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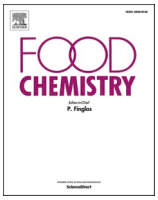

# Effect of whole foods on the microbial production of tryptophan-derived aryl hydrocarbon receptor agonists in growing pigs

Zhan Huang [a,b,*], Sonja de Vries [c], Vincenzo Fogliano [a], Jerry M. Wells [b], Nikkie van der Wielen [c,d], Edoardo Capuano [a,*]

[a] Food Quality and Design Group, Department of Agrotechnology and Food Sciences, Wageningen University & Research, P.O. Box 17, 6700 AA Wageningen, The Netherlands
[b] Host-Microbe Interactomics Group, Department of Animal Sciences, Wageningen University & Research, P.O. Box 17, 6700 AA Wageningen, The Netherlands
[c] Animal Nutrition Group, Department of Animal Sciences, Wageningen University & Research, P.O. Box 17, 6700 AA Wageningen, The Netherlands
[d] Division of Human Nutrition and Health, Department of Agrotechnology and Food Sciences, Wageningen University & Research, P.O. Box 17, 6700 AA Wageningen, The Netherlands

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ABSTRACT

Effects of whole foods on the microbial production of tryptophan-derived aryl hydrocarbon receptor (AhR) ligands in the intestine were investigated in a pig model. Ileal digesta and faeces of pigs after feeding of eighteen different foods were analyzed. Indole, indole-3-propionic acid, indole-3-acetic acid, indole-3-lactic acid, kynurenine, tryptamine, and indole-3-aldehyde were identified in ileal digesta, which were also identified in faeces but at higher concentrations except indole-3-lactic acid, together with skatole, oxindole, serotonin, and indoleacrylic acid. The panel of tryptophan catabolites in ileal digesta and faeces varied across different foods. Eggs induced the highest overall concentration of catabolites in ileal digesta dominated by indole. Amaranth induced the highest overall concentration of catabolites in faeces dominated by skatole. Using a reporter cell line, we observed many faecal samples but not ileal samples retained AhR activity. Collectively, these findings contribute to food selection targeting AhR ligands production from dietary tryptophan in the intestine.

## 1. Introduction

Aryl hydrocarbon receptor (AhR) is a well-known ligand-activated transcription factor (Gutiérrez-Vázquez & Quintana, 2018), which plays important physiological and homeostatic roles in many tissues and organs, as evidenced by AhR-deficient mice that suffer from developmental abnormalities (Fernandez-Salguero et al., 1995; Lahvis et al., 2000). In the intestine, AhR is expressed by intestinal epithelial cells and immune cells (Lamas, Natividad, & Sokol, 2018). AhR activation protects the intestine against pathogenic bacteria, maintains the epithelial barrier function, and regulates immunes tolerance and inflammation (Lamas et al., 2018; Moura-Alves et al., 2014). Lack of AhR ligands in the intestine compromises the maintenance of intraepithelial lymphocytes and results in increased vulnerability to epithelial damage (Li et al., 2011). In a mouse colitis model, administration of a potent AhR agonist, 6-formylindolo[3,2-*b*]carbazole, attenuated the severity of symptoms by down-regulating pro-inflammatory cytokines and producing interleukin (IL)–22 to increase epithelial barrier defences (Monteleone et al., 2011). IL-22 also aids in wound-healing by promoting tissue regeneration and producing antimicrobial peptides for innate defense (Ouyang & O'Garra, 2019).

A number of microbiota-derived metabolites have been shown to be agonists of AhR of which tryptophan (Trp) catabolites are of particular interest (Lamas et al., 2018). Gut microbiota catabolizes Trp into the AhR agonists indole (Ind) and its derivatives, such as tryptamine (TA), indole-3-aldehyde (I3A), indole-3-acetic acid (IAA), and indoleacrylic acid (IA) (Roager & Licht, 2018). Many studies have investigated the physiological functions of microbiota-derived Trp catabolites and their therapeutic effects in disease (Cervantes-Barragan et al., 2017; Natividad et al., 2018; Opitz et al., 2011). Indolic compounds from Trp also

*Abbreviations:* 5-HT, Serotonin; AhR, Aryl hydrocarbon Receptor; I3A, Indole-3-aldehyde; IA, Indoleacrylic acid; IAA, Indole-3-acetic acid; ILA, Indole-3-lactic acid; Ind, Indole; IPA, Indole-3-propionic acid; Kyn, Kynurenine; Oxi, Oxindole; Ska, Skatole; TA, Tryptamine; Trp, Tryptophan.

* Corresponding authors at: Food Quality and Design Group, Department of Agrotechnology and Food Sciences, Wageningen University & Research, P.O. Box 17, 6700 AA Wageningen, The Netherlands (Zhan Huang).
*E-mail addresses:* huangzhan1993@126.com (Z. Huang), edoardo.capuano@wur.nl (E. Capuano).

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have anti-angiogenic effects via the inhibition of vascular endothelial growth factor signaling (Cerezo, Hornedo-Ortega, Álvarez-Fernández, Troncoso, & García-Parrilla, 2017; Gallardo-Fernández, Cerezo, Hornedo-Ortega, Troncoso, & Garcia-Parrilla, 2022). Nevertheless, no study has yet investigated the formation of these Trp catabolites along the whole intestine and very limited information is available on the their presence in the small intestine. Previously, several Trp catabolites were reported in the ileal fluid samples of ileostomy patients, in which indole-3-lactic acid (ILA) was present at a relatively high concentration (Koper et al., 2020). ILA has anti-inflammatory activity in the immature intestine by interacting with AhR and preventing the transcription of pro-inflammatory cytokine IL-8 (Meng et al., 2020). A recent study identified an AhR-dependent subset of eosinophils in the small intestine with an immunomodulatory profile (Wang et al., 2022). Given the importance of the Trp catabolites to intestinal homeostasis, there is a great interest in identifying microbiota-derived Trp catabolites produced in the intestine, which may contribute to the design of dietary treatments to promote intestinal health.

The primary source of Trp in the intestine is from the digestion of food, as Trp is an essential amino acid. The chemical composition of food varies greatly, as well as the structural organization of its components at macro-, *meso*-, and microscopic level (Capuano, Oliviero, & van Boekel, 2018). We previously demonstrated that food matrix affects the *in vitro* microbial catabolism of Trp by influencing the microbial community structure and the accessibility of Trp to microbiota (Huang, Schoones, Wells, Fogliano, & Capuano, 2021). To date, no study has yet investigated the microbial production of Trp-derived AhR ligands in the intestine using the whole food. This information is of particular importance for selecting foods in terms of AhR ligand production in the intestine.

In this study, we investigated the microbial catabolism of dietary Trp from various foods including algae, fungi, seeds, cereals, animal products, and potato protein along the intestine (i.e. small intestine and colon). We used growing pigs as a model given the high anatomical and physiological similarity between human and porcine intestinal tracts (Ziegler, Gonzalez, & Blikslager, 2016). We collected samples from ileal digesta and faeces of pigs during the normal passage of whole foods along the intestine and quantified the Trp catabolites via LC-MS. We also characterized the AhR activation potential of ileal digesta and faeces of pigs using an AhR reporter cell line.

## 2. Experimental section

### 2.1. Ethiccal approval

The study was conducted at the research facilities of Wageningen University & Research (Wageningen, The Netherlands), and experimental procedures were approved by the Dutch Central Committee of Animal Experiments under the authorization number AVD104002015326.

### 2.2. Selection and preparation of foods

Eighteen foods were selected in this study that spanned the range of protein digestibility from low to high, including algae (spirulina and seaweed), fungi (mushrooms, yeast, and quorn), seeds (kidney beans, linseed, buckwheat, and amaranth), cereals (wheat flour, oatmeal, millet, rice crackers, cornflakes), animal products (chicken, fish, and eggs), and potato protein. They are food grade and were procured online or from local markets in Wageningen, The Netherlands (for details see Table S1). Each test food was used as the protein source in one test diet. The preparation of test foods was given in supplementary methods. In most cases, the test food was freshly prepared for each diet or stored frozen (−20 °C) and then mixed thoroughly with non-protein food ingredients, like purified maize starch, sucrose, vitamins and minerals, rapeseed oil, and purified cellulose, before being offered to pigs. The amount of test foods in the diet was adjusted to get a final protein concentration of 100 g/kg dry matter, except rice crackers and cornflakes because of their low protein content. Therefore, pigs fed with rice crackers and cornflakes were provided with additional amino acids on day 1 to 4 to meet the protein requirement. Basal and protein-free diets were also prepared. A basal diet was formulated to be structurally similar to a normal human diet and was fed to pigs during the period of acclimatization, recovery, and wash-out. A protein-free diet was used for correcting endogenous excretion of Trp catabolites. All diets were formulated to meet or exceed nutritional requirements for growing pigs, except protein, as prescribed by the National Research Council (National Research Council, 2012). The ingredient composition of basal diet, protein-free diet, and test diets was given in Table S2, together with the protein and Trp content in each diet. The true ileal Trp digestibility coefficients were determined as previously described (Hodgkinson et al., 2022) and presented in Table S3.

### 2.3. Animals and experimental procedures

The experimental protocol followed the guidelines as previously described (Hodgkinson, Stein, de Vries, Hendriks, & Moughan, 2020). A total of eighteen health growing pigs (Topigs TN-70) with an initial body weight of 19.7 ± 1.4 kg were used. Pigs were surgically fitted with a *T*-cannula (inner diameter 2.24 cm) in the distal ileum (±10-20 cm from cecum) according to the procedure described elsewhere (Hodgkinson et al., 2020). It allows sampling at the end of small intestine and a normal passage towards the large intestine. Pigs then were moved into individual metabolism pens (minimum 1.2 × 1.5 m) with slatted floors covered by plastics. Room temperature was controlled to be 21–24 °C and a 12-h light/dark cycle was applied. Each pen has a single-space feeder and a low-pressure bowl drinker for free access to water. Pigs were given 8–11 days to recover from surgery prior to starting the assay phase. The experimental feed design consisted of a triple 6 × 6 latin square design, where each pig was assigned to six diets each in six weeks, with halfway one week protein free diet followed by one week wash-out. Eighteen diets were tested and each test cycle had a duration of seven days, in which the initial five days were the adaptation period to the test diet, and samples were collected in the last two days starting immediately after the first meal of the day. Pigs were weighed weekly prior to a diet change. The daily dietary ration for each pig was 0.08 × bodyweight $(kg)^{0.75}$ calculated on a dry matter basis, which was adjusted according to the bodyweight of the pig. It was given in two equal meals at 08:00 and 17:00 of the day and fed as mash mixed with water in the feed trough. Feed refusals were recorded after each meal. Ileal digesta were collected from plastic bags attached to the *T*-cannula barrel using an elastic band. Bags were replaced whenever filled approximately 70% with digesta or at least once every 60 min from 08:00 to 17:00 of the day. Metabolism pens were cleaned every morning and faeces were collected from the floor if any from 15:00 to 16:00 of the day after the morning section of daily ration. Ileal digesta and faeces were pooled per pig within period and immediately frozen at −20 °C for further analysis. Pigs refused to eat yeast and sometimes showed clinical signs of sickness during the test week, resulting in some missing values. Therefore, samples from yeast diet were removed from the analysis. After the whole experiment, pigs were euthanized by Euthasol® (pentobarbital sodium and phenytoin sodium) injection and an autopsy was performed to evaluate the cannulation.

### 2.4. Extraction and quantification of microbiota-derived Trp catabolites

Freeze-dried ileal digesta (~50 mg) were mixed with ice-cold 80% methanol at a ratio of 1: 10 (w/v) and faeces (~100 mg) at 1: 6 (w/v). The mixture was homogenized by vortexing at 500 × *g* for 30 min and then centrifuged at 22000 × *g* for 10 min at 4 °C. The supernatants were collected and referred to as extracts. For quantification, extracts were diluted by 16-fold with Milli-Q water before being subjected to targeted

analysis for Trp catabolites by a Shimadzu Nexera XR LC-20ADxr UPLC system coupled with a Shimadzu LCMS-8050 mass spectrometer (Kyoto, Japan) using a Phenomenex Kinetex 1.7 μm EVO C18 100 Å LC column (100 × 2.1 mm). The operation conditions, mobile phase, and elution program were described elsewhere (Huang, Boekhorst, Fogliano, Capuano, & Wells, 2023). Catabolites were identified by comparing the transitions (*m/z*) and retention time (RT) with reference standards including TA (*m/z* 161.1 → 144.0; RT 2.08 min), Ind (*m/z* 118.2 → 91.1; RT 9.89 min), I3A (*m/z* 146.0 → 118.1; RT 9.36 min), IAA (*m/z* 176.0 → 130.1; RT 9.33 min), IA (*m/z* 188.0 → 115.1; RT 10.95 min), ILA (*m/z* 205.9 → 118.1; RT 9.05 min), indole-3-propionic acid (IPA, *m/z* 190.1 → 130.0; RT 9.88 min), oxindole (Oxi, *m/z* 134.0 → 77.1; RT 8.65 min), skatole (Ska, *m/z* 132.0 → 117.1; RT 11.52 min), kynurenine (Kyn, *m/z* 209.0 → 192.1; RT 1.91 min), and serotonin (5-HT, *m/z* 177.0 → 160.1; RT 1.63 min).

### 2.5. Recovery and matrix effect

Recovery of extraction and matrix effect on mass spectrometric response were determined as previously described (Dong et al., 2020). Briefly, an known amount of analyte was spiked into the ileal digesta and faeces before and after extraction, and then applied to LC-MS measurement. Recovery was calculated as follows: (peak area of the spiked analyte before extraction – peak area of the endogenous analyte) / (peak area of the spiked analyte after extraction – peak area of the endogenous analyte). The matrix effect was calculated as follows: (peak area of the spiked analyte after extraction – peak area of the endogenous analyte) / peak area of the spiked analyte in Milli-Q water.

### 2.6. Cell culture

HepG2-Lucia™ AhR reporter cells (InvivoGen, San Diego, CA) were cultured in Gibco™ Eagle's minimal essential medium (No. 31095029, Thermo Fisher Scientific) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (No. 10082147, Thermo Fisher Scientific), 1x non-essential amino acids (No. 11140050, Thermo Fisher Scientific), 100 U/mL penicillin-100 mg/mL streptomycin (No. P0781, Sigma-Aldrich), 100 μg/mL Normocin™ (No. ant-nr-1, InvivoGen), and 100 μg/mL Zeocin™ (No. ant-zn-05, InvivoGen). During the AhR induction, the cells were cultured in the test medium as above but without Normocin™ and Zeocin™. Cell lines were cultured in a humidified incubator at 37 °C and 5% $CO_2$.

### 2.7. Pre-treatment of samples for cellular assays

Extracts (100 μL) of ileal digesta and faeces obtained as described in section 2.4 were evaporated by nitrogen to remove the organic solution and then redissolved in 200 μL of the test medium of AhR reporter cells. After centrifugation at 11200 × *g* for 2 min at 4 °C, the supernatants were collected and used in the cellular assay. Trp catabolites and β-naphthoflavone (β-NAPH) were dissolved in DMSO, which was diluted to a final concentration lower than 1% (v/v) in the cell culture.

### 2.8. Measurement of AhR activity

AhR activity of ileal digesta and faeces was measured using a luciferase reporter assay on HepG2-Lucia™ AhR reporter cells as previously described (Koper et al., 2020). Briefly, AhR reporter cells were seeded into a 96-well plate with a volume of 180 μL per well containing about 20 000 cells and stimulated with tested samples (20 μL per well, in triplicate) for 48 h. Luciferase activity was quantified by QUANTI-Luc™ (No. rep-qlc2, InvivoGen) and measured via a Spectramax M5 (Molecular Devices, USA). Meanwhile, the cytotoxicity of test samples to AhR reporter cells was determined by the released lactate dehydrogenase (LDH) using the CytoTox 96 Non-Radioactive Cytotoxicity Assay (No. G1780, Promega) according to the manufacturer's instructions. The results of AhR activity were normalized on the basis of the luciferase activity of the vehicle. Cytotoxicity was expressed as the percentage of maximum LDH release achieved by adding10X Lysis Solution in the kit.

### 2.9. Statistical analysis

Statistical analysis was performed in GraphPad Prism 9.1.0 (GraphPad Software, La Jolla, CA). Concentrations of Trp catabolites in ileal digesta and faeces of pigs fed with test foods were normalized by protein-free diet and corrected for recovery and matrix effect. AhR activation of Trp catabolites was compared using one-way analysis of variance (ANOVA) with Tukey post-hoc analysis and AhR activation of ileal digesta and faeces was compared using Student's *t* test between each treatment group of test diets and vehicle (test medium). Concentrations of Trp catabolites in ileal digesta and faeces of the same pig were compared using paired Student's *t* test. Data are expressed as mean ± standard error of the mean (SEM). P value of $< 0.05$ was considered significant.

## 3. Results

### 3.1. Microbiota-derived Trp catabolites in ileal digesta and faeces of pigs

To understand the microbial catabolism of dietary Trp along the intestine, we quantified the Trp catabolites in ileal digesta and faeces of pigs and reported the results from the first week of feeding experiments across all pigs and all food matrices based on wet matter in Fig. 1. Most of Trp catabolites had a higher concentration in faeces than in ileal digesta with the exception of ILA (Fig. 1a). Ska, Oxi, 5-HT, and IA were quantifiable in faeces but not in ileal digesta (Fig. 1a). The overall concentration of Trp catabolites in faeces was over 5-fold higher than that in ileal digesta (Fig. 1b). According to the average abundance of individual catabolites relative to the sum of all identified Trp catabolites (Fig. 1c), Ind (44.99%), ILA (25.86%), IPA (12.05%), and IAA (11.13%) were dominating in ileal digesta, whereas Trp catabolites in faeces were dominated by Ska (55.13%), Oxi (17.90%), and Ind (16.16%). We also quantified these Trp catabolites in the test diets, but no Trp catabolites were detected (data not shown).

### 3.2. Microbiota-derived Trp catabolites from various whole foods

To explore the role of the food matrix in microbial catabolism of Trp, we fed pigs with various foods and reported the concentration of food-derived Trp catabolites based on dry matter (DM) in Fig. 2 for ileal digesta and in Fig. 3 for faeces. In ileal digesta, feeding pigs with eggs resulted in the highest concentration of Ind (~300 nmol/g DM), cornflakes in the highest concentration of IPA (~85 nmol/g DM), amaranth in the highest concentration of IAA (~30 nmol/g DM), chicken in the highest concentration of ILA (~70 nmol/g DM), seaweed in the highest concentration of Kyn (~7 nmol/g DM), and linseed in the highest concentration of I3A (~4 nmol/g DM) and TA (~2 nmol/g DM) (Fig. 2). In faeces, feeding pigs with amaranth resulted in the highest concentration of Ska (~1600 nmol/g DM), Ind (~600 nmol/g DM), IAA (~60 nmol/g DM), 5-HT (~28 nmol/g DM), and I3A (~10 nmol/g DM), millet in the highest concentration of Oxi (~500 nmol/g DM), buckwheat in the highest concentration of IPA (~110 nmol/g DM) and ILA (~6 nmol/g DM), mushrooms in the highest concentration of TA (~80 nmol/g DM), and kidney beans resulted in the highest concentration of Kyn (~5 nmol/g DM) and IA (~3 nmol/g DM) (Fig. 3).

Overall, feeding pigs with eggs resulted in the highest concentration of total Trp catabolites in ileal digesta compared with other test foods, whereas feeding pigs with amaranth resulted in the highest concentration of total Trp catabolites in faeces (Figs. 2 and 3). Spirulina, seaweed, mushrooms, and wheat flour induced a relatively low concentration of total Trp catabolites in both ileal digesta and faeces of pigs (Figs. 2 and 3).

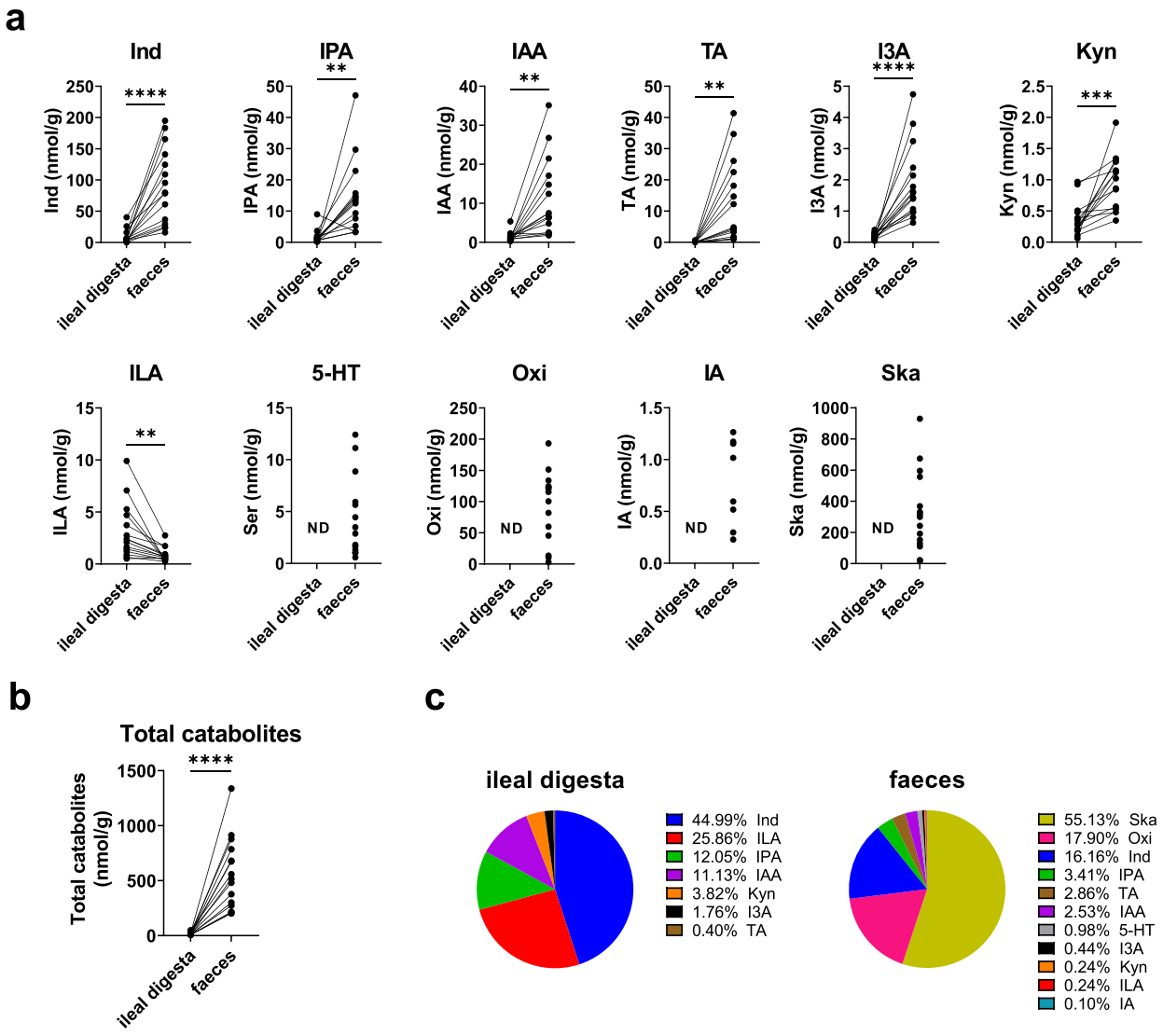


**Fig. 1.** Microbiota-derived Trp catabolites quantified in ileal digesta and faeces of growing pigs. a. Concentrations of individual catabolites. b. Concentrations of total identified catabolites. c. Average abundance of individual catabolites relative to the sum of all identified Trp catabolites. Data based on wet matter were from the first week of feeding experiments across all pigs and all food matrices. The lines connect samples for the same pig and data were analysed by paired Student's *t* test. Significance is reported as $^{**}p < 0.01$, $^{***}p < 0.001$, and $^{****}p < 0.0001$. ND: not detected. Ind: indole; IPA: indole-3-propionic acid; IAA: indole-3-acetic acid; TA: tryptamine; I3A: indole-3-aldehyde; Kyn: kynurenine; ILA: indole-3-lactic acid; 5-HT: serotonin; Oxi: oxindole; IA: indoleacrylic acid. Ska: skatole.

### 3.3. Food matrix affects the microbial catabolism of Trp in the intestine

To compare the pattern of Trp catabolites driven by different food matrices, we calculated the average abundance of each catabolite relative to the sum of all identified Trp catabolites and reported the results in Fig. 4. Trp catabolites in ileal digesta were dominated by Ind, especially in pigs fed with eggs, in which Ind represents more than 90% of the measured catabolites (Fig. 4). However, feeding pigs with millet and kidney beans inhibited the microbial biosynthesis of Ind in the small intestine and drove the catabolism to IAA, IPA, and ILA biosynthesis (Figs. 2 and 4). Decreases in the relative abundance of Ind were also observed in ileal digesta of pigs fed with mushrooms dominated by ILA (37.97%) and cornflakes dominated by IPA (48.02%). Trp catabolites in faeces were dominated by Ska, but feeding pigs with mushrooms shifted the microbial catabolism of Trp in porcine colon to Ind and TA biosynthesis and to Oxi, Ind, and IPA biosynthesis by feeding with buckwheat, which is reflected by the relative abundance of Trp catabolites in faeces (Fig. 4). Collectively, different foods induced distinct profiles of microbiota-derived Trp catabolites in ileal digesta and faeces of pigs (Fig. 4).

### 3.4. Microbiota-derived Trp catabolites as AhR activators

To determine the agonistic and antagonistic effects of Trp catabolites on the activation of AhR, we used a reporter cell from human HepG2 cells and reported the results in Fig. 5. Every examined Trp catabolite displayed a capacity to stimulate AhR activation in a dose-dependent manner, in which TA showed the highest AhR agonist activity at tested concentrations. Among the examined catabolites, TA together with Ind were potent AhR activators as they induced a significant activation of AhR at a relatively low concentration (1 μmol/L). Oxi, I3A, and IA induced a significant activation of AhR at a concentration of 10 μmol/L. Ser, Kyn, IAA, Ska, ILA, and IPA were less active AhR activators, which activated AhR at a concentration of 100 μmol/L.

Most of Trp catabolites had no antagonistic effects against the AhR activation induced by β-NAPH, a known potent AhR agonist, except Ska

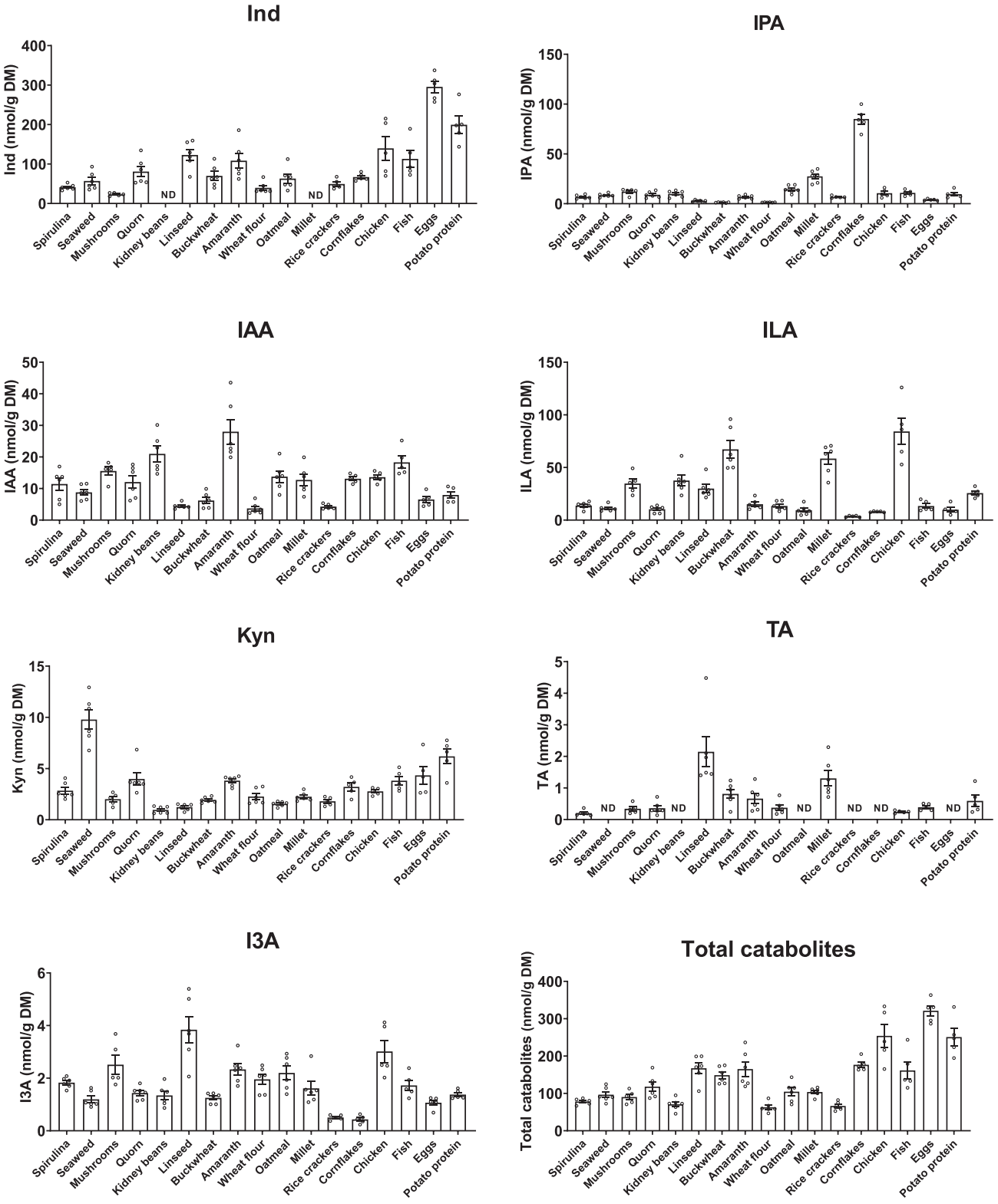


**Fig. 2.** Concentrations of microbiota-derived Trp catabolites produced from test diets in ileal digesta of growing pigs. Data are based on dry matter (DM) and presented as mean ± SEM. ND: not detected. Ind: indole; IPA: indole-3-propionic acid; IAA: indole-3-acetic acid; ILA: indole-3-lactic acid; Kyn: kynurenine; TA: tryptamine; I3A: indole-3-aldehyde.

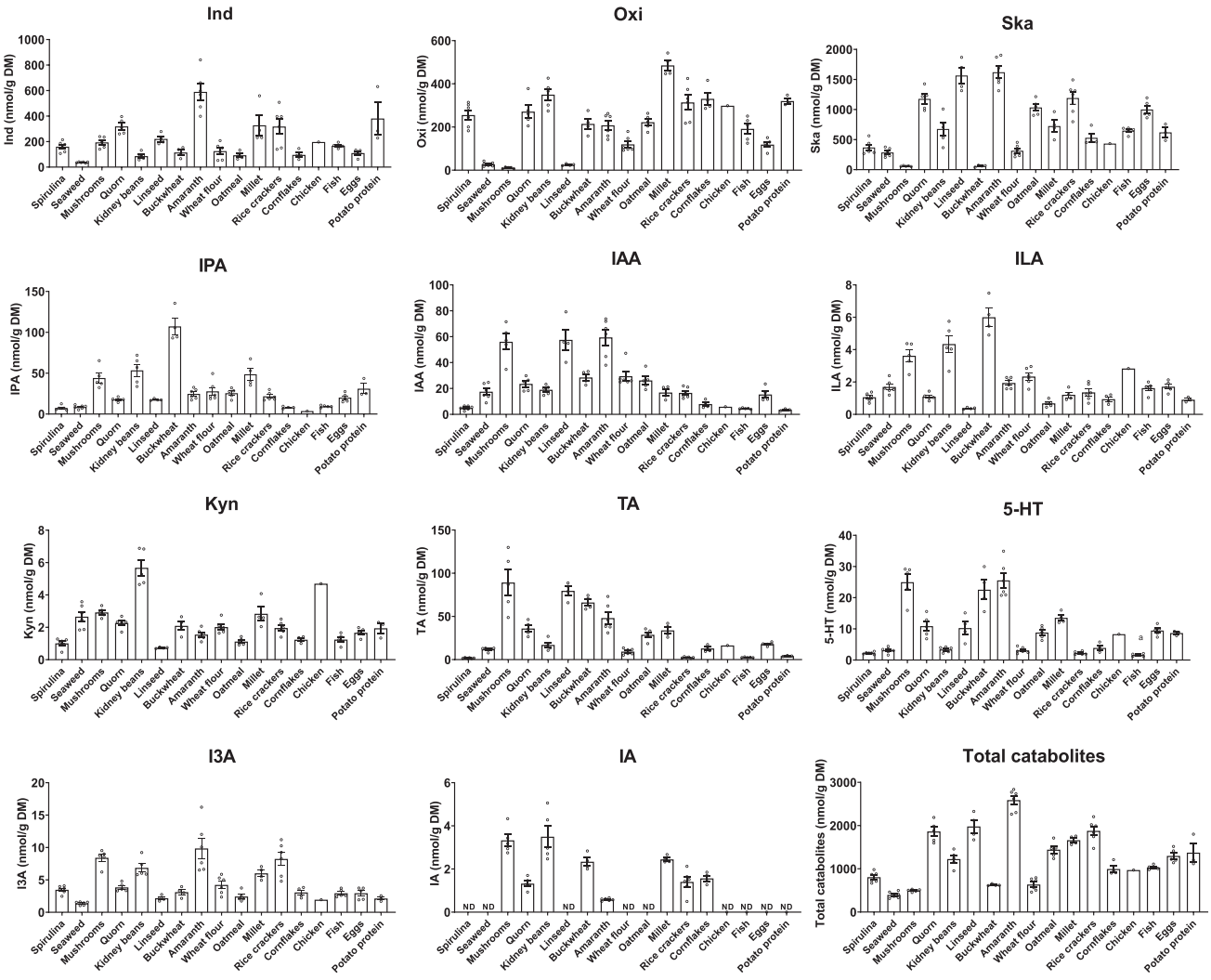


**Fig. 3.** Concentrations of microbiota-derived Trp catabolites produced from test diets in faeces of growing pigs. Data are based on dry matter (DM) and presented as mean ± SEM. ND: not detected. Ind: indole; Oxi: oxindole; Ska: skatole; IPA: indole-3-propionic acid; IAA: indole-3-acetic acid; ILA: indole-3-lactic acid; Kyn: kynurenine; TA: tryptamine; 5-HT: serotonin; I3A: indole-3-aldehyde; IA: indoleacrylic acid.

and Kyn showing antagonistic effects and TA and Ser showing agonistic effects in a dose-dependent manner.

### 3.5. AhR activation of ileal and faecal samples

The presence of AhR active Trp catabolites within the ileal digesta and faeces of pigs prompted us to examine their capacity to stimulate activity in an AhR reporter cell line. In the cellular assay, ileal and faecal samples were diluted 200 times to avoid cytotoxicity, but ileal samples from pigs fed with linseed still had a high cytotoxicity leading to cell inactivation (Fig. S1). As shown in Fig. 6, all ileal extracts failed to elicit a significant activation of AhR on the reporter cell line, but many faecal extracts significantly activated the AhR, showing a more than two-fold activation over the negative control (test medium).

## 4. Discussion

Gut microbiota-derived metabolites are able to promote host health, especially those produced from Trp with the capability to activate the AhR signalling in the intestine (Gutiérrez-Vázquez & Quintana, 2018; Lamas et al., 2018). Therefore, in this study, we used growing pigs as a model to investigate the concentration of Trp catabolites present in the ileum effluents and faeces upon feeding with different human dietary protein sources, and measured the AhR activity triggered by ileum effluents and faeces of pigs using a reporter cell line.

Small intestine is the most important site for nutrient digestion and absorption in the body, where Trp is released from hydrolysis of proteins by digestive enzymes and absorbed by the intestinal epithelium for peptide synthesis and endogenous metabolism (Gao et al., 2018; Snook & Meyer, 1964). This study shows that small bowel microbiota can compete with epithelial cells for Trp and generate several Trp catabolites, consistent with our previous findings in human ileal fluid samples (Koper et al., 2020). Bacterial genera commonly found in the small intestine of pigs include *Streptococcus*, *Clostridium*, and *Lactobacillus* (Crespo-Piazuelo et al., 2018). Several species from *Clostridium* are able to convert Trp into Ind, IAA, ILA, IPA, and TA, and some *Lactobacillus* spp. have a capacity to produce I3A and ILA (Roager & Licht, 2018). Since the microbial load in the small intestine is much lower than the colon (Sender, Fuchs, & Milo, 2016), and the transit time of food in the small intestine is shorter than the large intestine, it is not surprising to find lower concentrations of Trp catabolites in ileal digesta compared to faeces, except for ILA. This suggests that small intestine may be the main location for the microbial biosynthesis of ILA.

Given the extensive metabolic capacity of colonic microbiota

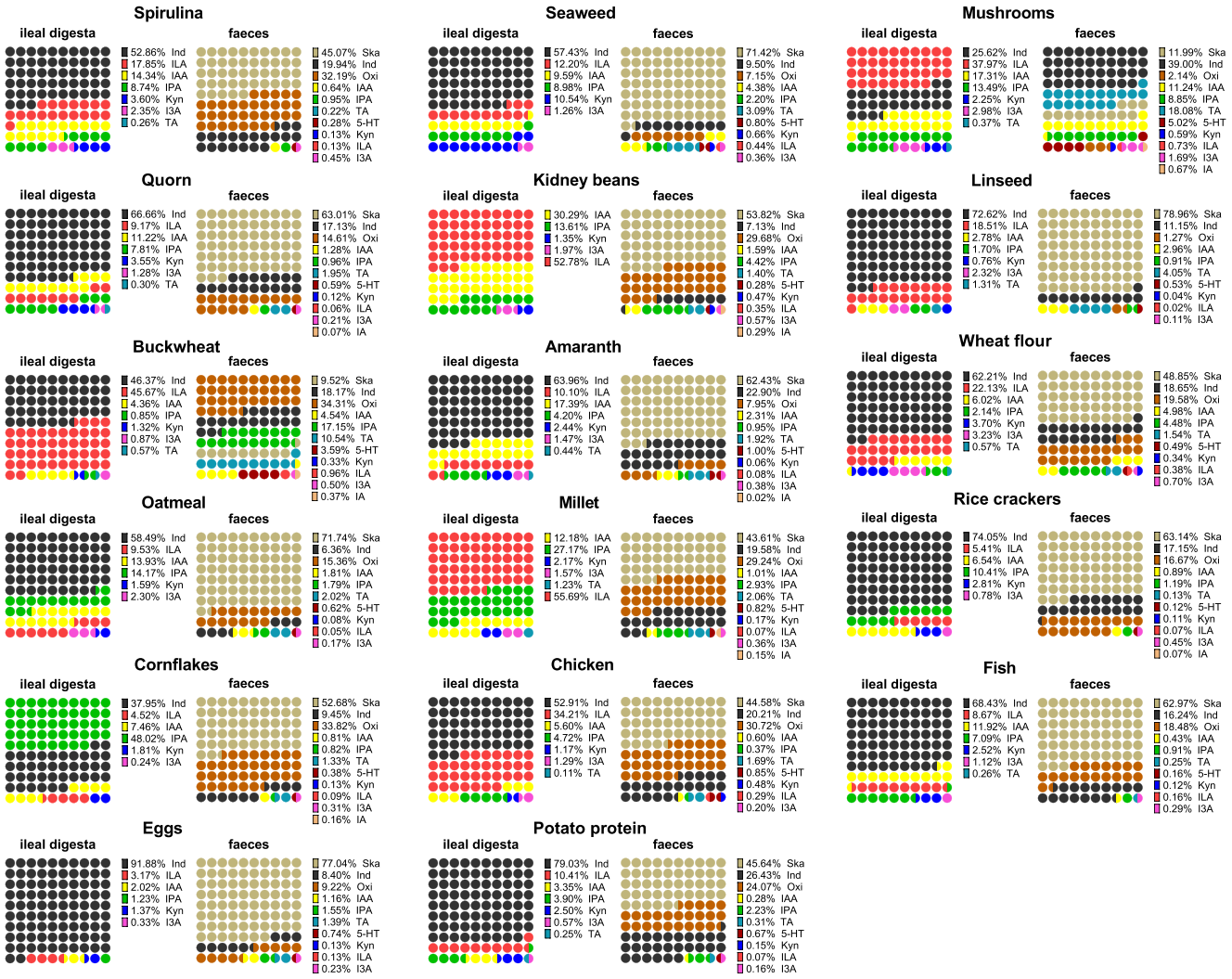


**Fig. 4.** Relative abundance of individual catabolites to the sum of all identified Trp catabolites in ileal digesta and faeces of growing pigs fed with different test diets. The dot plot only displays the major catabolites; the corresponding average abundances of all the pigs are indicated in the legend. Ind: indole; Oxi: oxindole; Ska: skatole; IPA: indole-3-propionic acid; IAA: indole-3-acetic acid; ILA: indole-3-lactic acid; Kyn: kynurenine; TA: tryptamine; 5-HT: serotonin; I3A: indole-3-aldehyde; IA: indoleacrylic acid.

(Martinez-Guryn, Leone, & Chang, 2019), a more diverse panel of Trp catabolites was observed in faeces compared to ileal digesta of pigs, in which Ska, Oxi, IA, and 5-HT were only identified in faeces. Different to human stool dominated by Ind (Dong et al., 2020), Ska was the major Trp catabolite in the faeces of pigs, which is produced by a few species mainly from *Clostridium* and *Bacteroides* genera via decarboxylation of IAA (Zamaratskaia & Squires, 2009). Oxi can also be measured in human stool at a concentration of 2–800 nmol/g (Dong et al., 2020). 5-HT together with Kyn are endogenous Trp metabolites, but recent studies suggest that they can also be produced by gut microbiota (Krautkramer, Fan, & Bäckhed, 2021; Valles-Colomer et al., 2019), which is further supported by our data in pigs.

As expected, feeding pigs with different food matrices induced different concentrations of microbiota-derived Trp catabolites in ileal digesta and faeces of pigs. The test diets were standardized for proteins which results in pigs received different amounts of Trp (Table S2) and the bioaccessibility of Trp to intestinal microorganisms is affected by the food microstructure (Aguilera, 2019). We speculated that the overall concentration of Trp catabolites in the intestinal lumen of small intestine and colon would be proportional to the available content of Trp to microbes which is determined by the Trp content in each diet and the Trp digestibility. We indeed found significant positive correlations between the determined content of Trp available to microbiota and the concentration of total Trp catabolites in ileal digesta of pigs ($r = 0.4893$, $p = 0.0462$), as well as the faeces ($r = 0.5845$, $p = 0.0137$) (Fig. S2). This hypothesis is further supported by the relatively lower concentration of total Trp catabolites in ileal digesta of pigs fed with most of the plant-based foods with determined low Trp digestibility compared to animal foods (Table S3), in which an intact plant cell wall and the presence of anti-nutritional compounds protect the intracellular protein against digestive enzymes in the small intestine (Capuano & Pellegrini, 2019). Food processing can improve the digestibility of plant proteins (Sá, Moreno, & Carciofi, 2020), and therefore increase the availability of Trp to small bowel microbiota. An extreme example was shown in pigs fed with potato protein having a high Trp digestibility and a high concentration of total Trp catabolites in the ileal digesta.

The content of Trp reaching the colon is negatively correlated to the Trp digestibility. The low Trp digestibility of unprocessed plant-based foods in the small intestine suggests that they could be suitable vectors for delivering Trp to the colon and producing Trp catabolites. However, we noticed that pigs feed with some plant-based foods, like seaweed and mushrooms, presented a low concentration of total Trp catabolites in the faeces. This could be due to that colonic microbiota preferentially utilizes fermentable carbohydrates over proteins as their

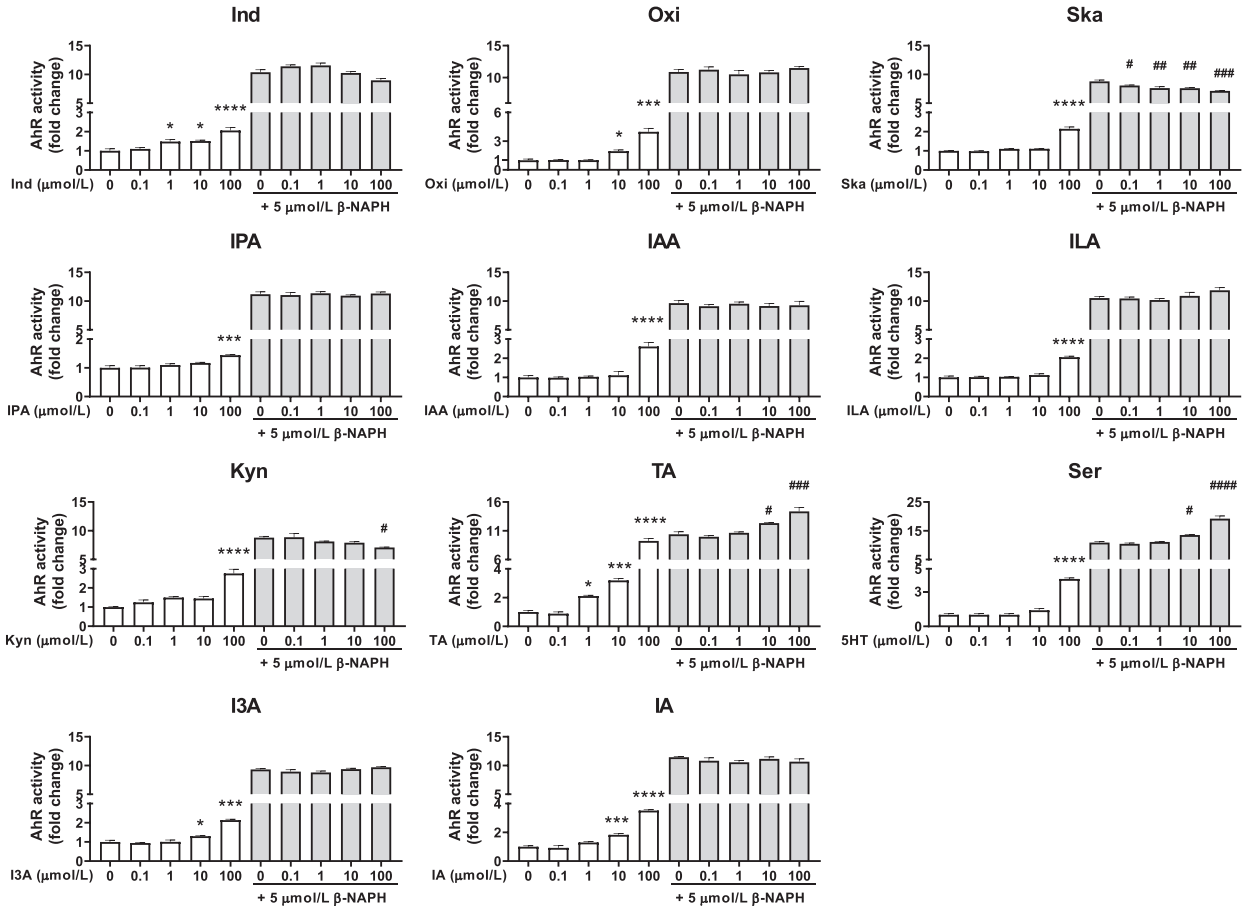


**Fig. 5.** AhR activation of microbiota-derived Trp catabolites. HepG2-LuciaTM cells were treated for 48 h with increasing concentrations of each catabolite in the absence (white bars) or presence (grey bars) of β-naphthoflavone (β-NAPH). Experiments were performed in two consecutive cell passages in triplicate. Results were expressed as luciferase fold change over 1% (v/v) DMSO (vehicle). Date are presented as mean ± SEM and analysed by one-way ANOVA with a Tukey post-hoc test. Significance is reported as */# $p < 0.05$, **/## $p < 0.01$, ***/### $p < 0.001$, and ****/#### $p < 0.0001$. Ind: indole; Oxi: oxindole; Ska: skatole; IPA: indole-3-propionic acid; IAA: indole-3-acetic acid; ILA: indole-3-lactic acid; Kyn: kynurenine; TA: tryptamine; Ser: serotonin; I3A: indole-3-aldehyde; IA: indoleacrylic acid.

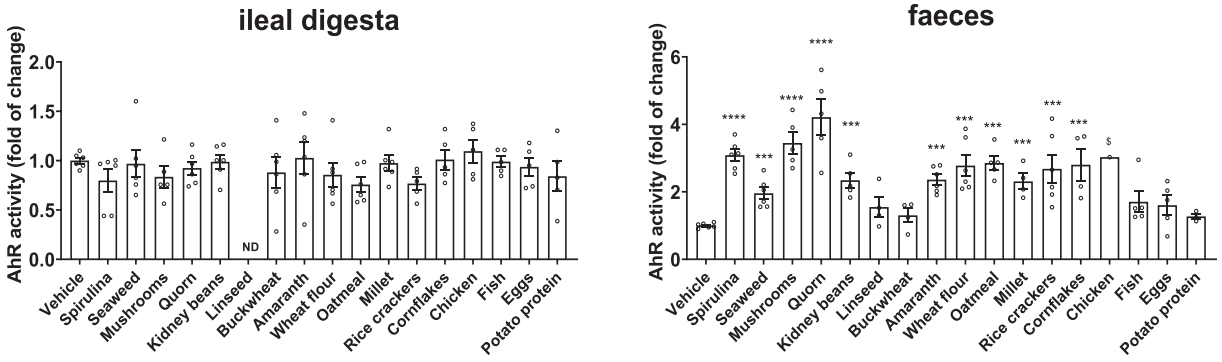


**Fig. 6.** AhR activation of ileal digesta and faeces of growing pigs. HepG2-LuciaTM cells were treated for 48 h with extracts from ileal digesta and fecal samples of pig after pre-treatment. Experiments were performed in two consecutive cell passages in triplicate. Results were expressed as luciferase fold change over cell test medium (vehicle). Data are presented as mean ± SEM. A Student's *t*-test was performed to determine significances between each treatment group of test diets and vehicle (test medium), except chicken diet ($) due to limited observations. Significance is reported as * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, and **** $p < 0.0001$. ND: not detected.

energy source (Oliphant & Allen-Vercoe, 2019), which is proved by the increased protein content in the faeces of pigs fed with plant-based foods after colonic fermentation (Table S4). This suggests that dietary fibers, especially fermentable fibers in plant-based foods may negatively affect the microbial production of Trp catabolites by reducing the protein fermentation in the colon. Despite a range of commensal species can secrete carbohydrate-active enzymes to utilize polysaccharides in the plant cell wall (Flint, Scott, Duncan, Louis, & Forano, 2012), we also found that the level of integrity of a plant matrix (i.e. the level of integrity of a cell wall) may reduce the amount of Trp catabolites from dietary substrates (Huang et al., 2021). This may reduce the actual accessibility of colonic microbiota to dietary Trp in some of the plant-

based foods tested here.

Apart from the effect of the food matrix on the amount of Trp available in the intestinal lumen, food components can modulate the gut microbiota composition, which may influence the microbial catabolism of Trp in the intestine. Although few studies have addressed how dietary shifts influence the microbiota composition and function in the small intestine, the distinct panel of Trp catabolites across different test foods in ileal digesta of pigs suggests that small bowel microbiota is likely very responsive to dietary perturbations, which influences the microbial production of Trp-derived catabolites in the small intestine. A recent study showed that a peptide-rich diet can enrich *Lactobacillus* species in the porcine small intestine compared to a protein-rich diet (Jing et al., 2022). A high-fat diet can increase the relative abundance of the family Clostridiaceae in the ileum of mice (Martinez-Guryn et al., 2018). Dietary fiber resists digestion in the small intestine, but in the colon, it can be fermented by colonic microbiota and increases the populations of fiber-degrading bacteria (Flint et al., 2012), some of which may have the capacity to catabolize Trp (Roager & Licht, 2018). Previously, we demonstrated that fiber supplementation (i.e. pectin and inulin) can promote the microbial production of IPA, IAA, and ILA by human gut microbiota (Huang et al., 2023). We also observed that pectin and inulin differently modulated the microbial catabolism of Trp, in which pectin specifically promoted the microbial production of I3A (Huang et al., 2023). Collectively, these observations suggest a combined effect of food matrix and gut microbiota in producing the differences in the concentration of Trp catabolites in the intestine.

The most documented beneficial effect of Trp catabolites is their capacity to activate the AhR (Lamas et al., 2018). Trp itself was reported being unable to activate AhR (Opitz et al., 2011), but it is an important precursor to endogenous AhR activators, especially those produced by gut microbiota, which is supported by our data in the reporter cell line. TA was the most AhR-active catabolite among others, but it was only present in low concentrations and mainly in the faeces. To understand the AhR activation induced by Trp catabolites within a physiologically relevant context, we quantified the AhR activation of ileal digesta and faecal samples. However, we failed to observe a significant activation of AhR when the reporter cell line was exposed to ileal extracts. One possible explanation is that the dilution of the ileal samples to avoid cytotoxicity reduced the concentration of Trp catabolites below the threshold concentration needed to activate AhR *in vitro*. Many faecal extracts were able to elicit a significant activation of AhR on the reporter cell line. We speculated that this could be due to the high concentration of Trp catabolites in faecal extracts. However, the spearman rank correlation analyses between the concentration of total as well as individual Trp catabolites and the AhR activity of faecal extracts appear to contradict this notion, in which we only found a positive correlation between I3A and AhR activity ($r = 0.2991$, $p = 0.007$) (Table S5). This suggests the presence of other molecules in faecal extracts that can modulate the AhR activation on the reporter cell line. We previously showed that propionate and butyrate retained AhR activity in the reporter cell line and have synergistic effects on the AhR activation (Huang et al., 2021). Additionally, we also cannot rule out the possibility that unknown inhibitors of AhR activity are similarly present in faecal extracts.

## 5. Conclusion

In conclusion, this study provides the first quantitative assessment of the levels of food-derived Trp catabolites in ileal digesta and faeces of pigs during the passage of a panel of whole foods along the intestine. The small intestine is known for nutrient digestion and absorption, but this study shows that, in pigs, the microbiota in this segment plays a role in the catabolism of dietary Trp to produce AhR active Trp catabolites and it is potentially sensitive to dietary pressures. Food selection aiming to promote the production of Trp-derived AhR ligands in the intestine should consider the intestinal segment and dietary factors influencing the microbial catabolism of Trp, specifically the amount of Trp available in the intestinal lumen and microbiota composition and function. Together, this study has important implications in the design of dietary approaches targeting the microbial production of Trp-derived AhR ligands in the intestine.

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## CRediT authorship contribution statement

**Zhan Huang:** Conceptualization, Methodology, Investigation, Formal analysis, Writing – original draft, Writing – review & editing. **Sonja de Vries:** Formal analysis, Writing – review & editing. **Vincenzo Fogliano:** Writing – review & editing, Supervision. **Jerry M. Wells:** Writing – review & editing, Supervision. **Nikkie van der Wielen:** Writing – review & editing. **Edoardo Capuano:** Writing – review & editing, Supervision.

## Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

## Data availability

Data will be made available on request.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodchem.2023.135804.

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