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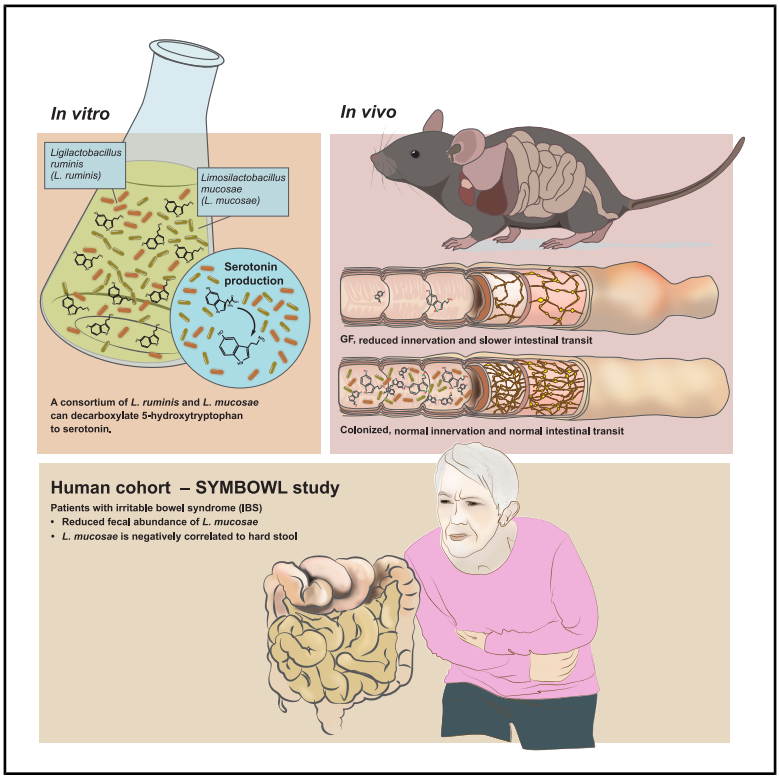
Identification of human gut bacteria that produce bioactive serotonin and promote colonic innervation

Graphical abstract



Highlights

- The gut microbiota is a source of bioactive serotonin
- *Limosilactobacillus mucosae* can decarboxylate 5-hydroxytryptophan
- Microbially produced serotonin normalizes colonic innervation

Authors

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In brief

Moretti et al. show a gut bacterial consortium (*L. mucosae* and *L. ruminis*) producing serotonin, increasing fecal serotonin and enteric neuron density and normalizing intestinal motility in mice. Reduced abundance of *L. mucosae* in patients with irritable bowel syndrome links microbial serotonin production in the gut.

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Identification of human gut bacteria that produce bioactive serotonin and promote colonic innervation

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SUMMARY

The gut microbiota regulates host intestinal serotonin synthesis, thereby promoting the development and maintenance of the enteric nervous system, which controls bowel motility. Functional bowel disorders, including irritable bowel syndrome, are associated with altered serotonin levels and gut microbiota composition. However, it is unclear if the gut microbiota can synthesize bioactive serotonin, which may affect enteric nervous system development. Here, we identify a consortium of the human gut bacteria *Limosilactobacillus mucosae* and *Ligilactobacillus ruminis* that synthesizes serotonin *in vitro* by decarboxylation of 5-hydroxytryptophan and elevates fecal serotonin levels, colonic neuronal density, and serotonin-immunoreactive neurons when introduced into germ-free, serotonin-deficient mice. The consortium normalizes intestinal transit time in germ-free wild-type mice, and we observe decreased fecal abundance of *L. mucosae* in individuals with irritable bowel syndrome. These findings suggest that specific members of the human gut microbiota synthesize bioactive serotonin that can contribute to gut health.

INTRODUCTION

Serotonin (5-hydroxytryptamine; 5-HT) is a key signaling molecule in the intestine, mediating essential gastrointestinal (GI) functions, including peristalsis, vasodilation, and visceral sensitivity.[1] Moreover, gut-derived serotonin is a trophic factor promoting the development and maintenance of the enteric nervous system (ENS),[2,3] which regulates intestinal motility and epithelial secretion.[1,4] Accordingly, aberrant serotonin signaling has been implicated in the pathogenesis of irritable bowel syndrome (IBS),[5–7] a multifactorial functional bowel disorder characterized by alterations in GI motility, secretion, and sensation.[5,8,9]

Approximately 95% of the body's serotonin pool originates from the gut, where it is synthesized, stored, and released by enterochromaffin (EC) cells.[10] Mammalian EC cells synthesize serotonin in a two-step reaction from the aromatic amino acid tryptophan (Trp). Trp hydroxylases (TPHs), belonging to the largest family of aromatic amino acid hydroxylases (AAAHs),[11] perform the first and rate-limiting step in serotonin synthesis. In mammals, TPH exists in two isoforms: TPH1, predominantly expressed in EC cells, and TPH2, expressed in the nervous system. The hydroxylation of Trp by TPH produces the serotonin precursor 5-hydroxytryptophan (5-HTP), which is further decarboxylated by aromatic L-amino acid decarboxylases (AAAD) to form serotonin. Finally, serotonin is degraded

to 5-hydroxyindoleacetic acid (5-HIAA) via monoamine oxidase enzymes (MAOs).[10]

Microbial regulation of host serotonin synthesis in the intestine was first proposed following the observation that germ-free (GF) mice have significantly lower serum serotonin levels compared to conventionally raised (CONV-R) mice.[12] Following these findings, studies have consistently shown that the gut microbiota modulates host serotonin synthesis by enhancing *Tph1* expression, both directly and indirectly via microbial metabolites such as short-chain fatty acids and secondary bile acids.[13–15] Supporting the studies in GF mice, perturbation of the gut microbiota through antibiotic administration reduces colonic *Tph1* expression and serotonin levels, inducing slower colonic motility and loss of enteric neurons.[16–18] These abnormalities are restored upon re-colonization.[18] Therefore, both the gut microbiota and serotonin play an essential role in the development and maintenance of the ENS and in the modulation of GI motility.[2,3]

Previous studies have detected serotonin in culture media of facultative anaerobic bacteria, such as *Escherichia coli* K-12,[19] *Morganella morganii*,[20] and *Staphylococcus pseudintermedius*,[21] as well as in mixed cultures of lactic acid bacteria with food-borne pathogens.[22] Recently, Sanidad et al. reported serotonin synthesis in cultures of human infant stools,[23] suggesting that serotonin may be produced by enteric bacteria. However, evidence is lacking for microbial synthesis and bioactivity of serotonin in the intestine, and human enteric bacteria producing serotonin have not been characterized. In this study, we identified human gut commensals that synthesize serotonin *in vitro* and *in vivo*, independent of host *Tph1*. Moreover, we investigated the physiologic activity of microbial serotonin on colonic innervation and intestinal motility.

RESULTS

Gut microbiota synthesizes serotonin

The gut microbiota can induce host serotonin production in the intestine.[14,15] Accordingly, adult GF mice have reduced circulating and fecal serotonin levels compared to CONV-R mice.[12,14,15] To determine if the gut microbiota directly can produce serotonin and contribute to serotonin pools independently of host serotonin production, we compared serum and fecal serotonin in 10-week-old, wild-type (*Tph1*$^{+/+}$) and *Tph1*-deficient (*Tph1*$^{-/-}$) CONV-R and GF mice. As expected, both fecal and serum serotonin levels were reduced in *Tph1*$^{-/-}$ compared to *Tph1*$^{+/+}$ CONV-R mice (Figures 1A–1C). Serotonin levels were also reduced in GF *Tph1*$^{+/+}$ mice compared to CONV-R *Tph1*$^{+/+}$ mice (Figures 1A–1C), which is consistent with previous findings.[12,14,15] Serum serotonin levels did not differ between GF and CONV-R *Tph1*$^{-/-}$ mice (Figures 1B and 1C), but we observed higher fecal serotonin concentrations in CONV-R *Tph1*$^{-/-}$ compared to GF *Tph1*$^{-/-}$ mice (Figure 1A).

To further confirm that the gut microbiota synthesizes serotonin, we conventionalized GF *Tph1*$^{-/-}$ mice (CONV-D) with cecum contents from age-matched CONV-R *Tph1*$^{+/+}$ mice and collected fecal samples after 15 days. Conventionalization significantly increased fecal serotonin in *Tph1*$^{-/-}$ CONV-D mice compared to the GF counterpart (Figure 1D) but did not affect systemic or portal serotonin levels (Figures 1E and 1F). These results indicate that the gut microbiota synthesizes serotonin and contributes to the intestinal serotonin levels.

Human gut microbiota synthesizes serotonin *in vitro*

Recent studies have indicated that *Tph1*$^{-/-}$ mice are not fully depleted of peripheral serotonin, and this phenomenon was attributed to the expression of aberrantly spliced *Tph1* transcripts, which may generate proteins with TPH catalytic activity.[24] To demonstrate that the adult human gut microbiota has the capacity to produce serotonin directly and independently of host *Tph1*, we assessed serotonin biosynthesis *in vitro* in batch cultures. Fecal microbiota from six healthy volunteers was cultured anaerobically in rich medium, and serotonin was quantified using targeted mass spectrometry in cell-free supernatants up to 48 h after inoculation. Albeit with individual variation, fecal microbiota produced serotonin in the growth medium after inoculum (time 0), reaching peak concentrations up to about 500 nM within the first 12 h (donor 1, 5, and 6; Figure 2A). The fecal microbiota from three donors (donor 2, 3, and 4) did not produce detectable levels of serotonin compared to the uninoculated medium and the baseline at time 0 (Figure 2A). Interestingly, the fecal levels of serotonin for these donors were among the lowest measured for the healthy volunteers, although the circulating levels were in the normal range (approx. 70–270 ng/mL)[25] (Table S1). No serotonin was detected in inoculum-free negative controls. Collectively, these data demonstrate that the adult human gut microbiota has the capacity to produce serotonin.

Isolation of lactobacilli strains able to synthesize serotonin

Several members of the human gut microbiota are known to catabolize Trp into a number of metabolites.[26,27] For serotonin, biosynthetic capacity has been described in Proteobacteria (e.g., *E. coli* and *M. morganii*) and lactic acid bacteria (e.g., *Enterococcus*, *Lactobacillaceae*, and *Streptococcus* species),[19,20,22] and aromatic amino acid decarboxylases have been described in *Latilactobacillus curvatus*[28] and *Levilactobacillus brevis*.[29]

To identify serotonin-producing lactobacilli from the adult human gut, we isolated bacterial strains from stools of healthy individuals using the De Man, Rogosa and Sharpe medium (MRS; Table S2), commonly employed for the isolation and cultivation of lactobacilli.[30] Several consortia were isolated, all containing at least one microbial species reported to metabolize Trp[26] but different species compositions (Table S3). To better support the growth of the different strains within the consortia and explore their potential for serotonin production, we cultured the consortia in another nutrient-rich broth (LYBHI; Table S2) and compared serotonin production with cultures of known Trp-metabolizing bacteria[26] (*Bifidobacterium pseudolongum* CCUG 34981T, *Lactobacillus acidophilus* ATCC 4356, *Lactiplantibacillus plantarum* ATCC 4381, and *E. coli* MG1655; Table S3).

Among the isolated consortia, only h1L12h and Ls produced detectable serotonin over 48 h (Figure 2B). Genomic analyses identified seven species in h1L12h and two in Ls (i.e., *Ligilactobacillus ruminis* and *Limosilactobacillus mucosae*; Table S3). Both L. *ruminis* and *L. mucosae* were also present in h1L12h,

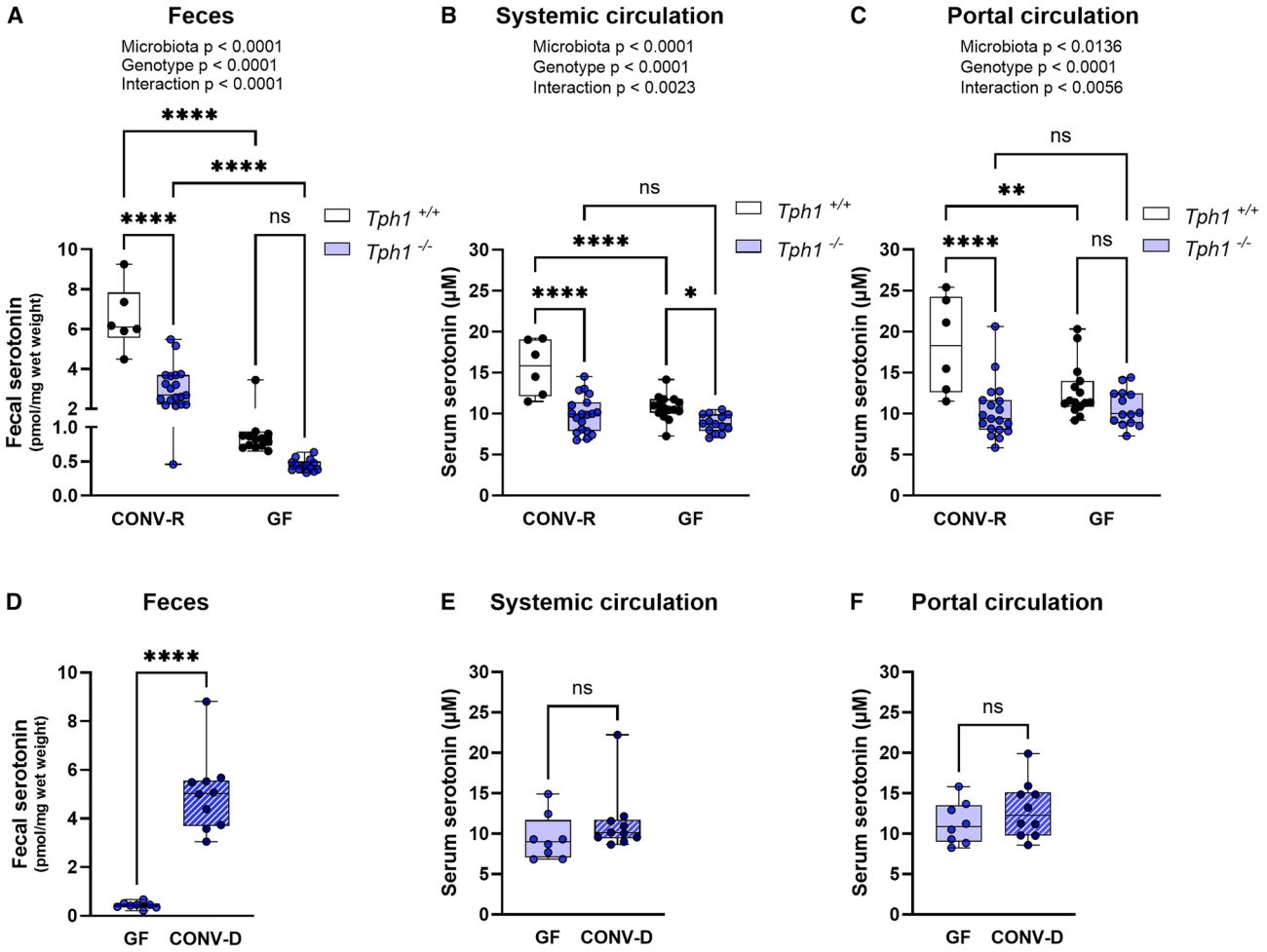

Figure 1. Gut microbiota synthesizes serotonin

(A) Serotonin levels in fecal samples of CONV-R *Tph1*$^{+/+}$ (n = 6), CONV-R *Tph1*$^{-/-}$ (n = 19), GF *Tph1*$^{+/+}$ (n = 15), and GF *Tph1*$^{-/-}$ mice (n = 14).

(B) Serotonin levels in serum samples from vena cava of the same mice as in (A).

(C) Serotonin levels in serum samples from vena porta of the same mice as in (A).

(D) Serotonin levels in fecal samples of vehicle-administered (GF; n = 8) and conventionalized (CONV-D) *Tph1*$^{-/-}$ mice (n = 10).

(E) Serotonin levels in serum samples from vena cava of the same mice as in (D).

(F) Serotonin levels in serum samples from vena porta of the same mice as in (D).

Data are presented as boxplots showing maximum, minimum, median, and interquartile range. Comparisons are by two-way ANOVA with Tukey's multiple comparison test (A–C) and two-tailed Mann-Whitney test (D–F); *p < 0.05, **p < 0.01 and ****p < 0.0001; ns, not significant.

while the other consortia lacked either one or both these species. Therefore, we concluded that *L. ruminis* and *L. mucosae* might be required for detectable serotonin production under the conditions tested.

To assess if *L. ruminis* and *L. mucosae* produced serotonin *in vitro* as monocultures, we isolated them from the Ls community as pure cultures (*L. ruminis*, WL43 and *L. mucosae*, WL68) in LYBHI. Under these experimental conditions, we did not observe detectable serotonin either in anaerobic (Figure 2C) or aerobic (Figure S1) conditions, suggesting that serotonin synthesis may require interaction between these two species. These results prompted us to test serotonin synthesis in co-cultures of the pure *L. ruminis* and *L. mucosae* strains, here referred to as reconstituted Ls. We observed that the reconstituted Ls did not yield detectable serotonin either in anaerobic (Figure 2C) or aerobic (Figure S1) conditions. This result suggests that the interactions (e.g., cross-feeding) between *L. ruminis* and *L. mucosae* may have been abrogated during isolation of the pure strains.

To identify enzymatic activities (Figure 2D) involved in serotonin production by Ls, we assessed serotonin synthesis from Trp and 5-HTP in resting cells assays. When Trp was added, neither 5-HTP nor serotonin was produced by Ls (Figure 2E), *L. mucosae* (Figure S2A), or *L. ruminis* (Figure S2B). However, we observed the production of tryptamine by Ls but not by *L. mucosae* or *L. ruminis* (Figure S3), indicating the presence of Trp decarboxylation activity in Ls cultures. In line with these results, Ls produced detectable levels of serotonin in the presence of 5-HTP (Figure 2F) and consumed 5-HTP within 24 h (Figure 2G). In

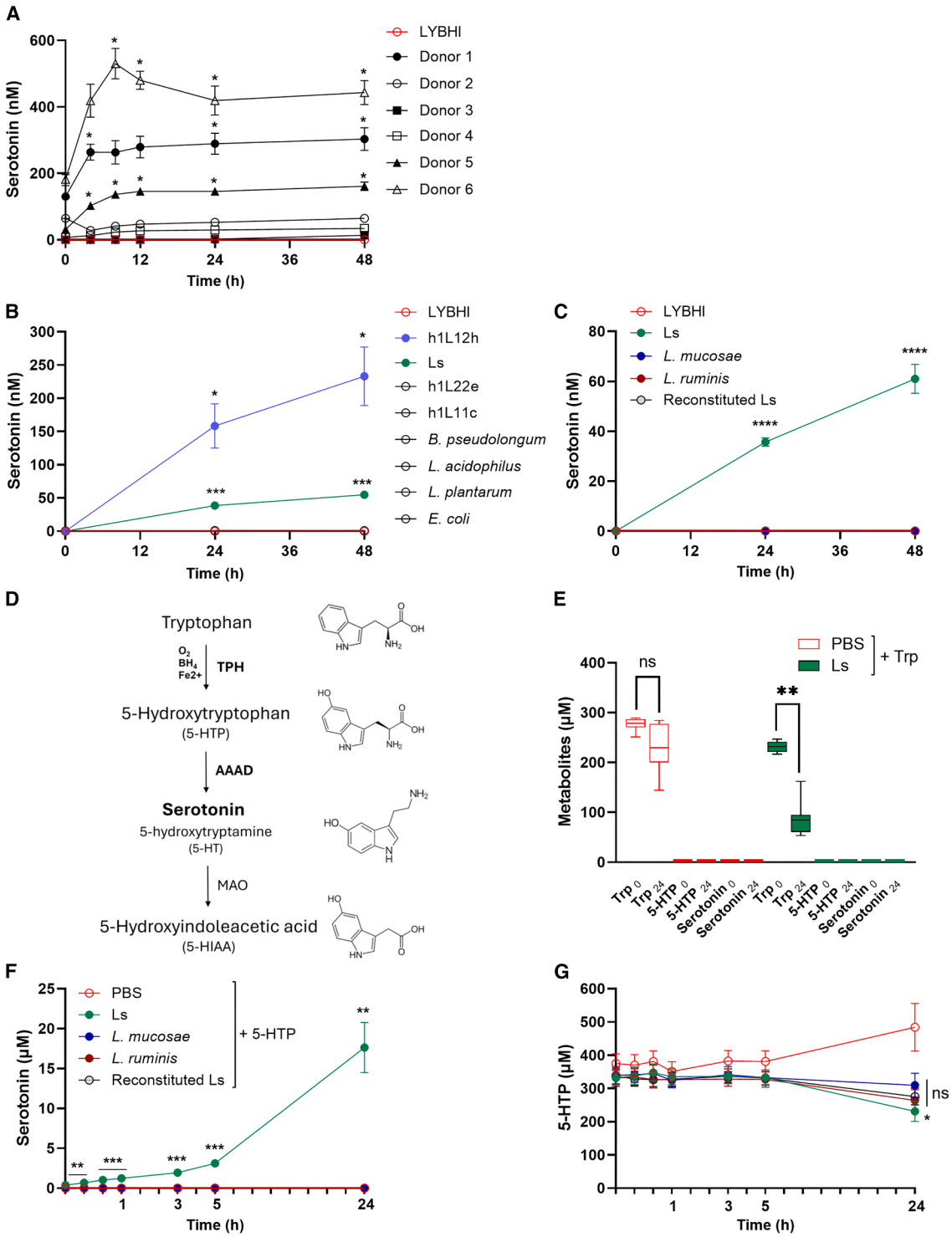

Figure 2. Human gut microbiota synthesizes serotonin *in vitro*

(A) Serotonin levels in cell-free supernatants of cultured fecal samples obtained from six healthy donors. Results are presented as mean ± SEM of 3 replicates for each donor. Differences in serotonin levels detected in uninoculated medium (LYBHI) and fecal cultures were tested by two-way ANOVA with repeated measures and Tukey's multiple comparisons test.

(B) Serotonin levels in cell-free supernatants from cultures of isolated microbial communities and Trp metabolizing bacteria in anaerobic conditions. Results are presented as mean ± SEM of 2–3 independent experiments with 3 technical replicates each. Differences in serotonin levels detected in uninoculated medium (LYBHI) and bacterial cultures were tested by two-way ANOVA with repeated measures and Tukey's multiple comparisons test.

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agreement with the results from batch cultures (Figure 2C), resting cells of the isolated *L. ruminis* and *L. mucosae* as well as reconstituted Ls did not produce serotonin from 5-HTP (Figures 2F and 2G) and did not yield tryptamine from Trp (Figure S3). Taken together, these findings demonstrate that two co-isolated human lactobacilli synthesize tryptamine and serotonin *in vitro* by decarboxylation of Trp and 5-HTP, respectively. We also observed that the decarboxylation of Trp and 5-HTP *in vitro* occurred in microbial communities (Ls) and not in monocultures.

Serotonin-producing lactobacilli strains increase enteric serotonin *in vivo*

To determine if Ls produced serotonin *in vivo*, we colonized 8-week-old GF *Tph1*$^{-/-}$ mice with Ls for 15 days. Colonization with Ls increased the fecal concentrations of tryptamine (Figure S4A) and serotonin (Figure 3A), as well as serotonin immunoreactivity within the colon tissue (Figures 3B and 3C). However, consistent with the results obtained in CONV-D mice (Figures 1E and 1F), colonization with Ls did not alter circulating serotonin levels (Figures 3D and 3E). Therefore, these data suggest that microbially produced serotonin contributes to intestinal but not circulating serotonin levels.

Next, we investigated if colonization with the isolated *L. mucosae* and *L. ruminis* or with the reconstituted Ls significantly influenced fecal serotonin levels in a *Tph1*-deficient host. In agreement with the *in vitro* results (Figures 2C–2F and S3), monocolonization with *L. ruminis* or *L. mucosae* did not increase fecal serotonin levels in GF *Tph1*$^{-/-}$ mice. However, the reconstituted Ls increased fecal serotonin *in vivo* (Figure 3F) despite no detectable production *in vitro* (Figures 2C and 2F) but did not increase fecal tryptamine (Figure S4B). Thus, our findings demonstrate that co-isolated and co-cultured *L. mucosae* and *L. ruminis* increase fecal serotonin *in vivo*.

Serotonin-producing lactobacilli strains promote enteric innervation and increase serotonin immunoreactivity within the ENS

The gut microbiota[2,18,31,32] and the serotonergic system[4,33] have essential roles in the neurogenesis and survival of enteric neurons. To investigate if colonization with serotonin-producing Ls affects colonic innervation, we stained proximal colon sections from Ls-colonized and GF *Tph1*$^{-/-}$ mice with the pan-neuronal marker Tuj1. The Tuj1-immunoreactive (IR) area was increased in the Ls group compared to GF controls (Figures 4A and 4B). Next, we sought to determine whether colonization with *L. mucosae*, *L. ruminis*, and the reconstituted Ls affects colonic innervation. Reflecting our previous results, we noted no effects by the isolated strains, while the reconstituted Ls produced an increase in colonic neuronal density (Figures 4C and 4D).

The myenteric plexus of the ENS and serotonin signaling are major regulators of intestinal motility.[34] Therefore, we determined whether colonization with Ls, *L. mucosae*, *L. ruminis*, or the reconstituted Ls modulates serotonin immunoreactivity within the myenteric plexus of the ENS. We specifically isolated the colonic myenteric plexus and stained myenteric neurons as whole-mount preparation to visualize the serotonin IR area within this specific set of neurons. We detected increased serotonin immunoreactivity within the isolated myenteric plexus of Ls-administered *Tph1*$^{-/-}$ mice compared to the vehicle-treated group (Figures 5A and 5B). Similarly, the reconstituted Ls elevated serotonin IR area within the myenteric plexus, while the isolated strains did not produce changes (Figures 5C and 5D). These findings indicate that not only a complex microbiota[3] but also specific serotonin-producing lactobacilli promote enteric innervation and increase serotonin levels within the ENS, potentially regulating intestinal motility.

Serotonin-producing lactobacilli strains normalize intestinal transit time in colonized GF mice

Next, we determined if colonization with Ls affected intestinal transit time in GF mice. As previously shown,[3,35] wild-type GF mice had reduced intestinal transit rate (i.e., slower transit) compared to wild-type CONV-R mice (Figure 6A). Colonization of wild-type GF mice with Ls normalized intestinal transit time to the levels of wild-type CONV-R mice (Figure 6A). Compared to GF mice, colonization with Ls significantly increased fecal serotonin levels (Figure 6B), which positively correlated with intestinal transit rate (Figure 6C). This correlation remained significant in females (Figure 6D) but not in male mice (Figure 6E) when analyzed separately, suggesting potential sex-specific effects. Importantly, colonization with Ls did not increase *Tph1* expression in proximal colon tissue in either the female (Figure 6F) or male group (Figure 6G), implicating Ls as a source of serotonin. Furthermore, colonic *Mao* expression was not decreased after colonization by Ls (Figure S5), indicating that the increase in fecal

(C) Serotonin levels in cell-free supernatants of lactobacilli strains (Ls), *L. ruminis* WL43, *L. mucosae* WL68, and reconstituted Ls anaerobic cultures. Results are presented as mean ± SEM of 3 independent experiments with 3 technical replicates each. Differences in serotonin levels between uninoculated medium (LYBHI) and bacterial cultures were tested by two-way ANOVA with repeated measures and Tukey's multiple comparisons test.
(D) Schematic view of serotonin synthesis and enzymatic activities potentially involved in the pathway investigated in resting cells experiments. TPH (tryptophan hydroxylase); AAAD (aromatic amino acid decarboxylase); MAO (monoamine oxidase).
(E) Tryptophan, 5-HTP and serotonin concentrations in control supernatants (PBS) and cell-free supernatants of Ls resting cells at the time of Trp addition (t_0) and after 24 h (t_{24}), in anaerobic conditions. Data are presented as boxplots showing maximum, minimum, median, and interquartile ranges of 3 independent experiments with 3 technical replicates each. Comparisons between t_0 and t_{24} are by Wilcoxon matched pairs signed rank test.
(F) Serotonin levels in cell-free supernatants of lactobacilli strains (Ls), *L. ruminis* WL43, *L. mucosae* WL68, and reconstituted Ls resting cells in the presence of 5-HTP in anaerobic conditions. Results are presented as mean ± SEM from 3 independent experiments with 3 technical replicates each. Differences in serotonin levels between uninoculated buffer (PBS) and bacterial cultures were tested by two-way ANOVA with repeated measures and Tukey's multiple comparisons test.
(G) 5-HTP levels in cell-free supernatants of lactobacilli strains (Ls), *L. ruminis* WL43, *L. mucosae* WL68, and reconstituted Ls resting cells. Results are presented as mean ± SEM of 3 independent experiments with 3 technical replicates each. Differences in serotonin levels between uninoculated buffer (PBS) and bacterial cultures were tested by two-way ANOVA with repeated measures and Tukey's multiple comparisons test. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ and ****$p < 0.0001$; ns, not significant.

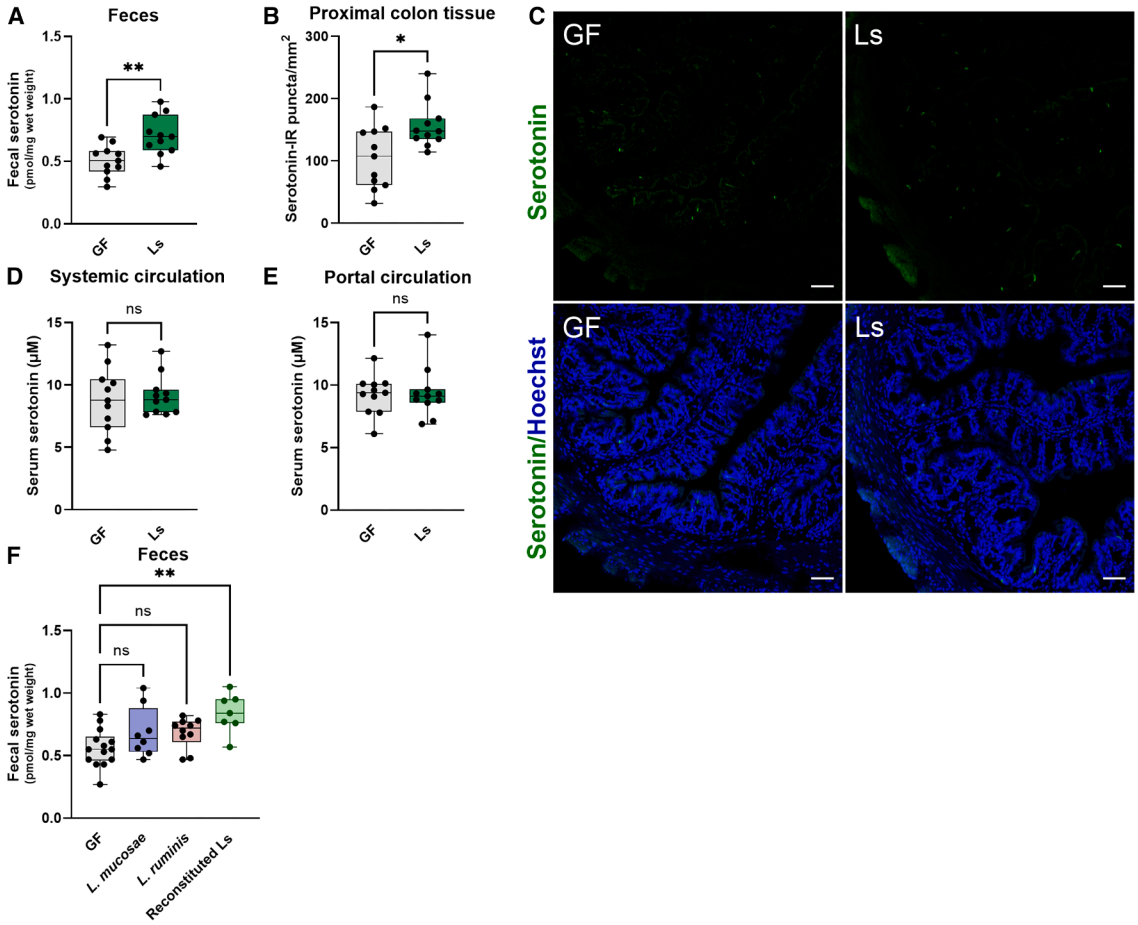

Figure 3. Serotonin-producing lactobacilli strains increase enteric serotonin *in vivo*

(A) Serotonin levels in fecal samples of GF *Tph1*$^{-/-}$ mice orally administered with vehicle (GF; $n = 11$) or lactobacilli strains (Ls; $n = 11$).

(B) Quantification of serotonin-immunoreactive (IR) puncta (green) per mm^2 of the total tissue area stained with the nuclear marker Hoechst (blue) in proximal colon sections of the mice in (A). For each mouse, 3 sections were imaged, and 2–4 pictures per section were taken.

(C) Representative images of stained colonic serotonin relative to (B). Scale bars, 50 μm.

(D) Serotonin levels detected in serum samples from vena cava of the same mice as in (A)–(C).

(E) Serotonin levels detected in serum samples from vena porta of the same mice in (A)–(D).

(F) Serotonin levels in fecal samples of GF *Tph1*$^{-/-}$ mice orally administered with vehicle (GF; $n = 14$), *L. mucosae* WL68 ($n = 8$), *L. ruminis* WL43 ($n = 10$), or reconstituted Ls ($n = 7$).

Data are presented as boxplots showing maximum, minimum, median, and interquartile range. Differences between GF and colonized mice are analyzed by two-tailed Mann-Whitney test (A)–(B) and (D)–(E) or Kruskal-Wallis test with Dunn's multiple comparisons test (F). *$p < 0.05$; **$p < 0.01$; ns, not significant.

serotonin was not due to a decreased host catabolism.[23] Our results suggest that Ls modulates intestinal transit time in GF mice independently of host gene expression of *Tph1* and *Mao*, which code for the main enzymes for host serotonin synthesis and catabolism, respectively.

Reduced abundance of serotonin-producing lactobacilli in patients with IBS

IBS has been associated with alterations in the gut microbiota, serotonin signaling, and intestinal transit time.[5–8,36] Since our results suggest that the gut microbiota contributes to enteric serotonin levels and modulation of intestinal transit in mice, we determined the fecal and circulating levels of serotonin, total oro-anal transit time, and stool form and consistency according to the Bristol Stool Form (BSF) scale and fecal abundances of the serotonin-producing lactobacilli *L. mucosae* and *L. ruminis* in 147 individuals with IBS recruited within the SYMBOWL (Symptom Generation in Functional Bowel Disorders) study and in 27 healthy controls (Table S4).

There were no significant differences in fecal (Figures S6A and S6B) and circulating (Figures S6C and S6D) serotonin levels in individuals with IBS compared to controls, also when considering IBS subtypes. Furthermore, fecal serotonin only weakly correlated with the total oro-anal transit time (in IBS patients only: Spearman rho 0.165, $p = 0.046$; with a trend in the full cohort: Spearman rho 0.147, $p = 0.053$). However, the fecal abundance of *L. mucosae* (Figures 7A and 7B), but not of *L. ruminis* (Figures 7C and 7D), was significantly lower in IBS patients

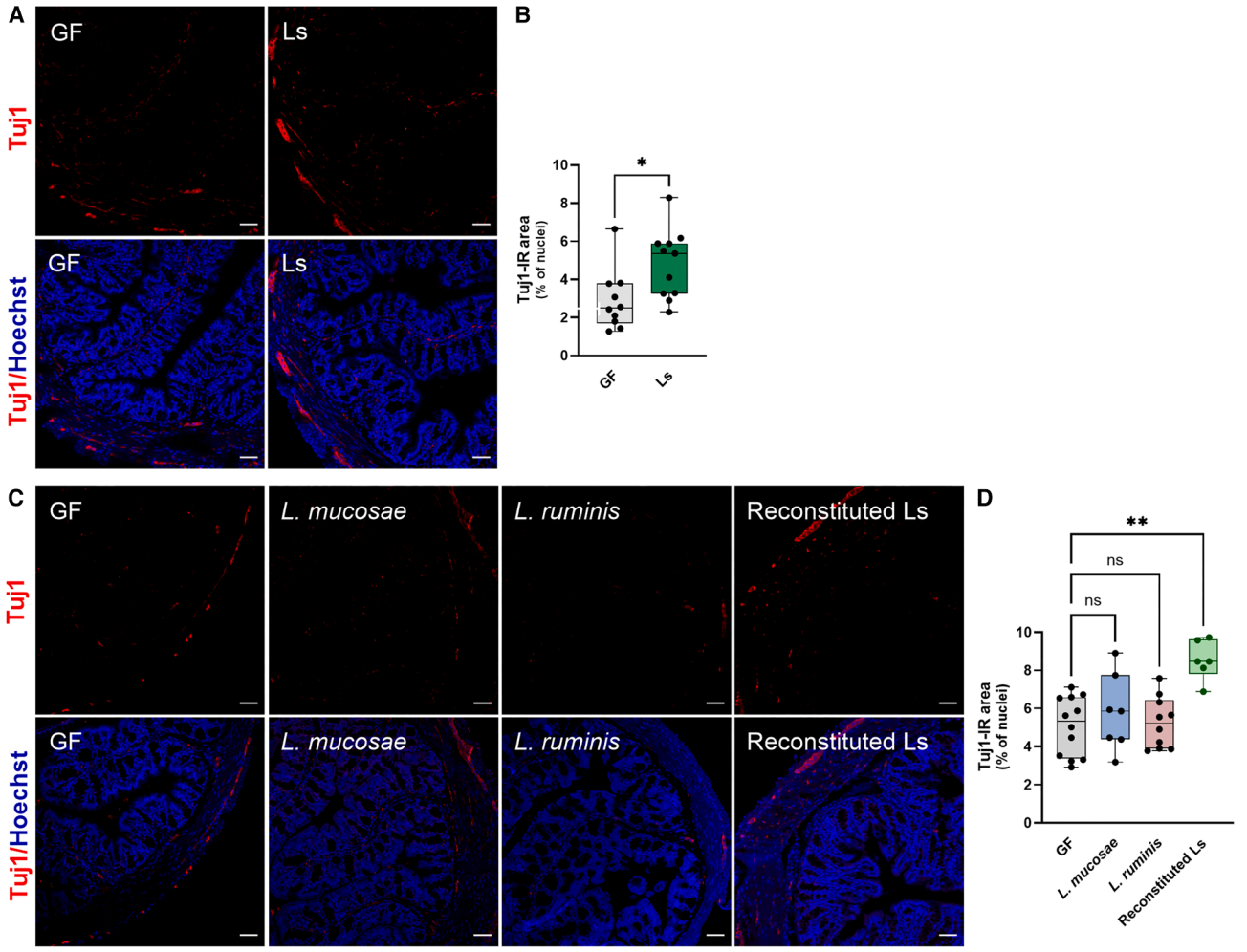

Figure 4. Serotonin-producing lactobacilli strains promote enteric innervation

(A) Representative images of Tuj1-immunoreactive (IR) neurons (red) in proximal colon sections of GF *Tph1*$^{-/-}$ mice orally administered with vehicle (GF; $n = 10$) or lactobacilli strains (Ls; $n = 11$). The total tissue area is highlighted by the nuclear staining Hoechst (blue). Scale bars, 50 μm.

(B) Quantification of colonic Tuj1-IR area (red, A). Values are expressed as percentage of the total, Hoechst-stained tissue area (blue, A).

(C) Representative images of Tuj1-IR neurons (red) in proximal colon sections of GF *Tph1*$^{-/-}$ mice orally administered with vehicle (GF; $n = 12$), *L. mucosae* WL68 ($n = 7$), *L. ruminis* WL43 ($n = 10$), or reconstituted Ls ($n = 6$). The total tissue area is highlighted by the nuclear staining Hoechst (blue). Scale bars, 50 μm.

(D) Quantification of colonic Tuj1-IR area (red, C). Values are expressed as percentage of the total, Hoechst-stained tissue area (blue, C).

Data are presented as boxplots showing maximum, minimum, median, and interquartile range. Differences are analyzed by two-tailed Mann-Whitney test; *$p < 0.05$; ns, not significant.

compared to healthy controls, also when considering IBS subtypes. Importantly, only the abundance of *L. mucosae* correlated negatively with hard stools (%BSF 1–2 of all stools) both in individuals with IBS (Spearman rho −0.226, FDR = 0.044) and in the total cohort (Spearman rho −0.239, FDR = 0.016).

Next, we investigated the expression of genes encoding for Trp or aromatic amino acid decarboxylases in *L. mucosae* and *L. ruminis*. Transcriptomic analysis of resting cells from the Ls consortium and reconstituted Ls exposed to 5-HTP showed expression of a gene fragment annotated as a pyridoxal-dependent decarboxylase uniquely in Ls (Figure S7A). Further sequence analysis allowed to reconstruct the full gene, which was found in the metagenome-assembled genome (MAG) belonging to *L. mucosae* (Figure S7A). To confirm its function, we cloned and expressed the gene and observed enzymatic activity yielding tryptamine from Trp (Figure S7B). Thus, our findings show that within the Ls consortium, *L. mucosae* encodes a Trp decarboxylase possibly involved in the production of serotonin from 5-HTP.

DISCUSSION

In this study, we show that members of the human gut microbiota produce serotonin in anaerobic cultures and *in vivo*. Furthermore, we show that lactobacilli strains from the human gut microbiota synthesize serotonin *in vitro* by decarboxylation of the precursor 5-HTP. We demonstrate that colonization with these lactobacilli strains increases enteric serotonin levels and

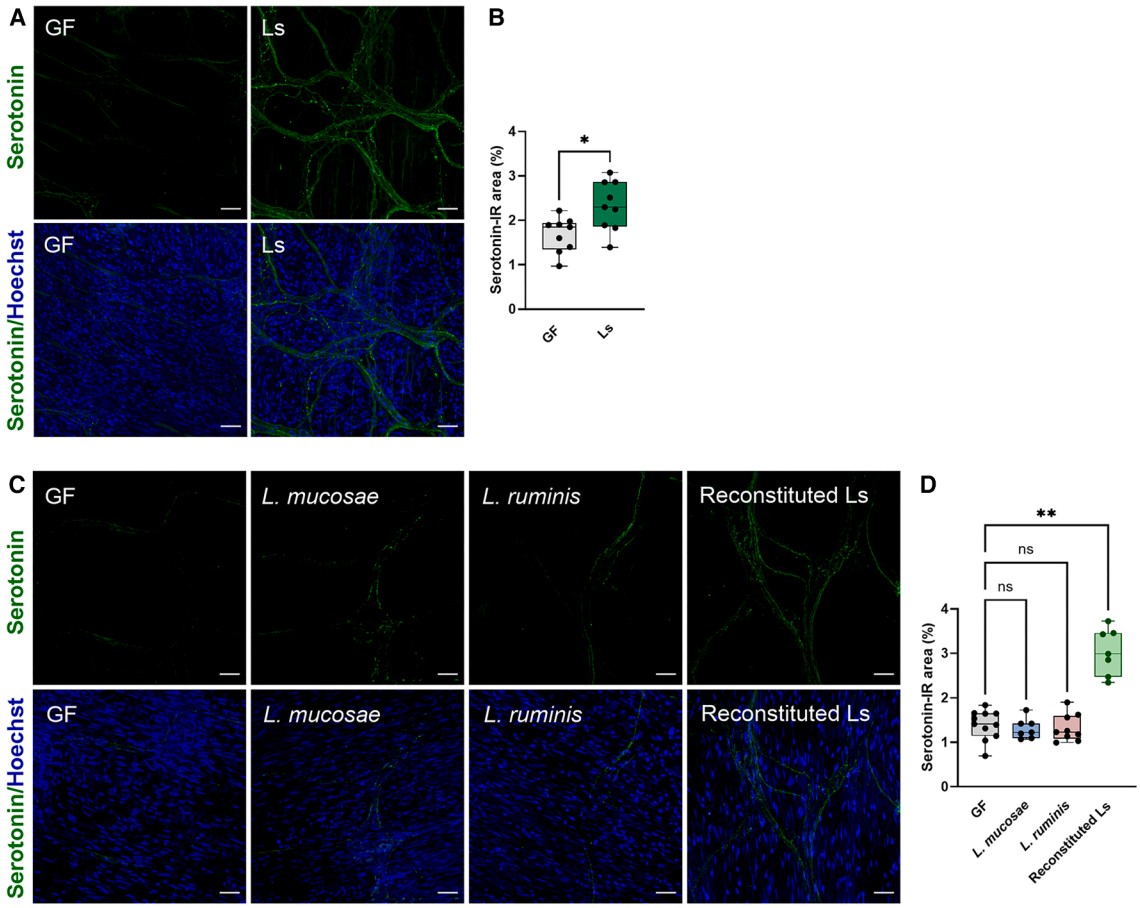

Figure 5. Serotonin-producing lactobacilli strains increase serotonin immunoreactivity within the enteric nervous system

(A) Representative images of serotonin-IR myenteric neurons (green) and Hoechst nuclear staining (blue) in the proximal colon of GF *Tph1*$^{-/-}$ mice orally administered with vehicle (GF; $n = 9$) or colonized with lactobacilli strains (Ls; $n = 9$). Scale bars, 50 μm.

(B) Quantification of the serotonin-IR area (green, A) in whole-mount preparations of the colonic myenteric plexus. Values are expressed as percentage of the entire tissue area that occupies the entire imaged field, as shown by Hoechst nuclear staining (blue, A).

(C) Representative images of serotonin-IR myenteric neurons (green) and Hoechst nuclear staining (blue) in the proximal colon of GF *Tph1*$^{-/-}$ mice orally administered with vehicle (GF; $n = 11$) or colonized with *L. mucosae* WL68 ($n = 7$), *L. ruminis* WL43 ($n = 9$), or reconstituted Ls ($n = 7$). Scale bars, 50 μm.

(D) Quantification of the serotonin-IR area (green, panel C) in whole-mount preparations of the colonic myenteric plexus. Values are expressed as percentage of the entire tissue area that occupies the entire imaged field, as shown by Hoechst nuclear staining (blue, C).

Data are presented as boxplots showing maximum, minimum, median, and interquartile range. Differences are analyzed by two-tailed Mann-Whitney test; *$p < 0.05$; ns, not significant.

promotes colonic innervation in GF *Tph1*-deficient mice, lacking peripheral endogenous serotonin production. Moreover, we show that the identified lactobacilli strains normalize intestinal transit time in GF mice in a *Tph1*-independent manner. Finally, we report decreased abundance of *L. mucosae* encoding a Trp and 5-hydroxytryptophan decarboxylase in feces from patients with IBS.

Previous studies have shown microbial regulation of host *Tph1* expression, which increases serum levels of serotonin following colonization of GF mice.[14,15] In this study, we used *Tph1*$^{-/-}$ mice lacking endogenous peripheral serotonin synthesis, to disentangle host and microbe contribution to serotonin production. Consistent with previous studies,[12,14,15] circulating serotonin levels were higher in CONV-R compared to GF wild-type mice. However, we did not observe a statistically significant difference in serum serotonin between CONV-R and GF *Tph1*$^{-/-}$ mice, possibly explained by the expression of aberrantly spliced *Tph1* transcripts, as previously shown,[24] or since Trp is more abundant under GF conditions,[12,37] other host AAAH may hydroxylate Trp to generate 5-HTP that is subsequently decarboxylated to serotonin.

Previous studies have reported serotonin synthesis *in vitro* by facultative anaerobic bacteria.[19,20,22] However, since the aim of these studies was to investigate biogenic amine production in fermented foods, the reported conditions did not mimic the conditions of the colon.[20,22] Later, Luqman et al. showed serotonin synthesis by *S. pseudintermedius* both during growth and in resting cells supplemented with 5-HTP. The staphylococcal AAAD SadA identified by Luqman and colleagues was found to decarboxylate Trp, phenylalanine, tyrosine, L-DOPA, and

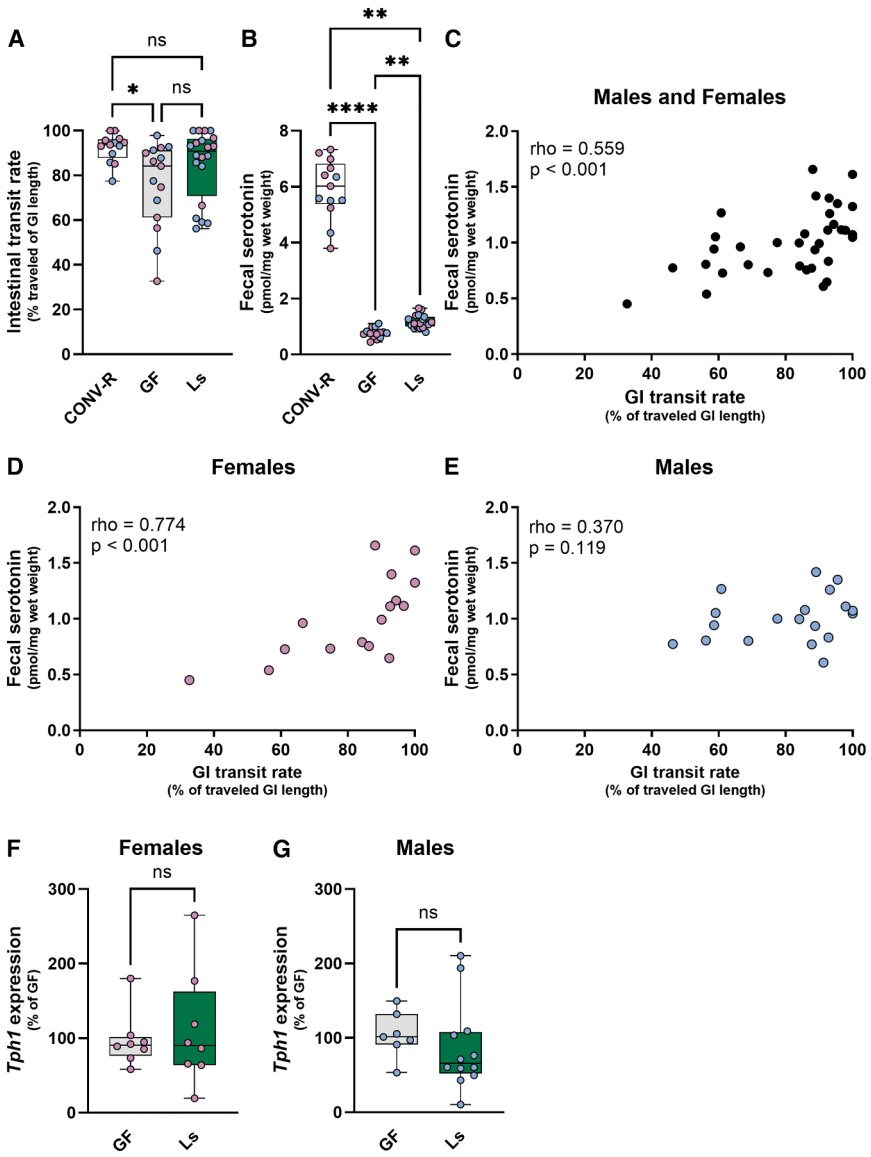

Figure 6. Serotonin-producing lactobacilli strains normalize intestinal transit time in colonized germ-free mice

(A) Intestinal transit rate of female (pink) and male (blue) wild-type conventionally raised (CONV-R; n = 13) and germ-free mice orally administered with vehicle (GF; n = 15) or colonized with lactobacilli strains (Ls; n = 20). Intestinal transit was assessed 5 h after an oral gavage with methylcellulose-containing Evans blue dye.

(B) Fecal serotonin levels for the mice in (A).

(C) Spearman correlations between fecal serotonin and intestinal transit rate for the GF and Ls groups in (A) and (B).

(D) Spearman correlations between fecal serotonin and intestinal transit rate for female mice administered with vehicle (GF; n = 8) or colonized with lactobacillus strains (Ls; n = 8) indicated in pink in (A) and (B).

(E) Spearman correlations between fecal serotonin and intestinal transit rate for male mice administered with vehicle (GF; n = 7) or colonized with lactobacillus species (Ls; n = 12) indicated in blue in (A) and (B).

(F and G) Colonic *Tph1* expression for the GF and Ls female (F) and male (G) groups.

Data in (A) and (B) and (F) and (G) are presented as boxplots showing maximum, minimum, median, and interquartile range, and differences between groups are analyzed by Kruskal-Wallis test with Dunn's multiple comparisons test (A and B) or two-tailed Mann-Whitney test (F and G); $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{****}p < 0.0001$; ns, not significant.

5-HTP *in vitro*, demonstrating non-substrate-specific activity.[21] Although *S. pseudintermedius* is not considered as a member of the human gut microbiota,[38] this study was the first to describe a microbial AAAD that directly synthesizes serotonin from 5-HTP.[21] Finally, recent research reported *in vitro* serotonin synthesis by bacteria isolated from infant stools.[23] Together, these findings suggested that some bacteria can produce serotonin by amino acid decarboxylation *in vitro*. However, it is unclear if human bacteria from the adult gut microbiota can decarboxylate 5-HTP to serotonin. Similarly, evidence of microbial serotonin synthesis in the host is lacking. Our study shows that gut bacteria from the adult human gut produce serotonin both *in vitro* and *in vivo*. Specifically, our *in vitro* findings demonstrate that the co-isolated Ls produce serotonin by decarboxylation of 5-HTP but do not hydroxylate Trp. Hydroxylation reactions require or are greatly favored by oxygen.[11] Accordingly, microbial AAAH genes have been reported in aerobic and facultative anaerobic non-gut bacteria[39–42] but are yet to be described in enteric bacteria. Thus, our results do not exclude that enzymatic Trp hydroxylation may take place also in the gut, potentially close to the mucosa where low levels of oxygen are present.[43] In addition, as 5-HTP biosynthesis can occur by non-enzymatic redox reactions,[44] we speculate that 5-HTP might also originate from reactions with free hydroxyl groups in the gut.

Our *in vitro* results suggest that microbial serotonin synthesis may require two or more bacteria and microbe-microbe interactions. Moreover, serotonin synthesis by Ls could not be reproduced by monocultures or co-cultures of isolated *L. ruminis* and *L. mucosae* strains *in vitro*. However, our experiments *in vivo* revealed that not only Ls but also co-cultured *L. mucosae* and *L. ruminis*, referred as to reconstituted Ls, increase fecal serotonin in the *Tph1*-deficient host. These results suggest that microbial serotonin synthesis might be favored by interactions within microbial communities and/or by gut environmental conditions. The latter may encompass nutrient availability, intestinal pH, oxygen levels, enzyme cofactors, and substrates, including Trp and diet- or host-derived 5-HTP. Collectively, these factors affect microbial communities and their metabolism, which, in

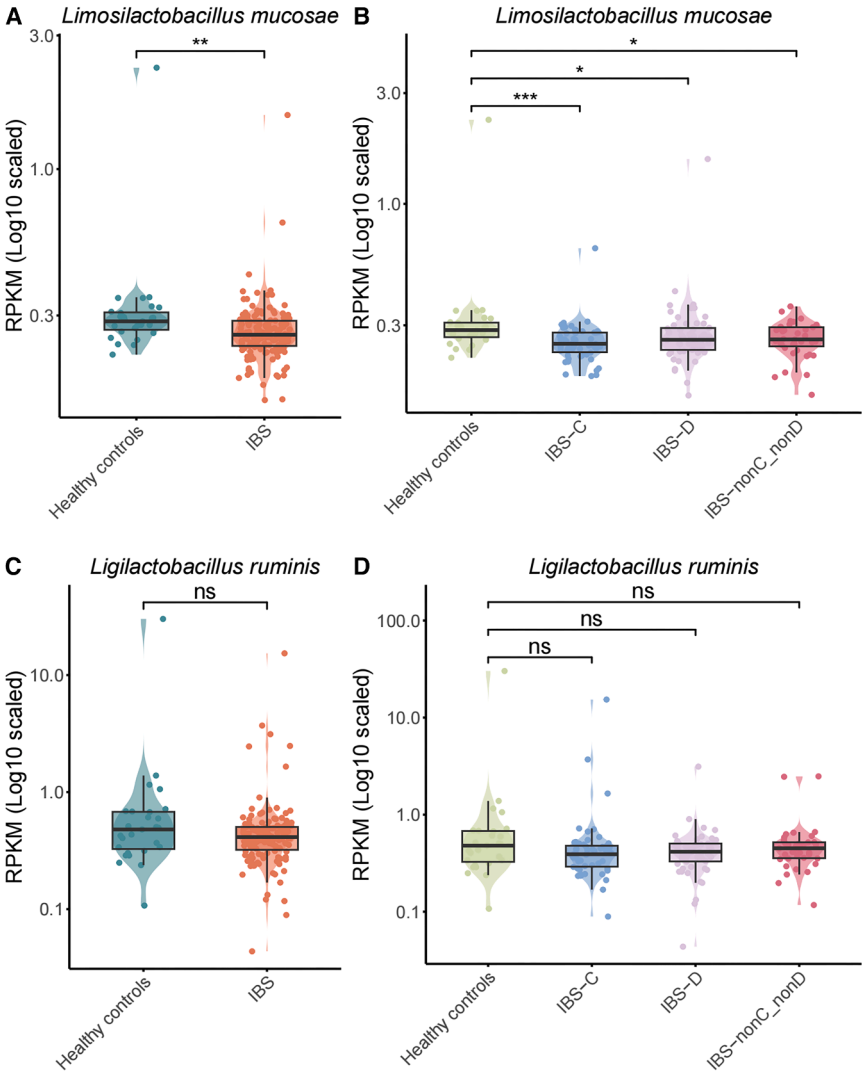

Figure 7. Fecal abundances of *L. mucosae* and *L. ruminis* in healthy individuals and patients with IBS

(A and B) Fecal abundance of *L. mucosae* in IBS patients (A) and IBS subtypes (B) compared to healthy individuals.

(C and D) Fecal abundance of *L. ruminis* in IBS patients (C) and IBS subtypes (D) compared to healthy individuals. Healthy controls, $n = 27$; IBS patients, $n = 147$. IBS-C, predominant constipation, $n = 54$; IBS-D, predominant diarrhea, $n = 54$; IBS-nonC_nonD, unspecified or mixed-type bowel habits, $n = 39$. RPKM, reads per kilobase per million mapped reads.

Differences between groups were tested by two-tailed Mann-Whitney test. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ns, not significant.

turn, affect gut environment and physiology.[45] Future studies are required to determine which specific conditions affect microbial serotonin synthesis in the intestine and if such conditions are altered in disorders of the GI tract, contributing to serotonergic dysregulation.

Notably, this study consistently shows that gut bacteria play a greater role in influencing fecal rather than circulating serotonin levels. Under physiological conditions, most EC cell-derived serotonin is secreted basally and enters the circulation. However, some serotonin is also released apically, into the gut lumen[46] and detected in feces.[47] Therefore, our study using *Tph1*$^{-/-}$ mice suggests that circulating serotonin might mainly reflect host-derived serotonin while microbially synthesized serotonin contributes to the luminal pool, which primarily regulates local gut functions.[47,48]

One of the most well-studied functions of gut-derived serotonin is the regulation of intestinal motility through its actions on the ENS and specifically on the myenteric plexus, which largely controls bowel movements.[34] We and others have demonstrated that GF mice have reduced neuronal density within the ENS and slower intestinal transit time.[2,3,17,18] Moreover, we have previously shown that the gut microbiota promotes ENS development via serotonergic signaling, thereby normalizing intestinal transit time in GF mice.[3] Here, we extend these findings to demonstrate that serotonin-producing Ls promote colonic innervation, increase serotonin immunoreactivity within the myenteric plexus, and accelerate intestinal transit time in GF mice. Importantly, we observed a correlation between fecal serotonin and intestinal transit time.

While fecal and circulating serotonin levels did not differ significantly between individuals with IBS and healthy controls, the reduced abundance of *L. mucosae* in IBS suggests a microbiota-related mechanism that may influence local serotonin biosynthesis or signaling. In addition, the negative association in humans between the fecal abundance of *L. mucosae* and the proportion of hard stools according to BSF, a commonly used surrogate marker of colonic transit time,[49] further supports the relevance for ENS function and intestinal motility. This is further supported by the observation that *L. mucosae* encoded a Trp and 5-HTP decarboxylase, which may be relevant for intestinal motility and function.

Limitations of the study

Our study has some limitations that warrant consideration. First, although we provide evidence implicating specific microbial taxa and their genes in host serotonin signaling, the regulatory mechanisms responsible for serotonin production remain undefined. Second, while decreased levels of *L. mucosae* were observed, we did not detect significant differences in fecal or serum serotonin levels between IBS patients and healthy controls, nor did we find strong correlations between *L. mucosae* abundance and serotonin concentrations. These discrepancies may be

partly explained by the modest cohort size, as well as by the complexity of serotonin dynamics, which involve not only production but also rapid uptake and metabolism. Thus, whether the observed reduction of *L. mucosae* in IBS reflects disrupted local serotonin homeostasis in the gut, in the absence of systemic changes, require further experiments. Accordingly, future studies with larger cohorts, improved functional assays, and pathway-level resolution will be required to address this question more definitively.

In conclusion, we identified human co-isolated *L. mucosae and L. ruminis* strains that synthesize serotonin *in vitro* and modulate enteric serotonin levels, enteric innervation, and intestinal transit time *in vivo*. Future research may investigate whether serotonin-producing bacteria could promote physiologic serotonin levels in patients suffering from dysfunctional intestinal motility.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to the lead contact, Fredrik Bäckhed (fredrik@wlab.gu.se).

Materials availability

This study did not generate new reagents.

Data and code availability

- The metagenomic sequencing data reported in this paper have been deposited in the European Nucleotide Archive (ENA) under accession number PRJEB96399. Demographic and clinical data for the SYMBOWL cohort are available upon request from M.S. (magnus.simren@medicine.gu.se), pending institutional and ethical approvals.
- This paper does not report original code; all software and bioinformatic packages used in this study are publicly available and listed in the STAR Methods section with version numbers and references.
- Additional information required to reanalyze the data in this paper are available upon request by the lead contact.

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AUTHOR CONTRIBUTIONS

Conceptualization, C.H.M., E.G., M.T.K., V.T., and F.B. Methodology, C.H.M., P.-O.B., J.-H.O., M.T.K., M.H., J.Z., G.Y., M.K., and V.T. Investigation, C.H.M., E.G., P.-O.B., J.-H.O., A.L., P.P., M.M., D.M., K.K., and N.K. Clinical investigation, T.v.G. and M.S. Formal analysis, C.H.M., J.Z., G.Y., P.-O.B., L.M.O., C.D., and V.T. Visualization, C.H.M. Resources, A.J.M., T.S., G.G., J.-P.v.P., S.R., and F.B. Writing – original draft, C.H.M., L.E.O., and F.B. Writing – reviewing & editing, C.H.M., L.E.O., V.T., and F.B. Supervision, F.B. and V.T. Funding acquisitions, F.B. All authors provided comments to the manuscript.

DECLARATION OF INTERESTS

M.T.K., G.G., and S.R. are employed or in part employed by BioGaia AB. L.M.O., M.T.K., and V.T. are co-founders and shareholders of Roxbiosens Inc. J.-P.v.P. receives research funding from BioGaia AB and is the founder and owner of Next-Gen Probiotics, LLC, a consulting company. F.B. receives research funding from BioGaia AB and Novo Nordisk A/S and is the co-founder and a shareholder of Roxbiosens Inc and Implexion AB. M.S. receives research funding from BioGaia AB and has been a consultant/advisory board member for Biocodex, Tillotts, BioGaia, Renapharma, and AlfaSigma and on the speaker's bureau for Tillotts, Takeda, Biocodex, Sanofi, Abbvie, Janssen Immunology, Pfizer, BioGaia, Renapharma, Mayoly, and Bromatech. However, these possible conflicts of interest have no direct relations to the content of the current paper. A patent application based on the findings has been submitted.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

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SUPPLEMENTAL INFORMATION

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Rat anti-serotonin	Abcam	Cat# ab6336; RRID: AB_449517
Rabbit anti-Tuj1	Abcam	Cat# ab18207; RRID: AB_444319
Donkey anti-rat 488	Life technologies	Cat# A21208; RRID: AB_141709
Donkey anti-rabbit 568	Life technologies	Cat# A10042; RIDD: AB_2534017
Bacterial and virus strains		
Lactobacilli strains (Ls)	This study	DSM 3361
Ligilactobacillus ruminis WL43	This study	N/A
Limosilactobacillus mucosae WL68	This study	N/A
h1L22e	This study	N/A
h1L12h	This study	N/A
h1L11c	This study	N/A
Bifidobacterium pseudolongum	Culture Collection University of Gothenburg (CCUG)	CCUG 34981T
Lactobacillus acidophilus	ATCC	ATCC 4356
Lactiplantibacillus plantarum	ATCC	ATCC 4381
Escherichia coli	ATCC	MG1655
Chemicals, peptides, and recombinant proteins		
Brain and Heart Infusion	Oxoid Fisher-Scientific	Cat# CM1135
Maltose	Sigma-Aldrich	Cat# 63418; CAS: 69-79-4
Cellobiose	Sigma-Aldrich	Cat# 22150; CAS: 529-67-9
Cysteine	Sigma-Aldrich	Cat# W326305; CAS: 52-90-4
Hemin	Sigma-Aldrich	Cat# 51280; CAS: 1406-30-6
Yeast Extract	Oxoid Fisher-Scientific	Cat# LP0021B
MRS	Sigma-Aldrich	Cat# 69966
Tween 80	Sigma-Aldrich	Cat# P8074; CAS: 9005-65-6
L-tryptophan	Sigma-Aldrich	Cat# T0254; CAS: 73-22-3
5-Hydroxytryptamine	Sigma-Aldrich	Cat# 14927; CAS: 50-67-9
Serotonin-α,α,β,β-d4 hydrochloride	Sigma-Aldrich	Cat# 747521; CAS: 2469263-61-2
5-Hydroxy-L-tryptophan-4,6,7-d_3	LGC Standards	Cat# QX158816 CAS: 1276197-29-5
L-Tryptophan-(*indole*-d_5)	Sigma-Aldrich	615862; CAS: 62595-11-3
Methanol	Sigma-Aldrich	Cat# 1.06035; CAS: 67-56-1
Acetic acid	Sigma-Aldrich	Cat# 695092, CAS: 64-19-7
Hydrochloric acid	Sigma-Aldrich	Cat# 258148; CAS: 7647-01-0
PFA	Histolab	Cat# HL96753
Triton X-100	Sigma-Aldrich	Cat# T8787; CAS: 9036-19-5
BSA	Sigma-Aldrich	Cat# A6003; CAS: 9048-46-8
Sodium Azide	Sigma-Aldrich	Cat# 71289; CAS: 26628-22-8
Hoechst 33342	Thermo Fisher Scientific	Cat# H3570; CAS: 23491-52-3
Donkey serum	Sigma-Aldrich	Cat# D9663
PBS	Cytiva	Cat# SH30028.02
Histolab Clear	Histolab	Cat# 14250
Ethanol 99.5%	KiiltoClean AB	Cat# 101100

(*Continued on next page*)

Continued		
REAGENT or RESOURCE	SOURCE	IDENTIFIER
Fluorescence mounting medium	Dako	Cat# S3023
Evans Blue	Sigma-Aldrich	Cat# E2129; CAS: 314-13-6
iQTM SYBR® Green supermix	BioRad	Cat# 1708887
Critical commercial assays		
Nucleospin® Soil kit	Techtum Lab AB	740780.50
HotStarTaq Master Mix 2× kit	QIAGEN	203645
NucleoSpin Gel and PCR Clean-up kit	Macherey-Nagel	740609.25
Serotonin ELISA kit	Enzo Life Sciences	ADI-900-175
RNeasy mini kit	QIAGEN	RNeasy mini kit
HighCapacity Reverse Transcription kit	Applied Biosystems	43-688-14
Datasets		
Metagenomic data	SYMBOWL	PRJEB96399.
Experimental models: Organisms/strains		
Conventionally raised $Tph1^{tm1Kry}/Tph1^{tm1Kry}$	Previous paper[50]	RRID:MGI:3837408
Germ-free $Tph1^{tm1Kry}/Tph1^{tm1Kry}$	Previous paper[50]	RRID:MGI:3837408
Germ-free C57BL/6J mice	Charles River	N/A
Oligonucleotides		
Primer: *Tph1* (*Tryptophan Hydroxylase 1*) Forward: AACAAAGACCATTCCTCCGAAAG Reverse: TGTAACAGGCTCACATGATTCTC	IDT	N/A
Primer: *Mao* (*Monoamine Oxidase*) Forward: GCCCAGTATCACAGGCCAC Reverse: GTCCCACATAAGCTCCACCA	IDT	N/A
Primer: *Rpl32* (*Ribosomal Protein L32*) Forward: CCTCTGGTGAAGCCCAAGATC Reverse: TCTGGGTTTCCGCCAGTTT	IDT	N/A
Software and algorithms		
Black Zen microscope software	ZEISS	https://www.micro-shop.zeiss.com/en/us/softwarefinder/software-categories/zen-black/
ImageJ (v. 2.0.0-rc-43/1.51k)	NHI	https://imagej.net/
Graphpad Prism v10	Graphpad Prism Inc	https://www.graphpad.com/
Minimap2 v. 2.14	Previous paper[51]	https://github.com/lh3/minimap2
MetaBat2/2.12.1	Previous paper[52]	https://bitbucket.org/berkeleylab/metabat/src/master/.
GTDB-Tk v1.5.0	Previous paper[53]	https://github.com/Ecogenomics/GTDBTk.

EXPERIMENTAL MODELS AND STUDY PARTICIPANT DETAILS

Murine studies

All mouse experiments were approved by the Ethics Committee on Animal Care and Use in Gothenburg, Sweden. CONV-R and GF C57BL6/J *Tph1*$^{+/+}$ and *Tph1*$^{-/-}$ mice were bred at the Experimental Biomedicine (EBM) Facility in Gothenburg. All animals were housed in a climate-controlled room (22 ± 2°C), subjected to a 12-h light/dark cycle and provided with autoclaved chow diet (Lab diet, St. Louis, MO; #5021) and water *ad libitum*. *Tph1*$^{-/-}$ mice have previously been described,[50] and heterozygous breeding couples were used to obtain mice with *Tph1*$^{+/+}$ and *Tph1*$^{-/-}$ genotypes. GF *Tph1*$^{-/-}$ mice were re-derived from CONV-R *Tph1*$^{-/-}$ mice by C-section and housed in gnotobiotic isolators. The GF status was assessed every second week by aerobic and anaerobic culturing of fecal samples and bedding as well as PCR analyses of the mouse feces.[54] For each experiment, adult aged-matched mice of both sexes (approx. 1:1 ratio) were euthanized at 10 weeks of age.

Study population

27 healthy individuals and 147 individuals with IBS were included in this study from the SYMBOWL (Symptom Generation in Functional Bowel Disorders) cohort, a cohort study focusing on the link between symptoms and pathophysiology in functional bowel disorders. The 174 individuals provided blood and fecal samples, data for oro-anal transit time and stool form and consistency (Bristol

Stool Form scale, BSF) at the same visit. All participants gave informed consent, and the study was approved by the Ethics Review Board in Gothenburg (988-14). The inclusion criteria were at least 18 years of age at baseline visit and symptoms suggestive of a functional bowel disorder or an established functional bowel disorder.[55,56] Exclusion criteria were: abnormal results on the screening laboratory tests, clinically relevant for study participation; other gastrointestinal disease(s); other severe disease(s) (i.e., malignancy, severe heart disease, kidney disease or neurological disease); symptoms indicating other severe disease(s) (i.e., gastrointestinal bleeding, weight loss or fever); severe psychiatric disease; previous history of alcohol abuse; consumption of antibiotics one month previous to the screening and throughout the study; consumption of cortisone, non-steroidal anti-inflammatory drugs or other anti-inflammatory drugs on a regular basis two weeks prior to screening and throughout the study; pregnant or lactating or wish to become pregnant during the period of the study.

Participants reported their bowel habits using the BSF scale during 14 consecutive days, as a measure of stool consistency and proxy measure of (colonic) transit time. The BSF ranges from type 1 to 7, and types 1–2 (hard stools) and 6–7 (loose stools) are considered as abnormal.[49] IBS patients were categorized into subtypes based on the BSF and clinician diagnosis[55,56]: IBS with predominant constipation (IBS-C, n = 54), IBS with predominant diarrhea (IBS-D, n = 54), and IBS with unspecified or mixed-type bowel habits (IBS-nonCnonD, n = 39).

Oro-anal transit time was determined with a validated and widely used method using radiopaque markers.[57,58] The subjects ingested 10 radiopaque markers every morning for five days. On day six, subjects ingested five radiopaque markers at 8 a.m. and an additional five at 8 p.m. to more precisely characterize those with a rapid oro-anal transit time. On day seven, the subjects came to the laboratory at 8 a.m. and the remaining radiopaque markers were counted using fluoroscopy (Exposcop 7000 Compact; Ziehm GmbH, Nüremberg, Germany). Oro-anal transit time (days) was calculated by dividing the number of remaining radiopaque markers by 10 (i.e., the daily dose of radiopaque markers).

Serum and fecal serotonin were analyzed in stool and serum sample for each patient. Samples were stored in −80°C until serotonin was measured.

METHOD DETAILS

Microbiota transplantation

Cecum content was obtained from four, 8-weeks old CONV-R, *Tph1*$^{+/+}$ mice and immediately transferred to an anaerobic jar after collection. In an anaerobic chamber (Coy Laboratory Products), a longitudinal incision of the cecum was performed using a sterile scalpel. Ceca from these four mice were transferred into a 50 mL falcon tube containing a solution of LYBHI medium (16 mL; Table S2) and glycerol (4 mL, Sigma-Aldrich). Upon thorough homogenization by vortexing at max speed for 2 min, the homogenate was allowed to sediment, transferred into 3 Hungate tubes and stored at −80°C until usage.[59] Vehicle solution (LYBHI) was prepared on the same day and stored under the same conditions (−80°C), until usage. Microbiota transplantations experiments were performed as two independent experiments, using one vehicle and one ceca suspension aliquot among those prepared. On the day of the experiment, 8-weeks old GF mice were fasted for 4 h before receiving 100 μL of ceca suspension or vehicle solution. Microbiota transplantation took place in a sterile hood system (Tecniplast, Italy). During the following 15 days of conventionalization period, mice were housed in a sterile iso-cage system (Tecniplast, Italy) to avoid contamination with environmental bacteria.

Colonization experiments

All microbial cultures for colonization experiments were performed using LYBHI medium (composition in Table S2) under strict anaerobic conditions at 37°C in a Coy chamber (Coy Laboratory Products) with a gas mix of 5% H_2, 10% CO_2, and 85% N_2. A standard inoculum (1:100) of an overnight culture was prepared and grown for 24 h until colonization of the mice. 8-weeks old male and female mice were fasted for 4 h before receiving 100 μL of microbial culture or vehicle (LYBHI) by oral gavage. Colonization was performed in a sterile hood, and mice were kept in sterile iso-cages until euthanized 15 days later.

Fecal and microbial cultures

For the fecal culture experiments, fecal samples were obtained from six adult male (n = 3) and female (n = 3) healthy subjects who had not undergone antibiotic treatment in the past 4 weeks (protocol approved by the ethics review authority in Sweden; approval Dnr 2022-02020-02). Samples were immediately transferred to an anaerobic chamber (Coy Laboratory Products) and incubated at 37°C under strict anaerobic conditions following inoculation and for the entire duration on the experiment. From each sample, three aliquots of similar weight (approximately 100 mg) were transferred into three separate sterile tubes and thoroughly homogenized by vortexing, in 7 mL of LYBHI growth medium (Table S2). LYBHI medium was selected to allow the growth of human gut bacteria, including anaerobic and oxygen-sensitive bacteria.[60] In parallel, three aliquots of control medium were prepared. Each fecal culture and control medium was sampled immediately after inoculation (t = 0), and after 4, 8, 12, 24 and 48 h. Each aliquot was centrifuged (10 000 g for 2 min at 4°C), the cell-free supernatant was collected into sterile tubes and stored at −80°C until metabolite detection by mass spectrometry.

All microbial strains and communities (Table S3) were cultured in LYBHI growth medium (Table S2) and kept in anaerobic or aerobic conditions (data shown in Figure S1 only) at 37°C.

The co-culture of *L. ruminis* WL43 and *L. mucosae* WL68 here referred as to reconstituted Ls was produced by inoculation (1:200 for each strain) of an overnight culture of each pure strain in fresh LYBHI medium followed by a 30-h incubation period. Reconstituted Ls was preserved as glycerol stock (LYBHI containing 20% glycerol) at −80°C. For each independent culturing experiment, three technical replicates were prepared. Samples obtained from these replicates at each timepoint were centrifuged (10 000 g for 2 min at 4°C), the cell-free supernatant was collected into sterile tubes and stored at −80°C until metabolite detection by mass spectrometry.

Isolation of microbial communities from human feces

Under strict anaerobic conditions maintained in a Coy chamber with a gas mix of 5% H_2, 10% CO_2, and 85% N_2, the microbial communities Ls, h1L22e, h1L11c and h1L12h were isolated from freshly voided fecal samples obtained from healthy volunteers. One loop of fecal sample (approximately 0.01 mg) was inoculated on De Man, Rogosa and Sharpe (MRS) agar plates, produced by addition of 2% agar (BD Bacto, DF0140-15-4) to the liquid MRS medium (Table S2), and incubated for 36 h. Isolated colonies/communities were visually selected and subsequently sub-cultured in MRS broth for 12–16 h under strict anaerobic conditions. Cultures were preserved as glycerol stocks (MRS containing 20% glycerol) at −80°C. MRS favors the growth of lactobacilli[30] and was thus chosen as medium for the isolations based on the observation that lactobacilli metabolize Trp[61–64] and produce serotonin *in vitro* especially when co-cultured with food-borne pathogens.[22] The choice of MRS was also based on the observation of aromatic amino acid decarboxylases in *L. curvatus*[28] and *L. brevis*.[29] Liquid cultures were centrifuged (10 000 g, 4°C, 2 min) to harvest cell pellets for DNA sequencing and subsequent identification of the bacteria.

Isolation of pure strains from Ls cultures

The pure L. *ruminis* WL43 and *L. mucosae* WL68 strains were isolated from 32-h cultures of the original Ls on LYBHI plates (liquid medium as in Table S2 with additional 2% agar). Well isolated colonies of different morphology were inoculated on fresh LYBHI plates and grown for 32 h until distinguished new colonies (approximately 0.5–1 mm) appeared. Single colonies were collected from plates for identification by full-length 16S rRNA sequencing (described below). Corresponding glycerol stocks were produced (LYBHI containing 20% glycerol) and stored at −80°C.

Full-length 16S rRNA sequencing

Colony PCR was performed by amplification of the full length 16S rRNA gene using 27F (5′- AGAGTTTGATCMTGGCTCAG - 3′) and 1492R (5′ – ACCTTGTTACGACTT – 3′) primers. DNA was amplified in 50 μL PCR reactions containing HotStarTaq Master Mix 2× (QIAGEN), 10 μM of each primer and Coral Load 10× (QIAGEN). PCR was carried out under the following conditions: initial denaturation for 5 min at 94°C, followed by 26 cycles of denaturation for 30 s at 94°C, annealing for 40 s at 52°C and elongation for 90 s at 72°C, and a final elongation step for 7 min at 72°C. PCR products were then purified with the NucleoSpin Gel and PCR Clean-up kit (Macherey-Nagel) and quantified using a NanoDrop spectrophotometer. Each sample was diluted to a final concentration of 10 ng/μL and prepared for 16S rRNA sequencing as requested by Eurofins Genomics LCC. The forward and reverse amplified sequences were obtained for each isolate and the consensus sequences were searched using the Basic Local Alignment Search Tool (BLAST; NIH) to identify the isolates.

Resting cells assays

Overnight cultures of Ls, *L. ruminis* WL43, *L. mucosae* WL68 and reconstituted Ls were diluted 1:100 in fresh LYBHI medium (Table S2) and grown until late mid-log phase ($OD_{600} \sim 0.5$) in anaerobic conditions at 37°C. Cells were pelleted by centrifugation (3000 g for 10 min at 4°C) and washed in sterile PBS containing 0.05% cysteine (Sigma-Aldrich). Cell pellets were resuspended in sterile PBS containing 0.05% cysteine with or without the serotonin precursors Trp and 5-HTP. Trp (L-tryptophan, 250 μM, Sigma-Aldrich) was used to test the hydroxylation reaction while 5-HTP (5-Hydroxytryptamine, 250 μM, Sigma-Aldrich) in the presence of pyridoxal phosphate (40 μM, Sigma-Aldrich) was used to test the decarboxylation reaction, as previously described.[21] Samples of the resting cells suspension were collected immediately after substrate addition (t = 0) as well as after 20 and 40 min, 1, 3, 5 and 24 h. Cell pellets were collected by centrifugation (10 000 g for 2 min at 4°C) and stored at −80°C until RNA extraction, while cell-free supernatants were stored at −80°C until metabolite detection by mass spectrometry.

RNA extraction and transcriptomic profiling of resting cells

Total RNA was extracted from cell pellets of Ls and reconstituted Ls resting cells incubated in either PBS or 5-HTP for 1 and 3 h. RNA extraction was performed according to the Macaloid isolation protocol using the Phase Lock Gel Heavy tubes (5 Prime GmbH) and the RNeasy mini kit with on-column DNaseI treatment (Qiagen) for purification, as previously described.[65,66] Libraries for transcriptome sequencing were prepared from rRNA-depleted total RNA and sequenced at BMKgene (Illumina Novaseq 6000, 150bp paired-end reads, 4GB raw sequencing data per sample). Mapping of reads was performed at BMKgene using the genomes of Ls and reconstituted Ls as references.

Genomic DNA extraction, sequencing and analysis

Total genomic DNA of Ls, *L. mucosae* WL68, *L. ruminis* WL43 and reconstituted Ls was extracted from 10 mL overnight cultures as previously described.[67] Total genomic DNA of the remaining communities and human gut isolates produced in this study and described in Table S2, was extracted from 1 mL overnight cultures using the Nucleospin Soil kit (Techtum Lab AB) with SL2 buffer and SX enhancer. Whole genome sequencing was performed at NOVOGENE by Illumina Sequencing in a NovaSeq instrument, with paired-end sequencing of 250 bp fragments and 1GB raw data per sample.

For genome assembly of *L. mucosae* WL68 and *L. ruminis* WL43, quality filtered reads were assembled into contigs using SPAdes v. 3.14.1 in metagenomic mode.[68] After aligning reads onto the assembled contigs using minimap2 v. 2.14,[51] the results were binned with MetaBat2/2.12.1.[52] Each bin was taxonomically classified using GTDB-Tk v1.5.0.[53]

Genome assembly of Ls was performed using SPAdes with the –meta parameter, followed by binning with MaxBin2 (version 2.2.6) using default settings. This process yielded two bins, which we assessed for quality using CheckM (version 1.0.12) and annotated using GTDB-Tk (version 2.4.0). The completeness scores for Bin.0 and Bin.1 were 99.48% and 99.18%, respectively, while their contamination levels were 0.26% and 0.00%, indicating that both genomes were of high quality. Taxonomic annotation revealed that Bin.0 corresponded to *L. ruminis* and Bin.1 to *L. mucosae*.

Identification of the decarboxylase gene

Differential gene expression analysis on the transcriptomic data from Ls and reconstituted Ls was performed by BMKgene. RPKM (Reads Per Kilobase per Million Mapped Reads) was used to quantify the levels of expressed gene and Deseq2 was used to assess genes differentially expressed. Significantly differentially expressed genes were defined for FDR <0.05 and absolute log2FC > 1. The analysis identified a gene fragment that was exclusively expressed in Ls and that was annotated as tyrosine decarboxylase [EC:4.1.1.25]. To further identify the gene, we performed a search with BLASTX (version 2.15.0) against a pre-built sequence database, including nr (a non-redundant protein sequence database combining GenPept, Swiss-Prot, PIR, PDF, PDB, and NCBI RefSeq), uniprot_sprot (a high-quality, manually curated Swiss-Prot database), and uniprot_trembl (a high-quality but unreviewed TrEMBL database). We filtered the BLASTX results based on the criteria: E-value < 1e-5, identity >50%, and alignment length coverage >80%, which yielded 18 significant protein hits related to pyridoxal-dependent decarboxylase or tyrosine decarboxylase. Next, we retrieved the corresponding complete gene sequences from NCBI of these 18 proteins and calculated the consensus gene sequence using the "msaConsensusSequence" function from the msa R package. To get the gene sequence in Ls, we mapped the short reads of Ls using Bowtie2 (version 2.5.4) with the parameters: –very-sensitive-local -N 1. The mapped reads were subsequently assembled using SPAdes (version 3.15.5), resulting in a newly assembled gene sequence of 752 bp. To determine the species in which the gene is present, we conducted a BLASTN search (version 2.15.0) to compare the newly assembled 752 bp gene against the assembled genomes of Ls. The results revealed that the gene mapped with 100% identity to a 1770 bp contig in Bin.1, indicating its potential presence in the *L. mucosae* genome.

To obtain the complete CDS region of the potential decarboxylase gene in the *L. mucosae* genome, we first used Prokka to predict CDS regions within the genome and identified the one overlapping with the assembled gene. To verify whether this CDS fully covered the entire decarboxylase enzyme, we performed a BLASTX search against the NCBI nr and nt databases using the predicted CDS sequence. This search yielded a single significant hit (WP_407418999.1; however, this sequence appears to have been recently removed from RefSeq), annotated as a pyridoxal-dependent decarboxylase protein in *L. mucosae* (genome accession number MY842818, also recently removed). Since the contigs in Bin.1 only partially covered this region, we mapped the short reads of Ls to the reference sequence WP_407418999.1 and assembled the mapped reads to reconstruct a more complete CDS region of the enzyme. This process resulted in two contigs. By aligning these contigs against the nucleotide sequence of WP_407418999.1 (NZ_JAZOPK010000041.1, retrieved from NCBI on January 8, 2025) using BLAST, we identified a 60 bp gap between them. To bridge this gap, we extracted the corresponding 60 bp sequence from NZ_JAZOPK010000041.1 and incorporated it into our assembly. Additionally, both contigs were trimmed based on the BLASTN alignment results to ensure the completeness of the gene sequence. As a result, we reconstructed the complete CDS region of the potential decarboxylase gene, with a total length of 1875 bp.

Plasmid construction

The decarboxylase (DC) gene of *L. mucosae* was amplified from genomic DNA in PCR reactions using primers designed in this study: Lmuco-DC-s (atggctagcggatccgaattcgagctccgtcgaATGAGTAATGTAAAAGATTATAAAGACA) and Lmuco-DC-a (gtggtgctcgagtg cggccgcaagcttgtcgacTTACAGAATGTTTTCGTGTTCAGC). The amplicon was resolved on agarose gel, excised, and purified (QIAquick Gel Extraction Kit, Qiagen). The purified fragment and pET-28a vector (His-tag expression vector) were digested with *Sal*-I and ligated to generate pET28a-Lmuco-DC. Correct insertion was confirmed by Sanger sequencing.

Protein expression and purification

Recombinant plasmids were transformed into E. coli BL21(DE3). Single colonies grown on LB agar with 50 μg/mL kanamycin were used to inoculate 2 mL LB (50 μg/mL kanamycin), incubated overnight at 37°C. This preculture was diluted 1:100 into 200 mL LB (50 μg/mL kanamycin) and grown at 37°C until $OD_{600} \approx 0.8$, at which point expression was induced with 1 mM IPTG at 16°C for 16 h. Cells were harvested (5,000 g, 10 min, 4°C), resuspended in lysis buffer (20 mM Tris-HCl pH 7.9, 10% glycerol, 500 mM KCl, 10 mM imidazole, 3 mM DTT), and lysed by sonication. After clarification (14,000 g, 1 h, 4°C), the supernatant was applied to

a Ni^{2+}–Sepharose 6 Fast Flow column (Invitrogen). The column was washed with 30 mL of buffer A (20 mM Tris-HCl pH 7.9, 10% glycerol, 500 mM KCl, 25 mM imidazole, 3 mM DTT) and eluted with 3 mL of buffer B (20 mM Tris-HCl pH 7.9, 10% glycerol, 500 mM KCl, 250 mM imidazole, 3 mM DTT). Eluted fractions were desalted via a PD-10 column (Sephadex G-25; GE Healthcare) into buffer (20 mM Tris-HCl pH 7.9, 500 mM KCl, 10% glycerol, 3 mM DTT) and further polished on a Superdex 200 size-exclusion column (GE Healthcare) equilibrated in 100 mM NaCl, 20 mM Tris-HCl pH 7.9, 3 mM FMN, 3 mM DTT. Purified protein was stored at −20°C in buffer containing 50% (w/v) glycerol.

Decarboxylase activity assay

Reactions were performed at 37°C in 50 mM sodium phosphate buffer (pH 6.5) containing 300 mM NaCl, 40 μM pyridoxal-5′-phosphate (PLP), and 1 mM tryptophan. Enzymatic reactions were initiated by adding 0.1 μM purified decarboxylase and quenched after 2 h by mixing aliquots with an equal volume of methanol.

Quantification of serotonin-producing bacteria in the SYMBOWL cohort

To quantify the abundance of serotonin-producing bacteria in the SYMBOWL cohort, we utilized the genome assemblies of Ls, which contained the gene coding for the tryptophan decarboxylase (Figure S7). Short reads from the SYMBOWL cohort were initially mapped to the *L. mucosae* and *L. ruminis* MAGs using Bowtie2.[69] To reduce potential bias from non-specific alignment to homologous regions in other bacterial genomes, we subsequently applied Kraken2 (version 2.1.3), a k-mer–based taxonomic classifier.[70] Specifically, to improve taxonomic resolution Kraken2 was used to reassign the reads mapped with Bowtie2 in the previous step, using a custom reference library constructed from the *L. mucosae* and *L. ruminis* MAGs. As Kraken2 classifies reads based on exact k-mer matches rather than full-sequence alignment, it can filter out misassigned reads that originated from conserved or homologous regions in other genomes, based on distinct k-mer patterns. Abundance estimates were derived from the Kraken2-assigned read counts, which were normalized using the RPKM method (Reads Per Kilobase per Million Mapped Reads) to account for differences in genome length and sequencing depth across samples.

Serotonin and tryptophan metabolites detection

Cell-free supernatants, serum and fecal samples homogenates were diluted in a solution of methanol and acetic acid [99/1; v/v] containing isotopically labeled standards 5-HT-d4 (247 nM), 5-HTP-d5 (300 nM) and tryptophan-d5 (455 nM), vortexed (1500 rpm, 5 min, RT), and centrifuged (3700 rpm, 10 min, RT). A standard curve was produced by 1:2 serial dilutions of the external standards serotonin, 5-HTP and tryptophan in methanol/acetic acid [99/1; v/v]. External analytical standards and isotopically labeled standards 5-HT-d4 and tryptophan-d5 were purchased from Sigma-Aldrich, 5-HT-d4 was purchased from LGC Standards. Samples and standard curve were evaporated under nitrogen at 40°C. Thereafter, samples and standard curve were reconstituted in an injection solvent solution of methanol:water [10:90; v/v] + 0.1% hydrochloric acid.

Samples and standards were injected (2 μL) into a Waters Acquity UPLC system equipped with a Waters BEH C18 column (2.1 × 100 mm; 1.7 μm particle size). Column temperature was maintained at 30°C and the flow rate was 0.4 mL/min. The temperature of the auto sampler was kept at 10°C. Serotonin, 5-HTP and tryptophan were detected using a Xevo TQ-XS (Waters, Milford, MA) using positive electrospray.

Serotonin in human serum samples was detected by ELISA kit (Enzo Life Sciences) following the manufacturer's recommendations.

Intestinal histology and immunohistochemistry

The myenteric plexus of the longitudinal muscle (LMMP) was dissected from the mouse proximal colon as previously described[71] and fixated in 4% PFA (Histolab, Askim, Sweden) overnight at 4°C. On the following day, tissues were washed 3 times in ice-cold PBS for 10 min and kept in NaN_3 solution (0.1% in sterile PBS without Ca^{2+} and Mg^{+}) until staining. Whole-mount LMMP preparations were stained by incubation of the tissues in 300 μL blocking solution (Triton X-100 0.5%, BSA 4% and donkey serum 4% in NaN_3 0.1% solution) for 1 h followed by incubation with the primary antibody (Table S5) diluted in blocking solution, overnight at RT. On the following day, the tissues were washed 3 times with PBS for 10 min and incubated with the secondary antibody (Table S5) for 1.5 h. The tissues were then washed with PBS 3 times for 10 min, incubated with the nuclear marker Hoechst 33342 (0.02%; Thermo Fisher Scientific) for 5 min and washed with PBS 3 times for 10 min. LMMP samples were mounted with fluorescent mounting solution (Dako, Agilent).

Proximal colon (~1 cm length from the cecum) was excised, gently cleaned from the contents and fixated for 24 h in 4% PFA at 4°C. The day after, the tissues were washed 3 times in ice-cold PBS and finally preserved in 70% ethanol until paraffin embedded. Sections (5 μm-thick) were deparaffinized by sequential steps (Histolab Clear 2 × 5 min; 99.5% EtOH 1 × 5 min; 95% EtOH 1 × 5 min; 70% EtOH 1 × 5min; PBS 1 × 5min). Antigen retrieval was performed by incubation with 10 mM sodium citrate/0.05% Tween 20 solution (pH 6.0) in a water bath at 95°C for 20 min followed by incubation at RT for 20 additional minutes. Sections were then rinsed twice with PBS +0.05% Tween 20, incubated in blocking solution (4% BSA, 4% donkey serum in PBS) for 1 h at RT and subsequently with primary antibodies (Table S5; in blocking solution) overnight, at 4°C. On the following day, sections were washed twice in PBS and incubated with the secondary antibodies (Table S5) for 1 h at RT. Finally, the slides were washed twice in PBS, stained with the nuclear marker Hoechst 3342 for 5 min at RT, washed with PBS three times for 5 min and mounted using Dako mounting medium. The

tissues were imaged by confocal microscopy using a Zeiss Laser Scanning Inverted Microscope LSM-700 equipped with 20×/0.8 NA objectives and Black Zen software (Carl Zeiss).

Serotonin-IR area within the whole mount myenteric plexus preparation and Tuj1-IR area within the proximal colon sections were normalized to the Hoechst-stained area. Within the same paraffin sections, numbers of serotonin positively stained puncta were counted as previously described[15] and normalized to the total Hoechst-stained area. For each whole mount preparation, 3 to 4 images per tissue were analyzed. For each paraffin-embedded colon sample, 3 sections, spaced 50 μm apart, were stained and 2 to 4 images were taken for each section. All the images were analyzed using ImageJ software (NHI) in a blinded fashion.

Gastrointestinal transit time

Intestinal transit time in mice was determined after a 2-h fasting period by oral gavage with 100 μL of 1.5% methylcellulose containing 5% Evans Blue dye (Sigma-Aldrich). The total length of the intestine and the length covered by the Evans Blue dye were measured 5 h after gavage and intestinal transit time was expressed as the percent of intestinal length covered by the dye. Fecal samples for mass spectrometry determination of serotonin levels were collected before the start of the experiment, snap frozen in liquid nitrogen and kept at - 80°C until metabolite detection by mass spectrometry.

RT-qPCR

Proximal colon tissue was immediately snap-frozen in liquid nitrogen after harvesting and kept at −80°C until RNA extraction. RNA was extracted using RNeasy mini kit (QIAGEN) following the manufacturer's instructions, and cDNA was synthetized using HighCapacity Reverse Transcription kit (Applied Biosystems). Quantitative PCR was then performed using iQTM SYBR Green supermix (BioRad) with the CFX96 Touch Real-Time PCR Detection System (BioRad). Gene expression was normalized to the expression level of the ribosomal protein L32 and calculated using the ΔΔCT method to obtain the relative gene expression. Primer sequences for qPCR are provided in the key resources table.

QUANTIFICATION AND STATISTICAL ANALYSIS

Statistical analysis was performed using GraphPad Prism 10. Differences between two groups were assessed using two-tailed, Mann-Whitney test for unpaired comparisons or Wilcoxon matched-pairs signed rank test for paired comparisons, as stated under each figure legend. Differences among more than two groups were assessed using Kruskal-Wallis test with Dunn's multiple comparisons test. Comparisons between multiple groups and two different variables were assessed by two-way ANOVA and Tukey multiple comparisons test. Comparisons between multiple groups over time were assessed by two-way ANOVA with repeated measures. Correlation analyses were performed by Spearman's correlation. Significant differences are indicated in the figures by $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, $****p < 0.0001$. Statistical details of experiments are found in the Figure legends.