Supplementary Material for

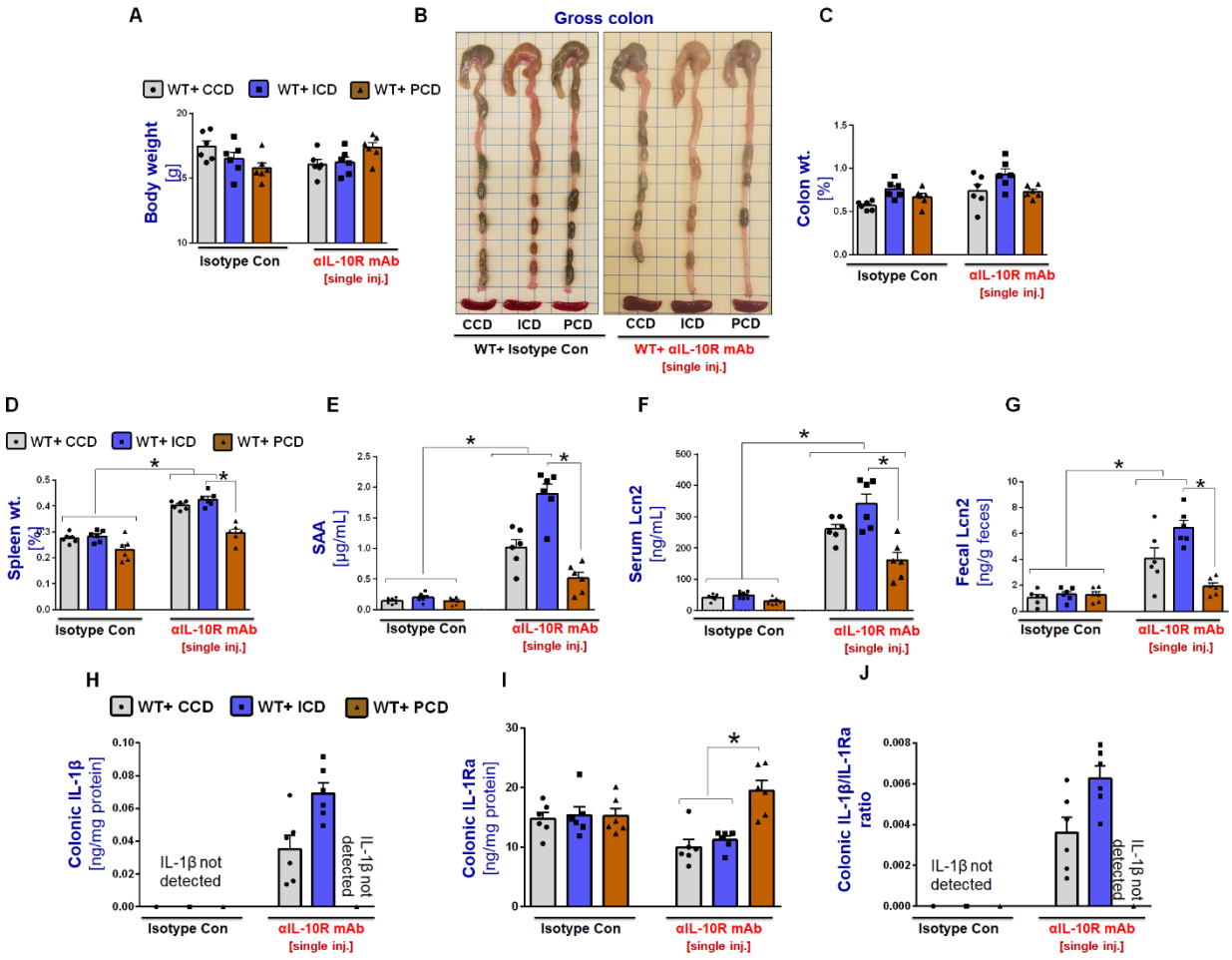
Microbiota Fermentation-NLRP3 Axis Shapes the Impact of Dietary Fibers on Intestinal Inflammation

1 - Supplemental Data

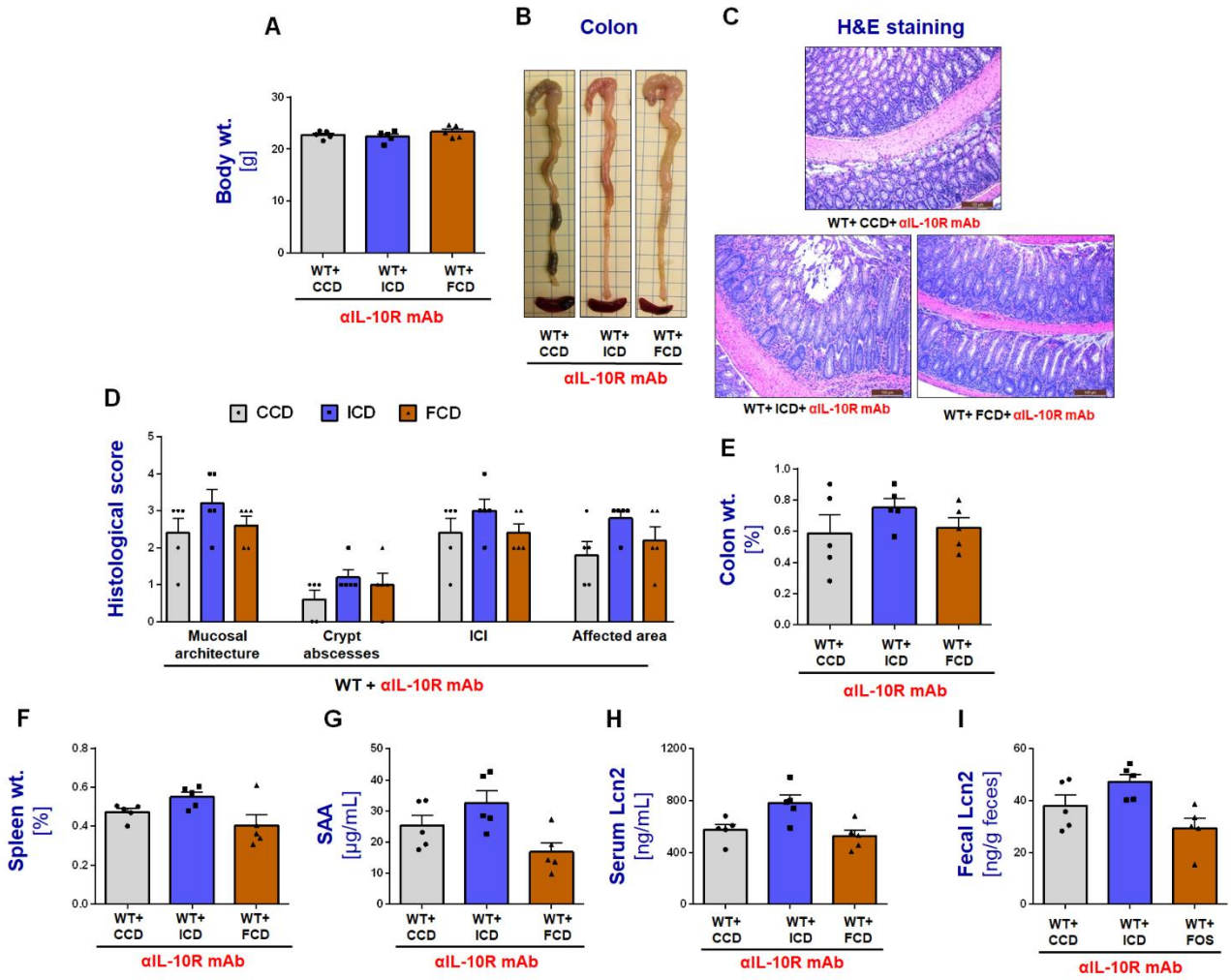
Supplementary Figures S1-S12

2 - Experimental Methods

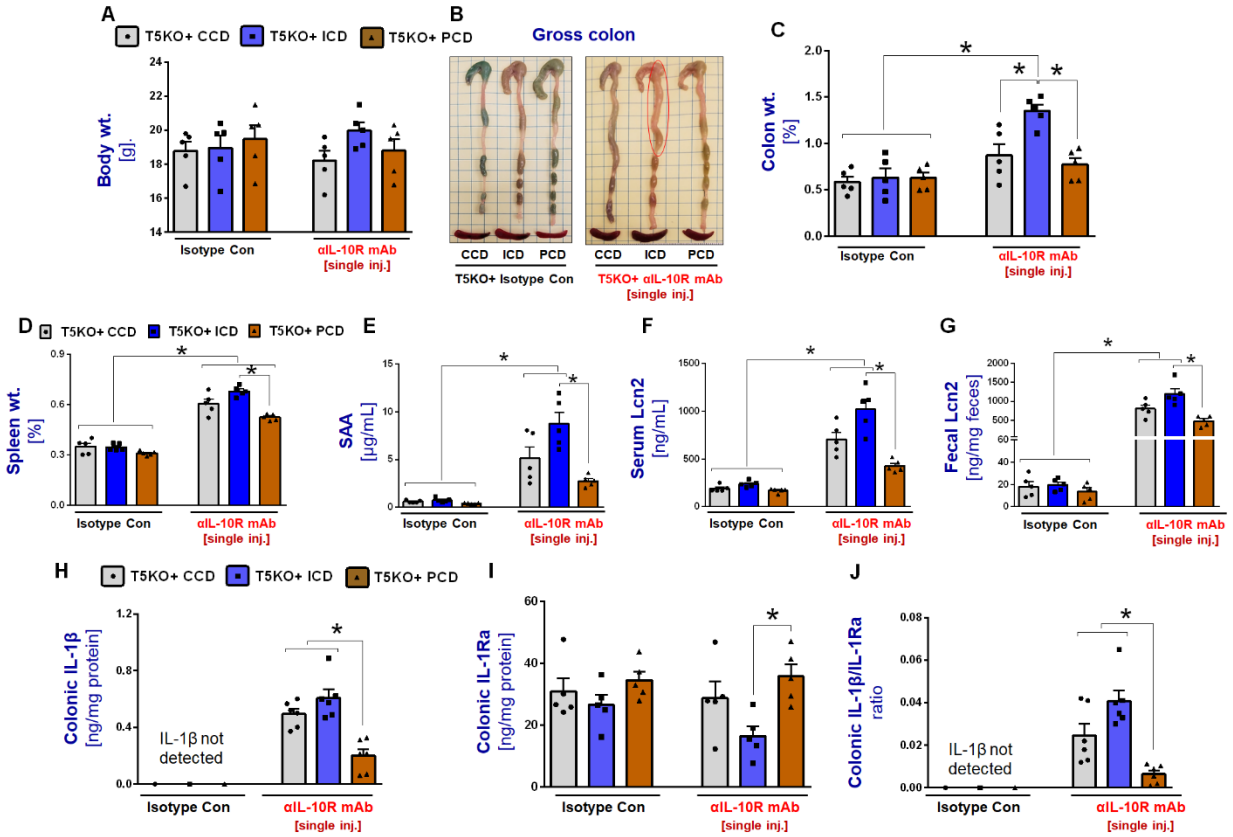
1 - Supplemental data



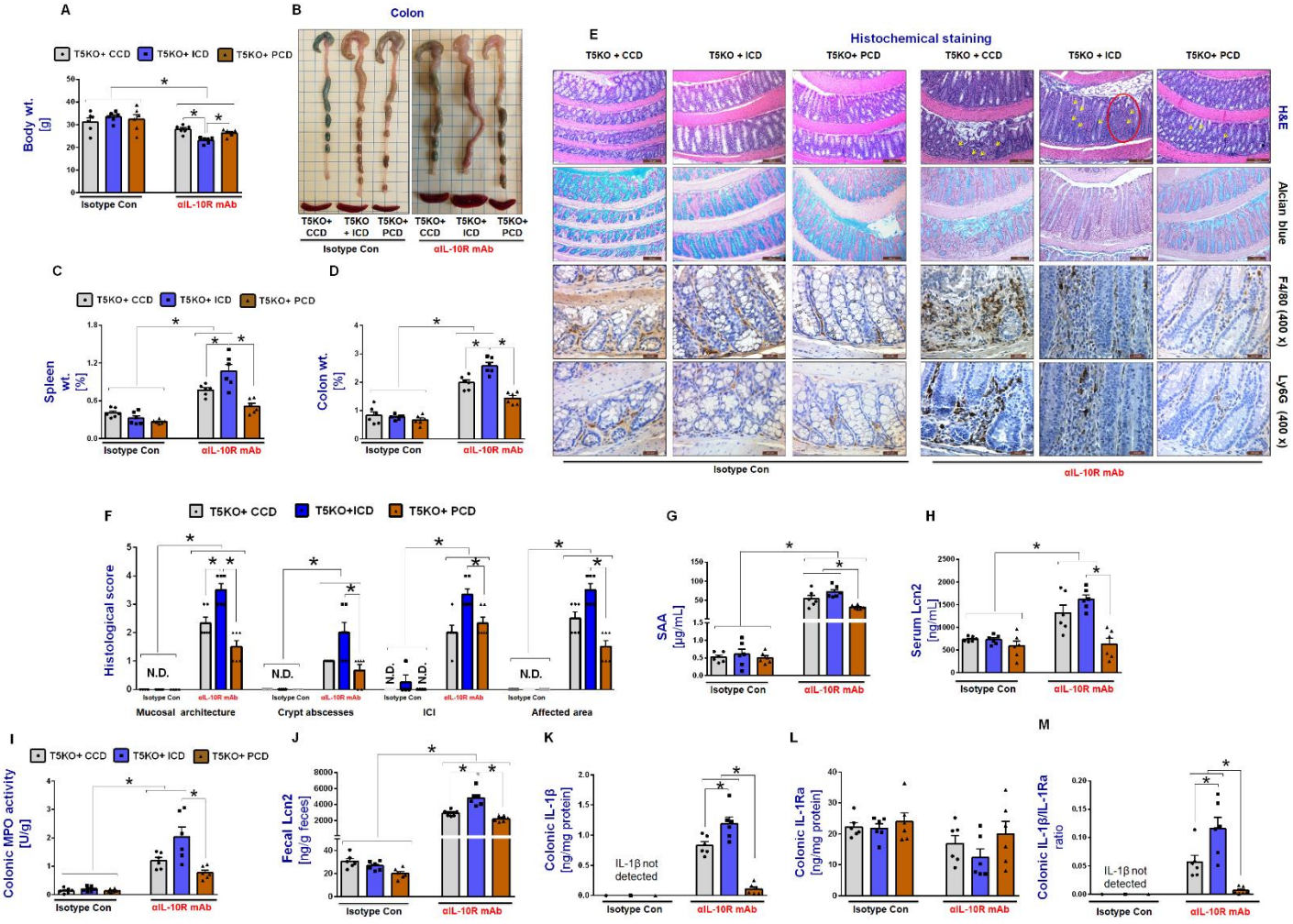
Supplementary Figure 1: Pectin mitigates IL-10R neutralization-induced acute colonic inflammation. C57BL6 (WT) male mice, after weaning, were maintained on lab chow for one week and then placed on purified diets comprised of either cellulose (100g/kg, CCD), inulin (75g/kg, ICD) or pectin (75g/kg, PCD); the remaining 25g/kg fiber in both ICD and PCD was made up with cellulose to constitute 10% total dietary fiber. Diets with 10% cellulose served as controls. On day 3, mice were given single injection of interleukin-10 receptor (IL-10R) neutralizing antibody (αIL-10R mAb, 1.0 mg/mouse, i.p.). The CCD, ICD or PCD-fed mice that received isotype control antibody (rat anti-mouse IgG1) were used as controls. After one-week, the mice were analyzed for colitis parameters: (**A**) Body weight. (**B**) Image of gross colon appearance. (**C**-**F**) Bar graphs represent percent (**C**) colon weight, (**D**) spleen weight, and serum levels of (**E**) serum amyloid A (SAA) and (**F**) lipocalin 2 (Lcn2). (**G**) Feces were collected before euthanasia, and Lcn2 was measured using ELISA and represented as ng/g feces. **(H**-**J)** A portion of proximal colon was collected and processed for protein extraction using RIPA buffer supplemented with protease inhibitors. Colonic cytokines were analyzed via ELISA. Bar graphs display the colonic level of (**H**) IL-1β, (**I**) IL-1Ra and (**J**) IL-1β/IL-1Ra ratio. The data (Mean ±SEM) is representative of 2 independent experiments. (*$p < 0.05$).



Supplementary Figure 2: **Fructooligosaccharides containing diet (FCD)-fed mice exhibit colitis similar to ICD group.** Male WT mice (4 weeks old) were fed CCD, ICD or FCD. On day 3, mice were treated with αIL-10R mAb (1.0 mg/mouse, 4 weekly injections, i.p.). (**A**) Body weight. (**B-C**) Images show the (**B**) gross colon, and (**C**) H&E-stained colonic section. (**D-I**) Bar graphs represent (**D**) colon histology score, (**E**) percent colon weight, (**F**) percent spleen weight, (**G**) serum levels of SAA and (**H**) Lcn2, and (**I**) fecal levels of Lcn2. The data represented as mean ±SEM. (*p< 0.05).



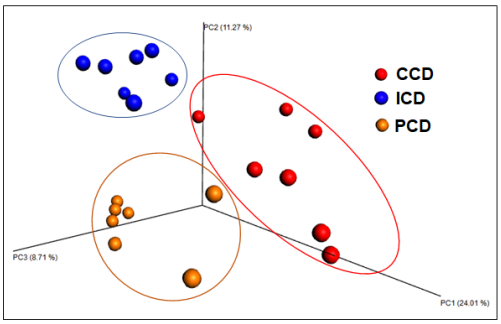
Supplementary Figure 3: PCD-fed *Tlr5*KO mice displayed reduced colonic inflammation. *Tlr5*KO (T5KO) mice (male, 4 weeks old) were maintained on purified diets of either CCD, ICD or PCD. On day 3, mice were treated with αIL-10R mAb (1.0 mg/mouse, single injection, i.p.). After 1-week, mice were analyzed for colitis parameters. (**A**) Body weight. (**B**) Images show the gross colon appearance. (**C-G**) Bar graphs represent (**C**) colon weight, (**D**) spleen weight, (**E**) serum SAA, (**F**) serum Lcn2, and (**G**) fecal level of Lcn2. (**H-J**) Colonic level of (**H**) IL-1β, (**I**) IL-1Ra and (**J**) IL-1β/IL-1Ra ratio. The data (Mean ±SEM) is representative of 2 independent experiments. (*p< 0.05).



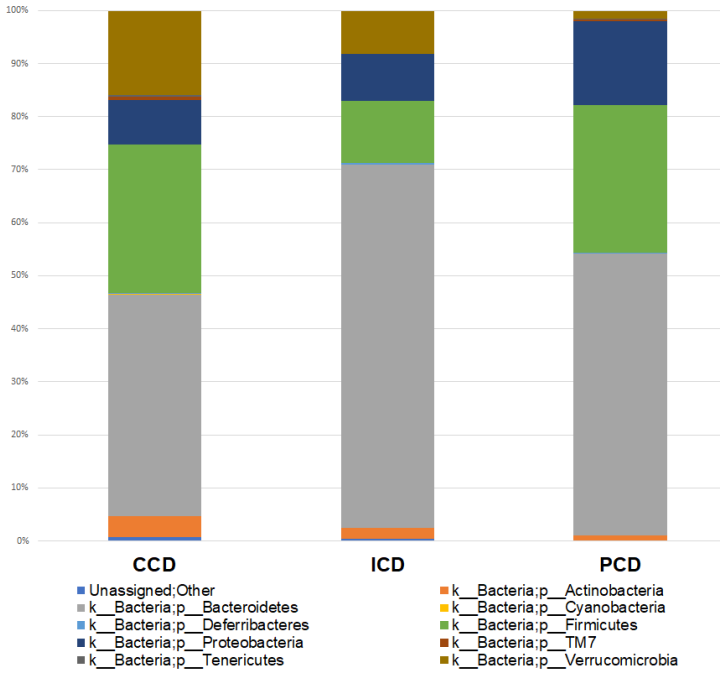
Supplementary Figure 4: PCD-fed *Tlr5*KO mice exhibit reduced colitis. Male *Tlr5*KO (T5KO) mice (n=6) after weaning on day 21, were maintained on lab chow for one week and then switched to either CCD, ICD or PCD. On day 3, each group received either αIL-10R mAb or isotype control (1.0 mg/mouse, 3 weekly injections; i.p.). Three weeks post last injection, the mice were euthanized and analyzed for colitis parameters. (**A**) Body weight. (**B**) Gross colon appearance. (**C-D**) percent weights of (**C**) spleen and (**D**) colon. (**E**) Histochemical staining of colons (**i**) H&E (**ii**) alcian blue, and immunohistochemical staining for **(iii)** macrophages (F4/80) and (**iv**) neutrophils

(Ly6G). (**F**) Colonic histology score. Serum levels of (**G**) SAA and (**H**) Lcn2. Bar graphs show (**I**) colonic MPO activity, (**J**) Lcn2 concentration in the feces, and colonic (**K**) IL-1β, (**L**) IL-1Ra and (**M**) IL-1β/IL-1Ra ratio. The data (Mean ±SEM) is representative of 3 independent experiments. (*$p < 0.05$).

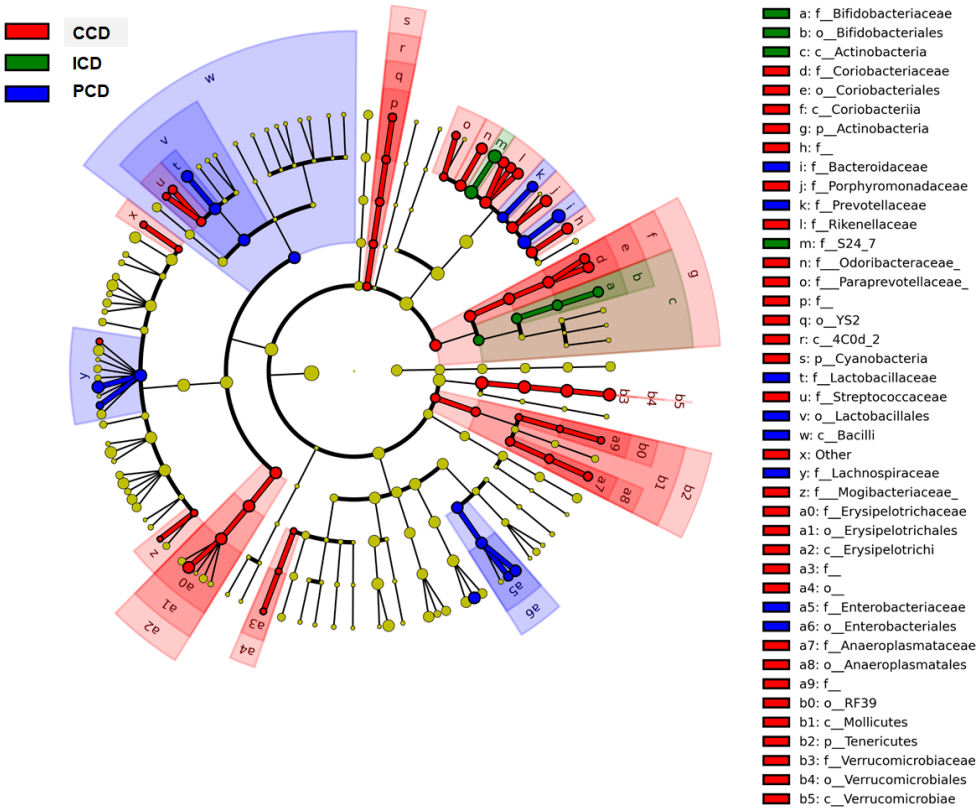
A


PC2 (11.27 %)
PC3 (8.71 %)
PC1 (24.01 %)
CCD
ICD
PCD

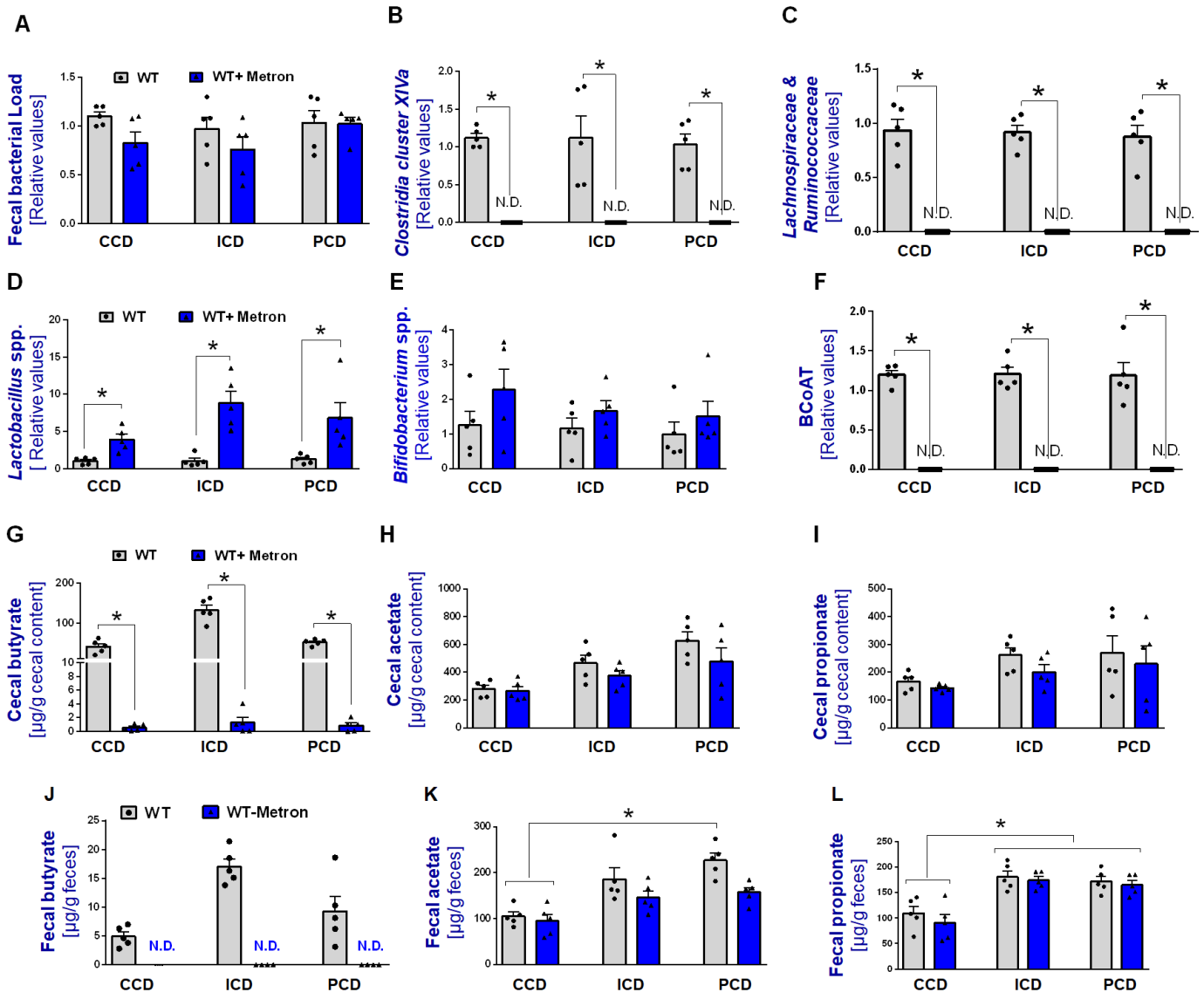
B


100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
CCD
ICD
PCD
Unassigned;Other
k__Bacteria;p__Bacteroidetes
k__Bacteria;p__Deferribacteres
k__Bacteria;p__Proteobacteria
k__Bacteria;p__Tenericutes
k__Bacteria;p__Actinobacteria
k__Bacteria;p__Cyanobacteria
k__Bacteria;p__Firmicutes
k__Bacteria;p__TM7
k__Bacteria;p__Verrucomicrobia

C

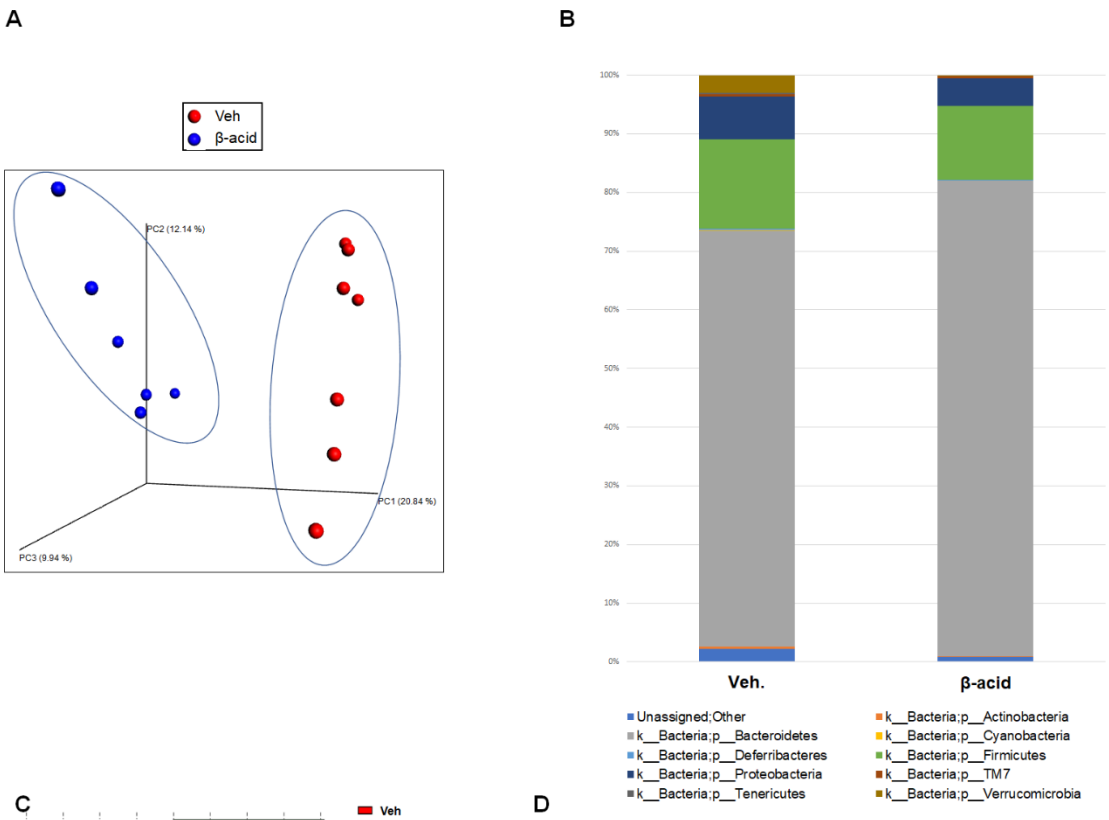

CCD
ICD
PCD
a: f__Bifidobacteriaceae
b: o__Bifidobacteriales
c: c__Actinobacteria
d: f__Coriobacteriaceae
e: o__Coriobacteriales
f: c__Coriobacteriia
g: p__Actinobacteria
h: f__
i: f__Bacteroidaceae
j: f__Porphyromonadaceae
k: f__Prevotellaceae
l: f__Rikenellaceae
m: f__S24_7
n: f__Odoribacteraceae_
o: f__Paraprevotellaceae_
p: f__
q: o__YS2
r: c__4C0d_2
s: p__Cyanobacteria
t: f__Lactobacillaceae
u: f__Streptococcaceae
v: o__Lactobacillales
w: c__Bacilli
x: Other
y: f__Lachnospiraceae
z: f__Mogibacteriaceae_
a0: f__Erysipelotrichaceae
a1: o__Erysipelotrichales
a2: c__Erysipelotrichi
a3: f__
a4: o__
a5: f__Enterobacteriaceae
a6: o__Enterobacteriales
a7: f__Anaeroplasmataceae
a8: o__Anaeroplasmatales
a9: f__
b0: o__RF39
b1: c__Mollicutes
b2: p__Tenericutes
b3: f__Verrucomicrobiaceae
b4: o__Verrucomicrobiales
b5: c__Verrucomicrobiae

Supplementary Figure 5. Structurally distinct dietary fibers differentially influence gut microbiota composition. Fecal microbial communities of CCD- [n=7 mice, two cages (4 mice+3 mice)], ICD- [n=7 mice, two cages (4 mice+3 mice)], and PCD- [n=7 mice, two cages (4 mice+3 mice)] fed mice were analyzed *via* 16S rRNA gene Illumina sequencing following induction of chronic colitis. **A.** Principal coordinate analysis (PCoA) of the unweighted Unifrac distance is represented, with samples colored by treatment. **B.** Taxa summarization performed at the phylum level. **C.** Taxonomic cladogram obtained from LEfSe (LDA Effect Size) analysis of fecal 16S rRNA genes sequencing, highlighting taxa significantly altered in one group compared to the other (LDA score > 2.0). **D** (enclosed as a separate file, labels **1**: CCD, **2**: ICD, **3**: PCD) List of taxa significantly altered in one group compared to the other (LDA score > 2.0).

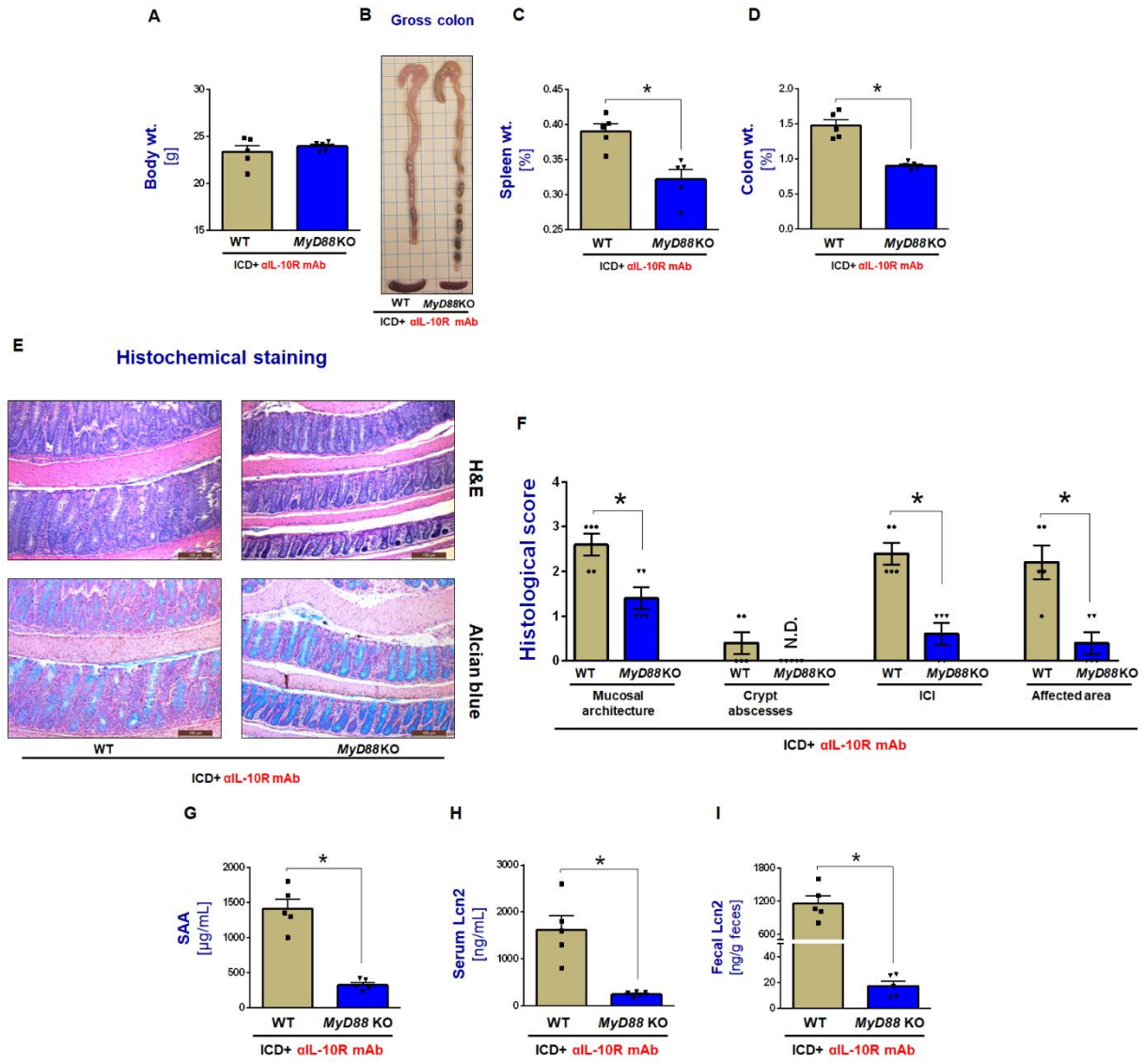


Supplementary Figure 6: Metronidazole targets butyrate producers and dampens butyrate levels in the gut. C57BL6 (WT) male mice (n=5) one-week post weaning were maintained on metronidazole (1g/L in drinking water). Mice were given purified diets CCD, ICD or PCD along with metronidazole. After 1-week, fecal bacterial DNA was isolated and subjected to qRT-PCR. Cecal contents were collected for short chain fatty acid quantification via GC-MS. (**A**) Fecal bacterial load, (**B**) *Clostridia cluster XIVa*, (**C**) *Lachnospiraceae* and *Ruminococcaceae,* (**D**)

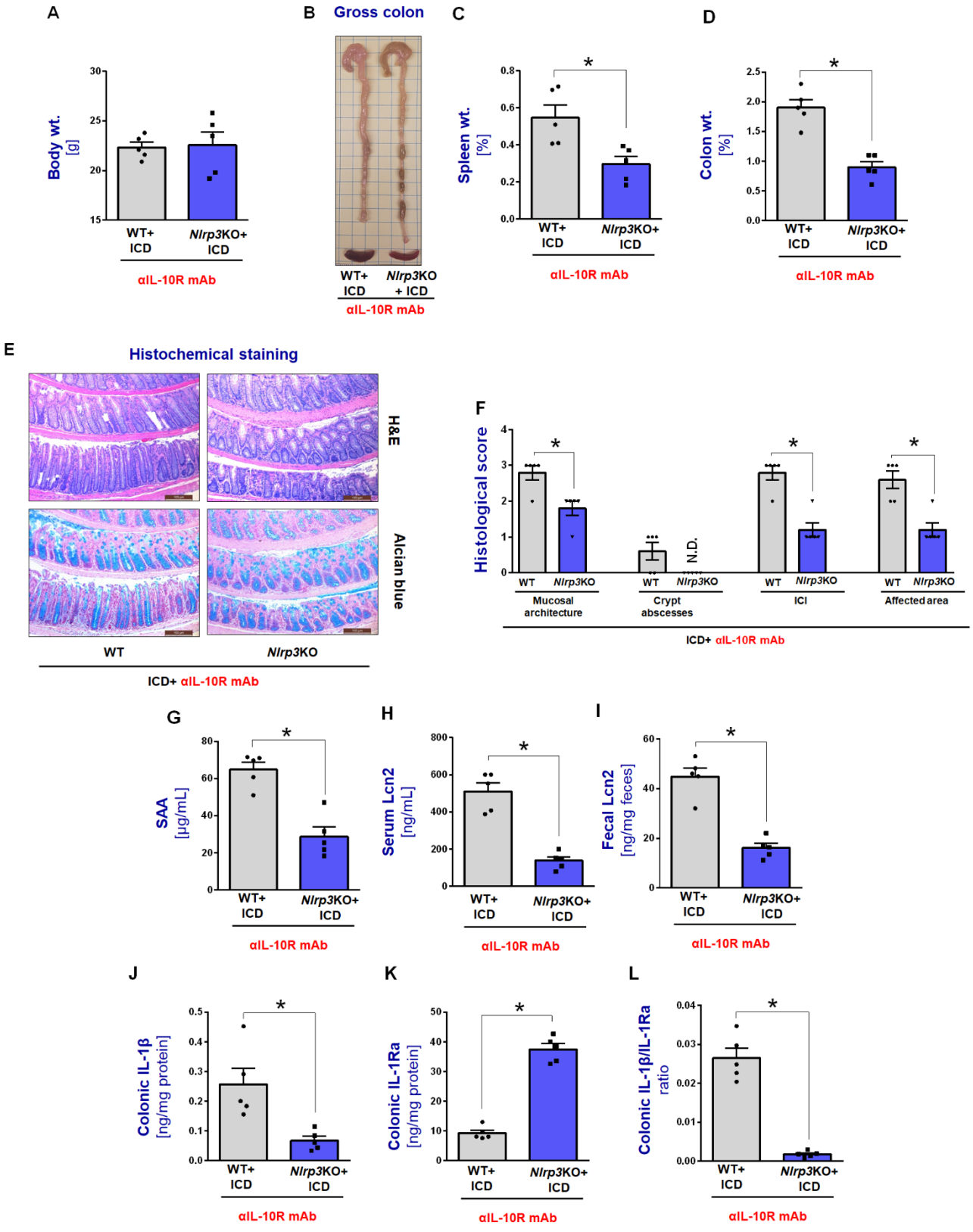
Lactobacillus species, (**E**) *Bifidobacterium* species, and (**F**) relative levels of *butyryl-CoA:acetate CoA-transferase* (BCoAT) gene. Bar graphs show the level of short chain fatty acids butyrate, acetate and propionate, respectively, in the cecal contents (**G-I**) and fecal contents (**J-L**). The data (Mean ±SEM) is representative of 2 independent experiments. (*$p < 0.05$). Each dot represents data from one mouse.


A
Veh
β-acid
PC2 (12.14 %)
PC1 (20.84 %)
PC3 (9.94 %)
B
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
Veh.
β-acid
k__Bacteria;p__Actinobacteria
k__Bacteria;p__Bacteroidetes
k__Bacteria;p__Cyanobacteria
k__Bacteria;p__Deferribacteres
k__Bacteria;p__Firmicutes
k__Bacteria;p__Proteobacteria
k__Bacteria;p__TM7
k__Bacteria;p__Tenericutes
k__Bacteria;p__Verrucomicrobia
Unassigned;Other
C
Veh
β-acid
f__S24_7
g__
k__Bacteria
g__Prevotella_
f__Paraprevotellaceae_
g__
f__Prevotellaceae
Other
g__Dorea
f__Streptococcaceae
g__Lactococcus
g__
f__Peptostreptococcaceae
o__Coriobacteriales
f__Coriobacteriaceae
c__Coriobacteriia
g__
Other
g__Paraprevotella
g__AF12
f__Lactobacillaceae
g__Lactobacillus
c__Actinobacteria
g__Bifidobacterium
o__Bifidobacteriales
f__Bifidobacteriaceae
g__Desulfovibrio
g__
p__Actinobacteria
g__
f__Christensenellaceae
f__Anaeroplasmataceae
g__Anaeroplasma
o__Anaeroplasmatales
p__Tenericutes
c__Mollicutes
g__Allobaculum
f__Erysipelotrichaceae
c__Erysipelotrichi
o__Erysipelotrichales
f__Veillonellaceae
Other
Other
Other
Other
Other
g__Odoribacter
f__Odoribacteraceae_
o__Verrucomicrobiales
g__Akkermansia
f__Verrucomicrobiaceae
c__Verrucomicrobiae
p__Verrucomicrobia
Unassigned
−4 −3 −2 −1 0 1 2 3 4 5
LDA SCORE (log 10)
D
a: Other
b: Other
c: Other
d: g__Bifidobacterium
e: f__Bifidobacteriaceae
f: o__Bifidobacteriales
g: g__
h: f__Coriobacteriaceae
i: o__Coriobacteriales
j: g__
k: f__Prevotellaceae
l: g__AF12
m: g__
n: f__S24_7
o: g__Odoribacter
p: f__Odoribacteraceae_
q: g__Paraprevotella
r: g__Prevotella_
s: f__Paraprevotellaceae_
t: g__Lactobacillus
u: f__Lactobacillaceae
v: g__Lactococcus
w: f__Streptococcaceae
x: g__
y: f__Christensenellaceae
z: Other
a0: Other
a1: g__Dorea
a2: g__
a3: f__Peptostreptococcacea
a4: f__Veillonellaceae
a5: g__
a6: g__Allobaculum
a7: f__Erysipelotrichaceae
a8: o__Erysipelotrichales

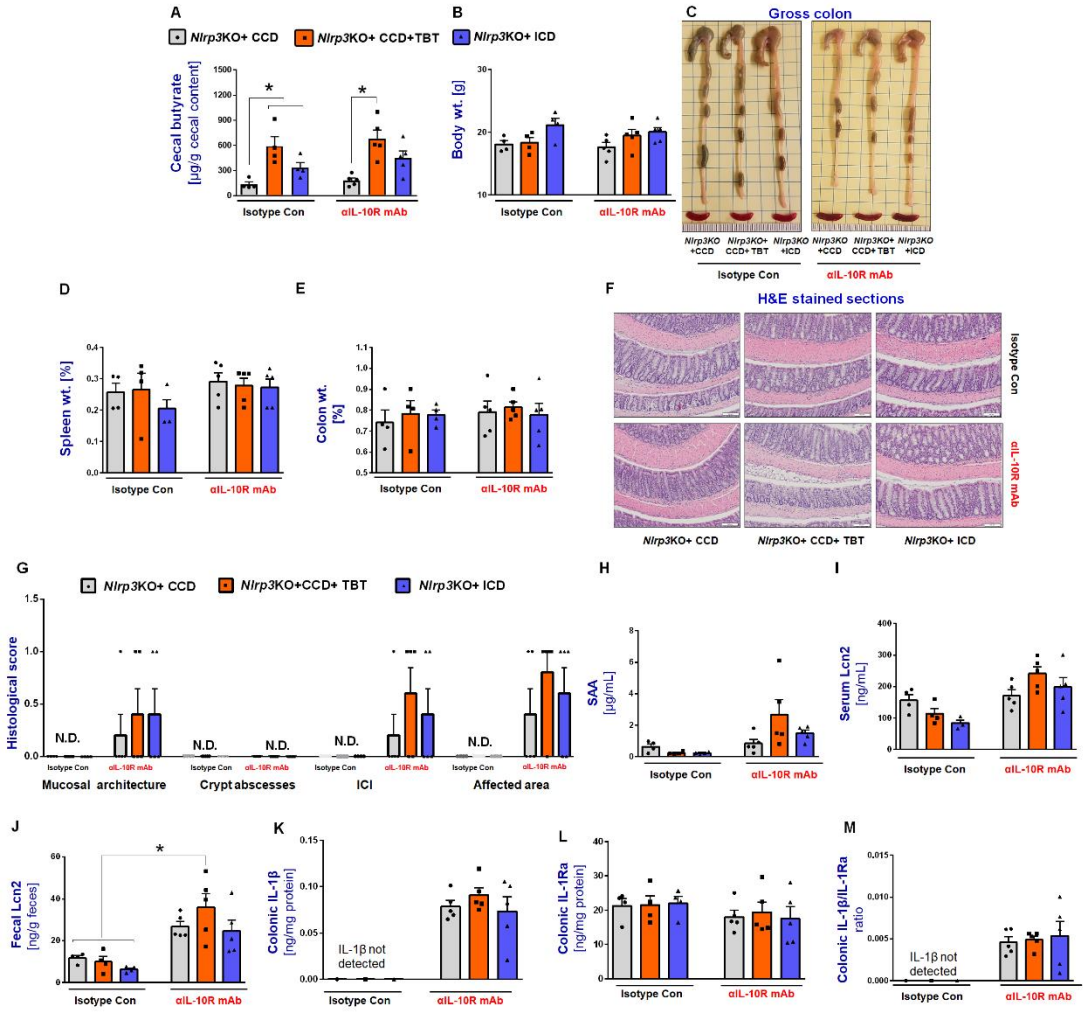
Supplementary Figure 7. β-acid influences gut microbiota composition. Four-week old male WT mice were maintained on ICD and β-acid (20 ppm) or vehicle (propylene glycol) simultaneously. At day 5, mice were treated with αIL-10R mAb (1 mg/mouse). After one-week, feces were collected [n=7 mice, two cages (4 mice+3 mice)] and processed for microbiota analysis by 16S rRNA gene sequencing. Fecal microbiota composition was analyzed by 16S rRNA gene Illumina sequencing. **A.** Principal coordinate analysis (PCoA) of the unweighted Unifrac distance is represented, with samples colored by treatment. **B.** Taxa summarization performed at the phylum level. Samples were grouped based on treatment. **C-D.** LEfSE (LDA Effect Size) was used to investigate bacterial members that drive differences between vehicle and β-acid group. **C.** List of taxa significantly altered in one group compared to the other (LDA score > 2.0). **D.** Taxonomic cladogram obtained from LEfSe analysis of 16S rRNA genes sequencing, highlighting taxa significantly altered in one group compared to the other (LDA score > 2.0).



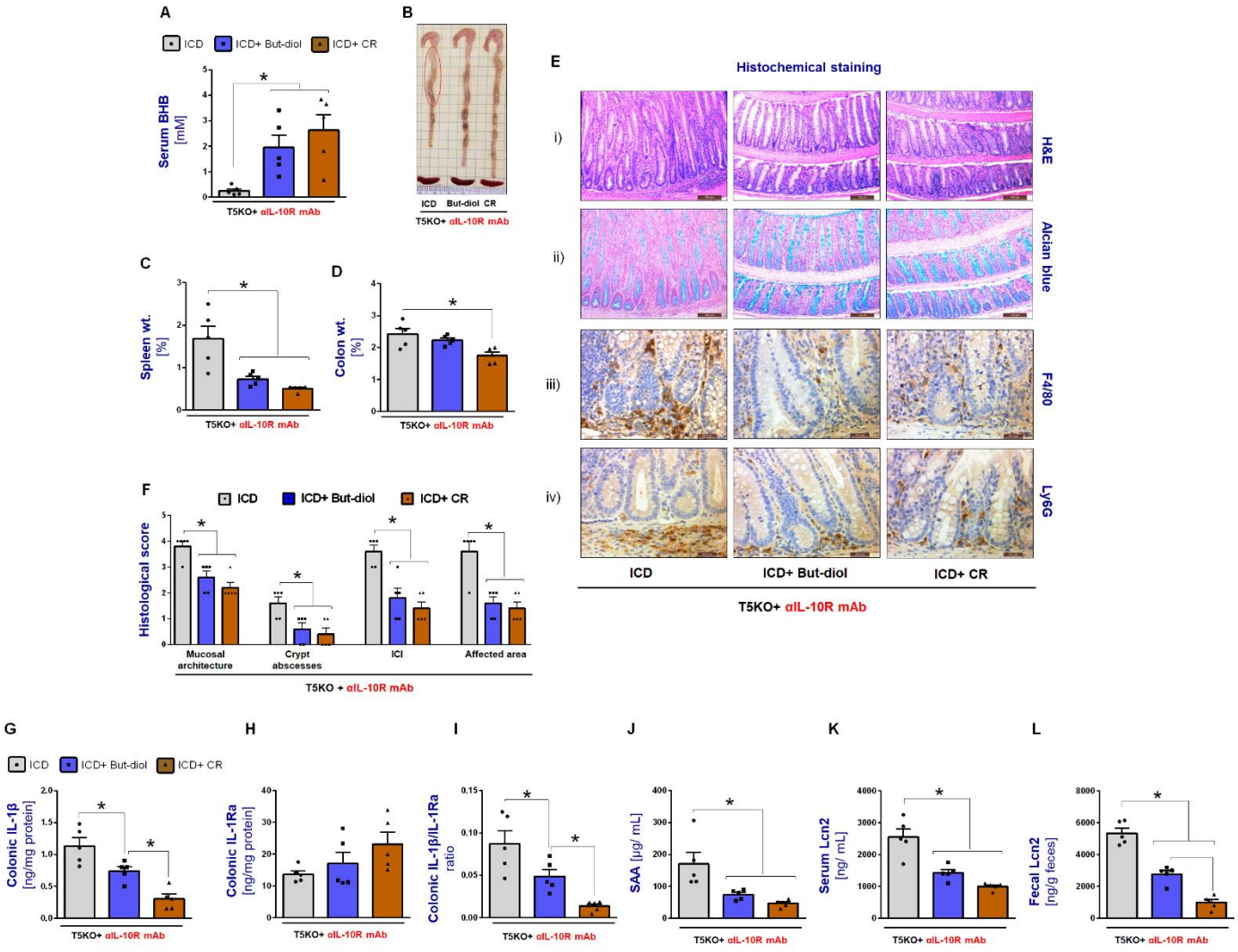
Supplementary Figure 8: Lack of innate immune response protects ICD-fed mice from colonic inflammation. MyD88-deficient (*MyD88*KO) and WT mice were maintained on ICD for 3 days and then challenged with αIL-10R mAb (1.0 mg/mouse, 4 weekly injections, i.p.). (**A**) Body weight. (**B**) Image displays the gross colon. (**C-D**) Percent weight of (**C**) spleen and (**D**) colon. (**E**) Image show histochemical staining for (**i**) H&E and (**ii**) alcian blue. (**F**) Colon histology score. Serum levels of (**G**) SAA and (**H**) Lcn2. (**I**) Fecal Lcn2 levels. The data is represented as mean ±SEM. (*p< 0.05).


A
Body wt. [g]
WT+ ICD
Nlrp3KO+ ICD
αIL-10R mAb
B
Gross colon
WT+ ICD
Nlrp3KO + ICD
αIL-10R mAb
C
Spleen wt. [%]
WT+ ICD
Nlrp3KO+ ICD
αIL-10R mAb
D
Colon wt. [%]
WT+ ICD
Nlrp3KO+ ICD
αIL-10R mAb
E
Histochemical staining
H&E
Alcian blue
WT
Nlrp3KO
ICD+ αIL-10R mAb
F
Histological score
WT
Nlrp3KO
N.D.
Mucosal architecture
Crypt abscesses
ICI
Affected area
ICD+ αIL-10R mAb
G
SAA [μg/mL]
WT+ ICD
Nlrp3KO+ ICD
αIL-10R mAb
H
Serum Lcn2 [ng/mL]
WT+ ICD
Nlrp3KO+ ICD
αIL-10R mAb
I
Fecal Lcn2 [ng/mg feces]
WT+ ICD
Nlrp3KO+ ICD
αIL-10R mAb
J
Colonic IL-1β [ng/mg protein]
WT+ ICD
Nlrp3KO+ ICD
αIL-10R mAb
K
Colonic IL-1Ra [ng/mg protein]
WT+ ICD
Nlrp3KO+ ICD
αIL-10R mAb
L
Colonic IL-1β/IL-1Ra ratio
WT+ ICD
Nlrp3KO+ ICD
αIL-10R mAb

Supplementary Figure 9: ICD-fed *Nlrp3*KO mice are protected from chronic colitis. Nlrp3-deficient (*Nlrp3*KO) and WT mice were maintained on ICD for 3 days and then treated with αIL-10R mAb (1.0 mg/mouse, 4 weekly injections, i.p.). (**A**) Body weight. (**B**) Image displays the gross colon. (**C-D**) Percent weight of (**C**) spleen and (**D**) colon. (**E**) Images show histochemical staining for (**i**) H&E and (**ii**) alcian blue. (**F**) Colonic histology score. Serum levels of (**G**) SAA and (**H**) Lcn2. (**I**) Fecal Lcn2 levels. Proximal colon tissues were processed to measure inflammasome cytokines using ELISA. (**J-L**) Bar graphs represent colonic (**I**) IL-1β, (**J**) IL-1Ra, and (**K**) IL-1β/IL-1Ra ratio. The data is represented as mean ±SEM. (*$p < 0.05$).



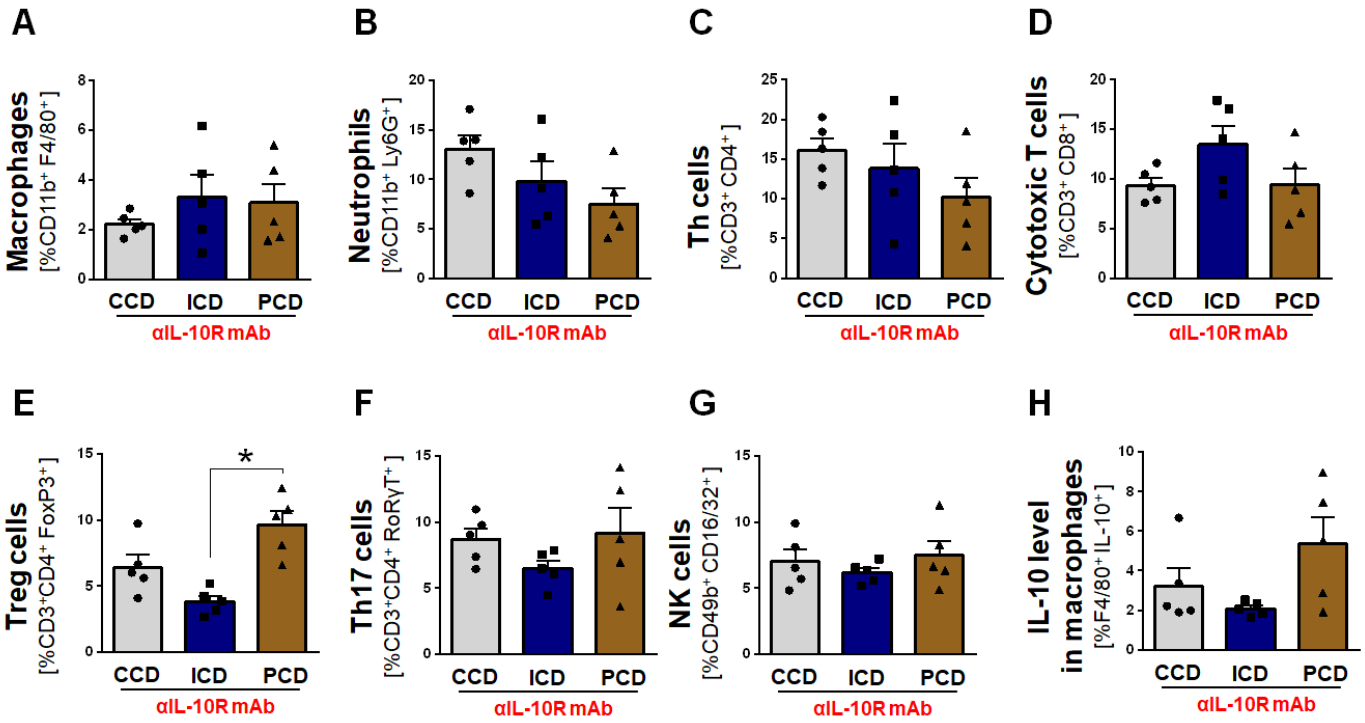
Supplementary Figure 10: Tributyrin supplementation failed to promote colonic inflammation in *Nlrp3*KO mice. Male *Nlrp3*KO mice (4 weeks old) were maintained on CCD, CCD plus TBT or ICD and received 3 weekly injections of αIL-10R neutralization antibody. Bar graphs represent (**A**) cecal butyrate, (**B**) body weight, (**C**) gross colon, (**D**) spleen weight, (**E**) colon weight, (**F**) H&E-stained colonic sections, (**G**) colonic histology score, (**H**) serum amyloid A (SAA), (**I**) serum Lcn2, and (**J**) fecal Lcn2. Bar graphs show cytokine level in proximal colon (**K**) IL-1β, (**L**) IL-1Ra and (**M**) IL-1β/IL-1Ra ratio. The data represented as Mean ± SEM (*p<0.05).



Supplementary Figure 11: Dietary and calorie restriction-induced β-hydroxybutyrate (BHB) suppresses colonic inflammation in *Tlr5*KO mice.

Male Tlr5-deficient (T5KO) mice (4 weeks old) were divided into 3 groups: group 1 received ICD, group 2 received ICD along with food grade 1,3 butanediol (20% v/v), and group 3 everyday received 5h fasting followed by controlled access (~70% of total daily food intake) to the diet. All mice were subjected to i.p. injection of αIL-10R mAb (1 mg/mouse, 3 weekly injections) and then analyzed for standard colitis parameters. (**A**) Serum β-hydroxybutyrate (BHB). (**B**) Image display

the gross appearance colons. (**C-D**) Percent weight of (**C**) spleen and (**D**) colon. (**E**) Images show the (**i**) H&E- and (**ii**) alcian blue staining, and immunostaining for (**iii**) macrophages (F4/80) and (**iv**) neutrophils. (**F**) Colon histology score. Proximal colon was processed in RIPA buffer and supernatant was used for cytokine estimation. (**G-I**) Colonic level of (**G**) IL-1β, (**H**) IL-1Ra and (**I**) IL-1β/IL-1Ra. Serum levels of (**J**) SAA and (**K**) Lcn2. (**L**) Lcn2 level in feces. The data is represented as mean ±SEM (*$p < 0.05$).



Supplementary Figure 12. Pectin promotes the expansion of regulatory T-cells in the lamina propria during acute colitis. Male WT mice maintained on CCD, ICD or PCD received single injection of αIL-10R mAb. After one-week mice were euthanized and colonic lamina propria cells were isolated to profile the immune cells. Bar graphs show the percentage of (**A)** macrophages, (**B**) neutrophils, (**C**) Th cells, (**D)** cytotoxic T cells, (**E**) regulatory T cells (Tregs), (**F**) Th17 cells, (**G**) natural killer (NK) cells, and (**H**) IL-10 levels in macrophages. Each dot represents data from one mouse. The data is represented as mean ±SEM (*p< 0.05).

2 - Methods

Mice

C57BL/6 background Toll-like receptor 5-deficient (*T5*KO), Nlrp3-deficient (*Np3*KO), and *T5* and *Np3* double knockout (*T5/Np3*-DKO) mice and their wild type (WT) littermates were bred and maintained under specific pathogen-free conditions at The Pennsylvania State University, University Park PA. Mice were housed in cages (n=4-5 mice/cage) containing sawdust bedding and nest lets, and fed *ad libitum* with unrestricted access to water throughout the study. The Institutional Animal Care and Use Committee at The Pennsylvania State University approved the animal experiments. Using an alpha of 0.05 (two-sided) and a power of 0.80, we calculated 5-7 mice per genotype as the minimum number necessary to obtain data that is statistically significant between the control and the experimental groups.

Diets

All the diets were prepared by the Research Diets, Inc. (New Brunswick, NJ). Composition of all diets including the source of dietary fibers inulin, cellulose and pectin are provided in supplementary table 1 (Table S1).

Induction of acute and chronic colitis by IL-10 receptor neutralization

Mice were weaned on day 24 and fed on chow diet for one week to acclimatize for solid food and stabilize their intestinal microbiota. After acclimatization, mice were switched to either inulin containing diet (ICD), cellulose containing diet (CCD), pectin containing diet (PCD), or fructoligosaccharide containing diet (FCD). On day 3, the colonic inflammation was induced by administering either single injection (acute colitis) or four weekly injections (chronic colitis) of αIL-10RmAb (1.0 mg/mouse, i.p.). All control mice received isotype control (Rat IgG, 1.0 mg/mouse) antibody. All mice were maintained on respective diets throughout the study. In acute

colitis, the mice were euthanized 1-week post first injection, whereas, in chronic colitis the mice were euthanized 2-week post fourth injection. In *Tlr5*KO mice chronic colitis was induced by 3 weekly injections and mice were euthanized 3-week post last injection. A subset of ICD-fed *Tlr5*KO mice exhibited hyperbilirubinemia[1]; those mice were excluded from the study. Immediately after euthanasia, blood, colon and cecum were isolated and processed for biochemical and histological analysis. The αIL-10R-induced colitis model offers several advantages over the routinely employed dextran sodium sulfate (DSS) model, including (i) a colitis driven by immune hyperactivation rather than chemical-induced injury, (ii) a viable model of chronic colitis rather than acute life-threatening illness, and (iii) a microbiota-dependent pathology, thus serving as a suitable model to study the interaction between the immune system and gut microbiota during the colitis. More importantly, we surmised that αIL-10R colitis is the most relevant and appropriate model to study the pathology observed in human IBD driven predominantly by the loss of IL-10 signaling.

Depletion of butyrate producers via metronidazole administration

To specifically deplete the major butyrate producers after one-week post weaning, mice were switched to drinking water containing metronidazole (1 g/L) for three days before placing them onto ICD. After another three days, mice received either αIL-10RmAb (1 mg/mouse, 3 weekly injections, i.p.) or isotype control and were maintained on ICD along with metronidazole. Post two weeks of fourth injection mice were euthanized and analyzed for standard colitis parameters.

Inhibition of bacterial fermentation via Hop β-acid administration

One-week post-weaning male WT and *T5*KO mice were simultaneously maintained on vehicle or β-acids from Hops (*Humulus lupulus*; in 45% w/w in food-grade propylene glycol at 0

or 20 ppm in drinking water) and ICD. After five days, mice were challenged with αIL-10RmAb (1 mg/mouse, i.p., 3 weekly injections) or isotype control antibody. After two weeks of third injection mice were euthanized and analyzed for standard colitis parameters.

Tributyrin treatment

CCD-fed WT mice were supplemented with tributyrin (TBT, 5g/kg body wt.; orally on every third day for a total period of 21 days, source: Sigma, Cat # W222305). TBT is a butyrate precursor, which has been shown to increase butyrate levels in the large intestine [2, 3]. Post three days of TBT treatment, mice were challenged with three weekly injections of αIL-10R neutralization antibody or isotype control. Mice were euthanized on day 21 (1-week post third injection) and analyzed for cecal SCFA and markers of colitis. The every third-day TBT treatment regime did not elicit a notable change in body weight and food intake when compared to the respective control.

Elevating systemic β-hydroxybutyrate (BHB) via either calorie restriction or 1,3 butanediol feeding in the drinking water

After weaning male WT and *T5*KO mice were maintained on lab chow for 1 week and then divided into 3 groups. First group had free access (*ad libitum*) to ICD. Second group was simultaneously maintained on drinking water supplemented with food grade 1,3 butanediol (20% v/v) along with ICD. The third group was subjected to a combination of 5h fasting every day plus limited (~30% less than average daily intake) access to the ICD. Our pilot experiments with aforementioned combinations exhibited ~5-fold elevation in systemic BHB. All mice received αIL-10R mAb (1.0 mg/mouse, 3 weekly injections, i.p.,) and then analyzed for standard colitis parameters.

Serum BHB quantification

Serum BHB was measured in the control (ICD-fed), butanediol supplement (ICD+ 1,3 butanediol) and 5h fasted groups using a colorimetric assay kit (Cayman Chemicals, catalogue # 700190). Briefly, sera were diluted to 1:5 (for ICD-fed control) and 1:15 (for butanediol supplement and fasted group) with kit assay buffer. Standards and samples were added to the respective wells, in duplicate, and the reaction was initiated by adding the developer solution. After 30 min. of incubation at 25ºC in the dark, optical density was recorded at 450 nm using a plate reader. The concentration of BHB in the serum was calculated by using a standard curve generated from BHB standard.

ELISA

The colons were flushed with PBS and proximal colons were homogenized in RIPA buffer (100 mg/mL) containing protease inhibitors (Roche's complete™ protease inhibitor cocktail). Interleukin (IL)-1β and IL-1 receptor antagonist (IL-1Ra) were measured in the colon homogenate via Duoset ELISA kits (R&D Systems; Minneapolis, MN) as per manufacturer's protocol. Feces (100 mg) were suspended in 1 mL of PBS containing 0.05% Tween 20, vortexed, and spun at 14000 g for 5 min and supernatant was used to measure lipocalin 2 (Lcn2) using ELISA kit from R&D Systems with appropriate dilution. Serum samples were also used to determine the circulating Lcn2 and serum amyloid A (SAA).

Colonic myeloperoxidase (MPO) assay

Colonic MPO activity was assessed as previously described in Singh *et al*., 2015 [4]. In brief, a portion of proximal colon (50 mg) was homogenized in 1 mL of potassium phosphate buffer (50 mM, pH 6.0) containing 0.5% hexadecyl trimethyl ammonium bromide (HTAB), freeze-thawed (thrice), sonicated and centrifuged (10000 *g*, 4°C). The clear supernatants were collected to

measure MPO activity by co-incubating with guaiacol (50 mM) and H_2O_2 (0.002%) in a 96-well plate (Corning). The change in absorbance at 470 nm was measured over a period of 10 min at 1-min intervals. One unit of MPO activity was defined as the amount that increases absorbance at 470 nm by OD of 1.0 per minute at 25°C, calculated from the initial rate of reaction.

Histochemical and immunohistochemical staining

The colons were emptied of fecal contents and opened longitudinally along the mesenteric border and formed a Swiss-roll from the proximal to distal end, then placed in 10% neutral-buffered formalin for 24 h. The Swiss-rolls were transferred to 70% ethanol and then processed to paraffin embedded blocks to generate 5 μm sections for histochemical and immunohistochemical staining. Hematoxylin & eosin staining was performed to observe the morphological and histopathological changes in crypt structure, ulceration and crypt loss, epithelial hyperplasia, mucosal thickening, and extent of immune cell infiltration (ICI) in mucosa and submucosa. Acidic mucus containing goblet cells were identified via alcian blue staining. Immunohistochemical staining was performed on paraffin-embedded colonic sections for neutrophil (Ly6G, Abcam, 1:200 dilution) and macrophage (F4/80, AbD Serotec, 1:50 dilution) infiltration. Immunoreactivity was revealed by using Vectastain Elite ABC kit and peroxidase substrate kit (Vector Laboratories Inc. Burlingame, CA).

Serum immunoreactivity to LPS and flagellin

Relative levels of serum LPS- and flagellin-specific immunoreactivity were examined by ELISA, as previously described [5]. Briefly, high binding ELISA plates were coated overnight with purified flagellin (100 ng/well) or LPS (2 μg/well; from *E. coli* 0128: B12, Sigma, Catolog No. 2887) in pH 9.6 bicarbonate buffer. Serum samples were serially diluted 1:200, 1:600 or 1:1200 and applied to wells coated with flagellin or LPS. After incubation and washing, the wells were incubated with HRP conjugated anti-mouse IgG (1:1000). Immunoreactivity of LPS and flagellin

were captured using the colorimetric peroxidase substrate tetramethylbenzidine (TMB), and optical density (OD) was read at 450 nm and 540 nm (the difference was taken to compensate for optical interference from the plate) with an ELISA plate reader. Data are reported as OD corrected by subtracting background (determined by readings in blank samples). Serum samples from *Rag1*-deficient mice was used as negative controls.

Quantification of bacteria using quantitative PCR

The abundance of total bacteria, γ-*Proteobacteria*, and major butyrate producers were analyzed using quantitative PCR assay. Alterations in abundance of butyrate producers were further confirmed by examining the levels of the butyryl-CoA:acetate CoA-transferase (*BCoAT*) gene that encodes the enzyme responsible for the final step in butyrate production. As mentioned in ref [6], total bacterial DNA was extracted from equal amounts of feces (50 mg) using the QIAamp DNA Stool Mini Kit (QiagenInc, Valencia, CA). Quantitative PCR was performed using Step One Plus Real-Time PCR System (Applied Biosystems) in a reaction mixture of fecal DNA, SYBR green master mix and bacteria-specific primers (Table S1).

SCFA quantification

Sample preparation

After euthanasia, the feces and cecum were collected and stored immediately in dry ice. The cecal contents were weighed and processed in the cold room to avoid loss of SCFA. Cecal SCFA was analyzed as described in ref [7]. In brief, ~80 mg cecal contents were mixed in 1 mL of 0.005M aqueous NaOH containing 5 µg/mL d3-caproic acid, vortexed using Fast-Prep-5^{G} homogenizer (M.P. Biomedicals), and spun at 20,000 g for 15 min. The supernatants (500 µL) were then mixed with equal volume of 1-propanol:pyridine (v/v=3:2). Next, 100 µL of propyl chloroformate was slowly added to the above mixture and incubated at 60°C for one hour. The

derivatized samples were extracted with hexane and the supernatant was transferred to a glass autosampler vial for GC-MS analysis.

GC-MS analysis

Analysis of the SCFA was performed using a Shimadzu GC-2010 gas chromatography system coupled with a QP2010 EI mass spectrometric detector (GCMS-QP2010, Shimadzu, Kyoto, Japan). Propylated derivatives of the SCFA were separated using a Supelco SPB-5 fused silica column. One microliter of derivative dissolved in hexane was injected using split mode (1:6 split ratio), a solvent delay of 2.5 minutes, and a gas flow rate of 0.42 mL/min with helium as the carrier gas. The injection port temperature was set at 220^{o}C. The initial oven temperature of 50^{o}C was held for 3 minutes and then increased at a rate of 7^{o}C/min until 150^{o}C was achieved. Then the rate was raised to 16^{o}C/min and held at 250^{o}C for 10 minutes. Mass spectral data was collected in scan mode, and both the interface and detector temperature were set at 200^{o}C.

Shimadzu Post-Run Analysis software was used to process raw chromatogram data (GC-MS solution, Shimadzu, Kyoto, Japan). Multiple ion counts (MIC) were used to reduce noise and to improve signal-to-noise ratios by selecting ions that were representative of the six targeted analytes. These ion counts were added together to obtain the MIC and peaks areas were calculated. Areas of analyte peaks were compared to the internal standard (d3-caproate) and a standard mix to determine concentrations.

Flow cytometric characterization of lamina propria immune cells:

The colons were obtained from CCD-, ICD- and PCD-fed WT mice treated with single injection of αIL-10R mAb. The tissue was dissociated to single-cell suspensions by combining mechanical dissociation with enzymatic degradation according to the manufacturer instructions (mouse Lamina Propria Dissociation Kit, Miltenyi Biotec).

Lamina propria immune cells ($2.0x10^5$ cells) were stained with fluorescent conjugated anti-mouse monoclonal antibodies directed against the following cell surface proteins: CD11b-FITC (BD Bioscience), F4/80-PE (BD Bioscience), Ly6G-APC (BD Bioscience), CD3-PE (BD Bioscience), CD4-PE-Cy7(BD Bioscience), CD8-FITC (BD Bioscience), CD49b-FITC (BD Bioscience) and CD16/32-APC (BD Bioscience) in staining buffer (BD Bioscience) and incubated for 30 min at room temperature in the dark. After surface staining, cells were washed in PBS, fixed and permeabilized with FoxP3 Staining Buffer Kit (BD Bioscience) according to manufacturer's instructions and incubated with FoxP3-Alexafluor and RORγt-Alexa Fluor 647 for 1h at room temperature. Cells were washed twice in FACS Buffer and were analyzed by Accuri c6 flow cytometer (BD Biosciences) with BD Accuri C6 Software (Becton Dickinson). Immune cells were defined by using surface markers: neutrophils $CD11b^+$ $Ly6G^+$ $F480^-$; macrophages $CD11b^+$ $Ly6G^-$ $F480^+$; helper-T cells (T_H) $CD3^+CD4^+CD8^-$; cytotoxic T cells (T_C) $CD3^+CD4^-$ $CD8^+$; regulatory T cells (T_{reg})$CD3^+CD4^+FoxP3^+$; T_H17 cells$CD3^+CD4^+ROR\gamma t^+$.

16S rRNA gene sequence analysis

16S rRNA gene sequencing was performed as previously described [8]. Briefly, DNA was extracted from feces of CCD-, ICD- or PCD-fed WT mice, which was collected 2-weeks post last αIL-10R injection, using a QIAamp®PowerFecal®DNA kit (Qiagen). Similarly, DNA was extracted from hops β-acid treated ICD-fed WT mice at two weeks post last αIL-10R injection. The 16S rRNA gene V4 region was amplified by the primers provided in table S1. Each PCR amplicon was purified using AMPure XP beads (Beckman Coulter Inc. Brea, CA). Purified PCR products were quantified by spectrophotometry and pooled in an equimolar manner. Pooled DNA was sequenced on an Illumina MiSeq 2*250 at Cornell University genomic core. Before analysis, sequences were demultiplexed and quality filtered using the Quantitative Insights Into Microbial

Ecology (QIIME, version 1.8.0) software package. Sequences were assigned to OTUs using the UCLUST algorithm [9] with a 97% threshold of pairwise identity (with or without the creation of new clusters with sequences that do not match the reference sequences) and classified taxonomically using the Greengenes reference database (13.8 version). Alpha diversity curves were determined through QIIME. Beta diversity were analyzed using principal coordinate analysis of the unweighted Unifrac distance [10]. LEfSE (LDA Effect Size) was used to investigate bacterial members that drive differences between groups[11109]. PICRUSt (Phylogenetic Investigation of Communities by Reconstruction of Unobserved States) was used to predict the metagenome-based on microbiota composition analysis.

Statistical analysis

All data are represented as Mean ± SEM. The D'Agostino-Pearson omnibus normality test was used to test the normal distribution of data. Statistical significance between two groups was calculated using unpaired, two-tailed t-test. Data from more than two groups was compared using a one-way ANOVA followed by Tukey's multiple comparison tests (when to compare the mean of each column with the mean of every other column). The Spearman correlation (r) was used to establish the association of the two variables. $P<0.05$ was considered statistically significant. All statistical analyses were performed with the GraphPad Prism 7.0 program (GraphPad, Inc.).

Supplementary Table 1: List of primers

	Forward (3------5')	**Reverse (3'-------5')**	**Annealing temperature**
16S ribosomal RNA (rRNA)	8F: AGAGTTTGATCCTGGCTCAG	338R: CTGCTGCCTCCCGTAGGAGT	52ºC
γ-Proteobacteria	TCGTCAGCTCGTGTYGTGA	CGTAAGGGCCATGATG	60ºC
Clostridium cluster XIVa	AAATGACGGTACCTGACTAA	CTTTGAGTTTCATTCTTGCGAA	50ºC
Lachnospiraceae & *Ruminococcaceae*	CGGTACCTGACTAAGAAGC	AGTTTYATTCTTGCGAACG	55ºC
BCoAT	GCIGAICATTTCACITGGAAYWSI TGGCAY ATG	CCTGCCTTTGCAATRTCIACRAANGC	53ºC
rpoB	AACATCGGTTTGATCAAC	CGTTGCATGTTGGTACCCAT	50ºC
16S rRNA sequence primers	5′AATGATACGGCGACCACCGAGATCTA CACTATGGTAATTGTGTGCCAGCMGCCG CGGTAA-3′	5′CAAGCAGAAGACGGCATACGAGATXXXXXX XXXXXXAGTCAGTCAGCCGGACTACHVGGGT WTCTAAT-3′	N/A
Lactobacillus spp.	AGCAGTAGGGAATCTTCCA	ATTYCACCGCTACACATG	60ºC
Bifidobacteria spp.	CTCCTGGAAACGGGTGG	GGTGTTCTTCCCGATATCTACA	60ºC

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