View Article Online
View Journal

Food & Function

Linking the chemistry and physics of food with health and nutrition

Accepted Manuscript

This article can be cited before page numbers have been issued, to do this please use: P. Tsai, E. Wu, H. Li, K. J. Senthilkumar and S.-Y. Wang, *Food Funct.*, 2023, DOI: 10.1039/D3FO01301D.

This is an Accepted Manuscript, which has been through the Royal Society of Chemistry peer review process and has been accepted for publication.

Accepted Manuscripts are published online shortly after acceptance, before technical editing, formatting and proof reading. Using this free service, authors can make their results available to the community, in citable form, before we publish the edited article. We will replace this Accepted Manuscript with the edited and formatted Advance Article as soon as it is available.

You can find more information about Accepted Manuscripts in the Information for Authors.

Please note that technical editing may introduce minor changes to the text and/or graphics, which may alter content. The journal's standard Terms & Conditions and the Ethical guidelines still apply. In no event shall the Royal Society of Chemistry be held responsible for any errors or omissions in this Accepted Manuscript or any consequences arising from the use of any information it contains.

View Article Online
DOI: 10.1039/D3FO01301D

ARTICLE

Hirami Lemon (*Citrus reticulata* var. *depressa*) modulates the gut-brain axis in chronic mild stress-induced depression mice model

Received 00th January 20xx,
Accepted 00th January 20xx

DOI: 10.1039/x0xx00000x

Po-Heng Tsai[a], Pei-Chen Wu[b], Hui-Ru Li[b], K.J. Senthil Kumar[c*], Sheng-Yang Wang[a,b,d,e,*]

Citrus reticulata var. *depressa*, commonly known as Hirami lemon, is a native citrus species found in the Taiwan and Okinawa islands of Japan. While several *Citrus* species are known to possess antidepressant activity by modulating gut microbiota, the antidepressant effect of Hirami lemon and its underlying mechanisms have not been thoroughly investigated. In this study, we explored the potential antidepressant efficacy of fruit extract (CD) and the essential oil (CDE) from Hirami lemon peel using chronic mild stress (CMS)-induced mice model and analyzed the association of gut microbiome changes. Our findings revealed that mice subject to CMS exhibited anxiety- and depression-like behaviors as assessed by elevated plus-maze and forced swimming tests, respectively. Significantly, oral administration of CDE and CD notably reversed CMS-induced depression- and anxiety-like behavior in CMS-induced mice. Moreover, compared to the non-stressed group, CMS significantly altered the gut microbiome, characterized by highly diverse bacterial communities, reduced *Bacteroidetes*, and increased *Firmicutes*. However, oral administration of CDE and CD restored the gut microbiota dysbiosis. We also performed a qualitative analysis of CD and CDE's using UPLC-MS and GC-MS, respectively. The CD contained 25 compounds, of which 3 were polymethoxy flavones and flavanones. Three major compounds, nobiletin, tangeretin and hesperidin accounted for 56.88% of the total relative peak area. In contrast, CDE contained 11 terpenoids, of which 8 were identified as major compounds, with D-limonene (45.71%) being the most abundant, followed by γ-terpinene (34.65%), linalool (6.46%), *p*-cymene (2.57%), α-terpineol (2.04%), α-pinene (1.89%), α-terpinolene (1.46%), and β-pinene (1.16%), accounting for 95.94% of the total oil. In conclusion, our study demonstrated the potential of Hirami lemon as a source of natural antidepressant agents for the prevention and treatment of major depressive disorders.

1 Introduction

Anxiety and depression are pervasive mental disorders that impact millions of individuals globally. The health burden of stress-related illnesses, including anxiety disorders and depression, is rising rapidly increasing, as estimated.[1] Moreover, the World Health Organization (WHO) has cautioned that depression will become one of the primary causes of death and disability in the coming years.[2] Consequently, it is, crucial to develop multiple strategies to prevent and treat mental disorders.

Over the past few years, an increasing number of studies have demonstrated the significant role that the gut microbiome plays in maintaining health and involvement in development.[3] This emerging evidence suggests that gut microbiome dysbiosis contributes to the pathogenesis of various chronic metabolic disorders, including diabetes, obesity, inflammatory bowel disease (IBD), mental disorders such as anxiety and depression, and even COVID-19 infection.[4]

Moreover, research on the gut-brain axis, which refers to bidirectional communication between the gut microbiota and the brain, has gained popularity in recent years. This complex interaction is responsible for regulating several essential functions, such as digestion, satiety, immunity, metabolism, and stress reactions, especially in the hypothalamic-pituitary-adrenal (HPA) axis.[5,6] Despite being in addition to the class of probiotics, psychobiotics have been found to offer significant health benefits, particularly in managing stress-related disorders, depression, and anxiety by modulating the gut-brain axis and promoting better mental health.[7] Research conducted on rodents, which are exposed to stress and tested for motivation, anxiety, and depression using stress-induced behavioral tests, has been a major focus in exploring the potential of psychobiotics in this field.[8]

Over time, an abundance of evidence has emerged indicating that a well-balanced diet, with a focus on plant-based foods, is crucial for achieving optimal health and nutrition. This is largely due to the presence of bioactive compounds derived from plants, which have been found to offer a range of mental health benefits. These benefits can be attained by consuming these bioactive compounds in their whole-food form or through supplements. As a result, plant-based therapies offer a

[a]*Ph. D. Program in Microbial Genomics, National Chung Hsing University, Taichung, Taiwan and Academia Sinica, Taipei, Taiwan*
[b]*Department of Forestry, National Chung-Hsing University, Taichung, Taiwan*
[c]*Bachelor Program of Biotechnology, National Chung Hsing University, Taichung, Taiwan*
[d]*Special Crop and Metabolome Discipline Cluster, Academy of Circle Economy, National Chung Hsing University, Taichung, Taiwan*
[e]*Agricultural Biotechnology Research Center, Academia Sinica, Taipei, Taiwan*
**Corresponding authors*
† Footnotes relating to the title and/or authors should appear here.
Electronic Supplementary Information (ESI) available: [details of any supplementary information available should be included here]. See DOI: 10.1039/x0xx00000x

promising alternative to traditional psychiatric medications for treating various mental conditions, including anxiety, depression, attention deficit hyperactivity disorder (ADHD), and schizophrenia.[9] Recent research has uncovered a strong link between gut microbiota composition and behavior/mood, and the discover of bidirectional communication between brain and gut microbiome has further highlighted the potential benefit of plant metabolites, or prebiotics, in reducing stress and preventing anxiety-/depression-like symptoms in high-risk individuals.[9] There is growing evidence supporting the use of dietary foods, medicinal herbs, and their secondary metabolites in regulating gut microbiome dysbiosis and improving overall mental health.[10, 11]

The *Rutaceae* family, commonly known as citrus species, is the most economically significant fruit crops globally. It includes oranges, lemons, grapefruits, pomelos, and limes.[12] Citrus fruits are rich in minerals, vitamins, and bioactive phytochemicals such as limonoids, carotenoids, flavonoids, and terpenes.[13] Several pharmacological studies have indicated that the bioactive compounds in *Citrus* fruits offer serval health benefits, including antioxidant, anti-inflammatory, anti-hypertensive, hypoglycemic, hypolipidemic, anti-diabetic, and chemo preventive activities.[13, 14] Previous research suggests that older adults who consumed flavanone-rich 100% orange juice for eight weeks experienced improved cognitive function.[15] Citrus polymethoxylated flavones, particularly nobiletin and tangeretin are known to have strong neuroprotective activities.[16, 17] Essential oils derived from citrus fruits and leaves exhibit a variety of pharmacological properties, including anti-inflammatory, antibacterial, analgesic, anti-asthmatic, anxiolytic, antidepressant, sedative, hypnotic, antispasmodic, anticonvulsant effects, and mood regulation by modulating the immune, endocrine, central nervous, and autonomic nervous systems.[18] Recent studies have even suggested that probiotics found in citrus fermentation products may enhance overall mental health by regulating the gut-brain axis.[19]

Citrus reticulata var. *depressa*, commonly known as Hirami lemon, is a native citrus species distributed at low elevations in Taiwan and Okinawa islands of Japan.[20] The fruits are small, oblate, sub-globose, yellow-orange to green when mature, with a rich and distinctive flavor.[21] It has been suggested that the consumption of a wild citrus fruit similar to lemon, which has been confirmed to be wild citrus from Taiwan is one of the reasons for the longevity of Okinawans. Despite this, no scientific study has yet been conducted to demonstrate its bioactive properties. Therefore, the present study, we investigated the regulatory effect of *C. reticulata* var. *depressa* fruit extract and essential oil on the central nervous system (CNS) and gut microbiota in a chronic mild stress-induced mice model.

2 Materials and methods

2.1 Plant material and sample preparation

The fruits of *C. reticulata* var. *depressa* were collected from Pingtung county, Taiwan, on September 2020. In order to prepare the fruit extract (CD) and essential oil (CDE), fresh fruits (4.7 kg) were thoroughly washed in water. Then, the peel (1.3 kg) and pulp (3.4 kg) were separated manually. The essential oil was extracted directly from the peel by hydro-distillation method, and the yield was 1.6% (v/w) of fruit peel. After squeezing the pulp, the insoluble part was removed by centrifugation and filtration. The fruit extract of the pulp was lyophilized and stored at −20°C until use. The yield of lyophilized fruit extract was approximately 10.7% (w/w) of fruit pulp.

2.2 Ultra-performance liquid chromatography- mass spectrometry (UPLC-MS) analysis

The chemical profiling of CD was performed on an Ultimate 3000 ultra-performance liquid chromatography (UPLC) system (Thermo Scientific, Waltham, MA, USA) coupled with a Bruker amaZon speed-ion trap mass spectrometer (Bruker Corporation, Billerica, MA, USA). The chromatographic separations were achieved with a Waters RP-18 column (150 × 2.1 mm i.d., 1.7 µm), using a 0.1% (v/v) acetic acid in water (A), acetonitrile (ACN) (B), and MeOH (C) as a mobile phase. The gradient started with 20% B and 80% A for 2 min, increased to 60% B and 40% A within 13 min, and changed to 100% B within 3 min. Then the gradient was quickly ramped to 0% B and 100% C within 2 min, maintained for 2 min, changed back to 20% B and 80% A in 3 min and re-equilibrated for 3 min.

2.3 Gas chromatography-mass spectrometry (GC-MS) analysis

GC-MS analysis was conducted with a Trace GC Ultra coupled to an ITQ 900 mass spectrometer chromatograph (Thermo Scientific) as described previously.[22] The DB-5MS column (30 m × 0.25 mm i.d., 0.25 µm) was used as the stationary phase and helium was used as a carrier gas at a flow rate of 1 ml/min. The instrument was set as follows: injector port temperature set to 270°C, interface temperature and detector temperature set as 250°C. The oven temperature program was started from an initial temperature of 30°C maintained for 3 min, 1°C/min to 40°C, 3°C/min to 180°C, held 3 min and then raised to a final temperature of 280°C held for 10 min at 10°C/min. The compounds were identified by Wiley/NBS Registry of mass spectral databases Ver. 8.0 (Hoboken, NJ, USA), National Institute of Standards and Technology (NIST) Ver. 2.0 GC/MS libraries and the Kovats indices were calculated for all volatile constituents using a homologous series of *n*-alkanes C_9-C_{24}. The major components were identified by co-injection with standards (wherever possible). The relative percentage of each component was calculated by the proportion of the corresponding peak area to the total area.

2.4 Animals

All animal procedures followed the Guideline for the Care and Use of Laboratory Animals approved by the Institutional Animal Care and Use Committee (IACUC) of National Chung Hsing University (IACUC NO. 108-005[R]). Six weeks old male BALB/c strain mice were obtained from BioLASCO (Yilan, Taiwan). The

View Article Online
DOI: 10.1039/D3FO01301D

mice were group-housed under controlled temperature (22 ± 2°C) and relative humidity (50-60%) with a 12 h light/dark cycle. Food and water were available *ad libitum*. The experiments were started after acclimatization for a week. Individual Body weights and cage food consumption was measured every two days.

2.5 Experimental groups and drug administration

Mice were randomly divided into eleven groups with different drug administrations, and drugs were administered daily after been challenged with stress. In oral administrative groups (n = 7), mice were orally administered with 10 mL/kg of PBS with 0.1% Tween 20 (groups, PBS and CMS-PBS), 10 mg/kg fluoxetine hydrochloride (group CMS-FLU), 500 mg/kg CD (group CMS-LCD), 1000 mg/kg CD (group CMS-HCD), 100 mg/kg (group CMS-LCDE), or 500 mg/kg CDE (group CMS-HCDE). The PBS with 0.1% Tween 20 and fluoxetine hydrochloride (FLU) was used as a normal control and drug control, respectively. For inhalation groups (n =6), mice were exposed to ethanol alone (group, ETOH), ethanol and CMS (group CMS-ETOH), 5% CDE (group CMS-CDE), or 5% lavender essential oil (group CMS-LAE). The ethanol and lavender essential oil (LAE) were used as normal control and drug control, respectively. The inhalation procedure was performed as described previously by Chioca et al. with slight modification.[23] The inhalation boxes (50 cm × 25 cm × 20 cm with a cover) were placed on cotton wool soaked in ethanol, 5% LAE, or 5% CDE in a constant volume of 3 mL. The mice were placed inside inhalation boxes for 2 h.

2.6 Chronic mild stress-induced depression in mice model

The procedure of CMS was followed as described previously with minor modifications.[23] Except for the control groups (PBS and ETOH), all mice were randomly treated with various stressors listed as follows, (1) restraint in a plastic tube for 1.5 h, (2) at 4°C for 1.5 h, (3) restraint in a plastic tube in 4°C for 0.5 h, (4) white noise (95 dB) stress for 3 h, (5) cage shaking (150 rpm) for 1 h, (6) confrontation with rats for 3 h. (7) inversion of the light-dark cycle for 24 h, (8) wet bedding for 12 h, (9) 45° cage tilting for 12 h, (10) removal of nesting for 12 h, and (11) continuous light for 24 h.

2.7 Mice behaviour evaluation

After six weeks of the CMS procedure, mice were subjected to behavioural testing that included the elevated plus-maze test (EPM), and forced swimming test (FST). For both behavioural testing, animals were habituated to the testing room in their home cages for at least 30 min before testing.

2.7.1 Forced swimming test

The FST was carried out as previously described.[24] Mice were individually placed into a cylindrical glass container (height 20 cm, diameter 14 cm) containing 10 cm water (24 ± 1.0°C). Mice were dropped individually into glass cylinders containing 10 cm of water for 6 min. After a 1 min habituation period, the duration of immobility was measured during the last 5 min. The mouse was considered immobile when floating in the water without swimming or struggling.

2.7.2 Elevated plus-maze test

The EPM was performed according to the previously described procedures with minor modifications.[25] The EPM apparatus consisted of two opposite open arms (32 cm × 6 cm) and two closed arms (32 cm × 6 cm × 15 cm), connected by an open central platform (6 cm × 6 cm). For testing, the mouse was placed in the central platform facing an open arm and observed for 5 min. The video tracking software recorded the time spent in the open arms (EthoVision XT, Noldus, Netherlands). After each test, the apparatus was cleaned with 70% ethanol to eliminate odour cues.

2.8 DNA extraction

The DNA was extracted from a 200 mg faecal sample using the QIAamp Fast DNA Stool Mini Kit (Qiagen, Hilden, Germany), zirconia/silica beads (BioSpec Product, Bartlesville, OK, USA), and a SpeedMill PLUS Bead Homogenizer (Analytik Jena, Jena, Germany). DNA concentration and quality were determined by spectrophotometry (ThermoFisher) and agarose gel electrophoresis, respectively.

2.9 Bacterial 16S rRNA analysis

The V3-V4 hypervariable regions of bacterial 16S rRNA genes were amplified using primers 341F (5'-CCTACGGGNGGCWGCAG-3') and 805R (5'-GACTACHVGGGTATCTAATCC-3') with sample-specific barcodes.[26] All PCR reactions were performed in a final volume of 25 µL containing 0.5 µL KAPA High-Fidelity PCR Master Mix (Sigma Aldrich, St. Louis, MO, USA), 0.5 µM forward and reverse primers and 1 ng DNA template. The PCR amplification was conducted with 30 cycles of denaturation at 95°C for 30 s, annealing at 57°C for 30 s, elongation at 72°C for 30 s, and final extension was carried out at 72°C for 5 min. Then, the PCR products were separated by gel electrophoresis using 2% (w/v) agarose gel, and the DNA fragments of 450-500 bp were purified with QIAquick Gel Extraction Kit (Qiagen). The final DNA concentrations of the purified products were measured with a Qubit 2.0 Fluorometer (Thermo Scientific). Subsequently, the purified products were mixed in equal molar concentrations to generate a library pool. The DNA libraries were prepared using the TruSeq Nano DNA library prep kit (Illumina, San Diego, CA, USA) and were validated using an Agilent 2100 Bio-Analyzer (Agilent Technologies, Santa Clara, CA, USA), and the qPCR analysis was performed (Applied Biosystems, Waltham, MA, USA). At last, the libraries normalized, pooled, and sequenced on the Illumina MiSeq platform (Illumina) with 2 × 300 bp paired-end reads.[27]

2.10 Bioinformatics analysis

The raw reads were first trimmed using Trimmomatics version 0.39[28], whereas low-quality sequence regions were removed. Then, the reads were demultiplexed according to the barcode

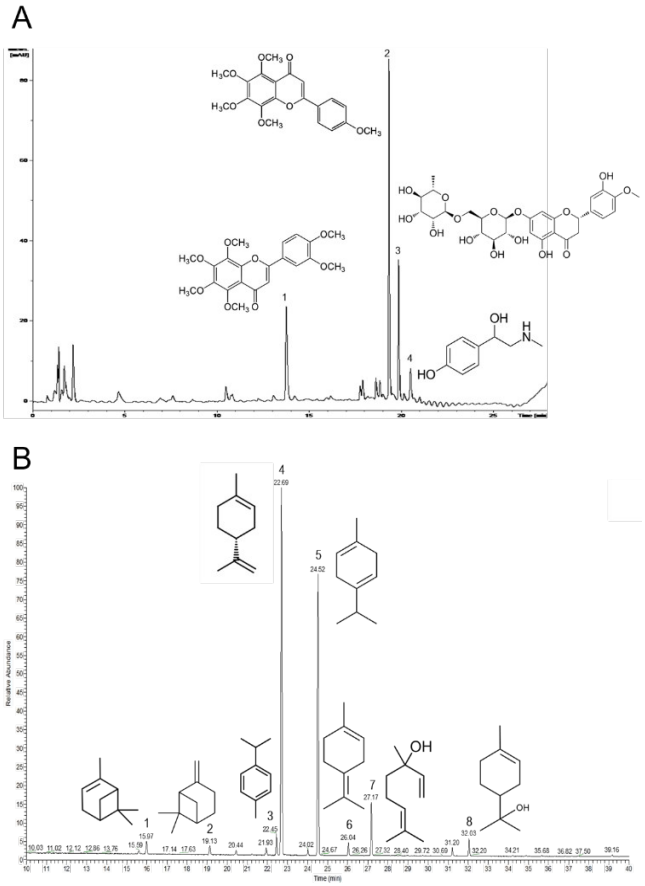

Fig. 1 The major components of CD and CDE. (A) UPLC-MS profile of CD (1) nobiletin, (2) tangeretin, (3) hesperidin (B) GC-MS profile CDE (1) D-limonene, (2) γ-terpinene, (3) linalool, (4) *p*-cymene, (5) α-terpineol, (6) α-pinene, (7) α-tepinolene (8) β-pinene.

sequences, and the primers and adaptors were trimmed using Cutadapt version 1.16.[29] Subsequently, FLASH version 1.2.11 was used to merge the paired-end sequences passed through the quality-filter according to the 10 bp overlaps.[30] Chimeric sequences were detected and removed by using the UCHIME algorithm based on the Gold database.[31,32] After processing, the qualified reads were assigned to species equivalent operational taxonomic units (OTUs) with a 97% pairwise identity threshold by Mothur version 1.39.5 software and SILVA version 132 database.[33,34] Alpha and beta diversity analysis were performed with QIIME version 1.9.0.[35]

2.11 Statistical analysis

All statistical analysis was performed using the GraphPad Prism 8 software version 8.0.2 (GraphPad Software, Boston, MA, USA). Data are expressed as mean ± standard error of the mean (SEM). Statistical analysis was performed using one-way analysis of variance (ANOVA), or two-way ANOVA repeated measures, followed by Tukey's post hoc test. However, the non-parametric statistical comparisons were made using the Kruskal–Wallis test followed by Dunn's post hoc test. Differences were considered statically significant if $P < 0.05$. *P* values of less than 0.05^{Δ} was considered statistically significant for the non-stressed control *vs*. CMS groups. *P* values of less than 0.05*, 0.01** and 0.001*** were considered statistically significant for the CMS *vs*. CD, CDE, FLU or LAE tratment groups. Statistical significance in the β-diversity analyses was determined by multi-response permutation procedures (MRPP) and analysis of similarity (ANOSIM). All graphs were generated using GraphPad Prism 6 (GraphPad Software).

3 Results

3.1 The chemical compositions of *C. reticulata* var. *depressa* fruit extracts and essential oils

The chemical compositions of fruit extract (CD) and essential oil (CDE) of *C. reticulata* var. *depressa* were analyzed by UPLC-MS and GCMS, respectively. The UPLC-MS profile of CD is presented in Figure 1A. The major compounds in CD were identified as nobilein, tangeretin and hesperidin, which were confirmed by using authentic standard spectrum (Table 1 and SI). The GC-MS profiling of CDE is presented in Figure 1B. The most abundant compounds in CDE were D-limonene (45.71%), followed by γ-terpinene (34.65%), linalool (6.46%), *p*-cymene (2.57%), α-terpineol (2.04%), α-pinene (1.89%), α-terpinolene (1.46%), and β-pinene (1.16%). The contents of major compounds and relative contents (%) in CDE are shown in Table 2.

Table 1 The major compounds of fruit extract from *C. reticulata* var. *depressa* by UHPLC analysis.

Peak NO.	Retention time (min)	Molecular weight	Exact mass (*m/z*) [M+H]$^+$	Compounds identified
1	15.97	402.4	403	Nobiletin
2	19.13	372.4	373	Tangeretin
3	22.45	610.2	611	Hesperidin
4	22.69	167.2	168	Synephrine

Table 2 The major components and their relative contents (%) of fruit essential oil from *C. reticulata* var. *depressa* by GC-MS analysis.

Peak NO.	Compounds	Retention time (min)	KI [a]	Relative contents (%)	Identification [b]
1	α-Pinene	15.97	934	1.89%	MS, KI, ST
2	β-Pinene	19.13	975	1.16%	MS, KI, ST
3	*p*-Cymene	22.45	1023	2.57%	MS, KI, ST
4	D-Limonene	22.69	1028	45.71%	MS, KI, ST
5	γ-Terpinene	24.52	1059	34.65%	MS, KI, ST
6	α-Terpinolene	26.04	1083	1.46%	MS, KI, ST
7	Linalool	27.17	1101	6.46%	MS, KI, ST
8	α-Terpineol	32.03	1194	2.04%	MS, KI, ST

[a]Kovats index on a DB-5ms column in reference to n-alkanes.

[b] MS, NIST, and Wiley libraries and literature; KI, Kovats index; ST, authentic standard compounds.

Journal Name ARTICLE

View Article Online
DOI: 10.1039/D3FO01301D

Table 3 Effect of *C. reticulata* var. *depressa* on the feed efficiency in CMS mice.

Group	2 weeks (%)	Compared with Con (%)	4 weeks (%)	Compared with Con (%)	6 weeks (%)	Compared with Con (%)
PBS	31.8	100.0	71.9	100.0	80.1	100.0
CMS-PBS	-1.0	-3.2 $^{\Delta\Delta}$	20.5	28.6$^{\Delta\Delta}$	40.8	51.0$^{\Delta\Delta}$
CMS-LCD	10.2	32.0	31.4	43.7	46.9	58.5
CMS-HCD	4.4	13.9	35.0	48.7	43.5	54.3
CMS-LCDE	11.0	34.7	28.5	39.6	54.2	67.6
CMS-HCDE	13.2	41.7	45.8	63.7	58.0	72.4
CMS-FLU	10.1	31.6	29.4	40.9	50.0	62.4

The feed efficiency is calculated as the percentage of weight gain divided by feed intake. The total feed intake by each cage of mice was recorded every day, and the body weight of each mouse was recorded every 2 days. PBS alone group represent the unstressed control group of mice without chronic mild stress induction in oral administration groups. Data were analyzed with two-way ANOVA with repeated measures followed by Tukey's post hoc test. *P* values of less than 0.01$^{\Delta\Delta}$ was considered statistically significant for the non-stressed control *vs*. CMS group. There is no statistical significance between CMS *vs*. CMS + sample treatment groups.

Table 4 Effect of *C. reticulata* var. *depressa* on the feed efficiency in CMS mice.

Group	2 weeks (%)	Compared with Con (%)	4 weeks (%)	Compared with Con (%)	6 weeks (%)	Compared with Con (%)
ETOH	36.9	100.0	61.1	100.0	71.4	100.0
CMS-ETOH	11.7	31.6	27.3	44.7$^{\Delta}$	26.7	37.4$^{\Delta\Delta}$
CMS-CDE	10.1	27.4	41.4	67.7	69.9	98.0*
CMS-LAE	6.5	17.7	49.4	80.9*	74.7	104.7**

The feed efficiency is calculated as the percentage of weight gain divided by feed intake. The total feed intake by each cage of mice was recorded every day, and the body weight of each mouse was recorded every 2 days. ETOH group represent the unstressed control group of mice without chronic mild stress induction in inhalation administration groups. Data were analyzed with two-way ANOVA with repeated measures followed by Tukey's post hoc test. *P* values of less than 0.05$^{\Delta}$ and 0.01$^{\Delta\Delta}$ were considered statistically significant for the non-stressed control *vs*. CMS group. *P* values of less than 0.05*and 0.01** were considered statistically significant for the CMS *vs*. CMS + sample treatment groups.

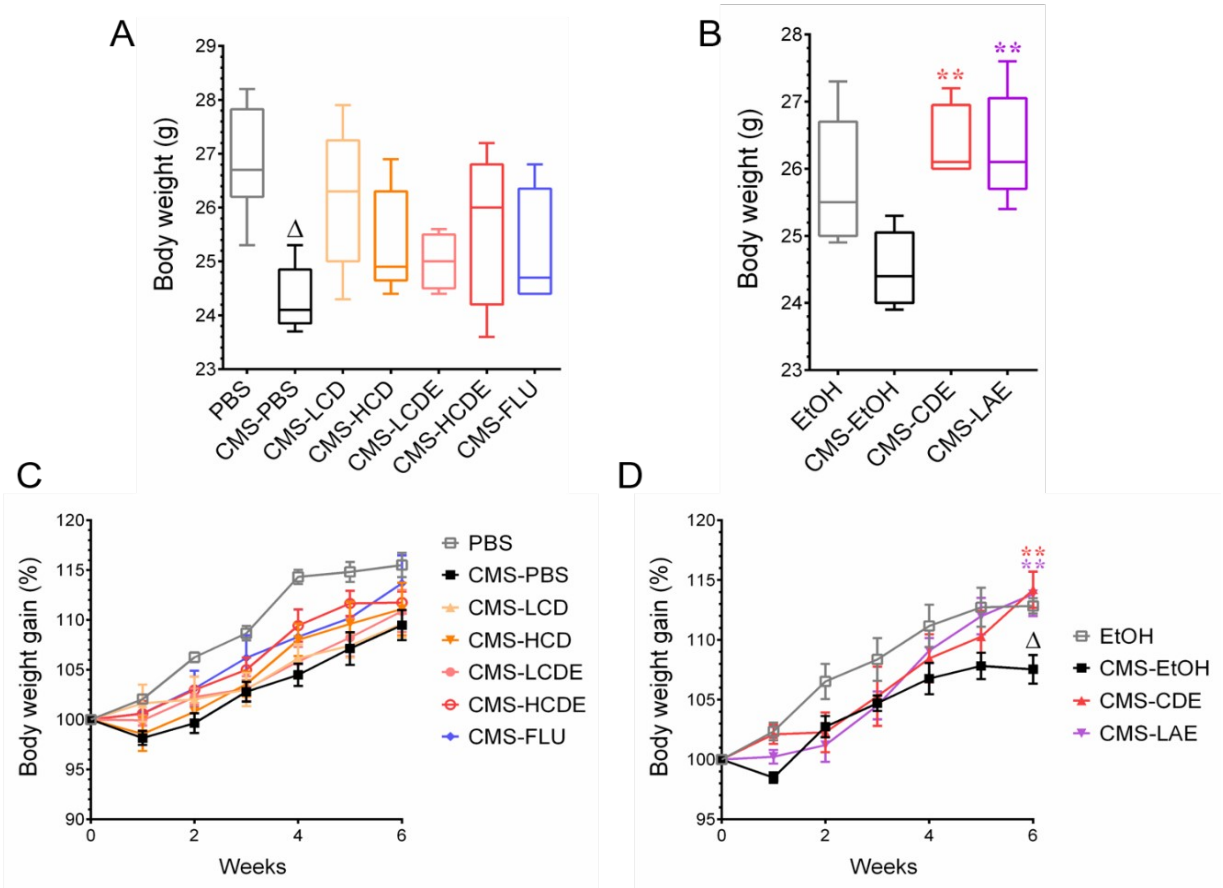

Fig. 2 Effect of CD and CDE on mice body weight. Comparison of overall body weight in oral administration groups (A) and inhalation group (B). Comparison of body weight gain in oral administration groups (C) and inhalation group (D). Data were analyzed with two-way ANOVA with repeated measures followed by Tukey's post hoc test. *P* values of less than 0.05$^{\Delta}$ was considered statistically significant for the non-stressed control *vs*. CMS groups. *P* values of less than 0.01** was considered statistically significant for the CMS *vs*. CMS + CDE or LAE treatment groups.

3.2 Effects of *C. reticulata* var. *depressa* fruit extracts and essential oils on body weight and feed efficiency in CMS-induced mice

At the end of 6-weeks treatment period, mice in unstressed control group (which received only oral administration of PBS) showed a significant increased body weight from 23.5 ± 0.79 g to 27.16 ± 0.74 g, when compared to their baseline weight in week 1. In contrast, the CMS-challenged mice did not experience any weight grain, and their average body weight decreased from 27.16 ± 0.74 g to 24.3 ± 0.61 (P = 0.0008). However, oral administration CD, CDE or FLU prevented CMS-induced weight loss in mice as indicated by their increased body weight of 26.16 ± 1.31, 25.36 ± 0.97, 25.0 ± 0.5, 25.6 ± 1.41, and 25.24 ± 1.06 g, respectively (Fig. 2A). In the CMS-EtOH group (which received only inhalation of EtOH), a significant reduction in body weight was observed, and the body weight decreased from 25.78 ± 0.96 g to 24.5 ± 0.56 g over the treatment period (P = 0.0335). However, treatment with CMS-CDE (P = 0.0006) or CMS-LAV (P = 0.0034) prevented the CMS-induced weight loss in mice, and the body weight of CMS-CDE and CMS-LAV treated mice were 26.4 ± 0.53 g and 26.32 ± 0.81 g, respectively (Fig. 2B). Interestingly, both CMS-CDE and CMS-LAV groups showed an even heavier weight trend than the unstressed control (ETOH) group after 6 weeks of treatment (Fig. 2B). Similar body weight gains were observed in the oral administration of CD and CDE (Fig. 2C) and inhalation of CDE (Fig. 2D) groups challenged with CMS. These results suggest that prolonged inhalation of essential oils can effectively slow down the effects of CMS-induced weight loss.

Feed efficiency (FE) is the conventional measure of livestock production efficiency that express percentage of the average weight gain (g) divided by the average weight (g) of the diet per animal. Table 3 shows that, after 2 weeks of experimentation, FE was negative (-3.2%) in mice fed with PBS alone and challenged with CMS (CMS-PBS). It is hypothesized that the initial chronic mild stress induction created a stressful environment for animals, resulting in the decline of gastrointestinal digestive function. However, after a 6-weeks treatment, the FE of the CMS-PBS group gradually improved their feed efficiency to 51.0%. Interestingly, mice challenged with CMS and given oral administration of LCDE (500 mg/kg) and HCDE (1000 mg/kg) had better FE (67.6% and 72.4%, respectively) than the PBS-only group and the group that was induced with chronic mild stress (CMS-PBS). The increase in FE with both LCDE and HCDE was even higher than that of positive drug control (CMS-FLU), which increased only 62.4%. Similarly, after 2-weeks of experimentation, compared with control group (unstressed), FE was lower in mice challenged with CMS and exposed to either EtOH or CDE or LAV through inhalation. After 6-weeks of treatment, FE gradually increased, and CMS-EtOH group reached 37.4%, while the FE of the mice challenged with

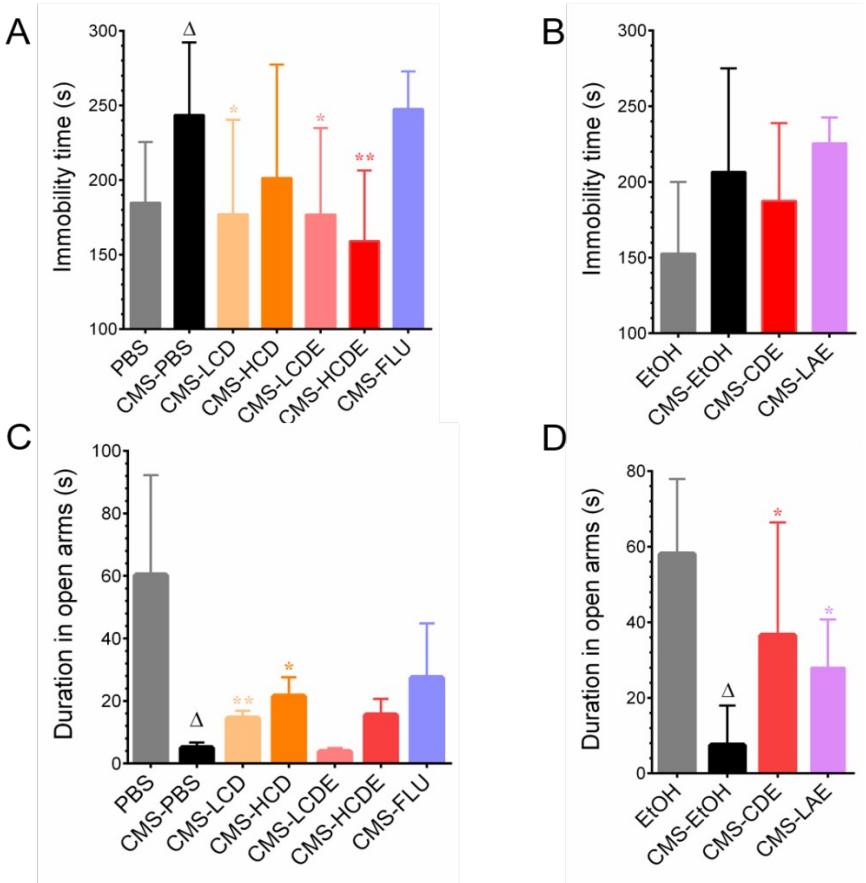

Fig. 3 Effect of CD and CDE on CMS-induced behavioral changes in mice. (A-B) Forced swimming test. (C-D) Elevated plus-maze test. Data were analyzed with one-way ANOVA followed by Tukey's post hoc test. P values of less than 0.05$^{\Delta}$ was considered statistically significant for the non-stressed control *vs*. CMS groups. P values of less than 0.05*, and 0.01** were considered statistically significant for the CMS *vs*. CMS + CD or CDE or FLU or LAE treatment groups.

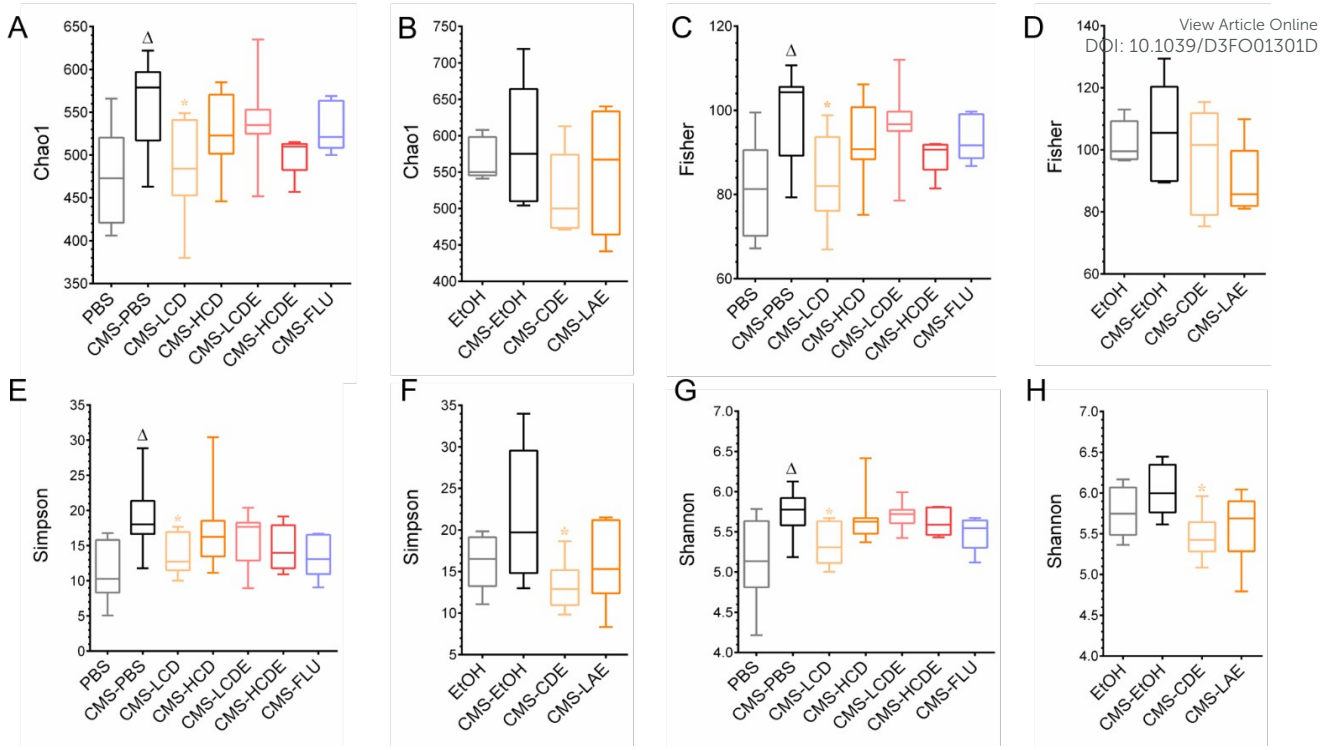

Fig.4 Effect of CD and CDE on the structure of gut microbiota with α-diversity. Chao1 index in oral administration groups (A). Chao1 index in inhalation group (B). Fisher index in oral administration groups (C). Fisher index in inhalation groups (D). Simpson index in oral administration groups (E). Simpson index in inhalation groups (F). Shannon index in oral administration groups (E). Shannon index in inhalation groups (F). Data were analyzed with Kruskal–Wallis test followed by Dunn's post hoc test. *P* values of less than 0.05^{Δ} was considered statistically significant for the non-stressed control vs. CMS groups. *P* values of less than 0.05* was considered statistically significant for the CMS vs. CMS + CD or CDE or FLU or LAE treatment groups.

CMS and exposed to CDE and LAV inhalation reached as high as 98% and 104%, respectively (Table. 4). These data suggest that inhalation of essential oil can improve the syndrome of decreased gastrointestinal digestive function caused by chronic stress.

3.3 Fruit extract and essential oil of *C. reticulata* var. *depressa* regulates CMS-induced behavioural changes in mice

To further evaluate the effects of CD and CDE on behavioural changes in CMS-induced mice, we conducted two behavioural tests, elevated plus-maze test (EPM), and forced swimming test (FST). Initially, we aimed to determine the effects of CD and CDE on depressive-like behaviours in CMS-challenged mice using the FST. Results from FST revealed that mice challenged with CMS displayed depressive-like behaviour as indicated by increased immobility time, which is reduced by CD and CDE treatments. In the oral administration groups, compared to unstressed control group (PBS, 184.8 ± 40.6 s), the immobility time of mice in the stress-induced group (CMS-PBS) was significantly increased to 258.5 ± 31.0 s (*P* = 0.0391). However, oral administration of LCD, HCD, LCDE, HCDE, and FLU decreased CMS-induced immobility to 177 ± 62.42 s (*P* = 0.0475), 201.3 ± 29.9 s (*P* = 0.2302), 176.9 ± 23.1 s (*P* = 0.0378), 159 ± 47.4 s (*P* = 0.0091), and 247.6 ± 25.2 s (*P* = 0.8691), respectively (Fig. 3A). In the inhalation treatment groups, compared with unstressed control group (EtOH, 152.7 ± 47.2 s), mice exposed to CMS increased immobility time to 206.6 ± 68.3 s (*P* = 0.1848), but this increase was not statistically significant. Neither inhalation of CDE (*P* = 0.5257) or LAV (*P* = 0.6089) altered CMS-induced depression-like behaviour in mice (Fig. 3B).

To determine the effect of CD and CDE on CMS-induced anxiety-like behaviour, we conducted an elevated plus-maze (EPM) test. In EPM test, mice with lower anxiety tended to spend more time in the open arms. As shown in Fig. 3C, the stress-induced control group of mice (CMS-PBS) exhibited an anxiety-like behaviour as evidenced by spending less time (5 ± 4.5 s, *P* = 0.0068) in the open arms compared to unstressed control group (PBS, 50.2 ± 68.5 s). Interestingly, oral administration of CD and CDE prevented CMS-mediated reduction in time spend in open arms and increased time spend in open arms to 14.5 ± 6.2 s (*P* = 0.0069), 21.5 ± 15.6 s (*P* = 0.0223), 3.8 ± 2.6 s (*P* = 0.5645), 15.5 ± 12.6 s (*P* = 0.064), and 27.4 ± 38.8 s (*P* = 0.1528) by LCD, HCD, LCDE, HCDE, and FLU, respectively. In the sample inhalation groups, compared with EtOH alone inhalation group (58.1 ± 39.7 s), mice challenged with CMS and inhaled EtOH significantly (*P* = 0.0515) lowered spending time on open arms (7.5 ± 11.5 s), whereas inhalation of either CDE or LAV increased spending time on open arms to 36.6 ± 28.9 s (*P* = 0.0336) and 28.0 ± 12.7 s (*P* = 0.0174), respectively. Notably, compared with LAV inhalation, CDE exhibited a pronounceable increase in time spend on open arms (Fig. 3D). Taken together these data indicate that both CD and CDE prevent CMS-induced anxiety-like behaviour in mice.

View Article Online
DOI: 10.1039/D3FO01301D

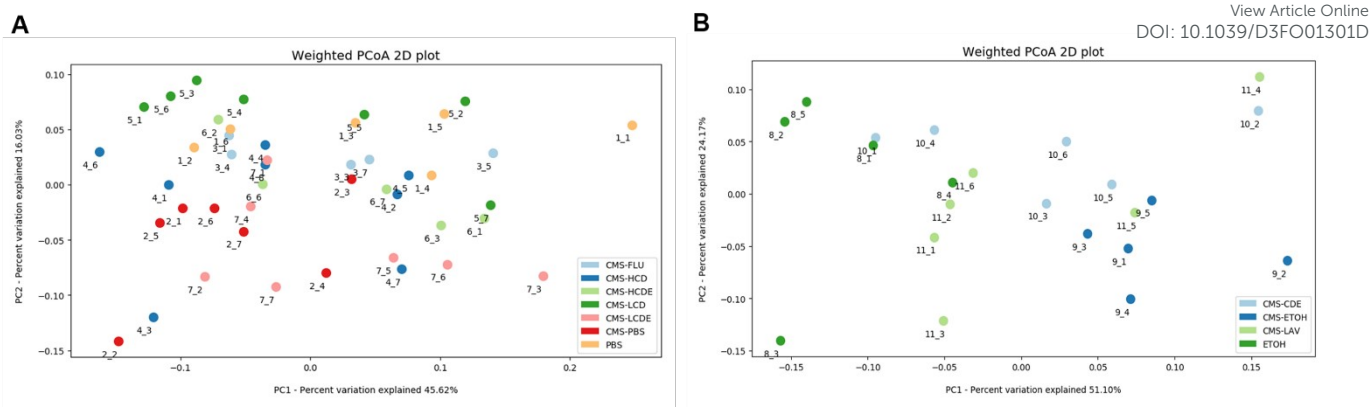

Fig.5 Effect of CD and CDE on the structure of gut microbiota with β-diversity. PCoA plots of weighted UniFrac distance showing the β-diversity clustering patterns of samples in (A). oral administration groups (ANOSIM: R = 0.1441, P < 0.005; MRPP: A =0.1000, P < 0.005) and (B). inhalation administration groups (ANOSIM: R = 0.3494, *P* < 0.005; MRPP: A =0.11718, *P* < 0.005).

3.4 Effect of fruit extract and essential oil of *C. reticulata* var. *depressa* on the gut microbiota α- and β-diversity

Recent studies have shown CMS play a significant role in emotional and behavioural changes, and closely related to alterations in the gut microbiome. To further analyze the relationship between the gut-brain axis and the effects of CD and CDE on behavioural changes, gut microbiota profiles in normal and stressed mice were analyzed by 16S rRNA sequencing. A total of 2,611,881 high-quality reads from 66 samples were generated, detecting a total of 1,78,569 OTUs at a similarity level of 97%. The species accumulation curves reached a saturation plateau, indicating that the sequencing samples were sufficient to further data mining. In microbial population, α-diversity determines the degree of species richness and evenness. Previous research has shown that α-diversity increases when mice were exposed to CMS.[36] Therefore, the α-diversity in CMS and CMS+CD/CDE treatments groups were measured by observed operational taxonomic units (OTUs), including Chao1, Fisher, Simpson and Shannon indices. Chao1 and Fisher diversity indices represented species richness and evenness, respectively,[37] while, Shannon and Simpson diversity indices provide information about the composition of the community rather than the richness and evenness of species.[38] As shown in Figure 4, all four α-diversity indices were significantly increased in CMS-PBS group compared with PBS group after 6 weeks of CMS exposure (*P* < 0.05). However, these indices showed a decreasing trend in the groups of mice that were orally administered LCD and HCDE. Chao1 and Fisher indices revealed significant differences (*P* = 0.0433 and *P* = 0.0254, respectively) in species richness between the control (PBS) and CMS-challenged group (CMS-PBS), suggesting that CMS can alter species richness and evenness in gut microbiota. Treatment with LCD or HCDE showed a notable difference with CMS alone group, while this difference was not statistically significant. In addition, treatment with HCD, LCDE, and FLU did not alter CMS-mediated elevation of species richness (Fig. 4A) and evenness (Fig. 4C). On the other hand, there was no significant difference between control (EtOH) or CMS-challenged (CMS-EtOH) or CMS with CDE (CMS-CDE) in oral administered groups (Fig. 4B,D). Taken together, these findings suggest that CD and CDE may play a role in regulating the gut microbiota in CMS-exposed mice, with LCD and HCDE showing promising effects on α-diversity indices. Our results showed that Shannon (Fig. 4E) and Simpson (Fig. 4G) values were significantly different (*P* = 0.0407 and *P* = 0.0432, respectively) between the control group (PBS) and CMS-challenged group (CMS-PBS), suggesting that CMS can increase the composition and diversity of the gut microbiota in mice. However, there were no significant differences in the orally administered CMS-LCD, CMS-HCD, CMS-LCDE, and CMS-HCDE groups. In the inhalation administration groups, Shannon (Fig. 4F) and Simpson (Fig. 4H) values were not significantly different between control (EtOH) or CMS (ECMS-EtOH) groups. However, CMS-CDE group showed a tendency for recovery in Simpson (*P* = 0.05) and Shannon (*P* = 0.05) indices compared with CMS-ETOH group.

PCoA of the weighted UniFrac distances revealed dissimilarities in the gut microbiota communities between the oral administration groups and inhalation administration groups (Fig. 5). In the oral administration groups (Fig. 5A), although sample overlapping occurred among the all groups, clustering tendencies were observed, which showed the samples from unstressed control group (PBS) were apparently separated from the CMS-challenged group (CMS-PBS). Furthermore, the gut microbiota structure of the CMS-LCD group showed greater similarity to that of the unstressed control (PBS) group and the positive drug control group (CMS-FLU). In the inhalation administration groups (Fig. 5B), the PCoA plots displayed that the microbial composition was predominantly differentiated between stressed (CMS-EtOH) and unstress control (EtOH) groups. In a same way, the microbial distribution in the LAV treatment group was close to that of the unstressed control group (EtOH), and distant from those of stress-induced group (CMS-EtOH). These results were confirmed using an analysis of similarities (ANOSIM) test on the unweighted Unifrac distances (10,000 permutations, *P* < 0.0001).

View Article Online
DOI: 10.1039/D3FO01301D

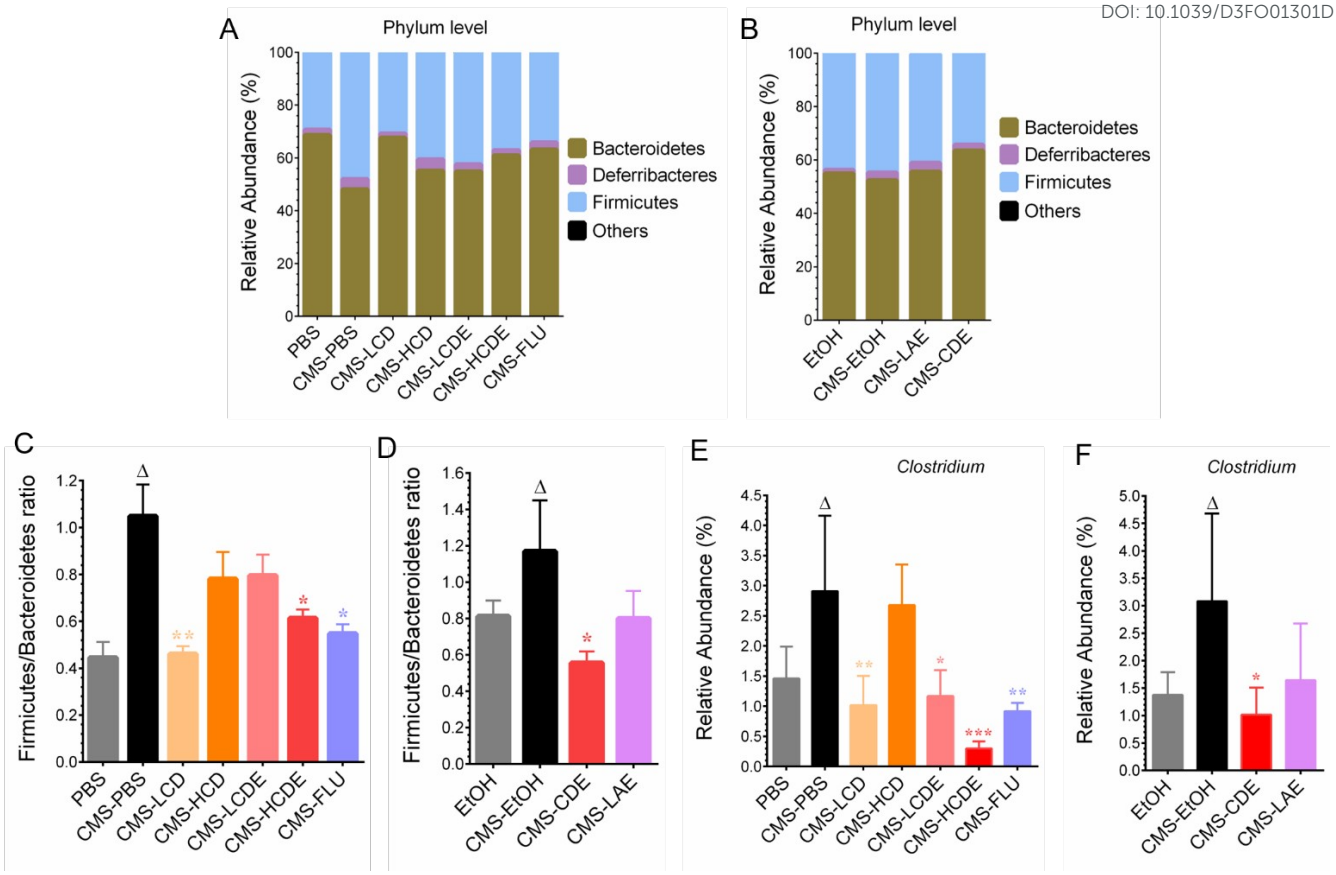

Fig.6 Effect of CD and CDE on CMS-induced changes in mice gut microbiota. (A) Relative abundance of gut microbiota at phylum level in oral administrative groups. (B) Relative abundance of gut microbiota at phylum level in inhalation group. (C) *Firmicutes* to *Bacteroidetes* (F/B) ratio in oral administrative groups. (D) *Firmicutes* to *Bacteroidetes* (F/B) ratio in oral administrative groups. (E) OUT species richness of genus *Clostridium* in oral administrative groups. (F) OUT species richness of genus *Clostridium* in oral administrative groups. Data were analyzed with two-way ANOVA with repeated measures followed by Tukey's test. *P* values of less than 0.05^{Δ} was considered statistically significant for the non-stressed control *vs.* CMS groups. *P* values of less than 0.05*, 0.01** and 0.001*** were considered statistically significant for the CMS *vs.* CMS + CD or CDE or FLU or LAE treatment groups.

3.5 Fruit extract and essential oil of *C. reticulata* var. *depressa* regulate CMS-induced dysbiosis in mice

To determine the community composition of the gut microbiota of mice in each group, we performed taxonomy-based analyses at the phylum and family levels. These 1,78,569 OTUs were divided into 19 phyla, 37 classes, 67 orders, 113 families, 228 genera, and 381 species. The results showed that the gut microbiota composition in control, CMS, and CMS with CD/CDE groups was similar. As shown in Figure 6A and B, the predominant bacterial phyla across all samples were *Bacteroidetes* (48.0%-68.5%), *Firmicutes* (29.4%-48.2%) and *Deferribacteres* (1.3%-4.3%), accounting for more than 70% of the total. A significant increase in *Firmicutes* and a decrease in *Bacteroidetes* were observed in the CMS-challenged groups (CMS-PBS and CMS-EtOH). However, oral administration of CD and CDE (Fig. 6A) and inhalation of CDE (Fig. 6B) significantly reversed such alteration in CMS-challenged groups. The Gut microbiome was further analysed by *Firmicutes* to *Bacteroidetes* (F/B) ratio. A significant increase in F/B ratio was observed in CMS-PBS challenged group (P = 0.0029). Oral administration of LCD (P = 0.0011) and HCDE (P = 0.0244) prevented this gut microbial alteration caused by CMS. Nevertheless, higher concentration of CD (P = 0.1504) and lower concentration of CDE (P = 0.1424) regulated CMS-induced dysbiosis, but these effects were not statistically significant (Fig. 6C). In addition, a similar trend of changes in the F/B ratio was observed in the sample inhalation groups (Fig. 6D). A group of mice inhaled EtOH and challenged with CMS (CMS-EtOH) exhibiting a dramatic alteration of gut microbiome, as evidenced by that F/B ratio, but the increase was not statistically significant (P = 0.2607) (Fig. 6D). However, inhalation of CDE significantly (P = 0.0451) prevented CMS-induced dysbiosis in mice. Interestingly, the effect CDE was highly comparable with LAV treatment group. Furthermore, the abundance of genus *Clostridium* was remarkably increased in both CMS-induced (CMS-PBS and CMS-EtOH) groups, while treatment with oral administration of LCD (P = 0.0019) and HCDE (P = 0.0004) exhibited significant reduction in the relative abundance of *Clostridium* species (Fig. 6E). A similar trend was also observed in the inhalation group, where CDE significantly

View Article Online
DOI: 10.1039/D3FO01301D

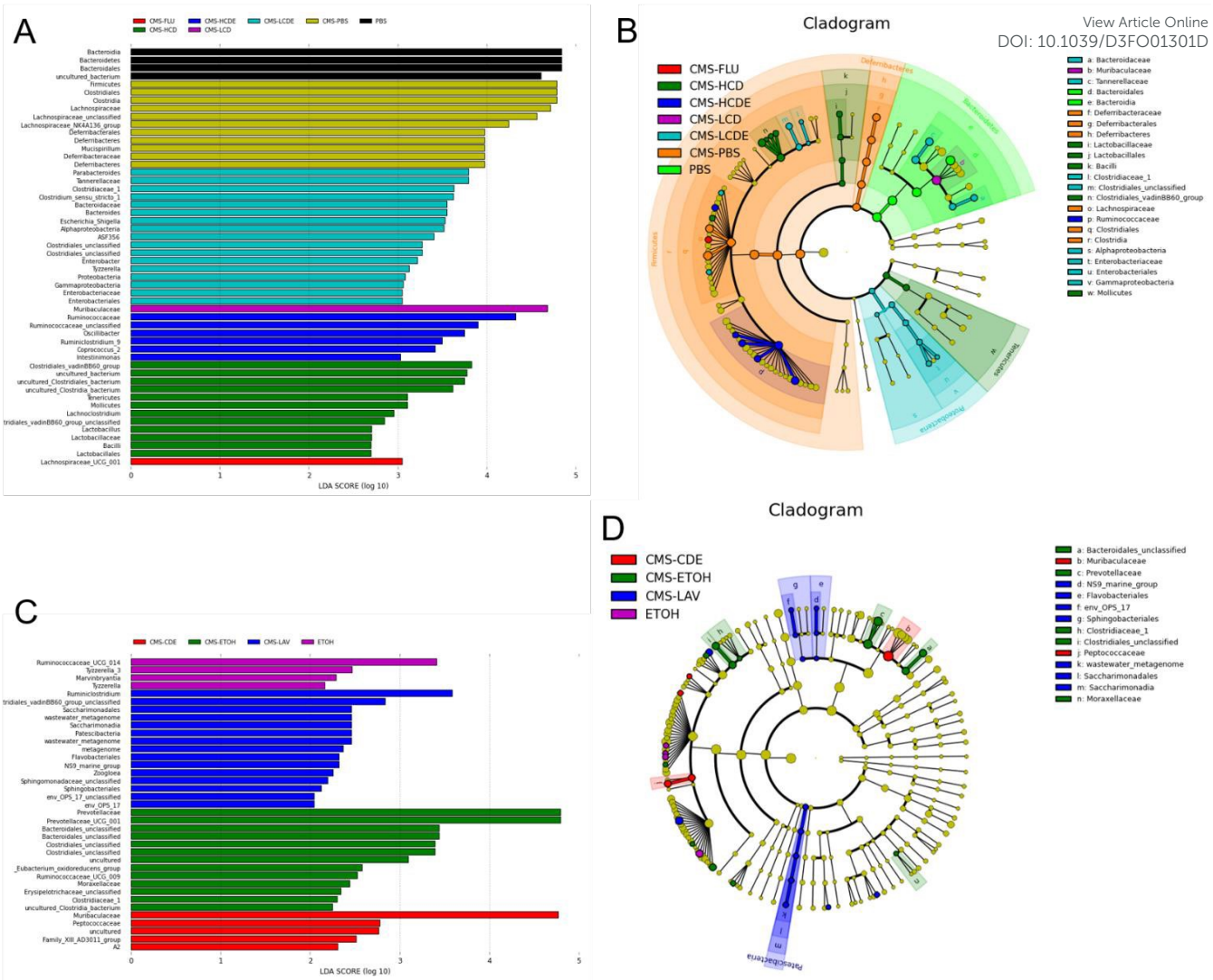

Fig. 7 LEfSe analysis identified the most differentially abundant taxa among oral administration groups (A-B), and inhalation administration groups (C-D). In cladograms, the different color indicates taxa enriched in each different treatment group while yellow indicates taxa with no significant change in relative abundance among groups. The size of node represents the proportion of abundance of taxa. The length of the bar represents the LDA score on the $\log_{10}$ scale, is indicated at the bottom. Only the taxa with LDA score higher than 2.0 are shown.

(*P* = 0.0122) decreased the relative abundance of *Clostridium* species (Fig. 6F). Interestingly, both CD and CDE exhibited discriminative for CMS-challenged group (CMS-PBS). However, strong reduction in *Clostridium* species, which were highly comparable with FLU and LAE treatment groups.

3.6 Analysis of difference in gut microbiota

To identify bacterial taxa that differed significantly between groups, we used linear discriminant analysis (LDA) coupled with effect size measurements (LEfSe). According to the LEfSe analysis, there were 52 and 37 bacterial taxa theat differed significantly in aboundance ($P < 0.05$, LDA score >2.0) between the oral administration and inhalation groups, respectively (Fig. 7). Members from the phylum *Deferribacters* to the genus *Mucispirillum* and the class *Clostridia* to the *Lachnospiraceae NK4A136* group, as well as the phylum *Firmicutes,* were four bacterial taxa showed increased abundance in the unstressed control group (PBS), with the highest LDA score attributed to the phylum *Bacteroidetes*, the class *Bacteroidia*, the order *Bacteroidalesas,* and the uncultured bacterium (*Lachnospiraceae* family). In the LCDE treatment groups, there was a significant abundance of bacterial taxa from the phylum *Proteobacteria* to the genera *Escherichia-Shigella* and *Enterobacter*. In addition, the class *Alphaproteobacteria*, the families *Tannerellaceae* and *Bacteroidaceae*, as well as the genus *Parabacteroides*, *Bacteroides*, *ASF356,* and *Tyzzerella*, were consistently abundant in the CMS-LCDE group. The CMS-HCDE group showed a higher relative abundance of the genus *Coprococcus 2* and the family *Ruminococcaceae*, including the genus *Oscillibacter*, *Intestinimonas,* and the unclassified genera. Furthermore, there were 12 dominant bacterial taxa in the CMS-HCD group, such as the classes *Mollicutes* and *Bacilli*, the family *Lactobacillaceae,* and the genera *Lactobacillus* and *Lachnoclostridium*. In contrast, CMS-LCD and CMS-FLU were characterized by a higher abundance of family *Muribaculaceae* and genus *Lachnospiraceae UCG-001*, respectively. In the oral

sample administration groups, members of the genera *Ruminococcaceae* UCG-014, *Tyzzerella*, and *Marvinbryantia* were identified as biomarkers of the normal control group (ETOH). In addition, the uncultured bacterium (*Clostridiales vadinBB60* group family) and *Prevotellaceae UCG-001* were potential microbial biomarkers of the CMS-challenged group (CMS-ETOH) (Fig. 7A, B). Interestingly, inhalation of the CDE group presented five discriminative features that were uniquely abundant versus all other groups, such as the genera *A2*, *Family XIII AD3011* group, and the unclassified genera belonging to *Peptococcaceae* (Fig. 7C,D).

4 Discussion

Flavonoids are widespread present in edible plants, such as fruits, vegetables, cereals, legumes, and beverages like tea and red wine, and are an essential part of the human diet. Based on their chemical structure, flavonoids can be categorized into various subclasses, including flavanones, flavones, isoflavones, flavans (flavanols), anthocyanins, and flavonols.[39] The majority of flavanones found in the human diet are found in citrus fruits, with lesser amounts in tomatoes and aromatic herbs like mint.[40] The most abundant Citrus flavanones, hesperidin and naringin, offer a variety befits, including antioxidative and anti-inflammatory properties.[40] Athough the number of flavones in fruits and vegetables is lower than that of flavanones and flavonols, there is a high concentration of polymethoxylated flavones, such as tangeretin, nobiletin, and sinensetin found in citrus peel.40 Several citrus species contain alkaloids, such as *p*-octopamine and synephrine, which are α-adrenergic agonists and widely used as medicinal and dietary supplements targeting weight loss.[41] In this study, we found that major componds in the fruit extracts were nobiletin, tangeretin, hesperidin, and synephrine. In addition, 8 terpenoids, including D-limonene, γ-terpinene, linalool, *p*-cymene, α-terpineol, α-pinene, α-terpinolene, and β-pinene were identified in essential oil of *Citrus reticulata* var. *depressa.*

The chronic mild stress (CMS) model of depression is a commonly employed technique in laboratory animals to engender behavioral changes. In this model, rats or mice are subjected to continuous exposure to unpredictable micro-stressors, resulting in the development of a range of behavioral changes, including anxiety, depression, eating problems, and anhedonia.[42] In the present study, we have demonstrated the antidepressant-like properties of fruit extract and essential oil obtained from *Citrus reticulata* var. *depressa* using CMS mice model. The experimental findings revealed that mice challenged with CMS exhibited physical and behavioral changes, such as reduction in body weight gain and increased anxiety and depression-like behaviors. Treatment with fruit extract and essential oil of *Citrus reticulata* var. *depressa* showed better antidepressant activity than fluoxetine hydrochloride, as evidence by a decrease in immobility time. However, the antianxiety activity of fruit extract and essential oil of *Citrus reticulata* var. *depressa* was comparably lower than that of fluoxetine hydrochloride. It is worth noting that fluoxetine is reported to have several side effects, such as tremors and hyperactivity, in human[43] and mice.[44] Nevertheless, the fruit extract and essential oil of *Citrus reticulata* var. *depressa* did not show any such effects in the animal in the present study, which is its advantage. As mentioned earlier, the fruit extract and essential oil of *Citrus reticulata* var. *depressa* containing of polyphenols and terpenoids. Therefore, being of natural origin, it is likely safe to use.

A recent study by Li et al., was reported that extracts and essential oil from *Citri reticulatae pericarpium* (sun-dried mandarin orange peel) possessed stronger antidepressant-like activity as evaluated by forced swim test and tail-suspension test in mice.[45] Nobiletin, tangeretin, and 3,5,6,7,8,3′,4′-hepta-methoxyflavone were found as major chemical components in extracts, while the essential oil contained 33 terpenoids, including D-limonene, β-elemene, germacrene D, and (Z, E)-α-farnesene, with D-limonene being a major component in the essential oil of *Citrus reticulata* var. *depressa*. Indeed, limonene exhibited potential neuroprotective effect and anticonvulsant activity via activation of the p38 MAPK pathway or modulation of adenosine A2A receptors on GABAergic neuronal function.[46,47] Further *in vivo* studies with limonene showed that it can upregulate the phospho-CREB levels in the hippocampus, which is demonstrated that CREB activation in the hippocampus with BDNF signalling can alleviate the pathogenesis the depression pathogenesis.[46,48] It was well demonstrated that the higher acetylcholinesterase (AChE) activity was found in plasma and hippocampus of CMS mice, which is caused by oxidative stress.[49-51] Indeed, γ-terpinene exhibited strong antioxidant activity[52] and anti-AChE activity[53]. Another study demonstrated that linalool, a major component in many essential oils possessed anxiolytic and antidepressant activities.[54,55] Further *in vitro* mechanistic studies revealed that the antidepressant effects of linalool may act by modulation monoaminergic, GABAergic and glutamatergic neurotransmissions inhibiting via interaction of HT1A receptors, GABAA receptors and NMDA receptors.[54,56,57] Yi et al reported antidepressant properties of nobiletin by a significant reduction in the duration of immobility in force swim test,[58] which is consistent with our results from behavioral tests. Additionally, tangeretin exhibited neuroprotective properties against lipopolysaccharide (LPS)-induced depressive-like behaviour deficits with an upregulation of BDNF.[59] Moreover, treatment with hesperidin effectively reversed the increase of immobility time of streptozotocin (STZ)-induced mice in FST and increased the levels of monoamine neurotransmitters, as well as BDNF expression in the brain.[60] These reports support our hypothesis that nobiletin , tangeretin, and hesperidin, which are abundant in the fruit extract; D-limonene, γ-terpinene and linalool were the major component in the essential oil of *Citrus reticulata* var. *depressa* may responsible for their anti-depressant properties.

Another study demonstrated the efficacy of "*gold lotion*", a natural product-based citrus peels (navel oranges, *Citrus hassaku*, *Citrus limon*, *Citrus natsudaidai*, *Citrus miyauchi*, and *Satsuma*) extract rich in flavonoids, inhibiting carrageenan-induced behavioral signs of inflammatory pain in mice, without

producing stress-related behavioral changes, such as anxiety and depression. The study evaluated the effects using the open field test and forced swim test, respectively.[61] The major chemical components in *golden lotion* were nobiletin, sinensetin, tangeretin, naringin, hesperidin, 3,5,6,7,8,3′,4′-hepta-methoxyflavone, 3,5,6,7,3′,4′-hexamethoxy-flavone, and 5,6,7,4′-tetramethoxy-flavone, as reported in another study.[62]

Over the past decade, emerging evidence from animal models and human patients has highlighted the potential contribution of the gut microbiota in pathogenesis of major depressive disorder and neuropsychiatric disorders. The gut microbiota is a core component of the gut-brain axis, which can influence Over the past decade, accumulating evidence from animal models and human patients' studies revealed that gut microbiota playing functional role in the pathogenesis of major depressive disorder (MDD) and neuropsychiatric disorders. The gut microbiota is a core component of the gut-brain axis that can alter behaviors through neurotransmitters and neuropeptides, low grade immune activation, hypothalamic-pituitary-adrenal axis activity, and neurogenesis.[36] Studies have shown that positive regulation of gut microbiota by probiotics, prebiotics, antibiotics, dietary supplements, and faecal microbiota transplantation can be effective treatments for MDD and neuropsychiatric disorders.[63]

In recent decades, there has been increasing attention given to the potential of dietary supplements to modulate the composition and activity of intestinal microbiota. Citrus polyphenols have been identified as having the ability to influence the gut microbiota composition and function, and exert potential neurological benefits.[64] Dysfunction in the brain-gut axis has been observed in the CMS animal model of depression. In this study, we provide evidence that the fruit extract and essential oil of *Citrus reticulata* var. *depressa* alleviate CMS-induced anxiety and depressive-like behaviors in mice as well as the distinct antidepressant effects between the inhalation and oral administration of CDE. However, the difference may be attributed to the fact that oral administration primarily targets the gastrointestinal digestive system, while inhalation primarily targets the central nervous system. Additionally, we also observed that the oral administration groups had better feed efficiency than inhalation administration groups after two weeks treatment. This phenomenon can be elucidated by the predominant influence of CDE on the gastrointestinal digestive system. Alongside these behavioral changes, we observed a significant regulation of the gut-microbiome in CMS-challenged mice, including increased species diversity and richness and significant community profile divergence from the unstressed groups. However, the fruit extract and essential oil of *Citrus reticulata* var. *depressa* were able to reverse these changes in microbiota composition. This positive regulation of gut microbiota was observed in parallel with antidepression-like activities.

It has been demonstrated that major depressive disorder is associated with significant changes in the composition of the intestinal microbiota, highlighting the importance of the microbiota in the aetiology of the disease.[65] The most common bacterial phyla in a typical gut microbiome include *Bacteroidetes*, *Firmicutes*, *Actinobacteria*, *Proteobacteria*, and *Fusobacteria,* which together constitute over 90% of the gut microbiota. However, several studies have shown there was no consensus on the abundance of *Firmicutes*, while an increased abundance of *Actinobacteria*, and a lower abundance of *Bacteriodetes* have been observed among individuals with depression.[66] In our study, we found a remarkable reduction in the abundance of *Bacteriodetes*, which has increased following the supplantation of fruit extract and essential oil of *Citrus reticulata* var. *depressa*. Interestingly, the long-term treatment with inhalation administration of CDE resulted in the highest feed efficiency, accompanied by a significant reduction in the F/B ratio. This finding suggests that although the inhalation of CDE primarily acts on the central nervous system, it can effectively modulate the gut-brain axis to improve the impaired gastrointestinal digestion function and ameliorate the gut microbiota dysbiosis caused by chronic mild stress. Furthermore, we observed a significant increase in *Clostridium* species in the CMS groups, which was significantly decreased following both oral administration and inhalation treatments of CD and CDE. Moreover, still it remains difficult to extrapolate how alterations in the composition of gut microbiome potentially yields various types of depressive disorders. Bailey et al. demonstrated that an increase in the relative abundance of *Clostridium* species in social disruption stress-induced mice.[67] The increase in *Clostridium* abundance following social disruption stress is in contrast to with a following work from Bharwani et al, who demonstrated decrease in the abundance of *Clostridium* species in social defeat stress in mice.[68]

5. Conclusion

Based on our results, we have demonstrated that mice subjected to CMS exhibit significant physiological and psychological changes, including a rapid reduction in body weight and depression-like behaviours. Oral administration of CD and inhalation of CDE significantly reversed CMS-mediated loss of body weight. Furthermore, both oral administration of CD and CDE effectively alleviated CMS-induced anxiety- and depression-like behaviours. However, inhalation of CDE only had an effect on anxiety-like behaviours but failed to modulate depression-like behaviour. We also found that structure and community of gut microbiome were significantly altered in CMS-challenger mice, highlighting the close relationship between the gut microbiota and depression-like behaviours. Indeed, both oral administration of CD and CDE and inhalation of CDE were able to modulate the gut microbiome and positive regulate gut-brain axis. This study supports that Hirami lemon-based supplements for stress-related disorders, and further research is needed to reveal the exact underlying molecular mechanisms.

Author Contributions

Conceptualization, S.Y.W. Methodology, P.H.T. Investigation, P.H.T, P.C.W and H.R.L. Validation, P.H.T. Formal analysis, P.H.T

View Article Online
DOI: 10.1039/D3FO02611D

and K.J.S.K. Writing original draft, P.H.T. Writing review & editing K.J.S.K and S.Y.W. Supervision, S.Y.W. Funding, S.Y.W.

Conflicts of interest

All authors declare no conflicts of interest.

Acknowledgements

This study was supported by grants from the National Science and Technology Council (NSTC), Taiwan (109-2313-B-005-043-MY3). The funding body did not have any role in the design of the study and collection, analysis, and interpretation of data and in writing the manuscript.

References

1 N. Salari, A. Hosseinian-Far, R. Jalali, A. Vaisi-Raygani, S. Rasoulpoor, M. Mohammadi, S. Rasoulpoor and B. Khaledi-Paveh, Prevalence of stress, anxiety, depression among the general population during the COVID-19 pandemic: a systematic review and meta-analysis, *Global. Health*, 2020, 16, 57.
2 Anonymous, Global burden of mental disorders and the need for a comprehensive, coordinated response from health and social sectors at the country level: report by the Secretariat. *World Health Assembly*, 2012, **65**, A65/10.
3 K. Hou, Z.X. Wu, X.Y. Chen, J.Q. Wang, D. Zhang, C. Xiao, D. Zhu, J. B. Koya, L. Wei, J. Li and Z.S. Chen, Microbiota in health and diseases, *Signal Transduct Target. Ther*, 2022, **7**, 135.
4 A. Vijay and A. M. Valdes, Role of the gut microbiome in chronic diseases: a narrative review, *Eur. J. Clin. Nutr*, 2022, **76**, 489-501.
5 J. A. Foster and K. A. McVey Neufeld, Gut-brain axis: how the microbiome influences anxiety and depression, *Trends Neurosci*, 2013, **36**, 305-312.
6 Y. Wang and L. H. Kasper, The role of the microbiome in central nervous system disorders, *Brain. Behav. Immun.*, 2014, **38**, 1-12.
7 T. G. Dinan, C. Stanton and J. F. Cryan, Psychobiotics: a novel class of psychotropic, *Biol. Psychiatry*, 2013, **74**, 720-726.
8 A. Sarkar, S. M. Lehto, S. Harty, T. G. Dinan, J. F. Cryan and P. W. J. Burnet, Psychobiotics and the manipulation of bacteria-gut-brain signals, *Trends Neurosci*, 2016, **39**, 763-781.
9 J. M. Jurek, O. Marcin, Psychobiotics and dietary supplementation in the clinical practice: A mini-review, *J. Biomed Res. Environ Sci*, 2022, **3**, 1093-1100.
10 C. T. Peterson, Dysfunction of the microbiota-gut-brain axis in neurodegenerative disease: the promise of therapeutic modulation with prebiotics, medicinal herbs, probiotics, and synbiotics, *J. Evid.-Based Integr. Med*, 2020, **25**, 2515690x20957225.
11 E.M. Pferschy-Wenzig, M. R. Pausan, K. Ardjomand-Woelkart, S. Röck, R. M. Ammar, O. Kelber, C. Moissl-Eichinger and R. Bauer, Medicinal plants and their impact on the gut microbiome in mental health: A systematic review, *Nutrients*, 2022, **14**, 2111.
12 D. A. Teigiserova, L. Tiruta-Barna, A. Ahmadi, L. Hamelin and M. Thomsen, A step closer to circular bioeconomy for citrus peel waste: A review of yields and technologies for sustainable management of essential oils, *J. Environ. Manage*, 2021, 280, 111832.
13 R. K. Saini, A. Ranjit, K. Sharma, P. Prasad, X. Shang, K. G. M. Gowda, Y. S. Keum, Bioactive compounds of citrus fruits: A review of composition and health benefits of carotenoids, flavonoids, limonoids, and terpenes, *Antioxidants*, 2022, **11**, 239.
14 X. Lv, S. Zhao, Z. Ning, H. Zeng, Y. Shu, O. Tao, C. Xiao, C. Lu and Y. Liu, Citrus fruits as a treasure trove of active natural metabolites that potentially provide benefits for human health, *Chem. Cent. J*, 2015, 9, 68.
15 R. J. Kean, D. J. Lamport, G. F. Dodd, J. E. Freeman, C. M. Williams, J. A. Ellis, L. T. Butler, and J. P. Spencer, Chronic consumption of flavanone-rich orange juice is associated with cognitive benefits: an 8-wk, randomized, double-blind, placebo-controlled trial in healthy older adults, *Am. J. Clin. Nutr*, 2015, **101**, 506-514.
16 K. P. Datla, M. Christidou, W. W. Widmer, H. K. Rooprai and D. T. Dexter, Tissue distribution and neuroprotective effects of citrus flavonoid tangeretin in a rat model of Parkinson's disease, *Neuroreport*, 2001, **12**, 3871-3875.
17 N. Braidy, S. Behzad, S. Habtemariam, T. Ahmed, M. Daglia, S. Mohammad Nabavi, E. Sobarzo-Sanchez, and S. Fazel Nabavi, Neuroprotective effects of citrus fruit-derived flavonoids, nobiletin, and tangeretin in Alzheimer's and Parkinson's disease, *CNS Neurol. Disord.-Drug Target*, 2017, **16**, 387-397.
18 N. S. Dosoky and W. N. Setzer, Biological activities and safety of citrus spp. essential oils, *Int. J. Mol. Sci*, 2018, **19**, 1966.
19 S. S. Monteiro, V. M. de Oliveira and M. A. de. B. Pasquali, Probiotics in citrus fruits products: Health benefits and future trends for the production of functional foods- A bibliometric review, *Foods*, 2022, **11**, 1299.
20 T. Tanaka, Wild Citri of the Japanese territories, *J. Fac. Agric. Kyushu Univ.*, 1926, **2**, 51-58.
21 S.Y. Lin and I.Z. Chen, Native Citrus in Taiwan and current status of research and utilization, *Seed. Nursery*, 2006, **8**, 1-12.
22 K.M. Lau, W.S. Su, S.C. Chien, S.Y. Wang and K. J. Senthil Kumar, *Melia azedarach* flowers and their volatile components improved human physiological and psychological functions, *J. Essent. Oil-Bear Plant*, 2021, **24**, 1200-1211.
23 I. A. Marin, J. E. Goertz, T. Ren, S. S. Rich, S. Onengut-Gumuscu, E. Farber, M. Wu, C. C. Overall, J. Kipnis and A. Gaultier, Microbiota alteration is associated with the development of stress-induced despair behavior, *Sci. Rep.*, 2017, 7, 43859.
24 J. Sun, F. Wang, X. Hu, C. Yang, H. Xu, Y. Yao, and J. Liu, *Clostridium butyricum* attenuates chronic unpredictable mild stress-induced depressive-like behavior in mice via the gut-brain axis, *J. Agric. Food Chem.*, 2018, **66**, 8415-8421.
25 C. Ducottet and C. Belzung, Behaviour in the elevated plus-maze predicts coping after subchronic mild stress in mice, *Physiol. Behav*, 2004, **81**, 417-426.
26 D. P. Herlemann, M. Labrenz, K. Jürgens, S. Bertilsson, J. J. Waniek and A. F. Andersson, Transitions in bacterial communities along the 2000 km salinity gradient of the Baltic Sea, *ISME J*, 2011, **5**, 1571-1579.
27 J. G. Caporaso, C. L. Lauber, W. A. Walters, D. Berg-Lyons, J. Huntley, N. Fierer, S. M. Owens, J. Betley, L. Fraser, M. Bauer, N. Gormley, J. A. Gilbert, G. Smith, and R. Knight, Ultra-high-throughput microbial community analysis on the Illumina HiSeq and MiSeq platforms, *ISME J*, 2012, **6**, 1621-1624.
28 A. M. Bolger, M. Lohse and B. Usadel, Trimmomatic: A flexible trimmer for Illumina sequence data, *Bioinformatics*, 2014, **30**, 2114-2120.
29 M. Martin, Cutadapt removes adapter sequences from high-throughput sequencing reads, *EMBnet. J*, 2011, **17**, 10-12.
30 T. Magoč and S. L. Salzberg, FLASH: fast length adjustment of short reads to improve genome assemblies, *Bioinformatics*, 2011, **27**, 2957-2963.

31 R. C. Edgar, B. J. Haas, J. C. Clemente, C. Quince, and R. Knight, UCHIME improves sensitivity and speed of chimera detection, *Bioinformatics*, 2011, **27**, 2194-2200.

32 B. J. Haas, D. Gevers, A. M. Earl, M. Feldgarden, D. V. Ward, G. Giannoukos, D. Ciulla, D. Tabbaa, S. K. Highlander, E. Sodergren, B. Methé, T. Z. DeSantis, J. F. Petrosino, R. Knight and B. W. Birren, Chimeric 16S rRNA sequence formation and detection in Sanger and 454-pyrosequenced PCR amplicons, *Genome Res*, 2011, **21**, 494-504.

33 E. Pruesse, C. Quast, K. Knittel, B. M. Fuchs, W. Ludwig, J. Peplies and F. O. Glöckner, SILVA: A comprehensive online resource for quality checked and aligned ribosomal RNA sequence data compatible with ARB, *Nucleic Acids Res*, 2007, **35**, 7188-7196.

34 P. D. Schloss, S. L. Westcott, T. Ryabin, J. R. Hall, M. Hartmann, E. B. Hollister, R. A. Lesniewski, B. B. Oakley, D. H. Parks, C. J. Robinson, J. W. Sahl, B. Stres, G. G. Thallinger, D. J. Van Horn and C. F. Weber, Introducing mothur: open-source, platform-independent, community-supported software for describing and comparing microbial communities, *Appl. Environ. Microbiol*, 2009, **75**, 7537-7541.

35 J. G. Caporaso, J. Kuczynski, J. Stombaugh, K. Bittinger, F. D. Bushman, E. K. Costello, N. Fierer, A. G. Peña, J. K. Goodrich, J. I. Gordon, G. A. Huttley, S. T. Kelley, D. Knights, J. E. Koenig, R. E. Ley, C. A. Lozupone, D. McDonald, B. D. Muegge, M. Pirrung, J. Reeder, J. R. Sevinsky, P. J. Turnbaugh, W. A. Walters, J. Widmann, T. Yatsunenko, J. Zaneveld and R. Knight, QIIME allows analysis of high-throughput community sequencing data, *Nat. Methods*, 2010, **7**, 335-336.

36 N. Li, Q. Wang, Y. Wang, A. Sun, Y. Lin, Y. Jin and X. Li, Oral probiotics ameliorate the behavioral deficits induced by chronic mild stress in mice via the gut microbiota-inflammation axis, *Front. Behav. Neurosci*, 2018, **12**, 266.

37 A. D. Willis, Rarefaction, Alpha Diversity, and Statistics, *Front. Microbiology*, 2019, **10**, 2407.

38 B. R. Kim, J, Shin, R. Guevarra, J. H. Lee, D. W. Kim, K. H. Seol, J. H. Lee, H. B. Kim, R. Isaacson, Deciphering diversity indices for a better understanding of microbial communities, *J. Microbiol. Biotechnol.*, 2017, **27**, 2089-2093.

39 Y. Stevens, E. V. Rymenant, C. Grootaert, J. V. Camp, S. Possemiers, A. Masclee and D. Jonkers, The intestinal fate of citrus flavanones and their effects on gastrointestinal health, *Nutrients*, 2019, **11**, 1464.

40 C. Manach, A. Scalbert, C. Morand, C. Rémésy and L. Jiménez, Polyphenols: food sources and bioavailability, *Am. J. Clin. Nutr*, 2004, **79**, 727-747.

41 S. Haaz, K. R. Fontaine, G. Cutter, N. Limdi, S. Perumean-Chaney and D. B. Allison, *Citrus aurantium* and synephrine alkaloids in the treatment of overweight and obesity: An update, *Obes. Rev*, 2006, **7**, 79-88.

42 P. Willner, The chronic mild stress (CMS) model of depression: History, evaluation and usage, *Neurobiol. Stress*, 2017, **6**, 78-93.

43 M. Serrano-Dueñas, Fluoxetine-induced tremor: clinical features in 21 patients, *Parkinsonism Relat. Disord*, 2002, **8**, 325-327.

44 T. Gosselin, A.-M. Le Guisquet, B. Brizard, C. Hommet, F. Minier and C. Belzung, Fluoxetine induces paradoxical effects in C57BL6/J mice: Comparison with BALB/c mice, *Behav. Pharmacol*, 2017, **28**, 466-476.

45 F. Li, K. Zhang, M. Yu, T. Chen, L. Ma, H. Zhang, H. Jia and Z. Zou, Antidepressant-like effect and phytochemical profile of supercritical CO2 extract from *Citri reticulatae pericarpium*, *Pharmazie*, 2021, **76**, 249-255.

46 S. Seo, Y. Song, S. M. Gu, H. K. Min, J. T. Hong, H. J. Cha and J. Yun, D-limonene inhibits pentylenetetrazole-induced seizure via adenosine A2A receptor modulation on gabaergic neuronal activity, *Int. J. Mol. Sci*, 2020, **21**, 9277.

47 M. Shinomiya, K. Kawamura, E. Tanida, M. Nagoshi, H. Motoda, Y. Kasanami, F. Hiragami and Y. Kano, Neurite outgrowth of PC12 mutant cells induced by orange oil and d-limonene via the p38 MAPK pathway, *Acta Med Okayama*, 2012, **66**, 111-118.

48 C. Björkholm and L. M. Monteggia, BDNF - a key transducer of antidepressant effects, *Neuropharmacology*, 2016, **102**, 72-79.

49 B. Youssef, K. S. Ramadan, S. El-Shebiney and E. A. Ibrahim, Antidepressant-like effects of aqueous extracts of miswak (*Salvadora persica*) and date palm (*Phoenix dactylifera*) on depression-like behaviors using CUMS model in male rats, *J. Food Biochem*, 2022, **46,** e14164.

50 I. O. Ishola, T. G. Olubodun-Obadun, O. A. Bakre, E. S. Ojo and O. O. Adeyemi, Kolaviron ameliorates chronic unpredictable mild stress-induced anxiety and depression: involvement of the HPA axis, antioxidant defence system, cholinergic, and BDNF signalling, *Drug Metab. Pers Ther*, 2022, **37**, 277-287.

51 X. Wang, P. Li, Q. Ding, C. Wu, W. Zhang and B. Tang, Observation of acetylcholinesterase in stress-induced depression phenotypes by two-photon fluorescence imaging in the mouse brain, *J. Am. Chem. Soc*, 2019, **141**, 2061-2068.

52 H. S. Choi, H. S. Song, H. Ukeda and M. Sawamura, Radical-scavenging activities of citrus essential oils and their components: detection using 1,1-diphenyl-2-picrylhydrazyl, *J. Agric. Food Chem*, 2000, **48**, 4156-4161.

53 M. Öztürk, Anticholinesterase and antioxidant activities of Savoury (*Satureja thymbra* L.) with identified major terpenes of the essential oil, *Food Chem*, 2012, **134**, 48-54.

54 H. Harada, H. Kashiwadani, Y. Kanmura and T. Kuwaki, Linalool odor-induced anxiolytic effects in mice, *Front. Behav. Neurosci*, 2018, **12**, 241.

55 A. C. Aprotosoaie, M. Hăncianu, I. I. Costache and A. Miron, Linalool: a review on a key odorant molecule with valuable biological properties, *Flavour Fragrance J*, 2014, **29**, 193-219.

56 S. L. Guzmán-Gutiérrez, H. Bonilla-Jaime, R. Gómez-Cansino and R. Reyes-Chilpa, Linalool and β-pinene exert their antidepressant-like activity through the monoaminergic pathway, *Life Sci*, 2015, **128**, 24-29.

57 L. Silva Brum, T. Emanuelli, D. Souza and E. Elisabetsky, Effects of linalool on glutamate release and uptake in mouse cortical synaptosomes, *Neurochem Res*, 2001, **26**, 191-194.

58 L. T. Yi, H. L. Xu, J. Feng, X. Zhan, L. P. Zhou and C. C. Cui, Involvement of monoaminergic systems in the antidepressant-like effect of nobiletin, *Physiol. Behav*, 2011, **102**, 1-6.

59 H.O. Lai, X.Y. Hu and Y.H. Liu, Neuroprotective potential of Tangeretin against lipopolysaccharide-evoked oxido-Nitrosative Stress, Depressive-Like Behavior, and neuroinflammation, *Lat. Am. J. Pharm*, 2016, **35**, 1154-1162.

60 S. A. El-Marasy, H. M. Abdallah, S. M. El-Shenawy, A. S. El-Khatib, O. A. El-Shabrawy and S. A. Kenawy, Anti-depressant effect of hesperidin in diabetic rats, *Can. J. Physiol. Pharmacol,* 2014, **92**, 945-952.

61 T. Iannitti, A. Di Cerbo, A. R. Loschi, S. Rea, M. Suzawa and J. C. Morales-Medina, Repeated administration of a flavonoid-based formulated extract from citrus peels significantly reduces peripheral inflammation-induced pain in the rat, *Food Sci. Nutr*, 2020, **8**, 3173-3180.

62 C. S. Lai, S. Li, C. B. Liu, Y. Miyauchi, M. Suzawa, C. T. Ho and M. H. Pan, Effective suppression of azoxymethane-induced aberrant crypt foci formation in mice with citrus peel flavonoids, *Mol. Nutr. Food Res*, 2013, **57**, 551-555.

63 S. Baldi, T. Mundula, G. Nannini and A. Amedei, Microbiota shaping - the effects of probiotics, prebiotics, and fecal microbiota transplant on cognitive functions: A systematic review, *World J. Gastroenterol*, 2021, **27**, 6715-6732.

View Article Online
DOI: 10.1039/D3FO01301D

64 M. G. Pontifex, M. M. A. H. Malik, E. Connell, M. Müller and D. Vauzour, Citrus polyphenols in brain health and disease: Current perspectives, *Front. Neurosci*, 2021, **15**.
65 M. A. Ortega, M. A. Alvarez-Mon, C. García-Montero, O. Fraile-Martinez, L. G. Guijarro, G. Lahera, J. Monserrat, P. Valls, F. Mora, R. Rodríguez-Jiménez, J. Quintero and M. Álvarez-Mon, Gut microbiota metabolites in major depressive disorder-deep insights into their pathophysiological role and potential translational applications, *Metabolites*, 2022, **12**, 50.
66 Z. A. Barandouzi, A. R. Starkweather, W. A. Henderson, A. Gyamfi and X. S. Cong, Altered composition of gut microbiota in depression: A systematic review, *Front. Psychiatry*, 2020, **11**, 541.
67 M. T. Bailey, S. E. Dowd, J. D. Galley, A. R. Hufnagle, R. G. Allen and M. Lyte, Exposure to a social stressor alters the structure of the intestinal microbiota: implications for stressor-induced immunomodulation, *Brain Behav. Immun*, 2011, **25**, 397-407.
68 A. Bharwani, M. F. Mian, J. A. Foster, M. G. Surette, J. Bienenstock and P. Forsythe, Structural & functional consequences of chronic psychosocial stress on the microbiome & host, *Psychoneuroendocrinology*, 2016, **63**, 217-227.