That gut instinct! Novel insights in the interplay between the microbiota, serotonin metabolism and gut function

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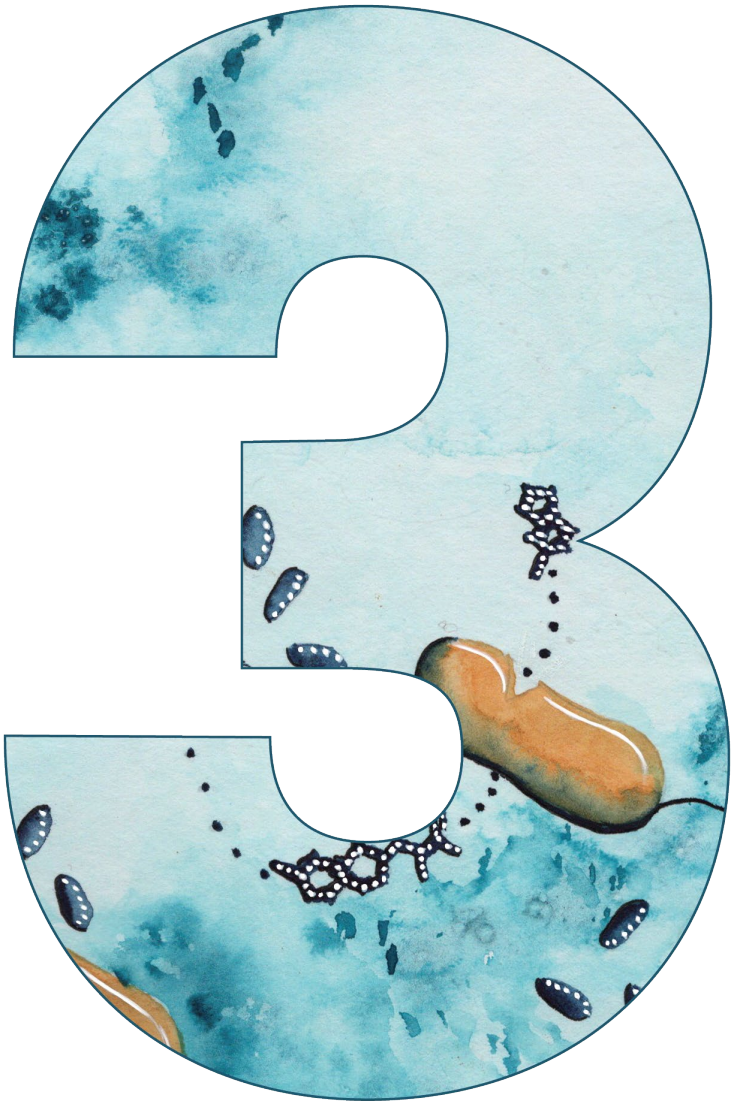
DOI:
10.33612/diss.215906461



CHAPTER 3

ORAL ADMINISTRATION OF THE GUT-BACTERIAL METABOLITE 5-HYDROXYINDOLE AND ITS SUBSEQUENT ACCELERATION OF GUT MOTILITY HAVE MARGINAL EFFECTS ON THE RAT MICROBIOTA

Submitted

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3. Oral administration of the gut-bacterial metabolite 5-hydroxyindole and its subsequent acceleration of gut motility have marginal effects on the rat microbiota

Abstract

Intestinal microbiota and microbiota-derived metabolites play a key role in regulating the host physiology. We have previously shown that a wide variety of gut bacteria can produce 5-hydroxyindole from its precursor 5-hydroxytryptophan. When 5-hydroxyindole was administered orally in rats, it significantly accelerated the total gut transit time. Here we show, using 16S rRNA gene sequencing, that 5-hydroxyindole has no effect on the bacterial richness. No separation between the treated and untreated groups was detected based on their microbial composition as shown by principal component analysis. A significant increase in the abundance of only two genera, *Shuttleworthia* and *Prevotellaceae_UCG-001*, which has been negatively associated with slow intestinal motility, was detected in the 5-hydroxyindole treated group compared to the vehicle group. No significant association was found between the reduced total gut transit time as a result of 5-hydroxyindole administration and the cecal bacterial composition. Together, our study infers a marginal effect of 5-hydroxyindole or its subsequent accelerated gut motility on the cecal microbiota composition of rats. The results urge further investigation of the impact of 5-hydroxyindole oral administration in humans, which will support its potential application as a treatment for slow intestinal motility disorders.

3.1. Introduction

The gastrointestinal tract is home to trillions of microbes. The gut microbiota produces a wide range of small bioactive molecules derived from various substrates, including dietary precursors and medications (Donia & Fischbach, 2015; van Kessel *et al.*, 2019). Such microbial conversion represents a significant regulatory mechanism by which gut microbes can alter intestinal host physiology, including gastrointestinal motility (Reigstad *et al.*, 2015; Yano *et al.*, 2015; Bhattarai *et al.*, 2018; Obata *et al.*, 2020). Recently, we have identified 5-hydroxyindole, a product of gut microbial conversion of the dietary supplement and antidepressant 5-hydroxytryptophan, as a potent accelerator of the gastrointestinal motility via its activation of L-type calcium channels located on the colonic smooth muscle cells and possibly also through its induction of serotonin production from enterochromaffin cells (Waclawiková *et al.*, 2021). These findings propose 5-hydroxyindole as a potentially safe treatment for gastrointestinal slow motility disorders. Slow gastrointestinal motility disorders, such as constipation, is a common, debilitating motility disorder affecting up to 27% of the population (Sanchez & Bercik, 2011). Constipation is also often associated with colorectal cancer, Parkinson disease, childhood attention-deficit/hyperactivity disorder, and autism spectrum disorder, as well as mood disorders (Hosseinzadeh *et al.*, 2011; Pang & Croaker, 2011; Mckeown *et al.*, 2013; Guérin *et al.*, 2014; Fasano *et al.*, 2015).

Indole (tryptophan) metabolites, one of the most studied gut microbiota-produced metabolites (Agus, Planchais & Sokol, 2018), are known for their effect on, for example, the aryl hydrocarbon receptors (AhR) (Lamas, Natividad & Sokol, 2018; Lamas *et al.*, 2020; Obata *et al.*, 2020), which in turn affects gastrointestinal barrier function (Bansal *et al.*, 2010; Shimada *et al.*, 2013; Venkatesh *et al.*, 2014) and host gut motility (Obata *et al.*, 2020). Thus, the influence of 5-hydroxyindole, being an indole derivative, can modify the fluid flow within the intestinal lumen, which is directly responsible for the transport and dispersion of the nutrients, bacterial colonization and the growth (Waclawiková *et al.*, 2022). This led us to hypothesize that since 5-hydroxyindole stimulates the gut motility it may impact the microbiota composition. Using cecal samples from rats treated orally with 5-hydroxyindole for 11 days, we show that, compared to the vehicle treated rats, 5-hydroxyindole has only a marginal effect on the gut microbiota composition.

3.2. Results & Discussion

5-hydroxyindole has a marginal effect on the richness and composition of the cecal microbiota in wild-type Groningen rats

Recently, we showed that a daily oral administration of the gut microbiota-produced 5-hydroxyindole (30 mg/kg) to wild-type Groningen (WTG) rats for 11 days results in a significant decrease of the total gut transit time (TGTT) (Waclawiková *et al.*, 2021). The dose for 5-hydroxyindole (30 mg/kg) was chosen based on a previous report (Mannaioni, Carpenedo & Moroni, 2003). To investigate the possible effect of 5-hydroxyindole and the subsequent change in the gut motility on the microbiota composition, 16 cecal samples were collected (5-hydroxyindole-treated group (n = 10); vehicle-treated group (n = 6)) after the TGTT was measured and amplicon sequencing of the V3-V4 regions of the bacterial 16S rRNA gene was performed. Microbial richness, assessed by the Chao1 index and observed number of OTUs, showed a marginal but not significant (*P* value = 0.056) increase in 5-hydroxyindole-treated rats compared to the control group (**Figure 1A; Supplementary Table 1**). Next, the microbiota diversity was determined by Shannon's H and Simpson's index, both indices are used to measure similar parameters of alpha diversity (Shannon, 1948; Simpson, 1949). The diversity index did not differ between the treated and untreated groups (**Figure 1A; Supplementary Table 1**). The data highlight that 5-hydroxyindole has a negligible effect on the richness and no effect on the diversity of the cecal microbiota. Despite the considerable recent progress in describing the effects of the indole (tryptophan) metabolites on the composition and diversity of the intestinal microbiota (Liang *et al.*, 2018; Menni *et al.*, 2020; Zhu *et al.*, 2020; Yusufu *et al.*, 2021), the overall impact of these metabolites on the host microbiota is contradicting and remains largely unknown. For example, Liang *et al.* showed that indole and its derivative, indole-3-acetic acid, significantly enhanced the richness, but not the diversity of the bacterial population in the cecal contents of piglets (Liang *et al.*, 2018). On the other hand, other studies showed no effect of high levels of indole metabolites produced from tryptophan and Mediterranean diet on the alpha diversity in mice and humans, respectively (Zhu *et al.*, 2020; Yusufu *et al.*, 2021).

As a general exploratory analysis, principal component analysis (PCA) was performed, explaining 21.3% and 16% of the variance respectively, and showed no significant difference between the 5-hydroxyindole and vehicle-treated groups

(PERMANOVA: *P* value = 0.175, stratified *P* value = 1; **Supplementary Table 2**). Next, LEfSe (Linear discriminant analysis Effect Size; (Segata *et al.*, 2011)) was employed to complement our differential abundance analysis. The main discriminant feature separating the groups (5-hydroxyindole and vehicle group) in WTG rats was the *Allobaculum* genus (**Figure 1C**). To support this analysis and investigate whether we can identify individual bacterial taxa to be affected by the 5-hydroxyindole treatment, pairwise comparisons of bacterial abundances were performed between 5-hydroxyindole-treated and vehicle-treated groups. Focusing on the phylum level, no significant changes were observed. On the family level, 5-hydroxyindole treatment seemed to only increase the abundance of family Yersiniaceae (*P* value = 0.03; **Supplementary Table 3**). On the genus level, 5-hydroxyindole treatment was associated with an increase in the abundance of *Allobaculum*, *Prevotellaceae_UCG-001*, *Serratia*, *Prevotellaceae_NK3B31_group*, *Shuttleworthia*, *Rikenellaceae_RC9_gut_group*, *Tuzzerella*, *Eubacterium_eligens_group*, *Parvibacter*, L*achnospiraceae_NK4B4_group*), while reduced the abundance of *Acetatifactor* (*P* value < 0.05, unpaired *t* test with Welch's correction) (**Figure 1D**; **Table 1**). Nonetheless, after multiple comparison correction (false discovery rate; FDR), only *Prevotellaceae_UCG-001* and *Shuttleworthia* (FDR < 0.05) showed a significant increase in their abundance. In agreement with our results, tryptophan enriched diets, and the consequent higher concentrations of indole metabolites in the cecal or fecal samples of piglets and mice, respectively, have been shown to increase the abundance of *Prevotella* genus (Liang *et al.*, 2018; Yusufu *et al.*, 2021). Taken together, the data analysis infers a minimal impact of 5-hydroxyindole treatment on the composition of the microbiota in the cecal samples of rats.

Table 1. Bacterial taxa on the genus level that were affected by the 5-hydroxyindole treatment. Significance was assessed by multiple comparison correction (FDR < 0.05). Marginal effect was assessed by unpaired t-test (*P* value < 0.05).

Genus	5-HI$_{Av}$	5-HI$_{Sd}$	Veh$_{Av}$	Veh$_{Sd}$	p.value	q.value
Prevotellaceae_UCG-001	0.0222	0.0040	0.0074	0.0023	< 0.0001	< 0.0001
Shuttleworthia	0.0016	0.0003	0.0003	0.0004	0.0002	0.0174
Tuzzerella	0.0012	0.0003	0.0006	0.0003	0.0014	0.0850
Allobaculum	0.0309	0.0238	0.0076	0.0030	0.0128	0.4308
Parvibacter	0.0002	0.0002	0.0000	0.0000	0.0136	0.4308

Continuation of Table 1.

Genus	5-HI$_{Avg}$	5-HI$_{Sd}$	Veh$_{Avg}$	Veh$_{Sd}$	p.value	q.value
*Prevotellaceae_NK3B31*_group	0.0027	0.0010	0.0011	0.0012	0.0205	0.4332
*Rikenellaceae_RC9*_gut_group	0.0026	0.0012	0.0015	0.0005	0.0197	0.4332
*Lachnospiraceae_NK4B4*_group	0.0001	0.0002	0.0000	< 0.0001	0.0283	0.4889
Serratia	0.0108	0.0071	0.0033	0.0054	0.0326	0.5174
*Eubacterium_eligens*_group	0.0008	0.0005	0.0004	0.0002	0.0419	0.6133
Acetatifactor	0.0004	0.0003	0.0010	0.0004	0.0163	0.4332

Abbreaviations: 5-HI, 5-hydroxyindole-treated group; Veh, Vehicle-treated group; Avg, average; Sd, standard deviation

Because the gut transit time was significantly affected in the 5-hydroxyindole-treated group (Waclawiková *et al.*, 2021), the TGTT was tested for its association with the abundance of genera using Spearman correlations. The correlation analysis revealed nine genera to be associated with the TGTT covariate (Spearman, *P* value < 0.05); **Figure 1D; Table 2**). Eight genera correlated negatively (*Lachnospiraceae_UCG-006*, *Lachnospiraceae_NK4B4_group*, *Tuzzerella*, *Prevotellaceae_NK3B31_group*, *Eubacterium_ventriosum_group*, *Prevotellaceae_UCG-001*, *Barnesiella*, *Bacteroides*) and one genus positively (*Anaerovibrio*). Nonetheless, none of these associations could be detected as significant after multiple comparison correction (FDR). Overall, the results indicate that the TGTT covariate, which was shown to be significantly enhanced by 5-hydroxyindole treatment (Waclawiková *et al.*, 2021), is not significantly associated with the rat cecal bacterial composition.

Table 2. Spearman correlations of bacterial taxa on the genus level that were marginally affected by the 5-hydroxyindole treatment.

Genus	Feature	Correlation	p.value	q.value
Lachnospiraceae_UCG-006	TGTT	-0.6702	0.0045	0.7847
*Lachnospiraceae_NK4B4*_group	TGTT	-0.6696	0.0045	0.7847
*Prevotellaceae_NK3B31*_group	TGTT	-0.6415	0.0074	0.8313
Tuzzerella	TGTT	-0.5670	0.0220	0.8457
*Eubacterium_ventriosum*_group	TGTT	-0.5457	0.0288	0.8457
Prevotellaceae_UCG-001	TGTT	-0.5404	0.0307	0.8457
Barnesiella	TGTT	-0.5200	0.0389	0.8457

Continuation of Table 2.

Genus	Feature	Correlation	p.value	q.value
Bacteroides	TGTT	-0.5107	0.0432	0.8457
Anaerovibrio	TGTT	0.6292	0.0090	0.8450

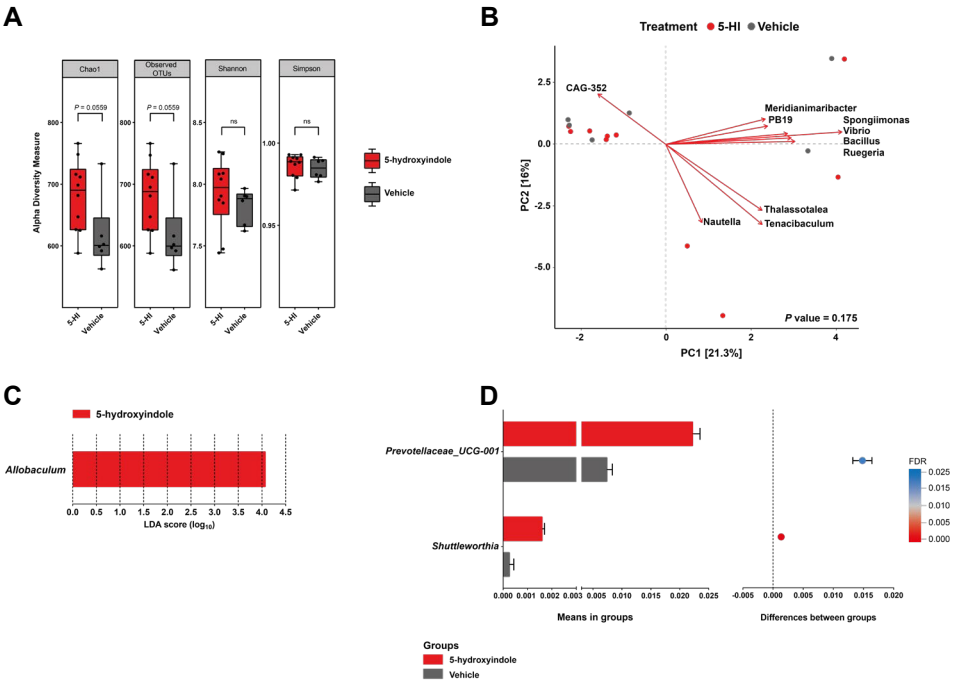

Figure 1. 5-hydroxyindole has a marginal effect on the cecal microbiota in wild-type Groningen rats. (**A**) Comparison of cecal microbiota alpha diversity between 5-hydroxyindole-treated group depicted by red bars, and vehicle-treated group depicted by grey bars, including species richness (represented by Chao1 and Observed OTUs) and diversity (represented by Shannon and Simpson index). Data were analyzed using the Mann Whitney test (*P* value is indicated above the box plots; ns = not significant). Error bars represent SEM. (**B**) Principal component analysis (PCA) indicating no separation of 5-hydroxyindole-treated and vehicle-treated group. Top 10 most contributing species are shown in the figure. (**C**) LEfSe (Linear discriminant analysis Effect Size) for the 5-hydroxyindole-treated group. The length of the bar represents the $\log_{10}$ transformed Linear discriminant analysis (LDA) score for genera significantly changed in the 5-hydroxyindole-treated group, indicated by vertical dotted lines. (**D**) Difference in the abundance of the *Prevotellaceae_UCG-001* and *Shuttleworthia* genera in 5-hydroxyindole-treated group (red bars) and vehicle-treated group (grey bars). Left panel represents means in groups. Right panel represents differences between groups, where each dot is colored by its FDR value < 0.05. Significance was assessed by multiple comparison correction. Error bars represent SEM.

3.3. Conclusion

Together, our current study suggests a marginal, presumably beneficial effect, of 5-hydroxyindole or its subsequent acceleration of the gut motility on the cecal microbiota composition when orally administered for 11 consecutive days to the WTG rats. Slow intestinal motility disorders, such as constipation, are highly prevalent gastrointestinal disorders in humans (Sanchez & Bercik, 2011). We speculate that potential future application of 5-hydroxyindole as a targeted drug, may have a marginal impact on the host gut microbiota. This gives an advantage for this microbiota-produced metabolite over several other available medications against constipation, which have been linked to significant changes in the gut microbiota composition (Vich Vila *et al.*, 2020). Additionally, *Prevotella* genus has been reported to exhibit significant reduction in the abundance in the gut microbiome of the constipated patients, compared to the controls (Zhu *et al.*, 2014). Thus, the observed significant increase in the abundance of *Prevotellaceae_UCG-001* upon the 5-hydroxyindole treatment, may provide another beneficial effect for the consideration of 5-hydroxyindole as a treatment for constipated patients. Nevertheless, human interventions aiming to investigate long-term impact of 5-hydroxyindole on the microbial composition and the gastrointestinal sensitization, are needed to support the present claim.

3.4. Materials and Methods

Cecal samples collection

All animal procedures were approved by the Groningen University Committee of Animal experiments (approval number: AVD1050020197786) and were performed in an adherence to the NIH Guide for the Care and Use of Laboratory Animals. Wild-type Groningen (WTG) male rats were orally administered either 30 mg/kg 5-hydroxyindole (H31859, Sigma) (n = 10) or vehicle (10% sucrose) (n = 6) for a period of 11 days and after measurement of TGTT (more detailed protocol can be found in (Waclawiková *et al.*, 2021)), the rats were anesthetized with isoflurane, killed and the whole cecum from every rat was collected and snap-frozen in a liquid nitrogen and stored in -80°C until further procedure.

DNA isolation

DNA isolation followed a previously proposed protocol by Yu and Morrison (2004). Briefly, about 500 mg of cecal material was resuspended in 750 µL of lysis buffer (500 mM NaCl, 50 mM Tris-HCl, 50 mM EDTA, 4% SDS) and transferred to a screw cap tube containing ~ 500 mg of 0.1 mm zirconium beads and four 3 mm glass beads. Samples were homogenized 3 × 1 min with 1-minute intervals on ice in a mini bead-beater (Biospec, Bartlesville, USA). Subsequently, samples were further incubated at 95°C for 15 min and centrifuged at 16000 x g for 15 min at 4°C. All subsequent centrifugation steps were conducted under the same conditions. The supernatant was transferred to a clean tube and 20 µL of 10 M ammonium acetate was added and incubated on ice for 10 min before centrifugation. The supernatant was again transferred to a clean tube and one volume of 100% ice cold isopropanol was added. Samples were incubated on ice for 1.5 - 2h and centrifuged. The supernatant was then aspirated and the pellet was washed with 200 µL of 70% ethanol with following centrifugation to collect the pellet. Ethanol was removed and tubes were left to air-dry for ~1h before resuspension of the pellet in 200 µL of TE buffer.

16S rRNA gene sequencing

Illumina 16S rRNA gene amplicon libraries were generated and sequenced at Novogene (Bioinformatics Technology Co., Ltd., Beijing, China). In short, 16S rRNA genes of distinct regions (16SV3-V4) were amplified used specific primers (341F and 806R) with the barcode. All PCR reactions were carried out with Phusion High-Fidelity PCR Master Mix with GC Buffer (New England Biolabs). The PCR products

were detected by 2% agarose gel electrophoresis, and the samples were mixed equally according to the concentration of PCR products. After full mixing, the 2% agarose gel electrophoresis was used for the detection again, and the target band was recovered by using the gel recovery kit provided by Qiagen company. The library was constructed by NEBNext Ultra IIDNA Library Prep Kit, and the constructed library was quantified by Qubit and Q-PCR. After the library was qualified, NovaSeq6000 was used for sequencing.

Microbiota analysis

Paired-end reads were trimmed of their barcodes and sequencing primers and subsequently merged using FLASH (v1.2.11) (Magoč & Salzberg, 2011). Fastp was used for quality control and read filtering (Chen *et al.*, 2018), while further VSEARCH was employed to detect chimera sequences by searching them against the Greengenes database (Rognes *et al.*, 2016). Filtered high-quality reads were then subjected to read denoising using DADA2 to obtain amplicon sequencing variants (ASVs), while making use of the QIIME2 software (Callahan *et al.*, 2016; Bolyen *et al.*, 2019). Sequences with less than 5 counts were removed and the remaining ASVs were classified with Classify-sklearn in QIIME2 leveraging the Greengenes database.

For downstream analysis, QIIME2 was further used to assess alpha diversity. We used the *phyloseq* and *microbiome* packages in the statistical programming language *R* to process our data further (Lahti & Shetty, 2012; McMurdie & Holmes, 2013). ASV absolute abundances were collapsed on genus level and CSS normalized (Paulson *et al.*, 2013) and the resulting abundance table was used for principal component analysis (PCA). Significance of the model was determined by an ANOVA-like permutation test implemented in the *vegan* package (Dixon, 2003) (PERMANOVA results can be found in **Supplementary Table 2**). Differential abundance between control and 5-hydroxyindole-treated groups was assessed by unpaired *t* test with Welch's correction. Significance was evaluated using FDR < 0.05. Marginal effect was assessed by P value < 0.05 (see main text for more details and **Table 1**). Additionally, LEfSe was used with default parameters to investigate differentially abundant taxa (Segata *et al.*, 2011). Pairwise correlations between microbial taxa and TGTT were performed using the *associate* function of the *microbiome* package, using Spearman correlations. Significance was evaluated using FDR < 0.05. Marginal effect was assessed by P value < 0.05 (see main text for more details, **Table 2**).

Statistical analysis

For alpha diversity, Mann Whitney test was used. For pairwise comparison (beta diversity) between groups, unpaired *t* test with Welch's correction was used. Data are presented as mean ± SEM. Data were evaluated with FDR < 0.05 for significance and *P* value < 0.05 for marginal effect (see main text for more details). For correlations between cecal microbiota, 5-hydroxyindole treatment and TGTT, Spearman correlation was used. Data were evaluated with FDR < 0.05 for significance and *P* value < 0.05 for marginal effect (see main text for more details).

Acknowledgments
We thank Amber Bullock, Host-Microbe Interactions group, Groningen Biomolecular Sciences and Biotechnology Institute (GBB), University of Groningen, Groningen, The Netherlands and Prof. Dr. Gertjan van Dijk, Department of Behavioral Neurosciences, Cluster Neurobiology, Groningen Institute of for Evolutionary Life Sciences (GELIFES), University of Groningen, Groningen, The Netherlands, for their help with the collection of the cecal samples.

Financial disclosure
S.E.A. is supported by Rosalind Franklin Fellowships, co-funded by the European Union and University of Groningen. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Data availability statement
All sequencing data is available at https://dataview.ncbi.nlm.nih.gov/object/PRJNA800624?reviewer=c9ag5ptv7vabikefplu16t8idt.

Author contributions
B.W. and S.E.A. conceptualized and designed the study.-B.W. and M.S. performed the analysis of the data. S.E.A. assisted with analysis of the data. B.W. and S.E.A. wrote the original manuscript that was reviewed by M.S. Funding was acquired by S.E.A.

Competing interests statement
The authors have declared that no competing interests exist.

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3.6. Supplementary Information

Supplementary Tables

Supplementary Table 1. Alpha diversity raw data for Chao1, Observed OTUs, Shannon and Simpson index. Marginal effect was assessed by Mann Whitney test (*P* value < 0.05).

Chao1		Observed OTUs		Shannon		Simpson	
5-HI	**Vehicle**	**5-HI**	**Vehicle**	**5-HI**	**Vehicle**	**5-HI**	**Vehicle**
647.00	599.00	647	597	7.87	7.62	0.99	0.98
748.25	562.50	747	561	8.26	7.97	0.99	0.99
698.46	602.38	695	602	8.09	7.67	0.99	0.98
588.13	733.00	588	733	7.90	7.87	0.99	0.98
716.00	592.00	716	592	8.25	7.90	0.99	0.99
625.18	616.00	625	616	7.48	7.90	0.98	0.99
711.71		711		8.04		0.99	
681.71		681		8.08		0.99	
626.40		626		7.44		0.97	
766.06		766		7.85		0.98	

Supplementary Table 2. PERMANOVA results of a PCA analysis.

NORMAL (adonis)						
	Df	SumsOfSqs	MeanSqs	F.Model	R2	Pr(>F)
Treatment	1	0.03408	0.034078	1.3803	0.08975	**0.175**
Residuals	14	0.34564	0.024688		0.91025	
Total	15	0.37971			1	

WITH STRATA (adonis2)					
	Df	SumOfSqs	R2	F	Pr(>F)
Treatment	1	0.03408	0.08975	1.3803	**1**
Residuals	14	0.34564	0.91025		
Total	15	0.37971	1		

Supplementary Table 3. Microbiota families marginally affected by 5-hydroxyindole treatment. Marginal effect was assessed by unpaired t-test (*P* value < 0.05).

Family	5-HI_{Avg}	5-HI_{Sd}	Veh_{Avg}	Veh_{Sd}	p.value	q.values
f__Yersiniaceae;	0.0108	0.0071	0.0033	0.0054	0.0326	0.8660
Others	0.0098	0.0042	0.0172	0.0065	0.0387	0.8660

Abbreviations: 5-HI, 5-hydroxyindole-treated group; Veh, Vehicle-treated group; Avg, average; Sd, standard deviation