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Dietary tryptophan, tyrosine, and phenylalanine depletion induce reduced food intake and behavioral alterations in mice

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ABSTRACT

Important precursors of monoaminergic neurotransmitters, dietary tryptophan (TRP), tyrosine, and phenylalanine (all referred to as TTP), play crucial roles in a wide range of behavioral and emotional functions. In the current study, we investigated whether diets devoid of TTP or diets deficient in TRP alone can affect body weight, behavioral characteristics, and gut microbiota, by comparing mice fed on these amino acids-depleted diets to mice fed on diets containing regular levels of amino acids. Both dietary TTP- and TRP-deprived animals showed a reduction in food intake and body weight. In behavioral analyses, the mice fed TTP-deprived diets were more active than mice fed diets containing regular levels of amino acids. The TRP-deprived group exhibited a reduction in serum TRP levels, concomitant with a decrease in serotonin and 5-hydroxyindoleacetic acid levels in some regions of the brain. The TTP-deprived group showed a reduction in TTP levels in the serum, concomitant with decreases in both phenylalanine and tyrosine levels in the hippocampus, as well as serotonin, norepinephrine, and dopamine concentrations in some regions of the brain. Regarding the effects of TRP or TTP deprivation on gut microbial ecology, the relative abundance of genus *Roseburia* was significantly reduced in the TTP-deprived group than in the dietary restriction control group. Interestingly, TTP was found even in the feces of mice fed TTP- and TRP-deficient diets, suggesting that TTP is produced by microbial or enzymatic digestion of the host-derived proteins. However, microbe generated TTP did not compensate for the systemic TTP deficiency induced by the lack of dietary TTP intake. Collectively, these results indicate that chronic dietary TTP deprivation induces decreased monoamines and their metabolites in a brain region-specific manner. The altered activities of the monoaminergic systems may contribute to increased locomotor activity.

1. Introduction

Aromatic amino acids (AAs) such as tryptophan (TRP), tyrosine (TYR), and phenylalanine (PHE) are important precursors of monoaminergic neurotransmitters, including serotonin (5-HT), dopamine (DA), norepinephrine (NE), and epinephrine. These monoamines are known to participate in a wide range of physiological processes and play crucial roles in regulating behavioral and emotional functions [1].

In general, to study the effects of the brain 5-HT system on the host physiology, acute TRP depletion is widely used to decrease the central nervous 5-HT synthesis via inhibiting the availability of TRP [2]. In fact, it can induce relapses in patients in remission from major depression and other psychiatric disorders [2], and also induce depressed mood in patients with a family history of depression [3]. In rats, acute TRP depletion changes behavioral activities such as aggression [4] and locomotion [5]. To evaluate the involvement of the brain catecholaminergic system, acute PHE and TYR depletion are often used. For example, Palmour et al. [6] reported that TYR and PHE depletion reduced the levels of homovanillic acid (HVA) and 3-methoxy-4-hydroxyphenylglycol (MHPG) that are derived from dopaminergic and noradrenergic activity, in the cerebrospinal fluid, and lowered voluntary alcohol consumption in vervet monkeys. In addition, such AAs-devoid mixtures are also useful when evaluating the role of brain catecholamine in behaviors and emotion in humans [7]. Thus, such acute TRP deprivation or acute PHE-TYR depletion are considered to significantly affect various metabolic and behavioral aspects of the host physiology. However, available information as to how and to what extent chronic TRP, TYR and PHE (all referred to as TTP) depletion could influence monoaminergic systems and behavioral characteristics, is still scant, especially in mice.

In addition to the well-established roles of the monoamines as a

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neurotransmitter, accumulating evidence suggests that the monoamines might act as important signal molecules between indigenous bacteria and the host in the gut [8–11]. More than 1000 species of microbes, referred to as the gut microbiota, are present in the human gut [12] and are involved in various host functions [13]. Recently, a great number of literatures indicate that gut microbes can play a role in regulating body weight [14] and the pathophysiology of the brain [15]. Indeed, the gut microbiome influences the central nervous system stress response [16] and other behaviors [17–20]. This concept, now called the "microbiota-gut–brain axis" [21–23], is a model indicating refined cross-talks between the microbiota, gut, and brain. Therefore, it is possible that aromatic AAs may affect the host behavioral properties via modulation by the "microbiota-gut–brain axis".

It is well known that the gut microbiota can synthesize free AAs from both undigested and partially digested proteins that are of alimentary and endogenous origins, e.g., pancreatic secretory products, desquamated epithelial cells, and mucous proteins [24–27]. In addition, some species of the gut microbes synthesize D- and L-amino acids [28,29]. Since several AA transporters are present in the colonic epithelial cells [30], it is interesting to speculate that dietary AAs may exert a significant impact on the host physiology by modulating the ability of the microbes to produce or metabolize AAs in the gut.

In this study, to clarify the role of dietary aromatic AAs in regulating metabolic and behavioral properties in terms of the bidirectional relationships between diets and gut microbes, we investigated the effects of the three aromatic AAs (TRP, TYR, and PHE) on body weight gain, behavioral characteristics, and gut microbiota by comparing animals fed TRP- or TTP-deficient diets to those fed normal AA-containing diets.

2. Methods

2.1. Animals

Male BALB/c mice were obtained at 4 weeks of age from KBT Oriental Co., Ltd. (Saga, Japan). All mice were kept individually and maintained under conditions described previously [9,31]. All animal experiments were approved by the Ethics Committee on Animal Experiments of the Graduate School of Medical Sciences, Kyushu University (A20–293–1), and were performed according to the Guidelines for Animal Experiments of the Graduate School of Medical Sciences, Kyushu University, and the Law (No. 105) and Notification (No. 6) of the Japanese Government.

2.2. Study protocol

As shown in **Table 1**, two experimental groups of mice received isocaloric diets devoid of either TRP or TTP, whereas the control group received an ordinary AA-supplemented diet (CON). In the first experiment, mice ($n = 7$ per group) that were provided TRP- or TTP-deficient diets exhibited a decrease in body weight concomitant with reduced food intake; therefore, in the second experiment ($n = 7$ per group), we established another dietary restriction control group (DIR) that was given the same amounts of AA-supplemented diets as the mean of the intake in the TTP and TRP groups. Each diet was started at 4 weeks of age and continued for 4 weeks. Body weight and food intake were measured twice weekly during the observation period. All mice were subjected to behavioral experiments from 7 to 8 weeks of age in the following order: open-field (OF) test, elevated plus-maze (EPM) test, and forced swimming (FS) test. After the behavioral experiments, blood samples were collected via cardiac puncture under anesthesia (0.3 mg/kg medetomidine, 4 mg/kg midazolam, 5 mg/kg butorphanol). After the mice were sacrificed by cervical dislocation, the brain and fecal materials were obtained and frozen on dry ice and were stored at −80 °C until further analyses.

Table 1
Amino acids composition of diets.[a].

	Control		TRP		TTP	
	gram	kcal	gram	kcal	gram	kcal
Corn Starch	550.5	2202	552.5	2210	564.5	2258
Maltodextrin 10	125	500	125	500	125	500
Cellulose	50	0	50	0	50	0
Corn Oil	50	450	50	450	50	450
Mineral Mix S10001	35	0	35	0	35	0
Sodium Bicarbonate	7.5	0	7.5	0	7.5	0
Vitamin Mix V10001	10	40	10	40	10	40
Choline Bitartrate	2	0	2	0	2	0
L-Arginine	10	40	10	40	10	40
L-Histidine-HCl-H_2O	6	24	6	24	6	24
L-Isoleucine	8	32	8	32	8	32
L-Leucine	12	48	12	48	12	48
L-Lysine-HCl	14	56	14	56	14	56
L-Methionine	6	24	6	24	6	24
L-Phenylalanine	8	32	8	32	0	0
L-Threonine	8	32	8	32	8	32
L-Tryptophan	2	8	0	0	0	0
L-Valine	8	32	8	32	8	32
L-Alanine	10	40	10	40	10	40
L-Asparagine-H_2O	5	20	5	20	5	20
L-Aspartate	10	40	10	40	10	40
L-Cystine	4	16	4	16	4	16
L-Glutamic Acid	30	120	30	120	30	120
L-Glutamine	5	20	5	20	5	20
Glycine	10	40	10	40	10	40
L-Proline	5	20	5	20	5	20
L-Serine	5	20	5	20	5	20
L-Tyrosine	4	16	4	16	0	0
Total	**1000**	***3872***	**1000**	***3872***	**1000**	***3872***

[a] Animals were given either diets containing 20 different kinds of amino acids (CON), diets without tryptophan (TRP), or diets without tryptophan, tyrosine, and phenylalanine (TTP).

2.3. Behavioral analyses

Behavioral analyses of mice were conducted using OF, EPM, and FS tests, as previously described [31–33]. These tests were conducted between 9:00 a.m. and 5:00 p.m. under low illumination (< 50 lx). They were conducted from days 22 to 25 after the beginning of dietary manipulation in the following order: OF (day 22), EPM (day 24), and FS (day 25) tests. In the first trial, the three groups (CON, TRP-, and TTP-depleted groups, $n = 7$ per group) were used for behavioral experiments. In the second trial, the DIR group was added, and the resultant four groups (CON, DIR, TRP-, and TTP-depleted groups, $n = 7$ per group) were analyzed using the same behavioral protocol. To minimize time-related variations, tests performed on the four groups were equally distributed and performed alternately.

OF tests were performed to evaluate the levels of anxiety and locomotor activity. In brief, mice were individually placed in the center of an OF box ($L \times B \times H$, 45 cm × 45 cm × 45 cm) [20,31]. The square bottom of the box was divided equally into 16 sub-squares (4 × 4). Behaviors were recorded and quantified using a computer system (SMART3.0, Panlab Harvard Apparatus, USA). The total distance traveled for 20 min and time spent in the 12 peripheral sub-squares over 20 min were automatically calculated as representative parameters for spontaneous locomotor activity and anxiety-like behavior, respectively.

The EPM tests were conducted as described previously [31,34]. Briefly, the apparatus comprised two open (30 × 5 cm) and two closed (30 × 5 × 15 cm) arms that extended from a central platform (5 × 5 cm). Testing was conducted in a quiet room illuminated only by dim light. The number of open or closed arm entries and the time spent in the open or closed arms were recorded during the test period. We used the time spent in open arm as a parameter of anxiety-like behavior. After each trial, the maze was cleaned with a 70% ethyl alcohol solution and dried with paper towels [35] before the next mouse was tested.

FS tests were performed according to the methods described

elsewhere [32]. Briefly, a mouse was placed in a cylindrical container filled with water. The time during which the mouse was immobilized in a 6 min session was used as an indicator of depressive-like behavior. A video camera-based computer tracking system (SMART3.0, Panlab Harvard Apparatus, USA) was used to record activities during each test.

2.4. Quantification of AA levels

All mice were euthanized by cervical dislocation at 8 weeks of age, and blood, brain, and fecal materials were collected as described previously. The brain that was removed was dissected into the medial prefrontal cortex, striatum, hippocampus, and brainstem.

For the AA analyses, using only the hippocampal specimens, each sample was prepared according to a previously described method [20,36]. Briefly, the samples were homogenized in 0.2 M perchloric acid containing 100 mM disodium EDTA. The homogenate was incubated for 30 min for deproteinization and then centrifuged at 20,000 × *g* for 10 min at 4 °C. The pH of the supernatant was adjusted to approximately 3.0 by adding 1 M sodium acetate, and the resultant supernatant was filtered through a 0.22 μm filter (Merck Millipore Ltd., Ireland). An automated method for high-throughput AA analysis, using precolumn derivatization high-performance liquid chromatography/electrospray mass spectrometry (HPLC/ESI-MS) was used in this study [37].

2.5. Measurement of monoamine levels in the brain

Brain samples (prefrontal cortex, striatum, hippocampus, and brainstem) were weighed and stored at −80 °C until assayed. The levels of monoamines and their metabolites were analyzed using a previously described method [10,20,38]. The samples were prepared using the same method as described under the subsection titled "Quantification of AA levels." A 30-μl portion of filtrate was applied in an HPLC system (Eicom, Kyoto, Japan) using a 150 mm × 3.0 mm octadecyl silane column (SC-5ODS, Eicom) and an electrochemical detector (ECD-300, Eicom, Kyoto, Japan) at an applied potential of +0.75 V vs an Ag/Ag Cl reference analytical electrode. The concentrations of monoamines and DA metabolites (dihydroxyphenylacetic acid [DOPAC] and HVA), NE metabolite (MHPG), and 5-HT metabolite (5-hydroxyindoleacetic acid [5-HIAA]), were determined, and their levels in the brain were calculated. For all monoamines, the detection limit of the system was 0.1 pg/sample.

2.6. Analysis of the fecal microbiome

DNA was extracted from fecal samples using a commercial extraction kit (QuickGene DNA tissue kit; Kurabo, Osaka, Japan) [39,40] and was processed by16S rRNA gene sequencing analysis according to a previously reported method [31,41]. In brief, after DNA extraction, the V3–V4 region of the bacterial 16S rRNA gene was amplified by polymerase chain reaction (PCR) with a TaKaRa Ex Taq 13 HS kit (TaKaRa Bio, Shiga, Japan) and a primer set of Tru357F 14 (5′-CGCTCTTCCGATCTCTGTACGGRAGGCAGCAG-3′) and Tru806R 15 (5′-CGCTCTTCCGATCTGACGGACTACHVGGGTWTCTAAT-3′). The DNA was amplified using the following protocol: 94 °C for 30 s, followed by 30 cycles of 94 °C for 30 s, 50 °C for 30 s, and 72 °C for 5 min. The amplified DNA was verified based on PCR product size using the QIAxcel system (Qiagen, Valencia, CA, USA). Thereafter, PCR products were amplified with a second primer set, which was adapted for the Illumina MiSeq (Illumina, San Diego, CA, USA) using the following protocol: 94 °C for 30 s and 8 cycles at 94 °C for 30 s, 50 °C for 30 s, and 72 °C for 5 min. Subsequently, equal amounts of amplicons from different samples were pooled, and primer dimers were removed by gel extraction with the QIAquick PCR Purification Kit. The pooled libraries were sequenced using Illumina MiSeq with a MiSeq v3 Reagent kit 3 (Illumina). The obtained sequences were filtered using the bowtie-2 program (ver 2–2.2.4) to remove the reads mapped to the PhiX174 sequence. Thereafter, the 3′ region of each read with a PHRED quality score of less than 17 was trimmed. Trimmed reads less than 150 bp in length with an average quality score of less than 25 or those lacking paired reads were also removed. The paired-end reads that passed the abovementioned quality filters were combined using the fastq-join script in EA-Utils (ver. 1.1.2–537). The sequences were analyzed using the Quantitative Insights into Microbial Ecology (QIIME) software package version 1.9.1. The sequences were assigned to operational taxonomic units (OTUs) by open-reference OTU picking with a 97% pairwise identity threshold and the Greengenes reference database.

2.7. Statistical analysis

Continuous data are expressed as mean ± SD. For non-normally distributed parameters using the Shapiro Wilk normality test, data are expressed as the median with interquartile range. All analyses of animal experiments were performed using the JMP PRO v.14.2.0 software package for Windows (SAS Institute, Japan).

Statistical differences in body weight or food intake among the CON, DIR, TRP-, and TTP-deprived groups were evaluated using repeated measures ANOVA. Then, differences between two groups at each time-point were assessed using post-hoc tests. Behavioral parameters and serum or hippocampal AA levels between groups that ingested diets with or without AAs were evaluated as follows: first, Kruskal-Wallis test was conducted among the three groups. When the p values of the Kruskal-Wallis test were less than those calculated by the Bonferroni correction based on the total number of tests, the Steel or Steel-Dwass test was applied to evaluate the difference in each variable between the two indicated groups. The association between total distance measured in the OF test and immobility time measured in the FS test was evaluated using Spearman's rank correlation coefficients.

QIIME [42,43] pipeline 1.9.1 (www.qiime.org) was used to generate relative abundance plots and to calculate α-diversity metrics (Shannon index, Chao 1, and observed species) and β-diversity parameters of both weighted and unweighted UniFrac metrics. Whereas the Shannon index depends on both species richness and evenness [44], the *β*-diversity parameter, including the unweighted UniFrac distance, reflects the difference in microbial composition between samples. Principal coordinate analysis (PCoA) plots were generated using the unweighted UniFrac method and visualized as a 3D graph using QIIME. Permutational multivariate ANOVA (PERMANOVA) [45,46] was also conducted to evaluate differences in bacterial composition among the four groups of mice. This was performed using the Adonis function in the vegan package in R studio with R 3.6.2. Differences in relative abundances of genera among all groups were evaluated using the Kruskal-Wallis test followed by the Bonferroni correction for multiple testing.

3. Results

3.1. Effects of AA deprivation on body weight gain

As shown in **Fig. 1a**, repeated measures ANOVA revealed a significant difference in body weight between the CON group and the other three groups ($F_{(3,45)} = 15.816$, $p < 0.0001$). Post-hoc analyses did not show any significant differences in body weight between the TRP-deprived, TTP-deprived, and DIR groups at each time-point. Food consumption was also lower in the TRP-deprived, TTP-deprived, and DIR groups than in the CON group (**Fig. 1b**; $F_{(3,45)} = 8.413$, $p < 0.0001$).

3.2. AA deprivation causes behavioral changes

As shown in **Fig. 2a**, the TTP-deprived group was more active than the CON and DIR groups when evaluated by OF tests (TTP vs CON, $z = 3.837$, $p = 0.0007$; TTP vs DIR, $z = 2.723$, $p = 0.0328$). Regarding anxiety-related behaviors, there was no significant difference among the four groups when examined by OF and EPM tests (**Fig. 2b and 2c**). In FS

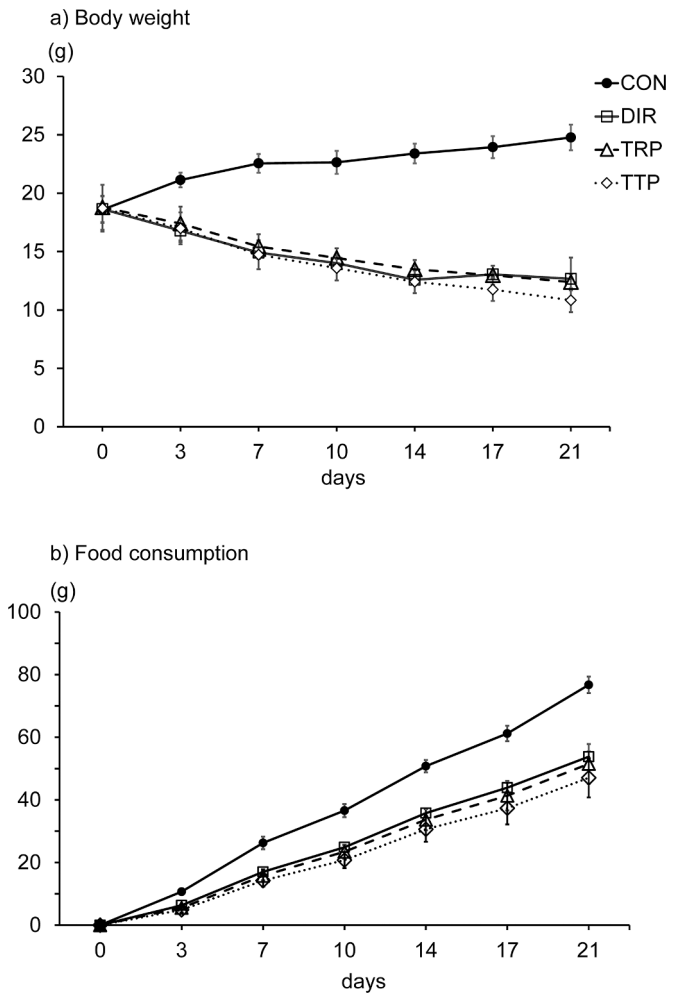

Fig. 1. Dietary TRP or TTP depletion reduces body weight and food intake. Body weight **(a)** and food consumption **(b)** in CON, TRP, and TTP groups (n = 14), and DIR group (n = 7) were measured twice a week for 3 consecutive weeks. All data are expressed as the mean ± SD. CON, control; DIR, a group fed restricted amounts of control diets; TRP, a group fed tryptophan-deficient diets; TTP, a group fed tryptophan-, phenylalanine-, and tyrosine-deficient diets.

tests, the immobility time was significantly shorter in the TTP-deprived group and, to a lesser extent, in the TRP-deprived group than in the CON or DIR groups (Fig. 2**d;** TRP vs CON, $z = -3.056$, $p = 0.012$; TRP vs DIR, $z = -2.650$, $p = 0.040$; TTP vs CON, $z = -4.435$, $p < 0.0001$; TTP vs DIR, $z = -3.621$, $p = 0.0017$). There was a negative correlation between immobility time and total distance measured by OF tests (Spearman's $\rho = -0.4011$, $p = 0.0043$). Such a correlation between immobility time and locomotion was also confirmed when analyzed using EPM tests (Spearman's $\rho = -0.3694$, $p = 0.0090$).

3.3. Diet-induced alterations in AA levels of the serum and hippocampus

Since behavioral characteristics did not show any significant difference between the CON and DIR groups, only the DIR group was used for further AA analyses.

As summarized in Table 2, serum TRP levels in the TRP- and TPP-depleted groups were significantly lower than those in the DIR group. Moreover, the TTP-deprived group showed a significant reduction in serum levels of PHE and TYR compared to the DIR group. Serum cystine levels in the TRP- and TTP-deprived groups were also significantly lower than those in the DIR group.

As summarized in Table 3, in the hippocampus, TRP concentrations were not reliably quantified in any group because they were below the detection limits. PHE and TYR concentrations were significantly lower in the TTP-deprived group than in the DIR group. In contrast, hippocampal alanine, aspartic acid, glycine, threonine, and valine levels in the TTP-deprived group, as well as hippocampal glycine and threonine levels in the TRP-deprived group, were significantly increased compared with the corresponding values in the DIR group.

3.4. Monoamine concentrations in various regions of the brain

As summarized in Table 4, TRP-deprived mice exhibited a significant reduction in both 5-HT and 5-HIAA levels in the cortex, striatum, and hippocampus, as well as in 5-HIAA levels in the brainstem, compared to the DIR group. Reduced 5-HT and 5-HIAA levels were also found in the cortex and hippocampus of TTP-deprived mice. Regarding the levels of NE and its metabolite MHPG, significant decreases in NE concentrations were detected in the cortex, brain stem, and hippocampus of TTP-deprived mice, as well as in the cortex and hippocampus of TRP-deprived mice, in comparison to those in the corresponding regions of DIR mice. In contrast, no significant difference in MHPG levels of any brain regions was found among the three groups. The TTP-deprived group also showed a significant reduction in brainstem DA concentrations, compared to the DIR group.

3.5. AA contents in fecal samples

As summarized in **Supplementary Table 1**, TTP was detected even in the feces of the TRP- and TTP-deprived groups. Fecal PHE levels were significantly lower in the TRP-deprived group than in the DIR group (PHE, $z = -2.556$, $p = 0.020$). Spearman's rank-order analyses showed a significant correlation between serum PHE and hippocampal PHE levels, as well as between serum PHE and hippocampal TYR levels (Table 5). Such a significant association was also found between serum TYR and hippocampal PHE levels and between serum TYR and hippocampal TYR levels. However, neither TRP, PHE, nor TYR in the feces showed any correlation with TTP in the serum. Regarding other AAs detected in the feces, some AA levels showed a significant alteration between the DIR and TRP-deprived groups or between the DIR and TTP-deprived groups (**Supplementary Table 1**).

3.6. AA-deficient diets alter gut microbial diversities

As shown in Fig. 3**a and** 3**b**, the Chao-1 values in the DIR group were significantly higher than those in either the CON or TTP group (CON vs DIR, $z = -2.555$, $p = 0.028$; DIR vs TTP, $z = -2.683$, $p = 0.020$). The DIR group also exhibited an increase in the Shannon index, compared with the CON group (CON vs DIR, $z = -2.555$, $p = 0.028$). A three-dimensional PCoA revealed a significant difference among the four groups (Fig. 3**c**; $f = 4.03$, $p < 0.0001$). The DIR group showed a significant difference in PCoA relative to the CON group ($f = 4.48$, $p = 0.0029$) or the TTP group ($f = 4.51$, $p = 0.00099$).

Taxonomic analysis showed a significant difference in the relative abundance of some bacterial genera among the four groups (**Supplementary Table 2**). The relative percentage of genus *Roseburia* was significantly lower in the TTP group than in the DIR group. No significant correlation was found between the bacterial relative percentage at the genus level and the behavioral parameters measured in this study. Interestingly, Spearman's rank-order analysis showed a significantly negative correlation between the relative percentage of the genus *Enterococcus* and fecal TYR concentrations (**Supplementary Table 3**, $\rho = -0.7516$, $p < 0.0001$).

4. Discussion

In the current study, mice subjected to TTP or TRP deprivation showed a reduction in food intake and body weight. TTP-deprived mice

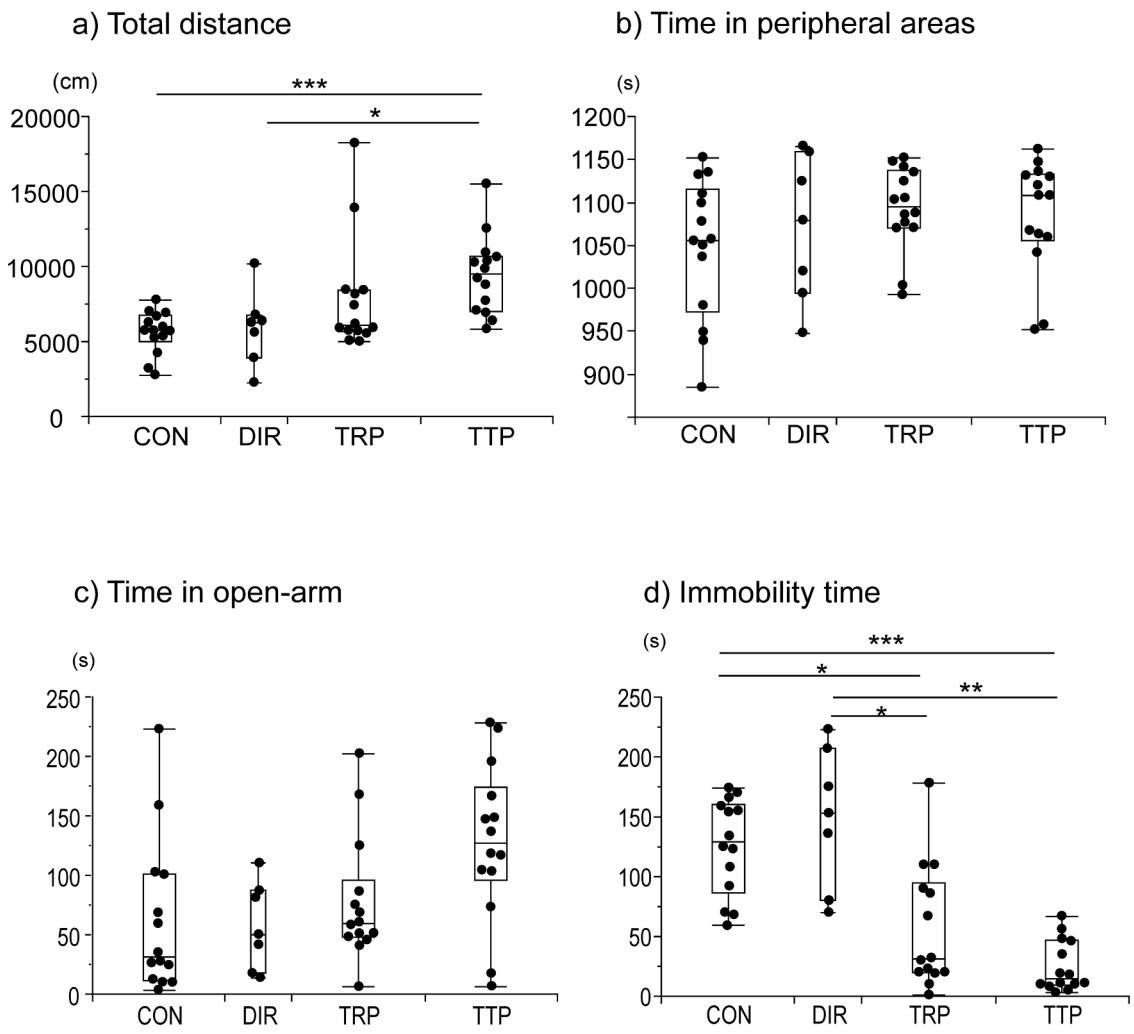

Fig. 2. Dietary TRP or TTP depletion induces behavioral alterations. (a) Locomotor activity was measured using the OF test ($n = 14$ per each CON, TRP, and TTP group; $n = 7$ per DIR group). The vertical bar indicates the total distance traveled during 20 min. **(b)** Anxiety-like behaviors were evaluated by both total time spent in peripheral areas during the OF test and **(c)** total time spent in the open arm during the EPM test ($n = 14$ per each CON, TRP, and TTP group; $n = 7$ per DIR group). **(d)** Depressive-like behaviors were assessed based on total time immobilized in the FS test ($n = 14$ per each CON, TRP, and TTP group; $n = 7$ per DIR group). Data for each group are visualized as box plots with medians (middle lines), first and third quartiles (box boundaries), and minimum and maximum values (whiskers). Statistical analyses were conducted using the Steel-Dwass test for multiple comparisons. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ indicate a significant difference between the indicated groups. CON, control; DIR, a group fed restricted amounts of control diets; EPM, elevated plus-maze; FS, forced swimming; OF, open-field; TRP, a group fed tryptophan-deficient diets; TTP, a group fed tryptophan-, phenylalanine-, and tyrosine-deficient diets.

were more active compared to CON and DIR mice. The TRP-deprived group exhibited a reduction in serum levels of TRP concomitant with a decrease in 5-HT and 5-HIAA levels in some regions of the brain, whereas the TTP-deprived group showed a reduction in TTP levels in the serum concomitant with decreases in hippocampal PHE and TYR levels and reduced 5-HT, DA, and NE concentrations in some regions of the brain. Regarding gut microbial ecology, the relative percentage of genus *Roseburia* was significantly lower in the TTP group than in the DIR group. Interestingly, TTP was still found in the feces of TTP- and TRP-deprived mice, indicating that gut luminal AAs produced by protein digestion did not compensate for the systemic AA deficiency caused by the lack of dietary AA intake. Collectively, these results suggest that chronic dietary TTP deprivation induced reduced 5-HT, catecholamine concentrations, and their metabolites in a brain-region specific manner. This reduction in brain monoaminergic activities may be responsible for increased locomotor activity.

In this study, the TRP or TTP deprived mice also exhibited a reduction in food intake, thus resulting in a decrease in body weight, although they did not show any apparent signs of illness. Similarly, Browne et al. [47] reported that TRP-deprived diets significantly decrease the body weight in BALB/c and C57BL/6 J mice. Considering these findings, it has been known for decades that rodents avoid diets that are devoid of even one essential amino acid, which would result in a strong decrease in food intake [48–50]. This response requires a sensory system that is located in the anterior piriform cortex of the brain to detect indispensable AA deficiency [2,51] and to develop conditioned aversion to cues associated with AA-deficient diets [52,53]. Collectively, the current results, in which there were no differences in both food intake and body weight between the AA-deprived mice and the DIR mice, indicated that the reduced body weight in the AAs-deprived mice was entirely due to the decreased food intake, and not due to specific effects of AA deprivation on energy harvest or metabolism of the host.

Dietary TRP deprivation induces behavioral alterations, such as an increased incidence of mouse-killing [4], a reduction in nesting behavior [54], and an increased locomotor activity [55] in rodents. In our results, TRP depletion did not exert a significant effect on motor activity evaluated by the OFT but reduced immobility time evaluated by the FS test, although to a lesser extent than TTP depletion. On the other hand, dietary TTP deprivation increased signs of behavioral activities and decreased depressive-like behavior in comparison to control mice. Since

Table 2
Effects of amino acid-deficient diets on serum amino acid profiles.[a].

	DIR (nmol/ml)	TRP (nmol/ml)	TTP (nmol/ml)	KW
TRP	69.0 (59.4–87.9)	**9.0 (8.5–10.7)****	**16.5 (7.4–19.6)****	**0.0008**
PHE	83.9 (74.2–162)	65.4 (49.3–76.7)	**33.2 (32.6–36.1)****	**0.0019**
TYR	43.5 (39.9–102)	37.0 (26.2–40.8)	**13.8 (12.5–14.8)****	**0.0009**
ALA	261 (229–1643)	598 (478–653)	956 (587–1024)	0.3499
ASN	27.5 (23.9–64.7)	33.1 (26.1–38.1)	40.7 (30.4–46.5)	0.4505
ARG	73.6 (51.7–95.8)	112 (99.5–141.2)	115 (110–131)	0.0479
ASP	27.0 (15.5–90.5)	25.0 (21.5–30.0)	114 (80.0–127)	0.0065
CYS	15.8 (12.4–16.3)	**5.9 (5.2–8.3)****	**6.6 (5.8–8.6)****	**0.0012**
GLN	388 (348–448)	553 (413–565)	538 (385–570)	0.1257
GLU	164 (98.5–272)	134 (112–207)	255 (207–261)	0.0640
GLY	231 (203–606)	273 (227–378)	368 (279–450)	0.3825
HIS	88.1 (68.8–135)	111 (103–144)	122 (103–126)	0.3237
ILE	83.9 (58.1–154)	52.4 (43.1–64.9)	96.9 (50.0–102)	0.1167
LEU	136 (97.1–240)	97.6 (82.1–121)	184 (113–213)	0.0506
LYS	269 (202–629)	324 (222–494)	356 (324–423)	0.8245
MET	40.9 (33.1–202)	45.1 (34.9–52.4)	59.6 (48.0–84.6)	0.2348
PRO	71.3 (66.9–274)	75.2 (56.6–81.8)	103 (72.6–115)	0.2379
SER	113 (98.0–305)	203 (152–219)	300 (263–349)	0.0116
THR	140 (102–735)	475 (331–580)	693 (451–708)	0.2907
VAL	163 (128–338)	177 (132–198)	330 (191–352)	0.1271

[a] All data ($n = 7$ per group) are expressed as the median with lower quartile and upper quartile. When a p value was less than 0.0025 in the KW test comparing the three groups, the Steel test was applied to evaluate differences in this amino acid level between the DIR and TRP groups or between the DIR and TTP groups. The p value of 0.0025 was calculated using the Bonferroni correction based on the total number of tests (20, $p = 0.05/20 = 0.0025$).

** $p < 0.01$ indicates a significant difference vs the DIR group. DIR, dietary restriction control; KW, Kruskal-Wallis; TTP, tryptophan, phenylalanine, and tyrosine; TRP, Tryptophan; PHE, Phenylalanine; TYR, Tyrosine; ALA, Alanine; ARG, Arginine; ASN, Asparagine; ASP, Aspartic acid; CYS, Cysteine; GLN, Glutamine; GLU, Glutamic acid; GLY, Glycine; HIS, Histidine; ILE, Isoleucine; LEU, Leucine; LYS, Lysine; MET, Methionine; PRO, Proline; SER, Serine; THR, Threonine; VAL, Valine.

Table 3
Effects of amino acid-deficient diets on hippocampal amino acid profiles.[a].

	DIR (nmol/g)	TRP (nmol/g)	TTP (nmol/g)	KW
TRP	ND	ND	ND	NT
PHE	145 (122–157)	141 (118–157)	**40.7 (0.1–57.0)****	**0.0021**
TYR	81.4 (47.5–90.8)	40.7 (37.2–105)	**ND****	**0.0010**
ALA	2999 (2404–3385)	3358 (2902–3546)	**4225 (3919–5208)****	**0.0025**
ASN	59.0 (54.9–83.8)	72.4 (59.0–83.8)	83.8 (61.3–93.7)	0.1313
ARG	124 (66.9–214)	221 (180–259)	261 (230–330)	0.0162
ASP	4998 (4720–5048)	6536 (5415–6962)	**9142 (6869–9925)****	**0.0014**
CYS	ND	ND	40.7 (0.1–57.0)	NT
GLN	10,240 (8448–11,160)	10,133 (8102–12,272)	9843 (5782–11,160)	0.9432
GLU	14,438 (13,627–15,377)	15,345 (14,370–18,433)	20,931 (18,549–23,458)	0.0034
GLY	1874 (1556–2116)	**4068 (3330–4180)****	**4657 (3866–5421)****	**0.0008**
HIS	197 (96.7–277)	348 (254–376)	336 (102–359)	0.0781
ILE	47.5 (31.7–81.4)	38.9 (31.7–45.0)	45.0 (32.9–57.0)	0.3046
LEU	190 (153–200)	157 (123–174)	217 (182–275)	0.0136
LYS	334 (293–418)	476 (416–487)	559 (508–642)	0.0026
MET	45.0 (31.7–177)	40.7 (37.2–127)	124 (104–153)	0.2408
PRO	27.2 (26.2–39.3)	245 (30.8–284)	322 (281–413)	0.0060
SER	1584 (1554–1736)	1483 (1475–1777)	1920 (1719–2384)	0.0550
THR	863 (734–1107)	**1421 (1341–1652)****	**1789 (1657–2288)****	**0.0006**
VAL	190 (153–200)	174 (106–197)	**267 (236–334)***	**0.0019**

[a] All data ($n = 7$ per group) are expressed as the median with lower quartile and upper quartile. When a p value was less than 0.0025 in the KW test comparing the three groups, the Steel test was applied to evaluate differences in this amino acid level between the DIR and TRP groups or between the DIR and TTP groups.

** $p < 0.01$ and * $p < 0.05$ indicate a significant difference vs the DIR group. DIR, dietary restriction control; ND, not detected; NT, not tested; KW, Kruskal-Wallis; TTP, tryptophan, phenylalanine, and tyrosine; TRP, Tryptophan; PHE, Phenylalanine; TYR, Tyrosine; ALA, Alanine; ARG, Arginine; ASN, Asparagine; ASP, Aspartic acid; CYS, Cysteine; GLN, Glutamine; GLU, Glutamic acid; GLY, Glycine; HIS, Histidine; ILE, Isoleucine; LEU, Leucine; LYS, Lysine; MET, Methionine; PRO, Proline; SER, Serine; THR, Threonine; VAL, Valine.

the immobility time measured by the FS test was negatively correlated with locomotor activity, such an antidepressive-like behavior may be ascribed to the increased locomotor activity of TTP-deprived animals. This observation was also confirmed by increased locomotion in TTP-deprived mice when evaluated by EPM testing. These results taken together suggest that PHE and TYR deprivation in combination with TRP deprivation may exert more profound effects on locomotor activities than those induced by TRP deprivation alone.

Dietary TRP deprivation resulted in reduced levels of 5-HT, 5-HIAA, and NE in several areas of the brain. Sánchez et al. [56] showed that acute TRP deprivation effectively reduced 5-HT and 5-HIAA levels in the frontal cortex, hippocampus, amygdala, nucleus accumbens, and caudate putamen of C57BL/6 J mice. Interestingly, this procedure also induced an alteration in catecholaminergic system, such as a decrease in TYR and HVA levels in the frontal cortex. Consistent with this, chronic TRP deprivation decreased cortical and hippocampal NE levels in this study. These findings indicate that TRP-free formulae can exert a significant impact on both 5-HT and catecholamine systems. The precise mechanism by which TRP deprivation affects brain catecholaminergic system is unclear.

In this study, dietary TTP deprivation affected monoamines and their metabolites in a region-specific manner. Certainly, the NE levels in the cortex, brain stem, and hippocampus, and the DA levels in the brainstem, were significantly lower in the TTP group than in the DIR group, whereas, the DOPAC and MHPG levels failed to show any difference between the two groups. Biggio et al. [57] demonstrated that oral administration of AA mixture lacking PHE and TYR induced a transient decrease in TYR, HVA, and DOPAC in the basal ganglia. In microdialysis experiments, McTavish et al. [58] reported that TYR-free AA mixture did not alter striatal extracellular DA levels under basal conditions, but reduced DA release when induced by amphetamine in a dose-dependent manner. Similarly, using the microdialysis method, Shnitko et al. [59] demonstrated that an intraperitoneal injection of PHE and TYR-free mixture decreased the frequency of DA transients in the nucleus accumbens, while not affecting DA and NE contents in the prefrontal cortex and ventral striatum under basal resting conditions. Importantly, Sánchez et al. [56] examined the effects of either acute TRP depletion, acute PHE and TYR depletion, or a mixed depletion of TRP, PHE, and TYR on brain 5-HT and DA systems in mice. The authors also showed that acute TTP depletion reduced 5-HIAA levels in several areas of the brain, DA in the amygdala, and HVA levels in the caudate putamen; however, it was less effective when compared with acute TRP depletion or acute PHE and TYR depletion alone. These results were also confirmed by the current study, in which chronic TTP depletion reduced 5-HT and 5-HIAA levels in several brain areas, but to the lesser extent than chronic TRP depletion alone. In this study, DA and NE concentrations in the cortex, brain stem, and hippocampus, were reduced by chronic TTP deprivation under basal conditions, and this has not been reported to be induced by acute TTP depletion previously. Therefore, these results indicate that prolonged TTP depletion may exert more profound effects on brain catecholaminergic system than acute TTP depletion. Nonetheless, the precise mechanism by which dietary AAs deprivation exerted a region-specific effect on brain monoamines and their metabolites remains to be clarified.

Increased locomotor activity, i.e., “hyperactivity,” is often seen in subjects with anorexia nervosa (AN) and is regarded as a key characteristic of the disorder [60–62]. In our recent studies using metabolomic analyses [63], serum AA levels, including TYR and TRP, were

Table 4
Levels of monoamines and their metabolites in various regions of the brain.

a) Cortex	DIR	TRP	TTP	ANOVA
5-HT	271 ± 45.7	**130 ± 51.7*****	**107 ± 50.9*****	**<0.0001**
5-HIAA	831 ± 115	**373 ± 215*****	**462 ± 162*****	**0.0002**
DA	28.5 ± 14.6	20.4 ± 13.2	14.3 ± 15.0	0.2027
DOPAC	123 ± 15.5	130 ± 62.9	65.7 ± 22.5	0.0133
NE	180 ± 34.3	**114 ± 45.8****	**30.6 ± 9.9*****	**<0.0001**
MHPG	45.7 ± 36.1	86.8 ± 38.4	61.0 ± 41.9	0.1654
b) Striatum	DIR	TRP	TTP	ANOVA
5-HT	446 ± 117	193 ± 69.4***	**299 ± 85.7***	**0.0003**
5-HIAA	2302 ± 185	**707 ± 365*****	1864 ± 980	**0.0004**
DA	254 ± 162	91.4 ± 41.4	215 ± 151	0.0771
DOPAC	693 ± 225	377 ± 101	847 ± 689	0.1341
NE	1483 ± 501	907 ± 391.1	1373 ± 657	0.1243
MHPG	87.8 ± 57.8	148 ± 63.0	249 ± 138	0.0155
c) Brain stem	DIR	TRP	TTP	ANOVA
5-HT	419 ± 145	200 ± 27.3	268 ± 146	0.0095
5-HIAA	767 ± 296	**253 ± 53.2****	625 ± 273	**0.0020**
DA	30.5 ± 7.4	25.9 ± 7.2	**13.8 ± 4.1*****	**0.0004**
DOPAC	128 ± 26.0	121 ± 29.8	92.3 ± 30.3	0.0723
NE	516 ± 43.4	473 ± 80.6	**329 ± 88.9*****	**0.0004**
MHPG	34.2 ± 34.5	33.1 ± 18.6	38.0 ± 23.1	0.9467
d) Hippocampus	DIR	TRP	TTP	ANOVA
5-HT	286 ± 57.4	**131 ± 40.0*****	**152 ± 40.7*****	**<0.0001**
5-HIAA	859 ± 176	**274 ± 114*****	**583 ± 141****	**<0.0001**
DA	16.7 ± 7.3	12.5 ± 2.1	11.3 ± 2.4	0.0975
DOPAC	115 ± 24.3	83.3 ± 17.0	95.0 ± 13.1	0.0159
NE	232 ± 67.3	**138 ± 20.2****	**83.2 ± 22.7*****	**<0.0001**
MHPG	19.1 ± 8.0	24.5 ± 8.1	31.3 ± 12.3	0.0879

All data ($n = 7$ per group) are expressed as the mean ± SD (ng/g). When a p value was less than 0.00208 in the ANOVA test comparing the three groups, Dunnett's test was applied to evaluate differences in this amino acid level between the DIR and TRP groups or between the DIR and TTP groups. The p value of 0.00208 was calculated using the Bonferroni correction based on the total number of tests (24, $p = 0.05/24 = 0.00208$).

*** $p < 0.001$,

** $p < 0.01$, and

* $p < 0.05$ indicate a significant difference vs the DIR group. 5-HT, 5-hydroxytryptamine; 5-HIAA, 5-hydroxyindoleacetic acid; DA, dopamine; DIR, dietary restriction control; DOPAC, 3,4-dihydroxyphenylacetic acid; NE, norepinephrine; MHPG, 3-methoxy-4-hydroxyphenylglycol; TRP, tryptophan; TTP, tryptophan, phenylalanine, and tyrosine.

significantly lower in the AN group than in the control group. In malnourished and emaciated individuals with AN, reduced plasma TRP availability [64–66] and reduced 5-HIAA levels in the CSF have been reported [67]. In addition, an impairment of the DA system is suggested to be involved in AN pathology because reduced CSF levels of DA metabolites occur in both ill and recovered individuals with AN [68]. These findings suggest that a lack of TRP and/or TYR may exacerbate AN-specific behavioral abnormalities, i.e., "hyperactivity," via modulating brain 5-HT and DA systems, subsequently inducing poor weight gain by increasing calorie expenditure. Nevertheless, it should be noted that decreased TRP and TYR levels in the sera may not be a main cause of developing AN, but just an epiphenomenon resulting from calorie restriction. Clearly, further studies are needed to unravel the role of dietary AAs in the pathogenesis of AN.

In the current study, substantial amounts of TTP were found even in the feces of mice receiving AA-deficient diets. Since several AA transporters are present in the colon [30], it is possible that the AAs generated in the gut lumen could compensate for dietary TTP deficiencies and, thus, restore altered functions, such as increased locomotion. However, this is unlikely because fecal TTP did not show any correlation with serum levels or behavioral parameters. These results indicate that colonic TTP originating from diets or host proteins is unable to exert a substantial effect on circulating TTP levels.

In this study, differences were found in microbial diversities among the CON, DIR, TRP-, and TTP-deprived groups. The TTP deprived group showed reduced Chao-1 values compared with the DIR group, and there was no difference in the Shannon values between the TTP and DIR groups. This may be related to the fact that the Chao-1 index reflects species richness whereas the Shannon index depends on both species richness and evenness. In addition, the TTP group also showed a distinct profile of PCoA, compared with the DIR group. Nonetheless, the functional outcome of these differences in α- and β-microbial diversities is unclear and should be examined in future studies. Concerning the relative abundance of bacterial composition, the genus *Roseburia* was significantly reduced in the TTP-deprived group than in the DIR group. Bacteria of the genus *Roseburia* are known to produce short chain fatty acid, especially butyrate which affects various metabolic pathways [69–71]. Moreover, the presence of this genus also serves as a potential biomarker for several diseases including irritable bowel syndrome [72], type 2 diabetes [73], and Parkinson's disease [73]. Interestingly, dietary fat or monosodium L-glutamate promotes the colonization of *RosebUria* spp. [74]; hence, dietary TTP may be an important factor for the growth of this bacteria.

Another interesting finding regarding the effects of AAs deficiency on gut microbes is that a significantly negative correlation was found between the relative percentage of the genus *Enterococcus* and fecal TYR concentrations. It has been reported that the genus *Enterococcus* contains TYR decarboxylase genes in its bacterial genome [75]. In general, TYR decarboxylase is an enzyme that converts L-TYR into tyramine; however, it also has the ability to decarboxylate levodopa to produce dopamine [76]. Quantification of dopamine or tyramine levels in the feces was not conducted in this study; however, it is interesting to clarify possible interactions between gut microbes and TYR or other AAs because not only AA transporters, but also several catecholamine receptors, such as α1 and/or α2 adrenergic and dopaminergic receptors, are known to be present in enterocytes [9,77].

The current study has some limitations. First, dietary TRP or TTP deficiency made mice more active; however, the mechanism underlying this phenomenon is unclear and should be clarified. Second, all included mice were male; therefore, future studies should be conducted using both sexes of mice. Third, we could not continue administering the AA-

Table 5
Correlations between serum amino acid and fecal or hippocampal amino acid levels.[a].

	Spearman	H-TRP	H-PHE	H-TYR	F-TRP	F-PHE	F-TYR
S-TRP	ρ P	NT	0.1046 (0.6608)	0.3955 (0.0844)	0.0045 (0.9849)	0.1098 (0.6450)	0.3625 (0.1162)
S-PHE	ρ P	NT	**0.6236 (0.0033)**	**0.7737 (<0.0001)**	−0.3512 (0.1289)	−0.2715 (0.2462)	−0.0504 (0.8328)
S-TYR	ρ P	NT	**0.6052 (0.0047)**	**0.8366 (<0.0001)**	−0.3569 (0.1224)	−0.3656 (0.1130)	−0.1396 (0.5573)

[a] Correlations between serum and hippocampal amino acid levels or between serum and fecal amino acid levels, evaluated using Spearman's rank-order test. The upper and lower figures in each cell indicate Spearman's ρ and p values, respectively. F, fecal; H, hippocampal; NT, not tested; PHE, phenylalanine; S, serum; TRP, tryptophan; TYR, tyrosine.

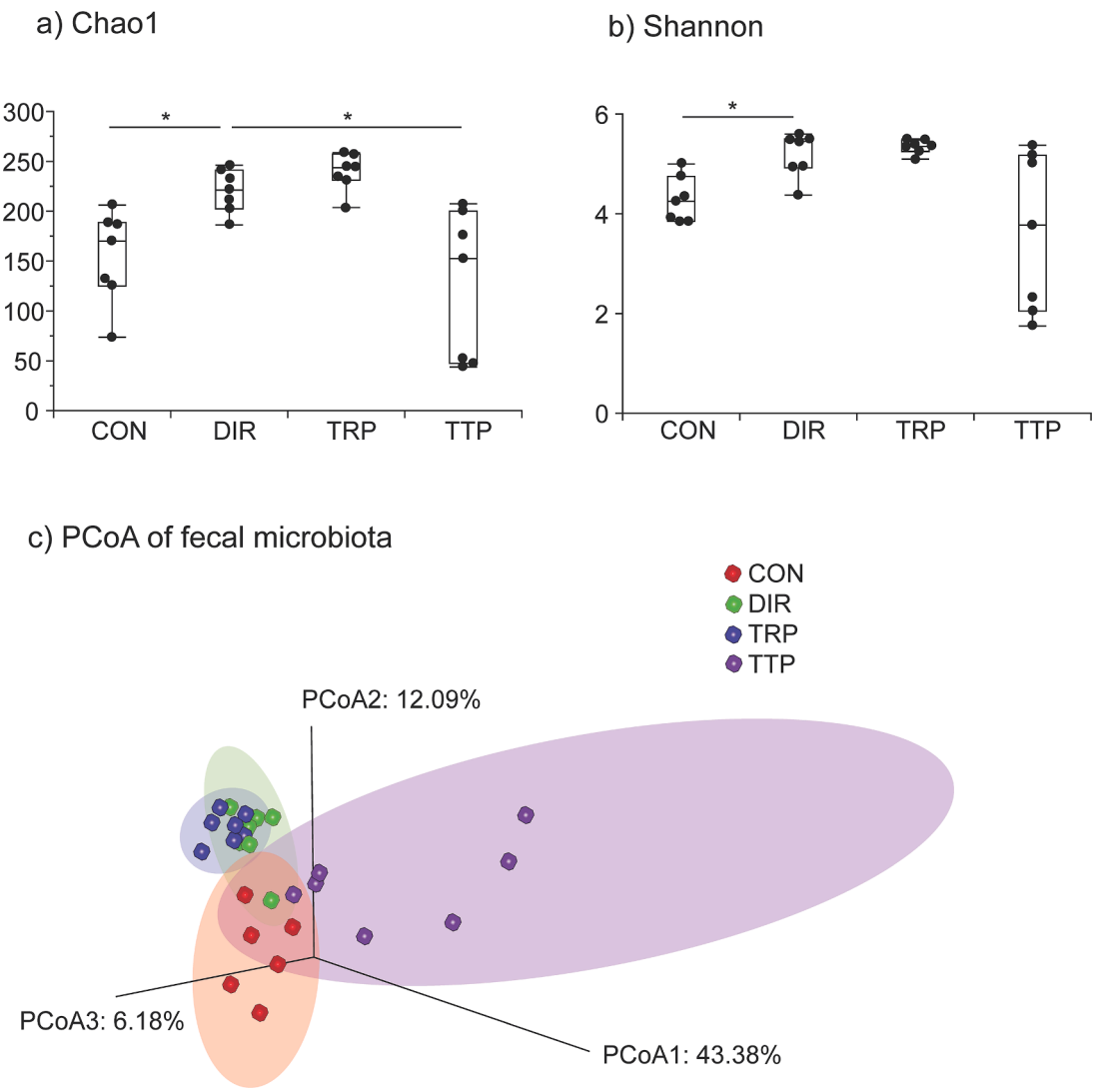

Fig. 3. Effects of AA-deficient diets on fecal microbiota. (a) Chao-1 values and **(b)** Shannon indexes are shown as a parameter of α-diversity. All data ($n = 7$ per group) are visualized as box plots with medians (middle lines), first and third quartiles (box boundaries), and minimum and maximum values (whiskers). **(c)** The principal coordinate analysis shows unweighted UniFrac distances for each group of mice ($n = 7$ per group). Each colored ellipse covers 95% of the samples belonging to a cluster. * $p < 0.05$ indicates a significant difference between the indicated groups according to Steel's multiple comparisons. AA, amino acid; CON, control; DIR, a group fed restricted amounts of control diets; TRP, a group fed tryptophan-deficient diets; TTP, a group fed tryptophan-, phenylalanine-, and tyrosine-deficient diets.

deprived food for a much longer time because of the ethical guidelines of animal experiments that prevent excessive body weight loss in animals. The duration of AA deprivation might have been insufficient to change the metabolic and behavioral characteristics. Fourth, the current study did not investigate the effects of dietary deprivation on the component of lean mass or body fat. Finally, we conducted the current experiments using young mice at 4 weeks of age; whether the effects of AA-deficient diet on behavioral phenotypes could be also found in mice during a different life stage is unclear and should be clarified in future studies. In addition, other measures of depression-like behavior, such as saccharin preference test and female urine sniffing test, would have provided an accurate information about AA deprivation-induced change in depression-like behaviors.

In conclusion, chronic dietary TTP deprivation reduced 5-HT, catecholamine concentrations, and their metabolites in a brain-region specific manner. The reduced activities of monoaminergic systems may be responsible for increased locomotor activity. The TTP depletion reduced the relative abundance of genus Roseburia, although the functional outcomes of this reduction remain unclear.

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Disclosure

The authors declare no conflicts of interest associated with this manuscript.

Author contributions

X.Z. and N.S. designed research; X.Z., K.Y., N.M., A.A., S.I., and T.H. performed experiments; X.Z., K.Y., T.H., S.T., and Y.A. analyzed data; X. Z., K.Y., N.M., A.A., and N.S. interpreted results of experiments; X.Z. and N.S. drafted manuscript; all authors read and approved the final version of manuscript.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.physbeh.2021.113653.

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Supplementary Table 1. Effects of amino acids-deficient diets on colon content acids profile[a]

	(nmol/g)			
	DIR	TRP	TTP	KW
Trp	52.2 (40.7-89.4)	22.2 (22.2-62.4)	91.8 (64.8-121)	0.0102
Phe	340 (275-387)	**153 (101-161)***	515 (392-648)	**0.0014**
Tyr	270 (242-341)	121 (79-151)	394 (103-520)	0.0265
Ala	960 (661-1067)	**377 (303-440)***	**1740 (1152-2700)***	**0.0006**
Asn	94.6 (83.6-130.9)	36.6 (16.2-39.6)	56.1 (0.1-128)	0.0244
Arg	332 (287-401)	157 (99.0-194)	568 (177-791)	0.0075
Asp	197 (85.2-209)	**51.6 (33.0-52.9)****	261 (133-366)	**0.0011**
Cys	0.1 (0.1-14.4)	ND	26.4 (16.5-36.3)	0.0032
Gln	298 (205-334)	**132 (96.0-146)****	479 (297-617)	**0.0011**
Glu	930 (486-1093)	**361 (309-444)***	1238 (1116-3400)	**0.0010**
Gly	329 (274-386)	**119 (95-170)****	770 (339-946)	**0.0005**
His	91.5 (48.4-109)	**31.2 (17.7-41.4)***	175 (110-452)	**0.0014**
Ile	360 (253-430)	139 (115-215)	534 (392-688)	0.0081
Leu	740 (550-1045)	363 (267-445)	1058 (983-1462)	0.0032
Lys	403 (350-481)	**205 (164-267)***	**959 (619-2363)****	**0.0003**
Met	250 (166-292)	109 (85.8-135)	314 (248-447)	0.0036
Pro	257 (205-291)	**80 (76.2-128)****	**482 (338-2500)***	**0.0004**
Ser	351 (278-417)	111 (80-154)	530 (200-566)	0.0026
Thr	303 (263-418)	**140 (121-161)****	691 (325-813)	**0.0010**
Val	538 (412-673)	233 (203-413)	878 (618-1463)	0.0047

[a] All data (n = 7 per group) are expressed as the median with Q1 (lower quartile) and Q3 (upper quartile). When a p value was less than 0.0025 in the KW test comparing the three groups, the Steel test was applied to evaluate differences in this amino acid level between the DIR and TRP groups or between the DIR and TTP groups. * $p < 0.05$ and ** $p < 0.01$ indicate a significant difference between the DIR and TRP groups or between the DIR and TTP groups. DIR, a group fed restricted amounts of diets; TTP, a group fed TRP, PHE, and TYR-deficient diets; TRP, a group fed TRP-deficient diets

Supplementary Table 2. Relative abundance at genus levels.

	CON	DIR	TRP	TTP	p value		p(steel)	
	MEDIAN (Q1-Q3)	MEDIAN (Q1-Q3)	MEDIAN (Q1-Q3)	MEDIAN (Q1-Q3)	KW	DIR vs CON	DIR vs TRP	DIR vs TTP
k__Bacteria;p__Actinobacteria;c__Coriobacteriia;o__Coriobacteriales;f__Coriobacteriaceae;g__Adlercreutzia	0.16 (0.10-0.27)	0.10 (0.07-0.18)	0.39 (0.22-0.46)	0.03 (0.02-0.15)	0.0337	0.8223	0.0563	0.4748
k__Bacteria;p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__Bacteroidaceae;g__Bacteroides	20.69 (5.99-26.28)	8.55 (6.34-10.37)	4.82 (3.02-5.10)	14.57 (5.72-42.42)	0.0524	0.6087	0.1041	0.6984
k__Bacteria;p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__Porphyromonadaceae;g__Parabacteroides	5.29 (1.79-19.61)	7.38 (5.20-13.61)	4.96 (3.97-13.71)	1.41 (0.57-6.83)	0.2778	0.9886	0.9205	0.1792
k__Bacteria;p__Deferribacteres;c__Deferribacteres;o__Deferribacterales;f__Deferribacteraceae;g__Mucispirillum	0.23 (0.01-0.40)	0.77 (0.53-3.06)	0.41 (0.27-2.72)	0.00 (0.00-0.00)	0.0139	0.2880	0.9637	0.0215
k__Bacteria;p__Firmicutes;c__Bacilli;o__Lactobacillales;f__Enterococcaceae;g__Enterococcus	0.00 (0.00-0.02)	0.03 (0.01-0.10)	2.03 (0.82-7.22)	0.05 (0.02-9.09)	0.0071	0.3359	0.0199	0.7805
k__Bacteria;p__Firmicutes;c__Bacilli;o__Lactobacillales;f__Lactobacillaceae;g__Lactobacillus	0.11 (0.07-0.30)	0.00 (0.00-0.02)	0.13 (0.06-0.27)	0.65 (0.39-1.02)	0.0032	0.1254	0.7026	0.0101
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__;g__	12.16 (8.33-14.23)	20.54 (18.12-23.35)	21.33 (17.79-25.11)	16.76 (0.03-30.29)	0.1505	0.0774	0.9637	0.7838
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__[Mogibacteriaceae];g__	0.24 (0.17-0.47)	0.23 (0.17-0.34)	0.24 (0.21-0.31)	0.27 (0.00-0.32)	0.9407	0.9203	0.9985	0.9884
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Dehalobacteriaceae;g__Dehalobacterium	0.36 (0.23-0.45)	0.41 (0.38-0.70)	0.46 (0.35-0.52)	0.12 (0.00-0.57)	0.5448	0.6047	0.9203	0.6047
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae;g__	10.93 (8.93-12.95)	11.18 (10.93-14.41)	12.02 (10.87-13.29)	9.40 (0.02-14.16)	0.5095	0.5196	1.0000	0.6087
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae;g__[Ruminococcus]	8.00 (5.38-10.07)	2.10 (2.07-2.69)	3.13 (2.06-3.85)	2.84 (0.01-4.14)	0.0778	0.0769	0.8223	1.0000
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae;g__Clostridium	0.28 (0.11-2.09)	1.21 (0.78-1.92)	1.03 (0.87-2.03)	1.06 (0.01-2.39)	0.7362	0.6087	1.0000	0.9203
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae;g__Coprococcus	0.92 (0.71-1.63)	2.18 (1.44-2.51)	1.16 (0.86-1.37)	0.18 (0.00-0.90)	0.0203	0.2888	0.2294	0.0194
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae;g__Dorea	0.15 (0.12-0.16)	0.14 (0.11-0.16)	0.15 (0.12-0.18)	0.13 (0.00-0.14)	0.4850	0.9985	0.9633	0.5562
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae;g__Roseburia	1.12 (0.57-1.73)	0.65 (0.37-1.02)	0.01 (0.00-0.07)	0.00 (0.00-0.00)	**0.0006**	0.6984	0.0620	**0.0176**
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae;Other	1.91 (1.00-2.14)	1.04 (0.77-1.22)	0.95 (0.77-1.21)	1.20 (0.67-2.36)	0.4882	0.4350	0.9985	0.9443
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Ruminococcaceae;g__	0.18 (0.06-0.31)	0.86 (0.71-1.61)	0.82 (0.36-2.36)	0.03 (0.00-0.18)	**0.0007**	**0.0407**	0.9205	**0.0059**
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Ruminococcaceae;g__Anaerotruncus	1.19 (0.53-3.84)	0.32 (0.20-0.56)	0.18 (0.14-0.35)	0.11 (0.01-0.31)	0.0063	0.1041	0.6984	0.2563
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Ruminococcaceae;g__Butyricicoccus	0.03 (0.01-0.04)	0.09 (0.05-0.15)	0.27 (0.27-0.30)	0.05 (0.00-0.17)	0.0031	0.1757	0.0765	0.8580
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Ruminococcaceae;g__Clostridium	0.53 (0.35-1.51)	0.34 (0.31-0.42)	0.37 (0.15-0.40)	0.05 (0.00-0.25)	0.0441	0.4757	0.8228	0.2263
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Ruminococcaceae;g__Oscillospira	8.11 (6.50-13.87)	17.17 (12.57-18.51)	11.84 (9.90-15.13)	3.21 (0.13-19.68)	0.4324	0.4350	0.6984	0.6984
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Ruminococcaceae;g__Ruminococcus	1.78 (1.10-2.58)	4.53 (3.03-4.96)	4.14 (3.14-5.08)	0.21 (0.00-3.04)	0.0622	0.3576	1.0000	0.1764
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Ruminococcaceae;Other	0.98 (0.70-1.78)	0.46 (0.36-0.80)	0.51 (0.38-0.69)	0.05 (0.00-0.36)	0.0105	0.4350	1.0000	0.0748
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;Other;Other	0.15 (0.02-0.66)	5.39 (2.99-7.67)	7.89 (6.77-8.56)	0.37 (0.00-3.32)	**0.0004**	**0.0136**	0.3576	0.0756
k__Bacteria;p__Firmicutes;c__Erysipelotrichi;o__Erysipelotrichales;f__Erysipelotrichaceae;g__Clostridium	4.43 (1.28-4.54)	0.81 (0.43-1.17)	1.25 (0.82-1.74)	3.46 (0.33-7.54)	0.3826	0.2294	0.6087	0.9205
k__Bacteria;p__Firmicutes;c__Erysipelotrichi;o__Erysipelotrichales;f__Erysipelotrichaceae;Other	0.26 (0.10-0.30)	0.01 (0.01-0.05)	0.23 (0.08-0.34)	0.01 (0.00-0.18)	0.1205	0.2838	0.1025	1.0000
k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Enterobacteriales;f__Enterobacteriaceae;g__Escherichia	0.06 (0.03-0.10)	0.06 (0.04-0.10)	0.07 (0.03-0.21)	2.42 (0.64-9.83)	0.0475	0.9998	0.9884	0.0752
k__Bacteria;p__Proteobacteria;Other;Other;Other;Other	0.00 (0.00-0.01)	0.01 (0.01-0.26)	0.09 (0.01-0.14)	0.01 (0.00-0.06)	0.2813	0.3775	0.9984	0.9144

All data (n = 7 per group) are expressed as the median with Q1 (lower quartile) and Q3 (upper quartile). Only genera that are detected in more than 50% samples were selected and processed for comparizons among the groups. When a p value was less than 0.00178 in the KW test comparing the three groups, the Steel test was applied to evaluate differences in this amino acid level between the DIR and TRP groups or between the DIR and TTP groups.This p value of 0.00178 was calculated using the Bonferroni correction based on the total number of tests (28, p = 0.05/28 = 0.00178). Bold figures indicate a significant difference between the indicated two groups.

Supplementary Table 3. Correlation between fecal amino acids and bacterial genera#

Bacterial genera	Amino acids	Spearman's ρ value	p value (Prob>|ρ|)
k__Bacteria;p__Firmicutes;c__Bacilli;o__Lactobacillales;f__Enterococcaceae;g__Enterococcus	**Tyrosine**	**-0.751603295**	**0.000133059**
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Ruminococcaceae;g__Oscillospira	Tyrosine	0.525009439	0.01745873
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae;Other	Tyrosine	0.400300978	0.08030509
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Ruminococcaceae;g__Anaerotruncus	Tyrosine	0.375470278	0.102813102
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae;g__Clostridium	Tyrosine	0.373824773	0.104451344
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Dehalobacteriaceae;g__Dehalobacterium	Tyrosine	0.365449781	0.113083697
k__Bacteria;p__Proteobacteria;Other;Other;Other;Other	Tyrosine	-0.32967943	0.155756985
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__;g__	Tyrosine	0.318916909	0.170528082
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Ruminococcaceae;g__Ruminococcus	Tyrosine	0.302106847	0.195468489
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__[Mogibacteriaceae];g__	Tyrosine	0.29427313	0.207884586
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae;g__Coprococcus	Tyrosine	0.288562829	0.217257298
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae;g__[Ruminococcus]	Tyrosine	0.275498702	0.239730939
k__Bacteria;p__Firmicutes;c__Erysipelotrichi;o__Erysipelotrichales;f__Erysipelotrichaceae;g__Clostridium	Tyrosine	-0.273787157	0.242782057
k__Bacteria;p__Actinobacteria;c__Coriobacteriia;o__Coriobacteriales;f__Coriobacteriaceae;g__Adlercreutzia	Tyrosine	-0.248400469	0.290960413
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae;g__Roseburia	Tyrosine	0.219315414	0.352870852
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Ruminococcaceae;g__Clostridium	Tyrosine	0.160768118	0.498337527
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae;g__	Tyrosine	0.152688992	0.520442511
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Ruminococcaceae;g__	Tyrosine	-0.134311512	0.572378817
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;Other;Other	Tyrosine	-0.124529722	0.600905887
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Ruminococcaceae;Other	Tyrosine	0.115761986	0.626961371
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae;g__Dorea	Tyrosine	0.096677427	0.685133825
k__Bacteria;p__Firmicutes;c__Bacilli;o__Lactobacillales;f__Lactobacillaceae;g__Lactobacillus	Tyrosine	0.04684589	0.844517407
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Ruminococcaceae;g__Butyricicoccus	Tyrosine	-0.03990965	0.867326838
k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Enterobacteriales;f__Enterobacteriaceae;g__Escherichia	Tyrosine	-0.035498743	0.881885694
k__Bacteria;p__Firmicutes;c__Erysipelotrichi;o__Erysipelotrichales;f__Erysipelotrichaceae;Other	Tyrosine	0.026485481	0.911744161
k__Bacteria;p__Deferribacteres;c__Deferribacteres;o__Deferribacterales;f__Deferribacteraceae;g__Mucispirillum	Tyrosine	-0.024252239	0.91916145
k__Bacteria;p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__Bacteroidaceae;g__Bacteroides	Tyrosine	-0.008273788	0.972383246
k__Bacteria;p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__Porphyromonadaceae;g__Parabacteroides	Tyrosine	-0.003760813	0.987445112
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Ruminococcaceae;g__	Tryptophan	-0.483387133	0.030834111
k__Bacteria;p__Firmicutes;c__Bacilli;o__Lactobacillales;f__Enterococcaceae;g__Enterococcus	Tryptophan	-0.424896712	0.061836603
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__;g__	Tryptophan	-0.364669056	0.113913783
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Ruminococcaceae;Other	Tryptophan	-0.363740008	0.114907268
k__Bacteria;p__Firmicutes;c__Erysipelotrichi;o__Erysipelotrichales;f__Erysipelotrichaceae;g__Clostridium	Tryptophan	0.355608955	0.123869251
k__Bacteria;p__Actinobacteria;c__Coriobacteriia;o__Coriobacteriales;f__Coriobacteriaceae;g__Adlercreutzia	Tryptophan	-0.350965383	0.129205282
k__Bacteria;p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__Bacteroidaceae;g__Bacteroides	Tryptophan	0.349568888	0.130841435
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;Other;Other	Tryptophan	-0.348945086	0.131577011
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae;g__	Tryptophan	-0.308043426	0.186397062
k__Bacteria;p__Firmicutes;c__Bacilli;o__Lactobacillales;f__Lactobacillaceae;g__Lactobacillus	Tryptophan	0.305271163	0.190597167
k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Enterobacteriales;f__Enterobacteriaceae;g__Escherichia	Tryptophan	0.26686884	0.255368355
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Ruminococcaceae;g__Anaerotruncus	Tryptophan	0.243581797	0.300723911
k__Bacteria;p__Proteobacteria;Other;Other;Other;Other	Tryptophan	0.234678104	0.319281982
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Ruminococcaceae;g__Butyricicoccus	Tryptophan	-0.232427366	0.324079229
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae;g__Dorea	Tryptophan	-0.228961334	0.331550118
k__Bacteria;p__Firmicutes;c__Erysipelotrichi;o__Erysipelotrichales;f__Erysipelotrichaceae;Other	Tryptophan	-0.213065329	0.367098298
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Ruminococcaceae;g__Ruminococcus	Tryptophan	-0.203173654	0.390270264
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Dehalobacteriaceae;g__Dehalobacterium	Tryptophan	-0.199850041	0.398234222
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae;g__Coprococcus	Tryptophan	-0.179004298	0.450178965
k__Bacteria;p__Deferribacteres;c__Deferribacteres;o__Deferribacterales;f__Deferribacteraceae;g__Mucispirillum	Tryptophan	-0.163561248	0.490803141
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Ruminococcaceae;g__Oscillospira	Tryptophan	-0.126086402	0.596327011
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae;g__[Ruminococcus]	Tryptophan	-0.096336031	0.686191361
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae;Other	Tryptophan	-0.089124503	0.70865896
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Ruminococcaceae;g__Clostridium	Tryptophan	-0.084656472	0.722697688
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae;g__Roseburia	Tryptophan	-0.067310587	0.777974907
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__[Mogibacteriaceae];g__	Tryptophan	0.064674902	0.786472094
k__Bacteria;p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__Porphyromonadaceae;g__Parabacteroides	Tryptophan	0.063420705	0.790523737
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae;g__Clostridium	Tryptophan	-0.046055512	0.847110854
k__Bacteria;p__Actinobacteria;c__Coriobacteriia;o__Coriobacteriales;f__Coriobacteriaceae;g__Adlercreutzia	Phenylalanine	-0.559819572	0.010263637
k__Bacteria;p__Firmicutes;c__Bacilli;o__Lactobacillales;f__Enterococcaceae;g__Enterococcus	Phenylalanine	-0.522985511	0.01797693
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Ruminococcaceae;g__	Phenylalanine	-0.4716059	0.035798053
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;Other;Other	Phenylalanine	-0.444528049	0.049559864
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Ruminococcaceae;g__Butyricicoccus	Phenylalanine	-0.404968267	0.076519776
k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Enterobacteriales;f__Enterobacteriaceae;g__Escherichia	Phenylalanine	0.345038837	0.136249845
k__Bacteria;p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__Bacteroidaceae;g__Bacteroides	Phenylalanine	0.336842105	0.146432943
k__Bacteria;p__Deferribacteres;c__Deferribacteres;o__Deferribacterales;f__Deferribacteraceae;g__Mucispirillum	Phenylalanine	-0.277280684	0.236580615
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae;Other	Phenylalanine	0.26852202	0.252323819
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Ruminococcaceae;Other	Phenylalanine	-0.255560633	0.276817024
k__Bacteria;p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__Porphyromonadaceae;g__Parabacteroides	Phenylalanine	-0.239097744	0.309986264
k__Bacteria;p__Firmicutes;c__Bacilli;o__Lactobacillales;f__Lactobacillaceae;g__Lactobacillus	Phenylalanine	0.237917851	0.312451758
k__Bacteria;p__Proteobacteria;Other;Other;Other;Other	Phenylalanine	-0.235233103	0.318105612
k__Bacteria;p__Firmicutes;c__Erysipelotrichi;o__Erysipelotrichales;f__Erysipelotrichaceae;Other	Phenylalanine	-0.233741042	0.321274059
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Ruminococcaceae;g__Oscillospira	Phenylalanine	0.213533835	0.366020638
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae;g__Dorea	Phenylalanine	-0.169121881	0.4759719
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae;g__	Phenylalanine	-0.12481203	0.600074418
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae;g__Clostridium	Phenylalanine	0.106766917	0.654141545
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Ruminococcaceae;g__Anaerotruncus	Phenylalanine	0.09853329	0.679394847
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Ruminococcaceae;g__Clostridium	Phenylalanine	-0.090327495	0.704894302
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae;g__Coprococcus	Phenylalanine	-0.060925164	0.798600819
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Dehalobacteriaceae;g__Dehalobacterium	Phenylalanine	0.051918751	0.827909676
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Ruminococcaceae;g__Ruminococcus	Phenylalanine	-0.051899214	0.827973506
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__[Mogibacteriaceae];g__	Phenylalanine	0.036911553	0.877218308
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae;g__[Ruminococcus]	Phenylalanine	-0.023325815	0.922240312
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae;g__Roseburia	Phenylalanine	0.019715193	0.934249575
k__Bacteria;p__Firmicutes;c__Erysipelotrichi;o__Erysipelotrichales;f__Erysipelotrichaceae;g__Clostridium	Phenylalanine	0.015037594	0.949827695
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__;g__	Phenylalanine	-0.003007519	0.98995973

Correlations between bacterial genera and tyrosine, between bacterial genera and tryptophan, or between bacterial genera and phenylalanine levels in sera were evaluated using the Spearman's rank-ordered analysis. Based on the Bonferroni correction, only the correlation between fecal tyrosine and genus Enterococcus was considered to be significant.