Butyrate Protects Mice from Clostridium difficile-Induced Colitis through an HIF-1-Dependent Mechanism

Graphical Abstract

Highlights
- Butyrate mitigates the pathogenicity of colitis induced by Clostridium difficile
- Butyrate increases expression of tight junctions in epithelial cells
- Butyrate reduces intestinal inflammation and bacterial translocation
- HIF-1 stabilization is necessary to the protection induced by butyrate

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In Brief
Fachi et al. demonstrate that butyrate is able to protect the intestinal epithelium from the damage caused by Clostridium difficile toxins by stabilizing HIF-1 and increasing tight junctions, which reduces intestinal epithelial permeability, thus inhibiting intestinal inflammation and bacterial translocation.
Butyrate Protects Mice from Clostridium difficile-Induced Colitis through an HIF-1-Dependent Mechanism

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SUMMARY

Antibiotic-induced dysbiosis is a key factor predisposing intestinal infection by Clostridium difficile. Here, we show that interventions that restore butyrate intestinal levels mitigate clinical and pathological features of C. difficile-induced colitis. Butyrate has no effect on C. difficile colonization or toxin production. However, it attenuates intestinal inflammation and improves intestinal barrier function in infected mice, as shown by reduced intestinal epithelial permeability and bacterial translocation, effects associated with the increased expression of components of intestinal epithelial cell tight junctions. Activation of the transcription factor HIF-1 in intestinal epithelial cells exerts a protective effect in C. difficile-induced colitis, and it is required for butyrate effects. We conclude that butyrate protects intestinal epithelial cells from damage caused by C. difficile toxins via the stabilization of HIF-1, mitigating local inflammatory response and systemic consequences of the infection.

INTRODUCTION

Clostridium difficile is a Gram-positive anaerobic bacillus that forms spores, conferring resistance to environmental factors and allowing persistence for several months on surfaces and food and in water. This bacterium is a common cause of intestinal infection, which mainly affects hospitalized patients, and is recognized as a serious public health problem (Martin et al., 2016). A continuous increase in the incidence, morbidity, and mortality of patients infected with C. difficile has been seen in recent decades (Martin et al., 2016). The development of clinical disease most commonly occurs after the ingestion of spores by a susceptible host. A major risk factor for C. difficile infection (CDI) is antibiotic use, which induces changes in indigenous intestinal microbiota, impairing resistance (Chen et al., 2008; Lamont and Hajishengallis, 2015; Rodriguez et al., 2015). Additional risk factors include age, immunological state, and comorbidities (Lamont and Hajishengallis, 2015; Rea et al., 2011).

CDI presents symptoms ranging from mild to moderate nonbloody diarrhea and intestinal discomfort to severe forms with intense diarrhea and abdominal pain, pseudomembranous colitis, and more serious complications such as toxic megacolon, peritonitis, respiratory distress, sepsis, and death (Johanesen et al., 2015; Lamont and Hajishengallis, 2015). These symptoms are...
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**A**

![Graph showing mean clinical score over days post-infection for Control and Butyrate conditions.](image)

**B**

![Graph showing body weight variation over days post-infection for Control and Butyrate conditions.](image)

**C**

![Bar graph showing histological score for Control and Butyrate at 2 and 4 d.p.i.](image)

**D**

![Images showing histological sections for Abx, Control, and Butyrate conditions.](image)

**E**

![Graph showing mean clinical score over days post-infection for Placebo and Tributyrin conditions.](image)

**F**

![Graph showing body weight variation over days post-infection for Placebo and Tributyrin conditions.](image)

**G**

![Graph showing mean clinical score over days post-infection for Control Diet and Insulin 25%.](image)

**H**

![Graph showing body weight variation over days post-infection for Control Diet and Insulin 25%.](image)

**I**

![Bar graph showing SCFA (mg/g) levels for different conditions.](image)

**J**

![Bar graph showing SCFA (mg/g) levels for different conditions.](image)

*(legend on next page)*
associated with the actions of the two major C. difficile virulence factors, toxin A (TcdA) and toxin B (TcdB) (Kuehne et al., 2010; Rodriguez et al., 2015). These toxins glycosylate Rho family proteins, keeping them in an inactive form and thus affecting downstream pathways, including cytoskeleton organization. The cytoskeletal effects lead to the disruption of the cellular actin structure and the death of intestinal epithelial cells (IECs), resulting in the loss of barrier function and, consequently, a profound inflammatory response (Cohen et al., 2011; Rodriguez et al., 2015).

The intestinal microbiota confers resistance against CDI. Studies conducted in humans and mice have shown that gut microbiota prevent intestinal colonization by C. difficile (Buffie et al., 2015; Stecher and Hardt, 2008; Theriot et al., 2014; van Nood et al., 2013) and that fecal microbiota transplant is an effective treatment for recurrent CDI (van Nood et al., 2013). The transfer of specific components from microbiota, such as secondary bile salt metabolites, has been found to confer resistance against CDI in mice (Buffie et al., 2015). However, the mechanisms involved in microbiota protection are not well understood.

A major link between microbiota and host cells is the production of short-chain fatty acids (SCFAs) through bacterial metabolism. These molecules (mainly acetate, propionate, and butyrate) are released into the intestinal lumen from fiber metabolization and absorbed by IECs. SCFAs have several immunomodulatory effects (Correa-Oliveira et al., 2016). Strategies that increase their intestinal concentrations are effective in reducing tissue damage and increasing immune system effector mechanisms. In this way, SCFAs improve the host response to inflammatory and infectious stimuli (Galvão et al., 2018; Kim et al., 2013; Maslowski et al., 2009; Vieira et al., 2017). Recent reports have noted that SCFA-producing bacteria are depleted and that SCFA concentrations are significantly reduced in the intestines of CDI patients (Antharam et al., 2013). In addition, CDI-susceptible mice have lower intestinal concentrations of SCFAs compared to CDI-resistant mice (Theriot et al., 2014). Here, we aimed to investigate the impact of administering the SCFA butyrate on an acute CDI mouse model.

RESULTS

Microbiota Changes after Antibiotic Treatment Affect SCFA Production

Samples were collected from mice that were either resistant (before antibiotic treatment) or susceptible to C. difficile infection (day 0). In addition, we collected samples from mice recovering from antibiotic treatment (day 6). A reduction in fecal bacterial load in CDI-susceptible mice compared to the other groups was observed (Figure S1E). After antibiotic treatment, mice showed a reduction in microbial diversity, found by comparing rarefaction curves with those from mice before antibiotic treatment. Microbial diversity partially recovered 7 days after clindamycin treatment (not shown). At the phylum level, we observed a reduction in Bacteroidetes (e.g., Bacteroides, Alstipes) and a less evident reduction in Firmicutes (e.g., Clostridium cluster XIVa, Lachnospiraceae) in mice after antibiotic treatment. A relative increment of Proteobacteria (e.g., components of the Enterobacteriaceae family, Parasutterella) and Verrucomicrobia (e.g., Akkermansia) was seen in antibiotic-treated mice (Figures S1A–S1D). These effects were accompanied by marked changes in SCFA production in the colon (Figure S1F). Five days after clindamycin administration (day 4), concentrations of SCFAs (apart from butyrate) were similar to those before antibiotic treatment (Figure S1F). Thus, butyrate production was persistently compromised, even though microbiota composition partially recovered after antibiotic treatment.

Oral Administration of Butyrate, Tributyrin, and Inulin Diet Protects against CDI

The addition of 150 mM butyrate to the drinking water of mice resulted in a protective effect against CDI. An improvement in clinical and colon histological scores was observed in butyrate-treated mice (Figures 1A–1C). A histological examination of the colon at the peak of the infection (day 2) revealed epithelial damage, moderate depletion of goblet cells, and evident mitosis at the intestinal crypts. Extensive infiltration of inflammatory cells (mainly granulocyte neutrophils) was observed in colonic lamina propria (LP) and submucosa in infected mice as compared to uninfected mice (Figure 1D). The changes were less evident on day 4 (Figure 1C). Butyrate-treated mice showed improved parameters, particularly epithelial ulceration and accumulation of cells in the LP and submucosa area (Figure 1D), at the peak of the infection. Administration of a pro-drug of butyrate, tributyrin, or an inulin-rich diet had the same effect on CDI, as both conditions resulted in protection of the mice (Figures 1E–1H). Both strategies increased butyrate intestinal concentrations in antibiotic-treated mice (Figures 1I and 1J). The inulin-rich diet also increased acetate and propionate concentrations, which may be relevant for the effect of the diet (Figure 1J).

Figure 1. Oral Administration of Butyrate, Tributyrin, and Inulin Diets Protects Mice against CDI

Mice were treated with antibiotic mixture for 4 days and then received a single dose of clindamycin. After 1 day, mice were infected with C. difficile (day 0). Mice received 150 mM butyrate during the entire protocol. (A and B) Mice were clinically monitored (A) and weighed (B) until day 6 after infection (n = 10). (C) Histological score of mice ± treatment with butyrate (n = 5). (D) Representative colon histological sections of antibiotic (Abx)-treated mice and C. difficile-infected mice at day 2 post-infection ± butyrate treatment. Arrows and asterisks in the sections indicate major histopathological differences between groups: polymorphonuclear infiltration of the LP and submucosa (white arrows), epithelial damage (black arrow), reduction of goblet cells (asterisks), and hyperplasia (red arrows). Upper scale bar: 100 μm, lower scale bar: 50 μm. (E–J) Mice treated with placebo (PBS) or tributyrin and infected with C. difficile were clinically monitored (E) and weighed (F) until day 5 after infection (n = 5). Mice treated with control or inulin-supplemented diet for 7 days and infected. They were clinically monitored (G) and weighed (H) until day 5 post-infection (n = 5). Results are means ± SEMs. Measurement of SCFA concentrations in the colon content of Abx-treated mice supplemented with tributyrin (I). Samples were collected after 30 or 120 min of tributyrin administration. Results are presented as means ± SEMs (n = 2–3). Measurement of SCFA concentrations in the fecal samples of mice treated with control or inulin-supplemented diet (J). Samples were collected before Abx treatment (day –7) and at the infection day (day 0). Results are presented as means ± SEMs (n = 5). nd, not detected.

*p < 0.05 compared to control. See also Figure S1.
Butyrate Does Not Interfere in C. difficile Colonization or Toxin Production

Since a recent study found that the consumption of a fiber-enriched diet modified gut microbiota, C. difficile burdens, and toxin production (Hryckowian et al., 2018), we tested the effect of butyrate on the microbiota composition of infected mice, C. difficile colonization, and toxin production. The administration of butyrate affected the overall microbiota community structure before infection (day 0, p = 0.01; analysis of similarities), but it did not affect specific phyla/genera (q > 0.05) or bacterial richness (Figures S2A–S2C). Butyrate still had a protective effect in germ-free mice infected with C. difficile, indicating that at least part of its effect is independent of microbiota changes (Figure S2D).

Next, we incubated bacteria with different butyrate concentrations and found that this limited growth of C. difficile only at a high concentration (50 mM) (Figure S2E) and that 10 and 50 mM butyrate increased the production of TcdA and TcdB (Figure S2F) due to cytotoxic and sporulation effects. However, butyrate treatment did not affect C. difficile colonization or toxin production in specific pathogen-free or germ-free mice (Figures S2G–S2J), indicating that the beneficial effects did not involve a direct effect on C. difficile burdens or toxin production. Tributyrin treatment also did not affect C. difficile colonization in mice (data not shown).

Intestinal Inflammation Is Attenuated by Butyrate, but It Is Not Required for Protective Effect in CDI

Consistent with the findings above, butyrate administration reduced the levels of pro-inflammatory cytokines (interleukin 6 [IL-6], IL-1β, and chemokine ligand 1 [Cxcl-1]) and increased the anti-inflammatory cytokine IL-10 in the colon at the peak of infection (Figure 2A). We next found that butyrate treatment increased the expression of Il-10 and Foxp-3 transcripts in colon and mesenteric lymph nodes (mLNs) (Figures 2B and 2C), accompanied by changes in the expression of several cytokines in these tissues (Figures S3A and S3B) indicating an effect on regulatory T [Treg] cells. No effect of butyrate on epithelial permeability in antibiotic-treated mice was observed (Figure S3C).

Previous studies showed that butyrate increases Treg numbers and function in the colon (Arpaia et al., 2013; Smith et al., 2013). Our results in Foxp-3 GFP mice treated with antibiotics and supplemented with butyrate corroborated these data (Figure 2D). We also found increased CD103+ dendritic cells in mLNs of mice treated with antibiotics and supplemented with butyrate compared to mice receiving antibiotic treatment only (Figures 2E and S3H–S3J). To gain insight into the role of T cells and the anti-inflammatory cytokine IL-10, we repeated the experiment in Rag1- and IL-10-deficient mice (Rag1–/– and Il10–/–). In both strains, butyrate protected against CDI (Figures 2F, 2G, S4A, and S4B), indicating that part of the effect of butyrate in this model is independent of the actions on T cells or IL-10.

Butyrate Reduces Intestinal Permeability and Microbial Translocation

As bacterial translocation after CDI damage in the intestinal barrier causes systemic inflammatory response and contributes to disease severity (Hasegawa et al., 2014), we tested the effect of butyrate on intestinal permeability in infected mice. Analyses of bacteria translocation to peripheral organs (livers, spleens, and mLNs) on day 2 after infection showed a high proportion of mice with bacteria present in these organs (Figures 3A–3D). Oral treatment with butyrate significantly reduced the colony-forming units (CFUs) in the liver (Figure 3A). We also found a higher proportion of livers that were positive for bacteria in control mice compared to those from butyrate-treated mice (54% versus 23% and 69% versus 54% under anaerobic and aerobic conditions, respectively). A similar pattern was observed for the spleen (Figures S4C and S4D). These results were confirmed by bacterial 16S rDNA qPCR (Figure 3D). Fluorescence in situ hybridization (FISH) analysis showed that butyrate-treated mice had a lower depletion of mucus and translocation of bacteria (Figure 3F). We also observed increased space between microbiota and IECs (Figure 3F). Next, we tested the effect of butyrate on intestinal epithelial permeability in CDI mice by fluorescein isothiocyanate (FITC)-dextran gavage on day 2 post-infection, with a measurement of translocation 4 h later. We consistently found a significant reduction in FITC-dextran translocation in butyrate-treated mice (Figure 3E). This was also observed in Rag-1-deficient mice (Figure S4B). Intestinal permeability, measured by bacteria and FITC-dextran translocation, was not different in uninfected mice (antibiotic + butyrate).

We found that butyrate treatment of infected mice increased the expression of genes associated with paracellular junction proteins, including Claudin-1 and Occludin (Figure S4E). The idea that butyrate improved the intestinal barrier was also corroborated by data obtained by immunostaining claudin-1, an important protein for maintaining epithelial integrity. This protein was increased in butyrate-treated mice as compared to mice receiving antibiotic treatment only (Figures 3G and 3H). These results indicate that butyrate acts directly on the intestinal barrier, and this may be relevant for the protection observed in the CDI model.

Activation of HIF-1 in IECs Is Required for Butyrate Effects

To investigate the direct effects of butyrate on IECs, we used HCT116 cells. The cytotoxic effect of C. difficile supernatant (containing high concentrations of TcdA/TcdB) was tested in vitro. Colon cells exposed to C. difficile supernatant showed reduced transepithelial electrical resistance (TEER), and butyrate partly prevented this effect (Figure 4A). Butyrate also attenuated the impact of C. difficile supernatant on viability and monolayer reduction (Figures 4B, 4C, and S4F).

Recent studies showed that butyrate stabilizes hypoxia-inducible factor 1α (HIF-1α) in IECs, an effect that is relevant to intestinal barrier integrity (Kelly et al., 2015; Rivera-Chávez et al., 2016). The expression of HIF-1 targets and genes related to the paracellular junction was higher in cells incubated with butyrate compared to controls (Figure 4D). In agreement with the idea that HIF-1 stabilization has a role in the effects, we found that HCT116 cells incubated with the HIF stabilizer BAY 85-3934 were more resistant to C. difficile supernatant-induced death (Figure 4E).

Oral administration of butyrate to infected mice increased the levels of HIF-1α mRNA and its target genes, including...
cathelicidin antimicrobial peptide (Camp), vascular endothelial growth factor (Vegfa), and trefoil factor 3 (Tff3) in the colons of infected mice (Figures 5A and 5B). Corroborating these data, we observed increased HIF-1α in the colon after butyrate administration to oxygen-dependent degradation domain (ODD)-luciferase mice (Figure 5C). We next tested a potential role for HIF-1 in the butyrate effect on CDI using Hif1aIEC mice. Co-housed villin-Cre+ (Hif1aIEC-KO) and Cre- (Hif1aIEC-WT) littermates from Hif1a flox/flox crosses were used in the experiments. Using these mice, we found that the effect of butyrate on intestinal permeability was lost, as shown by the absence of differences in FITC-dextran translocation between HIF-1-deficient mice ± treatment with butyrate (Figure 5D). This was corroborated by the finding that butyrate-treated deficient mice showed no changes in the translocation of bacteria compared to their controls (Figure 5E).

Figure 2. Intestinal Inflammation Is Attenuated by Butyrate, but It Is Not Required for Protective Effect in CDI

(A) Quantification of cytokines in the colons of C. difficile-infected mice ± butyrate treatment. Results were normalized by tissue weight (n = 4–5). Dashed line indicates the mean value obtained with samples from non-infected mice.

(B and C) qPCR analysis of Il10 (B) and Foxp3 (C) transcripts in colons and mLNs (n = 8). Results were normalized by values obtained with samples from non-infected mice and are presented as means ± SEMs.

(D) Percentage of Treg cells (TCRβ+CD4+FoxP3+) in colonic LP.

(E) Percentage of dendritic cells (CD11c+MHCII+CD103+) in mLNs. NC, control mice with no treatment; Abx, mice treated with antibiotics for 4 days; Abx + Bt, mice that received antibiotics and 150 mM butyrate for 4 days (n = 4–6).

(F) Clinical score of Rag1-deficient mice infected with C. difficile ± butyrate (n = 8).

(G) Clinical score of Il10-deficient mice infected with C. difficile ± butyrate (n = 9–10).

*p < 0.05 compared to control.
encoded by this gene plays a major role in the ubiquitination and degradation of HIF-β subunit of the hypoxia-inducible factor. Therefore, the deletion of Vhl results in the constitutive activation of HIF. Further corroborating the idea that butyrate acted through HIF-1, we found that the treatment of VhlIEC mice did not have a significant effect on clinical parameters after infection with C. difficile (Figures S5A and S5B).

Histologically, we observed that butyrate-treated Hif1aIEC presented increased numbers of infiltrating cells in the colonic LP and submucosa compared to their controls treated with butyrate (not shown). We next isolated IECs from butyrate-treated Hif1aIEC and their controls (WT) and compared their transcriptomes to identify the possible mechanisms behind the increased susceptibility to CDI. We observed that in the absence of HIF-1 signaling, 620 genes were downregulated and 460 were upregulated (p < 0.05; Figure 5H). To explore the functional annotation and pathway enrichments of DEGs (differentially expressed genes), we used DAVID Bioinformatics resources. DEGs downregulated in Hif1aIEC were associated with biological processes such as the immune response to microorganisms (virus and Gram-positive bacteria) and the cellular response to type I and type II interferons. In the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways analysis, terms associated with the response to microorganisms such as phagosome, pattern recognition receptors signaling, antigen processing and presentation, and the maintenance of the epithelial barrier (i.e., cell adhesion molecules) were downregulated in Hif-1αIEC. However, DEGs upregulated in Hif-1αIEC were associated with metabolism, mainly lipid metabolism (Figure 5I). These results indicate that HIF-1 activation by butyrate reduces the intestinal epithelium damage caused by CDI and improves the immune response against commensals, reducing their translocation to other tissues.
DISCUSSION

The maintenance of the host-microbiota balance is key for homeostasis in animals. CDI is a good example of the relevance of the microbiota-host equilibrium as disruptions in microbiota composition lead to the impairment of mechanisms involved in the resistance to \textit{C. difficile} colonization, growth, and production of toxins. These toxins can damage epithelial cells and activate inflammatory responses that together contribute to infection (Ba"umler and Sperandio, 2016).

SCFAs are important molecules in host-microbiota communication as they are directly associated with the modulation of the host immune system and metabolism (Correa-Oliveira et al., 2016; Koh et al., 2016). A drastic reduction in intestinal SCFAs

![Figure 4. Butyrate Increases the Resistance of IECs to \textit{C. difficile} Toxins](image-url)

(A) Analysis of barrier integrity by TEER of HCT116 monolayer cells incubated for 48 h with \textit{C. difficile} supernatant. NC, negative control (cells without toxins or treatment); Ct, cells incubated with \textit{C. difficile} supernatant; Bt, cells incubated with \textit{C. difficile} supernatant plus 1 or 10 \textmu M butyrate (n = 6).

(B and C) Analysis of epithelial cell cytotoxicity 48 h after exposure to \textit{C. difficile} supernatant and treatment with 10 \textmu M butyrate (n = 6–8). Percentage of dead cells (B) and representative examples of cells incubated with \textit{C. difficile} supernatant and butyrate (C) are shown. Scale bars, 100 \textmu m.

(D) qPCR analysis of HIF-1 target gene expression in HCT116 cells treated with \textit{C. difficile} supernatant and butyrate (n = 6–8).

(E) Percentage of dead epithelial cells after treatment with butyrate or Bay85-3934 (Bay) and incubation with \textit{C. difficile} supernatant (n = 4–5).

*p < 0.05 compared to control. See also Figure S4.
has been reported after antibiotic treatment and is associated with susceptibility to CDI (Antharam et al., 2013; Theriot et al., 2014). We observed that the restoration of intestinal concentrations of butyrate attenuated CDI in mice. This indicates that butyrate may be useful in preventing or attenuating CDI and relevant for the protective effect found with strategies such as the intake of dietary fiber (e.g., inulin, mixtures of microbiota-accessible carbohydrates) or SCFA-producing bacteria (e.g., *Bifidobacterium* spp.), associated with the reduction of *C. difficile* fitness in the intestinal tract (Hryckowian et al., 2018; Valdés-Varela et al., 2016).

Recent studies showed that SCFAs can diminish the severity and length of intestinal infections caused by different pathogenic bacteria such as *Salmonella enteric* serovar Typhimurium (Rivera-Chávez et al., 2016), *Shigella* (Raqb et al., 2006, 2012), and enterohemorrhagic *Escherichia coli* (EHEC) O157:H7 (Fukuda et al., 2012). Different molecular mechanisms and cell targets are involved, including direct/indirect effects on bacteria colonization and toxin production (Fukuda et al., 2012; Rivera-Chávez et al., 2016) and the stimulation of host-intestinal defenses (Raqb et al., 2006, 2012). Previous studies found that dietary fiber (the main source of SCFAs) or probiotic bacteria reduce CDI (Hryckowian et al., 2018; Valdés-Varela et al., 2016). Using butyrate supplementation, we found a protective effect against CDI, which was not dependent on the alterations in specific microbiota genera/phyla, *C. difficile* colonization, or toxin production, but on a direct effect on IECs.

A recent study found that butyrate restoration in mice led to improved IEC junction integrity and the mitigation of graft-versus-host disease (Mathewson et al., 2016). This effect of butyrate was independent of its impact on T cells, but it did involve the reduction of damage caused by allo-human leucocyte antigen (HLA)-reactive T cells in IECs (Mathewson et al., 2016), indicating that it increased their resistance to injury. As shown in other models, we found that butyrate had anti-inflammatory effects, including the inhibition of pro-inflammatory cytokines and attenuation of leucocyte recruitment to the inflammatory site (Smith et al., 2013; Vinolo et al., 2011). These effects may contribute to a balanced response that, while limiting tissue damage, is still effective in combating the infection (Buonomo and Petri, 2016). We also found that butyrate increased Treg and tolerogenic CD103+ dendritic cells, as described by others (Arpaia et al., 2013; Singh et al., 2014; Smith et al., 2013; Tan et al., 2014). Although we cannot rule out the contribution of these effects on the mitigation of CDI by butyrate, our results indicate that its direct effects on IECs play a role in CDI protection, and HIF-1 is relevant in this context. We found that butyrate administration to mice in the CDI model increased *HisTa* expression and stability. This explains the increased expression of HIF-1 target genes in vivo. Moreover, we found that the protective effect of butyrate in the CDI model was absent in *HisTa*-deficient mice with no differences in intestinal permeability, translocation of bacteria, or clinical score, and no additive effect of butyrate was observed in mice with constitutive activation of this transcription factor (*VhlIEC* mice). In contrast with the dependence of butyrate on HIF-1, acetate treatment exerted a protective effect that did not depend on the activation of this transcription factor (not shown).

Kelly et al. (2013) found that butyrate increases the stability of HIF-1 in IECs via the stimulation of metabolism and oxygen depletion. HIF-1 controls the expression of genes associated with inflammation, apoptosis, and intestinal barrier protection. Kelly et al. (2015) also found that butyrate treatment of Caco-2 cells reduced barrier permeability, a phenotype that was abrogated in HIF-1β knockdown cells. HIF-1 plays an important role in intestinal homeostasis and inflammatory conditions (Taylor and Colgan, 2017). In this context, Karhausen et al. (2004) observed the attenuation of the intestinal barrier during 2,4,6-trinitrobenzene sulfonic acid (TNBS) colitis in mice with the activation of HIF-1 and the opposite phenotype in *HisTaIEC*. Hirota et al. (2010) observed a reduction in intestinal injury and inflammation caused by *C. difficile* in mice that express *HisTa* in IECs compared to *HisTa*-deficient mice.

Our results expand these findings by demonstrating that butyrate increased the resistance of IECs to *C. difficile* toxins through an HIF-1α-dependent mechanism. We hypothesize that HIF-1 stabilization by butyrate may be protective during CDI, not only because it reduces intestinal epithelium damage but also because it improves the immune response against commensals, as indicated by the functional analysis of genes downregulated...
in the absence of HIF-1 signaling, reducing their translocation to other tissues. These results indicate that interventions that restore butyrate intestinal levels may be an alternative form of therapy to patients with CDI.

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**SUPPLEMENTAL INFORMATION**

Supplemental Information can be found online at https://doi.org/10.1016/j.celrep.2019.03.054.

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**AUTHOR CONTRIBUTIONS**


**DECLARATION OF INTERESTS**

The authors declare no competing interests.

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**REFERENCES**


# STAR★METHODS

## KEY RESOURCES TABLE

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## Chemicals, Peptides, and Recombinant Proteins

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CONTACT FOR REAGENT AND RESOURCE SHARING

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Marco Vinolo (mvinolo@unicamp.br).

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Mice

The Multidisciplinary Centre for Biological Investigation (CEMIB - UNICAMP) provided adult C57BL/6J male mice. Rag-1 deficient mice (Rag1−/−) were from the CEDEME (UNIFESP). Hif1αfl/fl, Vhlfl/fl, Villin-Cre, ODD-luciferase and Foxp3-GFP mice were purchased from Jackson Laboratories or provided by collaborators and maintained in the Animal facility of the Department of Genetics, Evolution, Microbiology and Immunology. These strains were maintained in a C57BL/6J background. ODD-luciferase mice had an FVB background. Experiments with germ-free animals (Swiss mice) were performed in the Department of Microbiology, Institute of Biological Science of the Federal University of Minas Gerais. All mice used in this study were 6-12 wks-old. Only male mice were used. All mice were kept in regular filter-top cages with free access to sterile water and food. All animal procedures were approved by the Ethics Committee on Animal Use of the Institute of Biology (protocol numbers 3230-1/3742-1).

CELL LINE CULTURE

Human colon carcinoma cells (HCT116) were cultivated in DMEM medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine and penicillin/streptomycin at 37°C with 5% CO2. Cells were used until passage 20.
METHOD DETAILS

Model of infection
The C. difficile VPI 10463 strain was cultivated in BHI blood agar supplemented with hemin (5 µg/mL) and menadione (1 µg/mL) at 37 °C in anaerobic atmosphere (AnaeroGen, Oxoid; ThermoFisher Scientific; Waltham, MA, USA) in jars. Mouse infections were performed as described (Chen et al., 2008). Briefly, mice were pre-treated with antibiotic mixture (0.4 mg/mL kanamycin, 0.035 mg/mL gentamicin, 0.035 mg/mL colistin, 0.215 mg/mL metronidazole and 0.045 mg/mL vancomycin; Sigma) added to drinking water for 4 days. Next, mice received one dose of clindamycin (10 mg/kg, i.p.) (Sigma). After 1 day, mice were infected with 1 × 10⁸ colony forming units (CFUs) of C. difficile by gavage. Mice were weighed and monitored daily during the entire protocol with a clinical severity score that varied from 0 (normal) to 15, as described (Li et al., 2012) (Table S1).

SCFA TREATMENT

Animals received oral pre-treatment with 150 mM butyrate or placebo, as reported in other studies (Smith et al., 2013; Vieira et al., 2017). Butyrate treatment started one day before addition of antibiotics and continued throughout the protocol. In parallel, mice were treated with 3 g/kg tributyrin by gavage on days –1, 0 and 1 of colitis induction. In dietary experiments, mice received food containing different amounts of soluble fibers: a control diet, based on American Institute of Nutrition (AIN93) recommendations containing 5% cellulose; and one with high fiber supplemented with 5% cellulose and 25% inulin. Mice were pre-fed the different diets for 7 days.

Determination of faecal bacterial load and 16S rRNA sequencing
Faecal samples (50 mg) were used for extraction of microbial genomic DNA using the PureLink™ Microbiome DNA Purification kit (ThermoFisher Scientific). For bacterial load measurement, DNA was quantified by quantitative polymerase chain reaction (qPCR) using primers complementary to Eubacteria 16S rDNA (Table S2). A standard curve was constructed with serial dilutions of E. coli genomic DNA. Results were normalized to controls. Amplification and sequencing of 16 s rRNA was performed using Illumina MiSeq (Institute of Chemistry, University of São Paulo). Sequences of primers used for amplification of 16S rRNA V3-V4 variable regions are given in Table S2. Raw reads were filtered using Prinseqlite v.0.20.4 (Schmieder and Edwards, 2011) by removing sequences with average quality scores below 20. Resulting paired-end reads were overlapped using the merge program PEAR (v.0.9.10). Primers and adapters were trimmed using cutadapt v.1.12 (Martin, 2011) allowing maximum frequencies of 0.12 and 0.10 mismatches and indels for forward and reverse primers, respectively. Sequences shorter than 300 bp or those which were untrimmed were discarded. Pre-processed sequences were clustered into Operational Taxonomic Units (OTUs) using UPARSE (Edgar, 2013) with 97% similarity threshold. Taxonomy was assigned using the RDP classifier (v.2.2) (Wang et al., 2007). To investigate alpha and beta diversity, OTU tables were rarefied according to number of sequences of the smallest sample (22,584) and sequences were aligned using FastTree (Price et al., 2009). Statistical analyses of beta diversity used the vegan R package function Adonis and its default settings (vegan v.1.9.1) (Caporaso et al., 2010b). A phylogenetic tree was built to calculate pairwise UniFrac (Lozupone and Knight, 2005) distances using FastTree (Price et al., 2009). Statistical analyses of beta diversity used the vegan R package function Adonis and its default value of 999 permutations (https://cran.r-project.org/web/packages/vegan/index.html). Differential abundance was calculated for differences between mean proportions of each treatment. Significance was determined using Welch’s t test and Benjamini-Hochberg FDR correction available in STAMP (Parks et al., 2014). The data is deposited at NCBI’s BioProject (ID: PRJNA486872).

Measurement of short-chain fatty acids
Fecal samples or colonic luminal content samples were harvested from mice as described (Fellows et al., 2018) for measurement of SCFAs. Chromatographic analyses were performed using a GCMS-QP2010 Ultra mass spectrometer (Shimadzu; ThermoFisher Scientific) and a 30 m × 0.25 mm fused-silica capillary Stabilwax column (Restek Corporation, Bellefonte, PA, USA) coated with 0.25-µm polyethylene glycol. Samples (1 µL) were injected at 250 °C using a 25:1 split ratio. High-grade pure helium was used as carrier gas at 1.0 mL/min constant flow. Mass conditions were as follows: ionization voltage, 70 eV; ion source temperature, 200 °C; full scan mode, 35–500 m/z with 0.2 s scan velocity. The runtime was 11.95 min.

Histological analyses
Mouse colons were harvested, opened longitudinally and fixed in 4% formalin/0.1% glutaraldehyde. Tissues were processed into historesin and 5-µm sections prepared for staining with Hematoxylin and eosin. Slides were analyzed using an Olympus Microscope (Mod. U-LH100HG). Samples were analyzed blindly using histological scores for each parameter (Table S3). Overall scores were the sums of each component and varied from 0 to 30.

Measurement of in vitro growth of C. difficile
Bacteria were incubated in 5 mL Brain Heart Infusion (BHI) agar containing 1-50 mM butyrate for 72 h in anaerobic atmosphere at 37 °C. Optical density of the medium was read at 600 nm and the bacterial suspension diluted and plated in BHI agar to check for possible contaminations. The culture supernatant was used for measurement of toxins.
Quantification of C. difficile TcdA/TcdB
Toxins were measured in faecal samples and bacterial culture supernatants using the Ridascreen® C. difficile Toxin A/B ELISA kit (R-Biopharm; Darmstadt, Germany). Faecal samples were harvested on day 2 post-infection, weighed and vortexed in 1 mL dilution buffer. Samples were left 10 min prior to decanting and the supernatant collected for measurement.

Isolation of C. difficile from faeces
Faecal samples harvested on day 2 post-infection were weighed, vortexed in 1 mL sterile PBS and left 10 min prior to decantation. Supernatants were diluted at $10^{-6}$ and $10^{-7}$ and plated on cycloserine-cefoxitin-fructose-agar supplemented with horse blood. Plates were incubated in an anaerobic atmosphere at 37°C for 4-5 days.

Measurement of cytokines in tissues
Colon samples (100 mg) were homogenized in PBS containing protease inhibitors (ThermoFisher Scientific). Samples were centrifuged 10 min at 2000 x g and supernatants used for measurement of TNF-α, IL-6, IL-1β, IL-10, Cxcl1 and Cxcl2 using the Duo Set ELISA kit (R&D System; Minneapolis, MN, USA).

QUANTITATIVE GENE EXPRESSION by qPCR
Total RNA was extracted from colon, cecum and mesenteric lymph nodes using the PureLink™ RNA kit (Ambion). RNA was converted to cDNA using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems; Foster City, CA, USA) and qPCR was performed using Power SYBR Green PCR Master Mix (Applied Biosystems) and primers indicated in Table S2. Quantification of gene expression was performed using a ΔΔ Ct method with β2-microglobulin as a reference gene.

Flow cytometry
Cells were isolated from colon, mesenteric lymph nodes (mLNs) and spleen as described by Hall et al. (2011). After removal of faecal contents, colon was incubated 20 min in RPMI-1640 medium containing 3% fetal bovine serum (FBS), 100 U/mL penicillin - 100 μg/mL streptomycin (Pen/Strep), 25mM HEPES, 50 mM β-mercaptoethanol (β-ME), 5 mM EDTA and 0.145 mg/mL of dithiothreitol (DTT) (Sigma). The epithelial layer was removed by agitation in serum-free RPMI containing 2 mM EDTA. Tissues were minced and digested with serum free RPMI containing: 25 mM HEPES, 50 μM β-ME, 1 mg/mL collagenase IV (Sigma) and 0.5 mg/mL DNase I (Sigma) by continuous stirring at 37°C for 25 min. Digests were diluted in RPMI containing 3% FBS, Pen/Strep, 25 mM HEPES and 50 mM β-ME, and mashed through 70- and 40-μm cell strainers. Cells were washed and suspended in complete RPMI. Dead cells were discriminated in all experiments using LIVE/DEAD fixable dead stain (ThermoFisher Scientific) and all staining steps carried out in media containing anti-CD16/32 (2.4G2 antibody). The following antibody clones were used: CD11b (M1/70), CD11c (N418), CD45.2 (104), CD64 (X54-517.1), CD103 (2E7), F4/80 (BM8), MHCII (I-A/I-E) (M5/114.15.2), Ly6C (HK1.4), Ly6G (1A8), CD19 (1D3), CD161 (NK1.1) (PK136), Lyve-1 (ALY7), Siglec F (E50-2440) and TCR-beta (H57-597). Cell acquisitions were performed on a BD LSR X20 cell analyzer (BD Biosciences) using FACSDiva software (BD Biosciences) and data analyzed using FlowJo software (TreeStar Inc.; Ashland, OR, USA).

Bacteria translocation
Spleen and liver were harvested on day 2 post-infection. Tissues were weighted and homogenized in 1 mL PBS using a tissue homogenizer under sterile conditions for 60 s. Homogenates were centrifuged 10 min at 2000 x g (4°C) and 100 μL supernatant plated on BHI agar plates supplemented with hemin and menadione. Plates were incubated in anaerobic jars or under aerobic conditions at 37°C. Colony counting was performed after 4-5 days. 16S rDNA gene levels were determined by qPCR.

Measurement of intestinal permeability with FITC-Dextran
Mice received 200 μL FITC-Dextran (70,000 Da; Sigma) suspension (250 mg/Kg) by gavage on day 2 of infection. After 4 h, mice were anesthetized, blood collected by caudal puncture and fluorescence readings performed in a Multi-Mode Microplate Reader (Synergy HT; Vermont, USA) at 485/528 nm (excitation/emission). A standard curve was prepared with serial dilutions of FITC-Dextran in PBS.

Measurement of luciferase activity in colon samples
Colon samples from ODD-luciferase mice were harvested and homogenized in lysis buffer. Luciferase activity was measured using Dual Luciferase Reporter Assay System (Promega Corporation; Madison, WI, USA). Protein concentrations were used for data normalization.
Immunostaining for tight-junction proteins
Colon fragments were harvested, washed with PBS and fixed 2 h in 4% formaldehyde. Tissues were embedded in Tissue-Tek OCT Compound (Sakura® Finetek, Torrance, CA, USA), snap frozen and stored at −80°C. Sections were cut (4 μm) using a cryostat and labeled overnight at 4°C with antibodies (1:50; anti-claudin-1 or 1:100; anti-occludin; ThermoFisher Scientific) after blocking non-specific binding sites with 1% BSA. A secondary antibody conjugated with AlexaFluor-488 (1:100; Sigma) was used for detection. DAPI (1:1000; Invitrogen; Carlsbad, CA, USA) was added and sections mounted in Vectashield medium (Vector Laboratories, Inc.; Burlingame, CA, USA). Markings were detected and photographed by confocal laser microscopy (CLSM; Bio-Rad MRC 1024;Bio-Rad, Richmond, CA, USA) through a 10x objective. To allow comparison between groups, green fluorescence intensity (485 ± 10/530 ± 12.5 nm excitation/emission) was measured using ImageJ software (National Institutes of Health, Bethesda, MD, USA) and expressed in arbitrary units.

Fluorescent in situ hybridization (FISH) preparation and analysis
FISH was performed as described by Molloy et al. (2013). Colon fragments were fixed in methacarn 3 h at 4°C and coronal slices (5-μm) obtained. After deparaffinization and rehydration, sections were incubated in hybridization buffer [20 mM Tris-HCl, 0.9 M NaCl and 0.1% SDS (pH 7.2)] 10 min at 50°C. Next, sections were incubated with 100 nM bacteria probes (5’- 3’: GCTGCTTCCGTAGGAGT; FITC-conjugated; Sigma) in hybridization buffer in the dark, 4 h at 50°C, washed with 20 mM Tris-HCl, 0.9 M NaCl (pH 7.2) and incubated 2 h at room temperature in 20 μg/mL Lectin–Ulex europaeus agglutinin-I (UEA-I; TRITC-conjugated, Sigma). Sections were washed again, incubated 10 min with 10 μg/mL Hoechst 33258 solution and mounted with SlowFade® Gold medium (ThermoFisher Scientific). Images were acquired using a Zeiss LSM-780 confocal microscope (Carl Zeiss; Oberko- chen, Germany). Samples were imaged with 63x/1.4NA oil-immersion objective at 3x with a 1024 × 1024 frame size. Qualitative analysis was performed and included the following features: presence/absence of mucus, bacterial load, bacterial translocation and epithelial morphology. Three apical extrusion zones of colon were randomly chosen to measure epithelium-luminal bacteria distance. The mean was used to calculate the distance between apical extrusion zones and the first bacterial focus in the gut.

Live/Dead assay
Cells were cultivated in 1:500 bacterial supernatant and 1, 10 or 100 μM butyrate in 96-well plates (1.0 × 10⁵ cells/well). After 48 h, cells were washed gently using Dulbecco’s (D)-PBS and media replaced by 100 μL Calcein-AM and propidium iodide (2 μM) in D-PBS and incubated 30 min at 37°C under 5% CO₂. Images were obtained using the Cytation 5 Cell Imaging Multi-Mode Reader and green fluorescence (485/530 nm, excitation/emission) of viable cells and red fluorescence (530/645 nm) of dead cells quantified using Gen5 software (Biotek, Winooski, VT, CA). The positive control was pre-incubation of cells with 0.1% Triton-X 15 min. To obtain C. difficile supernatants, the toxigenic strain VPI 10463 was cultured 24 h at 37°C in DMEM containing 10% FBS, 2 mM L-glutamine, and Pen/Strep in transwell permeable polyester supports (Corning Life Sciences; Tewksbury, MA, USA) (0.4 μm/pore, Costar) to approximate confluen-ce. Cells were incubated with 1:500 C. difficile supernatant and different concentrations of butyrate 48 h at 37°C under 5% CO₂. Transepithelial Electrical Resistance (TEER)
HCT116 cells (2.0 × 10⁵ cells/mL) were cultured 24 h at 37°C in DMEM containing 10% FBS, 2 mM L-glutamine, and Pen/Strep in transwell permeable polyester supports (Corning Life Sciences; Tewksbury, MA, USA) (0.4 μm/pore, Costar) to approximate confluen-ce. Cells were incubated with 1:500 C. difficile supernatant and different concentrations of butyrate 48 h at 37°C under 5% CO₂. Electric resistance was measured by potential difference using electrodes and the EVOM2 and Endohm 24-snap system (World Precision Instruments, Sarasota, FL). Values were expressed as Ω/cm² resistance.

RNA-seq
IECs were isolated from the colon of infected (2 d.p.i.) mice treated with butyrate. Briefly, colons were opened longitudinally and washed three times with ice cold DPBS. IECs were collected and washed with 2 mM EDTA/HBSS at 37°C with shaking for 60 min. The tubes with colons were shaken in vortex for 20 s after every 10 min. The material was then filtered through a 100 μm cell strainer. The extracted cells were pelleted at 475 g × 30 min at 4°C. Total RNA was extracted from 2 × 10⁶ IECs using PureLink™RNA Mini Kit (ThermoFisher). RNA quantification and integrity were measured on a BioAnalyzer RNA 6000 Pico chip (Agilent) and sent to BGI (Shenzhen, China) for cDNA library construction and sequencing. Sequencing was done using the BGISEQ-500 platform (read length 100 pb, paired-end) and, at least 20 million clean reads were obtained from each sample. The software SOAPnuke was used for removing adaptors reads, reads in which unknown bases are more than 5% and low quality reads. Reads were mapped to the mouse (GRCm38) reference genome and analyzed using SeqMonk software (version 0.34.1, Babraham Institute Bioinformatics - Cambridge, UK). Gene ontology analysis was done online in DAVID 6.8 (Database for Annotation, Visualization and Integrated Discovery).

Quantification and Statistical Analysis
Analyses were performed using GraphPad software 5.0 (San Diego, CA, USA). Differences were considered significant for p < 0.05. Results were first analyzed using D'Agostino/Shapiro-Wilk normality tests and compared by Student’s t-test or Mann Whitney test, as
appropriate. For more than two groups, differences were compared by one-way ANOVA followed by Tukey’s post hoc test or Kruskal-Wallis followed by Dunn’s test.

**DATA AND SOFTWARE AVAILABILITY**

The RNA-seq and 16S rDNA amplicon sequencing data reported in this paper have been deposited at BioProject NCBI, under accession numbers PRJNA515618 and PRJNA486872.