Colitis-Induced Microbial Perturbation Promotes Postinflammatory Visceral Hypersensitivity

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SUMMARY

Our understanding of the pathophysiological mechanisms underlying chronic pain in inflammatory bowel disease is incomplete. Here we show that microbial manipulation modulates the development of visceral, but not somatic, pain in a mouse model of postinflammatory dextran sodium sulfate colitis.

BACKGROUND & AIMS: Despite achieving endoscopic remission, more than 20% of inflammatory bowel disease patients experience chronic abdominal pain. These patients have increased rectal transient receptor potential vanilloid-1 receptor (TRPV1) expression, a key transducer of inflammatory pain. Because inflammatory bowel disease patients in remission exhibit dysbiosis and microbial manipulation alters TRPV1 function, our goal was to examine whether microbial perturbation modulated transient receptor potential function in a mouse model.

METHODS: Mice were given dextran sodium sulfate (DSS) to induce colitis and were allowed to recover. The microbiome was perturbed by using antibiotics as well as fecal microbial transplant (FMT). Visceral and somatic sensitivity were assessed by recording visceromotor responses to colorectal distention and using hot plate/automated Von Frey tests, respectively. Calcium imaging of isolated dorsal root ganglia neurons was used as an in vitro correlate of nociception. The microbiome composition was evaluated via 16S rRNA gene variable region V4 amplicon sequencing, whereas fecal short-chain fatty acids (SCFAs) were assessed by using targeted mass spectrometry.

RESULTS: Postinflammatory DSS mice developed visceral and somatic hyperalgesia. Antibiotic administration during DSS recovery induced visceral, but not somatic, hyperalgesia independent of inflammation. FMT of postinflammatory DSS stool into antibiotic-treated mice increased visceral hypersensitivity, whereas FMT of control stool reversed antibiotics’ sensitizing effects. Postinflammatory mice exhibited both increased SCFA-producing species and fecal acetate/butyrate content compared with controls. Capsaicin-evoked calcium responses were increased in naive dorsal root ganglion neurons incubated with both sodium butyrate/propionate alone and with colonic supernatants derived from postinflammatory mice.

CONCLUSIONS: The microbiome plays a central role in postinflammatory visceral hypersensitivity. Microbial-derived
SCFAs can sensitize nociceptive neurons and may contribute to the pathogenesis of postinflammatory visceral pain. (Cell Mol Gastroenterol Hepatol 2020;10:225–244; https://doi.org/10.1016/j.jcmgh.2020.04.003)

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Inflammatory bowel diseases (IBDs), including Crohn’s disease and ulcerative colitis, are chronic, debilitating illnesses with high socioeconomic burden and increasing prevalence. Despite achieving endoscopic remission, more than 20% of IBD patients experience chronic abdominal pain, which is associated with high levels of anxiety, depression and poor quality of life. Furthermore, studies demonstrate increased prevalence of widespread somatic pain in the absence of inflammation in IBD, indicating altered sensory neural processing in this condition. Unfortunately, effective treatments for chronic pain are severely limited, such that 5%-25% of IBD patients are on chronic narcotic therapy, use of which is not only ineffectual but leads to increased mortality.

The gut microbiome affects a wide variety of gastrointestinal processes. There is evidence that some IBD patients in remission exhibit persistent changes in the microbiome, although it is not known whether these changes are a cause or consequence of previous inflammation, dietary changes, or altered gastrointestinal transit. IBD patients in endoscopic remission with chronic pain exhibit elevated rectal transient receptor potential vanilloid-1 receptor (TRPV1) expression, which correlated with the severity of abdominal pain. Interestingly, there is evidence that microbial products can directly stimulate nociceptors via TRP channels, whereas microbial manipulation results in altered function and expression of molecular targets in pain signaling such as TRPV1. This suggests a potential link between dysbiosis and chronic visceral pain in the absence of inflammation in IBD through the microbial modulation of TRP receptors.

Our goal was to examine whether microbial perturbation in the postinflammatory state contributed to persistent visceral hypersensitivity. We chose to use the postinflammatory dextran sodium sulfate (DSS) mouse model of colitis because this is an established animal model of chronic visceral pain, in which animals display increased visceral hypersensitivity 5 weeks after DSS administration in the absence of inflammation. Postinflammatory visceral hyperalgesia was found to be dependent on TRPV1 in this model, because TRPV1 expression in afferent nerves was elevated in the postinflammatory state, similar to IBD patients in endoscopic remission with chronic pain. We also examined somatic pain in this model because of the data demonstrating increased prevalence of noninflammatory somatic pain in IBD. To manipulate the microbiome, we administered a broad-spectrum antibiotic cocktail to mice and also performed fecal microbial transplant (FMT). We set out to test whether the dysbiotic microbiome in the postinflammatory state drives nociceptor sensitization through TRPV1 regulation and thus may contribute to the transition from acute to chronic pain.

### Results

**Postinflammatory Microbial Perturbation Leads to Visceral Hyperalgesia But Not Somatic Hypersensitivity**

To evaluate the effects of inflammation-induced microbial perturbation on visceral pain, mice were treated with 2.5% DSS in the drinking water for 5 days (DSS) or water alone (control [CT]) and allowed to recover for 5 weeks. These mice were divided into 2 subgroups and treated with antibiotics (Abx) for the last 2 weeks of recovery (CT + Abx; DSS + Abx). Mice exposed to DSS showed signs of clinical disease including significant weight loss (Figure 1B) and loose stools but recovered after 5 weeks and did not exhibit macroscopic or microscopic intestinal inflammation at the time of death, as previously described (Figure 1C-E). Similarly, Abx treatment did not result in any macroscopic inflammation (Figure 1C and D).

The visceromotor response to colorectal distention was increased in postinflammatory DSS mice when compared with control mice given water only, similar to that seen previously (area under the curve for colorectal distention: control, 0.1 ± 0.01 vs DSS, 0.16 ± 0.01; P < .01; Figure 2A). Microbial disruption with Abx during colitis recovery induced visceral allodynia and hyperalgesia in Abx-treated mice (visceromotor response at 30 mm Hg: control, 0.01 ± 0.03 vs control + Abx, 0.05 ± 0.0; P < .05; area under the curve for colorectal distention: control, 0.1 ± 0.01 vs DSS + Abx, 0.22 ± 0.04; P < .01; Figure 2A), independent of DSS treatment.

To determine whether microbial manipulation was able to modulate somatic pain, we assessed both mechanical and thermal hypersensitivity in animals treated with DSS and Abx. Mechanical sensitivity was assessed by using the automated Von Frey test in the hind paw, and thermal sensitivity was evaluated by using the hot plate test. Mice treated with DSS developed somatic hyperalgesia in both mechanical (Von Frey test force: control, 7.7 ± 0.24 g vs DSS, 6 ± 0.23 g; P < .0001; control + Abx, 8.1 ± 0.25 g vs DSS + Abx, 6.6 ± 0.19 g; P < .001; Figure 2B) and thermal (time on 52°C hot plate: control, 10.6 ± 0.51 seconds vs DSS, 7.9 ± 0.40 seconds; P < .001; control + Abx, 10.8 ± 0.37 seconds vs DSS + Abx, 8.1 ± 0.25 g; P < .001; Figure 2B) paradigms.

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**Abbreviations used in this paper:** Abx, antibiotics; ASV, amplicon sequence variant; DMEM, Dulbecco modified Eagle medium; DRG, dorsal root ganglion; DSS, dextran sulfate sodium; FMT, fecal microbial transplant; HBSS, Hanks’ balanced salt solution; IBD, inflammatory bowel disease; IBS, irritable bowel syndrome; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; SCFA, short-chain fatty acid; TRPA1, transient receptor potential ankyrin-1 receptor; TRPV1, transient receptor potential vanilloid-1 receptor.

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Figure 1. Postinflammatory DSS mice exhibit no signs of macroscopic or microscopic inflammation. (A) Experimental protocol. Colitis was induced using 2.5% DSS for 5 days. Mice were allowed to recover for 3 weeks and then antibiotics (Abx) were administered in drinking water for 2 weeks before death. (B) DSS-treated animals initially lose weight but catch up to their control counterparts. Two-way analysis of variance, control (CT) vs DSS, ****P < .0001; CT vs DSS + Abx, +++++P < .0001. Controls (CT); n = 12, DSS; n = 15, CT + Abx; n = 11, DSS + Abx; n = 10. Macroscopic inflammation was assessed by using colon thickness (C) and length (D). CT: n = 17, DSS: n = 20, CT + Abx and DSS + Abx, n = 8. Microscopic inflammation was assessed by myeloperoxidase activity quantification (E) between control (n = 10) and DSS (n = 7) groups. DSS, dextran sulfate sodium.
To evaluate whether Abx exerted a direct effect on visceral pain, Abx were directly administered into the colon, and visceromotor responses were tested before and 1 hour after administration of either vehicle (sterile saline) or Abx into the colon.31 No differences were seen in visceral sensitivity between groups (area under the curve for colorectal distention: before vehicle: 0.63 ± 0.20, after vehicle: 0.73 ± 0.14, before Abx: 0.59 ± 0.13, after Abx: 0.73 ± 0.14; one-way analysis of variance, \( P = .65; n = 6/\) group; Figure 2D).

Microbial Reconstitution After Antibiotics Treatment Reverses Changes in Visceral, But Not Somatic, Hyperalgesia

To assess the role of Abx-induced microbial disruption in visceral pain, Abx-treated animals were given a 2-week
washout period in which they were given water only (Figure 3A). Seven weeks after DSS, the visceromotor response to colorectal distention in postinflammatory DSS mice was still significantly higher than controls (area under the curve for colorectal distention: control, 0.07 ± 0.01 vs DSS, 0.15 ± 0.04; P < .05; Figure 3B). Interestingly, visceral hypersensitivity recovered to control levels in control + Abx and DSS + Abx mice after 2 weeks of Abx washout. However, somatic hyperalgesia was unaffected by Abx washout (Figure 3C and D; Von Frey test force: control, 7.4 ± 0.16 g vs DSS, 5.5 ± 0.11 g; P < .001; control + Abx, 7.2 ± 0.27 g vs DSS + Abx, 5.8 ± 0.12 g; P < .001; time on 52°C hot plate: control, 13 ± 0.8 seconds vs DSS, 10.2 ± 0.6 seconds; P < .05; control + Abx, 13.9 ± 0.71 seconds vs DSS + Abx, 10.2 ± 0.34 seconds; P < .01), suggesting that microbial manipulation modulates the development of visceral, but not somatic, pain.

Gut Microbiota and Stool Short-Chain Fatty Acid Content Differ Between Control and Postinflammatory Dextran Sulfate Sodium Mice

Microbes and bacterial metabolites such as short-chain fatty acid (SCFA) are known to activate intestinal epithelial cells and extrinsic spinal neurons, suggesting that they may play a role in visceral hypersensitivity. Stool was collected from mice at baseline and at the time of death for microbial composition and SCFA analysis.

Examination of the microbiome composition demonstrated that the alpha and beta diversity were not significantly different when comparing control and postinflammatory DSS mice at the 5-week recovery time point, whereas Abx treatment resulted in a marked disruption characterized by a shift in both alpha and beta diversity at the operational taxonomic unit level (n = 6/group except DSS + Abx baseline, n = 7; control baseline, n = 5; and DSS + Abx group at death, n = 4; alpha diversity: linear mixed-effects model, P = .0072; beta diversity: permutational multivariate analysis of variance, P < .001; Figure 4A and B). When compared at the phylum and genus levels, the composition of all 4 groups was similar at baseline (Figure 4C). As expected, Abx treatment resulted in profound shifts in the microbiota characterized by an overgrowth of proteobacteria, primarily Klebsiella species (Figure 4C; beta-binomial regression, P < .01). Examined at the 5-week recovery period, DSS treatment resulted in a relative decrease in Bacteroidetes and an increase in Firmicutes phylum compared with controls, as previously described (Figure 4D); in addition, an increase in Verrucomicrobia, which has been linked with gut health in some studies, was seen over time in controls. At the genus level, a significant increase in Lachnospiraceae NK4A136 and FCS020 groups as well as Ruminococcus was seen, whereas a significant decrease in Akkermansia, Alistipes, Muribaculaceae, Ruminococcaceae, and Bacteroides species was apparent in postinflammatory DSS mice when compared with controls (Figure 4D; beta-binomial regression, P < .01). After antibiotic washout (Figure 3A), there was partial recovery of the microbiome in Abx-treated mice (Figure 5A and B) at the 7-week time point. However, DSS treatment resulted in a persistent increase in Lachnospiraceae NK4A136 and FCS020 groups as well as Ruminococcus groups at the 7-week time point (Figure 5C) when compared with controls.

The subtle microbial perturbation observed in postinflammatory DSS mice was associated with an increase in fecal acetate (acetate [mmol/L]: control, 2.2 ± 0.02 vs DSS, 4.4 ± 0.1; P < .05) and butyrate (butyrate [mmol/L]: control, 1 ± 0.02 vs DSS, 2.3 ± 0.04; P < .05) but not propionate when compared with controls (Figure 6A). SCFA concentration was below the limit of detection in Abx-treated animals. After antibiotic washout, SCFA concentration in Abx-treated animals was detectable but significantly lower for butyrate and propionate compared with the control and DSS groups, respectively (Figure 6B), suggesting partial restoration after Abx washout. No differences were observed between control and DSS groups.

Fecal Microbial Transplantation of Postinflammatory Dextran Sulfate Sodium Microbiota Transfers the Phenotype of Visceral, But Not Somatic, Pain

To determine whether the dysbiotic microbiome was necessary for the expression of visceral hypersensitivity, we performed FMT experiments. Mice were treated with 2 weeks of Abx before FMT to disrupt the basal microbiome (Figure 7A). Twenty-four hours after the cessation of Abx, mice received 4 days of oral gavage with homogenized stool derived from control mice, postinflammatory DSS mice, or vehicle alone (phosphate-buffered saline [PBS] + glycerol). Animals treated with postinflammatory DSS stools developed visceral hypersensitivity compared with mice given vehicle or control stool (area under the curve for colorectal distention: FMT PBS, 0.06 ± 0.007 vs FMT DSS, 0.12 ± 0.02; P < .01; FMT control, 0.05 ± 0.006 vs FMT DSS, 0.12 ± 0.02; P < .001; Figure 7B). Interestingly, mice given vehicle displayed hyperalgesia at 60 mm Hg compared with mice given control stool (visceromotor response at 60 mm Hg: FMT control, 0.03 ± 0.004 vs FMT PBS, 0.06 ± 0.007; P < .05; Figure 7B), suggesting that the sensitizing effect of Abx alone on visceral sensitivity could be reversed by FMT from control mice. In contrast, FMT did not transfer somatic hyperalgesia observed in postinflammatory mice (Figure 7C and D), suggesting that the microbiome plays a central role in the pathogenesis of postinflammatory visceral, but not somatic, hypersensitivity.

Examination of the SCFA profile of recipient mice revealed that FMT was associated with an increase in fecal propionate (propionate [mmol/L]: FMT PBS, 0.147 ± 0.044 vs FMT DSS, 0.6 ± 0.087; P < .0001; FMT control, 0.337 ± 0.041 vs FMT DSS, 0.6 ± 0.087; P < .05) and butyrate (butyrate [mmol/L]: FMT PBS, 0.073 ± 0.028 vs FMT DSS, 0.509 ± 0.103; P < .001; FMT control, 0.144 ± 0.021 vs FMT DSS, 0.509 ± 0.103; P < .01) in the stool of recipients given postinflammatory DSS stool compared with those given vehicle or control stool (Figure 7E). These data suggest that postinflammatory visceral hypersensitivity is associated
Figure 3. Microbial recovery after antibiotic administration reverses changes in visceral hyperalgesia. (A) Experimental protocol. Colitis was induced by using 2.5% DSS for 5 days, and animals were allowed to recover. Antibiotics (Abx) were administered in drinking water from days 28–42; animals were then given a 2-week washout period. (B) Visceral pain was assessed by using the visceromotor response to colorectal distention. One-way analysis of variance of area under the curve of DSS vs CT: *P < .05. CT: n = 11, CT + Abx and DSS: n = 12, DSS + Abx: n = 15. Somatic pain was assessed by using the automated Von Frey (C) and hot plate (D) tests. One-way analysis of variance: *P < .05; **P < .01; ***P < .001; ****P < .0001. CT and DSS: n = 7; CT + Abx and DSS + Abx: n = 8 for automated Von Frey test and n = 14/group for hot plate test (4 experiments). CT, control; DSS, dextran sulfate sodium.
with microbial shifts resulting in an increase in fecal SCFA, in particular butyrate.

**Supernatant From Postinflammatory Dextran Sulfate Sodium Mice Colon Increases Transient Receptor Potential Vanilloid-1 Receptor Function in Naive Dorsal Root Ganglion**

To evaluate in vitro correlates of nociceptor activation, we assessed the function of cultured mouse dorsal root ganglion (DRG) neurons expressing TRPV1 and transient receptor potential ankyrin-1 receptor (TRPA1) using calcium imaging and examined responses to the TRPV1 agonist, capsaicin, and the TRPA1 agonist, mustard oil.37–39 We incubated cultured T9-L2 (colonic projecting)40,41 DRG neurons with sterile-filtered colonic supernatants, derived from a total of 6 mice, 5 weeks after DSS or control administration (Figure 1A). Thus, we exposed naive TRP-expressing DRG neurons to the nociceptive mediators present in the colon.39 TRPV1 responses to 100 nmol/L capsaicin stimulation were significantly increased in naive colonic DRGs incubated with colonic supernatant derived from postinflammatory DSS mice compared with controls (area under the curve capsaicin 100 nmol/L: control, 202.4 ± 15.9 vs DSS, 309.8 ± 26.5; P < .05) (Figure 8A). In contrast, TRPA1 responses to mustard oil were not different between groups (Figure 8B). We also evaluated responses to capsaicin and mustard oil in hind paw projecting42 L4-L5 DRG neurons from postinflammatory DSS mice. TRPV1 responses in L4-L5 DRGs from DSS and DSS + Abx treated mice were significantly increased in response to 100 nmol/L capsaicin compared with control mice (area under the curve capsaicin 100 nmol/L: control, 99.5 ± 9.2 vs DSS, 133.4 ± 16.1; control, 99.5 ± 9.2 vs DSS + Abx, 128.5 ± 6.7; P < .05) (Figure 8C). No differences were observed in TRPA1 agonist responses between groups (Figure 8D).

Because fecal butyrate content was significantly elevated in the stool of postinflammatory DSS mice, as well as in recipient mice given postinflammatory DSS stool, we investigated the direct effect of SCFA on TRPV1 sensitization. Naive colonic DRG neurons were incubated with sodium acetate (5 mmol/L), sodium butyrate (1 mmol/L), and sodium propionate (1 mmol/L), and responses to capsaicin were examined. TRPV1 responses were increased in the presence of sodium butyrate and sodium propionate but not sodium acetate (area under the curve capsaicin 100 nmol/L: control, 136.7 ± 5.9 vs butyrate, 174.9 ± 11.2; control, 136.7 ± 5.9 vs propionate, 169 ± 7.2) (Figure 8E) when compared with media alone, suggesting that microbial-derived soluble mediators modulate TRP sensitization.

**Discussion**

IBD patients in endoscopic remission with chronic abdominal pain display decreased pain thresholds when compared with healthy controls,43,44 suggesting activation of pro-nociceptive pathways and/or suppression of anti-nociceptive pathways in these patients. It is well-known that the gut microbiome plays a key role in the pathogenesis of IBD;3 however, the role of the microbiome in the development of chronic visceral pain in IBD is poorly understood. We have demonstrated that microbial manipulation, through the use of Abx or FMT in the postinflammatory DSS mouse, modulates the development of visceral, but not somatic, pain. SCFA-producing species and fecal SCFA content were increased in postinflammatory DSS mice, whereas incubation of cultured DRG neurons with SCFAs in vitro leads to sensitization of TRPV1, suggesting that microbial-derived soluble products such as SCFA are able to sensitize nociceptive neurons.

Chronic visceral pain is a disorder of the gut-brain axis, and both central and peripheral mechanisms contribute to its pathogenesis.3 Painful sensation from the gut is relayed to the central nervous system by polymodal nociceptors, which transduce mechanical and chemical stimuli.45 TRPV1 is expressed on a subset of nociceptive peptidergic neurons and transduces inflammatory injury.45 TRPV1 sensitization has been shown to participate in the generation of neuropathic pain in IBD by provoking the release of neuropeptides such as substance P and calcitonin gene-related peptide from peripheral terminals.26,46 In conjunction with other inflammatory mediators released during injury or infection, neuropeptides have immunoregulatory properties and can in turn increase nociceptor excitability.45,46 Thus, TRPV1 sensitization is instrumental in the generation of inflammatory hyperalgesia, or exaggerated pain responses, and alldynia, or pain caused by innocuous stimuli.45 Furthermore, TRPV1 expression is increased in both patients with acute flares of IBD and experimental models of acute colitis,26,47 as well as in IBD patients with chronic abdominal pain and endoscopic remission.15 Because of the pivotal importance of TRPV1 in pain in IBD, we chose to use the postinflammatory DSS mouse model where TRPV1 sensitization was shown to play a crucial role in the generation of chronic postinflammatory visceral pain.24

We found that in vitro incubation of naive cultured T9-L2 DRG neurons (colonic projecting)40,41 with...
### A

**Alpha diversity (Shannon)**

- **Timepoint:**
  - Baseline
  - Washout

### B

**PCoA - Bray**

- **Groups:**
  - CT
  - CT + Abx
  - DSS
  - DSS + Abx

### C

| Genus                        | DSS | Family                     | Phylum            |
|------------------------------|-----|----------------------------|-------------------|
| Olsenella                    | 1   | Atopobiacaeae              | Actinobacteria    |
| Clostridium_sensu_stricto    | 1   | Clostridiaceae             | Firmicutes        |
| Unknown                      | 1   | Clostridiales_vadinBB60_group |               |
| Turicibacter                 | 1   | Erysipelotrichaceae        |                   |
| Unknown                      | 1   | Family_XIII                |                   |
| A2                           | 2   |                            |                   |
| Acetatitactor                | 2   |                            |                   |
| Anaerostipes                 | 1   |                            |                   |
| ASF356                       | 1   |                            |                   |
| Blautia                      | 1   |                            |                   |
| Eisenbergiella               | 1   |                            |                   |
| GCA-900066575                | 1   |                            |                   |
| Lachnoclostridium_5          | 1   | Lachnospiraceae            |                   |
| Lachnospiraceae_FCS020_group | 2   |                            |                   |
| Lachnospiraceae_NK4A136_group| 2   |                            |                   |
| Unknown                      | 3   |                            |                   |
| Romboutsia                   | 1   | Peptostreptococcaceae      |                   |
| Oscillibacter                | 1   |                            |                   |
| Ruminiclostridium            | 1   |                            |                   |
| Ruminiclostridium_9          | 2   |                            |                   |
| Ruminococcaceae_UCG-003      | 1   | Ruminococcaceae            |                   |
| Ruminococcaceae_UCG-014      | 1   |                            |                   |
supernatants from postinflammatory DSS mice, but not supernatants from Abx-treated animals, resulted in increased intracellular calcium in response to TRPV1 stimulation with capsaican. Furthermore, stimulation of L4-L5 DRGs (hind paw projecting\(^5\)) derived directly from postinflammatory DSS mice with capsaican also resulted in increased intracellular calcium responses when compared with neurons derived from Abx-treated mice alone. This parallels our in vivo data where previous inflammation resulted in somatic and visceral pain. Abx treatment induced visceral hyperalgesia alone; this effect was reversed by a 2-week antibiotic washout period. These data suggest that Abx treatment and previous inflammation cause visceral hyperalgesia through different mechanisms. It should be noted that we did not use retrograde tracers to specifically evaluate colonic projecting neurons, because this technique requires additional surgery/anesthesia and adds increased stress to mice, which itself could lead to visceral pain.\(^{16,49}\) Previous studies have demonstrated that incubation of naive cultured T9-L2 DRG neurons with sterile-filtered colonic supernatants results in similar findings to direct assessment of colonic projecting neurons.\(^{50}\)

Postinflammatory DSS mice exhibited somatic as well as visceral hyperalgesia, demonstrating both mechanical and thermal hyperalgesia in our model. This was not affected by Abx treatment. Similarly, FMT of postinflammatory DSS stool did not result in the transfer of somatic hyperalgesia when compared with control stool or vehicle, suggesting that microbial manipulation does not modulate somatic hyperalgesia. Instead, our data support the idea that the somatic hyperalgesia in our model results from the initial inflammatory insult and is maintained centrally. There is evidence that somatic hyperalgesia results from central sensitization in visceral pain models.\(^{22,25,51-53}\) For example, mice given DSS for 7 days were shown to display increased neuronal activation in the spinal cord, thalamus, hypothalamus, amygdala, and prefrontal cortex in a previous study.\(^{54}\) This was associated with mechanical and thermal hyperalgesia of the plantar skin in mice, suggesting that acute colitis caused central sensitization, which then resulted in somatic hyperalgesia.\(^{54}\) In another study of postinflammatory DSS mice, capsaican instillation in the colon caused neuronal activation in the dorsal horn, which correlated with somatic hyperalgesia of the abdominal wall, suggesting that in the postinflammatory model, referred somatic pain was secondary to central sensitization.\(^{25}\) A study in rats demonstrated that broad-spectrum Abx use did not result in thermal hyperalgesia,\(^{22}\) similar to our own data. Conversely, chemotherapy-induced mechanical hyperalgesia was reduced in Abx-treated as well as germ-free mice.\(^{25}\) In another study that examined somatic hyperalgesia, broad-spectrum Abx treatment alone resulted

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**Figure 5.** Postinflammatory DSS mice exhibit persistent changes in the microbiome. The alpha ($A$) and beta ($B$) diversity was assessed in controls, postinflammatory DSS, and mice given antibiotics and then allowed a 2-week washout period. Baseline samples were not different; antibiotic washout allowed some degree of microbial recovery, although there was still a significant difference in the alpha diversity when comparing CT vs CT + Abx groups (Tukey honestly significant difference; $P = .0067$) and in beta diversity when comparing CT vs CT + Abx groups and DSS vs DSS + Abx groups (permutational multivariate analysis of variance, CT vs CT + Abx, $P = .001$; DSS vs DSS + Abx, $P = .003$). Points represent individual samples, lines represent the median, and crosses represent the mean. ($C$) Comparison of bacterial content at the genus level between control and postinflammatory DSS mice at the washout time point. Blue symbols represent families significantly increased in the post-inflammatory DSS mice, and red symbols represent families significantly increased in controls (beta-binomial regression model, $P < .01$). $N = 6–8$ group. Abx, antibiotics; CT, control; DSS, dextran sulfate sodium.

|        | CT           | DSS          | CT+abx        | DSS+abx       |
|--------|--------------|--------------|---------------|---------------|
| Acetate (mM) | 2.1 ± 0.1   | 1.9 ± 0.1   | 2.3 ± 0.2     | 2.2 ± 0.2     |
| Butyrate (mM) | 3.2 ± 0.2   | 3.4 ± 0.3   | 3.6 ± 0.4     | 3.8 ± 0.5     |
| Propionate (mM) | 0.8 ± 0.1 | 0.7 ± 0.1   | 0.9 ± 0.2     | 0.8 ± 0.1     |

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**Figure 6.** Postinflammatory DSS mice exhibit elevated stool short-chain fatty acids. ($A$) Stool short-chain fatty acid content in postinflammatory DSS mice and controls. In antibiotic-treated animals (Abx), short-chain fatty acid concentration was below the limit of detection for butyrate and propionate. $N = 6$ group. ($B$) Stool short-chain fatty acid content after 2 weeks of antibiotic washout. CT and CT + Abx: $n = 7$, DSS and DSS + Abx: $n = 6$. One-way analysis of variance: *$P < .05$; **$P < .01$. Abx, antibiotics; CT, control; DSS, dextran sulfate sodium.
Figure 7. Fecal microbial transplant of postinflammatory DSS stool transfers the phenotype of visceral but not somatic pain and is associated with increased stool short-chain fatty acids. (A) Experimental protocol. Antibiotic-treated recipient animals received stool via gavage from postinflammatory DSS mice, controls (CT), or vehicle (sterile phosphate-buffered saline + glycerol) alone. (B) Visceral pain was increased in mice given postinflammatory DSS stool when compared with control and vehicle. Vehicle: n = 10, CT: n = 7, DSS: n = 11, 2 experiments. One-way analysis of variance of area under the curve: **P < .01; ***P < .001. Mice given vehicle displayed hyperalgesia at 60 mm Hg compared with mice given control stool (two-way analysis of variance, **P < .05). Somatic pain, assessed by using automated Von Frey (C) and hot plate tests (D), was unaffected after fecal microbial transplant. Vehicle: n = 9, CT: n = 7, DSS: n = 8, 2 experiments. (E) Fecal butyrate and propionate are increased in the stool of recipient mice given stool from postinflammatory DSS animals when compared with those that received stool from controls or vehicle. Vehicle: n = 12, CT: n = 7, DSS: n = 11. **P < .01; ***P < .001; ****P < .0001. DSS, dextran sulfate sodium.
in mechanical hyperalgesia to the von Frey hair test and the tail-flick test in rats.\textsuperscript{59} Taken together, these data suggest that although central sensitization may underlie somatic pain in the postinflammatory state, any role of the microbiome in this phenomenon needs to be examined more closely.

It was striking that Abx treatment alone results in profound visceral hyperalgesia. Metronidazole and neomycin in particular are known to have neurotoxic and ototoxic effects\textsuperscript{57–59}; however, visceromotor responses were unchanged after direct catheter infusion of Abx vs vehicle, suggesting that responses in Abx-treated animals were not solely due to neurotoxicity. Previous studies of Abx-treated animals have also found increased visceral pain after Abx treatment. In a seminal study where mice were treated with a 10-day course of oral Abx, animals developed hyperalgesia to colorectal distention, which was associated with increased colonic substance P expression.\textsuperscript{61} In a study where neonatal mice were treated with 10 days of Abx, animals developed persistent visceral pain at 10 weeks of age.\textsuperscript{60} Interestingly, this was associated with decreased TRPV1 expression in the lumbosacral spinal cord; no evidence of inflammation was seen.\textsuperscript{60} Conversely, when adult mice were treated with 6 weeks of broad-spectrum Abx, visceral pain to colorectal distention was markedly attenuated, and this was also associated with decreased TRPV1 expression in the lumbosacral spinal cord.\textsuperscript{62} Consistent with these data, germ-free mice displayed visceral hyperalgesia to colorectal distention, an effect that was reversed by bacterial colonization; TRPV1 expression was unchanged in this model.\textsuperscript{51} Together, these data suggest that commensal microbes may be anti-nociceptive; Abx treatment or absence of the microbiome seems to abrogate these anti-nociceptive effects. Indeed, probiotics, such as Lactobacillus and Bifidobacterium species, have been shown to decrease the visceromotor response to colorectal distention in animal models\textsuperscript{51,62}; the probiotic Lactobacillus reuteri DSM 17938 was able to decrease the capsaicin-induced increase in intracellular calcium in rat DRG neurons and reduce the response of distention evoked firing of spinal nerves, an effect that was abolished in TRPV1 knockout mice.\textsuperscript{63} Faecalibacterium prausnitzii, a butyrate-producing member of the Firmicutes phyla, was found to decrease the excitability of DRG neurons through a protease-dependent mechanism.\textsuperscript{15} In the current study, we observed that Abx-induced visceral hypersensitivity and dysbiosis were independent of SCFAs, whereas microbial shifts in the postinflammatory DSS model and subsequent visceral hypersensitivity were dependent on SCFA and TRPV1 sensitization. Thus, it is possible that Abx treatment results in dysbiosis and the loss of anti-nociceptive species/soluble mediators, resulting in visceral hyperalgesia.

Postinflammatory DSS mice exhibited microbial shifts in our study, with a relative decrease in Bacteroidetes and an increase in the Firmicutes phyla; close examination at the genus level revealed a relative increase in SCFA-producing species such as Lachnospiraceae and Ruminococcus. SCFAs such as butyrate are a crucial source of energy for colonicocytes and are important in maintaining gut barrier integrity and inhibiting inflammation.\textsuperscript{20,64} Our data are in agreement with a previous study that examined the microbiota after 3 cycles of DSS in mice, which also found an increase in Lachnospiraceae species.\textsuperscript{34} The increase in SCFA-producing Firmicutes is in marked contrast to the microbial profile of patients with active IBD,\textsuperscript{9,65–67} where a loss of these SCFA-producing species is more common. However, some patients with irritable bowel syndrome (IBS), a disorder of the brain-gut axis characterized by abdominal pain and altered bowel habits in the absence of overt inflammation,\textsuperscript{68,69} exhibit a similar increase in SCFA-producing species, in particular Lachnospiraceae,\textsuperscript{69,70} although this finding has not been universal, likely because of the heterogeneous nature of IBS.\textsuperscript{71} Interestingly, increased Lachnospiraceae species have been noted in the microbiota derived from the stool of both adults\textsuperscript{72–74} and children with IBS,\textsuperscript{75} and this increase was correlated with visceral pain\textsuperscript{72,75} and the activation of brain regions involved in the processing of painful sensorimotor input,\textsuperscript{72} suggestive of a causal relationship between this family of bacteria and visceral pain.

In our study, incubation of cultured naive DRG neurons with the SCFAs butyrate and propionate resulted in TRPV1 sensitization, whereas FMT of postinflammatory DSS stool resulted in a further increase in visceral hyperalgesia when compared with Abx alone. This suggests that in the postinflammatory DSS model, the microbiome may be pro-nociceptive. It should be noted that colonic supernatants contain soluble factors derived from both the host and the microbiota; hence, we performed experiments where naive DRG neurons were incubated with SCFA alone. This SCFA concentration was chosen on the basis of the quantification data from mouse stool. The evaluation of fecal SCFA levels is well-described\textsuperscript{76–79}; however, it should be noted that fecal/luminal SCFA concentration reflects a dynamic balance between SCFA production and absorption and may not necessarily reflect the true SCFA concentration at the mucosal barrier.\textsuperscript{80,81} As such, these levels should be

![Figure 8](see previous page). SCFAs modulate TRPV1 sensitization in dorsal root ganglia (DRGs) neurons. T9-L2 DRGs from naive mice (n = 6/group) were cultured overnight with supernatants from CT, Abx-treated, and DSS-treated mice. Cells were stimulated with the TRPV1 (A) or TRPA1 (B) agonists, capsaicin (100 nmol/L) or mustard oil (100 nmol/L), respectively, and calcium imaging was used to evaluate TRPV1 or TRPA1 sensitization. L4–L5 DRGs from postinflammatory mice (n = 6/group) were cultured overnight. (C) Capsaicin (100 nmol/L) or (D) mustard oil (100 nmol/L) was used to evaluate TRPV1 or TRPA1 sensitization using calcium imaging. (E) T9-L2 dorsal root ganglia (n = 6/group) were incubated directly with the short-chain fatty acids acetate (5 mmol/L), butyrate (1 mmol/L), or propionate (1 mmol/L). Cells were stimulated with capsaicin (100 nmol/L), and calcium imaging was used to evaluate TRPV1 sensitization. One-way analysis of variance of area under the curve, *P < .05 (3 experiments). Abx, antibiotics; CT, control; DSS, dextran sulfate sodium; TRPA1, transient receptor potential ankyrin-1 receptor; TRPV1, transient receptor potential vanilloid-1 receptor.
interpreted with caution. To date, there have been conflicting findings on the role of SCFAs, and in particular butyrate, in visceral pain. Our findings are in agreement with previous data from a rat model where butyrate enemas induced visceral hypersensitivity in control rats through activation of a mitogen-activated protein kinase–dependent mechanism in DRG neurons. Interestingly, in a rat model of acute colitis, butyrate enemas prolonged the pronociceptive effect of acute colitis on visceral pain. Furthermore, use of the low fermentable oligosaccharides, disaccharides, monosaccharides, and polyols (low FODMAP) diet, which is effective for the treatment of abdominal pain in IBD patients in remission, results in decreased fecal SCFA content. In contrast with our data, butyrate enemas in healthy patients and mouse models caused a dose-dependent reduction of abdominal pain to colorectal distention. Other studies that used Abx to modulate the microbiome, including our own, demonstrate a decrease in fecal butyrate content associated with an increase in pain behaviors, suggesting that although butyrate may be pro-nociceptive in some model systems, broad-spectrum Abx treatment results in a nonspecific loss in anti-nociceptive bacteria/soluble mediator(s). SCFAs exert their actions through both receptor-mediated and receptor-independent mechanisms. Future pharmacologic and genetic studies should focus on the mechanism whereby SCFAs cause nociceptor sensitization in postinflammatory DSS mice, which will in turn shed light on some of the conflicting data in the literature.

In conclusion, we have demonstrated that the microbiome appears to play an important role in post-inflamma-tory visceral, but not somatic, hypersensitivity. Microbial-derived soluble products, including sodium butyrate and sodium propionate, were able to sensitize nociceptive neurons in vitro, suggesting that these SCFAs may play a role in the pathogenesis of postinflammatory visceral pain.

Materials and Methods

Animals

Six-week-old male C57BL/6 mice were obtained from Charles River Laboratories (Montreal, Quebec, Canada). All mice were housed in plastic sawdust floor cages under standard conditions with free access to drinking water and food. All experiments were conducted on age-matched animals under protocols approved by the University of Calgary Animal Care Committee and in accordance with the guidelines for the ethical use of animals in research of the Canadian Council on Animal Care (AC16-0105). All animals were allowed to acclimatize for 1 week before any experimentation.

Induction of Inflammation and Microbial Manipulation

Colonic inflammation was induced by administration of 2.5% (wt/vol) DSS (36,000–50,000 MW; MP Biochemicals, Solon, OH) in drinking water for 5 days. Mice were separated into 2 groups, control untreated and DSS treated, and were allowed to recover for 5 weeks before the evaluation of visceral hyperalgesia. To manipulate the microbiome, mice received an Abx cocktail consisting of ampicillin, neomycin, metronidazole (1 g/L), and vancomycin (0.5 g/L) (all from Sigma-Aldrich Canada, Oakville, ON, Canada) administered in drinking water 2 weeks before the end of the recovery period. In some cases, mice were allowed to recover for a further 2 weeks after the discontinuation of Abx. Weight changes were measured daily in all mice. Colonic length and thickness were assessed at the time of death as previously described. Microbial manipulation was also performed by using FMT. Fresh fecal pellets from 6 donor mice were collected, pooled, and weighed. Pellets were homogenized for 5 minutes in 1 mL sterilized PBS supplemented with 10% glycerol by using 1 mm zirconium oxide beads (NextAdvance, Troy, NY) in a 4°C cold room using a bullet blender (NextAdvance). The volume was adjusted to give a concentration of 80 mg feces/mL. The fecal matter was centrifuged for 1 minute at 400g, and the supernatant was used for FMT. Recipient mice were pretreated with the previously described Abx cocktail to disrupt the basal microbiome. Subsequently, Abx-treated mice received 80 mg/mL homogenized stool in 200 μL sterile saline + 10% glycerol or sterile saline + 10% glycerol alone for 4 days via orogastric gavage.

In Vivo Measurement of Somatic and Visceral Pain

Visceromotor response to colorectal distention. The visceromotor response to colorectal distention was assessed as previously described. Briefly, 2 days before colorectal distention, mice were anesthetized with xylazine and ketamine, and electrodes were implanted bilaterally into the abdominal external oblique musculature (Bioflex AS-631; Cooner Wire, Chatsworth, CA). Electrodes were exteriorized at the back of the neck and protected by a plastic tube attached to the skin. Mice were allowed to recover for 48 hours after electrode implantation. For recording, electrodes were connected to a Bio Amplifier, which was then connected to an electromyogram acquisition system (both from ADInstruments, Colorado Springs, CO). A 10.5-mm-diameter balloon catheter (Fogarty arterial embolectomy catheter, 4F; Vygon USA, Lansdale, PA) was gently inserted into the rectum to a depth of 5 mm. Ten-second distentions were performed at pressures of 15, 30, 45, and 60 mm Hg by inflating the balloon in a stepwise fashion with water (20, 40, 60 and 80 μL, respectively) with 5-minute rest intervals. The electromyographic activity of the abdominal muscles was recorded, and visceromotor responses were calculated by using LabChart 7 software (ADInstruments).

To evaluate whether Abx exerted direct effects on visceral sensitivity, Abx or sterile saline (vehicle) were administered directly into the colon via a double lumen catheter, similar to a previous study. Visceromotor responses were tested before and 1 hour after administration of saline or Abx.
Somatic pain assessment. Somatic pain evaluation was performed by a blinded investigator. Mechanical and thermal sensitivities were tested simultaneously in all groups on the same day.

Mechanical sensitivity. Mechanical sensitivity was tested by measuring the threshold to withdrawal applied to the paw using the automated Von Frey hair test (Ugo Basile, Gemonio, Italy) as described. Mice were individually placed in custom-made plastic compartments on a metallic mesh for 1 hour, 2 days before testing, to minimize stress reactions. On the day of the experiment, 8 mice were allowed to habituate in individual opaque compartments until exploratory behavior was no longer observed (~20–30 minutes). The hind paw withdrawal threshold was measured by applying a small metal filament that automatically exerts constant progressive pressure perpendicular to the middle of the plantar surface of the hind paw until withdrawal. The force and latency values were recorded. This was repeated 5 times, and each trial was followed by a 10-minute resting period. In each animal, the left or right hind paw was chosen at random, and the withdrawal threshold was measured in all animals in a row, followed by evaluation of the opposite hind paw.

Thermal sensitivity. Thermal sensitivity was assessed by using the hot plate test (Bioseb, Pinellas Park, FL). Immediately after the automated Von Frey test, mice were placed on a metal hot plate set to 52°C ± 0.5°C. The latency from placement of the mouse on the heated surface until the first overt behavioral sign of nociception, such as lifting or licking a hind paw, vocalization, or jumping, was measured. The timer was stopped, and the mouse was immediately removed from the hot plate after responding or after a maximum cutoff of 20 seconds to prevent tissue damage.

In Vitro Evaluation of Somatic and Visceral Pain Supernatant generation. Colonic supernatants were generated as previously described. Briefly, full circumference colonic tissue sections of 5 mm from all mice used for somatic and visceral pain experiments were transferred and incubated for 1 hour in 96-well plate containing 300 μL Hanks’ balanced salt solution (HBSS) supplemented with calcium chloride and magnesium chloride (Gibco, Carlsbad, CA) in a humidified incubator at 37°C under 95% air and 5% CO2; samples were not standardized to the weight of segments. Supernatants were filtered (0.2 μm pore polyethersulfone membrane) and stored at −80°C. For in vitro experiments involving calcium imaging, pooled supernatants derived from mice in the same treatment group were used. Pooled supernatants from 2 animals of the appropriate treatment group were used for calcium imaging for each experiment, where naive DRGs were imaged from 2 mice at a time. Three total experiments were performed; naive DRGs from a total of 6 mice were used for the calcium imaging. These DRGs were incubated with pooled supernatants derived from a total of 6 different animals from the appropriate treatment group.

Dorsal Root Ganglion Dissection and Culture

Calcium Imaging

Calcium imaging was performed as previously described. Cultured DRG neurons were imaged by using a 10× objective on an Olympus IX51 microscope controlled by cellSens software (Olympus Canada, Toronto, ON, Canada). Cultured DRG neurons were loaded in HBSS (Gibco) with 2 μmol/L Fura-2AM (Life Technologies, Carlsbad, CA) for 30 minutes at 37°C and then washed with HBSS for 20 minutes at 37°C. The recording chamber was continually perfused at 37°C with external solution (in mmol/L: 140 NaCl, 5 KCl, 10 HEPES, 10 glucose, 1 MgCl2, 2 CaCl2; pH adjusted to 7.3–7.4 with 10N NaOH). To examine TRPV1 or TRPA1 activity, cells were perfused with doses of the TRPV1 agonist capsaicin (100 nmol/L and 1 μmol/L; Sigma) or the TRPA1 agonist mustard oil (100 nmol/L and 1 μmol/L; Sigma) diluted in external solution at a rate of 1 mL/min. Fluorescence was measured during excitation at 340 and 380 nm, and the ratio of the fluorescence emission at 510 nm was monitored. The baseline was monitored for 120 seconds before perfusion with agonist. Images were acquired every 1 second and processed by using ImageJ (NIH, Bethesda, MD) by drawing discrete regions of interest around cells that responded to KCl (70 mmol/L). Data are expressed as a fold change from baseline fluorescence normalized to background.
fluorescence (ΔF/F0) on raw traces and were analyzed by using area under the curve.

**Microbial Sequencing and Metabolomic Data**

**16S rRNA gene amplicon sequencing.** DNA extraction and purification from feces were performed by using QIAamp Fast DNA Stool extraction kit (Qiagen, Toronto, ON, Canada). The V4 hypervariable region of the 16S rRNA gene was amplified by using the following barcoded primers with MiSeq (Illumina, San Diego, CA) specific adaptors: 16SV4Fwd: AATGTAGCGGAGCACCCGAGTCTACACBARCODETATGCTTAATGTGTGCCCCCMGCCGCGTAA and 16SV4Rev: CAAGCAGAAGACGGCATACGAGATCACBARCODETATGCTTAATGTGTGCCCCCMGCCGCGTAA and 16SV4Rev. Qubit ScreenTape station (Agilent, Waldbronn, Germany) and on a High Sensitivity D1000 ScreenTape station (Agilent, Waldbronn, Germany) and on a Qubit fluorometer (ThermoFisher). The 16S rRNA v4 gene amplicon sequencing was performed by using a V2-500 cycle cartridge (Illumina) on the MiSeq platform (Illumina).

Raw fastq files were processed by using Cutadapt (version 1.16) and dada2 (R package, version 1.10.1). After removing standard Illumina V4 primers and reads shorter than 50 base pairs, quality filtering was performed by using the filterAndTrim function of dada2, removing reads with an expected error (EE = sum(10^-{-Q/10}) higher than 2 for both forward and reverse reads. A table of amplicon sequence variants (ASVs) was generated by using the standard dada2 workflow: generating an error model of the data, inferring sequence variants, merging forward and reverse reads, generating a count table, and removing chimeric sequences. Taxonomic classifications were assigned to ASVs by using the dada2::assignTaxonomy function, using the Silva 132 database as a reference.

**Liquid Chromatography–Mass Spectrometry Based Short-Chain Fatty Acids Quantification in Mouse Fecal Samples**

Samples were processed and analyzed as previously described. In brief, SCFAs were extracted (1:2 ratio wet sample weight [mg] to extraction solvent [μL]) from fecal samples with ice-cold extraction solvent (50% water/acetoni trile, v/v) spiked with 13C-labeled SCFA standards (acetic acid-1,2-13C2, propionic acid-13C3, and butyric acid-1,2-13C2), derivatized with N-(3-dimethylaminopropyl)-N’-ethylcarbodiimide hydrochloride and aniline, and then submitted to liquid chromatography–mass spectrometry analysis. The UHPLC-MS platform consisted of a Vanquish ultrahigh-performance liquid chromatography system coupled to a TSQ Quantum Access MAX triple quadrupole Mass Spectrometer (Thermo Scientific) equipped with an electrospray ionization probe. In short, SCFAs were separated on a HiperSIL GOLD TM C18 column (200 × 2.1 mm, 1.9 μm; Thermo Scientific) by using a binary solvent system composed of liquid chromatography–mass spectrometry grade water and methanol, both containing 0.1% (%v/v) formic acid, and monitored with the mass spectrometer operating in positive ionization mode and selected reaction monitoring mode. Data analyses, on the converted mzXML files, were conducted in MAVEN and the absolute quantification of native SCFA concentration was based on the 12C:13C signal intensity ratio and the respective 13C-in-internal standard concentration.

**Statistical Analysis**

All data are expressed as the mean ± standard error of the mean. Treatment effects were analyzed by Student t test using Prism 7 (GraphPad, La Jolla, CA). Data distribution was tested by using the D’Agostino and Pearson normality test. One-way or two-way analysis of variance with Ben-ferroni or Tukey post hoc test was applied for comparison of multiple groups. If the data were found to be not normally distributed, a Kruskal-Wallis test followed by a Dunn multiple comparison or a Mann-Whitney test was used (Prism 7). A P value <.05 was considered to be significant. The 16s rRNA sequencing analysis was carried out by using the R package phyloseq (version 1.24.2). Alpha diversity was estimated by using the Shannon index of diversity, and significance was determined by using a mixed-effects linear model, with treatment and time point as fixed effects and mouse ID as a random effect. Beta diversity was estimated by using a principal coordinates analysis on a matrix of Bray-Curtis distances and analyzed by using permutational multivariate analysis of variance. To test for significant differences in composition at the phylum level, a beta-binomial regression model was performed by using the R package corncob. Differential abundance analysis was carried out by using the R package DESeq2 to identify ASVs that were differentially abundant across treatments. ASV counts were modeled by using the negative binomial distribution, and significance was determined by a Wald test on log2 fold change values and an alpha = 0.01. P values were adjusted for multiple inference by using the Benjamini-Hochberg method.

**References**

1. Molodecky NA, Soon IS, Rabi DM, Ghali WA, Ferris M, Chernoff G, Benchimol EI, Panaccione R, Ghosh S, Barkema HW, Kaplan GG. Increasing incidence and prevalence of the inflammatory bowel diseases with time, based on systematic review. Gastroenterology 2012;142:46–54 e42; quiz e30.

2. Gracie DJ, Williams CJ, Sood R, Muntaz S, Bholah MH, Hamlin PJ, Ford AC. Negative effects on psychological health and quality of life of genuine irritable bowel syndrome-type symptoms in patients with inflammatory
bowl disease. Clin Gastroenterol Hepatol 2017; 15:376–384.
3. Regueiro M, Greer JB, Szigethy E. Etiology and treatment of pain and psychosocial issues in patients with inflammatory bowel diseases. Gastroenterology 2017; 152:430–439.
4. Jonefjall B, Ohman L, Simren M, Strid H. IBS-like symptoms in patients with ulcerative colitis in deep remission are associated with increased levels of serum cytokines and poor psychological well-being. Inflamm Bowel Dis 2016;22:2630–2640.
5. Jonefjall B, Strid H, Ohman L, Svedlund J, Bergstedt A, Simren M. Characterization of IBS-like symptoms in patients with ulcerative colitis in clinical remission. Neurogastroenterol Motil 2013;25, 756–e578.
6. Buskila D, Odes LR, Neumann L, Odes HS. Fibromyalgia in inflammatory bowel disease. J Rheumatol 1999; 26:1167–1171.
7. Targownik LE, Nugent Z, Singh H, Bugden S, Bernstein CN. The prevalence and predictors of opioid use in inflammatory bowel disease: a population-based analysis. Am J Gastroenterol 2014;109:1613–1620.
8. DeGruttola AK, Low D, Mizoguchi A, Mizoguchi E. Current understanding of dysbiosis in disease in human and animal models. Inflamm Bowel Dis 2016;22:1137–1150.
9. Halfvarson J, Brislawn CJ, Lamendella R, Vazquez-Baeza Y, Walters WA, Bramer LM, D’Amato M, Bonfiglio F, McDonald D, Gonzalez A, McClure EE, Dunklebarger MF, Knight R, Jansson JK. Dynamics of the human gut microbiome in inflammatory bowel disease. Nat Microbiol 2017;2:17004.
10. Shutkever O, Gracie DJ, Young C, Wood HM, Taylor M, John Hamlin P, Ford AC, Quirke P. No significant association between the fecal microbiome and the presence of irritable bowel syndrome-type symptoms in patients with quiescent inflammatory bowel disease. Inflamm Bowel Dis 2018;24:1597–1605.
11. Manichanh C, Rigottier-Gois L, Bonnaud E, Gloux K, Shutkever O, Gracie DJ, Young C, Wood HM, Taylor M, John Hamlin P, Ford AC, Quirke P. No significant association between the fecal microbiome and the presence of irritable bowel syndrome-type symptoms in patients with quiescent inflammatory bowel disease. Inflamm Bowel Dis 2018;24:1597–1605.
12. Lopez-Siles M, Enrich-Capo N, Aldeguer X, Sabat-Mir M, Duncan SH, Garcia-Gil LJ, Martinez-Medina M. Alterations in the abundance and co-occurrence of Akkermansia muciniphila and Faecalibacterium prausnitzii in the colonic mucosa of inflammatory bowel disease subjects. Front Cell Infect Microbiol 2018;8:281.
13. Martinez C, Antolin M, Santos J, Torrejon A, Casellas F, Borreau N, Guamer F, Malagelada JR. Unstable composition of the fecal microbiota in ulcerative colitis during clinical remission. Am J Gastroenterol 2008; 103:643–648.
14. Hotte NS, Salim SY, Tso RH, Albert EJ, Bach P, Walker J, Dieleman LA, Fedorak RN, Madsen KL. Patients with inflammatory bowel disease exhibit dysregulated responses to microbial DNA. PLoS One 2012;7:e37932.
15. Akbar A, Yangou Y, Facer P, Brydon WG, Walters JR, Anand P, Ghosh S. Expression of the TRPV1 receptor differs in quiescent inflammatory bowel disease with or without abdominal pain. Gut 2010;59:767–774.
16. Chiu IM, Heesters BA, Ghasemlu N, Von Hefn CA, Zhao F, Tran J, Wainger B, Strominger A, Muralidharan S, Horswill AR, Bubeck Wardenburg J, Hwang SW, Carroll MC, Woolf CJ. Bacteria activate sensory neurons that modulate pain and inflammation. Nature 2013;501:52–57.
17. Nohr MK, Egerod KL, Christiansen SH, Gille A, Offermanns S, Schwartz TW, Moller M. Expression of the short chain fatty acid receptor GPR41/FFAR3 in autonomic and somatic sensory ganglia. Neuroscience 2015; 290:126–137.
18. Stillings RM, van de Wouw M, Clarke G, Stanton C, Dinan TG, Cryan JF. The neuropharmacology of butyrate: the bread and butter of the microbiota-gut-brain axis? Neurochem Int 2016;99:110–132.
19. Sessenwein JL, Baker CC, Pradhananga S, Maitland ME, Petrof EO, Allen-Vercoe E, Noordhof C, Reed DE, Vanner SJ, Lomax AE. Protease-mediated suppression of DRG neuron excitability by commensal bacteria. J Neurosci 2017;37:11758–11768.
20. Lomax AE, Pradhananga S, Sessenwein JL, O’Malley D. Bacterial modulation of visceral sensation: mediators and mechanisms. Am J Physiol Gastrointest Liver Physiol 2019;317:G363–G372.
21. Meseguer V, Alpizar YA, Luis E, Tajada S, Deninger B, Fajardo O, Manenschijn JA, Fernandez-Pena C, Talavera A, Kichko T, Navia B, Sanchez A, Senaris R, Reeh P, Perez-Garcia MT, Lopez-Lopez JR, Voets T, Belmonte C, Talavera K, Viana F. TRPA1 channels mediate acute neurogenic inflammation and pain produced by bacterial endotoxins. Nat Commun 2014; 5:3125.
22. Hoban AE, Moloney RD, Golubeva AV, McVey Neufeld KA, O’Sullivan O, Patterson E, Stanton C, Dinan TG, Clarke G, Cryan JF. Behavioural and neurochemical consequences of chronic gut microbiota depletion during adulthood in the rat. Neuroscience 2016;339:463–477.
23. Guida F, Turco F, Iannotta M, De Gregorio D, Palumbo I, Sarnelli G, Furiano A, Napolitano F, Boccella S, Luongo L, Mazzitelli M, Ussiello A, De Filippis F, Iannotti FA, Piscitelli F, Ercolini D, de Novellis V, Di Marzo V, Cuomo R, Mainone S. Antibiotic-induced microbiota perturbation causes gut endocannabinoid-dome changes, hippocampal neuronal reorganization and depression in mice. Brain Behav Immun 2018; 67:230–245.
24. Lapointe TK, Basso L, Iftinca MC, Flynn R, Chapman K, Dietrich G, Vergnolle N, Altier C. TRPV1 sensitization mediates postinflammatory visceral pain following acute colitis. Am J Physiol Gastrointest Liver Physiol 2015; 309:G87–G99.
25. Eijkelkamp N, Kavelaars A, Eisenbruch S, Schedlowski M, Holtmann G, Heijnen CJ. Increased visceral sensitivity to capsaicin after DSS-induced colitis in mice: spinal cord c-Fos expression and behavior. Am J Physiol Gastrointest Liver Physiol 2007;293:G749–G757.
26. Engel MA, Khalil M, Mueller-Tribbensee SM, Becker C, Neuhuber WL, Neurath MF, Reeh PW. The proximodistal aggravation of colitis depends on substance P released from TRPV1-expressing sensory neurons. J Gastroenterol 2012;47:256–265.

27. Palm O, Moum B, Jahnson J, Gran JT. Fibromyalgia and chronic widespread pain in patients with inflammatory bowel disease: a cross sectional population survey. J Rheumatol 2001;28:590–594.

28. Rakoff-Nahoum S, Paglino J, Eslami-Varzaneh F, Edberg S, Medzhitov R. Recognition of commensal microbiota by toll-like receptors is required for intestinal homeostasis. Cell 2004;118:229–241.

29. Le Bastard Q, Ward T, Sidiropoulos D, Hillmann BM, Chun CL, Sadowsky MJ, Knights D, Montassier E. Fecal microbiota transplantation reverses antibiotic and chemotherapy-induced gut dysbiosis in mice. Sci Rep 2018;8:6219.

30. Bromberg JS, Hittle L, Xiong Y, Saxena V, Smyth EM, Li L, Zhang T, Wagner C, Fricke WF, Simon T, Brinkman CC, Mongodin EF. Gut microbiota-dependent modulation of innate immunity and lymph node remodeling affects cardiac allograft outcomes. JCI Insight 2018;3.

31. Verdu EF, Bercik P, Verma-Gandhu M, Huang XX, Blennnerhassett P, Jackson W, Mao Y, Wang L, Rochat F, Collins SM. Specific probiotic therapy attenuates antibiotic induced visceral hypersensitivity in mice. Gut 2006;55:182–190.

32. Amaral FA, Sachs D, Costa VV, Lagundes CT, Cisalpino D, Cunha TM, Ferreira SH, Cunha FQ, Silva TA, Nicoli JR, Vieira LQ, Souza DG, Teixeira MM. Commensal microbiota is fundamental for the development of inflammatory pain. Proc Natl Acad Sci U S A 2008;105:2193–2197.

33. Russo R, De Caro C, Avaglano C, Cristiano C, La Rana G, Mattace Raso G, Beni Canani R, Meli R, Calignano A. Sodium butyrate and its synthetic amide derivative modulate nociceptive behaviors in mice. Pharmacol Res 2016;103:279–291.

34. Berry D, Kuzyk O, Rauch I, Heider S, Schwab C, Hainzl E, Tachon S, Zhou J, Keenan M, Martin R, Marco ML. The chronic widespread pain in patients with irritable bowel syndrome. Gastroenterology 2016;150:875–887 e9.

35. van Hoboken EA, Thijssen AY, Verhaaren R, van der Week PP, Prins FA, Verspaget HW, Masclee AA. Symptoms in patients with ulcerative colitis in remission are associated with visceral hypersensitivity and mast cell activity. Scand J Gastroenterol 2011;46:981–987.

36. Faure C, Giguere L. Functional gastrointestinal disorders and visceral hypersensitivity in children and adolescents suffering from Crohn’s disease. Inflamm Bowel Dis 2011;17:1030–1033.

37. Yiangou Y, Facer P, Dyer NH, Chan CL, Knowles C, Williams NS, Anand P. Vanilloid receptor 1 immunoreactivity in inflamed human bowel. Lancet 2001;357:1338–1339.

38. Zheng G, Hong S, Hayes JM, Wiley JW. Chronic stress and peripheral pain: evidence for distinct, region-specific changes in visceral and somatosensory pain regulatory pathways. Exp Neurol 2015;273:301–311.

39. Hong S, Fan J, Kemmerer ES, Evans S, Li Y, Wiley JW. Reciprocal changes in vanilloid (TRPV1) and endocannabinoid (CB1) receptors contribute to visceral hyperalgesia in the water avoidance stressed rat. Gut 2009;58:202–210.

40. Cattaruzza F, Spreadsbury I, Miranda-Morales M, Grady EF, Vanner S, Bunnett NW. Transient receptor potential ankyrin-1 has a major role in mediating visceral pain in mice. Am J Physiol Gastrointest Liver Physiol 2010;298:G81–G91.

41. Boeckxstaens GE. Histamine receptor H1-mediated sensitization of TRPV1 mediates visceral hypersensitivity and symptoms in patients with irritable bowel syndrome. Gastroenterology 2015;149:2121–2131.
51. Gustafsson JK, Greenwood-Van Meerveld B. Amygdala activation by corticosterone alters visceral and somatic pain in cycling female rats. Am J Physiol Gastrointest Liver Physiol 2011;300:G1080–G1085.

52. Basso L, Lapointe TK, Ittincu M, Marsters C, Hollenberg MD, Kurrasch DM, Altier C. Granulocyte-colony-stimulating factor (G-CSF) signaling in spinal microglia drives visceral sensitization following colitis. Proc Natl Acad Sci U S A 2017;114:11235–11240.

53. Johnson AC, Greenwood-Van Meerveld B. Knockdown of steroid receptors in the central nucleus of the amygdala induces heightened pain behaviors in the rat. Neuropharmacology 2015;93:116–123.

54. Jain P, Hassan AM, Koyani CN, Mayerhofer R, Shen S, Lim G, You Z, Ding W, Huang P, Ran C, Johnson AC, Greenwood-Van Meerveld B. Amygdala induces heightened pain behaviors in the rat. Neuropharmacology 2015;93:116–123.

55. Gustafsson JK, Greenwood-Van Meerveld B. Amygdala induces heightened pain behaviors in the rat. Neuropharmacology 2015;93:116–123.

56. Selimoglu E. Aminoglycoside-induced ototoxicity. Curr Pharm Des 2007;13:119–126.

57. Aminoglycosides. LiverTox: clinical and research information on drug-induced liver injury. Bethesda, MD: National Institute of Diabetes and Digestive and Kidney Diseases, 2012.

58. Mahony SM, Felice VD, Gaynes RP. Clinical relevance of metronidazole and peripheral neuropathy: a systematic review of the literature. Int J Antimicrob Agents 2018;50:2–10.

59. Mahony SM, Dinan TG, Cryan JF. Microbiota of diarrhoea-predominant irritable bowel syndrome: present state and perspectives. Microbiology 2010;156:3205–3215.

60. Saito Y, Igarashi A, Yamanishi Y, Sugiyama T, Takada Y, Morikawa T, et al. Microbiota composition and functional changes in inflammatory bowel disease and irritable bowel syndrome. J Mol Diagn 2019;21:449–461.

61. Luczynski P, Tramullas M, Viola M, Shanahan F, Perez-Burgos A, Wang L, McVey Neufeld KA, Mao YK, Ahmadzai M, Janssen LJ, Stanisz AM, Bienstock J, Kunze WA. The TRPV1 channel in rodents is a major target for anticointective effect of the probiotic Lactobacillus reuteri DSM 17938. J Physiol 2015;593:3943–3957.

62. Tan J, McKenzie C, Potamitis M, Thorburn AN, Mackay CR, Macia L. The role of short-chain fatty acids in health and disease. Adv Immunol 2014;121:91–119.

63. Duvallet C, Gibbons SM, Gurry T, Irizarry RA, Alm EJ. Meta-analysis of gut microbiome studies identifies disease-specific and shared responses. Nat Commun 2017;8:1784.

64. Geirnaert A, Calatayud M, Grootaert C, Laukens D, Devriese S, Smagge G, De Vos M, Boon N, Van de Wiele T. Butyrate-producing bacteria supplemented in vitro to Crohn’s disease patient microbiota increased butyrate production and enhanced intestinal epithelial barrier integrity. Sci Rep 2017;7:11450.

65. Vich Vila A, Imhann F, Collij V, Jankipersadsing SA, Gurry T, Mujagic Z, Kurilshikov A, Bonder MJ, Jiang X, Tischelaar EF, Dekens J, Peters V, Voskuil MD, Visschedijk MC, van Dullemen HM, Keszthelyi D, Swertz MA, Franke L, Alberts R, Festen EAM, Dijkstra G, Masclee AAM, Hofker MH, Xavier RJ, Alm EJ, Fu J, Wijmenga C, Jonkers D, Zhernakova A, Weersma RK. Gut microbiota composition and functional changes in inflammatory bowel disease and irritable bowel syndrome. Sci Transl Med 2018;10.

66. Mearin F, Lacy BE, Chang L, Chey WD, Lembo AJ, Simren M, Spiller R. Bowel disorders. Gastroenterology 2011;13:501–502.

67. Rajilic-Stojanovic M, Biagi E, Heilig HG, Kajander K, Kekkonen RA, Tims S, de Vos WM. Global and deep molecular analysis of microbiota signatures in fecal samples from patients with irritable bowel syndrome. Gastroenterology 2011;141:1792–1801.

68. Bennet SM, Ohman L, Simren M. Gut microbiota as potential orchestrators of irritable bowel syndrome. Gut Liver 2015;9:318–331.

69. Pittayanon R, Lau JT, Yuan Y, Leontiadis GI, Tse F, Surette M, Moayyedi P. Gut microbiota in patients with irritable bowel syndrome: a systematic review. Gastroenterology 2019;157:97–108.

70. Labus JS, Osadchiy V, Hsiao EY, Tap J, Derren M, Gupta A, Tillisch K, Le Neve B, Grinsvall C, Ljungberg M, Ohman L, Tombloom H, Simren M, Mayer EA. Evidence for an association of gut microbial Clostridia with brain functional connectivity and gastrointestinal sensorimotor function in patients with irritable bowel syndrome, based on tripartite network analysis. Microbiome 2019;7:45.

71. Krogius-Kurikka L, Lyra A, Malinen E, Aamikunnas J, Tuimala J, Paulin L, Makivuokko H, Kajander K, Palva A. Microbial community analysis reveals high level phylogenetic alterations in the overall gastrointestinal microbiota of diarrhoea-predominant irritable bowel syndrome sufferers. BMC Gastroenterol 2009;9:95.

72. Salonen A, de Vos WM, Palva A. Gastrointestinal microbiota in irritable bowel syndrome: present state and perspectives. Microbiology 2010;156:3205–3215.

73. Hollier EB, Oezgun N, Chumpitazi BP, Luna RA, Weidler EM, Rubio-Gonzales M, Dahdouli M, Cope JL, Mistretta TA, Raza S, Metcalf GA, Muzny DM, Gibbs RA, Petrosino JF, Heitkermer M, Savidge TC, Shulman RJ, Versalovic J. Leveraging human microbiome features to diagnose and stratify children with irritable bowel syndrome. J Mol Diagn 2019;21:449–461.
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76. Primec M, Micetic-Turk D, Langerholc T. Analysis of short-chain fatty acids in human feces: a scoping review. Anal Biochem 2017;526:9–21.
77. Cox SR, Lindsay JO, Fromentin S, Stagg AJ, McCarthy NE, Galleron N, Ibraim SB, Roume H, Levenez F, Pons N, Maziers N, Lomor MC, Ehrlich SD, Irving PM, Whelan K. Effects of short-chain fatty acids in the human gut on colonocytes and markers of inflammation in patients with quiescent inflammatory bowel disease in a randomized trial. Gastroenterology 2020;158:176–188 e7.
78. Aden K, Rehnman A, Waschina S, Pan WH, Walker A, Lucio M, Nunez AM, Bharti R, Zimmerman J, Bethge J, Schulte B, Schulthe D, Franke A, Knaus S, Schroeder JO, Vandepute D, Raes J, Szymczak S, Waetzig GH, Zeuner R, Schmitt-Kopplin P, Kaleta C, Schreiber S, Rosenstiel P. Metabolic functions of gut microbes associate with efficacy of tumor necrosis factor antagonists in patients with inflammatory bowel diseases. Gastroenterology 2019;157:1279–1292 e11.
79. Halms EP, Christophersen CT, Bird AR, Shepherd SJ, Gibson PR, Muir JG. Diets that differ in their FODMAP content alter the colon luminal microenvironment. Gut 2015;64:93–100.
80. Sakata T. Pitfalls in short-chain fatty acid research: a methodological review. Anim Sci J 2019;90:3–13.
81. Gill PA, van Zelm MC, Muir JG, Gibson PR. Review article: short chain fatty acids as potential therapeutic agents in human gastrointestinal and inflammatory disorders. Aliment Pharmacol Ther 2018;48:15–34.
82. Xu D, Wu X, Grabauskas G, Owyang C. Butyrate-induced colonic hypersensitivity is mediated by mitogen-activated protein kinase activation in rat dorsal root ganglia. Gut 2013;62:1466–1474.
83. Tarrasias AL, Millecamp M, Alloui A, Beaugard C, Kemeny JL, Bourdu S, Bommelaer G, Eschalter A, Dapoigny M, Ardii D. Short-chain fatty acid enemas fail to decrease colonic hypersensitivity and inflammation in TNBS-induced colonic inflammation in rats. Pain 2002;100:91–97.
84. Vanhoutvin SA, Troost FJ, Kilkens TO, Lindsey PJ, Hamer HM, Jonkers DM, Venema K, Brummer RJ. The effects of butyrate enemas on visceral perception in healthy volunteers. Neurogastroenterol Motil 2009;21, 952–953.
85. Reijnders D, Goossens GH, Hermes GD, Neis EP, van der Beek CM, Most J, Holst JJ, Lenaerts K, Kootte RS, Nieuworp D, Groen AK, Olde Damink SW, Boekschoten MV, Smidt H, Zoetendal EG, Dejong CH, Blaak EE. Effects of gut microbiota manipulation by antibiotics on host metabolism in obese humans: a randomized double-blind placebo-controlled trial. Cell Metab 2016;24:63–74.
86. Song Z, Xie W, Chen S, Strong JA, Print MS, Wang JL, Shareef AF, Ulirch-Lai YM, Zhang JM. High-fat diet increases pain behaviors in rats with or without obesity. Sci Rep 2017;7:10350.
87. Barrot M. Tests and models of nociception and pain in rodents. Neuroscience 2012;211:39–50.
88. Cenac N, Andrews CN, Holzhause M, Chapman K, Cottrell G, Andrade-Gordon P, Steinhoff M, Barbara G, Beck P, Bunnett NW, Sharkey KA, Ferraz JG, Shaffer E, Vergnolle N. Role for protease activity in visceral pain in irritable bowel syndrome. J Clin Invest 2007;117:636–647.
89. Martin M. Cutadapt removes adapter sequences from high-throughput sequencing reads. EMBNet Journal 2011;17:10–12.
90. Callahan BJ, McMurdie PJ, Rosen MJ, Han AW, Johnson AJ, Holmes SP. DADA2: high-resolution sample inference from Illumina amplicon data. Nat Methods 2016;13:581–583.
91. Bihan D, Rydzak T, Wys M, Pittman K, McCoy KD, Lewis IA. Method for absolute quantification of short chain fatty acids via reverse phase chromatography mass spectrometry. ChemRxiv 2019.
92. Ciasquin MF, Melamud E, Rabinowitz JD. LC-MS data processing with MAVEN: a metabolomic analysis and visualization engine. Curr Protoc Bioinformatics 2012, Chapter 14:Unit14.11.
93. Melamud E, Vastag L, Rabinowitz JD. Metabolomic analysis and visualization engine for LC-MS data. Anal Chem 2010;82:9818–9826.
94. McMurdie PJ, Holmes S. phyloseq: an R package for reproducible interactive analysis and graphics of microbiome census data. PloS One 2013;8:e61217.
95. Love MI, Huber W, Anders S. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. Genome Biol 2014;15:550.

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