Supplemental Fig. 1. Experimental timeline and tissue preparation
(A) All mice used in this study were handled according to the depicted timeline, with the exception of the HM mice that were colonized with microbiota from a second human donor at 8 weeks and sacrificed at 12 weeks of age (Supplemental Fig. 2). (B) Regions of the mouse colon used in different applications are indicated.

Supplemental Fig. 2. Sex and colonization dependent differences in relative expression of *Tph1 and Chga* in proximal colon
Microbial colonization was associated with significant differences in *Tph1* and *Chga* expression ($P<.0001$), with lowest expression of both genes observed in GF mice; differential expression of these genes was not observed on the basis of sex.

Supplemental Fig. 3. Expression of serotonergic genes in the proximal colon of GF and HM mice using a second human donor
qRT-PCR was used to assess relative expression of selected serotonergic mRNAs in proximal colonic segments from 12-week-old, male GF and HM mice (4-5 mice/group; fecal donor was a healthy 37-year-old man). Relative mRNA expression was normalized to the housekeeping gene *Rpl32*, which encodes ribosomal protein L32. (A) *Tph1* mRNA encodes tryptophan hydroxylase 1, the rate-limiting enzyme for mucosal 5-HT production. (B) *Chga* mRNA encodes chromogranin A, involved in secretion of 5-HT from EC cells. (C) *Maoa* mRNA encodes monoamine oxidase A, the main enzyme for 5-HT catabolism in the gut. (D) *Slc6a4* mRNA encodes the mouse homolog of SERT (serotonin transporter). Student *t* test; * indicates $P<.05$; n.s., $P>.05$.

Supplemental Fig. 4. Sex and colonization dependent differences in relative expression of *Maoa, Slc6a4,* and *Htr4* mRNA in the proximal colon
(A) *Maoa* mRNA encodes monoamine oxidase A, the main enzyme for 5-HT catabolism in the gut (14-16 mice/group; 1-way ANOVA). (B) Sex and colonization state based differences in *Maoa* expression (7-10 males/group vs 6-7 females/group; 2-way ANOVA; *, $P<.05$;) . (C) *Slc6a4* mRNA encodes the mouse homolog of SERT, serotonin transporter (14-16 mice/group; 1-way ANOVA). (D) Sex and colonization state-dependent differences in *Slc6a4* mRNA expression in CR mice (7-10 males/group vs 6-7 females/group; 2-way ANOVA; *, $P<.05$;). (E) *Htr4* mRNA encodes serotonin receptor 5-HT_4 (14-16 mice/group; 1-way ANOVA). (F) Sex and colonization state-dependent differences in *Htr4* expression (7-10 males/group vs 6-7 females/group; 2-way ANOVA; *, $P<.05$;).

Supplemental Fig. 5. Proximal colonic 5-HT concentrations in male and female mice
Tissue 5-HT concentrations from emptied segments of proximal colon in males and females from GF, HM, and CR groups (8 mice/group [4 males]; Student *t* test).

Supplemental Fig. 6. Proximal colonic enterochromaffin cell density is not significantly different in GF, HM, and CR mice
Enterochromaffin cells were identified by immunohistochemical detection of 5-HT in the colonic mucosa (red, Cy3-labeled 5-HT^+ cells; blue, nuclei counterstained with DAPI). Similar densities were observed in proximal colon sections from GF, HM, and CR mice (6-7 mice/group; 7 cryosections and 21 high-power fields/mouse [ie, 3 HPFs/section]; 1-way ANOVA). Scale bars = 100 μM. 5-HT indicates 5-hydroxytryptamine; ANOVA, analysis of variance; CR, conventionally raised; DAPI, 4',6-diamidino-2-phenylindole; GF, germ free; HM, humanized.

Supplemental Fig. 7. Intestinal lengths and cecal sizes of GF, HM, and CR mice
(A) Small-intestinal length, (B) colonic length sans cecum, and (C) cecal weight of GF, HM, and CR mice 14-21mice/group;1-way ANOVA; ***, $P<.001$; ****, $P<.0001$).