted for a component of the sugar-phosphate uptake system were impaired in the ability to colonize C57BL/6 mice (Sit *et al*., 2015). The phenotype was only apparent in a competitive assay when mice were co-infected with an equal ratio of wild type to mutant bacteria or when infected mice were co-housed with their uninfected counterparts and the bacterial burden in the naïve mice after co-housing was evaluated (Sit *et al*., 2015). No difference in bacterial burden was detected in single infection experiments (Sit *et al*., 2015). To assess whether wild type and nitrate reductase deficient *S. flexneri* display differential colonization phenotypes under co-infection conditions, the competitive assay was tested *in vivo* by co-inoculating BALB/c mice with an equal ratio of *ΔtrbH* and *ΔnarGΔnarZΔnapA* mutant *S. flexneri*. The ratio of *ΔtrbH* to *ΔnarGΔnarZΔnapA* mutant *S. flexneri* recovered from the feces fluctuated to slightly above or below a CI (competitive index) value of 1 during the six day infection, however was only significantly different on day 2 post-infection. On day 2 post-infection, the CI value was approximately 0.5 indicating that slightly more *ΔnarGΔnarZΔnapA* mutant *S. flexneri* than *ΔtrbH* (representing wild type) bacteria were recovered from the feces of co-infected mice. In contrast, wild type *E. coli* outcompeted a nitrate reductase mutant strain by an approximate ratio of 10:1 at the end of a six day co-infection in the streptomycin treated mouse model (Spees *et al*., 2013). Equal numbers of bacteria were recovered from the cecal content of the *ΔtrbH* and *ΔnarGΔnarZΔnapA* mutant co-infected mice. Taken together, these data suggest that genes encoding components of the nitrate reductases essential for nitrate reductase activity in *S. flexneri* are dispensable during colonization in the streptomycin treated murine model of infection. There is no evidence to suggest that interruption of the *trbH* gene impacts any aspect of bacterial

physiology, virulence plasmid stability, invasion, or pathogenicity of *S. flexneri* (Radnedge *et al*., 1997), however few studies have directly addressed this issue. This mutant was selected since it has an alternative marker (the tetracycline resistance gene) replacing the *trbH* locus to allow easy recovery during co-infection and co-inoculation experiments with the *ΔnarGΔnarZΔnapA* mutant strain containing a marker for kanamycin resistance. An alternative approach would be to clone the antibiotic selection marker into one of three specific sites on the virulence plasmid of *S. flexneri* that our lab has previously identified as non-coding loci into which insertions would not interrupt the expression of any of the virulence plasmid genes.

Although the *narG*, *narZ*, and *napA* genes are conserved between *E. coli* and *S. flexneri*, these bacteria have notable differences that could explain the discrepancies observed in the use of nitrogen respiration to fuel colonization in the streptomycin treated mouse. The Spees *et al.* study used a commensal strain of *E. coli* (Nissle 1917), however findings can extend to related strains of pathogenic *E. coli* due to the conservation of the nitrate reductase genes. Pathogenic *E. coli* latch onto the apical membrane of target cells in the small or large bowel and with the exception of enteroinvasive strains, maintain an extracellular lifestyle while mediating disease through functions such as toxin secretion (Kaper *et al*., 2004). While *S. flexneri* also colonizes epithelial cells in the colonic mucosa, its lifestyle is unique from that of *E. coli*. *S. flexneri* crosses the epithelial barrier and interacts with cells at the apical membrane where invasion into the host cell is mediated. *Shigella* then ruptures the vacuole and maintains an intracellular lifestyle, while polymerizing host actin to spread to neighboring cells. Due to the differences in preferred niches within the host, it is possible that these bacteria rely on different

respiratory pathways to fuel colonization and achieve their respective lifestyles. While the findings from this study suggest that nitrate reduction is not a mechanism employed by *Shigella* to support colonization in the streptomycin-treated murine model, it does not rule out other forms of nitrogen respiration. For example, the *nirB* and *nirD* genes encode the large and small subunit of a nitrite reductase enzyme in *E. coli*, respectively, and a blast search reveals sequences with 100% identity to these genes across all serotypes of *Shigella*. In *E. coli*, the nitrite reductase genes along with a nitrite membrane transporter minimize toxicity resulting from the formation of metal-nitrosyl complexes associated with increased nitrite production during nitrate reductase activity (Cole, 1996; Jia and Cole, 2005; Stewart, 1994). The putative role of nitrite reductase encoding genes in *Shigella* has not been investigated.

4.3: Isolation of the Consortium of Commensal Bacteria

The third aim of this project was to investigate the involvement of commensal bacteria in colonization resistance to *S. flexneri*. Since streptomycin treatment is required to render mice susceptible to infection, we hypothesized that components of the murine microbiota are involved in maintaining resistance to colonization by *S. flexneri* in the undisturbed mouse gut. To explore this hypothesis, bacteria were cultured from the fecal matter of healthy mice and subjected to selective growth in the presence of increasing concentrations of butyrate and streptomycin. Increasing concentrations of streptomycin were introduced to select for streptomycin resistance among the cultured consortium in order to achieve compatibility with the streptomycin treated mouse model of *S. flexneri* infection. Butyrate was introduced with the aim of enriching for bacteria associated with the production of butyrate. A similar technique was applied in the detection and

enrichment of *Bifidobacterium* species, known SCFA producers, from the fecal matter of various animals using media supplemented with propionic acid (Beerens, 1991). Due to the effect of butyrate in restoring antimicrobial peptide levels and decreasing pathology following *S. flexneri* infection in the rabbit model (Raqib *et al*., 2006; Sarker *et al*., 2011), bacteria associated with the production of butyrate were of interest as a potential starting point in the search for mediators of colonization resistance.

The consortium of bacteria were cultured anaerobically because of the predominance of anaerobes in mammalian microbial gut communities and the relative lack of characterization of many of these species (Rigottier-Gois, 2013). While anaerobic culturing conditions were maintained to mimic the mammalian intestinal niche during growth of the bacterial consortium, discrepancies between the *in vitro* system used and the predicted luminal environment should be noted. To generate anaerobic conditions *in vitro*, GasPak EZ Anaerobe Container System Sachets were used. In a properly sealed anaerobic chamber, sachets create an atmosphere composed of less than 1.0% oxygen. In the lumen of the GI tract, studies have predicted a state of physiologic hypoxia however the oxygen content is subject to fluctuation and the exact oxygen composition is still being investigated (Colgan and Taylor, 2010). In this study, minimal media supplemented with butyrate was used to culture the consortia of bacteria in an attempt to narrow the selection process to include fewer bacteria that would be adapted to growth under these conditions. An alternative approach would be to use a richer growth media while maintaining the streptomycin and butyrate supplementation. Robust growth was not obtained on minimal media, particularly when the bacteria were recovered from frozen stocks of bacteria resuspended in PBS with glycerol. For this reason, fresh fecal pellets

were repeatedly obtained from untreated BALB/c mice and used as a source to culture consortia bacteria for subsequent experiments when robust recovery from frozen stocks was no longer obtained. It is possible that additional nutrients could support a more robust growth of the consortia bacteria and use of a richer media could address this issue. However, this approach would likely alter the composition of microbes cultured from the healthy mouse.

Other studies have differed in their approach to studying the interactions between commensals and pathogens underlying colonization resistance. In the *Vibrio cholerae* study by Jeff Gordon's group, fecal samples were obtained over time from individuals recovering from *V. cholerae* infection in an area of high disease burden in Bangaldesh. DNA extracted from the fecal samples was subjected to metagenomics analysis to identify bacteria that correlated with recovery from infection (Hsiao *et al*., 2014). From the metagenomics data, an artificial community of species was assembled and used in a gnotobiotic mouse model to study the effects during challenge with *V. cholerae* (Hsiao *et al*., 2014). Species constituting the artificial community were not obtained from cultures of the study participants' fecal bio specimens; rather they were obtained from public repositories (Hsiao *et al*., 2014). While the approaches vary, similar tools were applied in the research described in this thesis and the study of commensals involved in recovery from *V. cholerae*. In each case, next-generation sequencing was combined with a mouse model for disease challenge experiments.

In another study investigating the role of the microbiota in a murine *Salmonella* infection model, commensal organisms were selected based on their presence in a defined collection of commensal bacteria, known as the Microbial Ecosystem Therapeutic (MET-

1) used to treat recurrent *Clostridium difficile* infection (Martz *et al*., 2015). The MET-1 community was introduced into streptomycin treated C57BL/6 mice and the clinical outcome was evaluated upon oral challenge with *S. typhimurium.* Using this approach, protective phenotypes including the reduction of *Salmonella*-mediated destruction of host cell tight junctions, and decreased neutrophil infiltration in the cecum were observed (Martz *et al*., 2015). These studies provide a strong rationale for our approach to investigating the involvement of the commensal microbiota in colonization resistance to *Shigella.*

4.3.2 Characterization of Cultured Consortium of Bacteria by ^{1}H NMR

Following growth of the consortium of bacteria cultured from the feces of healthy BALB/c mice, the bacteria were tested for butyrate production using ^{1}H NMR analysis. This technique is commonly used along with ^{13}C NMR (carbon-13 nuclear magnetic resonance spectroscopy) to inform on the structure of complicated organic molecules. Briefly, ^{1}H NMR detects and plots electromagnetic signals emitted from nuclei that undergo a relaxation state following stimulation with radiation and an applied magnetic field (Folchetti, 2010). The number of electromagnetic signals emitted by a sample is dependent on the number of chemically equivalent protons within the compound, since protons in the same chemical environment experience the same magnetic force and yield overlapping signals (Folchetti, 2010). The position of a signal is determined by the chemical shift, a measure of how far the protons in a given chemical environment are from the most electronegative part of the molecule. The chemical shift is measured in parts per million (ppm). Thus, hydrogen atom-containing molecules can be recognized by the pattern of electromagnetic signals emitted during ^{1}H NMR analysis. This technique

has been used quantitatively to determine the metabolic footprint of the SCFA-producing anaerobe *Fusobacterium varium* (*F. varium*) in different chemically defined minimal medias (Resmer and White, 2011). In the present study, ^{1}H NMR analysis was applied as a qualitative approach to test for the presence of butyrate in the cultured consortium bacterial supernatant.

In this study, ^{1}H NMR was successfully used to demonstrate the presence of butyrate, and other metabolites, in the supernatant of the bacterial consortium (cultured consortium 2) cultured anaerobically under selective butyrate and streptomycin containing conditions. The ^{1}H NMR spectrum obtained from the cultured consortium 2 supernatant demonstrated the presence of butyrate as well as formate, lactate, and acetate. To demonstrate that the process of supplementing the culture medium with butyrate actively enriches for bacteria associated with butyrate production, I repeated the culturing and ^{1}H NMR analysis with a control consortium of bacteria grown up in the absence of butyrate. Fecal pellets were obtained again from healthy BALB/c mice and plated on both the original media used for growth of the cultured consortium 2 bacteria, and media lacking butyrate and supplemented instead with a slightly higher glucose concentration as a carbon source. Butyrate was not detected in the supernatant of the cultured consortium of bacteria grown with or without the butyrate enrichment. Since butyrate was not detected in the supernatant of bacteria grown on selective streptomycin containing, butyrate supplemented media, as it was before, the negative control consortium grown in the absence of butyrate does not help confirm the effects of butyrate enrichment.

Results suggest that the selective process of enriching for streptomycin resistant, butyrate producing anaerobes is not always successful. In the supernatant of bacteria

grown in the absence of butyrate, only unmetabolized glucose was detected on the ^{1}H NMR spectrum. On the spectrum obtained from bacteria grown under the butyrate selective conditions (cultured consortium 3), only low levels of acetate and lactate were visible in addition to unmetabolized glucose. It is possible that for unknown reasons, the cells used for ^{1}H NMR analysis were less metabolically active during the incubation period prior to the third ^{1}H NMR analysis experiment. Although culturing conditions were kept constant, the high levels of unmetabolized glucose suggest that cells may have reduced metabolism which could account for the discrepancies observed. Another potential explanation is the possible variation in the microbiomes of the mice used for fecal sample collection. While factors such as the age, sex, strain, and supplier of the mice were kept constant, microbiome differences between mice is a possible source of variation. A recent study demonstrated that relocating young mice between rooms within the same facility can result in shifts in the populations of microbes dominating the gut (Friswell *et al*., 2010). Ideally, the experiment should be repeated to ensure that the butyrate enrichment, streptomycin selective culturing technique can again result in a consortium of butyrate producing bacteria. In parallel to this experiment, a control consortium should be cultured in the absence of butyrate but from fecal matter of the same mice and tested for the lack of butyrate in the culture supernatant by ^{1}H NMR analysis. This future experiment is needed to confirm that the butyrate detected in the supernatant of cultured consortium bacteria is an effect of the butyrate supplementation during the culturing process. One possibility is that butyrate present in the agar plates used for growth of the consortium bacteria is transferred during the process of scraping the lawn of growth from the plate into the PBS solution prior to preparation of samples

for ^{1}H NMR analysis. To prevent this occurrence, bacteria were subjected to a PBS wash as part of the preparation of samples for ^{1}H NMR analysis. While it is possible that the wash step wasn't sufficient to remove all traces of butyrate before incubating bacteria in the low glucose solution to collect metabolites for NMR, this is unlikely since butyrate was not consistently detected in the supernatant of bacteria grown on butyrate-supplemented media. Isotopic labeling could be used to directly demonstrate the incorporation of carbon from the glucose source provided during the anaerobic incubation into butyrate by the cultured bacterial consortium.

4.3.2: Characterization of Cultured Consortia Bacteria by 16S rRNA Sequencing

To characterize the consortia of bacteria cultured under anaerobic growth in the presence of streptomycin and butyrate, 16S rRNA sequencing was conducted on extracted DNA from cultured bacteria and compared with the 16S rRNA profiles derived from DNA extracted from the fecal matter of the healthy BALB/c mouse. Although the ^{1}H NMR data accompanies only the cultured consortium 2 and cultured consortium 3 samples, the cultured consortium 1 sample was also included in the 16S rRNA sequencing analysis. All three selective growth experiments resulting in the cultured consortium 1, cultured consortium 2, and cultured consortium 3 samples, respectively, yielded genera that were common to and predominant among all three samples, while certain differences in predominant genera were also apparent. *Lactococcus*, *Paenibacillus*, *Bacteroides*, and *Parabacteroides* were reproducibly detected in the bacterial consortia grown in 3 independent experiments in the presence of butyrate. *Clostridium* or unclassified members of the order Clostridiales or family Clostridiaceae were also reproducibly found in the consortia of bacteria cultured in the presence of

butyrate. High proportions of *Lactobacillus* were detected in two out of the three consortia resulting from independent culturing events.

Due to the implication of butyrate in colon health (Hamer *et al*., 2008), butyrate-producing bacteria have been the focus of studies aimed at investigating the cultivability and diversity of butyrate-producing bacteria (Barcenilla *et al*., 2000; Louis *et al*., 2010; Vital *et al*., 2013). These studies have revealed that butyrate-producing bacteria can be phylogenetically diverse, a factor confounding the study of this functional group. To target butyrate-producing bacteria, some studies have focused on specific markers for butyrate production. For example, Louis *et al.* (2010) targeted butyryl-CoA:acetate CoA transferase, an enzyme involved in one of the two identified pathways responsible for butyrate production in bacteria. Using primers specific for this gene, sequences were amplified from fecal samples of healthy volunteers and effectively used to detect *Eubacterium* and *Roseburia*, two genera of bacteria previously isolated from human feces and known for butyrate production (Barcenilla *et al*., 2000; Duncan *et al*., 2007), as well as a cultured but unnamed species. Remaining sequences detected in this study were attributed to uncultured butyrate-producing strains (Louis *et al*., 2010). A potential next step to complement the ^{1}H NMR analysis data and assess whether the specific genera of bacteria cultured in this study correlate with the production of butyrate is the application of a recently developed computational approach called PICRUST (Langille *et al*., 2013). PICRUST (phylogenetic investigation of communities by reconstruction of unobserved states) is a method designed to predict the functionality associated with a microbial community (Langille *et al*., 2013). PICRUST uses marker gene data such as 16S rRNA sequences along with a database of reference genomes to predict the gene families

present in a given microbial community and estimate the associated functionality by combining the predicted gene families (Langille *et al*., 2013). The application of PICRUST to the sequencing data obtained in this study can potentially strengthen the association between the bacteria reproducibly cultured in the presence of butyrate and the ^{1}H NMR data suggesting that the bacterial community is able to produce butyrate. While a 16S rRNA sequencing approach was used in this study, metagenomics sequencing is an alternative method that would allow sampling the total DNA content of a microbial population. This approach would allow for the direct detection of genes involved in the production of butyrate and could also be used to complement the ^{1}H NMR analysis.

4.3.4: Administration of the Streptomycin-resistant, Butyrate-associated Consortium of Cultured Bacteria in the Murine Model of *S. flexneri* Infection

The cultured consortium of bacteria grown in the presence of streptomycin and butyrate was administered to mice prior to challenge with *S. flexneri* to test whether the consortium of bacteria could confer protection *in vivo.* Taken together, analyses of *Shigella* burden, mortality, and clinical outcome do not support a protective role for the cultured consortium 2 bacteria in conferring protection against *S. flexneri* in the mouse model of infection. Other studies have observed disease-reducing effects associated with commensal bacteria and a protective role has been demonstrated against the enteric pathogens *V. cholerae*, *S. Typhimurium,* and vancomycin-resistant *Enterococcus faecium* (Hsiao *et al*., 2014; Martz *et al*., 2015; Ubeda *et al*., 2013).

While the cultured consortium of butyrate-associated bacteria (cultured consortium 2) did not alter the clinical outcome of *S. flexneri* infection in mice, the process of culturing anaerobes from the feces of healthy mice in the presence of butyrate

did reproducibly yield consortia of bacteria with similar composition. Bacteria resulting from independent culturing events clustered in close proximity in a PCA plot and showed clear separation from samples representative of the healthy mouse gut. In one of the two ^{1}H NMR analyses, bacteria cultured using the butyrate selection technique demonstrated the production of butyrate. Variability in the microbiomes of mice used as the source for fecal samples from which bacteria were cultured could account for the discrepancies observed. Related to this work, an investigation of the variance in microbial communities between BALB/c mice matched for age and sex obtained from the same facility (Charles River Laboratories) would lend to a more complete understanding of this work.

DNA extracted from fecal samples of cultured consortium 2-treated mice was analyzed by 16S rRNA sequencing to test whether bacteria originally predominant in the cultured consortia could be detected in consortium-treated mice. Many genera that were present at high proportions in the cultured consortia of bacteria were no longer detected at high proportions in the feces of consortium 2 treated mice. These data suggest that colonization was likely not achieved by these particular genera including *Bacteroides* and *Parabacteroides*. In contrast, some genera predominant in the original cultured consortium were detected in the feces of mice treated with the cultured consortium 2 bacteria. Although levels varied between the two consortia treated samples representative of independent experiments, low levels of *Lactococcus* were detected in mice after treatment with the cultured consortium 2 as well as high proportions of *Lactobacillus* and *Clostridium*. While levels of *Lactococcus and Clostridium* were reduced in the feces of consortium-treated mice after infection with *S. flexneri*, *Lactobacillus* persisted at high levels. *Lactobacillus* has been associated with reducing inflammation via components in

the cell wall of the bacterium and is of interest for potential applications in treating patients diagnosed with IBD (Rigottier-Gois, 2013). Members of the genus *Paenibacillus,* which were low in samples representative of the healthy mouse, were detected at high levels in all other samples including those representative of the streptomycin treated mouse, the streptomycin-treated consortium treated mouse, the streptomycin *S. flexneri* infected mouse, and the streptomycin-treated mouse treated with consortium bacteria and infected with *S. flexneri*. One possibility is that *Paenibacillus* species thrive in the presence of streptomycin. Variables that could potentially improve the colonization of the cultured consortium of commensal bacteria include the timing and method of administrating the consortium of bacteria. A longer time period between treatment and *S. flexneri* infection might alter the ability of commensals to establish a niche within the host prior to challenge with *S. flexneri*. Repeated delivery of the commensal consortium throughout the infection might also improve colonization, however the acute nature of *S. flexneri* induced morbidity in the mouse and the short experimental design of our murine model would hinder this approach.

Strangely, 16S rRNA sequencing revealed a high proportion of unclassified Enterobacteriaceae in the sample from the control consortium grown in the absence of butyrate (data not shown). Due to the relatedness of *S. flexneri* and *E. coli*, of which there are many commensal strains, the region of the 16S rRNA gene used for analysis is not sufficiently different between these bacteria to allow for distinction. Therefore, these genera are grouped as unclassified members of the family Enterobacteriaceae. Using classical microbiological methods, we have excluded the possibility that this bacteria is

Shigella. We plan to culture the no butyrate control consortium again and repeat DNA extraction and sequencing to rule out the formal possibility that samples were mixed up.

4.3.5: Commensals Cultured from the Feces of Healthy AKR/J mice

To further investigate the potential role of commensal bacteria in colonization resistance to *S. flexneri*, bacteria were cultured from the feces of an alternative mouse strain, AKR/J, on selective Eggerth Gagnon (EG) media**.** EG media has been used to selectively culture *Barnesiella viscericola* from the cecal content of chicken (Sakamoto *et al*., 2007). Previous work in the Rohde Lab suggested that members of the *Barnesiella* genus might account for facility-associated differences observed in the susceptibility to *S. flexneri* infection in the AKR/J mouse (unpublished data). We aimed to culture *Barnesiella* as a first step to investigate whether this genus could be involved in colonization resistance to *S. flexneri.* Four different isolates were cultured from the feces of the healthy AKR/J mouse on selective EG media, however none were identified as *Barnesiella.* Instead, two species of *Enterococcus* and multiple species of *Clostridium* were identified by sequencing using universal primers for the V1-V3 region of the 16S rRNA gene. ^{1}H NMR profiles of these isolates revealed lactate and acetate, however no further metabolites were detected. One of these isolates had 99% sequence identity with both *C. nitrophenolicum* and *C. acidotolerans.* These bacteria have been isolated from soil (Suresh *et al*., 2007) and wetland sediment (Lee *et al*., 2007), respectively, however a literary search revealed that they have not previously been associated with the mammalian gut. The isolated bacteria from the feces of AKR/J mice represent potential starting points for continued study on the role of commensal bacteria in colonization resistance to *S. flexneri* in the Rohde Lab.

4.3.6 Conclusions:

This thesis addresses three areas of research related to colonization resistance to *S. flexneri:* the use of the intracellular carbon metabolism pathway to support *S. flexneri* colonization, the role of nitrate reduction in the ability of *S. flexneri* to colonize the streptomycin-treated mouse and overcome colonization resistance, and the role of commensal bacteria in colonization resistance to *S. flexneri*. The intracellular growth defect previously associated with a mutant interrupted for carbon flow through the PTA-ACKA pathway was confirmed after construction of the mutant and *in vitro* assays to measure intracellular proliferation. Results support a role for the PTA-ACKA pathway in proliferation of *S. flexneri* inside the host cytosol during infection. This work yielded mutant strains that can be used as tools to investigate the role of the PTA-ACKA pathway in *S. flexneri* infection *in vivo*.

The role of nitrate reduction in colonization in the murine model of *S. flexneri* was tested. A mutant defective for nitrate reductase activity behaved similarly to wild type *S. flexneri* during infection of the streptomycin-treated mouse in separate and co-infection experiments. We observed that the ability to reduce nitrate does not confer a competitive advantage to *S. flexneri in vivo* indicating that *S. flexneri* does not use nitrate reduction as a mechanism to overcome colonization resistance in the streptomycin-treated mouse.

Lastly, the role of the commensal microbiota in colonization resistance to *S. flexneri* in the murine model of infection was investigated. Commensal bacteria were cultured from the feces of healthy mice using a method of selective growth to enrich for streptomycin resistant, butyrate associated anaerobes compatible with the mouse model

of infection. ^{1}H NMR analysis demonstrated the production of butyrate by the cultured commensal bacteria, however butyrate production was not reproduced by a consortium of commensal bacteria cultured using the same method from the feces of a new set of mice. The butyrate-producing consortium of cultured bacteria did not confer protection upon challenge with *S. flexneri* in the streptomycin-treated mouse model. Characterization of the selectively grown bacterial consortia by 16S rRNA sequencing revealed similarities in the major genera cultured across the consortia derived from three independent experiments using feces from different mice. This work represents a proof-of-principal approach to enrich for butyrate-producing bacteria and addresses the underexplored theme of the role of commensal bacteria in colonization resistance to *S. flexneri.*

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