The Beneficial Effects of *Citrus kawachiensis* Peel on Neurogenesis in the Hippocampus and Gut Microbiota Changes in a Chronic Unpredictable Mild Stress Mouse Model

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Abstract: We previously reported that the dried peel powder of *Citrus kawachiensis*, a citrus product of Japan, exerted anti-inflammatory and neuroprotective effects in the brains of transient global cerebral ischemia model mice. It also ameliorated the hyperphosphorylation of Tau protein and the suppression of neurogenesis in the brains of the senescence-accelerated mouse-prone 8 aging model. Chronic unpredictable mild stress (CUMS) induces anxiety-like behavior, changes the composition of the gut microbiota and suppresses neurogenesis in the hippocampus. Therefore, we herein examined the effects of the dried peel powder of *C. kawachiensis* in a CUMS mouse model: CUMS enhanced locomotor activity, shown as the distance travelled in the open field test at the beginning of the test, while the *C. kawachiensis* treatment suppressed this increase. The *C. kawachiensis* treatment also prevented CUMS-induced decreases in hippocampal neurogenesis. The CUMS treatment changed the composition of the gut microbiota by reducing the abundance of *Lactobacillus* and increasing that of *Bacteroides*, whereas the *C. kawachiensis* treatment attenuated these changes. Collectively, the present results suggest that the dried peel powder of *C. kawachiensis* exerts neuroprotective effects in the brain and maintains the condition of the microbiome under mild stress.

Keywords: *Citrus kawachiensis*; brain; neurogenesis; gut microbiota; chronic unpredictable mild stress

1. Introduction

Stress is a major risk factor for the development of anxiety and/or depression. A characteristic feature of the stress response is the activation of the hypothalamic–pituitary–adrenal (HPA) axis, which increases adrenal glucocorticoid levels [1]. Several pathways connect the brain and the gut, such as the vagus nerve, HPA axis, and immune system and enteroendocrine signaling [2]; therefore, psychological stress may induce gut–brain axis dysfunctions and, ultimately, mental disorders by impairing one or more of these pathways [3]. Chronic stress influences not only brain function, but also peripheral tissues, including the gut microbiome, and many studies have investigated changes in the “gut–brain axis”, namely, the relationship between the gut microbiome and the brain function [4]. The gut microbiota is a key player in the regulation of this axis, even under stress conditions. Chronic mild stress was previously shown to induce anxiety-like behavior [5], while neurogenesis in the brain was suppressed [6]. Differences in anxiety-related behaviors are commonly reported in mice with altered gut microbiomes, indicating a role for the gut microbiota in stress and in behavior [7,8].
In recent years, not only probiotics, but also prebiotics have been attracting increasing attention due to their beneficial effects on mental health through interactions with commensal gut bacteria [1]. Burokas et al. showed that prebiotics exerted anxiolytic and antidepressant-like effects in a chronic stress mouse model [9]. Since diet is a major influence on the bacterial composition of the gut microbiota, food factors may contribute to the microbiota–gut–brain axis and the prevention or suppression of stress-related symptoms. Therefore, food factors as a prebiotic property with the suppression of chronic stress-induced anxiety and stimulation of neurogenesis are particularly important for mental health.

Citrus fruits contain a number of functional ingredients, and we have conducted research on Citrus kawachiensis, a citrus product of Japan. The peel of C. kawachiensis was previously shown to stimulate neurogenesis, protect neuronal cells, and exert anti-inflammatory effects in a senescence-accelerated mouse-prone 8 (SAMP8) model [10]. The peel of C. kawachiensis contains 3,5,6,7,8,3′,4′-heptamethoxyflavone (HMF), 0.27 mg/g in the dried peel [11], and it has many bioactive functions [12,13]. HMF was previously shown to attenuate depressive-like behavior and promote neurogenesis in a chronic unpredictable mild stress (CUMS) mouse model [13]. Therefore, we herein examined the effects of the peel of C. kawachiensis in mice exposed to CUMS with a focus on neurogenesis in the brain and the condition of the gut microbiome.

2. Materials and Methods

2.1. Sample Preparation

The fruit of C. kawachiensis was harvested in Yawatahama, Ehime, Japan. To prepare the dried peel, the peel of the fruit (1555 g) that was squeezed to obtain juice was chopped into small pieces and dried in vacuo at 60 °C for 1 day. Dried peel (ca. 300 g) was then milled and ground into a fine powder using a mill mixer (Iwatani IFM-660DG, Tokyo, Japan). After being passed through a 150-mesh sieve, the powder was used as the test sample. An experimental diet was prepared and mixed in the laboratory based on the AIN-93G formula including 20% casein (New Zealand Dairy Board, Wellington, New Zealand), 0.3% L-cystine (Nacalai Tesque, Kyoto, Japan), 53.2% α-corn starch (Sanwa Starch, Nara, Japan), 10% sucrose (Nippon Beet Sugar Manufacturing, Tokyo, Japan), 5% cellulose (International Filler Corp, North Tonawanda, NY, USA), 7% soybean oil (J-Oil Mills, Tokyo, Japan), 3.5% mineral mixture (each component were mixed in the laboratory based on AIN-93 mineral mixture composition), and a 1% vitamin mixture (each component was mixed in the laboratory based on AIN-93 vitamin mixture composition containing 25 g of choline bitartrate/100 g). Groups administered the C. kawachiensis peel received the basic experiment diet supplemented with 1 or 2% of dried peel powder.

2.2. Experimental Schedule and CUMS

Four-week-old male ICR mice were purchased from Japan SLC (Hamamatsu, Japan). The mice were kept at 23 ± 1 °C on a 12-h light/dark cycle (light on 8:00–20:00). Tap water and the experimental diet were freely available during the experimental period. All animal experiments were performed in accordance with the Guidelines for Animal Experimentation, approved by the Animal Care and Use Committee of Matsuyama University, and implemented by an approved protocol (#14-005).

After one week of habituation, mice (5 weeks age) were randomly divided into the following four groups (n = 12, respectively): the basal experiment diet without CUMS group (CON), the basal experiment diet with CUMS group (CUMS), 1% C. kawachiensis peel-supplemented experimental diet with CUMS group (CUMS + CK1), and 2% C. kawachiensis peel-supplemented experimental diet with CUMS group (CUMS + CK2). The following four stress conditions were prepared as CUMS: (1) Restraint stress (6 h); the tail and body were fixed with a band to a wooden board and made mice immobile, (2) Wet stress (24 h); sawdust in cages was moistened with water, (3) Tilt stress (24 h); the home cage was tilted (30°), and (4) Food restriction stress (24 h); food was removed. Mice received a day off after...
the food restriction stress; therefore, mice received one of the random stressors or a resting day in the consecutive experimental days for three weeks. The open field test (OFT) was performed on Day 22 (at 8 weeks of age). After OFT, mice were transcardially perfused with heparinized, ice-cold, phosphate-buffered saline through the left ventricle and the brains were removed.

2.3. OFT

The open field box had the following dimensions: 70 cm × 70 cm × 50 cm (L × W × H). Each mouse was placed in the center and allowed to move freely during a 10-min session. The apparatus was cleaned between trials using 70% ethanol. The total distance travelled was analyzed as locomotor activity using the ANY-maze Video Tracking System (Stoelting, Wood Dale, IL, USA) connected to a USB digital camera.

2.4. Western Blot Analysis

Hippocampal tissues were subjected to a Western blot analysis. Tissues were homogenized in 10 volumes (w/v) of RIPA buffer [20 mM Tris–HCl, pH 7.5, 150 mM NaCl, 0.1% SDS, 1% sodium deoxycholate, 1% NP-40, 2 mM EDTA, and a protease inhibitor cocktail (Roche, Mannheim, Germany)]. Lysates were centrifuged at 20,000 × g at 4 °C for 30 min and supernatant solutions were collected as the protein extract. Equal amounts of protein (30 µg) were separated on 12% SDS polyacrylamide gels and electroblotted onto an Immuno-Blot PVDF Membrane (Bio-Rad, Hercules, CA, USA). The primary antibody was against brain-derived neurotrophic factor (BDNF, 1:1000; Alomone Labs, Jerusalem, Israel) and actin (1:5000; Sigma-Aldrich, St. Louis, MO, USA), and the secondary antibody was horseradish peroxidase-linked anti-rabbit IgG (Cell Signaling, Woburn, MA, USA). Immunoreactive bands were visualized by ECL-plus (GE Healthcare, Little Chalfont, UK) and band intensities were measured using a ChemiDoc Touch MP imaging system and ImageLab software (Bio-Rad).

2.5. Immunofluorescence Staining

Thirty-micrometer-thick sagittal sections of frozen brains, postfixed in 4% paraformaldehyde, were cut using a cryostat (CM3050S; Leica Microsystems, Heidelberg, Germany) and incubated with goat anti-doublecortin (DCX, 1:50; Santa Cruz Biotechnology, Santa Cruz, CA, USA) as the primary antibody. Alexa Fluor 488 donkey anti-goat IgG (H + L) (1:300; Invitrogen, Carlsbad, CA, USA) was used as the secondary antibody. Sections were covered with a mounting medium (Vectashield; Vector Laboratories, Burlingame, CA, USA), and images were captured using a confocal fluorescence microscopy system (LSM510; Zeiss, Oberkochen, Germany). Six mice were randomly selected in each group, and two different sections were selected for each mouse for the quantitative analysis. DCX-positive cells in the dentate gyrus (DG) of the hippocampus in each section were counted.

2.6. Next-Generation Sequencing

Four mice were randomly selected, and DNA was extracted from their cecal contents using ISOFECAL for Beads Beating (Nippon Gene, Tokyo, Japan). Each DNA extract in TE buffer was adjusted to 10 ng/10 µL and pooled into one sample in each experiment group. Next-generation sequencing, PCR of the V3-4 region of bacterial 16s rRNA, and data analyses were outsourced to Macrogen Japan (Kyoto, Japan).

2.7. HPLC Analysis

The cecal contents of short-chain fatty acids (SCFAs) were measured by HPLC (LC-10AD; Shimadzu, Kyoto, Japan) using the internal standard method. Cecal contents were homogenized by vortex mixing in 1 mL of 10 mmol sodium hydroxide/L aqueous solution, containing 2.5 mmol crotonic acid/L (Nacalai Tesque, Kyoto, Japan) as an internal standard, in an ice-water bath, and then centrifuged at 16,800 × g at 4 °C for 15 min. Fat-soluble substances in the supernatant were removed by extraction with chloroform.
aqueous phase was filtered through a membrane filter (cellulose acetate, pore size 0.45 μm, DISMIC-13cp, Advantec Toyo Roshi, Tokyo, Japan). Using an ion exclusion column, SCFAs were separated and detected using the post-column pH-buffered electrical conductivity detection method. A 25-μL aliquot of the sample was applied to a H-type cation exchanger column (shim-pack SCR-102H, 8 mm i.d. × 30 cm long; Shimadzu, Kyoto, Japan) at 40 °C. The mobile phase was 5 mmol p-toluene sulfonic acid/L aqueous solution (flow rate: 0.8 mL/min). The SCFAs were detected using an electroconductivity detector of positive polarity at 40 °C (CDD-6A; Shimadzu) and pH buffering solution of 20 mmol bis-Tris/L aqueous solution containing 5 mmol p-toluene sulfonic acid/L and 100 μmol EDTA/L (flow rate 0.8 mL/min, 40 °C).

2.8. Statistical Analysis

Data for individual groups are expressed as means ± SEM. Data were analyzed using an unpaired t-test between two groups, or a one-way ANOVA followed by Dunnett’s multiple comparison test among three groups using Prism 6 (GraphPad Software, La Jolla, CA, USA). A p value < 0.05 was considered to be significant.

3. Results

3.1. Body Weight Changes and Behavioral Analyses

As shown in Figure 1A, the CON group constantly gained weight, and a significant difference was observed from that in the CUMS group at weeks 2 (** p < 0.01), 3 (** p < 0.001), and 4 (** p < 0.01). However, no significant differences were noted among the CUMS, CUMS + CK1, and CUMS + CK2 groups.

![Figure 1](image)

**Figure 1.** Effects of the dried peel powder of *C. kawachiensis* in body weight changes and in behavioral tests. (A) Body weight changes during the experiment. (B) Time course of the distance travelled during 10 min in the open field test. (C) Accumulation of distance travelled in the first 2 min in the open field test. Values are means ± SEM (n = 12, each group). (A) Symbols show significant differences between CUMS vs. CON at each time point (** p < 0.01, *** p < 0.001), (B) Symbols show significant differences between CUMS vs. CON at each time point (* p < 0.05, ** p < 0.001), and (C) CUMS vs. CON (** p < 0.001) or CUMS vs. CUMS + CK2 (# p < 0.05).

To assess anxiety-like behaviors in the present study, OFT was used, and CUMS was previously shown to induce hyperlocomotor activity in OFT [5]. The CUMS group exhibited hyperlocomotor activity during the first two minutes, and a significant difference was observed from that in the CON group at 1 min (** p < 0.001) and 2 min (* p < 0.05). However, no significant differences were noted among the CUMS, CUMS + CK1, and CUMS + CK2 groups (Figure 1B). On the other hand, accumulation of the distance travelled in the first two minutes significantly differed between the CUMS and CUMS + CK2 groups (# p < 0.05, Figure 1C).
3.2. Western Blot and Immunofluorescent Analyses of the Hippocampus

BDNF levels in hippocampal tissue were previously shown to decrease under CUMS [14]. Western blotting in the present study revealed that the ratio of mature BDNF (14 kDa) to proBDNF (32 kDa) was significantly lower in the CUMS group than in the CON group (* p < 0.05), whereas no significant differences were observed in the CUMS + CK1 and CK2 groups (Figure 2) at this time point. Mature BDNF is cleaved from proBDNF, and it binds to the tyrosine kinase B (TrkB) receptor.

The subgranular zone (SGZ) of DG is one of the areas at which neurogenesis occurs in the brain, and it is suppressed by CUMS [4]. The present results demonstrated that C. kawachiensis peel prevented stress-induced decreases in hippocampal neurogenesis in the SGZ of DG using DCX, a marker for immature neurons. Figure 3 shows the immunoreactivity of DCX in SGZ. While markedly increased in number in both the CUMS + CK1 and the CK2 groups (Figure 3A), DCX-positive cells (green) were rarely observed in the CUMS group. We counted the number of DCX-positive neurons in SGZ, and the results obtained showed significantly fewer of these neurons in the CUMS group than in the CON group (*** p < 0.001), but not in the CUMS + CK1 (## p < 0.01) or CK2 groups (### p < 0.001; Figure 3B).
Figure 3. Effects of the dried peel powder of *C. kawachiensis* on doublecortin immunoreactivity in the hippocampus. (A) Sagittal sections were stained with the anti-doublecortin antibody. Green signals show doublecortin-positive signals, and typical signals were shown with white arrows. (B) Quantification of doublecortin-positive signals in the hippocampus. (C) The location of the captured images is shown with a square in the figure. Values are means ± SEM (*n* = 12, each group). Symbols show significant differences for CUMS vs. CON (*** *p* < 0.001), CUMS vs. CUMS + CK1 (## *p* < 0.01) or CUMS vs. CUMS + CK2 (### *p* < 0.001).

3.3. Analysis of the Cecal Contents Using Next-Generation Sequencing and HPLC

The gut microbiota modulates adult neurogenesis, and a *Lactobacillus* strain was found to promote the survival of hippocampal neuronal progenitors [15]. Furthermore, previous studies showed that the abundance of *Bacteroidetes* in the microbiota of depressed animals increased, whereas that of *Lactobacillus* decreased [16]. Regarding the results of next-generation sequencing of the gut microbiota in the cecum in the present study, a comparison of the relative abundance of different phyla in the gut microbiota showed marked decreases in the abundance of *Firmicutes* and increases in that of *Bacteroidetes* in the CUMS group; in contrast, the CK-treated groups showed increases in *Firmicutes* and decreases in *Bacteroidetes* (Figure 4A). The abundance of *Bacteroides*, classified in *Bacteroidetes*, increased in the CUMS group; however, this increment was suppressed in the CUMS + CK group. In contrast, the abundance of *Lactobacillus*, classified in *Firmicutes*, markedly decreased in the CUMS group, and this reduction was suppressed in the CUMS + CK group (Figure 4B).

In the analysis of SCFAs in the cecal contents by HPLC, propionate and butyrate concentrations were both significantly decreased in the CUMS group, whereas no significant differences were observed in the CUMS + CK groups for improvement (Figure 5).
Figure 4. Effects of the dried peel powder of *C. kawachiensis* on the composition of the gut microbiota by next-generation sequencing. (A) Comparison of the relative abundance of phyla in the gut microbiota. (B) Comparison of the relative abundance of genera in the gut microbiota. A statistical analysis was not performed because each group had only one sample (four mouse samples were pooled into one sample) in the analysis.

**Figure 5.** Effects of the dried peel powder of *C. kawachiensis* in the cecal contents. Quantitative assessment by HPLC of cecal contents. Values are means ± SEM. The symbol shows a significant difference for CON vs. CUMS (** *p* < 0.01 or *** *p* < 0.001).

**4. Discussion**

Stress induces anxiety and/or depressive-like behavior, the atrophy of hippocampal neurons, and the suppression of neurogenesis in the adult hippocampus. A characteristic feature of the stress response is the activation of the HPA axis, which increases adrenal glucocorticoid levels [1]. Since glucocorticoid receptors are highly expressed in the hippocampus, this brain region is markedly affected by stress. Stress has been shown to alter BDNF levels in the hippocampus and other brain regions; the expression of BDNF in the hippocampus was markedly downregulated by both acute and chronic stress, which induced neuronal atrophy and suppressed neurogenesis [4]. Antidepressants were found to exert positive effects on behavior and to stimulate neurogenesis in the adult hippocampus [17]. Anxiety disorders show high comorbidity with depression, and they have also
been repeatedly associated with reduced levels of BDNF [18]. In the present study, serum glucocorticoid levels were not significantly changed in any experiment groups at this timepoint (on Day 22, data not shown). The expression of BDNF in the hippocampus identified by Western blotting was significantly decreased in the CUMS group; however, this reduction was slightly attenuated by the CK treatment. Further studies are needed to clarify whether the CK treatment significantly ameliorates this decrease at a different time point.

We previously reported that the peel of *C. kawachiensis* promoted the expression of DCX in the hippocampus of SAMP8 mice [6] and a streptozotocin-induced hyperglycemia mouse model [19]. In the present study, the number of DCX-positive cells was significantly lower in the CUMS group than in the CON group, and this decrease was significantly attenuated in both CK treatment groups. These results suggest that the CK treatment attenuated dysfunctions in hippocampal neurogenesis under CUMS. Although CUMS sensitizes the neuroinflammatory response [16], in our trial analysis showed that there were not significant differences in microglial and astrocytic activation in the hippocampus among all experiment groups in this experiment condition. Previous findings indicated that HMF altered depressive-like behavior, up-regulated BDNF expression, and stimulated neurogenesis in CUMS mice [13]; in addition, the peel of *C. kawachiensis* also contains a high amount of flavanone compounds, naringin, 44.02 mg/g in the dried peel [11], and naringin altered DCX suppression a streptozotocin-induced hyperglycemia mouse model [20]. Therefore, HMF and naringin in the peel of *C. kawachiensis* may partly contribute to these effects.

OFT is often employed to assess anxiety-like behaviors, and CUMS was previously shown to induce hyperlocomotor activity in OFT [5]. In the present study, the distance travelled during the first two minutes in OFT was significantly longer in the CUMS group than in the CON group. This result showed that mice in the CUMS group may have encountered difficulties adjusting to the new environment. The distance travelled was significantly shorter in the CUMS + CK2 group than in the CUMS group. Therefore, these results suggest that the CK treatment altered anxiety-like behavior in CUMS mice.

Several pathways connect the brain and the gut, such as the vagus nerve, HPA axis, and immune system and enteroendocrine signaling [2]; therefore, psychological stress may induce gut–brain axis dysfunctions and, ultimately, mental disorders by impairing one or more of these pathways [3]. Since the gut of mammals has its own enteric nervous system, it is often referred to as the “gut brain,” and it exerts independent responses to external signals. Exposure to stress disturbs the HPA axis, and it induces immune dysregulation; and, patients with depression frequently have “gut brain” dysfunctions, including metabolic disturbances, functional gastrointestinal disorders, and gut microbiota abnormalities [21]. The gut microbiota plays a key role in the “gut brain”. Differences have been reported in the microbiota of animal stress models, including a chronic variable stress model [22] and chronic restraint stress model [2]. Furthermore, the microbiota of depressed animals was found to be similar to that of patients with depression, namely, the abundance of *Bacteroidetes* was increased, whereas that of *Lactobacillus* was decreased [16]. A previous study reported that a chronic treatment with *Lactobacillus rhamnosus* (B-1) induced the expression of central gamma-aminobutyric acid receptors, reduced anxiety-like behavior, and attenuated the stress-induced corticosterone response, and the vagus nerve was suggested to contribute to these changes [23]. The gut microbiota has been shown to modulate adult neurogenesis, and the administration of a *Lactobacillus* strain promoted the survival of hippocampal neuronal progenitors [24]. Therefore, the effects of a *Lactobacillus* strain may involve multiple mechanisms, including the regulation of endocrine signaling, the HPA axis, and immunomodulation [15]. Based on these findings, we consider the beneficial effects of suppressing reductions in the abundance of *Lactobacillus* by administering *C. kawachiensis* under CUMS to be important.

Humoral signaling molecules and hormonal components also contribute to the regulation of the microbiota-gut–brain axis. Short-chain fatty acids (SCFAs), including propionate,
butyrate, and acetate, are key metabolic products of the gut microbiota [25], and they indirectly or directly exert central effects through G-protein coupled receptors [26]. Free fatty acid receptor (FFAR) 2 binds to the shorter aliphatic chains of acetate and propionate, whereas FFAR3 preferentially binds to propionate, butyrate, and valerate [27]. Propionate activates ganglion activity via FFAR3, which is strongly expressed in the sympathetic nervous system. Butyrate is an epigenetic modulator that acts through histone deacetylases to exert anti-depressant-like and anti-anxiety-like effects [28–32]. Furthermore, an intraperitoneal injection of butyrate was found to reduce immobility times in the forced swim test and up-regulate the expression of BDNF within the prefrontal cortex [33]. Butyrate produced in the intestines is released from monocarboxylate transporters in the gut epithelium, passes through the blood–brain barrier, and exerts its effects on brain tissue [30]. Cecal concentrations of SCFAs were previously reported to be lower in patients with depression than in controls [34,35]. In this experiment, propionate and butyrate concentrations in cecal contents were significantly suppressed following CUMS, but they were not observed in the CUMS + CK groups for improvement. The present data showed the changes between the gut microbiota and SCFAs in the cecal following CUMS were consistent, but the effect of C. kawachiensis treatments were not consistent between brain function and cecal SCFAs. The present data suggested the effect of C. kawachiensis in the brain may not be strongly reflected from cecal SCFAs. However, further studies are needed to measure the concentrations of SCFAs in blood.

The modulation of the gut microbiota in stress model animals has been shown to exert probiotic and prebiotic effects; however, only a few types of compounds have been examined in prebiotic trials on both humans and animals [36,37]. Polysaccharides, such as arabinoyxylans, fucoidan, glucans, fructo-oligosaccharides, and galacto-oligosaccharides, improved brain function in controlled human, animal, and in vitro studies after their oral, systemic, and localized administration [36]. Glucans have been suggested to enhance the growth of Lactobacillus strains in the human intestines through their fermentation in the cecum and colon [38]. Chronic psychosocial stress-induced changes suppressed Lactobacillus in the cecum; however, this was attenuated by fructo-oligosaccharides and galacto-oligosaccharides [9]. The prebiotic agent, sesamin suppressed changes in the abundance of Lactobacillus in the gut of stress-exposed mice [39]. Dietary supplementation with hesperetin, a flavanone rutinoside presents in citrus fruits, showed the potential to increase fecal Lactobacillales and cecal butyric acid levels [40]. A flavanone compound naringin in the peel of C. kawachiensis may similarly have contributed to the present experiment.

5. Conclusions

The present results demonstrated that the peel of C. kawachiensis accelerated brain neurogenesis and maintained the intestinal microbiota in mice subjected to CUMS; therefore, C. kawachiensis has potential as a prebiotic agent. However, we could not clearly show how C. kawachiensis participates in the gut–brain axis function. It currently remains unclear whether only one component in the peel of C. kawachiensis, such as HMF, directly affects neurogenesis in the hippocampus. However, food intake of this mouse strain is approximately 4 g/day, so the intake of HMF may be limited. Therefore, several components including naringin in the peel of C. kawachiensis may also be exerting direct effects on the brain and the gut function.

Furthermore, some factors exert their effects on brain function from the gut microbiota and the gut physiological function. Since there are many factors and parameters affecting the gut–brain axis, it is necessary to continue investigations on various parameters.

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Conflicts of Interest: The authors declare no conflict of interest.

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