Dietary Fructose Alters the Composition, Localization, and Metabolism of Gut Microbiota in Association With Worsening Colitis

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SUMMARY

The incidence of inflammatory bowel diseases is increasing worldwide, suggesting a potential role for dietary factors. Here, we demonstrate that a high fructose diet worsens colitis in a microbiota-dependent manner. Overall, this study highlights the importance of diet-microbe interactions in intestinal inflammation.

BACKGROUND & AIMS: The incidence of inflammatory bowel diseases has increased over the last half century, suggesting a role for dietary factors. Fructose consumption has increased in recent years. Recently, a high fructose diet (HFrD) was shown to enhance dextran sodium sulfate (DSS)-induced colitis in mice. The primary objectives of the current study were to elucidate the mechanism(s) underlying the pro-colitic effects of dietary fructose and to determine whether this effect occurs in both microbiologically driven and genetic models of colitis.

METHODS: Antibiotics and germ-free mice were used to determine the relevance of microbes for HFrD-induced worsening of colitis. Mucus thickness and quality were determined by histologic analyses. 16S rRNA profiling, in situ hybridization, metatranscriptomic analyses, and fecal metabolomics were used to determine microbial composition, spatial distribution, and metabolism. The significance of HFrD on pathogen and genetic-driven models of colitis was determined by using Citrobacter rodentium infection and IL10/− mice, respectively.

RESULTS: Reducing or eliminating bacteria attenuated HFrD-mediated worsening of DSS-induced colitis. HFrD feeding enhanced access of gut luminal microbes to the colonic mucosa
by reducing thickness and altering the quality of colonic mucus. Feeding an HFrD also altered gut microbial populations and metabolism including reduced protective commensal and bile salt hydrolase-expressing microbes and increased luminal conjugated bile acids. Administration of conjugated bile acids to mice worsened DSS-induced colitis. The HFrD also worsened colitis in Il10−/− mice and mice infected with C. rodentium.

CONCLUSIONS: Excess dietary fructose consumption has a pro-colitic effect that can be explained by changes in the composition, distribution, and metabolic function of resident enteric microbiota. (Cell Mol Gastroenterol Hepatol 2021;11:525–550; https://doi.org/10.1016/j.jcmgh.2020.09.008)

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The incidence of inflammatory bowel diseases (IBD) has increased worldwide over the last half century. Dietary changes are believed to help explain the rising incidence of IBD. In support of this possibility, consumption of a Western diet including fructose has dramatically increased over the last several decades. In fact, consumption of fructose has increased by approximately 30% over the last 3 decades in the United States alone, with sugary beverages and processed foods as major sources.1,2 Although multiple factors including diet have been implicated in the pathogenesis of IBD,3 high intake of fructose could be an important and underappreciated contributor. Rodent studies have provided direct evidence of a harmful role for fructose in the colon. These studies showed that dietary fructose reduced colonic mucus thickness and epithelial barrier function and increased plasma lipopolysaccharide levels, suggesting increased gut permeability.4,5 Recently, feeding a high fructose diet (HFrD) was reported to alter the gut microbiota in association with worsening dextran sodium sulfate (DSS)-induced colitis.5,6 However, it is not known whether the microbiota play a role in mediating this pro-colitic effect or whether such a diet worsens colitis in more clinically relevant T-cell–mediated or infectious models.

Intestinal microbiotas are important mediators of gut health and have been implicated in the pathogenesis of IBD.7–12 Dietary factors influence the composition of intestinal bacterial populations.13,14 This occurs, at least in part, by altering microbial nutrient sources, thus creating more or less favorable environments for certain species.15,16 Several mechanisms can explain how microbes exert their effects on the intestinal tract, including through modulating levels and profiles of small molecules such as bile acids (BAs) in the gut lumen.17 In fact, recent evidence suggests a link between dysbiosis and altered levels of fecal BAs in IBD patients.18–21

The current study examined the mechanisms by which feeding an HFrD impacts the etiopathogenesis of murine colitis. Here we show that an HFrD worsens experimental colitis in multiple murine models, an effect that was microbially mediated and associated with changes in the composition, distribution, and metabolic function of resident enteric microbiota. In addition, a role for conjugated BAs is suggested. Importantly, these findings suggest a connection between diet, microbial composition and metabolic activity, and intestinal inflammation.

Results

Feeding a High Fructose but not a High Glucose Diet Increases the Severity of DSS-Induced Colonic Injury

Because of the recent evidence that high fructose feeding worsens experimental colitis,6 we sought to determine whether this effect was selective for fructose or extended to other sugars. Therefore, we fed wild-type (WT) C57BL/6j male mice 3 isocaloric diets: (1) control diet (AIN-93G purified diet) containing corn starch, maltodextrin, and sucrose in ~60:20:20 ratio as the carbohydrate sources (64 kcal%), (2) high glucose diet (HGD) (based on AIN-93G) in which all carbohydrate content comes from glucose, and (3) HFrD in which all carbohydrate content comes from fructose, for 2 weeks (Table 1). Mice were then challenged with low dose (1%) DSS in drinking water to induce colitis while being continued on their respective diets, and colitis severity was assessed. As shown in Figure 1A–C, mice fed HFrD developed markedly worse colitis compared with mice fed the AIN-93G control diet or HGD including more severe weight loss, diarrhea, rectal bleeding, colonic shortening, and histologic injury. To characterize the inflammatory milieu during colitis in HFrD-fed mice, the expression of proinflammatory cytokines was measured in colonic tissues under basal conditions and after low dose DSS exposure in mice fed control or HFrD. The magnitude of increase in expression of multiple cytokines including Il1b, Il6, Il17a, and Il22 was greater in mice fed HFrD than control diet after 7 days of DSS exposure (Figure 1D). In the absence of DSS, consumption of HFrD did not increase colonic tissue inflammatory cytokine expression. Next, we evaluated immune cell populations in the colonic lamina propria in HFrD and control diet-fed mice after DSS exposure by fluorescence-activated cell sorting. This analysis showed increased numbers of several immune cell subsets including macrophages, neutrophils, dendritic cells, and natural killer cells on HFrD feeding (Figure 1E). Representative fluorescence activated cell sorting plots showing cell populations in each group are shown in Figure 2. The HFrD also exacerbated DSS-induced colitis in female mice (Figure 3). In addition, feeding the HFrD for either 1 or 2 weeks before DSS challenge led to very similar pro-colitic effects (Figure 4). To specifically compare the contributions of glucose vs fructose...
to colitis severity, isocaloric diets containing increasing amounts of fructose were compared with HGD (Table 2), which demonstrated a step-wise worsening of colitis as additional fructose was added (Figure 5). We next tested whether feeding mice a level of fructose comparable to high fructose consumers within the U.S. population would also exacerbate colitis. Therefore, we fed mice a diet containing 15 kcal% fructose for 1 week and challenged them with DSS. Impressively, this diet also worsened colitis (Figure 6). Taken together, these findings demonstrate that elevated dietary fructose intake, but not glucose, exacerbates experimental colitis.

**HFrD-Induced Worsening of Colitis Is Microbiota-Dependent**

Previous work has suggested that feeding a diet high in fructose alters the gut luminal microbiota, raising the possibility that this microbial shift is causally linked to worsening colitis. To evaluate this possibility, 3 complementary approaches were used in the DSS model of colitis. First, we administered broad-spectrum antibiotics (ABx) to test whether reduced bacterial abundance would attenuate HFrD-mediated worsening of colitis. We elected to carry out these and the remaining experiments in this study by using 64 kcal% fructose to better reveal fructose-mediated biological changes and to reduce the numbers of mice needed for studies. Mice were pretreated with ABx for 3 weeks in drinking water, which led to a 500-fold reduction in fecal bacterial load as determined by quantitative real-time polymerase chain reaction (qRT-PCR) using a general bacterial primer (Ct value: 16 ± 0.4 vs 25 ± 0.7; P < .001). Mice were then given control or HFrD for 1 week, followed by 1 week of DSS exposure, while being continued on control or HFrD. Control or ABx-containing drinking water was provided throughout the experimental period. As expected, HFrD-fed mice developed worse colitis than control diet-fed mice in the absence of ABx (Figure 7A–C). However, ABx treatment prevented HFrD-induced worsening of colitis (Figure 7A–C). To confirm the importance of the microbiota for mediating the effects of an HFrD, we next evaluated colitis severity in germ-free (GF) mice. Whereas specific pathogen-free (SPF) mice given HFrD developed worse colitis, this phenotype was completely prevented in GF mice (Figure 7D–F). To further confirm the role of microbes in this process, we transferred the colonic content of mice fed either control diet or HFrD to recipient mice and challenged them with DSS while being fed control diet. As shown in Figure 8, mice that received fecal material from HFrD-fed mice exhibited more severe colitis. Together, these data suggest that exacerbation of DSS-induced colitis by HFrD is dependent on the gut microbiota.

**HFrD Feeding Reduces Gut Barrier Integrity Resulting in Bacterial Access to the Mucosa**

To investigate the mechanism by which feeding an HFrD worsens acute experimental colitis via the microbiota, we evaluated the gut barrier and the bacterial-mucosal interface after HFrD feeding in the absence of colitis. Prior studies have shown that HFrD impairs the gut barrier, but the mechanism underlying this effect is unknown. Moreover, an impaired gut barrier is an important contributor to IBD pathogenesis, with relevance for the microbiota in this process. We first measured mucus thickness, which revealed a 20% reduction with HFrD feeding (Figure 9A and B). This decrease was not due to reduced

| Table 1. Composition of Control Diet, HFrD, HGD, and 15 kcal% Diet |
|---------------------------------------------------------------|
| **Control diet (AIN-93G)** | **HFrD** | **HGD** | **15 kcal% Fructose diet** |
| **gm% kcal%** | **gm% kcal%** | **gm% kcal%** | **gm% kcal%** |
| Protein | 20 | 20 | 20 | 20 | 20 | 20 | 20 | 20 |
| Carbohydrate | 64 | 64 | 64 | 64 | 64 | 64 | 64 | 64 |
| Fat | 7 | 16 | 7 | 16 | 7 | 16 | 7 | 16 |
| Total | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 |
| kcal/g | 4.0 | 4.0 | 4.0 | 4.0 | 4.0 | 4.0 | 4.0 | 4.0 |
| Ingredient | | | | | | | | |
| Casein | 200 | 800 | 200 | 800 | 200 | 800 | 200 | 800 |
| L-Cystine | 3 | 12 | 3 | 12 | 3 | 12 | 3 | 12 |
| Corn starch | 397.486 | 1590 | 0 | 0 | 0 | 0 | 347.486 | 1390 |
| Maltodextrin 10 | 132 | 528 | 0 | 0 | 0 | 0 | 132 | 528 |
| Dextrose | 0 | 0 | 0 | 0 | 629.486 | 2518 | 0 | 0 |
| Fructose | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Sucrose | 100 | 400 | 0 | 0 | 0 | 0 | 100 | 400 |
| Cellulose, BW200 | 50 | 0 | 50 | 0 | 50 | 0 | 50 | 0 |
| Soybean oil | 70 | 630 | 70 | 630 | 70 | 630 | 70 | 630 |
| t-Butylhydroquinone | 0.014 | 0 | 0.014 | 0 | 0.014 | 0 | 0.014 | 0 |
| Mineral Mix S10022G | 35 | 0 | 35 | 0 | 35 | 0 | 35 | 0 |
| Vitamin Mix V10037 | 10 | 40 | 10 | 40 | 10 | 40 | 10 | 40 |
| Choline bitartrate | 2.5 | 0 | 2.5 | 0 | 2.5 | 0 | 2.5 | 0 |
| Total | 1000.00 | 4000 | 1000.00 | 4000 | 1000.00 | 4000 | 1000.00 | 4000 |
Figure 1. HFrD increases the severity of DSS-induced colitis. WT C57BL/6J mice were given a control or HFrD for 2 weeks and then administered 1% DSS, while being continued on their respective diets. (A) Measurements of clinical scores associated with colitis severity were carried out including body weight, diarrhea, and bleeding. (B and C) At end of the experimental period, colon lengths (B) and histologic scores (C) were determined (N = 8/group), and representative histologic images are shown for each group (C). (D) Relative gene expression of cytokines measured from colons of mice fed either a control or HFrD for 2 weeks and then given plain drinking water or 1% DSS for 1 week, while being continued on their respective diets (N = 9–10/group). (E) Number of immune cells normalized to tissue weight was determined in colonic lamina propria of mice fed a control or HFrD for 1 week followed by 1 week of 1% DSS, while being continued on their respective diets, by flow cytometry (N = 4/group). Data are presented as mean ± standard deviation. *P < .05, **P < .01, ***P < .001. Data were generated from single experiments.
Elevated Dietary Fructose Leads to Change in Populations of Multiple Bacterial Species Including an Expansion of Akkermansia muciniphila

To determine which bacterial populations might be responsible for HFrD-induced worsening of colitis, we first carried out 16S rRNA profiling on feces from control or HFrD-fed mice. Principal coordinate analysis showed a marked shift in microbial populations after 1 week of HFrD feeding (Figure 10A). This shift was associated with increased fecal fructose levels (585 ± 263 vs 68 ± 25 μmol/L; P < .001). Of note, serum fructose levels were also elevated in HFrD-fed mice (539.4 ± 285.5 vs 257.4 ± 86.1 μmol/L; P = .001). When examined at the species level, the HFrD led to significant changes in the proportions of multiple species including increased A. muciniphila (Figure 10B). A. muciniphila is a bacterium known to degrade mucus and has been associated with colitis.27,28 Because of the observed thinning of colonic mucus after HFrD feeding, we further investigated this microbe. To directly evaluate its abundance in mice fed either diet, we carried out qRT-PCR on DNA extracted from feces by using A. muciniphila–specific primers. This analysis showed no difference in abundance (1.23 ± 0.65 vs 1.66 ± 1.62; P = 1.0). Although fecal levels of A. muciniphila did not change on HFrD feeding, we examined whether this microbe might be expanded at the mucus interface by carrying out FISH using an A. muciniphila–specific probe. This analysis demonstrated a strong clustering and expansion of this species at the mucus interface after HFrD feeding compared with control diet-fed mice (Figure 10C and D).

High Fructose Feeding Alters Bacterial Bile Acid Metabolism

In addition to an effect on bacterial composition and localization, HFrD feeding may also impact gut bacterial metabolism, which can in turn contribute to disease pathogenesis. To evaluate this hypothesis, we carried out metatranscriptomic analysis on bacteria derived from feces of control or HFrD-fed mice. No major differences in total number of genes were found at the phylum level (Table 3). Interestingly, when the number of transcripts was examined at the level of genus, there were significant changes associated with HFrD feeding across multiple genera (Figure 11). Analysis was next carried out on the metatranscriptomic profile to determine pathways that are altered by high fructose feeding. This investigation revealed the pathway “Bile acid metabolism” to be strongly decreased in the HFrD group, with genes from select phyla being affected (Figure 12A and B). Further analysis at the taxonomic level of order showed distinct clustering of bacteria based on BA metabolism-related gene expression (Figure 12C). Bacteria segregated into 3 distinct clusters on the basis of the relative abundance of transcripts for multiple genes, with a major contribution of choloylglycine hydrolases to the signature (Figure 12C). A more detailed description of gene changes in this pathway is shown in Supplementary Table 1. Choloylglycine hydrolase, also known as bile salt hydrolase (BSH), is the key enzyme that deconjugates BAs. Focused examination of choloylglycine hydrolase showed decreases in select orders of microbes (Bifidobacteriales and Erysipelotrichales) on HFrD feeding (Figure 13A). Relative levels of choloylglycine hydrolase in control and HFrD-fed mice segregated into 2 distinct clusters (Figure 13B), one with mixed effects (Figure 13B, cluster 1) and the other with a decrease within bacteria from HFrD-fed mice (Figure 13B, cluster 2). A more detailed description of choloylglycine hydrolase expression is shown in Supplementary Table 2.

Metatranscriptomic analysis showed alterations in the expression of BSH within bacteria of HFrD-fed mice, with mixed effects in terms of direction of change. Because BSH plays a key role in the deconjugation of BAs, we elected to next evaluate whether the observed changes in bacteria led to alterations in BA levels in the gut. Measurements of a panel of BAs in the feces of control and HFrD-fed mice by metabolomic analysis revealed a marked and selective increase in taurine-conjugated BAs in mice fed elevated fructose (Figure 14A). This finding along with our metatranscriptomic analysis suggested a defect in the ability of microbes to deconjugate BAs. Because this effect could result from reduced BSH expression within bacteria or reduced abundance of BSH-expressing bacteria, we next investigated the mechanism(s) that could account for the observed changes in BAs. Bifidobacterium pseudolongum and Lactobacillus johnsonii are key human commensal strains known to be major BSH expressers and suggested to be affected by an HFrD on the basis of our metatranscriptomic data (Figures 12 and 13). Moreover, 16S analysis suggested that the relative abundance of L johnsonii was
Figure 3. HFrD feeding worsens DSS colitis in female mice. (A–D) Measurements of colitis severity including body weight (A), diarrhea (B), bleeding (C), and colon length (D) were made in female mice fed control or HFrD for 2 weeks and then administered 1.5% DSS for 1 week, while being continued on their respective diets (N = 9–10/group). Data were generated from a single experiment.

Administration of Conjugated Bile Acids Reduces Mucus Thickness and Worsens Colitis

To determine whether increased gut luminal conjugated BAs after HFrD feeding might be causally linked to thinning of colonic mucus and worsening of colitis, we administered a cocktail of conjugated BAs by enema to mice fed control diet at a concentration that was intended to mimic the increase after feeding an HFrD. As shown in Figure 15, the BA enema reduced mucus thickness under non-colitic conditions and increased DSS-induced colitis severity when mice were fed a control diet. These data support a direct role for conjugated BAs in the phenotype observed after HFrD feeding.

Elevated Dietary Fructose Promotes the Growth of Citrobacter Rodentium and Worsens Colitis After Infection

Our in vivo and in vitro data suggest that HFrD impairs the growth of beneficial resident bacterial species (L johnsonii and B pseudolongum). It is possible that a loss of probiotic bacteria could facilitate the expansion of pathogenic species leading to worse infectious colitis. To test this concept, we infected mice with C rodentium, a commonly used model of infectious colitis in mice.29 After administration of the HFrD or control diet for 1 week, WT mice were inoculated with 5 × 10^7 colony-forming units C rodentium and continued on their respective diets. The abundance of C rodentium was measured by qRT-PCR in feces from mice every 2 days over a 6-day period after infection. A significant increase in abundance of this pathogen was detected in HFrD vs control diet fed mice on days 2 and 4 after inoculation (Figure 16A). To determine whether this increased abundance resulted in more severe infectious colitis, the same mice were examined for signs of colitis during the 6-day period. Those mice fed HFrD displayed attenuated weight gain and higher incidence of diarrhea and bleeding (Figure 16B). These changes were paralleled by shortening of the colon, worse histologic injury, and elevated expression of Il17a and Il22 (Figure 16C–E). In a separate experiment, mortality tracked over a 14-day period after C rodentium infection revealed that 40% of mice fed an HFrD died, whereas no deaths occurred in mice fed the control diet (P = .029). Taken together, these findings demonstrate that high dietary fructose consumption enhances the luminal growth of a pathogenic bacterium and worsens infectious colitis.

HFrD Exacerbates Colitis in Il10−/− Mice

The Il10−/− mouse model recapitulates the complex interplay of host genetic susceptibility, enteric microbiota, and chronic T-cell-mediated inflammation found in IBD. Therefore, we evaluated the role of dietary fructose in impacting colitis pathogenesis in this model. GF Il10−/− mice were inoculated with microbiota from SPF mice and then given control or HFrD. Although fecal lipocalin levels

Figure 2. (See previous page). Representative plots for fluorescence-activated cell sorter analysis of immune cell populations. Representative plots generated by fluorescence-activated cell sorter analysis used to identify and quantify colonic immune cell populations shown in Figure 1E are shown for control and HFrD-fed mice. All cell types were gated off of the live cell population, and specific immune cell subsets were gated off of the CD45+ population. NK, natural killer.
steadily increased over time in both groups, this increase was greater in those Il10−/− mice given HFrD at 4 and 6 weeks after inoculation (Figure 17A). Furthermore, the stool score (representative of diarrhea) was higher in HFrD-fed mice at 6 weeks (Figure 17B). After 6 weeks of dietary exposure, tissues were collected and examined for evidence of histologic injury. Mice fed HFrD exhibited a higher histology score, driven exclusively by colonic rather than small intestinal injury (Figure 17C and D). Consistent with these findings, the expression of several inflammatory genes was increased in the colons of mice fed the HFrD (Figure 17E). Moreover, colon explants from HFrD-fed mice produced higher levels of IL12p40 and interferon γ protein (Figure 17F).

**Discussion**

The rapidly rising incidence of IBD suggests that environmental factors such as diet could be casually linked. This study demonstrated that elevated consumption of the dietary component fructose worsened acute DSS-induced, infectious, and T-cell–mediated models of colitis. The exacerbation of colitis was likely microbiota-dependent and appeared to be mediated by changes in the composition, mucosal association, and functional activity of the microbiota. These findings demonstrate a potential connection between dietary fructose, microbes, and intestinal inflammation.

Dietary factors can elicit rapid and specific changes in gut microbes. Previous work showed that feeding rats a
diet high in fructose alters the luminal microbiota. A more recent study demonstrated that a diet enriched in fructose worsened DSS-induced colitis in association with altering the gut microbiota and disrupting features of the gut barrier. Moreover, direct exposure of fructose to colonic cells in culture reduced tight junction protein expression and increased cell permeability. This previous work suggested that elevated luminal fructose could mediate proinflammatory effects either through changes in the microbiota or via direct effects on epithelial cells. Our findings also show that an HFrD worsens experimental colitis but extends this phenomenon to multiple models, including T-cell–mediated chronic inflammation. Importantly, we demonstrate a potentially causal role for the resident microbiota by showing that when intestinal bacteria are substantially reduced (broad-spectrum ABx) or completely absent (GF), the effects of dietary fructose on colitis severity are lost. Moreover, the effects of microbial alterations were transferrable. Although our studies suggest a role for microbes in this process, we appreciate the limitations of the model systems that were used. It has been shown that mice given ABx or in the GF state experience worsened DSS-induced colitis compared with conventionally maintained mice, an effect that we also observed (Figure 7). Therefore, it is important to consider this issue when interpreting the findings that reduced microbial abundance attenuated the effects of HFrD on colitis severity. Regardless, our findings support the potentially important role that microbes play in mediating the pro-colitic effects of an HFrD.

Dysbiosis occurs frequently in IBD and is believed to contribute to disease pathogenesis. In addition to these changes in composition, bacterial localization is also relevant. In fact, direct exposure of luminal microbes to the intestinal mucosa has been observed in IBD patients, resulting from impaired gut barrier function. These alterations likely lead to a localized inflammatory response and contribute to disease pathogenesis. In the current study, we found alterations in both bacterial composition and mucosal localization after HFrD feeding. Interestingly, *Lactobacilli* and *Bifidobacteria*, which are reduced in abundance in HFrD-fed mice, are also decreased in IBD patients.

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Figure 5. Increasing amounts of dietary fructose progressively worsen experimental colitis. Mice were administered diets containing glucose as the sole carbohydrate source, glucose and fructose in a 1:1 ratio, glucose and fructose in a 1:3 ratio, or fructose as the sole carbohydrate source for 2 weeks. 2% DSS was then administered for 1 week, while mice were continued on their respective diets. Measurements of body weight (A), diarrhea (B), and bleeding (C) were carried out during DSS exposure, and colon length (D) was evaluated at end of the experiment. Data are combined from 2 independent experiments (N = 16 mice total per group). P value represents significance level when fructose is used as a continuous variable.

Figure 6. Consumption of 15 kcal% diet worsens DSS-induced colitis. Mice were fed control diet or diet containing 15 kcal % fructose for 1 week and then administered 1% DSS for 1 week, while being continued on their respective diets. Measurements of colitis severity including diarrhea (A), bleeding (B), colon length (C), and histologic injury (D) were made during DSS administration (A and B) or at end of the study (C and D). Data are combined from 2 independent experiments and presented as mean ± standard deviation (N = 25/group).
Note, these bacteria types are commonly used probiotics. Notably, we demonstrated that the growth of both of these strains of bacteria was suppressed in culture medium containing fructose compared with dextrose, which is consistent with their reduced abundance in the feces of HFrD-fed mice. Although our studies focused in part on these probiotic species, it is likely that other fructose-induced microbial changes contribute to the observed phenotype. For instance, FISH revealed a strong clustering of the mucolytic species *A. muciniphila* at the mucus interface after HFrD feeding. Therefore, it is possible that *A. muciniphila* contributed to the observed thinning of colonic mucus in response to an HFrD. Interestingly, this same HFrD diet augmented the luminal growth of the pathogenic...
Consistent with this by decreased expression of BSH, an enzyme that deconjugates BAs. Reduced levels of fecal conjugated BAs after HFrD feeding. BAs have been shown to adversely affect barrier function in vitro and in vivo. Moreover, elevated levels of fecal conjugated BAs have been observed in IBD patients. In fact, it has been suggested that the ability of BAs to disrupt the gut barrier contributes to IBD pathogenesis. In support of this possibility, we found that conjugated BAs given by enema exacerbated DSS-induced colitis. Our results also raise the intriguing possibility that probiotics expressing BSH could be useful in the prevention or treatment of IBD in subsets of patients. In addition to the potential importance of reduced bacterial BSH levels for explaining the exacerbation of colitis mediated by the HFrD, we appreciate that the observed microbial changes may have additional pro-colitic effects. For instance, gut bacterial alterations could also impact immune function by increasing inflammatory and decreasing regulatory pathways.

Glut5 in the plasma membrane of enterocytes is responsible for fructose absorption in the small intestine, and in fact, this transporter increases in expression in response to elevated consumption of fructose. We posit that feeding an HFrD to Glut5 knockout mice resulted in colon pathology, thus supporting the importance of efficient fructose absorption for gut homeostasis. Therefore, it is possible that dietary fructose may be especially harmful to those individuals who have reduced small intestinal Glut5 levels or function. In the future, it will be important to extend this line of investigation to humans. Perhaps Crohn’s disease patients who have had small intestinal resections (and an associated reduction in levels of Glut5) or have active small intestinal mucosal inflammation will be particularly sensitive to dietary fructose, leading to dysbiosis and consequences thereof. Certainly, our work strengthens the rationale for future human investigation and provides a potential basis for reducing fructose intake in both IBD patients and individuals with infectious colitis. Finally, the current findings have potential implications for nonalcoholic steatohepatitis (NASH). HFrD-induced changes in both the microbiota and gut barrier contribute to experimental NASH. The current findings raise the intriguing possibility that microbiota-mediated changes in BA metabolism may alter the gut barrier, thereby contributing to NASH as well as colitis.

**Materials and Methods**

**Diet Composition and Induction of DSS Colitis**

Mice were fed AIN-93G purified diet (control) containing a mixture of carbohydrates (64 kcal%), HGD (based on AIN-93G) in which all carbohydrate content comes from glucose, HFrD in which all carbohydrate content comes from fructose, or 15 kcal% fructose diet in which 15 kcal% comes from fructose. Additional diets were tested that contained glucose and fructose in ratios of 1:1 or 1:3. Diets were given for 1 or 2 weeks as indicated. The details of the diet compositions are shown in Tables 1 and 2.

To induce colitis, mice were administered 1%–2% DSS (MP Biochemical, Santa Ana, CA) in drinking water for 6–7 days as indicated. Male mice were used in most experiments except where indicated to ensure consistency in disease phenotype because of the known gender differences in...
susceptibility to DSS-induced colitis. For comparisons of colitis severity, measurements of body weight changes and the severity of rectal bleeding and diarrhea were carried out as previously described. Briefly, bleeding was assessed by detection of heme in stool using the Hemoccult Sensa test (Beckman Coulter, Brea, CA) or evidence of gross bleeding.
Diarrhea was assessed by measuring the softness or appearance of the stool on a scale from 0 to 3. At the end of the experiment, mice were euthanized, and colons were excised, length measured, and then flushed with ice-cold phosphate-buffered saline (PBS). Colons were either snap-frozen in liquid nitrogen or fixed in 10% formalin or 4% paraformaldehyde for 4–6 hours, Swiss-rolled, paraffin-embedded, and then sectioned. Sections were stained with H&E to evaluate the severity of histologic injury in a blinded manner by a gastrointestinal pathologist (M. Johncilla). All animal studies were approved by the Institutional Animal Care and Use Committee at Weill Cornell Medicine.

Immune Cell Profiling of the Colonic Lamina Propria

Colons were removed and flushed with ice-cold PBS, slit open longitudinally, and cut into 1-cm pieces. Isolation of cells from the lamina propria was carried out as previously described. Briefly, tissues were placed into dissociation solution (calcium/magnesium free Hank's balanced salt solution, 2% fetal bovine serum, 5 mmol/L EDTA, 1 mmol/L 1,4-dithiothreitol) and put into a shaking incubator for 20 minutes at 37°C to remove mucous and epithelial cells. The solution and tissue were vortexed and strained, and the epithelial dissociation step was repeated. The remaining tissue was placed into digestion solution (RPMI 1640 medium, 10% fetal bovine serum, 1 mg/mL collagenase III, 0.4 u/

### Table 1

| Species                        | Control (% Abundance) | HFrD (% Abundance) |
|--------------------------------|-----------------------|--------------------|
| Clostridium difficile*         | 11.48                 | 2.77               |
| Akkermansia muciniphila*       | 14.66                 | 11.11              |
| Bacteroides uniformis*        | 5.33                  | 4.33               |
| Lactobacillus johnsonii*      | 3.19                  | 0.31               |
| Robinsoniella pseudintermedius| 1.13                  | 1.44               |
| Clostridium sp.*              | 3.50                  | 6.90               |
| Bacteroides acidifaciens*     | 32.06                 | 2.56               |
| Ruminococcus flavefaciens     | 0.86                  | 1.14               |
| Lachnospiraceae pettenkoinii   | 1.25                  | 3.29               |
| Bacteroides uniformis*        | 0.77                  | 1.61               |
| Fusobacterium rectale         | 1.00                  | 1.90               |
| Clostridium sp.*              | 1.60                  | 4.59               |
| Roseburia sp.*                | 0.27                  | 1.20               |
| Lachnospiraceae pettenkoinii   | 0.85                  | 7.84               |
| Lachnospiraceae pettenkoinii   | 0.96                  | 2.45               |
| Allibacter sp.*               | 2.42                  | 0.62               |
| Fusobacterium coprostanolgenes| 0.77                  | 4.40               |
| Sporobacter tertisporus       | 0.75                  | 3.03               |
| Others                        | 17.15                 | 22.02              |

$$P < 0.001$$

Figure 10. Consumption of an HFrD alters fecal gut microbiota. (A and B) Proportions of fecal bacterial populations were determined in mice at baseline and after feeding control or HFrD for 1 week using 16S rRNA analysis. (A) Microbial abundance is shown by principal coordinate analysis (n = 8–9/group). (B) Proportion of fecal bacterial species in mice given control or HFrD for 1 week is shown as percent of total microbes. Those bacteria that are at least 1% of the total are listed by name, and those below 1% were placed into the “others” category. *Abundance is significantly different comparing samples from control vs HFrD. (C) FISH using A muciniphila-specific probe was carried out on sections from mice fed control or HFrD for 1 week. (D) Number of FISH positive A muciniphila at mucus interface was quantified in colonic sections from control or HFrD fed mice (N = 8 per group, median ± standard deviation). Data were generated from single experiments.

Figure 9. Consumption of an HFrD is associated with impaired gut barrier and altered bacterial localization. (A) Representative images showing mucus thickness of PAS/AB stained colons from mice fed control or HFrD for 1 week (original magnification, ×20). White bracket indicates surface mucus in images. (B) Quantification of mucus thickness for samples described in (A) (N = 20–22; median ± standard deviation). Data are combined from 2 separate experiments. $P$ value determined by nonparametric Wilcoxon rank-sum test. (C) Representative images showing PAS staining of colonic sections from mice fed control or HFrD for 1 week. Note the decrease in glycoproteins in mucus from HFrD fed mice compared with control fed mice. (D) Quantification of PAS intensity in colonic mucous from samples described in (D) (N = 6 samples/group). (E) Representative images of colonic sections stained by FISH using a general bacterial probe. White arrows indicate bacteria coming into contact with the colonic epithelium. (F) Quantification of number of bacteria present in the inner mucus layer (N = 5 samples/group; 3 fields per section). (G) Representative images of colonic sections stained by Gram stain. Data shown in (C–G) were generated in a single experiment.
mL dispase, 0.1 mg/mL deoxyribonuclease) and incubated at 37°C in a shaking incubator for 45 minutes to separate lamina propria immune cells. Solution and tissue were strained, the flow-through was centrifuged, and the pellet was resuspended in 40% Percoll and centrifuged. Debris was removed, and the pellet containing lamina propria cells was used for staining. Cells were first stained with brilliant violet 570 viability dye to identify live cells. Blocking of nonspecific binding of isolated cells was carried out by addition of anti-Fc monoclonal antibody, followed by incubation with the following fluorescently conjugated monoclonal antibodies at a dilution of 1:200 on ice for 30 minutes: CD45-Alexa700 (BioLegend, San Diego, CA), CD11c-Pacific Blue (eBioscience, San Diego, CA), CD11b- peridinin chlorophyll protein complex Cyan5.5 (Tonbo Biosciences, San Diego, CA), F/480-phycocerythin-Cyan7 (eBioscience), Ly6G-allophycocyanin (Tonbo Biosciences), MCHII-fluorescein isothiocyanate NK1.1-biotin (Invitrogen, Carlsbad, CA). Phycoerythrin-Texas Red secondary antibody (Invitrogen) was used to detect NK1.1. Flow cytometry was conducted on an LSRII flow cytometer (BD, Franklin Lakes, NJ) with data analyzed using FlowJo software (Tree Star, Ashland, OR). Comparisons of inflammatory cell types on the basis of surface markers using the antibodies described above were made between control diet and HFrD-fed mice to determine the average percentage of inflammatory cells expressing these markers in individual mice.

**Broad-Spectrum ABx Treatment**

To reduce bacterial abundance in the gastrointestinal tract of mice, drinking water containing ampicillin (0.5 g/L) (Millipore-Sigma, Burlington, MA), gentamicin (0.5 g/L) (Millipore-Sigma), metronidazole (0.5 g/L) (Millipore-Sigma), neomycin (0.5 g/L) (Millipore-Sigma), and vancomycin (0.25 g/L) (VWR International, Radnor, PA) was given for 3 weeks before HFrD feeding and DSS exposure and continued throughout the entire experimental period. Splenda (4 g/L) (Heartland Food Products Group, Carmel, IN) was included to mask the bad taste of the ABx and therefore maintain adequate water consumption. Control water contained Splenda only. This ABx cocktail has been successfully used to reduce the microbial population in other studies. Mice were continued on their respective treatments during DSS exposure. Reduced bacterial abundance in the ABx-treated group was confirmed by qRT-PCR using universal bacterial primers (Table 4), as described above.

**GF Mice**

GF mice on a C57BL/6J background were bred in the gnotobiotic facility at Weill Cornell Medicine. To carry out experiments, mice were transferred from the gnotobiotic facility to isolator cages and fed irradiated diet and autoclaved water for the duration of the experiment. Mice were assessed for parameters of disease severity in a sterile biosafety cabinet using sterile gloves to maintain them in a GF state.

**Fecal Transfer**

Eight-week-old C57BL/6j mice were fed control diet for 7 days, followed by either control diet or HFrD for 7 days (n = 5/group). Cecal content and feces were collected from mice fed either diet and resuspended in 5 mL sterile PBS, vortexed vigorously, and then centrifuged, and the clarified supernatant from each sample was pooled in an anaerobic chamber. Eight- to 10-week-old GF mice were orally inoculated with 200 μL of the pooled supernatant from donor mice fed either control diet or HFrD 3 days before 1.5% DSS administration and again on days 0, 2, 4, and 6 of DSS exposure. Recipient mice in both groups were fed control diet during the inoculations and DSS administration.

### Table 3. Total Number of Genes in Metatranscriptomes of Fecal Microbiota

| Phylum          | Control mice | HFrD mice |
|-----------------|--------------|-----------|
| Actinobacteria  | 4406         | 3306      |
| Ascomycota      | 29           | 376       |
| Bacteroidetes   | 27,807       | 25,816    |
| Firmicutes      | 124,686      | 122,382   |
| Proteobacteria  | 2645         | 2535      |
| Streptophyta    | 336          | 226       |
| Tenericutes     | 299          | 290       |
| Verrucomicrobia | 4257         | 4264      |
| Total           | 164,465      | 159,195   |

*Figure 11. Altered gene number in bacteria from mice fed HFrD. Mice were fed control or HFrD for 1 week, and metatranscriptomic profiling was carried out on fecal microbiota (N = 6/group). Total gene number was quantified across genera in both groups.*
Mice

GF Il10−/− 129SvEV colitis-susceptible mice from the National Gnotobiotic Rodent Resource Center, University of North Carolina were inoculated with fecal material from SPF-housed mice by resuspending 10–12 frozen fecal pellets in 10 mL sterile PBS. A sterile cotton swab was used to make fecal slurry. The slurry was then centrifuged at 1500 rpm for 5 minutes to remove the debris. Each mouse was inoculated with 200 mL supernatant by oral gavage twice over 3 days. At the time of fecal microbial colonization the mice were given HFrD or control diet for 6 weeks, and colitis severity was assessed by using numerous endpoints. Fresh stool was evaluated for softness using the stool scoring system as previously described.49 Briefly, stool samples from all mice were scored on the basis of the consistency of the fecal sample as follows: 0 = normal, 1 = loose stool, 2 = loose/some diarrhea, 3 = diarrhea, and 4 = severe watery diarrhea. At the end of the experiment, the small and large intestines were fixed in 10% neutral-buffered formalin (Fisher Chemical, Hampton, NH). Paraffin-embedded sections (5 μm) were stained with H&E, and ileal, cecal, proximal, and distal colon inflammations were quantified by using a well-validated histologic scoring system in a blinded fashion, as previously described.50 The evaluation of inflammation in the entire large intestine was performed by the summation of the scores for each region, with each region being graded from 0 to 4. The total histology score represents the sum of the scores for cecum, colon, and rectum, with a potential maximum score of 12.50

To assess cytokine production in colonic tissue, fresh tissues were thoroughly washed with PBS, shaken at room temperature in RPMI medium containing 50 μg/mL gentamicin (Millipore-Sigma) for 30 minutes at 250 rpm, and then cut into 1-cm pieces. Colonic tissue fragments were blotted dry, weighed, placed (50 mg/well) into 24-well plates (Costar, Corning, NY), and incubated in 1 mL RPMI medium supplemented with 5% fetal bovine serum, 50 μg/mL gentamicin, and 1% antibiotic/antimycotic (penicillin/streptomycin/amphotericin B; GIBCO, Dublin, Ireland) for 20 hours at 37°C in a CO2 incubator.51 Supernatants were collected and stored at −20°C before measurements of IL12p40 and interferon γ by enzyme-linked immunosorbent...
assay according to the manufacturer’s protocols (DuoSet; R&D Systems, Minneapolis, MN). The data were normalized to 50 mg of tissue weight. Lipocalin-2 (Lcn-2) levels were measured in fecal samples collected 0, 2, 4, and 6 weeks after SPF colonization by enzyme-linked immunosorbent assay (DuoSet; R&D Systems) in clarified supernatants generated from frozen fecal samples (5–20 mg) by overnight incubation in PBS with 0.1% Tween 20 (Thermo Fisher Scientific, Waltham, MA). Cytokine mRNA levels in colonic tissues were quantified as detailed below. The Il10−/− mouse study was approved by Institutional Animal Care and Use Committee at University of North Carolina.

**C. rodentium Infection**

*C. rodentium* strain DBS100 (ATCC 51459; American Type Culture Collection, Manassas, VA) was cultured overnight aerobically at 37°C in Lennox broth (Invitrogen). Bacterial concentration was evaluated by measuring absorbance at 600 nm. Before inoculation, mice were fasted for 8 hours and then inoculated with 5 × 10⁹ *C. rodentium* in a total volume of 200 μL. Mice were given access to food after the inoculation. The same culture was used to inoculate all mice in a single experiment to rule out effects of growth variation on disease pathogenesis. Disease severity was assessed over 6 days by monitoring body weight changes, diarrhea, and rectal bleeding (as described above). On day 6 after infection, mice were killed, and colon length was determined. The severity of colonic injury was determined by using a histology score as previously described. Cytokine expression in the colon was measured by qRT-PCR. To evaluate lethality, mice were treated as described for the 6-day study; however after infection, mice were observed for 14 days, and death was recorded.

**qRT-PCR**

In the DSS and *C. rodentium* models of colitis, total RNA was isolated using the RNeasy Mini Kit (Qiagen, Hilden, Germany), and RNA concentrations were measured using the NanoDrop system (Thermo Fisher Scientific). For RNA extraction of tissues exposed to DSS, messenger RNA (mRNA) was isolated using Oligotex mRNA Mini Kit (Qiagen), according to the manufacturer’s instructions. RNA was reverse transcribed to cDNA using the qScript cDNA Synthesis Kit (Quantabio, Beverly, MA). The resulting cDNA was amplified by using gene-specific designed primers or by QuantiTect Primer Assays (Qiagen) (Table 4). Glyceraldehyde-3-phosphate dehydrogenase (Gapdh) was used as an endogenous normalization control for both designed and commercial primers. The amplified products of designed primers were verified by sequencing. qRT-PCR was performed using SYBR No-ROX reagents (Bioline). PCR reaction cycles included 2 minutes at 95°C and 40 cycles of amplification (95°C, 5 seconds; 60°C, 10 seconds; 72°C, 20 seconds). The data were analyzed by the comparative Ct method (2−ΔΔCt).

For qRT-PCR based analysis of bacterial abundance and bacterial gene expression, buffer ASL was added to fecal samples collected 0, 2, 4, and 6 weeks after SPF colonization.
samples, followed by vigorous vortexing until thoroughly homogenized. DNA was extracted by using the QIAamp DNA Stool Mini Kit (Qiagen) per manufacturer’s instructions. For each sample, 8 μg of DNA was used to carry out qRT-PCR using Fast SYBR green PCR master mix and gene-specific primers (Table 4) on a StepOnePlus real-time PCR system. Abundance was reported relative to all bacteria using universal 16S primers by the ddCt method.

16S rRNA Analysis

Feces were collected from individual mice, snap-frozen in liquid nitrogen, and shipped to Molecular Research (Shallowater, TX) for 16S rRNA profiling. DNA extraction from feces was carried out using the Powersoil DNA Kit (Qiagen) per manufacturer’s instructions. After DNA extraction, the 16S rRNA gene V4 variable region was amplified by PCR primers 515/806 with barcode on the forward primer used in a 30-cycle PCR using the HotStarTaq Plus Master Mix Kit (Qiagen) under the following conditions: 94°C for 3 minutes, followed by 30–35 cycles of 94°C for 30 seconds, 53°C for 40 seconds, and 72°C for 1 minute and a final elongation step at 72°C for 5 minutes. The amplified DNA was purified by using calibrated Ampure XP beads for the construction of sequencing libraries. DNA sequencing was performed by using an Illumina HiSeq system (Illumina, San Diego, CA). Sequence data were processed by using MR DNA analysis pipeline. In summary, sequences were joined after depletion of barcodes and short sequences (<150 base pairs) or sequences with ambiguous base calls. Sequences were denoised, operational taxonomic units (OTUs) were generated, and chimeras were removed. OTUs were defined by clustering at 3% divergence (97% similarity). Final OTUs were taxonomically classified by using BLASTn against a curated database derived from RDPII and NCBI (www.ncbi.nlm.nih.gov, http://rdp.cme.msu.edu).

In Vitro Analysis of Bacterial Growth

*Lactobacillus johnsonii* was purchased from American Type Culture Collection (ATCC). The bacterium was maintained on deMan, Rogosa, and Sharpe (MRS) agar (Sigma-Aldrich, St Louis, MO). Liquid cultures were made in sugar-free Lactobacilli MRS Broth (L-MRS; US Biological Life Sciences, Salem, MA) supplemented with either dextrose or fructose at final concentration of 2%. *B pseudolongum* strain UMB-MBP-01 isolated from C57BL/6J mice was a gift from Dr Emmanuel Mongodin’s laboratory (University of Maryland School of Medicine).54 BSM agar (Sigma-Aldrich) was used for maintaining the strain, and L-MRS supplemented with 2% dextrose and 0.05% cysteine (Sigma-Aldrich; Cat. # 168149) was used for liquid cultures. Dextrose (Sigma-Aldrich) or D-fructose (Alfa Aesar; Haverhill, MA) was used for in vitro growth studies.

*L. johnsonii* was grown overnight in L-MRS broth containing 2% dextrose from a single colony on a MRS-agar plate at 37°C without shaking. This stock culture was

| Bile Acid                      | Fold Change (HFrD/Control) | P value |
|--------------------------------|-----------------------------|---------|
| Taurohyodeoxycholic acid       | 23.1                        | <.001   |
| Taurocholate                   | 20.5                        | <.001   |
| Taurodeoxycholate              | 19.1                        | <.001   |
| Tauro-beta-muricholate         | 11.8                        | <.001   |
| Taurochenodeoxycholate         | 10.3                        | <.001   |
| Taurooxycholate                | 5.5                         | <.001   |
| Taurothiolcholate              | 2.1                         | <.001   |
| Taurocholate sulfate           | 1.8                         | <.01    |

Figure 14. Fructose exposure increases fecal conjugated BAs and reduces abundance of BSH-expressing bacteria. (A) Fold change of significantly altered fecal BAs is shown for mice fed HFrD compared with control diet for 1 week (N = 8–9/group). (B) Relative abundance of *B pseudolongum* (left side) and *L johnsonii* (right side) in feces from mice fed control or HFrD for 1 week were determined by qRT-PCR (N = 8–9/group). (C) Expression of BSH in *B pseudolongum* (left side) and *L johnsonii* (right side) was measured by qRT-PCR in the same samples as (B). (D) *L johnsonii* and *B pseudolongum* were cultured in broth containing 2% dextrose or fructose as the carbohydrate source, and the area under the curve (AUC) for the growth rate was measured by optical density every 4–16 hours for up to 52 hours. Data shown in (A–C) were generated in a single experiment, and those in (D) are representative of minimum of 3 independent experiments.
diluted 1:30 in fresh L-MRS broth containing either 2% dextrose or fructose and subjected to growth experiments in an automatic data recording system (Bioscreen C System; Growth Curves, Helsinki, Finland) per manufacturer’s suggestions. Briefly, 200 μL of each diluted culture was transferred into corresponding wells in a 100-well Honeycomb plate (Bioscreen C System), followed by addition of 75 μL of mineral oil per well to achieve a microaerophilic environment-simulating intestinal milieu. The optical density (OD600) was recorded at 15-minute intervals for growth curve analysis.

B pseudolongum was cultured in a vinyl anaerobic chamber (Coy Lab Products, Grass Lake, MI). Because of the slow growth in BSM broth (3–4 days), bacteria were grown in L-MRS broth supplemented with 2% dextrose and 0.05% L-cysteine overnight as a stock culture. Thirty mL of fresh L-MRS supplemented with 0.05% L-cysteine and either 2% dextrose or fructose was inoculated with 1 mL of the overnight stock culture and mixed well. The diluted cultures were aliquoted into 5-mL culture tubes, 1.5 mL per tube, 18 tubes per sugar treatment (dextrose and fructose) (the remaining suspension from each treatment was used for OD600 measurement at time zero). These tubes were incubated in the anaerobic chamber at 37°C without shaking. At each time point, 3 tubes per group were taken out for OD600 measurement to record growth.

**FISH and Gram Stain**

Carnoy’s-fixed paraffin-embedded histologic sections (4 μm) were mounted on Probe-On Plus slides (Fisher Scientific, Pittsburgh, PA) and evaluated by FISH using a combination of eubacterial probe (EUB-338; Cy-3 5’-GCTGCCCTCCGTAGGAGT) and non-EUB-338 (6-FAM-5’-ACTCCTACGGGAGGCAGC) and A muciniphila (5’-S-MUC-1437-a-A-20: Cy-3-5’-CCCTGCGTGGCTCGATG-3’) and the eubacterial probe (EUB-338). Hybridized samples were washed in PBS, allowed to air dry, and mounted with a ProLong antifade kit (Molecular Probes Inc, Eugene, OR). Sections were examined on a BX51 (Olympus America, Melville, NY) epifluorescence microscope, and images were captured with an Olympus DP-7 camera. Probe specificity was controlled by using slides spotted with suspensions of cultured Escherichia coli.
DH5α, Proteus vulgaris, Klebsiella pneumoniae, Streptococcus equi, Streptococcus bovis, and A. muciniphila (ATCC BAA-835). It should be noted that A. muciniphila and other Verrucomicrobia are not recognized by the EUB338 probe. The number of A. muciniphila were manually counted in 8 fields per section across 8 samples per group at ×40 magnification, and quantification of the number of bacteria in the inner mucus layer was carried out on 3 fields per section across 5 samples per group and reported as number of bacteria per 100 μm². Gram staining was carried out as previously described.

**Measurements of Colonic Mucus Thickness and Quality**

A segment of colon containing feces was harvested and immediately preserved in fresh Carnoy’s fixative (methanol:chloroform:glacial acetic acid (60:30:10)) for 4–5 days. Tissues were washed twice in 100% methanol, followed by 2 washes in 100% ethanol. The fixed tissues were then washed in 1:1 ratio of 100% ethanol: xylenes for 15 minutes, followed by two 15-minute washes in xylenes and subsequently embedded in paraffin with a vertical orientation. Paraffin sections (5 μm thick) were cut and deposited on glass slides. AB/PAS staining was performed to stain mucus, as described at www.ihcworld.com. After staining, measurements of mucus thickness in 40–60 regions of each colon were carried out in a blinded manner using Fiji/ImageJ software (National Institutes of Health, Bethesda, MD) as previously described.

To determine differences in quality of mucus, PAS staining was carried out. Stained slides were scanned using an Aperio Image Scope (ScanScope, Aperio, CA). The mucus layer in scanned images was analyzed by Aperio Color Deconvolution algorithm by counting PAS positive pixels.

**Figure 16.** HFrD enhances growth of *C. rodentium* and exacerbates infectious colitis. WT C57BL/6J mice were fed control or HFrD for 1 week followed by infection with *C. rodentium* and continued on their respective diets for 6 days. (A) Abundance of *C. rodentium* was measured in feces every 2 days after infection (N = 6/group). (B) Clinical measurements of colitis severity were carried out during the 6-day period. (C–E) At the end of the experimental period, colon lengths were measured (C), histologic scores were determined and representative histologic images are shown for each group (D), and expression levels of cytokines were quantified (E) (N = 18–25/group). Data are combined from 2 separate experiments.
Quantification of Goblet Cells

After PAS/AB staining of tissue sections for goblet cells, the percent of mucosa occupied by goblet cells was calculated using the Fiji/ImageJ (NIH) through the Weill Cornell Medicine Microscopy and Image Analysis Core Facility. The entire region of interest was outlined and copied onto a separate file and then subjected to a color deconvolution algorithm. The color-deconvolved image representing the mucosa was saved and used to obtain 2 different measurements. To determine the total mucosal area, the color-deconvolved mucosa image was blurred with a large Gaussian blur (sigma = 20) and thresholded using the auto-threshold function “Huang.” This area was masked, measured, and labeled as “mucosal area.” To obtain the goblet cell area, the same color-deconvolved image was smoothed with a small Gaussian blur (sigma = 2) and thresholded using the auto-threshold function “Default.” This area was then masked, measured, and labeled as “goblet cell area.” The ratio of the 2 measurements provided the proportion of mucosal surface occupied by goblet cells.

Metatranscriptomic Analysis of Fecal Microbiota

Frozen fecal samples from 6 control and 6 HFrD mice were used for RNA isolation. Total RNA was purified using Trizol (Thermo Fisher) according to the manufacturer’s protocol. RNA sample quality was confirmed by spectrophotometry (Nanodrop) to determine concentration and chemical purity (A260/230 and A260/280 ratios) and with a Fragment Analyzer (Advanced Analytical, Parkersburg, WV) to determine RNA integrity. Ribosomal RNA was removed from total RNA samples by using the RiboZero Magnetic Gold Epidemiology/Bacteria Kit (Illumina) per manufacturer’s instructions. TruSeq-barcoded RNAseq libraries were generated with the NEBNext Ultra II Directional RNA Library Prep Kit (New England Biolabs, Ipswich, MA). Each library was quantified with a Qubit 2.0 (dsDNA HS kit; Thermo Fisher), and the size distribution was determined with a Fragment Analyzer (Advanced Analytical) before pooling. Libraries were sequenced on a NextSeq500 instrument (Illumina). A total of >20 M single-end reads (75 base pairs) were generated per library. To remove reads from the host (mouse), cleaned RNA reads were first mapped to the mouse genome (GRCm38.p6) using STAR program (https://academic.oup.com/bioinformatics/article/29/1/15/272537). The unaligned reads (bacterial reads) were then assembled by Trinity (http://evomics.org/learning/genomics/trinity/). The de novo assembled transcriptomes were further refined to remove redundant and contaminated transcripts. The longest transcript of each gene was selected as the representative transcript. Functional annotation of these representative transcripts was done by the AHRD pipeline (https://github.com/groupschoof/AHRD). An online annotation server GhostKOALA (https://www.kegg.jp/ghostkoala/) was used to assign each translated protein with a KEGG orthology and was used to reconstruct the KEGG pathways. Gene expression analysis was performed by aligning the bacterial reads to the final transcriptome assembly using Bowtie2 (http://bowtie-bio.sourceforge.net/bowtie2/index.shtml). The raw data were generated from a single experiment.
counts for each gene were calculated by the RSEM pipeline (https://deweylab.github.io/RSEM/) and fed to DESeq (https://bioconductor.org/packages/release/bioc/html/DESeq.html) to identify differentially expressed genes. Genes with false discovery rate < 0.05 and fold change ≥ 2 were defined as differentially expressed genes. To analyze the taxonomic composition of the transcriptome, representative transcripts were blasted against the nr database, and MEGAN (http://ab.inf.uni-tuebingen.de/software/megan6/) was used to place each transcript in its taxonomic position based on its nr hit. The taxonomic compositions were represented at both phylum and genus levels for the whole gene and differentially expressed genes set. Statistical analysis of metatranscriptomic data was performed with GraphPad Prism 8.2 (San Diego, CA) software using the tests as indicated.

**Fecal Metabolomic Analysis**

Frozen freshly collected fecal samples were shipped to Metabolon (Morrisville, NC) for detection of metabolites using a database of more than 4500 named molecules. Samples were lyophilized and then resuspended in water (20 μL/mg of dried sample) before homogenization. After homogenization, 100 μL of the fecal suspensions was used for metabolite extraction using the automated MicroLab STAR system (Hamilton Company, Reno, NV). A recovery standard was added before the first step in the extraction process for quality control purposes. To remove proteins, dissociate

| Table 4. Primer Sequences |
|----------------------------|
| **Primer sequences used for qRT-PCR of mouse tissues** |
| **Gene** | **Type** | **Sequence (5'-3')** |
| Il1b | Forward | TGGGCCCTCAAAGGAAAGAAT |
| | Reverse | CAGGGCTTGTGCCTGCTTTGG |
| Gapdh | Forward | AATGTGTCCTGGTCTGGAATC |
| | Reverse | CATCGAAGGTGGAAGAGTGG |

| **Commercial primers used for qRT-PCR of mouse tissues** |
| **Gene** | **Product name** | **Catalog number** |
| Il6 | Mm_Il6_1_SG QuantiTect Primer Assay | QT00098875 |
| Il17a | Mm_Il17a_1_SG QuantiTect Primer Assay | QT00103278 |
| Il22 | Mm_Il22_1_SG QuantiTect Primer Assay | QT00128324 |
| Gapdh | Mm_Gapdh_3_SG QuantiTect Primer Assay | QT01658692 |

| **Primer sequences for qRT-PCR carried out on tissues from Ifit1-/- mice** |
| **Gene** | **Type** | **Sequence (5'-3')** |
| Ifng | Forward | CTTCCCTAGGGCTTCTGG |
| | Reverse | ACGCTTATGTGTGCTATGG |
| Il1b | Forward | GGGACCTTGGATGAGGTG |
| | Reverse | CAGGGCTTGTGCCTGGAATC |
| Tnf | Forward | GCCTCGTAAAGCTCTGGT |
| | Reverse | ATCCATTGACCGCCTGCCAC |
| GAPDH | Forward | GTCCTCAGGAGGGAGAGTT |
| | Reverse | GTGACCGTACAGATAGACAG |

| **Primer sequences used for measuring relative abundance of bacterial populations** |
| **Gene** | **Type** | **Sequence (5'-3')** |
| Universal bacterial primer | Forward | ACTCCTACGGGAGGCCAGCAG |
| | Reverse | ATACCCCGGGCTGTGCT |
| L johnsonii-BSH | Forward | ATGCTGACTGTGGTCTGAC |
| | Reverse | ACCTTTGACGGCCCTGTCAC |
| L johnsonii | Forward | GCTGTAAGGGTCTGATTG |
| | Reverse | ATGCGCCAGATGGACAGCAG |
| C rodentium-espB | Forward | GCTGCAAGGGCTTCTGCA |
| | Reverse | GCTGACCGTACAGGAGAG |
| B pseudolonum-BSH | Forward | CTGACGGCAGCTTCTGCG |
| | Reverse | CAGGAGCAGGCTTCTGCA |
| B pseudolonum | Forward | ACTACCCGAGGGAGAGTT |
| | Reverse | TATGACTGACAGGTACAG |
| A muciniphila | Forward | AGAGGTCTCAAGGAGAGTT |
| | Reverse | AGAGGTCTCAAGGAGAGT |
| L johnsonii | Forward | TATGACTGACAGGTACAG |
| | Reverse | ACTATGTTGACCGCCTGAC |
small molecules bound to proteins or trapped in the precipitated protein matrix and to recover chemically diverse metabolites, proteins were precipitated with methanol under vigorous shaking for 2 minutes, followed by centrifugation. The resulting extract was divided into 5 fractions: 2 (ie, early and late eluting compounds) for analysis by ultra-high performance liquid chromatography-tandem mass spectrometer (UPLC-MS/MS) (positive ionization), 1 for analysis by UPLC-MS/MS (negative ionization), and 1 for the UPLC-MS/MS polar platform (negative ionization), and 1 sample was reserved for backup. Detection of metabolites was performed by using UPLC-MS/MS, as previously described. Briefly, the UPLC-MS/MS platform used a Waters Acquity UPLC with Waters UPLC BEH C18-2.1×100 mm, 1.7 μm columns, and a Q-Exactive high resolution/accurate mass spectrometer (Thermo Fisher Scientific) interfaced with a heated electrospray ionization (HESI-II) source and Orbitrap mass analyzer operated at 35,000 mass resolution. The sample extract was dried and then reconstituted in acidic or basic LC-compatible solvents, each of which contained 8 or more injection standards at fixed concentrations to ensure injection and chromatographic consistency. One aliquot was analyzed using acidic, positive ion-optimized conditions and the other using basic, negative ion-optimized conditions in 2 independent injections using separate dedicated columns (Waters UPLC BEH C18-2.1×100 mm, 1.7 μm). Extracts reconstituted in acidic conditions were gradient eluted using water and methanol containing 0.1% formic acid, whereas the basic extracts, which also used water/methanol, contained 6.5 mol/L ammonium bicarbonate. A third aliquot was analyzed via negative ionization after elution from a HILIC column (Waters UPLC BEH Amide 2.1×150 mm, 1.7 μm) using a gradient consisting of water and acetonitrile with 10 mmol/L ammonium formate. The MS analysis alternated between MS and data-dependent MS2 scans using dynamic exclusion, and the scan range was from 80 to 1000 m/z. Metabolites were identified by automated comparison of the ion features in the experimental samples to a reference library of chemical standard entries that included retention time, molecular weight, preferred adducts, and in-source fragments as well as associated MS spectra and curated by visual inspection for quality control using software developed at Metabolon. Identification of known chemical entities is based on comparison to metabolomic library entries of purified standards. Commercially available purified standard compounds have been acquired and registered into LIMS for determination of their detectable characteristics. Additional mass spectral entries have been created for structurally unnamed biochemicals, which have been identified by virtue of their recurrent nature (both chromatographic and mass spectral). These compounds have the potential to be identified by future acquisition of a matching purified standard or by classic structural analysis. Peaks were quantified using area under the curve. Raw area counts for each metabolite in each sample were normalized to correct for variation resulting from instrument inter-day tuning differences by the median value for each run-day, therefore setting the medians to 1.0 for each run. This preserved variation between samples but allowed metabolites of widely different raw peak areas to be compared on a similar graphical scale. Missing values were imputed with the observed minimum after normalization.

**Measurement of Fructose Levels**

Fecal samples were snap-frozen after collection and stored at –80°C before use. Feces were dried for 4 hours using an Eppendorf Vacufuge Concentrator 5301 (Eppendorf North America, Enfield, CT). On the basis of the dry weight, fecal samples were reconstituted in distilled water (40 mg/mL) and mechanically disrupted with a TissueLyser II (Qiagen) using stainless steel beads (Qiagen). After centrifugation (12,000 rcf, 4°C, 15 minutes), clarified supernatants were used to measure fructose levels using EnzyChrom Fructose Assay Kit (BioAssay Systems, Hayward, CA), according to the manufacturer’s instructions. Serum was analyzed using the same kit, but no extraction method was used.

**BA Enema**

Mice were anesthetized with 2% isoflurane (Henry Schein Animal Health, Dublin, OH), and the colon was flushed with PBS to clear fecal material. A 50 μL mixture of taurocholic acid (13 mg/mL), taurodeoxycholate (7.2 mg/mL), taurochenodeoxycholate (3.6 mg/mL), and tauro- glycineoxycholate (7.2 mg/mL) (Millipore-Sigma) dissolved in PBS or PBS alone was instilled into the colonic lumen using a p200 pipette tip with large orifice (VWR International, Radnor, PA), coated sparingly with petroleum jelly for a 20-second period. The concentration of BAs used was intended to reflect the increase observed in feces from mice fed an HFrD. Therefore, 20× the amount normally found in the feces of mice was used, an amount adapted from Sayin et al. After administration, the anal verge was sealed with Vetbond Tissue Adhesive (3M, St Paul, MN) to prevent luminal contents from being immediately excreted. Mice were then maintained under anesthesia for an additional 5 minutes. Six hours after the procedure, the anal canal was inspected to make sure it was patent.

**Statistical Analysis**

To evaluate differences in study endpoints, including colon length, histologic score, stool score, expression of cytokines, abundance of bacterial species measured using qPCR at a particular time point, and mucus thickness between 2 experimental groups, the nonparametric Wilcoxon rank-sum test was used. The Welch t test was used to compare the number of immune cells and the area under the bacteria growth curves in fixed time periods between 2 experimental groups. Differences in the continuous endpoints across multiple experimental groups were examined using analysis of variance. Linear contrasts of interest were examined using simultaneous tests of general linear hypotheses, and *P* values were adjusted for multiple comparisons by using Bonferroni-Holm method. Log transformation was used where appropriate to ensure the underlying model assumptions were satisfied. For relative mouse body weight, fecal lipocalin levels, relative abundance of bacterial species, and the occurrence of severe bleeding (score ≥2) or
severe diarrhea (score ≥ 2) measured over time, linear and
generalized linear mixed-effects models were used where
appropriate. Contrasts for parameters of interest including
the rate of change in weight, lipocalin levels, relative
abundance of bacterial species, and average proportion of
mice with severe bleeding or diarrhea were examined using
simultaneous tests of general linear hypotheses. $P$ values
were adjusted for multiple comparisons by controlling the
experiment-wise error rate using the “single-step” imple-
mentation in the R multcomp package. Correlation in the
abundance of different bacterial species measured using
qPCR was assessed using Spearman’s method. All the above
described analyses were carried out in R.

For analysis of 16S microbiota abundance data, an
empirical Bayes inference modeling framework was
used. Computational implementation was carried out
using a variant of the Expectation-Maximization Algorithm
for mixture models. Salerno et al developed the RRmix
using a variant of the Expectation-Maximization Algorithm
model of latent factors. The sparsity of the bacteria effects is
captured through the inclusion of latent factors. The sparsity of the bacteria effects is accounted for by a mixture component in the model. The
model’s inputs are the log-transformed, standardized bacteria
abundance values and the diet group indicator; the outputs of
the model are a probabilistic assessment of whether a particular bacterium is differentially abundant in the 2 diet
groups. If the estimated posterior probability of positive
identification is greater than a chosen threshold (eg, 0.8),
then the bacterium is said to be significantly different be-
tween the 2 study diets. This empirical Bayes approach
controls for false discoveries.

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