

Intimin Likely Used to Cause Disease During Competition with Commensal *Escherichia coli*

Dominique J. Richburg

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Randall Hubbard, Ph.D.  
Thesis Chair

---

James T. McClintock, Ph.D.  
Committee Member

---

Vernon M. Whaley, Ph.D.  
Committee Member

---

Marilyn Gadomski, Ph.D.  
Honors Assistant Director

---

Date

**ABSTRACT**

The intimin gene in the Locus of Enterocyte Effacement (LEE) island of pathogenicity is the primary attachment mechanism in *Citrobacter rodentium*. Intimin is a bacterial adhesin (protein) that attaches to obtain a niche/nutrient and thrive within the intestine. Intimin was deleted within *C. rodentium* to study colonization and pathogenesis in the murine intestine. Additionally, *C. rodentium* is an attaching/effacing pathogen, and a useful murine model in understanding Enterohemorrhagic *Escherichia coli* (EHEC) infection in humans. *E. coli* and *C. rodentium* cause gastroenteritis in humans and mice, respectively. *C. rodentium* is a murine pathogen commonly used to model gastrointestinal disease because EHEC cannot be studied within mice from its lack of pathogenicity. Results have shown that *C. rodentium* uses intimin through causing disease during competition with commensal *E. coli*. By studying the mechanisms and genes involved in pathogenic adhesion in *C. rodentium*, it will be easier to find out a cure or treatment for illness caused by the before mentioned *E. coli* strains such as Crohn's disease, ulcerative colitis and colonic tumorigenesis.

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## INTRODUCTION

*Citrobacter* species are often present in soil and water, and can be isolated from human and animal feces (1). *Citrobacter rodentium*, formerly known as *Citrobacter freundii* biotype 4280 is a non-invasive, gram-negative, enteric pathogen for mice that is similar to the human enteric pathogens, Enteropathogenic *Escherichia coli* (EPEC) and Enterohemorrhagic *E. coli* (EHEC) (1). Both EHEC and EPEC are poorly pathogenic in mice, yet can infect humans and other domestic animals (1). EPEC and EHEC O157:H7 are food-borne pathogens that cause diarrhea, hemolytic-uremic syndrome, and hemorrhagic colitis (2). As a non-invasive pathogen, *C. rodentium* is useful to in how the host recognizes and eliminates pathogens on the intestinal lumen, and differentiates the pathogens from the normal flora (1). *C. rodentium* is a member of the family of bacterial pathogens, and currently, the only known murine attaching and effacing (A/E) pathogen (3). Attachment is localized to the intestinal epithelium whereas effacement of the brush-border microvilli is characterized by the formation of pedestal-like structures underneath adherent bacterium (4, 5).

EHEC is a human pathogen that can cause attaching and effacing (A/E) lesions by colonizing the intestinal mucosa (4). EPEC is the main cause of infantile diarrhea, and results in high rates of illness and death in developing countries (4). *C. rodentium* is an essential model organism that can colonize the host—and initiating an immune response including an apoptotic and inflammatory response (4). In addition, *C. rodentium* is an effective model for studying inflammation as a result of mucosal immune responses to

infection (4). *C. rodentium* induces inflammation in the cecum and the colon (4). *C. rodentium* infections in mice present the same way as other similar gastrointestinal pathologies in mice (1).

*C. rodentium* is an unstable pathogen because of active rearrangements of mobile genetic elements and because of macro-scale genomic recombination. This study showed that genetic rearrangements caused *C. rodentium* to become less pathogenic because the gene deletion connected *C. rodentium* with pathogenicity. This showed that *C. rodentium* was the only known bacteria to cause disease in laboratory rodents. *C. rodentium* is also useful in studying gastroenteritis in other animals, such as rabbits and piglets. Infection caused by *C. rodentium* is useful for modeling several important intestinal disorders such as Crohn's disease, ulcerative colitis, and colon tumorigenesis so that mechanisms can be studied and cures can be found (4, 6, 7).

#### ***C. rodentium* DBS100 and Locus of Enterocyte Effacement (LEE)**

The two studied strains of *C. rodentium* are ICC168 (FN543502) and DBS100 (ATCC 51459), both of which were isolated from a disease outbreak of Swiss-Webster mice at Yale University School of Medicine in 1972 (7). DBS100 and ICC168 share a common ancestor with the primary difference where DBS100 is used in the United States, and ICC168 is used in Europe (7). DBS100 can induce pedestal formation *in vivo* (8). Studies have shown *C. rodentium* might require signals from the intestinal microbiota or from the host to regulate pathogenesis and facilitate colonization in the colon (4). The intestinal microbiota affects the development and activity of intestinal immune cells in mice that have increased resistance to colonization by *C. rodentium* (4). Additionally, nutrient supplementation with omega-6-polyunsaturated fatty acids (PUFAs), omega-3

PUFAs, or vitamin D regulates mucosal immune responses (4). Omega-3 PUFA and omega-6 PUFA supplementation reduces *C. rodentium*-induced inflammation but impedes epithelial intestinal alkaline phosphatase. Epithelial intestinal alkaline phosphatase detoxifies *C. rodentium* lipopolysaccharide (LPS) to limit inflammation, which causes increased mortality (4). Diet supplementation with monosaccharides provides an initial carbon source for *C. rodentium* to overcome lag and consequently outcompete the microbiota in the gastrointestinal tract (4).

Intestinal colonization by *C. rodentium* occurs in three stages: transient loose attachment, translocation of bacterial effectors into the cell via Type III secretion system (T3SS), and the formation of an A/E lesion (4). To form A/E lesions, the 41 genes of the LEE pathogenicity island are clustered into five operons: LEE1-5. The entire LEE pathogenicity island encodes transcript regulators *ler*, *glrA*, and *glrR*, the structural components of T3SS, effectors, their individual chaperones, and the outer membrane adhesion molecule, intimin (4). The translocated intimin receptor, Tir is considered the best characterized T3SS effector through inserting into the plasma membrane as a hairpin-loop (4).

### ***C. rodentium* Effacement**

Wanyin Deng *et al.* conducted a study in 2001 that focused on effacement caused by *C. rodentium*. EPEC and EHEC in a similar mechanism as *C. rodentium*, intimately attach to host intestinal cells, which induces effacement of brush border microvilli (3). *C. rodentium*'s is analogous to EPEC and EHEC with the secretion of several Esp proteins into the smallest culture medium and translocates some of the proteins into host cells. ES proteins are enterococcal surface proteins that aid in colonization and cause disease in

diverse hosts including humans, dogs, rabbits, and pigs (3). The Esp proteins include Esp A, Esp B, and Esp D (3). Esp A, Esp B and Esp D are virulence factors and three EPEC type III secreted proteins. An intimin mutant is a bacterium that lacks the intimin gene and consequently the protein. The *E. coli*, Tir gene also compliments *C. rodentium* (3).

### **Series of Infection of *C. rodentium***

Many gastrointestinal infection studies involve the inoculation of mice by feeding the mice lab-cultured bacteria resulting in a highly repeatable infection cycle (4). *C. rodentium* infected mice can colonize analogous to certain strains of *E. coli*, and cause severe gastroenteritis in humans. J.W. Collins *et al.* found that during lab infection, *C. rodentium* levels can reach 1-3% of the total intestinal microbiota, colonizing only in the distal colon. In addition, the normal course of *C. rodentium* infection in mice is multiplication of *C. rodentium* to a high level by day 7 post-infection that is cleared by day 21 (4). About two to three days later post-infection, bacteria should accumulate in the distal colon (4). Mice that lack T cells and B cells develop chronic infection whereas normal mice that have recovered from infection are more immune to repeated *C. rodentium* infection (4).

*C. rodentium* is an important model organism for human gastrointestinal disease research. *C. rodentium* is under the family of pathogenic bacteria that spreads via fecal-oral transmission, and causes colitis, or transmissible murine crypt hyperplasia (4). *C. rodentium* colonizes the major lymphoid structures in the colon first before traveling down the gastrointestinal tract (1). *C. rodentium* infected mice through natural transmission can rapidly colonize *C. rodentium* in the colon, and cause hyperplasia, without needing to adapt in the cecum (1). Development of colonic hyperplasia relates to an increased susceptibility

to carcinogens (1). The majority of mouse strains such as C57B1/6, NIH Swiss, and Balb/c show little to no mortality when infected with *C. rodentium*. This explains why the CD-1 mice are used to model infection (1).

### **Pathogenesis and Role of Intimin in Attachment**

*C. rodentium* shares over half of its genes with EPEC and EHEC, including the the locus of enterocyte effacement (LEE), a pathogenicity island, which codes the proteins to form A/E lesions (9). A main portion of LEE is the intimin gene, *eae*. The intimin-encoding *eae* gene was sequenced originally from EPEC strain E2348/69, and later from the EHEC strain EDL933 (10). It has been shown to have a conserved sequence across many *E. coli* and *Citrobacter* genomes. LEE also contains genes that encode for the LEE gene expression regulator, Ler (3). Ler is a type III secretion system (3). Schauer and Falkow validated that intimin expressed by *C. rodentium* is vital for forming intestinal A/E lesions in infected mice (11). Intimin's role in human disease was shown in human volunteer studies that ingested a void isogenic *eae* mutant of EPEC strain E2348/69 (10). The intimin family is expanding and there is confirmation that recombination has played a role in the history of *eae* (10). Further studies including pathogenetic and epidemiological investigations are needed to explain the mechanism and role of *eae* variability in human disease (10).

Intimin serves as the primary attachment mechanism in *C. rodentium* (8). Intimin was the first EPEC protein identified as vital for A/E lesion formation *in vitro* and for full virulence in human volunteer studies (8). *C. rodentium* carries intimin- $\beta$ , which is essential for attachment (8). Polymerase chain reactions (PCR) can be used to remove the intimin

gene (*eae*) to help further understand how essential attachment is to colonization and pathogenicity (9).

Studies have shown that exchanging intimin- $\alpha$  of EPEC with intimin- $\gamma$  of EHEC without exchanging Tir in a recombinant EPEC strain resulted in enhanced tropism to Peyer's patches (9). The gene intimin- $\alpha$  spread throughout the small intestine instead of intimin- $\gamma$  (9). The same study showed that a few intimin alleles are rare among strains related to severe human gastrointestinal disease (9). Further pathogenic studies and epidemiological investigations are now in progress to clarify the mechanism and the role of *eae* variability in human gastrointestinal disease (9).

### **The Role of Tir, the Intimin Receptor**

All A/E pathogens translocate their own intimin receptor, Tir (10). Tir was found initially as a 90-kDa tyrosine-phosphorylated protein in the target cell membrane, and was previously called Hp90 (10). Throughout the infection of *C. rodentium* within the colonic epithelium, adherent bacteria translocate Tir into the infected enterocyte by a type III secretion system (T3SS), which is further added into the plasma membrane in a hairpin-loop structure (4). *C. rodentium* puts Tir into the membrane of the enterocyte so that intimin can bind to it and thus allow the bacteria to adhere to the enterocyte (4). Tir is translocated to the apical side of colonic epithelial cells (4). During murine infections, Tir is expressed in *C. rodentium*, and then translocated into host intestinal epithelial cells (4). Post infection, the bacterial count of *C. rodentium* can reach from  $10^8$  –  $10^9$  Colony forming units (CFUs) per colon at day 10-14 in a few mouse strains and can be visualized through bacterial population abundances (4). Intimin binding causes the clustering of Tir and begins to assemble signaling complexes and actin polymerization (4).

The central actin polymerization pathway *in vitro* is initiated by phosphorylation of the tyrosine at amino acid 471 of Tir (4). This results in the formation of a binding site for non-catalytic region tyrosine-kinase (NCK) of the mammalian adaptor protein (4). NCK acts as an activator for the Tir-induced actin polymerization pathway at mucosal surfaces (4). The roles of Tir and intimin are vital for the pathogenicity of REPEC 0103 (rabbit EPEC), and *C. rodentium* (8). The study of rabbit EPEC serotype O103:H2 explained that Tir and intimin are required for REPEC to nucleate F-actin and cause A/E lesions (8). However, Tir is not necessary for A/E lesion formation, suggesting the induction of colonic hyperplasia in mice or intestinal colonization (8).

A *C. rodentium* Tir mutant that expresses either EHEC Tir or TirY471F can colonize in the mouse intestine and induce colonic hyperplasia (8). TirY471F is a mutant strain of *C. rodentium* Tir ( $\Delta tir + C. rodentium tir Y471F$ ) (8). DBS100 $\Delta tir$  phenotype infecting mice is analogous to the intimin deletion mutant DBS100 $\Delta eae$  in mouse colonization studies (8). The delta sign ( $\Delta$ ) indicates the gene  $\Delta tir$  or  $\Delta eae$  is missing. Intimin rather than Tir acts as the elicitor for inflammation, and causes colonic hyperplasia in mice infected with shown by W. Deng *et al.* (8). Tir continues to serve as the receptor in bacterial attachment for intimin so that the strain can connect to the mouse epithelial cell surface to induce inflammatory response (8).

### **The Interactions between Intimin and Tir**

The interactions between intimin and Tir are essential for bacterial adherence via large bowel-colonizing A/E pathogens, but not small bowel-colonizing A/E pathogens (8). In addition, interactions between intimin and Tir are the major pathways for colonic adherence of EHEC and *C. rodentium* (8). However, intimin mutants of small bowel-

colonizing A/E pathogens can retain their capability to colonize the host gastrointestinal tract, which implies that intimin is likely to be responsible for initial bacterial adherence (8). An intimin mutant implies that intimin was deleted within *C. rodentium*. However, intimin's interaction with Tir seems to be the main mechanism for colonic adhesion by EHEC and *C. rodentium* (8). Wanyin Deng *et al.* have shown that sequence analysis demonstrated that *Citrobacter* Tir is more comparable to EPEC Tir than EHEC Tir since both *Citrobacter* Tir and EPEC Tir have phosphorylated tyrosine residues and can support pedestal formation, unlike EHEC Tir (8). EHEC Tir is not tyrosine phosphorylated, and cannot supplement EPEC or *C. rodentium* without the protein Tir for pedestal formation (8).

### **Immune Responses**

The immune responses of the intestinal mucosa play a vital role in antimicrobial immunity and mucosal homeostasis (4). The myeloid differentiation primary response protein 88 (MYD88) is essential in innate immune signaling downstream of the Toll-like receptor (TLR) and the interleukin-1 receptor (IL1R), which mutually control transcriptional regulation of multiple immune-related genes (4). MYD88 regulates *C. rodentium* infection by recruiting neutrophils, macrophages, and dendritic cells to the mucosa by expressing the inducible nitric oxide synthase (iNOS) and generating the proliferation of epithelial cell, thus inducing colonic hyperplasia (4).

Deficient MYD88 mice lose the ability to limit bacterial replication within the host (4). The primary donors to MYD88-mediated responses against *C. rodentium* are two specific TLRs, TLR2 and TLR4, along with the whole TLR family at large as well as IL1R are the primary donors to MYD88-mediated responses against *C. rodentium* (4). TLR2

and TLR4 control *C. rodentium* infection by producing the pro-inflammatory cytokines, keratinocyte chemoattractant, tumor necrosis factor (TNF), and interleukin-6 (IL-6), and by causing upregulation of iNOS (4). TNF is the pro-inflammatory cytokines keratinocyte chemoattractant, during *C. rodentium* infection in TLR-2 deficient mice, fatal colonic pathology takes place with increased weight loss and mortality (4). TLR4 is vital for infiltration of the intestinal mucosa by neutrophils, macrophages, and for chemokine responses (4). TLR4 expression enhances colonization of the colon by *C. rodentium* during the initial stages of infection, which indicates that low-level inflammation is beneficial for the pathogen (4).

### **History of Intimin and Commensal *E. coli***

The most common cause of infantile diarrhea in industrialized countries during the 1940s and 1950s was EPEC, but now outbreaks are very rare (8). Previous studies have found that classical EPEC is the most common bacterial cause of diarrhea in children who are younger than 2 years old in non-industrialized countries (8). EPEC strains can be grouped as typical or atypical (9). Typical EPEC strains have a virulence plasmid including genes encoding the bundle-forming pilus (Bfp), which is needed for localizing adherence on cultured epithelial cells (9). Atypical EPEC strains do not have the EAF plasmid with the *bfp* gene (9). However, in industrialized countries, atypical EPEC strains are more regularly isolated from diarrheal cases than typical EPEC (9). While the actual cause of diarrhea remains undetermined, host responses to EPEC infection rather than the strain itself likely lead to diarrhea (10).

Intimin is involved in facilitating both tissue tropism, host specificity, and provides essential information on the association of EPEC and Shiga-toxin producing *E. coli* (STEC)

with specific bacterium-host related diseases (8). Countries such as Brazil, Bangladesh, Chile, and Uruguay have demonstrated a lower frequency of typical EPEC serotypes in stools from children with diarrhea (8). Since *C. rodentium* is similar to EPEC, and has been shown to be a useful murine model for studying gastrointestinal disease, current studies are being done to study colonization and pathogenesis (8). The intimin gene (*eae*) is essential to understanding colonization because diarrhea illness is a major public health problem worldwide (9). Statistics have shown that over 2 million people die each year due to diarrheal disease, especially infants younger than five years old (9).

### **Commensal Relationship of Enteropathogenic *E. coli* (EPEC) and Enterohaemorrhagic *E. coli* (EHEC)**

EPEC and EHEC O157:H7 within the mouse gastrointestinal tract have characteristics similar to commensal interaction instead of pathogenic interaction (12). Mice are used to study intestinal responses to EPEC infection and the role of virulence factors in EPEC-induced disease since the mouse gastrointestinal tract is similar to that of a human (12).

The Commensal *E. coli* 1917 Nissle was used as a control in this study to compare colonization dynamics of EPEC, EHEC, and *C. rodentium* (12). Nissle was used because the commensal has been shown to have the greatest stability in competitive fitness when colonizing in mice (12). EHEC can colonize more efficiently and cause greater intestinal inflammation than EPEC, which is unable to colonize effectively (12).

### **Understanding Quantitative Polymerase Chain Reactions**

Polymerase chain reaction (PCR) amplifies a specific DNA fragment from a convoluted pool of DNA (13). Performing PCR can be done using source DNA from a

various tissues and organisms, including hair, skin, peripheral blood, saliva, and microbes (13). Only picograms of DNA are required for PCR to create ample copies to be analyzed using standard laboratory procedures (13). Since only small amount of DNA are required, PCR is a sensitive assay (13).

Quantitative PCR (qPCR) gives information beyond the plain detection of DNA (13). It determines how much of a specific DNA or gene is present in a given sample. qPCR enables both the detection and quantification of the PCR product in real time, while it is being produced (13). Two conventional methods are used to detect and quantify the qPCR product (13). The first method is the use of fluorescent dyes that non-specifically interpolate with double-stranded DNA (13). The second method is the use of sequence-specific DNA probes, which consists of fluorescently labeled reports (13). Analysis of qPCR can be used to quantify the amount of a particular gene isolated, and investigate single cells and quantify various combinations of DNA, mRNA's, proteins (13).

Advantages of qPCR provide a simple way in understanding changes in gene expression levels in microbes, tumors, or other disease states (13). Additionally, qPCR produce results rapidly, and quantitatively demonstrate how much of a particular sequence is present (13).

## **Recent Studies**

### **Studies on Pretreatment with Probiotics**

Probiotics are useful in preventing and treating acute diarrhea caused by antimicrobial resistant intestinal pathogens (4). Probiotics are nonpathogenic, living microorganisms that create beneficial health effects, often from the capability to occupy a particular nutritional niche (14, 15). K.C. Johnson-Henry *et al* focused on the pretreatment

with probiotics for mice infected with *C. rodentium* (14). Mice were given sterile water, followed by  $10^9$  CFU/ml probiotics in sterile drinking water, and then followed by maltodextrin in sterile water (14). It was found that mice given sustainable probiotics or that were pretreated with probiotics remained in good health (14). However, mice without pretreatment of probiotics were highly susceptible to infection with *C. rodentium*. Probiotics decrease the severity of gastroenteritis in mice infected with *C. rodentium* (14). Therefore, probiotics are useful in lowering the extent of infectious diarrhea and reducing the fecal shedding of pathogens (16). There is a need for additional research in using probiotics for outbreaks of EHEC infection in humans (14).

Probiotics are useful in interrupting enteric infectious disease progression and are sufficient therapies for several human intestinal diseases such as infectious diarrhea, irritable bowel disease, and enterocolitis in premature newborns (16). Additionally, probiotics prevent binding of *C. rodentium* to host epithelial cells when provided before or at the time of infection (16).

In the Johnson-Henry study, pretreatment with any particular species for three or six hours before infection did not decrease the severity of infection (14). However, *Lactobacillus rhamnosus* and *Lactobacillus helveticus* are probiotic mixtures that prevent *C. rodentium* infection in neonatal mice (14). Resta-Lenert and Barrett showed that a probiotic mixture of *Streptococcus thermophilus* and *L. acidophilus* mitigates the unfavorable effects of enteroinvasive *E. coli* on host epithelial cells (14).

Other studies have shown the bactericidal effects of probiotics against pathogenic bacteria (14). Probiotic treatment of epithelial cells inhibits rearrangements of host

cytoskeletal proteins, inhibiting attaching-effacing lesions following EPEC and EHEC infection (14).

### ***C. rodentium* Infection Studies Using Polyunsaturated Fatty Acids (PUFAs)**

Dietary oils such as polyunsaturated fatty acids (PUFAs) are known to increase susceptibility to *C. rodentium* infection and induce colitis (4). Omega-6 PUFA is common in diet and an essential fatty acid (4). Dietary supplementation with omega-6 PUFA showed increased levels of Enterobacteriaceae, *Clostridium* species, and segmented filamentous bacteria (SFB) in mice, all of which have caused pro-inflammatory responses and irritable bowel disease (4). SFB is the first example of a commensal species that alters host adaptive immune cell homeostasis (17). Both omega-3 PUFA and omega-6 PUFA are precursors to various substances in the body and help regulate blood pressure, and inflammatory responses (4). Conversely, combining omega-6 PUFA with the omega-3 PUFA supplement increases the quantity of *Lactobacillus* and *Bifidobacteria* species but reduced the pro-inflammatory response in the microbiota during *C. rodentium* infection (4).

### ***C. rodentium* Infection Studies Using Deficient Antioxidants, Selenium and Vitamin E**

Selenium and Vitamin E are antioxidants that have been shown to increase *C. rodentium* infection (2). In addition, mice fed with vitamin E and selenium deficient diet for 6 weeks had enhanced loads of *C. rodentium* in the spleen and colon (2). *C. rodentium* was found to be an effective inducer of a Th1/Th17-type inflammatory response (2). A greater inflammatory response was demonstrated in increased cytokine and chemokine expression in infected mice fed the vitamin E and selenium deficient diet in comparison to those fed a controlled diet (2).

Vitamin E and selenium are essential in immune function and host antioxidant defense (2). Selenium is important for controlling oxidative stress and the oxidation balance (2). In addition, selenium is essential for protection against endotoxin-induced oxidative stress, respiratory bursts, and cytotoxic T-lymphocyte and natural killer cell activity (2).

Vitamin E is a potent peroxy radical scavenger that stops lipid peroxidation and is found to increase immune cell concentrations (2). The result of vitamin E deficiency is increased oxidative stress and damaged immune function (2). Understanding other methods used to increase the pathogenicity of *C. rodentium* infection is essential to revealing the mechanism of the pathogenesis of *C. rodentium* (2, 18, 19).

### **Future Research Studies**

Future studies on the intimin gene (*eae*) may aid in understanding how vital attachment is to pathogenicity and colonization (20, 21). With *C. rodentium*  $\Delta eae$  (intimin deletion mutant), attachment can be further analyzed on a new level. Another possible future study would be using qPCR to quantify bacterial population abundances in mice when co-colonized with *C. rodentium* and one of the three strains of *E. coli*: MG1655, HS, and Nissle. The degrees of pathogenicity differ for each *E. coli* strain (22). Since MG1655 is a commensal strain, the degree of pathogenicity will be higher compared to the probiotic, Nissle, and the normal flora, HS strain (22). Future experiments should be performed with *C. rodentium*  $\Delta eae$  and *E. coli* to further understand their role in competitive colonic colonization in mice. The interactions of *C. rodentium* through intimin along with the interactions of *C. rodentium* with *E. coli* and probiotics should be further analyzed.

Determining whether *C. rodentium* can co-colonize with *E. coli* strains MG1655, HS, and Nissle will show competitive fitness patterns in the mice gastrointestinal tract.

## METHODS

### **Experiment 1: Co-colonization of *C. rodentium* versus *E. coli* strains MG1655, Nissle, and HS**

***Antibiotic Treatment and Liquid Media.*** Eleven CD-1 male mice, 6 weeks of age, were given drinking water containing streptomycin sulfate (5g/L) for 24 hours prior to inoculation as well as throughout colonization, which opens the facultative anaerobic niche for streptomycin resistant *E. coli* and *C. rodentium* by removing the resident facultative microbiota while leaving anaerobic microbiota essentially intact. Nalidixic acid resistant *E. coli* strains and rifampicin resistant *C. rodentium* strains were used; appropriate antibiotics (50ug/ml each) were used in post-infection analysis to distinguish between the bacteria during the co-colonization. All strains were streptomycin resistant in addition to their distinguishing antibiotic resistances. A 1% tryptone lysogeny broth was used as a bacterial growth medium for fecal collections. MacConkey agar with appropriate antibiotics was used as a differential growth medium to select for the desired gram-negative antibiotic-resistant strains.

***Starvation and Inoculation of Mice.*** Eleven CD-1 male mice were starved 24 hours prior to inoculation to induce sufficient appetite for voluntary ingestion of bacterial suspension. The inoculate was composed of the bacterial culture of either the *C. rodentium* strain or *E. coli* strains suspended in the sucrose solution. A load of  $10^5$  CFU/mL was designated feeding high, while a load of  $10^8$  CFU/mL was designated feeding low.

***Co-colonization of 9 CD-1 Mice.*** *C. rodentium* versus *E. coli* strains MG1655, HS, and Nissle 1917 strains of *E. coli* were fed in a  $10^5$  CFU/ml bacterial suspension to 6 mice. The remaining 2 mice were used as the control and were inoculated with *C. rodentium* DBS100. Ten days post-inoculation, *E. coli* mice were few (low or high) DBS100 to induce competition. The addition of *C. rodentium* induces a co-colonization because two different strains of bacteria are competing for a single niche in the intestines of the mice. Control mice were colonized for 15 days. Co-colonized mice were colonized for 21 days: 10 days with *E. coli* and 11 days with *C. rodentium* competition.

***Fecal Collection.*** Feces were collected from each mouse 5 hours, 24 hours, and every second day thereafter throughout the course of the collection. One gram of feces from each mouse was diluted in 10mL of 1% Tryptone and homogenized. Ten-fold serial dilutions were prepared from the fecal homogenate and plated on MacConkey agar containing appropriate antibiotics. Agar plates were incubated for 24-48 hours at 37°C. The degree of intestinal colonization of each bacteria was determined by quantifying colony forming units persisting on the plated fecal homogenate.

***CFU/gram of Feces Analysis.*** Colonization fitness was quantified by counting the CFU/g of feces on each plated dilution and multiplying by the appropriate dilution factor. Strains with the highest CFU/g of feces were considered most fit as they outcompeted other strains for the facultative anaerobic niche in the intestine.

***Fecal Genomic DNA Preparation.*** Fecal genomic DNA was isolated using a Qiagen DNA Stool Mini Kit and quantified using a Thermo Scientific NanoDrop 2000 Spectrophotometer according to manufacturer's instructions (23). The concentration of DNA was typically around 75-300 ng/ul (23). The Qiagen DNA Stool Mini Kit provides

the most efficient, and easy purification of total DNA from frozen or fresh stool samples (23).

***Euthanization of the Mice.*** All mice were euthanized on day 21 post-infection, and the gastrointestinal tract was examined for inflammation. The colon and ceca for the mice were weighed with feces, and without feces to observe the inflammation and hydropsy in the gut. Fecal matter from the cecum and colon was diluted and plated as before to determine the quantity of *E. coli* and *C. rodentium* present in the distal gastrointestinal tract.

### **Experiment 2: *C. rodentium* $\Delta eae$ Colonization of 3 CD-1 Male Mice Infected with *C. rodentium* $\Delta eae$**

Three CD-1 male mice were inoculated with *C. rodentium*  $\Delta eae$  with rifampicin resistance using methods previously described. *C. rodentium*  $\Delta eae$  is a mutant version of *C. rodentium* that lacks of intimin gene (*eae*). Since intimin facilitates bacterial attachment, the purpose of this experiment was to observe colonization and inflammation to determine if attachment is necessary for pathogenesis. Mice were colonized for 15 days with fecal collections then euthanized as described previously.

### **Experiment 3: gDNA Community qPCR on Co-colonization Fecal Collections**

qPCR samples were prepared as described by Andrew Fabich (23). Genomic DNA from each mouse from days 7 and 17 of the co-colonization experiment were diluted to 0.5ng/mL using deionized water and prepared in a mixture containing SsoFast EvaGreen Supermix (BioRad #172-5200) and appropriate primers as described by the Finlay study (24). Primers for the following classifications of bacteria were included: *Eubacteria*,

*Bacteroidales*, *Bifidobacterium*, *Clostridium*, *Enterobacteriaceae* and *Lactobacillus* (Table 1). DNA samples were taken from feces on day 7 (7 days post *E. coli* inoculation) and day 17 (7 days post *C. rodentium* inoculation) to determine how the overall bacterial population in the gastrointestinal tract changed with the addition of a pathogenic bacterium.

**Table 1.** Primers for qPCR

Target 16S rRNA	Primer	Sequence	Reference
<i>Eubacteria</i> (total bacteria)	UniF340	ACTCCTACGGGAGGCAGCAGT	(24)
	UniR514	ATTACCGCGGCTGCTGGC	
<i>Bacteroidales</i>	BactF285	GGTTCTGAGAGGAAGGTCCC	(24)
	UniR338	GCTGCCTCCCGTAGGAGT	
<i>Bifidobacterium</i>	Bif164F	GGGTGGTAATGCCGGATG	(24)
	Bif662R	CCACCGTTACACCGGGAA	
<i>Clostridium</i> <i>coccoides</i>	UniF338	ACTCCTACGGGAGGCAGC	(24)
	CcocR491	GCTTCTTAGTCAGGTACCGTCAT	
Enterobacteriaceae	Coli F	GTGCCAGCMGCCGCGGTAA	(24)
	Coli R	GCCATAACGTTGAAAGATGG	
<i>Lactobacillus</i>	LabF362	AGCAGTAGGGAATCTTCCA	(24)
	LabR677	CACCGCTACACATGGAG	

## RESULTS

The three experiments performed aid in the understanding of *C. rodentium* growth for the purpose of further understanding of human infection. *E. coli* commensal strain MG1655 outcompeted *C. rodentium* infectious strain DBS100 in the mouse intestine. We found MG1655 is the best *E. coli* strain to use during co-colonization with *C. rodentium* because it shows maximal inflammation in the gastrointestinal wall in mice (Fig. 1b). Since *C. rodentium* infection in mice is analogous to *E. coli* infection in humans, it is used as an important model when studying gastroenteritis. However, Nissle also showed high competitive fitness during co-colonization because the colony forming units per gram of feces remained relatively higher throughout the course of infection (Fig. 5). The intimin mutant data showed that the cecum had minimal inflammation during colonization with an intimin mutant, showing that attachment plays an important role in pathogenesis (Fig. 8).

### **Co-colonization for *C. rodentium* and *E. coli***

Post-euthanization, the colon and ceca of all 9 mice were harvested from the mice and weighed. Larger ceca may indicate hydropsy or swelling (Fig. 1). Control mice showed no inflammation (a), Nissle and HS mice showed minimal inflammation (c and d), and MG1655 mice showed the most inflammation (Fig. 1). Figure 2 consists of the CFU/g feces analysis on the bacterial load during this 21-day experiment. *E. coli* HS had the greatest bacterial load for co-colonization in comparison to *E. coli* Nissle and *E. coli* MG1655 in competition with DBS100. Figure 3 showed the mice weights throughout the experiment. Post-infection, the weights of all 9 CD-1 male mice infected with DBS100 and MG1655, HS, and Nissle increased. While weights increased a minimal degree, post *E. coli* infection, the more substantial increase occurred post DBS100 infection; thus, the weight increase

was primarily due to infection by the pathogenic bacterium (Fig. 3). The weight increase may imply swelling and hydropsy as a result of infection (Fig. 3). Figures 4 and 5 demonstrates the ceca weights with feces and the bacterial plate counts of the ceca after euthanization. MG1655 showed the greatest competitive fitness in the cecum throughout infection, as demonstrated by consistency in the bacterial load (Fig. 5).

### ***C. rodentium* $\Delta eae$ Colonization**

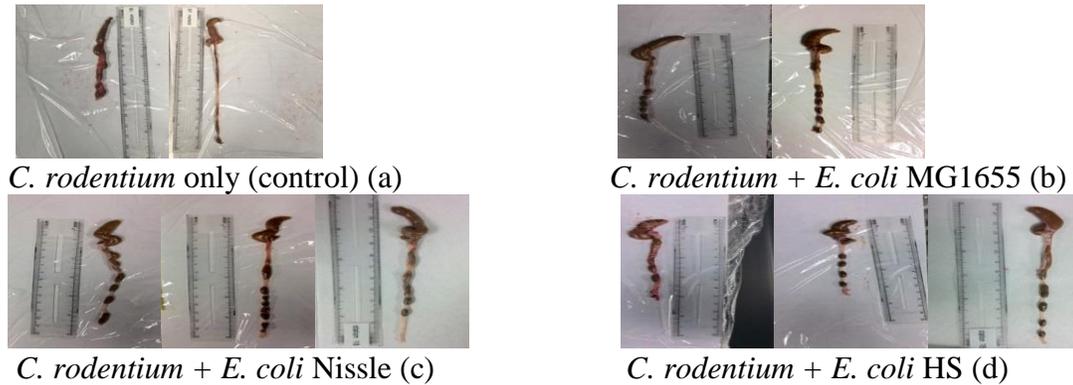
The weights remained consistent throughout the course of the colonization, implying that the mice appeared to remain relatively healthy throughout the infection (Fig. 6). Figure 7 shows the bacterial load of the *C. rodentium* intimin mutant mice, which remained fairly constant throughout the course of the infection. Figure 8 and Figure 9 compare the ceca weights with feces and without feces for all three mice as well as the CFU's from the ceca post-euthanization. Mouse 2 had the largest cecum and the greatest bacterial accumulation in the gastrointestinal tract in comparison to mouse 1 and mouse 3 (data not shown). All mice exhibited negligible inflammation, implying that attachment may be necessary for pathogenesis (data not shown).

### **gDNA Community qPCR for Co-colonized mice with strains *C. rodentium* and *E. coli* MG1655, Nissle, and HS**

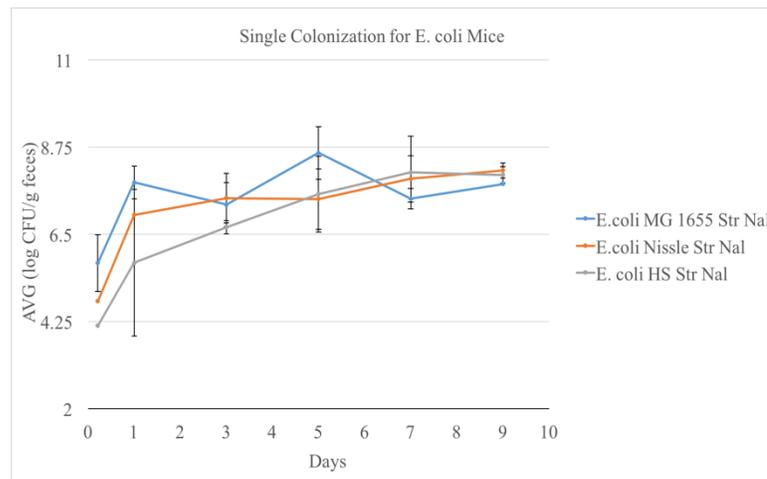
Competitive inhibition occurs when two bacteria compete for the same unique nutrients. The introduction of a novel bacterial species after successful colonization will illicit competition in the enteric biome. The qPCR for this experiment is indicated in table 2 and figure 10. Between day 7 and day 17 (7 days post *C. rodentium* infection), there was 6.72 and 6.82 fold change in *Lactobacillus* and *Clostridium*, respectively (Fig. 10, Table 2). This suggests that during competition between two bacterial populations, competition

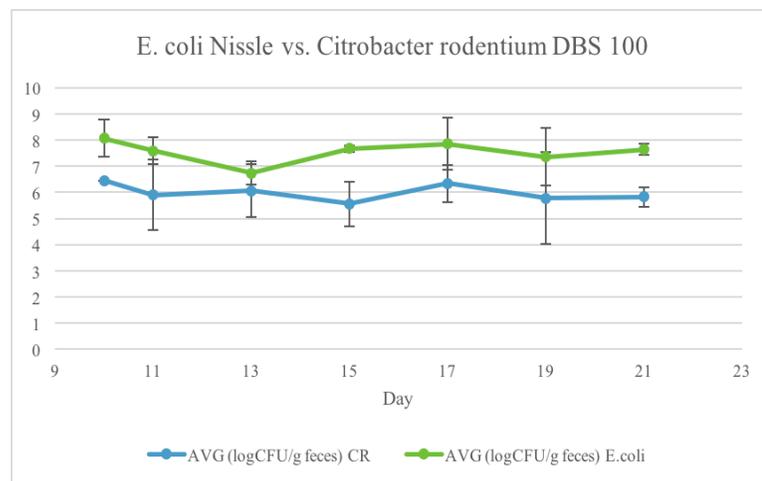
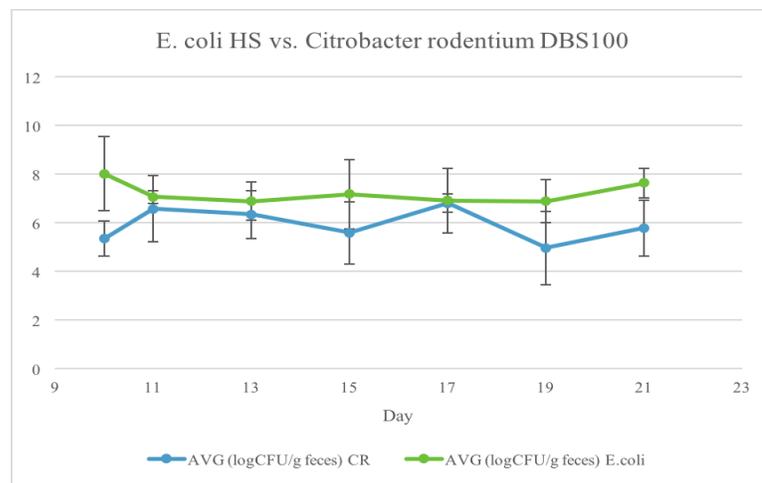
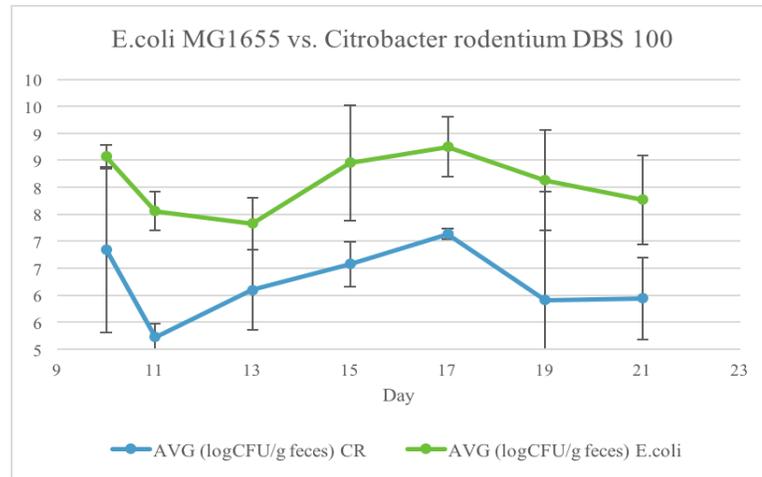
will increase amongst *Lactobacillus* and *Clostridium*. Furthermore, *Bifidobacterium* and *Bacterioidales* show very little to no enteric competitive increase versus *C. rodentium* throughout the course of infection (Fig. 10). *E. coli* and *C. rodentium* are in the family of Enterobacteriaceae, therefore, a slight increase in that category will be expected with infection (Fig. 10).

**Co-Colonization for *C. rodentium* and *E. coli* Data**



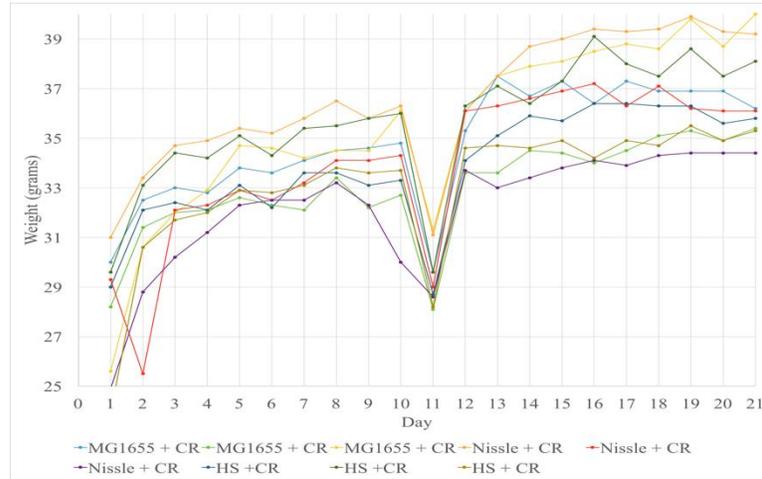
**Figure 1. Colon and Cecum length and observed inflammation.** *C. rodentium* only mice (a) exhibited less inflammation than co-colonized mice (b), (c), and (d). This suggests that when there is competition between bacterial populations in the intestine, the inflammation is greater.



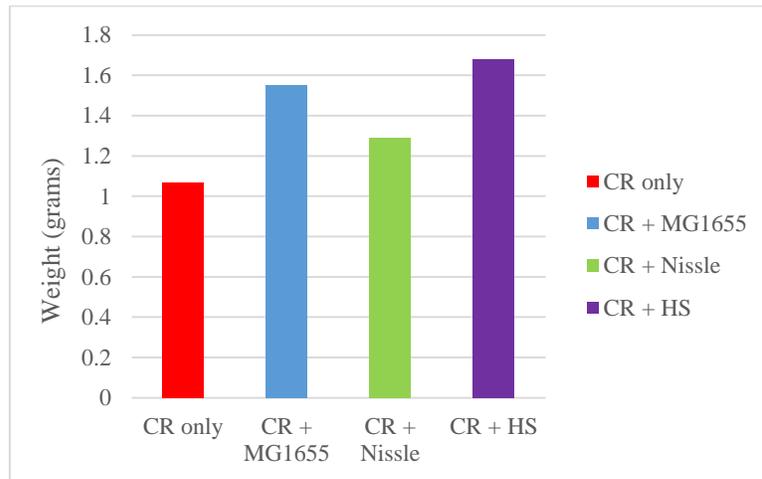


**Figure 2. Bacterial load for co-colonization (*C. rodentium* DBS100 versus *E. coli* strains) and single colonization (*E. coli*). *E. coli* MG1655 mice (b), HS (c), and Nissle**

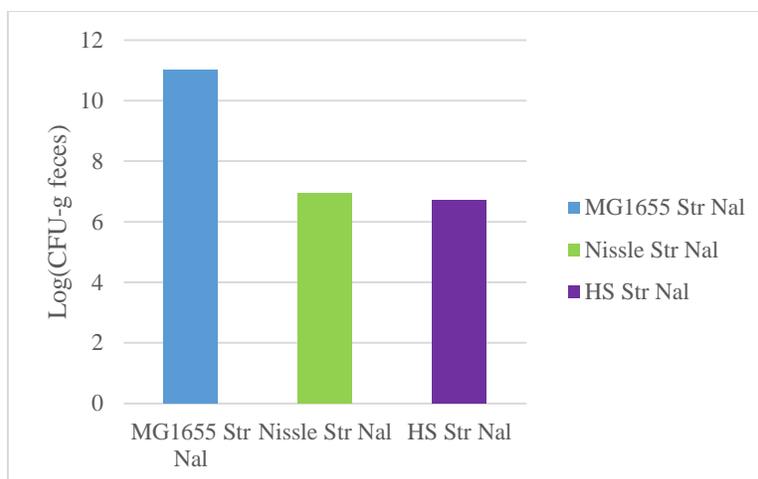
(d) outcompeted *C. rodentium* DBS100 in the mouse intestine. MG1655 mice showed greater fitness than *E. coli* HS and *E. coli* Nissle in competition with *C. rodentium* DBS100 as noted by the two-fold increase of CFU/g of feces.



**Figure 3. Co-colonized mice weights.** Mouse weights increased over the course of infection, possibly due to increased water retention. The drop at day 11 is due to starvation 24 hours prior to inoculation.

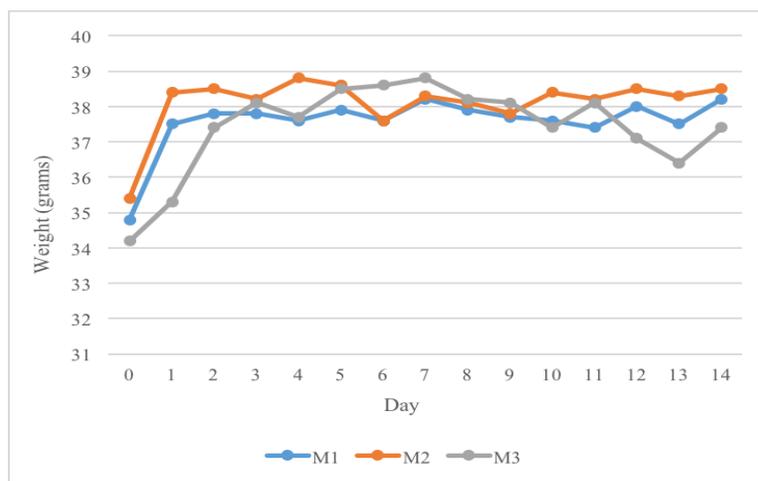


**Figure 4. Ceca weights of mice co-colonized with *E. coli* strains and *C. rodentium*.** HS *E. coli* strain showed greater ceca weight than the MG1655 and Nissle strains, possibly due to inflammation, hydropsy, or increased bacterial load.

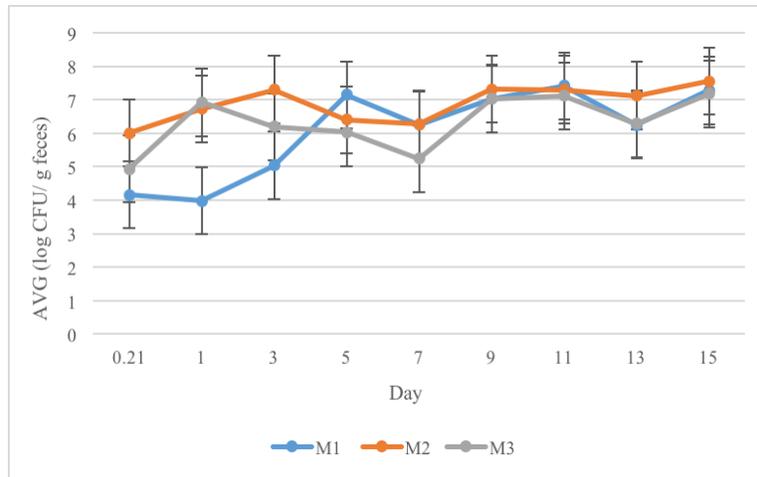


**Figure 5. Ceca plate counts for co-colonized *E. coli* mice.** Of the three *E. coli* strains used, MG1655 showed greatest competitive fitness, as exhibited by the greatest bacterial load.

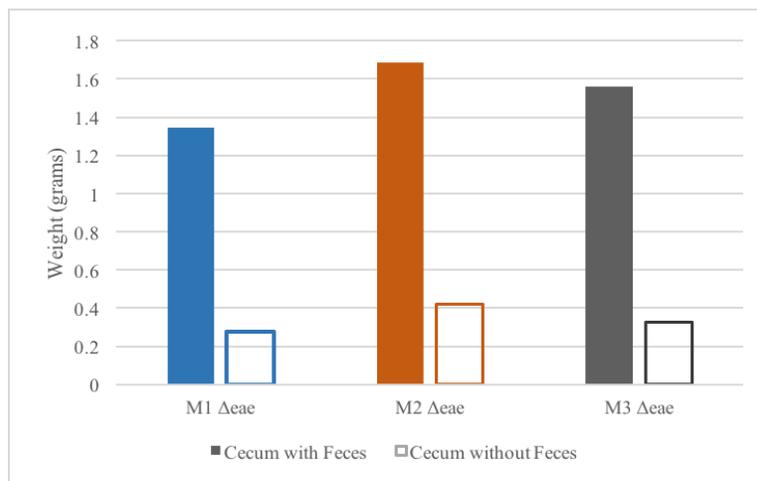
#### *C. rodentium* $\Delta$ ae Colonization Data



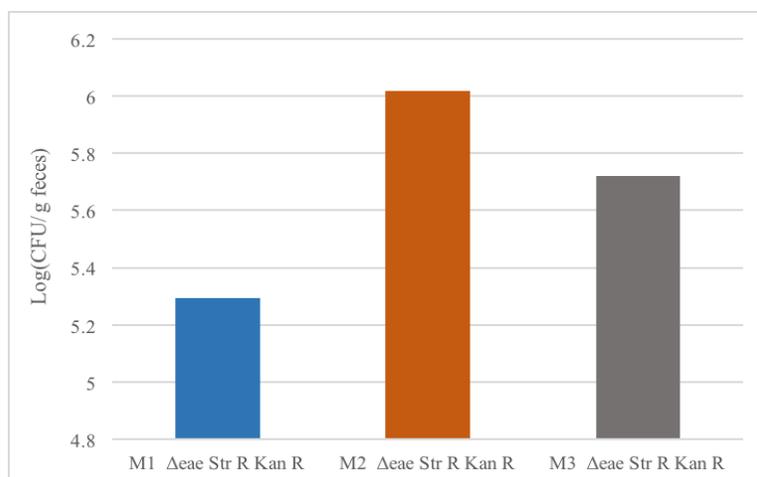
**Figure 6. Weights for mice colonized with *C. rodentium*  $\Delta$ ae.** Mice infected with *C. rodentium*  $\Delta$ ae exhibited consistent weight throughout the colonization. This implies that the mice remained in good health throughout the course of colonization. The increase at day 1 is due to weight loss caused by the starvation protocol used for inoculation.



**Figure 7.** Bacterial load for mice colonized with *C. rodentium*  $\Delta$ ae



**Figure 8.** Comparison of cecum with and without feces for the 3 mice infected with *C. rodentium*  $\Delta$ ae.

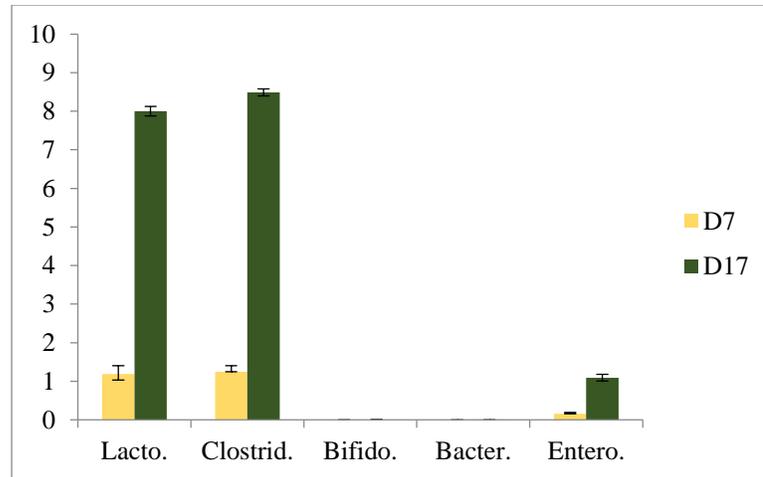


**Figure 9.** Ceca Plate Count for mice colonized with *C. rodentium* Δeae. Mouse 2 has the highest colonization of *C. rodentium* Δeae. Mouse 1 has the lowest *C. rodentium* accumulation in the gastrointestinal tract.

### gDNA Community qPCR Data

**Table 2.** Enteric Population Fold Changes Overtime for mice Co-colonize with strains *C. rodentium* and *E. coli* MG1655, Nissle, and HS.

Enteric Population Fold Changes Over Time		
	<u>D7</u>	<u>D17</u>
<i>Lactobacillus</i>	1.190793 ± 0.2145	8.000056 ± 0.1255
<i>Clostridium</i>	1.244915 ± 0.1566	8.486400 ± 0.0889
<i>Bifidobacterium</i>	0.000023 ± 0.0000	0.000001 ± 0.0000
<i>Bacteroidales</i>	0.000017 ± 0.0000	0.000107 ± 0.0000
Enterobacteriaceae	0.159952 ± 0.0281	1.092600 ± 0.0893



**Figure 10. Enteric Population Changes Overtime for Co-colonized mice with strains *C. rodentium* and *E. coli* MG1655, Nissle, and HS.** Once *C. rodentium* is introduced on Day 10 to the mice gastrointestinal tract, *Lactobacillus* and *Clostridium* show the greatest competitive increase, as indicated by the large fold change.

## DISCUSSION

The goal of this study was to understand and quantify bacterial population abundances in the intestinal microbiome of *C. rodentium* and *E. coli* infected mice. The long-term goal of applying the results is to aid in clinical practice and disease prevention. Nine mice were infected with three different strain of *E. coli*: MG1655, HS, and Nissle. MG1655 is a commensal lab strain, HS is a probiotic, and Nissle is a normal flora strain. The mice were subsequently co-colonized with *C. rodentium* strain DBS100, a murine pathogenic strain. The purpose of the co-colonization was (1) to determine the colonization fitness of the various strains and (2) to observe the effects of competition on the pathogenesis of DBS100. Unexpectedly, in each case, the *E. coli* strain outcompeted *C. rodentium* DBS100 (Fig. 2). This is putatively due to the colonization advantage given to the *E. coli* in the seven days prior to the DBS100 infection. Of the 3 *E. coli* strains used,

MG1655 showed the greatest fitness, as determined by the highest quantity of colony forming units. Following the addition of DBS100, mouse weights increased (Fig. 3). This increase is likely a result of inflammation and water retention; inflammation was shown upon euthanization of the mice (Fig. 3). This data implies that competition may instigate amplified pathogenesis (Fig. 5).

It was hypothesized that performing a colonization with an intimin mutant will show that *C. rodentium* induces minimal inflammation in the mouse intestine in the absence of intimin (its primary attachment protein). Three mice infected with bacteria lacking intimin showed negligible inflammation, which implies that attachment is necessary for pathogenesis. The results show that intimin is likely used to cause disease during competition with *E. coli*. *C. rodentium* is the best mouse model to use for infection and pathogenesis investigations of *E. coli* to develop cures for humans infected with gastroenteritis. Additionally, results showed that without intimin, minimal inflammation occurs in the mouse cecum, thus supporting my hypothesis that attachment is important in pathogenesis.

qPCR determined how much of a specific DNA or gene is present in a given sample. qPCR enabled both the detection and quantification of the PCR product in real time, while it is being produced. It can be inferred that at day 17 post infection (i.e. day 7 post *C. rodentium* infection), enteric competition is increased. The enteric competition of *Lactobacillus* and *Clostridium* are higher at day 7 post *C. rodentium* introduction (day 17 total) relative to day 7 post *E. coli* infection (Fig. 10). Both *Lactobacillus* and *Clostridium* show an eight-fold increase (*Lactobacillus* Day 7: 1.1908 Day 17: 8.0001) (*Clostridium* Day 7: 1.2449 Day 17: 8.464) from day 7 to day 17 (Table 2, Fig. 10). This is of interest

since both *Lactobacillus* and *Clostridium* are used as probiotics to lessen or inhibit *C. rodentium* infection. Thus, increase in the normal flora population of these bacteria may indicate competition to fight off infection. Further investigations of the mechanisms and behaviors of these bacteria in response to *C. rodentium* infection may aid in the development of preventative and therapeutic treatments.

Future work needs to be done to make any statistical conclusions for the qPCR study in relation to pathogenesis. However, these studies add to the studies investigated in the scientific community such as the Finlay study with antibiotic treatment altering intestinal microbiota infection (24). In order to develop cures or treatments for gastrointestinal diseases, it is important to understand the mechanisms behind infection. qPCR results imply that *Lactobacillus* and *Clostridium* are effective enteric competitors, suggesting that can be used to antagonistically treat bacterial infections with *C. rodentium* and *E. coli*.

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