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Probiotic-fermented blueberry juice prevents obesity and hyperglycemia in high fat diet-fed mice in association with modulating the gut microbiota†

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Blueberry dietary interventions have demonstrated remarkable potential against obesity and type 2 diabetes mellitus. However, the effects of fermented blueberry juice on metabolic syndrome, the gut microbiota, and insulin resistance have not yet been reported. This study aimed to investigate the potential of fermented blueberry juice against obesity, hyperglycemia, and gut microbiota dysbiosis in high fat diet (HFD)-fed mice. Our study findings revealed that supplementation with fresh blueberry juice (BBJ), and fermented blueberry juice with homemade probiotic starter (FBJ) or commercial starter (CFBJ) significantly decreased fat accumulation and low density lipoprotein cholesterol (LDL-C) levels in HFD-fed mice. FBJ showed relatively more potency to reduce body weight than BBJ and CFBJ. The percentage increase in the body weight of the FBJ group was almost the same as that in the normal chow diet (NCD) group, and was approximately 10% lower than the BBJ and CFBJ groups. Overall, all blueberry juices significantly ameliorated hyperlipidemia and insulin resistance in HFD-fed mice. Moreover, the dietary interventions with BBJ, FBJ, and CFBJ for 17 weeks significantly improved the community richness and diversity of the gut microflora along with an altered structure in the HFD-fed mice group. The FBJ treated mice group showed relatively low abundance of Firmicutes, obesity-related bacteria (*Oscillibacter* and *Alistipes*), and high abundance of lean bacteria (*Akkermansia*, *Barnesiella*, *Olsenella*, *Bifidobacterium*, and *Lactobacillus*) compared to the HFD-fed mice group. Furthermore, BBJ and FBJ treatments regulated the liver mRNA and protein expression levels involved in lipid and glucose metabolism. This study inferred that fermented blueberry juice could be used as a functional food to prevent the modern pandemics *i.e.*, obesity and insulin resistance.

1. Introduction

Globally, obesity and type 2 diabetes mellitus (T2DM) are the most prevalent public health problems which lead to many health complications and economic costs.[1] Several clinical studies indicated that insulin resistance was significantly associated with T2DM, obesity, and other glycolipid metabolism-related ailments.[2] Insulin resistance is generally accompanied by chronic low-grade inflammation in the body that promotes the pathogenesis of obesity and prediabetes.[3,4] The accumulation of adipose tissue macrophages results in a significant increase in the levels of various circulating pro-inflammatory cytokines leading to dysmetabolism by interfering with the insulin signaling pathways.[5] Therefore, controlling the expression of pro-inflammatory cytokines is essential to improve obesity-related inflammation and insulin sensitivity.

The mammalian gut is a remarkably complex digestive system that contains trillions of bacteria, which have important roles such as nutritional metabolism, absorption, and regulation of the immune system.[6] The intestinal ecosystem comprises a complex and highly characteristic microbial community and the imbalance of the gut microbiota can disrupt the normal metabolic pathways and weaken the immune system. Proven facts confirmed that the gut microorganisms have a crucial role in maintaining human health.[7] Many reports have demonstrated that dysbiosis of the intestinal microflora promotes the development of diabetes and

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obesity.[8–10] Sterile rodents were resistant to obesity induced by a high fat diet (HFD), suggesting that the gut microbiota affected mammalian adiposity by regulating the metabolic network that controls bioenergetics.[11] Another study found that translocation of the commensal gut microbiota was linked to diet-induced, early onset T2DM.[12] The change in the diversity and composition of the intestinal microbiota may trigger the development of obesity and T2DM. For example, the increased growth of pathogens results in the disruption of the gut barrier and increased gut permeability, leading to inflammation, hyperglycemia (insulin resistance), and obesity.[13] Furthermore, the abundance of some bacterial genera (*Enterobacter*, *Alistipes*, *Akkermansia, etc.*) was correlated (negatively or positively) with the body mass index (BMI), low density lipoprotein cholesterol (LDL-C) and glucose levels in the blood.[14–16]

Over the last few decades, the prioritization of medication to treat various ailments has been rapidly increased due to quick recovery compared to its traditional counterparts. Nevertheless, increasing awareness regarding drug side effects, shortage issues, and cost related restrictions has affected their clinical use. Moreover, dietary therapies (green interventions) are becoming popular among the masses, and therefore, the invention of effective functional foods is urgently required to counteract (care and cure) obesity and T2DM. In this context, blueberry, a fruit rich in phenolic compounds such as hydroxycinnamic acid, flavonoids, and proanthocyanidines, has exhibited beneficial effects such as lowering fasting serum glucose and preventing obesity.[17] Briefly, blueberry inhibited the inflammatory responses and modified the expressions of AMP-activated protein kinase (AMPK), insulin receptor substrate (IRS), and glucose transporter 4 (GLUT-4) which activated the energy metabolic pathways (*i.e.*, glucose uptake and metabolism). Moreover, significant improvements have been seen in the energy expenditure and insulin sensitivity in mice with induced obesity and T2DM. Some studies have also reported the biotransformation of blueberry juice with probiotics that increased its phenolic content and antioxidant activity, and subsequently modified its potential into antidiabetic biological activity *in vitro*.[18,19] In Asia, fermentation has been widely used for value addition and increase in the food function. It has been shown that plant substances can be fermented with probiotics to produce some functional bioactives (saccharides, peptides, aminobutyric acid, *etc.*) and eliminate some antinutrients (nitrates, phytates, *etc.*).[20] Several studies have demonstrated that fermentation improved the antiobesity and hypoglycemic effects of the plant matrix in mice.[21,22] This might be associated with short-chain fatty acids (SCFAs) and phytoceuticals produced by probiotic fermentation which promoted the ecological balance of the gut microbiota.

Nevertheless, the effects of fermented blueberry juice on HFD-induced hyperglycemia and the gut microbiota modulation have rarely been reported. This study aimed to assess the antiobesity and antidiabetic potential of fermented blueberry juice in a mouse model of HFD-induced obesity. C57BL/6 mice were orally supplemented with three different blueberry juices with or without fermentation for 17 weeks with the key objectives that include investigating the; (i) glycolipid metabolism profile, (ii) inflammatory cytokines and the gut microbiota, and (iii) liver mRNA and protein expression levels involved in lipid and glucose metabolism.

2. Materials and methods

2.1 Preparation of fermented blueberry juice

Fermented blueberry beverages were prepared from blueberries (*Vaccinium corymbosum*) grown in the middle-lower Yangtze hilly ground. Fresh blueberry was procured, drained to dry, and then placed under UV sterilization for 6 h after cleaning. Afterwards, the pellets were blended into syrupy pulp. Two kinds of starters (homemade and commercial) containing a variety of probiotics were added to the pulp to reach the viable count of 10^5 CFU mL^{-1}. Briefly, the homemade starter contains *L. plantarum*, *L. acidophilus, L. paracasei*, *L. rhamnosus, L. acidipiscis* and *S. cerevisiae* (70 : 18 : 6 : 3 : 1 : 2). The commercial starter containing *Bacillus coagulans*, *L. plantarum*, and *S. cerevisiae* (70 : 28 : 2) was kindly supplied by Zhejiang Quanzhi Biotechnology Co. (Hangzhou, China). The three kinds of blueberry products were defined as blueberry juice extracted from fresh blueberry (BBJ), fermented blueberry juice with homemade probiotic starter (FBJ), and fermented blueberry juice using commercial starter (CFBJ). These juices were fermented at 30 °C for 7 d, then at 37 °C for 7 d, and finally, stored at 30 °C for 46 d without stopping fermentation. After 60 days of fermentation, the juices were filtered and kept at −20 °C for further analyses. However, the fresh blueberry juice (BBJ) was processed without fermentation and kept at −20 °C. The physicochemical characteristics of these blueberry juices are provided in ESI Table S1.†

2.2 Chromatography and MS

Liquid chromatography-mass spectrometry (LC-MS) (Thermo, Ultimate 3000LC, Q Exactive) technique was used to analyze the samples following the method of Langer *et al.* with some modifications.[23] A Hyper Gold C18 column (100 × 2.1 mm, 1.9 μm) was employed for separation and identification of phenolic acids and anthocyanins using the gradient elution procedure as follows: 0–1.5 min with 100% A, 1.5–9.5 min with 80% A, 9.5–14.5 min with 0% A, 14.5–14.6 min with 100% A, and 14.6–18 min with 100% A. The column temperature was maintained at 40 °C, the flow rate was 0.3 mL min^{-1}, and the sample injection volume was 1 μL. Mobile phase A consisted of 5% acetonitrile and 0.1% formic acid in water, and mobile phase B was 0.1% formic acid in acetonitrile. The electrospray ionisation (ESI) positive mode was operated under the following conditions: heater temperature of 300 °C, sheath gas flow rate of 45 arb, Aux gas flow rate of 15 arb, sweep gas flow rate of 1 arb, spray voltage of 3.0 kV, capillary temperature of 350 °C, and S-Lens RF level set constant at 30%. However, in

the ESI negative mode, the spray voltage and S-Lens RF levels were set at 3.2 kV and 60%, respectively, while the other conditions remained the same as those in the ESI positive mode.

2.3 Bioevaluation trial using a mice model

Purposely, healthy male C57BL/6 mice (6 weeks old, n = 75) were obtained from the Shanghai SLAC Laboratory Animal Co., Ltd (Shanghai, China) and housed at 25 °C and 60% relative humidity, and allowed *ad libitum* access to food and water. After 1 week of acclimation, the experimental mice were randomly divided into five groups (n = 15 per group), and each group received different diets and drink solutions administered *ad libitum* for 17 weeks: (i) NCD group (NCD, water), (ii) HFD group (HFD, water), (iii) BBJ group (HFD, BBJ), (iv) FBJ group (HFD, FBJ), and (v) CFBJ group (HFD, CFBJ). The materials and energy compositions of the diets are listed in ESI Table S2.† All blueberry juices were diluted 1 : 10 in tap water (this dilution factor was calculated based on a proportional intake of 250 mL day^{-1} of orange juice, which is one serving in humans).[24] The body weights of mice were recorded weekly throughout the experimental period, and food and liquid intake were recorded twice a week. After 17 weeks, mice were fasted for 12 h and euthanized using pentobarbital sodium (100 mg kg^{-1}, *i.v.*) to enable tissue collection. Blood was centrifuged at 3000*g* for 15 min to collect the serum samples. The livers, kidney, spleen and fat pads were collected and weighed, and then stored at −80 °C. All procedures conformed to the guidelines of the Institutional Animal Care and Use Committee of Zhejiang Chinese Medical University (IACUC approval No. ZSLL-2016-131). The energy intake was expressed as kcal per mouse per day, and can be calculated using the following formula:

$$\text{Energy intake (kcal/mouse/day)} = \frac{\text{Total food and juice energy intake per cage (kcal)}}{\text{days of experiment (d)} \times \text{the number of mice per cage (mouse)}}$$

2.4 Glucose homeostasis

At week 15, mice were fasted for 12 h and an intraperitoneal glucose tolerance test (GTT) was conducted after glucose injection (2 g per kg body weight) according to the instructions of Zhao *et al.*[25] At week 17, mice were fasted for 6 h, and an intraperitoneal insulin tolerance test (ITT) was performed after insulin injection (0.75 U per kg body weight). The tail blood collection time was set at 0, 30, 60, 90 and 120 min, and blood glucose was determined using an Accu-Check glucometer (Roche, Mannheim, Germany). The fasting serum insulin level was determined using an ELISA kit (Jiyinmei, Wuhan, China) and the fasting serum glucose level was determined using a glucose oxidase assay kit (Jiancheng Bioengineering Institute, Nanjing, China). The homeostasis model assessment of insulin resistance (HOMA-IR) was calculated as:

$$\text{HOMA-IR} = \frac{\text{Fasting serum glucose (mmol/L)} \times \text{Fasting serum insulin (μU/mL)}}{22.5}$$

2.5 Serum and liver biochemical analysis

The serum insulin, leptin, adiponectin (ADP), hemoglobin A1c (HbA1c), peptide YY (PYY), glucagon-like peptide-1 (GLP-1), tumor necrosis factor alpha (TNF-α), interleukin 6 (IL-6), and interleukin 10 (IL-10) concentrations were measured using commercial ELISA kits (Jiyinmei, Wuhan, China). The serum levels of total cholesterol (TCHO), triacylglycerol (TG), high density lipoprotein cholesterol (HDL-C), low density lipoprotein cholesterol (LDL-C), hepatic malondialdehyde (MDA), total superoxide dismutase (SOD) and catalase (CAT) were determined using commercial kits (Jiancheng Bioengineering Institute, Nanjing, China).

2.6 Histological analysis

The epididymal adipose tissue and liver from mice (n = 6) were fixed in a 4% neutral formaldehyde solution for 24 h. Then, the samples were processed using a series of fractionated ethanol solutions to dehydrate. Then, they were embedded in paraffin, and sectioned into 5 μm thick sections. All tissues were stained with hematoxylin and eosin (H&E), and three photos were randomly taken using a microscope (Leica, Germany) for routine morphological evaluation at 40× magnification. The number and size of epididymal adipocytes were statistically analyzed using Image-Pro Plus software (version 6.0, Media Cybernetics, USA).

2.7 Q-PCR analysis of mouse liver genes

Total RNA from the liver tissues was extracted using an RNA extraction kit (R401-01, Vazyme Corp., Nanjing, China). cDNA was synthesized using a HiScript® III RT SuperMix Kit with gDNA wiper (R323-01, Vazyme Corp., Nanjing, China). The quantitative PCR (qPCR) test was performed using ChamQ™ SYBR® Color qPCR Master Mix (R411-02/03, Vazyme Corp., Nanjing, China) and an LC480 Real-Time PCR system (Roche Diagnostics, Penzberg, Germany). PCR was performed in duplicate at 95 °C for 30 s and subjected to 40 cycles at 95 °C for 10 s, and 60 °C for 30 s. The melting curve procedure was performed at 95 °C for 15 s, 60 °C for 60 s, and 95 °C for 15 s. The relative mRNA levels of target genes were normalized using the mRNA level of the β-actin gene in each sample. The relative expression of each gene was determined by the $2^{-\Delta\Delta Ct}$ method. All primers that were used for performing RT-qPCR are listed in ESI Table S3.†

2.8 Western blot

Liver tissues were lysed in RIPA buffer (Thermo Pierce, USA) at 4 °C. The tissue homogenates were centrifuged (6000*g*, 20 min, 4 °C), and the resulting supernatants (whole-tissue extracts)

were used for western blot analysis. The total protein content in the supernatants was determined using the Bradford assay (Bio-Rad, CA, USA). Equal amounts of total protein (60 μg) of samples were separated using 12% SDS-PAGE. Then, they were transferred onto a PVDF membrane and blocked in blocking buffer (Beyotime, China). The membrane was incubated with the corresponding primary antibodies (1 : 1000 dilution) (Abcam, U.K.) at 4 °C overnight. After incubation, T-TBS was used to wash the blotting membrane three times (5 min per time). The membrane was again incubated with the secondary antibodies (1 : 5000 dilution) for 2 h. Then, the bands were visualized using an ECL chemiluminescence detection kit (GE, U.S.A) on a FluorChem M imaging system. ImageJ software (version 1.8.0, Bethesda, MD) was used to calculate the strip densities. The following antibodies (Abcam, UK) were used: β-actin, anti-GAPDH, anti-glucokinase (GCK), anti-glucose transporter type 4 (GLUT-4), anti-low density lipoprotein receptor (LDL-receptor), anti-PPAR-γ, anti-PPAR-α, anti-insulin receptor (IR), anti-insulin receptor substrate (IRS), anti-pAKT, anti-AKT, anti-glycogen synthase kinase 3 (GSK3), anti-sterol regulatory element-binding protein 1c (SREBP1c), anti-fatty acid synthase (FAS), and anti-stearoyl-CoA desaturase (SCD).

2.9 16S rRNA analysis of the gut microbiota

Fresh fecal samples (*ca.* 30 mg) were frozen immediately using dry ice, and stored at −80 °C. Total DNA was extracted from the fecal samples using a QIAamp DNA Stool Mini Kit (Qiagen, Hiden, Germany) according to the manufacturer's instructions. The selected region of 16S rDNA was the V3–V4 region, and the universal primers were F341 and R806 for analysis. Library quality was examined using a Thermo NanoDrop 2000 ultraviolet microspectrophotometer and 2% agarose gel electrophoresis and Qubit were used for library quantification. Using an Illumina HiSeq2500 (PE250) to sequence on the computer, original paired-end (PE) reads were generated. The pairwise reads obtained by double-terminal sequencing were spliced into a sequence using Pandaseq software, and the long reads of hypervariable region were obtained. Then, the spliced reads were processed to obtained clean reads. After these reads were processed, the acquired sequences were clustered using Usearch (V10). For taxonomic classification, the GreenGene Database was used based on the RDP classifier V 2.2. α-Diversity (Chao1, Observed species, Shannon index and Simpson index), Venn diagrams were created using QIIME (V1.7.0), and the results were displayed using R software (V2.15.3). To compare β-diversity, the weighted UniFrac distances were calculated and included in a principal coordinate analysis (PCoA). Nonmetric multidimensional scaling (NMDS) was performed using OTUs for each sample based on Bray–Curtis. Linear discriminant analysis (LDA) combined with effect size (LEfSe) analysis was used for identifying significant differences among the groups (LDA > 2.0).

2.10 Analysis of SCFAs in caecal contents

Approximately 50 mg of thawed caecal sample was homogenized with 250 μL of sterile distilled water. 10 μL of 5 mol L^{-1} HCl was added to the sample and vortexed for 1 min. Afterwards, the mixture was kept for 5 min, vortexed again for 5 min, and centrifuged at 12 000 rpm for 20 min at 4 °C. After centrifugation, 200 μL of the supernatant was transferred to a new 1.5 mL Eppendorf tube and 0.5 μL of 20 fold diluted 2-ethyl butyric acid was added as an internal standard. The mixture was vortexed again for 1 min, and centrifuged at 12 000 rpm for 5 min at 4 °C. For gas chromatography, 1 μL of the upper layer was injected into a GC-2014 System (Shimadzu Corp., Japan) with a flame ionization detector and an automatic injector. Separation of SCFA occurred in an Agilent DB-FFAP 30 m × 0.52 × 0.5 mm PEG capillary column (125-3237, USA). After injection, the initial oven temperature of 100 °C was kept constant for 0.5 min, and it was increased to 180 °C at a rate of 8 °C min^{-1} and kept constant for 1 min. Then, it was increased to 200 °C at a rate of 20 °C min^{-1} and kept constant for 15 min. The detector temperature was 240 °C, the injector temperature was 200 °C, and the air and hydrogen flow rates were 300 mL min^{-1} and 30 mL min^{-1}, respectively. The standard solutions used included acetic acid, propionic acid, isobutyric acid, butyric acid, isovaleric acid and hexanoic acid. For short-chain fatty acids the results were expressed as micrograms per gram of caecal contents.

2.11 Statistical analysis

Statistical significance was determined using one-way analysis of variance (ANOVA) or two-way ANOVA with a *post hoc* Tukey's test. $P < 0.05$ indicated statistical significance and was represented by a superscript symbol (* *vs.* HFD, # *vs.* NCD). SPSS 22.0 (SPSS Inc., Chicago, IL, USA) and GraphPad Prism 6.0 (GraphPad Software Inc., San Diego, CA, USA) were used to perform statistical analysis and shape the data.

3. Results

3.1 Identification of bioactives in blueberry juices

The chemical characteristics of the blueberry juices showed that FBJ and CFBJ contained more total phenols and organic acids than BBJ (Table S1†). However, the ethanol content of CFBJ was much higher than BBJ and FBJ, reaching 11.2%. Fig. S1† illustrates representative UPLC chromatographs. Most of the detected compounds were phenolic acids and anthocyanins which are listed in Table 1. BBJ contained 13 major anthocyanins, and malvidin3-*O*-glucoside was the dominant anthocyanin. After fermentation, the amount of anthocyanins in BBJ was decreased, whereas the content of phenolic acids showed a significant increase. In particular, caffeic acid, gallic acid, chlorogenic acid, neochlorogenic acid and quercitrin were the principal phenolic acids in FBJ and CFBJ. In addition, the microbial colony counting assay revealed that bacteria were not present in the fermented blueberry juice samples (Table S1†).

3.2 FBJ prevented obesity in HFD-fed mice without affecting their energy intakes

At the initiation of trial (0 week), no significant difference was observed in the body weights of mice in the different groups (P

Table 1 Representative MS fragments of the major phenolic acids and anthocyanins in blueberry juice with or without fermentation

Peak no.	RT (min)	Class	Compound name	Formula	Mass (m/z)	ESI mode	BBJ (μg mL^{-1} [a])	FBJ (μg mL^{-1})	CFBJ (μg mL^{-1})
1	2.75	Anthocyanin	Delphinidin-3-*O*-galactoside	$C_{21}H_{21}O_{12}$	465.1	Pos	0.25	0.01	0.04
2	2.75	Phenolic acid	Quercetin-3β-D-glucoside	$C_{21}H_{20}O_{12}$	464.1	Pos	0.25	0.01	0.04
3	2.79	Phenolic acid	(*E*)-*p*-Coumaric acid	$C_9H_8O_3$	164.05	Pos	0.15	0.01	0.03
4	2.85	Anthocyanin	Delphinidin-3-arabinoside	$C_{20}H_{19}O_{11}$	435.09	Pos	0.09	0	0.01
5	2.88	Anthocyanin	Cyanidin-3-*O*-galactoside	$C_{21}H_{21}O_{11}$	449.11	Pos	2.24	0.02	0.05
6	2.92	Anthocyanin	Petunidin-3-galactoside	$C_{22}H_{23}O_{12}$	479.12	Pos	1.22	0.03	0.08
7	2.98	Anthocyanin	Cyanidin-3-*O*-α-arabinopyranoside	$C_{20}H_{19}O_{10}$	419.1	Pos	0.69	0.01	0.02
8	3.06	Anthocyanin	Peonidin 3-galactoside	$C_{22}H_{23}O_{11}$	463.12	Pos	2.12	0.02	0.09
9	3.06	Phenolic acid	Kaempferide	$C_{16}H_{12}O_6$	300.06	Pos	0.16	0	0.01
10	3.07	Anthocyanin	Malvidin-3-*O*-glucoside	$C_{23}H_{25}O_{12}$	493.13	Pos	12.22	0.24	0.87
11	3.1	Phenolic acid	*p*-Coumaric acid	$C_9H_8O_3$	164.05	Pos	0.15	0.11	0.22
12	3.16	Anthocyanin	Peonidin-3-glucoside	$C_{22}H_{23}O_{11}$	463.12	Pos	5.24	0.13	0.35
13	3.73	Anthocyanin	Delphinidin-3-arabinoside	$C_{20}H_{19}O_{11}$	435.09	Pos	0.4	0	0.01
14	3.79	Anthocyanin	Delphinidin-3-*O*-glucoside	$C_{21}H_{21}O_{12}$	465.1	Pos	0.07	0	0.01
15	3.81	Anthocyanin	Cyanidin-3-glucoside	$C_{21}H_{21}O_{11}$	449.11	Pos	0.87	0.05	0.18
16	4.01	Anthocyanin	Petunidin-3-*O*-glucoside	$C_{22}H_{23}O_{12}$	479.12	Pos	0.03	0.01	0.03
17	4.14	Anthocyanin	Malvidin-3-*O*-galactoside	$C_{23}H_{25}O_{12}$	493.13	Pos	0.74	0.06	0.17
18	1.43	Phenolic acid	Gallic acid	$C_7H_6O_5$	170.02	Neg	70.49	196.73	479.43
19	2.44	Phenolic acid	Protocatechuic acid	$C_7H_6O_4$	154.03	Neg	50.62	5.91	16.13
20	2.69	Phenolic acid	Neochlorogenic acid	$C_{16}H_{18}O_9$	354.1	Neg	11.87	136.68	243.12
21	3.02	Phenolic acid	Chlorogenic acid	$C_{16}H_{18}O_9$	354.1	Neg	39.31	197.11	419.33
22	3.23	Phenolic acid	Caffeic acid	$C_9H_8O_4$	180.04	Neg	217.09	333.79	912.51
23	3.81	Phenolic acid	Quercitrin	$C_{21}H_{20}O_{11}$	448.1	Neg	321.39	56.88	176.75

[a] μg mL^{-1} represents the relative content of juice.

> 0.05; Fig. 1A). After 3 weeks, the total body weight gain (TBWG) of the HFD group was significantly increased compared to the other groups (*i.e.*, NCD, BBJ, FBJ, and CFBJ). At the experiment termination point, the percent increase in the TBWG of mice fed with blueberry juice (HFD + BBJ), fermented blueberry juice (HFD + FBJ), and commercial fermented blueberry juice (HFD + CFBJ) was significantly lowered compared to the HFD group ($P < 0.0001$; Fig. 1A and B). The body weight percentage of the FBJ group had a relatively less increase than the BBJ group, and there was no significant difference when compared to NCD-fed mice. The liquid intakes of the NCD and BBJ groups were much higher than the other groups (HFD, FBJ, and CFBJ), but there was no significant difference between the FBJ and CFBJ groups. Though the liquid intake in each group was different, there were no significant differences among the groups in their daily energy intake, indicating that liquid consumption did not affect their total energy intake (Fig. 1C and D).

3.3 FBJ prevented fat accumulation in HFD-fed mice

After 17 weeks, there was a significant increase in the epididymal and inguinal fat accumulation in the HFD group (Fig. 2A). In contrast, supplementation with BBJ, FBJ, and CFBJ significantly decreased the epididymal fat weight, especially in the FBJ group. These results were in accordance with the body weight gain results (Fig. 1B). The adipocyte size in the HFD group was obviously increased compared to the NCD group, whereas BBJ, FBJ, and CFBJ treatments significantly reduced the increase in the epididymal adipocyte size caused by the HFD ($P < 0.05$), particularly in the FBJ group (Fig. 2B and C).

The histopathological changes in the liver tissue are shown in Fig. 2D, and the number of white lipid droplets in the liver biopsies of the HFD group was significantly larger than that of the NCD group, suggesting adverse hepatic lipid accumulation. In comparison with HFD-fed mice, a significant decrease in the fat accumulation of hepatocytes was observed after BBJ, FBJ, and CFBJ supplementation, especially after BBJ and FBJ treatments (Fig. 2D). Overall, CFBJ had a weaker inhibitory effect on fat mass development than BBJ and FBJ.

3.4 FBJ ameliorated hyperlipidemia and insulin-resistance in HFD-fed mice

As shown in Fig. 3A, compared to the HFD group, BBJ and CFBJ interventions increased serum TG, while the TCHO level was significantly decreased in the BBJ group ($P < 0.05$) (Fig. 3B). The mouse groups that received treatment with BBJ, FBJ, and CFBJ showed a significant decrease in the serum LDL-C concentration (Fig. 3D). Likewise, FBJ and BBJ interventions exhibited positive effects on the HDL-C levels compared to NCD-fed mice (Fig. 3C).

In the case of GTT and ITT, Fig. 3E and G indicate that HFD triggered insulin resistance and less glucose tolerance. However, in comparison with the HFD group, intervention with BBJ, FBJ, and CFBJ significantly improved their insulin sensitivity ($P < 0.05$) while showed no improvement in their glucose tolerance ($P > 0.05$) (Fig. 3F and H). In comparison with the HFD group, BBJ-, FBJ- and CFBJ-fed mice showed a significant decline in fasting serum glucose. Interestingly, there was no difference in fasting serum insulin in HFD-fed mice (Table S4†). The results of HOMA-IR and HbA1c indicated that intervention with BBJ, FBJ, and CFBJ significantly

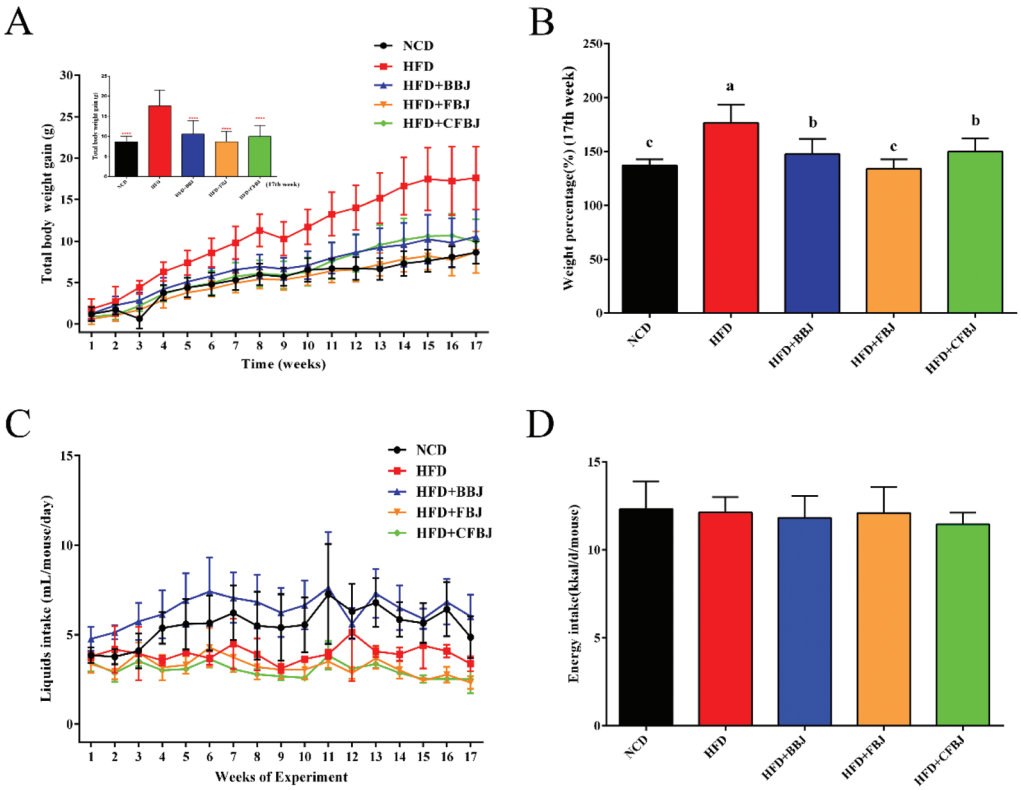

Fig. 1 Changes in the body weight gain, liquid intake, and energy intake of mice in the different groups. (A) Growth curve of total body weight gain of mice in different groups (n = 15), (B) weight percentage in different groups in the 17th week, (C) average liquid intake (mL per mouse per d) in different groups during the experimental period, and (D) average energy intake (kcal per mouse per d) in different groups. Data were expressed as mean ± SD. Mean values with the different letters differ significantly ($P < 0.05$) according to one-way ANOVA analysis followed by Tukey's tests.

increased insulin sensitivity and glucose homeostasis in HFD-fed mice (Fig. 3I and J).

3.5 FBJ improved serum hormones and cytokines in HFD-fed mice

To examine the modifications in the serum hormone levels after consuming blueberry juice with or without fermentation, the serum leptin, ADP, PYY and GLP1 levels were determined. As shown in Fig. 4A and B, the level of hormones secreted by adipocytes in the HFD group, compared to the NCD group, showed a significant increase in the serum leptin levels and a decline in the serum ADP levels ($P < 0.05$). These results indicated that a HFD induced a state of leptin resistance and less ADP in mice. However, the FBJ and CFBJ groups showed significantly lower leptin concentrations than the HFD group ($P < 0.05$), but there was no significant difference in their serum ADP levels ($P > 0.05$).

The circulating level of pro-inflammatory cytokine, TNF-α, in the HFD group was significantly increased ($P < 0.05$) (Fig. 4E). Supplementation with BBJ, FBJ, and CFBJ in HFD mice significantly decreased serum TNF-α ($P < 0.05$) (Fig. 4E). In contrast, the production of anti-inflammatory cytokine, IL-10, in the BBJ and FBJ groups was increased. The level of serum IL-6 remained almost unchanged among the groups. Although there were some changes in hepatic antioxidant enzymes (CAT) and oxidation marker (MDA) after intervention, there were no significant differences ($P > 0.05$) (Fig. 4I and J). Only the NCD group showed a significantly higher concentration of SOD than the HFD group, and BBJ + HFD-fed mice showed a slightly higher concentration of SOD (Fig. 4H).

3.6 FBJ modulated glycolipid metabolism-related gene transcription and protein expression in the liver

RT-qPCR and western blot assays were used to analyze the expression of glycometabolism-related genes and proteins, such as glucose transporter type 4 (GLUT-4) and glucokinase (GCK), and lipometabolism-related genes and proteins such as low density lipoprotein receptor (LDL-receptor) and peroxisome proliferator-activated receptors (PPARγ and PPARα). The results of RT-qPCR and western blot (Fig. 5A–G) showed that the mRNA and protein expression levels of GLUT-4, GCK, LDL-receptor, and PPARα were higher in FBJ-treated HFD-fed mice than in the HFD group ($P < 0.05$). However, the mRNA transcription levels and protein expression of PPARγ were remarkably lower in the liver tissues of the FBJ group than in those of the HFD group ($P < 0.05$). Moreover, FBJ increased the expression of key insulin signaling pathway proteins, IR and IRS, and reduced the expression of hepatic GSK3 (Fig. 5J). The phosphorylation of Akt in the FBJ group was upregulated compared to the HFD group (Fig. 5I). Furthermore, FBJ reduced

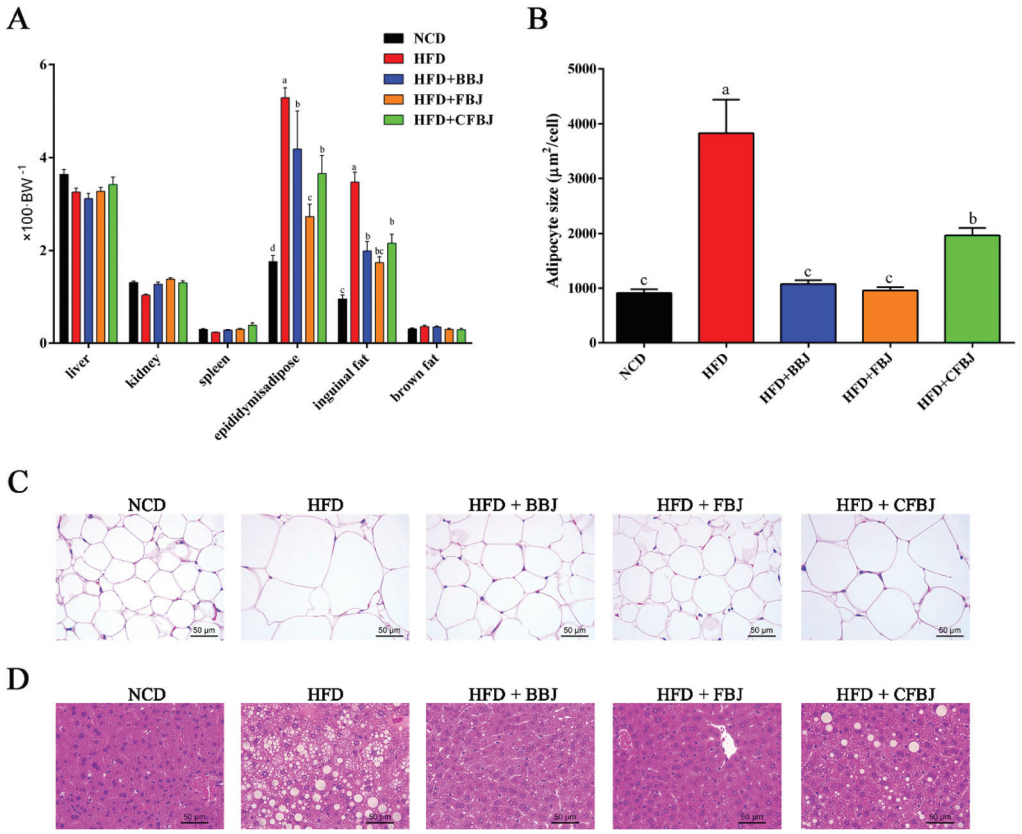

Fig. 2 Blueberry juice with or without fermentation prevented fat mass accumulation in HFD-fed mice. (A) The organ weight index of mice (the index = weight of organ/weight of every mouse × 100, n = 15, data were expressed as mean ± SEM. Mean values with the different letters differ significantly ($P < 0.05$) according to one-way ANOVA analysis followed by Tukey's tests, (B) adipocyte size of epididymal fat tissue (n = 6, 10 different visual fields were calculated for each H & E staining image), (C) H&E staining of epididymal fat and liver, and (D) of mice in the NCD, HFD, HFD + BBJ, HFD + FBJ, and HFD + CFBJ groups (scale bar: 50 μm).

the expression of lipid metabolism-related proteins such as SCD, SREBP1c and FAS (Fig. 5H and J).

3.7 FBJ increased the diversity and balance of the gut microbiota in HFD-fed mice

The α-diversity (the Chao 1 and Shannon's diversity parameter) of mice was significantly increased in the blueberry intervention groups compared to the HFD group (Fig. 6A–C), suggesting that the fermented blueberry juice increased the diversity of the gut microbiota. However, the results of Simpson diversity showed no significant difference among the groups (Fig. 6D). To estimate the structural alterations of intestinal microbes in each group based on the differences in their phylogenetic diversity, a principal coordinate analysis (PCoA) map was created to evaluate the dissimilarity and community composition of samples. HFD-fed mice had a distinct microbiota composition that clustered separately from the NCD group. BBJ- and FBJ-fed mice formed a distinct cluster compared to the HFD group. These results indicated that BBJ and FBJ altered the communities of intestinal microflora in a characteristic direction (Fig. 6E). As shown in Fig. 6F, OTUs of the HFD, BBJ, FBJ, and CFBJ groups shared with the NCD group were 247, 262, 250, and 248, respectively.

As shown in Fig. 6G, Firmicutes, Bacteroidetes, Verrucomicrobia, Actinobacteria, Proteobacteria and Deferribacteres were the common phyla and among these the contents of Firmicutes and Bacteroidetes reached above 90% in each sample. Moreover, the relative abundance of Firmicutes was increased ($P = 0.08$) and that of Bacteroidetes was significantly decreased ($P < 0.05$) in the intestines of HFD-fed mice compared to the NCD group. However, after 17 weeks of BBJ, FBJ, and CFBJ supplementation to HFD-fed mice, the relative abundance of Firmicutes was decreased, and that of Bacteroidetes was increased compared to the HFD group (Fig. 7B and C).

The main intestinal microbiota in all the groups consisted of 20 genera (the relative abundance of OTUs >0.25%, Fig. 7A). The phylum Firmicutes was dominated by genera *Oscillibacter*, *Clostridium XIVa*, *Flavonifractor*, *Clostridium XIVb*, *Peptococcus Lachnospiraceae incertae sedis*, *Clostridium sensu stricto*, *Lactobacillus*, and *Eubacterium*, and Bacteroidetes was dominated by genera *Bacteroides*, *Barnesiella*, *Alistipes*, and *Parabacteroides*. Likewise, the phylum Verrucomicrobia was dominated by the genus *Akkermansia*, and Actinobacteria was dominated by genera *Olsenella*, *Bifidobacterium*, and *Enterorhabdus*. Moreover, the phylum Deferribacteres was

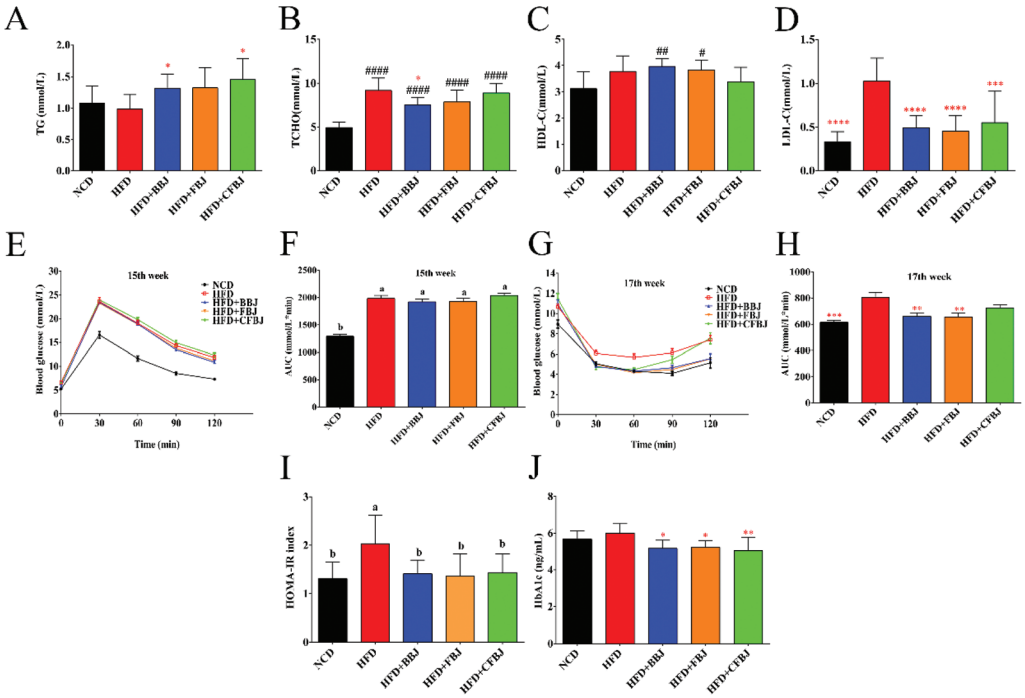

Fig. 3 Effect of blueberry juice with or without fermentation on insulin sensitivity and serum lipid level in HFD-fed mice. (A) TG, (B) TCHO, (C) HDL-C, (D) LDL-C, (E) glucose tolerance test (GTT), (F) area under curve (AUC) from GTT, (G) insulin tolerance test (ITT), (H) AUC from ITT, (I) HOMA-IR, and (J) HbA1c. Data were expressed as mean ± SD (n = 9–10). *P < 0.05, **P < 0.01, ***P < 0.001 and ****P < 0.0001 compared to the HFD group; $^{\#}P$ < 0.05, $^{\#\#}P$ < 0.01 and $^{\#\#\#\#}P$ < 0.0001 compared to the NCD group.

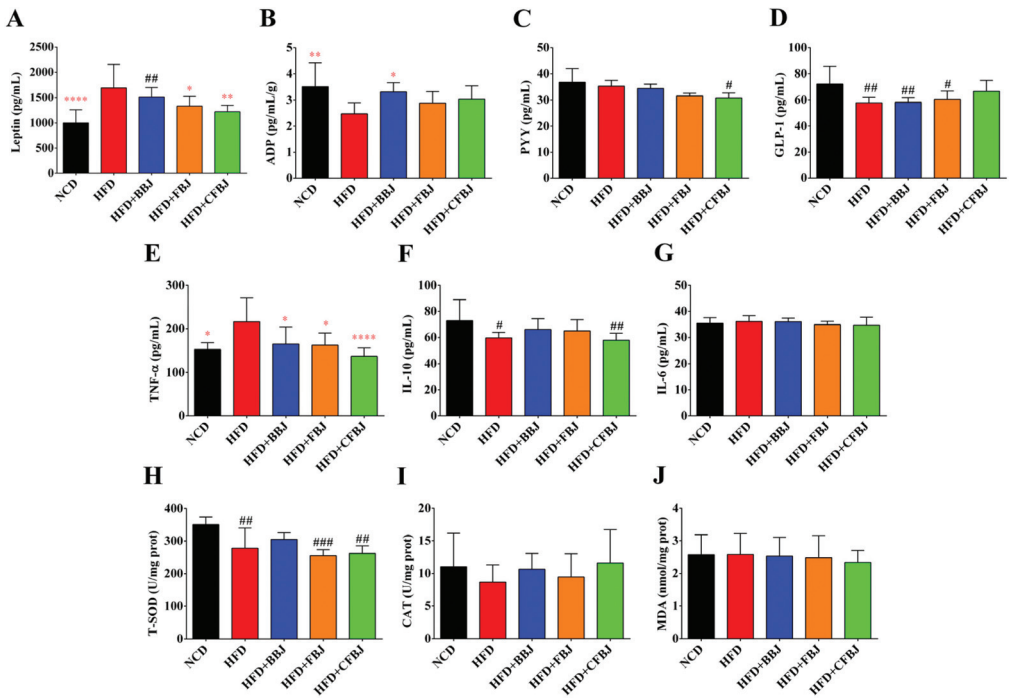

Fig. 4 Effect of blueberry juice with or without fermentation on serum cytokine and liver antioxidant enzyme activity in HFD-fed mice. (A) Leptin, (B) ADP (expressed as serum ADP content/bodyweight), (C) PYY, (D) GLP-1, (E) TNF-α, (F) IL-10, (G) IL-6, (H) liver T-SOD, (I) liver CAT, and (J) liver MDA. Data were expressed as mean ± SD (n = 8–10). *P < 0.05, **P < 0.01 and ****P < 0.0001 compared to the HFD group; $^{\#}P$ < 0.05 and $^{\#\#}P$ < 0.01 compared to the NCD group.

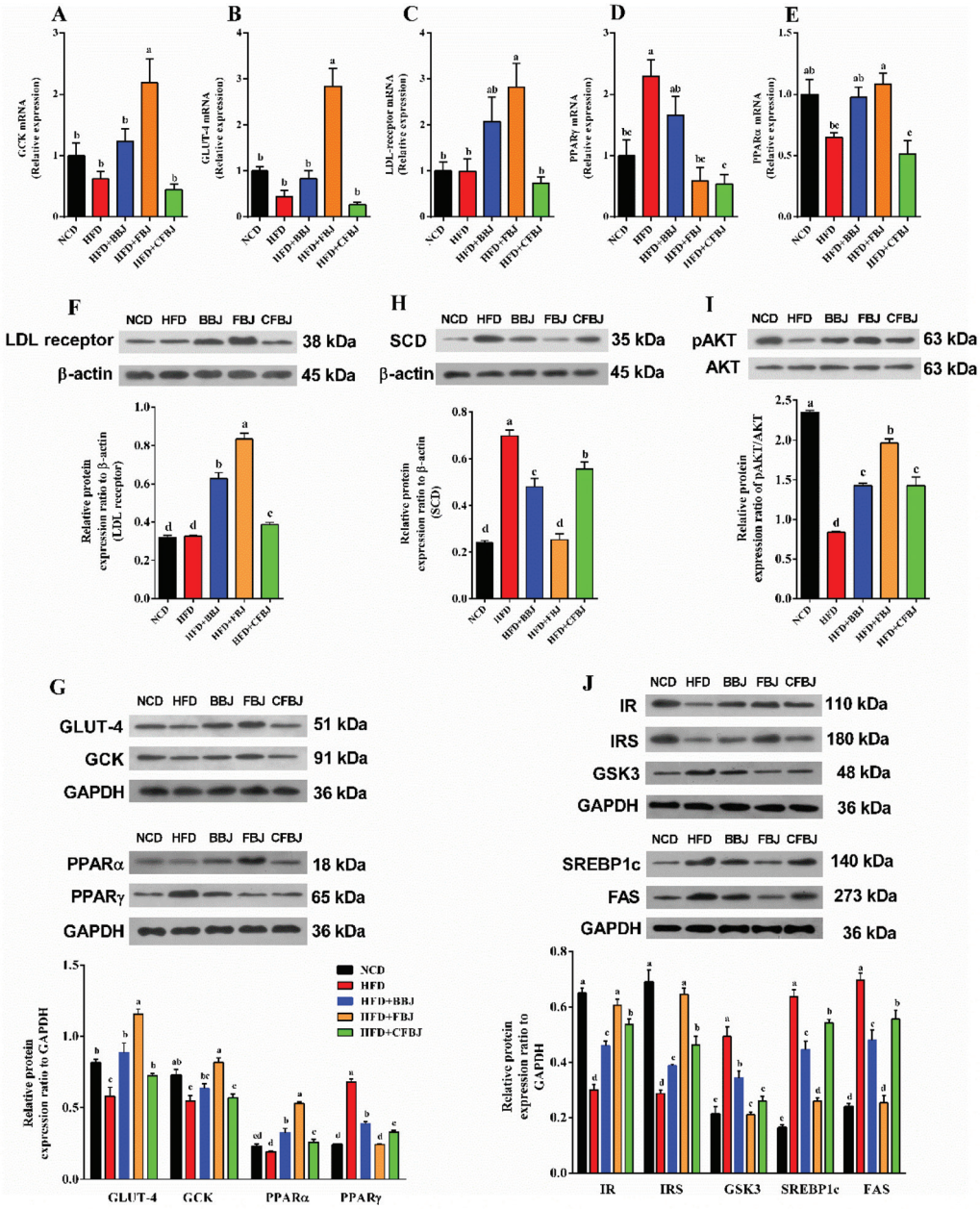

Fig. 5 Effect of BBJ, FBJ, and CFBJ on gene and protein expression in liver. (A) GCK, (B) GLUT-4, (C) LDL-receptor, (D) PPARγ, (E) PPARα, (F) densitometric quantification and western blot analysis of LDL-receptor, (G) densitometric quantification and western blot analysis of GLUT-4, GCK, PPARα, and PPARγ, (H) densitometric quantification and western blot analysis of SCD, (I) densitometric quantification and western blot analysis of pAKT and AKT, and (J) densitometric quantification and western blot analysis of IR, IRS, GSK3, SREBP1c, and FAS. Data were expressed as mean ± SE. Mean values with the different letters differ significantly ($P < 0.05$) according to one-way ANOVA analysis followed by Tukey's tests.

dominated by *Mucispirillum* and Tenericutes was dominated by *Anaeroplasma*, whereas *Desulfovibrio* was found dominant in the phylum Proteobacteria.

To further examine the effects of BBJ, FBJ, and CFBJ on the intestinal microbes in HFD-fed mice, an LEfSe analysis was performed to discriminate the gut microbiota among the five groups (Fig. S2†). According to the LEfSe results and their reported functions, the significantly different genera of bacteria are shown in Fig. 7D–J. The relative abundance of the anti-inflammatory genus *Akkermansia* in the FBJ and CFBJ groups was significantly increased ($P < 0.05$) (Fig. 7D). Interestingly, the relative abundance of lean microbiota *Barnesiella* was significantly increased after BBJ and FBJ interventions ($P < 0.05$) (Fig. 7E). The relative abundance of probiotics *Lactobacillus* and *Bifidobacterium* were also explored (Fig. 7F and G). FBJ supplementation significantly increased their relative abundance ($P < 0.05$) compared to the HFD group. The *Bifidobacterium* related genus *Olsenella* in the FBJ

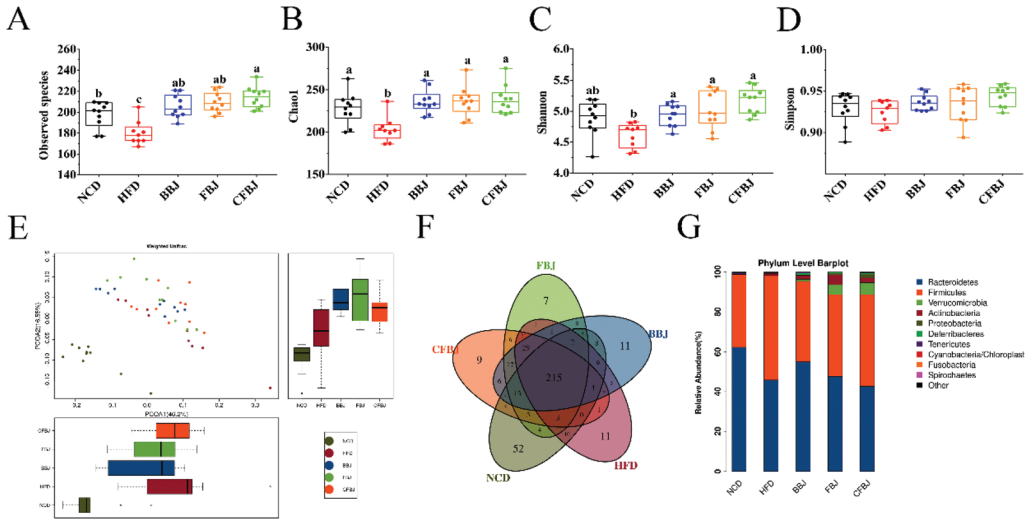

Fig. 6 Blueberry juice with or without fermentation increased the diversity and balance of A–D, α-diversity: observed species, the Chao 1 diversity parameter, Shannon's diversity parameter, and Simpson's diversity parameter, (E) PCoA plot based on weighted UniFrac distances, (F) OTUs Venn Diagram between treatments, (G) relative abundance of gut microbiota at the phylum level. Data were expressed as mean with min to max, and show all points (n = 9–10). Means with the different letters differ significantly (P < 0.05) according to one-way ANOVA analysis followed by Tukey's tests.

group was significantly increased (P < 0.05) compared to the other groups. In addition, the relative abundance of the obese-type gut microbiota (*Oscillibacter* and *Alistipes*, Fig. 7I and J) was significantly decreased (P < 0.05) after FBJ intervention in HFD-fed mice, and the change in their *Oscillibacter* content was consistent with the change in their body weights. Moreover, the correlations among genera were analyzed (Fig. 7K). Probiotics and lean microbiota showed a positive correlation (*Bifidobacterium* and *Barnesiella*), whereas *Bifidobacterium* and *Ruminococcus* showed a negative correlation. Finally, to further understand the potential relationship between the fermented blueberry juice-induced changes in the gut microbiota and the biochemical indicators, Spearman's correlation analysis was used (Fig. 7L). We found that microbes such as *Lactobacillus*, significantly enriched in the FBJ and CFBJ groups, were negatively correlated with TBWG, LDL-C, leptin, and HbA1c. However, HFD-related strains such as *Oscillibacter* and *Alistipes* were positively correlated with TNF-α, LDL-C, HOMA-IR, leptin, and HbA1c. In addition, the DNA abundances of the KEGG pathways indicated that FBJ increased the pyruvate, pyrimidine, glycolysis, and fatty acid metabolic pathways (Fig. S3†).

3.8 BBJ and FBJ increased the caecal SCFA content

The effects of NCD, HFD, BBJ, FBJ, and CFBJ on the production of SCFAs in the caecum are shown in Fig. 8. SCFAs, the end metabolites of carbohydrates fermented with the microbiota play a significant role in metabolic functions and are essential for the intestinal barrier health. In this study, the total content of SCFAs in the caecal samples was found to be the highest in the BBJ group (Fig. 8H). Our study results suggested that the administration of BBJ significantly increased the levels of SCFAs at the end of the experiment. In particular, the levels of acetate and valerate were significantly higher in the BBJ group than in the HFD group (P < 0.05). In addition, the FBJ group had a higher content of SCFAs than the HFD group (P = 0.16), and the levels of valerate in the FBJ group were significantly higher than in the HFD group (P < 0.05) (Fig. 8F).

4. Discussion

There is considerable evidence for proving the antiobesity and antidiabetic effects of blueberry juice and fermented blueberry juice in a HFD-fed murine model, but their potential mechanisms have not yet been clearly defined.[26–28] Based on the experimental trial, this study inferred that fermented blueberry juice prevented HFD-induced obesity, improved insulin resistance, and alleviated inflammation possibly by altering the composition and diversity of the intestinal microbiota. Moreover, this green intervention also played a crucial role in regulating the liver glycolipid metabolism-related genes and protein expression in the experimental mice. In recent years, a substantial number of studies have indicated the potential role of the intestinal microflora in glucose and lipid metabolism and related diseases. Therefore, the gut microbiota of mice fed with different blueberry juices was studied to explore the precise underlying mechanisms to prevent and relieve hyperglycemia using fermented blueberry juice as a green intervention.

Obesity includes the development of visceral fat accumulation, resulting in the corresponding metabolic dysfunction. In this study, the weight and cell size of the adipose tissue in

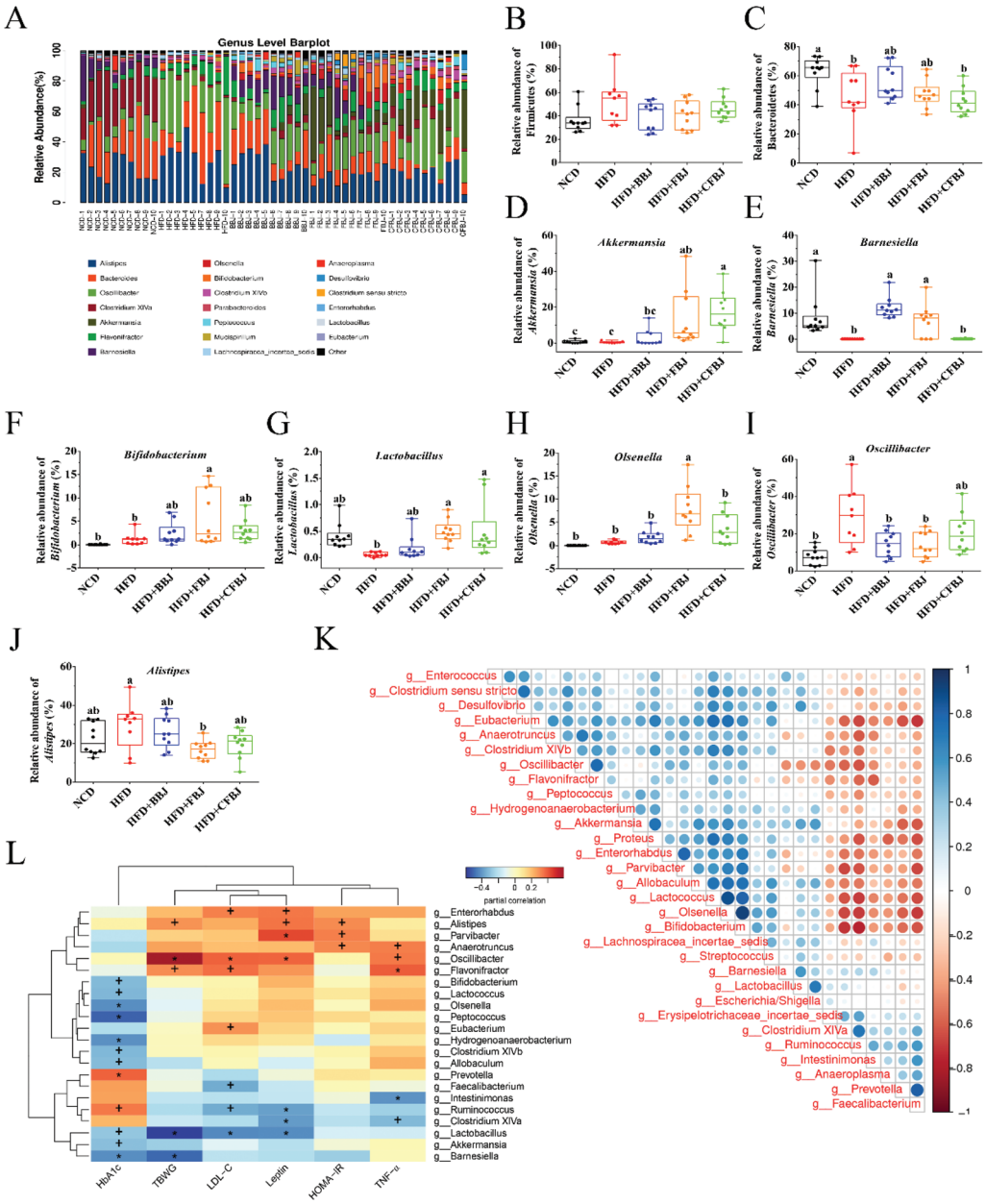

Fig. 7 Blueberry juice with or without fermentation improved the composition of the gut microbiota in HFD-fed mice. (A) Relative abundance of the intestinal microbiota at the general level (those rank top 20 are represented), (B) and (C) the relative abundance of the phyla *Firmicutes* and *Bacteroidetes*, (D–J) richness of some significantly enriched or depleted intestinal bacteria in mice (D, *Akkermansia*; E, *Barnesiella*; F, *Bifidobacterium*; G, *Lactobacillus*; H, *Olsenella*; I, *Oscillibacter*; J, *Alistipes*), (K) correlations between the microbiota at the general level, and (L) correlations between the caecal microbiota of significant differences and metabolic profiles. Data are expressed as mean with min to max, and show all points (n = 9–10). Mean values with the different letters differ significantly ($P < 0.05$) according to one-way ANOVA analysis followed by Tukey's tests. In Fig. 7L, significant correlation test was carried out, when $P < 0.05$ marked with "+", $P < 0.01$ marked with "*".

mice of the BBJ, FBJ and CFBJ groups were significantly reduced, particularly epididymal fat in HFD-fed mice (Fig. 1B, 2A and B). After supplementation with FBJ, hepatic lipid droplets, the size of adipocytes, and the body weight were reduced relatively more in the FBJ group than in the CFBJ and BBJ groups, indicating that FBJ intervention was more helpful in attenuating fat accumulation in the liver and fat tissues (Fig. 2A–D). Johnson *et al.* have reported similar findings that fermented berry beverage reduced fat mass in HFD-fed mice.[28] Likewise, Wen *et al.* have found that the fermentation of *Momordica charantia* polysaccharides promoted weight loss.[29] These researchers determined that the metabolism of phenolic compounds during fermentation was physiologically significant in lactic acid bacteria (LAB) and their metabolites, such as lactate, acetate, and other compounds that have been recently proved to suppress inflammation, inhibit adipogen-

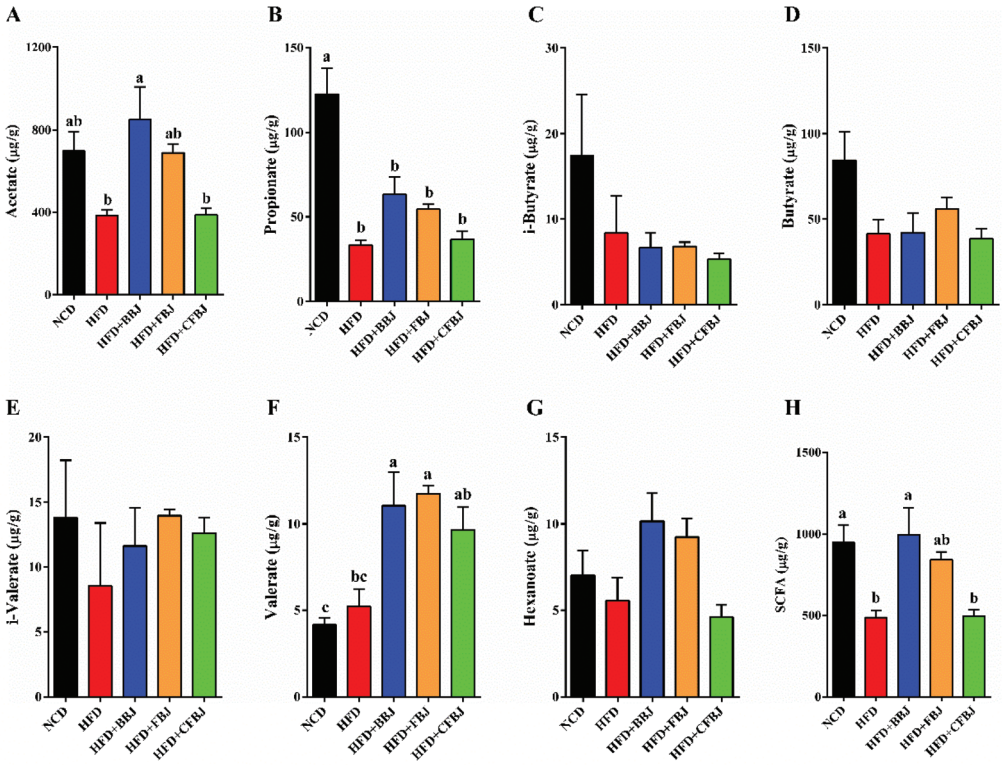

Fig. 8 Caecal content of SCFAs in groups supplemented with BBJ, FBJ, and CFBJ. Mean values with the different letters differ significantly ($P < 0.05$) according to one-way ANOVA analysis followed by Tukey's tests.

esis and accelerate energy metabolism.[18,30,31] In this study, probiotic-fermented blueberry juice interventions exhibited the antiobesity effects in HFD-fed mice. These properties may be attributed to organic and phenolic acids, such as acetate, caffeic acid, gallic acid, chlorogenic acid, and neochlorogenic acid, which are the principle bioactive components of FBJ (Table 1 and Table S1†).

In this study, HFD-fed mice showed significantly higher serum levels of TCHO and LDL-C, which was consistent with previous research.[32] Conversely, mice of the intervention groups (BBJ, FBJ and CFBJ) had lower serum LDL-C and TCHO levels than the HFD group (Fig. 3B and D). This might be because of BBJ, FBJ and CFBJ interventions, which either increased cholesterol excretion or reduced cholesterol synthesis. LDL-C and TCHO are considered to be the triggering factors in the development of atherosclerosis and are closely related to diabetes and metabolic syndrome.[33] Numerous studies have shown that blueberry supplementation improved the lipid profile in mice and humans.[34,35] Ren *et al.* have reported that the combination of blueberry juice and probiotics reduced the levels of TG and LDL-C.[36] The present study suggested that TG was increased in the BBJ and CFBJ groups, which was consistent with the study of Prior *et al.*, who reported that whole blueberries could not completely normalize the lipid parameters.[37] In view of the fact that hyperlipidemia is associated with insulin resistance, that leads to T2DM pathogenesis. Briefly, insulin resistance reduces the absorption and storage of glucose in metabolic tissues cell, which consequently results in an increase in the blood glucose levels. However, the effect of fermented blueberry juice in improving glucose homeostasis has not been completely explored, and thus its antidiabetic potential needs to be investigated. Based on the significant therapeutic effects of BBJ, FBJ, and CFBJ on the fat accumulation, high LDL-C and TCHO levels, we hypothesized that they might ameliorate insulin resistance as well as blood glucose in HFD-fed mice. As expected, FBJ and BBJ interventions improved insulin sensitivity in HFD-fed mice according to the ITT results (Fig. 3H). Previous studies have reported that daily intake of whole blueberries altered metabolic syndrome and reduced insulin resistance in obese rats.[38] To further confirm the modulatory effects on glucose homeostasis, we also determined fasting serum insulin, glucose and the Hb1Ac concentration and HOMA-IR (Table S3† and Fig. 3I and J), and found that BBJ, FBJ, and CFBJ exhibited positive effects on insulin sensitivity and hypoglycemic activity. These findings suggested that BBJ, FBJ and CFBJ interventions decreased the blood glucose levels by ameliorating insulin resistance and dyslipidemia in mice. Therefore, BBJ and FBJ supplementation prevented hyperlipidemia and hyperglycemia induced by a high fat diet.

Insulin resistance is related to adipocyte hormones (leptin and adiponectin) that mediate the insulin-sensitizing effects through the activation of adenosine monophosphate dependent kinase (AMPK), PPAR-α, and some other signaling path-

ways.[39] In the present study, FBJ supplementation increased the expression of GLUT-4 and GCK, indicating that FBJ promoted glucose transportation. Moreover, the protein expression of IR and IRS and the activation of AKT were increased, while GSK-3 was decreased in FBJ-fed mice (Fig. 5), which indicated that FBJ promoted the insulin-sensitivity signaling pathway. Furthermore, FBJ increased the expression of LDL-receptor and PPARα and decreased the expression of FAS, SREBP1c and SCD, showing that FBJ intervention decreased lipid synthesis. Thus, the FBJ group showed less body weight gain (Fig. 1B) and epididymal white adipose tissue (Fig. 2A) than the BBJ and CFBJ groups. Though the HFD group showed leptin resistance, the fermented blueberry juice significantly decreased the serum leptin levels. Obesity-induced insulin resistance may result from an imbalance in the expression of anti- (IL-10) and pro-inflammatory adipokines, such as TNF-α, IL-1β, and IL-6, which was mainly due to a gradual increase in plasma endotoxins, especially LPS.[40,41] The present study results showed that serum TNF-α significantly increased, whereas IL-10 decreased in the HFD group (Fig. 4E and F), suggesting that a high fat diet induced systemic low-grade inflammation. In this context, supplementation of BBJ, FBJ, and CFBJ significantly mitigated inflammation. Several studies have highlighted that blueberry phenolic compounds and fermentation metabolites exhibited protective effects against obesity and inflammation.[26,28,42] Thus, the potential mechanisms of fermented blueberry juice to attenuate insulin resistance and obesity may be through increasing the insulin-sensitivity signaling pathway, and reducing inflammation and lipid synthesis.

High fat diet-induced obesity and hyperglycemia were closely correlated with gut microbiota dysbiosis. Recent studies have proved that supplementation with blueberry or its extract showed significant effects, such as antiobesity and insulin sensitivity by restoring the balance of the diversity, richness, and composition of the gut microbiota in murines.[27,43] This study revealed that FBJ significantly increased the α-diversity and β-diversity compared to the HFD group (Fig. 6A–E), suggesting that it exerted positive effects on the intestinal microbial community in a characteristic direction. Notably, LPS-suppressing Verrucomicrobia in the FBJ and CFBJ groups was significantly improved compared to the HFD group. Many studies have demonstrated that obesity and insulin resistance were correlated and might be due to the imbalance of Firmicutes and Bacteroidetes, which had a crucial role in obesity-related inflammation.[15,44] In this case, BBJ and FBJ interventions increased Bacteroidetes and decreased Firmicutes.

Intriguingly, in accordance with previous studies, supplementation with a HFD increased the abundance of *Oscillibacter* and *Alistipes* in feces.[14,45] The FBJ group significantly reversed the increased *Oscillibacter* and *Alistipes*, which were found to be more abundant in patients with T2DM than in subjects with normal glucose metabolism.[14] We also speculated whether the growth of beneficial microbes could regulate lipid and glucose metabolic disorders by inhibiting the inflammatory reactions. One of the reasons may be that beneficial bacteria can enhance the conversion of dietary fiber to SCFAs. Mice fed with BBJ and FBJ produced more SCFAs, especially acetate (Fig. 8). Evidence indicated that SCFAs greatly improved glucose homeostasis and insulin sensitivity by inhibiting the production of proinflammatory cytokines.[46] Clinical works have demonstrated that *Akkermansia muciniphila* was negatively correlated with inflammation, obesity, and insulin resistance.[16,47] Our findings suggested that FBJ- and CFBJ-stimulated enrichment of *Akkermansia* was sufficient to reverse HFD-induced obesity (Fig. 7D). Fermented blueberry juices were rich in phenols and SCFAs, which were capable of enriching the *Akkermansia* population.[48,49] *Lactobacillus* and *Bifidobacterium*, as important intestinal probiotics, had crucial roles in maintaining the balance of the gut microbiota and guarding the intestinal epithelial cells against pathogenic microorganisms.[50] In the present study, FBJ supplementation significantly augmented the relative abundance of *Lactobacillus* and *Bifidobacterium* compared to HFD-fed mice. Recently, similar results have been reported to improve the gut microbiota using fermented orange juice.[51] A significant increase was observed in *Barnesiella* and *Olsenella*, which were negatively related to some obesity-related bacteria after FBJ treatment. These findings indicated that FBJ was a more potent alternative to improve the beneficial gut bacteria than CFBJ, which needs to be confirmed by further investigations. Furthermore, in a correlation analysis of the gut microbiota and different blueberry juice-induced metabolic effects (Fig. 7L), the abundances of *Akkermansia*, *Barnesiella*, and *Lactobacillus* were negatively associated with metabolic syndrome phenotypes. The studies of Haiping Du *et al.* and F. F. Anhe *et al.* indicated that increasing the abundance of *Akkermansia*, *Barnesiella* and *Lactobacillus* with prebiotics was associated with the augmented gut barrier and reduced intestinal endotoxins.[49,52] However, whether FBJ shows the same gut barrier effects needs further investigation. PICRUSt function prediction further indicated that the gut microbiota in the FBJ group had more capacity of fatty acid metabolism and glycolysis than the HFD group, that improved energy utilization and resulted in a significant decrease in the incidence of metabolic syndrome and T2DM.[53,54] Conclusively, based on our study findings, it is inferred that FBJ intervention can provide a robust modulation of the gut microbiota, thereby exhibiting antiobesity and antidiabetic (T2DM) effects in HFD-fed mice.

5. Conclusions

This study revealed that fermented blueberry juice was an effective green intervention in ameliorating the symptoms of metabolic syndrome in high fat diet-fed mice. Dietary intervention showed the dynamic aspects including suppressing fat accumulation, decreasing liver lesions, ameliorating insulin resistance, controlling systemic inflammation, and maintaining the gut microbiota balance. The gut microbiota was closely related to the changes in biochemical indices, and thus, sup-

plementation with fermented blueberry juice exhibited antiobesity effects, possibly through its ability to modulate the gut microbiota, liver mRNA, and protein expression. Overall, our study findings suggested that supplementation of fermented blueberry juice showed therapeutic effects and could be used to protect people against early onset of various ailments like insulin resistance, visceral fat accumulation, and chronic inflammation.

Conflicts of interest

There are no conflicts of interest to declare.

Acknowledgements

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