Colonization of Intestinal Pathogen Changes the Gut Microbiota

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Abstract

Enterohemorrhagic *Escherechia coli* is a serious human pathogen causing bloody diarrhea and hemolytic uremic syndrome. It is difficult to study in animal models, but pathogenesis may be modeled in mice with the similar murine pathogen, *Citrobacter rodentium*. *C. rodentium* does not cause disease in streptomycin-treated mice, suggesting that it is competition with other facultative anaerobes that triggers pathogenesis. Streptomycin-treated mice were co-colonized with *C. rodentium* and a commensal *E. coli* strain. The intestinal microbiota of each group was observed over a 15-day period using quantitative PCR. Colon weights were also measured over the same period. Results indicate that the disease caused by competition is not similar to normal *C. rodentium* pathogenesis. Further research is necessary to determine the precise mechanism of pathogenesis in this experimental model. The outcome may provide new insight into enterohemorrhagic *E. coli* prevention and treatment.

Acknowledgements

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Introduction

Enterohemorrhagic *Escherichia coli*

Enterohemorrhagic *Escherichia coli* (EHEC) is a rod-shaped Gram-negative facultative anaerobe that causes bloody diarrhea and hemolytic uremic syndrome. It is a member of the class of organisms known as attaching and effacing pathogens. In humans, EHEC infections may be life-threatening, especially if hemolytic uremic syndrome, which may lead to renal failure, develops. Perhaps the best known EHEC strain is *E. coli* O157:H7, a virulent strain that was first reported as an intestinal pathogen in 1982 (*1*). EHEC is commensal in ruminant animals, including cattle and deer, and is, therefore, often spread by contact with ruminants, their feces, or ingestion of contaminated meat or dairy products (*2, 3*).

Citrobacter rodentium as a Murine Model

The disease EHEC causes in humans is routinely modeled in mice with the murine pathogen *Citrobacter rodentium.* Like EHEC, *C. rodentium* is an attaching and effacing pathogen. EHEC does not cause disease in mice, and as has been noted, *C. rodentium* is similar to EHEC in virulence factors and pathogenesis (*4, 5*). This model has been proven effective and a further advantage in that *C. rodentium* is not pathogenic to humans (*5*). While *C. rodentium* does not carry all the same virulence factors as EHEC, the genes causing transmissible murine colonic hyperplasia are found in both *C. rodentium* and EHEC *(3).* In both species, those genes are found on the locus of enterocyte effacement (LEE), which is comprised of genes coding for a type III secretory system, intimin, translocated intimin receptor (Tir), and the LEE regulator Ler, as well as other gene products contributing to virulence (*5*).

Symptoms of Disease Caused by *Citrobacter rodentium*

Although the classic symptoms of disease caused by pathogens such as EHEC and *C. rodentium* include bloody diarrhea, mice infected with *C. rodentium* in the laboratory may not have diarrhea. In such cases, pathogenesis is determined via examination of feces and of the colon, as well as by histopathology. A conventional mouse infected with *C. rodentium* will have softened feces and increased colon weight when compared with a healthy mouse. These symptoms are due to breakdown of the intestinal epithelial barrier and edema caused by inflammation. Upon histopathological analysis, colonic hyperplasia and increased crypt height are visible (*5*).

Mechanism of Pathogenesis

Both *C. rodentium* and EHEC adhere to intestinal cells by means of attaching and effacing lesions. Bacteria attach directly to the outer membranes of intestinal epithelial cells, destroying brush-border microvilli in the immediate area. Successful attachment and subsequent colonization results in colonic hyperplasia as well as inflammation. To initiate attachment, effector proteins including Tir are injected into epithelial cells with type III secretion systems.

Tir is inserted in the outer membrane of host epithelial cells; the extracellular domain provides an attachment site for intimin, a bacterial adhesion factor. Intimin-Tir interaction causes phosphorylation of Tir, which promotes the formation of actin-rich pedestals to which the bacteria can attach. Tir is also instrumental in evading the infected cell's immune response and is upregulated in response to various stresses, including lack of nutrients (*6, 7*).

The disease caused by EHEC and *C. rodentium* disturbs the intestinal microbiota, affects colonic mucus layers, and may disrupt the integrity of the epithelial barrier. Intimin-Tir attachment is necessary for pathogenesis in EHEC and *C. rodentium*, but it is not always necessary for colonization (*2, 4, 8*).

Colonization resistance and the nutrient-niche theory

Colonization of the gastrointestinal tract is the first step necessary for pathogens to cause disease in a host. One of the primary barriers to pathogen colonization of the intestine is the intestinal microflora (*9*). As a diverse ecosystem, the commensal intestinal bacteria prevent colonization of new organisms by multiple forms of competition. Some commensal species secrete substances that change the local environment, making it more hostile to competitors. Other bacteria activate and then evade the host's immunity when presented with a new competitor (*9*).

Freter considered the most important part of colonization resistance to be competition for nutrients, in what is now known as his nutrient-niche hypothesis. According to Freter, invading bacterial species must grow faster than the washout rate for successful intestinal colonization. They must also be able to outcompete commensal species on at least one growth-limiting nutrient. Successful pathogens, then, are able to find a particular niche in the nutrients available and exploit that niche in order to colonize the intestine (*10*). Commensal bacteria that utilize the same nutrients as invading species are often able to completely prevent colonization. However, an organism's normal microbiota, though stable, is in continuous flux. Specific bacterial strains may colonize the intestine for long or short periods of time. When succession of strains occurs, a

nutritional niche is left open temporarily, and any bacterium able to exploit the niche has a chance to colonize.

Effect of pathogenesis on intestinal microbiota

About 800 species of bacteria are present in the human intestinal microbiota, forming a complex ecosystem that plays critical roles in host health. Most species in the intestine are obligate anaerobes, although some facultative anaerobes are present. Of the facultative anaerobes, *E. coli* is the most prominent species. Major genera include *Bacteroidales*, *Clostridium*, *Lactobacillus*, and *Enterobacteraciae*.

Relative abundances of each of these genera were previously measured in both conventional and streptomycin-treated mice infected with *C. rodentium*. *Bacteroidales* species showed an increase in relative population abundance in conventional mice infected with *C. rodentium* as compared to controls; the abundance of the same genus also increased in streptomycin-treated mice. *Lactobacilli* populations showed a significant increase associated with *C. rodentium* colonization in both conventional and streptomycin-treated mice, with the increase greatest in conventional mice. No significant change was noted in the relative abundances of *Clostridia, Bifidobacteria,* or *Enterobacteraciae* species with either streptomycin treatment or *C. rodentium* infection (Figure 1) (*11, 12*).

***Escherechia coli* Strains**

Three well-characterized commensal *E. coli* strains are commonly used in murine models of EHEC infections: MG1655, HS, and Nissle. MG1655 is a human-commensal K-12 strain of *E. coli* that has been studied in depth since it was first cultured in the early twentieth century. It is the typical strain of *E. coli* used as a model organism and as such

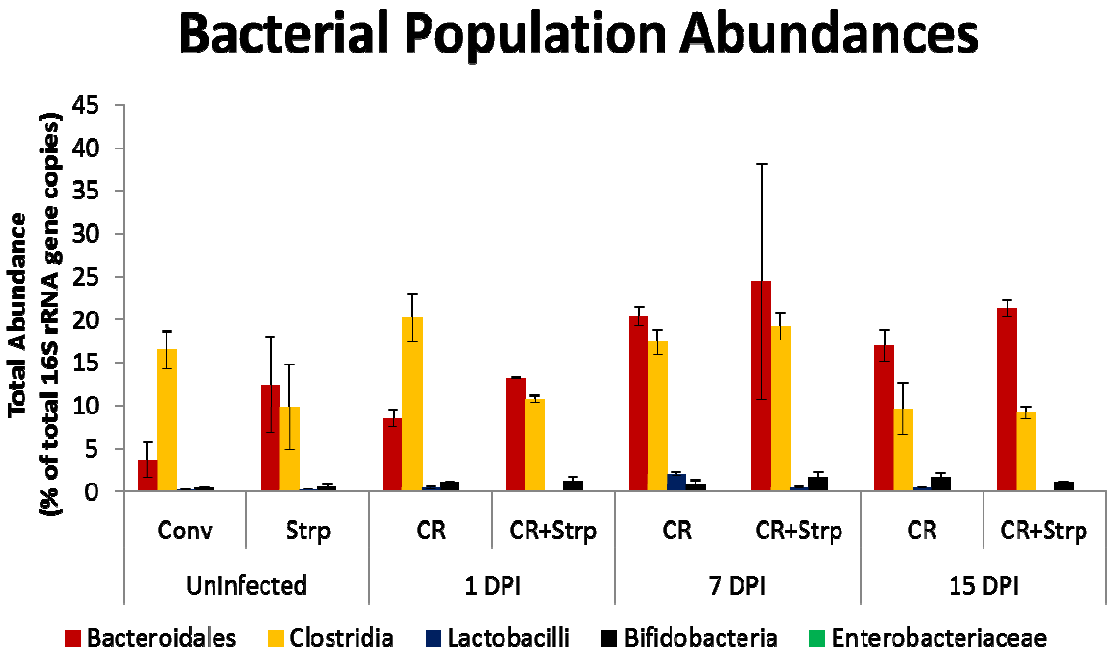

Figure 1. Comparison of bacterial population abundances in conventional and strep-treated mice with and without CR infection. Population abundances were quantified using qPCR over a 15-day period.

is present in most molecular biology laboratories. Nissle was initially cultured in 1917 from a German soldier who showed low susceptibility to intestinal disorders. A highly competitive strain in the mammalian intestine, it has been used as a probiotic and for treatment of ulcerative colitis (*13*). *E.coli* HS was isolated in 1958 from a healthy individual and is typically used to represent normal commensal strains. It is considered closer to wild-type commensal *E. coli* than MG1655 or Nissle (*2, 14*).

Murine Models

Because of the complexity of the intestinal environment and ecosystem, *in vivo* models are far superior. *In vitro* models are unable to precisely replicate the anaerobic environment, intestinal mucus, or the intestinal bacterial community. Rodent, particularly mouse, models are ideal for several reasons. Rodents and humans share similar intestinal anatomy. Both species possess a large and small intestine with a duodenum and jejunum, although mice have a large cecum when compared to the human appendix. Rodent diets and environments are easily controlled, and many studies have proven that results from rodent models are consistent with data obtained from humans (*5*).

Bacterial-host interactions are typically studied in gnotobiotic, conventional, or streptomycin-treated mouse models. Gnotobiotic mice are raised in sterile environments. They have no intestinal microbiota and so display no colonization resistance. This model may be useful for studying the interaction between a specific bacterium and a host, but it is unrealistic, as the complex interactions between the host and the intestinal community as well as the microbiota-pathogen interactions are absent. Gnotobiotic mice also often have a reduced immune system. Therefore, the gnotobiotic mouse model is not ideal for studying interaction between bacteria *in vivo* (*15*).

Conventional mice have their intestinal microbiota intact. This model is excellent for studying pathogenesis, as it represents a normal mammalian internal environment. Conventional mice, however, display colonization resistance as a result of their intact microbiota, which can make studies that require intestinal colonization difficult. 24 hours of streptomycin treatment before inoculation opens a nutritional niche, allowing facultative anaerobes such as *C. rodentium* to colonize the intestine more easily. The effects of streptomycin last about 48 hours, after which the normal facultative anaerobes begin to recolonize the intestine (*4, 5, 16*).

Streptomycin treatment may also be continued throughout experimentation. This approach has the advantage of causing minimal changes in the intestinal microbiota during experiments, as the disturbance due to antibiotic treatment occurs before inoculation. The streptomycin-treated mouse model reduces facultative anaerobes in the murine intestine to less than the lower limit of screening protocols (10^2 CFU), allowing study of specific facultative interactions *in vivo* while preserving interaction with other members of the intestinal microbiota (*4*).

Streptomycin is the antibiotic of choice because it targets facultative anaerobes as well as causing mild intestinal irritation, a state in which more nitrates are available for bacterial metabolism (*17*). Furthermore, when streptomycin is compared to other antibiotics such as metronidazole, it does not significantly alter either the gastrointestinal mucus layer or the intestinal microbiota (*16*).

C. rodentium causes disease in conventional mice; however, it does not do so in streptomycin-treated mice, as shown in Figure 2. Conventional mice given a burst of streptomycin treatment before inoculation show no pathogenesis initially. Disease

develops later and has been associated with normalization of the intestinal microbiota. Streptomycin-treated mice, then, are useful for studying the interactions between intestinal microbiota and bacteria such as *C. rodentium* before pathogenesis occurs.

Colonization versus pathogenesis

Colonization and pathogenesis are assumed to occur together in most models of intestinal disease. The ability of *C. rodentium* to colonize the streptomycin-treated mouse intestine without pathogenesis occurring is significant, demonstrating that colonization is not sufficient for pathogenesis. The primary difference between conventional and streptomycin-treated mice lies with the absence of facultative anaerobes in the latter. It is reasonable to assume, then, that interaction with other facultative anaerobes drives pathogenesis. Competition of *C. rodentium* with certain *E. coli* strains causes pathogenesis by a currently unknown mechanism. If the competition-induced disease state is similar to disease caused by *C. rodentium* in conventional mice, inducing pathogenesis via competition will be relevant to EHEC modeling. If competition-induced disease in streptomycin-treated mice is similar to the pathogenesis in conventional mice, it is possible that *C. rodentium* and, by extension, EHEC and other attaching and effacing pathogens, cause disease primarily through competition with commensal facultative anaerobes. One possible mechanism for competition-induced disease involves intimin and Tir. Intimin is necessary for *C. rodentium* to successfully colonize the murine intestine, while Tir is needed for attachment and pathogenesis, but not necessarily colonization (*6, 8*). Stress from direct competition with another facultative anaerobe for nutrients may trigger intimin-Tir attachment in *C. rodentium*, leading to pathogenesis.

I hypothesize that competition-induced disease in streptomycin-treated mice will

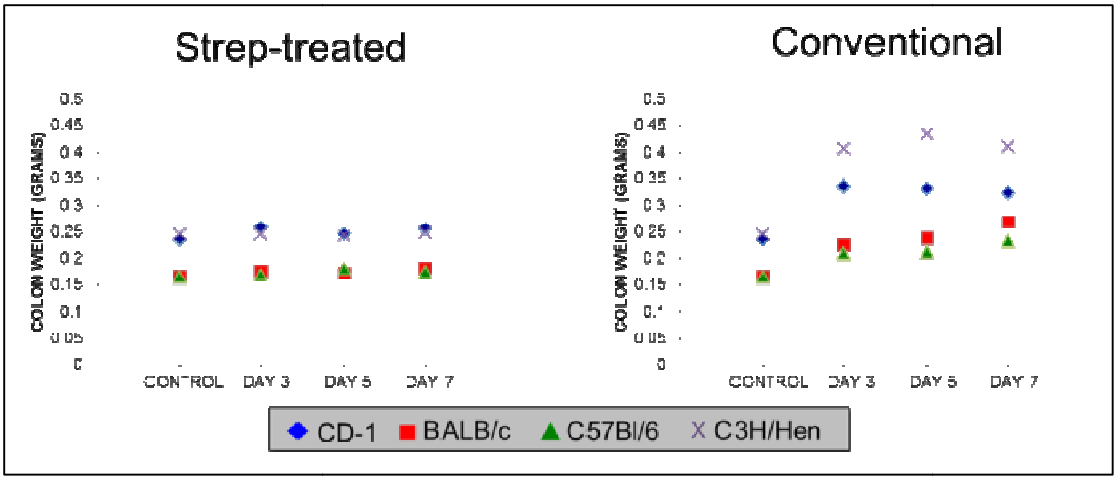

Figure 2. Colon weights from various conventional and streptomycin-treated mice. CD-1, BALB-C, C57B1/6, and C3H/HeN mice were fed 10^{10} CFU of *C. rodentium* , and the colons were harvested at days 3, 5, and 7 post infection. The increase in edema for the conventional mice is indicated by an increase in colon weights. There was no measurable edema in strep-treated mice. Edema is a measurable symptom of *C. rodentium* pathogenesis, and so this data indicates that streptomycin-treated mice do not experience disease due to *C. rodentium*.

resemble disease caused by *C. rodentium* in conventional mice and that changes in the intestinal microbiota will also be similar. Competition with all strains of *E. coli* is not expected to yield pathogenesis. Only facultative anaerobes in direct competition with *C. rodentium* should provide the necessary stress to *C. rodentium* for initiation of disease. The *E. coli* strains in this experiment most likely to cause competitive pathogenesis are HS and Nissle, which are better suited than MG1655 for competition within the host's intestine (*13*).

Materials and Methods

Streptomycin-treated Mouse Model

12 CD-1 male mice were given sterile water containing streptomycin sulfate (5g/L) starting at least 48 hours prior to inoculation and continuing throughout the experiment. All mice were ordered from Charles River Laboratories. 24 hours after streptomycin treatment began, the mice were starved of food and water for 18-24 hours. Oral inoculation was carried out via a suspension of bacteria in a 20% sucrose solution. Suspensions contained 10^5 CFU *C. rodentium* and one *E. coli* strain of the following: MG1655, HS, and Nissle. Strain combinations were as follows: *C. rodentium* and MG1655, *C. rodentium* and HS, and *C. rodentium* and Nissle. Nine mice were infected with each combination. Once the suspension was consumed, food and streptomycin water were returned. 1 gram of fecal matter was collected from each mouse on days 1, 3, 5, and 7 post-inoculation. One mouse from each group was euthanized by carbon dioxide inhalation and cervical dislocation after each collection, for a total of three mice euthanized per collection. The cecum and colon was removed from each euthanized mouse and weighed.

Bacterial Strains and Growth Conditions

E. coli strains MG1655, HS, and Nissle were used, as well as *C. rodentium* DBS100. All strains used were streptomycin-resistant. Bacteria were initially cultured in Luria-Bertani broth with streptomycin at 37 °C. Fecal samples were plated on MacConkey agar with streptomycin and incubated at 37 °C.

Fecal gDNA Isolation

Genomic DNA (gDNA) was isolated from stool samples from each collection with the QIAamp DNA Stool Mini Kit (Catalog #51504) (Qiagen), according to the manufacturer's instructions. Concentration of gDNA was determined by measuring the absorbance at 260 nm with a NanoDrop 2000 Spectrophotometer (Thermo Scientific).

Quantitative PCR of Genomic DNA

Quantitative PCR (qPCR) was performed on gDNA isolated from each stool sample. SsoFast EvaGreen SuperMix (BioRad #172-5200) was used, along with the primers shown in Table 1. 15 minutes of cycles at 95 °C were used, then 40 cycles of 94 °C for 15 seconds, 60 °C for 30 seconds, and 72 °C for 30 seconds (*16*). Primer accuracy was first tested on pure cultures. Amplification start was recorded at a cycle number (Cq)for every genus measured; the Cq for the total bacteria served as a standard against which to measure specific bacterial abundances. The change in Cq (ΔCq) was calculated and was then used to calculate relative population abundance by raising 2 to the value of the -ΔCq.

Results

Colon weight

Mice co-colonized with *C. rodentium* and *E. coli* HS or Nissle showed elevated colon weights (Figure 3). Mice co-colonized with *C. rodentium* and MG1655

Table 1. Primers for qPCR

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

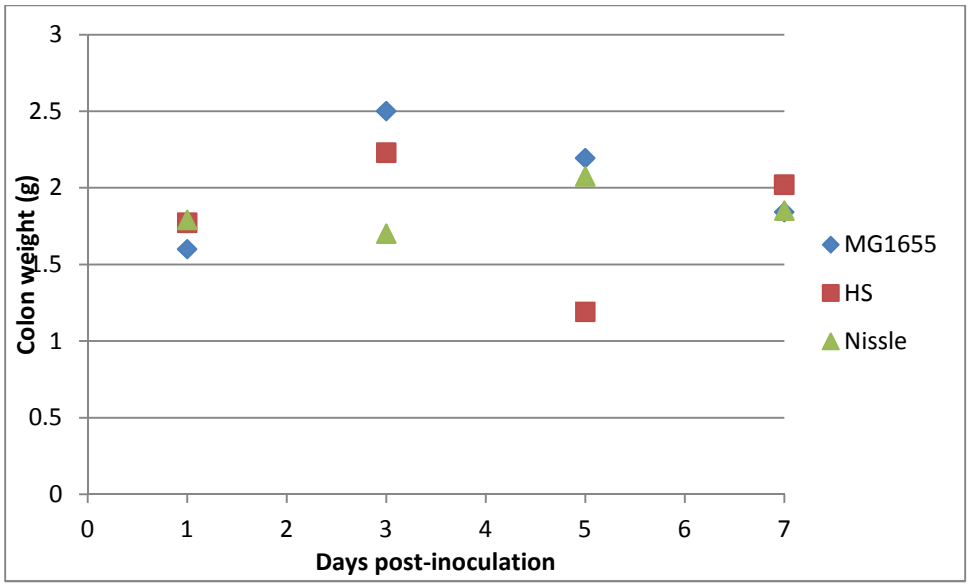

Figure 3. Colon weights for mice co-colonized with *C. rodentium* and HS, Nissle, or MG1655. Co-colonization of *C. rodentium* with all *E. coli* strains induces edema in the colon comparable to that found in conventional mice infected by *C. rodentium* alone.

also showed elevated colon weights, contrary to expectations. Elevated colon weight is associated with inflammation and edema and is thus a reliable indicator of pathogenesis for intestinal pathogens such as *C. rodentium*.

A notable spike in colonic weight occurred at day 3 in the mice colonized with both *C. rodentium* and MG1655; however, this is likely due to an unusual amount of feces observed in the colon at that time, rather than being caused solely by edema (Figure 3). Softened stools, another symptom of *C. rodentium* pathogenesis, were first observed on day 5 in mice infected with both *C. rodentium* and Nissle (Figure 4) (*18*).

Quantitative PCR results

Analysis of the commensal bacteria in mice co-colonized with HS or Nissle showed changes in relative distribution that were different from the changes in response to *C. rodentium* colonization and pathogenesis in conventional mice. At day 1 post-inoculation, each group of mice had a different genus dominant in the intestine; *Clostridia* for mice co-colonized with *C. rodentium* and MG1655, *Lactobacilli* for those with *C. rodentium* and HS, and both *Clostridia* and *Bacteriodales* in those colonized with *C. rodentium* and Nissle (Figures 5-8). Day 3 post-inoculation shows a marked increase in *Clostridia* species in mice colonized with *C. rodentium* and HS. *Lactobacilli* species also increased in the *C. rodentium* and MG1655 mice, but decreased in those co-colonized with *C. rodentium* and HS (Figures 5-8). At day 5 post-inoculation, *Clostridia* species have largely died off in mice colonized with *C. rodentium* and HS, but continue to be numerous in mice infected with *C. rodentium* and MG1655 or Nissle. *Bacteriodales* species have also bloomed in the *C. rodentium* and Nissle mice (Figures 5-8).

Figure 4. Comparison of ceca and colons to demonstrate pathogenesis. The colon on the left was taken from a mouse one day post-inoculation with *C. rodentium* and Nissle. The colon on the right was harvested five days post-inoculation from a mouse infected with the same bacteria. Discrete fecal pellets have formed in the entirety of the colon shown at left but only form in the last third of the colon at right.

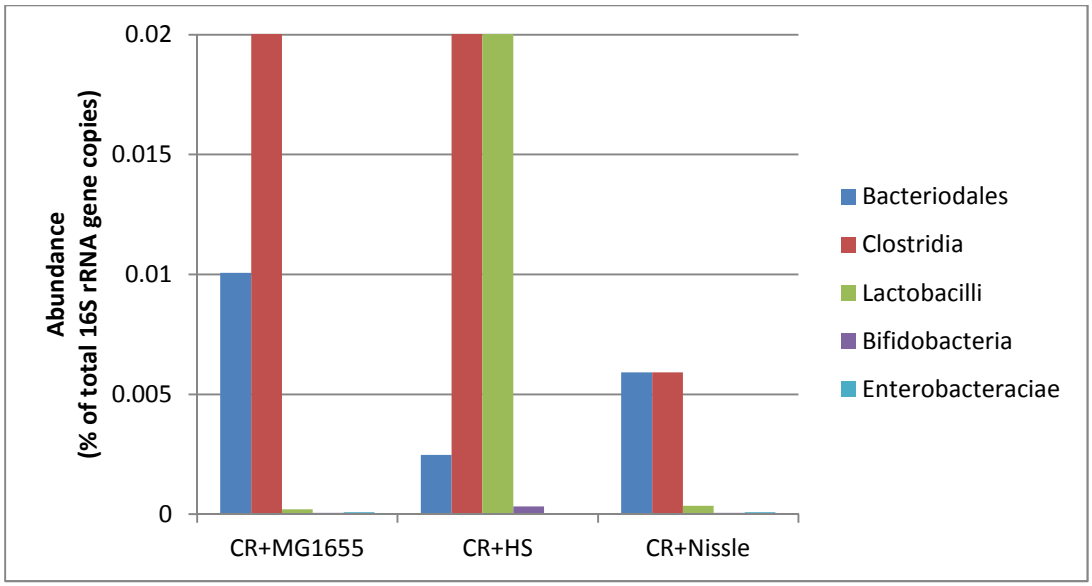

Figure 5. Relative abundances of major intestinal genera at day 1 post-inoculation. Mice in which *C. rodentium* is co-colonized with Nissle have relatively low abundances of *Clostridia* and high abundances of *Bacteriodales*. Co-colonization with HS and MG1655, however, produces high abundances of *Lactobacilli* and *Clostridia*, respectively.

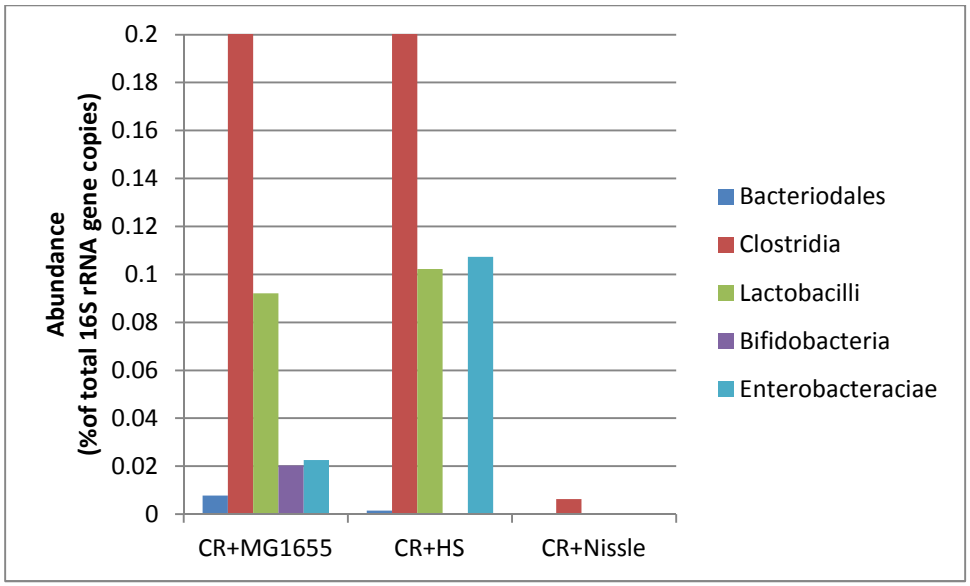

Figure 6. Relative abundances of major intestinal genera at day 3 post-inoculation. Compared to day 1 post-inoculation, *Lactobacillus* has increased relative to the total population in mice co-colonized with MG1655, although it has decreased in those co-colonized with HS. *Enterobacteraciae* show an increase in both. Mice co-colonized with Nissle show significantly more *Clostridia* than any other species.

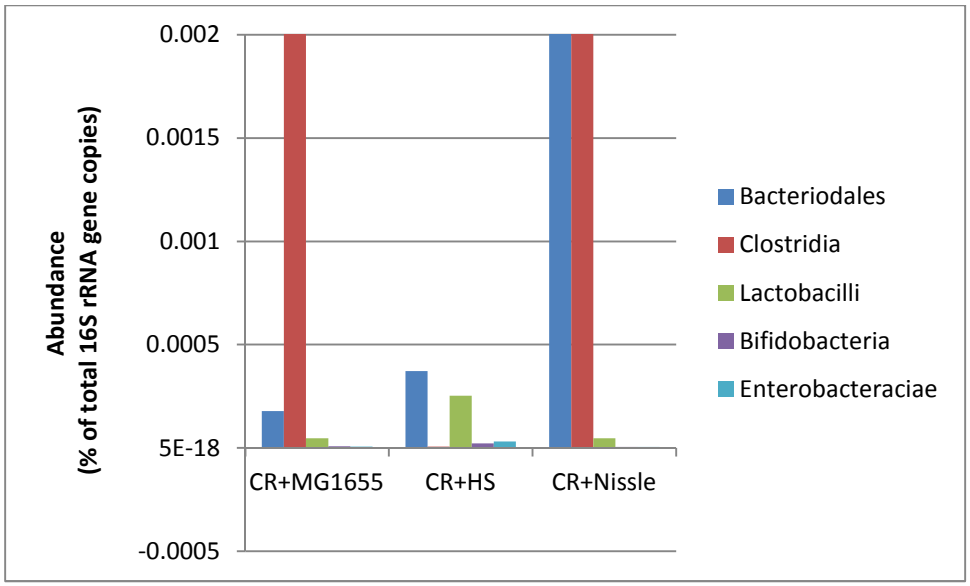

Figure 7. Relative abundances of major intestinal genera at day 5 post-inoculation. At day 7 post-inoculation, mice in which *C. rodentium* is co-colonized with MG1655 or Nissle have a bloom of *Clostridia* species, while the same genus is much reduced in HS. Mice colonized with *C. rodentium* and Nissle also had an increase in *Bacteriodales*.

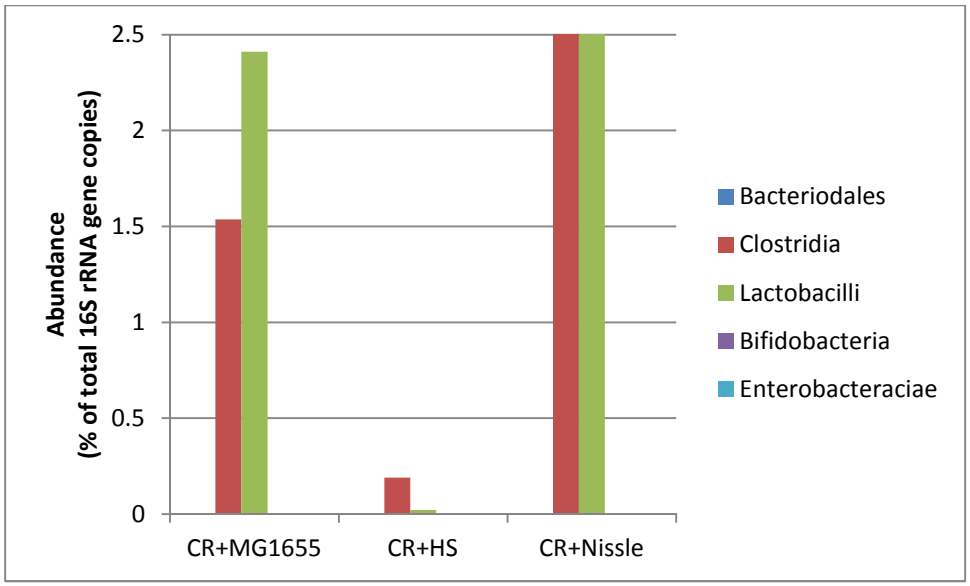

Figure 8. Relative abundances of major intestinal genera at day 7 post-inoculation. By day 7 post-inoculation, *Clostridia* and *Lactobacilli* species are dominant in the intestine. Mice colonized with both *C. rodentium* and MG1655 have higher proportions of *Lactobacilli* than *Clostridia*, while the reverse is true for mice co-colonized with *C. rodentium* and HS.

By the seventh day after inoculation, *Clostridia* and *Lactobacilli* are the dominant genera in all groups of mice. Mice colonized with both *C. rodentium* and MG1655 have higher proportions of *Lactobacilli* than *Clostridia*, while the reverse is true for mice co-colonized with *C. rodentium* and HS (Figures 5-8). Population abundances for the genera measured showed little similarity to those measured in *C. rodentium*-infected conventional mice treated with streptomycin.

Discussion

The characteristics of pathogenesis of *C. rodentium* intitiated by competition with *E. coli* strains indicates that in conventional mice, disease is caused by competition with commensal facultative anaerobes, including normal *E. coli* strains. The absence of disease in mice co-colonized with *C. rodentium* and MG1655 further supports this conclusion. MG1655 is a subset of the well-established *E. coli* laboratory strain K-12. HS and Nissle are equally well-known, but they are closer to wild-type intestinal *E. coli*.

Nissle in particular is known to have multiple traits that increase its ability to survive in the mammalian intestine, and in a host with a compromised intestinal ecosystem, Nissle has been observed to outcompete the present bacteria and cause pathogenesis(*13, 19*). HS is a typical commensal strain of *E. coli* , so pathogenesis by competition with HS supports the hypothesis that the diseased caused by *C. rodentium* is caused by competition in conventional mice as well as in streptomycin-treated models (*13*).

Conclusion

Competition-induced disease has similar physical effects on the intestine when compared with the disease caused in conventional mice by *C. rodentium;* however, it has

very different effects on the intestinal microbiota. Competition appears to affect the intestinal ecosystem in a way that decreases population stability, leading to rapid succession of commensal intestinal genera. These conditions appear particularly favorable to *Clostridia* species, judging by the frequency with which that genus was dominant in co-colonized mice. *Lactobacilli* species also appeared to benefit, although the populations were unstable. Co-colonization with *C. rodentium* and Nissle seems to produce conditions unfavorable to *Lactobacilli* growth in the early stages of infection (Figures 5-8). By day 7 post-inoculation, though, increased colon weights indicated disease in all groups, and the intestinal microbiota of each group was dominated by *Clostridia* and *Lactobacillus* species. It may be inferred that competitive disease produces an environment more favorable to those genera than to other intestinal bacteria, although confirmation would require analysis of samples taken beyond day 7 after inoculation.

This conclusion is based solely on measuring inflammation and edema via colon weight and on the changes in commensal intestinal microbiota as measured by quantitative PCR. Future collections of histopathological data are needed to gauge the true similarity of competition-induced disease to that caused in conventional mice.

The lack of similarity of competition-induced disease to pathogenesis of *C. rodentium* in conventional mice indicates that competition with commensal facultative anaerobes may not be the normal cause of pathogenesis. This conclusion can be extended to EHEC; however, further confirmation using a model susceptible to EHEC is necessary.

Several possible mechanisms are possible to explain competition-induced pathogenesis of attaching and effacing pathogens. Intimin is necessary for *C. rodentium* to colonize the murine intestine successfully, while Tir is needed for both attachment and

pathogenesis but not necessarily colonization (*7, 8*). Expression of LEE-encoded virulence factors has been observed to increase or decrease in response to environmental factors, including the presence of other members of the intestinal microbiota (*20*). Furthermore, proteins involved in stress responses, such as RegA, have been shown to upregulate LEE gene expression (*19, 21*). Therefore, stress from direct competition for nutrients with another facultative anaerobe may increase the expression of genes on the LEE, including Tir. Intimin-Tir attachment follows, leading to pathogenesis.

Another, similar possibility is that the type III secretion system present in attaching and effacing pathogens is activated by the stress of competition, injecting Tir and associated proteins into host cells in order for intimin-Tir attachment to occur (*19, 21*). Further studies on the gene regulation of Tir and other LEE genes during competition are necessary for a more detailed hypothesis.

Although competition-induced disease shows little similarity to the disease normally caused by *C. rodentium,* understanding various causes of pathogenesis for attaching and effacing pathogens may lead to effective treatments and preventions for disease caused by EHEC in humans. There is no treatment currently available for EHEC infections; many antibiotics cause an increase in virulence factor expression, which increases patients' risk of developing hemolytic uremic syndrome (*22*). Based on the results of this study, attaching and effacing pathogens such as EHEC and *C. rodentium* display virulence in the mammalian intestine, although not because of competition with other facultative anaerobes. The effects that competitive disease has on destabilizing the intestinal microbiota suggest that during rapid succession of intestinal strains during pathogenesis, other pathogens may be able to colonize the intestine and cause disease

(*10*). If the commensal microbiota, especially facultative anaerobes, can be manipulated to reduce competition with EHEC, the destabilization of the commensal ecosystem may be reduced or avoided. For such treatment to be viable, more studies on the normal intestinal microbiota and their interactions are necessary.

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