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Akkermansia muciniphila and its outer protein Amuc_1100 regulates tryptophan metabolism in colitis†

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Dietary interventions, including dietary ingredients, nutrients and probiotics, exert anti-inflammatory effects in ulcerative colitis (UC). Our previous study showed that *Akkermansia muciniphila* (*Akk*), a promising probiotic, could protect against colitis *via* the regulation of the immune response. However, whether it can restore aberrant tryptophan (Trp) metabolism during colitis remains unclear. In this study, untargeted serum metabolomics of patients with UC and colitis mice showed that Trp metabolism was activated, which was confirmed by quantification of Trp metabolites from a validation cohort and animal study. Integrative analysis of faecal metagenomes and serum metabolomes revealed significant associations between *Akk* and three Trp metabolites. Live *Akk*, pasteurised *Akk* and Amuc_1100 failed to restore the reduction in Trp metabolites involved in the serotonin pathway in colitis mice. However, live *Akk*, pasteurised *Akk* and Amuc_1100 increased kynurenine (Kyn) but decreased 2-picolinic acid (PIC) levels and the PIC/Kyn ratio without regulating any of the genes involved in Trp metabolism, suggesting that they could suppress the Kyn pathway (KP) independent of colon tissue. In addition, they could significantly restore the enrichment of Trp metabolism mediated by faecal microbiota. Specifically, live *Akk*, pasteurised *Akk* and Amuc_1100 could significantly offset the reduction in indoleacetic acid (IAA) levels. Pasteurised *Akk* significantly elevated the serum levels of indole acrylic acid (IA). In addition, live *Akk*, pasteurised *Akk* and Amuc_1100 could upregulate aryl hydrocarbon receptor (AhR) targeted genes, including *CYP1A1*, *IL-10* and *IL-22*, suggesting that *Akk* could activate AhR signaling by regulating Trp metabolism, thereby attenuating colonic inflammation.

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1. Introduction

Inflammatory bowel disease (IBD), encompassing Crohn's disease (CD) and ulcerative colitis (UC), is a chronic and relapsing inflammatory disorder. Although there is tremendous heterogeneity between the reported incidence rates in Western countries, newly industrialized countries and developing countries, the incidence of IBD has steadily increased over the past several decades.[1] Emerging epidemiological evidence has revealed that genetics, lifestyle and environmental factors contribute to the pathogenesis of IBD. Diet is a potentially modifiable environmental risk factor for IBD onset and severity through dysregulation of the immune system, alterations in intestinal permeability and the mucous layer, and contributing to microbial dysbiosis.[2] The gut microbiota contributes to many host physiological processes, including digestive and metabolic functions, regulation of the epithelial barrier, development and regulation of the host immune system, and protection against pathogen colonization.[3] Recently, imbalances in the gut microbiota have been associated with the development and progression of IBD.[4] Reduced microbiotal diversity and increased variability have been identified in patients with IBD compared with healthy controls.[5] Thus, rational therapeutic manipulation of microbiota might be useful in the treatment of IBD.

Dietary interventions, including dietary ingredients, nutrients and probiotics, have been reported to be beneficial for IBD, especially UC.[6] Long-term and continuous administration

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of *Bacillus subtilis* during remission effectively maintained the remission of IBD by protecting intestinal integrity, regulating epithelial proliferation, and reshaping microbial structure and function.[7] Our previous study showed that chlorogenic acid ameliorated the course of colitis in association with a proportional increase in *Akkermansia muciniphila* (*Akk*), which is a Gram-negative anaerobic bacterium that has been shown to be selectively decreased in the faecal microbiota of patients with IBD.[8] *Akk* improved the clinical parameters, including spleen weight, colon inflammation index and colon histological score in chronic colitis.[9] Supplementation with pasteurised *Akk* and a specific protein isolated from its outer membrane (Amuc_1100) could also improve colitis and colitis-associated colon cancer by modulating $CD8^+$ T cells.[10] Improvements in metabolic dysfunctions have been well described in humans who are obese and diabetic and mice treated with pasteurised *Akk*.[11,12] However, the role of *Akk* in host metabolism during the process of IBD remains unclear.

Gut microbiota-derived metabolites, notably metabolites of the essential amino acid Trp, have been implicated in the pathogenesis of IBD.[13] Trp metabolism follows three major pathways in the gastrointestinal tract: the microbial pathway, kynurenine pathway (KP) and serotonin (5-hydroxytryptamine, 5-HT) pathway.[14] Reduced serum levels of Trp and activated KP had been observed in patients with UC.[15] In addition to being biomarkers, the alterations and biological effects of Trp metabolites in UC suggest that they may be therapeutic targets. Dietary Trp alleviated dextran sodium sulfate (DSS)-induced colitis through the aryl hydrocarbon receptor (AhR) in mice.[16] Microbial Trp metabolites, including indole-3-ethanol, indole-3-pyruvate and indole-3-aldehyde (IAld) could protect against aberrant intestinal barrier function *via* AhR in mice with colitis.[17] Inoculation of mice with three *Lactobacillus* strains capable of metabolizing Trp could significantly attenuate colitis.[18] However, the regulation of Trp metabolism mediated by *Akk* in colitis has not yet been specifically addressed.

In this study, faecal metagenomes, untargeted serum metabolomes and colonic transcriptomes from patients with UC or mice with colitis were integrated to explore the effects of *Akk* on Trp metabolism. Metabolomics and the transcriptomes showed that Trp metabolism was activated in patients with UC and colitis mice, which was confirmed by the quantification of Trp metabolites. Integration of faecal metagenomes and untargeted serum metabolomes revealed significant associations between *Akk* and three Trp metabolites. In line with live *Akk*, pasteurised *Akk* and Amuc_1100 could increase Kyn and decrease PIC levels without regulating any of the genes involved in Trp metabolism, suggesting they could suppress KP independent of colon tissue. However, they could significantly restore the enrichment of Trp metabolism in faecal microbiota. Specifically, live *Akk*, pasteurised *Akk* and Amuc_1100 could significantly offset the reduction in IAA levels. Pasteurised *Akk* also significantly elevated the serum levels of IA. Collectively, these data indicated that *Akk* could regulate Trp metabolism by blocking KP and activating the microbial pathway in colitis mice.

2. Materials and methods

2.1. Chemicals and reagents

DSS (molecular weight of 36–50 kDa) was obtained from MP Biomedicals (Solon, OH, USA). L-Tryptophan (Trp), L-kynurenine (Kyn), 2-picolinic acid (PIC), quinolinic acid (QUI), indole acrylic acid (IA), indole-3-propionic acid (IPA), and tryptamine were obtained from Aladdin Biochemical Technology Co., Ltd (Shanghai, China). 5-Hydroxyindoleacetic acid (5-HIAA), indoleacetaldehyde (IAAld), indoleacetic acid (IAA) and serotonin (5-hydroxytryptamine, 5-HT) hydrochloride were obtained from Sigma Aldrich (MO, USA). 5-Hydroxy-L-tryptophan (5-HTP) was purchased from MAYA-Reagent (Jiaxing, China). Indole-3-aldehyde (IAld) was obtained from TCI (Shanghai) Development Co., Ltd. L-Tryptophan-d5 (Trp-d5) was obtained from Toronto Research Chemicals (Toronto, ON, Canada). The purity of Trp and its metabolites was greater than 98%.

2.2. Patients

Serum and stool samples were studied from a previously collected cohort, including 72 active patients with UC and 58 healthy controls. The metagenomic sequencing data of 130 faecal samples (PRJNA596333) were available from our previous study.[10] Note that for 54 healthy controls and 47 patients with UC serum was available for metabolomic analysis. The metagenomic and metabolomic data from these 101 participants were integrated into a multiomic signature.

For the validation cohort, 48 healthy controls (aged 49.6 ± 12.3 years) and 46 UC patients (aged 44.0 ± 13.2 years) were recruited from the Jiangsu Province Hospital of Chinese Medicine and the general population respectively. Participants were excluded from the study if they had had used antibiotics in the 4 weeks prior. All participants were requested to provide written informed consent and a blood sample under an approved institutional review board protocol. Serum from these blood samples was stored at −80 °C until analysis. All the participants signed the informed consent approved by the Institutional Review Board of Nanjing Medical University.

2.3. Mouse studies

All experimental protocols were performed in compliance with the Guidelines for the Care and Use of Laboratory Animals of Nanjing Medical University and approved by the Animal Ethical and Welfare Committee of Nanjing Medical University (IACUC-1812031). Male C57BL/6J mice (6–8 weeks) were obtained from the Animal Core Facility of Nanjing Medical University (SYXK(SU) 2020–0022) and maintained on 12/12 hours day/night cycle with free access to food and water under conditions of controlled humidity (50% ± 5%) and temperature (22 ± 2 °C). Colitis was induced in the mice by 2% DSS with or without pasteurised *Akk* or Amuc_1100 treatment as previously described.[10] The body weight, stool consistency and bleeding were scored to evaluate the severity of colitis. The metagenomic sequencing data of 24 pooled faecal samples

(6 each group) were available (PRJNA596333). A serum sample from each mouse was used for metabolomic analysis.

To further corroborate the critical role of Trp metabolism in the pathogenesis of colitis, mice with colitis were pretreated with live *Akk* (1.5×10^8 CFU), pasteurised *Akk* (1.5×10^8 CFU), Amuc_1100 (3 μg) in 100 μl of sterile PBS containing 2.5% glycerol by gavage or Trp (42 g kg^{-1} in diet) for 10 days until sacrifice. Blood samples were drawn from the orbit and allowed to clot at room temperature for 2 h before centrifugation (3000*g*, 4 °C, 10 min), and serum and snap-frozen colon tissues were collected for Trp metabolite quantification and RNA sequencing, respectively.

2.4. Serum metabolomes and data processing

Serum metabolic profiling analysis was performed with a UHPLC Ultimate 3000 system coupled with a Q Exactive hybrid quadrupole-orbitrap mass spectrometer (UPLC-MS) according to our previous study.[19] Thawed serum samples (50 μl) were spiked with ice cold methanol (200 μl). After 15 min of centrifugation (12 000*g*, 4 °C), the supernatant was collected for LC-MS analysis that was performed on a UPLC Ultimate 3000 system coupled with a Q Exactive hybrid quadrupole-orbitrap mass spectrometer (Q-Exactive UPLC-MS, Thermo Fisher Scientific, Bremen, Germany) in both positive and negative ion modes. The quality control (QC) sample was prepared by pooling an aliquot of the same volume from each sample to ensure reproducibility and stability during the whole procedure.

Data acquisition and analysis were performed by the SIEVE software (Thermo Fisher Scientific) and the SIMCA-P 14.0 software (Umetrics, Umea, Sweden). Partial least squares discriminant analysis (PLS-DA) was performed to detect the distributions of different groups, classifications and comparisons in each group. The variable importance in the projection (VIP) obtained from multivariate statistical analysis can provide significantly changed variables after drug intervention. The metabolites that showed VIP > 1.0 and $P < 0.05$ were considered statistically significant. The metabolites were identified using the HMDB library. Pathway analysis was performed by MetaboAnalyst 5.0 (http://www.metaboanalyst.ca) and the Kyoto Encyclopedia of Genes and Genomes (KEGG) database was also used to identify relevant metabolic pathways.

2.5. Integration of metagenomes and metabolomes

Gut microbiota from patients with UC and mice with colitis were analysed by 16S rRNA sequencing at the V3 hypervariable region on an Illumina MiSeq (PE300).[10] Sequences were clustered into operational taxonomic units (OTUs) using Mothur. The OTUs that reached 97% nucleotide similarity level were used for further analysis. Taxonomy was assigned using the Ribosomal Database Project (RDP) database (http://rdp.cme.msu.edu/) with a 70% bootstrap score. In this study, phylogenetic investigation of communities by the reconstruction of unobserved states (PICRUSt) was used to infer metagenomes based on the 16S marker data and predicted KEGG pathway abundances according to our previous study.[20] In addition, correlation between metagenomic and metabolomic data from humans were analysed using the Spearman rank test on the free online platform Majorbio Cloud Platform (http://www.majorbio.com).

2.6. Quantification of Trp metabolites

Trp and metabolites in serum (50 μl) from the validation cohort and colitis mice were extracted with 150 μl of methanol. Ten microlitres of Trp-d5 (1 mg L^{-1}) was added as the internal standard. After 15 min of centrifugation (11 000*g*, 4 °C), the supernatant was used for Trp and its metabolite detection by Q-Exactive UPLC-MS according to previous studies with minor modifications.[21,22] Representative UPLC chromatograms of Trp metabolites are presented in Fig. S1.† Separation was carried out on a Hypersile C_{18} column (100 mm × 2.1 mm, 1.9 μm) at 40 °C. The mobile phases consisted of 0.1% formic acid in water (A) and 0.1% formic acid in acetonitrile (B). The flow rate was set at 0.4 ml min^{-1} with an elution gradient as follows: 0 to 1% B (0–2 min); 1% to 99% B (3–13 min); 99% B (13–15 min); 1% B (15 min); 1% B (15–17 min). Data acquisition and analysis were performed with the Thermo XCalibur 2.2 software. All samples were analysed by positive electrospray ionization (ESI^+) full-MS scan mode. Nitrogen as the sheath, auxiliary, and sweep gas was set at 50, 10, and 2 units, respectively. Other conditions included resolution, 70 000 FWHM; AGC target $5E;^6$ maximum injection time, 200 ms; scan range, 110–800 *m*/*z*; spray voltage, 3.50 kV; and capillary temperature, 300 °C.

2.7. RNA sequencing (RNA-seq)

Total RNA was extracted from frozen colon tissue with TRIzol reagent (Invitrogen). Then, cDNA was synthesized for transcriptome sequencing on an Illumina NovaSeq 6000. The RNA-seq data were analysed with the free online platform Majorbio Cloud Platform (http://www.majorbio.com). Briefly, quantification of gene expression as transcript per million (TPM) values was performed using the RSEM algorithm. Differential analysis was performed with DESeq2. A gene with a fold change greater than 2 was considered significantly differentially expressed for *P*-value <0.005 after BH adjustment. Functional enrichment analysis was performed with GO and KEGG databases, and a *P*-value <0.05 was considered to be statistically significant.

2.8. Quantitative real-time polymerase chain reaction (qPCR)

Total RNA was extracted from frozen colon tissues using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the instructions of the manufacturer. The expression of the AhR targeted genes was measured by qPCR according to our previous study.[10] The primers were synthesized by GenScript Biotech Corp. (Nanjing, China). *Gapdh* was used as an internal control; primers are shown in the format of the gene, forward primer (F), and reverse primer (R): *IL-10*, (F) GCTGGACAA CATACTGCTAACC, (R) ATTTCCGATAAGGCTTGGCAA; *IL-22*, (F) ATGAGTTTTTCCCTTATGGGGAC, (R) GCTGGAAGTT GGACACCTCAA; *CYP1A1*, (F) GCCACATCCGGGACATCAC,

(R) GCTGGACATTGGCATTCTCGT; *Gapdh*, (F) AGGTCGGTGTG AACGGATTTG, (R) TGTAGACCATGTAGTTGAGGTCA.

2.9. Statistical analysis

Receiver operating characteristic (ROC) curve analysis was performed with the SPSS 22.0 software (Chicago, Illinois, USA). Mann–Whitney test was performed to compare the metabolomes and Trp metabolite quantification. The gene expression was analysed by ordinary one-way ANOVA with Tukey's multiple comparisons. All plots are shown as the means ± SEM. A *P*-value <0.05 was considered statistically significant.

3. Results

3.1. Metabolic profiling of serum from patients with UC and mice with colitis

PLS-DA showed clear differences between the controls and patients with UC (Fig. S2A†) or mice with colitis (Fig. S2B†). Serum metabolomes identified 76 differential metabolites (8 increased, 68 decreased) in patients with UC compared with healthy controls (Table S1†). For colitis mice, a total of 70 differential metabolites (14 increased, 56 decreased) were identified (Table S2†). Within the metabolome, colitis samples were largely defined by the depletion of metabolites, with >80% of the identified signature being metabolites present at higher abundances in healthy samples. Pathway analysis revealed that aminoacyl-tRNA biosynthesis, Trp metabolism, D-glutamine and D-glutamate metabolism and valine, leucine and isoleucine biosynthesis were enriched (Fig. 1A). Trp, Kyn, 5-hydroxy-tryptophan (5-HTP), 5-hydroxyindoleacetic acid (5-HIAA) and indoleacetaldehyde (IAAld) in the Trp metabolism pathway were significantly reduced in patients with UC (Fig. 1B). Trp metabolism was the only overlapping pathway enriched in patients with UC and colitis mice (Fig. 1C). Specifically, Trp, Kyn, 5-HT and 5-HTP corroborated the lower abundance in colitis mice compared with controls (Fig. 1D).

3.2. Integration of metagenomes and metabolomes in patients with UC

16S rRNA and metabolomic data were integrated into a multiomic signature. The correlation analysis of 12 metabolites identified in the 4 enriched pathways mentioned above between altered (richness top 50) bacteria at the genus level revealed 150 significant associations, and many of the involved genera were reduced in patients with UC (Fig. 2). Thirty-two genera were significantly associated with one of the 5 metabolites involved in the Trp metabolism pathway. Among these 32 genera, 23 were positively correlated and 4 were negatively correlated with 5-HTP levels. *Akkermansia* was the only genus from the phylum *Verrucomicrobia* identified as significantly

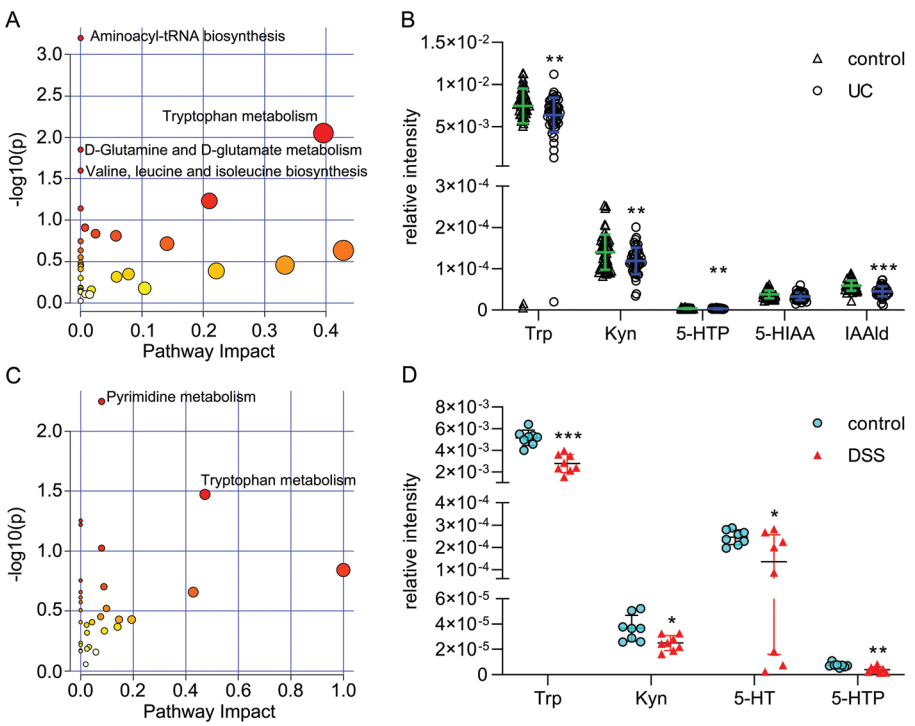

Fig. 1 Metabolic profiling of serum from patients with UC and mice with colitis. (A) Metabolic pathway analysis of differential metabolites from patients with UC. Enriched pathways with significant differences are noted ($p < 0.05$). (B) The relative intensities of metabolites involved in the tryptophan (Trp) metabolism pathway, including Trp, 5-hydroxy-L-tryptophan (5-HTP), 5-hydroxyindoleacetic acid (5-HIAA), L-kynurenine (Kyn) and indoleacetaldehyde (IAAld). (C) Metabolite pathway enrichment analysis of identified pathways altered in DSS-induced colitis mice compared with control mice. (D) The relative intensities of metabolites involved in Trp metabolism pathway, including Trp, serotonin (5-hydroxytryptamine, 5-HT), 5-HTP and Kyn in colitis mice. The plots labelled with specific pathway are significantly different ($p < 0.05$).

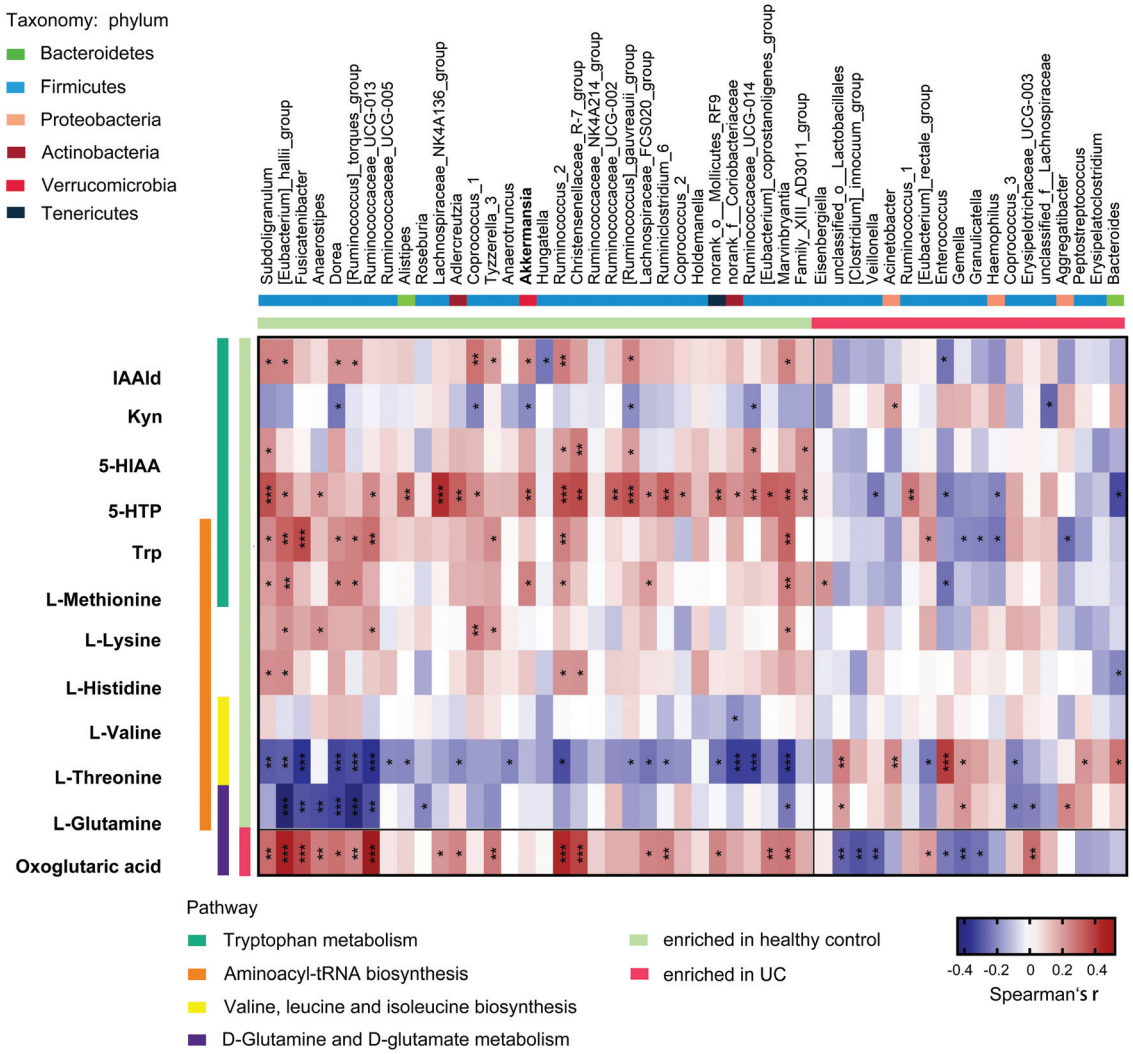

Fig. 2 UC-associated genera correlate with metabolites differentiating patients with UC and healthy controls. The genera (top 50) identified as significantly differential between patients with UC and healthy controls and serum metabolites involved in enriched pathways were included. Enrichment is indicated by coloured bars on the left and top of the plot. Significant correlations are denoted by stars (*$p < 0.05$; **$p < 0.01$, ***$p < 0.001$, Spearman test). Higher taxonomy of genera (phyla) and superpathways of metabolites are indicated by coloured bars.

differential between UC and healthy controls. Network analysis based on the integration of 16S rRNA sequencing and metabolomic data using genera (top 50) and metabolites involved in the Trp metabolism pathway, as described above, was performed to investigate the associations of the broader microbiome and UC-linked metabolites. *Akkermansia* was positively correlated with 5-HTP and IAAld, whereas it was negatively correlated with Kyn levels (Fig. 2 and S3†).

3.3. Effects of *Akk* on serum metabolic profiling in mice with colitis

To determine whether *Akk* regulates Trp metabolism, metabolomic profiling was applied to compare the global metabolites in colitis mice with or without pasteurised *Akk* or Amuc_1100 treatment. Alterations in Trp metabolism were characteristic of colitis mice treated with pasteurised *Akk* (Fig. 3A) but not Amuc_1100 (Fig. 3C) treatment. Pasteurised *Akk* significantly increased the serum levels of IAAld, 5-HIAA and 4,6-dihydroxyquinoline in colitis mice (Fig. 3B). In contrast, Amuc_1100 supplementation normalized the serum levels of Trp and Kyn (Fig. 3D). These data indicated that *Akk* could regulate Trp metabolism in colitis mice.

3.4. Effects of *Akk* on colonic transcriptomes in mice with colitis

To investigate whether *Akk* affects Trp metabolism related genes, colonic transcriptomes of colitis mice induced by DSS in response to pasteurised *Akk* or Amuc_1100 treatment were profiled by RNA sequencing. Compared with the control mice, 300 genes were significantly altered in colitis mice (Fig. 4A). A total of 123 and 28 genes in colitis mice were altered upon pasteurised *Akk* or Amuc_1100 treatment, respectively. Specifically, the expression levels of *Haao*, *Ido1*, *Il4i1* and *Kmo*, which are involved in the Trp metabolism pathway, were upregulated in

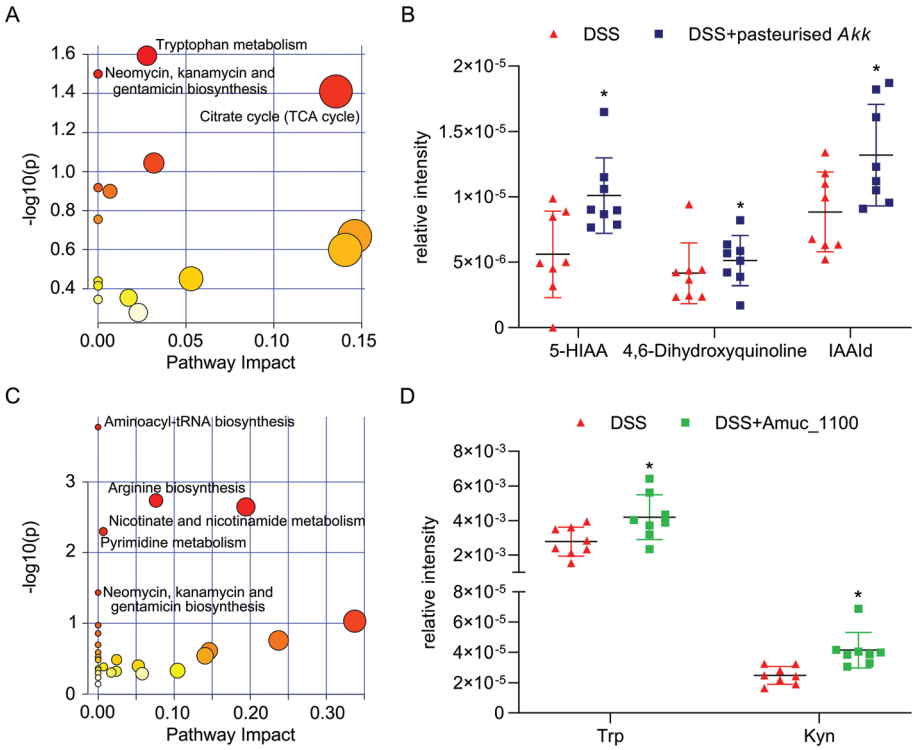

Fig. 3 Effects of *Akk* on serum metabolic profiling in mice with colitis. (A) Pathway analysis of differential metabolites between the DSS and pasteurised *Akk* + DSS groups. (B) The effects of pasteurised *Akk* on the relative intensities of metabolites involved in the Trp metabolism pathway, including 5-HIAA, 4,6-dihydroxyquinoline and IAAld. (C) Pathway analysis of differential metabolites in colitis mice upon Amuc_1100 treatment. (D) The effects of Amuc_1100 on serum levels of Trp and Kyn in mice with colitis. The entitled plots are significantly different ($p < 0.05$).

colitis mice (Fig. 4B). Sixty-three pathways were significantly enriched in colitis mice, such as in inflammatory bowel disease and Trp metabolism (Table S3† and Fig. 4C). GO enrichment analysis revealed that the *de novo* NAD biosynthetic process from Trp and Trp catabolic process and metabolic process were enriched in colitis mice (Table S6† and Fig. 4D). However, no effects from pasteurised *Akk* or Amuc_1100 were observed on genes involved in Trp metabolism pathway (Tables S4 and 5†) or GO terms (Tables S7 and 8†), indicating that pasteurised *Akk* and Amuc_1100 regulated Trp metabolism independent of colon cells. On inflammation, AhR targeted genes, including *IL-10*, *IL-22* and *CYP1A1* were downregulated in DSS-induced mice (Fig. 4E). Live *Akk*, pasteurised *Akk* or Amuc_1100 treatment could significantly increase these genes.

3.5. *Akk* normalized Trp metabolism by gut microbiota

Trp can be metabolized by either host cells or gut bacteria. Many Trp metabolites were derived from the gut microbiota; thus, the function of the gut microbiota was analysed. Trp metabolism pathway was more activated in patients with UC than the healthy controls (Fig. 5A). ROC analysis revealed that Trp metabolism markedly differentiated patients with UC from healthy controls (Fig. 5B). In line with a human study, the Trp metabolism pathway (Fig. 5C) was enriched in colitogenic mice. In addition, phenylalanine, tyrosine and tryptophan biosynthetic pathways were enriched in mice with colitis (Fig. 5D). However, pasteurised *Akk* and Amuc_1100 could only restore enrichment of the Trp metabolism pathway.

3.6. Validation of the microbial metabolism of Trp regulated by *Akk*

To validate the altered circulating metabolites involved in the Trp metabolism pathway, especially the direct transformation of Trp by gut microbiota, Trp and 6 microbial metabolites as well as 6 host metabolites (Fig. S3†) were accurately quantified in a validation cohort comprising 94 samples: 46 patients with UC and 48 healthy controls. The concentrations of Trp (Fig. 6A), Kyn (Fig. 6E), indole acrylic acid (IA, Fig. 6H), indole-3-propionic acid (IPA, Fig. 6I), tryptamine (Fig. 6J), and IAA (Fig. 6L) were significantly reduced, whereas the levels of 5-HT (Fig. 6C) and quinolinic acid (QUI, Fig. 6F) were increased in the serum of patients with UC. No statistical differences of 5-HTP (Fig. 6B), 5-HIAA (Fig. 6D), PIC (Fig. 6G), or IAAld (Fig. 6K) were observed between patients with UC and the controls. Further analysis revealed an increase in the 5-HT/Trp (Fig. 6M), Kyn/Trp (Fig. 6N), QUI/Kyn (Fig. 6O) and PIC/Kyn (Fig. 6P) ratios, confirming activated Trp metabolism in UC patients.

The serum levels of Trp (Fig. 7A), 5-HTP (Fig. 7B), 5-HT (Fig. 7C), 5-HIAA (Fig. 7D), QUI (Fig. 7F), PIC (Fig. 7G), IA (Fig. 7H), IPA (Fig. 7I), and IAA (Fig. 7L) were significantly decreased in colitis mice. A diet rich in Trp offset the reduction of these metabolites except for 5-HT, PIC and IPA. Pasteurised

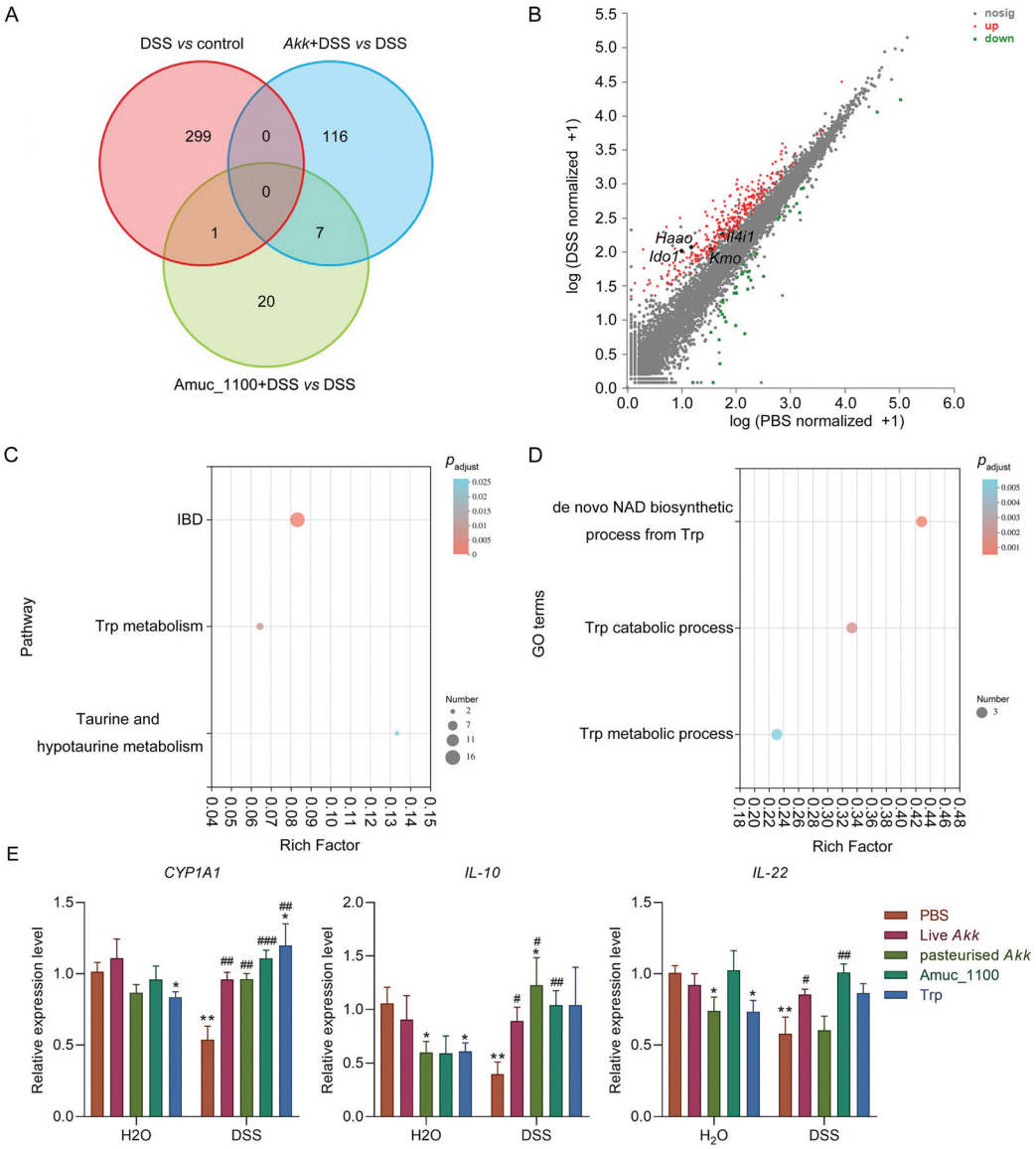

Fig. 4 Effects of *Akk* on colonic transcriptomes in mice with colitis. (A) The colonic transcriptomes in DSS-induced colitis mice treated with pasteurised *Akk* or Amuc_1100 were analysed by RNA sequencing (n = 3 each group). The differentially expressed genes (fold-changes 2, $p < 0.05$, BH adjusted) in these mice are represented in a Venn diagram. (B) Scatter plots of the genes between the control and mice with colitis induced by DSS. Differential genes involved in the Trp metabolism pathway are denoted. (C) Enriched KEGG pathways, including IBD and amino acid metabolism pathways are listed. (D) GO enrichment, including GO terms involved in Trp metabolism are listed. Functional enrichment of differentially expressed genes between the control and colitis mice was analysed, and BH adjusted $p < 0.05$ was considered statistically enriched. (E) The mRNA expression of Ahr targeted genes in colon tissue, including *CYP1A1*, *IL-10* and *IL-22*. Data are presented as means ± SEM and were analysed by ordinary one-way ANOVA with Tukey's multiple comparisons. *$p < 0.05$, **$p < 0.01$, compared with respective control; #$p < 0.05$, ##$p < 0.01$, ###$p < 0.001$, compared with the DSS group.

Akk restored the reduction in Trp in colitis mice. Live *Akk*, pasteurised *Akk* and Amuc_1100 significantly increased Kyn (Fig. 7E), whereas they decreased PIC levels as well as the PIC/Kyn ratio (Fig. 7P). Moreover, they could not restore the reduction of QUI or the QUI/Kyn ratio (Fig. 7O). Thus, the elevation of Kyn levels was mainly attributed to reduced degradation but not increased Trp degradation, as the Kyn/Trp ratio was not altered upon their treatment (Fig. 7N). They also failed to offset the reduction of the metabolites in the serotonin pathway. In addition, they could significantly restore the reduction of IAA, and pasteurised *Akk* also increased IA levels, indicating that *Akk* could regulate the microbial Trp metabolism.

4. Discussion

The Trp catabolites derived from gut microbiota could be used as biomarkers for dysbiosis and may be targeted for the devel-

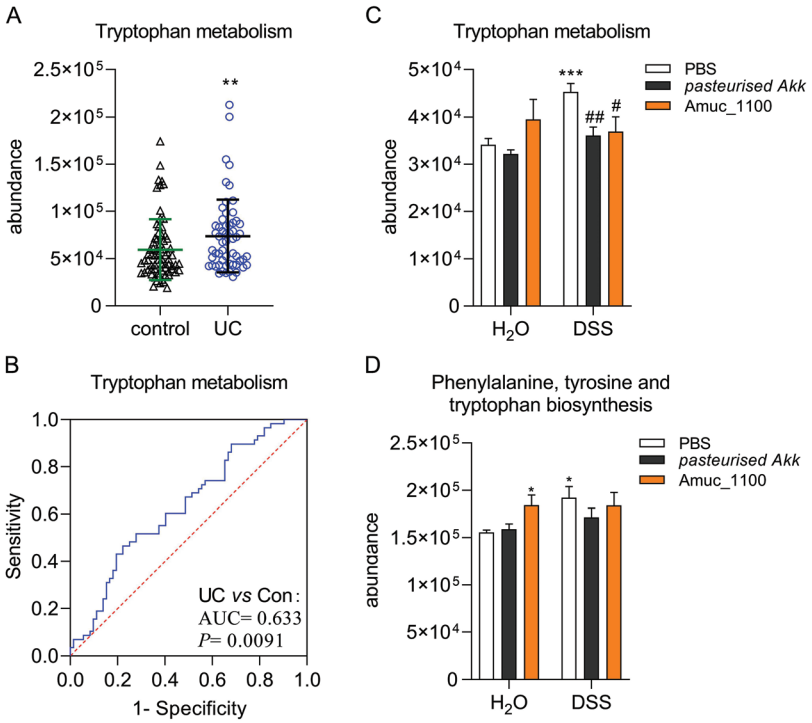

Fig. 5 The effects of *Akk* on impaired Trp metabolism in the gut microbiota. Predicted functions of the metagenome from 16S rRNA sequencing were conducted by PICRUSt and used to identify pathways. (A) Enrichment of Trp metabolism in the gut microbiota from patients with UC. (B) Receiver operator characteristic curves for evaluation of Trp metabolism as a biomarker for UC prediction. The effects of Amuc_1100 on the (C) Trp metabolism pathway and (D) phenylalanine, tyrosine and tryptophan biosynthesis pathway in the gut microbiota of mice with colitis. Data are presented as the mean ± SEM and analysed by Mann–Whitney test. *$p < 0.05$, ***$p < 0.001$, compared with respective controls; #$p < 0.05$, ##$p < 0.01$, compared with the DSS group.

opment of new therapeutic drugs for individuals with IBD.[18] In the present study, metabolic profiling of serum samples from patients with UC and experimental colitis mice revealed the enrichment of Trp metabolism, which was also confirmed by functional enrichment analysis of colonic transcriptomes. While bacterial Trp catabolism in the gut has received an increasing amount of attention in recent years, knowledge about the effects of *Akk* on Trp metabolism during colitis progression is limited. Our previous study showed that pasteurised *Akk* and Amuc_1100 could blunt colitis by modulating $CD8^+$ T cells.[10] Thus, serum metabolic profiling and prediction of the function of gut microbiota from these mice were conducted to investigate whether Trp metabolism was involved in their beneficial effects. Moreover, correlation analysis of metabolic profiling and gut microbiota suggested that the richness of *Akk* was positively correlated with 5-HTP and IAAld but negatively associated with Kyn. To verify the role of *Akk* (especially live bacterium) on Trp metabolism, Trp metabolites and colonic transcriptomes were further detected in colitis mice with or without live *Akk*, pasteurised *Akk* and Amuc_1100. In addition, as a control, colitis mice were fed a diet rich in Trp, and beneficial effects on colitis were well established.[16]

As an essential amino acid, Trp is a biosynthetic precursor of a large number of microbial and host metabolites that are involved in intestinal inflammation, epithelial barrier function, and energy homeostasis of the host.[23] As reported in previous studies,[15,24] serum Trp levels were decreased in patients with UC as well as colitis mice. Serum samples from fatigued patients with quiescent IBD had significant reductions in the levels of Trp and methionine.[25] Trp supplementation could improve the colonic immune response partly by reducing colonic 5-HT.[26] More Trp is then needed to synthesize proteins to heal the colonic wound in mice with colitis. The loss of histological structure and disruption of the epithelial barrier also suppress Trp absorption, which worsens the reduction in Trp. Most of the dietary Trp consumed by the host is engaged in protein synthesis, and the remainder is constantly involved in Trp metabolism.[27] Pasteurised *Akk* and Amuc_1100 could attenuate colonic injuries[10] and then increase Trp absorption and serum levels.

The Kyn pathway is responsible for ~95% of dietary Trp degradation, of which 90% is attributed to the hepatic Kyn pathway.[28] A previous study showed that activation of KP is based on high levels of QUI.[15] In the present study, the metabolic profiling and quantification of Trp metabolites revealed reduced serum Trp and Kyn levels but higher Kyn/Trp ratios in patients with UC and mice with colitis, suggesting increased synthesis of Kyn from Trp. Higher QUI levels and QUI/Kyn and PIC/Kyn ratios demonstrated that KP was activated in UC patients. Exogenous administration of Kyn and QUI led to the

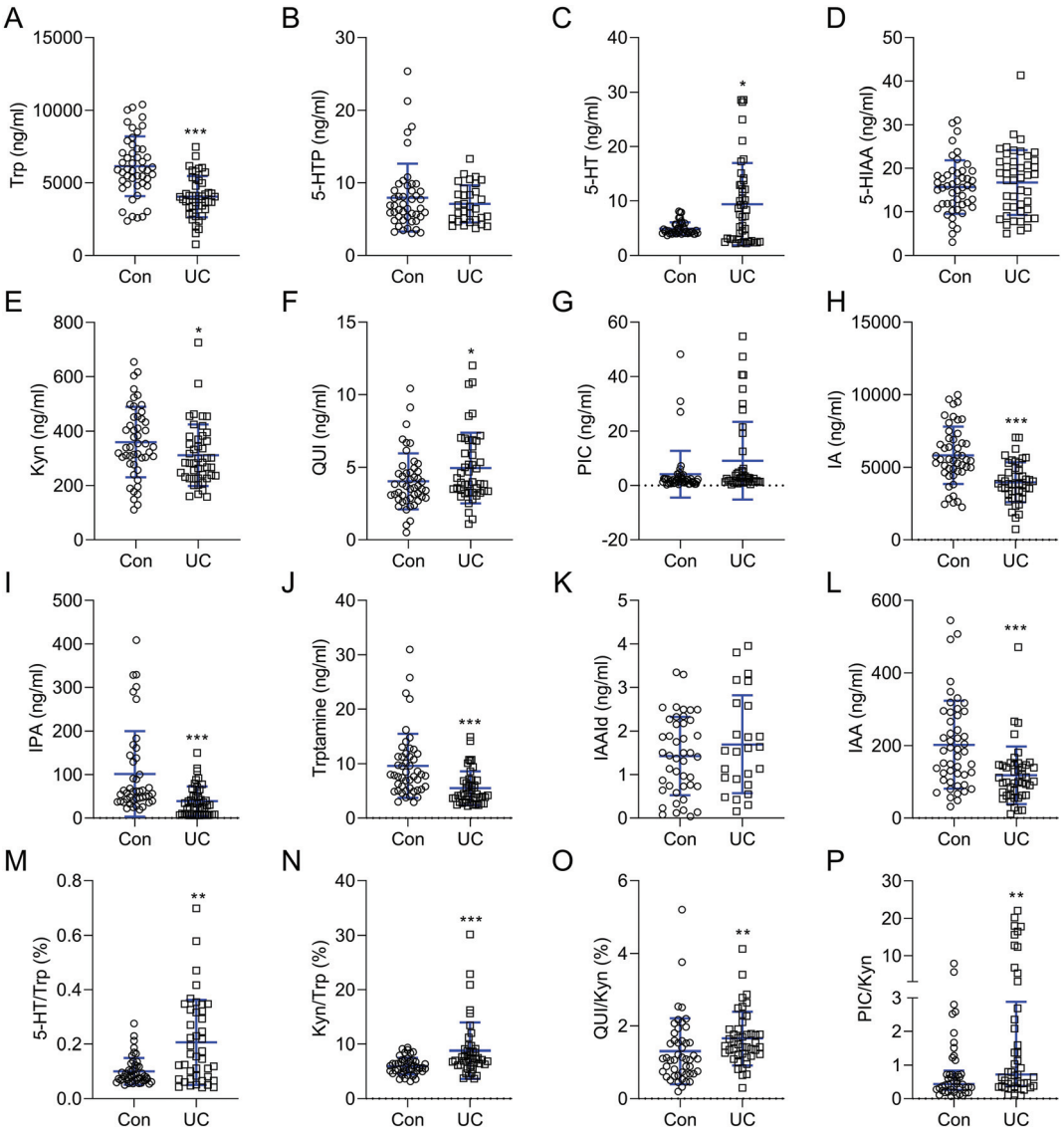

Fig. 6 Disruption of Trp metabolism in UC patients. Representative Trp metabolites in the serum from patients with UC and healthy controls were quantified by UHPLC-MS, including (A) Trp, (B) 5-HTP, (C) 5-HT, (D) 5-HIAA, (E) Kyn, (F) QUI, (G) PIC, (H) IA, (I) IPA, (J) tryptamine, (K) IAAld and (L) IAA. The ratios of (M) 5-HT/Trp, (N) Kyn/Trp, (O) QUI/Kyn and (P) PIC/Trp. Data are presented as mean ± SD and analyzed by Mann–Whitney test. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, compared with healthy controls.

activation of β-catenin and the proliferation of human colon cancer cells, and increased tumour growth in mice.[29] In addition, upregulation of *Ido1* and *Kmo* in colitis mice further confirmed this effect. These results indicated that the induction of these genes could not restore the reduction of Kyn, PIC or QUI due to a dramatic decrease in Trp absorption and catabolism. Unexpectedly, the QUI/Kyn and PIC/Kyn ratios were also decreased in colitis mice. Live *Akk*, pasteurised *Akk* and Amuc_1100 significantly elevated the serum levels of Kyn, but they failed to affect Kyn/Trp. Actually, they exacerbated the reduction in the PIC/Kyn and QUI/Kyn ratios without changing any of the genes involved in the KP pathway in the colon, indicating that *Akk* may block the hepatic KP pathway but not the colonic KP pathway.

More than 95% of the body's serotonin (5-HT) is produced by enterochromaffin cells in the intestine, where it affects gut physiology, including motor and secretory functions.[30] In the present study, higher 5-HT and 5-HT/Trp ratios were observed in UC patients, emphasizing the potential suitability of serum 5-HT as an auxiliary measure in diagnosing active UC. 5-HT exacerbated DSS-induced colitis *via* the upregulation of NADPH oxidase and interaction with the endocannabinoid system.[31,32] However, recent findings revealed that 5-HT deficiency leads to high levels of DNA damage in colonocytes, which is linked to inflammatory reactions and colorectal tumorigenesis.[33] Inconsistent with a human study, 5-HT levels decreased in colitis mice with substantial variation. Taken together, the state of the 5-HT pathway in IBD and colorectal

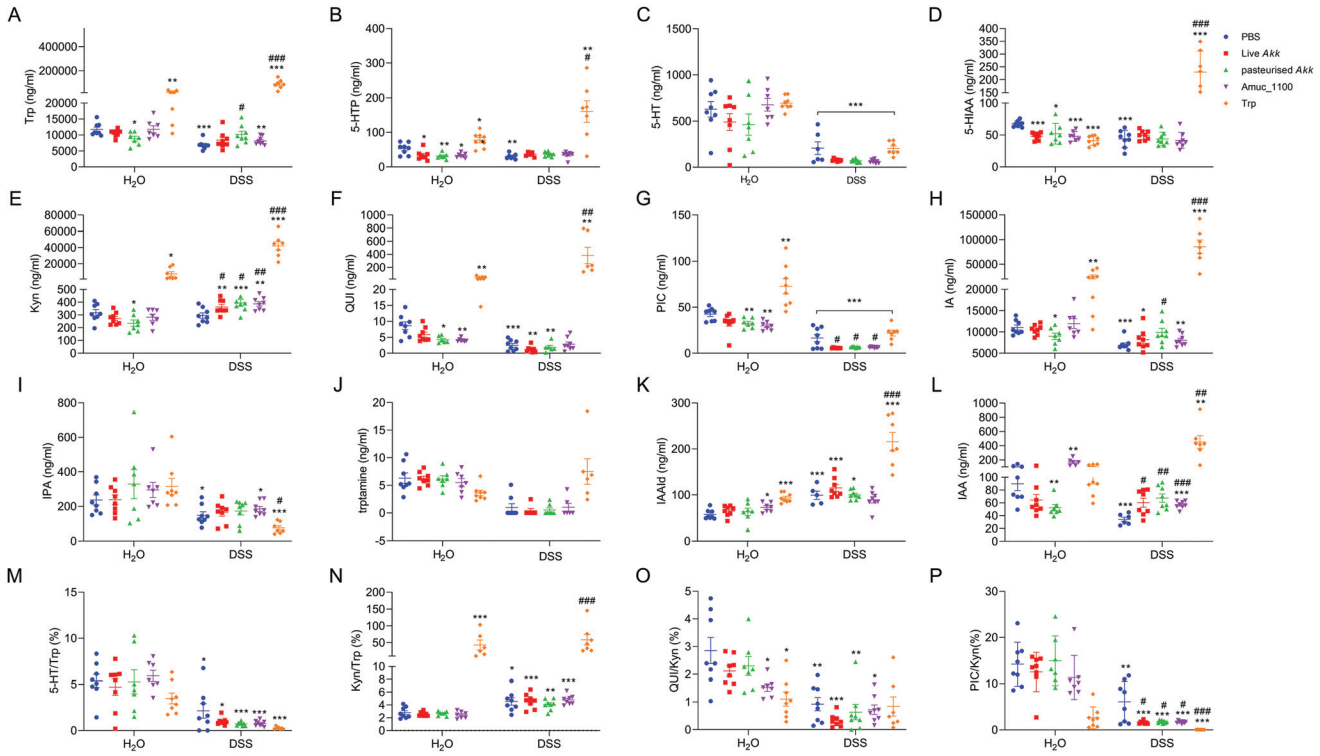

Fig. 7 The effects of *Akk* on Trp metabolism in the serum of mice with colitis. Trp metabolites in serum from colitis mice induced by DSS with or without live *Akk*, pasteurised *Akk*, Amuc_1100 and Trp were quantified by UHPLC-MS, including (A) Trp, (B) 5-HTP, (C) 5-HT, (D) 5-HIAA, (E) Kyn, (F) QUI, (G) PIC, (H) IA, (I) IPA, (J) tryptamine, (K) IAAld and (L) IAA. The ratios of (M) 5-HT/Trp, (N) Kyn/Trp, (O) QUI/Kyn and (P) PIC/Trp. Data are presented as mean ± SEM and analyzed by ordinary one-way ANOVA Tukey's multiple comparisons. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, compared with respective controls; #$p < 0.05$, ##$p < 0.01$, ###$p < 0.001$, compared with the DSS group.

cancer remains inconclusive.[14,34] Gavage with *Akk* or Amuc_1100 could markedly elevate the concentrations of 5-HT by upregulating the 5-HT synthesis rate-limiting enzyme Tph1 expression and inhibiting the serotonin reuptake transporter expression, which are decreased in colon tissues of antibiotic-treated mice.[35] However, live *Akk*, pasteurised *Akk* and Amuc_1100 failed to restore the reduction of 5-HT or the 5-HT/Trp ratio in colitis mice. In addition, no gene involved in the 5-HT pathway was affected in colitis mice upon treatment, suggesting that *Akk* could not regulate the 5-HT pathway during the colitis process.

AhR is widely expressed in the immune and intestinal cells of the gut and involved in the progression and outcome of IBD. AhR activation by Trp metabolites derived from gut microbiota, such as IA, IPA, IAld, IAA and IAAld, were reported to modulate the severity of acute colitis.[36] *Bacteroides thetaiotaomicron* promoted AhR activation by increasing IAA and IPA, thereby relieving colonic inflammation.[37] Thus, this study aimed to investigate whether AhR signaling regulated by Trp metabolites was involved in the protective effects of *Akk* on colitis. Microbial Trp metabolites were detected in the serum from patients with UC and colitis mice treated with live *Akk*, pasteurised *Akk* or Amuc_1100. IA, IPA and IAA levels were decreased in both patients with UC and colitis mice. However, live *Akk*, pasteurised *Akk* and Amuc_1100 failed to change the serum levels of IPA, IAAld, tryptamine or IAld. Intriguingly, they could significantly offset the reduction of IAA, indicating that IAA may contribute to the protective effects of *Akk* on colitis. Beyond IAA, pasteurised *Akk* could also significantly increase the serum levels of IA, which has been reported to promote intestinal epithelial barrier function and mitigate the inflammatory response in colitis mice.[38] *Lactobacillus plantarum* KLDS 1.0386 combined with Trp could protect against DSS induced colitis by elevating the level of IAA, which further upregulated AhR to activate the IL-22/STAT3 signaling pathway.[39] IL-10 and IL-22 are the best studied members of the IL-10 family. IL-10 serves as an important regulator in preventing pro-inflammatory responses while IL-22 plays a protective role in tissue damage by regulating the release of antimicrobial peptides and microbial composition.[40] Here, live *Akk*, pasteurised *Akk* and Amuc_1100 could upregulate the expression of CYP1A1 in colitis suggesting that *Akk* might trigger AhR signaling through increasing IA or IAA. Moreover, live *Akk*, pasteurised *Akk* and Amuc_1100 could restore the downregulation of IL-10 and IL-22, suggesting that *Akk* could exhibit protective effects on colitis *via* activating the AhR signaling.

Although many Trp metabolites were identified in patients with UC with substantial overlap with those identified in colitis mice, some of these metabolites showed different

trends. Inconsistent with quantitative data from colitis mice, the metabolic profiling of serum from patients with UC revealed elevation of IAAld. Moreover, the mouse study failed to validate the positive correlation between IAAld and *Akk*, indicating that the integration of multi-omic data is challenging due to the many variables that can influence associations, resulting in a suboptimal rate of validation in the laboratory.[41] In addition, this study was limited to metagenomes from pooled samples and therefore did not evaluate their associations with individual metabolic profiling in mice.

In conclusion, this study reveals a crucial role for *Akk* as a critical regulator of host metabolism. *Akk* could inhibit KP activation independent of colonocytes without reshaping the serotonin pathway in colitis mice. *Akk* could also increase the levels of IAA and IA in the microbial Trp metabolism pathway. In addition, live *Akk*, pasteurised *Akk* and Amuc_1100 restored the downregulation of CYP1A1, IL-10 and IL-22, suggesting that *Akk* could activate AhR signaling by increasing IA and IAA, thereby relieving colonic inflammation.

Data availability

The 16S rRNA sequencing data from human and mice faeces have been submitted to NCBI (Sequence Read Archive, SRA). The BioProject accession number is PRJNA596333 (https://www.ncbi.nlm.nih.gov/bioproject/PRJNA596333). The RNA sequencing data of mice colon have been deposited in NCBI under accession number PRJNA741389.

Author contributions

Z. Y. G., W. L. P. and Y. H. S. carried out most of the experiments and analyzed the data. Y. H. S., S. X. F., L. J. W. provided clinical samples and performed data analyses. Z. Y. G., Y. Z. and L. J. W. helped with animal experiments and analysis. J. Z., Q. W., Z. Z. and L. L. designed the experiments and analyzed the data. Z. Z. and L. L. conceived the study and participated in its design and coordination and helped to draft the manuscript. All authors have read and approved the final manuscript.

Conflicts of interest

The authors declare that they have no conflict of interest.

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References

1 G. G. Kaplan and J. W. Windsor, *Nat. Rev. Gastroenterol. Hepatol.*, 2021, **18**, 56–66.
2 A. N. Sasson, A. N. Ananthakrishnan and M. Raman, *Clin. Gastroenterol. Hepatol.*, 2021, **19**, 425–435.
3 R. Caruso, B. C. Lo and G. Nunez, *Nat. Rev. Immunol.*, 2020, **20**, 411–426.
4 M. Lee and E. B. Chang, *Gastroenterology*, 2021, **160**, 524–537.
5 A. G. Clooney, J. Eckenberger, E. Laserna-Mendieta, K. A. Sexton, M. T. Bernstein, K. Vagianos, M. Sargent, F. J. Ryan, C. Moran, D. Sheehan, R. D. Sleator, L. E. Targownik, C. N. Bernstein, F. Shanahan and M. J. Claesson, *Gut*, 2021, **70**, 499–510.
6 X. F. Zhang, X. X. Guan, Y. J. Tang, J. F. Sun, X. K. Wang, W. D. Wang and J. M. Fan, *Eur. J. Nutr.*, 2021, **60**, 2855–2875.
7 Y. Liu, F. Yin, L. Huang, H. Teng, T. Shen and H. Qin, *Food Funct.*, 2021, **12**, 2201–2210.
8 Z. Zhang, X. Wu, S. Cao, M. Cromie, Y. Shen, Y. Feng, H. Yang and L. Li, *Nutrients*, 2017, **9**, 677.
9 R. Zhai, X. Xue, L. Zhang, X. Yang, L. Zhao and C. Zhang, *Front. Cell. Infect. Microbiol.*, 2019, **9**, 239.
10 L. Wang, L. Tang, Y. Feng, S. Zhao, M. Han, C. Zhang, G. Yuan, J. Zhu, S. Cao, Q. Wu, L. Li and Z. Zhang, *Gut*, 2020, **69**, 1988–1997.
11 C. Depommier, A. Everard, C. Druart, H. Plovier, M. Van Hul, S. Vieira-Silva, G. Falony, J. Raes, D. Maiter, N. M. Delzenne, M. de Barsy, A. Loumaye, M. P. Hermans, J. P. Thissen, W. M. de Vos and P. D. Cani, *Nat. Med.*, 2019, **25**, 1096–1103.
12 A. Hanninen, R. Toivonen, S. Poysti, C. Belzer, H. Plovier, J. P. Ouwerkerk, R. Emani, P. D. Cani and W. M. De Vos, *Gut*, 2018, **67**, 1445–1453.
13 A. Lavelle and H. Sokol, *Nat. Rev. Gastroenterol. Hepatol.*, 2020, **17**, 223–237.
14 A. Agus, J. Planchais and H. Sokol, *Cell Host Microbe*, 2018, **23**, 716–724.
15 S. Nikolaus, B. Schulte, N. Al-Massad, F. Thieme, D. M. Schulte, J. Bethge, A. Rehman, F. Tran, K. Aden, R. Hasler, N. Moll, G. Schutze, M. J. Schwarz, G. H. Waetzig, P. Rosenstiel, M. Krawczak, S. Szymczak and S. Schreiber, *Gastroenterology*, 2017, **153**, 1504–1516.
16 J. Islam, S. Sato, K. Watanabe, T. Watanabe, Ardiansyah, K. Hirahara, Y. Aoyama, S. Tomita, H. Aso, M. Komai and H. Shirakawa, *J. Nutr. Biochem.*, 2017, **42**, 43–50.
17 S. A. Scott, J. Fu and P. V. Chang, *Proc. Natl. Acad. Sci. U. S. A.*, 2020, **117**, 19376–19387.
18 B. Lamas, M. L. Richard, V. Leducq, H. P. Pham, M. L. Michel, G. Da Costa, C. Bridonneau, S. Jegou, T. W. Hoffmann, J. M. Natividad, L. Brot, S. Taleb, A. Couturier-Maillard, I. Nion-Larmurier, F. Merabtene, P. Seksik, A. Bourrier, J. Cosnes, B. Ryffel, L. Beaugerie, J. M. Launay, P. Langella, R. J. Xavier and H. Sokol, *Nat. Med.*, 2016, **22**, 598–605.

19 Z. Zhang, D. Wang, S. Qiao, X. Wu, S. Cao, L. Wang, X. Su and L. Li, *Sci. Rep.*, 2017, **7**, 4508.
20 X. Y. Wu, L. J. Wang, L. Tang, L. Wang, S. Y. Cao, Q. Wu, Z. Zhang and L. Li, *J. Funct. Foods*, 2018, **46**, 312–319.
21 M. K. Saraf, B. D. Piccolo, A. K. Bowlin, K. E. Mercer, T. LeRoith, S. V. Chintapalli, K. Shankar, T. M. Badger and L. Yeruva, *Microbiome*, 2017, **5**, 77.
22 Y. Deng, M. Zhou, J. Wang, J. Yao, J. Yu, W. Liu, L. Wu, J. Wang and R. Gao, *Gut Microbes*, 2021, **13**, 1–16.
23 X. Li, Z. H. Zhang, H. M. Zabed, J. Yun, G. Zhang and X. Qi, *Mol. Nutr. Food Res.*, 2021, **65**, e2000461.
24 M. A. Borisova, O. A. Snytnikova, E. A. Litvinova, K. M. Achasova, T. I. Babochkina, A. V. Pindyurin, Y. P. Tsentalovich and E. N. Kozhevnikova, *Nutrients*, 2020, **12**, 445.
25 N. Z. Borren, D. Plichta, A. D. Joshi, G. Bonilla, V. Peng, F. P. Colizzo, J. Luther, H. Khalili, J. J. Garber, C. Janneke van der Woude, R. Sadreyev, H. Vlamakis, R. J. Xavier and A. N. Ananthakrishnan, *Clin. Gastroenterol. Hepatol.*, 2021, **19**, 519–527.
26 B. Wang, S. Sun, M. Liu, H. Chen, N. Liu, Z. Wu, G. Wu and Z. Dai, *J. Nutr.*, 2020, **150**, 1966–1976.
27 M. Sun, N. Ma, T. He, L. J. Johnston and X. Ma, *Crit. Rev. Food Sci. Nutr.*, 2020, **60**, 1760–1768.
28 A. A. Badawy, *Int. J. Tryptophan Res.*, 2017, **10**, 1178646917691938.
29 A. I. Thaker, M. S. Rao, K. S. Bishnupuri, T. A. Kerr, L. Foster, J. M. Marinshaw, R. D. Newberry, W. F. Stenson and M. A. Ciorba, *Gastroenterology*, 2013, **145**, 416–425.
30 E. Sugisawa, Y. Takayama, N. Takemura, T. Kondo, S. Hatakeyama, Y. Kumagai, M. Sunagawa, M. Tominaga and K. Maruyama, *Cell*, 2020, **182**, 609–624.
31 S. Dong, M. Chen, F. Dai, Q. Xuan, P. Chen, D. Feng, L. Gao, C. Zhu, Y. Chang, F. F. Chu and Q. Gao, *J. Cell. Biochem.*, 2019, **120**, 9230–9242.
32 M. Salaga, A. Binienda, F. Piscitelli, A. Mokrowiecka, A. I. Cygankiewicz, R. Verde, E. Malecka-Panas, R. Kordek, W. M. Krajewska, V. Di Marzo and J. Fichna, *Biochem. Pharmacol.*, 2019, **161**, 37–51.
33 J. Y. Sakita, M. Bader, E. S. Santos, S. B. Garcia, S. B. Minto, N. Alenina, M. O. Brunaldi, M. C. Carvalho, T. Vidotto, B. Gasparotto, R. B. Martins, W. A. Silva Jr., M. L. Brandao, C. A. Leite, F. Q. Cunha, G. Karsenty, J. A. Squire, S. A. Uyemura and V. Kannen, *J Pathol*, 2019, **249**, 102–113.
34 V. Kannen, M. Bader, J. Y. Sakita, S. A. Uyemura and J. A. Squire, *Trends Endocrinol. Metab.*, 2020, **31**, 611–625.
35 J. Wang, W. Xu, R. Wang, R. Cheng, Z. Tang and M. Zhang, *Food Funct.*, 2021, **12**, 3597–3610.
36 J. H. Shin, Y. K. Lee, W. J. Shon, B. Kim, C. O. Jeon, J. Y. Cho, H. C. Morse 3rd, E. Y. Choi and D. M. Shin, *Eur. J. Nutr.*, 2020, **59**, 3591–3601.
37 K. Li, Z. Hao, J. Du, Y. Gao, S. Yang and Y. Zhou, *Int. Immunopharmacol.*, 2021, **90**, 107183.
38 M. Wlodarska, C. Luo, R. Kolde, E. d'Hennezel, J. W. Annand, C. E. Heim, P. Krastel, E. K. Schmitt, A. S. Omar, E. A. Creasey, A. L. Garner, S. Mohammadi, D. J. O'Connell, S. Abubucker, T. D. Arthur, E. A. Franzosa, C. Huttenhower, L. O. Murphy, H. J. Haiser, H. Vlamakis, J. A. Porter and R. J. Xavier, *Cell Host Microbe*, 2017, **22**, 25–37.
39 J. Shi, P. Du, Q. Xie, N. Wang, H. Li, E. E. Smith, C. Li, F. Liu, G. Huo and B. Li, *Food Funct.*, 2020, **11**, 10736–10747.
40 H. X. Wei, B. Wang and B. Li, *Front. Immunol.*, 2020, **11**, 1315.
41 E. A. Franzosa, A. Sirota-Madi, J. Avila-Pacheco, N. Fornelos, H. J. Haiser, S. Reinker, T. Vatanen, A. B. Hall, H. Mallick, L. J. McIver, J. S. Sauk, R. G. Wilson, B. W. Stevens, J. M. Scott, K. Pierce, A. A. Deik, K. Bullock, F. Imhann, J. A. Porter, A. Zhernakova, J. Fu, R. K. Weersma, C. Wijmenga, C. B. Clish, H. Vlamakis, C. Huttenhower and R. J. Xavier, *Nat. Microbiol.*, 2019, **4**, 293–305.