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Nutrition and lifestyle medicine for neurodevelopmental and psychiatric disorders

Edited by
Nicholas Pang, Irene Hatsu and Krishnamachari Srinivasan

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Nutrition and lifestyle medicine for neurodevelopmental and psychiatric disorders

Topic editors

Nicholas Pang — Universiti Malaysia Sabah, Malaysia

Irene Hatsu — The Ohio State University, United States

Krishnamachari Srinivasan — St. John's Research Institute, India

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EDITED AND REVIEWED BY
Kiyotaka Nemoto,
University of Tsukuba, Japan

*CORRESPONDENCE
Krishnamachari Srinivasan
✉ srinivas@sjri.res.in

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Editorial: Nutrition and lifestyle medicine for neurodevelopmental and psychiatric disorders

Krishnamachari Srinivasan*

St. John's Research Institute, Bangalore, India

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Editorial on the Research Topic
Nutrition and lifestyle medicine for neurodevelopmental and psychiatric disorders

We are all aware of the phrase "you are what you eat." Over the years the relationship between an individual's diet and his/her behavior has been of interest to not just the scientific community but to the general population as well. Starting from the effect of choice of food on personality in ancient scriptures (1) to modern scientific research, there has been considerable interest in exploring this relationship. Academic papers in this special section are a testament to the increased research in both humans and animal models of neurodevelopmental disorders to identify specific mechanistic pathways that underpin the association between dietary intakes, nutrient status and emotional and cognitive outcomes. Most of the previous research in this area has focused on the effect of specific nutrients and lifestyle modifications on the brain and how that affects behavior and mental health (2). The most widely researched nutrients include omega-3 fatty acids (3), vitamins, especially B vitamins (4), folic acid (5) and iron (6) and their effect on cognition. Studies on treating psychiatric disorders using dietary and lifestyle modifications have been encouraging (7) with most studies till date done on depression and anxiety. The current issue presents eleven studies that further highlight the role of nutrients, dietary intakes and lifestyle factors in the aetiopathogenesis and treatment of neurodevelopmental disorders and mental health conditions.

An area of research that has received a lot of attention in recent times is the connection between the intake of prebiotics and probiotics and their effect on psychiatric disorders. Two review articles in this edition, by Ansari et al. and Yang et al. focus on studies in this area and conclude that pre and probiotics can influence the outcomes in a range of mental disorders. Though research is still in its infancy, these studies show the promise of dietary supplements being an important component of the therapeutic armamentarium in the treatment of psychiatric disorders. These two reviews examine the gut-brain axis as the conduit between the use of pre and probiotics and its impact on mental health outcomes. Other articles in this issue investigate the relationship between specific diets, nutrients and lifestyle factors and mental health. However, as research extends to more complex neurodevelopmental disorders or syndromes, the role of diets

in aetiopathogenesis and treatment may diminish as shown in the review by Pancheva et al. on autism spectrum disorders. Psychosocial issues become more salient in complex disorders, and a multidimensional model is often required to understand behavior and psychopathology. The article by Rochedy et al. illustrates this in children with Prader–Willi syndrome, a genetic disorder and suggests that problems in socialization impact eating behavior and eating disorders. In addition, measures that are used to study eating behavior, attitudes to food and diet intake need to take into account the local socio-cultural milieu and beliefs concerning food intake as highlighted in the study by Wider et al..

While experimental studies examining the link between nutrients and brain functions have relied on animal models, as in the studies by Li et al. and Chou et al. reported in this issue, this needs to be extended to studies involving human subjects to better inform the role of nutrients in shaping human behavior and psychopathology. Studies involving human subjects have begun to address the issue of the association between dietary patterns and lifestyle and mental health outcomes as reported by Hwang et al. and Wang et al..

Maternal factors such as nutrient status and emotional wellbeing during pregnancy and in the subsequent years are of critical importance in child development. Several studies using mother-child dyads have highlighted the influence of prenatal environment as well as the role of environment during the early childhood years on child neurocognitive development and behavior. However, identifying the specific mechanistic pathways that undergird the role of early maternal factors on child neurodevelopment and behavior are yet to be determined. Investigators have used animal models to better elucidate the biological underpinnings of the association between pre-and perinatal environment and child neurodevelopment and behavior. Two studies in this issue by Witek et al. and Benoit et al. report how the prenatal environment like a maternal diet of monosaccharides and postnatal environment like stress due to maternal separation can lead to long- term changes in the brain and consequently behavior problems that include emotional disturbances and increased motivation for food during adulthood.

The 11 articles in this issue thus provide multiple insights and further our understanding of the relationship between food, behavior and mental health and suggest possible areas that merit further investigation.

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Conflict of interest

The author declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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EDITED BY
Nicholas Pang,
Universiti Malaysia Sabah, Malaysia

REVIEWED BY
Emmanuel N. Pothos,
Tufts University, United States
Jeferson Jantsch,
Federal University of Health Sciences of Porto Alegre, Brazil

*CORRESPONDENCE
Kacper Witek
✉ witek@if-pan.krakow.pl
Małgorzata Filip
✉ mal.fil@if-pan.krakow.pl

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Maternal monosaccharide diets evoke cognitive, locomotor, and emotional disturbances in adolescent and young adult offspring rats

Kacper Witek*, Karolina Wydra, Agata Suder and Małgorzata Filip*

Department of Drug Addiction Pharmacology, Maj Institute of Pharmacology Polish Academy of Sciences, Kraków, Poland

Anxiety and depression are the most common mental disorders affecting people worldwide. Recent studies have highlighted that a maternal high-sugar diet (HSD) could be a risk factor for neurobehavioural dysregulations, including mood disorders. Increased consumption of added sugar in food such as refined fructose/glucose can increase the risk of metabolic disorders and impact susceptibility to mental disorders. Furthermore, a few papers have reported disabilities in learning and memory among offspring after maternal HSD, thus suggesting a relationship between maternal nutrition and offspring neurogenesis. In this study, we evaluated the impact of maternal monosaccharide consumption based on a glucose (GLU) or fructose (FRU) diet during pregnancy and lactation in adolescent and young adult offspring rats of both sexes on cognitive, locomotor, and emotional disturbances. Locomotor activity, short-term memory, anxiety-like and depressive-like behavior were evaluated in the offspring. We report for the first time that the maternal GLU or FRU diet is sufficient to evoke anxiety-like behavior among adolescent and young adult offspring. Moreover, we found that maternal monosaccharide diets lead to hyperactivity and depressive-like behavior in male adolescent rats. We also noticed that a maternal FRU diet significantly enhanced novelty-seeking behavior only in young adult male rats. Our novel findings indicated that the maternal monosaccharide diet, especially a diet enriched in FRU, resulted in strong behavioral alterations in offspring rats at early life stages. This study also revealed that male rats were more susceptible to hyperactivity and anxiety- and depressive-like phenotypes than female rats. These results suggest that maternal monosaccharide consumption during pregnancy and lactation is an important factor affecting the emotional status of offspring.

KEYWORDS

anxiety, behavior disorders, fructose, glucose, cognition, depression, hyperactivity

1. Introduction

Mental disorders are diseases characterized by a clinically significant disturbance in an individual's cognition, emotional regulation, or behavior that reflects a dysfunction in the psychological, biological, or brain developmental processes (1). Mental disorders are global problems in modern society, and they affect people across genders and ages, imposing significant barriers to a person's ability to function normally.

In 2019, one in every eight people, or 970 million people worldwide, lived with a mental disorder, among which anxiety and depressive disorders were the most common (2). On the other hand, it was estimated that in the United States, young adults aged 18–25 years had a higher prevalence of serious mental illness than adults aged 26–49 years and older (3). The development of mental disorders is due to biological, psychological, and social factors, and these risk factors may manifest differently at different ages and stages in life, with some of these risks appearing as early as the prenatal period (4). The Developmental Origins of Health and Disease hypothesis indicates that fetal exposure during a critical period of development and growth to external (environmental) and internal (maternal) factors can significantly impact the short- and long-term health of the offspring (5). Maternal diet during pregnancy and lactation can lead to metabolic or neurological diseases among offspring (6, 7).

Foods that contain sugar such as glucose (GLU) and fructose (FRU) are an important source of energy for cells and crucial for the maintenance of the body's biochemical processes. The brain consumes ∼ 20% of GLU-derived energy, making it the main consumer of GLU (8). Both GLU and FRU act as sweeteners and added sugars in many food products, such as FRU in high-fructose corn syrup (HFCS) or GLU in sugar-sweetened beverages (SSB). Importantly, youth and adults consume calories from added sugar in processed food (9, 10). Moreover, many adults exceed the daily intake of free sugars, which is limited by the World Health Organization to <10% of one's total energy intake (11). More than 10% of Americans' daily calories come from FRU consumption, which has potentially more harmful effects on the body than other major caloric sweeteners (12). Clinical observations have indicated that sugar-rich foods are desirable during pregnancy and were consumed too often during the study period, suggesting that many pregnant women were exposed to the adverse effects of a high-sugar diet (HSD) (13).

In a recent review, perinatal and postnatal HSD (such as sugar, sucrose, fructose, or glucose) consumption was considered a potential risk factor for neurobehavioural dysregulations such as mental disorders (14). Although several preclinical studies have evaluated the effects of maternal HSD on offspring behavior (15–18), most of these studies were performed using male or female rodents independently. It is also worth adding that most preclinical studies have focused on the relationship between postnatal HSD consumption and behavioral disturbances (19–24). However, maternal diet in the prenatal period also plays a crucial role in offspring neurodevelopmental disorders (25–27). Human observational studies have shown that stress-induced sugar or SSB consumption was correlated with increased anxiety and depression in adolescents and adults (28–31). Furthermore, HSD and sugar itself can be addictive and can predispose an individual to the risk of food addiction (32). Finally, sugar intake includes numerous neural mechanisms within the mesocorticolimbic system, hypothalamic orexigenic and anorexigenic pathways, and hypothalamic–pituitary–adrenal axis, all of which are strongly correlated with mental disorder development (33).

An increase in sugar consumption and several cognitive, locomotor, and emotional disturbances after maternal HSD suggest an interaction between these variables. However, there are no studies that have reported the effect of particular monosaccharides in the maternal diet on behavioral phenotypic changes in both sexes of offspring at later stages of life. Similarly, there are few preclinical studies have specifically investigated the impact of maternal HSD intake on offspring cognition and anxiety-like or depressive-like behavior development, while the impact of a maternal high-fat diet has been widely studied in this context. Hence, this study aimed to examine the effect of a maternal GLU or FRU diet during pregnancy and lactation on behavioral outcomes in adolescent and young adult unrelated offspring rats of both sexes.

2. Materials and methods

2.1. Experimental animal and maternal diets

Wistar rats obtained from the licensed animal Charles River breeder (Sulzfeld, Germany) were housed in standard plastic rodent cages in an animal breeding room at 21 ± 2°C and 40 ± 10% humidity with a 12-h light-dark (LD 12:12) cycle (lights on at 6:00 a.m.). Animals had free access to water and food *ad libitum*. Twenty-four virgin female rats (201–225 g), after the acclimatization period (14 days) and during the proestrus phase (the oestrous cycle phases determined by daily vaginal smears), were mated with six randomly assigned males (226–250 g) until the presence of sperm in the smears was confirmed. Pregnant females were individually housed and randomly assigned to one of three different experimental condition groups: standard control/VRF1 diet (SD), modified glucose (GLU) diet, or modified fructose (FRU) diet (Table 1). All diets were delivered by Special Diets Services (SDS; London, UK).

During pregnancy (21 days) and lactation (21 days), dams were given *ad libitum* access to water and food, depending on the diet group (Figure 1). One day after birth, the pups were weighed, and the litter sizes were counted and normalized to 8–10 pups. Next, at postnatal day (PND), 21 offspring were weaned and separated according to sex, marked, housed 5 per cage, and switched to SD. At the same time, we took two offspring sets (selected from 7 to 8 different dams and six different males) to minimize the parental genetic influence and reduce individual offspring predisposition by increasing their variability in cohorts (34). For behavioral studies, we used one of two subsets of each set randomly consisting of eight male and eight female rats for each diet. Two independent offspring subsets were used to assess spontaneous behavior in the early life stage periods without exposure to earlier experimental conditions. Female and male rats were tested separately. All experiments were carried out in conformity with the European Union Directive (2010/63/EU), Polish Act on the Protection of Animals (Dz.U. z 2020 r. poz. 638), and with approval of the Local Ethics Commission (Kraków, Poland; approval numbers 18/2021 and 54/2021) with the three Rs rule.

2.2. Offspring behavioral tests

2.2.1. Locomotor activity

Locomotor activity (LMA) was recorded for each non-habituated rat using the procedure described previously by Wydra et al. (35). Briefly, rats at PND 27 (subset from set I) and PND 59 (subset from set II) were placed individually in Opto-Varimex

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TABLE 1 Composition of main macronutrients (shown as a percentage of energy) and energy value of maternal experimental diets during pregnancy and lactation periods.

Diet		Control/VRF1 (SD)	Glucose (GLU)	Fructose (FRU)
Carbohydrates	Pectin (%)	1.37	0.00	0.00
	Hemicellulose (%)	8.76	0.13	0.13
	Cellulose (%)	3.95	6.40	6.21
	Lignin (%)	1.06	0.00	0.00
	Starch (%)	35.41	0.00	0.00
	Sugar (%)	4.64 (from sucrose)	53.76 (from glucose)	54.35 (from fructose)
Protein (%)		19.11	19.13	18.56
Fat (%)		4.75	5.06	4.91
Total energy (kcal/g)		3.40	3.44	3.43

The glucose (GLU) and fructose (FRU) diets are synthetic mimics of the control (SD) diet. The type of sugar was carefully modified and formulated to match the VRF1 diet.

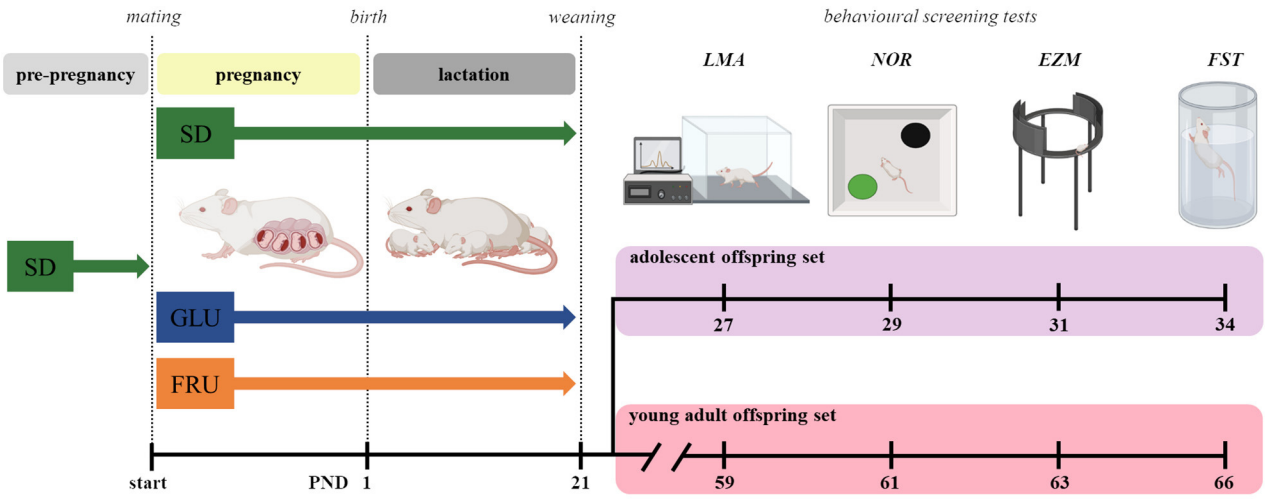

FIGURE 1
Scheme of the experiment. Dams before mating (pre-pregnancy) were fed a standard diet (SD) or, during pregnancy (~21 days) and lactation (~21 days), one of three diets: control (SD), glucose (GLU), or fructose (FRU). After weaning at postnatal day (PND) 21, the offspring were divided into two sets: adolescent and young adult. From each set, one offspring subset was used in behavioral screening tests: locomotor activity (LMA), novel object recognition (NOR), elevated zero maze (EZM), and forced swimming test (FST). All pups were fed SD chow after weaning.

(43 × 43 cm) cages (Columbus Instruments, Columbus, USA). Spontaneous locomotor activity, measured as horizontal activity, was recorded for 2 h using the Auto-Track system (Columbus Instruments, USA) every 5-min trial and presented as the distance traveled (cm).

2.2.2. Novel object recognition

To evaluate the ability to recognize a novel object (NOR) in the environment and working short-term memory in rats at PND 29 (subset from set I) and PND 61 (subset from set II), the novel object recognition test described previously by Gawlińska et al. (36) was used. On the first day, after 1 h of acclimatization in the experimental room, rats were placed separately for 10 min in the center of the empty, non-transparent plastic box (57 × 37 × 30 cm) arena for habituation. Next, for 5 min, the animals were presented with two identical objects—A, as a familiarization phase [A + A]. After a 1 h break in the home cages, rats again were placed in the same plastic box for 5 min and presented two objects, the familiar (F)—A and the first novel (N1)—B object, as a recognition phase I [A + B]. On the second day, 24 h after the familiarization phase, the rats were placed in the arena for 10 min and again presented with two objects: the previous (A) and the second novel (N2) (C) object as a recognition phase II [A + C]. Active object exploration was defined as time spent sniffing, touching, and close approach, not including the time spent sitting on the objects. The percentage of time spent exploring the novel object relative to the total time spent exploring both objects was measured using the recognition index (RI) formula: RI = TN/(TN + TF) × 100 (%), where T was the time spent exploring the novel (N1 or N2) and familiar (F) objects. An RI above 50% indicated novel object preference, below 50% indicated familiar object preference and 50% indicated

no preference (37, 38). The following objects were presented during the test: black (F) and orange (N1) metal cans (13.5 cm high, 5 cm in diameter) and a violet glass block (6 cm high, 3–5 cm in diameter) with a green plastic cone (N2; 2 cm high, 3–5 cm in diameter). Objects were placed on two opposite sides of the box, 13 cm from the center of the arena and 22 cm from each object. Plastic boxes and objects were cleaned using 20% ethanol to minimize olfactory cues before the next round. To reduce the stress of a bright lighting room, areas were illuminated with 170–230 lux light (1 m above the box). The test was video recorded by one investigator, while the results were manually extracted from videos by two independent blinded assessors.

2.2.3. Elevated zero maze

Anxiety-like behavior in rats was assessed using the elevated zero maze test (EZM) (39). The annular, black platform maze (105 cm in diameter, 10 cm path wide) consisted of four quadrants: two opposite open (with 1 cm high walls) arms and two opposite closed (with 25 cm high walls) arms, which were elevated to 65 cm above the floor. The rats were placed individually, alternately in one of the two closed arms of the maze, and a 5-min test was recorded. Behavioral measures comprised the time (s) spent in and the number of entries into open areas, the frequency of head dips from the dark to the light zone, beyond the wall in the open zone, and the frequency of stretch postures from closed to open quadrants. The maze was illuminated by a light source (120–150 lux) suspended 50 cm from the maze path. The test was conducted in the experimental, dark room after 1 h of acclimatization, 5 min of habituation to the surface, and color of the maze in the EZM box for PND 31 (subset from set I) and PND 63 (subset from set II) rats. The maze was cleaned using 20% ethanol before the next trial. All tests were performed and manually assessed by two independent investigators who remained at an appropriate distance of 2 m from the maze in the experimental room.

2.2.4. Forced swim test

The modified forced swim test (FST) was used to assess offspring rats' responses to stress at PND 34 (subset from set I) and PND 66 (subset from II) (40, 41). On the first day (pre-test), after 1 h of acclimatization, adolescent rats were placed individually in glass-transparent cylinders (41 cm high, 20 cm diameter) containing clean water (25 ± 1°C) at a depth of 30 cm (preventing touching the bottom) for 15 min. Then, all rats were removed from the water, dried, warmed, and returned to their home cages. Twenty-four hours after the pre-test, the rats were retested for 5 min under the same conditions, where one investigator recorded each animal's activity using a digital camera. Each cylinder was emptied and cleaned before the test for the next rat. Next, two independent blinded assessors manually extracted from the video the time spent swimming, climbing, and immobility. Immobility time was observed when the rat floated in water without struggling and made only the movements necessary to keep its head above water. Swimming time was defined as active swimming motions (displaced body around the cylinder), more than necessary to merely maintain head above water. Climbing time was measured when the rat was climbing, making active movements with forepaws in and out of the water.

2.4. Statistical analysis

All experimental data are expressed as the mean ± SEM (standard error of the mean). Statistical analyses were performed with Statistica 13.3 (TIBCO Software Inc., Palo Alto, CA, USA) and visualized in GraphPad Prism 9.4.1 (GraphPad Software, Inc., San Diego, CA, USA). The Shapiro–Wilk test and Levene test were used to test the normality and equal homogeneity of variance, respectively. For normally distributed data, two-way analyses of variance (ANOVA: diet + gender + diet × gender; F statistic) were performed. Alternative two-way ANOVA with Welch's correction (W statistic) was used for unequal variances. For non-normally distributed data, a non-parametric Kruskal–Wallis test by ranks (H statistic) was performed. In significant differences between diet (compared to SD), either parametric Dunnett's and Dunnett's T3 or non-parametric Dunn's multiple comparisons *post hoc* tests were performed. For the sex-specific changes, parametric Šidák's or Dunnett's T3 multiple comparisons *post hoc* tests were performed. "N" corresponds to the number of individuals. $p < 0.05$ was considered statistically significant.

3. Results

3.1. Effect of monosaccharide diets on body weight and caloric intake

During the experiment, dams showed a significant main effect of the period [$F_{(1.634,32.67)} = 106.90$; $p < 0.0001$] and interaction [$F_{(18,180)} = 2.346$; $p = 0.0024$] during 5-day measurements of body weight (Figure 2). During pregnancy (on day 15) and lactation (on day 5), SD dams had 8% and 9% higher body weights, respectively, than FRU dams (Figure 2). Moreover, we observed a main effect of the period [$F_{(1,40)} = 159.50$; $p < 0.001$], diet [$F_{(2,40)} = 6.44$; $p = 0.0038$] and interaction [$F_{(2,40)} = 7.13$; $p = 0.0023$] on total calorie intake (Figure 3A). The GLU and FRU dams showed 23 and 27%, respectively, less total calorie intake than SD dams in the lactation period (Figure 3A). The total calorie intake was similar in the pregnancy period between the groups. Assuming that 1 g of sugar has 4 kcal, and the percentage of sugar in the diet has been described (Table 1), we also assessed the contribution of calories from the sugar in the total calorie intake (Figure 3B). Here, period [$F_{(1,40)} = 164.70$; $p < 0.001$] and diet [$F_{(1,40)} = 4.87$; $p = 0.0128$] effects were observed. Conversely, for total caloric intake, both GLU and FRU dams showed 31% higher calorie intake than SD dams in the pregnancy period (Figure 3B). No changes were observed in the lactation period. Although we observed different litter sizes between dams in each diet, these changes were not statistically significant [$H_{(2,N=23)} = 1.32$, $p = 0.5146$; Figure 4A]. In turn, we observed significant differences in pup weight [$H_{(2,N=224)} = 30.69$, $p < 0.0001$; Figure 4B] between diet groups. The GLU and FRU pups weighed less by 7 and 11%, respectively than the SD

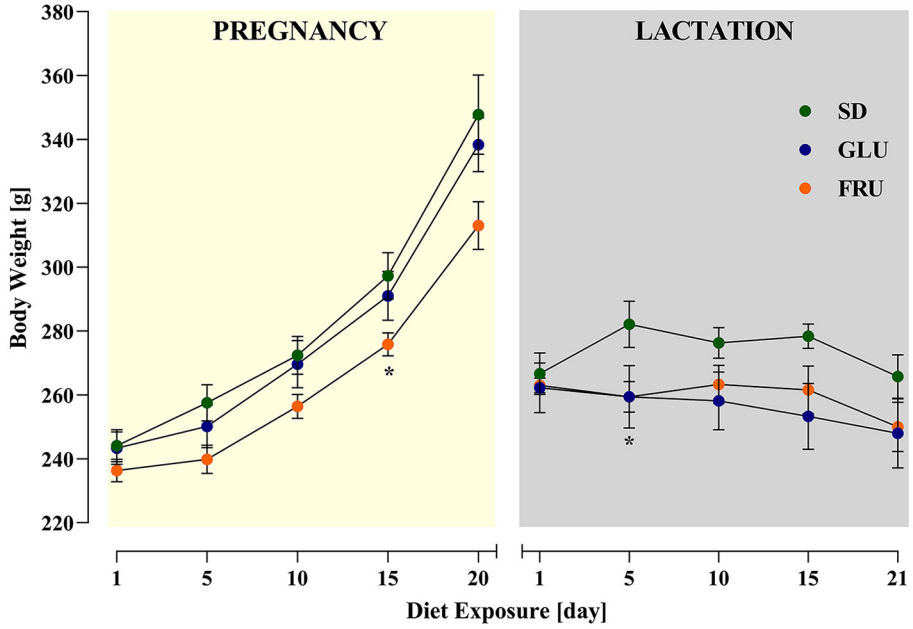

FIGURE 2
Effects of maternal glucose (GLU) or fructose (FRU) diet intake on dam body weight during pregnancy and lactation. At 15 days of pregnancy and 5 days of lactation, FRU dams showed less body weight than SD dams. $N = 8$ dams/SD and GLU, 7 dams/FRU. Data are comparable to the SD groups (*$p < 0.05$, two-way repeated measure ANOVA followed by Dunnett's multiple comparison test).

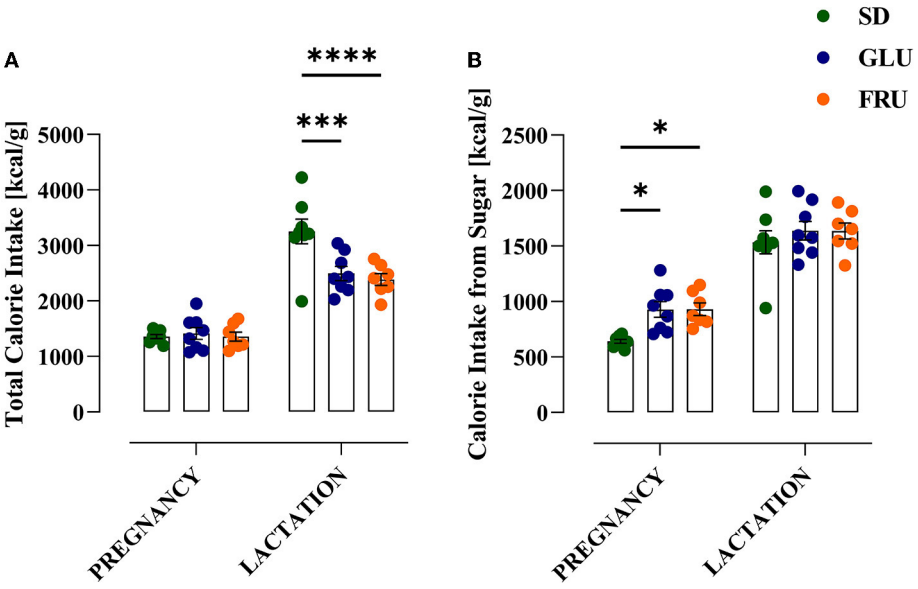

FIGURE 3
Effects of maternal glucose (GLU) or fructose (FRU) diet intake on dam calorie intake. **(A)** Total calorie intake during pregnancy and lactation. Both GLU and FRU dams had reduced total calorie intake during lactation but not during pregnancy compared to SD dams. **(B)** Calorie intake from a sugar source. Both GLU and FRU dams consumed more calories from sugar during pregnancy but not during lactation compared to SD dams. $N = 8$ dams/SD and GLU, 7 dams/FRU. Data are comparable to the SD groups (*$p < 0.05$, ***$p < 0.001$, ****$p < 0.0001$, two-way repeated measure ANOVA followed by Dunnett's multiple comparison test).

offspring (Figure 4B). Moreover, adolescent FRU male and GLU female offspring displayed 7 and 8%, respectively, higher body weights than SD rats [$W_{(5,18.66)} = 5.73$, $p = 0.0023$; Figure 4C]. Both GLU male and female young adult offspring had 6% higher body weight than SD offspring. Additionally, SD, GLU, and FRU female rats demonstrated 18%, 19%, and 20% lower body weight, respectively, than male rats in the same diet group [$W_{(5,19.14)} = 64.52$, $p < 0.0001$; Figure 4D].

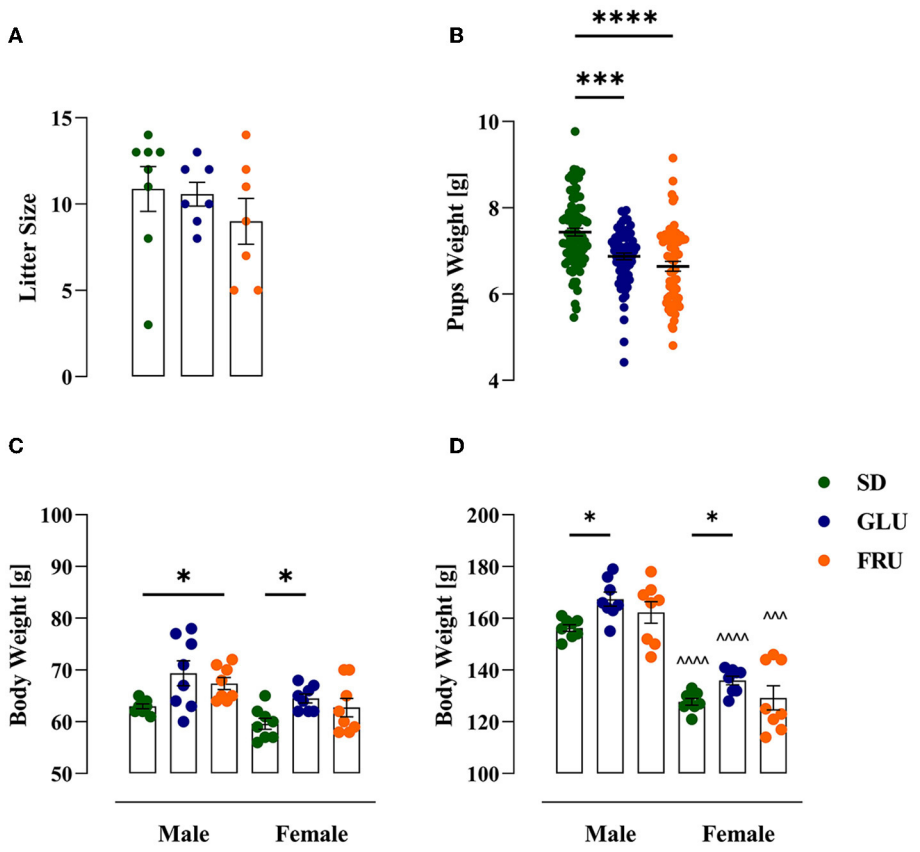

FIGURE 4
Effects of maternal glucose (GLU) or fructose (FRU) diet on litter size and offspring weight. **(A)** Litter size of each dam. Maternal diet affects litter size individually, but in total, no significant difference was observed. $N = 8$ dams/SD, 7 dams/GLU and FRU (Kruskal–Wallis tests). **(B)** Pups weight one day after birth (at PND2). GLU and FRU offspring rats weighed less than SD pups. $N = 87$ pups/SD, 74 pups/GLU and 63 pups/FRU. Data are comparable to the SD groups ($^{***}p < 0.001$, $^{****}p < 0.0001$, Kruskal–Wallis tests followed by Dunn's multiple comparison test). **(C)** Body weight of adolescent (at PND27) offspring. FRU male and GLU female adolescents had higher body weights than SD offspring. **(D)** Body weight of a young adult (at PND59) offspring. Both GLU male and female young adults had higher body weights than SD offspring. Young adult females had reduced body weight compared to males. $N = 8$ rats/group. Data are comparable to the SD groups ($^{*}p < 0.05$, two-way ANOVA with Welch's correction followed by Dunnett's T3 multiple comparison test) or between sexes in the same group ($^{\wedge\wedge\wedge}p < 0.001$, $^{\wedge\wedge\wedge\wedge}p < 0.0001$, two-way ANOVA with Welch's correction followed by Dunnett's T3 multiple comparison test).

3.2. Effects of maternal HMD on offspring locomotor activity

First, we assessed spontaneous locomotor activity measured as distance traveled in adolescent (at PND 27) and young adult (at PND 59) offspring (Figure 5). Adolescent rats showed a significant main effect of diet [$F_{(2,42)} = 9.05$; $p = 0.0005$] and sex [$F_{(1,42)} = 20.19$; $p < 0.0001$], as well as an interaction effect [$F_{(2,42)} = 3.88$; $p = 0.0283$] in 5-min trial measurements (Figure 5A). Both GLU and FRU male offspring showed 22 and 44% higher locomotor activity, respectively than SD male offspring. Moreover, FRU male rats demonstrated 29% higher activity than female rats. Likewise, after a 120-min trial (Figure 5B), GLU and FRU adolescent male offspring displayed 42 and 48%, respectively, enhancement in spontaneous locomotor activity [$H_{(5,N=48)} = 18.57$, $p = 0.0023$] compared with SD male rats. No significant interaction effects by diet and sex were observed in young adult offspring during 5- [$H_{(5,N=48)} = 3.57$, $p = 0.612$; Figure 5C] and 120- [$F_{(2,42)} = 0.81$, $p = 0.447$; Figure 5D] min trials.

3.3. Effects of maternal HMD on offspring memory

The effect of maternal diets on short-term memory and the ability to recognize a novel object in adolescent (at PND29) and young adult (at PND61) offspring were evaluated using the NOR test (Figure 6). In adolescent offspring, SD, GLU, and FRU females displayed 24%, 26%, and 28% higher recognition indices, respectively, than male rats [$W_{(5,19.11)} = 9.56$, $p = 0.0001$] 1 h after the familiarization phase (Figure 6A). Moreover, no interaction effect was observed 24 h after the familiarization phase [$F_{(2,42)} = 2.71$, $p = 0.078$; Figure 6B].

In young adult rats 1 h after the familiarization phase, only the sex effect was detected [$F_{(1,42)} = 7.39$, $p = 0.0095$] without significant differences between male and female rats (Figure 6C). On the other hand, 24 h after the familiarization phase, significant main diet [$F_{(2,42)} = 11.35$, $p = 0.0001$] and interaction [$F_{(2,42)} = 3.47$, $p = 0.040$] effects were

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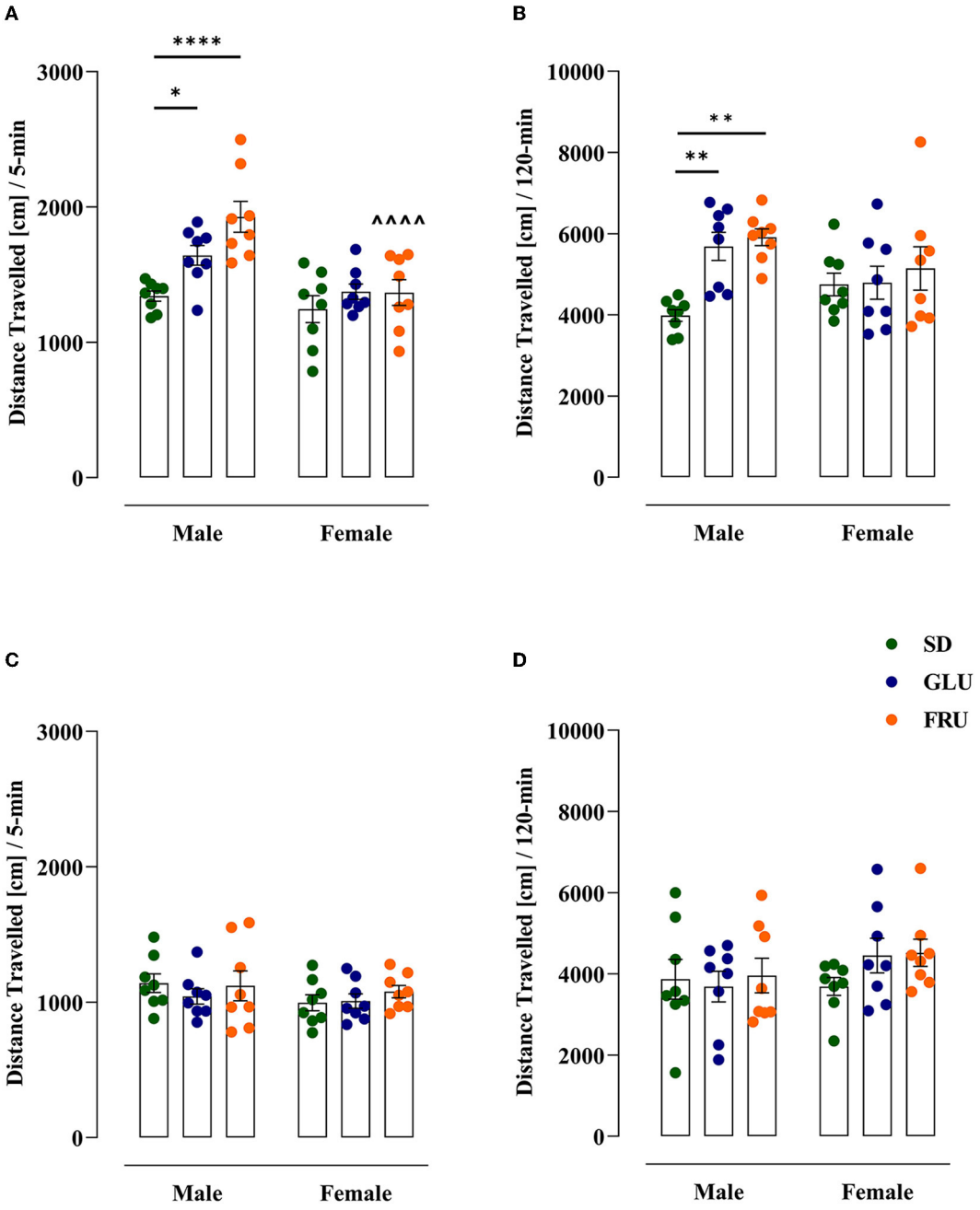

FIGURE 5
Effects of maternal glucose (GLU) or fructose (FRU) diet on adolescent (at PND27) and young adult (at PND59) offspring distance traveled. **(A, B)** Adolescent, but not young adult, GLU and FRU males showed hyperactivity during the 5-min trial. Adolescent FRU females had a reduced distance traveled compared to FRU males. **(C, D)** The 120-min trial indicated a significantly higher distance traveled in GLU and FRU adolescents but not in young adult rats. $N = 8$ rats/group. Data are comparable to the SD groups (*$p < 0.05$, **$p < 0.01$, ****$p < 0.0001$, two-way ANOVA or Kruskal–Wallis tests followed by Dunnett's or Dunn's multiple comparison test, respectively) or between sexes in the same group (^^^^$p < 0.0001$, two-way ANOVA followed by Šidák multiple comparison test).

noticed. Interestingly, only young adult FRU male rats manifested significant (26%) increases (Figure 6D) in the recognition index in comparison to SD male rats. After the 24-h familiarization phase, both adolescent and young adult offspring had RIs above 50%, suggesting a preference for the novel object.

3.4. Effects of maternal HMD on offspring anxiety-like behavior

We assessed anxiety-like behavior in offspring using an EZM test (Figure 7). In adolescent offspring, the time spent in an open area (Figure 7A) in FRU male and female offspring was 77% lower

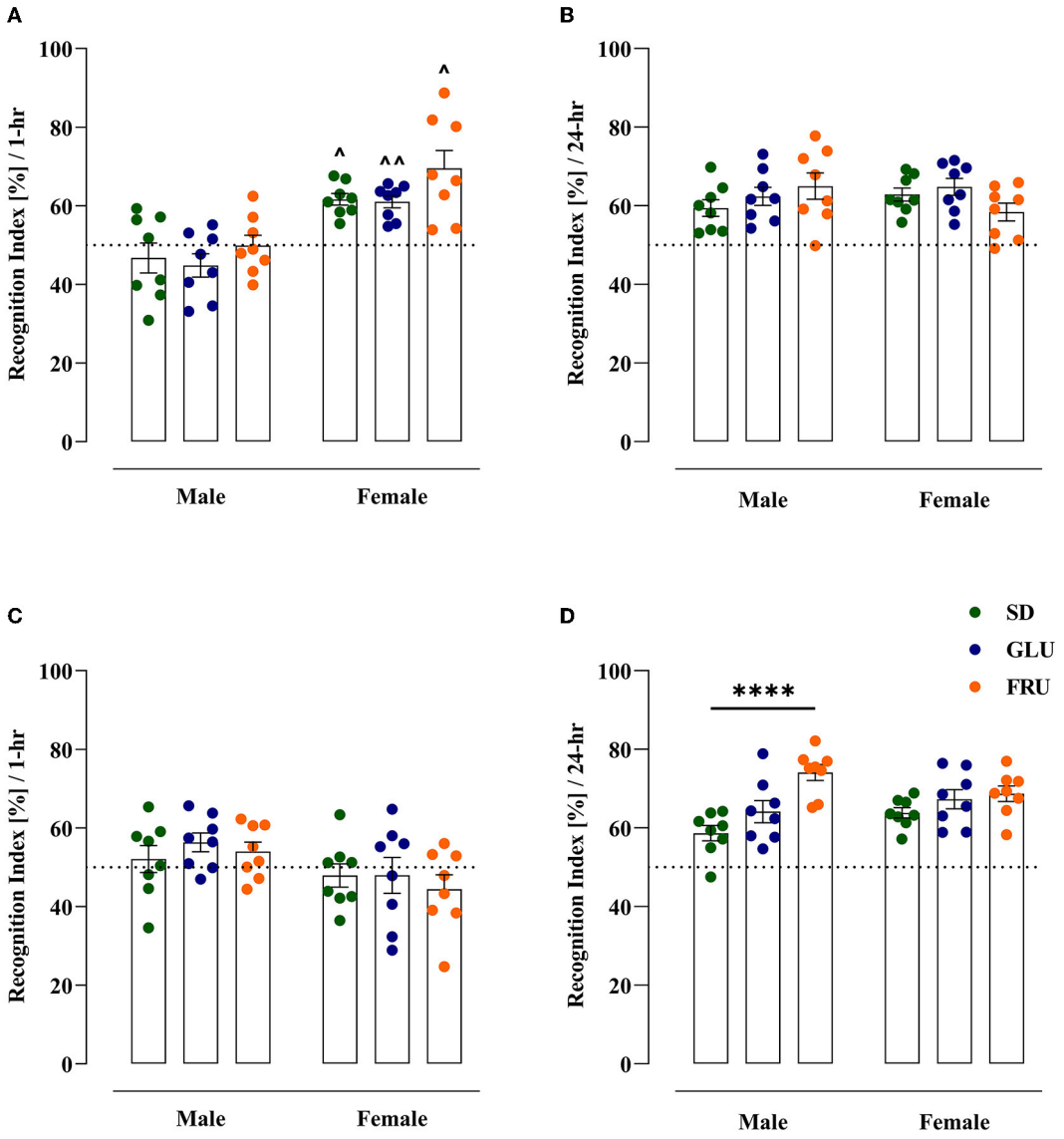

FIGURE 6
Maternal glucose (GLU) or fructose (FRU) diet changed the ability to recognize the new object in adolescent (at PND29) and young adult (at PND61) offspring. **(A)** Adolescent SD, GLU, and FRU female but not male rats showed a greater increase in the recognition index (RI) during the novel object recognition (NOR) test after a 1 h familiarization phase. FRU males and females were characterized by a higher time spent with novel objects than SD and GLU offspring. **(B)** Young adult SD, GLU, and FRU female, but not male, rats showed a decrease in RI after the 1 h familiarization phase. **(C)** After the 24-h familiarization phase, adolescent offspring showed no difference in RI. **(D)** Young adult FRU males showed a significantly increased recognition index in NOR after the 24-h familiarization phase. A larger number of offspring had an RI above 50%, which indicated a preference for the new object. $N = 8$ rats/group. Data are comparable to the SD groups (****$p < 0.0001$, two-way ANOVA followed by Dunnett's multiple comparison test) or between sexes in the same group (^$p < 0.05$, ^^$p < 0.01$, two-way ANOVA with Welch's correction followed by Dunnett's T3 multiple comparison test).

than that in SD rats [$H_{(5,N=48)} = 26.25, p < 0.0001$]. FRU male and female offspring had reduced entrance to the open area (Figure 7B) by 74% [$F_{(2,42)} = 27.83$, $p < 0.0001$] and had reduced stretched postures (Figure 7C) by 40% and 46% [$F_{(2,42)} = 16.22, p < 0.0001$], respectively, compared to SD offspring. In addition, FRU male, as well as FRU and GLU female offspring, showed significant (36%, 61% and 25%, respectively) decrease in head dips (Figure 7D) compared to SD [$F_{(2,42)} = 28.63, p < 0.0001$]. The average number of head dips in FRU female offspring was 38% lower than that in male offspring [$F_{(1,42)} = 4.89, p = 0.032$].

Furthermore, FRU and GLU young adult male offspring demonstrated significant decreases in time spent in the open area (Figure 7E) by 43 and 59%, respectively, compared to SD male offspring [$F_{(2,42)} = 3.70$, $p = 0.033$]. Moreover, FRU and GLU male rats entered the open area (Figure 7F) in 30 and 57%, respectively, less time than SD offspring [$F_{(2,42)} = 7.99, p = 0.001$].

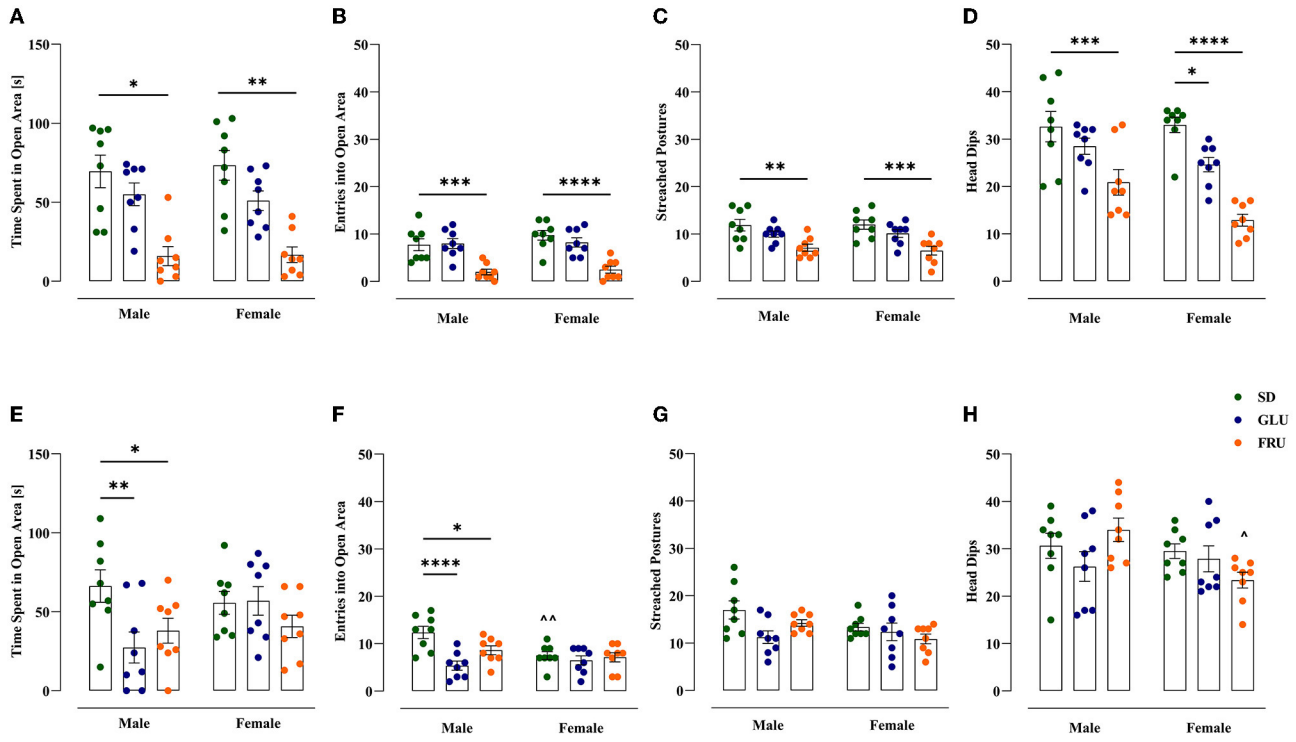

FIGURE 7
Maternal glucose (GLU) or fructose (FRU) diet increased anxiety-like behavior in adolescent (at PND31) and young adult (at PND 63) offspring. **(A–D)** Adolescent FRU males and female rats spent significantly less time in open arms of the elevated zero maze and had a reduced number of entries into the open area, the number of stretched postures, and head dips. Adolescent GLU females showed decreases in the number of head dips. **(E–H)** Young adult GLU and FRU male rats spent significantly less time in the open arms of the elevated zero maze and had a lower number of entries into the open area. $N = 8$ rats/group. Data are comparable to the SD groups (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$, two-way ANOVA or Kruskal–Wallis tests followed by Dunnett's or Dunn's multiple comparison test, respectively) or between sexes in the same group (^$p < 0.05$, ^^$p < 0.01$, two-way ANOVA followed by Šidák multiple comparison test).

Additionally, SD female rats showed 39% fewer entries into the open area (Figure 7F) than male rats [$F_{(1,42)} = 4.58$, $p = 0.038$]. No significance was observed in young adult stretched postures [$W_{(5,19.20)} = 2.45$; $p = 0.070$; Figure 7G]. However, FRU female offspring showed 31% lower head dips (Figure 7H) than male offspring [$F_{(2,42)} = 3.48$, $p = 0.039$].

3.5. Effects of maternal HMD on offspring depressive-like behavior

Finally, we examined offspring rat depressive-like behavior in the forced swim test (FST; Figure 8). In adolescent offspring, GLU and FRU males displayed enhanced (18 and 29%, respectively) time spent immobile [$W_{(5,19.32)} = 6.49$, $p = 0.001$] compared with SD males (Figure 8A). Additionally, compared to controls, FRU male offspring showed 24% decreased swimming behavior [$H_{(5,N=48)} = 16.25$, $p = 0.006$; Figure 8B]. Moreover, GLU male offspring and both FRU male and female offspring demonstrated a reduction (by 24%) in climbing time [$F_{(2,42)} = 6.41$, $p = 0.003$] compared with the SD groups (Figure 8C).

In young adult offspring, significant diet [$F_{(2,42)} = 5.48$, $p = 0.007$] and interaction [$F_{(2,42)} = 4.59$, $p = 0.015$] effects were observed for immobility time. Young adult GLU female offspring showed 16% less immobility than the SD group and a 17% decrease in comparison to GLU male offspring (Figure 8D). SD female rats had a 32% reduction in swimming time compared with SD male rats [$W_{(5,18.11)} = 4.13$, $p = 0.011$; Figure 8E]. Moreover, only FRU female offspring showed reduced climbing time by 55% [$H_{(5,N=48)} = 21.55$, $p = 0.0006$; Figure 8F] compared to SD female offspring.

4. Discussion

The present study showed changes in maternal monosaccharide diet intake and energy expenditure during pregnancy and lactation. For the first time, we presented significant associations between maternal FRU or GLU diet and cognitive, locomotor, and emotional dysregulation in two unrelated offspring cohorts.

4.1. Monosaccharide diet influences body weight and caloric intake

Our study illustrated an alteration in the dam's body weight across a high-GLU or high-FRU diet during pregnancy and lactation. Indeed, FRU female rats had significantly lower body weights than GLU and SD female rats during pregnancy. However, the FRU dam's weights oscillated with the GLU mother's weight

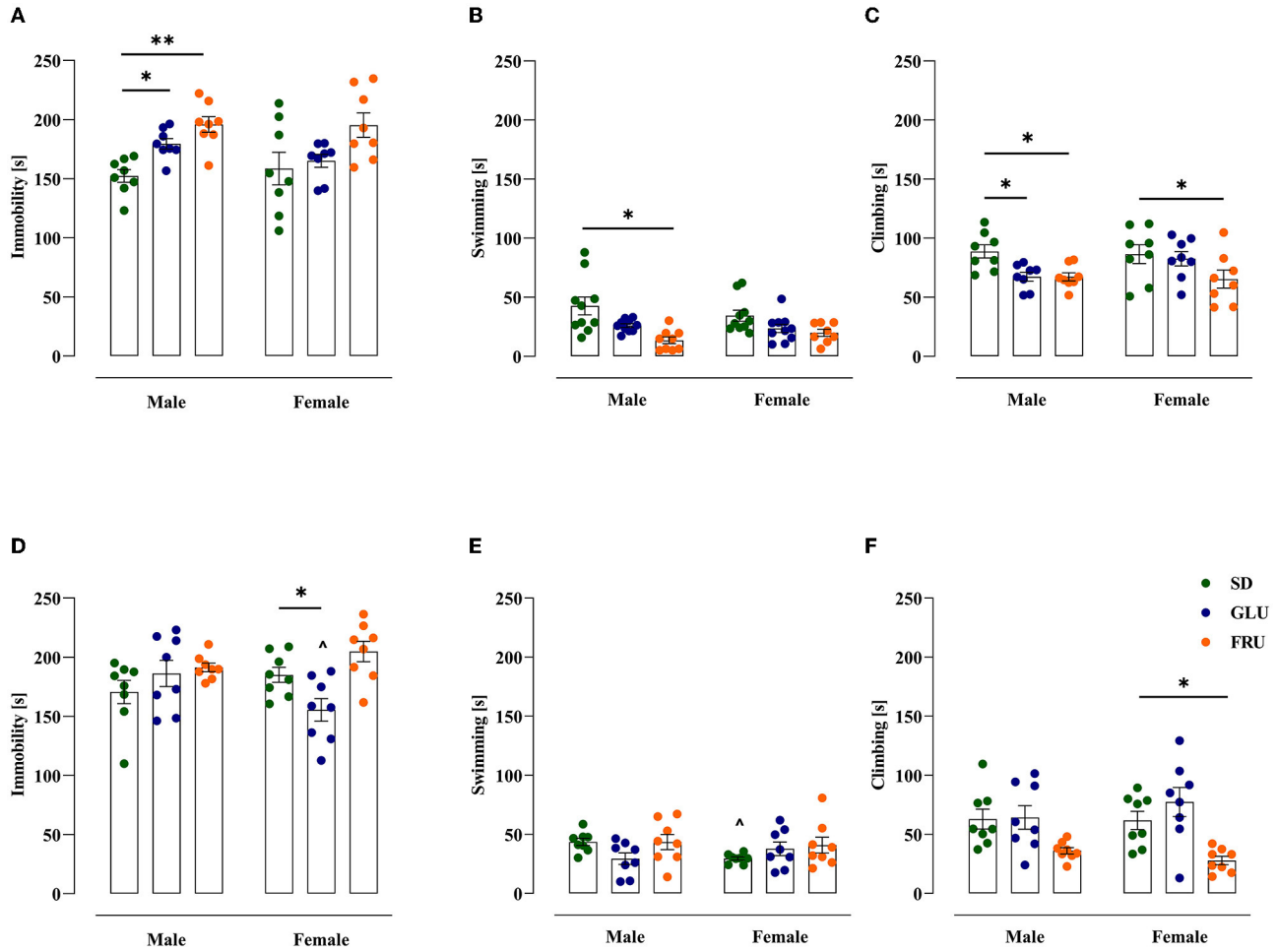

FIGURE 8
Maternal glucose (GLU) or fructose (FRU) diet display depressive-like behavior in adolescent (at PND34) and young adult (at PND66) offspring. **(A)** GLU and FRU diets changed adolescent male vulnerability to depressive-like behavior assessed as increased time spent immobile. **(B)** FRU adolescent male offspring showed decreased swimming time. **(C)** GLU and FRU adolescent male and FRU adolescent female rats reduced the time spent climbing. **(D)** Young adult GLU female rats demonstrated a reduction in time spent immobile in comparison to SD females and GLU males. **(E)** Maternal GLU and FRU diets do not change swimming time in young adult offspring. SD female offspring presented a decrease in time spent swimming compared to SD males. **(F)** Young adult FRU female offspring spent less time climbing. $N = 8$ rats/group. Data are comparable to the SD groups (*$p < 0.05$, **$p < 0.01$, two-way ANOVA with or without Welch's correction or Kruskal–Wallis tests followed by Dunnett's, Dunnett's T3 or Dunn's multiple comparison test, respectively) or between sexes in the same group (^$p < 0.05$, two-way ANOVA with or without Welch's correction followed by Šidák or Dunnett's T3 multiple comparison test, respectively).

during the lactation period. Moreover, neither of these changes correlated with the initial dam's body weight on the first day of pregnancy and lactation. Interestingly, there was no difference in total caloric intake during pregnancy, but as expected, GLU and FRU mothers received more calories from monosaccharides. In contrast to SD dams, GLU and FRU mothers consumed fewer calories during lactation, but no differences in calories from total sugar intake were observed during this period. We found that SD dams, which had a low percentage of monosaccharides in their diet (mainly from polysaccharides and disaccharides), sought to compensate for sugar calories by overeating only during lactation. These changes prove the high energy expenditure of mothers in these two different periods, where sugar is a crucial energy source for the growth and development of the fetus and later for the production of milk (42). Interestingly, GLU and FRU dam's litter sizes did not statistically differ. In turn, the postnatal weights of pups were significantly lower in GLU and FRU dams, indicating disturbances in the fetal stage of the pup's growth and development. Nevertheless, the GLU and FRU adolescent and young adult offspring's body weights during the experiment either balanced or were higher than the body weight of SD offspring. The observed sex-specific differences in body weight between diet groups were typical for any diet experiment (43).

Alternation in offspring body weight during the experiment could be due to maternal metabolic dysregulation during pregnancy and lactation or offspring fetal metabolic reprogramming by maternal GLU and FRU intake. Wistar rat dams fed 10% FRU in the water had increases in blood insulin, triglycerides, and GLU during pregnancy with a reduction in placental GLU transporter type one gene expression (44). Furthermore, FRU intake during pregnancy and lactation increased GLU transporter type five gene expression and glycogen

levels with a reduction in fructokinase gene expression in the Wistar rat liver and caused sex-dependent changes in lipid metabolism in offspring (45). Moreover, Sprague–Dawley offspring rats had a higher postnatal body weight after consuming the maternal 13 or 40% water FRU solution (46). Additionally, in C57BL/6J dam mice, a 20% (w/v) FRU solution increased plasma triglycerides and cholesterol levels during pregnancy and predisposed offspring to obesity and metabolic dysfunction by postnatal high-fat diet intake (47). In the same mouse strain, pups after maternal GLU or FRU intake before and during pregnancy had less fetal body weight that was enhanced after weaning and correlated with fat mass gain compared to control offspring (48, 49). The observed sugar-dependent metabolic dysregulation was strictly associated with the different GLU and FRU biodegradation mechanisms. GLU directly enters glycolysis in each body cell, whereas FRU must be converted to glycolysis GLU byproducts in the liver as the energy source mainly for the citric acid cycle. Sugar overconsumption leads to fatty acid and *de novo* triglyceride biosynthesis, contributing to the development of overweight and obesity. GLU is mainly stored as glycogen in the liver and muscle, while much more fat is formed from FRU and stored peripherally. In contrast to GLU, FRU is the substrate of uric acid production, does not have many intracellular transporters, and is non-regulated by pancreatic hormones (50, 51).

4.2. Monosaccharide diet-induced hyperactivity behavior

In LMA measurements, we demonstrated that a maternal monosaccharide diet significantly disrupted spontaneous locomotor activity in offspring. For the first time, we showed that perinatal FRU and GLU intake increased the distance traveled measured during 5- and 120-min trials in male adolescents compared to male control SD rats. However, in our study, locomotor activity alterations observed in adolescent males were not manifested in another cohort of female and young adult offspring rats. Our study extends the previous observations in another research group using different rat strains. In their study, male Sprague–Dawley offspring rats (at PND50) after maternal 30% FRU-enriched water during pregnancy and lactation had higher vertical activity than females and significantly higher stereotypic behavior than control males in the 5-min open-field test (OFT) (16). Similarly, ICR mouse offspring of both sexes exposed to maternal short-term (E6–E15) dietary manipulations with FRU (30% w/v) supplementation had a specific greater increase in total distance measured in the 20-min OFT compared to the control and sucrose (30% w/v) groups (17). Nevertheless, the effect of maternal HSD on offspring locomotor activity was measured after animal habituation in the experimental cage. Moreover, the dams were fed standard rodent chow with sucrose and FRU supplementation in water. Thus, we measured acute spontaneous locomotor activity in two non-habituated age-independent cohorts at 5- and 120-min trials after maternal FRU- and GLU-enriched diets. On the other hand, it has been shown that chronic postnatal sucrose (25% w/v) or FRU (55% in diet and 10–15% w/v) consumption also increased the distance traveled in the 10-min OFT in male rats (19, 52) and male mice (20).

Hyperactive behavior in rodent studies, characterized by increased or constant movement, is considered a symptom of attention deficit hyperactivity disorder (ADHD) diagnosis in humans. In fact, in cohort and case-control trials, chronic intake of SSBs, soft drinks, and sugar binging enhanced the risk of hyperactivity behavior and ADHD symptoms in children (53). Moreover, hyperactivity and ADHD more frequently occur in boys (54, 55). The mechanism of maternal sugar action on offspring hyperactivity behavior and ADHD development is not yet well-known. Maternal malnutrition or overnutrition during pregnancy and lactation might induce neurodevelopmental effects in offspring. In mouse offspring, maternal sucrose (9 g/kg/day) consumption in pregnancy induces ADHD-like symptoms and leads to a reduction in dopamine receptors (D1, D2, and D4) and increased dopamine active transporters (17). Moreover, the higher duration of the active state has been correlated with higher expression of dopamine receptors in the offspring rat hippocampus after the maternal FRU diet (46).

4.3. Monosaccharide diet-prone memory impairment

In the NOR test, only the FRU diet affected recognition memory associated with short-term memory in response to novel stimuli. In comparison to the SD group, the maternal FRU diet enhanced (by 26%) the memory recognition index in young adult male rats 24 h after familiarization recognition phase II. Moreover, both young adult offspring had RIs above 50% related to a novel but not familiar object preference. Additionally, sex-specific differences were observed in recognition phase I. Female rats had a higher recognition index than male adolescent rats, but these changes were not observed in young adult offspring. In the present study, a higher recognition index observed in FRU young adult male rats could be associated with short-memory impairment or novelty-seeking behavior as a higher ability to perceive novel signals from the environment. These findings are consistent with an earlier report that a maternal HSD (with 40% sucrose) enhanced the recognition index 24 h after the familiarization phase in the two-day NOR test in young adult (at PND65) offspring of both sexes compared to control rats (36). Our results may indicate that recognition memory may not be as sensitive as spatial memory to dietary intervention. Other studies have shown that maternal HSD (with 64% sugar) decreased the preference index in the four-day NOR test in adolescent (at PND28) male Wistar rats. Moreover, a decreased novel location preference index trend was observed in adolescent and young adult (at PND70) HSD male offspring (15). In addition to HSD, a maternal high-FRU (60%) diet resulted in longer escape latency to find the hidden platform of the Morris water maze in adult female offspring, which was reversed by enriched housing (18). Furthermore, maternal FRU supplementation during pregnancy and lactation decreased latency time in FRU Sprague–Dawley offspring rats of both sexes in the passive avoidance learning test (16). A more recent preclinical study indicated that early exposure to a cafeteria diet during pregnancy

and lactation impaired the performance of long-term memory tasks in adult (at PND120) male Wistar rats (56). The controversy in the findings of different maternal HSD studies may be explained by the different duration of NOR schedules, different recognition indexes measuring non-identical maternal diet composition, and offspring diversity (age, gender, and rat strain effect).

It has been well-documented that animals exposed to postnatal HSD intake had enhanced novelty-seeking behavior. Male Sprague–Dawley rats after 10% sucrose (w/v) intake for 24 days displayed an increase in exploration time with the novel object but not with a familiar object in comparison to control males (21). Moreover, 7.9% sucrose intake for 12 months significantly prolonged the time spent exploring a novel object in male Sprague–Dawley rats in the object recognition task (57). Furthermore, after 30% (w/v) FRU feeding for 9 weeks, male Swiss mice showed a higher recognition index than females and control males (22). Interestingly, female Wistar rats exposed to oral FRU (15%) supplementation for 24 weeks in a high fat-low protein diet showed increased time spent in the target quadrant and frequency of entries into the target quadrant in the Morris water maze (58). Despite the findings from these studies, several other studies have reported memory impairment after chronic, postnatal HSD consumption. Thus, postnatal sucrose consumption decreased the discrimination ratio measured in NOR (59), demonstrated poorer spatial memory in the Morris water maze (60), and decreased the amount of time exploring a novel object (61, 62) in rats.

Sugar and SSB consumption in humans influence memory and learning (63, 64). For instance, in a cross-sectional analysis of school children, higher consumption of SSB was associated with poorer performance on executive function and a high risk of executive dysfunction in children (65). Moreover, a higher intake of total sugars significantly impacted the lower prevalence of cognitive impairment scores in adult participants (66). Memory and learning processes are controlled by the hippocampus; thus, HSD can modify its neurodevelopment, neuronal plasticity, and metabolism. In a previous study, 12 weeks of maternal sucrose consumption altered episodic and spatial memory, which was correlated with a reduction in glial fibrillary acidic protein- and Nestin-expressing early-phase neurogenesis cells in the male mouse hippocampus (20). Moreover, FRU, but not GLU, consumption evoked memory impairment in rats and was associated with decreased brain-derived neurotrophic factor in the hippocampus (67). Additionally, male Wistar rats that received an FRU diet for 3 weeks showed increases in glial fibrillary acidic protein, FRU transporter (Glut-5), and tumor necrosis factor-alpha and decreases in synaptophysin, synaptotagmin, and postsynaptic density protein-95 protein levels in the frontal cortex associated with short-memory functions (68). Moreover, adolescent, but not adult, male Sprague–Dawley rats who consumed HFCS (11% w/v) for 30 days showed reduced correct hole investigation in the Barnes maze test with an increase in proinflammatory cytokine (IL-1β and IL-6) protein expression in the dorsal hippocampus (69). A recent paper also linked learning and memory impairment with the downregulation of the Wnt/β-catenin signaling pathway and changes in nervous system development gene expression in the hippocampus of offspring rats after maternal FRU intake (70).

4.4. Monosaccharide diet-induced anxiety-like behavior

For the first time, we demonstrated that maternal monosaccharide diet intake was sufficient to evoke anxiety-like behavior symptoms in offspring rats. As observed in our study, maternal FRU diet significantly evoked a decrease (by 77%) in time spent in the EZM with a reduced number of entries into the open area, diminished stretched postures, and head dips in adolescent offspring rats of both sexes. To be precise, the specificity of anxiety-like behavior results from the 5-min EZM test occurring in adolescent FRU offspring, especially in males, was associated with 5-min hyperactivity observed in the same rats in the LMA. Moreover, decreased time spent in the open area and reduced number of entries into the open area also manifested in unrelated young adult FRU male but not female rats. Interestingly, anxiety-like behavior appeared after the maternal GLU diet in young adult males, but it was not linked with previous GLU intake in adolescent offspring. Our results indicate that a maternal monosaccharide diet can predispose to anxiety-like disorders and/or manifest anxiety-like symptoms across the offspring's lifespan that were observed in male offspring. Similar to our findings, a few previous studies have shown that offspring exposure to a maternal FRU diet evokes anxiety-like behavior, but only when combined with saline treatment (71) or salt supplementation in the diet (72). Furthermore, maternal supplementation with 30% FRU decreased the exploratory activity of offspring of both sexes compared to control groups in the 5-min OFT (16). A recent study also showed that offspring (males and females together), after maternal 13 and 40% FRU in water intake, had higher active state duration and anxious state duration in a 5-min OFT (46).

As mentioned, our study has provided the first experimental evidence suggesting that the maternal monosaccharide diet induces anxiety-like behavior in the offspring. We also demonstrated age- and sex-dependent differences in behavioral phenotypes following the FRU or GLU diet. Our results complement recent studies that have established the relationship between postnatal sugar intake and anxiety-like behavior occurrence. Thus, male rats supplemented with 2 h of access to 10% sucrose (w/v) for 28 days demonstrated a decrease in time spent in the center area of the elevated plus maze (EPM) (23). In addition to sucrose, consumption of 30%, but not 15%, of FRU solution (w/v) for 9 weeks decreased the time spent in the open arms of the EPM in adult (at PND152) Swiss mice of both sexes (22). Likewise, chronic 18 weeks of FRU (23% w/v) feeding caused reduced entries and time spent in open arms of EPM (24), and preadolescent (at PND92) but not adult (at PND132) male rats fed a 55% high-FRU diet for 8–10 weeks reduced the time spent in the open arms of the EPM (19). Interestingly, 8 weeks of FRU supplementation in HFD (17% w/w) and water (10%) was sufficient to decrease the number of entries in OFT in male Wistar rats (73).

Notably, the maternal monosaccharide diet is sufficient to evoke anxiety-like behavior in offspring. Generalized anxiety disorders are characterized by uncontrollable anxiety, fear, and uncontrolled, exaggerated concern over endeavors. According to large population-based surveys, up to 33.7% of the population is affected by an anxiety disorder during their lifetime (74). Currently,

few studies have provided evidence of a clinical or observational trial relationship between higher sugar intake and anxiety disorder events in humans (75, 76). Cohort studies have shown that sugar intake from sweet food/beverages increases the chance of incident mood disorders in men, and there is limited evidence regarding recurrent mood disorders in both sexes (31). A recent study suggested that a maternal FRU diet during gestation and lactation can affect offspring neurodevelopment. Long non-coding RNA interferes with transcript and gene expression crucial for the growth of neuronal cells in the fetal brain (46).

4.5. Monosaccharide diet-induced depression-like behavior

In the FST, we demonstrated that a maternal monosaccharide diet enhanced responsiveness to an acute stressor. In adolescent male rats, both GLU and FRU diets increased immobility and decreased climbing time. Only the FRU diet reduced the swimming time in male and climbing time in female rats. Moreover, in young adult offspring, a specific diet effect was observed in female rats. A GLU diet decreased immobility time, while an FRU diet decreased climbing time. The specificity of the increased depressive-like phenotype observed in adolescent males in the FST confirms the hyperactivity and anxiety-like behavior observed in the same individuals previously tested. To the best of our knowledge, there is a lack of data in the literature showing the effect of maternal HSD on the development of depressive-like behavior in offspring. Thus, our novel results establish behavioral stress-responsiveness after the maternal monosaccharide diet during offspring maturation. Several studies have demonstrated the association between postnatal HSD (mainly fructose) intake and a reduction in struggling and floating time with increasing immobile time. For instance, female Wistar rats treated with 23% FRU for 18 weeks showed increased immobility time (24). Likewise, a 55% high-FRU diet for 10 weeks evoked greater immobility time correlated with an increase in blood corticosterone measured after a 10-min FST in rats (at PND 116–120) of both sexes (77). Similarly, preadolescent (at PND94) male rats supplemented with a 55% high-FRU diet for 8–10 weeks showed an increased immobility time associated with greater corticosterone ejection and decreased struggle (19). A 30% FRU solution (w/v) consumption for 9 weeks increased immobility time in the tail suspension test in adult (at PND151) Swiss mice of both sexes (22). At the molecular level, high-FRU diet intake changed the hypothalamic transcriptome in preadolescent rats, notably corticotropin-releasing factor signaling and pro-opiomelanocortin processing (19).

Behavioral despair is a visible predictor of depression disorder development in humans. Depression is a common mental disorder accompanied by low mood and aversion to activity. Approximately 280 million people, including 5% of adults in the world, suffer from depression symptoms. Depression results from a complex interaction of social, psychological, and biological factors. Human research has revealed that high consumption of foods rich in added sugars and sweetened drinks increased the risk of depression in subsequent years (78), resulting in a high incidence and recurrence of mood disorders (79). More recent studies have confirmed that excessive consumption of sugar-sweetened soft drinks was associated with an increased risk of depression in adults and adolescents in Asia (80). In overweight individuals, increased consumption of SSBs was associated with an increased incidence of a diagnosis of depression (81). A recent cross-sectional study indicated that more than medium SSBs or fast-food consumption may lead to increased stress, depressive symptoms, and suicidal ideation in Korean adolescents (82).

5. Conclusion

In conclusion, our findings highlight the important role of maternal nutrition during pregnancy and lactation in proper offspring development. The results from behavioral screening using LAM, NOR, EZM, and FST tests showed that a maternal monosaccharide diet is a sufficient factor for changes in the emotional status of offspring. Herein, we demonstrated for the first time that maternal GLU- and FRU-enriched diets evoke diet-specific and sex-dependent behavioral alterations in unrelated offspring rats. Since the effects of monosaccharide consumption have not been adequately studied in humans, we suggest that the maternal metabolic dysregulation caused by overconsumption of GLU or FRU during pregnancy and lactation can both reprogram offspring metabolism and/or predispose them to behavioral disorders associated with sugar-dependent mechanisms. Based on the behavioral data, future research focusing on maternal monosaccharide diets may provide important clues to mental disorder development and prevention mechanisms.

Data availability statement

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding authors.

Ethics statement

The animal study was reviewed and approved by 2nd Local Institutional Animal Care and Use Committee (IACUC) in Kraków.

Author contributions

MF: conceived, designed, coordinated the study, and contributed to writing the manuscript. KWi: designed and performed the study, analyzed the data, and wrote the manuscript. KWy: performed the study and analyzed the data. AS: participated in rat generation and behavioral assays. All authors read, revised, and accepted the final version of this manuscript.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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Nicholas Pang,
Universiti Malaysia Sabah, Malaysia

REVIEWED BY
Bintang Arroyantri Prananjaya,
Universitas Sriwijaya, Indonesia
Benedict Francis,
University of Malaya, Malaysia
Eugene Boon Yau Koh,
Universiti Putra Malaysia, Malaysia
Ruziana Masiran,
Universiti Putra Malaysia, Malaysia

*CORRESPONDENCE
Amandine Rochedy
✉ amandine.rochedy@univ-tlse2.fr

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Food socialization of children with Prader-Willi syndrome: an interdisciplinary problematization

Amandine Rochedy[1,2]*, Marion Valette[3,4], Maithé Tauber[3,5] and Jean Pierre Poulain[1,2,6,7]

[1]Université Toulouse—Jean Jaurès, Toulouse, France, [2]UMR5044 Centre d'Etude et de Recherche Travail, Organisation, Pouvoir (CERTOP), Toulouse, Midi-Pyrénées, France, [3]Reference Center of Prader-Willi Syndrome and Other Syndromes with Eating Disorders PRADORT, Children's Hospital, Toulouse, France, [4]UMR1295, Centre for Epidemiology and Research in Population Health (CERPOP), Toulouse, France, [5]INSERM UMR1291 Institut Toulousain des Maladies Infectieuses et Inflammatoires, Toulouse, France, [6]Chair of "Food Studies: Food, Cultures and Health", Taylor's Toulouse University Center, Taylor's University, Kuala Lumpur, Malaysia, [7]Faculty of Social Sciences and Leisure Management and Centre for Asian Modernisation Studies, Taylor's University, Kuala Lumpur, Malaysia

Eating "disorders" of people with Prader-Willi syndrome are frequently reported in the biomedical literature. The eating behaviors are presented as a syndrome-specific trajectory over the course of a lifetime. Infants initially show anorexic behavior, which then develops into hyperphagia that lasts from childhood to adulthood and is characterized by strong cravings for food and relentless thinking about it. However, the sociocultural determinants of these food practices are not fully understood. In the first section of this article, we carry out a literature review of medical articles published on disordered eating in children with PWS. The second section draws on a social science perspective and offers an interdisciplinary problematization using the concept of food socialization. To conclude, the third section explores the challenges facing research and new questions that emerge from the alternative problematization that is the PWS Food Social Norms Internalization (FSNI) theory.

KEYWORDS

food practices, food socialization, interdisciplinarity, neophobia, Prader-Willi syndrome, children, autism

Introduction

Prader-Willi syndrome (PWS) is a rare disease characterized by disordered eating. Manifesting as difficulty sucking and swallowing breast milk or formula in newborn infants, the disease primarily shows up as hyperphagia as the subject gets older, often causing severe obesity. Medical treatment focuses on the difficulties encountered by affected individuals in controlling their eating behavior and the consequences this has on family life. Such an approach emphasizes that, although sociable, children with PWS tend to experience issues surrounding socialization in large part due to their pervasive thoughts (1) about food and their compulsive eating (2, 3).

These issues pose a challenge for the sociological study of their food habits viewed through two aspects of socialization. The first of these relates to social interactions that take place during mealtimes. The second refers to the internalization of social norms that teach children to eat appropriately in a range of social settings. Thus, food socialization is central to a child's psychosocial development and in the development of their identity (4, 5).

The socialization issues experienced by this population can be examined based on: (i) how a child's food likes and dislikes are constructed in relation to the social groups to which the child

belongs and, more generally, the uses of food in wider society; (ii) the internalization of social norms, teaching children to behave "properly" at the table: rules of precedence, cleanliness, quantity ingested, etc.; and (iii) the process of building a social identity.

In children with PWS, we focus on the learning obstacles encountered during the socialization process. One of the frameworks for understanding food socialization is based on the theory of neophobia (6–11), which describes the cycle through which children experience a phase of narrowed food diversity following the construction of their own food repertoire. Recent studies on the socialization of children with autism spectrum disorders (ASD) have shown deviations in the typical process (11–13). Children are assumed to be emerging from the cycle of neophobia when they gain an awareness of other people and that their refusal to follow certain rules may lead to negative consequences.

Through an articulation of the PWS reading grid of nutrition with that of food learning developed by food sociologists, this article proposes to describe, identify and understand the (dys)functions within food socialization in these children whereby they develop the ability to improve their autonomy and enhance the quality of life for both them and their families.

This article is a "Conceptual Analysis" which aims to describe and to problematise in an interdisciplinary perspective the question of the eating disorders of the PWS children by focusing on the internalization of the social norms (5, 14–16). The internalization of food behavior norms allows a child to eat in society. Theses norms concern table manners, such as the use of tools, body control, postures, identification of common and individual spaces at table as well as chewing habits, control of body noises and the place given to pleasure. Learning of these rules takes place during social interactions both during and beyond mealtimes at home with immediate family members (i.e., parents, siblings, domestic helper). Learning also takes place outside of the home, with extended family members, friends, or even at school canteens which drives to internalization of these norms.

In the first section of this article, we carry out a literature review of medical articles published on disordered eating in children with PWS. The second section draws on a social science perspective and offers an interdisciplinary problematization using the concept of food socialization. To conclude, the third section explores the challenges facing research and new questions that emerge from the alternative problematization that is the PWS Food Social Norms Internalization (FSNI) theory.

The peculiar trajectory of food practices in children with PWS

PWS is a rare genetic neurodevelopmental disorder affecting around one in 20,000 newborn infants (17, 18). Eating disorders are a dynamic characteristic of the syndrome, with affected infants displaying anorexic behaviors, followed by the development of hyperphagic behaviors driven by strong cravings for and persistent thinking about food. Very early in childhood, this preoccupation with food pervades daily life to such an extent that some researchers have proposed looking at it as a "food addiction" (3, 19). There is currently no medical treatment available for this condition, and early multidisciplinary management is based on food control: first by optimizing calorie intake from birth, followed by stringent control over access to food in order to prevent severe obesity. Strategies for applying this control are offered and supported by healthcare professionals and implemented by the subjects' families on a day-to-day basis.

The work of the Florida group (20) identifies seven nutritional phases. *In utero* (phase 0), fetal movements are reduced and there is an excess of amniotic fluid (polyhydramnios) probably due to a lack of swallowing. At birth (phase 1a), severe hypotonia is observed, with a certain weakness in sucking, a lack of appetite and difficulty in gaining weight (failure to thrive), prompting nasogastric tube feeding in 80% of cases (18, 21–23). Between 9 months and about 2 years (phase 1b), feeding becomes easier and the child grows at a normal rate. Then, despite no increase in calorie intake, the child begins to gain excessive weight (phase 2a), followed by increased demand and searching for food (phase 2b). In the absence of control, severe obesity rapidly occurs due to excessive food intake, along with pervasive thoughts about food, active searching for food, overeating at meals and lack of satiety (phase 3). In adults (phase 4), some individuals are able to feel full. The overeating and lack of satiety in phases 3 and 4 have been studied extensively (24, 25). The overwhelming preoccupation with food and the impulsive urge to eat are manifested both at mealtimes and at other points during the day, leading to major behavioral issues such as temper tantrums, hitting out at others, repetitive behaviors, etc. (1).

Food therefore plays a central role in PWS, profoundly disrupting daily life. Families find themselves obliged to restrict access to food for children with PWS on an almost permanent basis, both in and outside the home. However, the seven nutritional phases of the eating trajectory are commonly described without consideration for the social environment of the person with PWS.

Three main paradigms are used to elucidate the food practices of people with PWS: genetic, endocrine, behavioral. They coexist without contradicting one another in any way and all take a developmental approach.

Genetic paradigm

Based on its original 1956 description (26), in 1989 PWS was the first condition to be identified as being caused by parental genomic imprinting (27–29). The clinical phenotype is due to a loss of expression of certain genes of paternal origin in the chromosomal region 15q11-q13. In infants diagnosed with PWS, this absence of expression results from a paternal deletion (around 50%), a maternal disomy (around 50%), or much more rarely a deficit of genomic imprinting or a translocation (less than 3%) (18). It is now well established that the mutation of a single gene on the paternal chromosome, *SNORD116*, reproduces the phenotype of PWS in humans and mice, with a developmental anorexia-hyperphagia trajectory (30–32). The expression of this gene is variable during development, while generally having a tightly regulated hypothalamic expression. In mice, the full developmental trajectory of anorexia-hyperphagia is achieved when *SNORD116* expression in the hypothalamus is blocked after weaning.

The mutation of another gene, *MAGEL 2*, reproduces the first phase of PWS in mice and humans, with neonatal hypotonia and anorexia. People with an isolated *MAGEL2* mutation have Schaaf-Yang syndrome, which is considered to be related to PWS, and are frequently diagnosed as having ASD.

Endocrine paradigm

The complete PWS phenotype can be explained by an impaired hypothalamic development and dysfunction. The *SNORD116* and *MAGEL2* genes are involved in the establishment, function and ontogeny of hypothalamic neurons, which secrete oxytocin (OT) (33–36). In addition, studies on neurons derived from induced pluripotent stem cells (iPSc) obtained from patient samples have demonstrated a failure to regulate the maturation of several hypothalamic hormones and other hormones such as insulin and ghrelin. This maturation defect is linked to a deficiency in the proconvertase 1/3 enzyme (PC1/3) and at least partially explains the endocrine abnormalities of PWS, particularly obesity with hyperphagia due to a defect in the maturation of proopiomelanocortin (POMC), other pituitary hormone deficits, and problems with insulin, OT, and ghrelin maturation (37). Eating disorders may be linked to abnormalities in OT and ghrelin: these hormones interact very closely and are strongly involved in brain development and the regulatory mechanisms of the dopaminergic system involved in the reward system. OT is secreted by hypothalamic neurons in the paraventricular and supraoptic nuclei, while ghrelin exerts most of its metabolic and appetite-regulating effects in the hypothalamus. The two hormones also have peripheral effects, particularly on the digestive, cardiac and bone systems, among others. In people with PWS, plasma total ghrelin levels are elevated from birth and remain high throughout their lives (38). Ghrelin comes in two forms: acylated (AG; binding of a fatty acid to the peptide chain) and un-acylated (UAG). AG has a strong orexigenic effect (leading to it being known as the "hunger hormone"), whereas UAG inhibits the effects of AG and therefore has an anorexigenic effect. The ontogeny of the ghrelin system is different in PWS and may explain the anorexia-hyperphagia trajectory and the metabolic disorders (39). From our perspective, hyperghrelinemia could also be involved in obsessive thinking about food and compulsive eating described in people with PWS. Indeed, ghrelin is involved in addictive behavior, kleptomania, and certain neurodegenerative pathologies. In mice, it has been shown that ghrelin peaks are regulated by the frequency of meals. The ghrelin system is involved in the "food entrainable oscillator"—a system that is modulated by food intake and induces a motor response that makes it possible to seek and obtain food. People with PWS have an "ultra-sensitive food clock," possibly related to high ghrelin levels, driving very precise anticipation of mealtimes. OT is involved in both the control of satiety and behavioral disorders (social skills, regulation of emotions). The abnormalities of these two very intricately connected hormonal systems in subjects suffering from PWS go some way to explaining their eating behaviors. OT plays a fundamental role in establishing the sucking reflex and the pathways involved in orality, as well as in the regulation of the neuro-vegetative system (40). These functions are deficient in PWS to varying degrees. Ghrelin and OT work through receptors in the brain, particularly the dopamine neurons involved in the reward system, and in many other organs (41).

Behavioral and eating disorders

Oral disturbance is present in PWS from birth and persists throughout the sufferer's lifetime. Regulation of the sucking-swallowing reflex is present from birth, and voluntary control subsequently occurs with learning. The maintenance and development of sucking skills help to establish the neural networks involved in the control of feeding and is also utilized in social learning. The orbitofrontal cortex contains neurons that respond to food stimuli and those that respond to social stimuli, with interactions between these two neuronal populations (42).

From the age of 7–8 years, food impulsivity echoes general impulsivity. In adults, compulsive eating can be present alongside other addictive behaviors, mainly smoking and, more rarely, alcohol abuse. Alongside the theft and storage of food, compulsive theft similar to kleptomania and the storing/collection of objects is often observed. Notably, these behaviors described in relation to food (frustration, bargaining, rigid thinking, difficulty in diverting attention, perseverance, theft, anger, etc.) have been observed to be intricately intertwined with all other areas of daily life. A hyperphagia questionnaire specific to PWS was developed (24, 25), dealing with: (i) behavior: describing the times, frequency, actions and bargaining involved in obtaining food; (ii) drive: describing the strong need or desire (impulsivity) to speak about or consume food, as well as the difficulties encountered in diverting the sufferer's attention from food, their perseverance, and avoiding frustration and anger; and (iii) severity: describing the extent to which the person is invaded on a daily basis by thoughts, words and actions relating to the search for or consumption of food. A high level of anxiety and restlessness before sitting down at the table has been observed, with individuals suffering from PWS becoming preoccupied about mealtimes and the quantity and quality of their food. Meal intake is either very rapid and even voracious, with significant risks of aspiration and suffocation or, conversely, excessively slow. People with PWS have been known to carefully collect every last crumb, entirely clean their plates, repeatedly scrub at the bottom yogurt containers and make exaggerated comparisons with the contents of the plates of other people sitting around the table.

Despite a proven deficit in executive (e.g., problem solving) and planning functions, people with PWS can develop highly elaborate strategies to carry out the steps necessary to access food that has been locked away. Parents often note that their child's intelligence is entirely directed toward finding and consuming food, although it is possible to divert their thoughts with other pleasant activities (games, television, etc.).

Family management of feeding practices

The announcement that a child is suffering from a serious pathological condition can significantly disrupt family life. The day-to-day strategies put into place to cope with disabilities or the child's differences have been studied within families (12, 43, 44), especially among mothers (45). With PWS, families and caregivers regularly discuss the adjustments that need to be made to avoid either imposing excessive restrictions on food, or relaxing these restrictions "too much" or "too frequently." According to the literature, the earliest family strategies thought to be "most effective" for food and weight management were: locking up food, constantly monitoring the child while he or she eats, and offering only low-calorie foods as snacks (46). More recent strategies have been suggested, including maintaining the child's involvement in activities and the use of routines within the family unit (47). An American team proposed an adage concerning

meals: "No doubt, no hopes, no disappointment"[1] to reassure and reduce the anxiety of people with PWS, especially those living in an institution. An environment of benevolent empathy has been shown to help contain, reassure and support such people. Conversely, a lack of a supportive framework or displays of excessive authority significantly worsen their behavior and anxiety levels. The important thing appears to be supporting them in their efforts to acquire autonomy in areas of their lives, but always with the understanding that the objective is not full autonomy. For example, talking about and anticipating any temptations or opportunities that may arise (in stores, vending machines, etc.) helps people to achieve a feeling of independence and a legitimate degree of freedom. In addition, the healthcare professionals at the PWS reference center[2] remain attentive to vulnerable families with poor adaptive skills (48).

Food socialization: a new point of entry for understanding eating practices

Eating behavior is a complex phenomenon. It results from the interactions between more or less "genetically" controlled or biologically overdetermined predispositions and social learning, which itself varies from one culture to another and can even be affected by a person's social position. From birth, the role of influence is powerful. The malleability of behavioral patterns allows for adaptation to a range of contexts. Moreover, learning affects predispositions, either amplifying or reducing their plasticity. The ability to implement eating behaviors suitable for a given social context and culture therefore results from the interaction between dispositions and learning. "Food socialization" refers to these interactions that occur over the course of a child's development and lead to the capacity to adopt behaviors adapted to social contexts.

The place of culture in eating habits

Human eating behavior is determined by biological and sociocultural factors. It is primarily determined by the biological status of the species as an omnivore. This is characterized by possibilities, digestive capacities, and constraints (the methods of breaking down food into nutrients, the synthesis and storage of certain nutrients, etc.). Certain reflexes should also be noted: sucking, swallowing and even preferences and dislikes for certain flavors. But eating behavior is also influenced by sociocultural processes. Although omnivore status defines predispositions and incapabilities, it has certain areas of freedom within which choices can be made without biological consequences (at least in the short term). Food can be ingrained in social mores. Within the broad set of potential foods, each culture selects specific foodstuffs and develops an "edible order" (4). How meals are presented and eaten, table manners, and rules of precedence are all ways a society presents its values at a given point in time (50–53). Meals provide opportunities for children to internalize the social norms and rules of conduct that prepare them to behave "properly." These "social food spaces" have areas of freedom, giving rise to the expression of cultural diversity where the processes of social differentiation take place (5).

Yet although the distinction between biological and sociocultural determinants is useful, it is also appropriate to examine the interactions between them. Culture has an impact on genetics by participating in modes of selection, transmission and dissemination of genes in society. Examples of this are kinship rules (54). Their specific details (prescriptions and prohibitions) affect the availability of certain characteristics at the level of population genetics and in the processes of phenotype expression or non-expression. Indeed, a new body of knowledge has arisen to examine these very issues: nutrigenetics (55–57). This field is restructuring the relationships between the biological sciences and the human and social sciences (58, 59). Interestingly, *in utero* conditioning has been highlighted, with studies finding that the taste of amniotic fluid changes with what the mother eats to the extent that the child becomes accustomed to frequent flavors in the food culture into which he or she will be born (60). The culture in which an individual is immersed therefore intervenes even before birth.

Food at the heart of the socialization process

Genetic heritage and the food model of the society into which a person is born are therefore somewhat established without any choice being made by the individual. Babies come into the world in a state of "dependence" and "incompleteness" (61). Initial programming enables them to suckle, digest breast milk and prefer sweet flavors, but everything else has to be learned, from using the senses to eating behaviors through to the acquisition of table manners. This learning takes place in sociocultural contexts and through social interactions. It is necessary to ensure the processing of information and especially to semanticize it, or to give it meaning (62). Learning about food also make it possible to attribute a scale of magnitude to sensations, which are both personal and derived from the norms of a person's social group. Food lies at the heart of the socialization system. When learning to eat, children set up behavioral modes useful for implementing and controlling this biological activity and simultaneously internalize the value system of the social group into which they were born.

Research in psychology has elucidated the modes of passing from sensation to perception, as this is essential for generalizing, categorizing, and ultimately constructing the "lived world." This transition is a decisive step in "learning to eat" as it allows an individual to build a repertoire of edible products and appropriate behaviors. This type of learning occurs through observation and imitation of adults and peers. The appropriation of food repertoires takes place over the course of the different socialization stages and in a range of social contexts (family, school, recreation center, etc.). Sociologists and psychosociologists emphasize that food learning begins early on in life within a web of emotional and relational contexts. Emotion and hedonism thus play a

1 https://pittsburghpartnership.com/downloadable_educational_materia2.htm

2 Since 2004, in France, PWS reference centre has three main goals: optimize patient's diagnosis, care and management throughout life, inform and train health professionals and develop and/or support clinical, epidemiological and basic research projects (49).

major role for children and remain the driving force behind their relationship with food over the course of their lifetime. For this reason, cognitive factors, though undeniably important, are not enough to learn or modify eating practices. Here, practices that are learned take precedence over those that are innate.

From the earliest days of sociology, food and food socialization have always occupied a special place. Émile Durkheim (63) sought to mark out the territory of this new discipline and distinguish it from biology and psychology. He proposed two methods for defining its focus on "social facts." The first definition specified what could not be deemed a "social fact." Food was the first example given because it was "too biological." The second definition listed the conditions for inclusion within sociology and among them was food once again, this time because of the rules of conduct that "are imposed on the individual from the outside." Food was therefore excluded from sociological study when it came too close to biology but included when it came to the customs, rules and social norms that defined its implementation in society. Durkheim's objective was to delimit an autonomous epistemological space in which he sought out the root of one social fact in another. Food is central to socialization and the transmission of these systems of norms from one generation to the next. It is thus part of a process of social integration and regulation that enables an individual to find his or her place in a social group and to be recognized as a member of the group. Sociology has therefore often focused on the social "institutions" that frame meals (16, 64, 65) and the social functions for which eating behaviors provide support.

Another tradition emerged from the work of Marcel Mauss (66) and his concept of the "techniques of the body" viewed in their social, psychological and biological dimensions. The conditions for an interdisciplinary approach came together in this pioneering work, but the focus remained on the influence of the social over the biological, with psychology relegated to a secondary role of articulation. These two traditions still weigh heavily on sociology today.

The traditional concept of the social fact has continued through Bourdieu's theory, with concepts of "incorporation" and "habitus" for identifying how social position influences bodies and tastes (67, 68). The concept of habitus juxtaposes two sides, one passive and the other active. The first refers to internalization during the socialization phase and the other ensures the organization and cognitive structuring of a situation after the end of this phase (69). From this perspective, the notion of "disposition" refers to a propensity to act. The emphasis, however, is more on the social origins of individuals and the consequences for their choices and tastes than on the construction of dispositions.

The second tradition is clearly interdisciplinary, and this is exemplified by the research on neophobia. A new definition of the concept of "incorporation" bases itself on what a person might imagine is happening during the act of ingesting food, thereby taking into account its psycho-sociological consequences. Incorporation from this perspective has two dimensions: (i) imaginary: symbolic appropriation, signs, norms, etc., and (ii) social: sharing norms and representations (7, 70).

However, both traditions are primarily concerned with the results of socialization and less with the dynamics of the process itself. It was not until the development of the sociology of childhood (71) and then of its connection with food (72–77) that the focus began to shift to the processes of socialization.

Psychology, however, began to examine childhood development very early on. Three major theories, all of which complement one another and which form one part of the whole, have dominated the landscape (78): a psychoanalytic approach focused on affective and sexual development (79–81), a cognitive theory of psychology (82) and a psychosociological perspective (83). These three theories share a conception of development as occurring in stages. This refers to a succession of stages during which a person's internal organization and functioning are more or less stabilized. The stages follow on from one another, each incorporating the features of the previous stage in an ever larger and more complex structure. Looking at these three theoretical perspectives from the outside reveals their dual complementarity. Firstly, when one theoretical framework becomes partially effaced, another increases in importance. One example of this is the lessening in importance of the psychoanalytic "latency period," which results in a reduction in the libidinal problematic and an increase in the importance of intellectual and cognitive development. Secondly, each theory produces information about the same stage that sheds light on aspects that complement each other (84). Erik Erikson (85) can be credited with the idea that social identity comes as a result of a developmental process through various successive stages that extends over a person's entire life.

Food neophobia, a tool for studying socialization

The concept of "food neophobia" has been used in two distinct analytical scales. From an anthropological perspective, it accounts for the ambivalence in the relationships that eaters maintain with food (fear of the unknown versus the search for novelty). Human eaters are faced with what has been called the "omnivore's paradox." The inability to synthesize certain nutrients requires them to have a varied diet, yet these foods may be potentially dangerous. Eating can therefore fluctuate between a fear of the unknown, prudence, the anxiety of incorporation (neophobia) and the search for novelty (neophilia). Human eating behaviors thus often manifest themselves in a back-and-forth between neophobia and neophilia, thereby generating a certain level of anxiety. Linked to incorporation, this is regulated by our culture surrounding food, which establishes a system of what is edible and associates symbolic representations with food (5).

The process of neophobia can be analyzed from two perspectives: the inter-individual variations in the development of a food repertoire and the intensity of neophobia. In the absence of a quantitative survey, our current knowledge about typical children offers an ideal-typical view (6). The process of neophobia can be broken down into several stages (Figure 1) (9): (1) A milk diet, (2) food diversification followed by the neophobia itself characterized by (3) closing off of the food repertoire, (4) stabilization, (5) reopening of the food repertoire and, finally, (6) the establishment of a more or less permanent food repertoire. Breaking down the neophobia process into stages makes it possible to identify pivotal moments and gain a deeper insight and therefore to act in more helpful ways.

Neophobic behavior in children is observed from the age of 2 years, albeit with individual variations (7). More than three quarters of children go through this stage of neophobia in a more or less pronounced way, classified into four degrees of severity (8).

Research in children with ASD showed that neophobia revealed a number of deviations from typical processes (13). In the first sub-population, children presented with "typical" neophobia, but the intensity varied in strength. In the second subset of population,

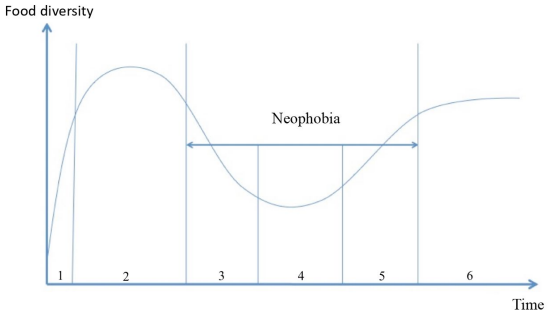

FIGURE 1
From milk to the adult food repertoire.

neophobia did not present in its classical way. In the "progressive" form, neophobia gradually increased from birth, while in its "late" form, neophobia started occurring at the age of 7, and in its "neophile" form, children did not exhibit neophobic behavior. This typology, in terms of the construction of the food repertoire, indicates that phenomena that have often been identified as specific to the autistic population in fact stem from deviations—prolonged, intensified, a combination of both, absence or delay—in the construction of food neophobia and its processes. It is therefore important to refer to food neophobias in the plural. The study highlighted a number of social and medical factors in the types of processes (12). ASD is associated with the genetic abnormalities of PWS (86) and similar behavioral symptoms are observed in the two populations (87). To cite just one example: altered social interactions with a specific weakness in interpreting social information and in responding to them. Therefore, it will be interesting to better understand the place of neophobia in the development of eating practices among children and adolescents with PWS.

Food neophobia and stages of development

Poulain (9) synthesized a number of theories surrounding the development of children with neophobia. To describe the dynamics of establishing a food repertoire, we can distinguish different stages, although they remain closely related to their preceding stages.

Initially, children consume only one type of food: milk. According to psychoanalytic theory, this is the first part of the oral stage, known as "gratifying" swallowing, during which the child and mother maintain a symbiotic relationship.

The second phase, the "transition" between milk and solid food is characterized by the food repertoire starting to open up. First, new foods are introduced in liquid or semi-liquid form (purée) alongside milk. Solid foods are then introduced. In this stage, many children will eat almost anything offered to them and show few food preferences. The food repertoire is wide open, only limited by the child's culture and family. This period is the second part of the oral stage, which begins with teething and during which the child experiences the pleasures of biting and chewing. During the two parts of the oral stage, the child puts many objects in its mouth and taste is marked by low discrimination, with an appetite for sweet flavors and a more or less clear rejection of sour and bitter tastes.

The onset of neophobia may occur during teething. These children will then experience the sucking/biting dilemma. They feel the urge to bite the mother's nipple, but if they do, she withdraws her breast. They thus enter a world of ambivalence about: (i) a desire that forms the basis for the "splitting of objects" and access to the "object world," and (ii) the objects which, from then on, can be "good" and "bad" at the same time (88). As opposed to the previous stage, neophobia is characterized by a narrowing of the edible space. At this stage, children refuse new foods and often temporarily abandon foods that were once deemed acceptable (11). In doing so, each child asserts personal tastes and preferences and a decision whether or not to eat (89). The intensity of the reduced number of foods and the duration of the phase varies from individual to individual. The neophobia cycle has three stages: narrowing, low stabilization and a reopening of the edible space. During these stages, social interactions take place with the adults who provide food and fellow eaters. They contribute to the socialization and construction of the personal identity of children through the internalization of a set of norms and rules relating to eating and their eating behavior itself. The appropriation of these norms contributes to the recognition of an individual as a member of the social group, the family first of all and then the other social groups to which he or she belongs. Children thus become recognized and accepted as members of the groups in which they socialize. Moreover, some leeway is acceptable in applying the group's norms and rules, and this allows children to develop a style that contributes to the process of constructing a personality. Developmental theories have identified phases of opposition to parental authority during which children assert themselves as individuals. Neophobia arises at the time of one of these phases and is therefore seen as an expression of opposition that is a normal stage of development. For Henri Wallon, for example, the period of 3 to 6 years old is the "stage of personalism," during which children tend to oppose adults in a kind of "negativist crisis." At this stage, the child is expressing ambivalence about a model embodied by adults. Wallon distinguished three sub-stages to this stage. In the first, children clearly oppose the adult. In the second, their behavior becomes much more accommodating, and in the third and final stage, they try to imitate the adult (83). Some have termed this period the "no phase" (90). The result of a long process of somato-psychic maturation, it opens the way to human communication and initiates the process of developing the personality (91). In this context, food plays a particularly important role. By refusing certain foods, children engage in power struggles with their parents and seek recognition as individuals with likes, dislikes and preferences. This recognition also involves the internalization of behavioral norms that frame the act of eating. By affirming personal choices, they begin constructing their food repertoire. Chiva (62) assumed that neophobia contributed to the process of attributing meaning to sensory experience and precipitated the passage from foods viewed as being "for us," referring to the family, toward the foods that are "for me." To semanticize means to attribute meaning and valence to a sensory experience on hedonic and moral scales. It is essential to the individual's orientation and development of future choices, and it complicates and gives meaning to the rudimentary basic flavors of sensory psychology.

After the reopening stage, children assert what will essentially be their permanent food repertoire. To do this, they select from the range of foods available within their culture and family. The origins of food preferences and dislikes have not been settled and may also be determined by social influences, psychological issues, allergies, deficits in digestive ability, and so on. Advances in epigenetics could help clarify this issue.

A systematic approach to the successive stages of the three main theories of child development with regard to the food neophobia cycle is put into perspective with the food peculiarities of children with PWS (Table 1).

TABLE 1 Neophobia processes, developmental stages in child psychology, and food practices of children with PWS.

Neophobia process		-	1. Feeding with milk/ diversification Accommodation, adaptation of breastfeeding	2. Food "for us" Accepts almost everything offered	Neophobia 3. Closing of the food repertoire 4. Low plateau 5. Reopening of the food repertoire		6. Food "for me" Construction of food categories Fixation of (dis)likes and personal categories			
Developmental psychology	**Affective & sexual**	-	Oral stage Harmony Symbiotic relationship with mother	Oral stage Sadistic Teething. Pleasure of biting. Splitting of objects	Anal stage Libidinal investment of the digestive system (from mouth to anus)	Phallic stage	Oedipus complex Triangulation of the relationship	Latency period	Adolescence Reactivation of the Oedipus complex	Adult
	Cognitive	-	Sensorimotor intelligence Cognitive development occurs while manipulating objects				Concrete intelligence Abstraction possible based on concrete data		Abstract intelligence Reasoning disconnected from the concrete	
	Psychosocial	-	Motor and emotional impulsivity	Sensorimotor projection 1. Practical intelligence manipulation 2. Representative intelligence	"Personalism" stage 1. Opposition to adults 2. Accommodation 3. Imitation		Categorization stage Intellectual activities of categorization and voluntary memorization activities		Adolescence	Adult
PWS	**Feeding phases**	Phase 0 Sucking disorders Hypotonia	Phase 1 A No sucking Nasogastric tube feeding	Phase 1 B Before the switch Bottle feeding improves, but with lower appetite than "normal"	Phase 2 A After the switch Appetite for food improves but weight gain exceeds what is "normal"	Phase 2 B Hyperphagia Increased interest in food	Phase 3 Adolescence Difficult phase of worsening hyperphagia			Phase 4 Diminution in hyperphagia and appetite in some cases
	Developmental stages	-	Does not breastfeed Does not touch the mother Weak interactions and difficulty in engaging (weak cry, few limb movements, pleasant)	Plays rarely with mouth or hands No apparent pleasure in biting Limited motor capacity (no crawling, late to walk, etc.)	No apparent feeling of the digestive system Little interest in cleanliness (no apparent anal stage)	No filter on food (will eat unprepared products, animal feed, pica) Fusion with mother more frequent Emotional lability Cognitive deficits Deficits in communication, pragmatics, social skills and theory of mind, temper tantrums (associated with frustration)	Intense affective demands, awareness of being different, low self-esteem Worsening behavioral and psychiatric disturbances Difficulty socializing and projecting self Some features of ASD			-

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This systematic approach to the theories and their dimensions has been fundamental in the development of our research questions and the problematization of PWS.

Toward a PWS food social norms internalization theory

This Food Social Norms Internalization (FSNI) theory articulates three main concepts: the internalization of food norms, neophobia and familialization. It claims that the incorporation of social food norms that takes place as part of social interactions with family members (parents, grandparents, siblings, etc.), is disrupted by PWS. This disruption is primarily derived from the bio-psycho-developmental side of the syndrome itself, mainly the trajectory of eating disorders, from anorexia to hyperphagia. Secondly, it comes from the structure of control systems set up by caregivers to manage the issues facing affected individuals in controlling their eating behavior.

Food Social Norms are rules related to table manners such as the use of tools (spoons, forks, knives, chopsticks, etc.), the identification of shared and personal spaces at the table, postures, chewing habits, body control, body noises, as well as the importance attached by families and cultures to pleasure. The appropriation of these social norms and rules allows a child to eat in society "normally." "Neophobia" refers to a developmental phase in which children may experience food restrictions, refusing certain foods and coming into conflict with their food caregivers. This "neophobia" phase plays an important role in the internalization of social norms and more generally in the socialization process. The concept of "familialization" was first used to analyze the transfer of activities and responsibilities from the family to state or private care systems. "De-familialization" describes the transfer of activities from the family to care systems and "familialization" from care systems to the family (92). This concept was used to develop international comparisons (93–95) and to study the consequences for gender equality (96). But "familialization" has also an alternative meaning. In a situation where one family member faces a chronic illness, their family members will typically use information and advice provided by health professional experts to re-structure their everyday life, including the distribution of parental roles (97, 98). This is the meaning to which we refer within FSNI theory. The familialization process therefore relates to how family members take on messages and advice formulated by health actors at the time of diagnosis and during the management of patient care.

For PWS children, the process of food socialization is much more complex than for their unaffected peers. The syndrome places eating behavior at the absolute center of family life. The syndrome itself modifies appetite and eating behavior, and disturbs the neophobia process. It also dictates the different ways to enact control over eating behavior as implemented by family interactions (familialization). This is the highly specific context of food socialization among PWS children (Figure 2). How do family members provide meaning to social norms surrounding food and reformulate them? How does this information then translate into a food management strategy? Who are the players and what are the roles played within the family to manage any food strategy? All these questions are linked to the role of familialization in shaping

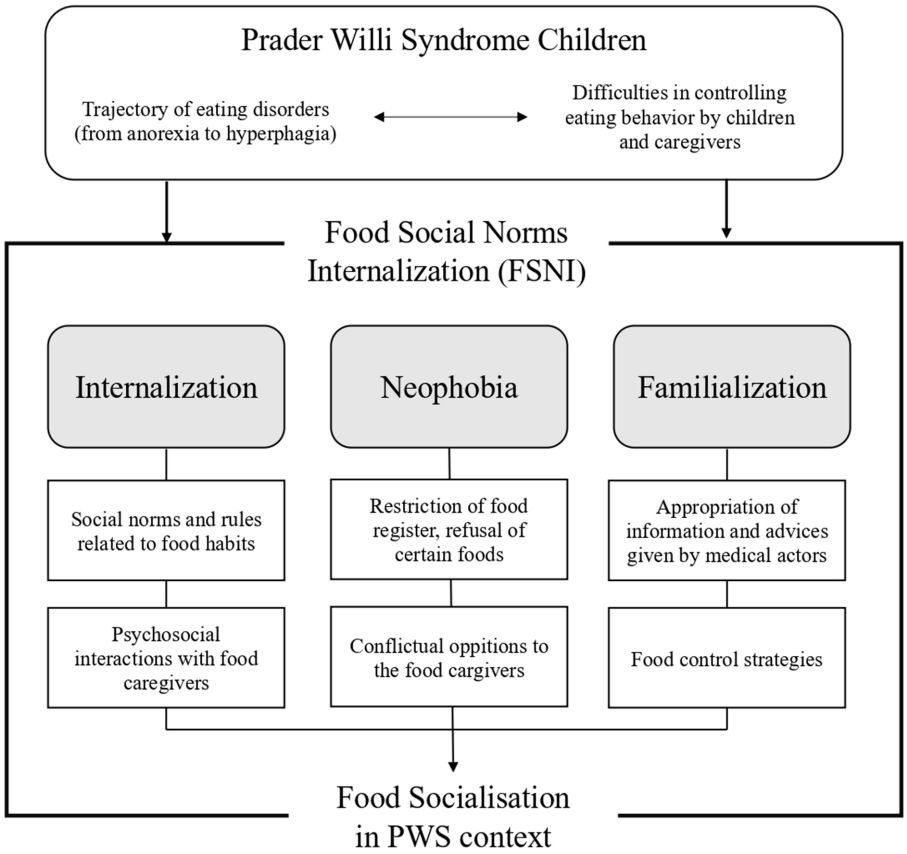

FIGURE 2
From eating disorder to food socialization. Internalization of food social norms in the context of Prader-Willi Syndrome.

food socialization among PWS children. Exploring these concerns may also provide an insight into the extent that strategies put in place by parents could either facilitate or disrupt learning, the internalization of norms and food empowerment among PWS individuals. This theoretical framework enables the development of new research focusing on the process of the internalization of social norms and their specific nature among PWS children. What creates an obstacle to learning in children's behavior and to their interaction with their surroundings.

This re-problematization opens up new avenues of research likely to enable the development of new ways of providing care. From research standpoint today, the four main active lines are:

- a genetic approach, identifying the specific genetic characteristics of people with PWS;
- the endocrine approach, which explores hormonal dysfunction due to abnormal hypothalamic development, which is said to more or less result from genetic configurations;
- a behavioral approach, describing the disorders and their changes running alongside psychosocial development; and finally,
- familial management of the care for PWS children.

By focusing on the issues posed by the internalization of social norms relating to food, the proposed re-problematization links the approach made to the eating disorder itself with the approach to family-based management. This new perspective emphasizes the role of social interactions in the process of internalization, including the conflictual ones.

Interdisciplinary questions will emerge from this view of the above classical approaches. For example, (i) the difficulties of internalization could be the starting point for a new description of the phenotype; or (ii) taking into account the stages of the PWS eating disorder trajectory, from anorexia to hyperphagia, developments could be made in adaptive and evolutive food education strategies. Last but not least is the traditional relationship between what is normal and what is pathological, which has shown how knowledge developed about a pathology can simultaneously further our knowledge on what is said to be normal (99, 100). As often, by studying pathology, we can expect a better understanding of normal processes. Examining eating disorders among PWS children as part of a general theory of socialization could be a way of advancing knowledge about the phenomenon of the internalization of food social norms among other eating disorders and even among typical children.

From the point of view of medical care and family management, the knowledge gained through such an examination could be used in at least two different ways. Firstly, on slowing down the pressure of external control by food caregivers and secondly on the "nutritionalization" of food experiences (the reduction of food to its weight and nutritional components) (101). Thus, food education and the daily food life of PWS children would leave more room for practices promoting the internalization of norms and the establishment of self-controlled routines. Conflicts between children and parents would not only be viewed as negative experiences, but also considered as a process of negotiation more likely to benefit the internalization of norms.

The perspective suggested would allow us to develop new methods of education and a comprehensive management for health professionals by integrating food social norms surrounding the concepts of internalization, neophobia and familialization. This would entail making efforts in training health professionals to include these concepts in routine care, by giving an insight into these concepts and taking into account all these dimensions for each child within a family context. This comprehensive approach may avoid or strongly mitigate any harmful effects of care and ensure the best possible level of food socialization.

Finally, some strategies already implemented successfully by some parents can be re-analyzed in the light of the FSNI theory and give rise to positive therapeutic education strategies involving the PWS community.

Conclusion

Many scientific and practical issues converge on the food socialization of children with PWS. The neophobia approach restructures the issues on "eating disorders" and how to act because it focuses on issues surrounding the acquisition and learning of the systems of norms that allow a child to eat "normally." By focusing on the difficulties of acquiring systems of norms allowing a child to eat "normally," the neophobic approach reorganizes the problem of "eating behavior disorders." This perspective opens up new ways of acting by inviting caregivers to move from external control to the search for conditions that could help the child to internalize norms and thus to empower, more or less depending on the case, his behavior. For example, this could be: patient family group discussion between parents on the question of neophobia and the function of "conflicts" in the food socialization process. Introduction into the clinical examination of questions relating to neophobia and food socialization by training health professionals who followed children and particularly young children and their families on the FSNI theory. This would help to facilitate and at least to prevent possible impaired neophobia.

This problematization invites to look at the interactions between biological determinants and determinants within the social environment. Hierarchies of determination may be revealed, opening up fresh insights and giving rise to new research questions on both sides. This will potentially create the conditions for an interdisciplinary dialog between professionals in food sociology, developmental psychology and pediatrics. The outcome of this interdisciplinary approach open news perspectives of research between medical sciences and social and human sciences. It may also help to redefine methods designed for intervention and care among PWS children and improve the support offered to their families.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary material, further inquiries can be directed to the corresponding author.

Author contributions

AR, MV, MT, and JP contributed to formulating the scientific question, designing the research protocol, and drafting the manuscript. MT and MV inventoried the knowledge on eating disorders in children with Prader-Willi syndrome from a medical perspective. JP and AR did the same for food socialization from the perspective of the human and social sciences. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

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Nicholas Pang,
Universiti Malaysia Sabah, Malaysia

REVIEWED BY
Walton Wider,
INTI International University, Malaysia
Eugene Boon Yau Koh,
Universiti Putra Malaysia, Malaysia

*CORRESPONDENCE
Shuang Zang
✉ zangshuang@cmu.edu.cn

†These authors share first authorship

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Associations of lifestyle with mental health and well-being in Chinese adults: a nationwide study

Xue Wang[1†], Yibo Wu[2†], Xinji Shi[1], Yu Chen[3], Yahong Xu[4], Hongbo Xu[5], Yanan Ma[6] and Shuang Zang[1*]

[1]Department of Community Nursing, School of Nursing, China Medical University, Shenyang, Liaoning, China, [2]School of Public Health, Peking University, Beijing, China, [3]School of Nursing, Southern Medical University, Guangzhou, Guangdong, China, [4]Department of Fundamental of Nursing, School of Nursing, Capital Medical University, Beijing, China, [5]Department of Medical Nursing, School of Nursing, Wenzhou Medical University, Wenzhou, Zhejiang, China, [6]Department of Biostatistics and Epidemiology, School of Public Health, China Medical University, Shenyang, Liaoning, China

Background: A healthy lifestyle is beneficial to individuals' health. However, little is known about the associations of lifestyle factors with mental health and well-being. This study examined the associations of lifestyle factors with mental health (i.e., depression, anxiety, loneliness, perceived pressure, and self-rated health status) and well-being in Chinese adults.

Methods: A nationally representative survey was conducted in China from 20 June 2022 to 31 August 2022. Data from the survey were analyzed using multiple linear regression to determine the associations of lifestyle with mental health and well-being in Chinese adults. Standardized regression coefficients (β) and 95% confidence intervals (CIs) were estimated using multiple linear regression.

Results: The survey included 28,138 Chinese adults. Multiple linear regression results showed that there were significant negative associations of lifestyle scores with scores of depression (β=−0.93, 95% CI: −0.98, −0.88), anxiety (β=−0.71, 95% CI: −0.76, −0.67), loneliness (β=−0.23, 95% CI: −0.24, −0.21), and perceived pressure (β=−0.19, 95% CI: −0.22, −0.16). Moreover, there were significant positive associations of lifestyle with self-rated health status (β=1.99, 95% CI: 1.79, 2.20) and well-being (β=0.96, 95% CI: 0.91, 1.02).

Conclusion: This study provides insight into the associations of lifestyle factors with mental health and well-being and highlights the importance of improving and maintaining healthy lifestyle behaviors for favorable mental health and well-being.

KEYWORDS

mental health, adults, China, lifestyle, well-being

Introduction

Mental health is the foundation for overall health and well-being (1). According to the World Health Organization (WHO), mental health problems are prevalent worldwide, affecting approximately one in four individuals at some point during their lifetime (2). Globally, approximately one-third of adult populations experience mental health problems annually, and these problems are a significant public health challenge (3, 4). As such, addressing mental health issues has become a vital priority for public health programs, including treatment, prevention, and health promotion efforts (5). Given the significant burden of mental health problems on

individuals and society as a whole, raising public awareness of the importance of mental health is crucial.

Literature review

Previous research has shown that some daily behaviors could be adapted to affect mental health (6, 7). Mental health programs that encourage individuals to manage their mental health by adjusting daily health behaviors are destigmatizing, empowering, and cost-effective in affecting the population (8). Several nationwide studies have identified some lifestyle factors associated with mental health, including physical activity, healthy eating behaviors, and adequate sleep, which are associated with reduced depression, anxiety, and stress levels (9–11). Conversely, some nationwide studies also found that increased consumption of smoking and alcohol is associated with unfavorable mental health outcomes (12–14). However, research on the association between lifestyle factors and positive psychological outcomes such as well-being is limited, despite some nationwide studies indicating that setting time, smoking, and diet are individually associated with well-being among adults (15–17). It is important to note that previous nationwide studies have mostly focused on solely behaviors, with limited research on the combination of these lifestyle factors. While analyzing these factors independently provides valuable information, combining them may better reflect real-life situations, as they often co-occur and may have synergistic effects (18, 19). Additionally, since lifestyle factors are multidimensional and complex, a comprehensive analysis of these factors may be more effective in capturing their association with mental health and well-being, compared to focusing on solely factors (20).

Theoretical underpinning

The present study is theoretically anchored in the biopsychosocial model of health and the health promotion model. These theoretical frameworks provide a comprehensive and holistic understanding of the intricate associations between lifestyle factors and mental health and well-being. The biopsychosocial model of health underscores the dynamic interplay among biological, psychological, and social factors in determining an individual's health status (21). In the context of this study, this model elucidates the potential correlation of lifestyle factors, such as diet and exercise, with mental health and well-being. The health promotion model accentuates the elements that facilitate and maintain healthy behaviors, highlighting the importance of individual perceptions, motivations, and behaviors in shaping health outcomes (22). This model offers insights into the psychological and behavioral mechanisms underlying the associations between lifestyle factors and mental health and well-being. Together, these two theoretical frameworks furnish a robust foundation for exploring the intricate associations between lifestyle and mental health and well-being in the general population and have significant implications for the development of effective interventions and policies to promote mental health and well-being in this population.

Thus, the aim of this nationally representative survey was to explore whether seven lifestyle factors (i.e., smoking, drinking, diet behaviors, physical activity, sitting time, sleep duration, and sleep quality) were associated with mental health (i.e., depression, anxiety, loneliness, perceived pressure, and self-rated health status) and well-being. This study aims to fill the research gap related to a nationwide survey and contribute to the development of effective health promotion programs and policies to improve mental health and well-being.

Methods

Survey design and study population

This survey was conducted by a multistage sampling method across 31 (91.17% of the total) provinces/autonomous regions/municipalities/special administrative regions in China from 20 June 2022 to 31 August 2022. In this survey, investigators issued one-on-one questionnaires to participants by using the online Questionnaire Star platform. The inclusion criteria for the study participants were as follows: Chinese people ≥18 years old who participated in the study voluntarily, understood the meaning of each questionnaire item, and completed the questionnaires independently. For participants with thinking ability but without sufficient mobility to complete the questionnaire, investigators assisted without intervening. The exclusion criteria for the study participants were as follows: people with confusion and mental disorders, participation in other similar studies, and refusal to participate. The participants who were diagnosed with mental illness by a doctor were identified through a combination of self-report and systematic record-keeping (available in community health service centers). During the survey, community workers (neighborhood committees or health service center staff) who were familiar with the local community also participated. Based on these criteria, respondents with psychological problems were excluded from the data pool. The specific survey process and quality control information were described in a previous study (23).

This study was approved by the Ethics Research Committee of the Health Culture Research Center of Shaanxi (No. JKWH-2022-02), and all participants provided informed consent before data collection.

Exposure measure

Lifestyle score

According to the WHO report and prior studies (24–26), we selected seven lifestyle factors to construct a lifestyle score. These lifestyle factors included smoking, drinking, diet behaviors, physical activity, sitting time, sleep duration, and sleep quality.

First, the smoking status of participants was measured by a single item classified into a lower-risk category (former and never smoking) or a higher-risk category (current smoking) (27). Second, the drinking status of participants was assessed by a single item classifying them into a lower-risk category (former and never drinking) or a higher-risk category (current drinking) (28). Third, diet behaviors were determined by examining breakfast behavior, tea-drinking behavior, sugar-sweetened beverage drinking behavior, eating out behavior, and water drinking behavior (29–31). For example, if the participants ate breakfast daily or 5–6 days/week, they were classified into a lower-risk category; otherwise, they were classified into a higher-risk category. Detailed information and coding methods on diet behavior ratings are presented in Supplementary Table S1. Then, a diet score was generated

according to the above diet behaviors, with a score ≥3 indicating low-risk diet behavior. Fourth, the participants' physical activity levels were evaluated by the International Physical Activity Questionnaire-7 (IPAQ-7) (32), and we then calculated individuals' basal metabolic time per week (minute). The following calculation method was used: (1) Mild-intensity activity metabolic equivalent of task (MET) = 3.3 × average time engaged in mild-intensity activity daily × weekly engaging in mild-intensity activity days. (2) Moderate-intensity activity MET = 4.0 × average time engaged in moderate-intensity activity daily × weekly engaging in moderate-intensity activity days. (3) Strenuous activity MET = 8.0 × average time engaged in strenuous activity daily × weekly engaging in strenuous activity days. Thus, basal metabolic time weekly (minute) = (1) + (2) + (3). Then, the physical activity of participants was classified into a lower-risk category [active (≥3,000 MET)] or a higher-risk category [minimally active (≥600 and <3,000 MET) and inactive (<600 MET)] (33). Fifth, the sitting time of participants was assessed by a single item, and the participants were classified into a lower-risk category (≤7 h/day) or a higher-risk category (>7 h/day) (34). Sixth, the sleep duration of participants was classified into lower-risk (>7 h/day) or higher-risk categories (≤7 h/day) (35). Finally, the sleep quality of participants was classified into lower-risk (relatively good and very good) or higher-risk categories (relatively bad and very bad) (36). The specific coding for these lifestyle factors is shown in Supplementary Table S2.

The above seven lifestyle factors were combined into a lifestyle score (ranging from 0 to 7), with higher scores representing healthier lifestyles. Due to the distribution of data, lifestyle was further categorized into five groups by the score (0–2, 3, 4, 5, and 6–7).

Outcome measure

Depression

The Patient Health Questionnaire-9 (PHQ-9) is used to measure participants' depression status (37). Each item is scored on a four-point Likert scale, ranging from 0 (never) to 3 (nearly every day). The total score of the PHQ-9 ranges from 0 to 27, with higher scores representing more severe depression. The Cronbach's α for the PHQ-9 was 0.920 in this study.

Anxiety

The Generalized Anxiety Disorder-7 (GAD-7) is used to assess participants' anxiety status (38). Each item is scored on a four-point Likert scale, ranging from 0 (never) to 3 (nearly every day). The total score of the GAD-7 ranges from 0 to 21, with higher scores reflecting more severe anxiety. The Cronbach's α for the GAD-7 was 0.944 in this study.

Loneliness

The Three-Item Loneliness Scale (T-ILS) is used to evaluate participants' loneliness (39). Each item is scored on a three-point Likert scale, ranging from 1 (never) to 3 (often). The total score of the T-ILS ranges from 3 to 9, with higher scores indicating higher levels of loneliness. The Cronbach's α for the T-ILS was 0.861 in this study.

Perceived pressure

The Perceived Stress Scale-4 (PSS-4) is used to measure participants' perceived pressure (40). Each item is scored on a five-point Likert scale, ranging from 1 (never) to 5 (always). The total score of the PSS-4 ranges from 4 to 20, with higher scores representing greater perceived pressure. The Cronbach's α for the PSS-4 was 0.681 in this study.

Self-rated health status

Participants rated their health status on a vertical scale of 0 (the least healthy) to 100 (the healthiest) (41).

Well-being

The World Health Organization Well-Being Index-5 (WHO-5) is used to evaluate participants' psychological well-being (42). Each item is scored on a six-point Likert scale, ranging from 0 (never before) to 5 (all times). The total score of the WHO-5 ranges from 0 to 25, with higher scores reflecting greater well-being. The Cronbach's α for the WHO-5 was 0.951 in this study.

Covariates

The following variables were included as covariates: age, sex, education level (junior high school and below, high school and junior college, bachelor's degree and above), career status (student, have a job, have no job), marital status (have no partner, have a partner), urban–rural distribution, whether having diagnosed chronic disease, family *per capita* monthly income (≤3,000 Chinese Yuan (CNY), 3001–6000 CNY, ≥6001 CNY), and self-rated family social status (scoring from 1 (lowest) to 7 (highest)).

Statistical analysis

First, Kolmogorov–Smirnov tests were performed to determine the normality of continuous variables. The continuous variables in this study were approximately normally distributed according to visual inspection of Q-Q plots. Second, the distributions of participant characteristics were examined based on the categories of lifestyle scores. Continuous variables were displayed as the mean and standard deviation (SD), and categorical variables were presented as numbers and percentages. The chi-squared test was performed to compare the categorical variables among lifestyle score groups, and variance analysis was conducted to compare the continuous variables. The collinearity between variables was determined by measuring the variance inflation factor (VIF). The multicollinearity test demonstrated no collinearity among the study variables in this study (maximum VIF = 2.53). Third, the associations of lifestyle with mental health and well-being were conducted using multiple linear regression adjusting for all covariates. Fourth, we generated fitting plots using generalized additive models to depict the associations of lifestyle with mental health and well-being, with adjustment for potential confounders. Fifth, the distribution of mental health and well-being scores in the lifestyle score groups was visualized using violin plots. Sixth, we also used violin plots to visualize the distribution of mental health and well-being scores in the lifestyle score groups stratified by categorical variables of covariates. Finally, the associations of lifestyle factors and detailed lifestyle behaviors with mental health and well-being were conducted by using multiple linear regression with all covariates adjusted.

All statistical tests were two-sided, and the significance level was set at $p < 0.05$. All statistical analyses were performed using Stata version 16.0 (StataCorp, College Station, TX, United States).

Results

Participant characteristics

A total of 28,138 Chinese adults were included in this survey. In this study, 43.00% of participants were male, 41.83% had a bachelor's degree and above, 49.80% had no partner, and 72.43% lived in urban areas. There were significant differences in demographic characteristics among the different lifestyle score groups (all $p < 0.05$) (Table 1). The mean score of the depression scale among the participants was 6.87 (SD: 5.61) points, the anxiety scale was 4.99 (SD: 4.72) points, the loneliness scale was 4.67 (SD: 1.63) points, the perceived pressure scale was 10.10 (SD: 3.09) points, the self-rated health status scale was 72.65 (SD: 22.40) points, and the well-being scale was 14.25 (SD: 6.09) points. Out of the 28,138 participants, 13.32% (n = 3,749) had a higher-risk smoking status, 21.81% (n = 6,138) had a higher-risk drinking status, 41.91% (n = 11,793) had higher-risk diet behaviors, 52.22% (n = 14,695) had higher-risk physical activity, and 47.36% (n = 13,325) had higher-risk sitting time. Additionally, 65.34% (n = 18,385) had higher-risk sleep duration, and 17.50% (n = 4,924) had higher-risk sleep quality (Table 2). Among the 28,138 participants, 2023 participants (7.19%) had a lifestyle score of 0–2, 4,436 participants (15.77%) had a lifestyle score of 3, 7,866 participants (27.96%) had a lifestyle score of 4, 8,231 participants (29.25%) had a lifestyle score of 5, and 5,582 participants (19.84%) had a lifestyle score of 6–7 (Table 2) (Supplementary Figure S1). Participants with lower lifestyle scores were more likely to have higher scores for depression, anxiety, loneliness, and perceived pressure. Participants with higher lifestyle scores tended to have higher scores for self-rated health status and well-being (Supplementary Figure S2). The distribution of mental health and well-being scores by lifestyle score stratified by categorical variables of covariates can be seen in Supplementary Figures S3–S9.

Associations of lifestyle with mental health and well-being

The multiple linear regression results showed that there were significant negative associations between lifestyle score (as a continuous variable) and scores of depression ($\beta = -0.93$, 95% CI: −0.98, −0.88), anxiety ($\beta = -0.71$, 95% CI: −0.76, −0.67), loneliness ($\beta = -0.23$, 95% CI: −0.24, −0.21), and perceived pressure ($\beta = -0.19$, 95% CI: −0.22, −0.16). Moreover, there were significant positive associations of lifestyle score (as a continuous variable) with scores of self-rated health status ($\beta = 1.99$, 95% CI: 1.79, 2.20) and well-being ($\beta = 0.96$, 95% CI: 0.91, 1.02). When the lifestyle score was treated as a categorical variable, all results were statistically significant (Table 3). After adjusting for possible confounders, negative associations between lifestyle score (as a categorical variable) and scores of depression, anxiety, loneliness, and perceived pressure were observed. Similarly, lifestyle scores were positively associated with scores of self-rated health status and well-being (see Figure 1 for details).

Additionally, after adjusting for potential confounders, the results of multiple linear regression indicated that significant associations between lifestyle factors and most of the outcomes remained robust. For example, when examining the associations of smoking status with scores of depression, anxiety, loneliness, perceived pressure, self-rated health status, and well-being, the β value (vs. higher-risk) was −1.19, −0.85, −0.18, −0.17, 2.16, and 1.31 for lower-risk, respectively. Similarly, lower-risk sitting time was associated with lower scores for depression ($\beta = -1.13$), anxiety ($\beta = -0.92$), and loneliness ($\beta = -0.28$) but with higher scores for self-rated health status ($\beta = 1.75$) and well-being ($\beta = 0.25$) than higher-risk sitting time. In addition, when analyzing the associations of sleep duration with scores of depression, anxiety, loneliness, perceived pressure, self-rated health status, and well-being, the β value (vs. higher-risk) was −1.24, −1.00, −0.34, −0.22, 3.24, and 1.55 for lower-risk, respectively. Lower-risk sleep quality was associated with lower scores of depression ($\beta = -3.69$), anxiety ($\beta = -2.93$), and loneliness ($\beta = -0.79$) but with higher scores of self-rated health status ($\beta = 8.96$) and well-being ($\beta = 3.15$) compared to the higher-risk sleep quality group (see Supplementary Table S3). Similarly, when analyzing the associations of detailed lifestyle behaviors with mental health and well-being, most results (e.g., diet scores and sitting time) were statistically significant (see Supplementary Table S4).

Discussion

The current study explored the associations of lifestyle with mental health and well-being. The results showed significant negative associations of lifestyle scores with scores of depression, anxiety, loneliness, and perceived pressure. Moreover, there were significant positive associations of lifestyle scores with scores of self-rated health status and well-being. In this field, Firth et al. (43) conducted a meta-analysis and found that lifestyle factors such as exercise, smoking, diet, and sleep were closely associated with mental health. This study was conducted with the primary objective of investigating the association between lifestyle and mental health and well-being, while considering multiple outcomes, including depression, anxiety, loneliness, and perceived pressure. Our study, to some extent, served to complement outcomes (e.g., loneliness and self-rated health status) that were not comprehensively addressed in Firth's meta-analysis. In addition, our study revealed positive associations between lifestyle scores and both self-rated health status and well-being. These novel findings provide deeper insights into the complex associations between lifestyle and mental health and well-being, enriching the understanding of this topic. Our study emphasizes the critical importance of combining lifestyle factors with mental health and well-being research and offers new evidence to support the existing studies.

The depression and anxiety scale scores in our study were slightly lower than the scores reported in previous nationwide population-based studies conducted in Turkey, Germany, and Poland (44–46). Regarding subjective well-being, the scores of our study were higher than those obtained in previous studies conducted in 15 European countries (47). The observed differences may be attributed to cultural variations, including the importance placed on interpersonal harmony and emotional restraint in China (48, 49). Additionally, variations in study design and sampling methods may also have played a role. As well, differences in socioeconomic context and healthcare systems

TABLE 1 Characteristics of participants according to lifestyle score (n=28,138).

Variables	Total sample	Lifestyle score					
		0–2	3	4	5	6–7	*p*
Age, mean (SD)	37.25 (17.83)	32.88 (15.80)	34.32 (16.81)	35.18 (17.36)	38.76 (18.19)	41.83 (18.18)	<0.001
Sex, *n* (%)							<0.001
Male	12,099 (43.00)	1,180 (58.33)	2,209 (49.80)	3,347 (42.55)	3,187 (38.72)	2,176 (38.98)	
Female	16,039 (57.00)	843 (41.67)	2,227 (50.20)	4,519 (57.45)	5,044 (61.28)	3,406 (61.02)	
Education level, *n* (%)							<0.001
Junior high school and below	6,334 (22.51)	305 (15.08)	792 (17.85)	1,498 (19.04)	2014 (24.47)	1725 (30.90)	
High school and junior college	10,034 (35.66)	787 (38.90)	1,579 (35.60)	2,786 (35.42)	2,943 (35.76)	1939 (34.74)	
Bachelor degree and above	11,770 (41.83)	931 (46.02)	2065 (46.55)	3,582 (45.54)	3,274 (39.78)	1918 (34.36)	
Career status, *n* (%)							<0.001
Student	10,023 (35.62)	816 (40.34)	1837 (41.41)	3,236 (41.14)	2,687 (32.64)	1,447 (25.92)	
Have no job	5,656 (20.10)	262 (12.95)	664 (14.97)	1,371 (17.43)	1908 (23.18)	1,451 (25.99)	
Have a job	12,459 (44.28)	945 (46.71)	1935 (43.62)	3,259 (41.43)	3,636 (44.17)	2,684 (48.08)	
Marital status, *n* (%)							<0.001
Have no partner	14,013 (49.80)	1,254 (61.99)	2,581 (58.18)	4,308 (54.77)	3,785 (45.98)	2085 (37.35)	
Have a partner	14,125 (50.20)	769 (38.01)	1855 (41.82)	3,558 (45.23)	4,446 (54.02)	3,497 (62.65)	
Urban–rural distribution, *n* (%)							<0.001
Rural	7,757 (27.57)	468 (23.13)	1,150 (25.92)	2060 (26.19)	2,357 (28.64)	1722 (30.85)	
Urban	20,381 (72.43)	1,555 (76.87)	3,286 (74.08)	5,806 (73.81)	5,874 (71.36)	3,860 (69.15)	
Whether having diagnosed chronic disease, *n* (%)							<0.001
No	21,501 (76.41)	1,450 (71.68)	3,318 (74.80)	6,062 (77.07)	6,308 (76.64)	4,363 (78.16)	
Yes	6,637 (23.59)	573 (28.32)	1,118 (25.20)	1804 (22.93)	1923 (23.36)	1,219 (21.84)	
Family *per capita* monthly income (Chinese Yuan), *n* (%)							<0.001
≤3,000	9,503 (33.77)	602 (29.76)	1,573 (35.46)	2,659 (33.80)	2,769 (33.64)	1900 (34.04)	
3,001–6,000	11,334 (40.28)	804 (39.74)	1720 (38.77)	3,115 (39.60)	3,397 (41.27)	2,298 (41.17)	
≥6,001	7,301 (25.95)	617 (30.50)	1,143 (25.77)	2092 (26.60)	2065 (25.09)	1,384 (24.79)	
Family social status (scores), mean (SD)	4.31 (1.31)	4.00 (1.36)	4.17 (1.31)	4.29 (1.32)	4.40 (1.28)	4.42 (1.27)	<0.001

Total percentages within categories may not equal 100% due to rounding.

could also impact mental health outcomes. Further investigation is needed to better understand the underlying factors contributing to these disparities. However, it is worth noting that variations in theoretical foundations and research themes may result in different studies incorporating diverse lifestyle indicators (50, 51), which can make cross-study comparisons challenging.

In this study, we found that participants with lower lifestyle scores tended to have higher scores of depression and anxiety, which was similar to other studies (50, 52). The evidence suggests that individuals' lifestyle factors, such as smoking status (53), diet behaviors (54), physical activity (55), sedentary behavior (56), and alcohol consumption (57), are associated with depression and anxiety status. Lifestyle factors may be associated with depression and anxiety via multiple pathways, including modifying neurotrophins essential to psychological disorders as well as nitrosative and oxidative stress pathways (58, 59). Additionally, individuals with depression and anxiety tend to have higher systemic inflammation levels (60). Higher systemic inflammation levels have also been demonstrated to be associated with unfavorable lifestyle factors, including unhealthy diet behaviors (61), low physical activity levels (62), and smoking (63). The present findings provide support for the growing evidence linking lifestyle factors to mental

TABLE 2 Characteristics of mental health, well-being, and lifestyle factors of the participants (n=28,138).

Variables	Value
Mental health and well-being	
Depression scale (scores), mean (SD)	6.87 (5.61)
Anxiety scale (scores), mean (SD)	4.99 (4.72)
Loneliness scale (scores), mean (SD)	4.67 (1.63)
Perceived pressure scale (scores), mean (SD)	10.10 (3.09)
Self-rated health status scale (scores), mean (SD)	72.65 (22.40)
Well-being scale (scores), mean (SD)	14.25 (6.09)
Lifestyle factors	
Smoking status, n (%)	
Higher-risk	3,749 (13.32)
Lower-risk	24,389 (86.68)
Drinking status, n (%)	
Higher-risk	6,138 (21.81)
Lower-risk	22,000 (78.19)
Diet behaviors, n (%)	
Higher-risk	11,793 (41.91)
Lower-risk	16,345 (58.09)
Physical activity, n (%)	
Higher-risk	14,695 (52.22)
Lower-risk	13,443 (47.78)
Sitting time, n (%)	
Higher-risk	13,325 (47.36)
Lower-risk	14,813 (52.64)
Sleep duration, n (%)	
Higher-risk	18,385 (65.34)
Lower-risk	9,753 (34.66)
Sleep quality, n (%)	
Higher-risk	4,924 (17.50)
Lower-risk	23,214 (82.50)
Lifestyle score	
0–2, *n* (%)	2,023 (7.19)
3, *n* (%)	4,436 (15.77)
4, *n* (%)	7,866 (27.96)
5, *n* (%)	8,231 (29.25)
6–7, *n* (%)	5,582 (19.84)

Total percentages within categories may not equal 100% due to rounding.

health outcomes and emphasize the critical role of lifestyle in the prevention of depression and anxiety. Encouraging individuals to adopt a healthier lifestyle, including smoking cessation, healthy dietary habits, regular physical activity, and limiting sedentary behavior and alcohol consumption, could help reduce the risk of depression and anxiety and improve overall mental health. Healthcare providers should also prioritize assessing and addressing lifestyle factors as part of their management approach to depression and anxiety.

The results of our study revealed that lifestyle was negatively associated with perceived pressure, which was in accordance with prior studies (64, 65). Individuals were inclined to engage in less tiresome activities during stressful times and avoided physical activity, probably due to time constraints and limited self-regulation capabilities (66, 67). Additionally, studies have also suggested that individuals often practice unhealthy behaviors to cope with emotion-focused stress, including smoking, drinking, reducing sleep duration, or avoiding physical activity (65, 68). Stress appears to be associated with eating behavior changes in an unhealthy direction (69). Moreover, work and academic stress are pervasive among adults (70). Therefore, public health policies ought to advocate for individuals to maintain or enhance healthy lifestyle practices to obtain maximum benefit from potential stress buffering and stress management. This goal can be realized through educational programs and interventions that aim to reduce unhealthy behaviors while promoting healthy habits.

Our study discovered that individuals with lower lifestyle scores tend to have higher loneliness levels. Research has reported that a sedentary lifestyle might increase the risk of loneliness (71). Several studies have also suggested that loneliness is associated with adverse health behaviors (e.g., less physical activity), poorer health practices (e.g., smoking and alcohol consumption) (72, 73), and sleep disturbances (e.g., decreased sleep duration and poorer sleep quality) (74, 75). In addition, as an important relevant factor for health, self-regulation ability may be one explanatory factor for poorer health behaviors in lonely individuals. A previous study showed that poorer self-regulation ability was associated with adults' loneliness (76). Poor self-regulation ability may contribute to loneliness-related health risks via reduced participation in health-promoting behaviors (73). Furthermore, poorer self-regulation ability often accompanies unhealthy lifestyles in adults (77). The results of our study highlight the importance of promoting individual responsibility for health among lonely populations, including participation in healthy lifestyle behaviors. These findings suggest that public health policies should focus on promoting healthy lifestyle practices to reduce loneliness levels and associated negative health behaviors. Targeted interventions are especially crucial for individuals with poor self-regulation abilities. Moreover, those in need of support should seek out resources such as social support and psychological counseling to decrease loneliness levels and improve overall health.

The study showed that lifestyle was positively associated with self-rated health status, consistent with previous findings (78, 79). Self-rated health status is a multidimensional concept. For individuals, it was associated with a multifactorial composite representing personal, psychological, social, medical, and behavioral characteristics (80, 81). Similarly, sedentary behaviors, sleep duration, diet behaviors, physical activity, alcohol, and smoking consumption were associated with individuals' health outcomes (82, 83). Thus, promoting healthy behaviors holistically rather than separately is an effective public health strategy for improving health, in general, and self-rated health status, in particular. In practice, this means that public health interventions

TABLE 3 Associations of lifestyle with mental health and well-being (n=28,138).

Items	Mental health										Well-being	
	Depression		Anxiety		Loneliness		Perceived pressure		Self-rated health status			
	β (95%CI)	*P*	*β* (95%CI)	*P*	*β* (95%CI)	*P*	*β* (95%CI)	*P*	*β* (95%CI)	*P*	*β* (95%CI)	*P*
Continuous												
Lifestyle score	−0.93 (−0.98, −0.88)	<0.001	−0.71 (−0.76, −0.67)	<0.001	−0.23 (−0.24, −0.21)	<0.001	−0.19 (−0.22, −0.16)	<0.001	1.99 (1.79, 2.20)	<0.001	0.96 (0.91, 1.02)	<0.001
Categorical												
Lifestyle score												
0–2	1 (ref)		1 (ref)		1 (ref)		1 (ref)		1 (ref)		1 (ref)	
3	−1.56 (−1.84, −1.28)	<0.001	−1.06 (−1.30, −0.82)	<0.001	−0.31 (−0.39, −0.23)	<0.001	−0.32 (−0.48, −0.16)	<0.001	2.85 (1.70, 4.01)	<0.001	1.32 (1.01, 1.63)	<0.001
4	−2.56 (−2.83, −2.30)	<0.001	−1.90 (−2.13, −1.68)	<0.001	−0.53 (−0.60, −0.45)	<0.001	−0.61 (−0.76, −0.46)	<0.001	5.07 (4.00, 6.15)	<0.001	2.38 (2.09, 2.67)	<0.001
5	−3.51 (−3.77, −3.24)	<0.001	−2.61 (−2.84, −2.39)	<0.001	−0.77 (−0.85, −0.70)	<0.001	−0.74 (−0.89, −0.59)	<0.001	7.47 (6.39, 8.55)	<0.001	3.50 (3.21, 3.79)	<0.001
6–7	−4.26 (−4.53, −3.98)	<0.001	−3.21 (−3.44, −2.97)	<0.001	−1.00 (−1.08, −0.92)	<0.001	−0.88 (−1.04, −0.72)	<0.001	8.78 (7.65, 9.92)	<0.001	4.22 (3.91, 4.52)	<0.001

All models were adjusted for age, sex, education level, career status, marital status, urban–rural distribution, whether having diagnosed chronic disease, family per capita monthly income, and family social status.

β, regression coefficients; CI, confidence interval; ref, reference.

should consider multiple dimensions of an individual's health and well-being. For example, interventions addressing both physical activity and social isolation, rather than treating them separately, could be more effective in promoting healthy behavior. Such a comprehensive approach may help public health practitioners develop more effective strategies for improving health outcomes and self-rated health status.

Moreover, our study revealed that lifestyle was positively associated with higher levels of well-being. Previous studies also found similar trends (7, 29). Individuals with high physical activity levels (84), lower-risk sitting time (85), and healthier dietary behaviors (17) were more likely to have increased well-being. The findings of this study underscored that more attention should be given to associations between lifestyle behaviors and individual well-being. This study provides valuable insights for healthcare professionals that adopting healthier lifestyles, including increased physical activity levels, reduced prolonged sedentary time, and healthier dietary habits, represents a meaningful avenue for enhancing individual well-being. By incorporating these behaviors into public health interventions, healthcare professionals can effectively promote positive health outcomes and improve overall well-being. Furthermore, by recognizing the associations between lifestyle behaviors and individual well-being, healthcare professionals can develop more comprehensive and holistic strategies to address the multifaceted nature of health and well-being.

Conclusion

In this nationwide study, we found negative associations of lifestyle scores with scores of depression, anxiety, loneliness, and perceived pressure and positive associations of lifestyle scores with scores of self-rated health status and well-being. These findings suggest that the adoption of a multi-behavioral healthy lifestyle, rather than just focusing on single behaviors, may be an effective approach to promoting and maintaining mental health and well-being.

The limitations of this study should also be acknowledged. First, it is essential to note that, similar to other studies, due to limitations of the cross-sectional design, causality cannot be identified. Thus, mental health and well-being could be the results or causes of a lifestyle. There is a necessity for further longitudinal and prospective studies to determine these associations. Second, all information was self-reported, meaning that it may not always reflect real situations. Some variables, such as smoking behavior, may tend to be underestimated. Third, while the findings from this study may be applicable to other countries' health promotion programs, it is still imperative that these findings are tested in other social contexts since the current findings were solely restricted to studying the Chinese adult sample. Finally, in our study, we analyzed PHQ and GAD scores as continuous variables, following the methods employed in previous studies (86, 87). However, it is crucial to exercise caution when using these scores as continuous variables, particularly with scores that fall

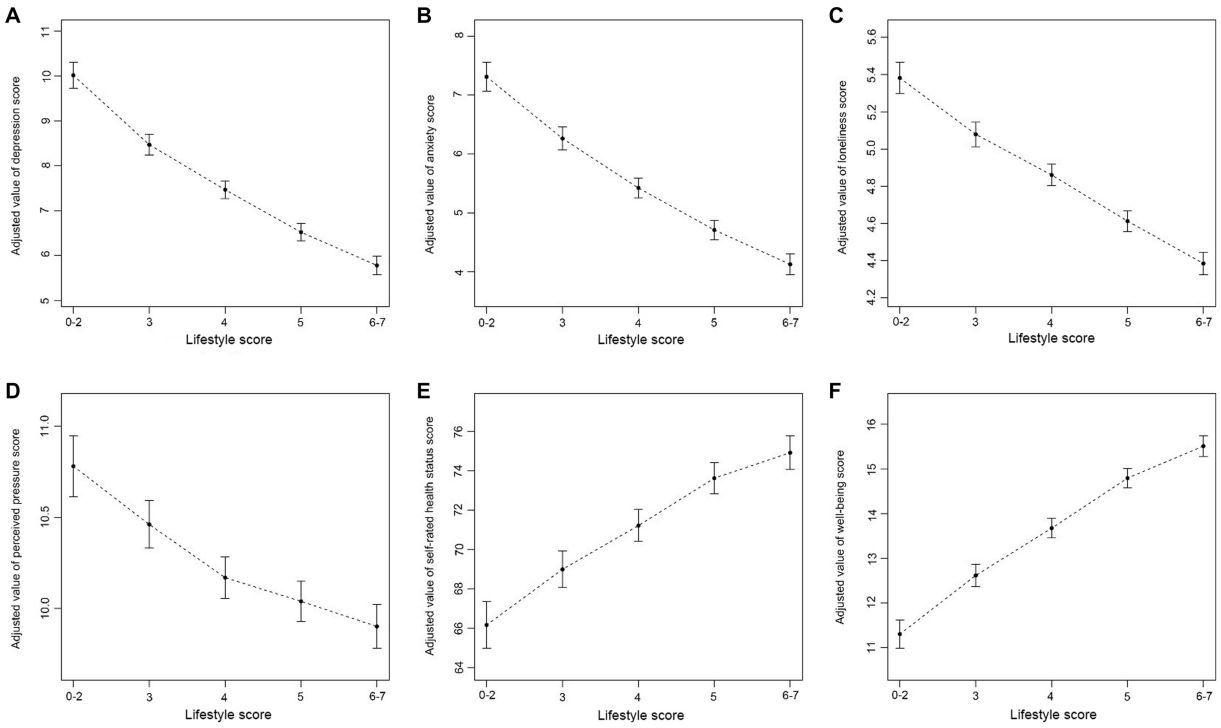

FIGURE 1
Associations of lifestyle with mental health and well-being. **(A)** Association of lifestyle with depression; **(B)** Association of lifestyle with anxiety; **(C)** Association of lifestyle with loneliness; **(D)** Association of lifestyle with perceived pressure; **(E)** Association of lifestyle with self-rated health status; **(F)** Association of lifestyle with well-being. All models were adjusted for age, sex, education level, career status, marital status, urban–rural distribution, whether having diagnosed chronic disease, family *per capita* monthly income, and family social status.

below the established cutoff points. Such scores may not always indicate the absence of depression and anxiety, and changes in scores cannot be simply interpreted as better or worse symptoms. Although using PHQ and GAD scores as continuous variables is a commonly utilized method, other measurement tools and methods may be explored in future research to enhance the accuracy and effectiveness of evaluating depression and anxiety symptoms.

Despite these limitations, our findings have important implications for the field of public health. These findings highlight the need to integrate the promotion of a healthy lifestyle into mental health promotion programs. Our results suggest that policymakers and healthcare professionals should take a comprehensive approach to promoting a healthy lifestyle and its positive effects on mental health and well-being. This includes encouraging individuals to engage in multiple healthy behaviors, such as physical activity, a healthy diet, and reducing sedentary behavior.

Our study contributes to the broader literature by providing evidence for the importance of a multi-behavioral healthy lifestyle in promoting mental health and well-being. The findings support and extend previous research in this field and underscore the need for further research on the association between lifestyle behaviors and mental health outcomes.

Overall, our study emphasizes the significance of promoting a healthy lifestyle and its potential to improve mental health and well-being. It underscores the need for healthcare professionals and policymakers to develop effective strategies to promote healthy behaviors, particularly in the context of mental health promotion programs.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary material, further inquiries can be directed to the corresponding author.

Ethics statement

This study was approved by the Ethics Research Committee of the Health Culture Research Center of Shaanxi (No. JKWH-2022-02). The patients/participants provided their written informed consent to participate in this study.

Author contributions

XW: conceptualization, methodology, formal analysis, writing–original draft, and reviewing and editing. YW: data collection, methodology, writing–original draft, reviewing and editing. XS, YC, YX, HX, and YM: and reviewing and editing. SZ: conceptualization and reviewing and editing. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnut.2023.1198796/full#supplementary-material

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EDITED BY
Nicholas Pang,
Universiti Malaysia Sabah, Malaysia

REVIEWED BY
Jean Pierre Poulain,
Université de Toulouse, France
Hurtado José Antonio,
University of Alicante, Spain

*CORRESPONDENCE
Ki-Hwan Yook
✉ cha99@cha.ac.kr
Chun Il Park
✉ chunil1001@cha.ac.kr

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Relationship between Mediterranean diet and depression in South Korea: the Korea National Health and Nutrition Examination Survey

Yeong-Geon Hwang[1,2], Chongwon Pae[1], Sang-Hyuk Lee[1], Ki-Hwan Yook[1]* and Chun Il Park[1]*

[1]Department of Psychiatry, CHA Bundang Medical Center, Seongnam-si, Republic of Korea, [2]Graduate School of Clinical Counseling Psychology, CHA University, Seongnam-si, Republic of Korea

Background: Several studies have shown that adherence to the Mediterranean diet is associated with a lower risk of depression; however, little is known about the Asian population. This study investigated the relationship between adherence to the Mediterranean diet and depression in a sample of the South Korean population.

Methods: In total, 5,849 adults from the 2014 and 2016 Korea National Health and Nutrition Examination Surveys were included in the study. The Mediterranean diet adherence was measured using a modified alternate Mediterranean diet score (mMED) developed to adjust for Korean dietary patterns. The mMED scores using the Food Frequency Questionnaire were divided into four categories (0–2, 3–4, 5–6, and 7–9 points). Subjects with depression were defined as having moderate-to-severe depressive symptoms using the Patient Health Questionnaire-9, with a cutoff value of 10. Logistic regression was used to estimate odds ratios (ORs) and 95% confidence intervals (CIs). A subgroup analysis was performed based on sex.

Results: The results of logistic regression analysis indicated that individuals with higher mMED were 42–73% less likely to report depression compared to individuals with the lowest mMED [ORs (95% CIs) =0.58 (0.37–0.90), 0.50 (0.31–0.80), 0.27 (0.15–0.47)] after adjusting for socio-demographic and health-related variables. In women, individuals with mMED of 7–9 had 71% lower odds of depression [ORs (95% CIs): 0.29 (0.13–0.64)]. In men, individuals with mMED of 5–9 had 55% [ORs (95% CIs): 0.45 (0.23–0.91)] to 79% [ORs (95% CIs): 0.21 (0.08–0.57)] lower odds of depression.

Conclusion: This study suggests that adherence to the Mediterranean diet is inversely associated with depression in both men and women among Korean adults. This study provides evidence that a Mediterranean diet is crucial in preventing depressive symptoms in Asian populations.

KEYWORDS

depressive symptoms, dietary patterns, KNHANES, Mediterranean diet score, sex difference

1. Introduction

It is estimated that approximately 256 million adults (5.02%) worldwide suffer from depression in 2019 (1), and the prevalence of depression has increased more than three times during the COVID-19 pandemic (2, 3). Depression is a leading cause of disability and contributes to the overall burden of disease worldwide (4). A longitudinal study demonstrated that depressive symptoms are associated with an approximately 8-fold increased risk of suicide attempts (5). Furthermore, South Korea has the highest suicide rate among Organization for Economic Cooperation and Development (OECD) countries (6). Effective treatment modalities, including pharmacotherapy for depression, have been established; however, half of the patients with depression show an inadequate response to antidepressants (7). Therefore, developing effective strategies to prevent depression is necessary.

Lifestyle modifications, such as dietary advice or exercise coaching, could be important in preventing depression (8, 9). Particularly, recent systematic reviews and meta-analyses indicated that adhering to a healthy diet can reduce the risk of clinical depression or depressive symptoms (10, 11). Diet quality has been reported to influence several biological processes associated with depression, including the production of monoamine neurotransmitters such as serotonin and dopamine, levels of oxidative stress, brain-derived neurotrophic factor (BDNF), and hypothalamic-pituitary-adrenal (HPA) activity (12).

The Mediterranean diet emerged from a classical study by Keys (13) and has been recognized by the United Nations Educational, Scientific, and Cultural Organization (UNESCO) as an intangible cultural heritage of humanity (14). The Mediterranean diet consists of a daily intake of whole grains, vegetables, legumes, fruits, nuts, dairy products, and olive oil; a weekly intake of fish (often); white meat (moderately); red and processed meat (less often); and moderate amounts of wine (15, 16). Previous evidence has shown that Mediterranean diet adherence is associated with a decreased risk of depression in the Mediterranean and other Western countries, including Greece, Italy, Spain, France, the U.K., the Netherlands, the U.S., and Australia (10, 17–19). Longitudinal studies have shown that the association between diet and depression is not significant in women (20, 21), suggesting sex-based differences.

Among the non-Caucasian population, a cross-sectional study of Iranian adults found an inversely significant association between adherence to a Mediterranean diet and depression (22). The Mediterranean diet index is commonly calculated using the median cutoff from each study sample; therefore, these results may be difficult to apply to other cultures (23). To the best of our knowledge, no evidence exists suggesting that adherence to a Mediterranean diet is associated with depression in Asian populations. Therefore, this study aimed to determine the relationship between adherence to the Mediterranean diet and depression in a nationally representative sample of Korean adults. We also investigated whether the association between depression and Mediterranean diet adherence differed between men and women.

2. Materials and methods

2.1. Data source and study population

Data were obtained from the National Health and Nutrition Examination Survey of South Korea (KNHANES), conducted by the Korea Centers for Disease Control and Prevention (KCDC). This nationwide survey comprised approximately 10,000 individuals over the age of 1 each year. The objectives of the KNHANES include monitoring trends in health-risk factors and their prevalence. The survey consisted of a health examination, health interview, and nutrition survey conducted by trained medical staff and interviewers. The available database and detailed descriptions can be found on the KNHANES website.[1]

Our primary sample included 15,700 individuals who completed all three surveys (health examination, health interview, and nutrition survey) in the 2014 and 2016 KNHANES. We excluded individuals with missing valid Patient Health Questionnaire-9 (PHQ-9) scores ($n = 4{,}990$), Food Frequency Questionnaire (FFQ) scores ($n = 4{,}614$), or covariate scores ($n = 34$) from this analysis. Individuals who were aged ≤19 or >69 years ($n = 93$), pregnant or breastfeeding ($n = 105$) (24), or had extreme energy intake (≤500 or > 6,000 kcal/day) ($n = 15$) (25) were excluded. The final sample included 5,849 individuals (Figure 1). All participants signed an informed consent form. The KNHANES was reviewed and approved by the KCDC Research Ethics Review Committee, which operates under domestic and international regulations, including the Declaration of Helsinki. The Institutional Review Board (IRB) of CHA Bundang Medical Center of CHA University exempted this IRB review because the databases provided publicly available secondary data (no. 2022-12-051).

2.2. Adherence to the Mediterranean diet

Adherence to the Mediterranean diet was measured using the modified alternate Mediterranean diet score (mMED) developed by Kim and Je (25) to adjust for Korean dietary patterns based on the alternate Mediterranean diet score (26). To calculate the mMED, dietary intake was evaluated using semiquantitative FFQ data from the KNHANES from 2012 to 2016. The FFQ consists of 112 items measuring the average frequency of food intake and service size over the past year. Regarding the frequency of food intake, nine categories were general options (from *less than once per month* to *more than three times per day*). For the serving size, the three categories were general options (0.5, 1, or 1.5 being the standard serving size), and the amount of alcoholic beverage was measured using open-ended questions. The consumption of each food item was estimated by multiplying the frequency of intake per week by the serving size (25). The reproducibility of the FFQ was acceptable, ranging from 0.54 to 0.61, and the validity was modest, ranging from 0.29 to 0.45 (27).

The mMED classifies 65 foods into nine groups: vegetables, legumes, fruits, whole grains, red or processed meats, white meat, fish/peanuts, dairy products, and alcohol (25). The mMED was calculated using sex-specific medians for the frequency of intake of each food group as cut-offs. The total mMED ranged from 0 to 9, with each food group scoring either 0 or 1. For vegetables, legumes, fruits, whole grains, white meats, fish/peanuts, and dairy products, the participants were awarded a point if they consumed more than the median intake. For red or processed meat, considering that the intake of red meat is relatively low in Korea (28), a point was awarded if the subjects

1 https://knhanes.kdca.go.kr

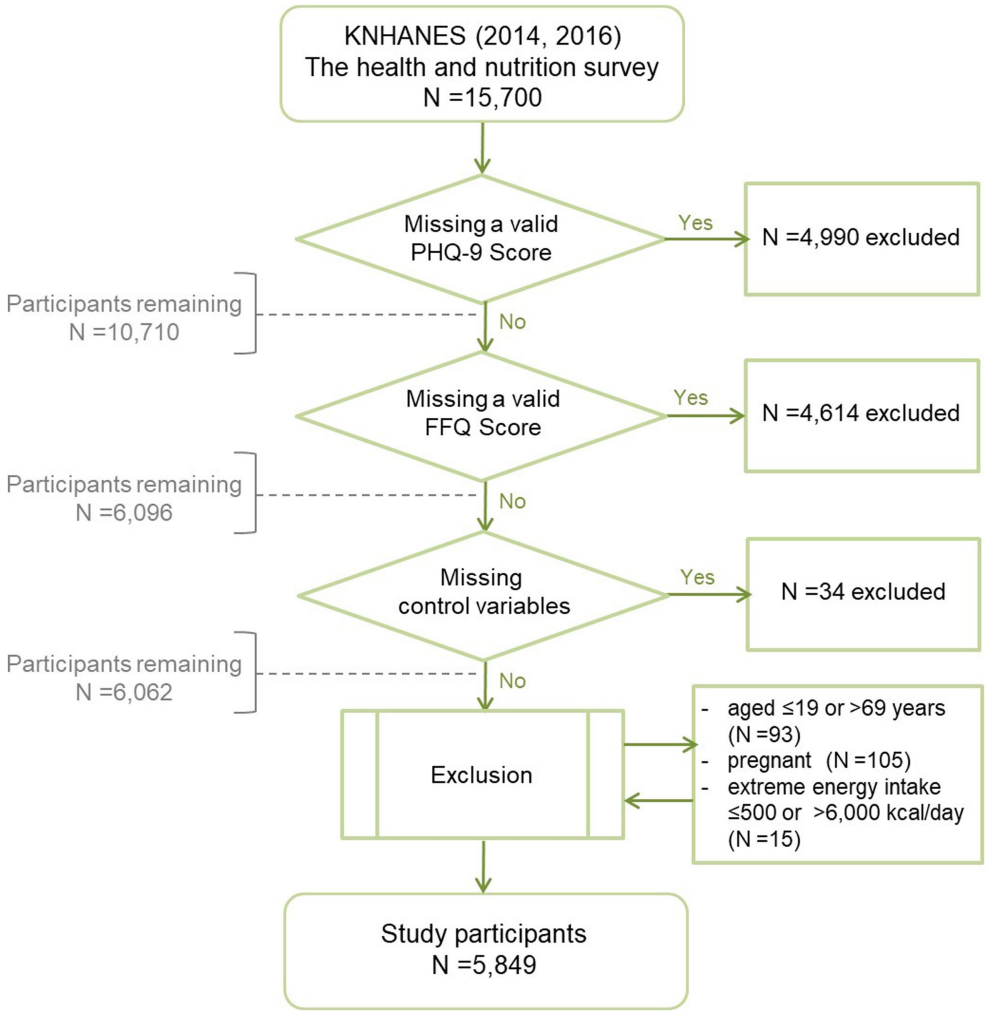

FIGURE 1
Flowchart of the study participants was presented. KNHANES, Korea National Health and Nutritional Examination Survey; PHQ-9, Patient Health Questionnaire-9; FFQ, Food Frequency Questionnaire.

consumed less than the 75th percentile. Ethanol consumption in alcoholic beverages was calculated according to the 8th Revision Korean Food Composition Table provided by the Korean Nutrition Society (29). A point was awarded for ethanol consumed of 10–50 g and 5–25 g per day for men and women, respectively. Otherwise, zero points were awarded. Finally, mMED was divided into four categories (0–2, 3–4, 5–6, and 7–9 points) for statistical analysis (25).

2.3. Depression

The PHQ-9, which has been administered biannually in the KNHANES since 2014, was used to evaluate depression severity (30). The PHQ-9 consists of nine criteria for depressive disorder from the Diagnostic and Statistical Manual of Mental Disorders, 4th Edition (DSM-IV), each rated on a scale from *not at all* (0), *several days* (1), *more than half the days* (2), and *nearly every day* (3), resulting in a total score ranging from 0 to 27. The PHQ-9 cut-point of 10 for major depressive disorder had a high sensitivity (88%) and specificity (88%) (30), and a validation study reported that the Cronbach's α of the Korean version of PHQ-9 was 0.81 (31). In this study, depression was defined as a PHQ-9 score of 10 (moderate or severe depression) (30).

2.4. Covariates

Sociodemographic (sex, age, residential area, household income, marital status, and education) and health-related (sleep, exercise variations, smoking, alcohol use, body mass index, chronic medical diseases, and energy intake) variables were selected as covariates that could affect diet quality or depression.

Household income was classified into quintiles based on the Korean population. Sleep duration was classified as *insufficient (<6 h/day)*, *normal, (<9 h/day)*, and *excessive (≥9 h/day)*. Exercise variation was classified as *none*, *one*, *two, or more*, depending on whether the three types of exercise (strength, aerobic, and walking) were performed at least once a week. Smoking and alcohol use were classified as *current* or *non-use*. Body Mass Index (BMI; kg/m^2) was classified into four categories: *underweight (BMI < 18.5)*, *normal weight (18.5 ≤ BMI < 23)*, *overweight (23 ≤ BMI < 25)*, and *obesity*

(25≤BMI) by calculating the weight and height measured by trained staff. Chronic medical diseases were classified as *none*, *one*, *two, or more*, and diabetes mellitus, hypertension, dyslipidemia, stroke, chronic renal failure, and coronary heart disease, which could directly or indirectly affect depression, were included as comorbidities (32). Energy intake was classified into quartiles according to sex based on the study data.

2.5. Statistical analysis

Descriptive statistics were calculated for the frequencies and percentages of sociodemographic and clinical characteristics. Differences between participants' characteristics were tested using the chi-square test. A False Discovery Rate (FDR) correction was performed to correct for type I error inflation. Using multivariate logistic regression analysis, odds ratios (ORs) and 95% confidence intervals (CIs) for depression were estimated in groups with mMED of 3–4, 5–6, and 7–9 when controlling covariates. Additional analysis was performed to investigate the ORs and 95% CIs for sex differences in the impact on depression using multivariable logistic regression analysis. All data processing and analysis were performed using Python (version 3.9), and a p-value < 0.05 was considered significant.

3. Results

3.1. Description of the study sample

The sociodemographic and clinical characteristics of the 5,849 participants based on the presence of moderate or severe depressive symptoms are summarized in Table 1. Among the participants, the prevalence of depression was 5.5% (4.0% for men and 6.4% for women; more details are presented in Table 1). The results of the chi-square test indicated that the higher the mMED, the lower the frequency of depression [9.7% vs. 6.5% vs. 5.5% vs. 3.3% (FDR-corrected $p < 0.001$)]. The residential area, alcohol consumption, and energy intake were not significantly associated with depression. In contrast, other sociodemographic and health-related variables, including sex, age, household income, marital status, education, sleep, exercise variations, smoking, BMI group, and chronic medical diseases, were significantly associated with depression (FDR-corrected $p < 0.05$).

3.2. Association between the Mediterranean diet and depression

The results of the multivariate logistic regression analysis of the association between the mMED and depression are presented in Table 2. The mMED significantly decreased the odds of depression [ORs (95% CIs): 0.65 (0.44–0.97), 0.54 (0.36–0.80), 0.32 (0.20–0.50); Model 1]. The association between the mMED and depression remained significant after adjusting socio-demographic variables [ORs (95% CIs): 0.56 (0.37–0.86), 0.50 (0.33–0.77), 0.30 (0.19–0.49); model 2] and all other covariates including sleep, exercise variations, smoking, alcohol use, BMI group, chronic medical disease, and energy intake [ORs (95% CIs): 0.58 (0.37–0.90), 0.50 (0.31–0.80), 0.27 (0.15–0.47); model 3]. This result indicates that individuals with higher mMED were 42 to 73% less likely to report depression compared to those with the lowest mMED.

3.3. Sex differences in the impact of Mediterranean diet adherence on depression

The results of the multivariate logistic regression analysis for sex differences in the impact of mMED on depression are shown in Figure 2. A significant association was found between depression and mMED after adjusting for all covariates in both men and women. In women, compared to the individuals in the lowest mMED group, individuals with mMED of 7–9 had 71% lower odds of depression [ORs (95% CIs): 0.29 (0.13–0.64)]. In men, individuals with a mMED of 5–9 had a 55% [ORs (95% CIs): 0.45 (0.23–0.91)] to 79% [ORs (95% CIs): 0.21 (0.08–0.57)] lower risk of depression.

Regarding the impact of sociodemographic and health-related factors on depression, men and women who were current smokers or reported having insufficient sleep were more likely to report depression. However, men and women who were married or had higher household incomes were less likely to report depression. Being underweight and high energy consumption were associated with depression only in men.

4. Discussion

This study investigated the relationship between adherence to the Mediterranean diet and depression using a representative sample of South Korean adults. We found that individuals with greater adherence to the Mediterranean diet had a lower risk of depression after adjusting for the confounding effects of sociodemographic and health-related variables. Furthermore, a significant association was observed between adherence to Mediterranean diet and depression in both men and women. To the best of our knowledge, this is the first study to examine the relationship between the Mediterranean diet and depression in an Asian population.

Our study found that individuals with greater adherence to the Mediterranean diet had a 42–73% lower risk of depression. This finding was consistent with previous studies suggesting that people with high Mediterranean diet scores were reported to have a 40–45% lower risk of depression (22, 33). Other meta-analysis studies showed adherence to the Mediterranean diet was associated with a 19% lower risk of depression (18, 19). Furthermore, randomized controlled trials have reported that Mediterranean dietary interventions significantly improved depressive symptoms (34, 35). Generally, these consistent results among non-Asian populations suggest the beneficial role of the Mediterranean diet in depression, and our results may confirm the relationship between the Mediterranean diet and depression in Asian cultures.

The mechanism underlying the association between the Mediterranean diet and depression is unclear; however, several potential explanations exist. The Mediterranean diet is rich in vitamin B and omega-3 polyunsaturated fatty acids (PUFA). Vitamin B can be involved in several methylation reactions, including serotonin and other monoamine neurotransmitters, and PUFA can play an important

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TABLE 1 Socio-demographics and clinical characteristics of study participants by the presence of depression.

Characteristics	Total (*n*=5,849)	Depressive (*n*=319, %)	Non-depressive (*n*=5,530, %)	*p*-value
Mediterranean diet score				**<0.001**
0–2	401	36 (8.98)	365 (91.02)	
3–4	1,684	114 (6.77)	1,570 (93.23)	
5–6	2,174	122 (5.61)	2,052 (94.39)	
7–9	1,590	47 (2.96)	1,543 (97.04)	
Sex				**<0.001**
Men	2,265	91 (4.02)	2,174 (95.98)	
Women	3,584	228 (6.36)	3,356 (93.64)	
Age (years)				**0.012**
20–29	837	61 (7.29)	776 (92.71)	
30–39	1,404	77 (5.48)	1,327 (94.52)	
40–49	1,467	58 (3.95)	1,409 (96.05)	
50–59	1,458	80 (5.49)	1,378 (94.51)	
60–69	683	43 (6.30)	640 (93.70)	
Residential area				0.772
Rural area	947	54 (5.70)	893 (94.30)	
Urban area	4,902	265 (5.41)	4,637 (94.59)	
Household income				**<0.001**
Quintile 1	603	93 (15.42)	510 (84.58)	
Quintile 2	1,098	63 (5.74)	1,035 (94.26)	
Quintile 3	1,302	61 (4.69)	1,241 (95.31)	
Quintile 4	1,362	54 (3.96)	1,308 (96.04)	
Quintile 5	1,484	48 (3.23)	1,436 (96.77)	
Marital status				**<0.001**
Married	4,301	168 (3.91)	4,133 (96.09)	
Separated/divorced/widowed	1,138	87 (7.64)	1,051 (92.36)	
Never married	410	64 (15.61)	346 (84.39)	
Education				**<0.001**
Primary or below	556	61 (10.97)	495 (89.03)	
Middle school	517	34 (6.58)	483 (93.42)	
High school	2,148	112 (5.21)	2,036 (94.79)	
College or above	2,628	112 (4.26)	2,516 (95.74)	
Sleep				**<0.001**
Insufficient (<6 h/day)	4,740	215 (4.54)	4,525 (95.46)	
Normal (<9 h/day)	714	78 (10.92)	636 (89.08)	
Excessive (≥9 h/day)	395	26 (6.58)	369 (93.42)	
Exercise variations				**<0.001**
None	655	45 (6.67)	610 (93.13)	
One	2,822	180 (6.38)	2,642 (93.62)	
Two or more	2,372	94 (3.96)	2,278 (96.04)	
Smoking				**<0.001**
Non-smoker	4,717	216 (4.58)	4,501 (95.42)	
Smoker	1,132	103 (9.10)	1,029 (90.90)	

(Continued)

TABLE 1 (Continued)

Characteristics	Total (*n*=5,849)	Depressive (*n*=319, %)	Non-depressive (*n*=5,530, %)	*p*-value
Alcohol use				0.61
No	2,441	138 (5.65)	2,303 (94.35)	
Yes	3,408	181 (5.31)	3,227 (94.69)	
Body Mass Index group				**0.021**
Underweight	252	24 (9.52)	228 (90.48)	
Normal weight	2,430	130 (5.35)	2,300 (94.65)	
Overweight	1,303	61 (4.68)	1,242 (95.32)	
Obesity	1864	104 (5.58)	1760 (94.42)	
Chronic medical disease				**0.009**
None	4,589	236 (5.14)	4,353 (94.86)	
One	804	44 (5.47)	760 (94.53)	
Two or more	456	39 (8.55)	417 (91.45)	
Energy intake				0.051
Quartile 1	1,463	98 (6.70)	1,365 (93.30)	
Quartile 2	1,462	65 (4.45)	1,397 (95.55)	
Quartile 3	1,462	74 (5.06)	1,388 (94.94)	
Quartile 4	1,462	82 (5.61)	1,380 (94.39)	

Depression was defined as a patient health questionnaire-9 score of 10. Bold values were defined as significant at *p*-values < 0.05.

TABLE 2 Odds ratios and 95% confidence intervals of the multivariable logistic regression for the association between modified Mediterranean diet score (mMED) and depression (PHQ-9≥10).

	Mediterranean diet score						
	0–2	3–4		5–6		7–9	
	ORs	ORs (95% CIs)	*p*-value	ORs (95% CIs)	*p*-value	ORs (95% CIs)	*p*-value
Model 1[a]	1.0 (ref)	0.65 (0.44–0.97)	0.034	0.54 (0.36–0.80)	0.002	0.32 (0.20–0.50)	<0.001
Model 2[b]	1.0 (ref)	0.56 (0.37–0.86)	0.007	0.50 (0.33–0.77)	0.002	0.30 (0.19–0.49)	<0.001
Model 3[c]	1.0 (ref)	0.58 (0.37–0.90)	0.015	0.50 (0.31–0.80)	0.004	0.27 (0.15–0.47)	<0.001

[a]Model 1 was unadjusted.
[b]Model 2 was adjusted for sex, age, residential area, household income, marital status, and educational level.
[c]Model 3 was additionally adjusted for sleep, exercise variations, smoking, alcohol use, BMI group, chronic medical disease, and energy intake.
Each modified Mediterranean diet score group was compared with the 0–2 group and *p*-value < 0.05 was defined as significant. PHQ-9, patient health questionnaire-9; ORs, odds ratios; CIs, confidence intervals; ref, reference group; BMI, body mass index.

role in central nervous system function and membrane fluidity, which could influence serotonin transport (36). Moreover, fruits, vegetables, and rice wine (37) in the Mediterranean diet may be rich sources of polyphenols with antioxidative and anti-inflammatory effects on depression (38, 39). Polyphenols can also induce BDNF expression by inducing cyclic adenosine monophosphate (cAMP) response element binding (CREB) (40). BDNF activation in the hippocampus and prefrontal cortex plays an important role in regulating depressive symptoms (41). The synergistic effects of these nutrients in the Mediterranean diet may contribute to its protective role against depression. Since this study could not identify the mechanism involved in nutritional biology, further research is needed to identify the mechanisms for the antidepressant properties of the Mediterranean diet.

We conducted further analyses to investigate sex differences in the effects of the Mediterranean diet and other demographic and health-related factors on depression. We found that moderate and high adherence to the Mediterranean diet was associated with a lower risk of depression in men, whereas women showed this relationship only in the high Mediterranean diet group. A previous large prospective cohort study showed that adherence to the Mediterranean diet was associated with incident depression in a male subgroup but not in a female subgroup (20), suggesting that there may be sex differences. Multiple factors, including biological, cultural, and reproductive events, can affect sex differences in the development of depression (42). Particularly, female sex hormones may induce changes in food intake and taste preference (43), as well as affect neuroinflammation and endocrine systems that contribute to depression (44–46). Body image perception by sex can also be considered as another possible explanation (47). Furthermore, longitudinal studies are required to investigate sex differences in the

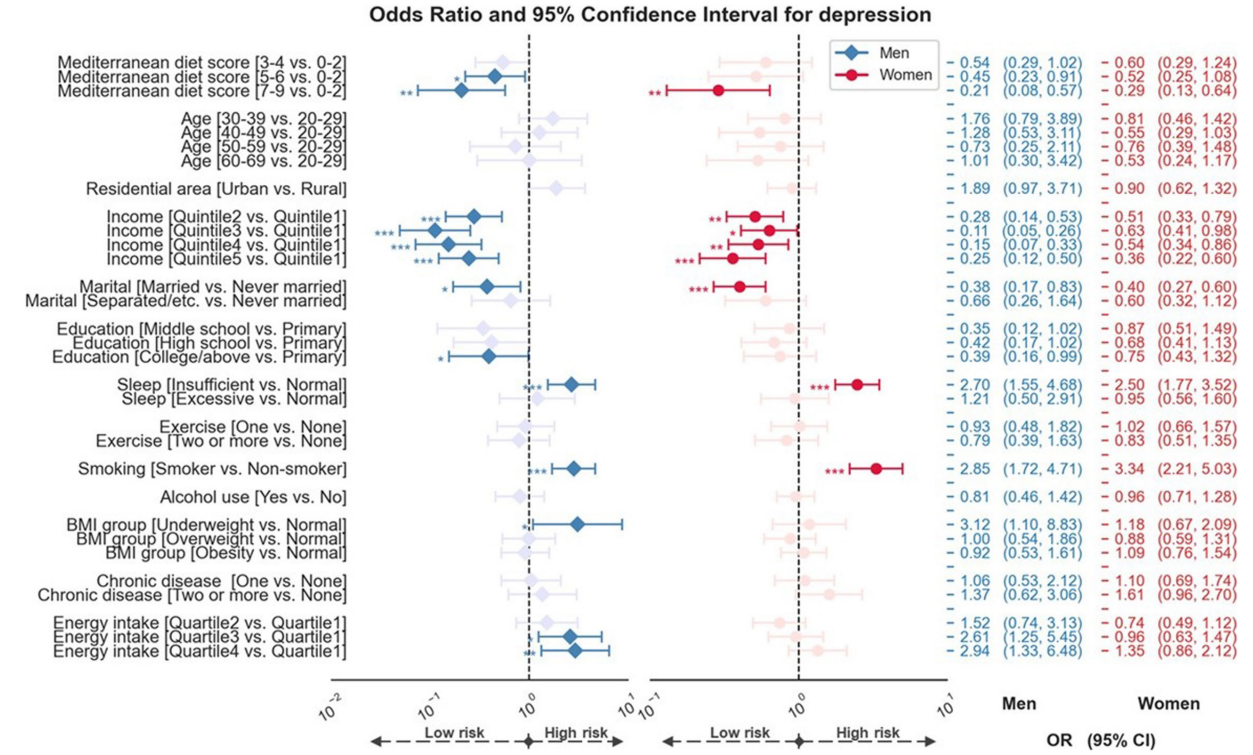

FIGURE 2
Forest plot showed odds ratios and 95% confidence intervals for sex difference in impact of the Mediterranean diet adherence on depression. Analyses were adjusted for age, residential area, household income, marital status, education, sleep, exercise variations, smoking, alcohol use, BMI group, chronic medical disease, and energy intake. *p*-value<0.05 was considered to be significant. *, **, and *** denote $p<0.05$, $p<0.01$, and $p<0.001$. OR, odds ratios; CI, confidence intervals; vs., versus; BMI, body mass index.

causal relationship between Mediterranean diet adherence and depression in Asian populations. Regarding sociodemographic and health-related factors, in both men and women, the risk of depression was associated with household income, marital status, sleep duration, and smoking, showing a pattern similar to that of previous studies (48–50). Interestingly, the risk of depression was significantly associated only with being underweight in the male group. Considering a previous result that men with depression are more likely to report decreased appetite and weight loss than women (51), low body weight may significantly impact depression in men.

Although this study investigated the adherence to the Mediterranean diet using the mMED index reflecting the Korean food culture, there are some points to consider the characteristics of the Korean dietary situation possibly affecting mental health. For example, most Koreans eat fermented vegetables, including Kimchi and soybean paste. Some studies suggested that fermented food modulates the composition of microbiota, especially via *Lactobacillus* (52, 53). As several studies examined the effect of the microbiome on mental health, such as mood disorders, anxiety disorder, and autism spectrum disorder (54, 55), adherence to fermented food might be linked with mental disorders. Future research investigating the effect of Korean food on depression would elucidate the association between diet and depression in Korean culture.

This study's key advantage is that it used large and homogeneous national data to analyze the relationship between adherence to the Mediterranean diet and depression among Asians. We used a validated measure of food intake frequency calculated from the Mediterranean diet scoring index based on Korean foods to assess diet adherence (25). Additionally, unlike previous studies on the Mediterranean diet and depression, our findings were adjusted for the effects of various sociodemographic and health-related variables to control for possible confounding effects. However, this study has several limitations. First, this was a cross-sectional study that could not clarify whether adherence to the Mediterranean diet could cause depression. Future prospective studies are required to confirm this causal direction. Second, the FFQ is a self-reported dietary assessment that may be biased toward dietary changes or recall errors. However, the FFQ has been commonly used to measure dietary patterns in epidemiological studies of diet and health and maybe more suitable than other dietary assessment methods for estimating usual intake (56). Third, this study investigated the Mediterranean diet according only to the frequency and amount of food. Therefore, we could not determine the effect of micronutrients. Furthermore, we could not control the impact of behavioral patterns related to food or alcohol consumption including solitary alcohol consumption. Previous studies reported that drinking alone was associated with depression (57, 58). Finally, this study was unable to explain the exact physiological mechanisms underlying the impact of the Mediterranean diet because biomarkers were not used. Future studies should include an assessment of biological mediators to identify the mechanisms by which the Mediterranean diet affects depression.

In summary, our study revealed that adherence to the Mediterranean diet was inversely associated with depression in both women and men in a nationally representative sample of Koreans. This

study provides evidence that a Mediterranean diet is crucial in preventing symptoms and supporting positive mental health, even among non-Caucasians.

Data availability statement

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding authors.

Ethics statement

The studies involving human participants were reviewed and approved by Institutional Review Board of CHA Bundang Medical Center. The ethics committee waived the requirement of written informed consent for participation.

Author contributions

Y-GH: conceptualization, formal analysis, data curation, and writing of the original draft. CP: methodology and validation. S-HL: supervision, conceptualization, project administration, and funding acquisition. K-HY supervised, validated, wrote, reviewed, and edited the manuscript. CIP: supervision, validation, study design, project administration, funding acquisition, and writing—review and editing. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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EDITED BY
Krishnamachari Srinivasan,
St. John's Research Institute, India

REVIEWED BY
Suhas Chandran,
St. John's Medical College Hospital, India
Juntao Kan,
Nutrilite Health Institute, China

*CORRESPONDENCE
Xiaobo Liu
✉ will.liuxb@gmail.com
Fu Li
✉ danlif@qq.com

†These authors share first authorship

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Prebiotics for depression: how does the gut microbiota play a role?

Yongde Yang†, Bi Zhou†, Sheng Zhang, Liang Si, Xiaobo Liu* and Fu Li*

Affiliated Wuhan Mental Health Center, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, China

Depression, a mood disorder characterized by persistent feelings of sadness and aversion to activity that can interfere with daily life, is a condition of great concern. Prebiotics, which are non-digestible substances selectively utilized by host microorganisms for health benefits, have gained attention for their potential to improve overall wellness and alleviate various disorders including depression. This study aims to review clinical trials utilizing carbohydrate-type prebiotics such as inulin-type fructans, galactooligosaccharides (GOS), human milk oligosaccharides, resistant starch, prebiotic phytochemicals including epigallocatechin gallate (EGCG), chlorogenic acids, resveratrol, and prebiotic lipids (n-3 polysaturated fatty acids) to determine their effects on depression. Our findings suggest that GOS at a daily dosage of 5g and eicosapentaenoic acid at or less than 1g can effectively mitigate depressive symptoms. While EGCG exhibits potential antidepressant properties, a higher dosage of 3g/d may be necessary to elicit significant effects. The plausible mechanisms underlying the impact of prebiotics on depression include the synthesis of neurotransmitters, production of short-chain fatty acids, and regulation of inflammation.

KEYWORDS

depression, prebiotics, microbiota, mood disorder, Fiber

1. Introduction

According to the International Scientific Association for Probiotics and Prebiotics, a prebiotic is defined as "a non-digestible substance that is selectively utilized by host microorganisms conferring a health benefit" (1). In addition, the health beneficial effects from the prebiotic need to be partially caused by microbial changes. To conclude, the four major elements for the consensus definition of prebiotics include nondigestibility, fermentability and selectivity, a health-related beneficial effect, and microbiota-mediated mechanism (Figure 1). Since the "prebiotic" concept was first put forward by Gibson and Roberfroid (2), the physiological benefits of prebiotics have been widely explored, especially in nutrient absorption, glucose management, lipid metabolism, and immune modulation.

Depression is one of the most common mental and mood health conditions, with an estimated 3.8% of the global population affected, generating negative consequences such as weight gain, inability to take care of oneself, impaired cognitions, and even suicidal ideations (3). In consistent with these findings, the lost productivity caused by depression and anxiety costs the global economy $1 trillion each year, and the economic burden of depression is projected to increase (4). In light of this, the World Health Organization released Mental Health

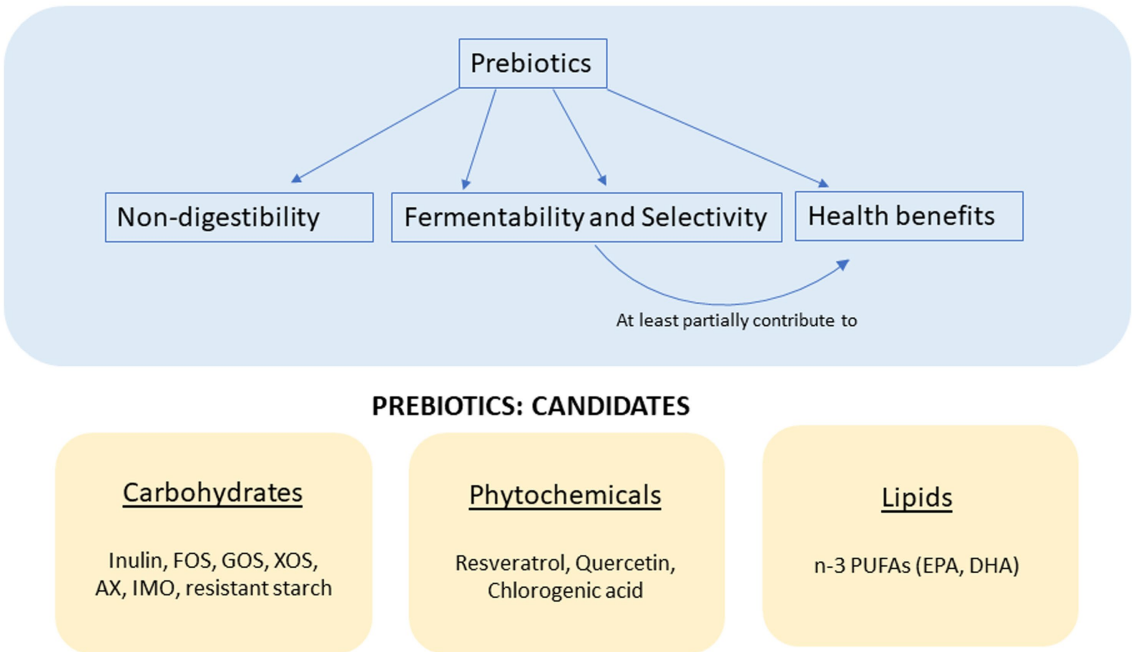

FIGURE 1
The definition of prebiotics and the candidates of prebiotics.

Action Plan 2013–2030 to emphasize the importance of providing appropriate interventions for patients with mental disorders including depression.

The pathophysiology of depression remains unclear due to its etiological heterogeneity. In recent years, accumulating evidence have shown that depression is linked to dysbiosis by the gut-microbiota-brain axis (GBA) (5, 6), which is a bidirectional network that connects the gastrointestinal (GI) system and the central nervous system through the interplay of multiple neuroimmune and neuroendocrine pathways (5). Despite a lack of deep understanding in the causal relationship between microbial changes and the development of diseases, the last decade witnessed a growing interest in utilizing microbiome manipulation to alleviate depressive disorders. Of several approaches that have garnered considerable interest, prebiotics attracted substantial attention for their broad applications in food and beverages, potential functionalities in improving texture (7), being free of addictive properties and generating less adverse effects (8).

There have been several review articles that discussed the role of prebiotics in depression (9–12). However, some of these publications lack mechanistic discussions on how prebiotics may alleviate depression, or mainly focused on dietary fibers and mental health instead of prebiotics and depression. Additionally, all these publications focused on carbohydrate-type prebiotics, whereas non-carbohydrate food ingredients that are also qualified for a prebiotic claim, such as fermentable phytochemicals (13), were left out from the studies. Chudzik et al. (11) synthesized the empirical evidence of pre-, pro-, and post-biotics for depressive symptoms, which provided the latest review on this topic. Nevertheless, with multiple animal studies and human studies published in the year 2022, it would be valuable to revisit the literature and update our knowledge.

Considering these, the objective of the current work is to summarize the animal and human studies that investigated the impact of prebiotics intake on depressive disorders. Since microbiota-mediated mechanism is a requisite for defining a substance to be prebiotic, this project highlights how the gut-brain-axis (GBA) moderates the effects of prebiotics in depression.

2. Prebiotics and their impact on the gut microbiota

The human gut harbors 10–100 trillion microorganisms, forming a dynamic and complex ecosystem (14). Within a "reference man" that weighs 70 kg, the total number of bacteria is approximately 3.8×10^{13}, and the ratio between human cells and resident microbes is approximately one-to-one (15). The gut microbiota is taxonomically classified by phyla, order, family, genus and species. Their genome consists of approximately over 3 million genes, encoding a large repertoire of biochemicals as a way of affecting the hosts' health and susceptibility to diseases (16). The gut microbiota can be altered by various environmental cues such as medication, health status, physical activity, as well as diets (16). Prebiotics hold substantial appeal due to their capability of selective fermentation, meaning that these compounds will exclusively stimulate the growth of beneficial bacteria, not pathogenic bacteria. Naturally occurring prebiotics and their respective food sources were listed in Table 1.

Inulin and inulin-type fructans, such as fructooligosaccharides (FOS), consist repetitive β–(2,1) fructosyl-fructose glycosidic linkages that are undigestible by the human intestinal brush boarder enzymes but readily fermentable by the gut microbiota (17). Although the chain

TABLE 1 Prebiotics and food sources.

Prebiotics	Food sources	Microbiota stimulated
Carbohydrates		
Inulin	Onion, banana, garlic, leek, wheat, asparagus, Jerusalem artichoke, chicory, and the blue agave plant (11, 17, 18)	*Bifidobacterium*, *Anaerostipes, Faecalibacterium, and Lactobacillus*
FOS		*Bifidobacterium*
GOS	Legumes, *Lycopus lucidus* and certain Traditional Chinese Medicine herbs (19–21)	*Bifidobacterium*
XOS	Fruits and vegetables, bamboo shoots, milk, and honey (22)	*Bifidobacterium*
AX	Major cereal grains (23)	*Bifidobacterium*
IMO	Miso, sake, soy sauce, and honey (22)	*Bifidobacterium*
RS	Starchy fruits and vegetables, legumes, cereal grains, and seeds (24), or synthesized starch	*Ruminococcus* and *Parabacteroidetes*
Phytochemicals		
Resveratrol	Grapes, wine, grape juice, peanuts, cocoa, and berries of Vaccinium species, including blueberries, bilberries, and cranberries (25–28)	*Bifidobacterium*
Quercetin	Onions, kale, apples, cherries, and red wine (29)	*Bifidobacterium*
Chlorogenic acid	Apples, artichoke, betel, burdock, carrots, coffee beans, eggplants, eucommia, grapes, honeysuckle (30)	*Bifidobacterium*
EGCG	Green tea	*Akkermansia*, *Bifidobacterium*
Lipids		
n-3 PUFAs	Marine organisms or deep-sea fish (31)	*Lachnospiraceae*, *Bacteroidetes*, *Roseburia*, *Coprococcus*, and *Blautia*

length of inulin, FOS, and short-chain FOS differ, these compounds exhibit similar biological effects due to the same type of glycosidic linkage. The most consistently reported microbial alteration with the consumption of inulin and FOS is the increase of *Bifidobacterium* (32, 33), and other concordant data indicate that inulin supplementation may stimulate the growth of *Lactobacillus*, *Faecalibacterium*, and *Anaerostipes* (33). Galactooligosaccharides (GOS), xylooligosaccharides (XOS), arabinoxylan (AX) and Isomaltooligosaccharides (IMO) are considered prebiotics with their bifidogenic effects (22, 34–36). All of the above-mentioned oligosaccharides are naturally contained in food, but they can also be prepared by using enzymatic or chemical methods (17, 22, 37). While other soluble fibers such as β-glucan and konjac glucomannan oligosaccharides have displayed certain prebiotic features, there is insufficient evidence that linked their physiological benefits to microbial changes. Resistant starch (RS) refers to a group of non-soluble carbohydrates that are resistant to upper GI digestion. RS is divided into four categories: RS1 is physically protected from digestive enzymes; RS2 is native granular starch consisting of a high amount of amylose; RS3 is retrograde starch; and RS4 is chemically modified starch. Notably, although RS are generally insoluble, certain RS molecules can be fermented by the gut microbiota, particularly by *Ruminococcus bromii* and *Parabacteroides distasonis* (38).

In addition to carbohydrates, certain phytochemicals are considered as prebiotics (13), since they are able to confer health benefits by selectively promoting the healthy gut microbiota. Resveratrol, quercetin, and chlorogenic acid, exemplified the most-studied phytochemicals that have shown health-promoting effects by increasing the abundance or proportional representation of *Bifidobacterium* strains and, therefore, are recognized as prebiotics (39). Besides, quercetin may reduce the opportunistic or pathogenic bacteria including *Listeria monocytogenes*, *taphylococcus aureus*, and *Vibrio parahaemolyticus*, which are clinically significant bacteria that may cause infection or diseases (29). Epigallocatechin gallate (EGCG), another well-studied phytonutrient, was found to enrich *Bifidobacterium* (40) and the SCFA-producing microbiota, such as *Akkermansia*, and exerted potent anti-inflammatory and anti-oxidative effects through enhancing the gut SCFA concentrations (41).

Intestine is the major site where lipid digestion and absorption occur. Lipid absorption is a complex process, which is almost fully completed in the small intestine. The lipids appearing in the colon and fecal materials are partly from dietary lipids that escape digestion in the upper GI system. As estimated, 95% consumed lipids are absorbed in the jejunum and ileum, leaving only approximately 5% dietary lipids entering the large intestine for further bacterial fermentation. Even with such a small quantity, the certain undigested lipids are able to induce changes in the gut microbiota. Omega-3 polyunsaturated fatty acids (n-3 PUFAs) are essential fatty acids because the limited quantity of *de novo* n-3 PUFAs synthesis is insufficient to satisfy the needs of humans. Eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), are long-chain n-3 PUFAs that are abundant in marine organisms or deep-sea fish, such as salmon, mackerel, and sardines (31). The influence of n-3 PUFAs on the gut microbiota was not widely studied. However, in the limited studies on adults, the authors showed a consistent effects of n-3 PUFAs in modulating the gut microbiota, with a decrease in *Faecalibacterium* and increase in *Lachnospiraceae* (42), *Bacteroidetes*, *Roseburia*, *Coprococcus*, and *Blautia*, which were associated with an enhanced production of SCFAs (43). Both DHA and EPA may attenuate multiple diseases by directly or indirectly affecting the gut microbiota (31). Conjugated linoleic acids (CLA) are isomers of linolenic acid and are heavily enriched in foods such as

meat and dairy products (44). Multiple animal studies reported that CLA could modulate the gut microbiota, especially in promoting *Prevotella, Akkermansia muciniphila* (45), *Lachnoclostridium*, *Roseburia*, *Dubosiella*, *Oscillibacter*, and *Anaerostipes* (46), as well as harboring a higher proportion of *Bacteroidetes* phylum in general (47). However, the efficacy of CLA on the gut microbiota has not been investigated in any clinical trials. The taxonomy of the promoted bacteria varied based on different animal models and different CLA isomers, causing a huge homogeneity in evidence. Therefore, more studies, especially in humans, are warranted to explore the efficacy of CLA in modulating the gut microbiota. Interestingly, certain gut microbiota, such as *Lactobacillus*, *Butyrivibrio*, and *Megasphaera* can feed on undigested linoleic acids and produce CLA (48, 49), which demonstrates a bidirectional relationship between CLA and the gut microbiota.

3. Depression and dysbiosis

Dysbiosis describes an imbalanced microbial profile characterized by loss of beneficial microbial abundance or signal and an augmentation of opportunistic and pathogenic microflora. Although the study of gut microbiota and mental health is a relatively new area that has caught researchers' attention in the past few years, converging clinical data already showed that patients diagnosed with depression may experience gut microbiome dysbiosis (50). The correlation between dysbiosis and depression has been portrayed in several observational studies. An overrepresentation of *Bacteroidales*, and an underrepresentation of *Lachnospiraceae* families, within the phylum *Firmicutes*, were observed in depressive patients (51). Patients with major depressive disorder (MDD) showed increased fecal bacterial α-diversity and enhanced *Bacteroidetes*, *Proteobacteria*, and *Actinobacteria*, but decreased *Firmicutes*. In particular, these patients had increased levels of *Enterobacteriaceae* and *Alistipes*, whereas their levels of *Faecalibacterium* were reduced and were negatively correlated with depressive symptoms (52). These results were, to a certain degree, consistent with the study by Naseribafrouei et al. (51) showing enrichment of the *Alistipes* in the subjects with depressive symptoms, indicating that depression patients may have a higher risk of undergoing dysbiosis.

Vice versa, the incidence of depression and other mental conditions were significantly higher in the patients with inflammatory bowel disease (IBD) than the subjects who had a healthy GI system (53, 54). A study by Chung et al. (55) explored the association between dysbiosis and depression by retrospectively utilizing a cohort where adult patients who were diagnosed with dysbiosis ($N = 552$) and their healthy counterparts ($N = 52$) were followed up for 5 years. The researchers found that the incidence of depression within 5 years of the index data was significantly higher in the patients who were diagnosed with dysbiosis, compared with the healthy controls (HR = 2.85) (55). Interestingly, the association was more potent in males than in females. Age was another important variable that modulated the degree of association, with a stronger association observed in the age group more than 60 years old (55). However, this retrospective study, along with the other observational studies, did not show causal relationships between the gut microbial changes and the onset or development of depression; instead, they focused solely on associations.

4. Prebiotics for depression

Since dysbiosis was closely linked to depression, and prebiotics are functional to alter the gut microbiota and mitigate dysbiosis, several well-designed clinical trials evaluated the effects of different prebiotics in alleviating depression. Clinical trials are the gold standard for evaluating the effectiveness of the compounds in certain conditions or diseases, as they represent the most rigorous method of examining causal relationships between interventions and outcomes.

4.1. Carbohydrates

Eleven clinical trials investigated the role of prebiotic carbohydrates in depression and depression-related parameters (Table 2). Among these studies, seven studies investigated inulin-type fructans that include inulin and FOS with different chain lengths. Three studies provided GOS treatment. The roles of HMO and RS in depression were investigated in one clinical trial, respectively.

In a cross-over study, Smith et al. (56) recruited 153 participants, and randomized them into two groups: the interventional group was provided with 10 g/d inulin, whereas the control group was given a placebo powder for 2 weeks, followed by a 2-week washout. As a result, the inulin supplementation did not alter any mental health related biomarkers, including mood, sleep quality, and memory, except for the surprisingly increased anxiety score (56). In 2015, the same group of researchers performed another clinical trial, in which 47 subjects were included and provided with one-time, 5 g inulin. In this trial, the researchers reported that inulin supplementation significantly enhanced the subjective mood and cognitive performance, with the most substantial effects on the episodic memory tasks, including improved accuracy on a recognition memory task and better recall performance (58). Similar beneficial effects of inulin-type fructan in improving mood health were observed in another clinical trial that used 5 g/d short-chain FOS (scFOS) for 4 weeks among 79 IBS patients with rectal hypersensitivity. In this study, researchers found that scFOS supplementation significantly decreased anxiety but did not change depression. Intriguingly, the subjects who took scFOS also experienced less rectal sensitivity and increased *Bifidobacteria*, indicating that scFOS might reduce anxiety by improving GI health (60). Among 48 patients with coronary heart disease, inulin supplementation at 15 g per day for 2 months did not affect patients' anxiety or depression scores, but the authors reported that the addition of inulin to a probiotic consisting of 1.9×10^9 colony-forming unit (CFU) of *L. rhamnosus* significantly improved psychological outcomes including decreased BDI and anxiety state (63). In a recent publication, researchers investigated the role of inulin on MDD patients at a dosage of 10 g/d for 8 weeks but failed to observe any significant effects in depression, but a trend of decreased anxiety score by using State-trait anxiety inventory II (STAII) (64). A pilot study which included 50 patients with alcohol use disorder depicted significant anti-depression and anti-anxiety effects of inulin supplementation for 19 days. The dosage of inulin was increased gradually from 4 to 16 g per day to reduce the GI adverse effects (65). Another clinical trial included 45 subjects and randomized them to receive one of two prebiotics (FOS, $N = 15$; Bimuno GOS, $N = 15$), or placebo at a dose of 5.5 g per day for 3 weeks. Results showed that GOS significantly decreased cortisol awakening response and attentional

TABLE 2 Prebiotic fibers for depression: study characteristics.

Study	*N*[a]	Mean age (years)	Healthy status	Prebiotic	Dosage	Study duration	Clinical measures	Mood health parameter changes	Bacterial change
Smith et al. (56)	142	32.0	Healthy	Inulin-type fructan	10 g/d	2 weeks	HADS-A, HADS-D	Mood (positive, negative, or depression): NC; anxiety: ↑ Psychomotor performance: NC Sleep quality: NC Memory: NC	Not available
Silk et al. (57)	44	54.0	IBS patients	GOS	3.5 or 7.0 g/d	4 weeks	HADS-A, HADS-D	Anxiety: lower dosage NC, higher dosage ↓ Depression: lower dosage NC, higher dosage NC	Not available
Smith et al. (58)	47	23.0	Healthy	Inulin-type fructan	5 g (one-time)	4 h	SSM	Subjective feeling of happiness: ↑ Subjective feeling of indigestion: ↓ Mood rating: NC Memory tasks (immediate free recal, delayed recall): ↑; delayed recognition memory: better accuracy but slower reaction times. Logistic reasoning, semantic processing and spatial memory: NC	Not available
Schmidt et al. (59)	45	23.7	Healthy	GOS, inulin-type fructan	5.5 g/d	3 weeks	STAI	Cortisol awakening response: GOS↓, FOS NC Attentional vigilance to negative vs. positive information: GOS↓, FOS NC Emotional categorization, recall and recognition: GOS NC, FOS NC Self-report anxiety: GOS NC, FOS NC Self-report perceived stress: GOS NC, FOS NC	Not available.
Azpiroz et al. (60)	79	41.7	IBS patients	Inulin-type fructan	5 g/d	4 weeks	HADS-A, HADS-D	Anxiety: ↓ Depression: NC	Total anaerobes: NC ↑Bifidobacteria

(Continued)

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TABLE 2 (Continued)

Study	N[a]	Mean age (years)	Healthy status	Prebiotic	Dosage	Study duration	Clinical measures	Mood health parameter changes	Bacterial change
Kazemi et al. (61)	72	36.7	Depressed patients	GOS	5 g/d	8 weeks	BDI-II	Depression: NC Biomarkers: kynurenine NC; tryptophan (Trp) NC; kynurenine/Trp ratio NC; Trp/BCAA ↑; Trp/isoleucine NC	Not available
Iribarren et al. (62)	60		IBS patients	HMO	5 or 10 g/d	4 weeks	HADS	Anxiety: NC Depression: NC	↑Bifidobacteria
Moludi et al. (63)	48	52.0	CAD patients	Inulin-type fructan	15 g/d	2 months	STAI BDI-II	Anxiety: NC Depression: NC	Not available
Vaghef-Mehrabani et al. (64)	45	39.8	MDD patients	Inulin-type fructan	10 g/d	8 weeks	STAI, STAII, HRDS, BDI-II	Anxiety: NC Depression NC	Not available
Amadieu et al. (65)	50	48.2	AUD patients	Inulin-type fructan	4–16 g/d	19 days	STAI, BDI	Anxiety: ↓ Depression: ↓	↓diversity and evenness Total anaerobes: NC ↑Actinobacteriota phylum, Bifidobacteriaceae family, Bifidobacterium genus; ↓Bacteroidaceae family, Bacteroides, Dorea and *Ruminococcus torques*
Becker et al. (66)	49	65.2	PD patients	RS	10 g/d	8 weeks	BDI-II	Depression: ↓	↑genus Rhodococcus

[a]Number of subjects included in the relevant statistical analyses.

AUD, alcohol use disorder; BDI, Beck depression inventory; CAD, coronary heart disease; IBS, irritable bowel syndrome; NC, not changed; PD, Parkinson's disease; STAI, State-trait Anxiety Inventory; HADS, Hospital Anxiety and Depression Scale; HDRS, Hamilton Depression Rating Scale.

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vigilance to negative vs. positive information, suggesting that GOS at 5.5 g per day may have anxiolytic effects by suppressing the neuroendocrine stress response. The administration of FOS did not generate any significant effects (59). In a crossover study where 44 IBS patients were included, the researchers found that GOS supplementation at both 3.5 and 7.0 g per day significantly alleviated IBS symptoms, but only the higher dosage significantly improved the anxiety scores without changing the depression severity (57). Consistently, a study with 72 depressed patients failed to observe significant anti-depressive effects of GOS at 5 g per day for 8 weeks, despite that GOS supplementation significantly increased the tryptophan to BCAAs ratio (61). These pieces of evidence led us to conclude that both inulin-type fructans and GOS have limited effects in depression on both healthy and diseased population, even though FOS appeared to exhibit certain acute anti-depressive benefits. However, it is intriguing that GOS supplementation consistently decreased the level of anxiety in different studies, which warrants to be further discussed.

The implications of HMO in psychological modification were investigated in one study with 60 IBS patients. At 10 g per day, 4-week HMO treatment significantly enhanced the abundance of Bifidobacteria, but did not affect anxiety or depression scores (62).

In an open-label clinical trial, researchers investigated the effects of an eight-week treatment with RS (type 3) at 10 g per day, and found the treatment to be effective in reducing depression scores and enhancing fecal butyrate levels (66). Nevertheless, this is the first and single study that we identified in exploring RS and depression. Therefore, translation requires caution as the totality of evidence is not sufficient to conclude the efficacy of RS in modulating mood health.

4.2. Phytonutrients

The benefits that phytonutrients can contribute to human health have been explored by various studies, targeting on a wide range of endpoints including cognitive performance and mood. In total, four studies focused on resveratrol, two studies used chlorogenic acid, and two studies leveraged EGCG treatments (Table 3).

Among 40 patients diagnosed with mild cognitive impairment, a mixture of resveratrol (200 mg/d) and quercetin (350 mg per day) for 26 weeks did not induce change in anxiety or depression (73). The independent effects of resveratrol in mood health were reported by three clinical trials. Evans et al. (72) recruited 80 postmenopausal women and randomized them to take *trans*-resveratrol (150 mg per day) or placebo for 14 weeks. The researchers found resveratrol supplements to be effective in reducing anxiety, but not functional in modulating depression, anger, fatigue, confusion, or vigor (72). The acute efficacy of resveratrol in modulating mood also appeared to be minor, as a group of researchers found that 250 mg resveratrol did not induce any changes in the subjective feelings of being alert, jittery, fatigue, relaxed, tense, or tired (71). The only clinical trial that reported a positive effect of resveratrol in depression was the one by Witt et al. (70), in which the 23 overweight subjects were provided resveratrol supplementation at 200 mg per day for 26 weeks and as a result, the supplementation was effective in reducing depression scores. However, the control group of this study was pairwise-matched to the intervention group, meaning that randomization and double-blinding were not achieved. Therefore, by summing up the above-mentioned clinical trials, the efficacy of resveratrol in modulating mood health including depression is weak and inconclusive.

Although the antidepressant effects of quercetin have been investigated in multiple animal studies, currently, few clinical trials have leveraged quercetin as a nutritional approach to improve depression. Interestingly, used as a part of the ingredients in the traditional Chinese medicinal formula, quercetin was reported to be functional in relieving depression by several clinical trials, and its efficacy even appeared to more potent than traditional antidepressants such as fluoxetine, paroxetine, and duloxetine (75).

Camfield et al. (69) examined the effects of a decaffeinated pure chlorogenic acids on the cognitive and mood performance of healthy adults aged 50 years or older. The participants were given either the chlorogenic acids or placebo in a double-blind acute cross-over design, and their cognitive and mood responses were assessed at pre-dose, 40-min and 120-min post-dose. While the study found no significant effects on the primary cognitive outcome measure, it did observe some improvements in jittery and headache with the chlorogenic acids in comparison to the placebo (69). In a study among healthy women with menopausal symptoms, researchers assigned the subjects to take either placebo or chlorogenic acids at a daily dosage of 270 mg for 4 weeks. Although findings showed significant improvements compared to the baseline values in both groups, no significant difference between the two groups was observed.

The role of EGCG in mood health was examined by two clinical trials. In the study that was conducted by Wightman et al. (68), 27 healthy adults received a placebo and two different doses (135 and 270 mg) of EGCG on separate days in a counterbalanced order. Their results showed that EGCG did not have significant effects in modulating relaxation, alertness, jitters, tiredness, tense and mental fatigue (68). However, in another study, Scholey et al. (67) found EGCG supplementation at 300 mg was effective in improving calmness and decreasing stress but did not affect alertness, contentedness and fatigue. Although insufficient data were available to substantiate EGCG's psychopharmacological effects, preliminary evidence suggests that EGCG may have a calming effect during the second hour post-dose. Given the fact that Scholey et al. (67) utilized a higher dosage in the study, it is possible that EGCG needs a higher dosage to exert its beneficial effect. However, such hypothesis needs to be further investigated in a dose-respondent study.

4.3. n-3 PUFAs

There is an increasing amount of evidence suggesting that omega-3 polyunsaturated fatty acids (omega-3 PUFAs) are effective in improving depression. The summary of the studies was based on a publication (76) that systematically summarized 26 studies, which included 2,160 subjects. The results showed that EPA and a formula with ≥60% EPA were effective in decreasing depression, and it was found that an EPA dosage ≤1 g/d resulted in significant benefits. On the contrary, DHA and a formula with ≥60% DHA did not show significant effects in alleviating depression. In addition to the effects in treating depression, PUFAs showed preventive effects against depression. A systematic review and meta-analysis of 20 RCTs on 7,682 participants showed that n-3 PUFAs supplementation showed a modest beneficial effect on depressive symptomology compared to the placebo groups. In spite of a potential publication bias, the subgroup

TABLE 3 Prebiotic phytonutrients for depression: study characteristics.

Study	N[a]	Mean age (years)	Healthy status	Prebiotic and dosage	Study duration	Clinical measures	Mood health parameter changes
Scholey et al. (67)	31	27.7	Healthy	EGCG: 300 mg	180 min	MVAS	Calmness ↑, stress ↓ Alertness, contentedness, fatigue: NC
Wightman et al. (68)	27	22.0	Healthy	EGCG: 135 or 270 mg	90 min	MVAS	Relaxation, alertness, jitters, tiredness, tense, mental fatigue: NC
Camfield et al. (69)	40	64.5[b]	Healthy	Chlorogenic acids: 540 mg	120 min	MVAS	Jitters and headache: ↓ Tiredness, alertness, clam, relaxation, mental fatigue, overall mood score: NC
Witte et al. (70)	46	64.3	Overweight	Resveratrol: 200 mg/d	26 weeks	BDI	Depression: ↓
Wightman et al. (71)	23	21.0	Healthy	Resveratrol: 250 mg/d	One-time	MVAS	Alert, jitters, mental fatigue, relaxed, tense, tired: NC Overall mood: NC
Evans et al. (72)	80	61.5	Healthy	Resveratrol: 150 mg/d	14 weeks	CES-D	Anxiety: ↓ Depression, anger, fatigue, confusion, vigor: NC
Köbe et al. (73)	40	67.2	MCI patients	Resveratrol: 200 mg/d and quercetin: 350 mg/d	26 weeks	BDI STAI-X1	Anxiety: NC Depression: NC
Enokuchi et al. (74)	82	51.0	Menopausal symptoms	Chlorogenic acids: 270 mg/d	4 weeks	STAI	Anxiety: NC

[a]Number of subjects included in the relevant statistical analyses.
[b]Median age.BDI, Beck's Depression Index; MCI, mild cognitive impairment; NC, not changed; MVAS, mood visual analog scales; STAI, State-trait anxiety inventory.

analysis of this study indicated that longer treatment duration may improve the effectiveness of n-3 PUFAs in preventing depression, especially depression with a lower degree of severity. However, a difference in favor of lower EPA dosage was observed, and the quality of evidence is relatively low (77). Several studies were published after the publication of these two meta-analyses. In a clinical trial with 61 MDD with high inflammation, only the 2 g/d dose of EPA decreased peripheral blood mononuclear cell tumor necrosis factor alpha (TNFα) levels, and EPA 4 g/d had a medium effect size for response rates compared to placebo. The study suggests that EPA 4 g/d may be effective in alleviating MDD in overweight individuals with elevated inflammatory markers, and changes in hs-CRP may be correlated with clinical response (78). The effects of a high n-3 plus low n-6 (H3-L6) dietary intervention in mood health was examined in a study with bipolar disorder (BD) patients, and the control diet contained usual U.S. level of n-6 and n-4 PUFA intakes. The results showed that H3-L6 group had reduced variability in mood symptoms compared to the control group, suggesting that the dietary intervention may be effective in improving mood stability in BD (79). One study aimed to investigate the effects of fish oil, which contains omega-3 polyunsaturated fatty acids, on emotion-generated corticolimbic functional connectivity in depressed youth at high risk for bipolar I disorder. The results showed that fish oil increased erythrocyte EPA + DHA composition and altered functional connectivity between the orbitofrontal cortex and superior temporal gyrus, and between the amygdala and inferior temporal gyrus. These changes were correlated with decreases in the severity of bipolar I disorder (80).

Apolipoprotein E (ApoE) is a protein involved in the metabolism and transport of lipids in the body. It is primarily produced in the liver, but it is also synthesized by other cells, including macrophages and neurons in the brain. ApoE plays a crucial role in the transportation of cholesterol and other lipids through the bloodstream and the regulation of lipid homeostasis (81). In elderly, a combination of 1,491 mg DHA and 351 mg EPA per day was effective in modulating the function of APOE ε4 carriers on depression and anxiety scores (82). APOE ε4 is a variant of the APOE gene that is associated with an increased risk for developing Alzheimer's disease. In terms of mood and cognition, APOE ε4 carriers have been found to have a higher risk for developing depression and anxiety compared to non-carriers (83). People with APOE ε4 may also experience cognitive decline at a younger age compared to non-carriers, although not all APOE ε4 carriers will develop Alzheimer's disease or experience significant cognitive decline (84). It is important to note that while APOE ε4 may increase the risk for certain health outcomes, it does not determine

them. Environmental factors, lifestyle choices, and other genetic factors can also play a role in determining an individual's overall health and cognitive function (85).

5. Mechanism: gut-brain-axis moderates the effects of prebiotics in depression

The gut microbiota, referred to as the "second brain" in humans due to its regulatory impact on the central nervous system through neuronal, chemical, and immune pathways, has been shown to establish a bidirectional communication channel between the gut and the brain (86). The gut-brain-axis (GBA) was discovered when gastrointestinal endocrine system changes were linked to alterations in neurons and brain cells. This concept was supported by studies that showed the contribution of gut microbiota to the development of cognitive diseases. Over 20 years ago, the observation of significant improvement in patients with hepatic encephalopathy after receiving oral antibiotics provided the most convincing evidence of a connection between gastrointestinal microbes and the brain in humans (87). Recently, emerging data suggested that the microbiota plays a role in influencing anxiety, depression, and other mood disorders. Patients with depression have different gut microbial profiles compared to healthy controls, while patients with gut inflammation experience mood and cognitive disturbances. *In vivo* studies show that gastrointestinal inflammation can induce anxiety-like behavior and alter the central nervous system (88). Germ-free animals have been used to determine causality, reporting that the absence of gut microbiota promotes anxiety and neuroendocrine response to stress (89). Prospective studies indicate the impact of dysbiosis on mental health disorders. Fecal microbiota transplantation studies have also shown that depressive symptoms can be transmitted to recipients, with those receiving fecal microbiota from depressed patients exhibiting more severe symptoms compared to those who received fecal microbiota from healthy subjects (90). These findings indicate a causal role of gut microbiota in the development of depressive symptoms.

5.1. Neurotransmitters

A growing body of evidence supports the notion that the interplay between the gut and brain, mediated by the gut microbiota, has been associated with the cause and development of depression and anxiety. Strains of Bifidobacteria, such as *B. adolescentis*, have the capability to produce Gamma-Aminobutyric Acid (GABA), a primary inhibitory neurotransmitter in the brain (91). In particular, *B. adolescentis* PRL2019 and *B. adolescentis* HD17T2H are the most distinguish GABA producers among the *B. adolescentis* strains (91). An animal study revealed that pretreatment with *B. adolescentis* resulted in anxiolytic and antidepressant effects in mice under chronic restraint stress. This was observed through an increase in time spent in open spaces, a decrease in immobility duration, and a reduction in inflammatory cytokine expression in the hippocampus, suggesting that the anxiolytic and antidepressant effects of *B. adolescentis* are related to its ability to reduce inflammation and rebalance the gut microbiota (92). In a study with children, researchers portrayed a negative correlation between *B adolescentis* and mental disorders, including depression and anxiety (93). It warrants further investigation whether the bifidogenic prebiotics function through enhancing GABA production.

In addition to GABA, other neurotransmitters including serotonin, norepinephrine, and dopamine can also be synthesized by the gut microbiota. Studies have demonstrated that *Streptococcus*, *Enterococcus*, and *Escherichia* are involved in the synthesis of the above-mentioned neurotransmitters (94–96). Norepinephrine, or noradrenaline, functions within the central nervous system and concurrently serves as a stress hormone. It contributes to the "fight or flight" response and is linked to mechanisms of arousal, attention, and focus (97). Dopamine partakes in reward processing and motivation, alongside movement control. It is affiliated with sensations of pleasure and contributes to the progression of addiction (98). The capacity of dopamine to act as a precursor in the biosynthesis of norepinephrine and epinephrine is well established in scientific literature (Figure 2). Serotonin, also known as 5-hydroxytryptamine (5-HT), is involved in regulating mood, appetite, sleep, and other bodily functions (97). Based on preclinical and clinical findings, it has been established that the dopamine, 5-HT, and norepinephrine in the central nervous system are disrupted in individuals experiencing depression (97, 99). Presently available antidepressants function mainly through one or more of the following mechanisms: hindering the reuptake of serotonin or norepinephrine, obstructing inhibitory presynaptic serotonin or norepinephrine receptors, or inhibiting monoamine oxidase (99). All of these mechanisms lead to elevated concentrations of serotonin and/or norepinephrine.

5.2. Short-chain fatty acids

Short-chain fatty acids (SCFAs) are a class of low molecular weight, organic monocarboxylic acids with a carbon chain length of up to six atoms (100). Acetate (C2), propionate (C3), and butyrate (C4) are the three major SCFAs in the gut, with an approximate molar ratio of 60:20:20 (101). SCFAs are energy substrates for colonocytes and hepatocytes, except for acetate, which cannot be oxidized in the liver (102). The concentrations of SCFAs are substantially lower in the circulating system compared with the colon. It has been shown that SCFAs can cross the blood brain barrier (BBB) while maintaining their integrity, although the SCFA levels in the brain are relatively low (103, 104). Nevertheless, the SCFAs are capable of exerting systemic effect by binding to G protein-coupled receptors (GPRs) or function by playing a role as histone deacetylase (HDAC) inhibitors (105). Accumulating evidence has shown that SCFAs possess anti-inflammatory and neuroprotective capabilities to maintain a robust microglial function through the inhibition of HDACs (104, 106). Butyrate supplementation showed effects in improving long-term memory consolidation and promoting the expressions of brain-derived neurotrophic factor (BDNF) and neurogenesis in rodents (107). Exploration into the underlying mechanisms that govern the modulation of neuronal function by SCFAs has illuminated that the activation of GPR41/GPR43 may mediate some of these effects. Dalile et al. (108) examined the effects of one-week SCFA-mixture supplementation on responses to psychosocial stress in healthy adults and demonstrated that both low (87.1 mmol acetate, 6.6 mmol propionate, and 26.2 mmol butyrate) and high (174.2 mmol acetate,

13.3 mmol propionate, and 52.4 mmol butyrate) SCFA mixture significantly mitigated the cortisol-induced psychosocial stress, but did not affect subjective mood ratings (108).

In humans, depressive symptoms were positively associated with fecal acetate levels and negatively associated with both butyrate and propionate levels (109, 110). It is noteworthy that these studies investigated fecal SCFAs. Although it is a valid surrogate measure of the gut SCFAs, it may be subject to various sources of bias (104). In animals, sodium butyrate supplementation led to a reversed depressive-like behavior in animal models of depression, potentially by improving the function of the mitochondrial respiratory chain complexes and the activity of tricarboxylic acid cycle enzymes in the stratum of rats (111). Consistently, another *in vivo* study reported that sodium butyrate showed antidepressant-like activity, which was, at least partially, attributed to an increase of BDNF and inositol depletion (112, 113) and increased histone acetylation in the hippocampus (114). The effects of butyrate supplementation for preventing or alleviating depression need to be further validated in humans.

5.3. Inflammation

Under normal physiological circumstances, the activation of immune cells and subsequent cytokine production may elicit only modest effects within the central nervous system (CNS). Nevertheless, sustained systemic inflammation, frequently precipitated by infectious agents, has been strongly linked with a range of behavioral and cognitive impairments (115, 116). Lipopolysaccharides (LPS) are outer membrane components of gram-negative bacteria. Increased levels of circulating LPS may prompt the upregulation of pro-inflammatory cytokines, including TNFα, monocyte chemoattractant protein-1 (MCP-1), interleukin-1 beta (IL-1β), and nuclear factor kappa B p65 (NF-κB p65) (117, 118). The precise mechanism through which peripheral LPS exerts its impact on the brain remains unknown. However, one plausible route involves the translocation of LPS across the BBB. Alternatively, LPS may trigger afferent nerves beyond the BBB, operate at circumventricular organs, and induce changes in the permeability and function of the BBB (119). Interestingly, some preclinical evidence suggests that LPS is a double sword for maintaining neural homeostasis, as low-dose LPS preconditioning suppressed the production of proinflammatory cytokines, whereas a high LPS concentration may induce potent inflammatory signaling cascade (120). Peripheral insults which elicit a systemic inflammatory response may serve to promote the mobilization of peripheral immune cells toward the brain, resulting in the excessive activation of the neuroimmune response (6). Activation of Toll-like receptor 4 (TLR4) by LPS results in the secretion of inflammatory cytokines and the enhancement of costimulatory molecules on antigen-presenting cells (121). But it is important to note that although LPS is recognized for producing Th1 responses, it exhibits pleiotropic properties as it is also capable of promoting differentiation into other T helper lineages given the appropriate conditions (121).

Meta-analyses of clinical studies have indicated a strong indication of pro-inflammatory cytokine involvement in depression, as demonstrated by higher concentrations of IL-6 and TNFα in the blood of depressed patients as compared to controls (122, 123). However, another prospective study reported that inflammatory cytokines were

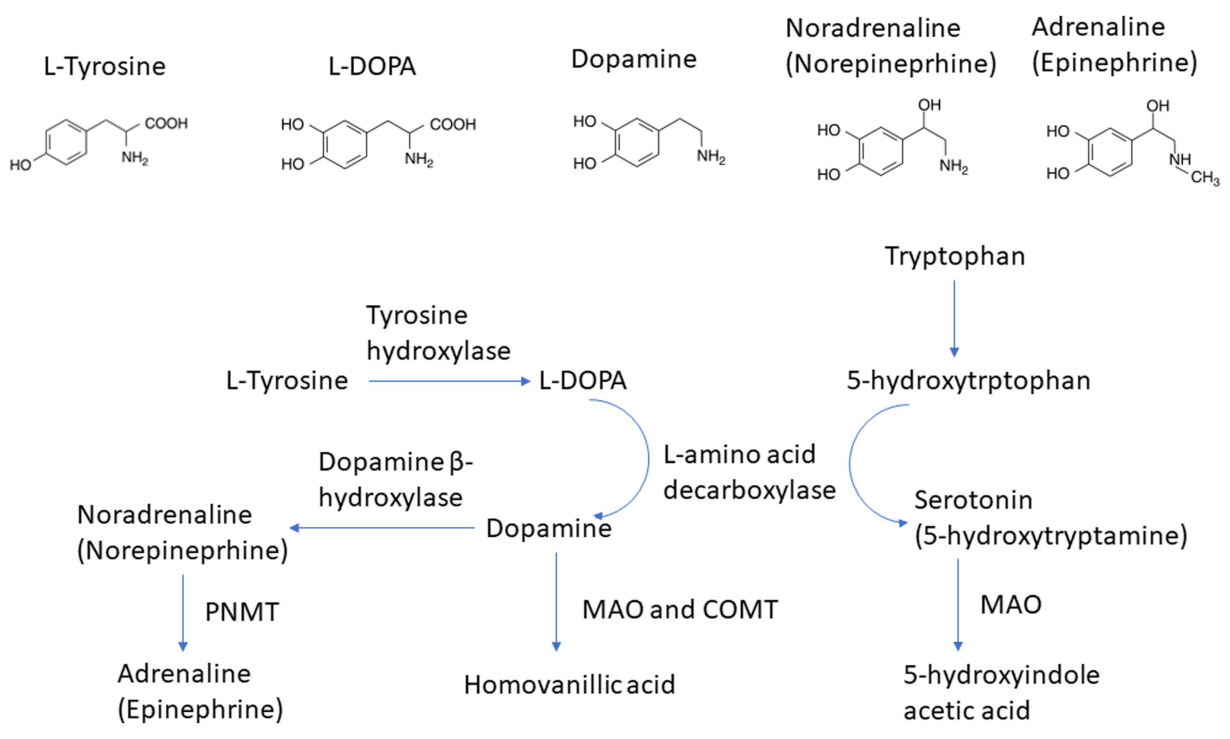

FIGURE 2
Pathway of norepinephrine and epinephrine biosynthesis.

not significantly associated with 10-year depression prognosis (124), indicating that an inflammatory status may impose acute effects on depression.

6. Conclusion and future directions

The current work summarized the role of promising prebiotics on modulating the development and severity of depression. By summarizing the existing clinical trials on prebiotics and depression, we concluded that GOS and n-3 PUFAs are effective in mitigating depressive symptoms. It is postulated that EGCG exhibits potential antidepressant properties, however, a higher dosage of EGCG may be required to elicit a significant effect. The plausible mechanisms that account for the impact of prebiotics are the synthesis of neurotransmitters, production of short-chain fatty acids (SCFAs), and regulation of inflammation (Figure 3). The impact of alternative prebiotics on depression has yet to be fully elucidated owing to several factors, including a dearth of clinical trials, disparate methodologies, variations in ingredient sources, and divergent extraction techniques.

While alterations in the microbial composition have been commonly documented in individuals with depression, there exists considerable variability in the specific microbiota that exhibit augmentation or reduction. The taxonomy that predominantly contributes to the onset and progression of depression is a subject of current investigation by several research teams; however, the answer to this question remains elusive. Multiple factors, such as age, gender, medication or antibiotic usage, geographical location, health status, physical activity, dietary patterns, and sleep duration, among others, may impact the diversity and profile of gut microbiota. Thus, it is crucial to record and report detailed subject characteristics to account for potential discrepancies between studies.

Considering that a beneficial microbial profile may function to prevent or alleviate depression and other mood disorders, it raises the possibility that probiotics may be functional for anti-depression as well. Prebiotics and probiotics differ in their mode of action, mechanisms, evidence, dosage, efficacy, side effects, study design, and future research directions. Prebiotics indirectly promote the growth of beneficial gut bacteria by providing nourishment, while probiotics introduce beneficial bacteria directly into the gut (125). Prebiotics affect depression through mechanisms such as neurotransmitter synthesis, short-chain fatty acid production, and inflammation regulation. Evidence for prebiotics is limited but promising, while probiotics have shown positive effects in numerous studies. Dosages vary for both, with prebiotics typically ranging from 2 to 10 g and probiotics ranging from 1 to 50 billion CFUs per day (10, 126, 127). Further research is needed to establish effectiveness, optimal dosages, and strains for both prebiotics and probiotics in depression treatment. The differences between the trials using prebiotics and postbiotics in depression are described in Table 4. A comprehensive evaluation of 10 clinical trials comprising a cumulative patient population of 1,349 subjects showed that no statistically significant difference in mood was observed between the intervention group with probiotics supplementation and the placebo group, although a subgroup analysis of studies conducted on depressed individuals exhibiting mild to moderate depressive symptoms revealed a noteworthy enhancement in the moods (127). The effects of probiotics on mitigating perinatal depression have also been controversial, and even the supplement of same probiotic strain showed different effects (6). The comparability of current clinical trials is hindered by inter-study discrepancies in probiotic dosing, bacterial strains, and strain combinations. Additionally, the generalizability of findings to depressed individuals is impeded by the fact that the majority of existing randomized controlled trials were performed on healthy populations. Nevertheless, it leads to the hypothesis that modulating the abundance of one group of gut microbiota may be insufficient to show anti-depressive effects. Thus, manipulating the gut microbiota as an integrated system by using different ingredients, such as prebiotics and fibers, may serve as a more effective approach to prevent or alleviate the development of depression.

Combined prebiotic and probiotic therapy and the use of prebiotics in combination with traditional antidepressants have garnered attention as potential treatment approaches for depression (128). This synergistic approach has the potential to improve gut microbiota composition and function, which may positively impact mental health. However, a recent RCT showed that in adults experiencing moderate psychological distress and low prebiotic intake, adopting a high-prebiotic dietary intervention shows potential in enhancing mood, reducing anxiety, alleviating stress, and improving sleep. However, the combination of a high-prebiotic diet and probiotic supplement, known as a synbiotic approach, does not seem to yield beneficial effects on mental health outcomes (129). More research is needed to explore the specific combinations, dosages, and strains that would yield optimal outcomes. Additionally, the efficacy and safety of combined therapy should be thoroughly evaluated through well-designed clinical trials.

Regarding prebiotics in combination with traditional antidepressants, the rationale behind this approach lies in the potential of prebiotics to modulate gut microbiota and influence the efficacy of antidepressant medications. By improving gut health and promoting the production of beneficial metabolites, prebiotics could potentially enhance the therapeutic effects of traditional antidepressants. However, the interactions between prebiotics and specific antidepressant medications need to be further investigated to understand potential synergies or adverse effects. Additionally, individual variability and personalized treatment strategies should be considered. Both combined prebiotic/probiotic therapy and the combination of prebiotics with traditional antidepressants offer intriguing possibilities for improving treatment outcomes in depression. Nevertheless, more robust clinical trials and mechanistic studies are required to determine the optimal protocols, dosages, and mechanisms underlying these approaches. The field of psychobiotics, which explores the interaction between the gut microbiota and mental health, holds promise for advancing our understanding and refining these combination therapies for depression. Currently, there are commercially available prebiotic antidepressant preparations that aim to harness the potential benefits of prebiotics in managing depression. These products typically contain specific types of prebiotics that are believed to support a healthy gut microbiota and potentially improve mental well-being. It is important to note that while these prebiotic antidepressant preparations are available, their efficacy and safety in treating depression have not been extensively studied or established. The scientific literature on the specific formulations and their effects on mental health outcomes is limited. Individuals considering the use of commercially available prebiotic antidepressant preparations should exercise caution and consult with healthcare professionals

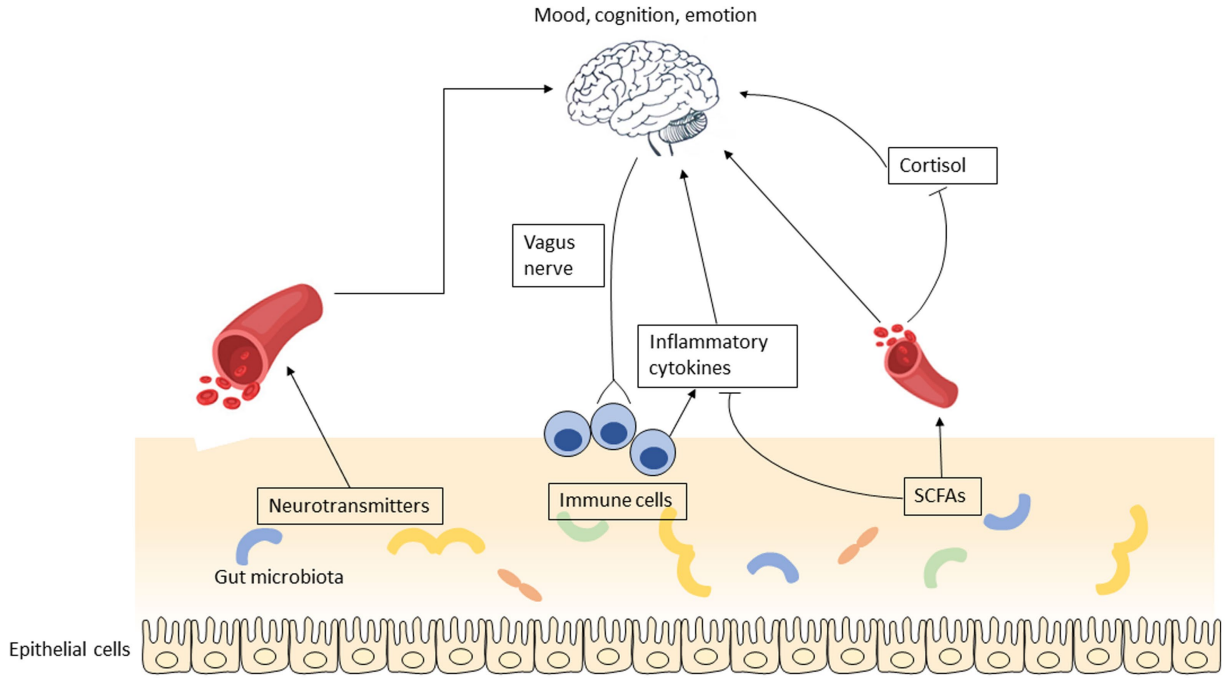

FIGURE 3
The putative mechanisms that account for the impact of prebiotics on depression involve several potential pathways, including the synthesis of neurotransmitters, production of short-chain fatty acids, and regulation of inflammation.

TABLE 4 Differences between prebiotics and probiotics.

Differences	Prebiotics	Postbiotics
Definition	Non-digestible substances that stimulate the growth and activity of beneficial bacteria in the gut and confer health benefits	Live microorganisms that confer health benefits when consumed in adequate amounts
Mode of action	Indirectly promote the growth of beneficial gut bacteria through providing nourishment	Directly introduce beneficial bacteria into the gut to restore microbial balance
Mechanisms	Synthesis of neurotransmitters—Production of short-chain fatty acids—Regulation of inflammation	Modulation of gut-brain communication—Immune system modulation—Anti-inflammatory effects
Evidence	Limited number of studies exploring the effects on depression, but show promising results	More extensive research demonstrating positive effects on depressive symptoms
Dosage	Varies depending on the specific prebiotic used, typically ranging from 2 to 10 grams per day	Varied strains and dosages, commonly ranging from 1–50 billion colony-forming units (CFUs) per day
Efficacy	Mixed results, with some studies reporting improvements in depressive symptoms, while others show no effect	Overall, positive effects observed in a significant number of studies, but efficacy may vary among individuals
Side effects	Generally well-tolerated, but may cause mild gastrointestinal symptoms such as bloating or gas	Minimal side effects reported, primarily gastrointestinal discomfort in rare cases
Study design	Limited number of randomized controlled trials (RCTs)	Numerous RCTs conducted, providing stronger evidence of efficacy
Future research	More rigorous and larger-scale RCTs needed to establish effectiveness and identify optimal dosages	Further exploration of specific strains, dosages, and treatment durations to enhance efficacy and consistency

before incorporating them into their treatment plan. It is advisable to seek evidence-based treatments and interventions that have undergone rigorous scientific evaluation and have a proven track record in managing depression.

Although the production of SCFAs is considered one of the primary mechanisms through which prebiotics alleviate depression and modulate mood, quantifying the dose of SCFAs delivered to the colon presents a significant challenge. Whole food or natural dietary sources cannot provide supraphysiological quantities of SCFAs, thereby impeding investigations into the potential benefits of SCFAs on brain function. To explore the dependent effects of SCFAs, animal studies often rely on oral administration, which may lead to distinct

physiological outcomes due to the absorption of SCFAs from the upper gastrointestinal tract. Intravenous administration of SCFAs bypasses the absorption and oxidation processes that occur in colonocytes and is thus non-physiological. Additionally, rectal administration is not suitable for chronic use (103). Consequently, future studies should be undertaken to establish the optimal dosage of SCFAs delivered to the gut through prebiotic supplementation. Moreover, research into identifying prebiotic sources that produce the most abundant SCFAs would be valuable in developing dietary strategies for preventing the onset of depression.

The existing literature on the relationship between prebiotics and depression exhibits several limitations, both in terms of methodological rigor and conceptualization of future research. These limitations highlight the need for further investigation to establish a clearer understanding of this topic. Methodologically, many studies suffer from small sample sizes, limiting the generalizability of their findings. Depression is a complex and multifaceted condition, and studying it requires large and diverse participant groups to capture its full spectrum. Moreover, the majority of studies rely on self-report measures for assessing depression symptoms, which can introduce biases and inaccuracies. The use of more objective and standardized diagnostic criteria, such as structured clinical interviews, would enhance the reliability of the results. Another limitation lies in the inconsistency of prebiotic interventions across studies. There is a lack of consensus on the optimal dose, duration, and type of prebiotics to administer. This heterogeneity hampers the ability to compare findings across studies and draw definitive conclusions. Future research should strive for standardized protocols to facilitate more meaningful comparisons and meta-analyses. Additionally, most studies focus solely on the effects of prebiotics on depressive symptoms, neglecting potential underlying mechanisms. Exploring the gut-brain axis and investigating the specific pathways through which prebiotics influence brain function would provide a more comprehensive understanding of their antidepressant effects. Last but not least, the generalizability of the findings might be impeded because the majority of existing studies were conducted on diseased population, so it would be challenging to leverage the current findings to indicate how healthy individuals may benefit from consuming the prebiotics. Overall, the existing literature on prebiotics and depression is limited by methodological shortcomings, including small sample sizes and inconsistencies in intervention protocols. Moving forward, it is essential to address these limitations by employing larger, more diverse samples, utilizing longitudinal designs, establishing standardized intervention protocols, and investigating underlying mechanisms. By addressing these gaps, future research can advance our knowledge of the potential role of prebiotics in the treatment and prevention of depression.

In conclusion, the results of our study indicate that a daily intake of 5 g of GOS and 1 g or less of EPA can be effective in alleviating depressive symptoms. Although EGCG shows promise as an antidepressant, a higher dosage of 3 g per day might be required to achieve notable effects. The potential mechanisms that explain the influence of prebiotics on depression include the synthesis of neurotransmitters, the production of short-chain fatty acids, and the regulation of inflammation.

Author contributions

YY and BZ performed literature search and data collection. YY, BZ, SZ, LS, FL, and XL conducted the study design and wrote the manuscript. FL and XL provided scientific proofreading and supervised the study. All authors contributed to the article and approved the submitted version.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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EDITED BY
Nicholas Pang,
Universiti Malaysia Sabah, Malaysia

REVIEWED BY
Sijin Duan,
Yantai University, China
Kacper Witek,
Polish Academy of Sciences, Poland

*CORRESPONDENCE
Muriel Darnaudéry
✉ muriel.darnaudery@u-bordeaux.fr

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Strain-specific changes in nucleus accumbens transcriptome and motivation for palatable food reward in mice exposed to maternal separation

Simon Benoit[1], Mathilde Henry[1], Sara Fneich[2,3], Alexia Mathou[1], Lin Xia[1], Aline Foury[1], Mélanie Jouin[2,3], Claudine Junien[2,3], Lucile Capuron[1], Luc Jouneau[2,3], Marie-Pierre Moisan[1], Cyrille Delpierre[4], Anne Gabory[2,3] and Muriel Darnaudéry[1]*

[1]Univ. Bordeaux, INRAE, Bordeaux INP, NutriNeurO, UMR 1286, Bordeaux, France, [2]Univ. Paris-Saclay, UVSQ, INRAE, BREED, Jouy-en-Josas, France, [3]Ecole Nationale Vétérinaire d'Alfort, BREED, Maisons-Alfort, France, [4]CERPOP, UMR1295, Inserm, Université Toulouse III Paul Sabatier, Toulouse, France

Introduction: In humans, adversity in childhood exerts enduring effects on brain and increases the vulnerability to psychiatric diseases. It also leads to a higher risk of eating disorders and obesity. Maternal separation (MS) in mice has been used as a *proxy* of stress during infancy. We hypothesized that MS in mice affects motivation to obtain palatable food in adulthood and changes gene expression in reward system.

Methods: Male and female pups from C57Bl/6J and C3H/HeN mice strains were subjected to a daily MS protocol from postnatal day (PND) 2 to PND14. At adulthood, their motivation for palatable food reward was assessed in operant cages.

Results: Compared to control mice, male and female C3H/HeN mice exposed to MS increased their instrumental response for palatable food, especially when the effort required to obtain the reward was high. Importantly, this effect is shown in animals fed *ad libitum*. Transcriptional analysis revealed 375 genes differentially expressed in the nucleus accumbens of male MS C3H/HeN mice compared to the control group, some of these being associated with the regulation of the reward system (e.g., *Gnas*, *Pnoc*). Interestingly, C57Bl/6J mice exposed to MS did not show alterations in their motivation to obtain a palatable reward, nor significant changes in gene expression in the nucleus accumbens.

Conclusion: MS produces long-lasting changes in motivation for palatable food in C3H/HeN mice, but has no impact in C57Bl/6J mice. These behavioral alterations are accompanied by drastic changes in gene expression in the nucleus accumbens, a key structure in the regulation of motivational processes.

KEYWORDS

early life stress, sex effect, operant conditioning, mesolimbic circuit, C3H/HeN mice

1. Introduction

In humans, early-life adversity, such as abuse, trauma or neglect may influence the development of child and exerts long-lasting effects on physiological functions and vulnerability to psychiatric disorders, notably depression, anxiety disorders, and substance abuse (1–3). Early-life adversity produces numerous physiological abnormalities including

hypothalamic–pituitary–adrenocortical (HPA) axis hyperactivity, low-grade inflammation, and affects brain areas involved in the regulation of cognitive and emotional processes such as the medial prefrontal cortex (mPFC), amygdala, hippocampus, and ventral striatum (4, 5). Additionally, early-life adversity exacerbates vulnerability to obesity in adult subjects (6), and this effect could be at least partially attributed to poor feeding habits and/or exacerbated motivation for high-calorie foods (7, 8). However, despite a large literature on the impact of early-life adversity on neuropsychiatric vulnerability, its effect on food motivation remains less explored (9).

In rodents, chronic maternal separation (MS) has been used as a *proxy* of early-life adversity. MS effects on emotional behaviors have been extensively documented in rats (10, 11). These effects include cognitive impairments, exacerbated anxiety-and depressive-like behavior as well as anhedonia associated with HPA axis alterations (12–14). Perinatal stress, both during prenatal and postnatal period, is also associated with metabolic disturbances (15–17), and a higher sensitivity to diet-induced obesity (18). Finally, a growing body of evidence suggests that early-life stress impairs reward processes (19). While MS has been associated with reduced sucrose preference, suggestive of an anhedonia phenotype (10, 20), early-life adversity was found to exacerbate addictive drug intake in rodents as well as in humans (21, 22). Strikingly, MS can also lead to lasting disruptions of the reward dopaminergic system (18, 23, 24). Thus, mice exposed to MS combined with unpredictable chronic mild stress or social defeat in adulthood showed transcriptomic changes in the ventral tegmental area and in the nucleus accumbens (NAc) (25, 26). However, the impact of MS on mice motivation for palatable food is still unknown. This is an important issue given that reward circuit alterations have been recurrently reported in the literature in both animal models and humans exposed to early-life adversity (24).

The aim of the present study was then to determine the impact of MS on motivation for palatable food in female and male mice offspring. Since mice are particularly resilient to early-life stress procedures (27, 28), we studied the effects of MS in C57Bl/6J and C3H/HeN mice, two mouse strains used for MS paradigms (25, 29–31). To further characterize the brain changes associated with MS, we also examined gene expression in the mPFC, the NAc, and the hypothalamus, brain areas sensitive to stress and playing a critical role in the regulation of reward processes.

2. Materials and methods

2.1. Animals

All experiments were carried out in accordance with French legislation (Directive 87/148, Ministère de l'Agriculture et de la Pêche) and European (Directive 2010/63/EU, 2010 September 22th) and approved by Institutional Regional Committee for animal experimentation (agreement #5012050-A). C57Bl/6J and C3H/HeN mice were obtained from Janvier Labs (Le Genest, Saint-Isle, France) and housed under standard laboratory conditions (23 ± 1°C; 12 h/12 h light/dark cycle; lights on at 7 a.m.; food and water *ad libitum*). After 1 week of habituation, two nulliparous female mice (11 weeks old) were placed with one male from the same strain during 1 week for breeding and then pregnant dams were single-housed in polycarbonate cages (48 × 26 × 21 cm) throughout gestation and lactation. The day of delivery was designated postnatal day 0 (PND0). At PND1, pups from all litters were pulled, sexed, and weighed. Two litters with abnormal number of pups (<3) or sex-ratio (only females) were excluded from the study. Litters were assigned to Maternal Separation (MS, C57Bl/6J, $n = 7$; C3H/HeN, $n = 7$) or control (C57Bl/6J, $n = 6$; C3H/HeN, $n = 6$) groups. The timeline of the experiment was shown in Figure 1.

2.2. Maternal separation combined with chronic unpredictable maternal stress

MS was carried out from PND2 to PND14 (180 min daily) (12, 31) and started randomly at 8:30, 9:00, 9:30, 10:15, 10:30, or 11:00 to minimize habituation. During separation sessions, pups were individually separated and kept at 32°C ± 2. During the separation, dams were exposed to a chronic unpredictable stress protocol (PND2: no bedding; PND3: sodden bedding; PND4: tilted cage 45°; PND5: soiled rat bedding; PND6: sodden bedding; PND7: no bedding; PND8: tilted cage 45°; PND9: no bedding; PND10: tilted cage 45°; PND11: sodden bedding; PND12: no bedding; PND13: soiled rat bedding; PND14: forced swim test) [adapted from Franklin et al. (29) and Rincel et al. (31)]. Control pups were left undisturbed with the dams. All pups were weaned on PND21 and grouped in 4–6 mice per cage by same strain, same sex, and same MS condition.

2.3. Behavioral assessment in operant chambers

At least 2 weeks before the beginning of the behavioral test, the light/dark cycle was inverted (lights off at 7 a.m.) in order to study mice behavior during the active phase of their cycle. Experiments were conducted during the dark phase between 09:00 and 17:00 h. Male and female offspring's food motivation for a palatable reward (10% condensed milk in water, 3.25 kcal/g), was assessed at 4–5 months of age in daily 60-min sessions (5 sessions per week) in operant chambers (Imétronic, Pessac, France) equipped with two levers, as previously described (32, 33). For the habituation and the initial training on fixed-ratio 1 (FR-1), mice were food restricted to 85% of their body weight. Then, animals were fed *ad libitum* throughout the experiment except for the concurrent choice test. *Ad libitum* access to food schedule was used in order to examine motivation for palatable food reward independently of the homeostatic state of the animals. Male and female cohorts were tested separately.

2.3.1. Habituation to the apparatus

Mice were placed into the operant chambers without lever for 30 min and milk reward delivered in the drinking cup every 60 s interval. A dose of milk was distributed only when the previous one had been consumed.

2.3.2. Fixed-ratio 1

Mice were initially trained to press one of the two levers (= active lever) on a fixed-ratio 1 (FR-1) schedule. Active lever press resulted in fluid (15 µL of milk solution) delivery associated with a 4 s cue (light above the lever) stimulus presence, in 60-min daily sessions.

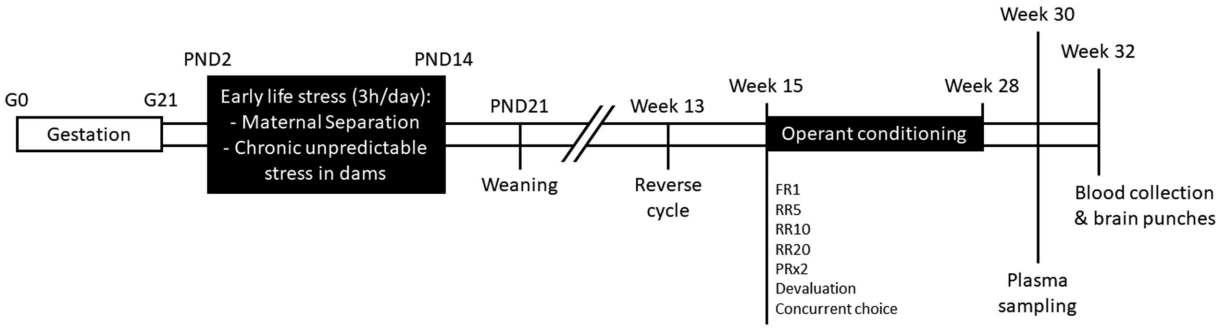

FIGURE 1
Schematic representation of the experimental timeline. G, gestational day; PND, post-natal day; FR1, fixed-ratio 1; RR5, random-ratio 5; RR10, random-ratio 10; RR20, random-ratio 20; PRx2, progressive-ratio x2.

Responses on the second lever (= inactive lever) were not associated with rewards and were recorded as a measure of non-specific activity. Mice received four FR-1 sessions under mild food deprivation, then mice were tested for an additional FR-1 session in *ad libitum* conditions.

2.3.3. Random-ratio

After FR-1, mice were submitted to random-ratio schedules (RR) in 60-min daily sessions: RR5 (12 sessions), RR10 (3 sessions), and RR20 (12 sessions), with, respectively, a probability of 1/5, 1/10 or 1/20 to be reinforced after one press on the active lever. RR schedules lead to reinforcement following an unpredictable average number of responses per rewards and result in high and consistent response rates that exceed those obtained with FR or interval schedules.

2.3.4. Progressive-ratio

Following training under RR20 schedule, mice were under a progressive ratio (PR) schedule for 2 daily sessions to assess the motivation for the palatable reward. During PR, the number of lever presses required to earn the next reward increased progressively and is multiplied by 2 (2, 4, 8, 16, 32 etc.) For each reinforced response, the animal received sweetened milk (15 µL). The cumulative active lever presses throughout 60 min the session and the total number of active lever presses until a 3 min cut-off without any presses were used as index of motivation. Two animals with very high values (one MS and one Control) were detected as statistical outliers using the Grubb's test and were removed from analyses.

2.3.5. Devaluation extinction test

Devaluation test allows assessing alteration of the representation of the outcome value. In this test, mice were pre-fed *ad libitum* to either milk or soy-bean oil emulsion Intralipid for 30 min in their home-cage. Immediately after, they were placed into the operant chamber to conduct a lever-press test in a 5-min extinction session. Although both levers were introduced, no reward was distributed. Then, mice received 2 days retraining using a RR20 procedure with free access before the second test. This second session was conducted as previously described, however this time, mice were pre-fed with the alternative outcome. The distribution order of the reward is randomly alternated for each mouse from one session to another.

2.3.6. Concurrent choice

Following one session under RR20 schedule with a food deprivation, a concurrent choice 60-min session test was conducted. In this task, fasted mice could press lever for milk (highly palatable), but their standard lab chow was also available on the floor of chamber opposite to the location of the lever. The total number of lever presses and the amount of lab chow consumed were recorded.

2.4. Plasma corticosterone

After the completion of the behavioral assessment, two blood samples were collected, one at the beginning of the active phase and one at the beginning of the inactive phase to assess circadian variation of plasma corticosterone levels. Blood was collected by tail nick using EDTA-coated tubes, were centrifuged (4,000 rpm, 4°C) for 10 min and stored at −20°C until use. Plasma corticosterone levels were determined with an in-house radioimmunoassay using a highly specific antibody as previously described (12). Cross reactivity with related compound such as cortisol was less than 3%. Intra-and inter-assay variations were less than 10% and less than 15%, respectively.

2.5. Metabolic hormones assay

Plasma resistin, leptin and insulin levels were measured in 12 h-fasted mice using a Mouse Metabolic Hormone Magnetic Bead-based immunoassay kits (Milliplex MAP Mouse Metabolic Magnetic Bead Panel, cat# MMHMAG-44 K-03, Millipore, Molsheim, FR) according to the manufacturer instructions on samples obtained at the sacrifice. Some samples were not included for technical issues (lack of plasma, out of range values).

2.6. Culling and samples collection

Mice were 12 h-food deprived and then were deeply anesthetized with isoflurane and sacrificed by decapitation. Blood was collected for metabolic hormones assessment. Whole brains were collected, medial prefrontal cortex (mPFC) and hypothalamus (HT) were dissected;

whereas nucleus accumbens (NAc) was punched on frozen slices and store at −80°C until use.

2.7. Microarrays

Total mRNA was extracted from mPFC, NAc, and hypothalamus using a TRIzol extraction kit (Invitrogen) according to the manufacturer's instructions. RNA concentration, purity and integrity were determined using a ND-1000 spectrophotometer (Nanodrop Technologies, Wilmington, DE, United States) and a bioanalyzer (Agilent, Les Ulis, France) (31). Gene expression profiles were performed at the GeT facility[1] using Agilent Sureprint G3 Mouse microarrays (8x60K, design 074809) following the manufacturer's instructions. For each sample, Cyanine-3 (Cy3) labeled cRNA was prepared from 200 ng (mPFC/HT) or 40 ng (NAc) of total RNA using the One-Color Quick Amp Labeling kit (Agilent Technologies, Santa Clara, CA) according to the manufacturer's instructions, followed by Agencourt RNAClean XP purification (Agencourt Bioscience Corporation, Beverly, Massachusetts). Dye incorporation and cRNA yield were checked using Dropsense™ 96 UV/VIS droplet reader (Trinean, Belgium). Six hundred ng of Cy3-labeled cRNA were hybridized on the Agilent SurePrint G3 Mouse GE microarray slides following the manufacturer's instructions. Immediately after washing, the slides were scanned on Agilent G2505C Microarray Scanner using Agilent Scan Control A.8.5.1 software and fluorescence signal extracted using Agilent Feature Extraction software v10.10.1.1 with default parameters.

Microarray data were analyzed using R (34) and Bioconductor packages (v 3.0)[2] (35) as described in GEO accession. Hierarchical clustering was performed using Pearson's correlation coefficient as distance function and Ward as linkage method. Partial least squares-discriminant analysis (PLS-DA) was performed for each tissue, with group (Ctrl or stress) as a Y response, using *ropls* package (36). Transformed signals were mean-centered and divided by the standard deviation of each variable. A model was fitted using the *limma lmFit* function (37). Pair-wise comparisons of biological conditions were applied using specific contrasts. Probes with Benjamini–Hochberg (BH) false discovery rate (FDR) < 0.05 were considered to be differentially expressed between conditions. Volcano plots were constructed with the *ggplot* function of the R *ggplot2* package. The differentially expressed gene datasets were uploaded into Ingenuity Pathway Analysis software (Qiagen IPA, content version 28,820,210) and a core analysis was performed, with the Agilent SurePrint G3 Mouse GE microarray as background. The canonical pathways with BH *p*s < 0.05 only and the upstream regulators with no flag "bias," activation z-score < −2 or > 2 and value of p of overlap < 0.05 only were considered. Another analysis was realized using ConsensusPathDB-mouse (38), with the differentially expressed gene list vs. the *mus musculus* database. KEGG pathways with a *q*-value < 0.05 were considered. For TLDA, analysis was performed with the R statistical software (34). For each gene, we compared the expression values within each maternal group in males and females, in C3H/HeN and C57Bl/6J apart. Pair-wise comparisons of maternal groups were conducted using a permutation test, as implemented in the oneway_test function of the *coin* package in R. For each set of tests (i.e., all tested genes for a given pair of maternal groups), *p*-values were BH adjusted for multiple testing. Differences were considered significant when *p*-adj < 0.05. The TLDA data clustering was performed using the ClustVis web tool for visualizing clustering of multivariate data[3] (39). Microarray data and experimental details are available in NCBI's Gene Expression Omnibus (40) and are accessible through GEO Series accession number GSE222781.

2.8. TaqMan low-density arrays

The top genes identified by IPA were selected for gene expression validation by TaqMan low-density arrays (TLDAs, Applied Biosystems): 44 genes for the *canonical pathway*; 21 genes for the *network (neurological and psychological diseases)*; 9 genes for *nervous system development and behavior*; and 13 genes for *behavior*. All samples were treated with DNase. The experiment was performed on the @BRIDGE platform (INRAE, Jouy-en-Josas, France) according to the manufacturer's instructions. Four samples were run on each TLDA card in simplicate. Each sample reservoir on the card was loaded with 100 µL of the reaction mix: cDNA template (600 ng) mixed with TaqMan Gene Expression Master Mix (Ap-plied Biosystems). After centrifugation (twice 1 min at 1200 rpm, Heraeus Multifuge 3S Centrifuge), the wells were sealed with a TLDA Sealer (Applied Biosystems). PCR amplification was performed on the 7900HT Real-Time PCR System (Applied Biosystems) using SDS 2.4 software with standard conditions: 2 min 50°C, 10 min 94.5°C, 30 s 97°C (40 cycles), 1 min 59.7°C. Threshold cycle (Ct) values were calculated with the ExpressionSuite v1.0.3 software (Applied Biosystems). The detection threshold was set manually for all genes and was the same for each assay in all tissues. Ct = 39 was used as the cut-off above which, expression level was set to 0. On the TLDA array, 96 genes were studied for each sample: 87 target genes and 9 reference genes (Supplementary Figure S2). Target genes were chosen among the differentially expressed genes found in the microarray experiment in different categories of pathways and biological function. Six on the 9 reference genes were defined as the best reference, using the GeNorm software (41). For each sample, Ct[ref] was the mean of the three Ct values of the reference genes. Then, expression level of target genes was calculated as 2 − (Ct[target gene] − Ct[ref]), as previously described (42).

2.9. Statistics

All data are expressed as the means ± SEM (standard error of the mean). Statistical analyses were performed with Statistica 6.0 (StatSoft, Tulsa, OK, United States) and visualized in Prism 9.0 (GraphPad Software, San Diego, CA, United States). Normality and homogeneity of variances were assessed using the Shapiro–Wilk test and Levene

1 https://www.genotoul.fr/

2 www.bioconductor.org

3 https://biit.cs.ut.ee/clustvis/

test, respectively. Statistical outliers were detected using Grubb's test. Body weight, hormones, and progressive ratio data were analyzed using a non-parametric Mann–Whitney U test. Behavioral data were analyzed using three-way or four-way ANOVAs with repeated measures with Strain (C57Bl/6J, C3H/HeN) and MS (Control, MS) as between factors and Lever (Active, Inactive), Session (1,2,3,4,5), RR (5,10,20), Devaluation (Non-devaluated, Devaluated) or Concurrent choice (Milk, Milk+Chow) as within factors. When significant interactions were detected, specific comparisons between groups were tested by planned comparisons. "n" corresponds to the number of individuals. $p < 0.05$ was considered statistically significant.

3. Results

3.1. Chronic maternal separation impairs C3H/HeN mice's body weight

MS procedure tended to decrease C57Bl/6J dams' body weight (Mann-Withney test, $U = 16$; $p = 0.059$), whereas stressed C3H/HeN dams displayed ~8% lower body weight compared to the undisturbed C3H/HeN dams ($U = 1.5$, $p < 0.001$) (Figures 2A,B). Similarly, at PND15, MS had no impact on male C57Bl/6J offspring body weight ($U = 20$, n.s., Figure 2C), while MS C3H/HeN male pups exhibited ~16% of decreased in their body weight compared to the control group ($U = 11$, $p < 0.04$, Figure 2D). MS C57BL/6J mice did not differ from controls in adulthood ($U = 66$, n.s., Figure 2E). The effect of MS on C3H/HeN male pups body weight was maintained at adulthood ($U = 14$, $p < 0.001$). MS C3H/HeN mice showed ~12% of decrease in their body weight in comparison to controls. In females, a similar effect of MS was observed in offspring at PND15 (Supplementary Figures S1A,B), but it was not maintained at adulthood (Supplementary Figures S1C,D).

3.2. Impact of maternal separation on corticosterone and metabolic hormones plasma levels in C57Bl/6J and C3H/HeN strains

The impact of MS on corticosterone and metabolic hormone plasma levels was studied in adult mice (Figure 3). Plasma corticosterone levels were determined at the beginning of the dark and light phases. During the dark period, C57Bl/6J MS male mice tended to have higher plasma corticosterone levels compared to C57Bl/6J controls ($U = 19$, $p = 0.062$, Figure 3A), but the effect of MS was significant in the C3H/HeN strain ($U = 23$, $p < 0.05$, Figure 3B). Indeed, MS C3H/HeN mice exhibited a ~150% increase in corticosterone plasma levels relative to levels in the control group. In contrast, during the light phase, regardless of strain, MS did not affect plasma corticosterone levels (C57Bl/6J, $U = 27$, n.s.; C3H/HeN, $U = 41$, n.s.). A similar profile was reported in females, but the MS effect on corticosterone levels was shown in both strains (Supplementary Figures S1E,F). Regarding metabolic hormone levels, MS significantly increased plasma resistin levels in the male C57Bl/6J strain ($U = 13$, $p < 0.05$, Figure 3C), but no difference was observed in the male C3H/HeN strain ($U = 31$, n.s., Figure 3D). Finally, MS had no effect on plasma insulin and leptin levels in males from all strains (all $U > 11$, n.s., Figures 3E–H).

3.3. Chronic maternal separation exacerbates the motivation for palatable food of C3H/HeN mice fed *ad libitum*

We used operant conditioning paradigm to examine the effect of MS on the motivation for palatable food (Figure 4). On the fixed-ratio 1 (FR-1) schedule, four-way ANOVA with repeated measures reveals significant strain effect [$F_{(1,44)} = 21.06$, $p < 0.001$], lever effect [$F_{(1,44)} = 545.96$, $p < 0.001$] and session effect [$F_{(4,176)} = 64.142$, $p < 0.001$] (Figure 4A and Supplementary statistics). Furthermore, the ANOVA indicates that the number of active lever presses differs across sessions between strains [Lever × Session × Strain effect, $F_{(4,176)} = 11.09$, $p < 0.001$]. In contrast, MS had no significant effect on lever presses in FR-1 whatever the strain considered [MS effect, $F_{(1,44)} = 0.14$, n.s.; Lever × Session × Strain × MS effect, $F_{(4,176)} = 1.11$, n.s.]. In both C57Bl/6J and C3H/HeN strains, the number of active lever presses was higher than the number of inactive lever presses ($p < 0.001$ for each session) and it significantly increased across the session 1 to 4 when animals were tested under fasted condition ($p < 0.001$, S1 vs. S2, S3, S4). C3H/HeN mice displayed a ~150% increase of their active lever presses compared to C57Bl/6J mice during the S3, S4, and S5 sessions ($p < 0.001$). When mice were fed *ad libitum* (5th session under FR1 schedule), the number of presses on the active lever decreased in both strains ($p < 0.001$, S4 vs. S5), but it remained higher than inactive lever ($p < 0.001$). Overall these results indicate that both strains progressively increased their number of active lever presses during the four sessions of FR-1 schedule conducted in fasted animals, this effect was exacerbated in C3H/HeN strain. Active lever presses were maintained in *ad libitum*-fed animals. Throughout the FR-1 schedule, the number of lever presses on the inactive lever was low. Finally, whatever the session, MS did not alter the performances neither in C57Bl/6J, nor in C3H/HeN strain.

A random ratio (RR) schedule with a 1/5, 1/10, and 1/20 probability (RR5, RR10, and RR20, respectively) to obtain one palatable food reward was next performed in *ad libitum* fed animals (Figure 4B and Supplementary statistics). Four-way ANOVA with repeated measures showed significant lever effect [$F_{(2,44)} = 160.04$, $p < 0.0001$] and RR effect [$F_{(2,88)} = 28.88$, $p < 0.0001$], but no main effect of strain [$F_{(2,44)} = 0.094$, n.s.] or MS [$F_{(2,44)} = 2.6207$, n.s]. Importantly, MS differentially affected the number of active lever presses across the RR sessions according to the strain [Strain × RR × Lever × MS, $F_{(2,88)} = 3.46$, $p < 0.05$]. Controls and MS C57Bl/6J mice increased their number of active lever presses according to the probability to obtain the reward (RR5 vs. RR10 or RR20, $p < 0.0001$). In contrast, whereas control C3H/HeN mice were stable across RR session (RR5 vs. RR10 or RR20, n.s.), MS C3H/HeN mice exhibited a significant escalation of their active lever presses (RR5 vs. RR10 or RR20, $p < 0.0003$). MS C3H/HeN mice showed an overall ~160% increment of their active lever presses in comparison to control C3H/HeN mice ($p < 0.05$). A similar profile was reported in females with no impact of MS in C57Bl/6J and a significant increase of lever presses in C3H/HeN mice exposed to MS (Supplementary Figures S1G,H).

To further study motivation, mice were then submitted mice to a progressive-ratio procedure during which the number of lever presses to obtain a single food reward progressively increased. Animals were tested in sated condition. Under a progressive-ratio schedule (PRx2), the cumulative number of lever presses were differentially affected by MS according to the strain across time [Figure 4C, three-way ANOVA, Strain effect, $F_{(1,42)} = 9.769$, $p < 0.01$; MS effect, $F_{(1,42)} = 0.816$, n.s; Time

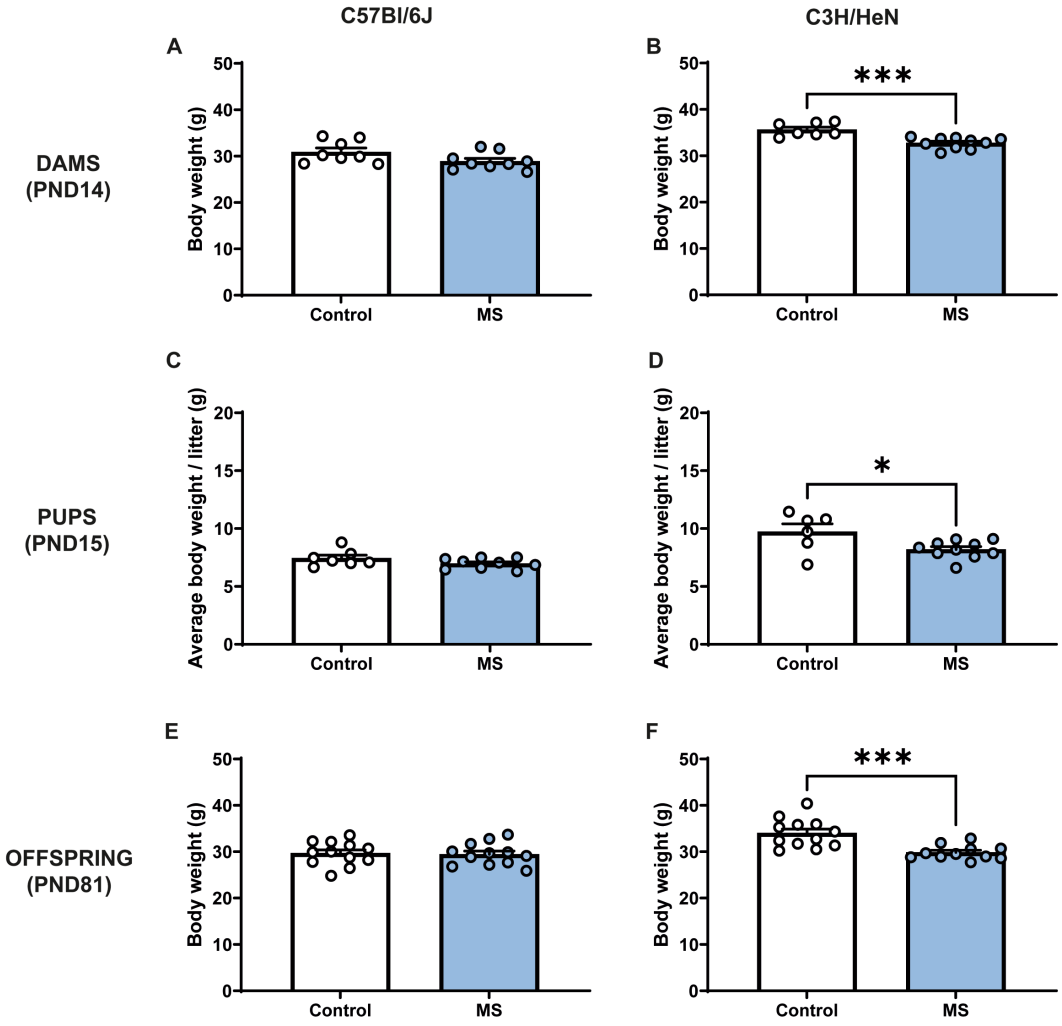

FIGURE 2
Impact of maternal separation on C57Bl/6J and C3H/HeN dam's and offspring body weight. Maternal separation decreases body weight of C3H/HeN in both dams and male offspring. Dams' body weight at PND14: **(A)** C57Bl/6J and **(B)** C3H/HeN. Male pups' body weight at PND15: **(C)** C57Bl/6J and **(D)** C3H/HeN. Male mice body weight at PND81: **(E)** C57Bl/6J and **(F)** C3H/HeN. $n = 6–10$ per group in dams and in pups; $n = 12$ per group at PND81. Mann–Whitney tests, $*p < 0.05$, $***p < 0.001$.

effect, $F_{(59,2478)} = 141.082$, $p < 0.001$; Strain × MS × Time effect, $F_{(59,2478)} = 2.646$, $p < 0.001$]. Control C57Bl/6J mice exhibited higher lever presses than Control C3H/HeN mice ($p < 0.01$), but MS exposure in C3H/HeN mice suppressed this difference. Additionally, while MS had no impact on the number of active lever presses before the cut-off (3 min without any lever presses) in C57Bl/6J mice ($U = 66$; n.s.), MS significantly increased the active lever presses in C3H/HeN mice ($U = 29$; $p < 0.05$) (Figure 4D). Overall, our results indicate that C57Bl/6J mice exhibit a high motivation for palatable food when fed *ad libitum*, and that MS leads to an exacerbation of the motivation for palatable food restricted to the C3H/HeN strain. In females, whatever the strain, MS had no significant impact on progressive ratio results (data not shown).

In order to determine whether the exacerbated motivation in MS male C3H/HeN was due to an alteration of the representation of outcome value, we studied their performances in a devaluation procedure. In the sensory-specific satiety test, a significant effect of pre-feeding was observed for the number of lever presses, this effect was not affected by MS and was similar between strains [three-way ANOVA, Devaluation effect, $F_{(1,44)} = 15.138$, $p < 0.001$; Strain effect, $F_{(1,44)} = 0.030$, n.s.; MS effect, $F_{(1,44)} = 0.058$, n.s; Devaluation × Strain × MS effect, $F_{(1,44)} = 0.1271$, n.s., Figure 5A]. Finally, in the concurrent choice test (conducted in fasted animals), the presence of an alternative reward less palatable (chow), but freely available in the operant chamber, reduced the number of lever presses for sweetened milk similarly between control and MS groups of both strain [Concurrent choice effect, $F_{(1,44)} = 10.787$, $p < 0.001$; Strain effect, $F_{(1,44)} = 3.25$, n.s., MS effect, $F_{(1,44)} = 2.46$, n.s.; Concurrent choice × Strain × MS effect, $F_{(1,44)} = 0.316$, n.s., Figure 5B]. The total amount of chow consumed during the test was similar between groups in both strains [Two-way ANOVA, Strain effect, $F_{(1,44)} = 0.0004$, n.s.; MS effect, $F_{(1,44)} = 0.72$, n.s., Strain × MS effect, $F_{(1,44)} = 0.59$, n.s., Figure 5C].

3.4. Maternal separation modifies the transcriptional profile in the nucleus accumbens of C3H/HeN

To investigate the molecular brain signature associated with exacerbated motivation in MS animals, after the operant task, we performed transcriptomic analysis in male C3H/HeN offspring.

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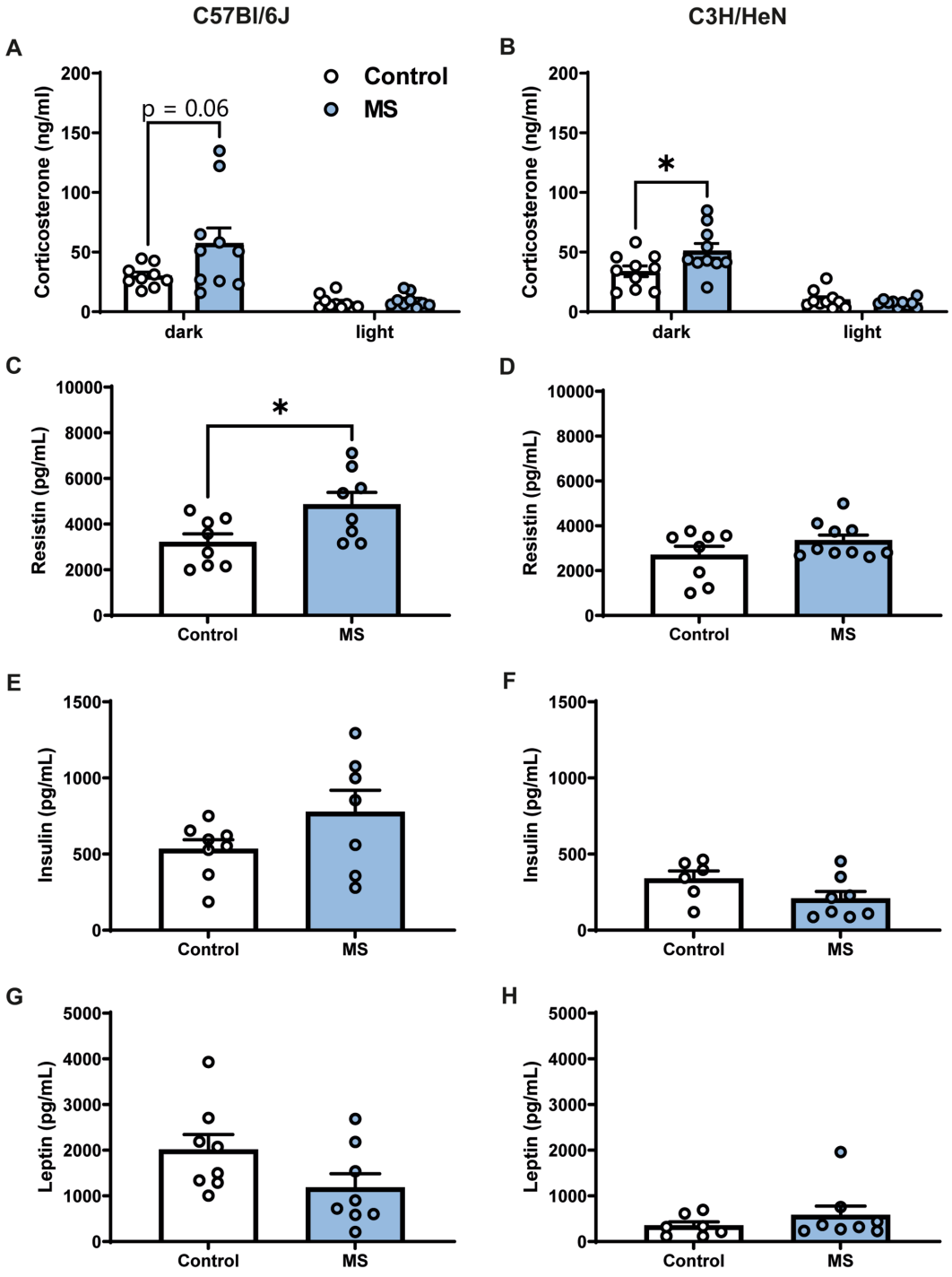

FIGURE 3
Impact of maternal separation on plasma corticosterone and metabolic hormones levels in C57Bl/6J and C3H/HeN male mice. Plasma corticosterone levels are elevated during the dark phase of the cycle compared to the light period in both **(A)** C57Bl/6J and **(B)** C3H/HeN groups. MS tended to exacerbate the rise of corticosterone during the dark period in C57Bl/6J mice. MS C3H/HeN group had higher plasma corticosterone levels than Control C3H/HeN group during the dark phase. Plasma levels of metabolic hormones in C57BL/6J and C3H/HeN mice: **(C,D)** Resistin levels were significantly increased by MS in C57Bl/6J mice, but not in C3H/HeN mice; **(E,F)** insulin and **(G,H)** leptin levels were not affected by MS. $n = 6–10$ per group. Mann–Whitney tests, $*p < 0.05$.

We hybridized RNA from whole-tissue micropunches of NAc, mPFC, and hypothalamus on Agilent microarrays. Descriptive analysis via hierarchical clustering showed a clear separation for NAc only (Figure 6A and Supplementary Figures S2A,D). PLS-DA analysis was able to build a model in each tissue but only the model in the NAc was validated (Figure 6B, $pR2Y = 0.014$, $pQ2 = 0.02$ for NAc, Supplementary Figure S2B, $pR2Y = 1$, $pQ2 = 0.84$ for mPCF, and Supplementary Figure S2E, $pR2Y = 0.78$, $pQ2 = 0.94$ for hypothalamus). No differentially expressed genes (DEGs) were found in the mPFC and hypothalamus (Supplementary Figures S2C,F),

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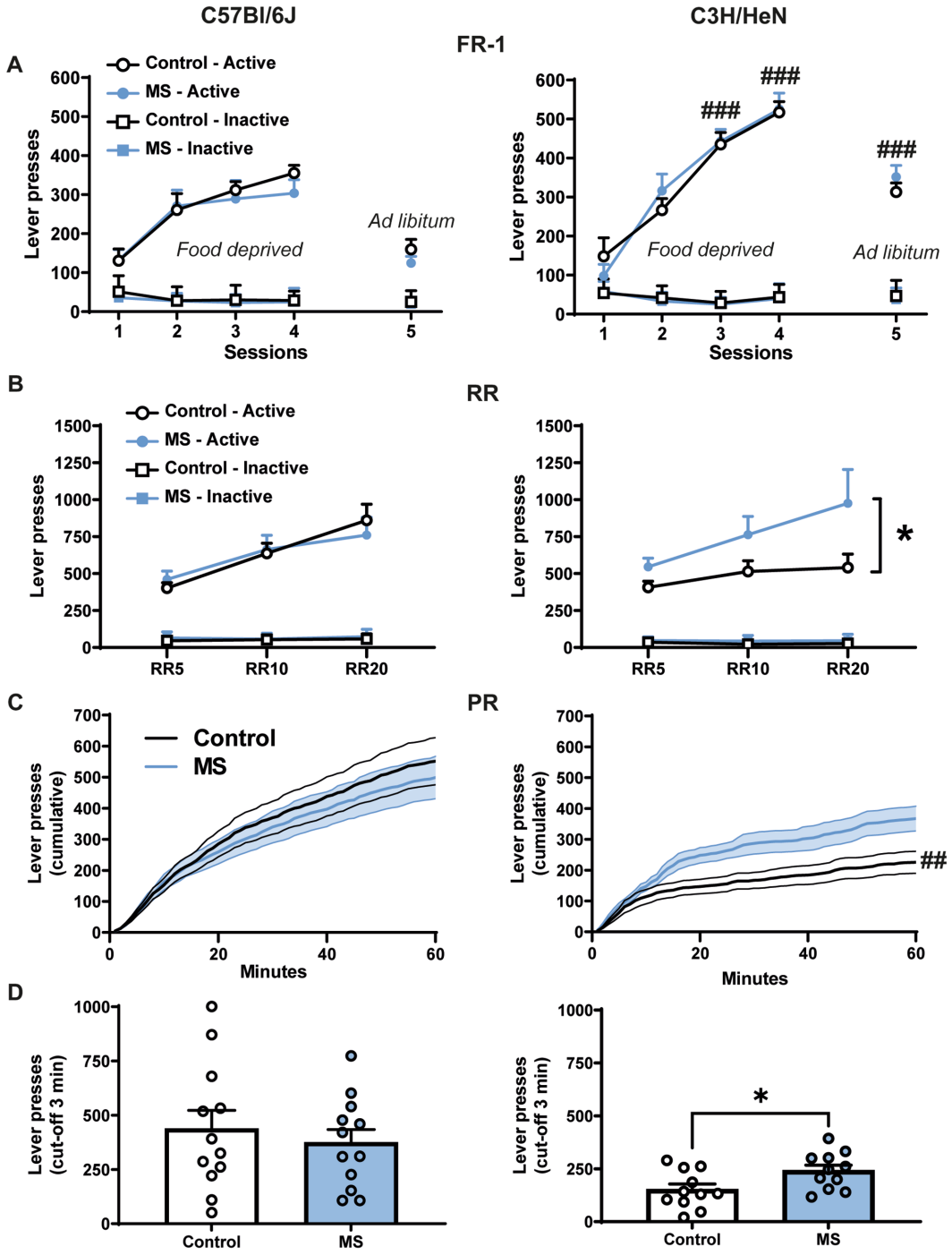

FIGURE 4
Impact of maternal separation on motivation for palatable food in C57Bl/6J and C3H/HeN male mice. **(A)** There is no impact of MS on mice behavior for the FR-1 schedule. Food deprived C7Bl/6J and C3H/HeN mice progressively increased their number of active lever presses during the 4 sessions in FR-1 schedule. Active lever presses were maintained in *ad libitum* fed animals during the 5th session. Throughout the FR-1 schedule, the number of lever presses on the inactive lever was low. $n = 12$ per group. Four-way ANOVA with repeated measures, followed by planned comparisons, $###p < 0.001$ C57Bl/6J vs. C3H/HeN. **(B)** During the random ratio schedules (RR5, RR10, RR20 *ad libitum* condition), MS increased the number of active lever presses in C3H/HeN mice but had no effect in C57Bl/6J mice. Motivation for palatable food was exacerbated in male C3H/HeN mice submitted to MS. $n = 12$ per group. Four-way ANOVA with repeated measures, followed by planned comparisons, $*p < 0.05$ Control vs. MS. **(C)** During the progressive-ratio schedule (PR), the increase of the cumulated number of lever presses over a 60-min session differed according the strain. $n = 11$ per group. ANOVA followed by planned comparisons, $##p < 0.01$ C57Bl/6J vs. C3H/HeN. **(D)** MS C3H/HeN mice displayed higher total active lever presses than control C3H/HeN during the PR (cut-off 3 min); control C57Bl/6J group was similar to the MS C57Bl/6J group. $n = 11$ per group. Mann Whitney test, $*p < 0.05$ Control vs. MS.

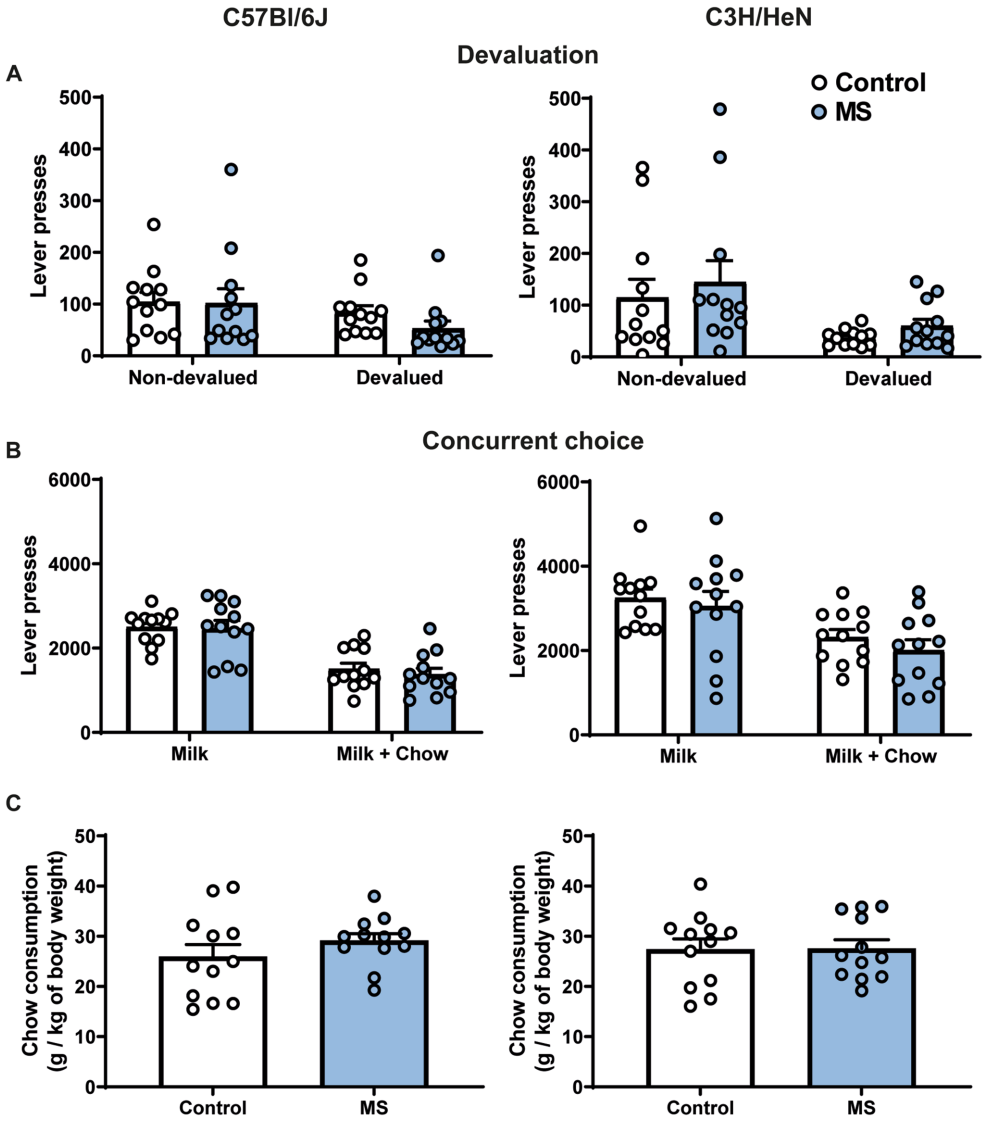

FIGURE 5
Impact of maternal separation on outcome devaluation and concurrent choice procedures in C57Bl/6J and C3H/HeN male mice. Maternal separation does not affect representation of outcome value and rate of lever presses in the concurrent choice procedure in C57Bl/6J and C3H/HeN mice. **(A)** Lever presses during a 5-min extinction test in a non-devaluated condition (Intralipid consumption before test) and in a devaluated condition (milk consumption before test). **(B)** Lever presses during a concurrent choice procedure: milk was provided as sole reward after instrumental response or standard chow was provided as free alternative to milk reward. Three-way ANOVA. **(C)** Amount of chow consumed during the concurrent choice test. $n = 12$ mice per group. Two-way ANOVA.

while 375 DEGs were found in NAc: 306 downregulated and 69 upregulated (Figure 6C and Supplementary Table S1). Therefore, the transcriptional effect is restricted to the NAc tissue. A bioinformatic analysis of functions and networks was carried on the 375 DEGs using the IPA software and the ConsensusPathDB-mouse (CPDB) website. This detected an overrepresentation of 8 canonical pathways with BH $ps < 0.05$ and 18 KEGG pathways with $q < 0.05$, respectively (Figure 6D). Among these pathways, 2 are common to the 2 analyses: GABA receptor signaling/GABAergic synapse and Glutamate receptor signaling/Glutamatergic/synapse. Pathways analysis also pointed out stress related pathways (α-adrenergic signaling and CRH signaling) and addiction pathways (nicotine, morphine cocaine). The IPA analysis also predicted upstream regulators, which may be causing the observed gene expression changes. Interestingly, among the 3 predicted upstream regulators, the L-Dopa had the lowest *p*-value and the highest *z*-score, with a predicted activation whereas the uncoupling protein 1 *Ucp1* and the histone deacetylases *Hdac* were predicted as inhibited (Figure 6E). Finally, using TLDA assays, we performed a RT-qPCR analysis on 84 genes of NAc samples from males and females of both strains. Microarray results obtained in C3H/HeN male mice were validated by TLDA (Supplementary Figure S2G, Spearman correlation $rho = 0.754$; $p < 2.2e^{-16}$). A clustering analysis of these TLDA results showed clearly the differential expression of the 84 genes between control and MS C3H/HeN males. In contrast, we did not observe any differential expression for the 84 genes identified in C3H/HeN mice when we compared control and MS C3H/HeN females or control and MS C57Bl/6J regardless of the sex (Supplementary Figure S2H).

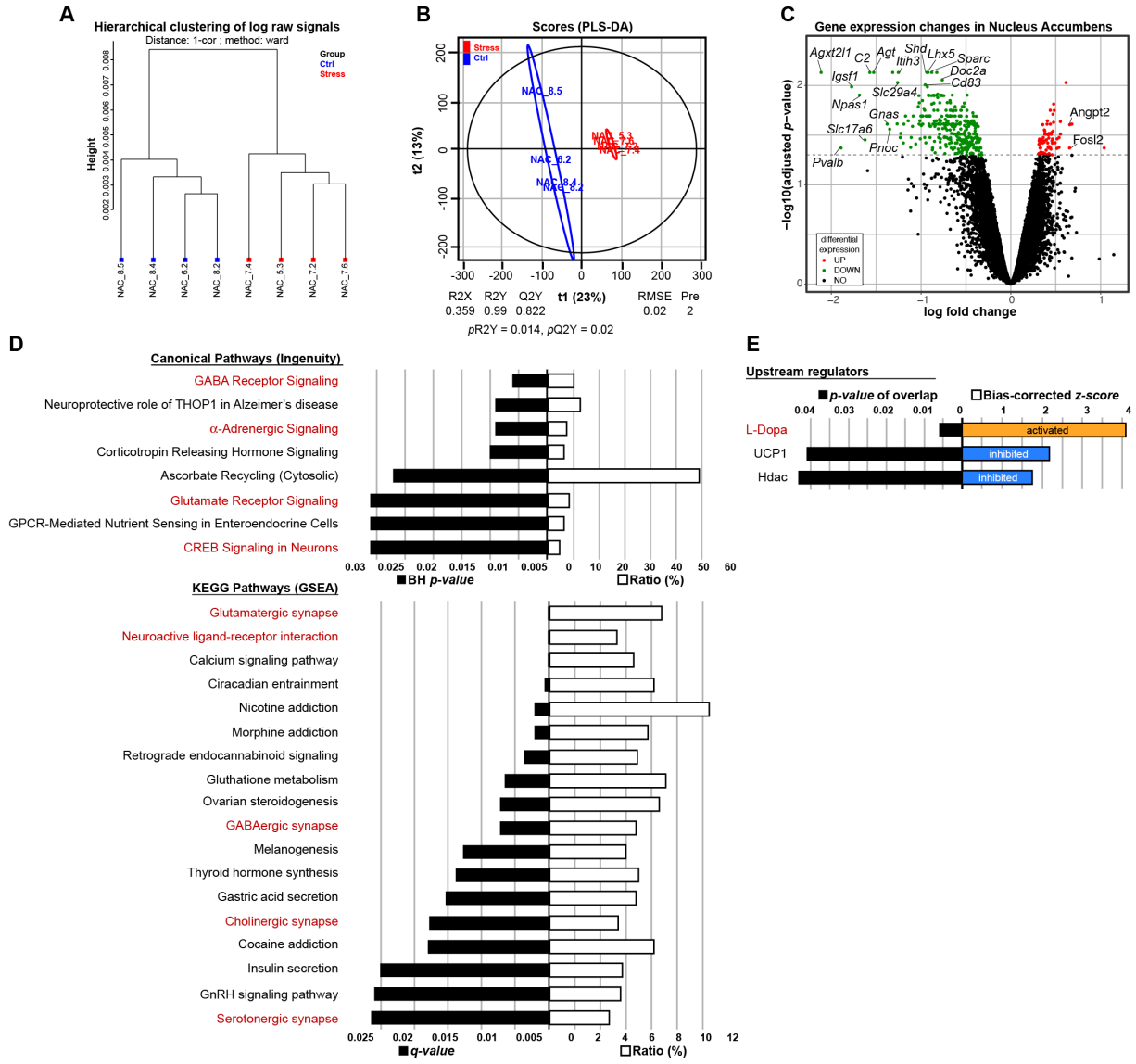

FIGURE 6
Impact of maternal separation on gene expression in the nucleus accumbens (NAc) of male C3H/HeN mice. Maternal separation drastically modifies the transcriptional profile in the NAc of male C3H/HeN mice. Clustering by Euclidean distance of the raw transcriptomic datasets of NAc from Control (blue) or MS (red) male adult offspring **(A)**. Score plots from the PLS-DA classification into Control (blue) and MS (red) groups. A model is considered robust when the response variance explained (R2Y = 0.99) is higher than the predictive performance of the model (Q2Y = 0.822). A model with a Q2Y > 0.5 is considered to have a good predictive performance **(B)**. Volcano plot depicting significantly differentially expressed genes in the NAc of C3H/HeN mice between the MS and Control conditions **(C)**. Top Canonical Pathways and KEGG pathways **(D)** and top upstream regulators **(E)** involved. In red are depicted the pathways or regulators associated with NAc neuronal function. *n* = 4 mice per group.

4. Discussion

MS models in mice have been widely used to examine the long-lasting effects of early-life stress on emotional function. Herein, we report evidence for a strain-dependent effect of MS on the motivation for a palatable nutritional reward in an operant conditioning paradigm. Our data show that MS combined to unpredictable chronic mild stress in lactating dams exacerbated motivation for a highly palatable food reward (sweetened milk) in both male and female C3H/HeN mice, but had no effect in C57Bl/6J strain. The transcriptomic analysis revealed that exacerbated motivation in MS C3H/HeN male mice was associated with marked changes in gene expression in the NAc, whereas no significant changes were reported in the PFC or hypothalamus.

A primary and key result of our study is the overall difference in behavioral and brain gene expression outcomes observed between C3H/HeN and C57Bl/6J strains in response to early life stress. Body weight and food motivation were specifically altered in C3H/HeN but not in C57Bl/6J suggesting that C3H/HeN strain is more susceptible to MS for these studied parameters. This comforts previous findings showing a resilience of the C57Bl/6J strain to the MS procedure effects (27, 43). Indeed, despite the fact that MS procedure has been fairly well-established in rats (10, 44), results in mice especially in the C57Bl/6J strain are very heterogenous. Previous work using an early-life stress

paradigm close to our study (3 h MS from PND1 to PND14, coupled to an unpredictable restraint stress or forced swim stress in dams during separation) showed increased depressive-like behaviors, reduced anxiety, but had no impact on serum insulin levels in C57Bl/6J offspring exposed to early stress (29, 45, 46). Regarding the HPA axis function, we demonstrated that MS increases plasma corticosterone at the beginning of the dark phase in both strains, consistent with previous results in the literature (27, 47). This finding is important since it demonstrates that despite differential strains' sensitivity to MS in food motivation, both strains are affected by MS. Most of previous works conducted in C57Bl/6J exposed to MS report long-term effects on behavior, only when animals are re-exposed to an additional stressor such as early weaning at PND17 (48), chronic social defeat or unpredictable chronic mild stress (UCMS) at adulthood (25, 26). Without additional stress, emotional behaviors in MS C57Bl/6J mice generally do not significantly differ from controls (25–27, 43, 49, 50). On the other hand, C3H/HeN strain displays gut dysfunction after MS (17, 30, 31) and emotional impairments in a multi-hit model combining MS, maternal UCMS and prenatal infection (31). The different susceptibility to MS procedure between the C57Bl/6J and C3H/HeN strains could be due to maternal care differences. Accordingly, C3H/HeN dams display more pups licking and nursing than other strains (51). This robust maternal behavior may be more affected by the disruption of the nest and pups' separation associated to MS procedure. Additionally, C3H/HeN strain are more anxious and show a higher sensitivity to stress compared to other mice strains; whereas C57Bl/6J strain has been recurrently described in the literature as a strain particularly resilient to stress (52). Taken together, this suggests that MS effects may vary depending of strains and behavioral dimensions.

Clinical literature suggests that early-life adversity is associated with higher risk to develop food addiction behaviors and obesity in adulthood (6, 7, 53). Although emotional behaviors have been extensively studied in MS literature, motivation for food reward has been less explored. Previous works in rodents exposed to early-life stress, demonstrate that MS exacerbates drugs of abuse motivation and ethanol intake (21, 54). Since addictive drugs and palatable food partially share common neurobiological substrates, we hypothesized that MS will affect food motivation and impact the mesolimbic circuit. Previous findings in rats showed that early-life stress exacerbates motivation for palatable food as indicated by their higher breakpoint in operant task and their lower latency to reach chocolate pellets in a runway task (18, 55). Here, we present data supporting an impact of early-life stress on palatable food motivation in operant conditioning test in C3H/HeN mice. This effect is strain-dependent, but it is observed in both male and female MS C3H/HeN mice. Interestingly, differences were reported in RR and PR schedules when the effort to obtain the palatable food reward is high. In contrast, FR-1 schedule performances, which reflect more the ability to learn the task, were similar between control and MS groups. Noteworthy, exacerbated motivation for palatable food in MS animals was found in mice non-submitted to food restriction during the instrumental task. These results suggest that regardless of their nutritional status, stressed animals may be more prone to seek and consume calorie-dense food. Accordingly, MS rats ate more palatable food when they have a free access in their home cage (18, 55, 56). Sweetened milk is highly palatable for mice and sugar has been shown to share numerous behavioral and neurophysiological processes with drug of abuse, suggesting the possibility of sugar addiction (57). Our results are in accordance and extend recent finding showing that a single long lasting (23 h) separation at PND3 in mice produces enhanced binge-eating behavior after repetitive cycles of re-exposure to a high-fat diet in adulthood (49) and clinical literature showing that early-life adversity is associated with a higher risk of developing food addiction behaviors and obesity in adulthood (6, 7, 53). Finally, in the present work, we also showed differences in operant response between C57Bl/6J and C3H/HeN strains. While C3H/HeN strains made higher active lever presses under fasting conditions, they were less motivated than C57Bl/6J strains when fed *ad libitum* in RR and PR patterns. We cannot exclude that these strain differences contribute to the variation in MS susceptibility between strains.

An important finding in the present work is that MS C3H/HeN males exhibiting exacerbated motivation for palatable food had marked changes of brain gene expression (adj *p*-values: 375 genes differentially expressed) specifically in the NAc, a key region for the regulation of motivation and reward processing. Notably, we validated a large amount of gene differentially expressed in MS C3H/HeN males using TLDA. Interestingly none of the genes significantly affected by early-life stress in C3H/HeN mice were changed in C57Bl/6J MS mice. However, given that C57Bl/6J results were not obtained using microarray, we cannot exclude that changes affecting other genes also occur in this group. Previous transcriptomic studies demonstrated a significant impact (with non-adjusted *p*-values) of early-life stress using RNAseq analysis in C57Bl/6 strain (25, 26). Using adjusted *p*-values, we did not detect a significant effect of MS on gene expression in the PFC and hypothalamus, indicating that NAc is a brain area particularly affected by MS in C3H/HeN. The lack of transcriptional change in the hypothalamus is quite surprising given the importance of this brain area in the effects of stress and in the control of food intake. Again, it is important to note that we used here the Benjamini-Hochberg adjusted *p*-values method which is highly conservative and may lead to under detection of change in gene expression between groups. Further studies should be conducted to examine the impact of MS on specific nuclei of the hypothalamus such as the lateral hypothalamus.

In the NAc, we identified several genes such as *Agt, Igsf1, Gnas, Pnoc, Npas1, Pvalb*, or *Fosl2* affected by early-life stress in C3H/HeN male mice which have been previously reported to be modified in the NAc or in the VTA after chronic stress procedures (25, 26). Interestingly, *Gnas* (Guanine Nucleotide-Binding Protein G Subunit Alpha) and *Pnoc* (prepronociceptin) have previously been linked to motivation and reward regulation (58–61). *Angpt2* (angiopoietin-2) plays a role in angiogenesis and its expression is induced by inflammatory markers (62). *Angpt2* has not been linked with motivational changes, but recent data suggest that up-regulation of immune factors within the NAc may correlate with addictive phenotype in rats (63) and compulsive sucrose seeking in mice fed with high-fat diet (64). The mechanisms underlying this marked impact of early-life stress on NAc transcriptome in C3H/HeN mice need to be explored. However, pathways analysis (using IPA and CPDB) on NAc genes revealed that the excitatory (Glutamate receptor signaling) and inhibitory ($GABA_A$ receptor signaling) pathways are both affected by early-life stress. GABAergic and glutamatergic systems play a major role in neurodevelopment and disruption of these systems have been involved in numerous neurodevelopmental disorders including autism, schizophrenia or ADHD (65). Furthermore, a large body of evidence has linked various perinatal stress paradigms with altered excitatory/inhibitory balance (66–69). Importantly, major upstream regulators identified by IPA are *L-Dopa*,

Ucp1 and *Hdac* suggesting that these factors may contribute to the effects of early stress. UCP1 (uncoupling protein 1, a proton carrier protein generating heat via non-shivering thermogenesis) has been recently identified in the brain, though its role still remains unclear (70). HDAC is an important epigenetic regulator and its inhibition in the NAc promotes drug self-administration (71). As epigenetic regulator, HDAC may be involved in numerous changes in the NAc transcriptome. Among the upstream regulators, the dopamine precursor L-Dopa may have an important function in the observed effects on motivation for a highly palatable reward. L-Dopa therapy in Parkinson disease has been shown to increase the risk of addictive behaviors, including compulsive eating (72). NAc dopamine is involved in palatable food-seeking behavior during instrumental task for schedules that have high-work requirements such as RR or PR (73). A limitation of the present study is that we cannot conclude a direct effect of MS on the NAc transcriptome that could lead to altered food motivation in C3H/HeN mice. Fasting prior to brain sampling can differentially modify gene expression in the NAc in MS CH3/Hen mice. Furthermore, a recent work demonstrates that operant training for highly palatable food in mice changes translating mRNA in dopaminoceptive neurons of the NAc (74). It is then possible that altered gene expression in the NAc results in part from a different rate of exposure to palatable food in C3H/HeN mice exposed to MS.

In conclusion, our work reveals that early-life stress increases motivation for palatable food in *ad libitum* fed adult C3H/HeN mice. This effect is associated with marked changes in gene expression within the NAc. Importantly, exacerbated palatable food motivation after MS is found in both male and female C3H/HeN mice, but this effect is strain dependent suggesting a relative resilience of C57Bl/6J. Overall, our study confirms that early-life adversity has enduring effects on reward circuits and highlights the importance to further explore the impact of early-life stress on motivational processes, in the context of food overconsumption and obesity.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found at: https://www.ncbi.nlm.nih.gov/, GSE222781.

Ethics statement

The animal study was reviewed and approved by Institutional Regional Committee for animal experimentation (agreement #5012050-A).

Author contributions

MD designed and supervised the study. SF, AF, MJ, M-PM, LJ, and AG performed and analyzed the transcriptomics. AM performed the behavioral tests. LX performed the metabolic measures. MH wrote the first draft of the manuscript. SB and MD wrote the manuscript. All authors discussed and commented on the manuscript.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnut.2023.1190392/full#supplementary-material

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EDITED BY
Nicholas Pang,
Universiti Malaysia Sabah, Malaysia

REVIEWED BY
Pradip Behare,
National Dairy Research Institute (ICAR), India
Phakkharawat Sittiprapaporn,
Mae Fah Luang University, Thailand

*CORRESPONDENCE
Hadi Pourjafar
✉ pourjafarhadi59@gmail.com

†These authors have contributed equally to this work

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The role of probiotics and prebiotics in modulating of the gut-brain axis

Fereshteh Ansari[1,2,3], Mehrdad Neshat[4†], Hadi Pourjafar[5*], Seid Mahdi Jafari[6,7,8†], Shohreh Alian Samakkhah[9†] and Esmaeel Mirzakhani[10†]

[1]Razi Vaccine and Serum Research Institute, Agricultural Research, Education and Extension Organization (AREEO), Tehran, Iran, [2]Research Center for Evidence-Based Medicine, Health Management and Safety Promotion Research Institute, Tabriz University of Medical Sciences, Tabriz, Iran, [3]Iranian EBM Centre: A Joanna Briggs Institute Affiliated Group, Tabriz, Iran, [4]Department of Clinical Science, Faculty of Veterinary Medicine, Tabriz Medical Sciences, Islamic Azad University, Tabriz, Iran, [5]Alborz University of Medical Sciences, Dietary Supplements and Probiotic Research Center, Karaj, Iran, [6]Department of Food Materials and Process Design Engineering, Gorgan University of Agricultural Sciences and Natural Resources, Gorgan, Iran, [7]Department of Analytical Chemistry and Food Science, Faculty of Science, Universidade de Vigo, Nutrition and Bromatology Group, Ourense, Spain, [8]College of Food Science and Technology, Hebei Agricultural University, Baoding, China, [9]Department of Food Hygiene and Quality Control, Faculty of Veterinary Medicine, Amol University of Special Modern Technologies, Amol, Iran, [10]Department of Food Science and Technology, Faculty of Nutrition and Food Sciences, Tabriz University of Medical Sciences, Tabriz, Iran

Pro-and prebiotics have been indicated to modulate the gut-brain axis, which have supportive impacts on central nervous systems, and decrease or control the incidence of some mental disorders such as depression, anxiety, autism, Schizophrenia, and Alzheimer's. In this review, complex communications among microbiota, gut, and the brain, and also recent scientific findings of the impacts and possible action mechanisms of pro-and prebiotics on mental disorders have been discussed. The results have shown that pro-and prebiotics can improve the function of central nervous system and play an important role in the prevention and treatment of some brain disorders; however, in order to prove these effects conclusively and firmly and to use these compounds in a therapeutic and supportive way, more studies are needed, especially human studies/clinical trials.

KEYWORDS

probiotics, prebiotics, gut microbiome, gut-brain axis, mental disorders, Alzheimer's, depression, anxiety

1. Introduction

The two-way communication between the gastrointestinal tract (GIT) and the brain has long been well known, with direct neural signals and indirect hormonal and enzymatic signals from the brain always being sent to the GIT lumen to control and regulate movement, secretion, and sensory transmission; on the other hand, similar signals are sent from the GIT to the brain affecting its functions and control and regulatory role of the brain. In fact, GIT is connected to the brain by about 200–600 million neurons (1, 2). In recent years, extensive studies have been conducted on the role and possible effects of the intestinal microbiome on brain functions as well as some central nervous system (CNS) disorders (3, 4). Mental diseases affect more than 1 billion people all over the world, and communal mental diseases refer to a range of depressive and anxiety disorders. According to the FAO/WHO, about 4.4 and 3.6% of the world's population suffer from depressive and anxiety disorders, respectively (2).

Today, the use of natural supplements that strengthen the intestinal microbiome and ultimately have a positive effect on brain functions has received more attention from researchers. The use of pro-and prebiotic dietary supplements is one of the most popular products that have a positive effect on the intestinal microbiome, improving intestinal and gut-brain axis functions, with the potential and ability to play an effective role in preventing and treating some mental disorders (5). By definition, "probiotics" are living microorganisms that in sufficient quantities cause one or more beneficial effects on the host. The most important probiotics belong to the genera *Lactobacillus* and *Bifidobacterium*. Foods containing probiotics should comprise at least 7 log CFU cells and should be eaten at a rate of 100 g or mL per day to have effective influences on health and control and treatment of diseases (6–8).

Prebiotics are compounds indigestible by the human GIT (resistant to secretions and intestinal enzymes) that travel through the intestine and reach the colon intact. Prebiotics in the colon are broken down by the gut microbiome (GM) or probiotic microorganisms that are eaten together to produce beneficial compounds. In fact, the breakdown of prebiotics not only produces therapeutic and health-promoting compounds, but also strengthens and functions colon-based probiotics as a food source. The most important prebiotics belong to carbohydrates and the family of galactooligosaccharides (GOS), fructooligosaccharides (FOS), and xylooligosaccharides (2). National Health and Nutrition Examination Survey (NHANES) reported that people at least 20 years of age in the United States consume only 61% of their recommended level, while there is no official information on the consumption of prebiotics, there are recommendations from researchers like consuming 10 g of FOS or 7 g of GOS per day (9, 10). Prebiotics exert their effect in low doses, for example, the effective amount of polydextrose is about 2 to 7.5 g per day (11), resistant starch is 2.5 to 5 g per day (12), and inulin is 1 to 6 g per day (13).

The combined use of pro-and prebiotics, called synbiotics, has a synergistic effect and plays an important role in controlling and reducing the risk of some diseases, including mental disorders. In the absence of prebiotics, which are considered a food source for probiotics, the number of probiotics decreases, causing problems with the intestinal and general immune systems of the host, as well as causing some abnormalities such as constipation. On the other hand, if there are no probiotics or their number is significantly low, then prebiotics will play a lesser role in host health and disease control (14). Numerous studies have shown that pro-and prebiotics, together or alone, play an important role in neuroimmune processes. It has also been shown that their health effects on the CNS are related to the interactions between GM and colon-based probiotics, the immune and nervous systems, which occur through the secretion of certain enzymes, hormones, immunological factors, and neurotransmitters (5, 15, 16). Also, animal, clinical and paraclinical studies have shown that there is a relationship between the presence and activity of pro-and prebiotics in the gut, CNS and immune systems and eventually the incidence of Alzheimer's, depression, schizophrenia, anxiety, autism, insomnia, severe stress, and other mental diseases (5, 17).

In this review, the possible role of pro-and prebiotics in regulating the immune and nervous systems, and finally the possible control and treatment of some mental disorders are discussed. The possible mechanisms involved in the healing process of CNS diseases by these supplements have also been investigated. Finally, the last part of this article provides an overview of the future prospects of using these compounds to treat mental disorders.

2. Gut microbiota and brain communications

It is recognized that the communication between the gut, the microbiota, and the brain is mediated by multiple signals from neural, immune, and endocrine pathways. The gut alone has a unique nervous system called the enteric nervous system (ENS), which is directly and permanently connected to the brain by the nerves. It is noteworthy that ENS is separated from the intestinal microbiota by the mucous cell layer; intestinal microbes do not have direct access to this local nervous system. It is possible that microbiota communicates indirectly with this nervous system by transmitting them from the intestinal lumen to the lamina propria via the microfold cells or dendritic cells, given the direct entry of resident microbes invasively causing ulceration and perforation in the intestine. Another possible communication pathway is intestinal bacterial secretions and metabolites such as short-chain fatty acids (SCFAs), exopolysaccharides (EPS), lipopolysaccharides (LPS), and glutamate that are able to cross the intestinal cell wall and directly affect the ENS, and are able to interact with some certain receptors; e.g., G-protein coupled receptors (GPCRs), and Toll-like receptors (TLRs) (3, 5).

GPCRs are the receptors in the CNS, especially in the striatum, which play an important role in regulating and controlling metabolism and the inflammatory process in mental disorders. SCFAs produced by the activity of GM, especially in the presence of prebiotics, stimulate and activate GPCRs at the ENS and CNS (GPR109A, GPR41, and GPR43 recognized as SCFAs receptors), as depicted in Figure 1. For example, it has been shown that the secretion of SCFAs such as acetic, butyric, and propionic acids with an effect on GPR43 plays an important role in regulating T cell homeostasis and preventing colitis. Similar effects have been reported in the prevention of some mental disorders caused by damage and inflammation in the brain (3), which is described in Section 4 in detail.

TLRs are stimulated and activated by some secretions and metabolites of GM such as exopolysaccharides and LPS, and then the immune system and ENS release cytokines and neurohormonal mediators that strengthen the intestinal and nervous systems to prevent some intestinal and mental disorders (Figure 1). For instance, it has been indicated that the activity and secretion of microbiota with effect on TLR2 strengthen and regulate ENS integrity, stimulate the emergence of a glial cell line-derived neurotrophic factor, enhance the number of glial cells and enteric neurons, and ultimately survive and strengthen several kinds of neurons (3, 18).

3. Impacts of pro-and prebiotics on the CNS

Many studies have shown that GM can affect the gut-brain axis and play an important role in preventing and controlling some brain diseases such as Alzheimer's, depression, and insomnia (4, 17–19).

Abbreviations: GIT, gastrointestinal tract; CNS, central nervous system; GM, gut microbiome; GOS, galactooligosaccharides; FOS, fructooligosaccharides; ENS, enteric nervous system; SCFAs, short-chain fatty acids; LPS, lipopolysaccharides; GPCRs, G-protein coupled receptors; TLRs, Toll-like receptors; GABA, gamma-aminobutyric; PSQI, Pittsburgh Sleep Quality Index; AD, Alzheimer's disease; ASD, Autism spectrum disorder; SCZ, Schizophrenia.

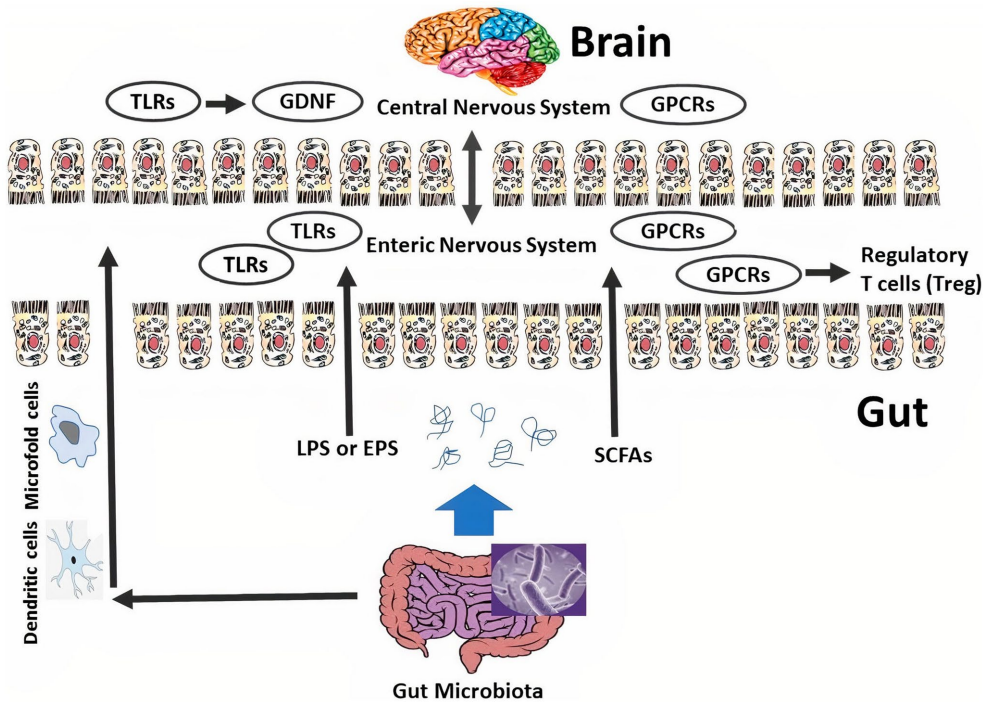

FIGURE 1
The connection among the microbiome, enteric nervous system/ENS, and central nervous system/CNS. Intestinal bacteria are transmitted from the intestinal lumen to the lamina propria by microfold cells and dendritic cells. Intestinal microbiome secretions like exopolysaccharides/EPS and short-chain fatty acids/SCFAs pass through the intestinal epithelium and directly affect the CNS. Intestinal bacteria and their metabolites can interact with certain receptors such as G-protein coupled receptors/GPCRs and Toll-like receptors/TLRs. The intestinal microbiome can adjust ENS function through TLRs, inspire the expression of glial cell line-derived neurotrophic factor/GDNF, and enhance the number of glial cells and enteric neurons. On the other hand, SCFAs adjust colonic regulatory T cell/cTreg homeostasis via affecting on GPCRs.

Although chemical drugs are used to treat these abnormalities, interest in applied studies and the use of natural compounds such as pro-and prebiotics, which have no specific side effects and have a preventive role, is increasing (Table 1). In fact, the presence of probiotics directly and by modulating the balance of intestinal microbiota toward positive function strengthens the gut-brain axis and has a positive effect on the treatment of some brain diseases. Also, the presence of prebiotics directly, and also, by modulating the balance of intestinal microbiota and strengthening and increasing the number of probiotics in the colon has the same therapeutic effect on mental disorders (2, 3, 5) (See Figure 2).

Optimal balance of intestinal microbiota and strengthening of ENS and vagus nerve with the presence of pro-and prebiotics alter and increase metabolites such as tryptophan and SCFAs that directly affect brain function, and the secretion level of some brain factors such as gamma-aminobutyric (GABA), serotonin/5 hydroxy tryptamine, brain-derived neurotrophic factor, and dopamine, ultimately affect mental disorders (2–5). The hypothalamic–pituitary–adrenal tension feedback, which regulates mood and emotion, is weakened by some probiotics, dropping corticosteroid amounts. The immune system, under the influence of pro-and prebiotics, causes the production and secretion of pro-inflammatory cytokines; then, by affecting the nerves and the hormonal system, the amount of inflammation in the target tissue, which is the brain, is reduced (3, 5). Studies have shown that the use of combined probiotics (e.g., *Lactobacillus*, *Enterococcus*, and *Bifidobacterium*) together with prebiotics (e.g., resistant starch, and inulin), called synbiotic products, produces a high level of neurotransmitters and neuropeptides, e.g., GABA and brain-derived neurotrophic factor, improving CNS function, counting psychiatric disease-related functions, such as anxiety, depression, stress, and memory ability (14, 15, 28). Table 2 shows some of the studies that have investigated the relationship between pro and prebiotics and mental disorders.

4. Portrayal of the effect of pro-and prebiotics on neurological disorders

4.1. Impact of pro-and prebiotics on anxiety

Epidemiological investigations have demonstrated that anxiety disorders are the main causes of functional impairment. A number of anxiety disorders include panic disorder, social anxiety disorder, obsessive–compulsive disorder, generalized anxiety disorder, post-traumatic stress disorder, and phobias (40).

Current studies have signified that pre-and probiotic supplementation has a potential impact to improve symptomology in mental ailments (41, 42). Prebiotics reach the colon, and GM can ferment them. On the other hand, prebiotics is the nutrient source for probiotics settling in the colon, and this cooperation surely improves GIT functionality. Specific pro-and prebiotics can confront infections and attenuate the risk of general diseases such as mental disorders (5).

TABLE 1 Summary of recent systematic reviews on the effects of pre and probiotics on mental disorders.

Ref	Title	Covered articles	Search databases	Intervention and comparison	Quality assessment	Population	Outcome	Studies	Patients	Subclass of Outcome	Hetero-geneity (L^2)	Data (95% confidence levels and p value)
(20)	From probiotics to psychobiotics – the gut-brain axis in psychiatric disorders	23	PubMed, Embase, Cochrane Central Register of Controlled Trials, and ClinicalTrials.gov	Pre or probiotic compared with placebo	Jadad scale	Diverse populations from students to the elderly and pregnant women	-A significant decrease in Beck depression inventory (BDI) scores after two months -Reduced depressive symptoms in patients with depression -Improvement of Hamilton Depression Rating Scale (HAM-D) scores	16	2,726	-	78%	−0.87 (−1.66–0.099)
(21)	Effect of Probiotics on Psychiatric Symptoms and CNS Functions in Human Health and Disease: A Systematic Review and Meta-Analysis	54	PubMed, Web of Science and Cochrane Library	Trials assessing the effectivity of viable and non-viable microorganisms or probiotics cell extracts with a blinded placebo control group	-	Adult men and women with different health conditions, including healthy people, people with mental disorders, or a specific functional CNS	- Reduction of depressive symptoms in both healthy and disordered groups	30	3,017	-	48%	−0.37 (−0.55, −0.20)
(22)	Effects of probiotics and paraprobiotics on subjective and objective sleep metrics: a systematic review and meta-analysis	15	PubMed (MEDLINE), Web of Science (via Thomas Reuters), Scopus and PsycINFO	Probiotics/ Paraprobiotics with placebo	Rosendale Scale, which combines the PEDro scale, Jadad scoring system, and Delphi List	People over 18 years who consumed probiotics	- Improving the quality of people's sleep - A decrease in the Pittsburgh Sleep Quality Index (PSQI) score	11	452	-	58%	0.78 (0.395–1.166)

(Continued)

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TABLE 1 (Continued)

Ref	Title	Covered articles	Search databases	Intervention and comparison	Quality assessment	Population	Outcome	Studies	Patients	Subclass of Outcome	Hetero-geneity (I^2)	Data (95% confidence levels and p value)
(23)	Efficacy of probiotics on stress in healthy volunteers: A systematic review and meta-analysis based on randomized controlled trials	25	Cochrane Library, Embase, Medline (Ovid), PsycINFO (Ovid), and CINAHL (EBSCOhost)	Probiotic compared with placebo	-	Participants with health conditions and no major health problems	-Reduce the subjective stress level of healthy people -Improvement of the subthreshold level of stress-related anxiety/ depression of healthy people	7	1,146	-	0%	−0.14 (−0.27, −0.01)
(24)	Effectiveness of Probiotic, Prebiotic, and Synbiotic Supplementation to Improve Perinatal Mental Health in Mothers: A Systematic Review and Meta-Analysis	54	MEDLINE (Ovid interface), EMBASE (Ovid interface), CINAHL Plus with Full Text (EBSCOhost interface), Cochrane Central Register of Controlled Trials (Wiley interface, which also includes ClinicalTrials.gov and the WHO International Clinical Trials Registry Platform) Scopus, Web of Science Core Collection, and BIOSIS (Web of Science Platform)	Pre/probiotics or synbiotic compared with no treatment/placebo	Grading of Recommendations Assessment, Development and Evaluation (GRADE) framework	Pregnant women with uncomplicated pregnancies	- Reduced anxiety scores in the STAI-6 questionnaire by almost 1 point at the end of follow-up	3	543	-	0%	−0.99 (−1.80, −0.18)

(Continued)

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TABLE 1 (Continued)

Ref	Title	Covered articles	Search databases	Intervention and comparison	Quality assessment	Population	Outcome	Studies	Patients	Subclass of Outcome	Hetero-geneity (L^2)	Data (95% confidence levels and p value)
(25)	Probiotics for Alzheimer's Disease: A Systematic Review	22 (18 animal studies and 4 clinical trials)	PubMed, Semantic Scholar, Nature, and Springer link	Probiotic compared with a control (placebo or standard treatment)	-	Patients with AD	-Improved Mini-Mental State Examination score. - Reduced serum high-sensitivity C-reactive protein (hs-CRP). - Reduced serum triglyceride. - Reduced serum MDA. - Effects on total antioxidant capacity. - Higher TYM score, cognitive function - Increased serum GSH. - Decreased serum 8-OHdG. - Lower concentration of fecal zonulin. - Increased Faecalibacterium - Prausnitzi in fecal. - Higher level of kynurenine in serum. - Higher level of neopterin and nitrite. - Higher RNA level in fecal bacteria	4	192	-	-	-

(Continued)

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TABLE 1 (Continued)

Ref	Title	Covered articles	Search databases	Intervention and comparison	Quality assessment	Population	Outcome	Studies	Patients	Subclass of Outcome	Hetero-geneity (L^2)	Data (95% confidence levels and p value)
(26)	Prebiotics and probiotics for autism spectrum disorder: a systematic review and meta-analysis of controlled clinical trials	3	PubMed, Web of Science, Embase, and Cochrane Library	Pre or probiotic compared with placebo	modified Jadad scale	ASD patients	-ADOS-CSS: Total ADOS Calibrated Severity Score. -6-GSI: 6-Gastrointestinal Severity Index. -CBCL: Child Behavior Check List. -ATEC: Autism Treatment Evaluation Checklist. -CGI-I: Clinical Global Impression-Improvement	3	144	Severity of overall ASD symptoms	0%	−0.23 (−0.56,0.11)
										Severity of GIT issues in ASD	85%	−1.14 (−3.56,1.31)
										Comorbid psychopathlology in ASD	0%	−0.06 (−0.37,0.25)
(27)	A Systematic Review of the Effect of Probiotic Supplementation on Schizophrenia Symptoms	3	PubMed, Medline, Embase, Google Scholar, ClinicalTrials.gov, Clinical Trials Register of the Cochrane Collaboration Depression, Anxiety and Neurosis Group (CCDANTR), and Cochrane Field for Complementary Medicine databases	Probiotic compared with placebo	Cochrane Collaboration's tool	patients with at least moderately severe psychotic symptoms of SCZ	PANSS (Positive and Negative Syndrome Scale)	3	172	-	0%	−0.09 (−0.38,0.20)

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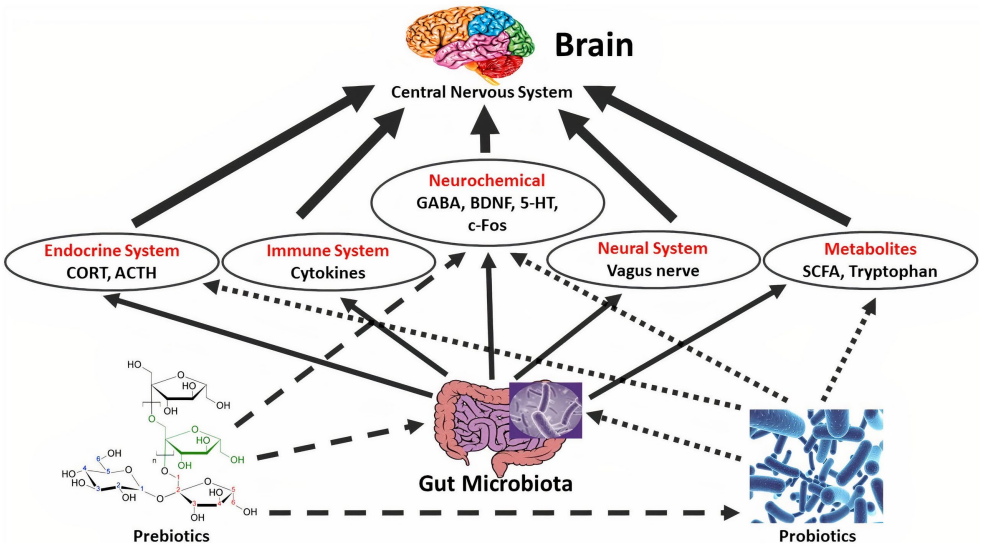

FIGURE 2
The effect of probiotics on the central nervous system/CNS through the effect on the microbiome-gut-brain axis. Probiotics affect brain function both directly and indirectly. Probiotic microorganisms affect the hypothalamic–pituitary–adrenal/HPA axis, through fluctuating corticosteroid/CORT and adrenocorticotropic hormone/ACTH ranges. The immune system is impacted by limited pro-inflammatory cytokine creation and inflammation and has stimuluses on the CNS. Probiotics can moreover straightly modify CNS biochemistry, for example by fluctuating 5-hydroxytryptamine/5-HT, brain-derived neurotrophic factor/BDNF, g-aminobutyric acid/GABA, Dopamine/DA, and c-Fos ranges, subsequently compelling mind and manners. The vagus and enteric nerves are also involved in gut-brain communications and are impacted by certain probiotic strains. Also, probiotic microorganisms regulate the gut microbiota by expanding microbiota variety and beneficial bacteria configuration. At that point, gut microbiota can adjust metabolites, such as, short-chain fatty acids/SCFAs, exopolysaccharides/EPS, and tryptophan, afterward, progresses CNS function indirectly. Also, the gut microbiota collaborates with the immune system, endocrine, and neural system.

The specific lactic acid bacteria such as *Lactobacillus rhamnosus* GG, *Lactobacillus casei*, *Lactobacillus plantarum*, and *Lactobacillus johnsonii*; or *Bifidobacteria* such as *Bifidobacterium bifidum* Bb12, and *Bifidobacterium lactis* or some yeasts like *Saccharomyces cerevisiae* Var. *boulardii* are the main members of probiotics (43).

Clinical researches have detected some psychobiotics with a good antidepressant, and anti-anxiety impacts. These ingredients can regulate GIT microbiota and improve the microbiota–gut–brain axis (20, 21, 44, 45). For example, the latest animal model research indicates that probiotics (such as *Lactobacillus* and *Bifidobacterium* genera) can effectively decrease anxiety-like behaviors in mice or rats assessed in the open field, the increased plus-maze, the light–dark box, and conditioned defensive burying. In addition, probiotics reduce plasma or serum corticosterone levels after severe stress. It is imagined that probiotics have anxiolytic-like impacts through vagal effects on the periaqueductal gray, central nucleus of the amygdala, caudal solitary nucleus, and bed nucleus of the stria terminalis. More investigations are needed to indicate the neurochemical anatomy under GM exerting effects through vagal or nonvagal pathways (44).

The results of an intervention study that was conducted for 4 weeks showed that GOS prebiotic supplement may be effective in improving pre-clinical anxiety indices (46). Moreover, in a meta-analysis it was shown that pro-and prebiotic supplement, as isolated therapies, incurred non-statistically significant results (45). Furthermore, the anxiety-reducing effects of probiotics in populations with anxiety was documented significantly (47). The probiotic impacts on the improvement of anxiety were exerted through several mechanisms, such as promoting the ENS or the immune system's stimulation through the bacteria, as well as affecting the psychophysiological markers of depression and anxiety in three different ways. They can decrease systemic inflammation and control the hypothalamic–pituitary–adrenal axis stress response. In addition, these substances induce the secretion of molecules such as neurotransmitters, proteins, and SCFAs can have a direct effect on the immune system (48).

4.2. Impact of pro-and prebiotics on depression

Regarded to WHO research, major depressive disorder leads to disability worldwide. GM is a factor that can be effective in depression and exerts its effect through the microbiota–gut–brain axis (1). Gut dysbiosis impairs mental health and mental health disorder interrupt gut microbiota. Depressive symptoms are usually associated with GIT disorders such as inflammatory bowel disease, metabolic syndrome, and irritable bowel syndrome. The concurrent occurrence of mental and GIT disorders enhances disease progression and intensifies the occurrence of poorer consequences, whereas, treatment of one of these two conditions can reverse the risk of the other. Moreover, the pathogenesis of depression is comorbid with alterations in the composition of GM (49).

Recent investigations have revealed probiotics positively affect individuals with pre-existing depressive symptoms, while, in healthier populations, mood symptoms are less significantly affected (5, 20, 50). Investigating the behavioral changes caused by LPS-induced in a rodent model to investigate the relationship between the absence of

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TABLE 2 The effect of pro and prebiotics on some mental disorders.

Ref	Probiotic strain(s)/ Prebiotic	Dose of probiotic/ prebiotic	Carrier	Model	Duration	Effect
(29)	*L. acidophilus*, *L. casei*, *L. fermentum*, and *B. bifidum*	2×10^9 CFU/g of each strain	Milk (200 mL/day)	Human	12 weeks	Improving cognitive function in AD patients
(30)	A mixture of lactobacilli (*L. acidophilus*, *paracasei, plantarum*, *bulgaricus*, *delbrueckii*), bifidobacteria (*B. longum*, *breve*, *infantis*), and Streptococci (*S. salivarius*, *thermophilus*)	8×10^8 CFU/g of lactobacilli,9×10^{10} of bifidobacteria, and 20×10^{10} Streptococci	VSL#3 (VSL Pharmaceuticals Inc., USA)	Human (case report)	4 weeks	Improvement of the core symptoms of ASD in a 12 years old child
(31)	*L. acidophilus*, *L. fermentum*, and *B. lactis*	1 mL of water containing 10^{10} CFU/g of the three bacteria	Drinking water (1 mL/day)	Rat	2 weeks	Improvement of stress-dependent behavioral disorders and the interaction between HPA and gut-brain-microbiota axes
(32)	*L. fermentum* NS9	10^9 CFU/mL	Drinking water	Rat	41 days	Reduction of anxiety-like behavior as well as reduction of memory retention disorders caused by ampicillin
(33)	*B. breve* CCFM1025	10^{10}CFU/day	Freeze-dried	Human	4 weeks	The probiotic group showed a better antidepressant-like effect than the control group (maltodextrin); so this is a promising strain that reduces depression.
(34)	S. *thermophiles, B. breve*, *B. lactis*, *B. infantis*, *L. plantarum*, *L. paracasei*, *L. helceticus*	9×10^{11} CFU/day	probiotic supplement (Vivomixx®, Mendes SA, Lugano, Switzerland)	Human	31 days	Probiotic treatment along with changes in gut microbiota, also reduced depressive symptoms
(35)	*L. plantarum* PS128	3×10^{10} CFU/capsule	Capsule (Two a day)	Human	30 days	Daily consumption of probiotics may reduce symptoms of depression, improve sleep quality, and reduce fatigue
(36)	Fructooligosaccharide as prebiotic, and probiotics, include *L. casei*, *L. acidophilus*, *L. bulgaricus*, *L. rhamnosus*, *B. breve*, *B. longum*, and *S. thermophilus*	100 mg fructooligosaccharide, and *L. casei* 3×10^8, *L. acidophilus* 2×10^8, *L. bulgaricus* 2×10^9, *L. rhamnosus* 3×10^8, *B. breve* 2×10^8, *B. longum* 1×10^9, and *S. thermophilus* 3×10^8 CFU/g	Capsule (One a day)	Human	6 weeks	Synbiotic is effective as an adjunctive treatment for moderate depression
(37)	Fructan	The amount of fructan received by each person from the number of times consumed and the fructan content of meals	Food consumed by each person	Human (cohort study)	-	A higher dietary intake of fructan is associated with a reduced risk of clinical Alzheimer's disease in people aged 65 years and older

(Continued)

TABLE 2 (Continued)

Ref	Probiotic strain(s)/ Prebiotic	Dose of probiotic/ prebiotic	Carrier	Model	Duration	Effect
(38)	Foods containing prebiotics (such as cereals, bread, root crops, milk products, and vegetables)	Amount of consumption of foods containing prebiotics by each person	Food consumed by each person	Human (cross-sectional exploratory study)	-	Lower consumption of prebiotic foods has a negative effect on anxiety, stress and depression
(39)	*Bifidobacterium infantis* and a bovine colostrum product (BCP) as a source of prebiotic oligosaccharides	2×10^{10} CFU/day of probiotics and 0.15 g/lb. body weight per day of colostrum powder	milk, juice, yogurt, or ice cream	Human	12 weeks	Particular aberrant behaviors were reduced in some autistic children treated with a combination of probiotics and prebiotics or prebiotics alone

GM and neuroinflammatory mechanisms has shown that the activation of pro-inflammatory mechanisms, the activation of the raphe nucleus, and depression-like behaviors are affected by GM function (51). Overall, the evidence represents that GM plays a potential role in the pathogenesis of depressive behavior and may be an antidepressant agent. In addition, molecules derived from microorganisms, such as SFCAs, indoles, bile acids, neurotransmitters, lactate, choline metabolites, and vitamins could be largely effective in stimulating emotional behavior. The neuroactive molecules (such as dopamine, tryptamine, GABA, acetylcholine, 5-hydroxytryptamine/ serotonin 5-HT, L-dopa, norepinephrine and histamine) are directly produced by the microbiome (52). Also, any changes in intestinal flora suppress hippocampal brain-derived neurotrophic factor expression in the neurons in the cortex and hippocampus leading to depression (53).

The intestinal microbiota can affect the brain tissue directly by regulating the secretion of hormones from brain-gut peptide production, intestinal endocrine cells, corticotropin, corticotropin-releasing factor, leptin and adrenocortical ketone. Furthermore, it was released that probiotics can play a role in changing the sensitivity of the intestinal tract, regulating the stimulation threshold of intestinal neurons and the secretory function of intestinal cells, maintaining the ecological stability of GM, and then influencing the CNS and improving depression (54). Prebiotics can also up-regulate the expression of the specific gene in the hippocampus and hypothalamus tissue, promote propionic acid and cecal acetic acid and reduce the isobutyrate value which is associated with behavioral improvement. Nevertheless, the mechanism of the microbiome-gut-brain interaction is still not fully elucidated (55).

On the other hand, some investigations have proved that pre-and probiotics have potential impacts on depression-like behavior through restoring cortisol values, attenuating the inflammatory mediators, and as well as regulating serotonin and CNS transmitters synthesis (56, 57). For instance, Schmidt et al. (57) reported that the awakening response of salivary cortisol declined significantly after B-GOS (Bimuno®-galacto-oligosaccharides) intake compared to placebo. Additionally, in a dot-probe task, it was observed that after taking B-GOS, there is a decrease in attentional vigilance toward negative information versus positive information. No significant results were found in healthy participants to intake FOS for 3 weeks (57). Zagórska et al. (20) revealed that probiotic consumption significantly reduced the symptoms of patients with depression after 8 weeks (20). Similarly, the meta-analysis of 34 controlled clinical trials, statistically showed that probiotics have significant effects on depression; however, the prebiotics did not differ from placebo for depression (49). In addition, treatment with *L. paracasei* strain Shirota for 12-week in eligible patients with bipolar disorder can reduce depression severity significantly evaluated by the Hamilton Depression Rating Scale (58).

4.3. Impact of pro-and prebiotics on stress

Stress is a major agent of the occurrence of horrible diseases such as heart disease. The healthy ways are believed to attenuate stress. The pro-and prebiotics potential effects on managing stressful conditions are very intriguing. The literature reviews have represented that GM has roles in the regulation of stress. The microbiome-gut-brain axis is a complex and bidirectional network that exists between the CNS and GM and any imbalance in this axis, induces various kinds of mental health disorders such as stress (20).

The bacteria are removed from the mucosa through the inherent and adaptive immune systems. Bacterial metabolites can induce the discharge of neuropeptides and other gut hormones from enteroendocrine cells. GM considerably affects the progress and sensitivity of the hypothalamic–pituitary–adrenal axis in responding to stressors. The grade of psychological stress may be progressed by dysbiosis of GM. Conversely, chronic psychological stress may exacerbate the degree of dysbiosis. It was demonstrated both probiotics and antibiotics can decrease psychological stress retorts (5, 59). Various prebiotics have enhanced the stress-protective microbial organism's activity and growth. Therefore, prebiotics in the diet increases the bacterial species to produce lactic acid and butyrate (stress-protective microbial species) and maintain the host from the neurobiological, behavioral, and physiological effects of stress (23, 60).

The long-term administration of a CP2305 tablet (containing heat-inactivated, washed *Lactobacillus gasseri*), in healthy young adults, significantly decreased stress and stress-induced instability in GM through the elevation of *Streptococcus* spp. the decline of *Bifidobacterium* spp. in feces (23). In addition, it was found that *Bifidobacterium longum* 1714™ modulated resting neural activity, enhanced mental vitality, and attenuated mental fatigue which leads to neural response modulation during stress (61). A diet containing prebiotics and regular exercise can be appropriate and practical

strategies to enhance stress-protective bacteria and resistance to the neurobiological effects of stress (60). On the other hand, (B)-GOS supplementation decreased the neuroendocrine stress response and improved emotional attention in healthy participants after 3 weeks (57).

4.4. Impact of pro-and prebiotics on sleep

Sleep disorders as a type of circadian rhythms sleep–wake disruption are characterized by insomnia or excessive sleepiness. People working night shifts have a circular rhythm disorder that generally shows less sleep time than the evening and day shift workers. The literature review has found probiotic administration can improve sleep quality which is related to balancing GM (62). The significant bidirectional connection between GIT and CNS (the gut-brain axis) plays a major role to regulate the GM composition. Therefore, probiotic supplementation may help to improve sleep quality by maintaining the balancing of the GM environment. Furthermore, probiotics promote the production of free tryptophan in the CNS, and promote melatonin formation from serotonin can regulate circadian rhythm (63). The prebiotics also can efficiently affect mental health and ameliorate cognitive function and sleep/wake cycle disruptions (2).

A clinical trial investigating which was conducted to evaluate the *Lactobacillus casei* strain *Shirota*/LcS effect on sleep quality under psychological stress, reported a significant positive effect of LcS supplementation and sleep quality. The results showed that the daily administration of LcS may maintain the quality of people's sleep during a period of increasing stress (64). In another investigation, consumption of a tablet containing heat-inactivated washed *Lactobacillus gasseri* CP2305 in healthy adults decreased sleep disorders (65). Similarly, a double-blind, placebo-controlled study with a probiotic mixture (containing *L. plantarum* LP01, *Lactobacillus fermentum* LF16, *L. rhamnosus* LR06, and *Bifidobacterium longum* BL04) for 6 weeks, significantly improved sleep quality in the probiotic group (66). Recently conducted meta-analytic studies indicate that probiotic supplements could be significantly effective in improving perceived sleep quality (21, 22).

4.5. Impact of pro-and prebiotics on Alzheimer's

Alzheimer's disease (AD), recognized as the most prevalent form of dementia currently affects around 50 million cases worldwide (67). At first, it was defined as a clinic-pathology status. Nowadays it is referred to as Alzheimer's clinical syndrome with a range of clinical manifestations and a multifactorial etiology that has several pathobiological subtypes. The basis of the definite diagnosis of AD is through pathological examinations and includes; observation of extracellular plaques with depositions of β-amyloid/Aβ, presence of Aβ in the brain vessels (cerebral amyloid angiopathy (CAA)), and protein forming neurofibrillary tangles associated with intraneural accumulation of abnormal hyperphosphorylated tau protein (67, 68). AD is a progressive neurodegenerative disorder characterized by memory loss, and problems with thinking, language, and problem-solving abilities. As it is an age-dependent situation, the problem will grow as the average age of the population increase. Another background factor is sex; AD is more common in women (68). During this review, we will discuss how the pathobiology of the disease can be described by the gut-brain microbiota connections.

Generally, we lack information about the exact cause of AD; however, there are some hypotheses about the etiology of the disease; (i) amyloid theory which has long been the main theory: Accordingly, alterations in the process of the Aβ cycle cause accumulation of Aβ protein in the brain. These plaques are harmful to the neurons and also cause oxidative damage. The generated Aβ cycles, in turn, induce forming neurofibrillary, and phosphorylation of tau protein which leads to further damage to the neural system (69). (ii) Presently, neurodegeneration caused by various mechanisms is considered to describe AD. Problems in the hemostasis of calcium, amyloid accumulation, imbalance of neurotransmitters, neuro-inflammation and astrocyte activation, and brain atrophy are some of the suggested mechanisms (19).

As discussed above, the neuropathology of AD has long been considered only a brain disease; however new evidence is supporting the idea of the effects of other organs in developing AD. Mainly the role of GM in the normal function of the brain and nervous system has been broadly studied. The findings suggest that GM can affect the structure and function of the brain directly. It can also change the immunity and behavior of the host, which indirectly affects brain function. There are some data available from experimental and clinical studies showing altered microbiome in neurodegenerative diseases such as AD. There are some mechanisms proposed for the effects; the transformed microbiome induces the release of neurotransmitters and pro-inflammatory factors leading to the increased permeability of the blood–brain barrier which in turn causes augmented neuro-inflammatory reactions and amyloid production and accumulation in the brain tissue. The dyes-biome allows the entrance of bacterial amyloid, LPS, and some toxic molecules in peripheral blood circulation and lately in the brain which in turn cause abnormal changes in the brain. Neurodegeneration may also be induced by dysfunction of the immune system related to the abnormal microflora. It should be mentioned that it is a chronic situation and the pathologic changes begin 10–20 years before the manifestation of the clinical disease. It can be concluded that restoring GM in patients with AD can no doubt slow down the progression of abnormal changes in the brain by reducing amylogenesis, and inflammation (19).

It is obvious that pro- and prebiotics may successfully be applied to cure patients with AD. For instance, the probiotic beverage containing *L. acidophilus*, *L. fermentum*, *L. casei*, and *B. bifidum* for 12 weeks significantly improved Mini-Mental State Examination score in 60 patients with AD, with a mean age of 80 (5). Gene profiling studies demonstrated that *Bifidobacterium breve* A1 can suppress inflammation in the hippocampus of the brain and also immune-reactive genes induced by amyloid (19). Lactopeptides and tryptophan-related dipeptides in fermented dairy products showed positive effects on memory and cognition function. In addition, there are some evidence which show consumption of dairy products such as cheese and milk reduce the risk of dementia and cognitive dysfunction (19). One systematic review conducted on the effects of the probiotics on AD reached plenty of evidence about promising effects of probiotics in improving the progression of the disease including *in vivo* studies and clinical trials. No side effects were reported (25).

4.6. Impact of pro-and prebiotics on autism spectrum disorder

ASD is a condition characterized by difficulties in social communication and interaction, repetitive and limited patterns of interests and behaviors, and changes in sensory processing related to neurobehavioral and neurodevelopment abnormalities (70). The results of ongoing research show that it is a growing concern with an increasing prevalence all over the world. Prevalence estimates published 10 years ago suggest around 100/10,000 morbidity with the male sex about 4 times more likely to get the disease (71). Due to the complex nature of the disease, it is known to have several etiological backgrounds including; anatomical changes in the brain, genetic abnormalities, and neurochemical dysfunctions. The altered pathways of many neurotransmitters including serotonin, dopamine, N-acetyl aspartate, oxytocin, GABA and glutamate, acetylcholine, arginine-vasopressin, vitamin D, melatonin, orexin, and opioids are supposed to have a role in the disease mechanism. However, the complex relationship between the abnormal neurotransmitters and the specific interaction system underlying the disease has not been recognized yet (70).

There are some evidences about the potential effects of GM on the pathogenicity of Autism. There is a high comorbidity of GIT symptoms such as abdominal pain, diarrhea, constipation, and the disease, and this, in turn, increases the behavioral problems in patients. The gut-brain interactions are related to the pathophysiology of ASD via the population and function of GM. It has been demonstrated that gut bacterial profile is different in patients with ASD compared to the normal controls. However, the altered microbiota may be the result of the special lifestyle of the patients such as diet and bowel habits. Based on the findings, the idea of the therapeutic effects of changing GM on ASD was developed. During a study, GM from patients with ASD was transferred to germ-free mice which induce autism symptoms such as repetitive behavior and decreased communication and locomotion. In addition, treatment with bacterial metabolites like 5-aminovaleric acid which is depleted in ASD patients can improve the function of the prefrontal cortex (related to social cognition) and consequently repetitive and social behavior. Among various therapeutic candidates to modulate the gut-brain axis in ASD, pro-and prebiotics have drawn special attention (28).

Several studies have been conducted to assess the effects of pro-and prebiotics on ASD. The main endpoints were ASD-related symptoms and GI wellbeing. Various strains of *Lactobacillus* such as *L. acidophilus*, *L. plantarum*, *L. paracasei*, as well as *Bifidobacterium* had been administered. Hydrolyzed guar gum, FOS, and maltodextrin were also applied to the patients as prebiotics. Some RCTs found no significant difference between probiotic and placebo groups regarding behavioral problems and symptoms severity after completion of the intervention. Other studies with significant differences between placebo and control groups were subject to bias distorting the effect. It can be concluded that the effect of probiotics on ASD symptoms has not been proven yet. However, studies on the effects of prebiotics and synbiotics show the beneficial effect of the treatment to improve some scales of the ASD-related symptoms. For instance, GOS containing prebiotic supplement (Bimuno®) can reduce anti-social behavior (72), and the combination of prebiotic oligosaccharides and *B. longum* subsp. *infantis* UCD272 on the lethargy of the patients showed positive effect (39).

According to the results of a systematic review on the RCTs, four of the trials showed no changes after consumption of probiotics. A significant reduction in GIT symptoms was demonstrated in two of the trials, and it was known to be associated with ASD behavioral symptoms. The main finding of the studies was the improvement in GIT symptoms such as constipation, diarrhea, and stool smell in the prebiotic compared to the control group. It should be noted that the treatment duration in the studies on prebiotics and synbiotics is longer than the probiotic studies, and it may be the reason for the observed effects in the prebiotic studies. Also, these studies are accompanied by various outcomes and comparisons such as sub-group analysis which increases the chance of statistically significant difference. So, some of the significant results may be simply due to the chance and not the real effects of the administered compounds. It can be concluded that we cannot still say for sure that probiotics, prebiotics, or synbiotics can make a positive change in ASD patients (28).

4.7. Impact of pro-and prebiotics on schizophrenia

Schizophrenia (SCZ) is a kind of psychiatric disorder with a global age-standardized prevalence of 0.28% and no sex difference in prevalence. The prevalence does not vary extensively across the countries. Though the low prevalence of the disease, it has a substantial burden on society due to the poor recovery outcome, and the decreased life expectancy and life quality. Suicide attempts and comorbid diseases (coronary heart disease, type II diabetes, respiratory, and malignancies) are from the problems of the individuals with SCZ (73). The symptoms are categorized into three main groups; positive, negative, and cognitive. Positive symptoms or the presence of psychotic symptoms are more responsive to the antipsychotic medication treatment than negative (e.g., social withdrawal) and cognitive (e.g., diminished abstract thinking) symptoms. The etiology of the disease has not been fully understood yet, however genetic and environmental factors are supposed to interact to induce the symptoms. Availability of proper medications is very important as early treatment, monitoring, suitable psychological management, and social support may lessen the symptoms or even lead to partial or full remission (74).

As discussed previously, there is much evidence on the effects of GM on brain functions and subsequently behavior and psychiatric problems. The mechanisms may also involve in SCZ. Studies on animal models suggest that some SCZ-associated behaviors such as social behaviors, cognition, and mood alterations can be influenced by GM. However, clinical studies on humans are still limited (74). The studies are focused on two main backgrounds; comparing the microbiome of the patients with SCZ and the healthy controls, and clinical trials to detect any therapeutic advances in the administration of pro-and prebiotics for schizophrenia. It has been demonstrated by several studies that the level of the family Lachnospiraceae is lower in individuals with SCZ compared to the healthy population which involves protecting the integrity of the intestinal barrier and producing beneficial compounds. However, the results of this type of studies are subject to biases due to the effects of psychiatric treatments and lifestyle on the microbiome (74).

Albeit promising effects of the pre and probiotics in experimental designs, a systematic review of the trials till 2018 revealed no beneficial

effects of probiotics on SCZ on meta-analysis. The authors concluded that regardless of the positive effects of the probiotics on bowel movement and ameliorating the metabolic effects of antipsychotic medications the administration of probiotics for SCZ is not recommended (27). We found no systematic review of the effects of prebiotics but the results of the trials imply potential beneficial effects. In one study, application of oligofructose-enriched inulin (OEI) increased serum butyrate in SCZ patients (75). Another prebiotic, lactosucrose, altered the fecal flora followed by improvement in the intestinal and psychotic symptoms of the patients (76).

5. Conclusion and future perspectives

The communication between GIT and the brain has long been well known. The direct neural signals and indirect hormonal and enzymatic connections are supposed to be responsible for the mutual effects. The idea developed to the application of pre-, pro-and synbiotics to modulate the CNS during mental disorders as a novel and natural treatment with very limited potential side effects. In this review we presented promising findings on the effects of pre-, pro-and synbiotics on a variety of mental disorders especially anxiety, depression, stress, sleep, and AD. Despite some studies on the positive effects of pre-, pro-and synbiotics on the other mental conditions including SCZ and ASD, the available data is not enough to support the idea of the application of such therapies for the above disorders. It is obvious that we need to expand our knowledge on this subject by conducting well design clinical trials using various kinds of pre-, pro-and synbiotics in well-defined and -as far as possible- large populations to get more specific and more reliable results. The present evidence is attractive enough to go ahead and design special formula of pre-, pro-and synbiotics for different mental disorders. This may also be accompanied by testing different drug regimens containing standard treatments and pre-, pro-, or synbiotics. In conclusion, it can be said that it is time to introduce a new generation of specific drugs based on the pre-, pro- and synbiotics for a variety of mental disorders. A need that should be met through conducting appropriate and rigorous research plans.

Author contributions

FA and HP conceived the idea. FA, MN, HP, SJ, SAS, and EM wrote sections of the manuscript. All authors contributed to the manuscript revision, read, and approved the submitted version.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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EDITED BY
Domenico Nuzzo,
National Research Council (CNR), Italy

REVIEWED BY
Ruth Naomi,
Universiti Putra Malaysia, Malaysia
Santhra Segaran Balan,
Management and Science University, Malaysia

*CORRESPONDENCE
Po-Hsien Li
✉ pohsien0105@pu.edu.tw
Ming-Fu Wang
✉ mfwang@pu.edu.tw

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Potential antidepressant effects of a dietary supplement from Huáng qí and its complex in aged senescence-accelerated mouse prone-8 mice

Ming-Yu Chou[1,2], Yue-Ching Wong[3], Shih-Yi Wang[2], Ching-Hsin Chi[2], Teng-Hsu Wang[4], Mao-Jung Huang[5], Ping-Hsiu Huang[6], Po-Hsien Li[7]* and Ming-Fu Wang[2,7]*

[1]School of Business, Qanzhou Vocational and Technical University, Jinjiang, China, [2]International Aging Industry Research & Development Center (AIC), Providence University, Taichung, Taiwan (R.O.C.), [3]Department of Nutrition, Chung Shan Medical University, Taichung, Taiwan (R.O.C.), [4]PhytoHealth Corporation, Taipei city, Taiwan (R.O.C.), [5]School of General Education, Hsiuping University of Science and Technology, Taichung, Taiwan (R.O.C.), [6]School of Food, Jiangsu Food and Pharmaceutical Science College, Huai'an, China, [7]Department of Food and Nutrition, Providence University, Taichung, Taiwan (R.O.C.)

Healthcare is an emerging industry with significant market potential in the 21st century. Therefore, this study aimed to evaluate the benefits of tube feeding Huáng qí and its complexes for 8weeks on 3-month-old senescence-accelerated mouse prone-8 (SAMP8) mice, 48 in total, randomly divided into 3 groups including control, Huáng qí extract [820mg/kg Body weight (BW)/day], and Huáng qí complexes (6.2mL /kg BW/day), where each group consisted of males (n=8) and females (n=8). Behavioral tests (locomotion test and aging score assessment on week 6, the single-trial passive avoidance test on week 7, and the active shuttle avoidance test on week 8) were conducted to evaluate the ability of the mice to learn and remember. In addition, after sacrificing the animals, the blood and organs were measured for antioxidant and aging bioactivities, including malondialdehyde (MDA) content and superoxide dismutase (SOD) activity and catalase activities (CAT), and the effects on promoting aging in SAMP8 mice were investigated. The findings showed that Huáng qí enhanced locomotor performance and had anti-aging effects, with positive effects on health, learning, and memory in SAMP-8 mice (p<0.05), whether applied as a single agent (820mg/kg BW/day) or as a complex (6.2mL/kg BW/day) (p<0.05). Based on existing strengths, a more compelling platform for clinical validation of human clinical evidence will be established to enhance the development and value-added of astragalus-related products while meeting the diversified needs of the functional food market.

KEYWORDS

senescence-accelerated mouse prone-8, anti-aging, functional food, Huáng qí, anti-depressant

Highlights

- The benefits of Huáng qí did not vary by mouse gender.
- The physiological status of the mice was not affected by Huáng qí.
- The antioxidant activity of the Huáng qí complex was superior to Huáng qí *in vivo*.
- Huáng qí enhanced locomotion and had anti-fatiguing, and antiaging effects in mice.
- Huáng qí increased antioxidant activity and minimized oxidative damage in mice.

1. Introduction

All organs and physiological functions are affected by aging, notably leading to a physical decline in the elderly with a high risk of malnutrition, infirmity, morbidity, and mortality (1–5). Several studies have reported that aging-induced loss of protein balance leads to a loss of muscle mass, which affects the functioning of the gastrointestinal tract, thus reducing nutrient absorption (3, 6). In addition, psychological and social factors lead to inadequate absorption of nutrients (7). However, as the most significant risk factor for all diseases, aging seriously challenges global social resources and health insurance systems. The growth rate of the elderly population over 60 years has been significantly higher than that of younger people (8–10), and the World Health Organization predicted a doubling in the proportion of the world's population over 60 years old by 2050 (2). Thus, healthy aging has become a concern despite the biological definition of aging as a progressive accumulation of diminished or complete loss of function (11). However, age-related phenomena, such as decreased immune system function, chronic inflammation, and disturbed gut microbiota, have also been extensively studied, with proper nutritional supplementation proven to improve or delay the onset, as food nutrition is one of the pillars of health (2, 4, 12–15).

Unfortunately, there are limitations of the approved and available medications (provide symptom relief rather than prevent or improve disease progression) (16). More recently, the role of dietary interventions or nutritional supplements to retard aging has become the focus (17–19). Therefore, it is important to address healthy aging and propose potential therapeutic approaches to limit aging-initiated disease and progression (5, 16, 20, 21). Several studies have been performed to repeatedly demonstrate the brain's vulnerability to the harmful effects of increased oxidative stress (OS), which also explains the increase in reactive oxygen species (ROS) and antioxidant defenses has been associated with brain structural changes, higher lipid content, rapid metabolic rate, and proinflammatory signaling pathways, thus contributing to the pathogenesis of depression (5, 19, 22–24). Consequently, identifying suitable natural sources of antioxidants to combat the overproduction of ROS and OS is an effective preventive strategy to ameliorate aging-related diseases.

The primary bioactive components in Huáng qí (the root of *Astragalus membranaceus* (Fisch.) Bge. var. mongholicus (Bge) Hsiao) are saponins, polysaccharides, and flavonoids (25–30). In China, Japan, Korea, and Central and Southeast Asia, Huáng qí is used as a tonic to treat hepatitis, nephritis, and diabetes or as a complementary therapy for cancer. The Huáng qí herbal supplement is used chiefly as an immune stimulant in the United States to protect against influenza and upper respiratory tract infections (29, 31–34). Specifically, it is traditionally used with other herbs (ginseng, atractylodes, tangerine peel, rhizoma cimicifugae, bupleurum, licorice, and angelica) to enhance the immune system (31, 34–36). Interestingly, Huáng qí (animal mode) has been reported to have immunological functions that stimulate and alleviate depression while avoiding the effects of toxins (35, 37, 38). Moreover, in the cellular mode (Caco2 cell line), *Astragalus* polysaccharides (APS) have been modified by gamma irradiation to enhance immunomodulatory activity without changing the functional groups (only improved physicochemical properties) (39). In addition, APS enhances the activities of macrophages, natural killer cells, dendritic cells, T- and B- lymphocytes, and microglia while inducing the expression of various cytokines and chemokines (40–44). Notably, pharmacological findings suggest that components in a Huáng qí extract increase telomerase activity and antiaging effects (29, 34). Hence, we aimed to evaluate the effects of Huáng qí on learning and memory in the brain as well as antiaging ability in SAMP8 mice. We expected to develop a potential formulation to prevent aging-related diseases or a complementary therapy to regular therapy.

2. Materials and methods

2.1. Materials

Huáng qí (root of *A. membranaceus* (Fisch.) Bge. var. *mongholicus* (Bge) Hsiao) was provided by PhytoHealth Co. (Taipei, Taiwan). It was washed in distilled water, wiped dry, frozen at −20°C for 24 h, and freeze-dried. The powder was dry -milled in a homogenizer (through an 80-mesh sieve). Next, 3.5 L of 95% alcohol and 210 g of Huáng qí powder were placed in a 5 L flask. The flask was shaken every 4 h for 24 h. The alcohol was removed by rotary vacuum evaporation, followed by freeze -drying. The freeze-dried sample was labeled the "Huáng qí extract." The Huáng qí complex formula was composed of the Huáng qí extract, red dates (*Ziziphus jujuba* Mill.), and *Acanthopanax* root [*Acanthopanax senticosus* (Rupr. Maxim.) Harms, which were mixed and flavored with anhydrous D-trehalose and pure water], were provided by PhytoHealth Co. All chemicals were purchased from Sigma-Aldrich® (Merck KGaA, Darmstadt, Germany) for direct use without any pre-treatment.

2.2. Experimental animal

A total of 48 (half male and half female) 3-month-old SAMP8 mice were purchased from the National Laboratory Animal Center (Taipei, Taiwan) and randomly distributed into control, Huáng qí extract, and Huáng qí complexes groups, each consisting of males ($n = 8$) and females ($n = 8$). SAMP8 mice were characterized by short life spans, lack of learning and memory, neuronal damage, and amyloid accumulation in the brain, indicating suitability for aging-related learning and memory studies. All mice were housed in plastic cages [30 (L) × 20 (W) × 10 (H) cm^3] by gender ($n = 8$), and the room was maintained at a specific pathogen-free with temperature (25 ± 2°C) and humidity (65 ± 5%). At the same time, the dark period (07:00–19:00) and light period (19:00–07:00). In addition, feed (AIN-93 M standard purified feed) and water were supplied *ad libitum,* which were renewed every morning. All the mice were randomly grouped according to gender ($n = 8$) following one week of adaptation. All groups consisted of a control group, a Huáng qí extract (820 mg/kg BW/day) group, and Huáng qí complexes (6.2 mL/kg BW/day) group. All samples were dissolved in distilled water and tube fed to each mouse once a day for 8 weeks. Moreover, the doses were based on "Guidelines for the Testing of Chemicals method, Section 4 Test No. 488: Transgenic Rodent Somatic and Germ Cell Gene Mutation Assays" from the Organization of Economic Cooperation and Development (OECD)'s recommended daily intake per kg BW of a 60 kg adult, namely the human equivalent dose, and then extended by a factor of 12.3 (45), i.e., one-fold for mice; the detailed calculations are as follows.

Huáng qí extract:

$$\text{Recommended daily BW intake per kg for a 60 kg adult (human equivalent dose)} = {}^{4\,g}\!/_{60\text{ kg BW / day}} = 66.7\text{ mg / kg BW / day}$$

$$1\text{ fold dose for mice} = 66.7\text{ mg / kg BW / day} \times 12.3 = 820\text{ mg / kg BW / day}$$

Huáng qí complexes:

$$\text{ecommended daily BW intake per kg for a 60 kg adult (human equivalent dose)} = {}^{30\,mL}\!/_{60\text{ kg BW / day}} = 0.5\text{ mL / kg BW / day}$$

$$1\text{ fold dose for mice} = 0.5\text{ mL / kg BW / day} \times 12.3 = 6.2\text{ mL / kg BW / day}$$

Finally, each BW of mice was used to calculate the daily sample tube feeding. Each group of BW, diet, and water intake changes were recorded weekly during the experiment. The locomotion and grading scores were evaluated at week 6, and the memory learning ability (passive avoidance task and active shuttle avoidance test) was assessed at weeks 7 and 8. Subsequently, mice were anesthetized with sodium pentobarbital (using a saline solution prepared to a concentration of 1% with a dose of 50 mg/kg), followed by cardiac blood harvesting and sacrifice (8 h fasting before surrender). Next, the serum biochemical analysis, brain, and organs were measured for bioactivity indicators related to aging, as detailed in the subsequent sections.

All animal procedures followed the standards outlined in the guidelines for the Care and Use of Experimental Animals by the Committee for the Purpose of Control and Supervision of Experiments on Animals and the National Institutes of Health. The Committee on Animal Research, Providence University, under code 220191211 A008 approved the protocol.

2.3. Grading score evaluation

The grading score was evaluated at week 6 and followed the grading score system by Takeda et al. (46). Each item had 5 levels according to the definition of each level, whereas high scores indicated that the mouse was aged seriously. Specifically, (I) Behavior: observe the reaction of mice to exploration (reactivity) within 30 s and the avoidance response (passivity) when the operator pinched the skin on the back of the neck. (II) Appearance: observe hair glossiness, coarseness, hair loss, and ulcers. (III) Eyes: observation of mucositis around the eyes and edema and redness of the eyelids (periophthalmic Lesions). (IV) Spine: observe the changes in the anterior and posterior curvature of the spine (spine lordokyphosis). The total score of each item was calculated.

2.4. Locomotor activity test

In this study, the locomotor activity test was performed as described by Weber et al. (47) with minor modifications. This evaluation was to observe the locomotion activity of mice in the local spatial plane. Briefly, a mouse was placed in the center of a 25 (L) × 25 (W) × 25 (H) cm^3 aluminum chamber and operated under weak light and quiet environments, whereas data were collected by video-recorded (FDR-AX700, Sony, Tokyo, Japan) observations, including the amount of activity and activity (rest time, exercise time, horizontal and vertical movement) of each mouse moving in a plane for 10 min. In addition, the interior space of the chamber was cleaned with a water-soaked Kimwipe to minimize the effect of odor following the completion of each mouse.

2.5. Passive avoidance task

The evaluation methodology was based on Lin et al. (48) with slight modifications. This study was conducted in a 35 (L) × 17 (W) × 20 (H) cm^3 shuttle cage (Model E10-15, Coulbourn Instruments, Massachusetts, USA). The inner part includes a light chamber and a dark chamber with a 7.5 (L) × 6.5 (W) cm^2 guillotine door (Model E10-15GD, Coulbourn instruments) in the center, which allows access to each other, while the bottom of the chamber has spaced (1 cm) and parallel metal rods with electric current. The mouse was initially placed in the light chamber, followed by 10 s of acclimatization, and then the guillotine door would be opened, allowing free exploration between the two chambers. However, by nature, the mouse is nocturnal behavior in the dark and will move to dark places. Upon entering the dark chamber, the guillotine door would be closed quickly, followed by an aversive stimulus (such as a foot shock) of 0.5 s (0.5 microamperes) at 5-s intervals for three consecutive times during the training period. Subsequently, the memory capacity of the mice was tested again at 24 h, 48 h, and 72 h. It was performed in the same way as described above, but without giving any electric shock. Meanwhile, a mouse staying in the light chamber was recorded, and the maximum duration of each test was 180 s. Lengthier time spent in the light room means the mouse has better memory capacity.

2.6. Active shuttle avoidance test

The evaluation followed the Weber et al. (47) described and was modified as appropriate. In brief, the same device as station 2.5 was used. However, a different device was set up with lights and sound at the top of the shuttle cage, controlled by a computer to stimulate the mice. Initially, the mouse was placed in a chamber within 10 s at the beginning of the test. Subsequently, light and sound-conditioned stimulus (CS) with a 10-s interval was given. In case the mouse remained in the same chamber during the CS demonstration, an electric shock of 0.3 mA for 5 s was automatically delivered as an unconditioned stimulus (UCS). In contrast, no shock was delivered once the mouse entered another chamber during the CS demonstration. Moreover, there is also a balance device at the center bottom, which utilizes the principle of stilts by sensing the left or right

side of the mouse's current position in the cage. The computer controls the experimental process for the time, sound, light, and electric shock, which finally records the results. During the process, the mouse stays on the same side, which means that the mouse has not yet learned to deliver the electric shock, which is a training process for learning. Conversely, a mouse moving to the other side indicated it had known without giving an electric shock, referring to its memory performance. The computer automatically determines whether the mice respond to the conditioned stimulus CS or UCS through the above balance device. Each mouse undergoes 5 rounds of the same test at each 15–20 min interval for four consecutive days. Notably, more successful mouse evasions were associated with better learning memory.

2.7. Relative organs weight (%)

This study's relative organs weight according to Chou et al. (49) described method. All SAMP8 mice were weighed for brain, heart, liver, spleen, lung, and kidney organs immediately after sacrifice while calculating relative organ weights according to the following formula. Simultaneously, each organ was evaluated with the naked eye, and any abnormalities such as abnormal color, enlargement, or hard masses were found, and further histopathological stations would be performed.

$$Relative\ organs\ weight\,(\%) = {Organ\ weight}/{BW} \times 100$$

2.8. Serum biochemical parameters analysis

Serum biochemical parameters analysis in this study was performed based on the approach described by Chou et al. (12). Blood from the mice, as mentioned earlier, obtained by cardiac blood collection was immediately centrifuged at 4°C and 12,000 × g for 10 min using a Microfuge 22R (Beckman Coulter Inc., Brea, Calif., USA). The analysis of plasma biochemical parameters included glucose, total protein, albumin, triglycerides, total cholesterol, high-density lipoprotein cholesterol (HDL-C), low-density lipoprotein cholesterol (LDL-C), glutamate-oxalate transaminase (GOT), glutamate pyruvate transaminase (GPT), blood urea nitrogen (BUN) and creatinine. The operation followed the manufacturer's specification with the Synchroh LX-20 system (Beckman Coulter Inc., Brea, Calif., USA).

2.9. Determination of the bioactivity indicators in brain and liver tissues

2.9.1. Determination of MDA content

2.9.1.1. Preparation of brain tissue

Whole brain tissue extraction was performed according to Chou et al. (12) and Lee et al. (13) described methods. Briefly, whole brain tissue was homogenized (for 30 s at 1,400 rpm in an ice bath) by adding 1 mL of 50 mM PBS (pH 7.4) using a Polytron® homogenizer (PT 3000, Thomas Scientific LLC, Swedesboro, NJ, USA). Next, remove unbroken tissue and debris at 15,000 × *g* for 30 min at 4°C via a centrifuge (HermLe Z383K, Benchmark Scientific Inc. Sayreville, NJ, USA). At the same time, the obtained supernatant was analyzed for MDA content as described in section 2.9.1.3.

2.9.1.2. Preparation of liver tissue

The collected mouse liver was cut 0.1 g, added to 2,000 μL of 50 mM sodium phosphate buffer (PBS, pH 7.0), and homogenized for 30 s at 1,400 rpm in an ice bath using a Polytron® homogenizer (PT 3000), while to the preparation of the liver tissue homogenization solution was for MDA content determination as described in next section.

2.9.1.3. Measurement of MDA content by the thiobarbituric acid reactive substances (TBARS) method

MDA content was determined according to the description of Ornoy et al. (50) with modifications. 150 μL of the above sample (in 2.9.1.1) was added with 300 μL of 2-thiobarbituric acid (TBA) colorant and 45 μL of butylated hydroxytoluene (BHT) solution, vortexed for 1 min, and reacted in a water bath at 90°C for 45 min. Afterward, the solution was cooled at room temperature, extracted with n-butanol (vortex shaking for 1 min), and centrifuged at 1,000 × *g* for 5 min at 4°C using a centrifuge (HermLe Z383K, Benchmark Scientific Inc). Finally, the absorbance value was measured with a UV/Vis spectrophotometer (DU530, Beckman coulter Inc., Brea, CA, USA) at wavelength 535 nm. In addition, standard curves were prepared using known concentrations (0.625, 1.25, 2.5, 5, 10, and 20 μM) of MDA, which were interpolated to calculate the sample MDA concentration (mM/g tissue).

2.9.2. Determination of SOD

This study used the SOD (Ransod) assay kit (SD125, Randox Laboratories Ltd., Moorgate, UK) as described in its protocol. It works on the xanthine oxidase (XO) principle to produce superoxide anion ($O_2^{-\bullet}$), followed by a reaction with 2-(4-iodophenyl)-3-(4-nitrophenol)-5-phenyltetrazolium chloride (I.N.T.) to make red formazan dye. One unit of SOD was used to produce a 50% inhibition of the reduction rate of I.N.T. under the assay conditions. Finally, the activity of SOD was measured by the degree of such reaction inhibition.

Specifically, 0.1 mL of liver tissue solution (as in 2.8.1) was mixed with 400 μL of cold deionized water and centrifuged at 4°C for 15 min. Then, 10 μL of supernatant was mixed with 490 μL of sample diluent solution (R1b Buffer), and the formazan dye content was determined using a spectrophotometer at a wavelength of 340 nm. The standard curve was prepared by serial dilution with the standard included in the kit using the same procedure described above. The SOD activity (U/g tissue) of the samples was calculated by interpolation.

2.9.3. Determination of CAT activity

This study determined the CAT activity by CAT peroxidation using an assay kit (Item No. 707002, Cayman Chem). The CAT will produce formaldehyde with methanol in the appropriate concentration of H_2O_2, whereby Purpald (4-amino-3-hydrazino-5-mercapto-1,2,4-triazole) with trans-formaldehyde formed a bicyclic heterocyclic structure with a colorless to purple color after oxidation, which measured the activity of CAT at wavelength 540 nm. 1 nmol of H_2O_2

degradation per min per g of tissue catalyzed was defined as 1 CAT activity unit (U).

Briefly, in a 96-well dish, 20 µL liver tissue solution (as in 2.8.1) was added to one well, followed by 100 µL assay buffer and 30 µL methanol. Next, 20 µL of hydrogen peroxide was rapidly added to initiate the reaction with 20 min (25°C) incubation on an oscillator followed by 30 µL of potassium hydroxide to terminate the reaction and subsequently 30 µL of Purpald reaction for 10 min (25°C). Finally, 10 µL potassium hydroxide was added and incubated on an oscillator for 5 min, followed by measurement of the absorbance at 540 nm. The standard and positive control operated in the same way. After preparing the standard curve, the sample's formaldehyde (µM) content was calculated by interpolation. Finally, the CAT activity of the sample was calculated by the following formula.

$$\text{CAT activity}\left(\text{U / g tissue}\right) = \frac{sample's\ formaldehyde\left(\mu M\right)content}{20\ \text{min}} \times Sample\ dilution\ ratio$$

2.10. Statistical analysis

All data were expressed as mean ± standard error (SEM), while all measurements were performed in triplicate with statistical analysis by IBM SPSS Statistics (version 22, IBM Corp., Armonk, N.Y., USA).

The differences between groups were examined using a one-way analysis of variance (ANOVA). Duncan's multiple allometric tests were used to compare the differences between groups, while the experimental results represented a significant difference ($p < 0.05$).

3. Results and discussion

3.1. BW variations, average diet, and water intake comparison

The overweight condition is positively associated with the risk of chronic disease and death, while long-term high BW, overweight, or obesity has been associated with the shortening of telomeres in the middle-aged and elderly populations (51, 52). Obesity has been linked to depleted leukocyte telomere length by other non-inflammatory mechanisms (53). Obesity and inflammation are associated with aging (51, 53). The BW changes, average diet, and water intake in the male and female groups ($n = 8$) of SAMP8 mice fed Huáng qí and the Huáng qí complex for 8 consecutive weeks (Table 1) were consistent, and no significant differences were observed between the groups. The mice were randomly grouped at trial entry with no significant difference in the initial BW; therefore, both Huáng qí (820 mg/kg BW/day) and the Huáng qí complex (6.2 mL/kg BW/day) had no adverse effects on the physiology, growth, or development of the mice. Notably, Pačesová et al. (54) reported the SAMP8 mice gradually gain weight from month two onwards, which is evidence of the aging process, where the trend in weight change was consistent with our study, which used 3-month-old SAMP8 mice at the beginning of the study. Furthermore, pathological features (e.g., decreased number of neurons and increased neuroinflammation) were reported in the published literature in the SAMP8 mice beginning at three months of age (54).

3.2. Evaluation of grading score (6th-week post-feeding)

Takeda et al. (46) developed a grading score system consisting of nine items scoring 0, 1, 2, 3, and 4 depending on the degree of aging. Finally, the total grading scores were used to evaluate the degree of aging in the senescence-accelerated mouse (SAM), where a higher score refers to more severe aging. In addition, SAMP8 mice developed normally but with rapid aging properties, such as hair loss and disturbances in day-night rhythm (55). In this study, male and female SAMP8 mice were fed Huáng qí and the Huáng qí complex for 6 consecutive weeks. The grading scores of the groups were evaluated, and the experimental group had a lower total grading score than the control group (Table 1). In particular, the total grading scores of the males and females fed the Huáng qí complex were significantly different ($p < 0.05$). Therefore, the grading score evaluation results indicate that providing the Huáng qí complex may reduce the age level of SAMP8 mice. Interestingly, the total grading score was better in females (5.50 ± 0.63) than in males (6.50 ± 0.42).

3.3. Locomotor (6th-week post-feeding)

Commonly known behavioral disorders, cognitive decline, psychiatric disorders, and dementia associated with depression are risk factors for AD (56–60). Therefore, deferring or minimizing these symptoms is essential to maintain the quality of life of the elderly (58). As a result, locomotion activity (sec/5 min) was not significantly different in the male and female groups of mice administered Huáng qí and its complex (Table 1). Moreover, locomotion decreased following the extended time during the 5 min post-test. This was attributed to the high locomotor activity of the initial exploratory response immediately after encountering an unfamiliar environment. However, as the mice acclimated to the environment, locomotor activity decreased, thus leading to a decrease in locomotion activity. Despite the decrease in locomotor activity in the SAMP8 mice, the effects of the Huáng qí complex were more pronounced. The potential mechanism of the antiaging effect of Huáng qí and its complex may be inhibiting OS in the brain. However, the mechanism remains unknown and warrants further study.

3.4. Learning and memory testing of the mice

Various studies have suggested that cognitive and motor deficits occur in aging rodents, apart from dysregulation of epigenetics during learning and memory processes, which has been associated with AD pathogenesis (61–63). The effect of epigenetic processes on learning and memory extends to the sensory system, where all core cue-evoking processes dependent on experience begin in the brain (64). Moreover, stress, fear, and anxiety-like behaviors have been associated with neurodegeneration, aging, and AD (62, 65, 66). Behavioral avoidance tests have been applied to assess learning and memory (48). Memory

TABLE 1 (1) Weight change, diet, and water intake; (2) grading score evaluation; (3) locomotor evaluation; (4) relative organs weight for the SAMP8 mice groups ($n = 8$) during the experimental period.

Genders		Male			Female		
Group ($n = 8$)		Control	Huáng qí (820 mg/kg BW/day)	Huáng qí complexes (6.2 mL/kg BW/day)	Control	Huáng qí (820 mg/kg BW/day)	Huáng qí complexes (6.2 mL/kg BW/day)
BW (g)	Initial	28.16 ± 0.44^{a}	28.09 ± 0.19^{a}	27.80 ± 0.20^{a}	28.34 ± 0.26^{a}	28.86 ± 0.30^{a}	28.52 ± 0.34^{a}
	Final	29.85 ± 0.37^{a}	29.95 ± 0.35^{a}	29.80 ± 0.26^{a}	29.03 ± 0.35^{a}	29.48 ± 0.37^{a}	28.81 ± 0.26^{a}
Gain/BW		1.70 ± 0.34^{c}	1.85 ± 0.26^{c}	2.00 ± 0.31^{d}	0.69 ± 0.15^{b}	0.62 ± 0.12^{b}	0.29 ± 0.13^{a}
Intake	Diet (g/day)	5.34 ± 0.04^{a}	5.43 ± 0.04^{a}	5.33 ± 0.03^{a}	5.03 ± 0.08^{a}	4.99 ± 0.11^{a}	5.05 ± 0.07^{a}
	Water (mL/day)	5.39 ± 0.03^{a}	5.35 ± 0.06^{a}	5.42 ± 0.02^{a}	5.04 ± 0.19^{a}	5.03 ± 0.17^{a}	5.06 ± 0.17^{a}
Grading score							
Behavior	Reactivity	1.25 ± 0.16	1.00 ± 0.19	1.00 ± 0.19	1.38 ± 0.32	1.13 ± 0.23	0.75 ± 0.25
	Passivity	1.38 ± 0.26	1.25 ± 0.16	0.88 ± 0.13	1.13 ± 0.35	1.00 ± 0.33	0.50 ± 0.19
Skin	Glossiness	1.00 ± 0.19	1.00 ± 0.19	0.88 ± 0.13	1.25 ± 0.25	1.13 ± 0.30	0.88 ± 0.30
	Coarseness	1.25 ± 0.16	1.25 ± 0.16	0.88 ± 0.23	1.38 ± 0.26	1.13 ± 0.30	0.75 ± 0.25
	Hair loss	1.38 ± 0.18	1.38 ± 0.18	1.25 ± 0.16	1.63 ± 0.18	1.13 ± 0.23	1.00 ± 0.27
	Ulcer	0.25 ± 0.16	0.13 ± 0.13	0.13 ± 0.13	0.13 ± 0.13	0.25 ± 0.16	0.25 ± 0.16
Eyes	Periophthalmic lesion	1.13 ± 0.13	1.25 ± 0.16	1.13 ± 0.23	1.38 ± 0.18	1.00 ± 0.27	0.63 ± 0.18
Spine	Lordokyphosis	0.50 ± 0.19	0.50 ± 0.19	0.38 ± 0.18	0.63 ± 0.18	0.50 ± 0.19	0.75 ± 0.16
Total		8.13 ± 0.55^{a}	7.75 ± 0.37^{ab}	6.50 ± 0.42^{b}	8.88 ± 1.19^{a}	7.25 ± 0.67^{ab}	5.50 ± 0.63^{b}
Locomotion (s/5min)							
0–5		112.38 ± 1.70 ^{a}	107.75 ± 1.57^{a}	109.88 ± 1.61^{a}	116.25 ± 1.88^{a}	105.75 ± 1.52^{a}	111.13 ± 1.87^{a}
6–10		82.38 ± 1.83^{C}	76.38 ± 1.25^{b}	76.50 ± 1.89^{b}	71.25 ± 1.81^{ab}	68.75 ± 1.51^{a}	75.00 ± 1.85^{b}
Relative organ weights (g/100g BW)							
Brain		1.44 ± 0.06^{a}	1.43 ± 0.03^{a}	1.51 ± 0.03^{a}	1.58 ± 0.03^{a}	1.50 ± 0.04^{a}	1.59 ± 0.03^{a}
Heart		0.52 ± 0.04^{a}	0.54 ± 0.04^{a}	0.56 ± 0.03^{a}	0.47 ± 0.03^{a}	0.52 ± 0.03^{a}	0.52 ± 0.03^{a}
Liver		4.52 ± 0.29^{a}	4.84 ± 0.38^{a}	4.88 ± 0.23^{a}	4.69 ± 0.22^{a}	4.52 ± 0.08^{a}	4.68 ± 0.13^{a}
Spleen		0.35 ± 0.02^{a}	0.38 ± 0.02^{a}	0.38 ± 0.02^{a}	0.36 ± 0.04^{a}	0.37 ± 0.04^{a}	0.32 ± 0.02^{a}
Lung		0.63 ± 0.04^{a}	0.66 ± 0.02^{a}	0.69 ± 0.03^{a}	0.67 ± 0.04^{a}	0.62 ± 0.03^{a}	0.64 ± 0.02^{a}
kidney		1.54 ± 0.08^{a}	1.72 ± 0.12^{a}	1.77 ± 0.07^{a}	1.74 ± 0.07^{a}	1.73 ± 0.04^{a}	1.69 ± 0.04^{a}

Values are mean ± SEM. Different lowercase letters on each line indicate a significant difference ($p < 0.05$). BW, Body weight.

is defined as a change in behavior caused by experience, and learning is the process of memory acquisition achieved by engaging neurotransmission and neurons (24). The passive avoidance task and active shuttle avoidance test were performed on weeks 7 and 8 in the SAMP8 male and female mice fed Huáng qí and its complex to evaluate the effects on learning and memory. The passive avoidance task was based on the duration in the light chamber. The active shuttle avoidance test was based on the escape response of the mice.

3.4.1. Passive avoidance task

The passive avoidance task is a fear-driven test, and mice with better memory and learning avoided entering the hazardous areas (48). All SAMP8 mice were fed Huáng qí and its complex during week 7 for the passive avoidance task (Figures 1C,D). As results, no significant differences in the learning results of male mice were detected on the first day of training in any of the groups (Figure 1C). However, the time in the length chamber increased significantly compared to the control group 24 and 48 h after training in both experimental groups of male mice ($p < 0.05$). The training results in female mice on the first day were the same as in males, with no significant differences between the groups (Figure 1D). However, the female mice in both experimental groups remained in the light chamber significantly longer than the control group ($p < 0.05$) after 24 h of training, with a satisfactory effect in the Huáng qí group. The time remaining in the light chamber increased after 48 h of training in both experimental groups of female mice. Significantly more time was spent in the light chamber by Huáng qí group than in the control group ($p < 0.05$). Notably, no significant difference in the time remaining in the light chamber was detected for the male or female mice 72 h after-training, which may have been due to the electric shock given during training, which caused a longer retention time in the light chamber. Unfortunately, memory-retaining ability decreased with time, resulting in a decrease in the time spent in the light chamber by 72 h post-training. Notably, Lee et al. (13) reported the

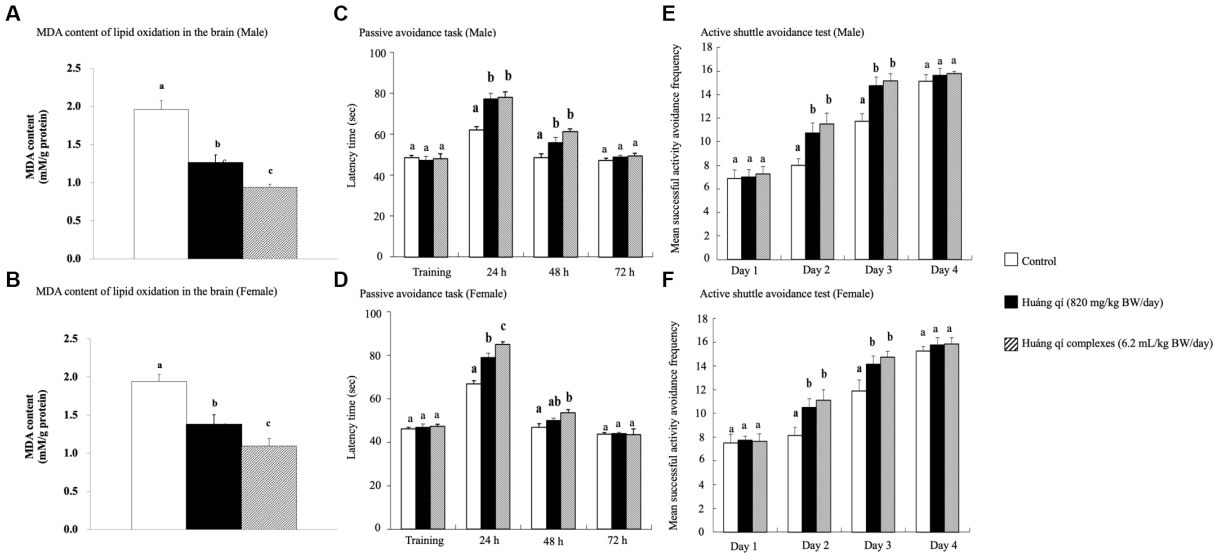

FIGURE 1
Effects of continuous 8-weeks of tube feeding Huáng qí and its complex on SAMP8 mice ($n = 8$) on **(A, B)** MDA content of lipid oxides in the brain; **(C, D)** the passive avoidance task; **(E, F)** the active shuttle avoidance test. Different lowercase letters represent significant differences ($p < 0.05$).

same findings for SAMP8 mice fed a diet supplemented with a liquid fermentation medium of *Hericium erinaceus* mycelium for 13 weeks; no improvement in learning performance was detected on the passive avoidance task training day, but improved learning and memory with the ability to retain this training memory were observed post-training.

3.4.2. Active shuttle avoidance test

An experimental animal moving to another chamber immediately preceding the onset of the conditioned stimulus was considered active avoidance, whereas if the animal ran to the other side after receiving an electric shock, the reaction was considered an escape movement (67). The active shuttle avoidance test used a double-sided shuttle. The experiment animals moved immediately to the other side regardless of where the animal was placed when the sound began or the lights went out, indicating that neither path is better than the other (67–69). The active shuttle avoidance test was performed on week 8 while feeding the Huáng qí and its complex. The number of successful avoidance attempts was not significantly different between the groups of mice on day 1 (Figures 1E,F). This occurred because all mice were still in the learning stage. However, the experimental groups (fed Huáng qí and its complex) were significantly more avoidant on days 2 and 3 compared with the control group ($p < 0.05$). The difference in response between training and testing was evidence for generating new memories (68). Moreover, this study showed an increase in successful response avoidance on day four but no significant differences between the groups. Along with other similar studies, the mice in the experimental groups experienced significantly lower retention than the control group regarding the frequency of electroshock while improving memory (56, 70, 71). Hence, this study showed that supplementation with Huáng qí or its complex for 8 consecutive weeks promoted learning and memory in the mice.

3.5. Variations in relative organ weights

The relative organ weight is useful for identifying normal or abnormal organ weights (72). Brain weight has no relationship with BW but has a strong negative correlation with age (72). The weights of the brain, heart, liver, spleen, lungs, kidneys, and the organs in all experimental groups fed Huáng qí, and its complex for 8 weeks were not significantly different compared with the control group (Table 1). However, no abnormalities, such as enlargement or hard masses, were observed with the naked eye, and the color remained normal. Thus, providing Huáng qí to the mice did not affect the organs or cause any damage.

3.6. Serum biochemical parameters

The serum biochemical results were not significantly different between the experimental and control groups after continuously feeding Huáng qí and its complex to male and female mice for 8 weeks (Table 2). Therefore, continuously supplementing with Huáng qí for 8 weeks did not affect the mice's serum biochemical parameters or physiology.

3.7. Aging-related liver indicators

3.7.1. Malondialdehyde in the brain and liver

Malondialdehyde (MDA) is an oxidized lipid product with neurotoxic properties, which represents ROS production. Sustained oxidant damage initiates the JNK/ERK signaling pathways to induce apoptosis (5, 22, 73). However, the free radical theory of aging refers to the slow production of ROS as an inevitable consequence of life with accumulated oxidative damage to cell membrane proteins and lipids (56, 74, 75).

TABLE 2 Serum biochemical parameters in the SAMP8 mice groups ($n = 8$) during the experimental period.

Gender	Group (n = 8)	GOT (U/L)	GPT (U/L)	Total Protein(mg/dl)	Albumin (g/dl)	BUN (mg/dl)	Creatinine (mg/dl)	Total Cholesterol (mg/dl)	Triglyceride (mg/dl)	HDL (mg/dl)	LDL (mg/dl)	Glucose (mg/dl)
Male	Control	113.50 ± 1.16[a]	50.00 ± 1.57[a]	6.91 ± 0.15[a]	3.75 ± 0.08[a]	30.88 ± 1.59[a]	0.25 ± 0.02[a]	149.00 ± 0.98[a]	114.38 ± 1.63[a]	49.63 ± 1.82[a]	9.50 ± 0.63[a]	148.88 ± 1.60[a]
	Huáng qí (820 mg/kg BW/day)	112.13 ± 1.44[a]	50.25 ± 1.56[a]	6.86 ± 0.08[a]	3.78 ± 0.09[a]	32.23 ± 1.29[a]	0.24 ± 0.02[a]	149.13 ± 1.76[a]	111.38 ± 1.66[a]	48.50 ± 1.20[a]	9.25 ± 0.67[a]	150.38 ± 1.12[a]
	Huáng qí complexes (6.2 mL/kg BW/day)	112.63 ± 1.41[a]	49.13 ± 1.94[a]	6.88 ± 0.14[a]	3.79 ± 0.10[a]	30.58 ± 1.11[a]	0.26 ± 0.02[a]	149.88 ± 1.30[a]	111.75 ± 1.82[a]	49.63 ± 1.31[a]	9.75 ± 0.70[a]	149.88 ± 1.57[a]
Female	Control	112.00 ± 1.31[a]	51.13 ± 1.57[a]	6.85 ± 0.12[a]	3.81 ± 0.07[a]	30.90 ± 1.18[a]	0.33 ± 0.03[a]	150.50 ± 1.22[a]	111.88 ± 1.75[a]	48.25 ± 1.21[a]	9.25 ± 0.45[a]	149.50 ± 1.31[a]
	Huáng qí (820 mg/kg BW/day)	114.75 ± 1.79[a]	51.13 ± 1.96[a]	6.89 ± 0.11[a]	3.78 ± 0.09[a]	30.88 ± 1.10[a]	0.29 ± 0.01[a]	151.75 ± 1.44[a]	109.38 ± 1.93[a]	49.25 ± 1.35[a]	9.13 ± 0.67[a]	150.50 ± 1.50[a]
	Huáng qí complexes (6.2 mL/kg BW/day)	112.75 ± 1.21[a]	51.63 ± 1.34[a]	6.86 ± 0.11[a]	3.91 ± 0.11[a]	31.19 ± 0.89[a]	0.31 ± 0.01[a]	151.50 ± 1.25[a]	108.75 ± 1.88[a]	47.50 ± 1.61[a]	9.38 ± 0.68[a]	149.75 ± 1.50[a]

Values are mean ± SEM. Different lowercase letters on each column indicate a significant difference ($p < 0.05$). BW, Body weight.

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The brain MDA contents of male and female mice in all experimental groups decreased after 8 weeks of treatment with Huáng qí and its complex (Figures 1A,B) and were significantly lower ($p < 0.05$) than the control group. The Huáng qí and its complex reduced the MDA levels in the brains of SAMP8 mice for 8 consecutive weeks. Xu et al. (5) reported that administering spermidine, spermine, and rapamycin (0.78 mg/kg/d) for 8 consecutive weeks alleviated OS in the SAMP8 mouse brain by decreasing MDA levels and increasing brain SOD activity, which agreed with the present study results.

Continuous feeding of Huáng qí and its complex for 8 weeks resulted in a significant decrease in MDA contents in the livers of male and female mice compared to the control group ($p < 0.05$), and the effects were better in the groups fed the Huáng qí complex (Figures 2A,B). However, the implication was that administering either Huáng qí or its complex for 8 weeks reduced MDA levels in the liver of mice. The Huáng qí compounds reduced MDA levels the most in the liver, and the effect was better in males than females. ROS damaged the neuronal membrane components and are associated with the pathophysiology of neurological diseases (age -related), such as SAMP8 mice and AD, where mice have altered free radicals, and SOD activity and MDA levels increased, while glutathione levels (in the liver or brain) decreased (13, 55, 76–78). In addition, several reports have indicated that MDA may contribute to producing highly immunogenic MDA acetaldehyde compounds under sustained OS. These compounds participate in secondary reactions by promoting intra- or inter-molecular protein/DNA cross-linking and chronic inflammation, disease, aging, and DNA damage (15, 73, 79–83).

3.7.2. Superoxide dismutase in the liver

Superoxide dismutase (SOD) is part of the primary defense system that scavenges free radicals to reduce OS (5). Damage caused by OS leads to neurodegenerative diseases, and *in vivo,* antioxidant capacity affects memory capacity (70, 84). This study showed that liver SOD activity increased significantly in the experimental groups ($p < 0.05$) compared to the control group after feeding Huáng qí and its complex for 8 consecutive weeks (Figures 2C,D). Thus, supplementing male and female mice with the Huáng qí complex enhanced SOD activity.

3.7.3. Catalase activity in the liver

Significant increases in liver CAT activity were detected in all male and female experimental groups compared to the control group after feeding male and female mice Huáng qí and its complex for 8 weeks (Figures 2E,F). Ornoy et al. (50) reported that valproic acid (VPA) and S-adenosylmethionine (SAM, with saline) increase SOD and CAT activities, respectively. However, a post -VPA injection of SAM prevented autistic -like behavior in mice, suggesting that the *in vivo* antioxidant capacity originated from the nutritional supplement while effectively mitigating oxidative damage in the brain exposed to OS. Alternatively, severe oxidative damage (liver, micronucleus, and DNA) occurs due to decreased antioxidant level/activity (85). These results indicate that continuously feeding either Huáng qí or its complex for 8 weeks reduced OS in SAMP8 mice *in vivo* (brain and liver).

4. Conclusion

This study showed that Huáng qí and its complex reduced aging and improved learning and memory in the SAMP8 mouse model after continuous supplementation for 8 weeks. In addition, the enhanced SOD and CAT activities in the liver and reduced MDA content to achieve antioxidant effects may have reduced oxidative damage (liver and brain), thus promoting anti-aging effects. Therefore, Huáng qí and its complex may be more effective for age-related brain impairment than conventional agents as a novel nutritional intervention or as a nutritional supplement with full nutritional potential for cognitive health. Overall, Huáng qí is a safe and effective herbal supplement targeting various pathologies. In particular, APS is a valuable immunomodulatory agent, but further studies are necessary to substantiate clinical efficacy in humans.

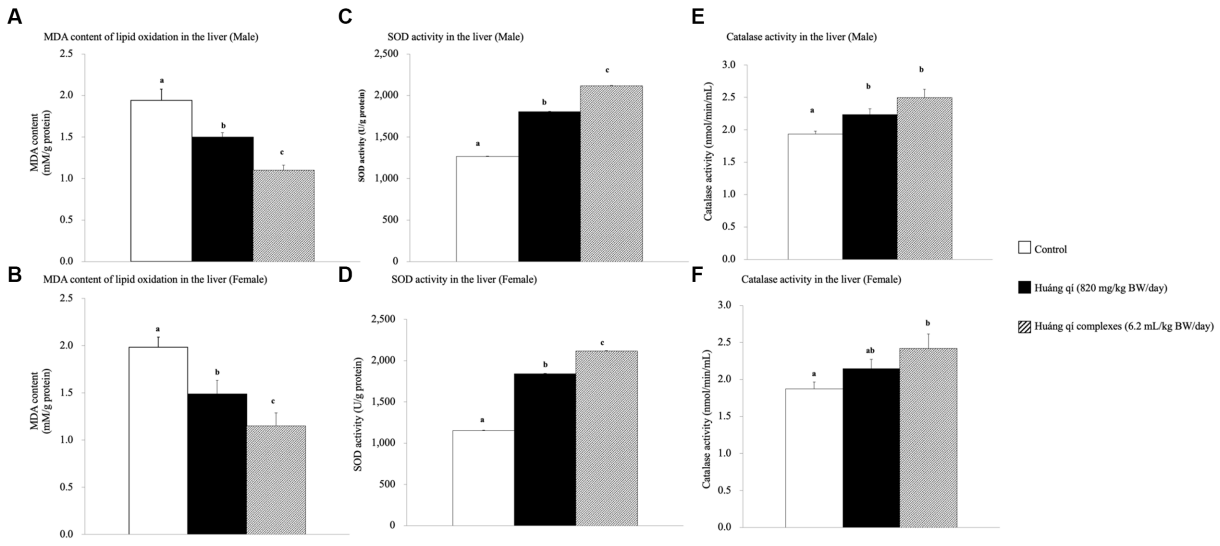

FIGURE 2
Effects of a continuous 8-weeks of tube feeding Huáng qí and its complex on SAMP8 mice ($n = 8$) malondialdehyde (MDA) content **(A,B)**, superoxide dismutase (SOD) activity **(C,D)**, and catalase (CAT) activity **(E,F)** in the liver. Different lowercase letters represent significant differences ($p < 0.05$).

Data availability statement

The original contributions presented in the study are included in the article/Supplementary files, further inquiries can be directed to the corresponding authors.

Ethics statement

The animal study was reviewed and approved by the Committee on Animal Research, Providence University, under code 220191211 A008 approved the protocol.

Author contributions

M-YC, Y-CW, and S-YW: conceptualization. C-HC, T-HW, and M-JH: data curation and methodology. P-HH, Y-CW, and S-YW: investigation. P-HH, P-HL, and M-FW: writing —original draft. P-HL and M-FW: writing—review and editing. All authors have read andagreed to the published version of the manuscript.

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Conflict of interest

T-HW was employed by PhytoHealth Co.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnut.2023.1235780/full#supplementary-material

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EDITED BY
Krishnamachari Srinivasan,
St. John's Research Institute, India

REVIEWED BY
Suhas Chandran,
St. John's Medical College Hospital, India

*CORRESPONDENCE
Rouzha Zlatanova Pancheva
✉ rouzha.pancheva@gmail.com

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Evidence or no evidence for essential fatty acids in the treatment of autism spectrum disorders?

Rouzha Zlatanova Pancheva[1]*, Silviya Nikolova[2], Asena Serbezova[3], Krassimira Zaykova[4], Desislava Zhelyazkova[4] and Lubomir Dimitrov[1]

[1]Department of Hygiene and Epidemiology, Faculty of Public Health, Medical University of Varna "Prof. Dr. Paraskev Stoyanov", Varna, Bulgaria, [2]Department of Social Medicine, Faculty of Public Health, Medical University of Varna "Prof. Dr. Paraskev Stoyanov", Varna, Bulgaria, [3]Department of Health Policy and Management, Faculty of Public Health, Medical University of Sofia, Sofia, Bulgaria, [4]Faculty of Medicine, Medical University of Varna "Prof. Dr. Paraskev Stoyanov", Varna, Bulgaria

Autism spectrum disorders (ASDs) are a group of neurodevelopmental disorders that affect social communication, behavior, and sensory processing, in which PUFAs are considered important. This mini-review article aims to investigate the current evidence regarding the use of essential fatty acids (EFAs) in the treatment of autism spectrum disorders (ASDs). The study examines various research studies, related to EFAs, their benefits, and their role in ASD treatment. The article focuses on exploring the potential mechanisms underlying the effects of EFAs on ASDs, including their anti-inflammatory, antioxidant, and neuroprotective properties. Furthermore, the study discusses limitations and challenges associated with the use of EFAs in ASD treatment, including variability in dosage and duration of treatment. The results of this review indicate that while some studies suggest a positive effect of EFAs on ASD symptoms, there is currently insufficient evidence to support their routine use as a stand-alone treatment for ASD. The need for further research to better understand the potential benefits and limitations of EFAs in ASD treatment is highlighted.

KEYWORDS

essential fatty acids, autism, supplementation, child, intervention

Introduction

Autism spectrum disorders (ASDs) are a group of neurodevelopmental disorders that affect social communication, behavior, and sensory processing. The prevalence of ASDs has increased significantly in recent years, with current estimates suggesting that 1 in every 36 (2.8%) 8-year-old children was estimated to have ASDs in 2020 (1).

Despite the growing prevalence of ASDs, effective treatments for the disorder are limited. Traditional therapies, such as behavioral interventions and medications, have shown some efficacy, but they often have significant limitations and may not be suitable for all individuals with ASDs (2). Therefore, there is a need for novel treatment approaches that can effectively address the core symptoms of ASDs.

One potential avenue for exploring new treatments for ASDs is through the use of dietary supplements (3). In particular, there has been growing interest in the potential role of fatty acids in the treatment of ASDs (1). Fatty acids are essential components of cell membranes and play important roles in the development and function of the brain (1).

Research in recent years has suggested that certain types of fatty acids, such as omega-3 and omega-6 fatty acids, may have therapeutic potential for individuals with ASDs (4). A high omega-6/omega-3 ratio in the cell membrane has been associated with inadequate brain development (5). This ratio [known as the "fatty acid (FA)" index] has started to be used as a biomarker of treatment efficacy in human diseases. The evidence supporting the use of fatty acids in the treatment of ASDs is still evolving, and there is a need for a comprehensive review of the available literature (6).

Therefore, this research article aimed to analyze the evidence supporting the use of fatty acids in the treatment of ASDs. This mini-review will examine the current literature on fatty acids and ASDs, with a focus on the potential benefits and limitations of fatty acid supplementation as a treatment avenue for children with autism.

Essential fatty acids and brain development

Polyunsaturated fatty acids (PUFAs) are significant components of phospholipids, which are required for cell membrane structure and function. Linoleic acid (LA), an omega-6 acid, α-linolenic acid (ALA), an omega-3 acid, and their metabolic derivatives, namely arachidonic acid (AA), eicosapentaenoic acid (EPA), and docosahexaenoic acid (DHA), are essential structural and functional components of cellular and intracellular membranes in the human body (7).

Docosahexaenoic acid (DHA) is found in high concentrations in neural tissue, and research indicates that omega-3 fatty acids are crucial for the growth and functional development of the brain. Additionally, omega-3 fatty acids have anti-inflammatory effects (8).

The influence of essential fatty acids (EFAs) on brain development has been a subject of significant research interest. EFAs, including omega-3 and omega-6 polyunsaturated fatty acids (PUFAs), play key roles in various biological processes, including neuronal growth, synaptogenesis, and neurotransmitter signaling (9). Understanding the interactions between EFAs and brain development is crucial for gaining insights into their potential implications for neurodevelopmental disorders, such as autism spectrum disorders (ASDs).

During critical periods of brain development, EFAs serve as structural components of cell membranes and contribute to the formation and function of neural networks (10). Omega-3 PUFAs, such as docosahexaenoic acid (DHA), are particularly abundant in the brain and are crucial for neuronal membrane integrity, synaptic plasticity, and neurogenesis (11). Omega-6 PUFAs, such as arachidonic acid (AA), also play vital roles in brain development, modulating inflammatory responses and gene expression related to neuronal development (11).

Research investigating the impact of EFAs on brain development, including animal studies and observational studies in humans, has provided valuable insights. For instance, studies have shown that EFA deficiencies during pregnancy and early postnatal life can negatively affect brain development and cognitive function (12). Additionally, prenatal exposure to appropriate levels of omega-3 PUFAs has been associated with improved cognitive and behavioral outcomes in an offspring (13).

The interplay between EFAs and brain development in the context of ASDs has also been explored. Evidence suggests that individuals with ASDs may have altered EFA metabolism and imbalances in the omega-6 to omega-3 ratio (8). These imbalances may contribute to disruptions in neuronal signaling, synaptic plasticity, and inflammation, potentially influencing ASD symptomatology.

Furthermore, DHA is involved in cognitive functions, neurite formation, membrane fluidity, neurotransmission, endothelial function, neuronal survival, and neurodegeneration prevention. One study by Parletta et al. (14) reported lower levels of omega-3 PUFA EPA and DHA and a higher AA/EPA ratio in children with ADHD and ASD compared with typically developing controls, and these levels were associated with greater severity of symptoms. Individuals with ASD have altered PUFA metabolism, which results in increased production of proinflammatory cytokines, increased oxidative stress, and an imbalance in the formation and action of neurotransmitters (7).

While the relationship between EFAs and brain development shows promising scientific advances, it is important to note that the available evidence is not yet conclusive. Further research, including randomized controlled trials and longitudinal studies, is necessary to better understand the specific mechanisms by which EFAs interact with brain development and their potential implications for ASDs.

Methodology

The methodology for this narrative review adhered to the Scale for the Quality Assessment of Narrative Review Articles (SANRA) guidelines (15). A comprehensive literature search was conducted in May–June 2023 across several electronic databases, including PubMed, Web of Science, and Scopus. The search strategy used the following keywords and boolean operators: "autism" OR "ASD" AND "omega-3′" OR "omega-6′" AND "supplementation" OR "effect" OR "intake".

The initial search yielded a total of 639 articles. The articles were screened for duplicates, resulting in the exclusion of several duplicate entries. The subsequent screening was based on the titles and abstracts of the remaining articles, with the exclusion of reviews and non-relevant articles such as studies with no comparison or control group or qualitative-only designs. The inclusion criteria for the review were as follows: (1) randomized controlled trials (RCTs) or pre-post studies involving children with autism or ASD; (2) study participants in the study samples had to be under 18 years of age; (3) studies were included if they were reported on EFA outcomes; (4) if studies were interventions; and (5) articles had to be published in English.

The exclusion criteria were as follows: (1) studies focusing exclusively on animal models; (2) studies with no pre-post design

Abbreviations: AA, arachidonic acid; ADD, attention deficit disorder; ADHD, attention deficit hyperactivity disorder; ALA, α-linolenic acid; ARA, arachidonic acid; ASDs, autism spectrum disorders; ATEC, Autism Treatment Evaluation Checklist; DHA, docosahexaenoic acid; EFAs, essential fatty acids; EPA, eicosapentaenoic acid; GLA, gamma linolenic acid; LA, linoleic acid; NDD, neurodevelopmental disorder; PUFAs, polyunsaturated fatty acid; RCT, randomized controlled trial.

and comparison groups; (3) review articles; and (4) articles in other than the English language.

Following this rigorous screening process, a total of 12 studies met all the inclusion criteria and were included in the final review. The selected studies underwent a thorough analysis and synthesis of their findings to inform the conclusions of this narrative review.

Supplementation with omega-3 and omega-6 fatty acids in ASD

Table 1 provides a summary of studies and a comprehensive overview investigating the effects of omega-3 and omega-6 fatty acid supplementation on autism spectrum disorder (ASD) symptoms and behaviors.

In total, 12 clinical trials across seven countries were considered, with participant sample sizes ranging from 13 to 73. In terms of study design, all trials followed a double-blind, randomized, placebo-controlled trial design, with the exception of one (18) that was simply described as a randomized clinical trial. One study was a crossover trial (5). The duration of the selected studies ranged from 6 weeks to 6 months, with dosages varying considerably between the trials.

Significant ASD symptom improvements were reported in seven out of 12 studies. For instance, Amminger et al. (16) in Australia noted improvement in ASD symptoms and psychopathology after administering 1.5 g/day of omega-3 over a 6-week period. Similarly, Boone et al. (19) in Ireland reported positive behavioral and sleep changes in toddlers born at $\leq$29 weeks' gestation following 12 weeks of omega-3 supplementation. Furthermore, in Poland, Doaei et al. (21) demonstrated improved social, verbal, and behavioral activities in ASD children after providing them with 1,000 mg/day of omega-3 for 8 weeks. Similarly, in the United States, Sheppard et al. (22) observed reduced ASD symptoms and increased gesture use, respectively, in their study participants after supplementing them with a combination of omega-3, omega-6, and omega-9 fatty acids. Parellada et al. (5) reported significant improvement in social motivation and social communication subscale scores, with a moderate-to-large effect size ($p = 0.004$, $d = 0.73$ and $p = 0.025$, $d = 0.79$, respectively).

On the contrary, in Spain, two studies conducted by Bent et al. (18) and de la Torre-Aguilar et al. (20) did not find significant behavioral improvements despite the supplementation with omega fatty acids. Two studies conducted by Mazahery et al. (23) and Voigt et al. (25) in the USA and one study by Yui et al. (26) in Japan showed mixed or questionable effects. The longest supplementation study was performed on Spanish children with ASD, who exhibited an appropriate omega-3 FA status in plasma and erythrocytes without clinical improvement of ASD, or a better anti-inflammatory or fatty acid state has been found after an intervention with DHA/EPA for 6 months (20).

In terms of dosage, all studies provided specific information about the amount of omega-3 or omega-6 fatty acids used, with the highest dose being 1.5 g/day (16) and the lowest dose being 200 mg/day of DHA (25).

When examining the difference between omega-3 supplementation alone and a combination of omega-3 and omega-6, the analysis of four studies that exclusively utilized omega-3 supplementation yielded outcomes that were varied and inconclusive. Amminger et al. (16) and Doaei et al. (21) reported significant improvements in ASD symptoms with the use of omega-3 alone, while Voigt et al. (25) and Bent et al. (17) found no significant improvement.

Studies using a combination of omega-3 and omega-6 also showed mixed results. Boone et al. (19) and Keim et al. (24) observed significant improvements in caregiver-reported behavior, sleep, and ASD symptoms. Additionally, Sheppard et al. (22) reported an increase in gesture use in the treatment group. However, Mazahery et al. (23), who used a combination of omega-3 and Vitamin D, found no significant improvement.

Discussion

Our study shows that irrespective of the criteria used to assess diverse outcomes–whether it pertains to the effectiveness of omega-3 alone versus a combination of omega-3 and omega-6, varying dosages, or differing durations of supplementation–the enhancement of behavioral symptoms in children with autism remains inconclusive. This ambiguity could potentially stem from a multitude of factors, including the specific dosage administered, the duration of the treatment, the particular symptoms targeted for improvement, and the inherent individual differences among the children. Furthermore, it's important to acknowledge that some studies incorporated additional elements like vitamin D or omega-9 in their combinations, introducing the possibility of these elements also exerting an influence on the observed outcomes (23).

The observed differences in the effects of omega-3-6 supplementation on ASD symptoms and behaviors could be attributed to several factors.

First, variations in the characteristics of the study populations, such as age range, severity of ASD symptoms, and comorbidities, can contribute to the heterogeneity of the findings. The response to omega-3 supplementation may vary among individuals with different profiles, leading to contrasting outcomes across studies.

Second, differences in the dosage and duration of omega-3-6-9 supplementation may influence the results. Studies have utilized varying dosages of omega-3 fatty acids and administered them for different lengths of time, which can impact the efficacy and magnitude of any observed effects. The optimal dosage and duration of supplementation are yet to be determined.

Additionally, the choice of outcome measures and assessment tools used in the studies can contribute to the discrepant findings. There is a lack of standardized measures for assessing ASD symptoms and behaviors, which can lead to variability in the interpretation of results. The use of diverse assessment tools across studies may affect the ability to compare and combine the findings. These measures encompassed a range of variables, such as autism symptom severity, maladaptive behaviors, social interaction, communication, psychopathology, behavior and sleep in toddlers, plasma and erythrocyte FA profiles, and inflammation markers. The variability in outcome measures adds complexity to the interpretation of the results and underscores the need for standardized assessments in future studies.

Moreover, variations in the composition and quality of the omega-3 supplements used in the studies may also contribute to the differences in outcomes. The types and ratios of omega-3 fatty

TABLE 1 Summary of studies included in the review.

References	Country	Sample size	Design of the study	Variables/outcome measures	Dosage	Duration	Improvement in ASD symptoms	Statistical significance
Amminger et al. (16)	Australia	13	Double-blind, randomized, placebo-controlled trial	ASD symptoms, psychopathology	1.5 grams/day of omega-3	6 weeks	Improved hyperactivity and stereotypy	Yes
Bent et al. (17)	USA	27	Double-blind, randomized, placebo-controlled trial	Hyperactivity, stereotype	1.3 grams/day of omega-3	6 weeks	Improvement in hyperactivity, as measured by the Aberrant Behavior Checklist	Yes
Bent et al. (18)	USA	57	Randomized clinical trial		1.3 g of omega-3 fatty acids or an identical placebo	6 weeks	No statistically significant reduction in hyperactivity	No
Boone et al. (19)	Ireland	31	Double-blind randomized clinical trial	Caregiver-reported behavior and sleep in toddlers born at ≤29 weeks gestation	706 mg total omega-3 fatty acids: 338 mg EPA, 225 mg DHA; 280 mg total omega-6 fatty acids: 83 mg GLA; and 306 mg total omega-9 fatty acids	12 weeks	Mixed effects on measures of caregiver-reported behavior and sleep	Yes/no
de la Torre-Aguilar et al. (20)	Spain	54	Double-blind, randomized placebo-controlled	Plasma lipids, cytokines, and FA profiles in plasma and erythrocytes at baseline and after 6 months of treatment in ASD children and at baseline in the reference group.	252 mg DHA, 60 mg EPA	6 months	No clinical improvement	No
Doaei et al. (21)	Poland	54	Double-blind randomized clinical trial	Social, verbal, and behavioral activities in ASD children	1,000 mg/day of omega-3	8 weeks	Improved stereotype behaviors, social communication	Yes
Sheppard et al. (22)	USA	31 preterm born	Double-blind randomized controlled trial	Language abilities in children at risk for ASD	338 mg EPA, 225 mg DHA, 280 mg total omega-6 fatty acids (including 83 mg GLA), and 306 mg total omega-9 fatty acids/day	3 months	Increase in gesture use for children in the treatment group	Yes
Mazahery et al. (23)	New Zealand	73	Double-blind, randomized, placebo-controlled trial	Vitamin D and omega-3 supplementation; ASD symptoms (ATEC scores)	610 mg EPA, 405 mg DHA/day	16 weeks	Improvement in some core symptoms of ASD but no definitive conclusions	Yes/No
Parellada et al. (5)	Spain	68	Randomized crossover, placebo-controlled study	Erythrocyte membrane FA composition, plasma antioxidant status (TAS), social responsiveness and clinical global impression severity	962 and 1,155 mg/d for children and adolescents, respectively	8 weeks	Improved erythrocyte membrane without changing TAS; significant improvement in social motivation and social communication subscale scores.	Yes
Keim et al. (24)	USA	31	Double-blind randomized controlled trial	ASD symptoms and related behaviors, as reported by parents	338 mg EPA, 225 mg DHA, and 83 mg GLA/day	90 days	ASD symptoms reduced in the group receiving Omega-3-6-9 junior	Yes
Voigt et al. (25)	USA	48	Double-blind, randomized, placebo-controlled trial	Autism symptoms, maladaptive behaviors	200 mg/day of DHA	12 weeks	Teachers reported a higher average rating of functional communication; opposite for parent reported social skills	Yes/no
Yui et al. (26)	Japan	13	Double-blind, placebo-controlled, randomized trial	Social interaction, communication	40 mg of DHA, 40 mg of ARA/day	16 weeks	Improved social withdrawal and communication	Yes

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acids, as well as the purity and formulation of the supplements, can vary between studies, potentially influencing their effectiveness.

Gaps, clinical implications, and future research

The current landscape of research on the use of essential fatty acids (EFAs) in ASD presents an intricate web of possibilities and challenges. The significant strides made in understanding the impact of omega-3 and omega-6 on ASD symptoms have opened the door to broader investigations. However, the complexity of ASD, combined with the multifaceted nature of EFAs, introduces a series of gaps that need to be explored further.

A prominent gap is observed in the investigation of the role of EFAs in other neurodevelopmental disorders including attention deficit disorder and ADHD. The heterogeneity of ASDs and their frequent co-occurrence with other conditions introduces a layer of complexity that necessitates a broader perspective.

Moreover, comparisons between EFAs' impact on ASDs' core symptoms and other complementary and alternative medications, such as oxytocin, secretin, elimination diets, or other biomedical treatments, offer an avenue for future exploration. Such a comparative analysis could unravel nuanced understandings of EFAs' unique contributions and synergistic effects.

The current literature also falls short of studies examining EFAs in combination with early intervention programs as opposed to stand-alone interventions. The interplay between EFAs and other therapeutic measures could lead to more personalized and effective strategies, opening new horizons in clinical practice.

Further in-depth analysis of the potential role of EFAs in psychiatric comorbidities in ASDs vs. ASDs without comorbidities could shed light on specific applications and tailored interventions. This could be particularly illuminating, given the established literature on EFAs' role in depressive disorders.

Another vital aspect is the assessment of the isolated effect of EFAs from conventional medications and therapy, especially regarding hyperactivity and restlessness in children with ASD and ADHD. Such isolation could provide clearer insights into the specific benefits of EFAs.

A broader economic evaluation concerning the cost-effectiveness of EFA use also emerges as a significant gap. Such an assessment could guide policy and practice, aligning therapeutic choices with economic realities.

Finally, an exploration of the short-term vs. long-term side effects of EFA use, as well as the necessary monitoring, needs to be integrated into the research framework. The holistic understanding of EFAs' impact necessitates a balance between benefits and potential risks.

Limitations

It is important to note that the evidence from these studies is diverse and not definitive. The sample sizes also varied, with some studies having small sample sizes. Additionally, the publication dates ranged from 2007 to 2022, indicating a span of research over several years.

Additionally, methodological differences, such as study design, sample size, blinding, and control groups, can impact the robustness and reliability of the findings. Studies with larger sample sizes, well-controlled designs, and appropriate blinding are generally considered to provide more reliable results.

Our review also uncovers additional limitations that warrant mention. An understanding of the role EFAs play in improving outcome measures is complicated by a lack of isolation from conventional medications and therapy. This intermingling calls for separate investigations that unravel EFAs' unique contributions. The benefits of EFA in children with ASD and ADHD warrant separate attention, particularly in improving hyperactivity and restlessness. The potential overlap and distinctiveness between ASD and ADHD in response to EFAs require nuanced exploration. There is a need for more studies to assess the cost-effectiveness of EFA use and evaluate the short-term vs. long-term side effects and the monitoring required.

Conclusion

While both omega-3 alone and a combination of omega-3 and omega-6 have shown potential benefits in some studies, more research is needed to definitively understand their relative effectiveness in treating behavioral symptoms associated with autism. Considering many additional factors, it is crucial to interpret the findings of the studies on omega-3-6-9 supplementation in ASD with caution and acknowledge the complexities and nuances involved in understanding the effects of supplementation on ASD symptoms and behaviors. More randomized controlled clinical trials with longer follow-up periods that address these factors and utilize rigorous methodologies are needed to provide more definitive conclusions.

Author contributions

RP conceived the idea for this article and identifying the need to explore a specific topic within the field of study. RP, SN, AS, KZ, DZ, and LD collaborated in retrieving relevant articles and conducting extensive research. SN took the lead in developing the methodology and devising a robust framework for data analysis and interpretation. The collective efforts of all authors contributed to the comprehensive exploration of the subject matter and the generation of insightful findings. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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EDITED BY
Nicholas Pang,
Universiti Malaysia Sabah, Malaysia

REVIEWED BY
Rouzha Zlatanova Pancheva,
Medical University of Varna, Bulgaria
Ann-Katrin Kraeuter,
Northumbria University, United Kingdom

*CORRESPONDENCE
Jian He
✉ jianhe@yili.com
Yingqian Zhang
✉ 18190727710@163.com

†These authors have contributed equally to this work

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Combination of Walnut Peptide and Casein Peptide alleviates anxiety and improves memory in anxiety mices

Qinxi Li[1,2†], Xiuzhen Jia[3,4†], Qixing Zhong[1,2†], Zhihui Zhong[1,2], Yu Wang[1], Cheng Tang[1], Bangcheng Zhao[1,2], Haotian Feng[3,4], Jingyu Hao[3,4], Zifu Zhao[3,4], Jian He[3,4]* and Yingqian Zhang[1,2]*

[1]Laboratory of Nonhuman Primate Disease Modeling Research, Department of Neurology, West China Hospital, Sichuan University, Chengdu, China, [2]State Key Laboratory of Respiratory Health and Multimorbidity, West China Hospital, Sichuan University, Chengdu, China, [3]Inner Mongolia Dairy Technology Research Institute Co. Ltd., Hohhot, China, [4]Yili Innovation Center, Inner Mongolia Yili Industrial Group Co., Ltd., Hohhot, China

Introduction: Anxiety disorders continue to prevail as the most prevalent cluster of mental disorders following the COVID-19 pandemic, exhibiting substantial detrimental effects on individuals' overall well-being and functioning. Even after a search spanning over a decade for novel anxiolytic compounds, none have been approved, resulting in the current anxiolytic medications being effective only for a specific subset of patients. Consequently, researchers are investigating everyday nutrients as potential alternatives to conventional medicines. Our prior study analyzed the antianxiety and memory-enhancing properties of the combination of Walnut Peptide (WP) and Casein Peptide (CP) in zebrafish.

Methods and Results: Based on this work, our current research further validates their effects in mice models exhibiting elevated anxiety levels through a combination of gavage oral administration. Our results demonstrated that at 170 + 300 mg human dose, the WP + CP combination significantly improved performances in relevant behavioral assessments related to anxiety and memory. Furthermore, our analysis revealed that the combination restores neurotransmitter dysfunction observed while monitoring Serotonin, gamma-aminobutyric acid (GABA), dopamine (DA), and acetylcholine (ACh) levels. This supplementation also elevated the expression of brain-derived neurotrophic factor mRNA, indicating protective effects against the neurological stresses of anxiety. Additionally, there were strong correlations among behavioral indicators, BDNF (brain-derived neurotrophic factor), and numerous neurotransmitters.

Conclusion: Hence, our findings propose that the WP + CP combination holds promise as a treatment for anxiety disorder. Besides, supplementary applications are feasible when produced as powdered dietary supplements or added to common foods like powder, yogurt, or milk.

KEYWORDS

walnut peptide, casein peptide, anxiety, memory-improving, serotonin, neurotrophic factor

1. Introduction

Anxiety disorders are the most common category of mental disorders characterized by feelings of unease, worry, fear, tension, and apprehension (1). According to the World Health Organization (WHO), approximately 380 million individuals worldwide are affected by anxiety disorders (2). It is associated with a decreased quality of life and overall functioning. Besides emotional symptoms, anxiety leads to brain dysfunction, such as depression and dementia (3). Research has found that chronic stress can lead to alterations in both the neuroendocrine and neurotransmitter systems, which in turn impact the creation and management of memories (4, 5). In a 2015 study, Luiz Pessoa explored the multiple interactions between anxiety and cognition functions within the brain. In particular, he looked at how these interactions in the prefrontal cortex (PFC) can minimize response conflicts and selectively affect working memory. Additionally, negative emotions have been shown to influence processes related to memory disproportionately (6).

Although anxiolytic drugs such as antidepressants and Benzodiazepines have been developed to address anxiety symptoms, their efficacy differs among individuals and may lead to addiction or other adverse effects (7). It is worth noting that the Food and Drug Administration has released no new anxiolytic agents since 2007 (8). In addition to drugs, there is growing interest in the potential anxiety-alleviating properties of food like yogurt combined with specific ingredients. This natural, secure, uncomplicated, and budget-friendly approach can be conveniently managed by individuals. Consequently, utilizing food to alleviate anxiety presents a strategy that mitigates the risks linked to psychotropic medications.

Walnut peptide (WP) is a bioactive peptide extracted from walnut protein, a nutritious food rich in polyunsaturated fatty acids, proteins, and minerals (9). WP has been reported to enhance sleep quality memory and cognition in mouse models and human clinical trials (10). Meanwhile, in numerous preclinical and clinical investigations, casein peptide (CP), an essential bioactive peptide derived from milk, has exhibited anxiety-reducing properties, establishing its potential as a therapeutic intervention (11). Due to WP's and CP's nutritional benefits and functional properties, they are widely used in food and beverage products. Adding WP and CP to powder, yogurt, or milk, the dairy products people consume daily is a convenient way to intake various nutrients. Combining WP and CP as the nutrient combination could show antianxiety and memory-improving effects as WP and CP-only exhibits and reduce production costs. WP and CP may relieve anxiety through neurotransmitters, such as dopamine (DA), serotonin, gamma-aminobutyric acid (GABA), and acetylcholine (ACh), which regulate emotions, cognitive functions, and memory formation (12). However, the roles of walnut peptide and casein peptide in neurotransmission remain unclear. Our previous study discovered that the nutrient combination WP + CP at 56.7 + 100 μg/mL showed effects of antianxiety, antioxidants, neuroprotection, and memory improvement in zebrafishes (13), whether they exhibit synergistic effect and alleviates anxiety in anxiety mice model is currently unknown.

This study aims to investigate and highlight the antianxiety and memory-improving effects of WP + CP as a cost-effective nutrient combination in mice. Furthermore, we seek to elucidate the underlying mechanisms involved, including neurotransmitters, neurotrophic factors (BDNF), and microglial cells. We established the chronic anxiety model in mice by elevated open platform. Then, we tested the antianxiety and memory-improving effects of the WP + CP combination and the combination added in powder, yogurt, or milk. Still, we could determine whether the effects of WP and CP combinations are consistent in powder, yogurt, and milk consumed by anxiety mice. This was the first time that the effects of WP + CP combinations were evaluated on anxiety-relieving and memory improvement in rodents.

2. Materials and methods

2.1. Animals

A total of 100 male C57BL/6 J mice (6–8 weeks old) were acquired from Charles River Laboratories Animal Technology Co., Ltd. (Beijing, China) for this study. The mice were housed in standard cages under specific pathogen-free (SPF) conditions, with a consistent 12-h light–dark cycle, and given standard laboratory chow and distilled water (Manufacturer: Beijing Keao Xieli Feed Co., Ltd., Production License: SCXK (jing) 2015–0013, Batch Number: 20229811). Kangcheng Biotech, Ltd., Co. (Sichuan, China) facilities were used to maintain the mice in a room with ambient temperature (21°C–24°C) and humidity (40%–60%). In each cage, a group of four mice is accommodated. The animal production license number was SYXK (Chuan) 2019-215. Four mice were housed in each cage. All procedures followed the guidelines of the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) and were approved by the Institutional Animal Care and Use Committee (IACUC) of the West China Hospital, Sichuan University (Approval No. 2019194A).

2.2. Model establishment and grouping

The anxiety model was established by the elevated open platform (EOP) with a slight adjustment based on a previous study (14). Mice were exposed to the clear square Plexiglas board (10 cm × 10 cm) at 1 m, 1 h per day for 30 days (Figure 1; Supplementary Figure 1). The control mice were not exposed to the elevated open platform (EOP) modeling, yet their living conditions and environment were consistent with those of the other experimental groups.

Mice were randomly assigned to one of 10 groups based on their body weight on day one: control, model, buspirone, powder, yogurt, milk, C, C + powder, C + yogurt, and C + milk. Control, model, and buspirone were designed to test the stability of our modeling system. C was short for the WP + CP combination. Since the combination will enter the powder, yogurt, or milk market, we set the groups of products containing C as C + powder, C + yogurt, and C + milk. The base for the product was powder, yogurt, and milk, respectively. All the nutrients were given by intragastric administration. The protocols utilized for administering intragastric (IG) treatment to mice subjected to the EOP model are described herein (Figure 1). Mice arriving at the laboratory were designated as "eligible mice" if they weighed between 21–25 g and covered 3,000–5,000 cm in the open field test (OFT; Supplementary Figure 2). This criterion ensured a consistent and standardized selection process. Locomotor activity, assessed by distance in the open field test, served as a prerequisite for inclusion in the study and minimized potential confounding factors affecting the reliability and validity of outcomes. Behavioral assessments were conducted from day 30 through day 41, after which

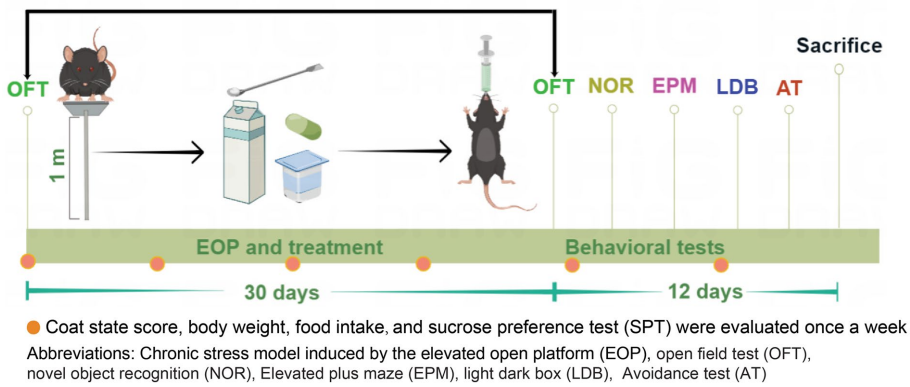

FIGURE 1
Experimental design and timeline. The experimental design for EOP and the subsequent treatment was illustrated in a timeline extending over 30 days. The mice were subjected to adaptive feeding for 7 days, after which they were randomly assigned to 10 groups based on body weight and total distance traveled. Each group comprised 10 mice. Following the assignment of groups, the mice were subjected to a daily, one-hour session on an elevated platform. They were orally administered with nutrients before being returned to their cages. Behavioral tests (OFT, NOR, EMP, LDB, and AT) were performed after 30 days of model administration to assess anxiety-relieving and cognitive improvement. Additionally, parameters such as body weight, food intake, coat state scores, and SPT were measured every week (as indicated by red dots in the figure). Finally, on day 42 of the experiment, the mice were humanely euthanized, and their brain tissues and serum were collected for analysis.

the mice from each group were humanely sacrificed and sampled a week following the conclusion of the behavioral experiment.

2.3. Sample information

Buspirone hydrochloride (Sigma Chemical Company, St. Louis, MO, United States), a 5-HT1A receptor agonist, was dissolved in saline and given intraperitoneally (IP) once daily for 30 days at 2 mg/kg, as previously reported (15). The powder, yogurt, and milk doses were determined according to the recommended human dose (16–18). The information of combination and products containing combination are shown in Table 1.

2.4. Assessment of animals' physical states

Body weight, food intake, and coat state score were evaluated every Wednesday between 10 and 12 a.m. (Figure 1). More details were shown in Supplementary material.

2.5. Behavioral assessment

2.5.1. Open-field test

Each mouse was carefully placed in the center of an open field box (50 × 50 × 50 cm) and allowed to explore for 10 min, following established procedures (20). The software was used to record and analyze the distance traveled within the open field and the times each mouse entered the central area of the arena (measuring 25 × 25 cm). The Topscan Package provided by Clever Sys Inc. based in the United States was used to quantify these parameters accurately.

2.5.2. Elevated plus-maze test

The Elevated plus-maze (EPM) consisted of two arms, open and closed, with the closed arms having 20 cm high walls. Both arms were 30 cm in length and 5 cm in width, and the EPM was elevated to a height of 60 cm above the ground, forming a 90° angle between the arms. Before the test, the mice could explore the central area for 5 min while facing the open arm. The open arm entry percentage (OE%) was calculated by dividing the number of entries into the open arm by the total number of entries into both arms and multiplying by 100. The open arm time percentage (OT%) was calculated by dividing the time spent in the open arm by 300 s (the total test duration) and multiplying by 100 (21).

2.5.3. Light–dark box test

As previously mentioned, anxiety was tested in the light/dark box (LDB) (22). A Plexiglas box with two equal chambers (18 × 12 × 12 cm) and an opening in the middle (5 cm length × 5 cm width) was used. The experiment was conducted in a dimly lit room (60 lx), and an infrared camera recorded the dark box. The mice were placed in the lightbox facing the dark box, and their exploration behaviors were recorded for 10 min after they crossed the opening (22).

2.5.4. Novel object recognition test

Recognition was assessed using the Novel object recognition (NOR) test, which comprised a learning trial and a subsequent test trial (23). In the former, two identical objects were presented to the mouse, which was allowed to explore them for 10 min in an open field box (60 × 25 × 25 cm). After a one-hour interval, the mouse underwent a test trial, during which one of the objects was replaced with a novel object. The mouse was given 5 min to explore both objects. The discrimination ratio of the recognition index was calculated using a specific formula (23).

$$Discrimination\ ratio = \frac{Ratio\ of\ total\ time\ spent\ with\ the\ novel\ object\ divided}{Total\ time\ spent\ on\ exploring\ either\ object\ multiplied} \times 100\%$$

TABLE 1 The information of samples tested.

Group	Name	Origin	Human dose	Mice dose	Calculation procedure
Positive drug	Buspirone Hydrochloride	Sigma Chemical Co	20–40 mg	2 mg/kg	
Base	Powder	Inner Mongolia Yili Industrial Group Co., Ltd	25 g/d	3791.67 mg/d·kg	25 g/d ÷ 60 kg × 1,000 mg/g = 416.67 mg/d·kg × 9.1 = 3791.67 mg/d·kg
	Yogurt		200 mL/d	30.33 mL/d·kg	200 mL/d ÷ 60 kg × 9.1 = 30.33 mL/d·kg
	Milk		200 mL/d		
Combinations	C	WP: The plant extract, a bioactive peptide extracted from the protein of walnut residues (Sinphar Group Co.) CP: Milk (Guangdong Huapeptides Biotechnology Co., Ltd.)	170 + 300 mg	25.87 + 45.50 mg/kg	WP: 170 mg/d ÷ 60 kg = 2.83 mg/d·kg (Human dose) × 9.1 = 25.75 mg/d·kg (Mice dose) CP: 300 mg/d ÷ 60 kg = 5 mg/d·kg (Human dose) × 9.1 = 45.50 mL/d·kg (Mice dose)
Products	C + powder	Inner Mongolia Yili Industrial Group Co., Ltd	Powder 25 g/d, containing combinations 170 + 300 mg	3791.67 mg/kg	25 g/d ÷ 60 kg × 1,000 mg/g = 416.67 mg/d·kg (Human dose) × 9.1 = 3791.67 mg/d·kg
	C + yogurt		Yogurt 200 mL/d, containing combinations170 + 300 mg	30.33 mg/kg	200 mL/d ÷ 60 kg × 9.1 = 30.33 mL/d·kg (The products were ready to administer, requiring no preparation.)
	C + milk		Milk 200 mL/d, containing combinations 170 + 300 mg	30.33 mg/kg	

The general packaging size for liquid dairy products is 200 mL; the powder is around 25 g dissolved in 180–200 mL water. The administration dosage for the mice was converted according to the surface area of a 60 kg human body. According to the conversion ratio between humans and mice (9.1) (19), the daily combination for mice can be calculated as the following formula: conversion ratio of surface area between mice and humans (9.1) × a daily dose of an adult (25 g/d powder)/average weight of adults (60 kg), that is 9.1 × 25 g/60 kg = 3.79 g/kg. The feeding volume was 20 mL/kg for each mouse. The combinations of WP + CP were tested in three dosages: 85 + 200 mg, 170 + 300 mg, and 170 + 600 mg. The open field test (OFT) and elevated-plus maze (EPM) showed that a combination with 170 + 300 mg had the best antianxiety-improving effects (Supplementary Figures 3A–C). Furthermore, there were discernible outcomes observed in the light–dark box (LDB), novel object recognition (NOR), and passive avoidance test (PAT) tests for both the C-medium and C-high groups (Supplementary Figures 3D–F). Therefore, the medium dosage (170 mg WP + 300 mg CP) was selected for the following studies.

2.5.5. Avoidance test

The Avoidance test (AT) protocol was conducted in a light/dark shuttle box with minor adjustments from day 36 to day 41, as previously described (24). The AT, including the active avoidance test (AAT) and passive avoidance test (PAT), involved three consecutive days, starting with an adaptation day where the mouse explored both compartments for 2 min. On the training day, the mouse received an electric shock upon entering the dark compartment, while on the PAT test day, the mouse explored the bright compartment without shock. The fourth day was designated the AAT, where the mouse explored the dark compartment without shock.

2.6. Sucrose preference test

To prepare for the sucrose preference test (SPT), a 1% sucrose solution and distilled water were simultaneously introduced into each mouse's cage. This allowed for 2 days of taste adaptation. On the third day, after a six-hour fasting and water deprivation period, the mice were placed individually in cages that contained pre-weighed bottles of 1% sucrose solution and distilled water. Six hours later, the positions of these bottles were swapped. Following an additional 12 h, both bottles were removed. The remaining liquid was measured to calculate the sucrose preference of the mice using the following formula (25):

$$Sucrose\ preference\ percentage = \frac{Sucrose\ consumption}{Total\ liquid\ consumption} \times 100\%$$

SPT was performed weekly, consistent with the time points of body weight, food intake, and coat state scores (Figure 1).

2.7. Serum corticosterone and adrenocorticotropic hormone assay

After blood collection between 7:00–8:00 a.m., mice were euthanized by carbon dioxide inhalation. Serum concentrations of corticosterone and adrenocorticotropic hormone (ACTH) were determined using an enzyme-linked immunosorbent assay (ELISA) kit from TECAN, Germany (RE52211) and Abcam, United Kingdom (ab263880), respectively.

2.8. Liquid chromatograph mass spectrometer

The brains of mice were weighed and homogenized in RNase-free water at a 1:4 ratio for detection. The samples were centrifuged at 12,000 g for 5 min at a temperature of 4°C. Protein precipitation was

TABLE 2 The chromatographic gradient of mobile phase (A: water, and B, acetonitrile).

Time (min)	Flow rate (mL/min)	A (%)	B (%)
Initial	0.6	90	10.0
0.30	0.6	90	10.0
2.00	0.6	10.0	90.0
2.50	0.6	10.0	90.0
2.60	0.6	90.0	10.0
3.50	0.6	90.0	10.0

performed using 50 μL of the brain homogenate, 5 μL of blank, and 200 μL of acetonitrile internal standard. A standard curve was prepared using a 1 mg/mL mother solution diluted with 50% acetonitrile. Standard curve and biological samples ranging from 50~2,000 ng/mL were prepared. Quality control samples were also prepared at 5, 10, 50, 800, and 1,600 ng/mL. The protein precipitator containing IS was added to each standard working solution, QC working solution, and unknown concentration samples, which were then vortexed for 30 s. After centrifugation, the supernatant was diluted with water. Brain extracts were injected into the Analyst® System, and the data are expressed as ng per g tissue. HPLC-MS, using Analyst® software, detected serotonin, ACh, GABA, and DA in the prefrontal cortex (PFC). A Waters column was utilized with mobile phases A and B. The chromatographic gradient is presented in Table 2, and the injection volume was set at 5 μL, with dexamethasone and verapamil as internal standards.

2.9. RNA extraction and real-time reverse transcription polymerase chain reaction analysis

The modified guanidine isothiocyanate-phenol-chloroform method, incorporating RNX+ reagent, was used to isolate RNA from the right hemisphere hippocampus, following the manufacturer's protocol, with subsequent treatment using RNase-free water to prevent DNA contamination. Spectrophotometry, using a Thermo-Nano Drop 2000c-spectrophotometer, was used to quantify the concentration and purity of all RNA samples and determine the mean absorbance ratio and optical density (OD) at 260/280 nm. The cDNA was synthesized using HiScript III RT SuperMix for qPCR (+gDNA wiper; Vazyme, R323, China) and ChamQ Universal SYBR qPCR Master Mix (Vazyme, Q711, China). The primers used to analyze β-actin and brain-derived neurotrophic factor (BDNF) were β-actin, 5′-CCACCATGTACCCAGGCATT-3′ (forward), and 5′-CAGCTCAGTAACAGTCCGCC-3′ (reverse); BDNF, 5′-TCCG GGTTGGTATACTGGGTT-3′ (forward) and 5′-GCCTTGTCCGTG GACGTTT-3′ (reverse). RT-PCR analyses were conducted using the Bio-Rad CFX Manager (Bio-Rad, CFX Connect, United States) according to the manufacturer's protocol. The DNA amplification process consisted of an initial cycle at 95°C for 30 s, followed by 40 cycles of denaturation (95°C for 10 s), annealing (60°C for 30 s), and extension (95°C for 15 s). The $2-\Delta\Delta Ct$ method was used to calculate the expression of β-actin and BDNF, with a single calibrated sample serving as the reference for comparison with the expression of all unknown samples.

2.10. Ionized calcium-binding adapter molecule 1 immunohistochemistry

Ionized calcium-binding adapter molecule 1 (Iba1) is a protein that marks microglial cells. The mouse brains were fixed in 10% buffered formalin and then embedded in paraffin. For immunohistochemical analysis, four μm sections were prepared. The anti-Iba1 antibody from Wako (Richmond, VA) was used at a concentration of 1:1,000 to detect Iba1. We quantified the number of Iba1-positive cells, the total number of cells, and the number of positively stained cells in the areas with the highest tumor-cell density in 10 non-overlapping microscopic fields (at 400 × magnification) of tumor-bearing brains taken from mice in each group.

2.11. Statistical analysis

The statistical analysis was conducted using SPSS 26.0 software (IBM Ltd., United Kingdom). Data conforming to normal distributions were represented as mean ± standard errors of the mean (SEM). Unpaired *t*-tests were utilized to analyze the differences between the control and model groups. A one-way Analysis of Variance (ANOVA) followed by the *post hoc* Tukey Dunnett's multiple comparison tests was used to analyze differences among the model, buspirone, and each treatment group when the data followed a normal distribution and the variances were equal. A repeated measures ANOVA was employed to examine group variations in body weight, food intake, coat state, sucrose preference, and fecal amount. Interaction effects between repeated indicators and days were tested by Pillai's trace. In the case of interactions observed between a specific variable and days, the differences among each group were compared at the final time point. If no interactions were present, post-hoc analysis using Bonferroni's multiple comparison tests was conducted. The correlations between each effect were performed using Pearson's correlation. For all analyses, two-sided p-values < 0.05 was considered statistically significant.

3. Results

3.1. WP and CP combination showed apparent antianxiety effects

3.1.1. Changes of general states in each group

During the anxiety model establishment and combination administration, the body weight, food intake, coat state score, and sucrose preference test (SPT) were determined weekly (Figure 1). We tested three WP + CP combination dosages, 85 + 200 mg, 170 + 300 mg, and 170 + 600 mg, respectively. During the anxiety experiment, the C-low group demonstrated markedly reduced scores on the Open field test (OFT) and EPM assessments compared to the C-medium and C-high groups. In the cognitive tests, discernible disparities in the NOR and PAT tests were solely observed with the C-medium and C-high groups when contrasted against the control group, thereby substantiating the decision to establish the combination dosage as the medium dose: 170 + 300 mg (Supplementary Figure 3). The body weights were maintained steady, slightly decreasing in some groups after EOP, and there was an interaction effect between body

weight and days (Group * day, $F = 3.027$, df = 63, $p < 0.001$; Figure 2A). The simple effect of body weight was shown in Supplementary Tables 1A,B. On day 49, the model group had the lowest body weight compared with the control ($t = 3.054$, df = 18, $p = 0.007$; Figure 2B). One-way ANOVA was employed to analyze the difference between the model group and all the treatment groups (Buspirone, powder, yogurt, milk, C, C + powder, C + yogurt, and C + milk) in the present study. Since differences were evident among these eight groups [$F_{(8, 81)} = 13.04$, $p < 0.001$], a *post hoc* Bonferroni test was conducted, revealing that buspirone, yogurt, C + yogurt, and C + milk led to a tremendous increase in mice's body weight compared to the model group ($p < 0.001$, $p = 0.029$, $p < 0.001$, $p < 0.001$, respectively; Figure 2B). Compared with yogurt and milk, C + yogurt and C + milk increased body weight ($p = 0.032$, $p = 0.001$; Figure 2B). Besides body weight, the food intake decreased gradually in general, especially in the model group, possibly due to anxiety-induced appetite loss. Due to the interaction effects between food intake and days (Group*day, $F = 4.906$, df = 54, $p < 0.001$; Figure 2C), we only compared changes in food intake on the last time point, Day 42. The simple effect of food intake was analyzed in Supplementary Table 2B. Food intake was the least in the model group, significantly lower than control ($t = 4.358$, df = 18, $p < 0.001$; Figure 2D). Differences existed in the model and treatment groups [$F_{(8, 81)} = 4.337$, $p = 0.001$]. The *post hoc* Bonferroni test revealed that buspirone, C, C + powder, C + yogurt, and C + milk could all increase the food intake of anxiety mice ($p < 0.001$, $p = 0.005$, $p = 0.037$, $p < 0.001$, $p < 0.001$; Figure 2D). The simple effects of food intake were analyzed in Supplementary Tables 2A,B.

The EOP represents a prominent approach to anxiety modeling. Our study evaluated coat condition and anhedonia in mice following 30 days of long-term intragastric therapy and modeling. The interaction effect was found between coat state scores and days (Group*days, $F = 2.173$, df = 45, $p < 0.001$; Supplementary Tables 3A,B), and we could see the highest level of coat state scores in the model group (Figure 2E). SPT was performed six times, and an interaction effect existed between sucrose preferences and days (Group*days, $F = 5.643$, df = 45, $p < 0.001$; Supplementary Tables 4A,B). It showed that the sucrose preference was consistently decreased during the early stages of the modeling process. However, in the later stages (21 days later), almost all groups showed varying degrees of recovery (Figure 2F), and the simple effect of groups. When comparing changes in sucrose preference on day 35, the model was lower than the control ($t = 2.402$, df = 18, $p = 0.027$). One-way ANOVA was employed to analyze the model group and treatment groups in the present study [$F_{(8, 81)} = 8.492$, $p < 0.001$]. Following the analysis, a *post hoc* Bonferroni test was conducted, revealing that buspirone, yogurt, milk, C + yogurt, and C + milk help increase sucrose preference ($p = 0.033$, $p = 0.006$, $p = 0.002$, $p < 0.001$, $p < 0.001$; Figure 2G).

Anxiety mice exhibited lower activity in the open-field experiment and preferred the periphery over the central area. Correspondingly, mice in an anxious state produced more fecal particles (26). Therefore, we aimed to measure mice's anxiety levels by quantifying fecal particle

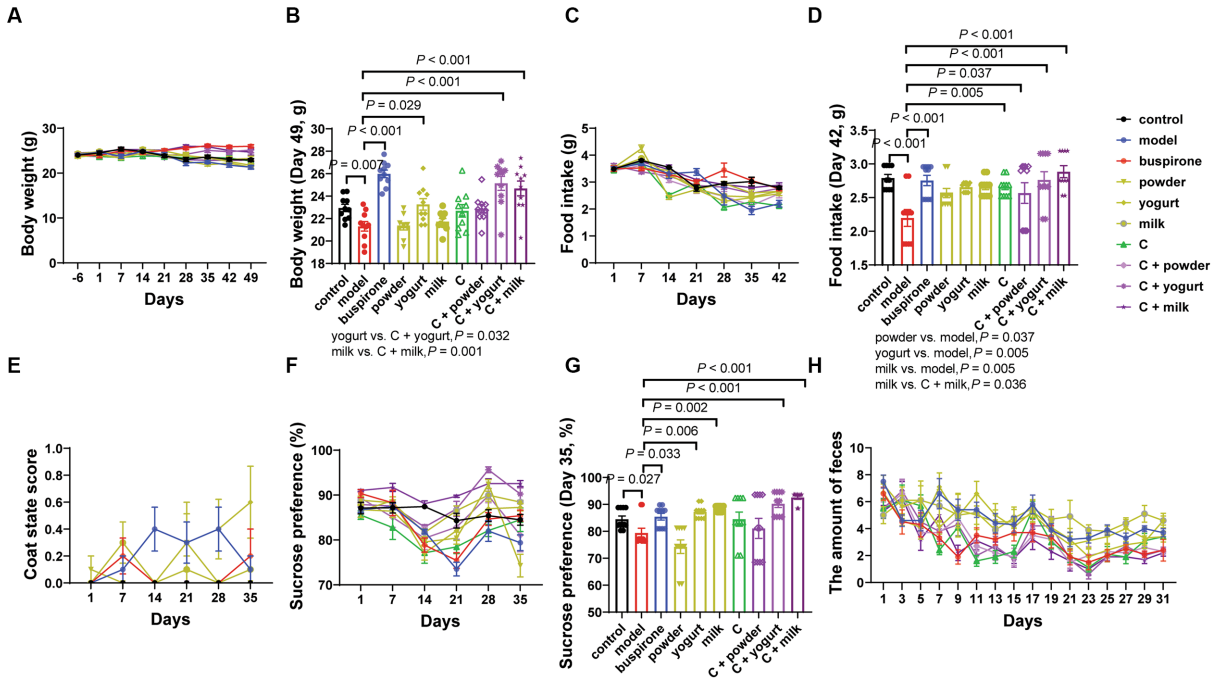

FIGURE 2
The influence of the combined administration of WP + CP on the fundamental physiological data in mice. Throughout the experiment, changes in body weight **(A)**, and at the experimental endpoint of 49 days, discernible variations in body weight were observed among the different groups of mice **(B)**. Food intake **(C)** was monitored weekly, and at the 42-day experimental endpoint, conspicuous disparities in food consumption were observed across the various mouse cohorts **(D)**. Changes in coat state score **(E)**, SPT **(F)**, and the amount of feces **(H)** were recorded every week. On the 35th day of the experiment, notable variations in the percentage of water preference among the different groups were detected **(G)**. The data, representing mean values ± SEM, $n = 10$ in each group, were analyzed using repeated measures analysis of variance (ANOVA) to investigate potential differences among groups. If the interactions between one index and days existed, we compared the difference among each group of the last time points **(B,D,F)**. If the interactions did not exist, post-hoc analysis (Bonferroni's multiple comparison tests) could be done next.

production on an elevated platform. Clearly, there was an interaction effect between feces amount and days (Group*days, $F = 1.746$, df = 120, $p < 0.001$; Supplementary Tables 5A,B). The C + milk group exhibited a lower fecal particle count than the model group. In contrast, the milk group demonstrated significantly higher particle levels than the C + milk group (Figure 2H).

3.1.2. Results of behavioral tests in evaluating anxiety states

During days 30–35, a sequential battery of tests was conducted to assess mice's anxiety-like behaviors, including the OFT, EPM, and LDB. The percentage of time spent in the inner zone was lower in the model group than in the control ($t = 2.654$, df = 18, $p = 0.016$). At the same time, buspirone treatment increased the center's exploration of mice compared to the model ($t = 2.654$, df = 18, $p = 0.016$; Figures 3A,B). The model group exhibited significantly fewer rearing times than the control ($t = 3.557$, df = 18, $p = 0.002$). One-way ANOVA was employed to analyze the model group and treatment groups in the present study [$F_{(8, 81)} = 5.186$, $p < 0.001$]. Following the analysis, a *post hoc* Bonferroni test was conducted, revealing that buspirone, C + yogurt, and C + milk induced more rearing times relative to the yogurt and milk ($p = 0.004$, $p = 0.002$, $p = 0.010$; Figure 3C). Supplementary Table 6 shows cases of the additional metrics of the open field activity.

During the EPM test, the model group exhibited the lowest percentage of open-arm entries and time spent in the open arms. However, treating with buspirone, milk, C, and C + powder/yogurt/milk increased open-arm exploration compared to the model (Figures 3D–F). This suggests that the combination and its products effectively alleviate anxiety-like behavior in mice.

We employed the LDB test as a third behavioral assay to assess the anxiety state. The model group showed a remarkably shorter time spent in the light box than the controls ($t = 4.632$, df = 18, $p < 0.001$). One-way ANOVA was employed to analyze the model group and treatment groups in the present study [$F_{(8, 81)} = 8.536$, $p < 0.001$]. Following the analysis, a *post hoc* Bonferroni test was conducted, revealing that buspirone C, C + powder, C + yogurt, and C + milk prolonged the time spent in the lightbox (all p-value < 0.001). In particular, the C + powder and C + yogurt groups exhibited a substantially longer time spent in the light box, suggesting a reduced anxiety-like phenotype (Figures 3G,H). Trace of OFT, EPM, and LDB in the treatment group was shown in Supplementary Figure 4.

3.1.3. Changes in stress-related hormones

We subsequently investigated the alterations in stress-related hormones in the serum, the commonly used biomarkers in clinical settings that reflect changes in the hypothalamic–pituitary–adrenal axis. Notably, the levels of corticosterone and ACTH were elevated in the model compared with the control ([corticosterone]: $t = 5.927$, df = 18, $p < 0.001$; [ACTH]: $t = 4.119$, df = 18, $p < 0.001$, Figures 3I,J). Excluding the control group, a one-way ANOVA analysis was conducted on the remaining groups [$F_{(8, 81)} = 13.23$, $p < 0.001$]. Regarding serum corticosterone levels, group C demonstrated elevated concentrations in comparison to C + yogurt ($p = 0.043$) and C + milk ($p < 0.001$), followed by a subsequent decrease in serum corticosterone upon the introduction of C to the three bases (Figure 3I). One-way ANOVA was employed to analyze the model group and treatment groups in the present study [$F_{(8, 81)} = 6.446$, $p < 0.001$]. As the upstream regulatory factor, changes in serum ACTH were discovered to be more responsive than corticosterone reductions in serum ACTH were observed in C + powder/milk compared to the model ($p = 0.006$, $p = 0.022$). Furthermore, C + powder and C + milk triggered lower serum ACTH expression levels than the two bases ([powder vs. C + powder]: $p = 0.010$; [milk vs. C + milk]: $p = 0.008$; Figure 3J).

3.2. The WP + CP combination enhanced memory In mice with anxiety

We further conducted the NOR and avoidance (PAT and AAT) tests to evaluate memory impairment in anxiety mice. On day 32, the NOR test indicated a decrease in the recognition index in the model group compared to the control ($t = 6.071$, df = 18, $p < 0.001$). This suggests that the persistent stress led to a decline in memory function. The administration of buspirone was efficacious in improving memory (Figures 4A,B).

In the PAT test, we observed a reduced latency in entering the dark box in the model and the three basal groups compared to the control (Figures 4C,D). One-way ANOVA was employed to analyze the model group and treatment groups in the present study [$F_{(8, 81)} = 8.646$, $p < 0.001$]. Additionally, the C, C + yogurt, and C + milk groups had longer latency times than the model group ($p = 0.040$, $p = 0.012$, $p = 0.048$), with the C + milk group displaying a longer latency time than the yogurt group ([powder vs. C + powder]: $p = 0.001$; [yogurt vs. C + yogurt]: $p = 0.004$; Figures 4C,D). During the AAT, a heightened latency was noted in the model group and the three basal groups upon entering the light box compared to the control (Figures 4E,F). Excluding the control group, a one-way ANOVA analysis was conducted on the remaining groups [$F_{(8, 81)} = 8.201$, $p < 0.001$]. Moreover, the C + powder group showed a more extended incubation period than the C + milk group ($p = 0.035$). For additional trajectory plots illustrating the traces of treatment groups receiving the combination, kindly consult Supplementary Figure 4.

3.3. WP + CP combination improved the imbalanced neurotransmitters and BDNF expression caused by anxiety

The occurrence and development of anxiety are closely related to changes in neurotransmitters, such as Serotonin, GABA, DA, and ACh. Serotonin and GABA are the targets of commonly used antianxiety medications. We assayed neurotransmitter concentrations in mice's PFC, revealing a significant reduction in serotonin levels in the model compared to the control ($t = 7.751$, df = 18, $p < 0.001$). Excluding the control group, a one-way ANOVA analysis was conducted on the remaining groups [$F_{(8, 81)} = 3.007$, $p < 0.001$]. Notably, C + powder/yogurt induced higher serotonin levels than the model ($p = 0.004$, $p = 0.020$). In contrast, C + yogurt also caused higher serotonin concentration than yogurt ($p = 0.037$; Figure 5A). Furthermore, the model observed lower GABA concentrations than the control ($t = 2.785$, df = 18, $p = 0.012$). Excluding the control group, a one-way ANOVA analysis was conducted on the remaining groups [$F_{(8, 81)} = 4.187$, $p < 0.001$]. However, C and C + powder/milk significantly increased GABA

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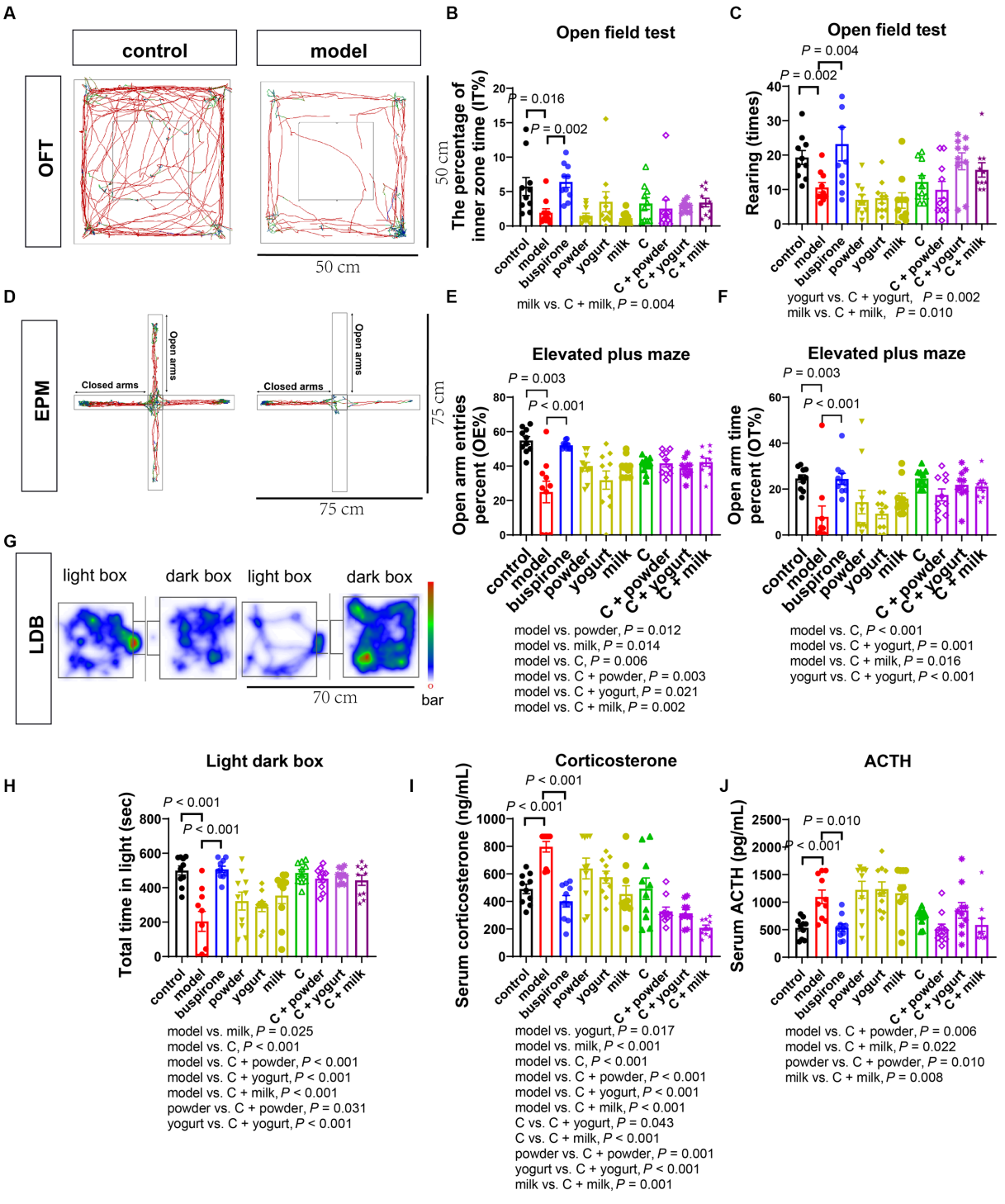

FIGURE 3
Effects of WP + CP on anxiety relieving in mice. **(A–C)** OFT performed on the 30th day. The representative pictures of control and model **(A)**, changes of the percentage of inner zone time **(B)**, and rearing times **(C)** by OFT in each group. **(D–F)** EPM performed on the 32nd day. Representative pictures of EPM **(D)**, changes of OE% **(E)**, and OT% **(F)** by EPM in each group. **(G)** LDB by trajectories on the 35th day. **(G,H)** LDB performed on the 35th day. Representative pictures of LDB **(G)** and changes of total time in light of each group **(H)**. **(I,J)** Serum concentrations of corticosterone **(I)** and ACTH **(J)** in each group. Data represent mean ± SEM; $n = 10$ in each group.

concentrations compared to the model ($p = 0.030$, $p = 0.005$, $p = 0.006$; Figure 5B). In addition, dopamine (DA) concentration was higher in the control compared to the model ($t = 3.270$, df = 18, $p = 0.004$). Excluding the control group, a one-way ANOVA analysis was conducted on the remaining groups [$F_{(8, 81)} = 3.648$, $p < 0.001$]. The DA concentration in the C group was considerably higher than the model ($p = 0.005$; Figure 5C). Lastly, in comparison to the control group, the model group displayed a slight decrease in ACh

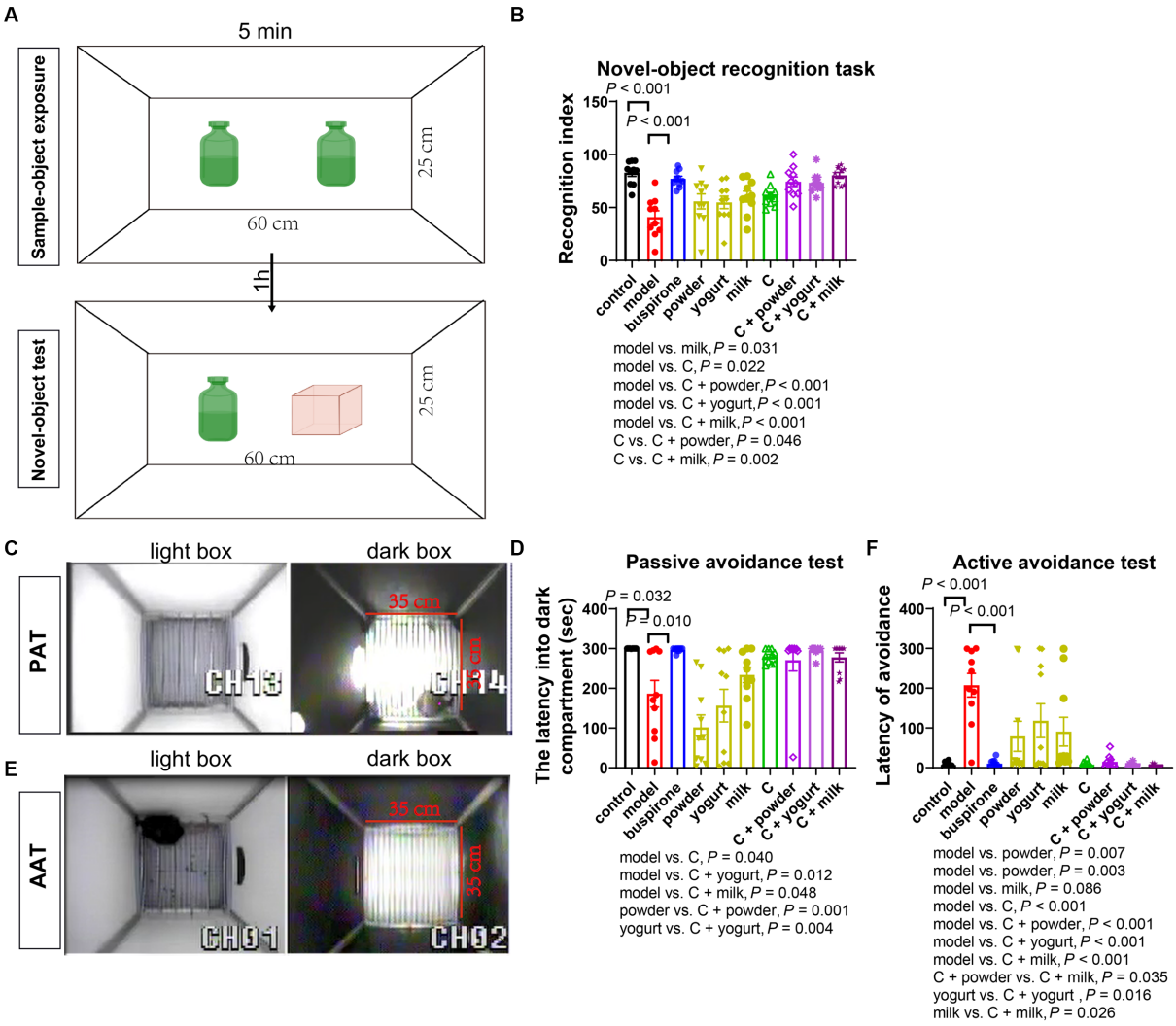

FIGURE 4
The memory improvement effects of the combination. The NOR test performed on the 32nd day. **(A)** Schematic of novel object recognition test. The recognition index analyzed by NOR in each group **(B)**. On the 36th, the avoidance test was conducted. Schematic of the PAT **(C)**. The latency to enter the dark compartment by PAT for each group on the 36th day is presented in panel **(D)**. On the 41st day; the avoidance test was conducted. Schematic of the AAT **(E)**, while the latency to avoidance by AAT for each group is shown in panel **(F)**. The data are expressed as mean + SEM with $n = 10$ in each group.

concentration ($t = 5.116$, df = 18, $p < 0.001$). Excluding the control group, a one-way ANOVA analysis was conducted on the remaining groups [$F_{(8,81)} = 5.284$, $p < 0.001$]. Differences existed among the model and the eight treatment groups ($p < 0.001$). Moreover, the C + powder group exhibited a higher concentration of ACh when compared to the powder group ($p = 0.004$; Figure 5D).

It is widely observed that reduced BDNF protein expression is induced by chronic stress (27); we investigated the BDNF expressions in the hippocampus. BDNF expression was inhibited in the model compared to the control ($t = 3.283$, df = 18, $p = 0.004$). Excluding the control group, a one-way ANOVA analysis was conducted on the remaining groups [$F_{(8, 81)} = 7.136$, $p < 0.001$]. Conversely, buspirone treatment effectively elevated the BDNF expression ($p < 0.001$; Figure 5E). Moreover, a marked increase in the relative expression level of BDNF was observed in the C/C + yogurt/C + yogurt/C + milk group compared to the model group.

3.4. The correlations between behavioral tests and serotonin or BDNF expressions were highly related

Through the comprehensive analysis of the data presented, we have verified the anxiolytic and memory-enhancing effects of WP + CP. As alterations in neurotransmitter and BDNF expression have been observed in response to anxiety, we aimed to explore the interplay among behavior, serum corticosterone, ACTH, neurotransmitters, and BDNF expression in mice (Figure 6A). Notably, a negative correlation was found between serotonin concentration in PFC and anxiety-like behaviors, such as total time in light in LDB (r = 0.646, $p = 0.044$, Figure 6B) and RI (r = 0.632, $p = 0.050$; Figure 6B). Furthermore, OE% exhibited a positive correlation with relative BDNF expression (r = 0.773, $p = 0.009$; Figure 6C), which was consistent with the correlation observed

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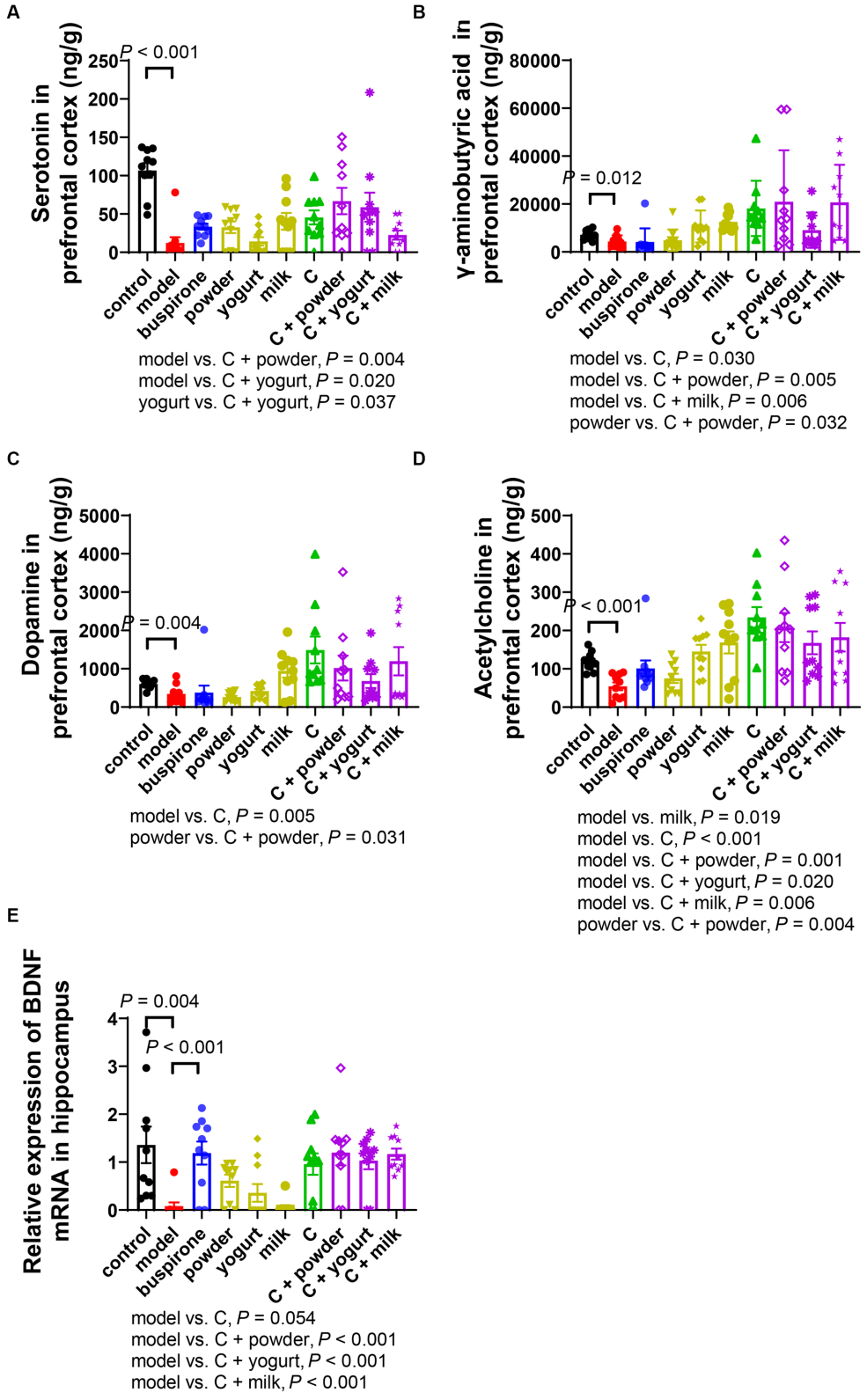

FIGURE 5
Changes of neurotransmitters in PFC and BDNF mRNA expression in the hippocampus after anxiety **(A-D)**. The concentration of serotonin (5-HT) **(A)**, γ-aminobutyric acid (GABA) **(B)**, dopamine (DA) **(C)**, and acetylcholine (ACh) **(D)** in the right prefrontal tested by LC–MS. **(E)** BDNF mRNA relative expression changes in the hippocampus. Concentrations are given in ng/g wet tissue. Data represent mean ± SEM; $n = 10$.

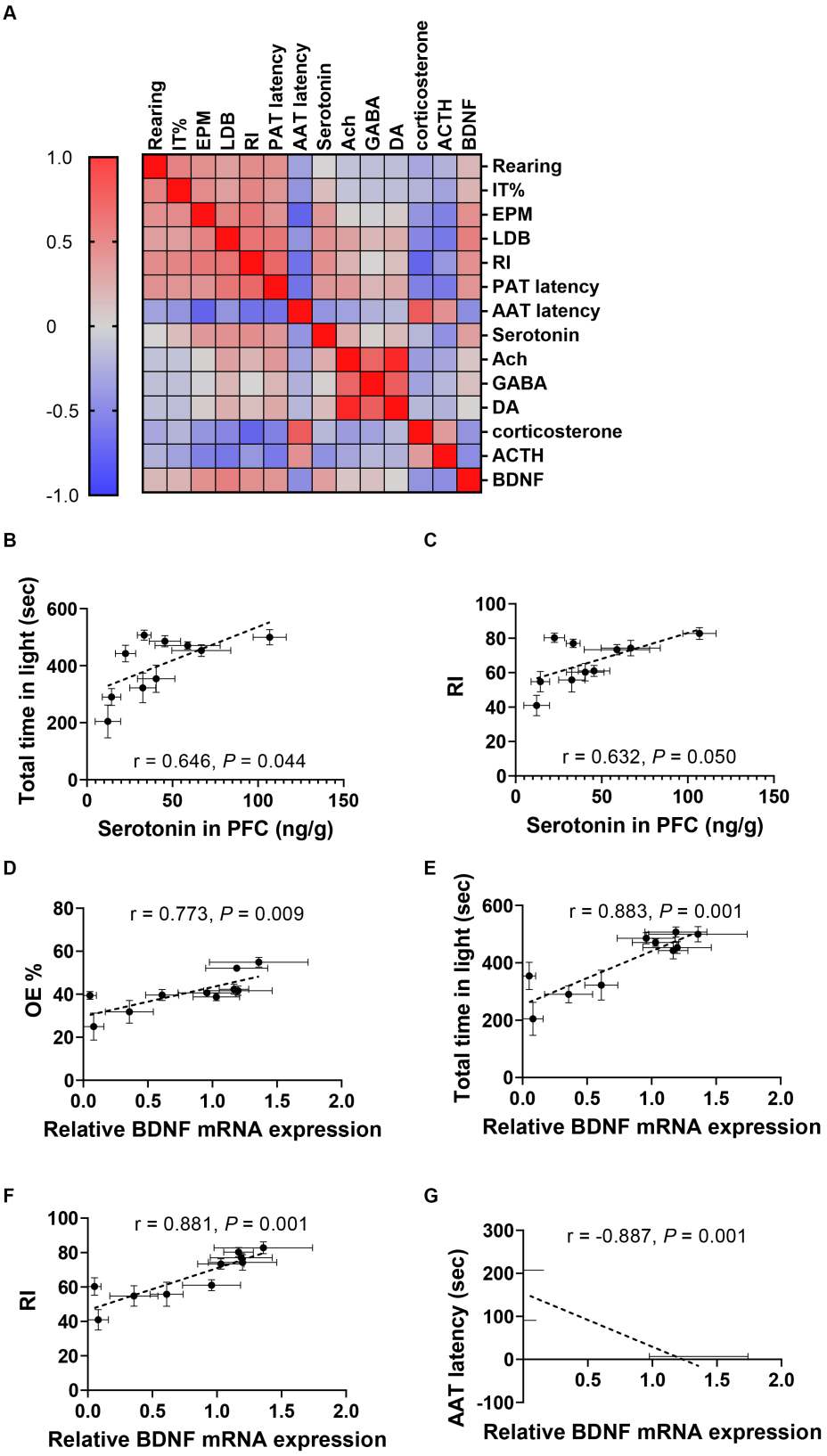

FIGURE 6
Correlations between neurotransmitters, BDNF, and anxiety-related indices. **(A)** Heatmap of the Correlation coefficient matrixes of the responses of various indicators for each mouse. The correlation coefficient matrices display a spectrum of colors, wherein intensifying shades of red correspond to heightened correlation levels while deepening shades of blue denote decreased correlation levels. **(B,C)** Correlations between 5-HT concentration in PFC and total time in light in LDB **(B)** and RI in NOR **(C)**. **(D–G)** Correlations between relative BDNF mRNA expression and OE% in EPM **(D)**, total time in light of LDB **(E)**, RI in NOR **(F)**, and AAT latency **(G)**. Data were represented as mean ± SEM, $n = 10$ in each group.

between total time in light in LDB (r = 0.883, $p = 0.001$; Figure 6D). Impressively, a strong correlation was also observed between the relative expression level of BDNF and NOR (r = 0.881, $p = 0.001$; Figure 6E) and AAT experiments (r = −0.887, $p = 0.001$; Figure 6F).

Given the observed correlation between fecal particle production and behavioral outcomes, we further investigated the potential connections between behavior, BDNF expression, and fecal excretion, as outlined in Supplementary Figure 5. Notably, a negative correlation emerged between fecal production and behaviors such as total time spent in the lit area of the LDB (r = −0.848, $p = 0.004$; Supplementary Figure 5A), AAT latency (r = 0.840, $p = 0.005$; Supplementary Figure 5B), and RI (r = −0.827, $p = 0.006$; Supplementary Figure 5C). Additionally, the number of feces positively correlated with BDNF mRNA expression relative to control levels (r = −0.958, $p < 0.001$; Supplementary Figure 5D). These correlation analysis data indicated the relatively strong reciprocity between behaviors and neurotransmitters or behaviors and BDNF expressions. The correlation matrix of behavioral and biological indicators is shown in Supplementary Table 7.

Besides neurotransmitters, numerous studies have highlighted the importance of low-grade inflammation in the pathophysiology of anxiety (28). We tested Iba1 expression, the biomarker of microglia activation, in the left hemisphere by immunohistochemistry. There were no significant differences in Iba1 microglial cell count (Supplementary Figure 6) between the control and model groups, indicating that the increase in Iba1 expression and coverage could not be ascribed to variances in the number of cells.

4. Discussion

The current study reported the anxiety-relieving and memory-improving effects of the WP + CP at 170 + 300 mg (human dose), both as a combination and their derived products (powder, yogurt, and milk). The dosage was designed by integrating the results from the previous human trials (29, 30), zebrafish experiments (13, 31), and the current mouse experiments. Utilizing a combined nutrient combination of WP and CP in this study is essential as it not only exhibits antianxiety and memory improvement effects, as observed with WP and CP alone, but also presents a potential cost-reduction advantage. This study investigates the rationale behind utilizing this combination and presents the necessity for further research in this area.

We employed the EOP paradigm to establish anxiety models in mice. Although foot shock stress is commonly used to establish an anxiety model, inconsistent stress induction procedures can lead to anhedonia and learned helplessness (32, 33). Therefore, we chose the EOP paradigm, which utilizes a comparatively milder physical stimulus, to construct our mouse anxiety model. This approach provides a controlled and consistent method for inducing anxiety-like behaviors in mice, which helps investigate anxiety disorders' neurobiological basis and assess potential treatments.

In this study, we evaluated the anxiety states of mice using the OFT, EPM, and LDB tests. The results indicated that the EPM and LDB tests had more positive outcomes than the OFT test. The study confirmed the sensitivity of EPM and LDB in detecting anxiolytic compounds, proving their reliability (34). Previous research has shown that OFT is less reliable due to the administration operation's interference with the actual experimental results, primarily when orally administered to mice fed different diets (35). In contrast, rearing times were the most sensitive index on OFT as it reveals a mouse's exercise capacity in vertical directions (36). In our study, we observed that incorporating the combination compound C + milk yielded superior antianxiety effects compared to the milk group, as evidenced by significant differences in OFT, serum corticosterone, and ACTH indicators. These findings strongly suggest a synergistic interaction between the combined compound and milk, enhancing its potential to alleviate anxiety (37, 38).

Wang et al. and Zhao et al. investigated the neuroprotective effects of WP against memory deficits induced by lipopolysaccharide and walnut-derived peptide mechanism and pathway of mitophagy in mice (9, 39). The studies of CP on stress were started 30 years ago in animal experiments and clinical trials, showing the effects of insomnia and anxiety-improving properties (40–42). Our previous studies in zebrafish revealed that the nutrient combination of WP + CP at 56.7 + 100 μg/mL exhibited antianxiety, antioxidant, neuroprotection, and memory improvement properties (13). This is the first time to evaluate the effect of WP and CP combination. Based on dose conversion between zebrafish and mice, the current study utilized WP + CP at a dosage of 25.87 + 45.50 mg/kg, equivalent to 170 + 300 mg of the equal human dose, which proved effective in improving anxiety and memory in mice.

Studies have shown that ACTH secretion is regulated by various factors, including stress stimuli. When exposed to stress, the pituitary gland releases ACTH, which stimulates the synthesis and release of corticosterone from the adrenal cortex. Subsequently, the elevated levels of corticosterone suppress ACTH secretion through a negative feedback mechanism, maintaining the homeostasis of the endocrine system (43). The dysfunctional neurotransmitter systems exist in anxiety regulation (44). In primates, the PFC, the chief executive officer of the brain, regulates anxiety by engaging high-level regulatory strategies aimed at coping with and modifying the experience of anxiety (45). Based on these theories, we determined the concentrations of four neurotransmitters in the PFC of each mouse. In the chronic stress rodents, reduced 5-HT, GABA, and DA levels in PFC were reported (46–48), which is consistent with our model. We found that the product of combination (C + powder/yogurt) significantly increased the 5-HT concentration in PFC, demonstrating its antianxiety effects through 5-HT. However, the treated groups had even higher GABA concentrations than the control in our study. This could be due to the long-term administration of dairy products, which may promote intestinal absorption and affect immunity, the microbiome, and the gut-brain module (49–51). ACh plays a significant role in regulating muscles, the heart, the digestive tract, and the nervous system (52). Our study found that treating anxiety mice with the combinations and its products could increase ACh levels in PFC, which was in line with the memory-related behavioral tests.

Previous studies have demonstrated reduced BDNF protein expression in the hippocampus induced by chronic stress (27), consistent with our experimental results. Recent research has revealed that the amygdala, known for its role in emotions, also processes non-conditioned stimuli (53–55). This sheds new light on the intricate relationship between cognition and emotion, traditionally associated with the hippocampus (56, 57). The hippocampus is one of the key brain structures for emotional response and is particularly susceptible to endogenous stressors. Meanwhile, BDNF exerts its activity most in the hippocampus (58–60). Brain-derived neurotrophic Factor (BDNF) plays a pivotal role in the nervous system by facilitating neuronal growth, differentiation, and survival, thus serving as a critical neurotrophic factor (61). Ma et al. found that adult neurogenesis

persists in the dentate gyrus of rodents and is stimulated by chronic treatment with conventional antidepressant drugs through the BDNF/Tropomyosin receptor kinase B (TrkB) signaling pathway (56).

Numerous studies have highlighted the importance of low-grade inflammation in the pathophysiology of anxiety, such as increased levels of proinflammatory cytokines in the brain (28). Inflammatory conditions promote tryptophan metabolism along the kynurenine pathway at the expense of the 5-HT pathway (62). We did not find a difference between the model and control, which may be due to the time of sampling and the brain region we chose to perform IHC assays. Additionally, activated microglia exhibited phenotypes termed M1 and M2 phenotypes. M1 microglia contribute to the development of inflammation, while M2 microglia exert anti-inflammation effects (63). We may find more precise and meaningful results if we detect M1 and M2 microglia phenotypes separately.

One of the limitations of this study was the absence of BDNF protein expression. In the following studies, we will conduct in-depth studies on the specific mechanism of WP + CP on anxiety-relieving and memory improvement, particularly the serotonin and BDNF pathways. Besides, administrating buspirone by intraperitoneal (IP) injection rather than intragastric (IG) administration might cause a slight difference in anxiety levels. Previously, we proved that IP exhibited superior therapeutic efficacy, which is more suitable for administering positive control (35). However, it did not affect the evaluation of combinations in this study. Since this study was a basic animal research project, further investigation is required to determine the effective dose of WP and CP in humans.

5. Conclusion

Overall, the study investigated the impact of a WP + CP combination, administered at a human dose of 170 + 300 mg, on anxiety relief and memory improvement in mice with anxiety. The combination, either alone or dissolved in products such as powder, yogurt, or milk, exhibited similar efficacy, possibly through the modulation of neurotransmitters or the BDNF pathway.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary material, further inquiries can be directed to the corresponding authors.

Ethics statement

The animal study was approved by Institutional Animal Care and Use Committee (IACUC) of the West China Hospital, Sichuan University (Approval No. 2019194A). The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

QL: Writing – original draft, Methodology, Validation. XJ: Validation, Writing – review & editing, Conceptualization, Resources. QZ: Validation, Project administration, Writing – review & editing. ZhZ: Writing – review & editing, Investigation, Project administration. YW: Methodology, Software, Writing – original draft. CT: Methodology, Software, Writing – original draft. BZ: Methodology, Software, Writing – original draft. HF: Resources, Writing – review & editing. JHao: Resources, Writing – review & editing. ZiZ: Resources, Writing – review & editing. JHe: Data curation, Supervision, Writing – review & editing. YZ: Conceptualization, Project administration, Writing – original draft.

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Conflict of interest

XJ, HF, JHao, JHe, and ZiZ were employed by Inner Mongolia Yili Industrial Group Co., Ltd. and Inner Mongolia Dairy Technology Research Institute Co. Ltd.

The remaining authors declare that the research was conducted without any commercial or financial relationships constructed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnut.2023.1273531/full#supplementary-material

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EDITED BY
Jamilah Hanum Abdul Khaiyom,
International Islamic University Malaysia,
Malaysia

REVIEWED BY
Claudia Hunot-Alexander,
University of Guadalajara, Mexico
Anna Cecília Queiroz De Medeiros,
Universidade Federal do Rio Grande do Norte,
Brazil

*CORRESPONDENCE
Jiaming Lin
✉ 605737714@qq.com
Jasmine Adela Mutang
✉ jasmine@ums.edu.my

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Assessing the factor structure of the Eating Attitude Test-26 among undergraduate students in Malaysia

Walton Wider[1], Jasmine Adela Mutang[2]*, Bee Seok Chua[2], Jiaming Lin[1]*, Assis Kamu[3] and Nicholas Tze Ping Pang[4]

[1]Faculty of Business and Communications, INTI International University, Nilai, Negeri Sembilan, Malaysia, [2]Faculty of Psychology and Education, University Malaysia Sabah, Kota Kinabalu, Malaysia, [3]Faculty of Science and Natural Resources, University Malaysia Sabah, Kota Kinabalu, Malaysia, [4]Faculty of Medicine and Health Sciences, University Malaysia Sabah, Kota Kinabalu, Malaysia

The objective of this study was to assess the factor structure of the 26-item Eating Attitude Test (EAT-26) through confirmatory factor analysis (CFA) among 1,084 undergraduate students in Malaysia. The initial findings indicated a lack of support for the proposed three-factor structure. Model modifications were made due to the inadequate initial fit. The fit of the model was significantly improved by excluding items with factor loadings below 0.40 and integrating residual covariances. In conclusion, it is necessary to make contextual modifications to the EAT-26 in order to effectively utilize it among Malaysian undergraduates. This highlights the significance of cultural adaptations in psychological instruments.

KEYWORDS

confirmatory factor analysis, Eating Attitude Test, eating disorder, undergraduate students, obesity

Introduction

Eating disorders among young adults have increased in recent years as a result of the ongoing pandemic (1). Eating disorders are defined as a persistent irregularity of eating or eating-related behaviors that leads to abnormal food consumption and severely harms physical and psychological health in the Diagnostic and Statistical Manual of Mental Disorders, 5th edition (DSM-5). University students who had previously relied on outside food may have developed eating disorders as a result of the reinforcement of several strict lockdowns. Furthermore, a lack of physical activity during the pandemic may have resulted in abnormal food consumption among university students (2). Other negative effects of the pandemic on young adults included disruption of routines and increased psychological distress, both of which had a negative impact on food consumption (3).

Globally, when young adults graduate from high school and begin university, many of them will be living away from home for the first time (4). As a result, university life is an important time for shaping their eating attitudes and behaviors, which may be linked to weight gain and poor health in their emerging adulthood (5). South-East Asia's lower- and upper-middle-income countries have a higher prevalence of disordered eating than other regions of the world (6). Many issues concerning eating habits confront university students. According to a Malaysian study, students preferred to eat foods that were easy to consume and were less expensive than those that were healthy and nutritious (7). Furthermore, it was

discovered that, while the majority of the students ate meals on a daily basis, they had a tendency to skip meals, particularly breakfast. Another local study revealed a link between disordered eating and negative body image in young adults (8). University students face various academic and social challenges. These factors can exacerbate pre-existing personal issues, such as negative body image perceptions, which frequently originate from childhood experiences. Perceptions during childhood and adolescence can be intensified by influences from peers, media, and familial expectations. Entering university can intensify existing insecurities due to additional stressors. It is crucial to recognize and confront the possible exacerbation of body image issues in university settings.

A separate locally conducted study found that adolescents who were dieting and suffering from Post-Traumatic Stress Disorder (PTSD) were more likely to develop eating disorders (9). According to the study's findings, 13.9% of university students in the sample were at risk of developing an eating disorder. A more recent Malaysian study found that one in every five university students had an eating disorder (5). Female students exhibited higher rates of disordered eating than male students. The study revealed that 22.9% of female university students had disordered eating, compared to 13.3% of their male counterparts. Among male students, only depressive symptoms were a reliable predictor of disordered eating. In females, however, depressive symptoms were the most important factor, followed by body size satisfaction and appreciation. These findings provide preliminary evidence that eating disorders increase the risk of depression, possibly due to an inability to control eating habits such as binge eating and caloric restraint. This can lead to a failure to achieve a desired physique, which can lead to depression.

The Eating Attitudes Test (EAT) was initially developed as a 40-item questionnaire designed to serve as a global index for symptoms of anorexia nervosa (10). The updated version, known as the Eating Attitudes Test-26 (EAT-26), is a more concise self-report questionnaire consisting of 26 items. Created by Garner et al. (11), the EAT-26 is geared toward examining attitudes and behaviors related to disordered eating. It measures three factor scores: (a) dieting, which assesses avoidance of fattening foods and preoccupation with weight loss; (b) bulimia and food preoccupation, which gauges intrusive thoughts about food and symptoms of bulimia; and (c) oral control, which measures self-control around eating and perceptions of societal pressure to gain weight. Studies have shown that the 14 items removed from the original EAT-40 were redundant, as evidenced by a strong correlation between EAT-26 and EAT-40 scores (12). Additionally, the third factor (oral control) is inversely related to weight and bulimia, while the second factor (bulimia and food obsession) is positively related to bulimia symptoms and higher body weight—both of which have implications for outcomes (13). The EAT-26 has been extensively utilized both in clinical and non-clinical settings to identify individuals at risk for eating disorders (14). The EAT-26 has been used by psychologist and clinical practitioners worldwide (15). It is worth noting that the tool has been translated into multiple languages and adapted for various cultural contexts, including Italian (16), Lebanese (17), Brazilian (18), Chinese (19), and Japanese (20).

Eating Attitudes Test-26 has increasingly been the instrument of choice in epidemiological studies examining eating disorders. In a study by Khaled et al. (21) involving 2,692 young female university students from Qatar—a Muslim-majority country in the Middle East—the factor structure and measurement invariance of the EAT-26 were examined across language and BMI categories. While the EAT-26 demonstrated strong internal consistency overall, the study revealed issues with the scale's measurement properties related to language and BMI. These limitations suggest that when using EAT-26, or its shorter versions, to assess disordered eating among adolescent Arabic-speaking females with varying body weights, caution should be exercised due to these significant measuring difficulties.

A cross-sectional survey conducted at a large North Carolina university targeted undergraduates aged 18–25 years. The majority of participants were White (22). To calculate their BMI, the participants self-reported their weight and height. In addition to demographic information, participants completed the eating attitudes (EAT 26) scale to determine their proclivity for dieting. Only 12% of the subjects had disturbed eating behavior, which was lower than previously reported. Nonetheless, the findings support the widely held belief that improper dieting and disordered eating habits are common among college students, particularly females. At the same time, 10% of college males reported having disordered eating attitudes, demonstrating that the problem is not limited to young female students. In a Malaysian study by Edman and Yates (23), the researchers examined the relationship between eating habits and BMI among ethnic Chinese and ethnic Malay college students. The study utilized the EAT-26 and included 187 Malay and 80 Chinese students as participants. The study found no gender-based differences in disordered eating attitudes within either ethnic group. However, Malay students exhibited higher EAT-26 scores compared to their Chinese counterparts. This contrasts with research commonly conducted in Western settings, where gender differences in attitudes toward disordered eating are often observed.

Inconsistent and ambiguous factor-analytic results have fueled debate over the EAT-26's factorial validity, as well as its general structure and utility as a screening tool in non-clinical samples. The differences in eating habits and body-image standards between English-speaking and non-English-speaking countries have been attributed primarily to cultural differences (24). Maïano et al. (25) investigated the factor structure of the EAT-26 in one of the largest groups (n = 1,779) of ethnically diverse Europeans and Africans in a series of experiments. This study's sample included adolescent males and girls in France, aged 11–18 years, who could communicate in French. Using exploratory structural equation modeling (ESEM) and confirmatory factor analysis (CFA), the authors replicated the best-fitting six-factor model with 18 items from the EAT-26. These included social pressure to gain weight, fear of becoming overweight, eating-related control, eating guilt, food obsession, vomiting-purging behavior, and food preoccupation. Another less studied but critical feature is the demonstration of measurement invariance or equivalence across significant subgroups, i.e., the same construct is measured in each subgroup. While a few studies have found measurement inconsistency for the EAT-26 in English across ethnic groups, this has rarely been investigated for translations (26). Before presenting evidence of cultural group differences and similarities, linguistic measurement invariance in the mother tongue should be demonstrated. Without it, it is impossible to rule out the

possibility that observed group differences are not transferable across languages or cultures.

Eating Attitudes Test-26 factorial validity and utility as a screening tool for eating disorders in non-clinical samples have been called into question due to its inconsistent and ambiguous factor-analytic results. It has been hypothesized that cultural differences, such as varying eating habits and body image standards between English-speaking and non-English-speaking countries, may contribute to these inconsistencies. In light of these considerations, the aim of this study is to examine the factor structure of the EAT-26 in the context of undergraduate students in Malaysia.

Methods

Participants and procedure

The participants in this study consisted of 1,084 undergraduates from one of the public universities in Sabah who were recruited through the use of a convenience sample. Prior to data collection, the EAT-26 was translated into Malay language by the researchers, who then asked two psychologists who are fluent in both English and Bahasa Malaysia in translating the translated instrument (the Malaysian version) back into English using a back translation technique (27). Informed consent was obtained from each student, ensuring they understood the purpose of the study and their rights as participants. The data was collected from first to third-year students who took the faculty's compulsory courses. The data collection was conducted in one of the lectures of their compulsory course and the questionnaires were distributed to the students by the researcher after the lecture ended.

Measure

The Malay version of EAT-26 was used to validate its applicability for assessing disordered eating attitudes and behaviors among undergraduate students in Malaysia. The EAT-26 is a 26-item questionnaire that assesses three major areas of disordered eating attitudes and behaviors: dieting behavior (e.g., "I am preoccupied with a desire to be thinner"), bulimia and food preoccupation (e.g., "I give too much time and thought to food"), and oral control (e.g., "I find myself eating when I'm not hungry"). The items are scored on a 6-point Likert scale, with higher scores indicating more severe disordered eating attitudes and behaviors. The EAT-26 is a validated and reliable measure of disordered eating attitudes and behaviors. Cronbach's alpha coefficients ranged from 0.77 to 0.91 across various populations, indicating good internal consistency (11, 28, 29). Over a 4-week period, the EAT-26 was also shown to have good test-retest reliability, with intraclass correlation coefficients ranging from 0.77 to 0.92 (11, 28). The EAT-26 has been used in a variety of settings to identify people at risk for eating disorders and to evaluate the efficacy of interventions. It has been demonstrated to be sensitive to changes in eating attitudes and behaviors following treatment (30) and to have good predictive validity for identifying people at risk for eating disorders (11, 28).

TABLE 1 Demographic information about respondents (N = 1,084).

	Frequency	Percentage (%)/mean (SD)
Age		21.7 years (1.00)
Gender		
Male	346	31.9
Female	738	68.1
Ethnicity		
Malay	225	20.8
Chinese	121	11.2
Indian	70	6.5
Bumiputera Sabah	559	51.6
Bumiputera Sarawak	81	7.5
Others	28	2.6
Academic year		
Year 1	57	5.4
Year 2	147	13.6
Year 3	802	74.0
Year 4	78	7.2

Data analysis

Confirmatory factor analysis was used to test whether the suggested three-factor model of the EAT-26 could be confirmed among Malaysian undergraduate students, using the IBM SPSS AMOS 23 Program (maximum likelihood estimation method). To evaluate the model's fit to the data, Hu and Bentler (31) suggested using Comparative Fit Index (CFI > 0.90), Tucker-Lewis Index (TLI > 0.90), Root Mean Square Error of Approximation (RMSEA 0.05–0.08), and Standardized Root Mean Square Residual (SRMR 0.08). Furthermore, the EAT-26's reliability was assessed using the internal consistency Cronbach alpha method.

Results

Respondents' demographic characteristics

The participants' mean age was 21.7 years (SD = 1.00). The majority of participants (68.1%) were female, with 31.9% being male. In terms of ethnicity, the largest group was Bumiputera Sabah (51.6%), followed by Malays (20.8%), Chinese (11.2%), Indians (6.5%), Bumiputera Sarawak (7.5%), and others (2.6%). In terms of academic year, Year 3 (74.0%) had the most participants, followed by Year 2 (13.6%), Year 4 (7.2%), and Year 1 (5.4%). **Table 1** summarizes the sample's demographic characteristics.

Confirmatory factor analysis

Confirmatory factor analysis with maximum likelihood estimation was used to evaluate the EAT-26 factor structure.

TABLE 2 Standardized coefficients and associated data.

Factor	Indicator	Symbol	Estimate	SE	*z*-Value	*p*	95% Confidence interval	
							LL	UL
Factor 1	DIET1	λ11	1.129	0.043	26.092	<0.001	1.044	1.214
	DIET2	λ12	0.699	0.040	17.366	<0.001	0.620	0.778
	DIET3	λ13	0.653	0.034	19.208	<0.001	0.587	0.720
	DIET4	λ14	0.786	0.035	22.553	<0.001	0.718	0.855
	DIET5	λ15	1.338	0.043	31.380	<0.001	1.255	1.422
	DIET6	λ16	1.148	0.043	26.659	<0.001	1.064	1.233
	DIET7	λ17	1.182	0.043	27.464	<0.001	1.098	1.267
	DIET8	λ18	0.567	0.036	15.730	<0.001	0.497	0.638
	DIET9	λ19	0.614	0.033	18.433	<0.001	0.549	0.679
	DIET10	λ110	0.525	0.036	14.614	<0.001	0.454	0.595
	DIET11	λ111	0.836	0.033	25.235	<0.001	0.771	0.901
	DIET12	λ112	0.604	0.035	17.228	<0.001	0.535	0.672
	DIET13	λ113	0.231	0.048	4.803	<0.001	0.137	0.325
Factor 2	BFP1	λ21	0.616	0.045	13.576	<0.001	0.527	0.705
	BFP2	λ22	0.669	0.039	17.170	<0.001	0.592	0.745
	BFP3	λ23	0.217	0.023	9.288	<0.001	0.171	0.263
	BFP4	λ24	0.779	0.036	21.523	<0.001	0.708	0.850
	BFP5	λ25	0.781	0.038	20.758	<0.001	0.707	0.854
	BFP6	λ26	0.265	0.028	9.426	<0.001	0.210	0.320
Factor 3	ORC1	λ31	0.545	0.032	17.228	<0.001	0.483	0.607
	ORC2	λ32	0.521	0.042	12.457	<0.001	0.439	0.604
	ORC3	λ33	0.266	0.053	5.039	<0.001	0.163	0.370
	ORC4	λ34	−0.063	0.060	−1.042	0.298	−0.180	0.055
	ORC5	λ35	0.487	0.046	10.524	<0.001	0.396	0.578
	ORC6	λ36	0.582	0.037	15.942	<0.001	0.511	0.654
	ORC7	λ37	0.507	0.039	12.929	<0.001	0.430	0.584

However, the results revealed that the hypothesized factor structure did not match the current dataset well. The fit indices, in particular, did not meet the acceptable thresholds established by Hu and Bentler (31). The CFI was calculated to be 0.662, the TLI to be 0.629, the RMSEA to be 0.102, and the SRMR to be 0.087.

Despite the misalignment of the overall structure, a closer examination of individual items yielded more encouraging results. Except for the ORC4 item, all factor loadings ranged from 0.15 to 0.82 and were statistically significant (as shown in Table 2 and Figure 1). These findings indicate that the majority of the items were successfully loaded onto their designated factors. Furthermore, factor correlations were found to be statistically significant, indicating a moderate to high level of interdependence between the factors. Table 3 details these correlations. The significance of these correlations necessitates additional investigation to ensure that there is no undue overlap that could affect the discriminant validity of the factors.

Cronbach's alpha was used to assess internal consistency for each of the factors. With a value of 0.867, factor 1 demonstrated commendable internal consistency. With a value of 0.670, factor 2 demonstrated acceptable consistency. Factor 3's consistency, on the other hand, was relatively low and questionable, with a value of 0.587. The lower consistency score for factor 3 suggests that the items in this factor may need to be refined or reconsidered in order to improve their reliability.

Model plot

Modifications were deemed necessary due to the poor model fit. As shown in Figure 2 and Table 4, two major adjustments were made. To begin, items with factor loadings less than 0.40 were removed to improve model fit. Second, residual covariances were added based on modification indices. Following these changes, the model's fit improved significantly: CFI = 0.870, TLI = 0.847, RMSEA = 0.075, and SRMR = 0.059. Internal consistency of the revised model improved significantly for factors 1 and 2. Factor 1's consistency increased to 0.883 and factor 2's to 0.701. However, factor 3's consistency dropped slightly to 0.553, indicating that it should be scrutinized further or modified in future iterations.

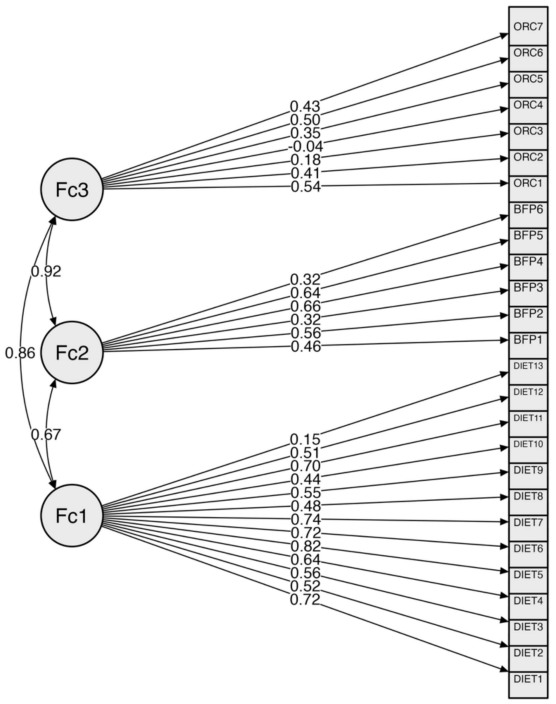

FIGURE 1
Factor structure with maximum likelihood estimation.

Discussion

The findings of this study suggest that the hypothesized three-factor model of the EAT-26 did not achieve an acceptable fit with the data from Malaysian undergraduate students. The fit indices (CFI = 0.662, TLI = 0.629, RMSEA = 0.102, SRMR = 0.087) fell short of the recommended criteria for an acceptable fit, as outlined by Hu and Bentler (31). This outcome aligns with previous research that has questioned the validity of the EAT-26 in various non-Western cultures, including those in Iran, Pakistan, Hong Kong, Israel, and the Arab world (21, 32–35). Notably, five items (ORC3, ORC4, BFP3, BFP6, and DIET13) on the EAT-26 had low factorial loadings (<0.40). This observation is consistent with the findings of Ocker et al. (36), who also reported poor fits for the three-factor models of the EAT-26. The inadequate fit may be attributable to a variety of factors, including cultural nuances, the use of non-clinical samples, and inherent limitations of the instrument itself. As a result, items with low factorial loadings may need to be revised or excluded from the questionnaire. Further corroborating the concerns about the EAT-26, multiple studies have identified poor internal consistency for the scale [e.g., (36, 37)]. Taken together, these findings cast doubt on the reliability and validity of the EAT-26 as a measure of disordered eating attitudes and behaviors among Malaysian undergraduate students.

Eating disorders are a worldwide problem that can have a significant impact on an individual's overall health and quality of life including university students. Several studies conducted on the incidence of eating disorders among Malaysian university students have revealed that a range of 11–43% of the student population is susceptible to developing an eating disorder (9, 38–40). The EAT-26 is a self-report measure that has been extensively researched to assess symptoms and concerns that are typical of eating disorders, and has frequently been employed as a screening instrument for evaluating the likelihood of developing an eating disorder. The EAT-26 assessment tool has the potential to be utilized in both clinical and non-clinical environments, without a specific emphasis on eating disorders.

In light of the results we've discussed earlier, it is crucial to consider the broader context of eating disorders, notably among university students. The EAT-26 assessment tool, which we utilized in our study, is designed to screen for symptoms and concerns typical of eating disorders. It is a versatile tool applicable in both clinical and non-clinical settings.

As detailed in our results section, the EAT-26 assesses three key factors: dieting, bulimia, and oral control. Although the tool was originally validated in Western contexts, it has been adapted for various cultures (41–43). Its psychometric properties have been both lauded and criticized in academic literature. While some studies affirm its robustness (36, 41, 43, 44), others question its reliability and validity (21, 32, 36, 42). One area that remains ambiguous is the cultural applicability of the EAT-26 in Malaysia, especially among university students—a primary focus of our study. Although we did not employ qualitative methodologies in this research, future studies could consider using "Think-aloud" techniques to probe deeper into the language and cultural nuances affecting the EAT-26's reliability and validity in non-Western contexts (45).

Our findings suggest that the cultural milieu in Malaysia might significantly impact the EAT-26's validity. The cultural norms around food and hospitality in Malaysia, as highlighted by the concept of "Third Space" (46) and work by Perry (47), could render some items in the Oral Control factor incongruent with local customs. Phrases like "please eat more and enjoy the food" are indicative of these deeply rooted cultural norms. Another aspect to consider, as revealed in our analysis, is the challenge of linguistic translation. The EAT-26 underwent adaptation from its original English version to the Malay language via back to back translation. However, this translation process might not have adequately captured the subtleties of local cultural nuances and lack of comprehensive cross-cultural adaptation process. In

TABLE 3 Correlation between factors.

	Estimate	SE	*z*-Value	*p*	95% Confidence interval	
					Lower	Upper
Factor 1 ↔ factor 2	0.666	0.026	26.088	<0.001	0.616	0.716
Factor 1 ↔ factor 3	0.863	0.031	27.525	<0.001	0.802	0.925
Factor 2 ↔ factor 3	0.916	0.030	30.178	<0.001	0.856	0.975

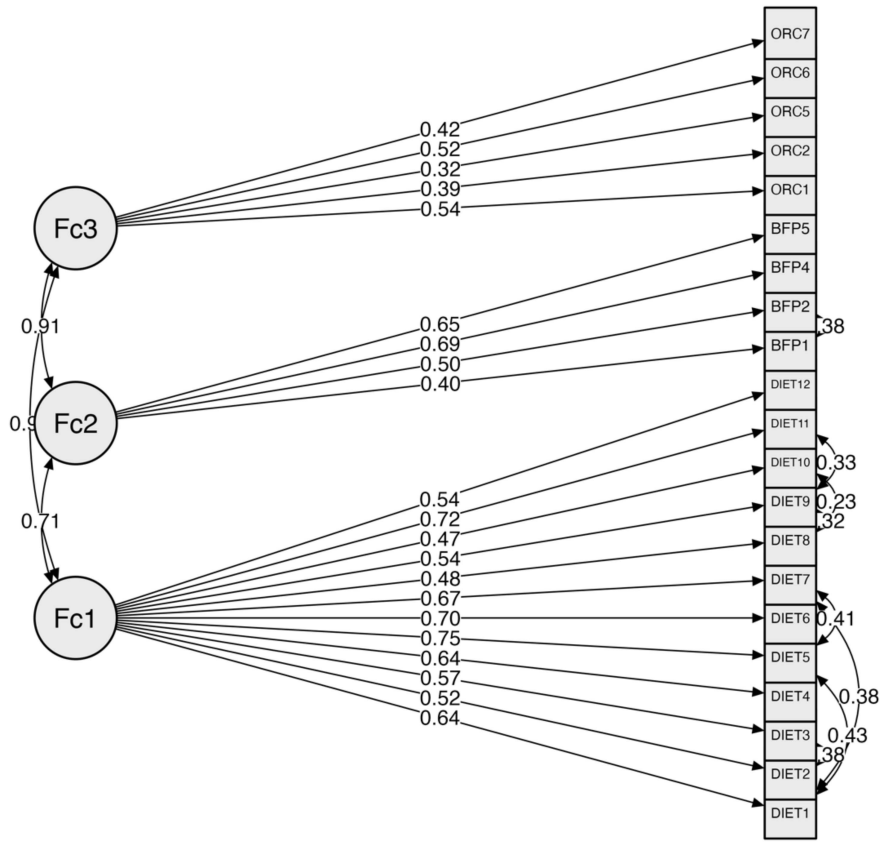

FIGURE 2
Factor structure with maximum likelihood estimation (after modification).

TABLE 4 Correlation between factors (after modifications).

	Estimate	SE	*z*-Value	*p*	95% Confidence interval	
					Lower	Upper
Factor 1 ↔ factor 2	0.709	0.027	26.356	<0.001	0.656	0.762
Factor 1 ↔ factor 3	0.937	0.025	37.672	<0.001	0.888	0.986
Factor 2 ↔ factor 3	0.908	0.033	27.775	<0.001	0.844	0.972

this approach, the questionnaire was translated from its original English version into, potentially resulting in certain questionnaire items becoming less relevant or difficult to interpret within the Malaysian culture context. Consequently, this limitation could pose significant concerns regarding the validity of the tool within this cultural setting, potentially being the primary factor hindering the interpretation of the CFA results. The absence of a more thorough cross-cultural adaptation process could compromise the questionnaire's reliability and validity in the Malaysian context. This concern was supported by our results, which found poor factor loadings for items in the Bulimia and Food Preoccupation factors, likely due to our non-clinical sample. This aligns with recent studies that question the EAT-26's applicability in non-clinical settings (48, 49). Moreover, critics argue that the EAT-26 may be overly complex for use in general health surveys (50).

Finally, the limitations of our study deserve acknowledgment. The cross-sectional design and convenience sampling method limit the findings' generalizability. Additionally, the original validation sample for the EAT-26 differed significantly from ours, being a clinical female sample diagnosed with anorexia nervosa. Furthermore, the self-reported nature of our data introduces the potential for social desirability and recall biases, which could either underreport or exaggerate the psychopathology associated with eating disorders.

Conclusion

The implications of this research warrant careful consideration, particularly in the context of shifting lifestyle and dietary patterns influenced by rapid urbanization and economic growth (51). Recent observations point to increasing rates of obesity (52), growing preferences for fast food and processed meals, more sedentary lifestyles, and heightened exposure to Western beauty standards that glorify thinness. Implementing early detection of eating disorders could serve as a cost-effective public health

strategy, notably in educational environments where interventions are both practical and economically viable (53).

Our study contributes to a better understanding of the applicability of the EAT-26 questionnaire within a Malaysian context, which is crucial for the development of culturally relevant interventions and policies for treating eating disorders in this particular demographic. However, it is important to note that while the EAT-26 is a widely used tool for assessing abnormal eating behaviors, its factorial structure appears to be less robust, especially among the non-clinical sample of university students that we examined.

The homogeneity of our sample—comprised solely of university students, largely of the same age and predominantly single—poses limitations on the generalizability of our findings to the broader population. Additionally, our results raise questions about the potential influence of cultural factors on how individuals interpret the EAT-26 items. Further research is warranted to validate these preliminary observations and to assess the utility of the original three-factor structure of the questionnaire in various cultural contexts within Malaysia.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

Ethical approval was not required for the studies involving humans because the study was conducted according to the guidelines of the Declaration of Helsinki and following academic ethics. The studies were conducted in accordance with the local legislation and institutional requirements. The participants provided their written informed consent to participate in this study.

Author contributions

All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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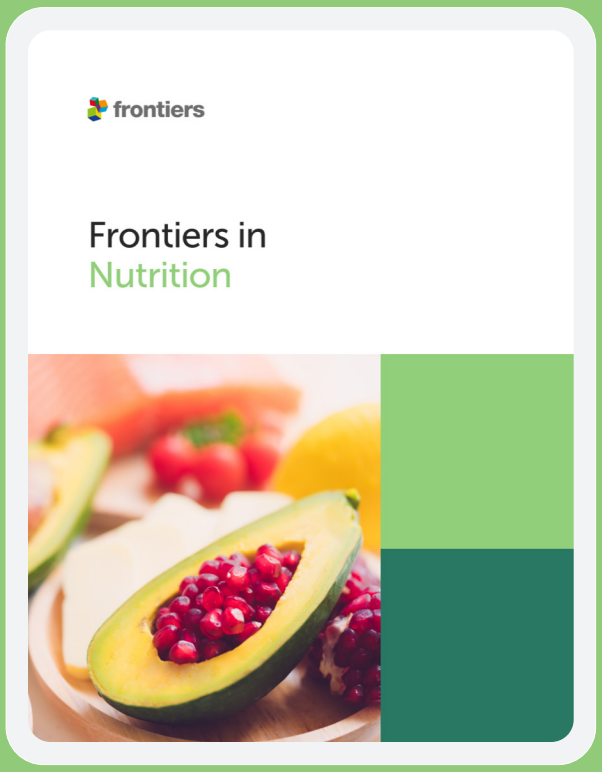

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