Research Article

Effects of long-term antibiotic treatment on mice urinary aromatic amino acid profiles

Xuehang Zhu1,2,*, Bin Fu3,*, Manyuan Dong1,2, Yangyang Guo1,2, Zheng Cao1,2 and Junfang Wu4

1State Key Laboratory of Magnetic Resonance and Atomic and Molecular Physics, Key Laboratory of Magnetic Resonance in Biological Systems, Wuhan Center for Magnetic Resonance, Wuhan Institute of Physics and Mathematics, Innovation Academy for Precision Measurement Science and Technology, Chinese Academy of Sciences, Wuhan, China; 2University of Chinese Academy of Sciences, Beijing, China; 3Hubei Provincial Hospital of Integrated Chinese and Western Medicine, Hubei University of Chinese Medicine, Wuhan, China; 4Division of Cardiology, Department of Internal Medicine, Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, China

Correspondence: Junfang Wu (junfang.wu@tjh.tjmu.edu.cn)

Introduction

There are numerous gut microbes that exist in the digestive tract, which constitute a complex ecosystem [1]. The cross-talk between host and gut microbiota has been well established in a bi-direct manner. That is, symbiotic microbes rely on the nutrient-rich environment provided by the gut. On the other hand, gut microbes play an important role in regulating the host’s physiological functions, including processing nutrient substances, maintaining energy balance [2], and improving the immune system [3]. Studies have found that the disruption of the microbial ecological balance or the loss of bacterial diversity would lead to pathophysiological processes in the host. For example, lack of gut microbes increased the incidence of hyperglycemia, adiposity, and insulin resistance in the germ-free mice model [4]. In turn, regulation of the microbial flora could modulate the gastrointestinal physiology, promote intestinal inflammation, and alleviate the non-alcohol fatty liver disease or neuropsychiatric diseases [5,6]. But the effects of long-term microbial disturbance on host remain unclear.

Previous studies have shown that the metabolites are good indicators for representing the cross-talk between the host and the gut microbiota. Gut microbiota can regulate the gut peptides (such as serotonin (5-HT) and dopamine (DA)) through neuroendocrine or the vagal afferent pathway via the microbiota–gut–brain axis [7]. Those metabolites produced under the existence of microbiota, such as bile acids [8], short-chain fatty acids, and trimethyl-N-oxide can play physiological roles in the host [6,9]. Besides, the aromatic

© 2021 The Author(s). This is an open access article published by Portland Press Limited on behalf of the Biochemical Society and distributed under the Creative Commons Attribution License 4.0 (CC BY).
amino acids (AAAs), including phenylalanine, tryptophan, and tyrosine, are kind of essential amino acids that participate in the cross-talk between host and its microbiota. Some of the AAAs, which mainly come from dietary food, undergo digestion in the intestine, and the rest escape intestinal digestion could serve as fermentable substrates for further metabolism by gut microbiota [10]. The increased AAA is a risk that associated with insulin resistance and diabetes [11]. Dodd et al. found Clostridium sporogenes could modulate serum AAA levels and affect intestinal permeability and systemic immunity in a gnotobiotic mice model [12]. Taken together, there is accumulating evidence that levels of AAA are associated with the disturbance of the intestinal microbial community.

In this work, two representative antibiotics, gentamicin and ceftriaxone, were selected to establish a mice model for observing the effects of long-term antibiotics on host and its intestinal flora metabolism. Of them, gentamicin belongs to aminoglycosides and its antibacterial spectrum is mainly Gram-negative bacteria. It can combine with bacterial 30S ribosome subunits for blocking the synthesis of bacterial proteins. Ceftriaxone is a β-lactam antibiotic that inhibit cell wall formation and facilitate its cracking. It has a strong antibacterial effect on both Gram-negative and -positive bacteria. The reasons for choosing these two antibiotics are: first, these two antibiotics are not absorbable by the host following oral administration [13], which are suitable for establishing a mice model with local intestinal flora disturbance and investigating the metabolic changes on local microbial dysbiosis. Second, both of them are broad-spectrum antibiotics that can widely inhibit intestinal flora in a certain complementary way to investigate the effects of combined antibiotics on host intestinal flora. Third, these two antibiotics metabolize very quickly by oral administration so that it has less impact on other organs except for intestinal tracts to avoid antibiotic toxicity (such as gentamicin-induced renal toxicity and hearing loss) at the greatest extent. Our previous works have found a 4-day short-term combined gentamicin and ceftriaxone treatment suppressed bacterial fermentation and stimulated protein degradation [14]. However, the long-term antibiotic effects on metabolic profiling remain unknown.

Here we established a 4-month continuous long-term antibiotic treated mice model which started from 3 weeks since weaning. To reveal the relationship between the host and its gut microbes, the urinary AAA metabolites and cecal gut microbial profiling were measured by a combination of targeted liquid chromatography-tandem mass spectrometry (LC-MS/MS) and 16S ribosomal RNA gene sequencing. The interactions between changed AAA and gut microbiota were calculated to illustrate their correlation.

Materials and methods
Animal experiments and sample collection
Animals in experiments were bred by mating male and female C57BL/6 mice in SPF animal facility under standard conditions (temperature, ∼20°C; humidity, 40–70%; 12-h/12-h light/dark cycle) with free access to chow diet and water at Wuhan Institute of Physics and Mathematics (SYKK 2015-0051). The F1 hybrid male and female C57BL/6 mice were purchased from Hunan Slyk Jingda Experimental Animals Co., Ltd. The experiment started with mice aged 3 weeks at their weaning and received the same standard diet according to laboratory animals’ nutrients for formula feeds of China (GB14924.3-2010) all through the experiment. The mice were divided into two groups (Group C: control, n=8, Group A: control mice treated with antibiotics, n=8). The mice in group A received combined antibiotics at low doses by oral gavage for consecutive 13 weeks (sulfate gentamicin: 180 mg/kg/day; ceftriaxone sodium: 500 mg/kg/day). Mice in the control group were treated with equal vehicle (0.9% saline) once a day until killing at the 16th week. All experiments and procedures were adhering to the National Guidelines for Experimental Animal Welfare and the ethics approval were confirmed by the research ethics committee at Wuhan Institute of Physics and Mathematics, Chinese Academy of Sciences.

The urine samples were collected 1 day before killing at week 16. Mice were killed by cervical dislocation under isoflurane anesthesia to obtain serum. The colon was quickly cut out for freezing in liquid nitrogen. Cecal contents were gently squeezed out of the excised cecum into the Eppendorf tube and flash-frozen. All samples were stored at −80°C until further analysis.

16S ribosomal RNA gene sequencing
Microbial DNA was extracted from the cecal content of the mice, and the bacteria 16S ribosomal RNA gene amplicon sequence library was prepared. The equimolar amounts of purified DNA amplicons were further amplified on an Illumina MiSeq platform for paired-end sequencing (2 × 300 bp) according to the standard protocols by Shanghai Major Bio-Pharm Technology Co., Ltd. The raw fastq files were then demultiplexed and quality-filtered (Phred quality score ≥ 20) using QIIME (version 1.9.1). The Operational Taxonomic Units (OTUs) table was clustered with a 97% similarity cutoff based on the open-reference approach using UCLUST.
Targeted metabolite quantification by LC-MS/MS

Targeted host–microbiota related co-metabolites in urine were measured on Agilent 6490 triple quadrupole LC-MS/MS equipped with electrospray ion source. Briefly, The internal standard (IS) mixture was prepared with benzoic acid-C_{13} (200 μM), homovanillic acid-d_{5} (200 μM), 4-hydroxybenzoic acid-C_{13} (25 μM), 4-hydroxy-3-methoxymandelic acid-d_{3} (25 μM), indole-3-acetic acid-d_{3} (10 μM), nicotinamide-d_{4} (1.25 μM), 3-hydroxytryptamine-d_{3} (5 μM), l-phenylalanine-N_{15} (50 μM), l-tyrosine-C_{13} (25 μM), hippuric acid-N_{15} (400 μM), and l-tryptophan-d_{3} (100 μM). Then 20 μl of IS mixture and 170 μl of deionized water was added into 10 ml of the urine sample. After vortex and centrifugation (12000 rpm, 4°C, 10 min), the supernatant was filtered by 0.22-μm filter membrane before LC-MS/MS measurement. The metabolites were quantified with the ISs. At the same time, the creatinine concentration was tested to be a reference in the quantification of host–microbiota co-metabolites in urine samples. The concentration of urinary co-metabolites was expressed as μmol per liter urine relative to mmol/l of creatinine.

To further investigate the tryptophan pathway-related metabolites in serum and cecal contents, 10 μl of serum was mixed with 10 μl of IS (trp-d_{5}, 2 μg/ml) and 10 μl of 50% acetonitrile (ACN) containing 0.1% formic acid. Subsequently, 150 μl ice-cold methanol was added into this mixture and stood for 30 min at −20°C for protein precipitation. After centrifugation for 15 min (3000 × g, 4°C), the supernatants were collected and evaporated to dryness with a concentrator [15]. The dried extracts were reconstituted in 100 μl of 50% ACN containing 0.1% formic acid. Two microliter was injected into the LC-MS system. For sample preparation in cecal contents, ~10 mg samples were mixed with 400 μl ice-cold methanol, 50 μl of 50% ACN containing 0.1% formic acid, and 10 μl of IS (trp-d_{5}, 2 μg/ml). Then the mixture was homogenized by tissuelyzer for 90 s at 25 Hz. After centrifugation and evaporation [15], the dried extracts were reconstituted in 200 μl of 50% ACN containing 0.1% formic acid. Quantification of tryptophan metabolites was performed using calibration curves and ratios of the integrated peak areas of their ISs.

RNA extraction and RT-qPCR of colon tissue

Total RNA was extracted from colon tissue using RNAiso Plus (TAKARA, JAPAN) according to the manufacturer’s instructions. Then 1 μg RNA was extracted for reverse transcription reaction. The complementary DNA (cDNA) was synthesized with transcript all-in-one first-strand cDNA synthesis supermix for qPCR (Transgene, Beijing). The cDNA was diluted with 1:10, and then 1 μl was used for each qPCR. The qPCR was performed using SYBR Green reagent (Applied Biosystems, CA, U.S.A.) on a Step-One Real-Time PCR System (Applied Biosystems) with the following specific gene primers were prepared for Aryl hydrocarbon receptor (AHR): Forward Prime 5′-TCGGGGTACCAGTTCATCCA-3′, Reverse Prime 5′-ACCTCCAGCGACTGTGTTTT-3′. The qPCR conditions were set to 40 cycles of 95°C for 20 s, 95°C for 30 s, and 60°C for 30 s. The GAPDH was chosen as a housekeeping gene for each experiment. The relative expression was calculated using the formula $2^{-\Delta\Delta C_t}$ for analysis.

Statistical analysis

Statistical analyses and data mapping were performed using SPSS 25 (SPSS Inc., Chicago, IL, U.S.A.) and GraphPad Prism 8 statistical software (La Jolla, CA, U.S.A.). Independent Student’s t test (two-tailed) was performed for comparison between two groups. Otherwise, the non-parametric Mann–Whitney U test was used for those data that did not pass the homogeneity of variance test. The $P<0.05$ with FDR correction was considered statistically significant. All values were expressed as Mean ± S.D. The statistical analysis about 16S ribosomal RNA gene sequencing was performed on the I-Sanger cloud platform, and data visualization was conducted in the R environment.

Results

Effects of long-term antibiotics treatment on mice bodyweight and gut microbial profiling

Compared with the control group, the body weight of mice treated with long-term antibiotics did not show significant differences at an early stage. Since 11 weeks after antibiotics treatment, bodyweight in group A mice started to display a significant decrease compared with control ones (Figure 1A).

To investigate the effects of long-term antibiotics treatment on microbial profiling, the 16S gene sequence in mice cecal contents were measured in both groups. The gut microbiota richness was measured by numbers of observed OTU. The principal coordinated analysis (PCoA) based on the Bray–Curtis distance algorithm revealed a clear cluster for mice treated with antibiotics and the control ones (Figure 1B). From the phylogenetic diversity index, mice in group A showed significant down-regulation of α diversity, indicating that the suppressed gut microbiota diversity following by antibiotics treatment (Figure 1C). The relative abundance of taxa was assessed at different levels of
Effects of long-term antibiotics treatment on mice bodyweight and gut microbial profiling

Figure 1. Effects of long-term antibiotics treatment on mice bodyweight and gut microbial profiling
(A) Bodyweight changes of mice treated with antibiotics and its equal vehicle in 16 weeks. (B) The PCoA plot for the compared two groups on genus level. (C) The α diversity in the gut microbiome of mice treated with vehicle and antibiotics (P=0.0007). (D, E) Community abundance bar plot analysis in the gut microbiome of mice treated with vehicle and antibiotics on phylum (D) and genus (E) levels. (F) The F/B ratio decreased in mice treated with antibiotics compared with those treated with vehicle (P=0.0148). Keys: C, control mice; A, control mice treated with antibiotics. Data are presented as mean ± SD; n=8 per group; *P<0.05, ***P<0.001 by two-tailed Student’s t test or Mann–Whitney test. Abbreviation: PCoA, principal coordinated analysis.

Effects of long-term antibiotics on metabolic profiling by targeted LC-MS measurement

The AAA and their downstream metabolites produced with the existence of gut flora in urine were quantified by targeted LC-MS measurement to explore the interaction between the host and its gut microbiota.

Of the 26 quantified urinary metabolites, 15 were changed significantly in mice treated with long-term antibiotics compared with the controls. There were five changed metabolites related to tyrosine metabolism (Figure 2A and Table 1). The levels of tyrosine, 3-(3,4-dihydroxy phenyl) propionic acid (3,4-HPAA), and 4-hydroxyphenyl acetic acid (4-HPAA) were increased in group A whereas the levels of DA and 3-methoxytyramine (3-MT) were decreased significantly. There were eight changed metabolites involved in phenylalanine metabolism. The increased levels of urinary phenylalanine, 4-hydroxybenzoic acid (4-HBA), and p-hydroxyhippuric acid (p-HHA), whereas the decreased levels of phenylacetyl glycine (PA-Gly), hippuric acid (HA), 3-hydroxyphenyl acetic acid (3-HPAA), phenylacetyl glutamine (PA-Gln), and 3-(3-hydroxyphenyl) propanoic acid (3-HPPA) were found in antibiotics-treated mice (Figure 2B and Table 1). The levels of tryptophan and indole-3-lactic acid (I3LA) in tryptophan metabolism were increased significantly in group A (Figure 2C and Table 1). Such changes implied the accumulated AAA and the re-distribution of the host–microbiota co-metabolites following antibiotic treatment.

To further investigate the pathways involved in tryptophan metabolism, a total of seven metabolites were measured by targeted LC-MS/MS in serum and cecal contents in both groups (Table 2). The metabolites in serum showed
### Table 1

The concentration of measured urinary host–microbial AAA co-metabolites in two groups

| Concentration (μM) | C       | A       |
|-------------------|---------|---------|
| **Tyrosine pathway** |         |         |
| Tyr               | 0.83 ± 0.19 | 1.33 ± 0.29 |
| DA                | 0.22 ± 0.06 | 0.15 ± 0.04 |
| 3-MT              | 0.1 ± 0.03  | 0.05 ± 0.01 |
| 3,4-HPPA          | 2.06 ± 0.47 | 3.61 ± 0.86 |
| 4-HPA             | 2.55 ± 0.69 | 4.67 ± 1.86 |
| **Phenylalanine pathway** |         |         |
| Phe               | 0.6 ± 0.22  | 0.83 ± 0.18 |
| p-HHA             | 1.82 ± 1.21 | 3.92 ± 0.71 |
| 4-HBA             | 1.07 ± 0.61 | 2.58 ± 0.33 |
| HA                | 53.31 ± 25.52 | 3.12 ± 0.79 |
| PA-Gln            | 0.06 ± 0.03  | 0.02 ± 0.01 |
| PA-Gly            | 29.05 ± 11.89 | 10.89 ± 3.15 |
| 3-HPAA            | 0.67 ± 0.15  | 0.22 ± 0.03 |
| 2-HHA             | 0.18 ± 0.08  | 0.17 ± 0.04 |
| 3-HPPA            | 0.74 ± 1.29  | N.D.      |
| PPA               | 0.31 ± 0.17  | 0.14 ± 0.09 |
| 2-HPAA            | 0.4 ± 0.13   | 0.33 ± 0.14 |
| **Tryptophan pathway** |         |         |
| Trp               | 0.15 ± 0.05  | 0.2 ± 0.03  |
| XAN               | 1.13 ± 0.25  | 1.07 ± 1.26 |
| Tryptamine        | 0.04 ± 0.01  | 0.04 ± 0.01 |
| KYN               | 0.59 ± 0.3   | 0.69 ± 0.29 |
| Tryptophanol      | 0.005 ± 0.003 | N.D.       |
| 5-HIAA            | 1.13 ± 0.25  | 1.24 ± 0.19 |
| 5-HT              | 0.08 ± 0.02  | 0.08 ± 0.01 |
| I3LA              | 0.59 ± 0.16  | 1.36 ± 0.26 |
| I3AA              | 0.08 ± 0.03  | 0.05 ± 0.03 |
| NAM               | 2.87 ± 1.13  | 2.18 ± 1.67 |

All values were presented as mean ± SD. Abbreviations: C, control; A, control mice treated with antibiotics; I3AA, indole-3-acetic acid; KYN, kynurenine; NAM, nicotinamide; ND, not detected; PAA, phenylacetic acid; Phe, phenylalanine; Trp, Tryptophan; Tyr, Tyrosine; XAN, xanthurenic acid; 2-HHA, 2-hydroxyhippuric acid; 2-HPAA, 2-hydroxyphenylacetic acid; 5-HIAA, 5-hydroxyindole-3-acetic acid.

### Table 2

The concentration of measured tryptophan-related metabolites in two groups

| Concentration (μM) | C       | A       |
|-------------------|---------|---------|
| **Metabolites in serum** |         |         |
| Indole            | 3.83 ± 0.48 | 4.42 ± 1.13 |
| KA                | 4.97 ± 0.72  | 5.22 ± 0.67 |
| KYN               | 34.82 ± 4.23 | 44.82 ± 5.83 |
| PA                | 4.95 ± 0.8   | 5.23 ± 1.78 |
| 5-HT              | 150.86 ± 26.23 | 206.44 ± 20.83 |
| Trp               | 1635.29 ± 306.2 | 1881.65 ± 272.59 |
| XAN               | 12.86 ± 0.93 | 33.71 ± 25.6 |
| **Metabolites in cecum** |         |         |
| Indole            | 0.21 ± 0.05  | 0.3 ± 0.14 |
| KA                | 0.46 ± 0.13  | 0.95 ± 1.39 |
| KYN               | 1.92 ± 0.34  | 29.02 ± 27.84 |
| PA                | 4.16 ± 4.37  | 2.86 ± 2.37 |
| 5-HT              | 0.88 ± 0.43  | 11.07 ± 9.92 |
| Trp               | 39.96 ± 9.98 | 41.55 ± 35.94 |
| XAN               | 1.86 ± 0.54  | 3.44 ± 4.28 |

All values were presented as mean ± SD. Abbreviations: C, control; A, control mice treated with antibiotics; KA, kynurenic acid; PA, picolinic acid; Trp, tryptophan; XAN, xanthurenic acid.
Figure 2. Quantification of three kinds of AAAs co-metabolism metabolites in mice treated with vehicle and antibiotics

Quantification of changed urinary metabolites involved in (A) tyrosine metabolism, (B) phenylalanine metabolism, and (C) tryptophan metabolism. (D,E) Quantification of tryptophan-related metabolites in serum sample (D) and cecum content (E) of mice treated with vehicle and antibiotics. (F) AHR mRNA expression in the colon of mice treated with vehicle and antibiotics. Keys: C, control mice; A, control mice treated with antibiotics; PAA, phenylacetic acid; Phe, phenylalanine; Trp, tryptophan; Tyr, tyrosine. Data are presented as mean ± SD; n=8 per group; *P<0.05, **P<0.01, and ***P<0.001, by two-tailed Student’s t test or Mann–Whitney test.

Asimilar pattern with those in cecal contents. The levels of kynurenine (KYN) and serotonin in serum, as well as the cecal content of mice treated with antibiotics, were significantly increased (Figure 2D,E). Even we did not find significant changes in indole; however, its receptor, the AHR mRNA expression declined significantly in colon tissue from antibiotics-treated mice (Figure 2F), which indicated stimulated conversion from tryptophan into KYN and 5-HT pathway under the suppressed intestinal micro-ecology following long-term antibiotics treatment.

Correlation between the gut microbiome and metabolic profiles

The connection between changed gut microbiota and AAA was presented in a heat map to present the correlation between bacterial species and AAA metabolites in measured biofluid samples of antibiotic-treated mice (Figure 3). The increased abundance of Bacteroides showed a highly positive correlation with increased AAA and their downstream products 4-HPAA, I3LA, 4-HBA, and 3,4-HPPA. Except for the above positive correlation, the Provitellaceae, Blautia, and Lachnoclostridium that belong to the Bacteroidetes phylum also showed a negative correlation with 3-MT and DA which are involved in the tyrosine pathway, as well as a negative correlation with PA-Gln, PA-Gly, HA, and 3-HPPA that are involved in the phenylalanine pathway. The decreased abundance of several genera (such as Lachnospiraceae and Erysipelotrichaceae) belong to the Firmicutes phylum showed a highly positive correlation with PA-Gln, PA-Gly, HA, and 3-HPPA involved in the phenylalanine pathway, together with a negative correlation with 4-HBA in the phenylalanine pathway, 3,4-HPPA in the tyrosine pathway, and KYN, XAN (Xanthurenic acid), I3LA that are involved in the tryptophan pathway.
Figure 3. Correlations between gut microbiota on genus level and metabolites involved in the AAA pathway
The correlation was calculated by the Spearman correlation. *$P<0.05$, **$P<0.01$ and ***$P<0.001$. Abbreviations: C, Cecal content; I3AA, indole-3-acetic acid; PAA, phenylacetic acid; S, serum; Trp, tryptophan; Phe, phenylalanine; Tyr, tyrosine; U, urine.

Discussion
The gut microbiome is known to play a vital role in human health. To investigate the metabolic relationship between host and microbiome in long-term gut microbiota disturbance, we investigated the gut microbial composition and urinary AAA profiles of mice following combined gentamicin and ceftriaxone treatments after weaning for nearly 4 months by integrating metagenomics and metabolomics. Our study showed that long-term antibiotic treatment
induced the re-distribution of the gut microbiome and unbalanced host–microbiota co-metabolism of AAA, which provide direct evidence for the gut microbiota that participates in AAA metabolism.

The F/B ratio comes from the most abundant phyla (Firmicutes and Bacteroidetes) in the gut microbiome, is associated with several pathological conditions. The increased F/B ratio has been found in non-alcoholic fatty liver related to obese youth due to a lower abundance of Bacteroidetes, Prevotella, Gemmiger in phylum Bacteroidetes [16]. Ley et al. bring out the point that the F/B ratio could be a very first index derived from the dysbiotic microbiota of obesity [17,18]. The imbalances in the F/B ratio implied the disturbed microbial diversity or abundance in the antibiotics treated mice model.

The increased levels of urinary tyrosine, 3,4-HPPA, and 4-HPAA, and the decreased levels of DA and 3-MT revealed the effect of gut microbial disorder on tyrosine metabolism, which indicated the long-term antibiotic intervention stimulated conversion from tyrosine into 3,4-HPPA and 4-HPAA, and inhibited the synthesis of DA and 3-MT (Figure 4). DA is a naturally occurring catecholamine of neurotransmitters participating in various physiological functions [19]. Its deficiency can result in depression [20], anxiety [21], fatigue [22], and memory impairment [23], which have been reported in previous antibiotics-treated animal models [24] and patients [25]. This implied the possible mechanisms for neurological defects or symptoms after long-term antibiotics treatment. Maini et al. found that Enterococcus can help tyrosine decarboxylase (TDC) to degrade tyrosine into DA [26]. Here we found Enterococcus is not only shows a negative correlation with DA and 3-MT significantly but also shows a positive correlation with 4-HPAA (Figure 3). It may be because TDC not only participates in the transition from tyrosine into DA and 3-MT but also involves the conversion from tyrosine into 4-HPAA. Besides, the Bacteroides, Clostridium, Eubacterium, and Parabacteroides also participate in the conversion of tyrosine into 4-HPAA [27], which consisted of the positive correlation between Enterococcus, Bacteroides, Eubacterium, Parabacteroides and 4-HPAA in our results.

The conversion from phenylalanine into 4-HBA and p-HHA, together with the suppressed levels in phenylacetic acid (PAA), phenylpropionic acid (PPA) and benzoate (BA) pathway that indicated the involvement of the phenylalanine pathway after antibiotics treatment (Figure 4). Previous studies have shown that phenylalanine enrichment mediated by gut microbiota can promote immune cells to infiltrate the brain, leading to pathological neuroinflammation...
Desulfovibrio, Bacteroides, Eubacterium, Enterococcus, and form tryptophan into indole and its derivatives such as the up-regulated KYN and 5-HT in the antibiotics mice. Several bacterial species have been reported to help trans-intestinal microorganisms, the indole-related metabolites produced by tryptophan should be suppressed, along with CXCL1, IL-17, and IFN-γ-treatment of ampicillin and vancomycin mice model [37]. Knoop et al. found the increased inflammatory cytokines in the gut. Zarrinpar et al. proved that the up-regulation of pro-inflammatory cytokines IL-6 and IL-12 in a combined cecum may suggest stimulated inflammatory status [35] and immune reaction [36] in long-term antibiotics treatment into KYN in the gut [32]. Here the up-regulation of KYN and IDO (KYN/tryptophan ratio [33,34]) in serum and cecum can activate IDO (Indoleamine 2,3-dioxygenase 1), a rate-limiting enzyme for transforming tryptophan pathway of tryptophan, mice with altered intestinal flora lack sufficient AHR ligands and further aggravate the inflammatory state and impaired immune system in the long-term antibiotics model. It is known that the Clostridium, Lactobacillus, and Bacteroides [10,31]. The urinary metabolite 3-HPPA/PA-Gln/PA-Gly were also found to highly correlate with Clostridium/Bacteroides in our results. Except for this, the phenylalanine also could metabolize into phenolic, benzoic, and phenyl derivatives such as BA, HA, and 4-HBA with help of Clostridium, Roseburia, Ruminococcus, and Lactobacillus [27,31]. The Clostridium, Roseburia, Ruminococcus, and Lactobacillus were positively correlated with decreased HA in our results. In contrast, the Eubacterium was positively correlated with the increased levels of 4-HBA and p-HHA (Figure 4). This suggests that the decreased abundance of Clostridium, Roseburia, Ruminococcus, Lactobacillus, and increased abundance of Eubacterium affected the catabolism from phenylalanine into PAA, BA, 4-HBA, and PPA after the long-term antibiotics intervention.

Tryptophan is another essential AAA that can mediate the interaction between the host and its gut microorganisms by three major pathways: KYN pathway, 5-HT pathway, and indole pathway [32]. The KYN pathway is the main pathway of tryptophan metabolism with the aid of Pseudomonas [33]. Agus et al. found the release of inflammatory cytokines can active IDO (Indoleamine 2,3-dioxygenase 1), a rate-limiting enzyme for transforming tryptophan into KYN in the gut [32]. Here the up-regulation of KYN and IDO (KYN/tryptophan ratio [33,34]) in serum and cecum may suggest stimulated inflammatory status [35] and immune reaction [36] in long-term antibiotics treatment. Zarrinpar et al. proved that the up-regulation of pro-inflammatory cytokines IL-6 and IL-12 in a combined treatment of ampicillin and vancomycin mice model [37]. Knoop et al. found the increased inflammatory cytokines CXCL1, IL-17, and IPN-γ in single dose of a combination of four antibiotic treatments [38], which confirmed the antibiotics promoted inflammation through the bacteria translocation. Besides, under the condition of diminished intestinal microorganisms, the indole-related metabolites produced by tryptophan should be suppressed, along with the up-regulated KYN and 5-HT in the antibiotics mice. Several bacterial species have been reported to help transform tryptophan into indole and its derivatives such as Clostridium, Lactobacillus, Ruminococcus, Anaerostipes, Desulfovibrio, Bacteroides, Eubacterium, Enterococcus, and Parabacteroides [39]. However, because of the difficulty in coverage of detecting all the indole and its derivatives in our results, we did not find the direct correlation between indole and its derivatives with bacterial species. But the negative correlation between metabolites in the KYN/5-HT pathway and the Clostridium, Lactobacillus, Ruminococcus, Anaerostipes, Desulfovibrio support it indirectly. It was known that indole and its derivatives are endogenous ligands of AHR [40], which mediates a variety of physiological functions involving immunity and intestinal homeostasis [41,42]. Based on the decreased levels of metabolites in the indole pathway, mice with altered intestinal flora lack sufficient AHR ligands and further aggravate the intestinal inflammation [36]. The decreased expression of AHR in the colon tissue after antibiotic treatment further confirmed such hypothesis.

In conclusion, the long-term antibiotic treatment significantly changed the composition of the gut microbiota and destroyed the homeostasis in the intestinal metabolism. The host–gut microbiota disturbance manifested as accumulated AAA and re-distribution of their down-streaming metabolites. The correlation between gut microbiota and AAA metabolites could provide essential information for selecting the functional bacteria, which could be helpful in optimizing therapeutic strategies for long-term antibiotics induced dysbiosis in the near future. Our results also revealed the AAA profiles could be an indicator for exploring interactions between host and gut microbiota.

**Data Availability**
The data used to support the findings of the present study are available from the corresponding author upon request.

**Competing Interests**
The authors declare that there are no competing interests associated with the manuscript.

**Funding**
This work was supported by the Natural National Science Foundation of China [grant number 31971358]; and the Hubei Provincial Natural Science Foundation of China [grant number 2017CBF536].

© 2021 The Author(s). This is an open access article published by Portland Press Limited on behalf of the Biochemical Society and distributed under the Creative Commons Attribution License 4.0 (CC BY).
Author Contribution

X.H.Z. and J.F.W. performed animal experiments. X.H.Z., M.Y.D., Y.Y.G., and Z.C. conducted data analysis. X.H.Z., B.F. and J.F.W. contributed to data interpretations and manuscript preparation.

Abbreviations

3,4-HPPA, 3-(3,4-dihydroxy phenyl) propionic acid; 3-HPAA, 3-hydroxyphenyl acetic acid; 3-HPPA, 3-(3-hydroxyphenyl) propanoic acid; 3-MT, 3-methoxytyramine; 4-HBA, 4-hydroxybenzoic acid; 4-HPAA, 4-hydroxyphenyl acetic acid; 5-HT, serotonin; AAA, aromatic amino acid; AHR, aryl hydrocarbon receptor; BA, benzoate; cDNA, complementary DNA; DA, dopamine; F/B, Firmicutes/Bacteroidetes; FDR, false discovery rate; HA, hippuric acid; I3LA, indole-3-lactic acid; IDO, indoleamine 2,3-dioxygenase 1; IS, internal standard; KYN, kynurenine; LC-MS/MS, liquid chromatography-tandem mass spectrometry; OTU, operational taxonomic unit; p-HHA, p-hydroxyhippuric acid; PA-Gln, phenylacetyl glutamine; PA-Gly, phenylacetyl glycine; PAA, phenylacetic acid; PPA, phenyl propionic acid; TDC, tyrosine decarboxylase.

References

1. Clemente, J.C., Ursell, L.K., Parfrey, L.W. and Knight, R. (2012) The impact of the gut microbiota on human health: an integrative view. Cell 148, 1259–1270, https://doi.org/10.1016/j.cell.2012.01.035
2. Martin-Gallausiaux, C., Marinelli, L., Blottiere, H.M., Larraufie, P. and Lapaque, N. (2020) Scfa: Mechanisms and functional importance in the gut. Proc. Nutr. Soc. 1–13, https://doi.org/10.1017/S0029665120006916
3. Vangolsenhoven, R. and Cresci, G.A.M. (2020) Role of microbiome and antibiotics in autoimmune diseases. Nutr. Clin. Pract. 35, 406–416, https://doi.org/10.1002/ncc.10489
4. Logan, I., Bohe, G., Miranda, C., Vasquez-Perez, S., Choi, J., Lowry, M. et al. (2020) Germ-free swiss webster mice on a high-fat diet develop obesity, hyperglycemia, and dyslipidemia. Microorganisms 8, 520, https://doi.org/10.3390/microorganisms8040520
5. Dan, Z., Mao, X., Liu, G., Guo, M., Zhuang, Y., Liu, Z. et al. (2020) Altered gut microbiota profile is associated with abnormal metabolism activity of autism spectrum disorder. Gut Microbes 11, 1246–1267, https://doi.org/10.1080/19490976.2020.1747329
6. Powell, N., Walker, M.M. and Talley, N.J. (2017) The mucosal immune system: master regulator of bidirectional gut–brain communications. Nat. Rev. Gastroenterol. Hepatol. 14, 143–159, https://doi.org/10.1038/nrgastro.2016.191
7. Collins, S.M., Surette, M. and Berck, P. (2012) The interplay between the intestinal microbiota and the brain. Nat. Rev. Microbiol. 10, 735–742
8. Vrieze, A., Out, C., Fuentes, S., Jonker, L., Reuling, I., Kootte, R.S. et al. (2014) Impact of oral vancomycin on gut microbiota, bile acid metabolism, and insulin sensitivity. J. Hepatol. 60, 824–831, https://doi.org/10.1016/j.jhep.2013.11.034
9. Lepage, P., Leclerc, M.C., Joossens, M., Mondot, S., Blottiere, H.M., Raes, J. et al. (2013) A metagenomic insight into our gut's microbiome. Gut 62, 146–158, https://doi.org/10.1136/gutjnl-2011-301805
10. Liu, Y., Hou, T., Wang, G., Zheng, X. and Hao, H. (2020) Gut microbiota composition of aromatic amino acids as signals in host-microbe interplay. Trends Endocrinol. Metab. 31, 818–834
11. Wang, T.J., Larson, M.G., Vasan, R.S., Cheng, S., Rhee, E.P., McCabe, E. et al. (2011) Metabotype profiles and the risk of developing diabetes. Nat. Med. 17, 448–453, https://doi.org/10.1038/nm.2307
12. Dodd, D., Spitzer, M.H., Van Treuren, W., Merrill, B.D., Higginbottom, S.K. et al. (2017) A gut bacterial pathway metabolizes aromatic amino acids into nine circulating metabolites. Nature 551, 648–652, https://doi.org/10.1038/nature24661
13. Rudin, A., Healey, A., Phillips, C.A., Gump, D.W. and Forsyth, B.R. (1970) Antibacterial activity of gentamicin sulfate in tissue culture. Appl. Microbiol. 20, 989–990, https://doi.org/10.1128/AM.20.6.989-990.1970
14. Zhao, Y., Wu, J., Li, J., Zhou, N.-Y., Tang, H. and Wang, Y. (2013) Gut microbiota composition modifies fecal metabolic profiles in mice. J. Proteome Res. 12, 2987–2999, https://doi.org/10.1021/pr400263n
15. Dong, M., Xu, X., Huang, Q., Lei, H., Xu, G., Ma, J. et al. (2019) Dose-dependent effects of tricloacarbon exposure on lipid homeostasis in rats. Chem. Res. Toxicol. 32, 2320–2328, https://doi.org/10.1021/acs.chemrestox.9b00316
16. Kravetz, A.M., Testerman, T., Galuppo, B., Graf, J., Pierpont, B., Siebel, S. et al. (2020) Effect of gut microbiota and pnpla3 rs738409 variant on non-alcoholic fatty liver disease (nafld) in obese youth. J. Clin. Endocrinol. Metab. 105, e3575–e3585
17. Tseng, C.-H. and Wu, C.-Y. (2019) The gut microbiome in obesity. J. Formos. Med. Assoc. 118, S3–S9, https://doi.org/10.1016/j.jfma.2018.07.009
18. Ley, R.E., Turnbaugh, P.J., Klein, S. and Gordon, J.I. (2006) Human gut microbiota associated with obesity. Nature 444, 1022–1023, https://doi.org/10.1038/4441022a
19. Eisenhofer, G., Aneman, A., Friborg, H., Hooper, D., Fändriks, L., Lonroth, H. et al. (1997) Substantial production of dopamine in the human gastrointestinal tract. J. Clin. Endocrinol. Metab. 82, 3864–3871, https://doi.org/10.1210/jcem.82.11.4339
20. Grace, A.A. (2016) Dysregulation of the dopamine system in the pathophysiology of schizophrenia and depression. Nat. Rev. Neurosci. 17, 524–532
21. Dedic, N., Kühne, C. and Jakovcevski, M. (2018) Chronic crh depletion from gabaergic, long-range projection neurons in the extended amygdala reduces dopamine release and increases anxiety. Nat. Neurosci. 21, 803–807, https://doi.org/10.1038/s41593-018-0151-z
22. Cordeiro, L.M.S., Rabelo, P.C.R., Moraes, M.M., Teixeira-Coelho, F., Coimbra, C.C., Wanner, S.P. et al. (2017) Physical exercise-induced fatigue: The role of serotoninergic and dopaminergic systems. J. Med. Biol. Res. 50, e4632, https://doi.org/10.1590/1414-431x20176432
23. Bahari, Z., Meftahi, G.H. and Meftahi, M.A. (2018) Dopamine effects on stress-induced working memory deficits. Behav. Pharmacol. 29, 584–591, https://doi.org/10.1097/FBP.0000000000000429

© 2021 The Author(s). This is an open access article published by Portland Press Limited on behalf of the Biochemical Society and distributed under the Creative Commons Attribution License 4.0 (CC BY).
24 Koutzoumis, D.N., Vergara, M., Pino, J., Buddendorff, J., Khooshbooei, H., Mandel, R.J. et al. (2020) Alterations of the gut microbiota with antibiotics protects dopamine neuron loss and improve motor deficits in a pharmacological rodent model of parkinson's disease. *Exp. Neurol.* **325**, 113159, https://doi.org/10.1016/j.expneurol.2019.113159

25 Missale, C., Nash, S.R., Robinson, S.W., Jaber, M. and Caron, M.G. (1998) Dopamine receptors: from structure to function. *Physiol. Rev.* **78**, 189–225, https://doi.org/10.1152/physrev.1998.78.1.189

26 Maini Rekdal, V., Bess, E.N., Bisanz, J.E., Turnbaugh, P.J. and Balskus, E.P. (2019) Discovery and inhibition of an interspecies gut bacterial pathway for levodopa metabolism. *Science* **364**, 1055–1062, https://doi.org/10.1126/science.aau6323

27 Wang, X., Sun, G., Feng, T., Zhang, J., Huang, X., Wang, T. et al. (2019) Sodium oligomannate therapeutically remodels gut microbiota and suppresses gut bacterial amino acids-shaped neuroinflammation to inhibit Alzheimer's disease progression. *Cell Res.* **29**, 787–803, https://doi.org/10.1038/s41422-019-0216-x

28 Scott, N.A., Andrusaite, A., Andersen, P. and Lawson, M. (2018) Antibiotics induce sustained dysregulation of intestinal T cell immunity by perturbing macrophage homeostasis. *Sci. Transl. Med.* **10**, 1–15, https://doi.org/10.1126/scitranslmed.aao4755

29 Vujkovic-Cvijin, I., Dunham, R.M., Iwai, S., Maher, M.C., Albright, R.G., Broadhurst, M.J. et al. (2013) Dysbiosis of the gut microbiota is associated with hiv disease progression and tryptophan catabolism. *Sci. Transl. Med.* **5**, 193ra191, https://doi.org/10.1126/scitranslmed.3006438

30 Agus, A., Planchais, J. and Sokol, H. (2018) Gut microbiota regulation of tryptophan metabolism in health and disease. *Cell Host Microbe* **23**, 716–724, https://doi.org/10.1016/j.chom.2018.05.003

31 Li, H., Bullock, K., Gurjao, C., Braun, D., Shukla, S.A., Bosse, O. et al. (2019) Metabolomic adaptations and correlates of survival to immune checkpoint blockade. *Nat. Commun.* **10**, 4346, https://doi.org/10.1038/s41467-019-12361-9

32 Lamas, B., Natividad, J.M. and Sokol, H. (2018) Aryl hydrocarbon receptor and intestinal immunity. *Mucosal Immunol.* **11**, 1024–1038, https://doi.org/10.1038/s41385-018-0019-2

33 Hubbard, T.D., Murray, I.A. and Perdew, G.H. (2015) Indole and tryptophan metabolism: Endogenous and dietary routes to ah receptor activation. *Drug Metab. Dispos.* **43**, 1522–1535, https://doi.org/10.1124/dmd.115.064246

34 Roager, H.M. and Licht, T.R. (2018) Microbial tryptophan catabolites in exercise, inflammation, and mental health. *Science* **357**, 369–376, https://doi.org/10.1126/science.aaf9794

35 Lamas, B., Richard, M.L., Leducq, V., Pham, H.P., Michel, M.L., Da Costa, G. et al. (2016) Card9 impacts colitis by altering gut microbiota metabolism of tryptophan into aryl hydrocarbon receptor ligands. *Nat. Med.* **22**, 598–605, https://doi.org/10.1038/nm.4102

36 Roager, H.M. and Licht, T.R. (2018) Microbial tryptophan catabolites in exercise, inflammation, and mental health. *Science* **357**, 369–376, https://doi.org/10.1126/science.aaf9794

37 Hubbard, T.D., Murray, I.A. and Perdew, G.H. (2015) Indole and tryptophan metabolism: Endogenous and dietary routes to ah receptor activation. *Drug Metab. Dispos.* **43**, 1522–1535, https://doi.org/10.1124/dmd.115.064246

38 Roager, H.M. and Licht, T.R. (2018) Microbial tryptophan catabolites in exercise, inflammation, and mental health. *Science* **357**, 369–376, https://doi.org/10.1126/science.aaf9794

39 Lamas, B., Richard, M.L., Leducq, V., Pham, H.P., Michel, M.L., Da Costa, G. et al. (2016) Card9 impacts colitis by altering gut microbiota metabolism of tryptophan into aryl hydrocarbon receptor ligands. *Nat. Med.* **22**, 598–605, https://doi.org/10.1038/nm.4102

40 Roager, H.M. and Licht, T.R. (2018) Microbial tryptophan catabolites in exercise, inflammation, and mental health. *Science* **357**, 369–376, https://doi.org/10.1126/science.aaf9794

41 Roager, H.M. and Licht, T.R. (2018) Microbial tryptophan catabolites in exercise, inflammation, and mental health. *Science* **357**, 369–376, https://doi.org/10.1126/science.aaf9794

42 Roager, H.M. and Licht, T.R. (2018) Microbial tryptophan catabolites in exercise, inflammation, and mental health. *Science* **357**, 369–376, https://doi.org/10.1126/science.aaf9794

43 Roager, H.M. and Licht, T.R. (2018) Microbial tryptophan catabolites in exercise, inflammation, and mental health. *Science* **357**, 369–376, https://doi.org/10.1126/science.aaf9794

44 Roager, H.M. and Licht, T.R. (2018) Microbial tryptophan catabolites in exercise, inflammation, and mental health. *Science* **357**, 369–376, https://doi.org/10.1126/science.aaf9794