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Special Topic: Dietary Amino Acids and Intestinal Microbiota

Title: Emerging Effects of Tryptophan Pathway Metabolites and Intestinal Microbiota on Metabolism and Intestinal Function

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Abbreviations:

3-HK 3-hydroxykynurenine; AADAT, aminoadipate aminotransferase; ACMSD, aminocarboxymuconate semialdehyde decarboxylase; AhR, aryl hydrocarbon receptor; DDC, DOPA decarboxylase; FITC, fluorescein isothiocyanate; GF, Germ-Free; HFD, high fat diet; IDO, I3AA, indole-3-acetic acid; IDO, indoleamine 2,3-dioxygenase; IFN-γ, interferon γ; IPA, indole-3 propionic acid; ISA, indoxyl-3-sulfuric acid; KA, kynurenic acid; KAT, Kynurenine aminotransferase/cysteine conjugate beta-lyase (CCBL); KMO, kynurenine 3-monooxygenase; KYN, kynurenine; kynA, tryptophan 2,3-dioxygenase (bacterial); KYNU, kynureninase; NAD, nicotinamide; RFU, relative fluorescence units; T2DM, type 2 diabetes; tam1, tryptophan amino transferase 1 (bacterial); TDO, tryptophan 2,3-dioxygenase; tnAa, tryptophanase (bacterial); TNF-α, tumor necrosis factor-α; TPH1, tryptophan hydroxylase 1; TTX, tetrodotoxin; XA, xanthurenic acid

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Abstract:

The metabolism of dietary tryptophan occurs locally in the gut primarily via host enzymes, with ~5% metabolized by gut microbes. Three major tryptophan metabolic pathways are serotonin (beyond the scope of this review), indole, kynurenine and related derivatives. We introduce the gut microbiome, dietary trypophan and the potential interplay of host and bacterial enzymes in tryptophan metabolism. Examples of bacterial transformation to indole and its derivative indole-3 propionic acid demonstrate associations with human metabolic disease and gut permeability, although causality remains to be determined. This review will focus on less well-known data, suggestive of local generation and functional significance in the gut, where kynurenine is converted to kynurenic acid (KA) and xanthurenic acid (XA) via enzymatic action present in both host and bacteria. Our functional data demonstrate a lack of effect on intestinal epithelial cell monolayer permeability and in healthy neurogenically-mediated secretion in mouse ileum. Other data suggest a modulatory effect on the microbiome, potentially in pathophysiology. Supportive of this, we found that the expression of mRNA for three kynurenine pathway enzymes were increased in colon from high fat-fed mice, suggesting that this host pathway is perturbed in metabolic disease. These data, along with bacterial genomic analysis and germ-free mice, confirms expression and functional machinery of enzymes in this pathway. Therefore, the host and microbiota play a significant dual role in either the production or regulation of these kynurenine metabolites which, in turn, can influence both host and microbiome, especially in the context of obesity and intestinal permeability.

Introduction: Tryptophan and the Microbiome:

The metabolism of dietary tryptophan occurs locally in the gut, although most tryptophan is absorbed by the small intestine for enzymatic transformation in the liver, or elsewhere. In humans, protein can 'escape' into the large intestine (Evenepoel et al. 1999) providing an energy source for the colonic microbiome. Overall, it has been estimated that ~5% of dietary tryptophan is metabolized by gut microbes, and pathway analysis has identified specific resident bacteria that can metabolize aromatic amino acids, including tryptophan (Dodd et al. 2017; Gao et al. 2018).

The human microbiota is a community of bacteria, archaea, protists, fungi and viruses that live in and on the human body (Group et al. 2009). The gut microbiota comprises over 5000 bacterial species and 3 million genes in a typical individual (Frank et al. 2007; Pasolli et al. 2019). In the gut, it is dominated by the phyla Firmicutes and Bacteroidetes (Eckburg et al. 2005; Quigley 2013). The composition, however, changes from the small intestine, which is enriched with Bacillus and Actinobacteria, distal toward the large intestine which is characterised by Bacteroidetes and Lachnospiraceae (Frank et al. 2007). Establishment of the early-life microbiota is informed by birth mode and is defined by Bifidobacterium species after vaginal delivery (Huurre et al. 2008). The microbiota then stabilizes at approximately 1–2.5 years of age (Voreades et al. 2014) and remains largely stable through adulthood but can be influenced by environmental factors including long-term dietary changes (Turnbaugh et al. 2008). Further changes to the microbiota are occur later in life, observed as a reduction in diversity and an increase in enteric bacteria which may be associated with age-related physiological decline (Turnbaugh et al. 2008; Claesson et al. 2012)

In the next section we will briefly describe the effects of tryptophan deficiency on subsequent production of catabolites. The contribution of the host intestinal enzymes to derivatives of serotonin, indole, and kynurenine pathways is well known. The host metabolizes tryptophan to serotonin via tryptophan hydroxylase 1 (TPH1) expressed in enterochromaffin cells, reviewed in (Grifka-Walk et al. 2021). Since serotonin has been well-studied in gut physiology, with links to metabolic disease (Jones et al. 2020), it is considered too large a topic for the scope of the current review. However, we will cover the recent studies on the indole pathway where bacterial enzymes are largely responsible for several indole metabolites. These insights are part of the reason for increasing interest and research on the contributions of microbially-derived, alone or in combination with host-derived, tryptophan metabolites and intestinal function. The rest of the review will focus on the recent data in less well-studied metabolites of the kynurenine pathway – Kynurenic Acid (KA) and Xanthurenic Acid (XA) and any associations these have locally with gut permeability and metabolic diseases.

Dietary Tryptophan

Tryptophan is a key intra-kingdom signalling molecule that can benefit both host and bacteria, as recently reviewed (Grifka-Walk et al. 2021). The guideline for human tryptophan intake is 250-425 mg daily but adults typically ingest 1000 mg or more each day (Sainio et al. 1996). Recent reviews have covered the effects of tryptophan, for example, on mood disorders, or altered intestinal absorption of nutrients due to pathogenic bacteria (Peng et al. 2020). Mice given a tryptophan-depleted diet showed neuronal inflammation in disease models due to microglial activation and cytokine production (Rothhammer et al. 2018). Feeding aging mice a low tryptophan diet reduces intestinal bacterial diversity, as well as elevating circulating pro-inflammatory cytokine levels (Yusufu et al. 2021). In contrast, tryptophan-rich diets may have a protective role in the gut epithelial layer; for example, by preventing chemically induced in a mouse colitis model (Islam et al. 2017).

Dietary tryptophan is required to maintain gut health and intestinal immunity and increased kynurenine metabolites have been associated with host inflammatory responses, neurological disorders, and immunity regulation (Gao et al. 2018). There are examples where intestinal bacteria and host locally compete enzymatically for tryptophan, which influences host metabolic and intestinal function (Roager & Licht 2018). Gut bacteria can alter the rate and availability at which tryptophan metabolism occurs through the kynurenine pathway and this can both protect the host from excess tryptophan, as well as produce beneficial kynurenine metabolites (Badawy 2017). Locally in the intestine, upregulation of an enzyme diverting tryptophan's catabolites down the kynurenine pathway modulates the immune response to infection and serves to increase local tryptophan deprivation in the intestine (Zelante et al. 2021).

In healthy subjects, acute tryptophan depletion decreases circulating tryptophan and kynurenine levels concomitantly (Kennedy et al. 2015), though the downstream consequences on kynurenine metabolites were not established. In another healthy human study, acute tryptophan depletion reduced plasma serotonin levels, but was without effect on duodenal mucosal kynurenic acid (KA) levels, which is downstream in the kynurenine pathway (Keszthelyi et al. 2012). In the same study there were no changes in gut permeability (by differential sugar absorption), or in the expression of genes involved in maintaining intestinal barrier integrity (Keszthelyi et al. 2012). Notably, assessment of gut barrier function was carried out during the period of divergence in plasma KA profile between control and experimental subjects. The results suggest that local, rather than plasma KA levels, may perhaps play a more important role gut barrier function.

Enzymes Involved in Kynurenine Pathway Metabolites

About 90% of tryptophan is metabolised along the kynurenine pathway. Of two enzymes transforming tryptophan to kynurenine, indoleamine 2,3-dioxygenase (IDO) is expressed ubiquitously, with highest activity detected in intestine, lung, and spleen (Kudo & Boyd 2000) whereas tryptophan 2,3-dioxygenase (TDO) is expressed in the liver (Roth et al. 2021) and brain (Badawy 2017). Currently there are two known forms of IDO, IDO1 and IDO2 with evidence for an immunoregulatory function under certain conditions (Badawy 2017; Van der Leek et al. 2017). Intestinal IDO conversion to kynurenine is induced by cytokines, such as interferon γ (IFN-γ), tumor necrosis factor-α (TNF-α) and lipopolysaccharide (LPS) and in metabolic diseases and chronic inflammation, IDO1 is upregulated in (Oxenkrug 2010; Ciorba 2013), whereas anti-inflammatory cytokines decrease IDO expression (Badawy 2017). This upregulation may protect the host by creating a local tryptophan deficiency as well as having direct cellular effects through kynurenine and its metabolites (Stone 2016).

Tryptophan entering the kynurenine pathway leads ultimately to the synthesis of nicotinamide (NAD) and other bioactive molecules, including KA and Xanthurenic Acid (XA) (Tanaka et al. 2021). Kynurenine aminotransferase (KAT) activity results in the production of KA while the activity of kynurenine 3-monooxygenase (KMO) drives production of 3-hydroxykynurenine (3-HK), which can be further metabolised by KATs to XA. Both kynurenine and 3-HK can be transaminated to KA and XA although there are four isoforms of KAT and they exist in different organs and across species. KAT activity can be enhanced after inhibition of KMO (Badawy 2017). Alternatively, 3-HK is metabolized by kynureninase (KYNU) to quinolinic acid, which subsequently enters the NAD+ pathway (Maddison et al. 2020; Tanaka et al. 2021). This pathway from dietary tryptophan via quinolinic acid to NAD+ is the de novo synthetic pathway, but there are also salvage pathways to generate NAD+ (Yang & Sauve 2016). Several key bacterial and host enzymes contributing to the derivatives in the kynurenine pathway are illustrated (Figure 1).

In the gastrointestinal tract, KA has been detected in the mucus of healthy human subjects (Walczak et al. 2011). This, combined with the presence of KATI and KATII protein in human epithelial cell lines (Walczak et al. 2011) further supports the presence of local host-derived source of kynurenine metabolites in the gastrointestinal tract. Moreover, the systemic activity of the kynurenine pathway appears to be influenced by the intestinal microbiome (Gheorghe et al. 2019). For example, in male germ-free animals hippocampal KAT1 expression was reduced, and a number of micro RNAs involved in the regulation of the kynurenine pathway influenced by the colonisation status of these mice (Moloney et al. 2017).

The enzyme KMO synthesizes the metabolite 3-HK and regulates the balance between several neuroactive metabolites, which has made it a popular target for potential therapeutic agents for neurodegenerative diseases (Maddison et al. 2020). Kynureninase hydrolyses 3-HK to 3-hydroxyanthranilic acid, and kynurenine to anthranilic acid. *Escherichia coli*, which express KAT, are capable of increasing kynurenine pathway derivatives within gut (Han et al. 2001). The activity of IDO appears to be influenced by *Lactobacilli* in particular (Gheorghe et al. 2019). There also appears to be a degree of interplay between diet, IDO and the microbiome, with HFD fed IDO knockout mice exhibiting a Firmicutes/Bacteroidetes ratio more like wildtype mice maintained on a control diet but dissimilar to that of IDO knockout mice maintained on the same control diet (Laurans et al. 2018). These data would suggest, therefore, that under normal feeding conditions, tryptophan metabolism along the kynurenine pathway, and derivatives, can shape the 'normal' gut microbiome, and that diet-induced perturbations on the microbiome are also IDO dependent.

Bacterial- and Host-derived Indoles in Intestinal and Metabolic Function

Three main bacterial pathways are involved in intestinal microbiome–derived tryptophan metabolism: the tryptophan-indole pathway, tryptophan--indole-3 propionic acid (IPA) pathway and tryptophan--indole-3-aldehyde pathway, recently reviewed (Li et al. 2021b). The bacterial enzyme tryptophanase (tnAa) converts dietary tryptophan into indole, which has been demonstrated in over 85 species, and includes *Escherichia coli* (Lee & Lee 2010). Indole can be converted to tryptamine by either bacterial e.g. *Ruminococcus gnavus* and *Clostridium sporogenes*, or mammalian decarboxylases (Williams et al. 2014). In conventional mice, tryptamine concentration in feces is 200% greater than germ free mice (Marcobal et al. 2013). *Escherichia coli* (Bansal et al. 2010) and a higher prevalence of *E.coli*, *Bacillus spp*., and *Clostridium spp* is associated with higher fecal indole concentrations, ranging from high µM to low mM (Chappell et al. 2016). Indole in the intestine increased tight junction protein expression and epithelial resistance *in vitro*, consistent with a beneficial effect in response to pathogens (Bansal et al. 2010). Indole is a signalling molecule in intestinal-liver axis and appears to directly act in the liver to reduce hepatic damage and associated inflammatory response in genetically obese mice (Knudsen et al. 2021).

Tryptophan-catabolizing bacteria producing indole-derivatives include those from the genera *Anaerostipes, Bacteroides, Clostridium, Bifidobacterium and Lactobacillus* (Roager & Licht 2018). Both bacterial and mammalian enzymes are required to convert indole to indoxyl-3-sulfuric acid (ISA) and indole-3-acetic acid (I3AA); however, microbial metabolism alone converts indole to indole-3 propionic acid (IPA) (Wikoff et al. 2009). A simplified diagram of bacterial and host enzymes in this pathway is shown (Fig. 1).

Indole derivatives mediate biological activities partly through both the aryl hydrocarbon receptor (AhR) and the Pregnane X Receptor (PXR). For example, the potential anti-inflammatory mechanisms of IPA are due in part to activation of AhR (Zelante et al. 2013; Hubbard et al. 2015) and it regulates intestinal barrier function partly via activating PXR (Venkatesh et al. 2014). When given unrestricted tryptophan availability, species of *Lactobacilli* increase and produce an indole derivative that locally activated AhR-dependent IL22 transcription (Zelante et al. 2013).

Oral administration of IPA functionally improved the intestinal permeability in mice fed with HFD (Jennis et al. 2018). IPA-treated mice that were exposed to radiation injury had improved gastrointestinal function and epithelial barrier function (Xiao et al. 2020). Circulating IPA levels are reduced in patients suffering from Inflammatory Bowel Disease and increased levels are associated with remission (Alexeev et al. 2018). Dosing mice with *C. sporogenes*, or oral supplementation with IPA, is anti-inflammatory and improves intestinal barrier function in germ-free mice (Venkatesh et al. 2014).

Bacterial-derived indoles protected against cytokine-induced changes in permeability in a mouse model of colitis and *in vitro* (Scott et al. 2020). Moreover, indole reduces intestinal mucosal inflammation (Whitfield-Cargile et al. 2016) and after long term exposure, glucagon-like peptide 1 release (Chimerel et al. 2014). Interestingly, in a cohort of Finnish subjects with T2D, higher serum IPA levels were associated with better insulin secretion, possibly through the preservation of β-cell function (de Mello et al. 2017). In obese individuals, inflammatory markers are elevated and correlated with a decrease in indole production (Cussotto et al. 2020). The levels of microbially-derived IPA are reduced in individuals with Type 2 Diabetes (T2DM) in Finland (Tuomainen et al. 2018) and in morbidly obese patients with T2DM in US who underwent Roux-en-Y gastric bypass surgery (Jennis et al. 2018). In the latter study, by three months after gastric bypass surgery, circulating IPA levels were significantly higher than the pre-surgery baseline of these T2DM obese patients (Jennis et al. 2018) although a correlation analysis between extent of weight loss and IPA levels was not reported. Bariatric surgery in morbidly obese patients itself changes gut microbiota composition (Ejtahed et al. 2018), though concurrent dietary changes confound the relationship between surgery, microbiome and the altered production of indoles. In any event, beneficial effects include reduced endotoxin signalling and improved intestinal permeability (Tuomi & Logomarsino 2016).

In rodent diet-induced models of obesity and diabetes, oral supplementation with IPA reversed the worsened intestinal permeability, reduced metabolic endotoxemia, reduced weight gain, or improved glucose homeostasis (Abildgaard et al. 2018; Jennis et al. 2018; Konopelski et al. 2019). Increased IPA was also noted in genetically obese mice but supplementation with IPA in diet induced

obesity did not reverse the increased liver triglycerides and makers of inflammation (Lee et al. 2020). Although supplementing the diet with IPA in diet-induced obese models may not always be sufficient to reverse disease (Lee et al. 2020) there is increasing interest in enhancing the production of indole derivatives. For example, there are novel probiotics that may have beneficial effects through modulating the composition of the microbiome and the tryptophan metabolites produced. For example, *Bacillus sp*. DU-106 supplementation changed indole derivative profiles, decreased the body weight, liver index, and total cholesterol in high-fat diet rats (Huang et al. 2021). An indole derivative from *Bifidobacterium infantis* has anti-inflammatory effects in the intestine (Meng et al. 2020; Walker & Meng 2020).

The above summarizes some of the evidence for beneficial effects of tryptophan via indole- and derivative producing bacteria associated with disease pathologies, and some functional effects to account for these, including improvements in intestinal permeability. This provides the context for the focus of the rest of this review on new evidence for kynurenine derivatives, and their effects on metabolic function and gut physiology. This has generally been less well-studied but, based on the emerging literature, may be also influenced by host gut and bacterial metabolism.

Kynurenine Pathway Host and Microbiome Derivatives in Metabolism

In silico analysis of bacterial genomes and publicly available microbiome data identified microbial tryptophan metabolism pathways, including those involved in the production of kynurenine (Kaur et al. 2019). Some bacteria express a version of the TDO and kynurenine formamidase, termed *kynA* and *kynB*, respectively (Kaur et al. 2019). Computational analysis of 8392 bacterial genomes revealed that at the phylum level Bacteroidetes, Actinobacteria, and Proteobacteria were enriched for the kynurenine pathway, while at the genus level, *Bacteriodes, Candidatus, Paenisporosarcina, Ralstonia, Serratia, Burkholderia, Bacillus, Streptomyces, Pseudomonas, Staphylococcus* and *Providencia* were identified to have a probable role in utilising tryptophan for kynurenine production (Kaur et al. 2019). Moreover, it has been functionally demonstrated that the pathogen *Pseudomonas aeruginosa* has the metabolic machinery to further metabolise kynurenine to KA (Bortolotti et al. 2016).

In germ-free (GF) mice, caecal KA levels were reduced and XA levels were below detectible levels, suggesting that the microbiota plays a significant role in either the direct or indirect production of these kynurenine metabolites (Dong et al. 2020). Both metabolites were also detected in human fecal samples, and, as in the mouse, these were proportionally less than indole levels (Dong et al. 2020). In antibiotic-treated GF mice, kynurenine was increased in both the serum and cecum, and cecal KA and XA levels were doubled (Zhu et al. 2021). Although a beneficial action of KA is

neuroprotective for glutamate excitotoxicity (Toth et al. 2021) , the functional significance of these local, luminal metabolites on the gut has yet to be fully explored, although some of our unpublished data are reported below.

Due to the evidence for KA actions in the gut to modulate inflammation (Kaszaki et al. 2012), we compared the effects of KA and XA on *in vitro* human colonic epithelial intestinal permeability. Intestinal cells (T84) were grown to polarized monolayers on transwells and kynurenine metabolites, KA and XA were assessed after 24 hour incubation on baseline and a cytokine-induced increase in permeability (IFN-γ and TNF-α both 5ng/mL). The concentrations of cytokines used were the same as used to enhance mouse GI permeability *ex vivo* (Jennis et al. 2018) and lower than used in human tissue *ex vivo* experiments designed to show an inflammatory-mediated effect of Toll-Like receptor 3 ligand on human bronchiole contractility (Cooper et al. 2009). Elevated circulating concentrations of TNF-α (range: 0 to <5 pg/mL range) and IFN-γ (range: 0 to <80 pg/mL) are associated with being overweight or obese (Koelman et al. 2019). Unfortunately, circulating levels may not accurately depict local levels in the gut. Tryptophan metabolites in human serum are typically in the range of 1-100 nM (Hu et al. 2017) and we chose concentrations of KA and XA based on X1, X10 or X100 fold more than circulating blood levels (Wishart et al. 2018) to approximate a range of possible gut luminal concentrations. As a positive control, the short chain fatty acid butyrate (0.5, 5.0 and 50 mM) reversed the cytokine-enhanced permeability in this assay, as did IPA (Jennis et al. 2018). After 20 hours FITC-dextran (MW 4kDa 1 mg/mL) was added to the mucosal side and relative fluorescence units (RFU) measured on the basal/serosal side of the transwell. Neither incubation of KA (Fig. 2A) nor XA (Fig. 2B) altered epithelial cell permeability at baseline, nor reduced the increased permeability after IFN-γ/TNF-α. At low nanomolar concentration, XA (2.4 nM) caused a modest increase in permeability in the presence of IFN-γ and TNF-α, but had no effect in the absence of these inflammatory cytokines. The effect of XA and KA on gene expression of selected cytokines and transporters was not investigated since previous attempts to identify pathways that might contribute to IPA reversal of IFN-γ permeability in these cells were not successful (Jennis et al. 2018).

Next, we explored the effect of KA and XA on short circuit current (Isc), a surrogate measure of neurosecretory activity, in *ex vivo* tissues to demonstrate any potential neural activity. All experiments were conducted in accordance with the European Directive 86/609/EEC, Recommendation 2007/526/65/EC, and approved by the Animal Experimentation Ethics Committee of University College Cork. Muscle-stripped ileal segments from healthy C57BL/6 mice were mounted in Ussing-Type Flux chambers. Mucosal side administration of KA (0.2 & 2μM) or XA (0.25 & 2.5μM) did not alter the Isc effect of serosal bethanechol (100 μM) or forskolin (10 μM), which are cholinergic calcium-mediated, and cAMP-mediated ion secretion activators, respectively (data not

shown). Neither mucosal KA nor XA alone altered Isc (Fig. C & D) or the conductance of the tissue (Fig. 2 E & F). The lack of effect on Isc indicates that neither metabolite influenced neurogenically-mediated ion secretion, and since there was no effect to abolish, the neurotoxin TTX did not alter the Isc in response to either metabolite (Fig. 2 C & D). Notwithstanding that there is a wide concentration range reported for these metabolites in mouse cecal contents (Dong et al. 2020; Zhu et al. 2021), the concentrations tested in our study are likely to be physiologically relevant. It may be that the metabolites did not reach sufficiently high enough levels of exposure on the serosal side where the majority of gut neuronal innervation is present.

Given that KA and XA can also activate receptors widely expressed throughout the enteric nervous system (see Table 1), and that the ENS exerts regulatory control over gut barrier function (Neunlist et al. 2003) , we also examined the effects of luminal KA and XA on tissue conductance in the presence of TTX (Fig. 2E-F). Luminal administration of KA and XA alone did not functionally affect conductance but in the presence of TTX, XA reduced the conductance relative to control (Fig. 2F). We could speculate that, in the absence of a potentially XA-mediated protective influence on conductance through enteric TTX-sensitive pathways, luminal concentrations of XA may influence conductance via neurogenic epithelial mechanisms. If the effect on tissue conductance are confirmed in in future studies, this could support XA having permeability effects in the nM range, which is likely too low to activate AhR. From the above initial experiments, we conclude that there is no effect of KA or XA on neurogenically-mediated secretion in healthy mouse ileum *ex vivo,* but additional work is necessary to demonstrate effects on permeability through epithelial neurogenic pathways that may be protective. Other studies report that neither KA nor XA influenced permeability in human epithelial cells, but XA appeared to increase cytokine-induced barrier disruption at a concentration which fails to elicit an AhR transcriptional response (Dong et al. 2020). This might suggest that augmentation of cytokine-induced disruption occurs independent of AhR, which otherwise would be protective when activated at higher concentrations. The absence of on effect on tissue conductance might also indicate that concentrations in excess of those tested in our study may be required to activate AhR (Dong et al. 2020). Alternatively, it may involve pathophysiological conditions since AhR activation *per se* does not appear to significantly influence gut barrier function in healthy animals, but appears to be physiologically relevant following high-fat feeding (Natividad et al. 2018).

In obese individuals, inflammatory markers are elevated and correlated with an increase in the tryptophan:kynurenine ratio (Cussotto et al. 2020). The levels of KA and XA are increased in the plasma of patients with T2DM (Oxenkrug 2015) and, in morbidly obese patients who undergo Roux-en-Y gastric bypass surgery, elevated KA levels plasma levels declined 1-year post-surgery (Favennec

et al. 2016). In mice fed a "Western" Diet, IDO levels are decreased in the small intestine (Ohland et al. 2016). Therefore, we investigated whether expression of kynurenine pathway enzymes are also altered in the colon of diet-induced obesity in mice. Expression of tryptophan metabolism enzyme mRNA was measured in full thickness distal colon from mice fed for 12 weeks either Low Fat Diet (10%kcal D12450B; n=9) or HFD (45% kcal D12451; N=11). Gene expression levels by RT-PCR were calculated as the average of three technical replicates for each biological sample, normalized to the reference gene (GAPDH), and then expressed relative to the vehicle-treated control for the following genes of interest in the tryptophan pathway: TPH1, KMO, CCBL1, AADAT, CCBL2 (KAT), IDO1, IDO2, KYNU. Fold changes were calculated using the double delta Ct (ΔΔCt) method and compared by students t-test for each gene relative to control. HFD fed mice had increased colonic gene expression of IDO1, KMO and KYNU (Fig. 2G). In contrast to the small intestine (Ohland et al. 2016), colonic IDO1 was increased in HFD- fed animals This increased expression of all three enzymes in the colon of obese HFD-fed mice in our study is supportive of enhanced host tryptophan-kynurenine pathway metabolism which may ultimately increase both 3-hydroxykynurine, quinolinic acid and NAD+.

The transcriptional changes we observed in the intestine of diet-induced obese animals, characterized by increased expression of IDO1, KMO and KYNU might also suggest that pathways involved in tryptophan metabolism are shunted toward kynurenine metabolism in obesity which may be protective by promoting local production of AhR ligands.

In GF mice, blood levels of kynurenine metabolites were lower despite higher tryptophan blood levels and when these mice were colonized with conventional-raised microbiota, the kynurenine:tryptophan ratio was increased (Clarke et al. 2013). This suggests that either the microbiota can directly generate kynurenine metabolites, or indirectly regulate the host pathways responsible for their generation. Gut bacteria encode the enzymatic machinery to metabolize tryptophan through the kynurenine pathway. Unfortunately, the diversity and complexity of the gut microbiota make it difficult to determine which tryptophan metabolites specific bacteria produce. One approach to get around this has been the analysis of genomic and proteomic annotations from UniProt Consortium to identify which bacterial genera have the catabolism machinery homologous to tryptophan catabolism. The majority of these reside within the phylum, Proteobacteria. Proteobacteria have been associated with dysbiosis in hosts with metabolic or inflammatory disorders (Vujkovic-Cvijin et al. 2013) and aging in C57Bl/6 mice (Wu et al. 2021). Furthermore, gut-resident bacteria with capacity to catabolize tryptophan through the kynurenine pathway were found to be enriched in HIV-infected subjects, strongly correlated with kynurenine levels in HIV-infected subjects and were capable of kynurenine production in vitro (Vujkovic-Cvijin et al. 2013).

Therefore, under such conditions, the microbiota may compete with the host for utilisation of tryptophan along the kynurenine pathway.

On the other hand, activity of the host kynurenine metabolic pathway may be increased by the gut microbiota. In a study of gluten-sensitive patients, duodenal biopsies had increased levels of IFN-γ after the subjects ate gluten-containing bread. It was suggested that the undigested gluten was a food source for the gut microbiota, leading to an increase in IFN-γ that would induce of IDO expression (Brottveit et al. 2013). Conversely, feeding rats with *Lactobacillus johnsonii* decreased ileum IDO mRNA levels and serum kynurenine concentrations; it also increased ileum H_2O_2 (an indicator of inhibition of IDO1) that would decrease kynurenine relative to serotonin (Valladares et al. 2013). Finally, IDO activity may itself regulate metabolic health by shaping the intestinal microbiota (Zhou et al. 2021). In a study performed in IDO knock out mice fed HFD, mice were protected against obesity as they exhibited a microbiome profile more similar to wildtype mice maintained on a control diet (Laurans et al. 2018).

To summarize, the microbiota plays a significant role in either kynurenine metabolite production, tuning of host metabolic pathways and modulation or perturbation of the microbiome, which itself can modify the fecal kynurenine metabolite profile. However, whether the inflammation is a characteristic of the underlying disease and results in activation of the tryptophan-kynurenine pathway, and whether this is a protective or detrimental response, remains to be fully determined. The topic of gut immunity is too broad for this review; however, in the section below we summarize the receptor mediated effects of KA and XA.

Source and Availability of Substrates:

As well as host- and possible microbiome-derived sources of KA, a large variety of foods contain KA with the highest concentrations found in bee propolis, pollen and honey (nanomolar) as well as in milk, yoghurts, meats and fruits, with vegetables having lower concentrations especially when cooked (Turski et al. 2009). Fermented foods were also found to contain KA (Yilmaz & Gokmen 2018). Dietary KA is bioavailable, since following intragastric gavage, KA was readily detectible in the circulation and tissues (Turski et al. 2009). Dietary modification can significantly influence the circulating levels of kynurenine and its metabolites, with caloric restriction reducing kynurenine, KA, XA and QA levels, although tryptophan levels were also reduced suggesting that these observations may relate to substrate availability (Heischmann et al. 2018). Moreover, a similar pattern, albeit to a greater extent, was also observed in response to a ketogenic diet in which carbohydrate intake is drastically reduced (Heischmann et al. 2018).

As noted above, a high-fat diet increased intestinal levels of kynurenine and this elevation in local kynurenine was absent in IDO knockout mice (Laurans et al. 2018). Xanthurenic acid levels were modified by diet such that volunteers eating *ad libitum* had higher fecal levels than those maintained on a defined diet (Dong et al. 2020). These studies would suggest that where energy intake is restricted, activity of the tryptophan-kynurenine pathway is reduced, whereas in response to increased energy intake in the form of a HFD the opposite occurs. However, the mechanisms by which this occurs are not understood. Such significant dietary changes are likely to impact the gut microbiome as well as the host directly. It is also unclear whether activation of the kynurenine pathway represents a pathophysiological response, or, perhaps, a beneficial one.

Eight-week tryptophan supplementation in mice was associated with a significant increase in kynurenine and associated metabolites, KA and QA (Valente-Silva et al. 2021) (PMID: 34436450). However, there was no significant impact of tryptophan supplementation on bodyweight, lean mass, fat mass or on adipose tissue expression of genes related to energy expenditure (Valente-Silva et al. 2021) (PMID: 34436450). In contrast, bariatric surgery-associated reductions in circulating kynurenine and downstream metabolites, KA, XA and QA were associated one year later with a significant reduction in body mass index and inflammatory markers (Christensen et al. 2018) (PMID: 29401505). Similarly, caloric restriction in overweight adults significantly reduced circulating tryptophan and kynurenine levels as well as body mass index but had no effect on inflammatory cytokines (Strasser et al. 2015) (PMID: 24687684). These data perhaps suggest that in the absence of obesity, tryptophan supplementation does not appear to significantly impact metabolic and inflammatory measures, irrespective of a change in circulating kynurenine metabolites, but that in the context of metabolic syndrome a reduction in kynurenine and kynurenine metabolites may be beneficial. Whether these effects are coincidental or are mechanistically linked warrants further investigation. Tryptophan supplementation is associated with significant changes in the gut microbiome, particularly influencing taxa with the ability to metabolize tryptophan via tryptophanase to indoles (Liang et al. 2018). However, it is not known whether kynA expressing bacteria respond in a similar manner to increased tryptophan availability with bacterial metabolism along the kynurenine pathway. Nonetheless, *Bacteroidetes*, a phylum with predicted kynurenine metabolic activity is increased in abundance in tryptophan supplemented piglets (Liang et al. 2018). Both tryptophan deficient and enriched diets similarly increased abundance of phyla with predicated kynurenine metabolic activity in aged mice, but no genera with proposed kynurenine metabolic activity were significantly influenced by tryptophan availability (Yusufu et al. 2021).

Supplementation of KA in HFD-fed mice reduced their body weight gain (Agudelo et al. 2018) suggesting that activation of the kynurenine pathway toward KA production could be a beneficial

host response to HFD. In adult rats no effect on body weight was observed (Turski et al. 2014), but the responses may depend on when supplementation begins. Decreased body weight resulted from chronic treatment initiated earlier in life (Tomaszewska et al. 2019). An obvious question is whether this was due to altered food intake or energy expenditure - in mice KA did not affect food intake but did increase energy expenditure and after prolonged administration the decrease in body weight was associated with reduced serum QA (Agudelo et al. 2018). Interestingly, in GPR35 deficient mice the same parameters were not affected by KA supplementation (Agudelo et al. 2018) suggesting GPR35 was responsible for KA effects.

Pharmacological Targets associated with Kynurenine Metabolites

In obese human subjects a negative correlation between AhR target gene expression and obesity-associated inflammation in the jejunum was observed. Furthermore, AhR appears to protect against gut epithelial barrier disruption (Postal et al. 2020). Again, it is difficult to interpret whether the elevations in KA in the context of obesity may be a protective response to restore gut barrier function in light of a decreased expression of its receptor, AhR. In support of such a concept, addition of relevant KA fecal concentrations, stimulated expression of AhR target genes in *in vitro* human epithelial cell line, while XA was without such an effect (Dong et al. 2020). KA also protected against HFD-induced dysfunction of the gut barrier *in vivo* and against cytokine-induced changes in epithelial resistance in vitro (Natividad et al. 2018). In addition, IDO prevented HFD-induced increases in permeability, suggesting that the kynurenine pathway plays are modulating the HFD-induced inflammatory response, since as IDO knockout *per se* did not affect barrier function (Laurans et al. 2018). These data may point to a role of kynurenine metabolites in the maintenance of the gut epithelial barrier which may only become apparent in a challenged system or as consequence of inflammation.

Kynurenine metabolites that act on targets modulating gastrointestinal neurotransmission (Kaszaki et al. 2012) are summarized in Table 1. Excitotoxicity has also been considered as a mechanism underlying HFD-induced enteric neuropathy. *In vitro* studies using ketamine (NMDA receptor antagonist) did not support this hypothesis (Voss et al. 2013), but the concept that neuroprotective kynurenine metabolites with activity at peripherally expressed glutamatergic receptors is one worthy of further consideration in a more complex system. KA does protect against inflammation-associated changes in gut motility, possibly because of glutaminergic NMDA receptor antagonism (Varga et al. 2010), and, at micromolar concentrations, KA attenuated glutamatergic contractions in *ex vivo* guinea pig ileum (Moroni et al. 1989). Whether luminal KA can access the enteric nervous system at relevant concentrations to influence contractility *in vivo* remains to be determined. In weaning piglets diarrhea was positively correlated with levels of XA, as well as the

presence of several microbial taxa (Liang et al. 2021). However, XA is also an agonist at mGlu2/3 metabotropic glutamate receptors (Fazio et al. 2015), which are widely expressed in the enteric nervous system (Larzabal et al. 1999), and could account for changes in gastrointestinal motility.

Intestinal Kynurenine Derivative effects on the Microbiome

In the rat, micromolar concentrations of KA were detected in small intestinal fluid increasing in a proximal to distal direction (Kuc et al. 2008) where resident ileal bacteria are found. These concentrations were significantly greater than those present in the intestinal tissue leading the authors to speculate that the KA is of microbial origin (Kuc et al. 2008). Using similar concentrations of KA as that detected in the small intestinal lumen, Dolecka and colleagues (Dolecka et al. 2011) reported concentration-dependent effects on the viability of different probiotic mixes and bacterial strains. At lower concentrations KA either had no effect on bacterial viability or, in some cases, stimulated growth (*Lactobacillus reuteri; Lactobacillus rhamnosus).* At higher concentrations KA negatively impacted the growth of most products and strains tested, but not all (Dolecka et al. 2011). In a small number of HFD-fed animals, supplementation with KA purified from the marine horseshoe crab, decreased food and energy intake without a change in body weight (Li et al. 2021a). In the same study, KA (at the highest dose tested) supplementation in HFD-fed mice restored the abundance of *Lactococcus* and *Lactobacillus* toward that observed in control mice and normalized the *Firmicutes/Bacteroidetes* ratio (Li et al. 2021a). It is tempting to speculate that elevated intestinal kynurenine and its metabolites are utilised by the microbiota or affect microbial composition similar to indole, which favors the growth of symbiotic bacteria adapted to the higher concentrations in the intestine (Yang et al. 2020).

Perspective:

In contrast to evidence that favors a protective role of bacterial-derived indoles in obesity and metabolic disease, at least by a positive associated with reduced intestinal permeability, the role of kynurenine derivatives KA and XA may be more complex. This may be a consequence of whether the derivatives are due to host metabolism, the potential for microbial-derived sources, or whether there is microbial regulation of the host metabolic pathway (or vice versa) in the gut. Disease-associated shifts in the microbiome may further increase this complexity particularly in the context of dysbiosis associated with gut inflammation, which will drive metabolism along the kynurenine pathway. Kynurenine metabolites, even if bioavailable, may have their effect luminally where they may influence the composition of the gut microbiome, which in turn influences the host response. Alternatively they may enter the circulation, and if at sufficient levels to engage their pharmacological targets, could directly influence host physiology. Our own data would suggest that

luminal KA and XA exert limited effects on the gastrointestinal physiological parameters measured, but further work in this area is warranted to better understand their presence in a complex system which incorporates gut-brain signalling and to further characterise their effects on the gut microbiome. It is clear, however, that kynurenine metabolites are more than mere biomarkers of disease, and this is supported by their pharmacological activity across several receptor families and their observed effects on gut physiology and metabolism *in vivo* and *ex vivo*. The further utilisation of tools like GF animals and combined approaches using genetically modified mice (e.g. IDO deficient) raised in a GF environment might provide further insight into the host-microbiota interactions which clearly influence kynurenine metabolism. It is also apparent that tissue-specific changes in the metabolic activity of the host kynurenine pathway may occur concurrently and may diverge in terms of activity. This, combined with the potential involvement of the microbiome as a further source of kynurenine metabolites, perhaps limits the usefulness of only measuring circulating levels of kynurenine metabolites. A more holistic approach incorporating the microbiota-gut-brain axis as a bio-functional unit would perhaps be of more value to tease apart what is clearly emerging as a complex but physiologically relevant metabolic pathway in the context of metabolic disease and gastrointestinal physiology.

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FIGURE 1

A simplified diagram of tryptophan metabolism with key metabolites in the indole and kynurenine pathways along with the host and bacterial (indicated in lower case) enzymes.

Abbreviations: ACMSD aminocarboxymuconate semialdehyde decarboxylase DDC DOPA decarboxylase/ aromatic L-amino acid decarboxylase; IDO, indoleamine 2,3-dioxygenase; IPA, indole-3 propionic acid; ISA, indoxyl-3-sulfuric acid; KA, kynurenic acid KAT; kynurenine aminotransferase/cysteine conjugate beta-lyase (CCBL); KMO, kynurenine 3-monooxygenase, KYN, kynurenine; kynA, tryptophan 2,3-dioxygenase (bacterial) KYNU, kynureninase; NAD, nicotinamide; tam1, tryptophan amino transferase 1 (bacterial); TDO, tryptophan 2,3-dioxygenase TPH1, tryptophan hydroxylase 1; tnAa, tryptophanase XA, xanthurenic acid

FIGURE 2

A, B Fold change in FITC-Dextran RFU on basal side of T84 cell monolayers relative to vehicle after incubation with (A) KA (2, 20 and 200 nM) or (B) XA (2.4, 24.4 and 244 nM) alone and in the presence increase permeability due to proinflammatory cytokines (IFN-γ/TNF-α) to *P<0.05.

C, D. Change in Short Circuit Current (Isc) of mouse ileal muscle-stripped mucosa in response to (C) KA (0.2 and 2.0 uM) and (D) XA (0.25 and 2.5 uM) in the presence and absence of TTX. Data are shown as Box and Whisker plots.

E, F. Change in conductance in response to 2mV measured at termination, 30 minutes after incubation with metabolites and drugs on mouse ileal muscle-stripped mucosa. The response to (E) KA (0.2 and 2.0 uM) and (F) XA (0.25 and 2.5 uM) alone and in the presence of neurotoxin TTX are shown. A small, but significant decrease in conductance compared to vehicle tissue was noted after incubation with XA in the presence of TTX. Data are shown as box and whisker plots. * P<0.05 by 2way ANOVA

G. Tryptophan derivative enzyme mRNA expression (relative to GAPDH) of full thickness distal colon from C57Bl/6 mice fed Low vs High Fat Diet. Significant increases in mRNA expression of IDO1, KMO and KYNU were noted in high fat fed group. * P<0.05.

Abbreviations: AADAT, aminoadipate aminotransferase; FITC, fluorescein isothiocyanate; KAT/CCBL, Kynurenine aminotransferase/cysteine conjugate beta-lyase; KMO, kynurenine 3-monooxygenase, KYN, kynurenine KYNU, kynureninase NAD, nicotinamide RFU, relative fluorescence units; TPH1, tryptophan hydroxylase 1; TTX, tetrodotoxin