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Supplemental Information

Bacterially Derived Tryptamine Increases Mucus Release by Activating a Host Receptor in a Mouse Model of Inflammatory Bowel Disease

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Supplemental Information

Figure S1: Measurement of mucus release using PAS-H staining in mouse proximal colon. Related to Figure 1. The 40X raw PAS-H image was deconvoluted in FIJI (Image J v 2.0.0-RC-69/1.52i) software to identify goblet cells within the glandular region of proximal colon. Intensity threshold were manually adjusted to identify mucus containing intact goblet cells and cavitated goblet cells. Percent of cavitated goblet cells was determined using the formula described below.

% of intact goblet cell =

(Low Intensity Threshold (Intact goblet cell) /High Intensity Threshold (Total goblet cell))*100%

% of goblet cell cavitated = 100 - % of intact goblet cell

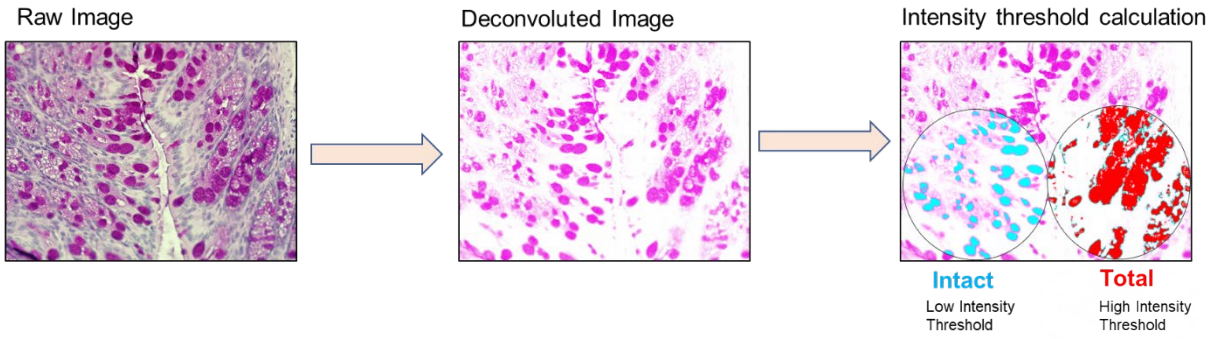

Figure S2: Experimental outline and study design. Related to Figure 2, 3, 4, and 5. (A) 100 µl of frozen *B. thetaiotaomicron* Trp D+/- (*B. theta+/-*) stocks were plated in tryptone yeast extract glucose (TYG) agar plates. After 2 days of incubation, individual bacterial colonies were identified on plates and cultured in a 3ml TYG medium. After 2 days, TYG bacterial cultures were transferred into the gnotobiotic isolator, 300 µl of TYG bacterial solution was then gavaged to GF mice. (B) Drinking water with 0.25% tryptophan was provided to 7-8 week old GF C57BL/6J monocolonized mice followed by 5 days of 3% DSS supplementation to induce colitis. Change in body weight and DAI was measured over the course of 7 days.

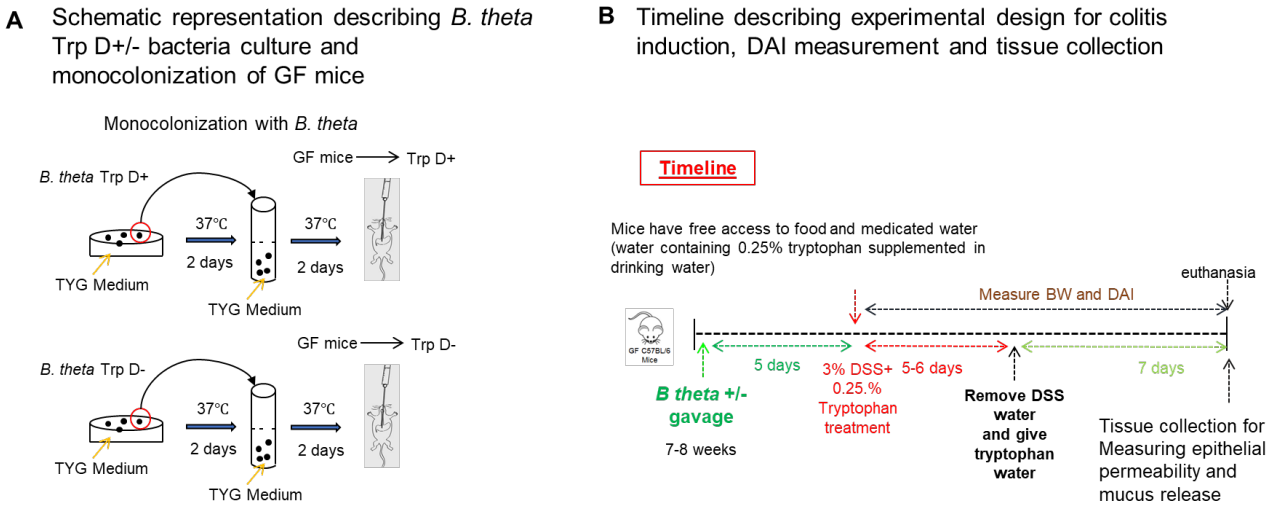

Figure S3: Pilot experiments to determine optimal DSS concentration for induction of colitis in gnotobiotic mice. Related to Figure 2. Figure shows percentage weight loss following administration of 1% (A; n=3-4), 2% (B; n=4) and 2.5% (C; 5-6 males, D; 12-13 females) DSS in mice colonized with *B. thetaiotaomicron* Trp D-. Data are mean±SEM.

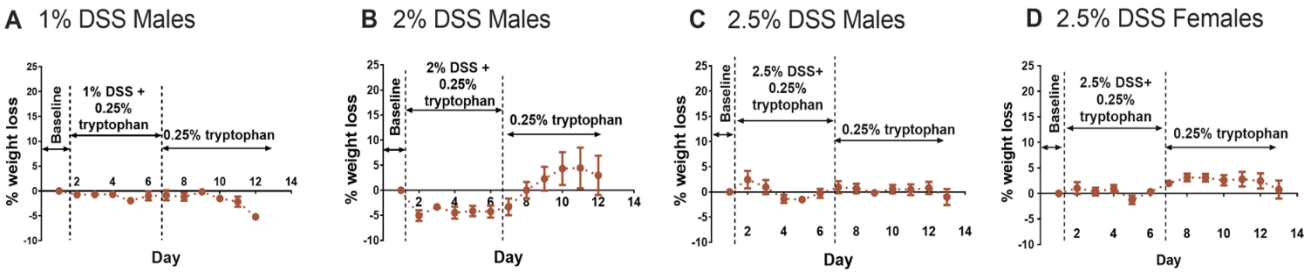

Figure S4: Survival curve for GF and gnotobiotic mice after 3% DSS administration.

Related to Figure 2, 3. Survival curve showing mortality following DSS administration in (A) GF mice and (B) GF mice colonized with *B. thetaiotaomicron* Trp D+/-.

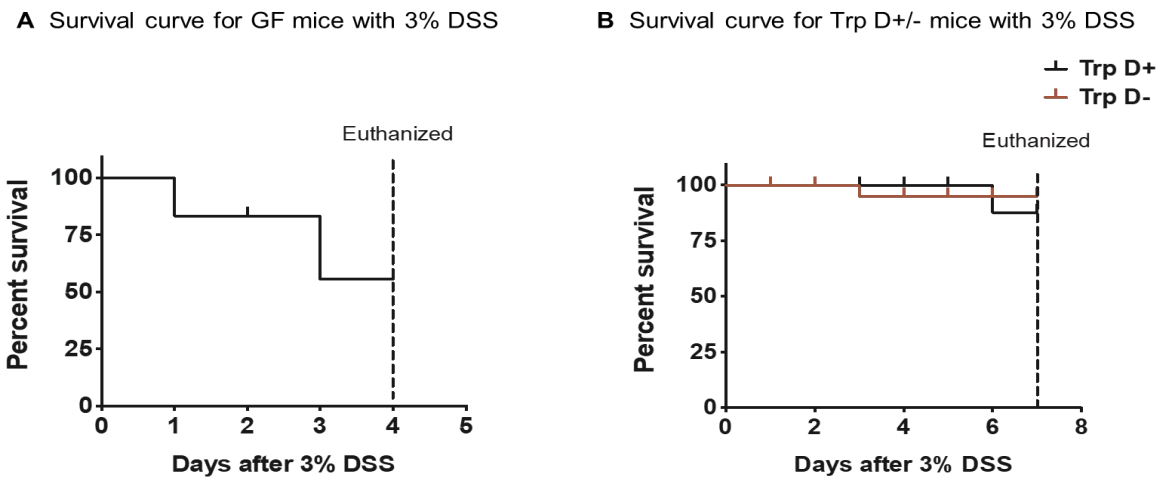

Figure S5: Bacterially-derived tryptamine does not affect severity of inflammation in DSS-challenged mice based on histology. Related to Figure 3. Figures (A-C) quantification of histopathological damage in male and female *B. thetaiotaomicron* Trp D+ and Trp D- monocolonized mice. n=12-13 (7-8 males, 4-6 females). Bars indicate the median and error bars the interquartile range.

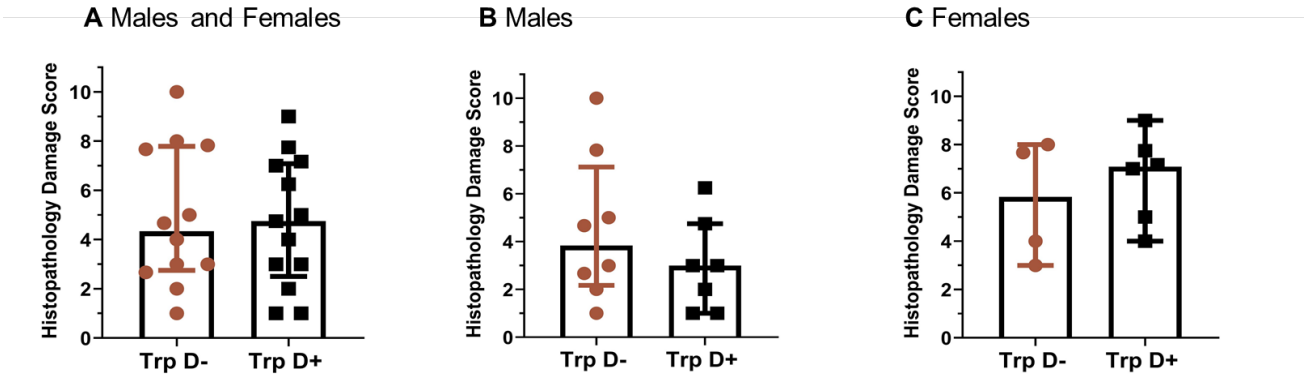

Table S1: Change in body weight after 6 days of 3% DSS administration in Trp D+ and Trp D- monocolonized mice. Related to Figure 2. Change in body weight post DSS administration in (A) male and (B) female *B. thetaiotaomicron* Trp D+ and Trp D- monocolonized mice.

A. Males

	Trp D-			Trp D+		
Day	Mean	SEM	n	Mean	SEM	n
BASELINE (DAY 0)	27.625	0.65894	8	27.1571	0.94336	7
1 DAY AFTER DSS	28.1375	0.6716	8	27.4714	1.0087	7
2 DAYS AFTER DSS	27.95	0.63864	8	27.4857	0.94754	7
3 DAYS AFTER DSS	28.25	0.72161	8	27.4143	0.93641	7
4 DAYS AFTER DSS	28.4875	0.74221	8	27.7714	0.94962	7
5 DAYS AFTER DSS	28.575	0.65921	8	28.0229	1.07705	7
6 DAYS AFTER DSS	28.25	0.64003	8	27.4286	0.88498	7
1 DAYS POST WATER SWITCH	27.825	0.65948	8	26.9714	0.93776	7
2 DAYS POST WATER SWITCH	27.1375	0.66088	8	26.6571	0.98823	7
3 DAYS POST WATER SWITCH	26.6375	0.70355	8	26.1286	0.89382	7
4 DAYS POST WATER SWITCH	25.6	0.82245	8	25.3714	0.90599	7
5 DAYS POST WATER SWITCH	24.325	0.97756	8	24.6429	0.8799	7
6 DAYS POST WATER SWITCH	23.075	1.02395	8	23.7857	0.87843	7
7 DAYS POST WATER SWITCH	22.0875	0.89949	8	22.9857	0.89534	7

B. Females

	Trp D-			Trp D+		
Day	Mean	SEM	n	Mean	SEM	n
BASELINE (DAY 0)	23.1167	0.56534	6	22.68	0.47896	5
1 DAY AFTER DSS	23.3333	0.73242	6	22.54	0.64467	5
2 DAYS AFTER DSS	23.55	0.53588	6	22.34	0.54461	5
3 DAYS AFTER DSS	23.4	0.5379	6	22.4	0.56833	5
4 DAYS AFTER DSS	23.9	0.54099	6	22.78	0.60778	5
5 DAYS AFTER DSS	23.6167	0.52752	6	22.92	0.72829	5
6 DAYS AFTER DSS	23.1333	0.64533	6	22.26	0.63451	5
1 DAYS POST WATER SWITCH	22.8333	0.55297	6	21.88	0.68659	5
2 DAYS POST WATER SWITCH	21.9667	0.66767	6	21.58	0.42708	5
3 DAYS POST WATER SWITCH	20.9167	0.81544	6	21.2	0.43359	5
4 DAYS POST WATER SWITCH	20.45	0.8582	6	20.88	0.414	5
5 DAYS POST WATER SWITCH	19.8667	0.99488	6	20.4	0.30166	5
6 DAYS POST WATER SWITCH	19.4167	1.13149	6	20.2	0.34496	5
7 DAYS POST WATER SWITCH	18.5167	1.06534	6	19.78	0.34409	5

Table S2: Table highlighting method for disease activity Index (DAI) scoring. Related to Figure 3.

Score	Weight loss	Stool Consistency	Bleeding
0	None	Normal	Normal
1	1-5%		
2	5-10%	Loose stool	Hemoccult +
3	10-20%		Visible blood in stool only
4	>20%	Diarrhea	Gross bleeding (visible)

Table S3: Table highlighting key criteria and method for scoring histopathological damage in DSS challenged *B. thetaiotaomicron* Trp D+/- monocolonized male and female mice. Related to Figure 3.

Histopathology Damage Score Key:	**Severity score**
Destruction of Normal Architecture	
Normal appearance	0
Hyperplasia	1
Loss of glands in focal spot	2
Loss of glands throughout section	3
Infiltration	
Normal appearance	0
Focal expansion of immune cells	1
Cellulitis in submucosal space	2
Infiltration throughout layers	3
Muscle Thickening	
Normal appearance	0
Slight thickening	1
Thick muscle	2
Maximal thickening	3
Crypt Abscess - neutrophils in crypt	
Not present	0
Present	1
Goblet Cell Depletion	
Normal	0
Depleted	1

Transparent Methods

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Mouse Husbandry and Ethics

Specific pathogen free (SPF) conventionally raised (CR) founder 129/Sv WT and 5-HT4R knockout (KO) mice used for *ex vivo* mucus measurement were previously obtained from our collaborator (Compan et al., 2004). 129/Sv WT and 5-HT4R KO mice were bred, co-housed, and maintained in a micro-isolator caging system using conventional caging and husbandry practices. C57BL/6J germ-free (GF) breeders were obtained from Taconic Farms (Germantown, NY). GF and gnotobiotic C57BL/6J mice were housed within the flexible film vinyl (Class Biologically Clean, Madison, WI) isolators in a temperature-controlled gnotobiotic facility room at 69-70°F in 12 hrs light-dark cycle. Mice were housed in open top cages with autoclaved Sani-Chips bedding and were given ad libitum access to autoclaved diet (Purina Lab diet, 5K67, Collins feed and seed center, Rochester, MN), and autoclaved nano-pure water. Feed and bedding were changed every week or earlier if needed. GF status was confirmed prior to start of experiments with two consecutive negative cultures of fecal pellet, feed and bedding on brain heart infusion (BHI), Sabouraud dextrose, and nutrient media under both anaerobic and aerobic conditions, as well as PCR of 16S rRNA gene using fecal DNA as a template using universal primers and *Turicibacter* primers. GF male and female mice aged 7-8 weeks were used for the study. We were limited by the number of mice we could maintain within the confined space of gnotobiotic isolators, we therefore based our sample size on our previous study using similar methodology (Bhattarai et al., 2018). Mice were randomly assigned within each litter to experimental or controls group. Male and female

mice in each group were co-housed in their respective cages within the gnotobiotic isolator for the entirety the experiment. Since staging mice with in these isolators is challenging and significantly increases both the risk of contamination as well as stress for the mice, female mice were not controlled for estrous cycle in our study. All mice studies were approved by Mayo Clinic Institutional Animal Care and Use Committee (Protocol# A00004100-18) and conducted in compliance with regulatory guidelines.

METHOD DETAILS

Histochemistry to assess *ex vivo* mucus release and histopathological damage in DSS challenged mice

Full-thickness proximal colon segments were obtained 1-2 cm below the cecum. Luminal contents were quickly but gently flushed with chilled Krebs solution (composition in mM/l: 11.5 d-glucose/d-mannitol, 120.3 NaCl, 15.5 $NaHCO_3$, 5.9 KCl, 1.2 NaH_2PO_4, 2.5 $CaCl_2 \cdot 2H_2O$, and 1.2 $MgCl_2$; pH 7.3–7.4) using a 30 ml syringe hooked to a 200 µl pipette tip. The proximal colon segments were then divided into four equal sections. The sections were equilibrated for 10 minutes in oxygenated 37°C Krebs solution, followed immediately by 30 minutes of incubation under various experimental conditions. After incubation, the tissues were rinsed and placed in an Eppendorf microcentrifuge tube containing 10% neutral buffered formalin at room temperature. Tissues were shipped overnight to the Mayo Clinic histopathology core facility located in Scottsdale, Arizona. Tissues were paraffin-embedded, sectioned, and stained with Periodic Acid-Schiff and hematoxylin (PAS-H) counterstain, and with hematoxylin and eosin (H&E) stain to assess mucus release and histopathological damage post DSS challenge respectively.

In short, the PAS-H protocol involved the following steps. First, cross section paraffinized colonic tissue blocks were sliced into 4 μm thickness. Sliced tissues were then deparaffinized using a series of washes with histological grade xylene (Fisher Scientific #SS32 500, Toronto, Ontario, Canada) and reagent grade ethanol and finally hydrated in distilled water. Tissues were incubated in 1% periodic acid solution for 5 minutes and rinsed with distilled water. Tissues were placed in Schiff's reagent (Fisher Scientific #SS32 500, Toronto, Ontario, Canada) for 45 min and afterwards washed under running tap water for 10 minutes. Tissues were finally counterstained with hematoxylin (Richard Allen Scientific, Ref#7221) for 15 seconds and dehydrated beginning with 95% alcohol and mounted. H&E staining was performed using a standard H&E protocol on tissues from *B. thetaiotaomicron* monocolonized DSS challenged mice. The stained slides were evaluated using a bright-field microscope (Olympus, BX51W1, Olympus Life Science Solutions, Center Valley, PA, USA).

PAS-H images were obtained at 40x magnification and analyzed using FIJI (Image J v 2.0.0-RC-69/1.52i) software. Images were deconvoluted to identify goblet cells within the glandular region of proximal colon. The deconvolution vectors were calculated from PAS-H specific region of interests (ROI) within the individual RGB images. Images were subsequently deconvoluted in accordance with the Beer-Lambert law $I = I_0 \exp(-\varepsilon LC)$, where I is the intensity of the stain as recorded by the charge coupled device camera, I_0 is the intensity of the background white light, ε is the molar attenuation coefficient of the stain, L is the sample thickness and C is the concentration of the stain. The percentage of cavitated and non-cavitated goblet cells was evaluated by the particle analysis function on the PAS channel images after manually adjusting the pixel intensity thresholds to identify mucus-containing intact and mucus-depleted goblet cells. While establishing these methods for assessing

goblet cell cavitation, we found that goblet cell staining in the distal colon was diffuse and harbored poor demarcation compared to proximal colon samples. We thus decided to quantify goblet cell cavitation only in the proximal colon to assess mucus release with greater accuracy and confidence. Representative images and the calculations for this analysis are shown in **Figure S1**.

H&E stained slides were evaluated by a blinded scorer. Histopathological damage assessment as a result of DSS insult was scored based on five histological parameters; destruction of tissue architecture (severity score range: 0-3), immune infiltration (0-3), muscle thickening (0-3), crypt abscess (0-1) and goblet cell depletion (0-1). The severity scores were then summed to get a total damage score. Key criteria for damage scoring and severity scoring are tabulated in **Table S3**.

Generation of bacterial strains and growth conditions

Previously described engineered *Bacteroides thetaiotaomicron* that encodes a phage promoter driving the tryptophan decarboxylase (Trp D+) gene capable of producing tryptamine, and a vector-only control strain of *B. thetaiotaomicron* (Trp D-) were used in this study (Whitaker et al., 2017). Successful integration of the plasmid into the *B. thetaiotaomicron* chromosome was confirmed in the previous study by PCR (Whitaker et al., 2017) and through *in vitro* production of tryptamine in tryptone-yeast extract-glucose (TYG) culture media containing tryptophan (Bhattarai et al., 2018). Bacterial gavage sample were prepared from engineered *B. thetaiotaomicron* Trp D+ and Trp D- frozen stocks from previous study (Bhattarai et al., 2018). Frozen stocks stored in crimp top chromatography vials in -80^{o}C freezer were first slowly thawed in a small container containing sparse amount of dry ice. 100

µl of thawed stock was withdrawn using a tuberculin syringe and streaked on pre-reduced tryptone yeast extract glucose (TYG) agar plates. The plates were incubated inside an anaerobic growth chamber, kept at 37°C, and supplied with 15% CO_2, 5% H_2 and 80% N_2 (Coy Laboratory Products, Grass Lake, MI). After two days of culture, single bacterial colonies were inoculated into liquid TYG culture medium using sterile inoculating loops. After 24 hours of liquid culture, the culture medium was aliquoted into sterile 2 ml cryovials and sealed before removing from the anaerobic chamber to prevent oxygen exposure. The sealed aliquots were transferred into two separate sterile experimental isolators containing GF mice. 300 µl of TYG aliquot containing *B thetaiotaomicron* Trp D+ and Trp D- strains was gavaged into seven to eight weeks old male and female GF mice (**Figure S2A**).

Induction and evaluation of DSS induced colitis in mice

GF mice were co-housed inside the gnotobiotic isolators based on their sex and monocolonized between 7-8 weeks with either tryptamine producing *B. thetaiotaomicron* Trp D+ strain or with *B. thetaiotaomicron* Trp D- control strain. Drinking water was supplemented with 0.25% tryptophan immediately after colonization. After five days of monocolonization, freshly prepared 3% (wt/vol) dextran sulfate sodium salt (DSS, molecular weight 40 kDa, Catalog#42867, Sigma Life Science, USA) was introduced into drinking water to induce colitis. Mice were maintained on 1-3% DSS and 0.25% tryptophan water for a period of 5-6 days. Freshly prepared DSS tryptophan water was switched on day 3 and completely removed by day 5-6. Mice were then continuously maintained on 0.25% tryptophan water for additional 7 days (**Figure S2B**). During this period change in body weight was determined by trained individuals blinded to the treatment groups (**Table S1**). In case body weight loss

exceeded >20% of the initial body weight on 7th day post DSS removal, the protocol was terminated on 7th day per humane endpoint guidelines set in the IACUC protocol (Mayo Clinic IACUC Protocol# A00004100-18). Colitis severity was assessed using a disease activity index (DAI) score, which was determined by calculating sum of scores for change in percentage weight loss, stool consistency score and hematochezia score. The scoring system for DAI is presented in **Table S2**.

Tissue preparation

Mice were euthanized by day 7 post DSS administration. Mice were asphyxiated with CO_2, and euthanasia was confirmed by cervical dislocation. For every mouse, a segment of proximal colon ~ 2 cm below the cecum was removed. The luminal contents were flushed with chilled (4°C) Krebs solution. Segment of proximal colon tissue used to analyze ex-vivo epithelial permeability in Ussing chambers were opened along the mesenteric border. Another piece of proximal colon tissue was stored in 10% formalin for histochemistry as described above.

Ussing chamber to measure *ex-vivo* epithelial permeability

To measure change in *ex vivo* epithelial permeability, the opened proximal colon segment was pinned flat on Sylgard with the mucosa side facing up. Full thickness tissue was transferred and mounted on an Ussing cassette (Physiologic Instruments, San Diego, CA) with an aperture of 0.31 cm^2 under a dissecting microscope with fine forceps. The chamber on the submucosal side was bathed with 4 ml of glucose-containing Krebs solution while the mucosal side was bathed with 4 ml of mannitol Krebs solution. The Krebs solution was

bubbled with 97% O_2 and 3% CO_2 mixture. Flux of 4 mg/ml 4KDa FITC (Fluorescein isothiocyanate), a measure of paracellular transport, was determined over a period of two hours with readings recorded every 30 minutes.

Tryptamine measurement in fecal samples post DSS challenge

To confirm that engineered *B. thetaiotaomicron* survives DSS challenge and produces tryptamine during the course of the study, tryptamine levels in fecal pellets on the last day of DSS challenge were determined essentially as previously described by Sangwan et al (Sangwan et al., 1998). Briefly, fecal pellet samples were weighed in pre-weighed bead beating tubes filled with ~100 µl of 0.1 mm glass beads. Per mg of fecal pellet 8 µl nanopure water was added and tubes were homogenized using beat beating (40 seconds, 6 meter/second). The tubes were centrifuged at max speed for 10 minutes at 4^{o}C and 50 µl of cleared extract was transferred to a clean microcentrifuge tube. To this extract 50 µl of assay buffer was added consisting of 5 mM beta-mercaptoethanol, 5 mM thiourea, and 1 mM ethylenediaminetetraacetic acid (EDTA) in 0.1 M sodium phosphate buffer (pH 7.5). Samples were alkalized with 0.2 ml of 4 N NaOH and phase extracted with 350 µl of ethyl acetate. After vortexing and allowing 10 minutes of phase separation, 100 µl of the upper ethyl acetate phase was added to a solvent-compatible 96 well plate and directly subjected to spectrofluorometric fluorescence monitoring in a Biorad Synergy plate reader (excitation 290 nm / emission 360 nm). A tryptamine standard curve was generated by serial dilution of 1 mg/ml tryptamine (Sigma 193747 Sigma-Aldrich Corp., St. Louis, MO, USA) in assay buffer and processed in parallel. Extraction specificity was verified using mixes of tryptophan and tryptamine.

QUANTIFICATION AND STATISTICAL ANALYSIS

Statistical Analysis

All statistical analyses (Student's *t test* and *ANOVA*) were conducted with Prism 7.0a software (GraphPad San Diego, CA, USA). All bar graphs were presented either as Mean ± SD or median ± interquartile range, while line graphs were presented as Mean ± SD. $P < 0.05$ was considered statistically significant.

SUPPLEMENTAL REFERENCES

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