The Gut Reaction: How the Intestinal Microbiota Respond to *Citrobacter rodentium* Colonization

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ABSTRACT

The human intestine hosts a diverse community of bacteria known as the intestinal microbiota. The intestinal microbiota have a symbiotic relationship with the host organism. Current research does not clearly define the effect these commensal microorganisms have on the overall maintenance of gastrointestinal health, including protection from the invasion and pathogenesis of foreign bacteria known as pathogens. EHEC O157:H7 causes severe sickness and can be life-threatening, but is difficult to study *in vivo*. One challenge is that EHEC does not colonize the preferred animal model for human GI studies: the murine intestine. However, the murine pathogen *Citrobacter rodentium* has been shown to colonize the mouse intestine and is used in current research as a model for EHEC colonization and infection. However, no one knows how the intestinal microbiota respond during the colonization *C. rodentium* and is addressed in this thesis. The colonization of a streptomycin-resistant strain of *C. rodentium* was observed over a 15-day period and quantified in both conventional and streptomycin-treated mouse models using genus-specific primers and quantitative PCR. The day 7 results indicate that there is a significant increase of *Bacteroidales* in the uncolonized, streptomycin-treated model, which is consistent with previous studies. The characterization of the intestinal microbiota has yet to be fully described for the colonization of *C. rodentium* in the mouse intestine as data analysis for this study continues. The anticipated outcomes may lead to the prevention and clinical care of EHEC on a global scale.

The Gut Reaction: How the Intestinal Microbiota Respond to Colonization by *Citrobacter rodentium*

INTRODUCTION

1. Gastrointestinal tract and its microbes

Gastrointestinal (GI) health is becoming increasingly popular in science and health-related fields, especially concerning disease prevention and overall human health. Within the GI tract exists a dynamic microcommunity that contains a diverse number of species each with a unique role in maintaining the health of the host (1). This microcommunity is known as the intestinal microbiota. At least 800 different bacterial species comprise the intestinal microbiota with 10^{13} to 10^{14} total bacteria present at a given time (2-4). The mutually beneficial relationship between the intestinal microbiota and the host is not completely understood, although the interactions that exist microbe-to-microbe as well as microbe-to-host may contribute to preventing disease and promoting health (5). Effectively relating GI health to clinical practice is impossible without understanding the basic nature, i.e. relationships among different microbes, genetic and metabolic attributes, fluctuations occurring in the onset of infection, etc., of the microbial community occupying the GI tract.

2. Models for study

A proper enteric model is needed to accurately study the dynamic interactions of the intestinal microbiota. An *in vivo* model has proven superior to other modes of study because it not only accounts for the physiological conditions of a real-life situation but also for the anaerobic nature of the commensal gut bacteria (6). Rodents are used to model the human GI tract because they share similar anatomical structures: both species

have a duodenum, jejunum, large and small intestine, and cecum, although the cecum of rodents is more pronounced than that of humans (7, 8). Rodents are preferred over other mammals because their diet and environment are easy to control and monitor. In addition, many research studies have obtained physiologically consistent results to those observed in humans (9).

In current research, there exist three murine models to study the interactions between a host and a given bacterium: gnotobiotic, conventional, and streptomycin-treated. The efficacy and practical application to *in situ* conditions must be considered when choosing a model. Gnotobiotic (germ-free) mice have no commensal microbes to impede the introduction of an invading microbe into the system. In practical use, this model is used to introduce specific elements back into a sterile system. However, introducing a bacterial cocktail back into the GI tract post-infection does not account for the complex interaction between a commensal microbial community and an invading bacterium; elements of real-world situations are lost entirely. Thus, the gnotobiotic mouse model may not be the model of choice for applications to clinical research in relation to the other two models.

The conventional mouse model maintains the commensal microbial community the gnotobiotic mice lacks. The presence of the normal gut microbiota makes the conventional model ideal for studying pathogenesis; this model represents the conditions that an invading microbe would face *in situ.* However, its use for other types of studies (i.e. colonization studies) is limited for the same reason; the normal gut microbiota makes the survival of a foreign microbe in the system difficult. Hence, the need exists for an *in*

vivo model that allows for the introduction of an invading microbe into a system while maintaining the physiological conditions of an *in situ* model.

Studies have shown that pre-treating mice with antibiotics (i.e. streptomycin) allows for enteric pathogens to grow competitively within the mouse intestine, although the mechanism by which this occurs is poorly understood (10, 11). It has been hypothesized that streptomycin treatment decreases the intestinal mucosal layer of commensal facultative anaerobes, freeing up nutritional niches, and vacating space (12-14). In addition, streptomycin does not alter the mucosal layer of the GI tract as severely as other antibiotics, which reduce the mucosal layer and in effect initiate a pathogenic state (15). Many studies report only pre-treating mice with streptomycin and then discontinuing the treatment for the rest of the study. These mice were asymptomatic in the beginning of the study, but showed signs of pathogenesis at the end of the study. Therefore, a given pathogen cannot be properly studied in the pre-pathogenic state with pre-treatment alone.

Contrary to pre-treatment with streptomycin, continuous streptomycin treatment maintains a pre-pathogenic state. Recent studies suggest that continuous streptomycin treatment interferes with microbe-host interactions by increasing nitrate availability via a slight inflammatory response, which may affect the persistence of the invading microbe in the intestine (16, 17). Thus, the continuous streptomycin-treated mouse model is the best model to describe how a foreign microbe maintains growth and survives within the intestine without inducing pathogenesis (14, 18).

3. Commensal bacteria within the intestine

In the intestine there exist a number of microhabitats where both commensal and pathogenic bacteria reside. Commensal, non-pathogenic bacteria typically reside in two places: either in the mucus layer covering the intestinal epithelial cells or in the luminal contents. The intestinal epithelial cells secrete a mucus layer that covers the entire GI tract. This mucus layer assumes an important role in maintaining and protecting both the microbiota as well as the host cells from invading pathogens. In addition to acting as a protective covering over the intestinal epithelia, the mucus layer separates the cell wall of the intestine from the lumen, and serves as a source of nutrients for commensal strains of bacteria (1, 6, 19). In general, the normal microbiota are found in the mucus layer because they cannot maintain a growth rate equal to or greater than the turnover rate of the intestinal epithelial cells (3). Sloughing and degradation of the mucus layer results in the presence of commensal bacteria in the luminal contents, offering another form of protection against invading microbes (19). Protection from invading microbes is the basis of disease prevention, with the intestinal microbiota being the most fundamental source of protection in the mammalian host.

4. Pathogenic bacteria within the intestine

In contrast to commensal bacteria, pathogenic bacteria are generally found in close proximity to the intestinal epithelia; however, they do not necessarily grow faster than either their commensal counterparts or the turnover rate of the intestinal epithelia. The pathogenic nature of bacteria is a result of their ability to produce an array of virulence factors otherwise lacking in commensal strains (20). Through the use of virulence factors, pathogenic bacteria compromise the integrity of the intestinal epithelia;

the physical separation of the luminal contents from the rest of the body is no longer functional. Thus, disease is promoted throughout the host. The pathogenicity of a foreign microbe to the host intestine is not only affected by its location within the intestine, virulence factors, and genetic differences from commensal bacteria, but it is also a function of the ability of a given pathogen to grow, survive, and maintain stable existence in a particular niche of the intestine (3, 6).

5. Colonization within the intestine

Colonization may be described as the ability of a bacterium to compete for nutrients and maintain a growth rate equal to or greater than the turnover rate of the intestinal epithelia (8). The degree of pathogenicity of a given bacterium not only depends on the virulence and successful competition for space, nutrients, and host receptors of the bacterium but it also depends on its colonization success. Colonization is the first step to pathogenesis, making the need for a suitable model of the colonization period even more critical, especially as it relates to disease prevention (1, 21, 22).

6. Microbial composition in colonic mucus

Facultative anaerobes, including *Escherichia coli,* make up about 0.1% of the intestinal microbiota (23). *Escherichia coli* is the most abundant facultative anaerobe found in the mucosal layer of the intestine and is the most studied microorganism (6, 21). Although it only makes up a small percentage of the total microbiota, *E. coli* is found in nearly every mammal in similar abundances. The potential protective role of commensal *E. coli* against pathogenic strains of *E. coli* has also been implicated (18).

The dominant enteric species inhabiting the large intestine include *Bacteroides* spp., *Bifidobacterium* spp., *Clostridium* spp., *Lactobacillus* spp., and *Enterococcus* spp.,

but their roles in the functional stability of the complex microbial community of the GI tract is not well known (19). The populations of obligate anaerobes *Bacteroides, Clostridia* and *Bifidobacteria* are not altered significantly when treated with streptomycin, but the populations of *Lactobacillus* and facultative anaerobes typically decreased under the same conditions (10). *Bacteroides* and *Clostridium* are the most abundant obligate anaerobes, with *Bacteroides* having an eclectic composition of different species (4).

7. Colonization resistance

Colonization resistance describes the challenge a microbe faces to colonize a given system. The GI tract asserts a level of colonization resistance against foreign bacteria via the function and presence of host commensal microbes. In the conventional mouse model, commensal bacteria elicit host immunological responses via their interaction with the intestinal epithelia to ward off infection by a foreign microbe, such as pathogenic *E. coli* (24). In contrast, the streptomycin-treated mouse model eliminates many factors impeding the colonization success of a given enteric pathogen. Factors contributing to colonization resistance include competition for mucosal carbon sources among commensal and pathogenic strains, competition for space, and respiration needs. Some pathogens, such as Enteropathogenic *E. coli,* may overcome colonization resistance via the utilization of multiple carbon sources and the co-metabolism of several carbon sources to maintain colonization (25). Overcoming colonization resistance is necessary for pathogenesis to ensue.

8. Pathogenic *Escherichia coli*

Enteropathogenic *Escherichia coli* (EPEC) and Enterohemorrhagic *Escherichia coli* (EHEC) are Gram-negative enteric pathogens. EPEC colonizes the small intestine while EHEC colonizes the large intestine. In humans, EPEC and EHEC share many virulent factors and can cause colitis with instances of bloody diarrhea (26). EHEC O157:H7 causes life-threatening hemolytic uremic syndrome in humans, but exhibits commensal-like behavior in cattle and streptomycin-treated mice (27). Also, EHEC colonization is poorly understood partly due to its inability to colonize the conventional mouse intestine (28). Its commensal-like behavior and its poor colonization present a unique challenge for studying the colonization of EHEC O157:H7 in an animal model, and, therefore, requires another means by which it can be studied in the mouse intestine.

9. *Citrobacter rodentium* as a model for EHEC

A member of *Enterobacteriaceae,* the enteric murine pathogen *Citrobacter rodentium* is genetically similar to EHEC and EPEC, but does not induce pathogenesis in humans (9, 29). However, it is considered highly infectious to mice. *C. rodentium* targets the cecum and colon during infection and causes colonic hyperplasia (9). Not only do genetic similarities exist between *C. rodentium* and EHEC and EPEC, but also similarities in virulence factors (i.e. attachment mechanisms) have been observed among the pathogenic strains (9, 30-32).

In mice infected with *C. rodentium,* the colonic response is similar to the response induced in humans by EHEC infection (9, 30, 33); in short, *C. rodentium* is to mice what pathogenic *E. coli* is to humans. However, the known virulence mechanisms of *C. rodentium* in the murine intestine are not well-defined (32, 34). Because of its genetic

and virulent similarities to EHEC, *C. rodentium* in the murine intestine serves as a suitable model for EHEC in the human intestine. There is a gap in current research regarding the microbial response to enteric colonization of EHEC and the potential role of the intestinal microbiota in preventing the disease caused by the pathogen. *I hypothesize that* Bacteriodales *spp. and* Clostridium *spp. will have significantly altered population abundances when streptomycin-treated mice are colonized with C.* rodentium*.* The results from this thesis will be the first time the bacterial community is quantified and examined with the colonization and infection of *C. rodentium* in the streptomycin-treated mouse model. Inferences may then be made concerning the interaction between the human intestinal microbiota and pathogenic *Escherichia coli*. The anticipated outcomes of this study may have applications in regard to future preventative measures made in response to the global health threat and disease caused by EHEC.

MATERIALS AND METHODS

1. Conventional mouse model

The conventional mouse model was the model for studying the pathogenesis induced by infection with *C. rodentium.* Three male, 6-week old CD-1 mice (Charles River) were starved of food and water for 18-24 hours before being orally fed a 20% sucrose suspension containing 10^5 CFU of *C. rodentium* strain DBS100 Str^r (streptomycin-resistant strain) (ATCC 51459). After the bacterial suspension was ingested, food and water were returned *ad libitum*. Fecal samples were collected and bacterial load was quantified 5 hours, 24 hours, and every other day post infection for 15 days. The mice were then euthanized by CO_2 gas asphyxiation and cervical dislocation

before the ceca and colons were harvested. Three male, 6-week old CD-1 mice that were not given the bacterial suspension served as controls.

2. Streptomycin-treated mouse model

The streptomycin-treated mouse was the model for studying the colonization of the mouse large intestine by *C. rodentium*. Three male, 6-week old CD-1 mice (Charles River) were given drinking water containing streptomycin-sulfate (5 g/L) to decrease the resident facultative anaerobic microbiota. Mice were then starved of food and water for 18-24 hours before being orally fed a 20% sucrose suspension containing 10^5 CFU of *C. rodentium* DBS100 Str^r. After the bacterial suspension was ingested, food and streptomycin water were returned *ad libitum*. Fecal samples were collected, bacterial load was quantified, and mice were euthanized as was done with the conventional mice. Three male, 6-week old CD-1 mice that were treated with streptomycin and not given the bacterial suspension served as controls.

3. CFU determination

To determine the number of colony forming units (CFU), the fecal samples were homogenized and serially diluted in 1 mL of 1% tryptone broth for every 0.1 g feces, and plated on MacConkey agar containing streptomycin only (100 µg/ml). The fecal plates were incubated overnight at 37°C, and bacterial colonies were counted the following day. Colonies that were round with a red center and white rim were identified as *C. rodentium*. In reality, fecal samples contain a smaller amount of the population of interest when compared to cecal samples; however, collecting fecal samples is standard procedure for bacterial quantification and is less intrusive compared to cecal sampling (35). The $\log_{10}$ mean number of CFU per gram of feces for either strain in the mice was calculated for

each time point. The statistical significance for each CFU/g feces result was determined using a Student's *t* test (two-tailed with unequal variance).

4. Fecal genomic DNA isolation

Genomic DNA was extracted from the collected fecal samples from days 1, 3, 7, and 15 of the colonization period using the QIAamp DNA Stool Mini Kit (Catalog #51054) (Qiagen) according to the manufacturer's instructions. Genomic DNA concentration was determined at a wavelength of 260 nm using a NanoDrop 2000 Spectrophotometer (Thermo Scientific).

5. Relative quantitative PCR of genomic DNA

Relative quantitative PCR (qPCR) was employed using SsoFast EvaGreen SuperMix (Bio-Rad #172-5201) and the primers listed in Table 1 for the day 7 samples for both control and experimental groups. qPCR was performed with cycles consisting of 95°C for 15 min and 40 cycles of 94°C for 15 s, 60°C for 30s, and 72°C for 30 s (15). qPCR was performed first on pure cultures to test the accuracy of the primers. The start of amplification was recorded at a particular cycle number (Cq) for each genus, and the Cq value for the Eubacteria (total bacteria) was used a standard against the specific bacterial abundances. The change in Cqs (ΔCq) was determined and was then used to calculate the relative population abundance by raising 2 to the -ΔCq.

RESULTS

1. Colonization Results

The genotypic and phenotypic profiles of the gastrointestinal microbiota may be altered by changes occurring in the intestinal microenvironment. The introduction and

Table 1. Quantitative PCR Primers

Target 16S rRNA	Primer	Sequence	Reference
***Eubacteria* (total bacteria)**	UniF340 UniR514	ACTCCTACGGGAGGCAGCAGT ATTACCGCGGCTGCTGGC	(15)
Bacteroidales	BactF285 UniR338	GGTTCTGAGAGGAAGGTCCC GCTGCCTCCCGTAGGAGT	(15)
Clostridium coccoides	UniF338 CocR491	ACTCCTACGGGAGGCAGC GCTTCTTAGTCAGGTACCGTCAT	(15)
Lactobacillus	LabF362 LabR677	AGCAGTAGGGAATCTTCCA CACCGCTACACATGGAG	(15)
Enterobacteriaceae	Coli F Coli R	GTGCCAGCMGCCGCGGTAA GCCATAACGTTGAAAGATGG	(36)
Bifidobacterium	Bif164F Bif662R	GGGTGGTAATGCCGGATG CCACCGTTACACCGGGAA	(15)

colonization of *C. rodentium* strain DBS100 may be considered an important change in the intestinal microcommunity; this change as well as other physiological conditions may affect its colonization *in vivo*. Therefore, the pathogenesis and colonization of *Citrobacter rodentium* strain DBS100 Str^r were observed by using both conventional and streptomycin-treated mouse models over a 15-day period. The log CFU/g feces during the *C. rodentium* DBS100 Str^r colonization assay of both experimental groups were determined (Figure 1). The degree to which each respective strain colonized the intestine was assessed via the number of CFUs that persisted in the feces.

The first day shows no statistical difference in log CFU/g feces between the conventional and streptomycin-treated models. However, a significant divergence between the two models is observed beginning at day 7 and continuing to day 15 of the experiment (Figure 1). Quantifying the population abundance of the most prominent bacteria in the intestinal microbiota will increase the understanding of the colonization results and the role the bacterial community has in the infection with *C. rodentium* strain DBS100 Str^r.

2. Relative qPCR results for day 7

To describe the gastrointestinal community dynamics associated with day 7 of the colonization period, relative qPCR was used with genera-specific primers of the bacterial 16S rRNA gene (Table 1). The respective bacterial population abundances were normalized to the total bacteria 16S rRNA gene copies in each sample. The *Bacteroidales* population was significantly higher in the streptomycin-treated mouse control compared to the conventional control, having over a three-fold increase in

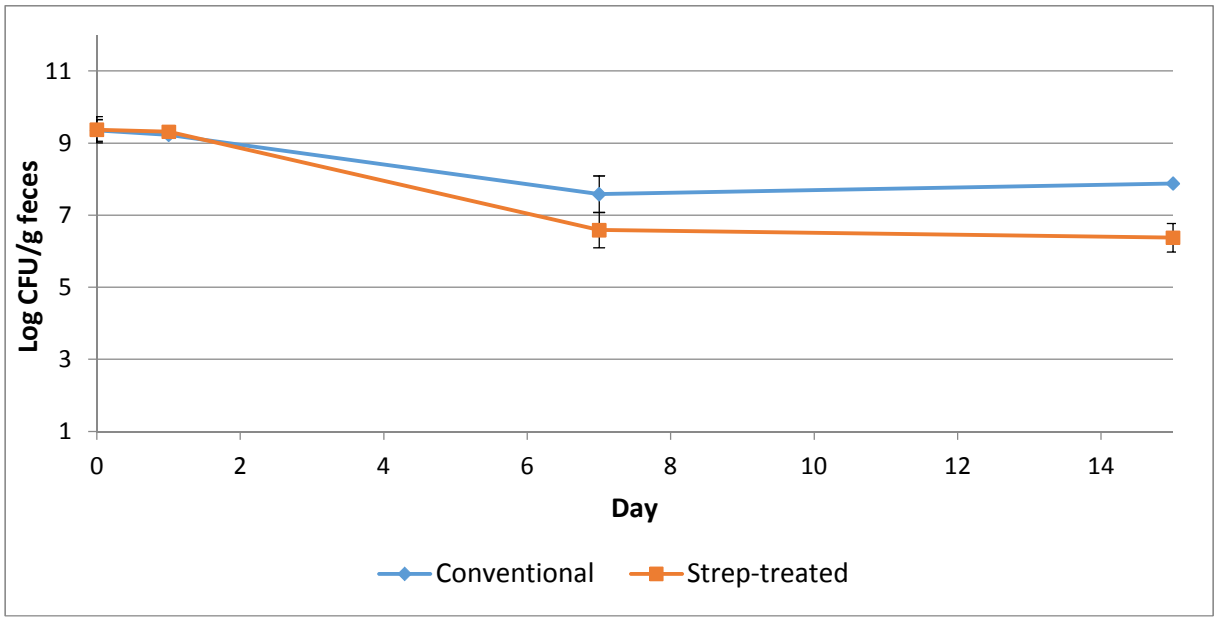

Figure 1. The colonization of *C. rodentium* strain DBS100 Str^r in conventional and streptomycin-treated mice. The log CFU/g of feces for both groups are nearly indistinguishable for day 1 and then begin to display a significant difference on day 7, with the conventional mice having significantly higher colonization than the streptomycin-treated mice by the end of the colonization period.

the streptomycin-treated intestine. There was a five-fold increase in *Bacteroidales* population abundance in the colonized, conventional model versus its control, and nearly a two-fold increase in the colonized, streptomycin-treated intestine versus its control (Figure 2). However, neither one of these results are considered significant differences. The *Clostridia* population had a two-fold decrease in the streptomycin-treated intestine control versus the conventional control (Figure 3). There was not a significant increase in population abundance in the colonized, conventional model, and less than a two-fold increase in the streptomycin-treated mouse model. Neither the conventional nor the streptomycin-treated mouse intestine had significant *Clostridia* differences with the colonization of *C. rodentium* when compared to their respective controls. Also, *Clostridia* population abundances in the presence of *C. rodentium* did not alter significantly with the antibiotic treatment (Figure 3).

The *Lactobacilli* population abundance did not change with antibiotic treatment alone (i.e. the controls had almost the same abundance). The conventional group colonized with *C. rodentium* had a significant increase of over seven-fold compared to its control, and a four-fold increase compared to the streptomycin-treated group colonized with *C. rodentium* (Figure 4). The *Bifidobacteria* population did not display a significance difference between the control groups or between the streptomycin-treated control and the colonized streptomycin-treated mouse intestine (Figure 5). The *Enterobacteriaceae* population abundances did not significantly differ between any of the experimental groups, including the controls (Figure 6). Figure 7 displays the collective relative abundances of each of the chosen genera.

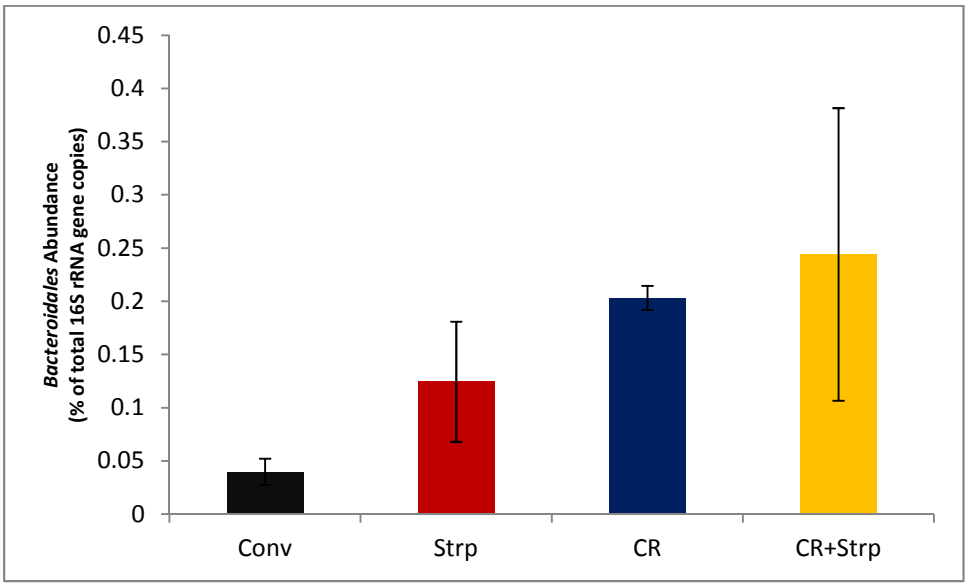

Figure 2. The bacterial abundance percentage for *Bacteroidales*. Quantitative PCR of *Bacteroidales* populations using group-specific primers on DNA extracted from fecal samples. The abundance of *Bacteroidales* was normalized to the total bacterial 16S rRNA gene copies in each sample. Results had n = 3 mice per group. Student's t-test reveals a significant difference between control groups ($P<0.05$). Error bars represent standard errors of the mean (SEM).

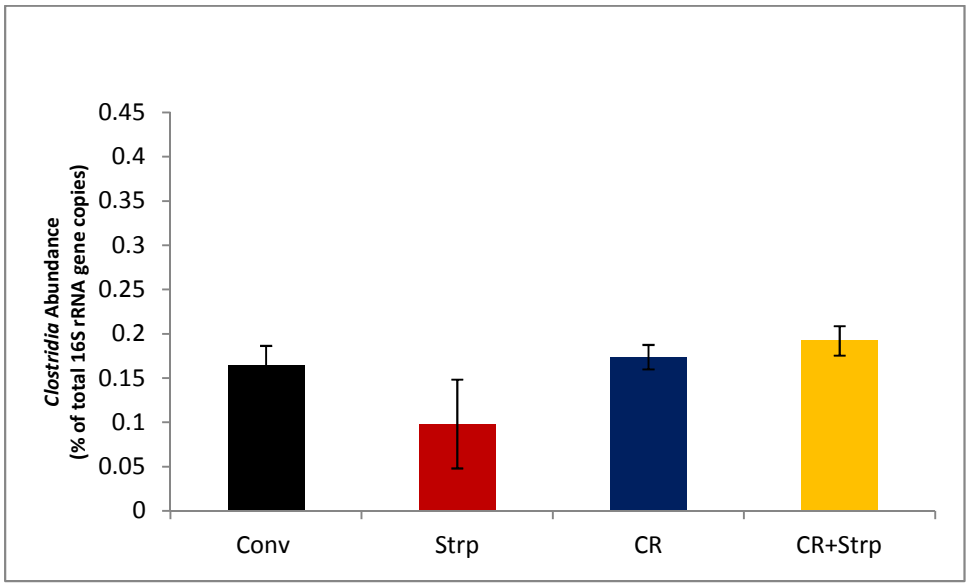

Figure 3. Bacterial abundance percentage of *Clostridium*. Quantitative PCR of *Clostridia* populations using group-specific primers on DNA extracted from fecal samples. The abundance of *Clostridia* was normalized to the total bacterial 16S rRNA gene copies in each sample. Results had n = 3 mice per group. Student's t-test reveals no significant difference between control groups or between the colonized/streptomycin model ($P>0.05$). Error bars represent SEM.

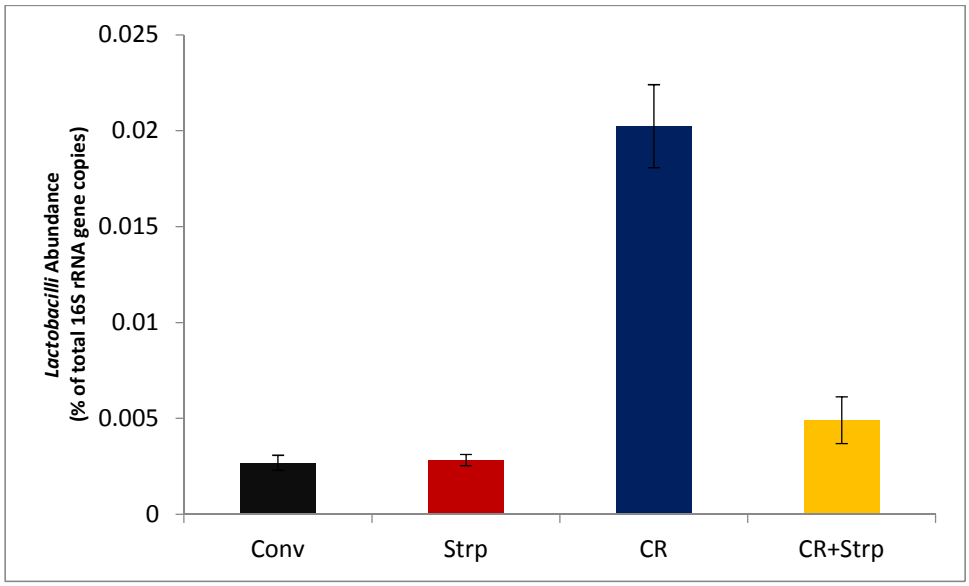

Figure 4. The bacterial population percentage of *Lactobacilli*. Quantitative PCR of *Lactobacilli* populations using group-specific primers on DNA extracted from fecal samples. The abundance of *Lactobacilli* was normalized to the total bacterial 16S rRNA gene copies in each sample. Results had n = 3 mice per group. Student's t-test reveals no significant difference between control groups or between the experimental groups versus their respective controls (P>0.05). Error bars represent SEM.

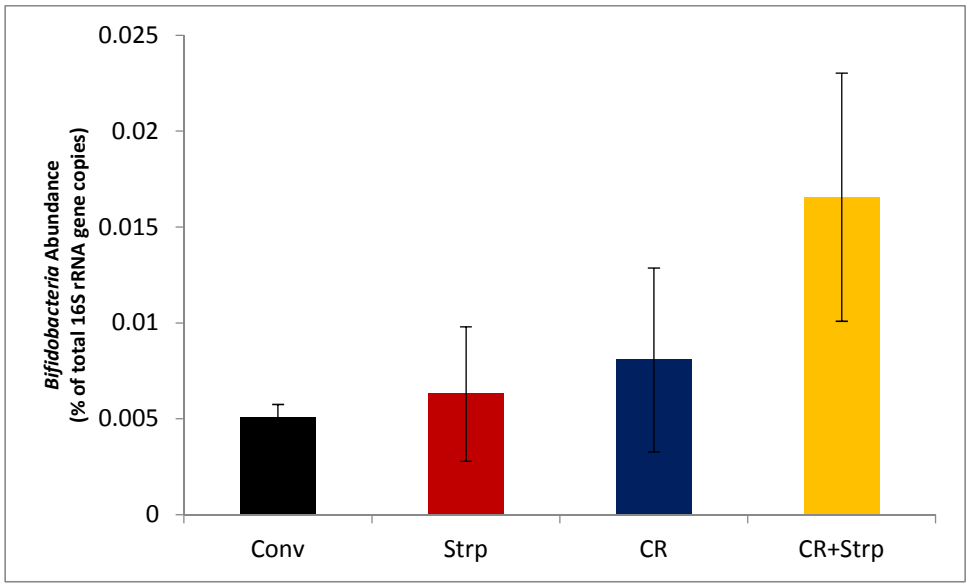

Figure 5. The bacterial population percentage of *Bifidobacterium*. Quantitative PCR of *Bifidobacterium* populations using group-specific primers on DNA extracted from fecal samples. The abundance of *Bifidobacteria* was normalized to the total bacterial 16S rRNA gene copies in each sample. Results had n = 3 mice per group. Student's t-test reveals no significant difference between control groups or between the experimental groups versus their respective controls (P>0.05). Error bars represent SEM.

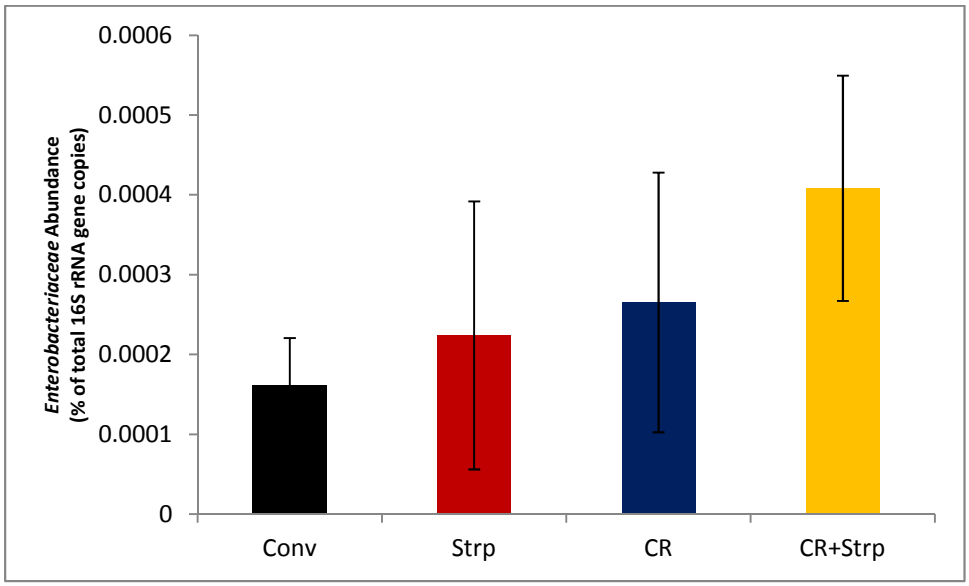

Figure 6. The bacterial population percentage of *Enterobacteriaceae*. Quantitative PCR of *Enterobacteriaceae* populations using group-specific primers on DNA extracted from fecal samples. The abundance of target groups was normalized to the total bacterial 16S rRNA gene copies in each sample. Results had n = 3 mice per group. Student's t-test reveals no significant difference between control groups or between the experimental groups versus their respective controls ($P>0.05$). Error bars represent SEM.

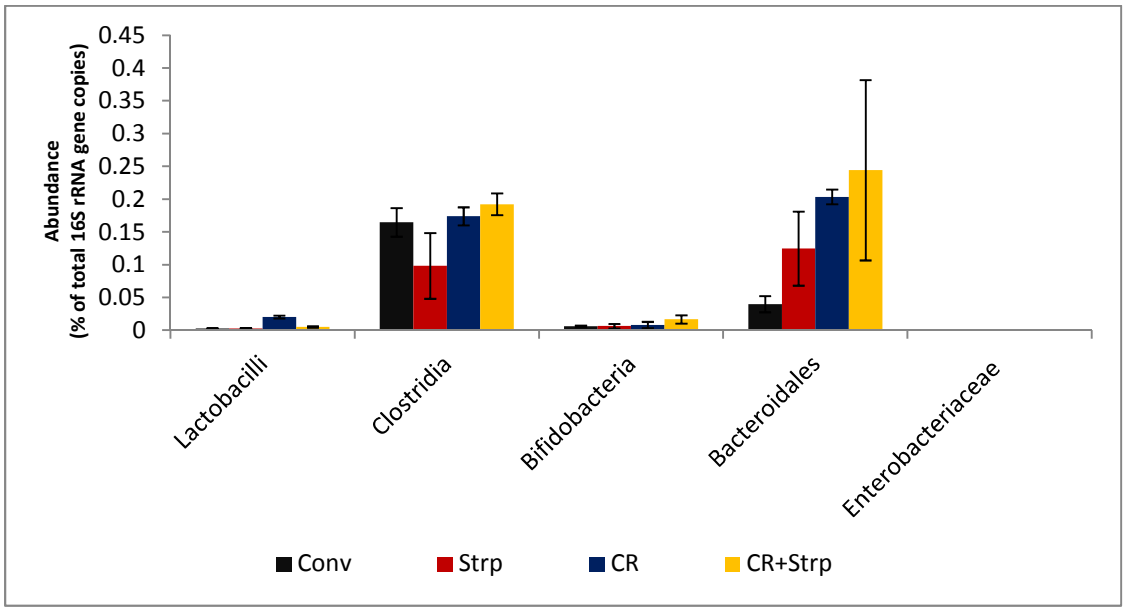

Figure 7. The collective display of bacterial abundance percentages. Quantitative PCR of select bacterial populations using group-specific primers on DNA extracted from fecal samples. The abundance of target groups was normalized to the total bacterial 16S rRNA gene copies in each sample. Error bars represent SEM.

DISCUSSION

The goal of this study was to characterize the changes that occur among the predominate genera of the intestinal microbiota in the presence of *Citrobacter rodentium*, with the long-term goal of applying the results to clinical practice and disease prevention. Unexpectedly, the colonization data revealed that *C. rodentium* DBS100 Str^r was able to colonize (i.e. maintain stable growth) better in the conventional model than in the streptomycin-treated mouse intestine. The colonization of *C. rodentium* in the conventional mouse intestine was unanticipated in consideration of previous studies, which have shown that colonization of *E. coli* in the conventional model is not displayed due to colonization resistance (37). Recall that the slight inflammatory response induced by streptomycin treatment is a contributing factor to colonization success. The colonization advantage observed in the conventional model of this project may be explained by the inflammatory response previously observed in the intestines of conventional mice infected with *C. rodentium* strain DBS100 (unpublished data). It was observed that the enteric inflammatory response of the conventional mice was greater than that observed in streptomycin-treated mice infected with the same bacteria. While the exact source of inflammation in the conventional mouse is unknown, the response of the intestinal microbiota to lipopolysaccharide has been implicated to induce an exaggerated inflammatory response in humans (38). The reason for a colonization advantage in the conventional model for *C. rodentium* is unclear in current research and is beyond the scope of this study.

Concerning the control groups, the qPCR results are consistent with previously published data that *Bacteroidales* populations are elevated with an approximately three-

fold increase under streptomycin treatment (13). Curiously, the results indicate that there was no significant change in any of the bacterial population abundances for the groups colonized with *C. rodentium* in either model compared to their respective controls. Both *Bacteroidales* and *Clostridia* bacteria maintained growth with the colonization of *C. rodentium* even with antibiotic treatment (Figures 1 and 2). The day 7 results (Figure 4) support the previously shown result that *Lactobacilli* are not significantly altered by streptomycin treatment alone. The seven-fold increase of the *Lactobacilli* population abundance in the colonized, conventional mouse intestine compared to its control has not been observed in previous studies. Unfortunately, the dynamic relationship is not fully understood between *C. rodentium* and *Lactobacilli,* and would be worth exploring in a follow-up experiment. In the streptomycin-treated mouse intestine, the nearly two-fold increase in *Lactobacilli* population abundance was not considered a significant change, which was an expected result since streptomycin-treatment has not had any previous indication of affecting the Gram-positive bacterial populations. However, this nearly two-fold increase cannot be ignored as a change to the gut microbial composition for future studies since the influence *Lactobacilli* have on the rest of the microbiota as well as the host has not been described.

The results of this study indicate that the absence of antibiotic treatment in addition to the presence of *C. rodentium* may have a direct impact on other factors leading to the promotion of Gram-positive bacterial growth. The results of the *Clostridia* and *Bifidobacteria* population abundances (Figures 2 and 5) do not support the conclusion that all Gram-positive bacteria benefit (in terms of increased growth) from the presence of *C. rodentium* in the absence (or presence) of continuous antibiotic treatment.

To ensure the validity of the unpredicted finding of increased growth of *Lactobacilli* in the conventional and not in the streptomycin-treated model compared to their respective controls may warrant a repeated analysis of the genomic DNA with qPCR.

The colonization differences between EHEC and *C. rodentium* in the conventional model cannot be fully explained by current research, but one contributing factor may include the dynamic relationship between *C. rodentium* and the commensal bacterial populations. Overall, the integrity of these results is called into question in part because of the very low abundance percentage values; the collective population abundances should comprise the majority of the microbial community. Notice in each figure that the percentages do not sum to 100% (or even come close to this value). A particular example proceeds from the low abundance percentage of *Enterobacteriaceae* (Figure 6). Especially concerning the amplification of the DNA in the qPCR analysis, there are many factors that could affect the results, including the purity of the genomic DNA.

The microbial composition was defined for only day 7 of the colonization period, but the changes in commensal bacteria abundances occurring for the entire duration of the colonization experiment must be considered to better characterize the results shown in Figure 1. The genomic DNA quantification of days 1 and 15 will spread light on the bacterial population fluctuations and their contribution to the colonization differences between both models throughout the 15-day colonization period, and is the goal of future work for this study.

Notice that the colonization data from day 3 was not recorded; this was due to misplating, resulting in undetermined CFUs. In addition, the genomic DNA from the day

3 samples extraction is unreliable as the purity of the genomic DNA was compromised during the extraction process. As a result, the day 3 colonization data was not recorded and the quantification from day 3 will not be performed in the future.

The colonization results encourage histopathological scoring of the ceca and small intestines of the mice under study. The histopathology scoring will elucidate whether or not the colonization of *C. rodentium* caused colonic stress, especially that leading to the pathogenesis phenotype. The ceca and colons of the mice used in this study have already been isolated in preparation for the continuance of the project along the immunohistochemistry branch.

Changes occurring in the *Bacteroidales*, *Clostridia*, and *Enterobacteriaceae* population levels throughout the colonization period for the colonized, streptomycin-treated mice when compared to the uncolonized, streptomycin-treated mice are expected because of the antibiotic effect on facultative anaerobes. Based on the day 7 results, significant fluctuations for the *Lactobacilli* population levels in the colonized, streptomycin-treated mice when compared to the uncolonized, streptomycin-treated mice are not anticipated. Altered population levels in the colonized, conventional mice when compared to the uncolonized, conventional mice are expected for the five bacterial genera under study, particularly with the *Lactobacilli* abundance. We expect a greater significant change in bacterial population levels overall in the streptomycin-treated mouse model in relation to the conventional mouse model. The divergence in colonization of *C. rodentium* is not clearly explained by the present data, which further emphasizes the need for the future quantification of all colonization samples of this colonization experiment. The data obtained from the quantification of all of then samples

will provide better insight into the interactions between the intestinal microbiota and the colonization of *C. rodentium* in the conventional versus streptomycin-treated mouse models.

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