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RESEARCH ARTICLE

Figures and figure supplements

Bidirectional interactions between indomethacin and the murine intestinal microbiota

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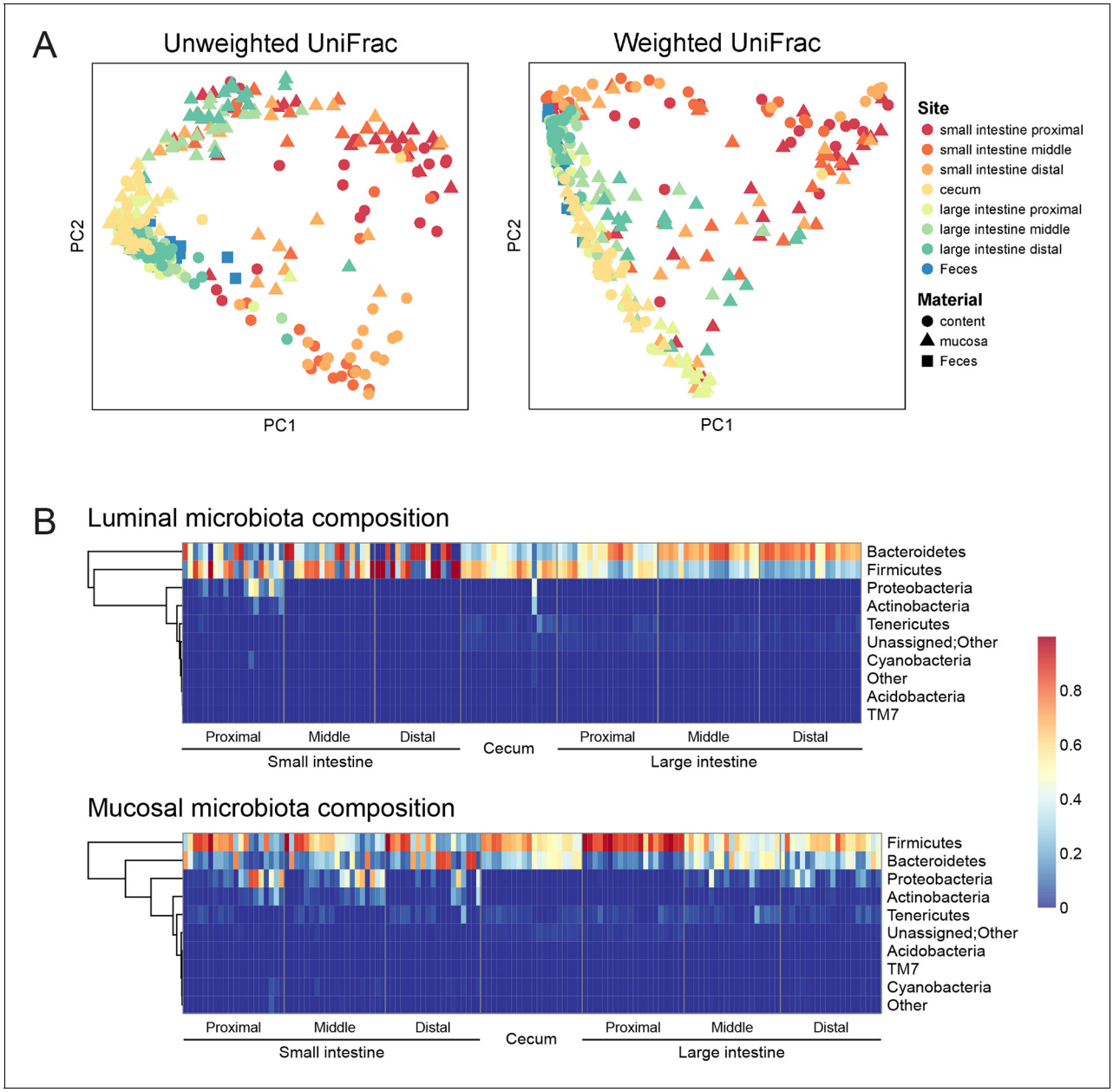

Figure 1. Geographic heterogeneity of basal intestinal microbiota composition along the intestine in mice. Bacterial communities colonized in the mouse intestine were profiled using 16S rRNA gene sequencing and analyzed using QIIME (***Caporaso et al., 2010b***). (**A**) Principal coordinates analysis (PCoA) of unweighted (left) and weighted (right) UniFrac values (***Lozupone et al., 2011***), depicting the comparison of microbial communities from luminal content (round), mucosal tissue (triangle), or feces (square). The base line microbiota compositions along the intestine are heterogeneous at anatomical sites. Each point represents a sample, and each sample is colored according to the habitat sites in the intestine. N=17–20. (**B**) Heat map of the microbiota composition in luminal content (upper) and mucosal tissue (lower) along the intestine. Each column represents sample, and each row represents one phylum. The proportions of phyla are indicated by the color code to the right. Anatomical sites of the intestine are indicated at the bottom. N=17–20.

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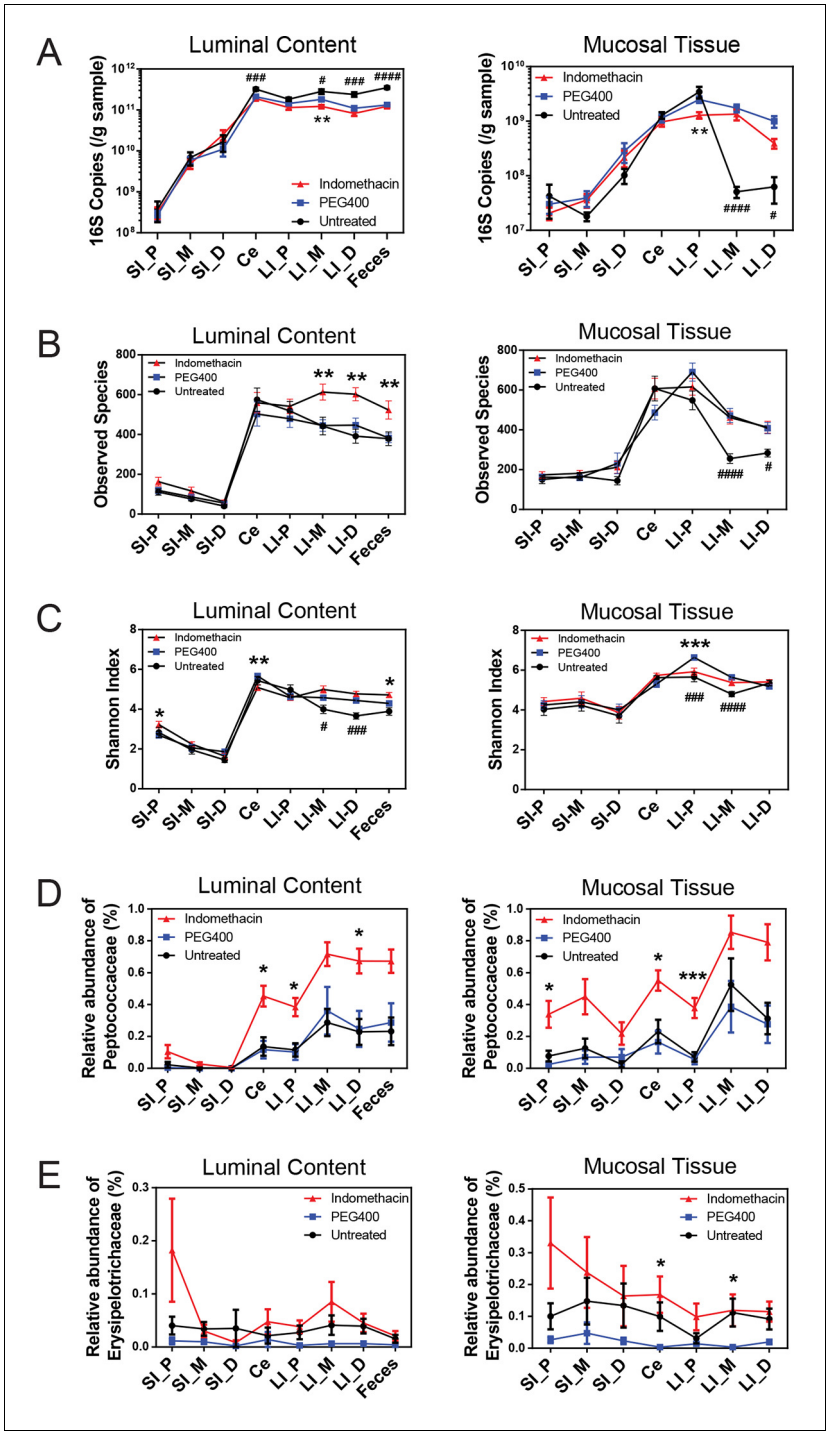

Figure 2. Indomethacin induces changes in microbial composition along the intestine in mice. Bacterial load in samples were inferred from 16S rRNA gene quantitative PCR. Bacterial communities colonized in the mouse intestine were profiled using 16S rRNA gene sequencing and analyzed using QIIME (***Caporaso et al., 2010b***). (**A**) 16S rRNA gene copies per gram of luminal contents (left) and mucosal tissues (right) at anatomical sites along the intestine in indomethacin (red), PEG400 (blue), and untreated (black) groups. Microbial loads at anatomical sites along the intestine are barely different between PEG400 and indomethacin groups, although PEG400 causes changes by itself. **p<0.01 by multiple t test comparing PEG400 versus indomethacin groups, FDR corrected. #p<0.05, ###p<0.001, ####p<0.0001 by multiple t test comparing untreated versus PEG400 groups, FDR corrected. N=20/group. Mean ± S.E.M. shown. SI, small intestine; Ce, cecum; LI, large intestine. P, proximal; M, middle; D, distal. Observed Species (**B**) and Shannon Index (**C**) are used to estimate richness and diversity of

Figure 2 continued on next page

Figure 2 continued

microbial communities in luminal content (left) and mucosal tissue (right) at anatomical sites along the intestine in indomethacin (red), PEG400 (blue), and untreated (black) groups. Indomethacin altered microbial diversity in the distal intestine, although PEG400 also causes changes in the distal intestine by itself. *p<0.05, **p<0.01, ***p<0.001 by multiple t test comparing PEG400 versus indomethacin groups, FDR corrected. #p<0.05, ###p<0.001, ####p<0.0001 by multiple t test comparing untreated versus PEG400 groups, FDR corrected. N=20/group. Relative abundance of *Peptococcaceae* (**D**) and *Erysipelotrichaceae* (**E**) at anatomical sites along the intestine are significantly elevated in indomethacin (red) group than in PEG400 (blue) and untreated (black) group in both luminal content and mucosal tissues of the distal gut. *p<0.05, ***p<0.001 by QIIME analysis, FDR corrected. Mean ± S.E.M. shown. SI, small intestine; Ce, cecum; LI, large intestine. P, proximal; M, middle; D, distal.

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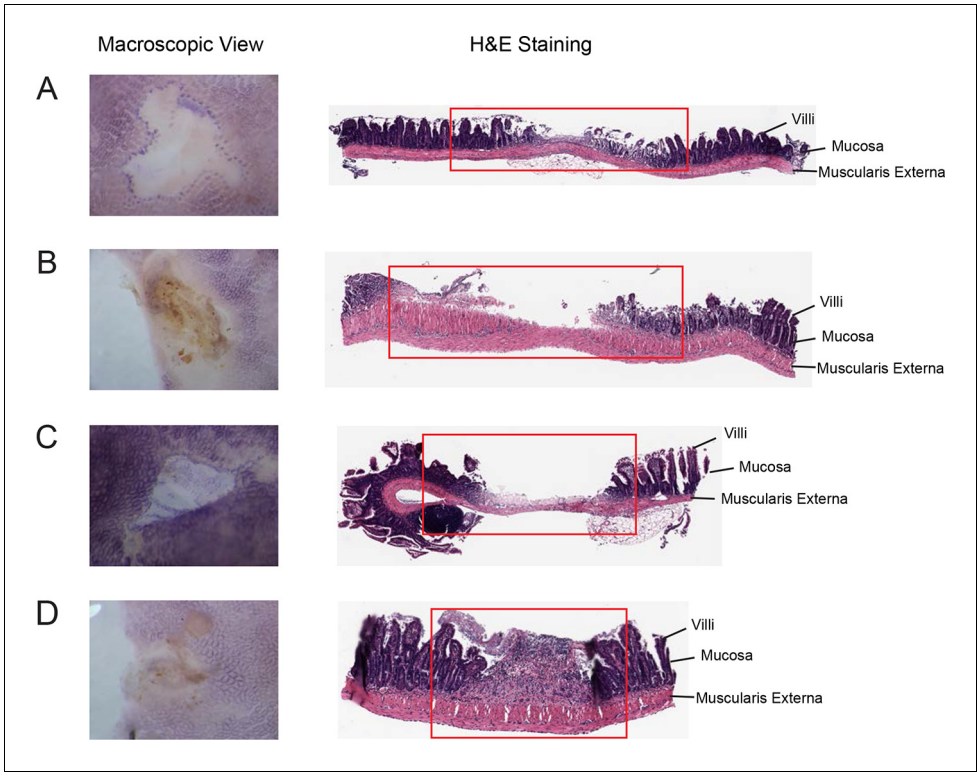

Figure 2—figure supplement 1. Indomethacin induces small intestinal damage in C57BL/6 mice. Representative sections of small intestinal injuries 24 hr after 10 mg/kg indomethacin treatment, including macroscopic views (left) and hematoxylin and eosin (H&E) staining (right). Macroscopically identified lesion areas were cut out for histopathology analysis by H&E staining at the center of the area. Red rectangle outlines the mucosal erosion (**A**) and ulcerations (**B-D**) observed.

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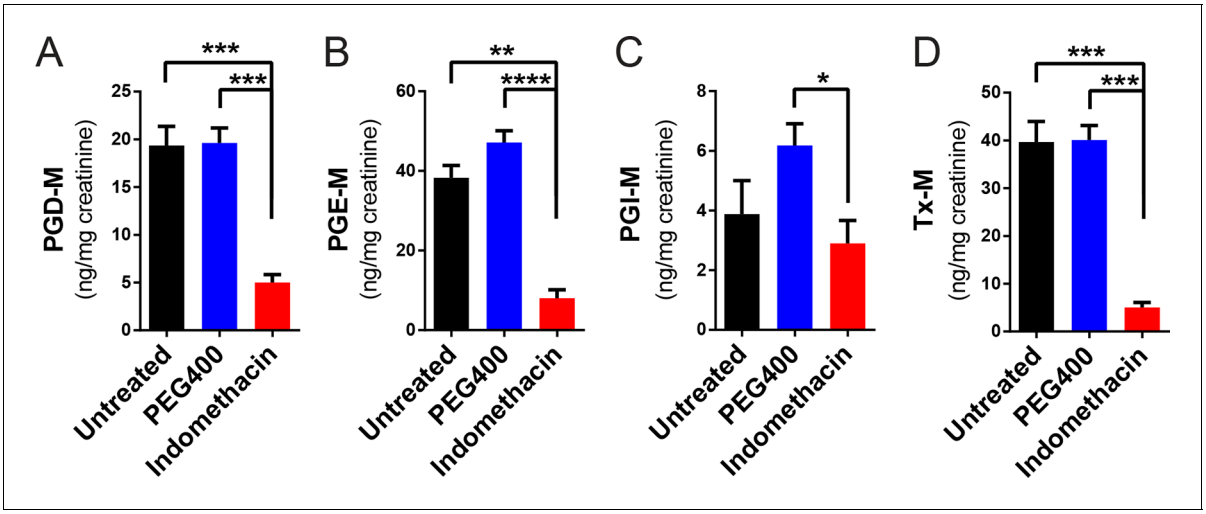

Figure 2—figure supplement 2. Inhibitory effects of acute indomethacin treatment on COX-1 and COX-2 in C57BL/6 mice. Mice were administered by gavage with or without 10 mg/kg indomethacin (red) or PEG400 (blue) and urine were collected for the analysis of prostanoid metabolites. PGD-M (**A**), PGE-M (**B**), PGI-M (**C**), and Tx-M (**D**) are reduced in indomethacin-treated mice. N=6/group. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 by Mann-Whitney test, multiplicity adjusted. Mean ± S.E.M. shown.

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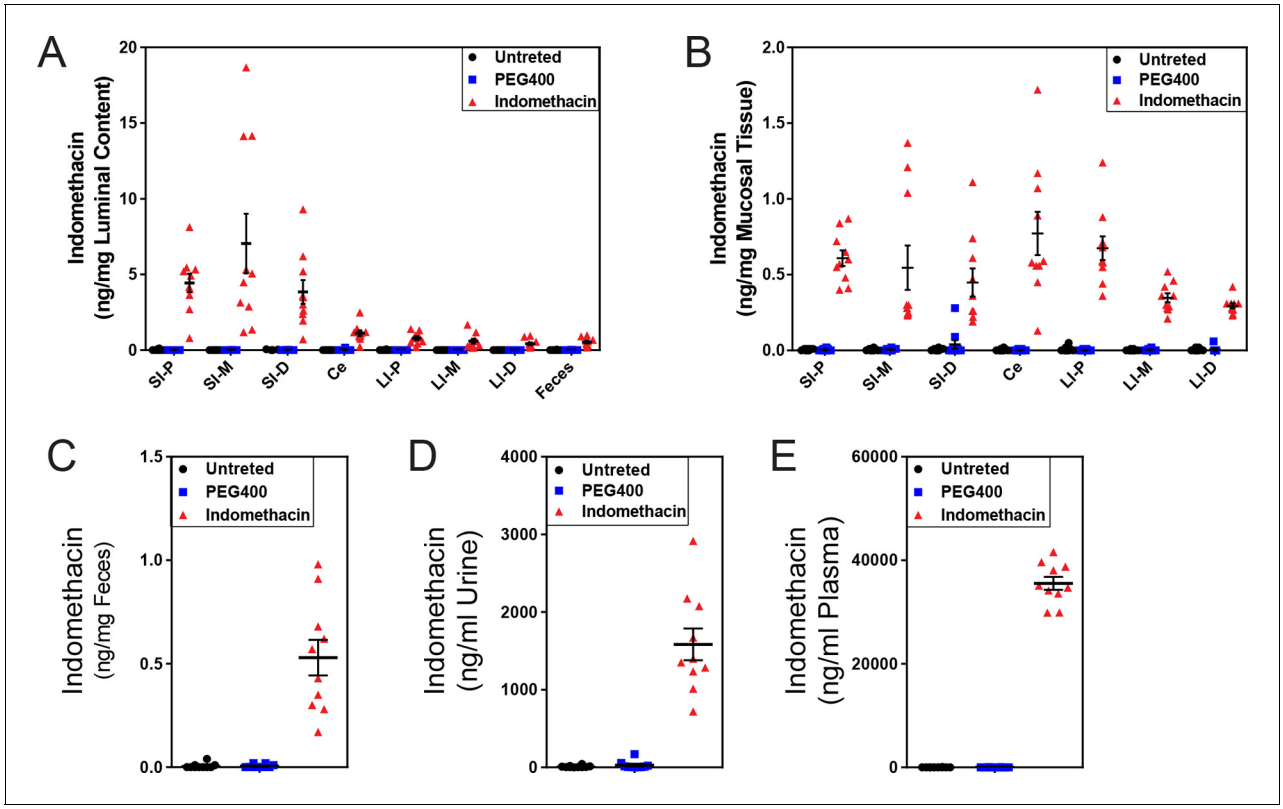

Figure 2—figure supplement 3. C57BL/6 mice are systemically and locally exposed to indomethacin. Mice were administered by gavage 10 mg/kg indomethacin in PEG400 (red) or PEG400 alone (blue). Urine, feces, plasma, and intestines were collected from mice at 6 hr after drug administration. Indomethacin concentrations were measured in samples and corrected by sample weight. Indomethacin is detected along the intestine in both luminal content (**A**) and mucosal tissue (**B**) in mice of indomethacin (red) group, but not in those of PEG400 (blue) or untreated (black) groups. In feces (**C**), urine (**D**), and plasma (**E**), indomethacin is also detected in mice of indomethacin (red) group, but not in those of PEG400 (blue) or untreated (black) groups. N=10/group. Mean ± S.E.M. shown. SI, small intestine; Ce, cecum; LI, large intestine. P, proximal; M, middle; D, distal.

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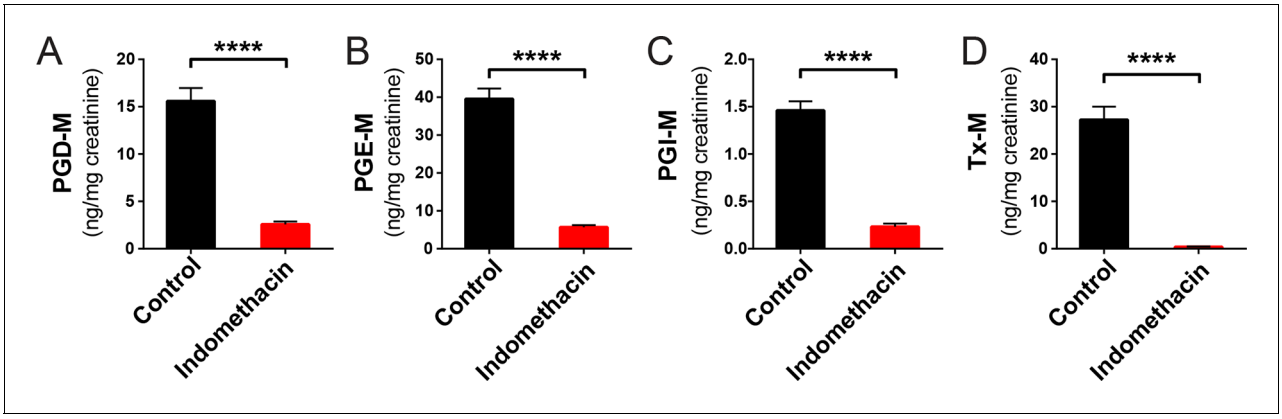

Figure 2—figure supplement 4. Inhibitory effects of chronic indomethacin treatment on COX-1 and COX-2 in C57BL/6 mice. Mice were receiving control diet (black) or indomethacin diet (20 ppm, red) for 7 days and urine were collected for the analysis of prostanoid metabolites. PGD-M (**A**), PGE-M (**B**), PGI-M (**C**), and Tx-M (**D**) are reduced in indomethacin-treated mice. N=10/group. ****p<0.0001 by Mann-Whitney test, multiplicity adjusted. Mean ± S.E.M. shown.

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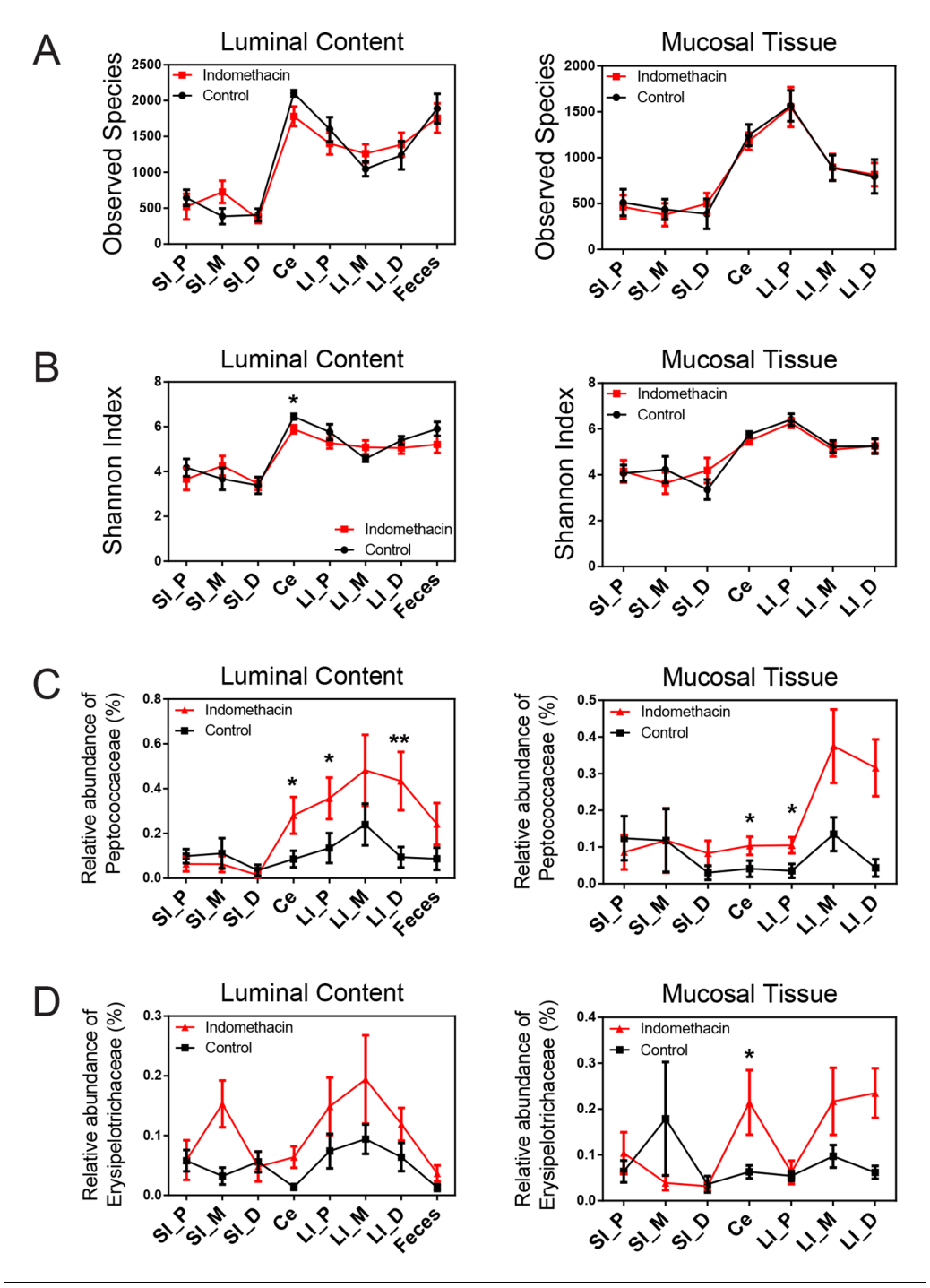

Figure 2—figure supplement 5. Chronic indomethacin treatment induces changes in microbial composition along the intestine in mice. Bacterial communities colonized in the mouse intestine were profiled using 16S rRNA gene sequencing and analyzed using QIIME (***Caporaso et al., 2010b***). Observed Species (**A**) and Shannon Index (**B**) are used to estimate richness and diversity of microbial communities in luminal content (left) and mucosal tissue (right) at anatomical sites along the intestine in indomethacin (red) and control (black) groups. Indomethacin altered microbial diversity in the cecum lumen. *p<0.05 by one-tailed Mann-Whitney test. N=9–10/group. Relative abundance of *Peptococcaceae* (**C**) and *Erysipelotrichaceae* (**D**) at anatomical sites along the intestine are significantly elevated in indomethacin (red) group than in control (black) group in both luminal content and mucosal tissues of the distal gut. *p<0.05, **p<0.01 by one-tailed Mann-Whitney test. Mean ± S.E.M. shown. SI, small intestine; Ce, cecum; LI, large intestine. P, proximal; M, middle; D, distal.

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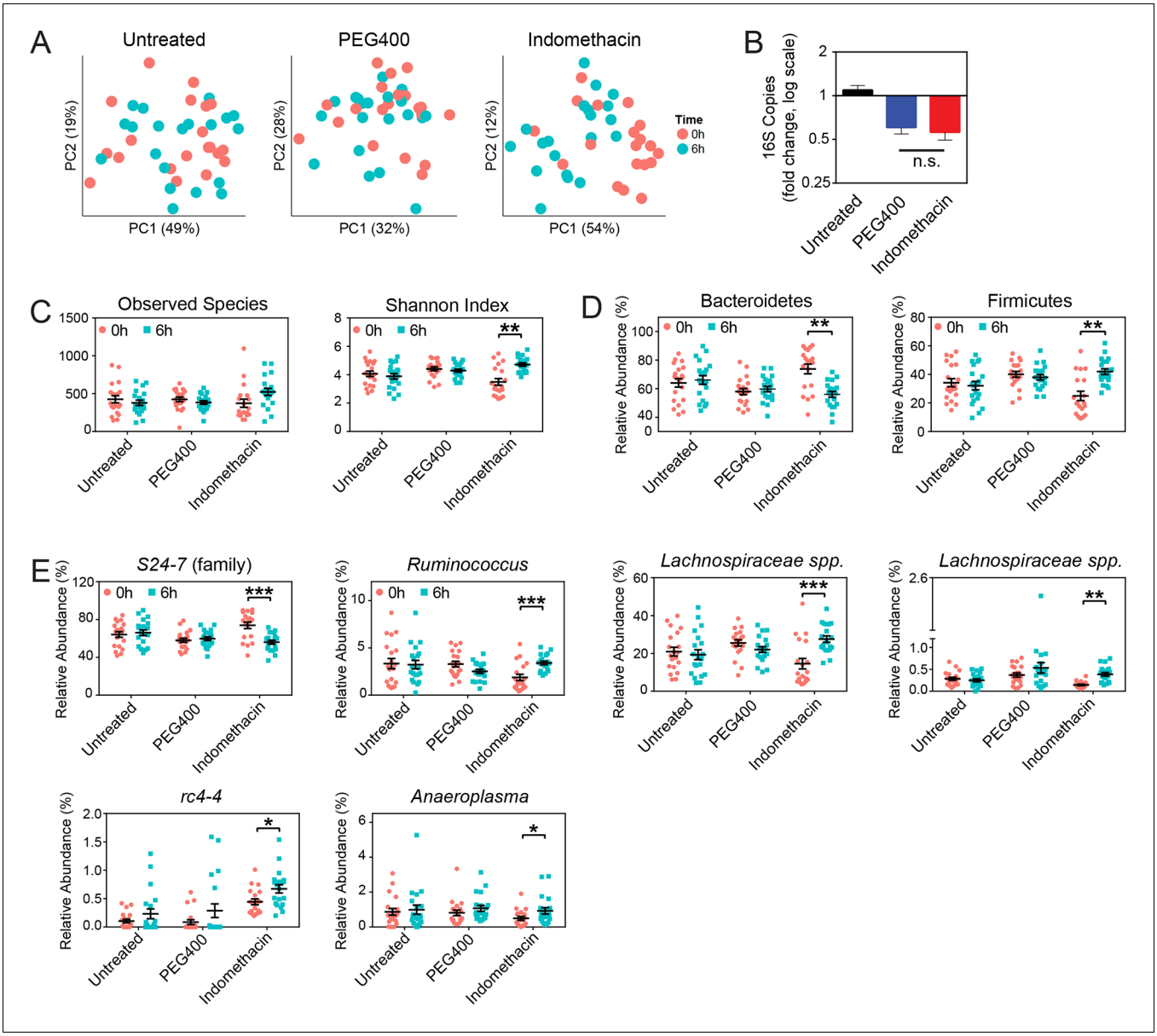

Figure 3. Indomethacin induces longitudinal changes in fecal microbiota composition. Microbiota composition in fecal pellets before (0 hr) and after (6 hr) treatment with or without indomethacin or PEG400 is analyzed by 16S rRNA gene profiling, including sequencing and quantitative PCR. (**A**) Principal coordinates analysis (PCoA) of weighted UniFrac values (***Lozupone et al., 2011***), comparing the fecal microbial communities at 0 hr (black) versus 6 hr (blue) of untreated (left), PEG400 (middle), and indomethacin (right) groups. Each point represents a sample. Fecal microbial communities at 0 hr and 6 hr are not separated in untreated group ($p>0.5$), and significantly clustered in PEG400 group ($p<0.5$) and in indomethacin group ($p<0.01$). Clustering was analyzed by ADONIS test. (**B**) 16S rRNA gene copies per gram of feces at 0 hr and 6 hr (left), and Fold changes (right) in indomethacin (red), PEG400 (blue), and untreated (black) groups. Both PEG400 and indomethacin groups have lower bacterial loads at 6 hr, whereas these are no between-group differences at 0 hr or 6 hr. ****$p<0.0001$ by Mann-Whitney test comparing 0 hr versus 6 hr. N=20/group. Mean ± S.E.M. shown. (**C**) Both Observed Species (left) and Shannon Index (right) are increased at 6 hr in indomethacin-treated mice, while unchanged in Untreated and PEG400 groups. **$p<0.01$ by multiple t test, FDR corrected. N=19–20/group. Mean ± S.E.M. shown. (**D**) The relative abundance of Bacteroidetes (left) is decreased and that of Firmicutes (right) is increased at 6 hr (blue) after indomethacin treatment. **$p<0.01$ by multiple t test, FDR corrected. N=19–20/group. (**E**) Indomethacin induced a decrease in the relative abundance of *S24-7* (family), and increases in those of *Ruminococcus, Lachnospiraceae sp., Lachnospiraceae sp., rc4-4*, and *Anaeroplasma* at 6 hr (blue). *$p<0.05$, **$p<0.01$, ***$p<0.001$ by QIIME analysis, FDR corrected. N=19–20/group. Mean ± S.E.M. shown.

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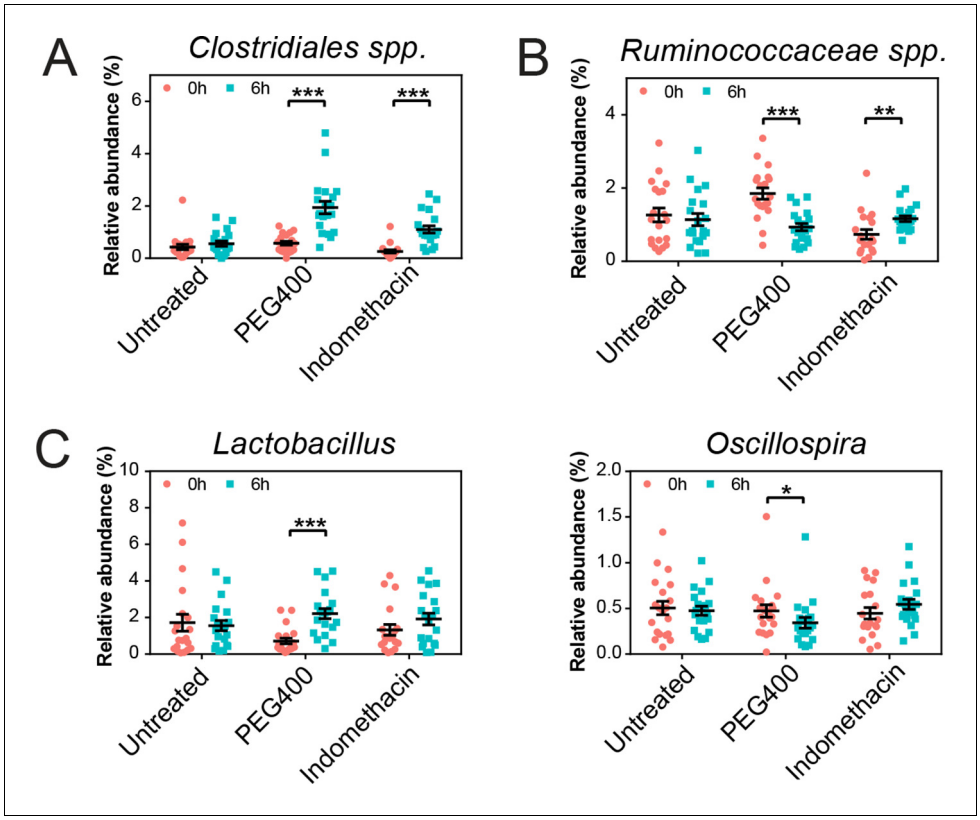

Figure 3—figure supplement 1. Longitudinal effects of acute indomethacin treatment in fecal microbiota composition. Microbiota composition in fecal pellets before (0 hr, black) and after (6 hr, blue) treatment with or without indomethacin or PEG400 is analyzed by 16S rRNA gene profiling. (**A**) The relative abundance of *Clostridiales sp.* is increased in both PEG400 and indomethacin groups at 6 hr. (**B**) The relative abundance of *Ruminococcaceae sp.* is decreased in the PEG400 group but increased in the indomethacin group at 6 hr. (**C**) PEG400 induced an increase of *Lactobacillus* (left) and a decrease of *Oscillospira* (right), whereas there is no change in the untreated or indomethacin treated groups. *p<0.05, **p<0.01, ***p<0.001 by QIIME analysis, FDR corrected. N=19–20/group. Mean ± S.E.M. shown.

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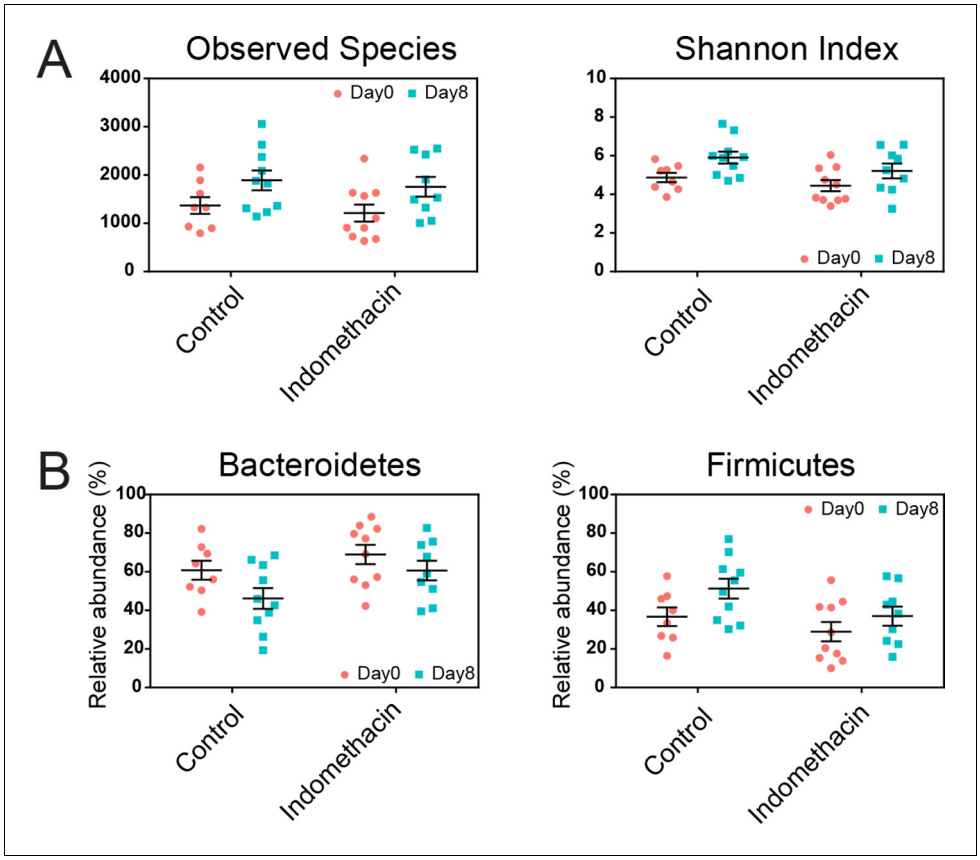

Figure 3—figure supplement 2. Longitudinal effects of chronic indomethacin treatment in fecal microbiota composition. Fecal microbiota composition before (day 0) and after (day 8) treatment in mice receiving control or indomethacin diet is analyzed by 16S rRNA gene profiling. (**A**) Observed species (left) and Shannon Index (right) showed no significant difference after indomethacin treatment in both control and indomethacin groups. (**B**) The relative abundance of Bacteroidetes (left) and Firmicutes (right) showed no significant difference after indomethacin treatment in both control and indomethacin groups. Mann-Whitney test. N=9–10/group. Mean ± S.E.M. shown.

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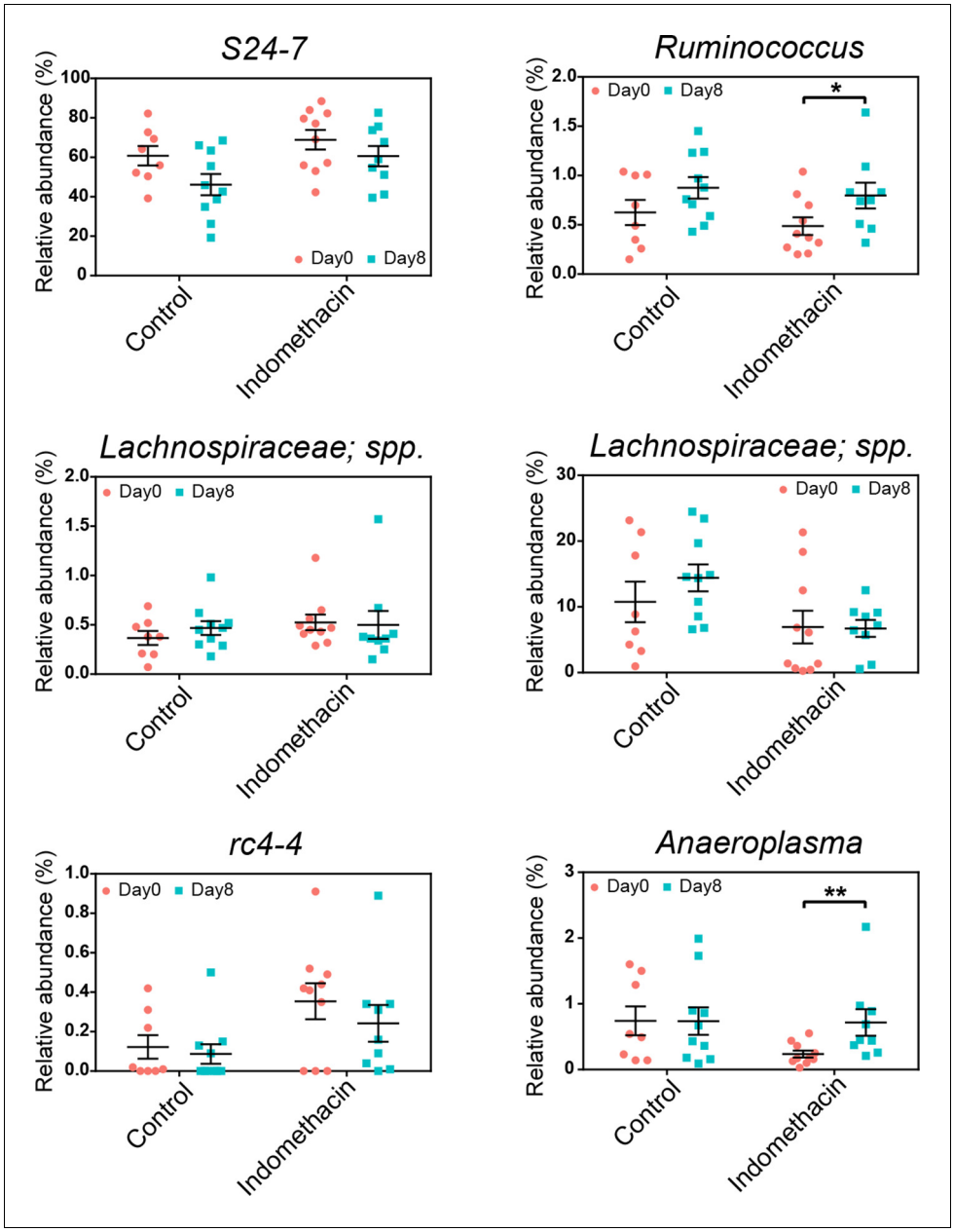

Figure 3—figure supplement 3. Longitudinal effects of chronic indomethacin treatment on genera abundance in fecal microbiota. Fecal microbiota composition before (day 0) and after (day 8) treatment in mice receiving control or indomethacin diet is analyzed by 16S rRNA gene profiling. Indomethacin induced increases in the relative abundance of *Ruminococcus* and *Anaeroplasma* at day 8. *p<0.05, **p<0.01 by two-tailed Mann-Whitney test. N=9–10/group. Mean ± S.E.M. shown.

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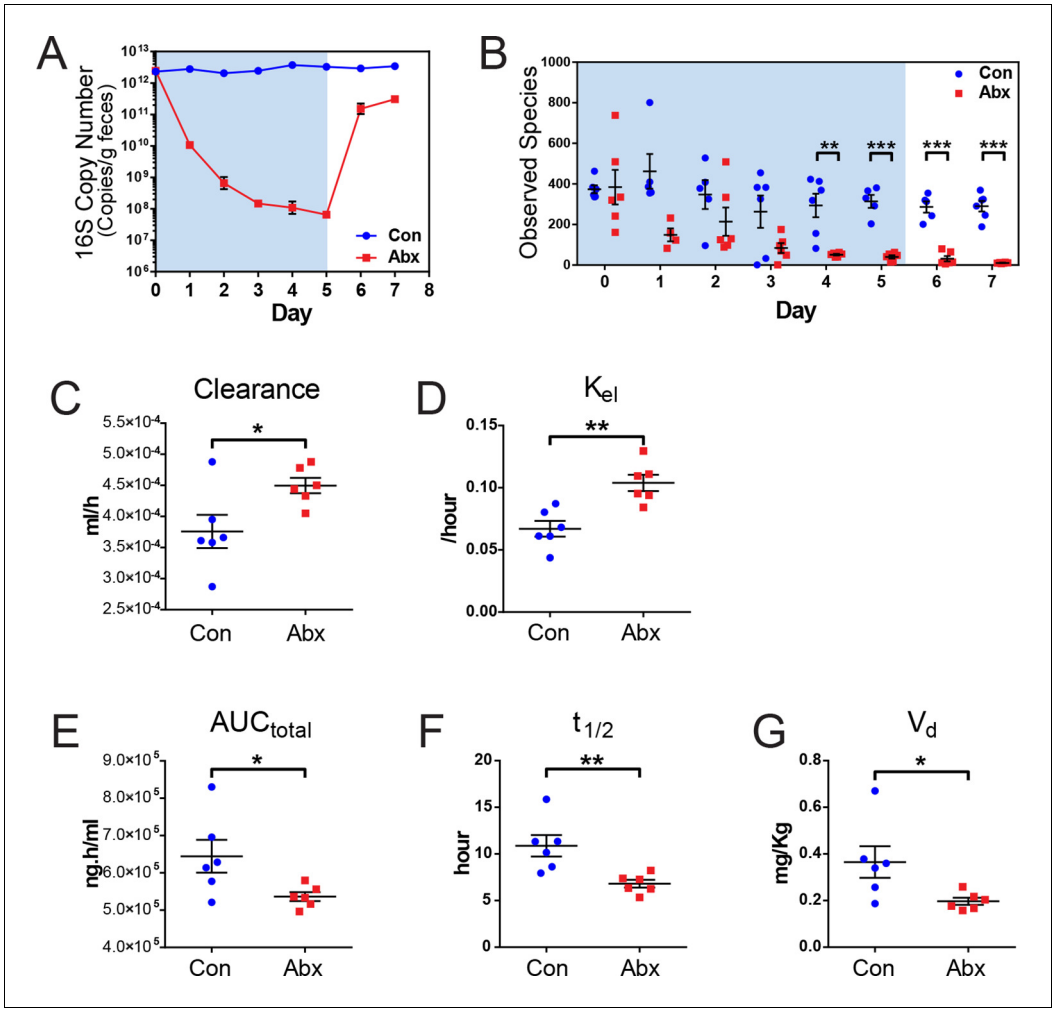

Figure 4. Microbiota-depletion with antibiotics alters the pharmacokinetics of indomethacin in mice. Mice were subjected to control water (Con) or antibiotic cocktail (Abx, neomycin and vancomycin) for 5 days (blue-shaded area). Upon the cessation of 5-day treatment, mice were administered by gavage with 10 mg/kg indomethacin. Plasma was collected sequentially for pharmacokinetic analysis. Fecal microbiota compositions over time were analyzed using 16S rRNA gene profiling. (**A**) Longitudinal analysis of 16S rRNA gene copies per gram of feces reveals a significant reduction in microbial load in Abx group (red). (**B**) Longitudinal analysis of observed species reveals decreased microbial richness in Abx group (red). **p<0.01, ***p<0.001 by multiple t test, FDR corrected. N=4–6/group. Mean ± S.E.M. shown. In antibiotic-treated mice (red), indomethacin has increased oral clearance (**C**) and elimination rate constant (K_{el}) (**D**), as well as decreased area under the curve (AUC_{total}) (**E**), half-life ($t_{1/2}$) (**F**), and apparent volume of distribution (V_d) (**G**). *p<0.05, **p<0.01 by Mann-Whitney test. N=6/group. Mean ± S.E.M. shown.

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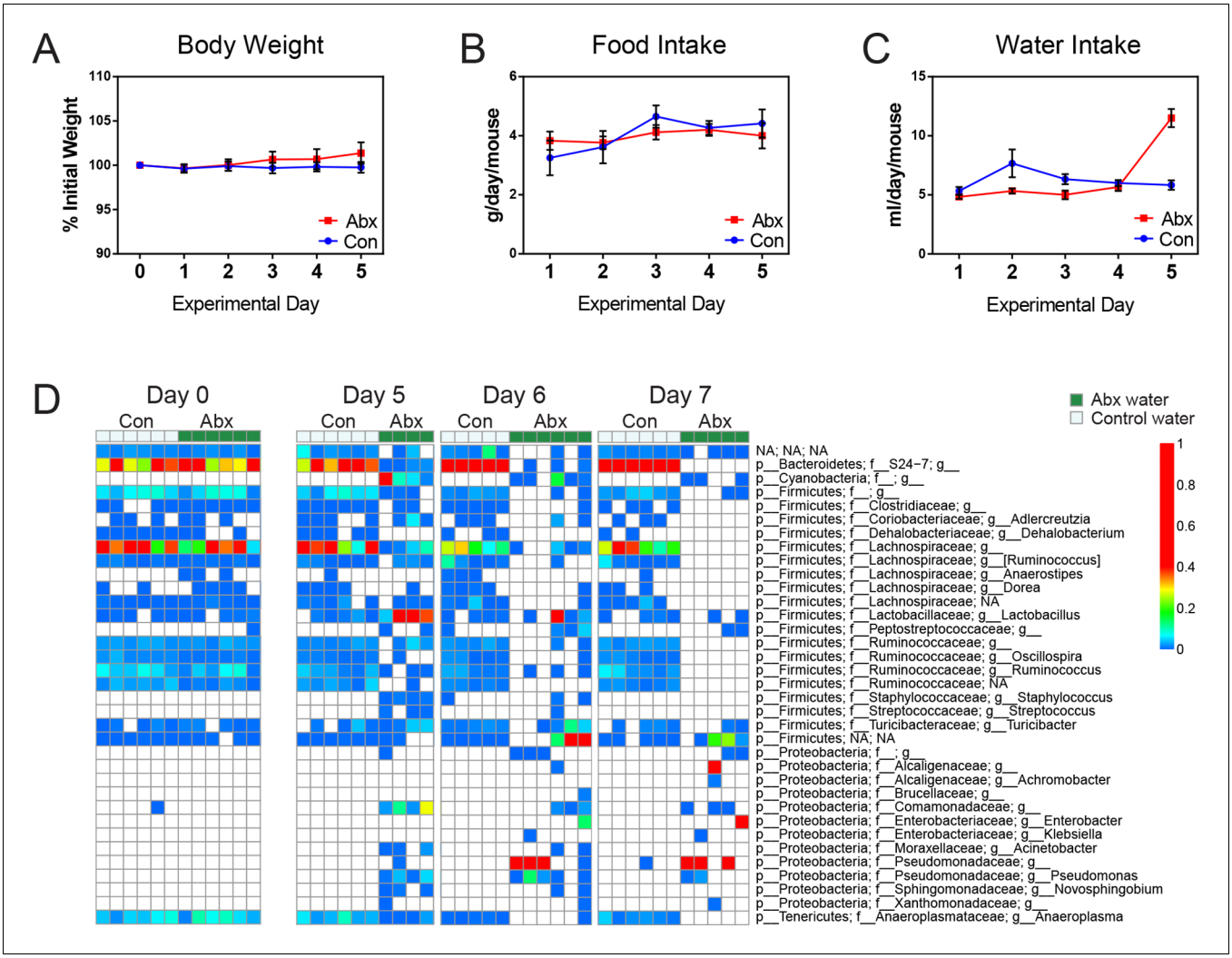

Figure 4—figure supplement 1. Antibiotic-treatment causes compositional changes in intestinal microbiota without affecting body weight, food intake, and water intake in C57BL/6 mice. Mice were subjected to antibiotic water (Abx, neomycin and vancomycin) or control water (Con) for 5 days (blue-shaded) and the body weight, food intake, and water intake were monitored daily. Fecal pellets were collected for the analysis of microbiota composition. Body weight (**A**), Food intake (**B**), and Water intake (**C**) are not affected by antibiotic treatment. Note: on day 5, animal cages were changed by facility staff and the movement caused water loss. N=6/group. Mean ± S.E.M. shown. (**D**) Heat map of the longitudinal analysis of bacterial lineages detected in feces of control (light green) or antibiotic treated (dark green) mice before (day 0) and after (Dday 5, 6, and 7) treatment. Each column represents one individual mouse of the time and treatment group indicated. Microbial composition is shifted in the Abx group but is stable in Con group. The proportions of bacterial lineages are indicated by the color code to the right.

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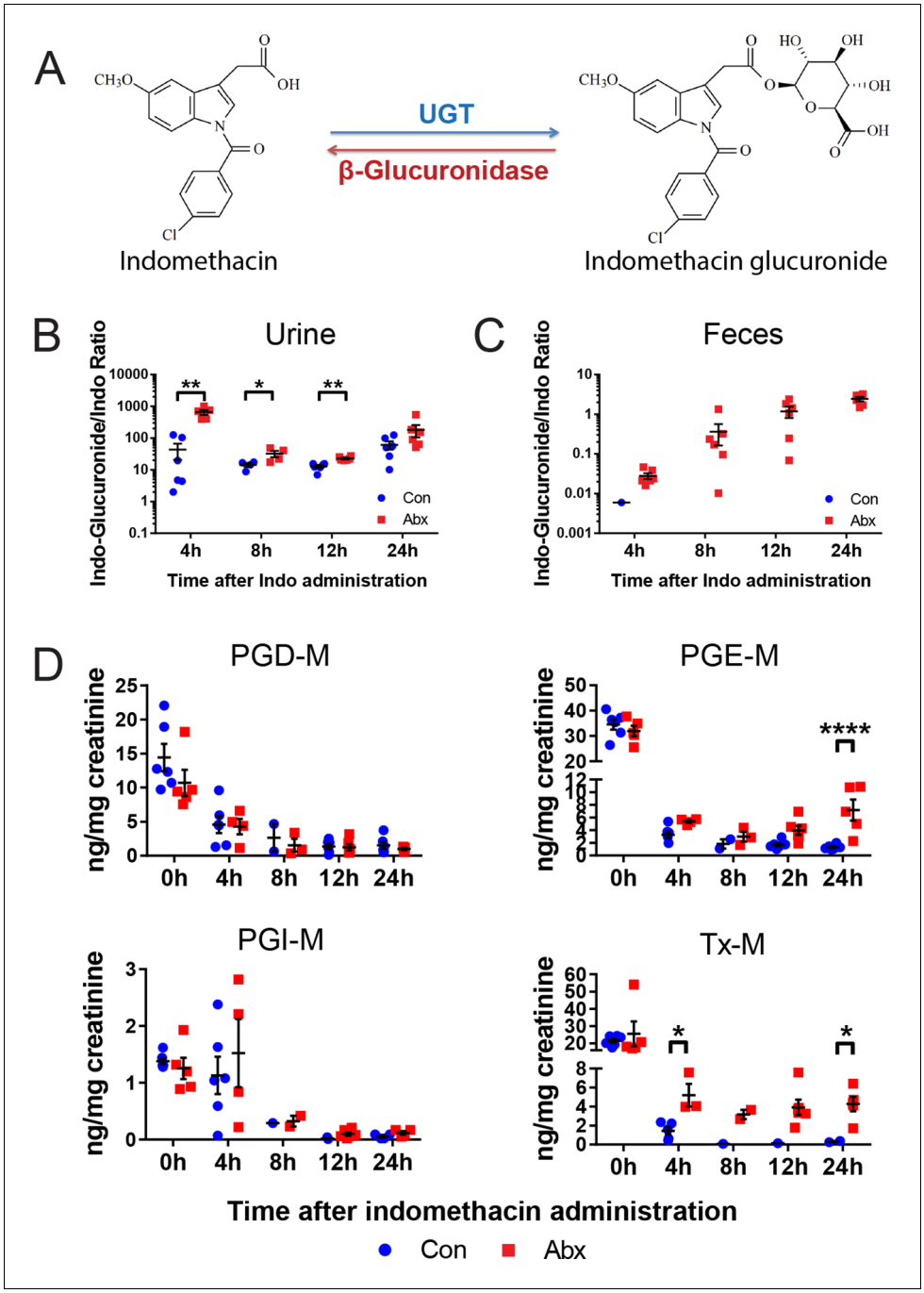

Figure 5. Metabolism and efficacy of indomethacin in antibiotic-treated mice are altered. Upon the cessation of 5-day treatment with antibiotic cocktail (Abx, neomycin and vancomycin) or control water (Con), mice were administered by gavage with 10 mg/kg indomethacin. Urine and feces were collected at indicated time for metabolic analysis. (**A**) Chemical structures of indomethacin (left) and indomethacin glucuronide (right). Enzyme catalyzing the glucuronidation is UDP-glucuronosyltransferase (UGT), and the one catalyzing the de-glucuronidation is β-glucuronidase. The ratio of indomethacin-glucuronide to indomethacin in urine (**B**) and feces (**C**) are higher in Abx group (red) than in Con group (blue). *p<0.05, **p<0.01 by Mann-Whitney test. N=6/group. Mean ± S.E.M. shown. (**D**) Urinary prostanoid metabolites were analyzed with LC/MS and values are corrected by creatinine. In Con mice (blue), all metabolites were reduced time-dependently. In Abx mice (red) PGD-M and PGI-M remained suppressed 24 hr after indomethacin, whereas PGE-M and Tx-M concentrations recovered more quickly. Two-way ANOVA revealed significant effect of time in PGD-M (p=0.001) and PGI-M (p=0.0004), and significant antibiotic effect of PGE-M (p<0.0001) and Tx-M (p=0.0002).In Abx mice, PGE-M was higher mice at

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24 hr, and Tx-M was higher at 4 hr and 24 hr. N=6/group. *p<0.05, **p<0.01 by multiple comparison test, adjusted. Mean ± S.E.M. shown.

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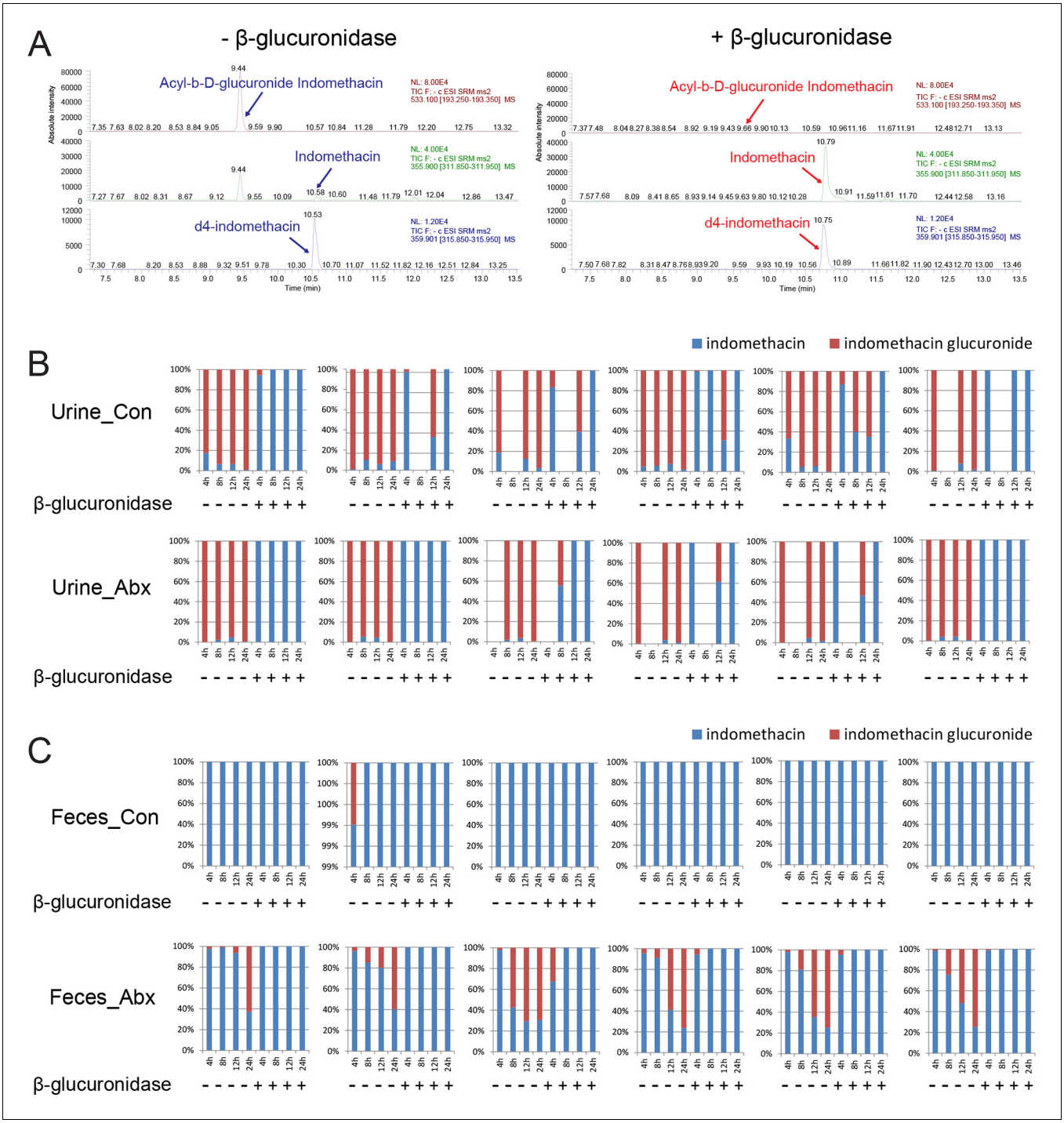

Figure 5—figure supplement 1. *β*-glucuronidase catalyzes de-glucuronidation reaction. Mice were administered by gavage 10 mg/kg indomethacin in PEG400 after 5 days of antibiotic treatment. Urine and feces were collected at indicated times for the analysis of glucuronidation by in vitro by incubation with or without β-glucuronidase. (**A**) Representative spectra of LC/MS measurements of indomethacin and its metabolites. The peak denoting Acyl-b-D-glucuronide Indomethacin (indomethacin glucuronide) is larger without β-glucuronidase (left) than with β-glucuronidase (right). The peak denoting indomethacin is smaller without β-glucuronidase (left) than with β-glucuronidase (right). In urine (**B**) and feces (**C**) samples of control (upper) and antibiotic (lower) groups, the proportions of indomethacin glucuronide are smaller with β-glucuronidase added. Similarly, the proportions of indomethacin are larger with β-glucuronidase added. Each graph shows the longitudinal changes in one mouse. N=6/group.

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