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RESEARCH ARTICLE

Oral tryptophan activates duodenal aryl hydrocarbon receptor in healthy subjects: a crossover randomized controlled trial

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Abstract

Tryptophan is an essential amino acid transformed by host and gut microbial enzymes into metabolites that regulate mucosal homeostasis through aryl hydrocarbon receptor (AhR) activation. Alteration of tryptophan metabolism has been associated with chronic inflammation; however, whether tryptophan supplementation affects the metabolite repertoire and AhR activation under physiological conditions in humans is unknown. We performed a randomized, double blind, placebo-controlled, crossover study in 20 healthy volunteers. Subjects on a low tryptophan background diet were randomly assigned to a 3-wk L-tryptophan supplementation (3 g/day) or placebo, and after a 2-wk washout switched to opposite interventions. We assessed gastrointestinal and psychological symptoms by validated questionnaires, AhR activation by cell reporter assay, tryptophan metabolites by liquid chromatography and high-resolution mass spectrometry, cytokine production in isolated monocytes by ELISA, and microbiota profile by 16S rRNA Illumina technique. Oral tryptophan supplementation was well tolerated, with no changes in gastrointestinal or psychological scores. Compared with placebo, tryptophan increased AhR activation capacity by duodenal contents, but not by feces. This was paralleled by higher urinary and plasma kynurenine metabolites and indoles. Tryptophan had a modest impact on fecal microbiome profiles and no significant effect on cytokine production. At the doses used in this study, oral tryptophan supplementation in humans induces microbial indole and host kynurenine metabolic pathways in the small intestine, known to be immunomodulatory. The results should prompt tryptophan intervention strategies in inflammatory conditions of the small intestine where the AhR pathway is impaired.

NEW & NOTEWORTHY We demonstrate that in healthy subjects, orally administered tryptophan activates microbial indole and host kynurenine pathways in the small intestine, the primary metabolic site for dietary components, and the richest source of immune cells along the gut. This study provides novel insights in how to optimally activate immunomodulatory AhR pathways and indole metabolism in the small intestine, serving as basis for future therapeutic trials using L-tryptophan supplementation in chronic inflammatory conditions affecting the small intestine.

aryl hydrocarbon receptor; indole; kynurenine; microbiome; tryptophan

INTRODUCTION

Modulation of host and microbial pathways through dietary intervention and natural supplements is a central topic in biomedical research (1). Natural supplements are widely adopted by the general population, often with little evidence or mechanistic support of their effectiveness. Among these, tryptophan, one of 20 essential amino acids (2), has gained central attention given the complexity of its metabolism by host and gut microbiota, and the biological effects of these metabolites (3). Once ingested within foods such as poultry and cruciferous vegetables, tryptophan is used for protein synthesis or becomes the substrate of three major metabolic pathways: serotonin, indoleamine 2,3-dioxygenase (IDO)/kynurenine, and indole synthesis (4). Serotonin is a neurotransmitter with complex biological functions, including mood regulation (5), while kynurenine and its metabolites are involved in immune regulation and gut-brain signaling (6–8). Both serotonin and IDO/kynurenine pathways are mainly host-dependent, with tryptophan being degraded by 90% of its total to kynurenine products by the key enzyme IDO (9). In contrast, indole metabolites are produced by the gut microbiota, many of them being ligands of aryl hydrocarbon receptor (AhR), which is expressed throughout the

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gastrointestinal tract and has important homeostatic functions (10–12).

AhR activation leads to downstream homeostatic mechanisms that participate in intestinal epithelial renewal and barrier integrity, and modulate many cell types, including intraepithelial lymphocytes and Th17 cells (4). Changes in AhR activation can lead to an imbalance of the other two host-mediated metabolic pathways of tryptophan and immune dysregulation (13, 14), favoring inflammatory, rather than homeostatic mechanisms. Indeed, impaired activation of the AhR pathway has been demonstrated in chronic inflammatory conditions associated with gut dysbiosis, such as inflammatory bowel disease, metabolic syndrome, or celiac disease (15–17).

Animal models of colitis and celiac disease have demonstrated that the indole pathway can be rescued by tryptophan supplementation through microbial production of indoles (18, 19), raising possibility of preventative or therapeutic potential of oral tryptophan in chronic inflammation. However, whether tryptophan supplementation has any impact on host and bacterial metabolites with physiological impact in humans is unknown. Thus, the objective of this study was to determine the effects of tryptophan supplementation on immune homeostasis in the gut, IDO/kynurenine, indole and serotonin metabolic pathways, gastrointestinal symptoms, and mood in healthy volunteers. We hypothesized that oral tryptophan supplementation will increase the production of bacterial indoles and increase AhR activation.

MATERIALS AND METHODS

Study Population

Twenty-two healthy subjects (18 to 75 yr) of both sexes in overall good health and not fulfilling Rome IV criteria for functional gastrointestinal disorders (20) were recruited between October 2017 and July 2019 at McMaster University Medical Centre. We included volunteers of a wide range of age to ensure generalizability and effectiveness of the interventions across different age groups. For detailed demographics, see Table 1.

Table 1. *Demographic information*

	Healthy Subjects (n = 20)
Age, yr	28 (19–57)
Sex	
Female	11 (55)
Male	9 (45)
Body mass index, kg/m^2	23.2 (18.6–29.1)
Race	
White	15 (75)
Asian	5 (25)
Smoking	
Nonsmoker	12 (60)
Smoker	5 (25)
Ex-smoker	3 (15)
Chronic medication	
Yes	1 (5)
No	19 (95)
Alcohol consumption	
Yes	19 (95)
No	1 (5)

Data presented as median (IQR), or number (percent).

Study Design

This was a randomized, double-blind, placebo-controlled, crossover study. Subjects were instructed by a research dietician to follow a diet restricted in tryptophan for two periods of 3 wk, separated by a 2-wk washout period when their regular diet was resumed (Supplemental Fig. S1). The washout period allows to minimize the potential carryover effect, ensuring that the results of the subsequent opposite intervention are not influenced by the previous intervention (21). Participants were randomized and equally allocated in two groups: L-tryptophan (3 g/day) or placebo, and instructed to consume six capsules daily for 3 wk during the two study periods. A randomization list was generated including the following sequence: block identifier, block size, sequence within block, treatment, and code without any stratification. Duodenal aspirate, blood, urine, and stool samples were collected, and clinical parameters were assessed at baseline and at the end of each treatment period. The trial protocol was conducted in accordance with the Declaration of Helsinki, approved by Hamilton Integrated Research Ethics Board (Project No. 2820), and registered in clinicaltrials.gov (Trial ID: NCT03059862). Written informed consent was obtained from all study participants.

Interventions

Enteric-coated capsules, each containing either 500 mg L-tryptophan (Tryptan, Bausch Health, Canada, Inc.) or 500 mg of leucine (MEDISCA, Inc.), were prepared by the McMaster University Medical Centre Pharmacy, after approval from the Ethics Committee and applicable regulatory authorities of Health Canada. The identity of the specific product was blinded to subjects and investigators, distinguishable only by a deidentifiable code, known only by the pharmacy staff. Study participants were instructed to take two capsules (1,000 mg) three times daily with meals, with a total daily dose of 3 g. Medication compliance was assessed by counting residual capsules in the retrieved containers at the end of every period. Appearance of adverse events (AE) or serious adverse events (SAE) was monitored during the entirety of the study.

Tryptophan-Restricted Diet

The diet provided adequate protein (50 g/day) and energy (1,800 kcal/day) intake while containing less than 500 mg of L-tryptophan per day. Subjects were provided with a list of allowed low-tryptophan foods, as well as high-tryptophan foods to avoid, with outlined serving limits per day. Subjects recorded the amount of the food consumed during the intervention periods in the form of a diary, including the servings per meal during each day, which was strictly monitored by the dietician. A regular diet was followed during the baseline and washout periods, with an estimated amount of 1,000 mg L-tryptophan intake per day.

Primary and Secondary Study Outcomes

The primary outcome was a change in aryl hydrocarbon receptor (AhR) activation level in stool and duodenal content samples before and after intervention. As secondary outcomes, we assessed microbial and host metabolites of tryptophan in blood, urine, and stool, cytokine production by isolated peripheral blood mononuclear cells, microbiota profiles, as well as gastrointestinal and psychological symptoms.

Assessment of Clinical Parameters

Gastrointestinal and psychological symptoms were assessed at baseline and at the end of each intervention by validated questionnaires, including Gastrointestinal Symptom Rating Scale (GSRS) (22), Hospital Anxiety and Depression Scale (HAD) (23), and Depression, Anxiety and Stress Scale-21 (DASS-21) (24).

Sample Collection

All samples were obtained at the day of the endoscopy procedure, at baseline (before starting the tryptophan-restricted diet), and at the end of each intervention. Upper gastrointestinal endoscopy was performed after an overnight fasting, using conscious sedation with midazolam and fentanyl. After entering the second portion of the duodenum, available luminal fluid was aspirated into a sterile mucus specimen trap (Medline), and then using waterjet with sterile water, we dislodged the intestinal mucus and aspirated the luminal contents. Duodenal aspirates (total of approximately 15 mL) were then frozen on dry ice. Stool samples and first morning midstream urine samples were collected in sterilized containers. Blood was drawn using BD Vacutainer K2 EDTA and serum tubes (BD Biosciences), then centrifuged and aliquoted into sterilized microtubes. All samples were stored in −80°C until further analysis.

Cell Line and Culturing Conditions

H1L1.1c2 cells containing the plasmid pGudLuc1.1 (25) were thawed from a frozen stock and grown in culturing media (Dulbecco's modified Eagle's media, DMEM, supplemented with 10% heat-inactivated FBS, 50 IU/mL penicillin, 50 IU/mL streptomycin, and 50 mg/mL of geneticin; Sigma-Aldrich) at 37°C, 5% CO_2. After 24 h of growth, spent media was removed and replaced with fresh media. Cells were grown until 80%–90% confluency. At this point, cells were passaged by aspirating spent media, washing the cells with PBS, and detaching the cells using trypsin-EDTA (0.5%). Cells were then added to fresh media at a 1:10 dilution and grown to desired confluency. This process was repeated until the desired number of cells was produced.

Aryl Hydrocarbon Receptor Activity Assay

AhR activity in duodenal aspirate and stool samples was assessed using a luciferase reporter assay method (12). H1L1.1c2 cells, containing the dioxin response element-driven firefly luciferase reporter plasmid pGudLuc1.1 (26), were stimulated with human stool suspensions or small intestinal contents for 24 h. Luciferase activity was measured and expressed as fold changes relative to the control. Cytotoxicity was monitored by lactate dehydrogenase (LDH) release; highly cytotoxic wells were excluded from the analysis.

AhR activity in duodenal contents was normalized using the osmolality of the sample. Two duodenal aspirate samples were excluded from the analysis as luciferase activity not measurable, likely due to high dilution. Resulting values (delta) of placebo and tryptophan arms in relation to the baseline are shown as percentages.

Tryptophan Metabolite Levels

Metabolites were extracted from each sample after addition of the internal standards and MeOH/H_2O in different concentrations. The samples were vortexed and homogenized at −20°C for 30 min and centrifuged at 11,000 rpm for 10 min. The supernatant was transferred to 96-well plates for Liquid chromatography coupled with high-resolution mass spectrometry (LC-HRMS) analysis. Chromatography was conducted using a Phenomenex Kinetex 1.7 μm XB—C18 column (150 mm × 2.10 mm, 100 Å) kept at 55°C. The solvent system consisted of mobile phase A (0.5% formic acid in water) and mobile phase B (0.5% formic acid in methanol). Multiple reaction monitoring (MRM) analyses were carried out on a UPLC Ultimate WPS-3000 system (Dionex, Germany) coupled with a Q-Exactive mass spectrometer (Thermo Fisher Scientific, Bremen, Germany) operating in positive (ESI +) and negative (ESI −) electrospray ionization modes (one run for each mode). The collected data were processed using Xcalibur software (Thermo Fisher Scientific, San Jose, CA) by integrating selected product ion chromatographic peak area to calculate calibration curves and samples concentration (27).

Peripheral Blood Mononuclear Cells and Cytokines in Culture Supernatants

Peripheral blood mononuclear cells (PBMCs) were isolated from whole blood, and cytokines were measured using in culture supernatants using the Bioplex Pro Assay (Bio-Rad). Whole blood was layered over Histopaque-1077 and centrifuged (30 min, 400 *g*) to collect PBMCs. After washing, cells were counted with trypan blue, resuspended (1×10^7 cells/mL) in RPMI with 10% FCS. Freezing media (20% DMSO in FCS) was added, cryovials were slow-frozen at −80°C, then transferred to liquid nitrogen after 24 h. Thawed PBMCs (1×10^6 cells/mL) were then plated (0.18×10^6 per well) in 96-well plates, rested for 12 h, and stimulated (triplicate) with phorbol 12-myristate 13-acetate (PMA; 500 μg/mL) and ionomycin (1 μg/mL) for 24 h at 37°C, 5% CO_2. Supernatants were stored at −80°C for analysis. Cytokines were measured in cultured supernatants using the Bioplex Pro Assay (Bio-Rad). Briefly, magnetized beads with detection antibodies for cytokines [interferon γ (IFN-γ), tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6), interleukin-8 (IL-8), and interleukin-22 (IL-22)] were combined with the samples, standards, and antibodies in a 96-well plate. After incubation and washing steps, streptavidin-PE was added, followed by a final measurement using the MAGPIX reader (Luminex).

Fecal Microbiota Sequencing

For DNA extraction, each stool sample was placed in a tube with ceramic and glass beads, GES solution (guanidium thiocyanate, EDTA, and Sarkosyl), and sodium phosphate buffer. The samples were mechanically homogenized for 3 min at 3,000 rpm for 2 cycles and then incubated at 37°C for 1 h with lysozyme and RNaseA (mutanolysin was excluded). After a second incubation at 65°C for 1 h with SDS (sodium dodecyl sulfate), NaCl, and Proteinase K, the samples were centrifuged at 13,000 rpm for 5 min. The supernatant was mixed with an equal volume of phenol:chloroform:isoamyl for extraction and purification and centrifuged again (13,000

rpm for 10 min). The resulting aqueous layer was combined with 200 μL of DNA binding buffer and purified using the Zymo DNA Clean and Concentrator-25 kit. The DNA was purified and eluted with 50 μL of ultrapure water (28). The V3–V4 regions of the 16S rRNA gene were PCR-amplified with adapted primers (29). Sequencing utilized the MiSeq Illumina platform. Sequences were processed using Cutadapt (30) and DADA2 (31) with the SILVA reference database v. 138.1. A phylogenetic tree was generated with FastTree 2 (32). Data exploration used the phyloseq package and custom R scripts (33). One sample with <1,000 reads was excluded. Of the remaining samples, 1,787,525 reads were obtained, averaging 45,833 per sample (range: 1,674–72,808).

Statistical Methods

Data are presented as median (interquartile range). Wilcoxon test was used to compare metabolites and clinical parameters using GraphPad Prism v. 9. Spearman's rank correlation between clinical parameters, cytokine expression, and metabolites parameters was analyzed using R v. 3.5.2. Cytokine data were analyzed using the MAGPIX xPONENT software, Bio-Plex Manager v. 5.0.

For microbiota analysis, differences in beta-diversity were evaluated using a PERMANOVA. Differences for alpha-diversity genera were evaluated with a generalized linear mixed model using a negative binomial distribution (34), performed using glmmTMB package for R (v. 4.1.1) (35). Treatment group was modeled as a fixed effect and individual subject as a random effect, with number of reads used as an offset. Statistical differences between groups were identified using estimated marginal means and custom contrasts using emmeans and contrast functions of the emmeans package for R (36), followed by Šidák correction for multiple comparisons.

First author and co-authors had access to the study data, had reviewed, and approved the final manuscript

RESULTS

Study Subjects

Twenty-two healthy subjects were enrolled in the study. Two subjects were excluded (one for presence of asymptomatic eosinophilic esophagitis and the second one for not adhering to the study protocol) before the randomization. No participant withdrew from the study due to self-reported adverse effects from the intervention. Therefore, 20 subjects were included in the analysis (for demographics see Table 1).

L-Tryptophan Increased Duodenal but Not Fecal AhR Activation

Luciferase cell reporter assay showed that although there was no significant change from baseline in AhR activity in feces between placebo and L-tryptophan supplementation, AhR activity in the duodenal aspirates increased during L-tryptophan supplementation compared with placebo ($P = 0.03$, Fig. 1).

L-Tryptophan Altered Production of Urine and Serum Metabolites but Not Serum Cytokines

Using LC-HRMS, 15 tryptophan metabolites were assessed, focusing on IDO/kynurenine and indole pathways. In urine, several indole pathway metabolites, including indole-3-acetic acid, indole-3-aldehyde, indole-3-lactic acid, and tryptamine were increased after L-tryptophan supplementation compared with placebo (Fig. 2*A*). Similarly, urine levels of kynurenine, kynurenic acid, and several other IDO/kynurenine pathway metabolites were higher after L-tryptophan supplementation (Fig. 2*B*). In parallel, serum levels of total IDO metabolites, kynurenic acid, 3-OH-kynurenine, 3-OH-anthranilic acid, and quinolinic acid were increased after L-tryptophan supplementation, together with total levels of indoles and indole-3-sulfate (Fig. 3, *A* and *B*).

No differences in fecal indole or kynurenine metabolites were found between placebo and L-tryptophan supplementation (Supplemental Table S1). No differences in serotonin pathway metabolites were found in serum, urine, or feces (Supplemental Table S2).

The AhR plays a critical role in immune homeostasis by affecting the expression of various cytokines, namely IL-6 (37), IL-8 (38), IL-22 (13), TNF-α, and IFN-γ (39). Therefore, we investigated the production of these cytokines by stimulated PBMCs, finding no significant changes after L-tryptophan supplementation compared with placebo (Supplemental Fig. S2).

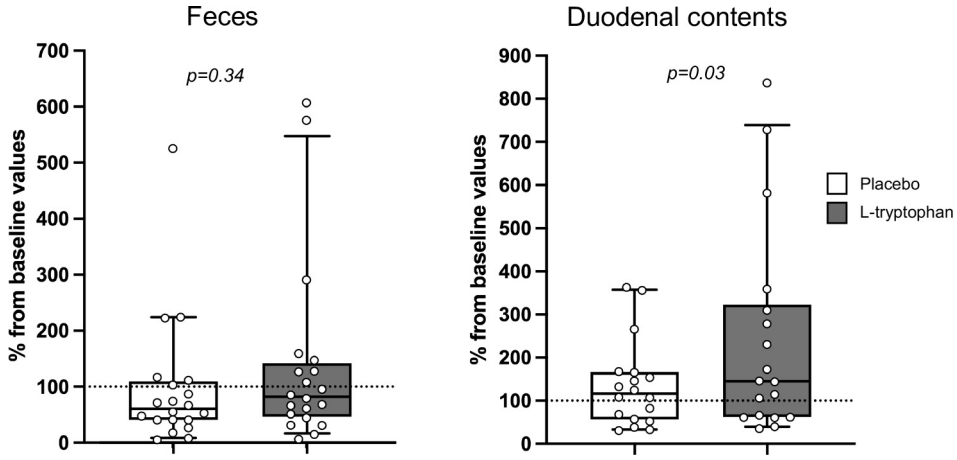

Figure 1. Effect of L-tryptophan supplementation on aryl hydrocarbon receptor (AhR) activity in feces and duodenal contents. AhR activation measured with luciferase activity in fecal samples (*left*) and duodenal contents (*right*) was expressed as a percentage of change relative to the baseline. Statistical significance was assessed by the Wilcoxon signed-rank test.

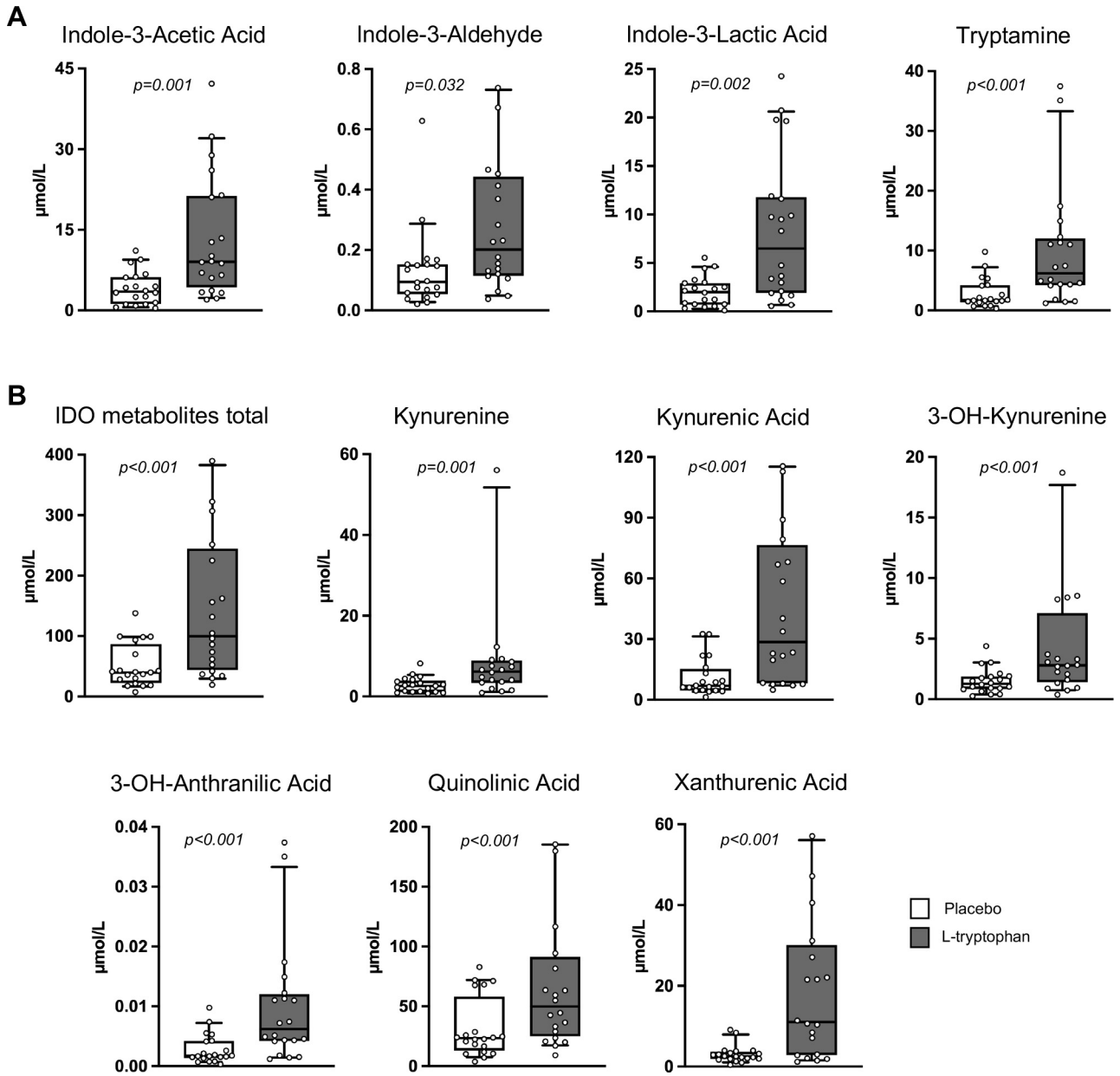

Figure 2. Effect of L-tryptophan supplementation on urine metabolites. Indole pathway metabolites (*A*) and IDO pathway metabolites (*B*) as measured by liquid chromatography coupled to high-resolution mass spectrometry (LC-HRMS). Statistical significance was assessed by the Wilcoxon signed-rank test. IDO, indoleamine 2,3-dioxygenase.

L-Tryptophan Did Not Affect Clinical Scores

Abdominal symptoms scores were within normal range and were not affected by L-tryptophan supplementation compared with placebo (Fig. 4; Supplemental Fig. S3*A*). Similarly, scores of depression, anxiety, and stress were low and not affected by L-tryptophan supplementation (Fig. 4; Supplemental Fig. S3, *B* and *C*).

Correlation between Tryptophan Metabolites, Serum Cytokines, and Clinical Scores

Although metabolites in feces were similar between L-tryptophan supplementation and placebo groups (Supplemental Table S1), several fecal tryptophan metabolites, such as indole-3-lactic acid, 3-OH-anthranilic acid, and kynurenine, correlated positively with clinical parameters, including anxiety, depression, stress, abdominal pain, and diarrhea (Supplemental Fig. S4*A*). Similarly, levels of cytokines IL-6 and IL-22 positively correlated with age and BMI, as well as with anxiety, indigestion, and overall abdominal symptoms (Supplemental Fig. S4*B*). In addition, there was a negative correlation between serum IDO/kynurenine pathway metabolites, namely kynurenine, 3-OH-kynurenine and quinolinic acid, and cytokine IL-8 (Supplemental Fig. S5*A*) during L-tryptophan supplementation, but not in placebo group (Supplementary Fig. S5*B*).

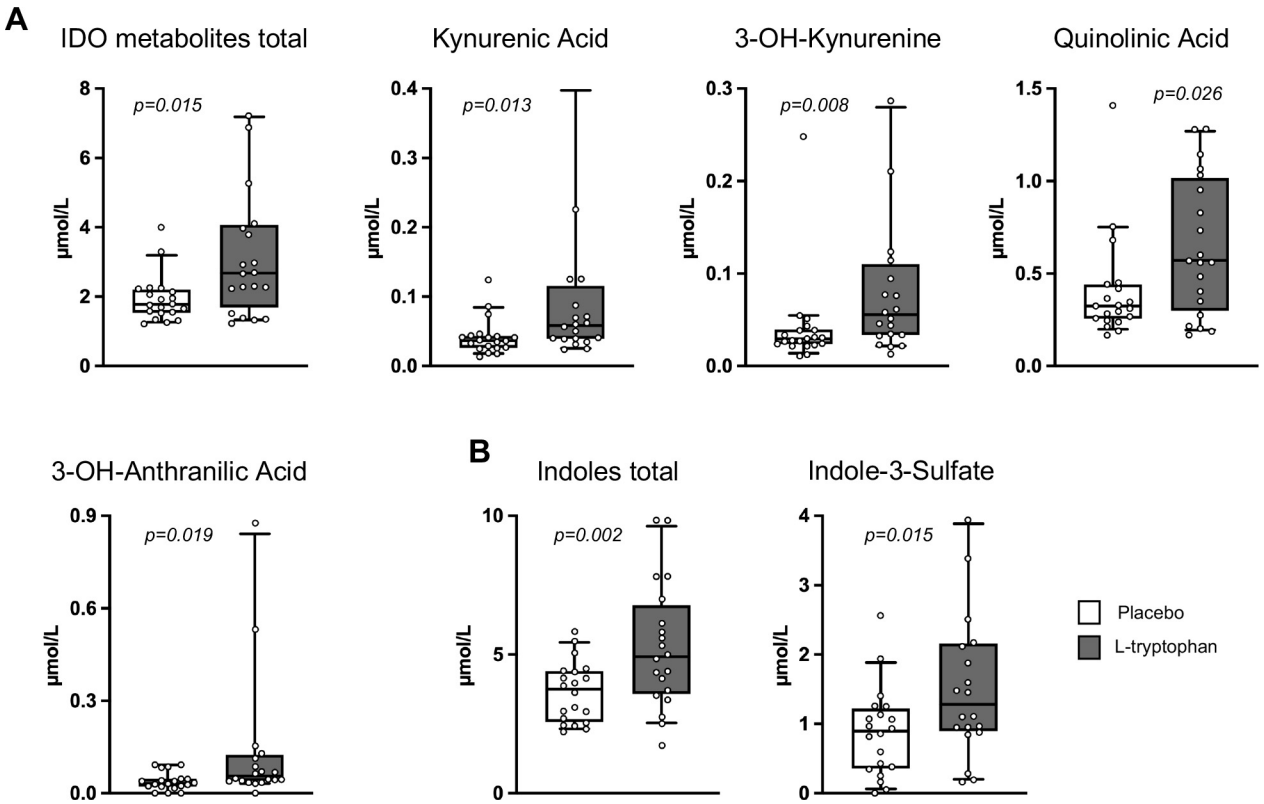

Figure 3. Effect of L-tryptophan supplementation on serum metabolites. Indole pathway metabolites (*A*) and IDO pathway metabolites (*B*) as measured by liquid chromatography coupled to high-resolution mass spectrometry (LC-HRMS). Statistical significance was assessed by the Wilcoxon signed-rank test. IDO, indoleamine 2,3-dioxygenase.

L-Tryptophan Supplementation Had Modest Impact on Fecal Microbiota Profiles

Fecal microbiota sequencing identified five genera, namely *Erysipelatoclostridium*, Family XIII–UCG 001, Monoglobus, NK4A214 group, and *Colidextribacter*, which differed between L-tryptophan and placebo groups, but otherwise no major differences in composition or abundance were found (Fig. 5*A*). Alpha and beta diversity were similar between the two groups (Fig. 5, *B* and *C*). However, several genera including *Coprococcus*, *Ruminococcus*, *Streptococcus*, and *Oscillospiraceae* UCG 002 correlated with several clinical parameters (scores for anxiety, depression, stress, reflux, indigestion, and diarrhea), fecal tryptophan metabolites, and cytokines (Supplemental Fig. S6).

DISCUSSION

In humans, oral L-tryptophan supplementation at the doses used in this study was safe and well tolerated with no adverse events reported. Tryptophan increased duodenal content, but not fecal, capacity for AhR activation and enhanced tryptophan metabolites of both microbial and host origin, with increased concentrations of indole and IDO/kynurenine metabolites, respectively, in urine and serum. Thus, oral delivery of L-tryptophan activates AhR-related metabolic pathways in the small intestine of healthy subjects.

Fecal tryptophan metabolites were not affected by L-tryptophan supplementation, suggesting that either the supplemented L-tryptophan did not reach the colon, being fully

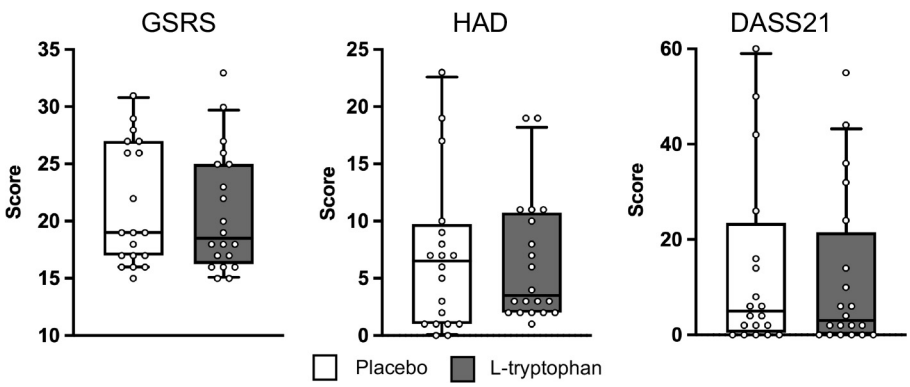

Figure 4. L-tryptophan supplementation had no effect on gastrointestinal symptoms or mood. Gastrointestinal symptoms are assessed by the Gastrointestinal Symptom Rating Scale (GSRS) (*left*), mood and anxiety assessed by the Hospital Anxiety and Depression (HAD) scale (*middle*), and the Depression, Anxiety and Stress Scale-21 (DASS21) (*right*). Statistical significance was assessed by the Wilcoxon signed-rank test.

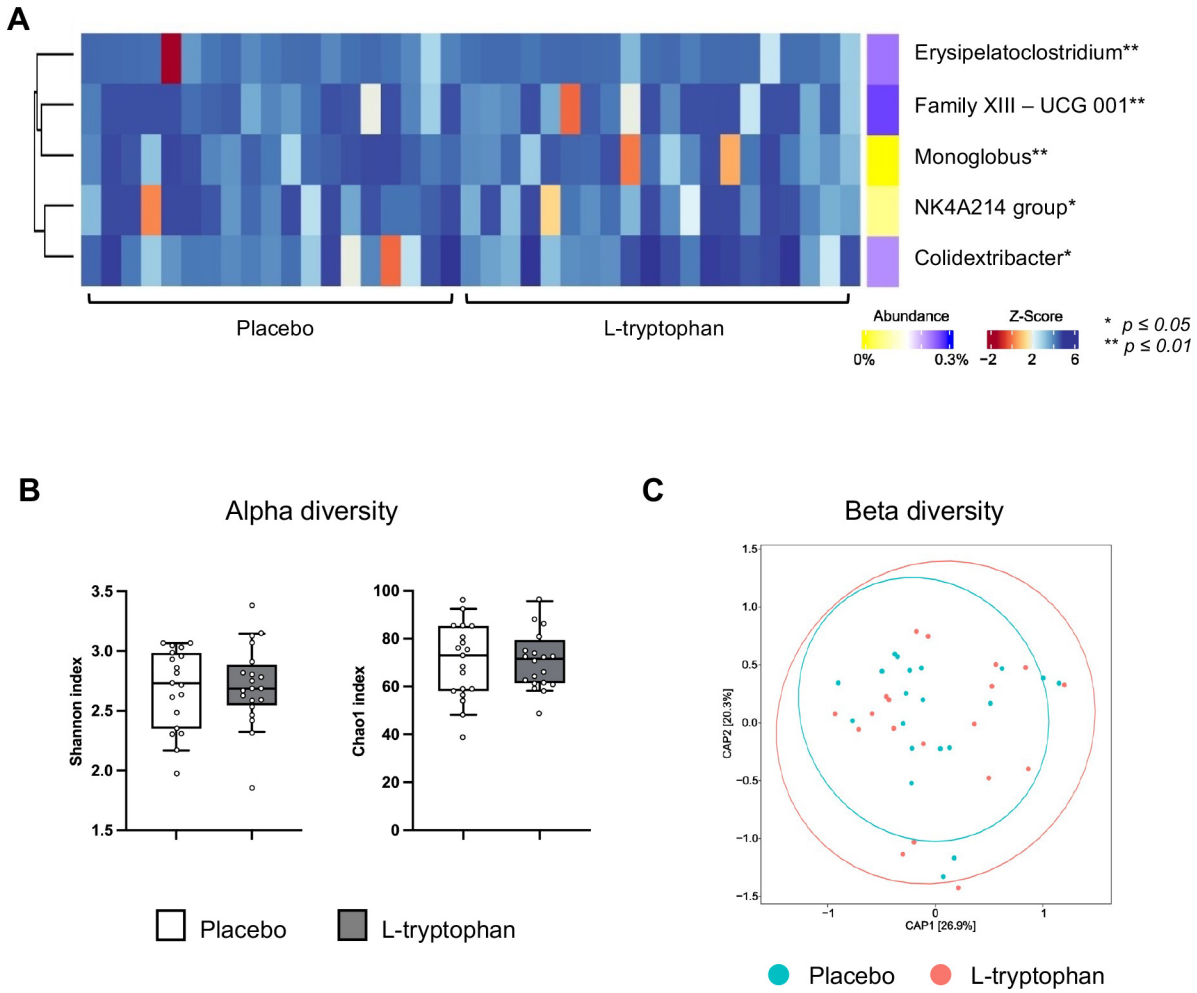

Figure 5. Effect of L-tryptophan supplementation on gut microbiota. *A*: differential taxa abundance changes assessed by16S rRNA sequencing, associated with placebo and L-tryptophan supplementation. Each column represents a subject, rows represent genera statistically different between the two groups, and color represents the corresponding Z score. Squares next to genera indicate median abundance of each genus in the dataset; *B*: alpha diversity indicated by the Shannon and Chao1 index; *C*: beta diversity: ordination plots generated using the weighted UniFrac metric. Data points represent subjects receiving placebo (blue) or L-tryptophan (orange). Statistical analysis was performed using estimated marginal means and custom contrasts using emmeans followed by Šidák correction for multiple comparisons, Wilcoxon signed-rank test, and PERMANOVA. CAP, canonical analysis of principal coordinates.

metabolized in the small intestine, or the microbial metabolites were absorbed before reaching the distal colon. This finding is clinically relevant, as there is growing interest into how to best implement tryptophan metabolite therapies during chronic intestinal conditions (40). In this study, L-tryptophan and placebo were administered as enteric-coated capsules, which are rapidly dissolved and fully absorbed and/or metabolized in the small intestine, thus explaining the higher levels in urine and blood, with no effects on colonic metabolites. A measurable effect of L-tryptophan supplementation on the level of indoles in both serum and urine demonstrates active metabolism by the human small intestinal microbiota. In accordance with the absence of changes in fecal metabolites, we did not see any differences in fecal AhR activity. However, there was higher AhR activity in the duodenum, which strongly suggests increased bacterial indole production in the small intestine. It is possible that in more distal intestinal segments, such as jejunum or proximal ileum, where the microbial density is higher and where supplemented L-tryptophan is available, AhR activity would be even more pronounced.

Gastrointestinal symptoms, as well as mood and anxiety scores did not change with L-tryptophan supplementation, which is not surprising as this study was performed in healthy volunteers. The vast majority of these subjects scored low and within normal range for all the parameters measured. However, we found that fecal tryptophan and several of its metabolites, likely produced by gradual digestion of dietary tryptophan contained within complex food matrix that ultimately reaches the colon, correlated with clinical parameters, including gastrointestinal symptoms, depression, and anxiety scores. The clinical significance of this finding is unclear, and it remains to be determined whether they represent active players or are only passive bystanders in these processes.

L-Tryptophan supplementation did not affect cytokine production by PBMC with placebo, suggesting no systemic immune impact. However, within the L-tryptophan

supplemented group, we found that higher serum levels of tryptophan and several IDO/kynurenine metabolites (total IDO metabolites, kynurenine, 3-OH-kynurenine, quinolinic acid) negatively correlated with IL-8. Although in general the IDO activity is considered to be proinflammatory and related to poor clinical outcomes in anxiety and depression (41), some studies suggested that IDO activity can have tolerogenic and immunosuppressive effects, reducing not only allergy-related inflammation and food sensitivities (42–44), but also colitis severity in mouse models, by increasing xanthurenic and kynurenic acid, known AhR ligands (40).

The indole pathway of tryptophan metabolism is gut microbiota dependent (45). In our study, L-tryptophan supplementation had a modest effect on fecal microbiota composition, in agreement with our metabolomic results, suggesting that most of the observed effect was exerted by the small intestinal microbiome. Indeed, the metabolic capacity of the upper gastrointestinal tract microbiome is gaining attention (46, 47). Although our previous study in mice showed that a high tryptophan diet favors the growth of *Lactobacillus*, known metabolizer of tryptophan (16), none of the five altered genera detected in this human study has been previously associated with tryptophan metabolism (48), thus warranting further investigation of their activity as indole producers.

Our study has several limitations, including lack of metagenomic assessment of the duodenal microbiome to link the AhR activation and tryptophan metabolism to specific bacteria, due to technical difficulties associated with the analysis of low microbial mass. We recruited a relatively low number of subjects, and thus the results should be validated in a larger cohort. Most importantly, the study was performed in healthy individuals, therefore L-tryptophan supplementation should be investigated in patients with chronic inflammatory conditions with impaired activation of the AhR pathways (15–17).

In summary, we demonstrate that in physiological conditions, oral supplementation of L-tryptophan contained with enteric-coated capsules increased serum and urine levels of multiple metabolites related to IDO activity and indole production, reflecting both host and microbial metabolism of tryptophan, which occurred in the small intestine as assessed in our reporter cell line. Our study provides novel insight into the effects of dietary tryptophan in humans, mode of administration and dose, to optimally and safely activate AhR pathways and indole metabolism in the small intestine. The study will encourage and provide basis for therapeutic trials in inflammatory conditions of the small intestine, such as nonresponsive celiac disease.

DATA AVAILABILITY

Data will be made available upon reasonable request.

SUPPLEMENTAL DATA

Supplemental Figs. S1–S6 and Supplemental Tables S1 and S2: https://doi.org/10.6084/m9.figshare.24915486.v1.

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GRANTS

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

N.C.-C., M.I.P.-S., P.L., S.M.C., H.S., E.F.V., and P.B. conceived and designed research; G.H.R., N.C.-C., R.B., A.N., M.I.P.-S., M.C., J.L., L.M.P.L., and H.S. performed experiments; G.H.R., N.C.-C., M.C., J.L., V.M., L.M.P.L., S.M.C., H.S., E.F.V., and P.B. analyzed data; G.H.R., A.N., M.I.P.-S., M.C., J.L., V.M., P.L., L.M.P.L., J.M.W., S.M.C., H.S., E.F.V., and P.B. interpreted results of experiments; G.H.R., M.C., J.L., V.M., and L.M.P.L. prepared figures; G.H.R., L.M.P.L., E.F.V., and P.B. drafted manuscript; R.B., A.N., M.I.P.-S., P.L., J.M.W., S.M.C., H.S., E.F.V., and P.B. edited and revised manuscript. R.B., M.I.P.-S., P.L., J.M.W., S.M.C., H.S., E.F.V., and P.B. approved final version of manuscript. All authors read and approved the final manuscript.

REFERENCES

1. **Zmora N**, **Suez J**, **Elinav E.** You are what you eat: diet, health and the gut microbiota. *Nat Rev Gastroenterol Hepatol* 16: 35–56, 2019. doi:10.1038/s41575-018-0061-2.
2. **Bender DA.** Biochemistry of tryptophan in health and disease. *Mol Aspects Med* 6: 101–197, 1983. doi:10.1016/0098-2997(83)90005-5.
3. **Gao J**, **Xu K**, **Liu H**, **Liu G**, **Bai M**, **Peng C**, **Li T**, **Yin Y.** Impact of the gut microbiota on intestinal immunity mediated by tryptophan metabolism. *Front Cell Infect Microbiol* 8: 13, 2018. doi:10.3389/fcimb.2018.00013.
4. **Agus A**, **Planchais J**, **Sokol H.** Gut microbiota regulation of tryptophan metabolism in health and disease. *Cell Host Microbe* 23: 716–724, 2018. doi:10.1016/j.chom.2018.05.003.
5. **Jenkins TA**, **Nguyen JC**, **Polglaze KE**, **Bertrand PP.** Influence of tryptophan and serotonin on mood and cognition with a possible role of the gut-brain axis. *Nutrients* 8: 56, 2016. doi:10.3390/nu8010056.
6. **Metghalchi S**, **Ponnuswamy P**, **Simon T**, **Haddad Y**, **Laurans L**, **Clément M**, **Dalloz M**, **Romain M**, **Esposito B**, **Koropoulis V**, **Lamas B**, **Paul J-L**, **Cottin Y**, **Kotti S**, **Bruneval P**, **Callebert J**, **den Ruijter H**, **Launay J-M**, **Danchin N**, **Sokol H**, **Tedgui A**, **Taleb S**, **Mallat Z.** Indoleamine 2,3-dioxygenase fine-tunes immune homeostasis in atherosclerosis and colitis through repression of interleukin-10 production. *Cell Metab* 22: 460–471, 2015. doi:10.1016/j.cmet.2015.07.004.
7. **Takamatsu M**, **Hirata A**, **Ohtaki H**, **Hoshi M**, **Hatano Y**, **Tomita H**, **Kuno T**, **Saito K**, **Hara A.** IDO1 plays an immunosuppressive role in 2,4,6-trinitrobenzene sulfate-induced colitis in mice. *J Immunol* 191: 3057–3064, 2013. doi:10.4049/jimmunol.1203306.
8. **Schwarcz R**, **Bruno JP**, **Muchowski PJ**, **Wu HQ.** Kynurenines in the mammalian brain: when physiology meets pathology. *Nat Rev Neurosci* 13: 465–477, 2012. doi:10.1038/nrn3257.
9. **Chen L-M**, **Bao C-H**, **Wu Y**, **Liang S-H**, **Wang D**, **Wu L-Y**, **Huang Y**, **Liu H-R**, **Wu H-G.** Tryptophan-kynurenine metabolism: a link between the gut and brain for depression in inflammatory bowel disease. *J Neuroinflammation* 18: 135, 2021. doi:10.1186/s12974-021-02175-2.

10. **Lamas B**, **Natividad JM**, **Sokol H.** Aryl hydrocarbon receptor and intestinal immunity. *Mucosal Immunol* 11: 1024–1038, 2018. doi:10.1038/s41385-018-0019-2.
11. **Alexeev EE**, **Lanis JM**, **Kao DJ**, **Campbell EL**, **Kelly CJ**, **Battista KD**, **Gerich ME**, **Jenkins BR**, **Walk ST**, **Kominsky DJ**, **Colgan SP.** Microbiota-derived indole metabolites promote human and murine intestinal homeostasis through regulation of interleukin-10 receptor. *Am J Pathol* 188: 1183–1194, 2018. doi:10.1016/j.ajpath.2018.01.011.
12. **Rothhammer V**, **Mascanfroni ID**, **Bunse L**, **Takenaka MC**, **Kenison JE**, **Mayo L**, **Chao C-C**, **Patel B**, **Yan R**, **Blain M**, **Alvarez JI**, **Kébir H**, **Anandasabapathy N**, **Izquierdo G**, **Jung S**, **Obholzer N**, **Pochet N**, **Clish CB**, **Prinz M**, **Prat A**, **Antel J**, **Quintana FJ.** Type I interferons and microbial metabolites of tryptophan modulate astrocyte activity and central nervous system inflammation via the aryl hydrocarbon receptor. *Nat Med* 22: 586–597, 2016. doi:10.1038/nm.4106.
13. **Zelante T**, **Iannitti RG**, **Cunha C**, **De Luca A**, **Giovannini G**, **Pieraccini G**, **Zecchi R**, **D'Angelo C**, **Massi-Benedetti C**, **Fallarino F**, **Carvalho A**, **Puccetti P**, **Romani L.** Tryptophan catabolites from microbiota engage aryl hydrocarbon receptor and balance mucosal reactivity via interleukin-22. *Immunity* 39: 372–385, 2013. doi:10.1016/j.immuni.2013.08.003.
14. **Qiu J**, **Heller JJ**, **Guo X**, **Chen Z-M. E**, **Fish K**, **Fu Y-X**, **Zhou L.** The aryl hydrocarbon receptor regulates gut immunity through modulation of innate lymphoid cells. *Immunity* 36: 92–104, 2012. doi:10.1016/j.immuni.2011.11.011.
15. **Lamas B**, **Richard ML**, **Leducq V**, **Pham H-P**, **Michel M-L**, **Da Costa G**, **Bridonneau C**, **Jegou S**, **Hoffmann TW**, **Natividad JM**, **Brot L**, **Taleb S**, **Couturier-Maillard A**, **Nion-Larmurier I**, **Merabtene F**, **Seksik P**, **Bourrier A**, **Cosnes J**, **Ryffel B**, **Beaugerie L**, **Launay J-M**, **Langella P**, **Xavier RJ**, **Sokol H.** CARD9 impacts colitis by altering gut microbiota metabolism of tryptophan into aryl hydrocarbon receptor ligands. *Nat Med* 22: 598–605, 2016. doi:10.1038/nm.4102.
16. **Lamas B**, **Hernandez-Galan L**, **Galipeau HJ**, **Constante M**, **Clarizio A**, **Jury J**, **Breyner NM**, **Caminero A**, **Rueda G**, **Hayes CL**, **McCarville JL**, **Bermudez Brito M**, **Planchais J**, **Rolhion N**, **Murray JA**, **Langella P**, **Loonen LMP**, **Wells JM**, **Bercik P**, **Sokol H**, **Verdu EF.** Aryl hydrocarbon receptor ligand production by the gut microbiota is decreased in celiac disease leading to intestinal inflammation. *Sci Transl Med* 12: eaba0624, 2020. doi:10.1126/scitranslmed.aba0624.
17. **Natividad JM**, **Agus A**, **Planchais J**, **Lamas B**, **Jarry AC**, **Martin R**, **Michel M-L**, **Chong-Nguyen C**, **Roussel R**, **Straube M**, **Jegou S**, **McQuitty C**, **Le Gall M**, **da Costa G**, **Lecornet E**, **Michaudel C**, **Modoux M**, **Glodt J**, **Bridonneau C**, **Sovran B**, **Dupraz L**, **Bado A**, **Richard ML**, **Langella P**, **Hansel B**, **Launay J-M**, **Xavier RJ**, **Duboc H**, **Sokol H.** Impaired aryl hydrocarbon receptor ligand production by the gut microbiota is a key factor in metabolic syndrome. *Cell Metab* 28: 737–749.e4, 2018. doi:10.1016/j.cmet.2018.07.001.
18. **Islam J**, **Sato S**, **Watanabe K**, **Watanabe T**, **Hirahara K**, **Aoyama Y**, **Tomita S**, **Aso H**, **Komai M**, **Shirakawa H.** Dietary tryptophan alleviates dextran sodium sulfate-induced colitis through aryl hydrocarbon receptor in mice. *J Nutr Biochem* 42: 43–50, 2017. doi:10.1016/j.jnutbio.2016.12.019.
19. **Scott SA**, **Fu J**, **Chang PV.** Microbial tryptophan metabolites regulate gut barrier function via the aryl hydrocarbon receptor. *Proc Natl Acad Sci USA* 117: 19376–19387, 2020. doi:10.1073/pnas.2000047117.
20. **Sperber AD**, **Bangdiwala SI**, **Drossman DA**, **Ghoshal UC**, **Simren M**, **Tack J et al**. Worldwide prevalence and burden of functional gastrointestinal disorders, results of Rome Foundation Global Study. *Gastroenterology* 160: 99–114.e3, 2021. doi:10.1053/j.gastro.2020.04.014.
21. **Lim CY**, **In J.** Considerations for crossover design in clinical study. *Korean J Anesthesiol* 74: 293–299, 2021. doi:10.4097/kja.21165.
22. **Kulich KR**, **Madisch A**, **Pacini F**, **Piqué JM**, **Regula J**, **Van Rensburg CJ**, **Ujszászy L**, **Carlsson J**, **Halling K**, **Wiklund IK.** Reliability and validity of the gastrointestinal symptom rating scale (GSRS) and quality of life in reflux and dyspepsia (QOLRAD) questionnaire in dyspepsia: a six-country study. *Health Qual Life Outcomes* 6: 12, 2008. doi:10.1186/1477-7525-6-12.
23. **Zigmond AS**, **Snaith RP.** The hospital anxiety and depression scale. *Acta Psychiatr Scand* 67: 361–370, 1983. doi:10.1111/j.1600-0447.1983.tb09716.x.
24. **Lovibond PF**, **Lovibond SH.** The structure of negative emotional states: comparison of the Depression Anxiety Stress Scales (DASS) with the Beck Depression and Anxiety Inventories. *Behav Res Ther* 33: 335–343, 1995. doi:10.1016/0005-7967(94)00075-u.
25. **He G**, **Zhao B**, **Denison MS.** Identification of benzothiazole derivatives and polycyclic aromatic hydrocarbons as aryl hydrocarbon receptor agonists present in tire extracts. *Environ Toxicol Chem* 30: 1915–1925, 2011. doi:10.1002/etc.581.
26. **Zhao B**, **Bohonowych JES**, **Timme-Laragy A**, **Jung D**, **Affatato AA**, **Rice RH**, **Di Giulio RT**, **Denison MS.** Common commercial and consumer products contain activators of the aryl hydrocarbon (dioxin) receptor. *PLoS One* 8: e56860, 2013. doi:10.1371/journal.pone.0056860.
27. **Lefèvre A**, **Mavel S**, **Nadal-Desbarats L**, **Galineau L**, **Attucci S**, **Dufour D**, **Sokol H**, **Emond P.** Validation of a global quantitative analysis methodology of tryptophan metabolites in mice using LC-MS. *Talanta* 195: 593–598, 2019. doi:10.1016/j.talanta.2018.11.094.
28. **Szamosi JC**, **Forbes JD**, **Copeland JK**, **Knox NC**, **Shekarriz S**, **Rossi L**, **Graham M**, **Bonner C**, **Guttman DS**, **Van Domselaar G**, **Surette MG**, **Bernstein CN.** Assessment of inter-laboratory variation in the characterization and analysis of the mucosal microbiota in Crohn's disease and ulcerative colitis. *Front Microbiol* 11: 2028, 2020. doi:10.3389/fmicb.2020.02028.
29. **Bartram AK**, **Lynch MD**, **Stearns JC**, **Moreno-Hagelsieb G**, **Neufeld JD.** Generation of multimillion-sequence 16S rRNA gene libraries from complex microbial communities by assembling paired-end illumina reads. *Appl Environ Microbiol* 77: 3846–3852, 2011 [Erratum in Appl Environ Microbiol 77: 5569, 2011]. doi:10.1128/AEM.02772-10.
30. **Martin M.** Cutadapt removes adapter sequences from high-throughput sequencing reads. *EMBnet J* 17: 10, 2011. doi:10.14806/ej.17.1.200.
31. **Callahan BJ**, **McMurdie PJ**, **Rosen MJ**, **Han AW**, **Johnson AJ**, **Holmes SP.** DADA2: high-resolution sample inference from Illumina amplicon data. *Nat Methods* 13: 581–583, 2016. doi:10.1038/nmeth.3869.
32. **Price MN**, **Dehal PS**, **Arkin AP.** FastTree 2–approximately maximum-likelihood trees for large alignments. *PLoS One* 5: e9490, 2010. doi:10.1371/journal.pone.0009490.
33. **McMurdie PJ**, **Holmes S.** phyloseq: an R package for reproducible interactive analysis and graphics of microbiome census data. *PLoS One* 8: e61217, 2013.doi:10.1371/journal.pone.0061217.
34. **Zhang X**, **Pei Y-F**, **Zhang L**, **Guo B**, **Pendegraft AH**, **Zhuang W**, **Yi N.** Negative binomial mixed models for analyzing longitudinal microbiome data. *Front Microbiol* 9: 1683, 2018. doi:10.3389/fmicb.2018.01683.
35. **Brooks ME**, **Kristensen K**, **van Benthem KJ**, **Magnusson A**, **Berg CW**, **Nielsen A**, **Skaug HJ**, **Maechler M**, **Bolker BM.** glmmTMB balances speed and flexibility among packages for zero-inflated generalized linear mixed modeling. *R J* 9: 378–400, 2017. doi:10.32614/RJ-2017-066.
36. **Lenth RV**, **Buerkner P**, **Herve M.** *emmeans: estimated marginal means, aka least-squares means (1.7.2)*. CRAN, 2014. https://CRAN.R-project.org/package=emmeans.
37. **Domínguez-Acosta O**, **Vega L**, **Estrada-Muñiz E**, **Rodríguez MS**, **Gonzalez FJ**, **Elizondo G.** Activation of aryl hydrocarbon receptor regulates the LPS/IFNγ-induced inflammatory response by inducing ubiquitin-proteosomal and lysosomal degradation of RelA/p65. *Biochem Pharmacol* 155: 141–149, 2018. doi:10.1016/j.bcp.2018.06.016.
38. **Vogel CF**, **Sciullo E**, **Li W**, **Wong P**, **Lazennec G**, **Matsumura F.** RelB, a new partner of aryl hydrocarbon receptor-mediated transcription. *Mol Endocrinol* 21: 2941–2955, 2007. doi:10.1210/me.2007-0211.
39. **Monteleone I**, **Rizzo A**, **Sarra M**, **Sica G**, **Sileri P**, **Biancone L**, **MacDonald TT**, **Pallone F**, **Monteleone G.** Aryl hydrocarbon receptor-induced signals up-regulate IL-22 production and inhibit inflammation in the gastrointestinal tract. *Gastroenterology* 141: 237–248.e1, 2011. doi:10.1053/j.gastro.2011.04.007.
40. **Michaudel C**, **Danne C**, **Agus A**, **Magniez A**, **Aucouturier A**, **Spatz M**, **Lefevre A**, **Kirchgesner J**, **Rolhion N**, **Wang Y**, **Lavelle A**, **Galbert C**, **Da Costa G**, **Poirier M**, **Lapière A**, **Planchais J**, **Nádvorník P**, **Illes P**, **Oeuvray C**, **Creusot L**, **Michel ML**, **Benech N**, **Bourrier A**, **Nion-Larmurier I**, **Landman C**, **Richard ML**, **Emond P**, **Seksik P**, **Beaugerie L**, **Arguello RR**, **Moulin D**, **Mani S**, **Dvorák Z**, **Bermúdez-Humarán LG**, **Langella P**, **Sokol H.** Rewiring the altered tryptophan

metabolism as a novel therapeutic strategy in inflammatory bowel diseases. *Gut* 72: 1296–1307, 2023. doi:10.1136/gutjnl-2022-327337.

41. **Huang YS**, **Ogbechi J**, **Clanchy FI**, **Williams RO**, **Stone TW.** IDO and kynurenine metabolites in peripheral and CNS disorders. *Front Immunol* 11: 388, 2020. doi:10.3389/fimmu.2020.00388.
42. **Van der Leek AP**, **Yanishevsky Y**, **Kozyrskyj AL.** The kynurenine pathway as a novel link between allergy and the gut microbiome. *Front Immunol* 8: 1374, 2017. doi:10.3389/fimmu.2017.01374.
43. **Bussmann C**, **Xia J**, **Allam JP**, **Maintz L**, **Bieber T**, **Novak N.** Early markers for protective mechanisms during rush venom immunotherapy. *Allergy* 65: 1558–1565, 2010. doi:10.1111/j.1398-9995.2010.02430.x.
44. **Xu H**, **Oriss TB**, **Fei M**, **Henry AC**, **Melgert BN**, **Chen L**, **Mellor AL**, **Munn DH**, **Irvin CG**, **Ray P**, **Ray A.** Indoleamine 2,3-dioxygenase in lung dendritic cells promotes Th2 responses and allergic inflammation. *Proc Natl Acad Sci USA* 105: 6690–6695, 2008. doi:10.1073/pnas.0708809105.
45. **Lee JH**, **Lee J.** Indole as an intercellular signal in microbial communities. *FEMS Microbiol Rev* 34: 426–444, 2010. doi:10.1111/j.1574-6976.2009.00204.x.
46. **Constante M**, **Libertucci J**, **Galipeau HJ**, **Szamosi JC**, **Rueda G**, **Miranda PM**, **Pinto-Sanchez MI**, **Southward CM**, **Rossi L**, **Fontes ME**, **Chirdo FG**, **Surette MG**, **Bercik P**, **Caminero A**, **Verdu EF.** Biogeographic variation and functional pathways of the gut microbiota in celiac disease. *Gastroenterology* 163: 1351–1363.e15, 2022. doi:10.1053/j.gastro.2022.06.088.
47. **Folz J**, **Culver RN**, **Morales JM**, **Grembi J**, **Triadafilopoulos G**, **Relman DA**, **Huang KC**, **Shalon D**, **Fiehn O.** Human metabolome variation along the upper intestinal tract. *Nat Metab* 5: 777–788, 2023. doi:10.1038/s42255-023-00777-z.
48. **Lu Y**, **Chong J**, **Shen S**, **Chammas JB**, **Chalifour L**, **Xia J.** TrpNet: understanding tryptophan metabolism across gut microbiome. *Metabolites* 12: 10, 2021. doi:10.3390/metabo12010010.