Effects of black rice anthocyanins on the behavior and intestinal microbiota of mice with chronic unpredictable mild stress

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Abstract. In order to study the effect of black rice anthocyanins (BRACs) on the behavior and intestinal microbiota of depression model mice, depression model mice were induced by chronic unpredictable mild stress (CUMS). The anhedonia of BRACs treated mice was evaluated by sucrose preference test. The open field experiment was used to analyze the behavior of the mice. The species differences of intestinal microbiota were analyzed by 16S rRNA gene sequence. The results showed that compared with the normal group, the body weight and sugar water consumption rate of mice in the model group were significantly decreased, and the voluntary activities were significantly weakened. The species richness and diversity of intestinal microbiota of mice in the model group were significantly decreased. The levels of Bacteroidetes and Proteobacteria at the phylum level were increased, the levels of Firmicutes were decreased, and the levels of Lactobacillus at the genus level were decreased. Compared with model group, the body weight and sugar water consumption of mice in BRACs treated group were significantly increased, the autonomous activity was significantly increased and the difference was extremely significant, the species richness and diversity of intestinal microbiota increased, and the levels of Bacteroidetes and Proteobacteria decreased, while the levels of Firmicutes increased. The level of lactic acid bacteria increased at the genus level. In summary, BRACs achieve antidepressant effects by regulating intestinal microbiota homeostasis.

1. Introduction
Depression is one of the main types of mental disorders, is a variety of general emotional disorders of the emotional mental illness, it seriously affects the health of the human body, clinical can be seen in low mood, depression, grief, and even suicidal thoughts or behavior misanthropy, serious people can appear hallucinations, delusions and other psychiatric symptoms. Nowadays, the incidence of depression is increasing year by year, and it has become a global health problem. At present, antidepressants have some problems such as narrow spectrum of antidepressants and obvious adverse reactions, so it is necessary to further study the effective treatment methods and drugs for the prevention and treatment of depression[1,2].

In recent years, some achievements have been made in the treatment of depression with natural products. Anthocyanin is a secondary metabolite unique to plants, which has a variety of biological activities related to human health, such as anti-oxidation, anti-inflammation and improving atherosclerosis. Therefore, vegetables rich in anthocyanin have become a pursuit of food consumption. The health effects of black rice are closely related to the anthocyanins (BRACs) in its husk. As a
health food, black rice is digested and absorbed through intestinal tract, and it is worth exploring whether it has a regulating effect on gut microbiota. In this study, an animal model was used to study the antidepressant activity of BRACs, the behavioral and gut microbiota changes of mice were evaluated by intragastral administration of BRACs, so as to explore whether BRACs can improve the antidepressant ability of mice, providing experimental basis for the development and utilization of BRACs[3,4].

### 2. Materials and methods

#### 2.1. Extraction and purification of anthocyanins from black rice

Remove the impurities visible to the naked eye in the black rice, crush it with a high-speed grinder, sieve it through 60 mesh, get black rice flour. The extraction temperature was 50 °C, pH was 2.2, and the extraction time was 2.5 h, with the ratio of solid to liquid 1:18. The extract was centrifuged at 4000 r/min for 15 min to remove large particles and obtain the supernatant. The supernatant is passed through a 30 kDa membrane separator to remove starch, protein and other macromolecules, and the filtrate is collected. The filtrate was concentrated with 200 Da nanofiltration membrane to obtain a concentrated solution. X-5 macroporous resin was used to further purify the concentrated solution. The pH value of the sample solution was 3.0, the adsorption flow rate was 2.0 mL/min, and the equilibrium time was 3.0 h. The elution was carried out with 80% ethanol and the eluent was collected. The eluent was concentrated with 200 Da nanofiltration membrane to obtain a concentrated solution of anthocyanin. The concentrated solution was freeze-dried at -60 °C and -1.0 MPa until the moisture content was less than 0.5% to obtain black rice anthocyanin powder.

#### 2.2. Experimental animals

A total of 30 SPF grade male C57BL/6 mice, weighing ~25g, were purchased from Beijing Vitong Lihua Experimental Animal Technology Co., Ltd. Mice were reared in the Mice Laboratory of Barrier Area, Medical Experimental Animal Center, Jianghan University. SPF grade mice were fed with growth and reproduction feed at room temperature of 20 ~ 26 ℃ and relative humidity of 40% ~ 70%, with free water and diet. All Laboratory animal-related operations in this study are strictly in accordance with the Guide for the Care and Use of Laboratory Animals.

#### 2.3. Model establishment and administration program

After 1 week of animal adaptive feeding, the sugar water consumption test was carried out. Ten mice with similar scores were randomly selected as the normal group, with 5 mice in each cage, and the rest mice were raised alone. Except for the normal group, the isolated mice were subjected to chronic unpredictable mild stress (CUMS) modeling within 4 weeks [5], and the stress factors included water ban (24h), fasting (24h), day-night reversal (24h), tail clamping (5min), swimming in ice water at 4℃ (5min), damp padding (24h), and heat stress at 45℃ (5min). 1 kind of stimulus is given daily and applied randomly, so that the animals can not predict the next stimulus and avoid the animals to produce adaptability. After 28 days of modeling, the mice were randomly divided into 3 groups with 10 mice in each group, namely, normal group (C), model group (M) and BRACS group (BRACs). Normal group and model group were given water, and BRACS group was given 100 mg/kg by intragastric administration for 30 days.

#### 2.4. Sugar water consumption test

Sugary water consumption test is a potent indicator of anhedonia, a central symptom of depression, in animals.

All mice were given 1 bottle of pure water and 1 bottle of 1% (W/V) sucrose water for the sugar water consumption experiment. The positions of the two bottles were placed alternatively regularly to eliminate the possibility of side or position preference. After 24h, the consumption was weighed and the proportion of sugar water consumption was calculated. Mice were trained before the formal test. In
brief, the mice were given two bottles of 1% sucrose solution in each cage for the first 24 hours, followed by a bottle of pure water for the next 24 hours. After the acclimation, the mice were fasted and deprived of water for 24 hours, after which the baseline test for 12 hours began.

2.5. Open field test
The open field test was carried out in a 40cm×40cm×50cm behavior test box, with the bottom divided into 4×4 square squares. The mice were placed in the center square at the bottom of the open box, and a camera was fixed in the middle of the top of the box for recording and timing. The computer was used to record the number of mice entering the center, moving distance and standing times within 5min. At the end of a single experiment, the urine and feces of each animal were cleaned, and the bottom of the box was wiped with alcohol. The next animal experiment was carried out after the odor dissipated.

2.6. 16S rRNA gene sequencing
Fecal samples were taken from mice in each group, and total DNA of fecal flora was extracted for amplification. 16S rRNA basic targeted sequencing technology was used for sequencing. The diversity and distribution of flora were analyzed, and the species differences of samples at different levels were analyzed. The number of OTU and the main parameters of α diversity were calculated by QIIME software. The β-diversity differences were analyzed by PCOA and the species differences were evaluated by QIIME analysis.

2.7. Statistical analysis
All data are expressed as mean ± standard deviation. SPSS 19.0 statistical software was used to analyze the data. Chi-square test was used for statistical processing, α=0.05 was used as the test level, \( P < 0.05 \) indicates that the difference is statistically significant.

3. Results

3.1. Body weight and sugar water consumption of mice
After 30 days of administration, compared with the control group, body weight and sugar water consumption rate of mice in the model group decreased, and the differences were significant \( (P < 0.05) \), but there were no significant differences in body weight and sugar water consumption rate of mice in the BRACS group \( (P > 0.05) \). The body weight of mice in BRACS group was compared with that in model group and sugar water consumption rate were significantly different \( (P < 0.01) \). These results indicate that BRACS can improve the pleasure and appetite of depressed mice, increase food intake, maintain the growth of body weight and improve the symptoms of depression.

![Figure 1](attachment:image.png)

Figure 1. Effects of BRACs on body weight and sucrose consumption in mice. (A) Body weight was reduced after 30 d of exposure to UCMS; (B) Sucrose preference was lower in the UCMS group. Data were shown as the mean ± SD \( (n = 10) \).
3.2. Open field test

After 30 days of administration, compared with the control group, the movement distance, the times of entering the center and the times of standing of mice in the model group and the BRACs group were decreased, and the difference between the model group and the control group was extremely significant ($P < 0.01$), while the difference between the BRACS group and the control group was not significant ($P > 0.05$). Compared with the model group, the movement distance, the times of entering the center and the times of standing were increased in the BRACS group. By comparing the movement trajectories of each group of mice in the open field experiment, the results showed that the spontaneous activity of mice was significantly reduced after chronic unpredictable stress stimulation. After BRACS treatment, the spontaneous activity of mice was significantly increased compared with the model group.

![Graphs showing data](image)

Figure 2. Effect of BRACs on the total distance of mice in open field test. Data are represented as mean ±SD (n = 10). (A) total travel distance; (B) number of rearings; (C) number of grooming episodes.

3.3. Gut microbiota

3.3.1. Species composition analysis

From phylum level and genus level, the species composition of fecal flora of three groups of mice was compared. At the phylum level, the dominant bacteria were mainly Firmicutes, Bacteroidetes and Proteobacteria. In addition, relatively low abundances include Actinobacteria, Tenericutes, and undefined bacteria. Compared with the control group, the relative abundance of the fecal flora in the three groups was significantly different. The relative abundance of Bacteroidetes and Proteobacteria in the model group increased, while the relative abundance of Firmicutes decreased. The Bracs group was similar to the control group. Compared with the model group, the relative abundance of Bacteroidetes and Proteobacteria decreased in the Bracs group, while the relative abundance of Firmicutes increased (Figure 3).
3.3.2. Alpha diversity

Alpha diversity analysis is often used to reveal the abundance and diversity of microbial communities. Shannon index was used to evaluate species diversity. The larger the value, the more uniform the individual distribution and the higher the degree of bacterial diversity. The Chao 1 index was used to evaluate species richness. By examining the Shannon index and Chao 1 index of Alpha diversity in each group, the Shannon value in control group and Bracs group was higher than that in model group ($P < 0.05$). Similarly, the Chao1 value in control group and BRACs group was higher than that in model group ($P < 0.05$). The above results indicated that the faecal flora diversity of the control group and the BRACS group was higher than that of the depressed mice induced by CUMS in the model group.

3.3.3. Beta diversity

The first two dimensions of the PCOA chart describe the weighted Unifrac distance between different groups. The PCOA chart shows that PCOA1 and PCOA2 are the two characteristics that cause the largest differences among the three groups of samples, with their contribution rates of 16.09% and 13.56%, respectively. On the whole, the model group could be separated from the control group along PCOA1, and the BRACS group could be separated from the model group along PCOA2.
Figure 5. Principal coordinate analysis based on weighted UniFrac distance

4. Conclusion
In conclusion, BRACs can increase the body weight and sugar water consumption rate of depressed mice, enhance the pleasurable phenomenon of mice, and improve the ability of autonomous activity. Meanwhile, the anti-depressive effect can be realized by enhancing the species richness and diversity of fecal flora of mice and regulating the intestinal species structure.

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