


RESEARCH PAPER



Removal of the cecum affects intestinal fermentation, enteric bacterial community structure, and acute colitis in mice

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ABSTRACT

The murine cecum is a major site of fermentation of dietary materials, and production of short chain fatty acids (SCFAs). To examine the role that the cecum plays in acute bacterial infection in mice, the cecum was surgically removed, and changes in bacterial communities and production of SCFAs were analyzed relative to surgical sham animals. To incite bacterial colitis, mice were orally challenged with *Citrobacter rodentium*. The impact of butyrate administered directly into the colon was also examined. Concentrations of SCFAs in feces were substantially lower in mice with an excised cecum. Bacterial communities were also less diverse in cecectomized mice, and densities of major SCFA-producing taxa including bacteria within the Ruminococcaceae and Lachnospiraceae families were reduced. Colonization of the intestine by *C. rodentium* was not affected by removal of the cecum, and the bacterium equally incited acute colitis in mice with and without a cecum. However, cecectomized mice exhibited lower body weights at later stages of infection indicating an impaired ability to recover following challenge with *C. rodentium*. Furthermore, removal of the cecum altered immune and inflammatory responses to infection including increased inflammatory markers in the proximal colon (*Tnfα*, *Il10*, β *d1*), and heightened inflammatory response in the proximal and distal colon (*Ifnγ*, *Tnfα*, *Relmβ*). Exogenous administration of butyrate was insufficient to normalize responses to *C. rodentium* in cecectomized mice. The murine cecum plays a critical role in maintaining intestinal health, and the murine cecectomy model may be a useful tool in elucidating key aspects of intestine-pathogen-microbiota interactions.

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Introduction

THE CECUM is an intraperitoneal pouch located at the most cranial aspect of the large intestine. While the function of the cecum is not fully understood, it is thought to play a role in production of short chain fatty acids in many mammals,¹ and it has been proposed to serve as a reservoir of anaerobic bacteria that populate the colon. The composition of the bacterial community that colonizes intestines are thought to be critical in mitigating responses to infection,² yet mechanisms of this ‘colonization resistance’ are enigmatic at present.

Short-chain fatty acids (SCFAs) are bi-products of bacterial fermentation of dietary fermentable materials.³ The cecum is the main site of fermentation in mice and SCFA production decreases along the colon as a function of distance from the cecum due to absorption

by colonocytes (i.e. epithelial cells lining the colon) for energy (e.g. butyrate), or for use in cholesterol, fat, and sugar metabolism (e.g. acetate and propionate).⁴ As the cecum has been identified as a primary site of colonic fermentation¹ and a reservoir of anaerobic bacteria, we hypothesize that the removal of the cecum will be detrimental to intestinal health (e.g. inflammation) as a result of decreased SCFA availability to colonocytes and a greatly decreased richness of the ‘commensal’ colonic bacterial community. Our recent investigation demonstrated that colonic infusion of butyrate can ameliorate intestinal inflammation in mice.⁵ Others also have indicated that butyrate modulates bacterial communities within the colon of mice during periods of aberrant inflammation (e.g. colitis)^{6,7}; however, the mechanisms by which butyrate affects intestinal health

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(e.g. amelioration of enteric inflammation) remain poorly understood.

In 1987, Voravuthikunchai and Lee⁸ reported that the removal of the cecum in mice severely compromised production of a SCFAs and lowered resistance to infection by *Salmonella enterica* serovar Enteritidis. This pathogen typically incites typhus in mice,⁹ and the mechanisms involved in loss of colonization resistance including changes in host responses (i.e. enteritis) and commensal bacterial communities that were associated with reduced SCFA production and pathologic changes were not determined.⁸ In the current study, cecectomy surgery was performed in mice, and fecal SCFA and changes in colon bacterial communities were characterized; Illumina sequencing was employed. The implications of cecectomy surgery and associated changes in response to enteric inflammation were determined by inciting acute colitis using *C. rodentium* and measuring histological and molecular indicators of inflammation. Finally, butyrate was administered directly to the colon to ascertain whether exogenous butyrate can compensate for the low concentrations of butyrate within the colon (i.e. as result of the inability of cecectomized mice to ferment carbohydrates).

Results

Cecum removal decreases fecal short chain fatty acids in feces

Concentrations of total SCFAs, acetic acid, butyric acid, isobutyric acid, valeric acid, isovaleric acid, and propionic acid were substantially lower ($P < 0.001$) in feces from cecectomized mice following surgery and post-inoculation (p.i.) with *C. rodentium* (Figure 2A–G). Cecum removal had no effect ($P = 0.375$) on concentrations of caproic acid in feces (Figure 2H). Inoculation with *C. rodentium* increased production of total SCFAs ($P < 0.001$), particularly acetic ($P < 0.001$), butyric ($P < 0.001$), and propionic ($P < 0.001$) acids in feces from cecectomy or sham mice. Colonic administration of butyrate did not increase ($P = 0.714$) concentrations of this SCFA within the cecum (Figure S1) or excreted in feces (Figure S2).

Cecum removal alters bacterial communities in the colon \pm colitis

A lower diversity of bacteria associated with mucosa ($P \leq 0.001$) was observed in both the

proximal and distal colon of cecectomized relative to sham mice regardless of colitis (Figure 3). The lower diversity was reflected in Simpson's metric of species richness, as well as Shannon's diversity index, which also accounts for species evenness. Furthermore, the composition of the microbiota differed conspicuously ($P \leq 0.002$) in cecectomized mice in both the proximal (Figure 4A) and distal (Figure 4B) colon. At both sites there was an increase in the abundance of Enterobacteriaceae and Erysipelotrichaceae, and decreased abundance in Lachnospiraceae and Ruminococcaceae families in cecectomized mice (Figure 5). Within the Lachnospiraceae family, an increase in abundance of *Clostridium* cluster XIVb and *Dorea* spp. was noted in the distal colon, while decreases in abundance of *Lachnospira*, *Marvinbryantia* and *Roseburia* spp. were observed in both the proximal and distal colon (Figure 6). Within the Ruminococcaceae family, an increase in abundance of *Anaerotruncus* was observed in the distal colon, and decreases in abundance of *Butyricicoccus*, *Clostridium* cluster IV, *Flavonifractor*, *Oscillobacter*, *Ruminococcus*, and *Sporobacter* spp. occurred in the proximal and distal colon (Figure 7). No differences in alpha diversity ($P \geq 0.520$), beta diversity ($P \geq 0.202$), or relative abundance of individual taxa were observed as a result of butyrate administration to mice with or without a cecum (Figure S3; Figure S4).

Infection by *C. rodentium* may have differentially affected the abundance of several bacterial taxa, such as *Clostridium* cluster XIVb and *Anaerotruncus* spp. in the proximal colon of Cec+ mice, and *Oscillobacter* spp. in the proximal and distal colon of Cec- mice (Figure 6–7).

Cecum removal does not affect intestinal colonization by *Citrobacter rodentium*

To incite colitis, we inoculated mice with *C. rodentium*. In cecectomized mice inoculated with the pathogen (\pm colonic butyrate administration) there was no difference ($P \geq 0.247$) in densities of *C. rodentium* cells shed in feces over the duration of the experiment (Figure 8A). Furthermore, examination of the colonic tissues of mice inoculated with the pathogen by fluorescence *in situ* hybridization (FISH) showed an equal abundance and distribution of *C. rodentium* cells associated with the colonic

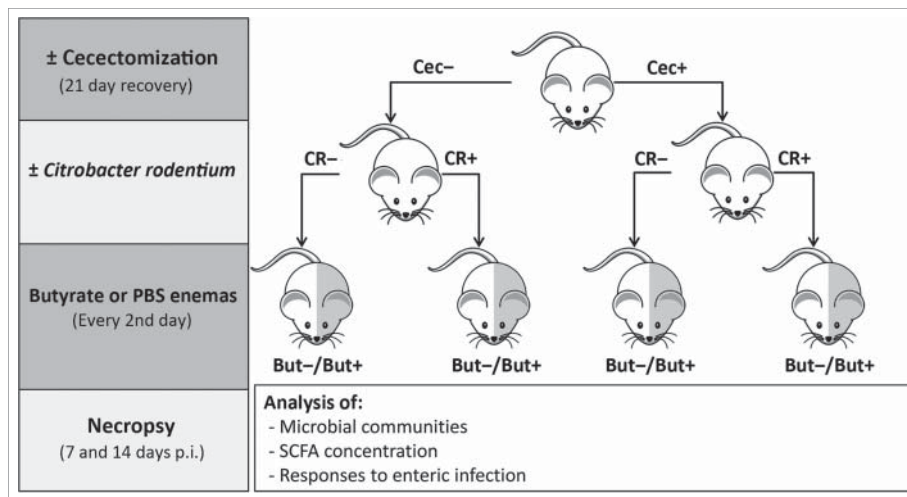


Figure 1. The experiment was designed as four factor factorial experiment with two levels of time (7 and 14 days post-inoculation [p.i.]), two levels of cecum (i.e. mice that underwent sham [Cec-] or cecectomy [Cec+] surgery), two levels of inflammation (mice that were orally administered phosphate buffered saline [CR-] or *Citrobacter rodentium* [CR+]), and two levels of butyrate (i.e. mice were administered phosphate buffered saline [But-] or butyrate [But+] via enemas every second day p.i.). Thus, 16 mice were included in each replicate, and four replicates were conducted on separate occasions equaling 64 mice in total.

mucosa (Figure 8B-E). No *C. rodentium* was isolated from feces, nor was the bacterium visualized in the colons of mice not inoculated with the pathogen by FISH.

Cecum removal alters host responses to *Citrobacter rodentium* infection

While body weights were similar ($P \geq 0.137$) between cecectomized and sham mice at the early and peak stages of infection (Figure 9A), at late infection, mice without a cecum had lower ($P \leq 0.031$) body weights (Figure 9B). It is noteworthy that 13 days following inoculation with *C. rodentium*, sham mice had recovered to the same body weight as uninfected mice; however, inoculated mice with no cecum exhibited lower body weights relative to the infected sham control mice (Figure 9B). Colonic administration of butyrate had no effect ($P = 0.269$) on relative weight loss following *C. rodentium* inoculation in either cecectomized or sham mice.

Cecum removal altered inflammatory response to *Citrobacter rodentium* infection

Indicators of inflammation within the spleen and intestine were analyzed to determine whether the presence of the cecum within mice (and associated effects) alters host responses to infection. In mice that

received sham and cecectomy surgery, equally prominent responses to infection at peak infection (i.e. 14 days p.i.) were observed including increased size and number of germinal centres, increased numbers of apoptotic and necrotic lymphocytes, and increased infiltration of inflammatory cells into the spleen ($P = 0.010$). At early infection (i.e. 7 days p.i.) in contrast, expression of markers of inflammation in splenic tissue were observed in cecectomized mice ($P = 0.001$) but not in sham treated mice ($P = 0.215$) (Figure 10). Colonic injury was determined by histopathologic scoring for epithelial cell hyperplasia, crypt height, epithelial injury, inflammatory cell infiltration, mitotic activity, and goblet cell depletion. In the distal colon, higher ($P \leq 0.013$) total histopathologic scores were observed in infected versus non-infected mice at both 7 and 14 days p.i. (Figure 11B). In the proximal colon, higher total scores were found only in infected mice that had their cecum removed at 14 days p.i. ($P = 0.031$); however, a similar trend for higher histopathologic scores was observed in infected mice at 7 days p.i. and for infected sham mice at 14 days p.i. (Figure 11A). There was no difference ($P \geq 0.141$) in the severity of histopathologic changes in infected cecectomized versus infected sham mice, nor were there changes in histopathologic scores as a result of butyrate administration ($P \geq 0.243$).

To assess molecular mechanisms involved in inflammation within the intestines, expression of

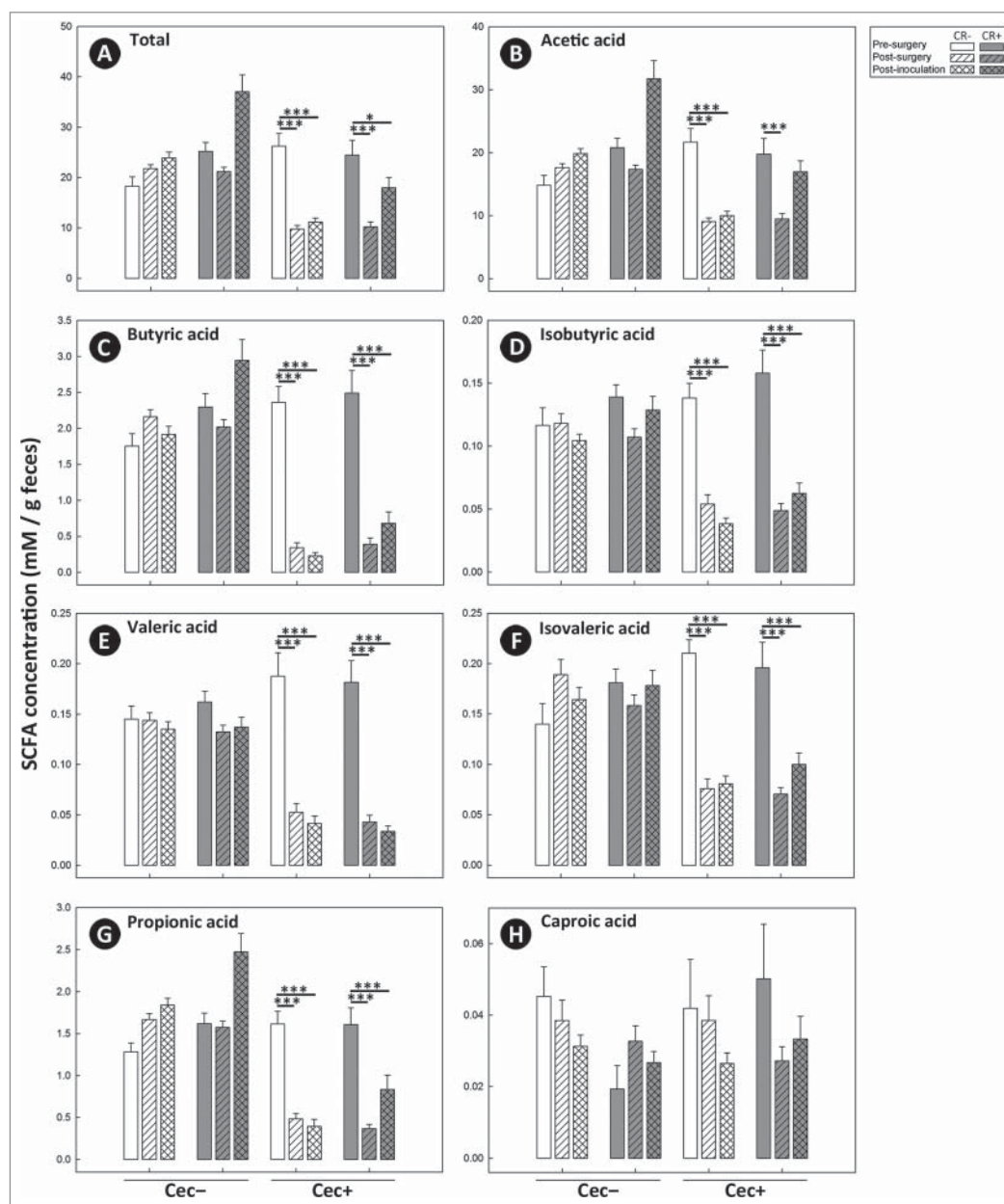


Figure 2. Concentration of: (A) total short chain fatty acids; (B) acetic acid; (C) butyric acid; (D) isobutyric acid; (E) valeric acid; (F) isovaleric acid; (G) propionic acid; and (H) caproic acid in feces from mice that received sham (Cec-) or cecectomy (Cec+) surgery and were orally administered PBS (CR-) or *Citrobacter rodentium* (CR+). Fatty acids were measured in feces collected prior to surgery (pre-surgery), between surgery and inoculation (post-surgery), and after administration of *C. rodentium* or PBS. Vertical lines associated with histogram bars represent standard error of the means (n = 10 to 15 mice/treatment). * $P \leq 0.050$, *** $P \leq 0.001$.

mRNA of genes encoding proteins involved in the detection of SCFA, intestinal inflammation, and intestinal repair and recovery were quantified. Several markers were differentially expressed in non-infected mice that had their cecum removed, including *Tnfa* ($P = 0.025$), *Il17a* ($P = 0.022$), *Il10* ($P = 0.023$), and $\beta d1$ ($P \leq 0.028$) in the proximal colon (Figure 12), and *Tnfa* ($P = 0.017$) and *Relm β* ($P \leq 0.048$) in the distal colon (Figure 13). Infection with *C. rodentium* affected expression of

several markers in the proximal colon, including *Ifn γ* ($P \leq 0.043$), *Tnfa* ($P = 0.036$), *Il17* ($P < 0.043$), *Tgf β* ($P \leq 0.050$), and *Ffar3* ($P = 0.021$) (Figure 12), and in the distal colon, including *Ifn γ* ($P < 0.001$), *Tnfa* ($P \leq 0.002$), *Il17a* ($P < 0.001$), *Relm β* ($P < 0.001$), *Muc2* ($P \leq 0.009$), and *Ffar3* ($P < 0.001$) (Figure 13). Differential responses to infection by *C. rodentium* were also observed in cecectomized mice. In this regard, in the proximal colon of infected mice, higher levels of expression

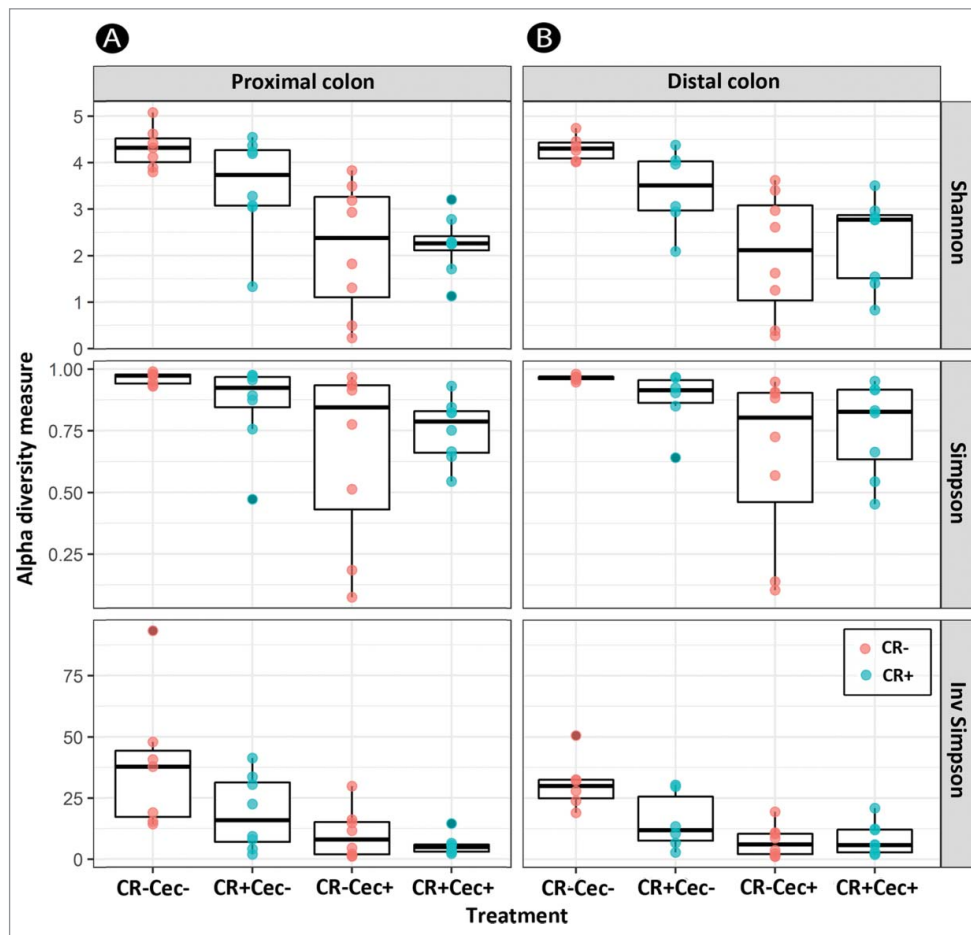


Figure 3. Alpha diversity of bacterial communities in the proximal (A) and distal (B) colon of mice that received sham (Cec-) or cecectomy (Cec+) surgeries and were orally administered PBS (CR-) or *Citrobacter rodentium* (CR+) 14 days post-inoculation. Vertical lines associated with histogram bars represent standard error of the means ($n =$ six to eight mice/treatment).

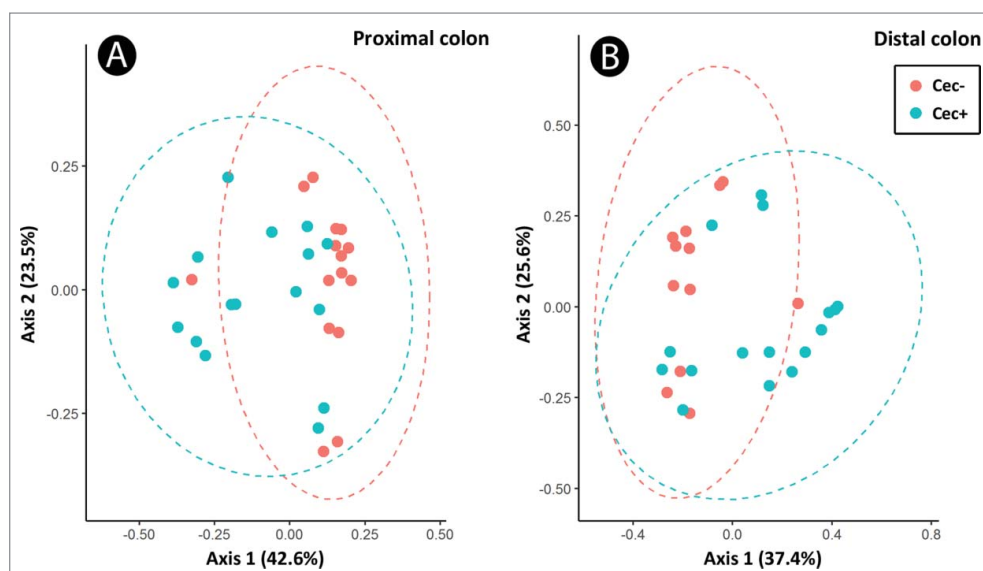


Figure 4. Principal component analysis based on Bray-Curtis dissimilarity in bacterial communities in the proximal (A) and distal (B) colon of mice that received sham (Cec-) or cecectomy (Cec+) surgeries 14 days post-inoculation.

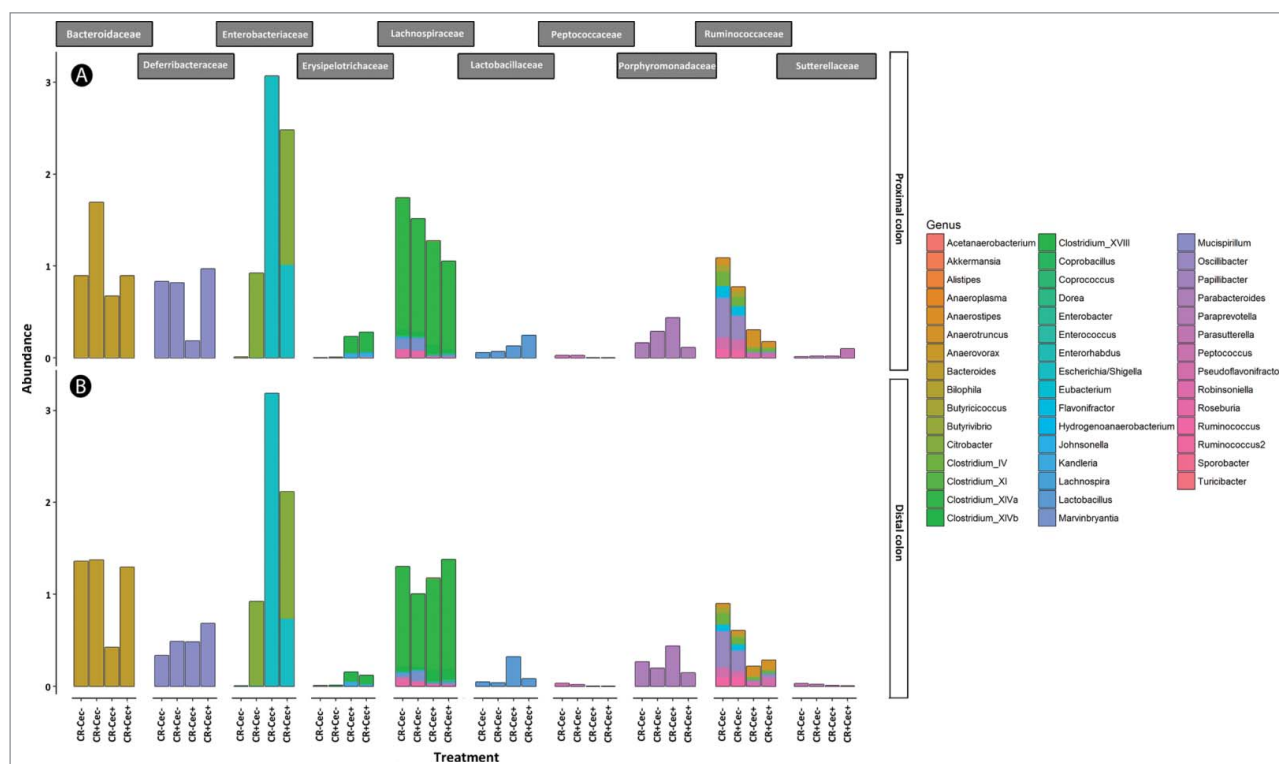


Figure 5. Relative abundance of bacteria genera within different families in the proximal (A) and distal (B) colon of mice that received sham (Cec-) or cecectomy (Cec+) surgeries and were orally administered PBS (CR-) or *Citrobacter rodentium* (CR+) 14 days post-inoculation. Bars indicate the mean ($n =$ four mice/treatment).

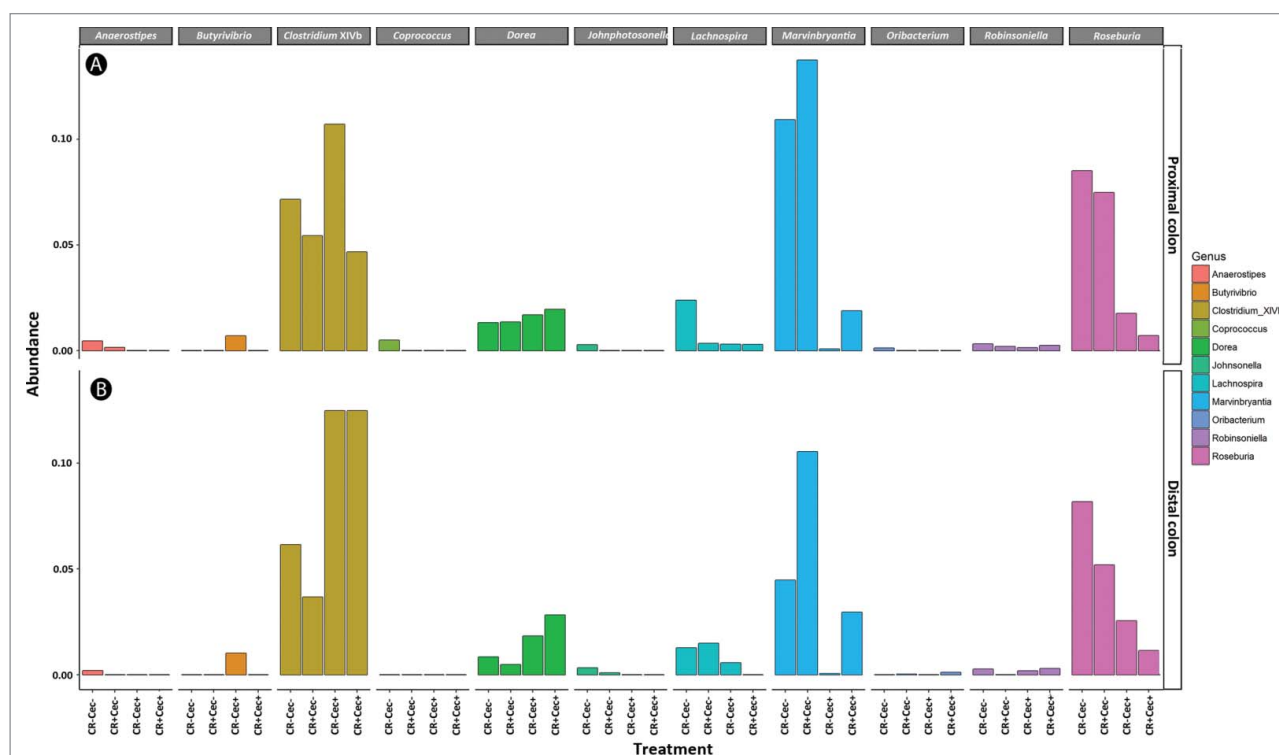


Figure 6. Relative abundance of genera within the Lachnospiraceae family in the proximal (A) and distal (B) colon of mice that received sham (Cec-) or cecectomy (Cec+) surgeries and were orally administered PBS (CR-) or *Citrobacter rodentium* (CR+) 14 days post-inoculation. Data represents the mean ($n =$ four mice/treatment).

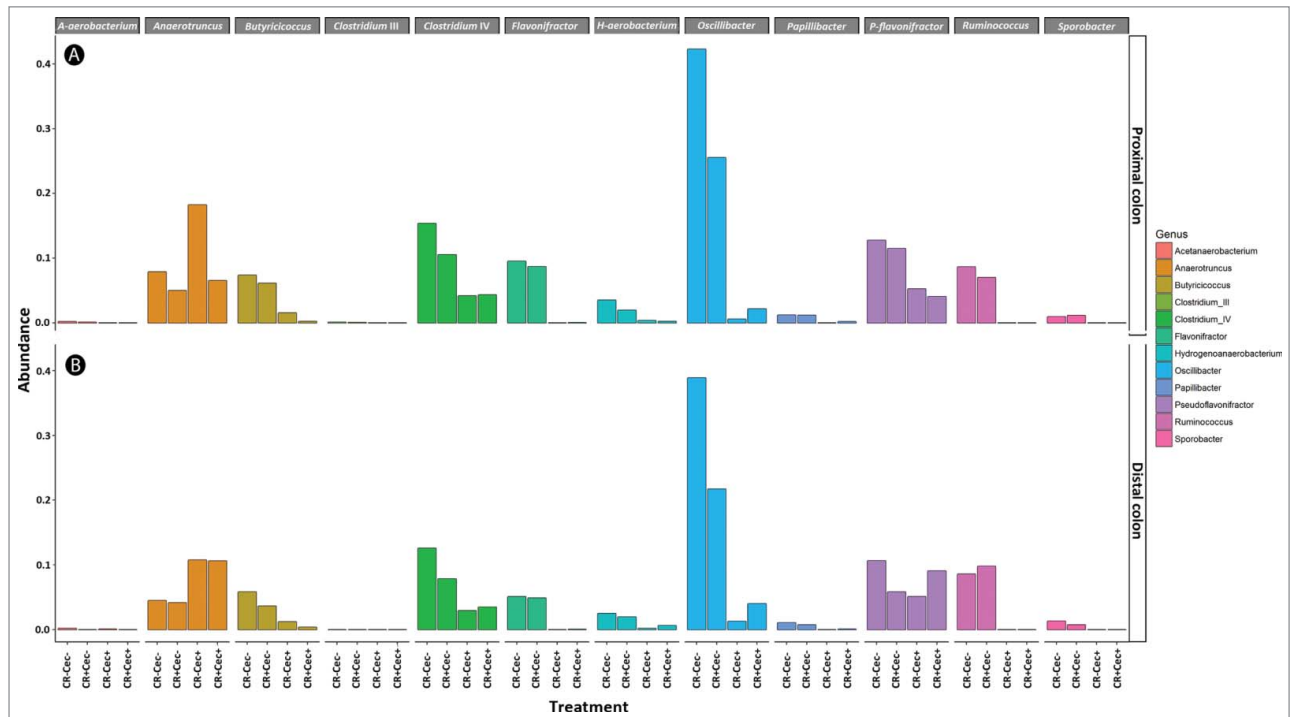


Figure 7. Relative abundance of genera within the Ruminococcaceae family in the proximal (A) and distal (B) colon of mice that received sham (Cec-) or cecectomy (Cec+) surgeries and were orally administered PBS (CR-) or *Citrobacter rodentium* (CR+) 14 days post-inoculation. Data represents the mean (n = four mice/treatment).

of *Ifn γ* were observed at 7 ($P = 0.002$) and 14 ($P = 0.009$) days p.i., and higher expression of *Tnfa* ($P = 0.041$) and *Tgf β* ($P = 0.041$) was observed at 7 days p.i. (Figure 12). Furthermore, expression of *Ffar2* was higher ($P = 0.012$) in the proximal colon of infected mice without a cecum at 7 days p.i. In the distal colon, intestinal inflammation was generally more severe but appeared less affected by the removal of the cecum although higher levels of expression of both *Ifn γ* ($P = 0.032$) and *Relm β* ($P = 0.007$) was observed in infected mice without a cecum (Figure 13).

Exogenous administration of butyrate did not affect bacterial communities or colitis

Concentrations of butyric acid in feces ranged from 0.28 ± 0.05 to 2.32 ± 0.16 mM, and the colonic administration of butyrate did not increase ($P = 0.714$) the concentration of butyric acid excreted in feces (Figure S1). Administration of butyrate also had no effect on the concentration of SCFAs in cecal contents at the time of animal euthanization (Figure S3) Moreover, no effects of butyrate administration on expression of inflammatory markers in the proximal ($P \geq 0.222$) or distal ($P \geq 0.280$) colon were observed

(Figure 12–13). In the distal colon, a trend for lower total histopathologic scores was observed in mice rectally administered butyrate with colitis (i.e. Cec- mice at 7 days p.i., and Cec+ mice at 14 days p.i.), suggesting that butyrate may have imparted a mild protective effect within the colon.

Discussion

The cecum is considered the primary site of SCFA production in mice, and as such, surgical removal of the cecum may provide insights into colonization resistance within the colon and factors that contribute to intestinal health. In the current investigation, we removed the cecum in mice using a surgical procedure described previously,⁸ and we applied modern analytical tools not available to Voravuthikunchai and Lee⁸ to obtain insight into the possibility of using the murine ‘cecectomy model’ for elucidating key aspects of the host-pathogen-microbiota interaction. Furthermore, we used a pathogen that produces localized colitis in mice (i.e. *C. rodentium*)¹⁰ in contrast to *S. Enteritidis*.⁸

The intestine is colonized by a complex community of bacteria, which varies in both density and complexity along its length.^{11,12} In mice, the cecum represents

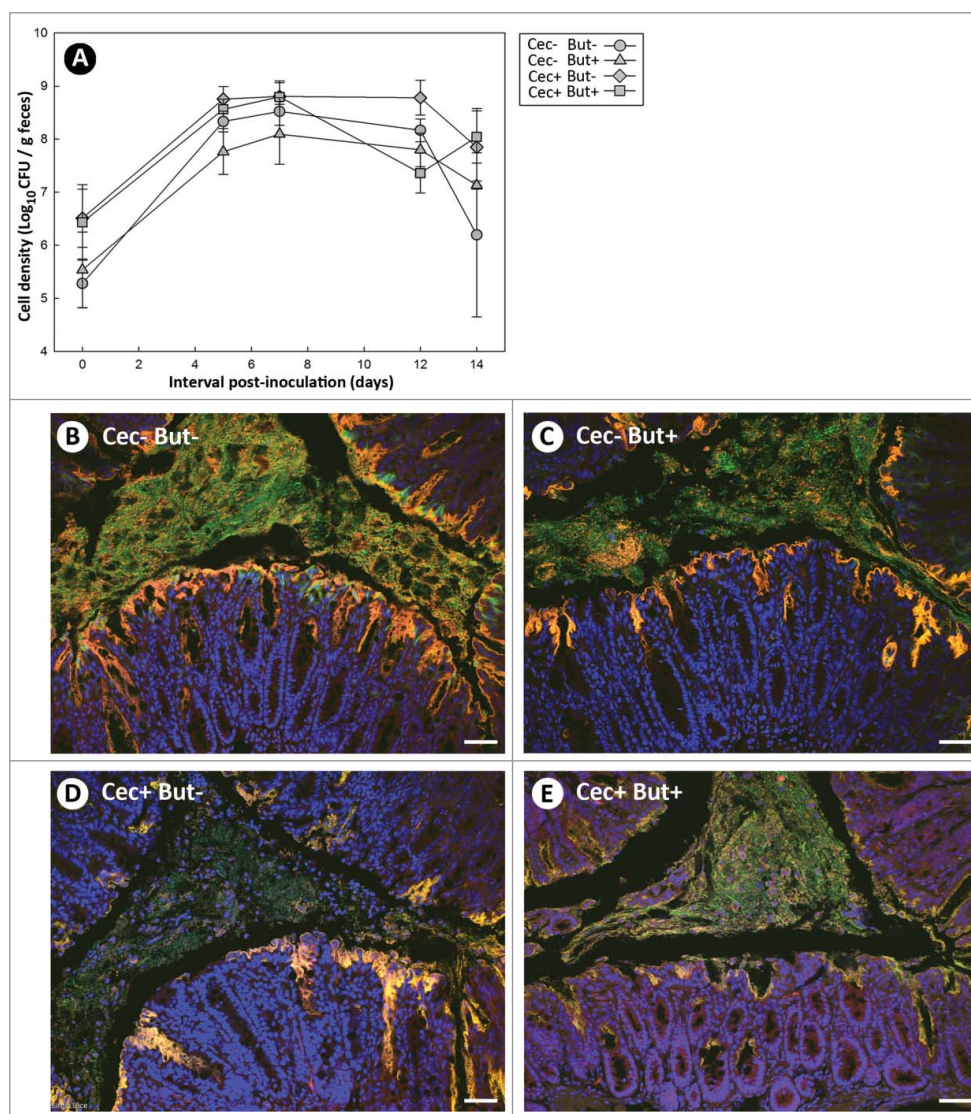


Figure 8. (A) Densities of viable *C. rodentium* (log CFU/g) in feces as determined by dilution plating for mice that received sham (Cec⁻) or cecectomy (Cec⁺) surgeries, were orally administered *Citrobacter rodentium* (CR⁺), and received enemas containing PBS (But⁻) or butyrate (But⁺). Vertical lines associated with markers indicate standard error of the means (n = four mice/treatment). (B-E) Visualization of *C. rodentium* in distal colon tissues at peak infection (i.e. 7 days post-inoculation); (B) Cec⁻ and But⁻; (C) Cec⁻ and But⁺; (D) Cec⁺ and But⁻; (E) Cec⁺ and But⁺. Cell nuclei are stained with DAPI (blue), total bacteria are stained with Alexa-488 (green) and γ -Proteobacteria (*C. rodentium*) are stained with Alexa-594 (red). Scale bar = 50 μ m.

a junction between a lower diversity of bacterial communities within the small intestine and a higher diversity of bacterial communities within the colon.¹¹ The variations in bacterial populations cranial and distal to the ileal-cecal junction, in addition to high diversity of bacteria within the blind-ended cecum, have led to the hypothesis that the cecum functions as a bacterial reservoir from which the colon microbiota is populated. At present, however, there is a paucity of conclusive evidence to support this hypothesis. In our study we demonstrated that removal of the cecum resulted in a conspicuous decrease in both richness and evenness of bacterial communities of the colon, as well as a

pronounced change in the composition of the bacterial community structure. A more diverse microbiota is considered to provide beneficial effects to the host, whereas a reduced diversity community is often associated with intestinal disease.¹³ Thus, it is plausible that the cecum may play an important role in maintaining intestinal homeostasis and overall health of the host by influencing both the amount and diversity of bacteria within the large intestine.

Bacteria that contain SCFA-producing taxa (e.g. members of the Ruminococcaceae and Lachnospiraceae families)¹⁴ were conspicuously reduced in relative abundance as a result of cecal removal. Within the

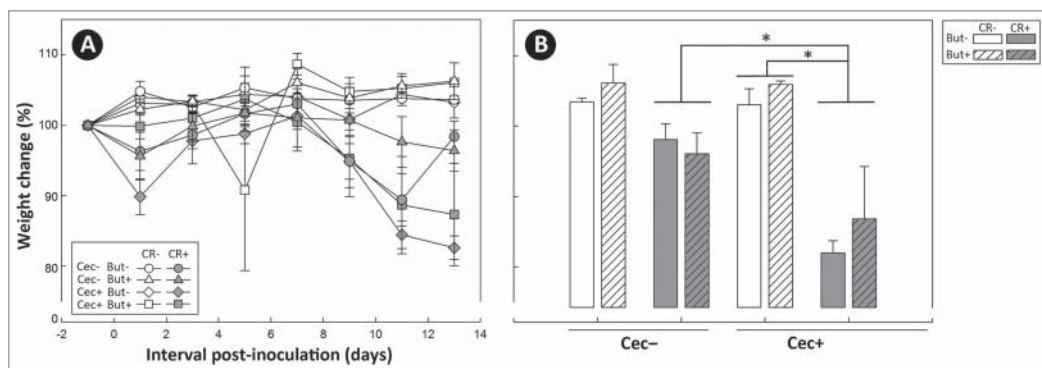


Figure 9. (A) Temporal change in body weight, and (B) change in body weight at 13 days after administration of *Citrobacter rodentium* or PBS. Mice that received sham (Cec-) or cecectomy (Cec+) surgeries, were orally administered PBS (CR-) or *C. rodentium* (CR+), and received enemas containing PBS (But-) or butyrate (But+). Vertical lines associated with markers or histogram bars represent standard error of the means ($n =$ three to five mice/treatment). * $P \leq 0.050$.

Ruminococcaceae family, *Butyricoccus*, *Ruminococcus*, *Flavonifractor* and *Sporobacter* were among the genera that were decreased in abundance as a result of cecum removal. Within the Lachnospiraceae family, a decrease in *Marvinbryantia* and *Roseburia* spp. was observed in mice without a cecum. Little is known about the functional capacity of these bacteria individually; however, bacteria within these groups have been found to be positively correlated with SCFA-associated improvement in health outcomes including reduced severity of disease in Crohn's disease patients¹⁵ and protection from colorectal cancer in rats.¹⁶ In concert with the observation of reduced abundances of SCFA-producing taxa, we found that prominent SCFAs in feces, including acetic, propionic, butyric, isobutyric, isovaleric, and valeric acids were markedly lower in mice with excised cecums. Significant decreases in concentration of acetic,

propionic, and butyric acid in the colon of cecectomized mice were noted in a previous cecectomy study with a similar trend of reduction in isobutyric, isovaleric, and valeric acids.⁸ We observed that the concentration of caproic acid was unaffected by cecum removal. Short chain fatty acids are thought to provide several beneficial effects on host tissues within the intestine

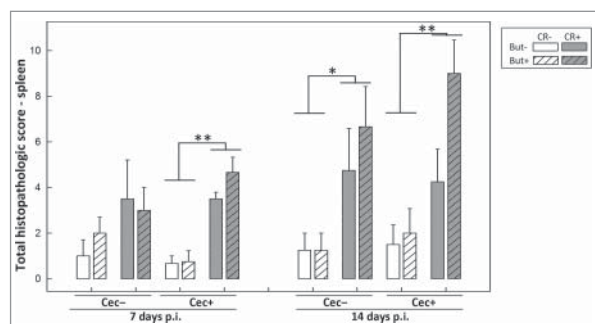


Figure 10. Histopathological scores of spleen tissues from mice that received sham (Cec-) or cecectomy (Cec+) surgeries, were orally administered PBS (CR-) or *Citrobacter rodentium* (CR+), and received enemas containing PBS (But-) or butyrate (But+). Data is shown 7 and 14 days after administration of *C. rodentium* or PBS. Vertical lines associated with histogram bars represent standard error of the means ($n =$ three to five mice/treatment). * $P \leq 0.050$, ** $P \leq 0.010$. The maximum possible score is 12.

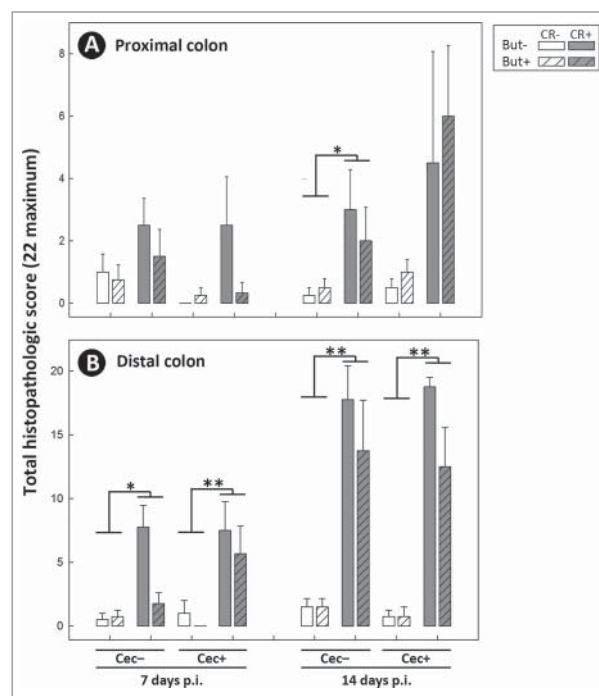


Figure 11. Histopathological scores of tissue sections from the (A) proximal and (B) distal colon of mice that received sham (Cec-) or cecectomy (Cec+) surgeries, were orally administered PBS (CR-) or *Citrobacter rodentium* (CR+), and received enemas containing PBS (But-) or butyrate (But+). Data is shown 7 and 14 days after administration of *C. rodentium* or PBS. Vertical lines associated with histogram bars represent standard error of the means ($n =$ three to five mice/treatment). * $P \leq 0.050$, ** $P \leq 0.010$.

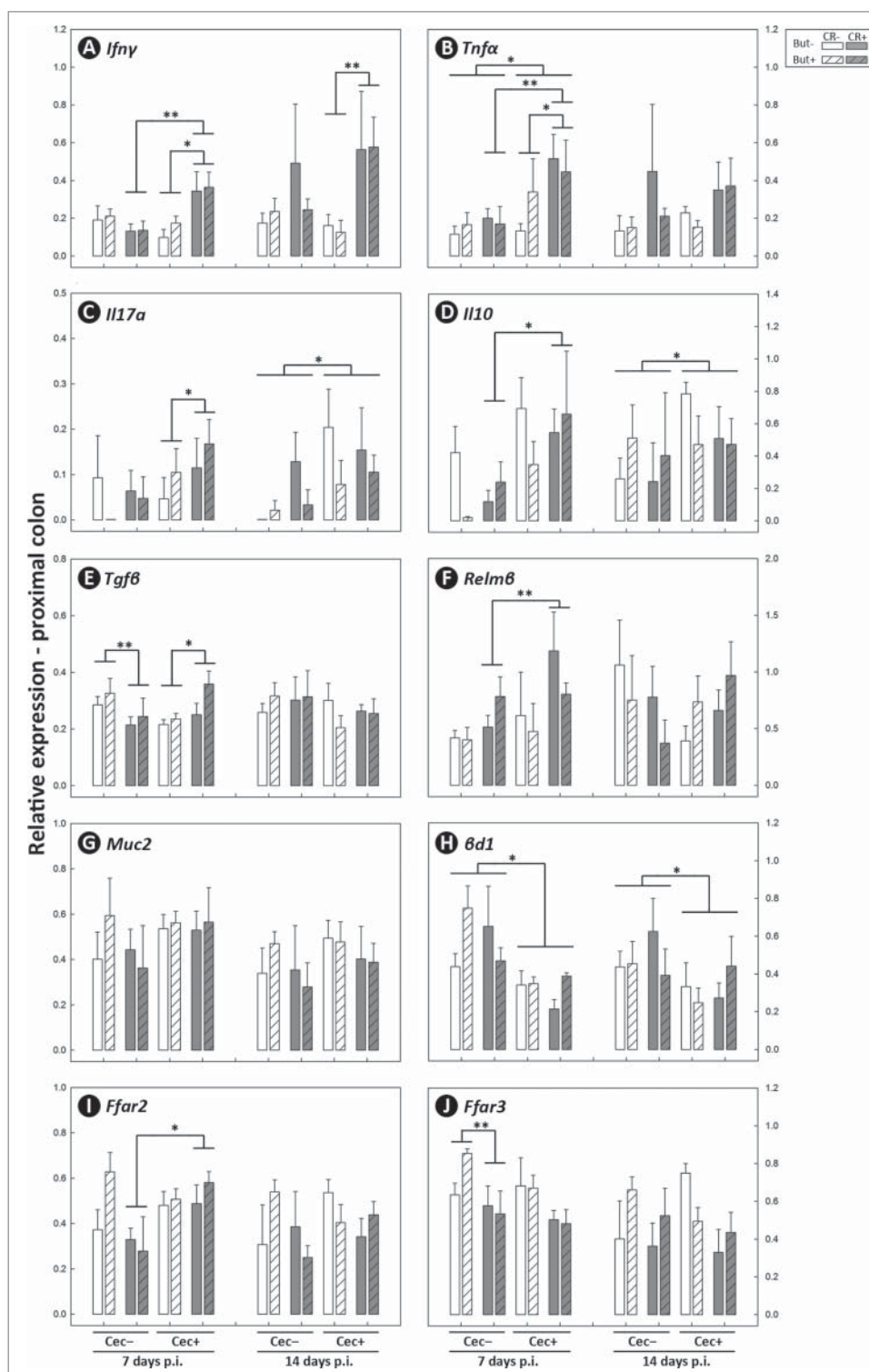


Figure 12. Expression of mRNA transcripts which code for: (A) *Ifn* γ ; (B) *Tnf* α ; (C) *Il17a*; (D) *Il10*; (E) *Tgf* β ; (F) *Relm* β ; (G) *Muc2*; (H) $\beta d1$; (I) *Ffar2*; and (J) *Ffar3* in proximal colonic tissues of mice that received sham (Cec-) or cecectomy (Cec+) surgeries, were orally administered PBS (CR-) or *Citrobacter rodentium* (CR+), and received enemas containing PBS (But-) or butyrate (But+). Data is shown 7 and 14 days after administration of *C. rodentium* or PBS (p.i.). Vertical lines associated with histogram bars represent standard error of the means (n = three to five mice/treatment). * $P \leq 0.050$, ** $P \leq 0.010$.

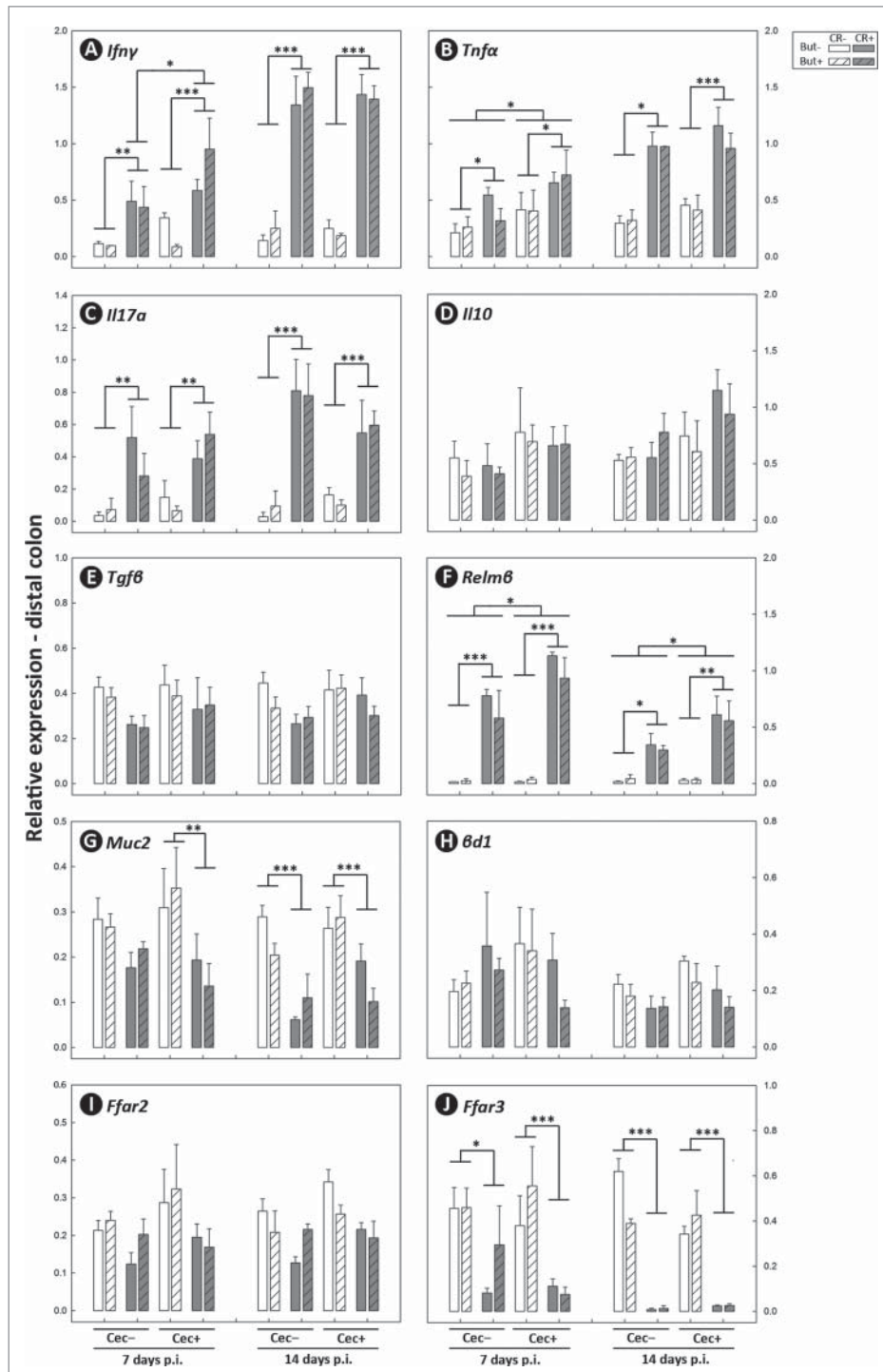


Figure 13. Expression of mRNA transcripts which code for: (A) *Ifny*; (B) *Tnfα*; (C) *Il17a*; (D) *Il10*; (E) *Tgfβ*; (F) *Relmβ*; (G) *Muc2*; (H) *βd1*; (I) *Ffar2*; and (J) *Ffar3* in distal colonic tissues of mice that received sham (Cec-) or cecectomy (Cec+) surgeries, were orally administered PBS (CR-) or *Citrobacter rodentium* (CR+), and received enemas containing PBS (But-) or butyrate (But+). Data is shown 7 and 14 days after administration of *C. rodentium* or PBS (p.i.). Vertical lines associated with histogram bars represent standard error of the means (n = three to five mice/treatment). * $P < 0.050$, ** $P < 0.010$, *** $P < 0.001$.

and in extra-intestinal tissues of the body.¹⁷ For example, higher concentrations of SCFAs decrease luminal pH, which inhibits the growth of pathogenic organisms and increases nutrient absorption.¹⁸ Furthermore,

butyrate is an energy source for colonocytes, and this SCFA may enhance intestinal health by increasing mucin production¹⁹ and improve epithelial integrity.²⁰ Additionally, decreased abundance of SCFA-producing

bacteria and lower concentrations of fecal SCFA have been observed in individuals with inflammatory diseases of the intestine.²¹ Given our expanding knowledge on the role of individual SCFAs on intestinal health,²² the information obtained in the current study supports the emerging hypothesis that the murine cecum is an essential organ for maintaining a diverse and physiologically beneficial microbiota in the colon. Furthermore, the cecectomy model may prove useful to elucidate other key aspects of the microbiota-host interactions not measured in the current investigation (e.g. colonization resistance) in mice and other monogastric mammals (e.g. pigs).

To determine how these cecum-derived changes in the intestinal environment alter host responses to enteric stress, we incited intestinal inflammation with the murine pathogen *C. rodentium*. It has been reported that lymphoid tissue within the cecum (i.e. the cecal patch) is the location within the intestinal tract where *C. rodentium* initially colonizes during infection.²³ We observed that the densities of live *C. rodentium* in feces was the same in mice that received cecectomy and sham surgeries and thus, our data indicates that the cecum is not required for the initiation of *C. rodentium* infection. Despite equivalent patterns of colonization with *C. rodentium* within the colon, differences were noted in the colonic responses to infection by *C. rodentium* in cecectomized mice. Mice with an excised cecum failed to recover from *C. rodentium* infection, as underscored by an inability to return to normal body weight at later stages of infection. In general, non-surgically manipulated mice infected by *C. rodentium* commenced gaining weight by the peak to late stages of colitis.²⁴ In contrast, we observed that infected mice without a cecum weighed less at late infection than they did before inoculation with *C. rodentium*. These observations suggest that the cecum or cecum-derived microbiota is directly or indirectly important to the ability of mice to recover from *C. rodentium* infection.

To assess whether removal of the cecum alters immune and inflammatory responses to infection with *C. rodentium*, intestinal tissues were examined for histopathological changes as well as molecular markers of intestinal health. As well, effects on the bacterial community structure were examined. Histopathological effects of *C. rodentium* infection were similar in both the proximal and distal colon; however, several molecular markers of inflammation

incited by the pathogen were differentially expressed in cecectomized mice. In general, removal of the cecum had a greater effect on tissues harvested from the proximal colon, as there was a decrease in expression of defensins (β *dl*), and an increase in expression of both pro-inflammatory (*Tnfa* and *Ifn γ*) and anti-inflammatory (*Il10*) genes. There was also a more pronounced response to *C. rodentium* inoculation in cecectomized mice as indicated by increased expression of immune regulators, *Relm β* , *Ifn γ* , and *Tgf β* , and the free fatty acid receptor, *Ffar2*. Although colitis incited by *C. rodentium* did not appreciably affect bacterial community composition in the colon, *Clostridium* cluster XIVb, *Anaerotruncus*, *Oscillibacter* spp. may have been differentially affected as a result of colitis in cecectomized or sham surgery mice. There are some reports of these taxa being affected by enteric inflammation or barrier function,²⁵⁻²⁹ but little is currently known about their impacts on host health.

In our investigation, we used *C. rodentium* as an incitant of self-limiting colitis. In contrast, Voravuthikunchai and Lee⁸ examined the effects of *S. Enteritidis* on weight gain and mortality in normal, cecectomized, and sham mice. They observed that cecectomized mice inoculated with *S. Enteritidis* exhibited conspicuous weight loss, and possibly higher mortality (89% to 100%) relative to normal (50% to 78% mortality) and sham (67% to 83% mortality) mice. This was proposed to be due to cecectomy-induced dysbiosis of the colonic microbiota, characterized by an increase in coliforms, and a decrease in fusiform bacteria and total anaerobes. However, their characterizations of the colonic microbiota relied on culture-based enumeration methods, and beyond weight gain and mortality the authors did not report any indicators of disease. Moreover, *S. Enteritidis* incites typhoid-like systemic disease in mice with high rates of mortality.⁹ Thus, this model is of limited value to study physiologic mechanisms of enteritis or colonization resistance within the intestine of mice. In this regard, *S. enterica* serovar Typhimurium would be a better choice since this pathogen is capable of inciting enterocolitis in mice with similar pathology to that observed in human beings.^{30,31} However, the current murine model of enteritis incited by *S. Typhimurium* requires pre-treatment of the animals with streptomycin,³¹ which is a broad-spectrum antibiotic. Antibiotics, including streptomycin, can impart both direct and indirect impacts on the host,^{32,33} and it is possible that the murine cecectomy model will

prove to be a useful alternative to the streptomycin dysbiosis model to explore the mechanisms of pathogenesis of enteric salmonellosis and colonization resistance. This may prove particularly useful considering emerging evidence which indicates that several classes of antibiotics modulate inflammatory responses in host tissues within the intestine.^{34,35}

To determine if of butyrate could compensate for the loss of endogenous production of this SCFA by the enteric microbiota in cecectomized mice, butyrate was administered to mice by colonic infusion. Bacteria-derived butyrate is known to promote the differentiation of T regulatory cells in mice,^{36,37} and direct administration of butyrate to the intestine has shown promising beneficial results in some clinical trials. For example, 2 week treatment with 100 mmol/L sodium butyrate decreased symptoms of ulcerative colitis,³⁸ although these results have proved inconsistent and difficult to replicate,³⁹ potentially due to variability in intestinal transit time and inconsistency in contact time of butyrate with the intestinal mucosa. Moreover, Jiminez et al.⁵ showed that colonic administration of butyrate increased feed consumption and weight gain, ameliorated *C. rodentium*-induced cell injury via enhanced mucus production and tissue repair mechanisms (i.e. *Relmβ*, *Tff3*, *Myd88*), and increased the abundance of butyrate-producing bacteria in mice with enteritis. Furthermore, butyrate concentrations measured in feces from mice with enteritis were higher than control mice⁵ suggesting that the SCFA was more rapidly absorbed by colonocytes of animals without enteritis.^{40,41} In contrast to the above studies, we found that colonic administration of butyrate at a concentration of 100 mM had no effect on the structure of the bacterial communities in the colon, nor did it increase the concentration of butyric acid in the feces or reduce the severity of colitis in *C. rodentium* challenged mice. Concentrations of total SCFAs are higher in the cecum (131 ± 9 mmol/kg), but similar in the descending colon (80 ± 11 mmol/kg) and feces (77.6 ± 4.5 mmol/kg) of human beings,^{42,43} with similar trends observed in mice.⁵ Although we did not measure SCFAs in ingesta or tissues within the colon, as it requires the euthanization of additional mice, our results suggest that direct administration of SCFAs to the colon using a rigid gavage needle may not be the most effective technique to deliver butyrate to the colon. Others have administered

butyrate via colonic infusions to rodents.^{44,45} However, the use of flexible infusion tubes for direct colonic delivery of butyrate may be a superior strategy in future studies in mice. Alternatively, *per os* or intra-gastric administration of butyrate could be used.^{46,47} We chose to deliver butyrate directly to the colon to ensure equal targeting between cecectomized and sham treatment mice; however, effective and equal passage of butyrate through the proximal alimentary canal to the colon, especially in mice \pm a cecum may be problematic. The *per os* administration of phenylalanine-butyramide to overcome the poor palatability of sodium butyrate is an option in subsequent studies.⁴⁸

In conclusion, we observed that the removal of the murine cecum disrupted bacteria fermentation as evidenced by greatly reduced SCFA production. Furthermore, cecal removal resulted in a conspicuous dysbiosis in the colonic microbiota, and acute inflammation incited by *C. rodentium* was enhanced in cecectomized mice. However, the administration of butyrate directly to the colon did not ameliorate inflammation. With respect to our hypothesis, we showed that the removal of the cecum decreased richness of the 'commensal' colonic bacterial community and resulted in shifts in bacterial communities including decreased SCFA-associated bacteria. While the changes in SCFA were insufficient to modulate enteric inflammation resulting from a *C. rodentium* infection, our study indicates that the cecum in mice, and possibly other mammals, is a critical organ for maintaining bacterial diversity and SCFAs in the colon. Importantly, the murine cecectomy model may prove to be an effective tool to study the impact of dysbioses and SCFAs on various aspects of intestinal physiology and host well-being, including mechanisms of colonization resistance.

Materials and methods

Ethics statement

The study was carried out in strict accordance with the recommendations specified in the Canadian Council on Animal Care Guidelines. The project was reviewed and approved by the Lethbridge Research and Development Centre (LRDC) Animal Care Committee (Animal Use Protocol Review 1423), and the LRDC Biosafety and Biosecurity Committee before commencement of the research.

Surgical protocol

The surgical procedure used to remove the cecum was described previously.⁸ Mice were anesthetized with isoflurane and placed in dorsal recumbency while receiving continuous anaesthetic. The abdomen was shaved and scrubbed twice with a chlorhexidine surgical solution, rinsed with 70% ethanol, and a final scrub of prepodyne solution was applied just prior to surgery. A surgical drape was placed on the abdomen and a 1.5 to 2.0 cm incision was made along the lower abdomen. The cecum was gently exteriorized, and a sterile barrier drape rinsed in phosphate buffered saline (PBS; pH 7.2) was placed under the cecum, the distal ileum, and the proximal colon. The cecum was ligated at the ileocecal junction, and the cecum was excised. Any remaining excess cecal tissue was trimmed. Care was taken to ensure that cecal contents were not released into the peritoneal cavity, and any residual ingesta on mucosal surfaces was irrigated with sterile PBS. The intestine was kept moist throughout the procedure and following the cecectomy, the intestine was returned into the abdominal cavity. The abdomen muscle layers were then closed with 4-0 or 5-0 Vicryl sutures and skin was closed with Michel suture clips (7.5mm x 1.75 mm). Clips were removed 7 to 10 days post-surgery. Each surgery lasted \approx 10 min. For sham control mice, the surgical procedure described is above; the cecum was exteriorized and left outside the abdominal cavity for 2 min, and the cecum was then replaced in the peritoneal cavity and the abdomen and skin closed. During surgical induction, mice were provided meloxicam and buprenorphine subcutaneously while under general anaesthesia. A second dose of buprenorphine was administered 2 to 3 hr after surgery based on the level of discomfort exhibited by individual mice. Upon recovery from anesthesia, mice were administered a subcutaneous injection of warmed saline. Meloxicam was administered once daily to all mice for 2 days post-surgery. Mice were fed a conventional low fiber diet (Prolab RMH 3500, Canadian Lab Diets, Leduc, AB), and allowed to drink *ad libitum*. The surgical incision sites were monitored daily until fully healed, and animals were examined daily for changes in body temperature and behavioural manifestations of post-surgical distress.

Experimental design and treatment administrations

The experiment was conducted as a two (cecectomization) by two (inflammation) by two (butyrate

administration) by two (sample time, 7 and 14 days p.i.) factorial experiment arranged as a completely randomized design (Figure 1). Four time-independent replicates were conducted on separate occasions including a total of 64 mice. Mice were individually housed within individually ventilated cages attached to a HEPA filter unit operated in containment mode (Techniplast, Montreal, QC). Twenty-one days after surgery, cecectomized and sham control mice were colonically administered butyrate in PBS or PBS alone. Butyric acid (Sigma-Aldrich, Oakville, ON) was diluted with PBS to attain final concentrations of 100 mM butyrate; the pH was adjusted to 7.4 ± 0.2 with sodium hydroxide. The butyrate solution was prepared the day prior to administration, and stored at 4°C until used. Solutions were warmed to room temperature (RT) for 30 min before administration. Butyrate and PBS were administered via enemas (200 μ L) at 2-day intervals for the duration of the experiment. To administer enemas, mice were anesthetized with isoflurane, inverted at a 45° angle, a 22G x 2.5 cm-long rigid gavage 'needle' with a 1.25 mm ball tip was gently inserted into the colon, the liquid was slowly injected, and mice were maintained in an inverted position for 30 sec after administration of the enema.⁵ Animals were monitored for discomfort/pain for 4 hr after the enemas were administered. On the same day of butyrate administration, mice were orally gavaged with *C. rodentium* (ATCC 51459; 2×10^9 cells/ml) suspended in PBS or PBS alone on two consecutive days according to an established protocol.²⁴

Data acquisition and tissue collection

Mice were monitored daily for changes in health status and these included changes in body temperature, food consumption, behavioral and the presence of diarrhea. Recently voided feces were collected at intervals before and after inoculation of *C. rodentium* to measure the presence of *C. rodentium* and amounts of SCFAs. Seven and 14 days p.i. (i.e. corresponding to peak and late stages of disease, respectively), animals were anaesthetized with isoflurane and humanely euthanized by cervical dislocation. The intestine, liver and spleen were aseptically harvested, and segments of the intestine and spleen were frozen at -80°C for analysis of bacterial communities and fixed in 10% neutral buffered formalin for histopathological analysis. Tissue samples (liver, ileum, cecum, proximal colon, and distal colon) were

stored in RNeasy Lysis Buffer (Sigma-Aldrich, Oakville, ON) for quantitative PCR analysis.

Enumeration of *C. rodentium*

Densities of *C. rodentium* cells were determined by homogenizing fecal or colonic mucosal samples in Columbia broth (Oxoid, Nepean, ON), and spreading serial dilutions of the homogenate onto MacConkey agar (Becton, Dickinson and Company, Mississauga, ON). Cultures were incubated overnight at 37°C, enumerated at the dilution yielding 30–300 colony forming units per culture, and adjusted by initial sample weight.

Histopathological scoring

Tissue samples from the cecum, proximal colon, distal colon, and spleen were fixed in 10% buffered formalin for 12–24 hr, dehydrated, embedded in paraffin, sectioned ($\approx 4 \mu\text{m}$ thick), and stained with hematoxylin and eosin (H&E) according to a standard procedure.²⁴ Scoring of tissues for histopathologic changes was performed by a veterinary pathologist (RREU) blinded to the treatments, with scoring criteria adapted from previously described methods.²⁴ Briefly, colonic sections were graded 0 to 4 for epithelial cell hyperplasia, crypt height, epithelial injury, extent of inflammatory infiltrates, and 0 to 3 for mitotic activity of epithelial cells, and goblet cell depletion. Spleen tissues were graded 0 to 3 for germinal centre number, germinal centre size, cell infiltration, and apoptotic and necrotic cells. The total histopathologic score was obtained by calculating the sum of scores for all categories for each mouse; the maximum total scores were 22 and 12 for colonic and splenic tissues, respectively.

Quantification of mRNA expression

Total RNA was extracted from proximal and distal colon sections using the RNeasy Mini Kit (Qiagen Inc., Toronto, ON) according to manufacturer's instructions with the addition of a DNase step to remove genomic DNA. RNA quality and quantity was determined using Bioanalyzer RNA 6000 Nano kit (Agilent, Mississauga, ON) and cDNA was reverse transcribed using Quantitect Reverse Transcription Kit (Qiagen Inc.). Quantitative PCR was performed using Quantitect SYBR Green Master Mix (Qiagen Inc.) with primers specific to genes encoding cytokines, chemokines, defensins, and mucins. Relative

expression was calculated using qBase software (Biogazelle, Gent, Belgium) relative to reference genes Peptidylprolyl isomerase A, hypoxanthine-guanine phosphoribosyltransferase, and beta-glucuronidase.

Analysis of bacterial communities

DNA from mucosal plugs (3-mm in diameter) from the proximal and distal colon of mice in the 14 days after administration of *C. rodentium* or PBS was extracted using DNeasy Tissue Kit (Qiagen Inc.) according to manufacturer's instructions. Standard protocols for library preparation were used according to the manufacturer's recommendations (Illumina Canada, Victoria, BC), including primers which flank the V3/V4 region.⁴⁹ Sequencing was performed on a MiSeq instrument (Illumina Canada) using a MiSeq Reagent Kit v3 (600-cycle) (Illumina Inc.). Primer removal and quality trimming was performed in Cutadapt⁵⁰ with a threshold quality score of 18, and analysis of the sequencing reads was performed using dada2⁵¹ and phyloseq⁵² packages within R.⁵³ Within dada2, forward reads were trimmed to 240 base pairs and reverse reads to 210 base pairs, dereplicated sequences were merged, and chimeras identified and removed using the removeBimeraDenovo function. Taxonomy was assigned based on the RDP database and a neighbor joining phylogenetic tree was generated using the Phangorn package.⁵⁴ Sequence depth varied between ≈ 2000 to $\approx 50,000$ sequences (Table S1) and depth was rarefied to 9000 sequences per sample which captured bacterial diversity within the samples (Figure S5). Within sample diversity was calculated using Shannon, Simpson, and Inverse Simpson metrics. Beta diversity was calculated using the weighted UNIFRAC metric⁵⁵ and differences among groups was identified using permutational analysis of variance (PERMANOVA).⁵⁶

Short chain fatty acid quantification

Fecal samples were collected, weighed, and homogenized in PBS (1 mg/ml). Meta-phosphoric acid (Sigma Aldrich, Oakville, ON) was added to the homogenate at a concentration of 20% v/w and samples were incubated at room temperature (RT) for 30 min. Samples were centrifuged at RT for 75 min at 16000 x g, and the supernatants were collected and stored at -20°C until further processing. Concentrations of total SCFAs, acetic acid, butyric acid, isobutyric acid,

valeric acid, isovaleric acid, propionic acid, and caproic acid were quantified using a gas chromatograph (Agilent Technologies, Model 6890N with 7683 Series Injector) according to established protocols.^{57,58}

Fluorescence in situ hybridization

Formalin fixed and paraffin embedded colonic tissue sections were deparaffinized with xylene and subsequently rehydrated. An Alexa-fluor 594 labelled probe for Gammaproteobacteria (GAM42a: 5'-GCC TTC CCA CAT CGT TT-3') and an Alexa-fluor labelled 488 probe for Eubacteria (EUB338: 5'-GCT GCC TCC CGT AGG AGT-3') prepared at a concentration of 2.5 ng/ μ l in hybridization solution (52.6 g NaCl, 12.2 g Trizma base, 300 mL formamide, 1 g SDS in 1 L; pH 7.2) was incubated with the tissues at 37°C for \approx 18 hr in the dark. Slides were washed for 15 min in hybridization solution, then washed for 15 min in a wash buffer (52.6 g NaCl, 12.1 g Tris base in 1 L; pH 7.2) then placed in deionized water. Slides were mounted with Fluoroshield Tm with DAPI (Sigma Aldrich) and visualized and images using a T81X confocal microscope (Olympus Canada Inc., Toronto, ON).

Statistical analysis

All parametric statistical analyses were performed using Statistical Analysis Software (SAS Institute Inc. Cary, NC). Continuous data was checked for normality and analyzed using the MIXED procedure of SAS. Where applicable (i.e. fecal *C. rodentium* densities and body weights), collection time was treated as a repeated measure; the appropriate covariance structure was utilized according to the lowest Akaike's Information criterion. In the event of a main treatment event effect ($P \leq 0.050$), the least squares means test was used to compare treatments within factors. Histopathologic measurement data were analysed using the Chi squared (NPAR1WAY) procedure of SAS. Data is represented by mean \pm standard error of the means (SEM), and asterisks represent the following probability values: * $P \leq 0.050$; ** $P \leq 0.010$; and *** $P \leq 0.001$.

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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Authors' contributions

G.D.I. conceived the research; K.B., R.R.E.U. and G.D.I. designed the research; G.D.I. obtained ethics approvals; K.B., R.R.E.U., and G.D.I. performed experiments; K.B. and G.D.I. analyzed data; K.B., R.R.E.U. and G.D.I. interpreted results of experiments; K.B. and G.D.I. prepared figures; K.B. and G.D.I. generated the initial draft of the manuscript; K.B., D.W.A., R.R.E.U. and G.D.I. edited the manuscript and approved final version of manuscript

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