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Research Article

Black Rice Anthocyanidins Regulates Gut Microbiota and Alleviates Related Symptoms through PI3K/AKT Pathway in Type 2 Diabetic Rats

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Black rice anthocyanins (BRAs) have extremely high nutritional value and health care effects. This study investigated the intervention effect of BRAs on type 2 diabetes mellitus (T2DM) and the regulation effect on intestinal microbiota imbalance in T2DM rats. This study established successfully a T2DM model in a high-fat and high-glucose diet combined with streptozotocin (STZ). BRAs intervention reduced significantly the fasting blood glucose level of T2DM rats, improved the glucose tolerance of rats, reduced the blood lipid level and inflammation state, and repaired liver, oxidative stress, and other injuries. In addition, BRAs's intervention enhanced the expression of phosphoinositol 3-kinase (PI3K)/protein kinase B (AKT), activated the expression of adenosine 5'-monophosphate-activated protein kinase(AMPK), and the downstream acetyl-CoA carboxylase (ACC) and carnitine palmitoyl transferase (CPT1) in the liver. 16S rRNA sequencing showed that BRAs significantly decreased the abundances of *Bifidobacterium* and *Clostridiaceae_Clostridium*, and promoted the abundances of *Akkermansia* and *Lactobacillus*. Accelerate the recovery of gut microbiota diversity. BRAs play an antidiabetic role by regulating the PI3K/AKT signaling pathway and intestinal microbiota in T2MD rats.

1. Introduction

In recent years, diabetes has become one of the most common diseases in the world. According to the International Diabetes Federation (IDF), the total number of people with diabetes will rise to 643 million in 2030, and 73.6 million more people will have diabetes worldwide in 2045. Among them, T2DM patients account for more than 90% of all diabetic patients [1]. The primary features of T2DM are chronic hyperglycemia, significant dyslipidemia [2, 3], a low level of the inflammatory response, and insulin resistance [4]. T2DM is a complex metabolic disease, and the phosphatidylinositol 3-kinase (PI3K)/protein kinase B (AKT) signaling pathway plays an important role in regulating insulin resistance and lipid metabolism as an intracellular signaling pathway [5], the imbalance of which leads to obesity and T2DM [6]. Dysregulation of hepatic lipid metabolism causes the accumulation of triglycerides [7], which in turn induces insulin resistance. PI3K/AKT and its downstream signaling factor AMPK can stimulate lipid oxidation and reduce the synthesis of ACC [8, 9], thus improving lipid metabolism and achieving the effect of alleviating T2DM. Dysbiosis of the gut microbiota and impaired intestinal barrier function are emerging factors contributing to the development of T2DM. Ge et al. [10] showed that dysbiosis of the intestinal flora affects lipid metabolism, increases the risk of metabolic disease development and intestinal permeability, and produces low-grade inflammation [11]. The gut microbiota plays a crucial role in metabolism by producing a variety of metabolites such as

short-chain fatty acids and endotoxins or bacterial products to regulate host physiology [12]. Currently, clinical drugs for the treatment of diabetes may have certain side effects and their application is limited. More evidence shows that dietary modifications can alleviate dyslipidemia and hyperglycemia to some extent [9, 13], so the risk of developing T2DM and its complications can be reduced by consuming plants and their bioactive ingredients with efficacy and safety.

Black rice, a genus of rice in the family Gramineae, is a kind of rice used for both food and medicine, with remarkable edible and medicinal values. Black rice contains anthocyanins, phenolic acids, dietary fiber and minerals, and other bioactive components [14]. Among them, BRAs are a natural plant pigment, a water-soluble flavonoid, mainly found in the bran layer and embryo of black rice, which has high nutritional value and health functions [15, 16]. It has been shown that BRAs have antioxidant, antibacterial [17], weight loss [18], anti-inflammatory [19], and organ-protective properties [20]. The interaction of anthocyanins with microorganisms shapes the composition of the intestinal microbiota [21]. Kim et al. [9] demonstrated that phytochemicals and dietary fiber complexes in coarse grains could alleviate T2DM by reducing lipid accumulation and improving lipid metabolism. Recent studies have shown that anthocyanins reduce obesity by modulating the gut microbiota [14]. Therefore, gut microbiota may be a potential target for anthocyanins to improve related diseases including T2DM.

BRAs can be used as a functional food for the prevention of obesity, hyperglycemia, and inflammation-induced diseases, and little is known about their hypoglycemic and hypolipidemic effects by improving gut microbiota. In this experiment, we evaluated the hypoglycemic effect of BRAs by gavage of different doses of BRAs extract and compared the body weight, blood glucose level, lipid metabolism, liver damage, inflammatory factors, oxidative stress level, and intestinal flora balance of T2DM rats. This study will provide a theoretical basis for the prevention and intervention of T2DM by BRAs.

2. Materials and Methods

2.1. Materials and Instruments. Yis Laboratory Animal Technology Co., Ltd. (Jilin, China) delivered the authors 40 male Sprague–Dawley (SD) rats of SPF grade, weighing 180 ± 20 g, with license number SCXK (Ji)-2018–0007.

Black rice was acquired from the local market in Changchun (Jilin, China); STZ was purchased from Shanghai Yuanye Biotechnology Co., LTD; metformin(met) was purchased from Sino-American Shanghai Squibb Pharmaceutical Co., LTD. The serum total cholesterol (TC), triglycerides (TG), low-density lipoprotein (LDL), low-density lipoprotein cholesterol (LDL-C), high-density lipoprotein cholesterol (HDL-C), catalase (CAT), superoxide dismutase (SOD) SOD, malondialdehyde (MDA), aspartate transaminase (AST), alanine aminotransferase (ALT), and alkaline phosphatase (ALP) assay kits were purchased from Nanjing Jiancheng Bioengineering Institute (Jiangsu, China); Tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), interleukin-10 (IL-10), interleukin-6 (IL-6), assay kits, and insulin (Fins) enzyme-linked immunosorbent assay (ELISA) kit were purchased from Jiangsu Enzyme Labeled Biotechnology Co., LTD(Jiangsu, China); RNA extraction kit was purchased from Vazyme Biotechnology Co., LTD(Jiangsu, China). All other chemical reagents were analytically pure.

2.2. Extraction of Anthocyanins from Black Rice. Black rice was crushed into powder and extracted with ethanol/water (80 : 20, v/v) containing 1% citric acid at room temperature until anthocyanin pigmentation disappeared. The water-alcohol anthocyanins were filtered under reduced pressure and then concentrated by a rotary evaporator at 35°C. The anthocyanins were lyophilized and stored at −20°C. The content of total anthocyanins and phenols in the lyophilized powder was determined by liquid chromatography–mass spectrometry and Folin–Ciocalteu method, respectively [22, 23].

2.3. Animal Experimental Design. Forty SPF male SD rats were fed for 7 days under the conditions of temperature 20–22°C, humidity 50–55%, and light 12 hours/dark 12 hours cycle. After one week of adaptive feeding, 8 rats were randomly selected as Con (maintenance feed (MF)), and the other 32 rats were used as Mod (fed with high-fat and high-sugar diet (HFHS)), the detailed formulation of the rat chow is detailed in Table S1. After 8 weeks, the rats in the Mod were injected intraperitoneally with STZ (35 mg/kg) and Con with the same amount of normal saline (NS). After that, the rats were fed with MF and HFHSD feed, respectively. After fasting for 12 hours for one week, fasting blood glucose was measured through the tail vein. The rats whose fasting blood glucose was higher than 16.7 mmol/L were regarded as T2DM model rats.

After the establishment of the T2DM model, the successfully established rats were randomly divided into four groups with 8 rats in each group and fed continuously for 4 weeks. During the experiment, the general state of the rats was observed, and the body weight of the rats was recorded. The grouping diagram is shown in Figure 1.

2.4. Oral Glucose Tolerance Test (OGTT). After 4 weeks, fasted the rats for 12 hours without limitation of drinking water. All rats were given oral glucose (2 g/kg BW). Blood glucose was measured at 0, 0.5, 1, 1.5, and 2 hours, respectively. The OGTT results were expressed as the area under the curve over 120 min.

2.5. Sample Collection and Preservation. At the end of the 4-week gavage period, the rats were fasted without water for 12 h and anesthetized with sodium pentobarbital. Collecting the blood samples by cardiac puncture and centrifuging by 3000 rpm for 15 min at 4°C to collect serum. Killing the rats by cervical spondylolysis, the contents of the cecum were collected and placed in a 2.0 mL aseptic tube. Collecting the small intestine and put all samples in the tissue fixation solution and store them at −80°C.

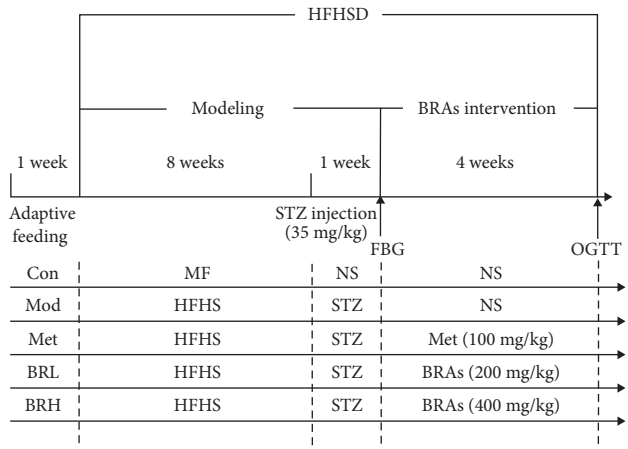

Figure 1: Schematic diagram of animal experiment process. Con: normal group, Mod: model group, Met: positive control group, BRL: black rice anthocyanins low dose group, and BRH: black rice anthocyanins high dose group.

2.6. Determination of Serum Biochemical Indexes. The levels of TC, TG, LDL-C, HDL-C, TNF-α, IL-1β, IL-6, and IL-10 were detected by the corresponding kits.

2.7. Determination of Liver Biochemical Indexes. The levels of CAT, SOD, MDA, GSH-Px, AST, ALT, and ALP were measured by the corresponding kits.

2.8. Histological Study. The small intestine was stained with hematoxylin and eosin (HE), and the histological changes were observed under a light microscope.

2.9. Real-Time Quantitative PCR Analysis of mRNA Expression. Total RNA was extracted from the liver using an RNA extraction kit and detected using a nanophotometer. Quantitative real-time polymerase chain reaction (qRT-PCR) results were normalized by glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression using the QuantStudioTM 6 Flex real-time fluorescence quantification system and HiScript II One Step qRT-PCR fluorescence kit. qRT-PCR conditions were reverse transcription at 50°C for 3 min, followed by 30 cycles of predenaturation at 95°C for 30 s, 95°C for 10 s, and 60°C for 30 s. PCR primer sequences are shown in Table S2.

2.10. Microflora Analysis Based on 16SrRNA Sequencing. About 0.25 g cecal contents were stored in dry ice and sent to Shanghai Paiseno Biotechnology Co., Ltd for 16s rRNA testing. 16s rRNA V3-V4 region was selected to design polymerase chain reaction primers (forward primer: ACT CCTACGGGGAGGAGCA; reverse primer: GGACTACHVGGWTCTAAT). The original sequencing data are processed by the Illumina NovaSeq platform. Using the Divisive Amplicon Denoising Algorithm (DADA2) for depriming, quality filtering, denoising, splicing, and dechimerism, high-quality read data are divided into operational taxons (operational taxonomic unit, OTU) with 97% similarity. Alpha diversity analysis (chao1 index, Shannon index, and Simpson index), Beta diversity analysis (principal coordinate analysis, PCoA), community structure analysis of each group at phylum and genus level, species composition heat map, and other methods to obtain microbial-related information. The sequencing sample information has been submitted to NCBI, and the accession number is PRJNA919151.

2.11. Statistical Analysis. The significance analysis of all experimental data was analyzed by analysis of variance of SPSS 26 statistical software, expressed as mean $\pm s$ standard deviation, $P < 0.05$ represents the significant difference, $P < 0.01$ represents the extremely significant difference, and Origin software was used to draw relevant charts.

3. Results

3.1. Contents of Total Anthocyanins and Phenols in Black Rice. The total phenolic content of the crude extract of black rice was 338.9 mg Gallic acid equivalent (GAE)/g, and the total anthocyanin content of 237.6 mg-3muro-glucoside (Cy3GE)/g. HPLC-MS results showed that the main component of the crude extract of black rice was anthocyanin Cy3GE, which is circled in red (Figure S1). According to the literature [24], other ingredients are procyanidin 3-rutin and epigallocatechin and anthocyanin 3-gentianin, paeoniflorin 3-glucoside, and anthocyanin 3-gentianin (or anthocyanin-3mine5-glucoside, m/z611287).

3.2. Effects of BRAs on Body Weight, Water Intake, and Diet of Rats. During the experiment, the rats in the Con had a regular diet and water, while the rats in the Mod had a significant decrease in body weight, drinking more water and eating more, and were slow and insensitive, which belonged to the typical symptoms of T2DM. As shown in Table 1, after 4 weeks of gavage, the body weight of rats in the Mod decreased significantly, and compared with the Mod, the body weight of rats in the Met and the BR increased significantly, indicating that BRAs have a certain improvement in the living status and body weight of T2DM rats.

Table 1: Effects of BRAs on body weight of T2DM rats.

Group	The body weight of the eighth week	Final body weight	Water intake in the first week of gavage	Water intake in the 4th week of gavage	Dietary amount in the first week of gavage	Dietary amount in the 4th week of gavage
	G		mL/100 g (body weight)		g/100 g (body weight)	
Con	469.81 ± 6.83	560.18 ± 7.74**	21.58 ± 2.83**	20.08 ± 1.79**	5.30 ± 1.06**	7.13 ± 1.39**
Mod	487.85 ± 8.20	311.30 ± 10.73##	121.92 ± 8.31##	136.92 ± 10.80##	20.21 ± 1.34##	18.32 ± 1.05##
Met	491.19 ± 5.23	413.47 ± 6.72**	105.51 ± 9.46*	94.28 ± 5.38**	18.69 ± 1.54	15.26 ± 0.72**
BRL	489.34 ± 4.15	368.84 ± 11.09**	115.10 ± 10.69	107.66 ± 8.30**	19.79 ± 1.47	17.59 ± 0.80
BRH	490.32 ± 6.93	393.16 ± 9.35**	110.51 ± 8.20	98.80 ± 9.70**	20.21 ± 0.91	16.52 ± 0.79*

Data are expressed as means ± SD. Compared with the model group, #was significantly different ($P < 0.05$) and ##was extremely significant ($P < 0.01$). *was significantly different ($P < 0.05$) and **was extremely significant ($P < 0.01$). Con: normal group, Mod: model group, Met: positive control group, BRL: black rice anthocyanins low dose group, and BRH: black rice anthocyanins high dose group.

3.3. Effect of BRAs on the Symptoms of T2DM in Rats. As can be seen from Figure 2(a), the fasting blood glucose concentration of Con rats was maintained between 5 and 6 mmol/L, and the other four groups were significantly increased, above 16.7 mmol/L, indicating that the model of T2DM rats was successful. The concentration of fasting blood glucose decreased in both BRL and BRH, which was significantly different from that of Mod, which indicated that BRAs could improve T2DM to a certain extent, and the effect of BRH was better.

The blood glucose concentration of Con rats maintained a gentle trend, and the blood glucose of each group peaked at 0.5 h after the oral administration of glucose. Among them, the blood glucose concentration of Mod was the highest, reaching 33.27 mmol/L, which is shown in Figure 2(b). Compared with Mod, the blood glucose of BRL rats decreased more rapidly at 0.5–1 h and decreased more slowly at 1-2 h, and the final blood glucose concentration was 25.67 mmol/L at 2 h ($P < 0.05$). The decreasing trend of blood glucose concentration in BRH rats was significantly lower than that in Mod rats from 0.5 h to 2 h ($P < 0.01$).

As shown in Figure 2(c), compared with Mod, BRH, and Met have similar physiological effects and have a very significant tendency to reduce the area under the blood glucose curve, but there is no significant difference between BRL and Mod. It is suggested that BRAs can reduce the level of blood glucose in T2DM rats and alleviate glucose tolerance in T2DM rats to some extent.

Figure 2(d) shows the HOMA-IR of rats. The insulin resistance index of Mod was significantly increased, and other groups after the intervention of Met and BR were significantly lower than that of Mod ($P < 0.01$), indicating that BRAs have some alleviating effect on insulin resistance.

3.4. Effect of BRAs on Serum Levels of TC, TG, LDL-C, and HDL-C in T2DM Rats. Compared with Con, the levels of TC, TG, and LDL-C in Mod increased significantly, while the level of HDL-C decreased significantly ($P < 0.01$) (Figure 2(e)). Compared with Mod, the BR was similar to Met. The levels of TC, TG, and LDL-C were significantly decreased ($P < 0.01$ or $P < 0.05$). For HDL-C level, BRH had a significant increase ($P < 0.05$), while BRL had no significant difference in the increase of HDL-C level. The results showed that BRAs could improve dyslipidemia in rats fed a high-fat diet.

3.5. Effect of BRAs on Inflammatory Factors in Serum of T2DM Rats. As shown in Figure 2(f), compared with Con, the levels of proinflammatory factors TNF-α, IL-1β, and IL-6 in Mod rats were significantly increased, and the level of anti-inflammatory factor 1L-10 was significantly decreased ($P < 0.01$). Compared with Mod, the levels of TNF-α, IL-1β, and IL-6 in the BR were significantly decreased ($P < 0.05$), and the level of IL-10 was significantly increased ($P < 0.05$), indicating that BRAs have a certain regulatory effect on inflammatory factors in T2DM rats. To some extent, it can alleviate the inflammatory state of T2DM rats.

3.6. Effect of BRAs on the Antioxidant Level of Liver in T2DM Rats. CAT, SOD, and GSH-Px levels in the liver of Mod rats were significantly lower than those of Con rats in Figure 3(a), while the level of MDA was significantly higher than that of Con rats ($P < 0.01$), indicating that the antioxidant level in the liver of T2DM rats was significantly abnormal. Compared with the Mod, the SOD level in each treatment group was significantly increased, and the MDA level was significantly decreased ($P < 0.01$ or $P < 0.05$). BRH and Met had similar effects on CAT and GSH-Px levels ($P < 0.01$), but there was no significant difference between BRL and Mod. The results showed that BRH could significantly improve the antioxidant level of T2DM rats.

3.7. Effects of BRAs on AST, ALT, and ALP Levels in the Liver of Diabetic Rats. The levels of AST, ALT, and ALP in Mod rats showed a very significant increase trend ($P < 0.01$), indicating that T2DM can cause liver damage in rats. As shown in Figure 3(b), BRL had a significant trend to reduce the levels of the three enzymes ($P < 0.05$), while BRH had a more obvious trend ($P < 0.01$). It indicates that BRAs have a reversing effect on liver damage.

3.8. Effect of BRAs on Pathological Sections of Rat Small Intestine. Rat small intestine pathological HE staining results are shown in Figure 4, Con neat small intestinal villus, tight, goblet cells without damage phenomenon, Mod small intestinal villus damage is serious, arranged loosely, goblet cells arranged

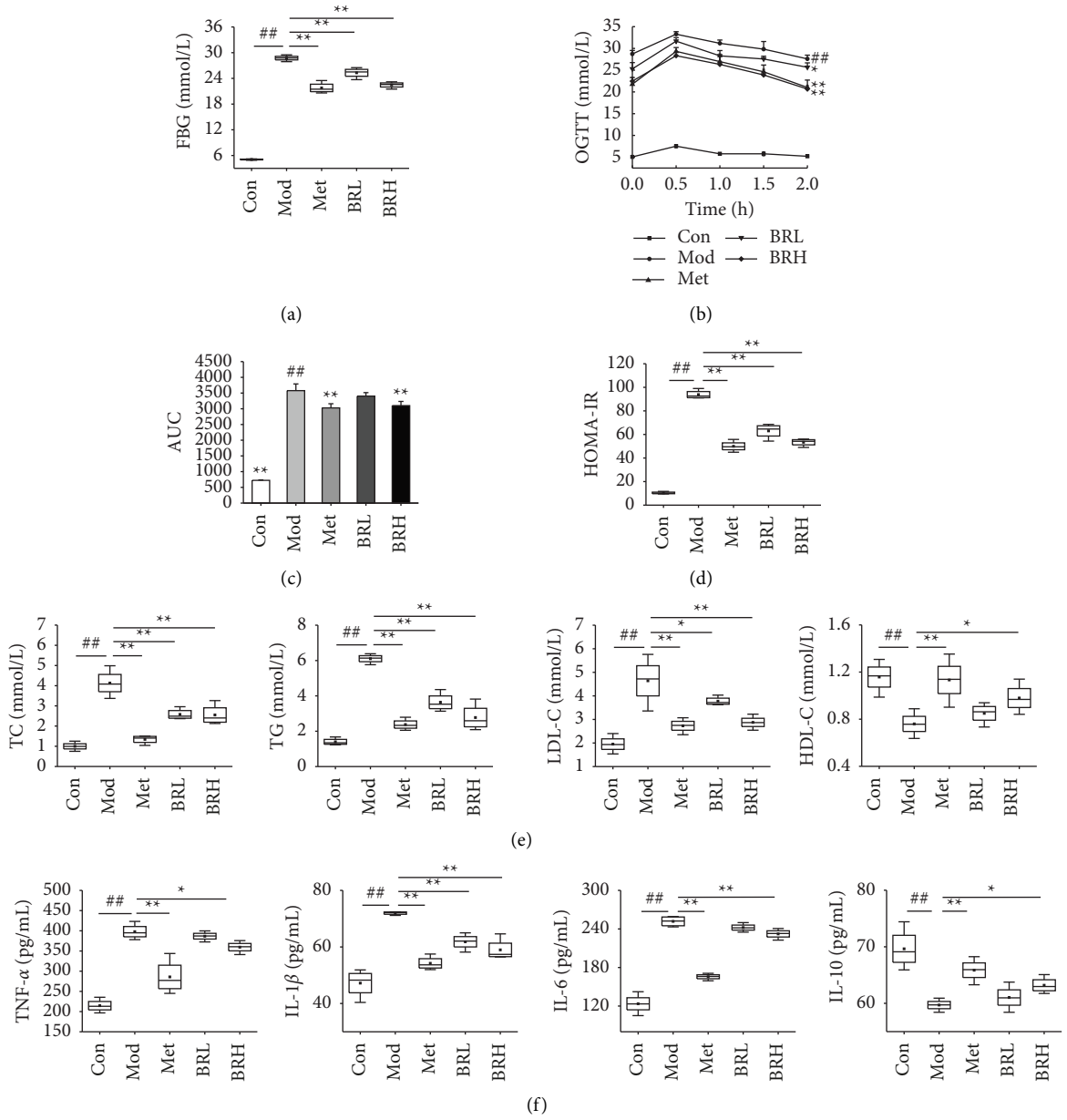

Figure 2: Effects of BRAs on serum-related indicators in T2DM rats: (a) effects of BRAs on FBG in T2DM rats, (b) effects of BRAs on oral glucose tolerance in rats, (c) the effect of BRAs on the area under the curve of blood glucose in rats, (d) effects of BRAs on insulin resistance index in T2DM rats, (e) effects of BRAs on blood lipids in T2DM rats, including TC, TG, LDL-C, and HDL-C, and (f) effects of BRAs on inflammatory factors in T2DM rats, including TNF-α, IL-1β, IL-6, and IL-10. Data are expressed as means ± SD. Compared with the model group, $^{\#}$was significantly different ($P < 0.05$) and $^{\#\#}$was extremely significant ($P < 0.01$). *was significantly different ($P < 0.05$) and **was extremely significant ($P < 0.01$). Con: normal group, Mod: model group, Met: positive control group, BRL: black rice anthocyanins low dose group, and BRH: black rice anthocyanins high dose group.

disorder, compared with the Mod, BRL, and BRH of small intestinal villus with different degrees of recovery shows that BRAs effectively restore the intestinal damage.

3.9. Effect of BRAs on Gene Expression Levels in Rats. Compared with Con, the mRNA expression of PI3K, AKT, AMPK, and CPT1 in the liver of Mod rats was significantly decreased ($P < 0.01$), and the mRNA expression of ACC was significantly increased ($P < 0.01$) (Figure 3(c)). The mRNA expressions of PI3K, AKT, AMPK, and CPT1 in BR were significantly increased ($P < 0.05$), and the mRNA expression of ACC was significantly decreased ($P < 0.05$). These results suggest that BRAs can regulate AMPK, PI3K/AKT, and downstream signaling molecules to improve the symptoms of T2DM.

3.10. Effect of BRAs on Intestinal Microbiota in T2DM Rats

3.10.1. Alpha and Beta Diversity Analysis. 16S rRNA sequencing revealed the intervention effect of BRAs on gut microbiota abundance (Figure 5(a)). The Alpha diversity of gut

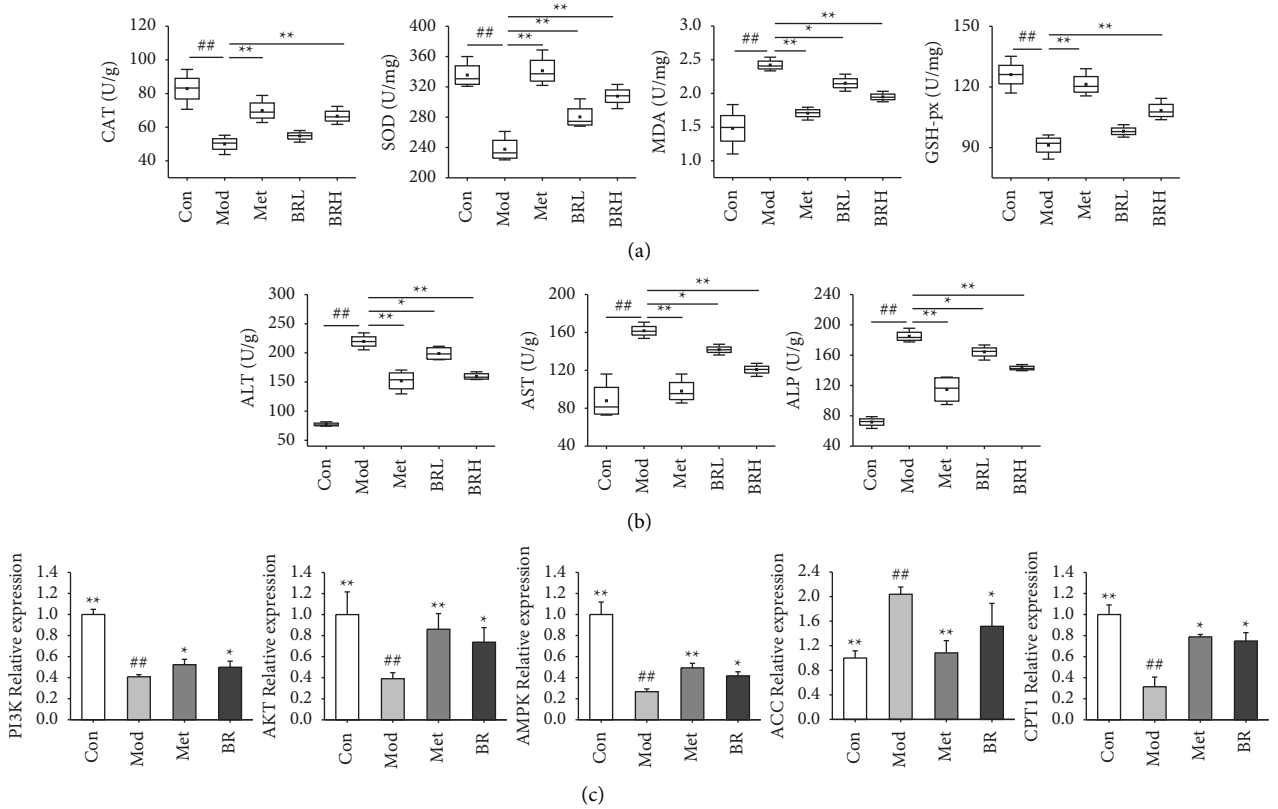

Figure 3: Effects of BRAs on liver-related parameters in T2DM rats: (a) effects of BRAs on the antioxidative ability of T2DM rats, including CAT, SOD, MDA, and GSH-px, (b) effects of BRAs on AST, ALT, and ALP in T2DM rats, and (c) effect of BRAs on the expression of related mRNA in rats, including PI3K, AKT, AMPK, ACC, and CPT1. Data are expressed as means ± SD. Compared with the model group, $^{\#}$was significantly different ($P < 0.05$) and $^{\#\#}$was extremely significant ($P < 0.01$). *was significantly different ($P < 0.05$) and **was extremely significant ($P < 0.01$). Con: normal group, Mod: model group, Met: positive control group, BRL: black rice anthocyanins low dose group, and BRH: black rice anthocyanins high dose group.

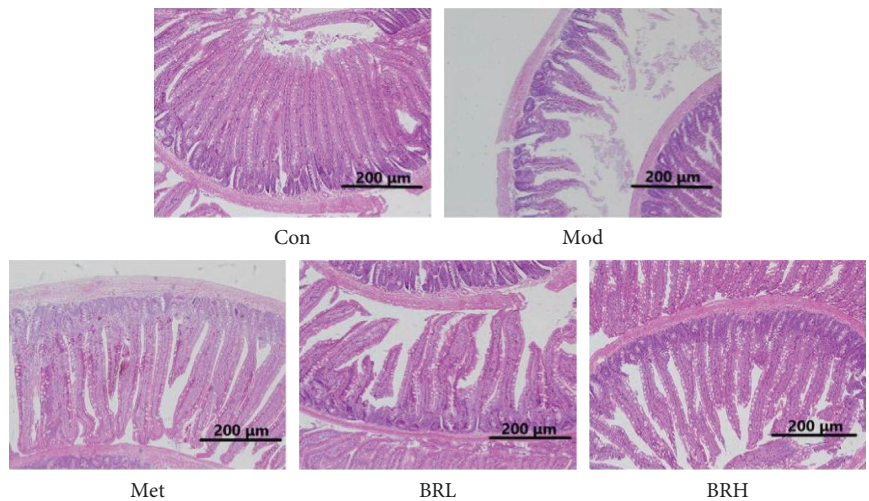

Figure 4: Effect of BRAs on intestinal pathological changes in T2DM rats. Con: normal group, Mod: model group, Met: positive control group, BRL: black rice anthocyanins low dose group, and BRH: black rice anthocyanins high dose group.

microbiota in each group of rats is shown in the figure. Among them, Chao1 and Shannon of Mod were significantly lower than those of Con ($P < 0.05$), indicating that the diversity of cecal microbiota in T2DM rats was significantly reduced. Compared with Mod, the Alpha diversity index of BR had certain improvement but no significant difference, indicating that the BRAs improved the intestinal flora diversity to a certain extent.

PCoA analysis showed in Figure 5(b), Mod was far away from Con, indicating that the microbial flora of Mod rats was significantly different from Con, and the intestinal

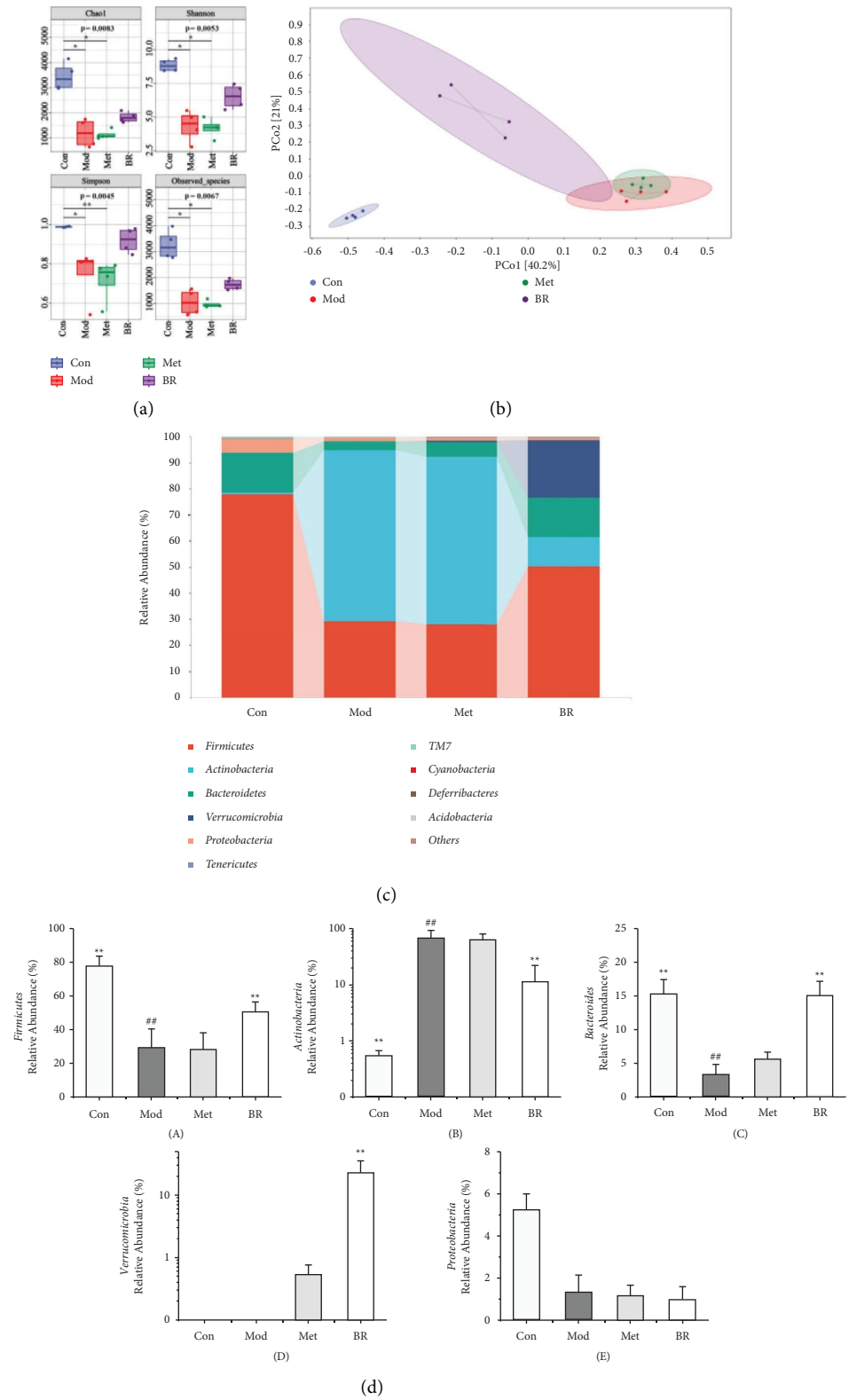

FIGURE 5: BRAs on the diversity and composition of the gut microbiota: (a) Alpha diversity indexes, (b) distance matrix and PCoA analysis, (c) map of the species composition of gut microbiota at the phylum level, and (d) relative abundance of gut microbiota species at the phylum level, including (A) Firmicutes, (B) Actinobacteria, (C) Bacteroidetes, (D) Verrucomicrobia, and (E) Proteobacteria. Data are expressed as means ± SD. Compared with the model group, $^{\#}$was significantly different ($P < 0.05$) and $^{\#\#}$was extremely significant ($P < 0.01$). *was significantly different ($P < 0.05$) and **was extremely significant ($P < 0.01$). Con: normal group; Mod: model group; Met: positive control group; BR: black rice anthocyanins group.

microbial community of T2DM rats was already disordered. Compared with Mod, the microbiota in BR significantly deviated from Mod and shifted to Con, but there was a small part of overlap with Mod, indicating that the intestinal microbiota of rats in BR was affected by diabetes and BRAs, and the intervention had a certain improvement on intestinal microbiota imbalance.

3.10.2. Analysis of Gut Microbiota Composition at the Phylum and Genus Levels. At the phylum level, compared with Con, the relative abundance of *Firmicutes* and *Bacteroidetes* in Mod was significantly decreased ($P < 0.01$), while that of *Actinobacteria* was significantly increased ($P < 0.01$) (Figures 5(c) and 5(d)). Compared with Mod, the relative abundance of *Firmicutes, Verrucomicrobia*, and *Bacteroidetes* in BR was increased to varying degrees ($P < 0.01$). The relative abundance of *Actinobacteria* was significantly decreased ($P < 0.01$).

At the genus level, compared with the Mod, the relative abundance of *Bifidobacteriu*m in BR was significantly decreased in Figure 6 ($P < 0.01$). The relative abundance of *Clostridiaceae_Clostridium* and *Allobaculum* also showed a downward trend. BRAs could interfere with the relative abundance of *Akkermansia, Coprococcus, Ruminococcaceae_Ruminococcus*, and *Phascolarctobacterium*. It has a significant upward trend ($P < 0.01$ or $P < 0.05$). The relative abundance of *Lactobacillus* also increased to some extent.

3.11. Correlation between Gut Microbiota at Genus Level and Blood Lipids, Inflammatory Factors, and Antioxidant Related Factors in T2DM Rats. We selected Con, Mod, and BR for Spearman analysis. As shown in Figure 7, a heatmap of the correlation between gut microbiota at genus level and body weight, HOMA-IR, blood lipids, inflammatory factors, and antioxidant factors of T2DM rats is shown. *Coprococcus* and *Oscillospira* were significantly positively correlated with body weight. *Bifidobacterium* and *Allobaculum* were positively correlated with HOMA-IR, serum TC, TG, LDL-C, proinflammatory factors, and liver MDA, and negatively correlated with weight, HDL-C, anti-inflammatory factors, and liver SOD, CAT, and GSH-Px. *Coprococcus, Oscillospira*, and *Prevotella* were negatively correlated with HOMA-IR, serum TC, TG, LDL-C, proinflammatory factors, and liver MDA. It was positively correlated with HDL-C, anti-inflammatory factors, and SOD, CAT, and GSH-Px in the liver.

4. Discussion

Diabetes mellitus has become one of the most common diseases in the world due to many factors such as high fat and sugar eating habits and lifestyle disorders. Among them, the determinants of T2DM include insulin resistance, dyslipidemia, hypertension, oxidative stress, and obesity, which is called "metabolic syndrome" [25]. This study shows that BRAs can restore the levels of blood glucose, inflammatory cytokines, oxidative stress, and liver injury in T2DM rats induced by a high-fat and high-sugar diet combined with STZ to some extent. Moreover, BRAs can activate the gene expression of PI3K, AKT, and AMPK, and regulate the gene activity of ACC and CPT1, thus promoting lipid oxidation and reducing fat accumulation, and the regulation of gut microbiota is associated with the mechanism of BRAs improving T2DM.

There is a complex relationship between gut microbiota and T2DM, especially gut microbiota disorder [26], inflammation, and gut barrier disruption [27]. The composition of the gut microbiota of Con, Mod, and BR was different from that of the diversity of Alpha and PCoA, indicating that the gut microbiota of rats was affected by disease and BRAs intervention. After intervention with BRAs, T2DM rats showed an increased abundance of *Firmicutes, Bacteroidetes, and Verrucomicrobia* and decreased abundance of *Actinobacteria*, and the ratio of *Firmicutes/Bacteroidetes* decreased significantly. Some studies have shown that the number of *Firmicutes* in diabetic patients is small, and the ratio of *Firmicutes* to *Bacteroidetes* is positively correlated with blood glucose and insulin resistance [28, 29], which is consistent with the experimental conclusion. *Verrucomicrobiae* is thought to prevent T2DM and regulate glucose intolerance [30]. After BRAs intervention, the diversity and total abundance of gut microbiota were restored to a certain extent.

The interaction between anthocyanins and microorganisms can shape the composition of the gut microbiota [21]. In order to further study the potential mechanism of BRAs improving the gut microbiota in T2DM rats, we will discuss the genus-level strains. After BRAs intervention, compared with the Mod, the content of *Bifidobacterium* was significantly reduced. It is generally believed that the abundance of *Bifidobacterium* is usually negatively correlated with T2DM, but it is also controversial, such as Lin et al. [31] found that the abundance of *Bifidobacterium* is positively correlated with T2DM, which is similar to the results of this study. The mechanisms by which BRAs regulate the gut microbiota remain to be elucidated but may involve both direct and indirect interactions, with compounds in BRAs that can directly stimulate or inhibit bacterial growth. Meanwhile, the relative abundance of *Clostridiaceae_Clostridium* in the BR was significantly lower than that in the Mod. *Clostridiaceae_Clostridium* is a pathogenic bacterium that can enhance the effect of diet-induced obesity and promote the occurrence of T2DM [32, 33], which in turn can cause an increase in the abundance of *Clostridiaceae_Clostridium*. *Clostridium* spp. is positively correlated with chronic inflammation, serum TG, TC, and TNF-α levels [34], and TNF-α can activate various signaling cascades and induce insulin resistance. *Akkermansia* is negatively correlated with obesity and diabetes. It can reduce body weight and lipid accumulation induced by high-fat and high-sugar diets, reduce serum endotoxin production, enhance intestinal barrier [35], improve glucose tolerance and insulin resistance [36–38], negatively correlated with inflammation, and has immunomodulatory effects. *Akkermansia* of BR was significantly increased, indicating that BRAs could alleviate the symptoms of T2DM rats by increasing *Akkermansia*. In addition, BRAs increased the

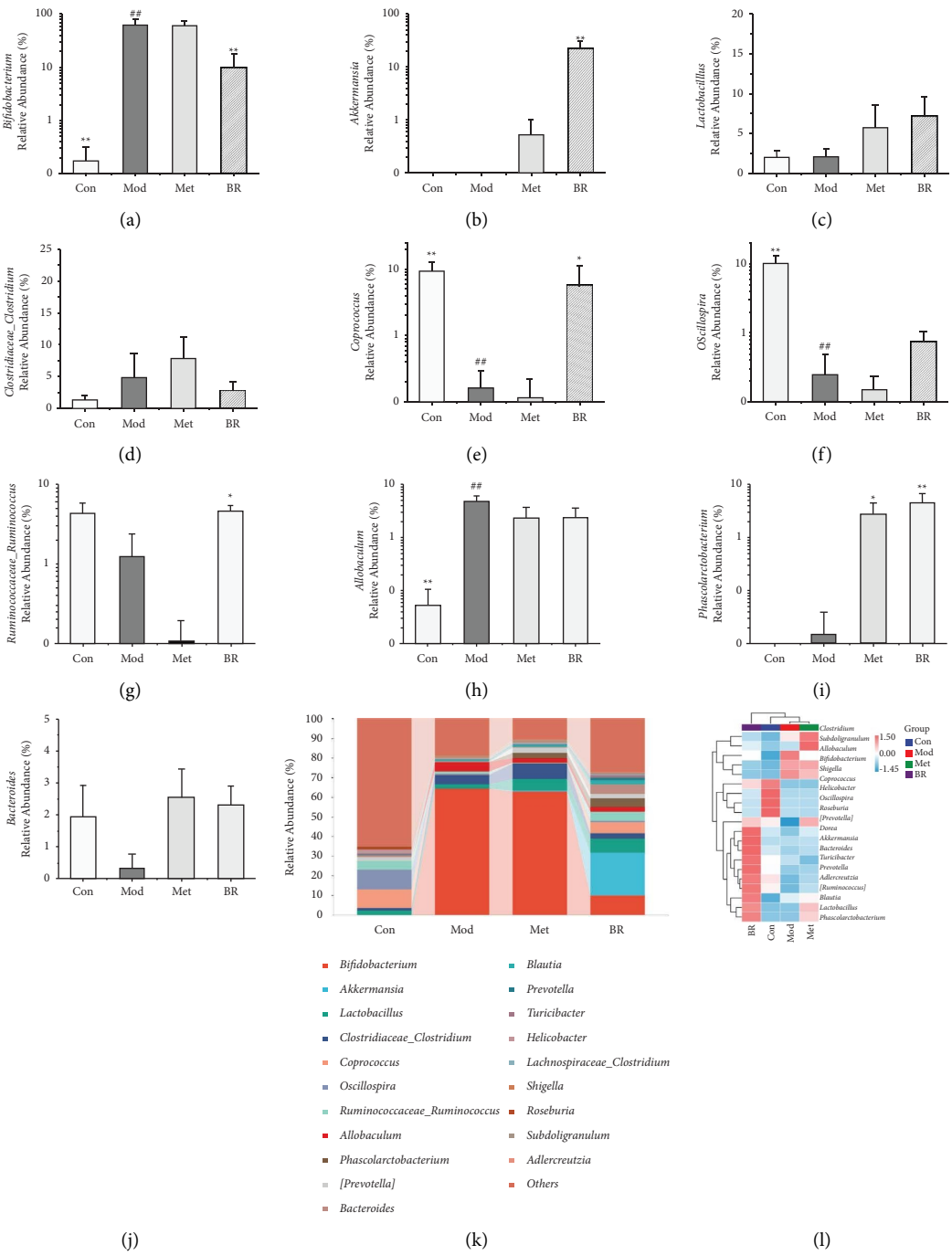

Figure 6: Species heat map, species composition map, and species abundance map of gut microbiota at the genus level, including (a) *Bifidobacterium*, (b) *Akkermansia*, (c) *Lactobacillus*, (d) *Clostridiaceae_Clostridium*, (e) *Coprococcus*, (f) *Oscillospira*, (g) *Ruminococcaceae_Ruminococcus*, (h) *Allobaculum*, (i) *Phascolarctobacterium*, (j) *bacteroids*, (k) map of the species composition of gut microbiota at the genus level, (l) genus level heat map of gut microbiota. Data are expressed as means ± SD. Compared with the model group, $^{\#}$was significantly different ($P < 0.05$) and $^{\#\#}$was extremely significant ($P < 0.01$). *was significantly different ($P < 0.05$) and **was extremely significant ($P < 0.01$). Con: normal group, Mod: model group, Met: positive control group, and BR: black rice anthocyanins group.

abundance of SCFAs-producing bacteria such as *Lactobacillus, Coprococcus, Oscillospira,* and *Prevotella*, which played a significant role in improving diabetes-related symptoms such as blood glucose and blood lipids, anti-inflammation, and antioxidation. *Coprococcus, Oscillospira*, and *Prevotella* were negatively correlated with HOMA-IR, serum TC, TG, LDL-C, proinflammatory factors, and liver MDA. It was positively correlated with weight, HDL-C, anti-inflammatory factors, and SOD, CAT, and GSH-Px in the liver. Among them, *Coprococcus* can regulate the intestinal tract and maintain intestinal health by strengthening the intestinal barrier function and inhibiting inflammation [39, 40]. *Oscillospira* can produce butyrate, which can induce fatty acid oxidation, fat decomposition, and thermogenesis,

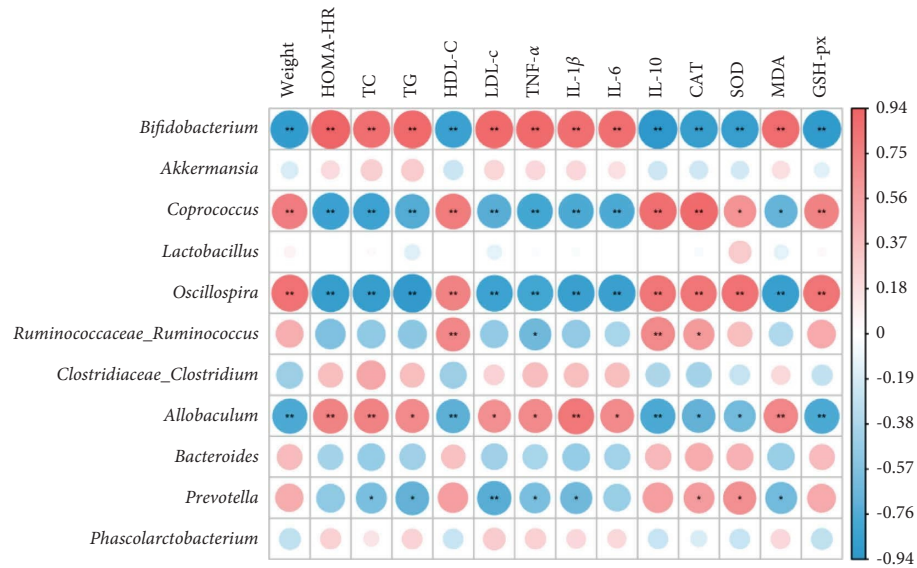

Figure 7: Spearman analysis of genus-level gut microbiota and serum lipids, inflammatory factors, and antioxidant factors in T2DM rats. *was significantly different ($P < 0.05$) and **was extremely significant ($P < 0.01$).

reduce fat accumulation and stimulate the activity of mitochondria in the liver [41], which plays an active regulatory role in relieving chronic inflammation-related diseases. *Prevotella* is a propionate-producing bacteria that can reduce serum cholesterol and liver fat production, thereby preventing weight gain [42]. *Lactobacillus* can promote the intestinal colonization of specific bacterial groups, protect the intestinal barrier [43], and also play a powerful role in improving the antioxidant status and fasting blood glucose status of T2DM patients. The above results were consistent with the results of TC, TG, LDL-C in serum, oxidative stress indexes of SOD, CAT, GSH-px, and MDA in the liver, and remission of symptoms of ALT, AST, and ALP in liver injury. And similar studies have shown that dehydroxyphenolic acids metabolized by proanthocyanidins microorganisms can reduce the secretion of IL-6, IL-1 β, and TNF-α in healthy subjects [44], polyphenol metabolites, such as ferulaldehyde, induce an anti-inflammatory response by reducing MAPK activation, thereby inhibiting the production of NF-kappa B and ROS [45], and flavonol and proanthocyanidins from cranberry extracts can reduce HFD-induced obesity and related metabolic changes [46]. It can be seen that there is a two-way interaction between BRAs and intestinal microorganisms, which requires microorganisms to degrade BRAs and regulate gut microflora through BRAs and their metabolites, so as to stimulate the increase of beneficial bacteria and hinder the increase of pathogenic bacteria, so that a variety of beneficial bacteria work together, which can help to alleviate T2DM-related symptoms.

The liver is the main organ regulating glucose homeostasis. As for the related pathways affected by BRAs intervention, we studied the PI3K/AKT signal pathway, one of the main pathways of insulin action. Among them, PI3K can promote the activation of the downstream gene Akt, and the activated Akt can phosphorylate various downstream factors [47]. Activation of AMPK in the liver can inhibit glucose production, and lipogenesis and stimulate fatty acid oxidation [48], which plays a central role in inhibiting lipogenesis and lipid metabolism and is one of the most important methods to improve hyperglycemia. Some studies have shown that blueberry polyphenols can activate AMPK in the liver and reduce the expression of genes related to lipogenesis regulation in mouse liver [11]. AMPK can regulate the downstream acetyl-CoA carboxylase (ACC) and carnitine palmitoyltransferase (CPT1) pathways, in which the activation of AMPK can induce significant phosphorylation of ACC, while the phosphorylation of ACC can activate CPT1 and stimulate fatty acid oxidation [49]. In this study, after the intervention of BRAs, the gene expression of PI3K, AKT, and AMPK was activated, AMPK inhibited the activity of ACC and restored the activity of CPT1, and the lipid content in BR decreased significantly, along with the significant decrease of serum TC and TG concentration, indicating that BRAs can reduce lipid content by enhancing fatty acid oxidation, thus reduce lipotoxicity, improve insulin sensitivity and achieve the anti-T2DM effect. Czank et al. labeled anthocyanins by using $^{13}C_5$, the relative bioavailability of cyanidin-3-O-glucoside was $12.3\% \pm 1.3\%$ [50]. However, due to the wide variety and high instability of anthocyanins, their metabolism, and bioavailability are still uncertain [51]. Large amounts of anthocyanins at the recommended dose can mediate a range of mechanisms, most likely due to alterations in the gut microbiota. Microbiota is involved in the metabolism of anthocyanins and degrades the unstable form of anthocyanins to phenolic acids [52]. Polyphenols can affect molecular signal transduction pathways, such as inflammatory cascades, cell proliferation/migration, oxidative stress, and metabolic disorders [53], and anthocyanins in polyphenols have a variety of biological properties. Tsuda et al. studies have found that blueberry fruits contain large amounts of anthocyanins, of which (anthocyanin-3muro-glucoside C3G) is absorbed into the blood in an intact form and metabolized as methoxy

derivatives in the liver and kidney [54]. Other researchers have also detected complete forms of blueberry anthocyanins in plasma and metabolites in the liver and kidneys, suggesting that anthocyanin metabolites may also regulate metabolism [55–58]. We believe that BRAs can regulate lipid metabolism and reduce T2DM through PI3K/AKT pathway.

5. Conclusions

In conclusion, this study showed that BRAs improved the indexes related to T2DM in T2DM rats. We conclude that the modulation of gut microbiota by BRAs plays a key role in improving T2DM, meanwhile, BRAs alleviate T2DM symptoms by activating the hepatic adipose oxidation PI3K/AKT pathway. Our results provide a biochemical basis for the use of BRAs and also have important implications for the prevention and intervention of T2DM.

Data Availability

The data sets used and/or analyzed during the study are available from the corresponding author upon reasonable request.

Additional Points

BRAs alleviate the symptoms of T2DM by regulating gut microbiota and activating the PI3K/AKT pathway of liver fat oxidation. It is expected that as a functional food, it will provide an experimental theoretical basis for the prevention and improvement of T2DM in the later stage of BRAs.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Authors' Contributions

Xuejing Zhang was involved in writing the original draft and formal analysis. Mubai Sun was involved in the investigation, conceptualization, and methodology. Da Li was involved in methodology and visualization. Xinyu Miao was responsible for visualization and writing, reviewing, and editing. Mei Hua was involved in writing, reviewing, editing, and data curation. Ruiyue Sun was responsible for data curation and writing, reviewing, and editing. Ying Su was involved in data curation and investigation. Yanping Chi was responsible for validation. Jinghui Wang was responsible for supervision, validation, and conceptualization. Honghong Niu was involved in supervision, validation, writing, reviewing, and editing.

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Supplementary Materials

Table S1. feed formulation for rats. *Table S2.* PCR primer sequences. *Figure S1.* HPLC-MS Atlas of total anthocyanin content in black rice. (*Supplementary Materials*)

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