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Proton Pump Inhibitors Exacerbate NSAID-Induced Small Intestinal Injury by Inducing Dysbiosis

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BACKGROUND & AIMS: Proton pump inhibitors (PPIs) and nonsteroidal anti-inflammatory drugs (NSAIDs) are among the most commonly used classes of drugs, with the former frequently coprescribed to reduce gastroduodenal injury caused by the latter. However, suppression of gastric acid secretion by PPIs is unlikely to provide any protection against the damage caused by NSAIDs in the more distal small intestine. **METHODS:** Rats were treated with antisecretory doses of omeprazole or lanzoprazole for 9 days, with concomitant treatment with anti-inflammatory doses of naproxen or celecoxib on the final 4 days. Small intestinal damage was blindly scored, and changes in hematocrit were measured. Changes in small intestinal microflora were evaluated by denaturing gradient gel electrophoresis and reverse-transcription polymerase chain reaction. **RESULTS:** Both PPIs significantly exacerbated naproxen- and celecoxib-induced intestinal ulceration and bleeding in the rat. Omeprazole treatment did not result in mucosal injury or inflammation; however, there were marked shifts in numbers and types of enteric bacteria, including a significant reduction (~80%) of jejunal Actinobacteria and *Bifidobacteria* spp. Restoration of small intestinal Actinobacteria numbers through administration of selected (*Bifidobacteria* enriched) commensal bacteria during treatment with omeprazole and naproxen prevented intestinal ulceration/bleeding. Colonization of germ-free mice with jejunal bacteria from PPI-treated rats increased the severity of NSAID-induced intestinal injury, as compared with mice colonized with bacteria from vehicle-treated rats. **CONCLUSIONS: PPIs exacerbate NSAID-induced intestinal damage at least in part because of significant shifts in enteric microbial populations. Prevention or reversal of this dysbiosis may be a viable option for reducing the incidence and severity of NSAID enteropathy.**

Keywords: Ulcer; Bleeding; Acid Secretion; Microflora; Enteropathy

The ability of nonsteroidal anti-inflammatory drugs (NSAIDs) to cause damage in the stomach is well-known, but these drugs also have the capacity to cause clinically significant injury in the small and large intestine. Approximately 70% of chronic NSAID users exhibit small intestinal inflammation,[1] which is associated with bleeding, strictures, and occasionally perforations.[2] The pathogenesis of NSAID enteropathy appears to be distinct from that of NSAID gastropathy.[3] Suppression of prostaglandin synthesis by NSAIDs renders the intestinal mucosa more susceptible to injury and less efficient in undergoing repair,[4,5] but, unlike the case for the stomach, a primary role of cyclooxygenase (COX) inhibition in the mechanism of NSAID-induced enteropathy is not clear.[4] On the other hand, the enterohepatic recirculation of NSAIDs and their secretion in bile are primary factors in the production of intestinal damage, coupled with their direct cytotoxic actions on enterocytes.[4,6,7] Enteric gram-negative bacteria also contribute significantly to NSAID-induced intestinal damage.[8] Germ-free mice do not develop intestinal ulcers when given NSAIDs,[9] but, when colonized by conventional bacteria, they become susceptible to NSAID-induced intestinal ulceration.[10] Broad-spectrum antibiotics have been shown to markedly reduce NSAID-induced small intestinal ulceration in animals.[11,12] Furthermore, mice that lack the receptor for bacterial endotoxin (Toll-like receptor 4) do not develop intestinal damage when given an NSAID.[13]

Proton pump inhibitors (PPIs) substantially reduce the incidence of NSAID-induced gastroduodenal damage.[14] On the other hand, acid does not appear to contribute significantly to NSAID-induced damage distal to the ligament of Treitz, and protective effects of PPIs against NSAID-induced small intestinal damage in humans have not been reported.[15] Recent video capsule endoscopy studies suggest a very high incidence of small intestinal damage in young, healthy, human subjects taking both an NSAID and a PPI for 2 weeks (55%–75% vs 7%–11% in placebo treated).[16–19] This suggests that the PPI conferred little, if any, protection to the mid- and distal small intestine, which are major sites of NSAID-induced bleeding.[1,20]

Gastric acid can kill most bacteria, and chronic suppression of acid can lead to bacterial overgrowth in the stomach and small intestine.[21–23] Given the apparent importance of gram-negative bacteria in the pathogenesis of

Abbreviations used in this paper: CBS, cystathionine β-synthase; CFU, colony-forming units; COX, cyclooxygenase; CSE, cystathionine γ-lyase; DGGE, denaturing gradient gel electrophoresis; MRS, Man, Rogosa & Sharpe; NSAID, nonsteroidal anti-inflammatory drugs; PCR, polymerase chain reaction; PG, prostaglandin; PPI, proton pump inhibitor; mRNA, messenger RNA.

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NSAID enteropathy, it is possible that suppression of acid secretion by a PPI could exacerbate NSAID-induced small intestinal damage. In the present study, we tested this hypothesis using an established animal model of NSAID-induced enteropathy and using doses of the test drugs that were effective in blocking their target enzymes.

Materials and Methods

Animals

Male Wistar rats weighing 180–220 g were obtained from Charles River (Montreal, QC, Canada) and were housed in the Central Animal Facility at McMaster University. The rats were fed standard chow and water ad libitum. Germ-free National Institutes of Health (Bethesda, MD) Swiss mice (male, 8 weeks of age) were raised in the Farncombe Institute Axenic Gnotobiotic Facility, as described previously.[24] All experimental procedures described herein were approved by the Animal Care Committee of the Faculty of Health Sciences at McMaster University, and the studies were carried out in accordance with the guidelines of the Canadian Council on Animal Care.

Suppression of Acid Secretion

To confirm that omeprazole and lansoprazole, at the selected dose (10 mg/kg twice daily), were significantly suppressing gastric acid secretion, rats treated for 5 days with these drugs or with vehicle were anesthetized with isoflurane, and the pylorus was ligated (the rats were deprived of food, but not water, for 18 hours prior to this procedure). The rats were allowed to recover from the anesthetic. Three hours later, the volume and titratable acidity of the luminal fluid were determined as described previously.[25]

NSAID-Induced Enteropathy

Following collection of a blood sample (75 μL) from the tail for determination of initial hematocrit, rats were treated twice daily with omeprazole, lansoprazole (both at 10 mg/kg intraperitoneally) or vehicle for a total of 9 days. In some experiments, naproxen (10 mg/kg) or vehicle was administered orally twice daily for the final 4 days of PPI/vehicle administration. The dose of naproxen was selected based on previous studies that demonstrated that it was effective in reducing inflammation in a rat adjuvant arthritis model,[26] that is suppressed systemic COX-1 activity by >95%, and that it suppressed gastric prostaglandin synthesis by >85%.[27] Moreover, on a per kilogram basis, the selected dose is similar to that most commonly used by humans with osteoarthritis (500 mg twice daily). Four hours after the final administration of drug or vehicle, hematocrit was measured, and the extent of hemorrhagic damage in the small intestine was blindly measured (the cumulative length, in millimeters, of all lesions). Additional studies were performed in which rats were treated with celecoxib (10 mg/kg) instead of naproxen.

Pharmacokinetics of Naproxen

The effect of omeprazole on plasma and biliary naproxen levels was determined as described in the legend of Supplementary Figure 1. Concentrations of naproxen in the bile and plasma samples were determined by high-performance liquid chromatography.[28]

Effects of Omeprazole on Intestinal Mucosal Integrity

Rats treated with omeprazole (10 mg/kg) or vehicle twice daily for 9 days then anesthetized with isoflurane. A blood sample was taken from the inferior vena cava for measurement of whole blood thromboxane synthesis, as an index of systemic COX-1 activity.[29] Formalin-fixed jejunal tissue was fixed for blind histologic examination (H&E staining). Additional jejunal tissue was snap frozen in liquid nitrogen for quantitative real-time polymerase chain reaction (PCR) analysis of messenger RNA (mRNA) expression for COX-1, COX-2, endothelial nitric oxide synthesis, tumor necrosis factor (TNF) α, cystathionine γ-lyase (CSE), and cystathionine β-synthase (CBS)[30] and for measurement of prostaglandin (PG)E_2 and hydrogen sulfide synthesis.[29,31] Blood samples were collected for measurement of serum levels of various cytokines and chemokines (Quansys Biosciences, Logan, UT).

Effects of a PPI on Enteric Microflora

Preliminary studies were focused on aerobic bacteria and are described in the legend of Supplementary Figure 1.[32] Subsequently, more extensive analysis of colonization of jejunum and colon by aerobic and anaerobic bacteria was performed for analysis of any marked changes in the microbiota after administration of omeprazole or vehicle for 9 days, as above. Samples of the jejunum and colon, with the luminal contents preserved, were flash frozen in liquid nitrogen. The tissue samples (and luminal contents) were further processed for denaturing gradient gel electrophoresis (DGGE), as described below.

Bacterial DNA/RNA was extracted from biologic samples as previously described.[33] The hypervariable v4 region of the bacterial 16S ribosomal DNA gene was amplified using PCR or reverse-transcription polymerase chain reaction (RT-PCR) with universal bacterial primers (HDA1-GC, HDA-2) (Mobixlab, McMaster University core facility, Hamilton, Canada) as previously described.[34] DGGE was carried out as previously described.[35,36] A scanned image of an electrophoretic gel was used to measure the staining intensity of the fragments using Quantity One software (version 4.2; Bio-Rad Laboratories, Hercules, CA). The intensity of fragments is expressed as a proportion (%) relative to the sum of the intensities of all of the fragments in the same lane of the image.[37]

Identification of bacterial phylogenies from DNA bands or bacterial colonies was performed as previously described.[34] PCR products were first checked by DGGE before being sent for sequencing using the didoexy method.[38] The retrieved sequences was compared with sequences among the Ribosomal Database Project (RDP)-II and National Center for Biotechnology Information GenBank (Bethesda, MD) databases using the maximum likelihood algorithm, and the sequences were used to represent phylotypes. In addition, real-time PCR was performed to determine the presence of Actinobacteria, *Bifidobacter* spp, and various specific *Bifidobacteria* species in jejunal samples from rats that had been treated with omeprazole or vehicle, as above (see Supplementary Tables 1 and 2).

Effects of Administration of Selected Commensal Bacteria on PPI-Induced Dysbiosis and NSAID Enteropathy

Commensal bacteria were isolated from samples of jejunal luminal contents from healthy rats by culture on Man, Rogosa & Sharpe (MRS) complemented with cysteine (0.5 g/L) and mupirocin (50 mg/L) and grown anaerobically at 37°C for

48 hours, which selectively promotes growth of *Bifidobacteria*.[39] PCR analysis of the broth (see list of primers in Supplementary Table 1) confirmed the presence of *Bifidobacter* spp, *Bifidobacteria breve*, and *Bifidobacteria longum* in the "enriched" broth but not in the control MRS broth. Rats were treated with omeprazole, twice daily, for a total of 9 days. Subgroups of 6 rats each were randomly assigned to be treated with a suspension of the selected commensal bacteria or with sterile MRS broth once daily the final 4 days. The selected commensal bacteria were administered orally at a dose of 10^9 colony-forming units (CFU) each day. In another series of experiments, rats (n = 12 per group) received omeprazole or vehicle, twice daily, for a total of 9 days, and received naproxen (10 mg/kg) twice daily beginning on the final 4 days. Subgroups of 6 rats each were randomly assigned to be treated once daily with a suspension of the selected commensal bacteria (10^9 CFU each day) or with sterile MRS broth 3 hours after the morning administration of naproxen each day. Four hours after the final administration of naproxen, the extent of gastric and intestinal damage was blindly scored, and jejunal tissue and contents were collected for PCR-DGGE analysis.

Effects of Colonization of Germ-Free Mice With Microflora From PPI-Treated Rats

Groups of 5 rats each were treated with omeprazole or vehicle, for 5 days, as described above. They were killed by an overdose of isoflurane, and the contents of a segment of jejunum were collected. For each group, the jejunal contents were pooled. Two groups of germ-free mice (n = 8/group) were given jejunal contents orally: each mouse in 1 group receiving 0.1 mL of jejunal contents from the PPI-treated rats, and each mouse in the other group receiving 0.1 mL of jejunal contents from vehicle-treated rats. One week later, 3 mice from each group were killed by an overdose of isoflurane, and the small intestine was examined (blindly) for signs of injury. The remaining mice were given naproxen (10 mg/kg) orally twice daily for 4 days and were then killed by an overdose of isoflurane. A segment of jejunum was fixed in neutral-buffered formalin and processed for blind histologic evaluation (H&E staining). The following scoring system was used: 0, normal; 1, mild sloughing of surface epithelial cells; 2, moderate sloughing of surface epithelial cells; 3, extensive mucosal edema or mucosal injury extending deeper than the gastric pits; 4, extensive mucosal injury.

Statistical Analysis

Groups of data were compared with one another using a 1-way analysis of variance followed by the Dunnett's Multiple Comparison test (for parametric data) or with the Mann–Whitney *U* test (for nonparametric data). An associated *P* value of <5% was considered significant.

Results

Effective Target Enzyme Inhibition Was Achieved With PPIs and Naproxen

Twice daily administration of omeprazole or lansoprazole resulted in > 99% suppression of gastric acid secretion by the fifth day when twice daily administration of naproxen was initiated in subsequent experiments (Figure 1*A* and *B*). The mean pH in the vehicle-treated group was 1.6, and that in the omeprazole and lansoprazole groups was 3.6 and 3.9, respectively (n ≥ 6 per group).

Naproxen inhibited systemic COX-1 activity (whole blood thromboxane synthesis) by > 90% after a single dose and by 99% after twice daily dosing for 4 days. Naproxen inhibited intestinal PGE_2 synthesis by > 85% after a single dose and > 95% after 4 days of twice daily dosing. There was no significant difference in the degree of suppression of thromboxane or PGE_2 synthesis by naproxen between the groups cotreated with vehicle or with a PPI.

PPIs Exacerbated Small Intestinal Damage and Bleeding Induced by NSAIDs

Naproxen administration over 4 days (twice daily) resulted in very low levels of hemorrhagic damage in the stomach and small intestine. When naproxen was given to rats receiving a PPI, no gastric damage was observed, but intestinal damage was significantly worsened (Figure 1*C* and *D*). At the time of death, blood was evident in the lumen of all rats treated with naproxen and a PPI, and ulcers were clearly evident (Figure 1*E*). Consistent with the luminal blood, coadministration of naproxen with a PPI resulted in a significant decrease in hematocrit (Figure 1*F*), whereas naproxen alone or a PPI alone had no significant effect.

In rats given a selective COX-2 inhibitor (celecoxib) rather than naproxen, a similar exacerbation of small intestinal injury was observed in the animals coadministered a PPI. The mean small intestinal damage score in rats treated with celecoxib alone was 0.5 ± 0.3, whereas that in rats treated with omeprazole and celecoxib was 35.1 ± 4.6 ($P < .001$). Blood was present in the lumen of the small intestine of rats treated with omeprazole and celecoxib, and a significant decrease in hematocrit was observed (−6.2% vs 2.2% vs −0.5% vs 0.3% in rats treated with vehicle + celecoxib; $P < .05$).

Omeprazole Did Not Cause Intestinal Damage or Inflammation

The jejunum of rats treated only with omeprazole for 9 days did not exhibit any histologic signs of inflammation or damage. Tissue myeloperoxidase levels were similar in vehicle- and omeprazole-treated rats (50.0 ± 6.8 vs 48.9 ± 4.6 U/mg tissue, respectively), and there was no histologic evidence of inflammation or damage. Jejunal PGE_2 synthesis was not affected by omeprazole treatment (Supplementary Figure 2*A*). However, jejunal hydrogen sulfide synthesis was increased by ~100% in rats treated with omeprazole (Supplementary Figure 2*B*). Expression of mRNA for enzymes PG synthesis (COX-1 and COX-2; Supplementary Figure 2*C* and *D*) and hydrogen sulfide synthesis (CSE and CBS; Supplementary Figure 2*E* and *F*) was not affected by omeprazole. Similarly, omeprazole did not affect expression of mRNA for endothelial nitric oxide synthase (1.38- ± 0.24-fold change vs vehicle treated) or TNF-α (1.55- ± 0.51-fold change vs vehicle treated). Omeprazole also did not significantly change serum

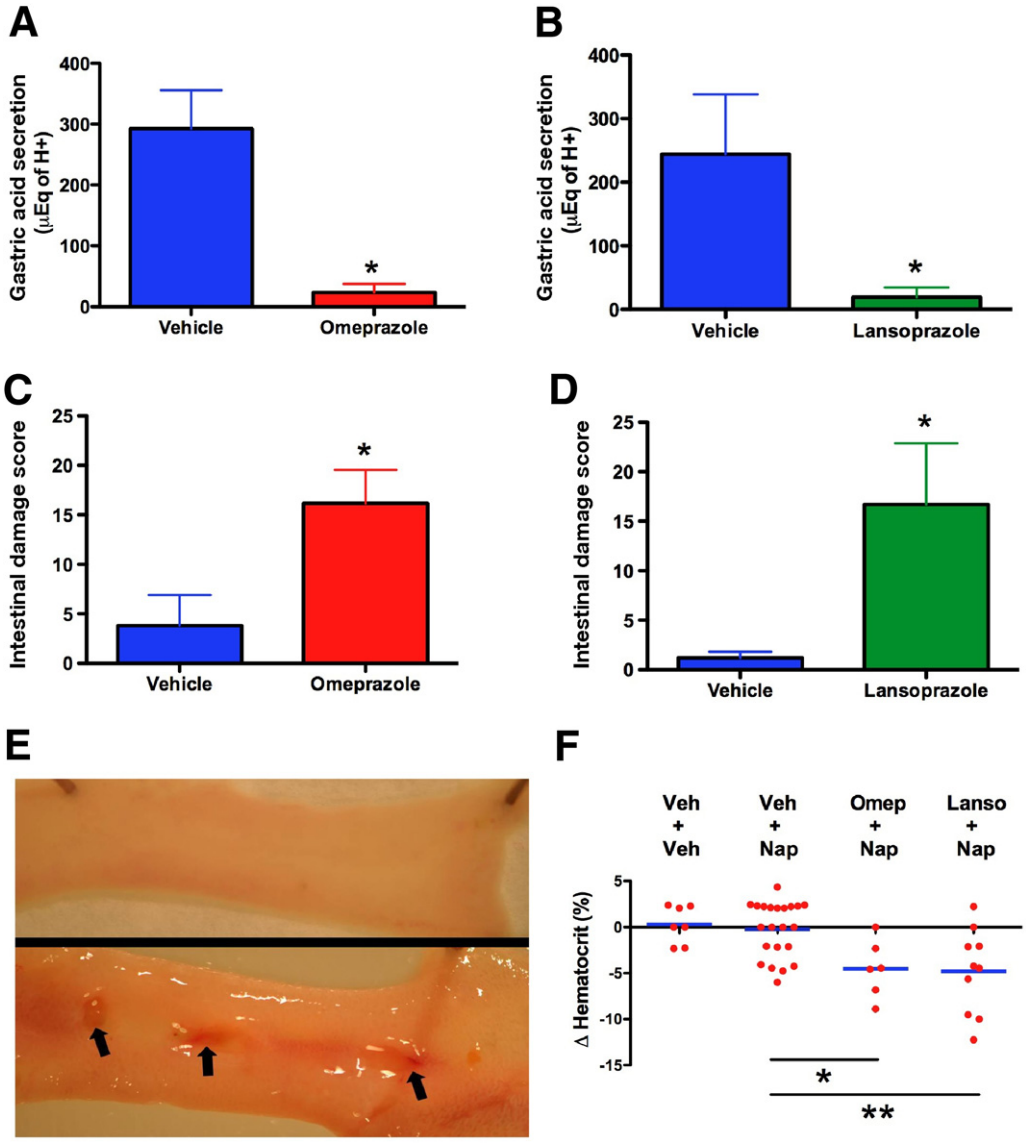

Figure 1. PPIs suppress gastric acid secretion, but they exacerbate small intestinal injury and increase bleeding. Both omeprazole (*red bars*) and lansoprazole (*green bars*), each at 10 mg/kg twice daily for 5 days, significantly reduced gastric acid secretion (*A* and *B*, respectively). *Panels C* and *D* show the exacerbation of small intestinal injury. Naproxen was given at 10 mg/kg twice daily orally for 4 days. *Bars* represent the mean ± standard error of mean. *Panel E* shows jejunal tissue from a naproxen-treated rat (*top panel*), with no macroscopic damage, whereas ulcers (*arrows*; *lower panel*) are apparent in the jejunum of a rat treated with naproxen and omeprazole. There was no significant change in hematocrit with naproxen alone (*F*; *blue lines* represent the average), but coadministration of omeprazole or lansoprazole resulted in a significant decrease in hematocrit (*$P < .05$, **$P < .01$ vs the vehicle + naproxen group).

levels of several cytokines and chemokines (Supplementary Table 3).

Omeprazole Did Not Alter Naproxen Pharmacokinetics

The exacerbation of naproxen-induced intestinal injury by omeprazole was not related to altered absorption or biliary excretion of naproxen (Supplementary Figure 1*A* and *B*). The slightly lower levels of naproxen in bile from omeprazole-treated rats would be expected to be associated with less severe intestinal damage, but the opposite was the case.

Omeprazole Treatment Resulted in Dysbiosis

Treatment of rats with omeprazole for 9 days resulted in significant increases in the numbers of aerobic bacteria (both gram negative and gram positive) in the jejunum (Supplementary Figure 1*C*). PCR-DGGE analysis revealed that omeprazole-treated rats had significantly lower proportion of Actinobacteria (~75%; $P < .05$) in the jejunum as compared with vehicle-treated rats (Figure 2*A* and *B*; Supplementary Figure 3). *Bifidobacteria* likely accounted for a significant component of the Actinobacteria (operational taxonomic unit sequences, based on the 16S ribosomal DNA database, are attributed to Actinobacteria at 97% identity

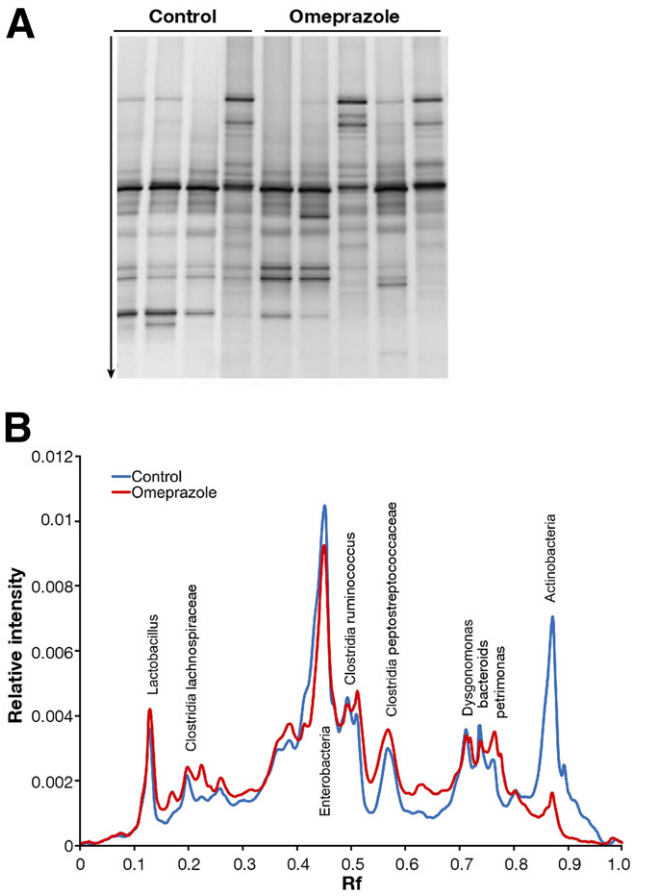

Figure 2. Treatment with omeprazole caused significant intestinal dysbiosis. PCR-DGGE revealed a key difference in the jejunal microbiota between rats treated with vehicle (control) vs omeprazole (10 mg/kg) twice daily for 9 days (each *lane* in *panel A* represents 1 rat, whereas the *lines* in *panel B* represent the mean of 4 or 5 rats). Rf, retention factor. Omeprazole-treated rats exhibited a relative absence of Actinobacteria in the jejunum compared with the control rats (*lower panel*). There were no significant differences in the numbers of any of the other subgroups of bacteria between the control and omeprazole-treated groups.

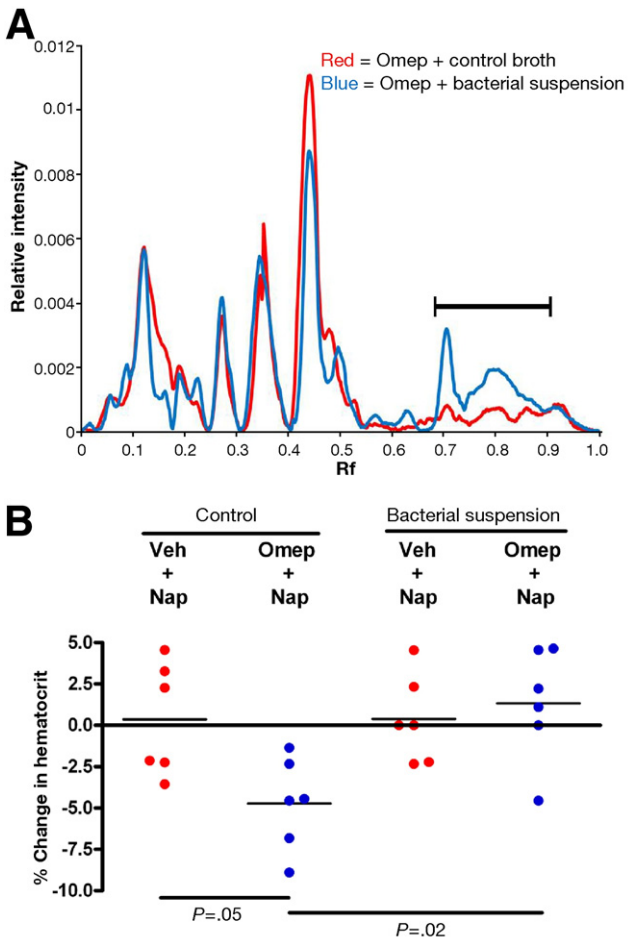

Figure 3. Administration of selected commensal bacteria (*Bifidobacteria* enriched) reversed the PPI-induced dysbiosis and the increase in naproxen-induced bleeding. (*A*) Administration of selected commensal bacteria to rats treated with omeprazole resulted in an increase ($P < .05$) in jejunal Actinobacteria (*black bar*) but had no effect on the relative proportion of any other subgroups of bacteria (ie, no significant difference between the controls and bacterial suspension-treated rats). Rf, retention factor. (*B*) Treatment with the selected commensal bacteria abolished the increase in bleeding (decreased hematocrit) associated with administration of omeprazole and naproxen.

and correspond to *Bifidobacteria* at a 94% identity threshold). RT-PCR analysis of jejunal tissue and contents confirmed a marked reduction (80%; $P < .001$) of the levels of Actinobacteria and *Bifidobacteria* spp (Supplementary Table 2) in omeprazole-treated rats.

Recolonization With Bifidobacteria-Enriched Commensal Bacteria Prevented Intestinal Damage and Bleeding

Administration of selected commensal bacteria (*Bifidobacteria* enriched) to rats receiving omeprazole partially reversed the PPI-induced dysbiosis (Figure 3*A*). Whereas naproxen elicited widespread damage in omeprazole-treated rats that were administered the sterile MRS broth (mean damage score of 17 ± 5), the daily administration of 10^9 CFU of selected commensal bacteria to omeprazole/naproxen-treated rats reduced intestinal damage to a level (mean score of 2 ± 2) not different from that observed in rats treated with vehicle/naproxen and sterile MRS broth (mean score of 1 ± 1). Consistent with the lack of intestinal damage in rats treated with selected commensal bacteria, this treatment abolished the decrease in hematocrit that was seen when naproxen and omeprazole were coadministered (Figure 3*B*).

PPI-Induced Exacerbation of NSAID Enteropathy Is Transferrable via Microbiota

Germ-free mice have been reported to be resistant to NSAID-induced enteropathy.[9,10] In our study, germ-free NIH Swiss mice colonized for 1 week with jejunal bacteria from rats treated with vehicle exhibited only mild hyperemia when administered naproxen twice daily for 4 days (Figure 4*A*). Mucosal structure was largely intact (Figure 4*B*). However, germ-free mice colonized with jejunal bacteria from PPI-treated rats developed significant small intestinal damage when given naproxen (Figure 4*A*). The small intestine was friable, with mucosal damage (Figure 4*C*) and extensive subepithelial edema (Figure 4*D*).

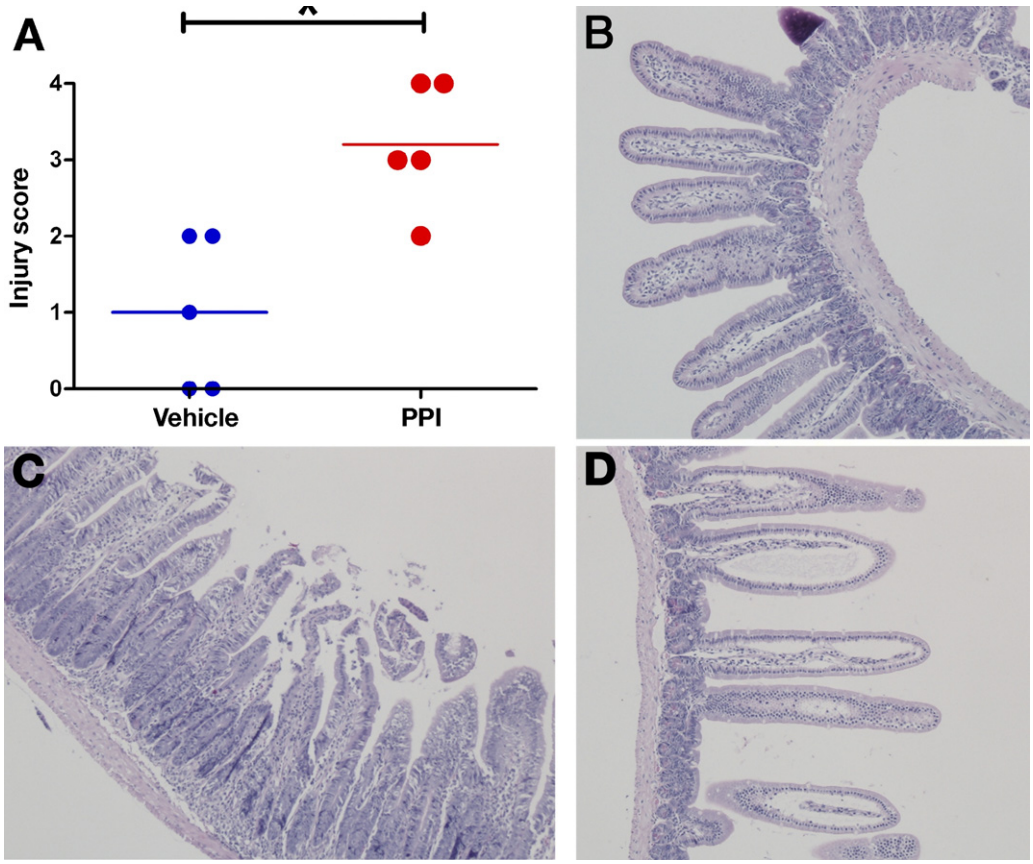

Figure 4. PPI-induced exacerbation of NSAID enteropathy is transferrable via intestinal microflora. (*A*) Germ-free mice colonized by intestinal flora from PPI-treated rats developed more severe naproxen-induced small intestinal damage than germ-free mice colonized by intestinal flora from vehicle-treated rats (*$P < .05$). (*B*) In the mice colonized with intestinal flora from vehicle-treated rats, mucosal structure was largely intact following twice daily naproxen administration over 4 days. (*C*) In mice colonized with intestinal flora from PPI-treated rats, twice daily naproxen administration over 4 days resulted in epithelial damage in the small intestine and, in some mice (*D*), extensive subepithelial edema. *Scale bar,* 10 μm.

Granulocyte infiltration was not evident histologically, and this was confirmed by the near absence of myeloperoxidase activity in germ-free mice, regardless of the source of intestinal microbiota with which they were colonized (data not shown). Neither group of germ-free mice exhibited intestinal injury in the absence of administration of naproxen.

Discussion

NSAIDs and PPIs are 2 of the most widely used classes of drugs, and, increasingly, patients taking the former for treatment of inflammatory conditions are also advised to take the latter to reduce the risk of gastric ulceration and bleeding.[40] This approach is rational and evidence based for reducing the incidence of gastric injury[14,40,41] but questionable as a strategy for producing beneficial effects in the small intestine.[20,42] The results of the present study, which used doses of NSAIDs and PPIs that produced effective inhibition of their target enzymes (COX and H^+/K^+-ATPase, respectively), demonstrate that a reduction of gastric injury by the PPI was accompanied by a marked exacerbation of small intestinal ulceration and bleeding. The increase in intestinal damage was not due to altered pharmacokinetics of naproxen. The enhancement of NSAID enteropathy was confirmed with a second PPI (lansoprazole) and with second NSAID (celecoxib; selective COX-2 inhibitor).

These observations in rodent models suggest that a careful evaluation of the use of PPIs together with NSAIDs is warranted. Assessment of the impact of these frequently used drugs on intestinal damage has not been directly addressed in humans. However, several recent studies[16–19] in which healthy human volunteers were given both an NSAID and a PPI demonstrated a very high incidence of damage in the small intestine (55%–70%), as detected by video capsule endoscopy. It is noteworthy that this high incidence of small intestinal damage was observed with short-term NSAID administration (up to 2 weeks) in a population with a low risk of NSAID-induced gastroenteropathy (young, healthy individuals). Of course, it is possible that the observed worsening of NSAID enteropathy is more apparent in rodents than in humans because of different microbiota and hygiene (eg, rodents being coprophagic).

NSAID-induced enteropathy has a pathogenesis distinct from that of the gastric damage induced by these drugs.[3] Acid is unlikely to play a significant role in the production of intestinal injury beyond the proximal du-

odenum. Whereas inhibition of mucosal COX-1 and COX-2 is of paramount importance in the development of gastric lesions,[29] the small intestinal damage caused by NSAIDs is more closely tied to their enterohepatic circulation and the consequent repeated exposure of the intestinal epithelium to these drugs in the presence of bile.[2,3,6,7] Studies using antibiotics, germ-free animals, and Toll-like receptor 4–deficient mice suggest an important role of enteric bacteria (particularly gram negative) in the development of NSAID-induced lesions in the small intestine.[8–13] Interestingly, monocolonization of germ-free rats with *Escherichia coli* or *Eubacterium limosum* restored the susceptibility to NSAID-induced intestinal damage, but monocolonization with *Bifidobacter adolescentis* or *Lactobacillus acidophilus* did not.[10] Our studies demonstrate that treatment with omeprazole resulted in significant dysbiosis. A striking effect of omeprazole was the significant reduction in the proportion of Actinobacteria in the jejunum. Based on sequencing, *Bifidobacter* ssp were the most prominent members of the Actinobacteria phylum in the jejunum. RT-PCR confirmed this finding, and the significant (80%) reduction of Actinobacteria and *Bifidobacter* spp in omeprazole-treated rats. Administration of a suspension of selected commensal bacteria (*Bifidobacteria* enriched) reversed the omeprazole-associated decrease in Actinobacteria and abolished the increased susceptibility to damage in omeprazole-treated rats. Moreover, our studies using germ-free mice demonstrated that the elevated susceptibility to NSAID enteropathy could be transferred to the mice through the microbiota. Together, these data strongly suggest that the dysbiosis induced by a PPI is a major contributing factor to the increased susceptibility to NSAID-induced small intestinal injury. Although detailed studies of PPI-induced changes in relative proportions of bacterial species in humans have not been reported, several studies have documented significant increases in small intestinal bacterial numbers in PPI users.[21–23] Interestingly, elevated numbers of small intestinal bacteria has been linked to significant increases in small intestinal permeability,[43] which is widely accepted as an early event in the pathogenesis of NSAID enteropathy.[2,4]

Whereas treatment with a PPI alone caused significant changes in small intestinal bacteria, it had little detectable effect on the intestinal mucosa. Histologically, the tissue was not damaged or inflamed. There were no significant changes in the expression of genes for a number of enzymes involved in mucosal defense.[3] Expression of the key enzymes for synthesis of PGs (COX-1 and COX-2), endothelial nitric oxide (eNOS), and hydrogen sulfide (CSE and CBS) were not altered by omeprazole treatment. Mucosal expression of mRNA for TNF-α, which has been implicated in the pathogenesis of NSAID enteropathy,[44] was also not changed by omeprazole. However, a significant increase in mucosal hydrogen sulfide synthesis was observed. Increased mucosal hydrogen sulfide synthesis has been observed following induction of injury in the stomach[45] and colon[31] and has been shown to contribute significantly to the healing of that damage and to resolution of inflammation. On the other hand, reduced mucosal hydrogen sulfide synthesis has been associated with an increase in mucosal inflammation, impaired tissue repair, and increased susceptibility to NSAID-induced damage.[31,45,46]

Selective COX-2 inhibitors cause small intestinal damage, albeit somewhat less frequently than NSAIDs that inhibit both COX-2 and COX-1 at therapeutic doses.[47] We observed that an effective anti-inflammatory dose of celecoxib caused negligible small intestinal damage, but, when given together with omeprazole, there was extensive small intestinal ulceration, overt bleeding, and a significant decrease in hematocrit. The magnitude of the decrease in hematocrit was greater than the threshold (2%) that is considered "clinically significant" in humans.[47]

The absence of clinical studies examining the potential impact of PPIs on NSAID-induced enteropathy is somewhat surprising given the widespread use of these 2 classes of drugs. It is most likely related to the difficulty, with conventional endoscopy, of adequately and easily assessing damage in the more distal parts of the small intestine. Clinical trials using video capsule endoscopy to evaluate the impact of PPIs on NSAID-induced enteropathy would be informative.

If it is the case in humans, as in rats, that PPIs do not protect the small intestine from NSAID-induced injury, or can even exacerbate that injury, other approaches have to be considered to reduce the incidence of this serious adverse effect.[40,42] However, there are few obvious strategies available at present. Given the role of bacteria in the pathogenesis of NSAID enteropathy, antibiotics are another prophylactic option, but their use is limited by adverse effects and the potential for development of drug resistance.[40,42] Appropriate prebiotics or probiotics may be a viable approach to prevention of NSAID enteropathy, but this requires further study. Until such a time as a viable, cost-effective method of preventing NSAID-associated small intestinal injury is available, the results of the present study suggest that caution should be exercised in the combined use of PPIs and NSAIDs. Benefits achieved in terms of reduced gastroduodenal damage and bleeding may be offset by a significant increase in damage more distally, which is more difficult to detect and treat in a clinical setting.

Supplementary Material

Note: To access the supplementary material accompanying this article, visit the online version of *Gastroenterology* at www.gastrojournal.org, and at doi: 10.1053/j.gastro.2011.06.075.

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Conflicts of interest

These authors disclose the following: M.B. and E.O. are employees of NicOx S.A., a company developing anti-inflammatory drugs. J.L.W. is a founder and shareholder of Antibe Therapeutics Inc, a company developing anti-inflammatory drugs. The remaining authors disclose no conflicts.

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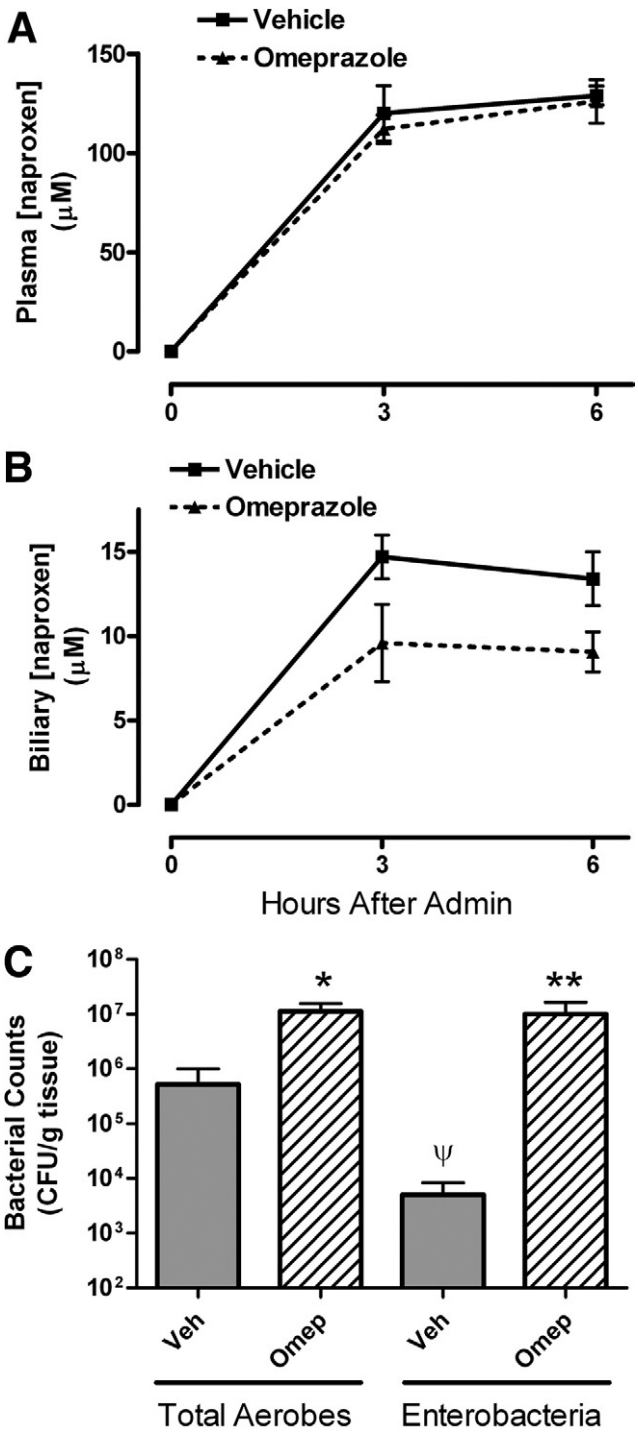
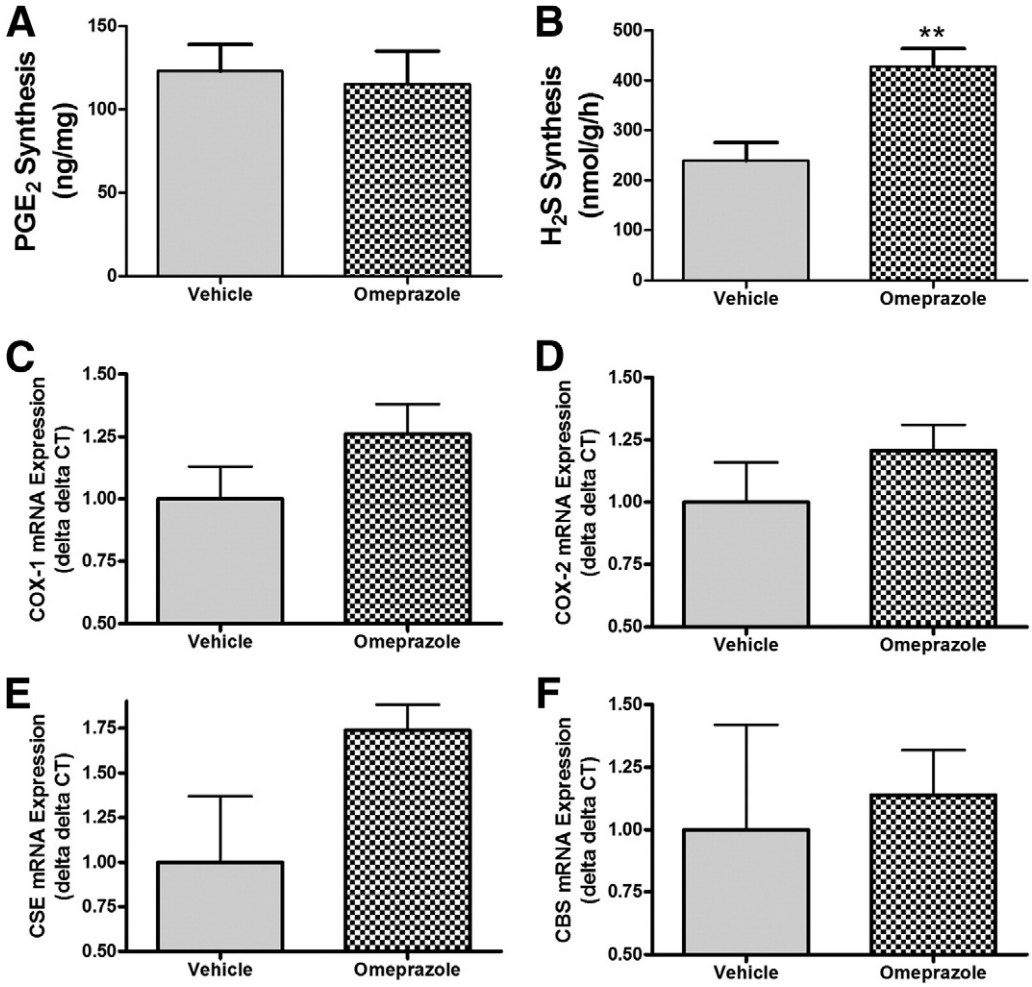
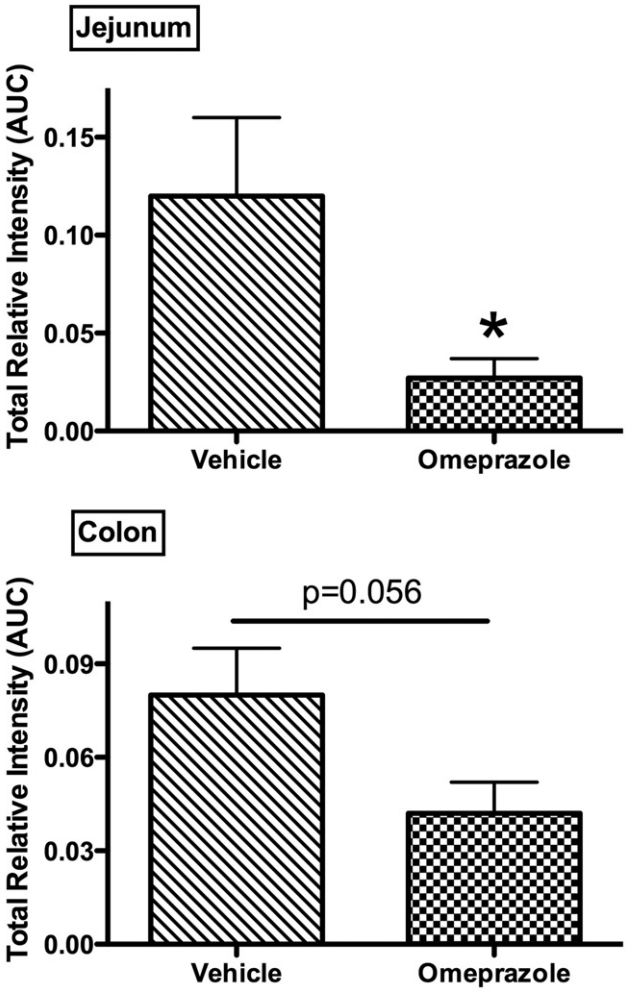


Supplementary Figure 1. Omeprazole does not alter the pharmacokinetics of naproxen but does trigger bacterial overgrowth in the small intestine. Rats were given omeprazole (10 mg/kg) orally twice a day for 5 days then were given a single dose of naproxen (10 mg/kg) orally. Subgroups (n = 3 each) of rats were anesthetized with isoflurane 3 or 6 hours later. The bile duct was cannulated,[1] and bile was collected for 30 minutes, after which a blood sample was drawn from the inferior vena cava. *Panels A* and *B* show the plasma and biliary levels of naproxen, respectively, in rats treated twice daily with omeprazole (10 mg/kg) or vehicle for 5 days prior to naproxen administration. There were no significant differences between the groups. *Panel C* shows the number of total aerobes and of enterobacteria in the jejunum of rats treated with vehicle or omeprazole. $^{*}P < .05$, $^{**}P < .01$ vs the corresponding vehicle-treated group. $^{\Psi}P < .05$ vs the number of total aerobes in vehicle-treated rats. In this experiment, rats were treated twice daily with omeprazole (10 mg/kg) or vehicle and were then anesthetized with isoflurane. A sample (~150 mg) of jejunum was flash frozen in liquid nitrogen. The contents of the jejunum were subsequently diluted serially and plated onto MacConkey agar or blood agar then incubated for 18–24 hours under aerobic conditions.[2] Plates containing between 20 and 200 colony-forming units (CFU) were analyzed to determine bacterial levels, and the results expressed as CFU per gram of tissue.



Supplementary Figure 2. Omeprazole treatment resulted in elevated jejunal hydrogen sulfide (H_2S) synthesis but did not affect PG synthesis or expression of messenger RNA for several key enzymes for synthesis of PGs and H_2S. *Panels A* and *B* show jejunal PGE_2 and H_2S synthesis, respectively (**$P < .01$ vs the vehicle-treated group). *Panels C* through *F* show expression of messenger RNA for COX-1 and -2, cystathionine γ-lyase (CSE), and cystathionine β-synthase (CBS), respectively. CT, threshold cycle.



Supplementary Figure 3. Omeprazole treatment (10 mg/kg), twice daily for 9 days, significantly reduced the numbers of Actinobacteria in the jejunum (*$P < .05$). The ~40% reduction of Actinobacteria in the colon of omeprazole-treated rats did not reach statistical significance ($P = .056$; n = 4 or 5 per group).

Supplementary Table 1. Reverse-Transcription Polymerase Chain Reaction Primers Used to Detect Actinobacteria and *Bifidobacteria*

Target bacterial group/species	Sequence (5′–3′)
Actinobacteria phylum	CGCGGCCTATCAGCTTGTTG
	CCGTACTCCCCAGGCGGGG
Bifidobacterium group	CTCCTGGAAACGGGTGG
	GGTGTTCTTCCCGATATCTACA
B longum group	TTCCAGTTGATCGCATGGTC
	TCACGCTTGCTCCCCGAT
B bifidum	CCACATGATCGCATGTGATTG
	CCGAAGGCTTGCTCCCAAA
B breve	CCGGATGCTCCATCACAC
	ACAAAGTGCCTTGCTCCCT
B adolescentis	CTCCAGTTGGATGCATGTC
	TCCAGTTGACCGCATGGT
B catenulatum group	CGGATGCTCCGACTCCT
	CGAAGGCTTGCTCCCGAT
B angulatum	CAGTCCATCGCATGGTGGT
	GAAGGCTTGCTCCCCAAC
B lactis	GTGGAGACACGGTTTCCC
	CACACCACACAATCCAATAC

NOTE. These primers were used to detect the target bacterial groups/species in samples of jejunal tissue and in the broth containing selected commensal bacteria.[3] In all cases, the annealing temperature was 55°C.

Supplementary Table 2. Prevalence of Bacteria in Jejunal Samples: Effects of Omeprazole

	Vehicle			Omeprazole		
	Detection incidence	Median number (log)	Interquartile range (log)	Detection incidence	Median number (log)	Interquartile range (log)
Actinobacteria	5/5	4.96	5.13–4.89	6/6	4.25[a]	4.58–4.09
Bifidobacteria spp	5/5	3.80	4.72–3.38	0/6		
B longum	2/5			1/6		
B breve	2/5			0/6		
B bifidum	0/5			0/6		
B adolescents	0/5			0/6		
B catenulatum group	0/5			0/6		
B angulatum	0/5			0/6		
B lactis	0/5			0/6		

NOTE. Jejunal tissue samples and luminal contents were collected from each rat and immediately frozen in liquid nitrogen. Bacterial DNA was then extracted from samples and from bacterial strains used as ladder using the QIAamp DNA stool Mini kit (Qiagen, Toronto, Canada) following the manufacturer's instructions. PCRs were performed to determine the presence of Actinobacteria and *Bifidobacterium* populations within the Actinobacteria phylum using phylum-, genus-, and species-specific primers (Supplementary Table 1). Real-time quantitative PCR was used to quantify the different bacterial groups. The PCR amplification and detection were performed with an Eppendorf Realplex4 (Eppendorf Ltd, Mississauga, ON, Canada) using Perfecta SYBR Green PCR Master Mix (Applied Biosystems Ltd, Streetsville, ON, Canada). The bacterial concentration from each sample was calculated by comparing the threshold cycle values obtained from standard curves of reference strains. Standard curves were created using serial 10-fold dilutions of pure culture DNA corresponding to 10^2 to 10^9 cells, as determined by plate counts.
[a]$P < .001$ vs the vehicle-treated group.

Supplementary Table 3. Effects of Omeprazole on Serum Levels of Various Cytokines and Chemokines

Treatment	IL-2	IL-17	IFN-γ	GM-CSF	RANTES
Vehicle	321 ± 38	693 ± 84	14 ± 6	254 ± 26	4476 ± 326
Omeprazole	351 ± 47	770 ± 103	26 ± 7	280 ± 27	6791 ± 2421

GM-CSF, granulocyte macrophage colony-stimulating factor; IFN, interferon; IL, interleukin; RANTES, regulated on activation normal T cell expressed and secreted; SEM, standard error of mean; TNF, tumor necrosis factor.
NOTE. Serum levels are measured as picograms/mL. Blood was collected from rats (n = 6/group) treated with omeprazole (10 mg/kg) or vehicle orally twice daily for 9 days. Serum was frozen for subsequent measurement, in triplicate, of various cytokines and chemokines by enzyme-linked immunosorbent assay. The levels of IL-1β, IL4, IL-6, IL-10, IL-12p70, and TNF-α in most samples were below the limits of detectability. Results are shown as the mean ± SEM. There were no significant differences between the 2 treatment groups.