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Research Article

Investigation on Quality Characteristics and Antidiabetic Properties of Mulberry Leaf Fu Brick Tea

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Mulberry leaves (*Morus alba* L.) exhibit antidiabetic properties; however, their taste is unappetizing, leading to limited practical applications. Although Fu brick tea has a favorable flavor, its hypoglycemic effects are weak. Hence, we combined the two to create mulberry leaf Fu brick tea (SF) and investigated its sensory perception, safety, microorganism content, key metabolites, and impact on hyperglycemic mice using the same raw materials and techniques to prepare Fu brick tea (F) and mulberry leaf brick tea (S) individually. Our study showed that SF, which comprises blended mulberry leaf primary dark tea and primary dark tea (1 : 3 g/g), was sweeter and smoother, with a median lethal dose of 22.3 g/kg, indicating that it is safe for consumption. Compared to F, caffeine levels were reduced in SF and 1-deoxynojirimycin and aminobutyric acid levels were increased. Phenolic substances and flavonoids showed increased levels in SF compared with S. These changes contribute to the taste and functionality of SF. The primary microorganisms in SF were *Bacillus*, *Lactobacillus,* and *Aspergillus*, which contributed to the improved quality. SF demonstrated its antidiabetic effects by reducing the blood sugar levels of hyperglycemic mice, restoring the function of pancreatic cells, reducing lipid levels, and improving the antioxidant capacity of the liver. Overall, SF exhibited a better taste and antidiabetic properties than F and S, highlighting its potential as a functional beverage.

1. Introduction

Diabetes mellitus is a multifactorial metabolic disease characterized by chronic hyperglycemia and the disruption of fat, protein, and carbohydrate metabolism. The common symptoms include polyuria, polydipsia, and polyphagia [1]. Hyperglycemia, a key clinical feature of diabetes, is caused by the abnormal synthesis and secretion of insulin and insulin resistance. Abnormal insulin production and regulation can lead to chronic hyperglycemia, which is a hallmark of diabetes. Poor regulation of blood glucose levels can lead to serious complications, such as cardiovascular, kidney, eye, and foot diseases, as well as an increased risk of cancer [2]. Although many drugs have been developed to treat diabetes, almost all are chemical or biological agents, and there is a lack of naturally effective, side-effect-free drugs. Therefore, the focus of this research was to identify a natural drug for treating diabetes without side effects.

Mulberry leaf (*Morus alba* L.) is a traditional Chinese medicine that has been used since the 16th century to treat diabetes. Li Shizhen documented in his work, "Compendium of Materia Medica" (in Chinese "Ben Cao Gang Mu"), that mulberry leaves were used to treat diabetes. Mulberry leaves are regularly consumed in South Korea and Japan as anti-hyperglycemic supplements [3]. Mulberry leaves contain active ingredients, such as polysaccharides, flavonoids, alkaloids, and polyphenols [4], and therefore, they have health benefits, such as the prevention of cardiovascular diseases, anti-hypertension and antioxidant abilities, and the regulation of the gut flora [5, 6]. Despite their numerous advantages, the astringent taste of mulberry leaves impedes their use in commercial beverages.

Tea (*Camellia sinensis* (L.) Kun) is an ancient Chinese beverage with a reputation as a healthy drink. Ethnic minorities in Northwest China have enjoyed dark tea, a unique fermented tea, for centuries. One such example is Fu brick tea (F), a typical Hunan Anhua dark tea that has been found to have antiobesity and hypolipidemic properties [7]. Fu brick tea contains numerous active ingredients including polysaccharides, tea polyphenols, catechins, amino acids, and alkaloids [8]. Its health benefits include anti-hyperlipidemia, antimutation, antioxidation, antitumor, antiobesity, and antitoxicity effects. The unique floral fragrance and mellow taste of Fu brick tea are enjoyed by local and international consumers alike [7, 9, 10]. However, the hypoglycemic effect of Fu brick tea is weak [11].

Mulberry leaf Fu brick tea is prepared by introducing mulberry leaves to traditional Fu brick tea; however, there is a lack of research on this blend. In this study, we followed the same process [12] and used identical raw materials to create three different types of tea: Fu brick tea (F), mulberry leaf Fu brick tea (SF), and mulberry leaf brick tea (S). This study aimed to answer two questions. First, what changes occur in the taste and safety of SF when mulberry leaves are added to F? Second, what are the differences in the quality and antidiabetic properties of SF, F, and S? To this end, we conducted sensory evaluations for all three types of tea and assessed the safety of SF. Microbiome analysis and nontargeted metabolomics were used to study the microorganisms and key metabolites in the three samples. Additionally, we used animal models to study the effects of tea on diabetes. Therefore, our study aimed to provide further insights into the potential health benefits and risks of mulberry leaf Fu brick tea and its hypoglycemic effect.

2. Materials and Methods

2.1. Experimental Materials. Primary dark tea was supplied by Anhua Yuntange Tea Co. Ltd. (Xiaoyan Town, China), which was first-class primary dark tea of Anhua. Mulberry primary dark tea was provided by Hunan Southern Cocoon Co. Ltd. (Pushi Town, China), which was first-class primary dark tea, and the variety was Yuesang11. The method used to produce the tea samples is shown in Figure S1. We prepared F from primary dark tea, S from mulberry leaf primary dark tea, and SF from blended mulberry primary dark tea (0.25kg) and primary dark tea (0.75kg)(1:3g/g). The preparation methods for the SF, F, and S extracts were based on those used in a previous study [7]. Briefly, three samples were extracted separately. Fist, samples were crushed and extracted for 40 min at a tea-water ratio of 1 : 10 at 100°C, followed by filtration with absorbent cotton. A second extraction step was performed for 30 min at a tea-water ratio of 1 : 8 at 100°C, followed by filtration. The two filtrates were combined, concentrated using rotary evaporation, and freeze-dried into tea powder for further experiments.

2.2. Chemicals and Reagents. We obtained the following chemicals from the designated suppliers: catechins, γ-aminobutyric acid (GABA), and 1-deoxynojirimycin (DNJ) (Shanghai Yuanye Biotechnology Co., Ltd., Shanghai, China); metformin (Guizhou Tianan Pharmaceutical Co., Ltd., Guiyang, China); 9-fluorenylmethyl chloroformate (FMOC-Cl) (Shanghai Yuanye Biotechnology Co., Ltd., Shanghai, China); Magnetic Soil and Stool DNA Kit (TIANGEN); streptozotocin (STZ; Sigma, St. Louis, MO, USA); ethanol (Shanghai Sinopharm Group, Shanghai, China); and assay kits for total cholesterol (TC), total triglycerides (TG), low-density lipoprotein cholesterol (LDL-c), superoxide dismutase (SOD), glutathione peroxidase (GSH-Px), and malondialdehyde (MDA) (Nanjing Jiancheng Bioengineering Institute, Nanjing, China).

2.3. Sensory and Quality Analyses of SF, F, and S. The sensory evaluation of SF, F, and S was performed using the "Tea Sensory Evaluation Method" (GB/T 23776-2018). Five senior tea reviewers (Hunan Agricultural University, China) participated in sensory evaluations. Briefly, 150 mL of boiled water was poured into a tea cup containing a 3 g tea sample and was steeped for 8 min. The tea juice was then filtered into a tea bowl. The aroma, soup color, taste, and leaf bases were evaluated. The quality in terms of polyphenol and free amino acid contents were evaluated referring to "Testing Methods for Tea Polyphenols and Catechins in Tea" (GB/T 8313-2018) according to a previous study [13]. Analyses were performed using a high-performance liquid chromatography (HPLC) system (Waters 590; Waters Corp., Milford, MA, USA) equipped with a Hypersil ODS 2 C18 column (5 mL, 4.6 mm × 250 mm). Detection was performed by measuring the absorbance at 280 nm and 35°C. Solvents A (2% acetic acid) and B (acetonitrile) were run using a linear gradient for 20 min, from 93% to 55% A, with a constant flow rate of 1.4 mL/min. The catechin content was determined by comparing the peak areas of the samples with those of known standards. Polysaccharide content was analyzed as follows. The extract was diluted, and 1 mL aliquots were added to a volumetric flask containing 8 mL anthrone reaction solution (6×10^{-3} g/mL). ddH_2O was used as a blank control. The flask was placed in boiling water for 3 min and, after cooling, the absorbance was measured at 620 nm using a spectrophotometer. The polysaccharide content was calculated according to a standard curve generated using glucose solutions of different concentrations [13]. The total flavonoid content was determined using the aluminum trichloride colorimetric method. The extract was diluted,

and 0.5 mL aliquots were added to a volumetric bottle. Ten milliliters of 1% $AlCl_3$ aqueous solution was added, with 1% $AlCl_3$ aqueous solution used as a blank, and after 10 min, the absorbance was measured at 420 nm using a spectrophotometer. The rutin content was calculated according to the standard curve generated by standard rutin solutions of different concentrations. The DNJ content was determined according to the method described by Nitra et al. [14]. Briefly, 1 g of double-extracted sample in 10 mL of 0.05 M HCl was derivatized with FMOC-Cl and detected using HPLC (LC-20AT, Shimadzu, Japan). The GABA content was determined according to the method described by Teng et al. [15]. Briefly, GABA was derivatized with ortho-phthalaldehyde and detected by HPLC. The mobile phase was 0.2 mol/L sodium citrate buffer, pH 3.2–10.0, and the gradient elution was performed at a flow rate of 0.3 mL/min, with a 55°C column temperature.

2.4. Evaluation of the Acute Toxicity of SF. The acute toxicity of SF was tested in healthy ICR mice (18–22.9 g) obtained from Hunan Slack Jingda Experimental Animal Co. Ltd. (Changsha, China). A pretest was first performed to ascertain the lethal dose, followed by a formal acute toxicity test. Sixty mice were divided into six groups (five males and five females in each group; 10 mice per group), including a blank control group and five treatment groups with different dosages. The mice were fasted for 12 h and allowed to drink water freely. According to preliminary data, the concentrations of SF after oral administration to animals in the five treatment groups were determined as (i) 14.60 g/kg, (ii) 18.90 g/kg, (iii) 24.90 g/kg, (iv) 32.60 g/kg, and (v) 42.90 g/kg at a dose of 20 mL/kg. The observed and recorded activities and survival status of the mice were used to determine the median lethal dose (LD_{50}). The Bliss method was used to calculate the LD_{50} and 95% confidence intervals. Animal experiments adhered to National Institutes of Health guidelines (No.85-23 Rev.1985) and the "Guidelines for Animal Care and Use" of the Committee on Ethics of Hunan Agricultural University (No. of registration: 015063506, Changsha, China).

2.5. Microbiota in the SF, F, and S. To identify the microbial type and count in SF, F, and S, metagenomic analysis was performed in triplicate. Taxonomic analysis of bacterial and fungal communities was carried out. Universal primers were used to amplify the V3-V4 region of the bacterial *16S rRNA* gene (338F, 5′-ACTCCTACGGGAGGCAGCA-3′; 806R, 5′-GGACTACHVGGGTWTCTAAT-3′) and the fungal internal transcribed spacer (ITS) 1 region (ITS1F, 5′-CTTGGTCATTTAGAGGAAGTAA-3′; ITS2R, 5′-GCTGCGTTCTTCATCGATGC-3′). A Magnetic Soil and Stool DNA Kit was used to extract DNA of high quality and yield from the samples, according to the manufacturer's instructions, and metagenomic analysis was performed using a NovaSeq 6000 platform (Illumina, San Diego, CA, USA) at Shanghai Biotree Biotech Co. Ltd. (Shanghai, China).

Data quality was filtered using Trimmatic software (version 0.33). Primer sequences were identified and removed using Cutadapt software (version 1.9.1). FLASH (version 1.2.11) was used to splice double-ended reads and remove chimeras (UCHIME version 8.1) to obtain high-quality sequences. USEARCH (version 10.0) was used for operational taxonomic unit (OTU) clustering, with 97% similarity. The Silva Database (http://www.arb-silva.de/) was used based on the Mothur algorithm to annotate taxonomic information. Alpha diversity is applied in analyzing complexity of species diversity for a sample through 5 indices, including observed-species, Chao1, Shannon, Simpson, and ACE. All these indices in our samples were calculated with QIIME (Version 1.9.1) and displayed with R software (Version 3.6.3, http://www.biotree.cn/). Beta diversity analysis was performed using R vegan (version 3.6.3, https://www.biotree.cn/).

2.6. Metabolomics Experiments. Metabolites were extracted from the SF, F, and S teas and assessed using an ultra-high-performance liquid chromatography (UPLC) system (Vanquish; Thermo Fisher Scientific, Waltham, MA, USA) with a UPLC HSS T3 column (2.1 mm × 100 mm, 1.8 μm) coupled to a Q Exactive HFX mass spectrometer (Orbitrap MS, Thermo Fisher Scientific). Each sample was tested six times. The sample powder (10 mg) was added to 1,000 μL of extraction solution (methanol : water = 3 : 1, with an isotopically labelled internal standard mixture) in an Eppendorf tube. The mixture was vortexed for 30 s at 35 Hz for 4 min and sonicated for 5 min in an ice-water bath. The preceding step was repeated three times. The treated samples were centrifuged for 15 min at 12,000 rpm at 4°C and the resulting supernatant was tested. A quality control (QC) sample was mixed with all the samples and also tested. Phase B was acetonitrile and phase A was an aqueous solution of 5 mmol/L ammonium acetate and 5 mmol/L acetic acid. The autosampler temperature was 4°C and the injection volume was 3 μL. Tandem mass spectrometry (MS) spectra were acquired in the information-dependent acquisition mode using a Thermo Q Exactive HFX mass spectrometer with Xcalibur, version 4.0.27 software (Thermo Fisher Scientific).

Raw data were converted to the mzXML format using ProteoWizard and processed with an in-house program, which was developed using R vegan (version 4.1.0, https://www.biotree.cn/) and based on XCMS, for peak detection, extraction, alignment, and integration. Subsequently, an in-house MS2 database (BiotreeDB) was used to annotate the metabolites. The cutoff for annotation was set as 0.3. Logarithmic conversion and centralized formatting of the data were performed using SIMCA software (V16.0.2; Sartorius Stedim Data Analytics AB, Umea, Sweden), followed by automatic modeling analysis.

2.7. Diabetic Animals and Experimental Design. Four-week-old C57BL/6 pathogen-free male mice (18–22 g) were purchased from Hunan Slack Jingda Experimental Animal Co. Ltd. A climate-controlled environment was provided for the animals (25 ± 2°C and 60%~80%). The animal breeding room had a 12-hour dark/light cycle and was ventilated with fresh air ≥15 times/h. The mice were acclimatized to the animal housing conditions for 1 week and were provided with ad libitum access to food and water. Five consecutive days of daily intraperitoneal injections of STZ (50 mg/kg

body weight) were used to induce diabetes. Blood glucose levels were measured after fasting for 6 h. The STZ-induced diabetes model was considered successful if the blood glucose level exceeded 11.1 mmol/L.

All mice were randomly divided into six groups, including a control group (CON) of untreated nondiabetic mice, and five groups of STZ-induced diabetic mice with the following treatments: (i) DB group, control treatment with physiological saline; (ii) metformin group (MET), positive control treatment with metformin (125 mg/day/kg body weight); (iii) SF group; (iv) F group; and (v) S group. Groups SF, F, and S received 520 mg/kg of their respective aqueous extracts daily (the dose of samples = 18 g ÷ 60 kg (body weight) × 7.8 (the conversion factor) × 22.5% (the leaching rate is approximate) ≈ 520 mg/kg). The CON and DB groups received equivalent volumes of physiological saline instead of the aqueous extract. The intervention lasted 4 weeks. Body weight and fasting blood glucose (FBG) levels were measured on the fifth day of each week.

Oral glucose and insulin tolerance tests were performed using a blood glucose meter (Roche Diagnostics, Shanghai, China) to measure blood glucose levels in the tail tips. Oral glucose and insulin tolerance assessments were conducted as described by Liu et al. [16]. For serum analyses, 5 groups of mice were fasted for 6 h after treatment and subsequently anesthetized. Blood was collected from the eyeballs and centrifuged at 3,000 rpm for 10 min to obtain serum, which was then aliquoted for various assays. A mouse insulin enzyme-linked immunosorbent assay kit was used to measure serum insulin content according to the manufacturer's instructions. The insulin resistance index was calculated using the HOMA2-IR model calculator (https://www.dtu.ox.ac.uk/homa). In accordance with the manufacturer's instructions, serum levels of TC, TG, and LDL-C were measured using commercial assay kits. The livers were excised and washed with a 0.9% saline solution and fixed in 10% formalin, embedded in paraffin, sectioned, and stained with hematoxylin and eosin for histopathological examination according to the procedure described by Teng et al. [17]. To prepare the sample for oxidative stress analysis, 100 mg liver specimens were added to 900 μL of normal saline and homogenized using a tissue homogenizer. The protein concentrations of GSH-Px, SOD, and MDA in the liver samples were subsequently measured using commercial assay kits according to the manufacturer's instructions. Differential results were assessed using Duncan's test, with $P < 0.05$ considered statistically significant.

3. Results and Discussion

3.1. Sensory Evaluation and Major Chemical Components of SF, F, and S. The sensory attributes and chemical compositions of SF, F, and S tea samples were investigated in this study. The scores for SF, F, and S were 91.54, 89.52, and 82.89 (Table S1). SF has a bright orange-red color with a profound aroma. It tastes mellow, thick, and sweet. Similarly, F has a bright orange-red color with a unique fungal aroma and a mellow and thick taste. In contrast, S has a dull black color with a grass odor and a sweet and smooth taste. The tea reviewers agreed that SF had a more agreeable taste than F and S. The principal components in each sample were polyphenols, polysaccharides, and flavonoids (Table 1). Compared to F, SF possessed lower caffeine content (1.79% and 2.43%, respectively) but higher contents of DNJ (63.67 mg/100 g and not detected (ND), respectively) and GABA (62.19 mg/100 g and 31.03 mg/100 g, respectively). Compared to S, SF possessed higher phenolic (5.50% and 1.06%, respectively), flavonoid (0.61% and 0.48%, respectively), and gallic acid (0.2% and ND, respectively). To summarize, F had a low content of functional ingredients and S had a poor taste. Thus, SF can compensate for the shortcomings of both individual teas. As a result, SF had an enhanced taste and also contained more potent bioactive compounds.

3.2. Acute Toxicity of SF. The mortality of the animals showed a positive correlation with the amount of SF administered. Our findings revealed that the oral LD_{50} of SF was 22.3 g/kg, with a 95% confidence interval of 19.3–25.6 g/kg, as presented in Table S2. According to the "China National Food Safety Standard Acute Toxicity Oral Test," an LD_{50} greater than 5 g/kg indicates nontoxicity. Similarly, the "Health Food Inspection and Evaluation Technical Specifications" classify an LD_{50} > 15 g/kg as nontoxic. The LD_{50} of an aqueous extract of mulberry leaves has been shown to be higher than 15.0 g/kg [18]. The acute oral LD_{50} of Fu brick tea is 14,700 mg/kg in male mice and 19, 600 mg/kg in female mice [19]. Previous studies have shown that mulberry leaves and tea leaves are nontoxic, and our results demonstrated that SF is nontoxic. Therefore, SF is considered a safe beverage.

3.3. Microbiota in SF, F, and S. From the nine tea samples, we obtained 720,493 bacterial *16S rRNA* and 720,214 fungal ITS sequences, and most microbes were captured under the analytical conditions employed (Figures 1(a) and 2(a)). Different samples showed significant differences in bacterial abundance and diversity. As shown in Table 2, the bacterial abundance and diversity were significantly higher in SF and F than in S ($P < 0.05$), but fungal abundance and diversity were not significantly different. Based on the relative abundance (RA) of the OTUs, principal component analysis (PCA) showed significant differences in the abundance of bacteria but no significant differences in the abundance of fungi (Figures 1(b) and 2(b)).

The bacteria were grouped into 26 phyla based on their OTUs (Dataset S1, Sheets 1 and 2). Proteobacteria and Firmicutes were the two dominant phyla (Figure 1(c)). The results of a previous study on the bacterial phyla in Fu brick tea were consistent with this conclusion [20]. The proportions of *Bacillus* spp. in SF, F, and S were 39.27%, 1.81%, and 90.35%, respectively. *Lactobacillus* and *Weissella* accounted for 2.45%, 2.24%, and 0.13% and 0.86%, 0.41%, and 0.01%, respectively (Figure 1(d)). *Bacillus* and *Lactobacillus* were significantly more abundant in SF than F and S, which was helpful for improving its quality. *Bacillus* spp. are vital microorganisms present in fermented foods. They have strong adaptability and are capable of producing proteases that break down proteins, as well as antibiotics

Table 1: Chemical composition of SF, F, and S ($n = 3$).

	SF	F	S
Polyphenols (%)	5.50 ± 0.30^{b}	8.02 ± 0.52^{a}	1.06 ± 0.32^{c}
Flavonoids (%)	0.61 ± 0.02^{b}	0.65 ± 0.01^{a}	0.48 ± 0.01^{c}
Polysaccharides (%)	2.46 ± 0.10^{b}	2.20 ± 0.08^{b}	5.26 ± 0.20^{a}
Theobromine (%)	0.13 ± 0.00^{b}	0.17 ± 0.00^{a}	ND
Gallic acid (%)	0.20 ± 0.01^{b}	0.42 ± 0.02^{a}	ND
Caffeine (%)	1.79 ± 0.01^{b}	2.43 ± 0.00^{a}	0.10 ± 0.00^{c}
EGC (%)	ND	1.00 ± 0.00	ND
DL-C (%)	0.08 ± 0.00^{b}	0.19 ± 0.00^{a}	ND
EC (%)	0.05 ± 0.00^{b}	0.17 ± 0.00^{a}	ND
EGCG (%)	0.07 ± 0.00^{b}	0.37 ± 0.00^{a}	ND
GCG (%)	0.07 ± 0.00^{b}	0.21 ± 0.00^{a}	ND
ECG (%)	ND	0.08 ± 0.00	ND
DNJ (mg/100g)	63.67 ± 0.22^{b}	ND	249.84 ± 5.57^{a}
GABA (mg/100g)	62.19 ± 0.66^{b}	31.03 ± 0.62^{c}	154.74 ± 0.45^{a}
Fructose	0.30 ± 0.04^{b}	0.18 ± 0.00^{c}	0.83 ± 0.06^{a}
Glucose	0.23 ± 0.09^{b}	0.21 ± 0.03^{b}	1.68 ± 0.15^{a}
Sucrose	0.40 ± 0.02^{b}	0.20 ± 0.08^{c}	1.48 ± 0.11^{a}
Maltose	0.16 ± 0.03^{b}	0.13 ± 0.02^{b}	0.23 ± 0.01^{a}

Mulberry leaf Fu brick tea, SF; Fu brick tea, F; mulberry leaf brick tea, S. EGC, (−)-epigallocatechin; DL-C, (−)-catechin; EC, (−)-epicatechin; EGCG, (−)-epicatechin-3-O-gallate; GCG, (−)-gallocatechin-3-O-gallate; ECG, (−)-epicatechin-3-O-gallate; DNJ, 1-deoxynojirimycin; GABA, γ-aminobutyric acid. Different letters indicate significant differences ($p < 0.05$). The same letter indicates that the difference is not significant.

that inhibit harmful bacteria [21]. Exopolysaccharides produced by members of *Bacillus* improve hyperglycemia, dyslipidemia, and cardiovascular disease risk in rats with STZ-induced diabetes [22]. *Lactobacillus* spp., which can ferment sugars to produce lactic acid, are important probiotics and they have a variety of probiotic effects, such as improving gastrointestinal function by regulating the balance of the intestinal flora and participating in immune system regulation. They are widely used in industrial production, food fermentation, medical care, and other fields [23]. *Lactobacillus* spp. can be used to produce functional nutraceutical supplements with antidiabetic activity. *Lactobacillus* spp. have been used to develop functional fermented milk products, which can reduce blood glucose and α-amylase concentrations in diabetic rats [24]. Therefore, we speculated that the bacterial species present in SF may have potential health benefits and play a significant role in the quality of the final product.

The fungal OTUs were divided into four phyla (Dataset S1, Sheets 3 and 4). The dominant phylum was *Ascomycota,* at 99.81%, 99.95%, and 99.79% in SF, F, and S, respectively (Figure 2(c)). At the genus level, *Aspergillus* accounted for 99.31%, 99.69%, and 99.31% of the OTUs of SF, F, and S, respectively (Figure 2(d)). The dominance of *Aspergillus* in Pu-erh and Fu brick teas has been previously reported [25, 26]. *Aspergillus* is a crucial genus of economically important fungi that can produce and secrete different enzymes and metabolic products to improve food quality during fermentation [27]. Previous studies have shown that, in Fu-break tea, members of *Aspergillus* can secrete and regulate the activities of various enzymes, such as amylase, polyphenol oxidase, cellulase, and pectinase, which play crucial roles in the decomposition of sugars, proteins, and lipids [13, 28]. Therefore, we believe that mulberry leaves added to SF do not affect the growth of *Aspergillus* and that the growth of *Aspergillus* can transform the substances in SF.

3.4. Metabolites in SF, F, and S. Metabolomic analysis of the three samples (Dataset S2, Sheets 1 and 2) detected a total frequency of 14,150 m/z. PCA with 88.4% variation revealed that the metabolites identified in SF, F, and S were distinct (Figure 3(a)). By comparing with a reference and data from relevant databases (https://biodb.swu.edu.cn/mmdb/), 142 metabolites were identified in the three samples, including caffeine, (−)-epigallocatechin, moracin M, gamma-aminobutyric acid, and epicatechin. Most of these metabolites were flavonoids (27 metabolites), carboxylic acids and their derivatives (16 metabolites), organooxygen compounds (15 metabolites), and fatty acids (12 metabolites) (Dataset S2, Sheets 3 and 4). Of these metabolites, 78 were annotated to 38 KEGG pathways, with highly represented pathways of purine metabolism (map00230), flavonoid biosynthesis (map00941), and glyoxylate and dicarboxylate metabolism (map00630), which included nine, nine, and three metabolites, respectively (Datasets S2 and 5).

The relative levels of 23 or 31, 68 or 47, and 72 or 45 metabolites decreased (Variable Importance in the Projection (VIP) >1.0, $p < 0.05$, and fold change (FC) <0.84) or increased significantly (VIP >1.0, $p < 0.05$, and FC >1.2) in SF/F, SF/S, and F/S, respectively (Figure 3(b)). Among them, hypoxanthine, chlorogenic acid, quercitrin, and morin increased significantly, whereas caffeine, catechin, and theobromine decreased significantly compared to SF/F (Figure 3(c), Dataset S2, Sheet 6). SF may be more suitable than F for individuals who are sensitive to caffeine. Kaempferol, caffeine, quercetin, gallic acid, epigallocatechin gallate (EGCG), L-theanine, theaflavin, epicatechin (EC), theobromine, and rutin increased significantly, whereas gamma-aminobutyric acid, vitamin A, and chlorogenic acid decreased significantly in comparison with SF/S (Figure 3(d); Dataset S2, Sheet 7). Previous studies have shown that kaempferol can stimulate glycogen synthesis [29], quercetin can protect pancreatic cells [30], EGCG binds alpha-amylase active sites to inhibit starch hydrolysis [31], and rutin inhibits tissue gluconeogenesis and increases tissue glucose uptake [32]. Therefore, we concluded that SF had a stronger hypoglycemic effect than S.

3.5. Effect of SF, F, and S on Blood Glucose Homeostasis in STZ-Induced Diabetic Mice. SF, F, and S were used as interventions in STZ-induced diabetic mice. The FBG levels of the mice in the SF, F, and S groups gradually decreased, and there was a significant difference between the SF, F, and S groups and DB group after four weeks, which decreased by 24.21%, 23.60% and 17.95%, respectively ($P < 0.05$; Table 3). The weights of mice in the SF, F, and S groups significantly exceeded those in the DB group ($P < 0.05$; Table S3). Tea and mulberry leaves are known to regulate glycolipid digestion, absorption, and metabolism [33]. Therefore, SF, F, and S may improve the physical condition of diabetic mice. The glucose tolerance test assesses the function of pancreatic cells

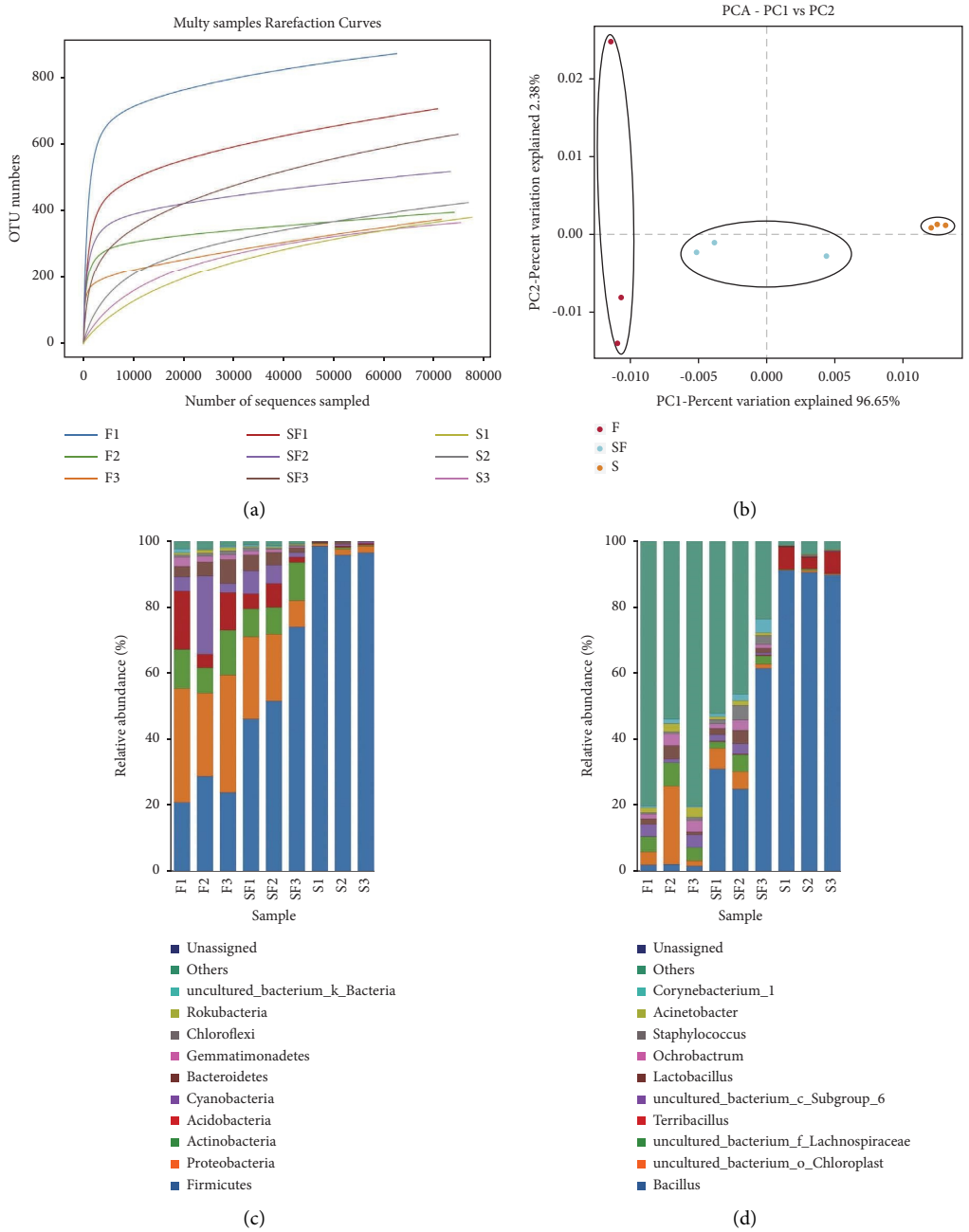


Figure 1: Results of bacterial abundance in SF, F, and S. (a) Rarefaction curve of all samples. (b) PCA of relative abundance (RA) of bacterial OTUs in each sample. Bacterial species distribution bars at the phylum level (c) and at the genus level (d).

and the effectiveness of the body at maintaining normoglycemia [34]. Our findings revealed that the area under the curve (AUC) for the oral glucose tolerance test (OGTT) was significantly lower in the SF group than the DB group ($P < 0.05$; Figures 4(a) and 4(b)), suggesting a pronounced beneficial effect of SF on glucose tolerance in diabetic mice. However, SF was less effective than metformin. The insulin tolerance test results showed that the AUC of the insulin tolerance test (ITT) was significantly lower in the F group than the DB group ($P < 0.05$; Figures 4(c) and 4(d)), indicating that F markedly enhanced insulin sensitivity in diabetic mice.

The hormone insulin plays a crucial role in lowering blood glucose levels within the body [35]. Consequently, fasting insulin levels are considered an essential indicator of both the secretory function and reserve capacity of pancreatic β-cells [36]. Our results revealed that the SF group had considerably higher serum insulin levels than the DB, MET, and S groups ($P < 0.05$; Figure 5(a)). These findings suggested that SF promotes insulin secretion. To evaluate the degree of peripheral insulin resistance and homeostatic β-cell function, we calculated the insulin resistance (HOMA-IR) score and homeostatic β-cell function (HOMA %B) based on FBG and fasting insulin levels [37]. Our findings

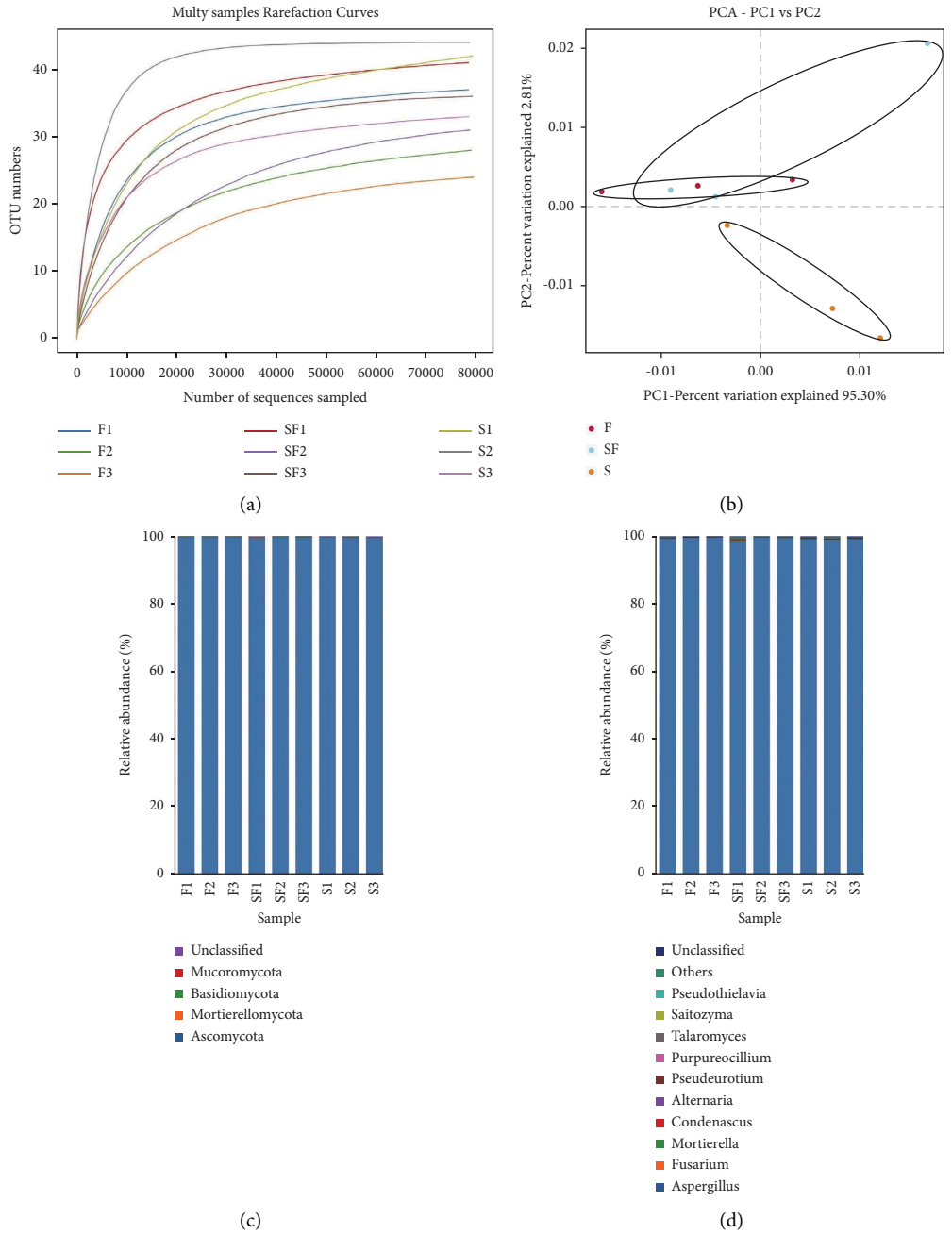
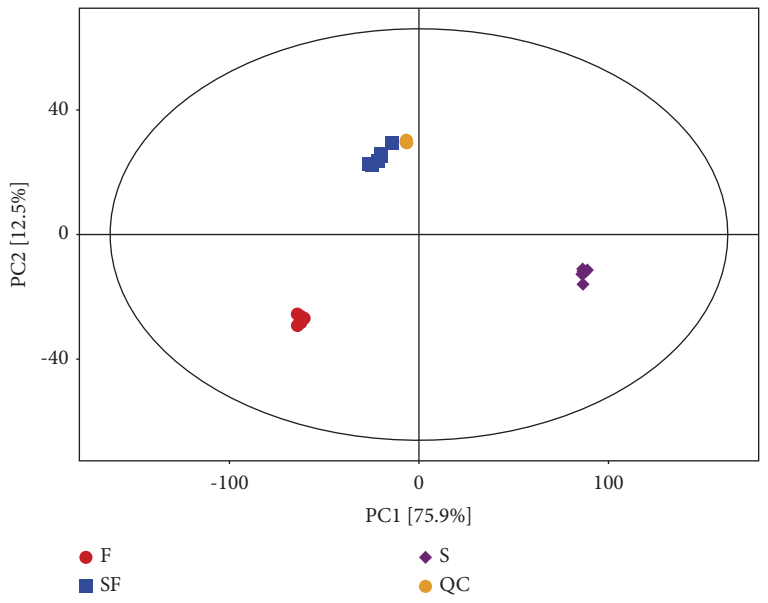


Figure 2: Results of fungal abundance in SF, F, and S. (a) Rarefaction curve of all samples. (b) PCA of relative abundance (RA) of fungal OTUs in each sample. Fungal species distribution bars at the phylum level (c) and at the genus level (d).

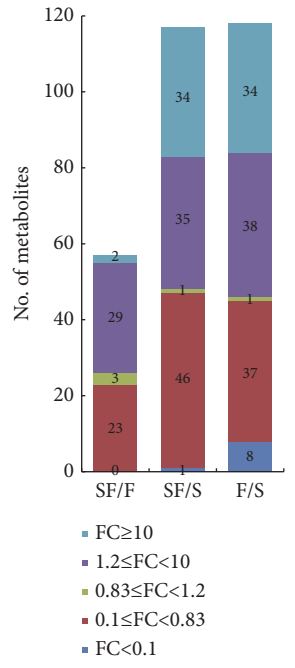
Table 2: Alpha diversity analysis of microorganisms in samples ($n = 3$).

	Sample ID	ACE	Chao1	Simpson	Shannon
Bacterial	SF	1089.17 ± 159.84^{a}	862.41 ± 172.82^{a}	0.82 ± 0.17^{a}	5.52 ± 1.58^{a}
	F	1117.57 ± 104.74^{a}	765.38 ± 231.75^{a}	0.97 ± 0.03^{a}	7.37 ± 1.23^{a}
	S	573.08 ± 145.55^{b}	535.35 ± 109.06^{a}	0.19 ± 0.02^{b}	0.86 ± 0.19^{b}
Fungal	SF	37.83 ± 4.69^{a}	37.46 ± 5.04^{a}	0.01 ± 0.02^{a}	0.09 ± 0.09^{a}
	F	31.43 ± 6.25^{a}	30.92 ± 6.42^{a}	0.01 ± 0.00^{a}	0.04 ± 0.03^{a}
	S	41.57 ± 5.94^{a}	43.78 ± 7.67^{a}	0.02 ± 0.01^{a}	0.12 ± 0.04^{a}

Different letters indicate significant differences ($P < 0.05$). The same letter indicates that the difference is not significant.

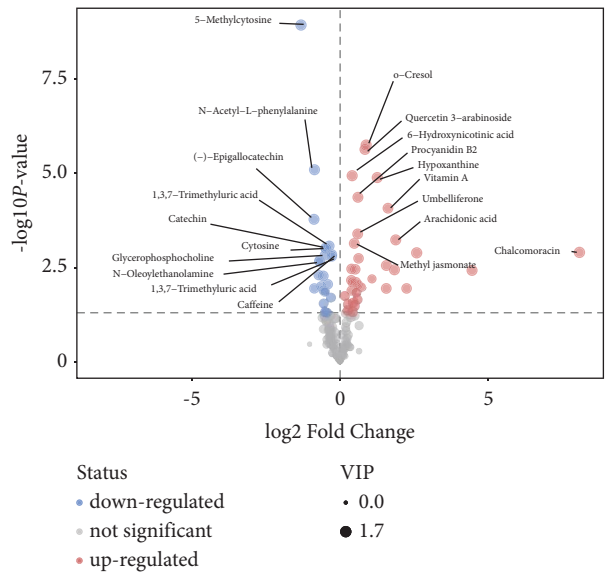


(a)

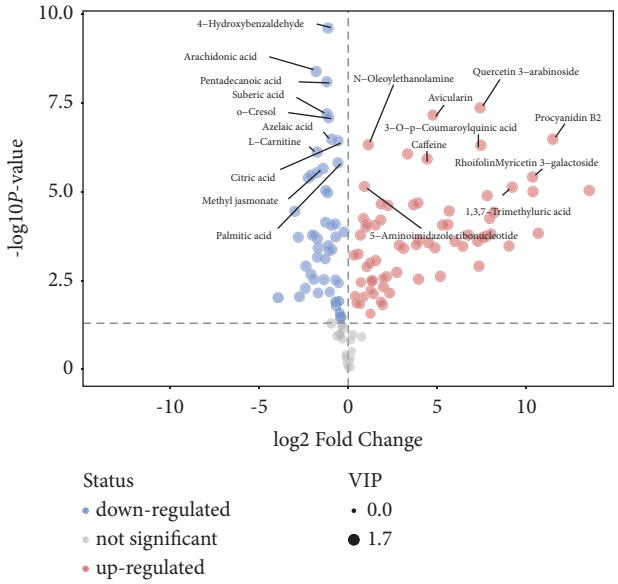


(b)

Figure 3: Continued.



(c)



(d)

Figure 3: Results of microbiota and metabolomic analysis in SF, F, and S. (a) Principal component analysis (PCA) of the items detected by positive and negative date in the metabolomic analysis. (b) Distribution of fold changes (FC) of metabolites. (c) Volcano plot with label of SF/F. (d) Volcano plot with label of SF/S.

Table 3: Effects of SF, F, and S on fasting blood glucose in model mice ($n = 10$).

Group	Diabetes model	1 week	2 weeks	3 weeks	4 weeks
CON	5.48 ± 0.66^{b}	5.45 ± 0.62^{b}	5.97 ± 0.70^{b}	5.28 ± 0.92^{b}	7.33 ± 0.44^{c}
DB	21.82 ± 1.98^{a}	20.07 ± 2.10^{a}	22.61 ± 2.15^{a}	22.87 ± 2.24^{a}	24.78 ± 1.46^{a}
MET	22.4 ± 1.60^{a}	21.88 ± 1.19^{a}	21.82 ± 1.29^{a}	20.73 ± 1.76^{a}	18.27 ± 0.91^{b}
SF	21.65 ± 0.88^{a}	20.08 ± 1.31^{a}	20.72 ± 0.51^{a}	20.18 ± 1.70^{a}	18.78 ± 1.02^{b}
F	22.28 ± 2.39^{a}	21.38 ± 2.48^{a}	20.48 ± 2.95^{a}	20.55 ± 5.05^{a}	18.93 ± 2.12^{b}
S	22.15 ± 3.23^{a}	21.83 ± 1.73^{a}	21.97 ± 2.54^{a}	21.00 ± 2.68^{a}	20.33 ± 2.67^{b}

Normal control group (CON); model (DB); metformin group (MET); mulberry leaf Fu brick tea (SF); Fu brick tea (F); and mulberry leaf brick tea (S). Different letters indicate significant differences ($P < 0.05$). The same letter indicates that the difference is not significant.

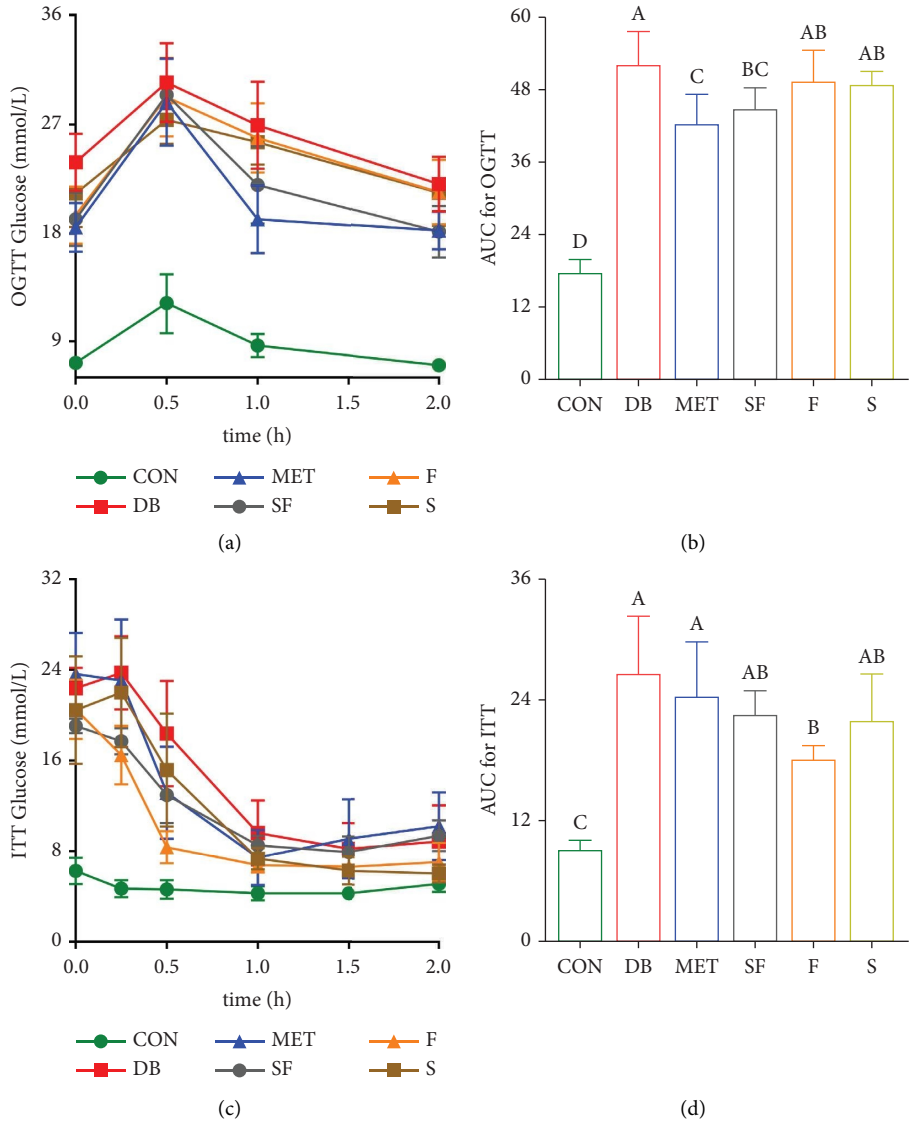


Figure 4: The effects of SF, F, and S on oral glucose tolerance and insulin tolerance in model mice. (a) OGTT. (b) OGTT AUC levels. (c) ITT. (d) ITT AUC levels ($n = 5$). Normal control group (CON); model (DB); metformin group (MET); mulberry Fu brick tea (SF); Fu brick tea (F); and mulberry leaf brick tea (S). Different letters in the same column indicate significant differences ($P < 0.05$). The same letter indicates that the difference is not significant.

revealed significant reductions in HOMA-IR and HOMA % B scores across all intervention groups compared to those in the DB mice ($P < 0.05$; Figures 5(b) and 5(c)). Furthermore, in the SF group, homeostatic β-cell function was markedly higher in the SF group than the S group ($P < 0.05$; Figure 5(c)), indicating that SF can restore islet cell function to a greater extent than S. We speculate that the reconstruction of pancreatic islet cell function may be one of the primary antidiabetic mechanisms of SF.

3.6. Effects of SF, F, and S on Serum Lipid Levels and Antioxidation of the Liver in STZ-Induced Diabetic Mice. Diabetic mice exhibit abnormal lipid metabolism and lipid disorders, and the worsening of dyslipidemia due to uncontrolled hyperglycemia is an underlying cause of insulin resistance [38]. Additionally, cardiovascular diseases and other complications of diabetes arise due to the accumulation of fatty deposits in the heart and blood vessels [39]. Therefore, maintaining normal serum lipid levels is critical for preventing diabetes. We observed that TC, TG, and LDL-c levels were significantly higher in the DB group than the CON group ($P < 0.05$; Figures 5(d)–5(f)). After the intervention, lipid levels in the SF and F groups were significantly lower compared to the DB group ($P < 0.05$), and these effects were comparable to the effect of metformin. Previous studies have demonstrated the hypolipidemic effects of F [7], and these were confirmed in our study. Based on our results, SF supplementation was found to exert hypolipidemic effects in diabetic mice. Therefore, SF administration prevented hyperlipidemia in diabetic mice.

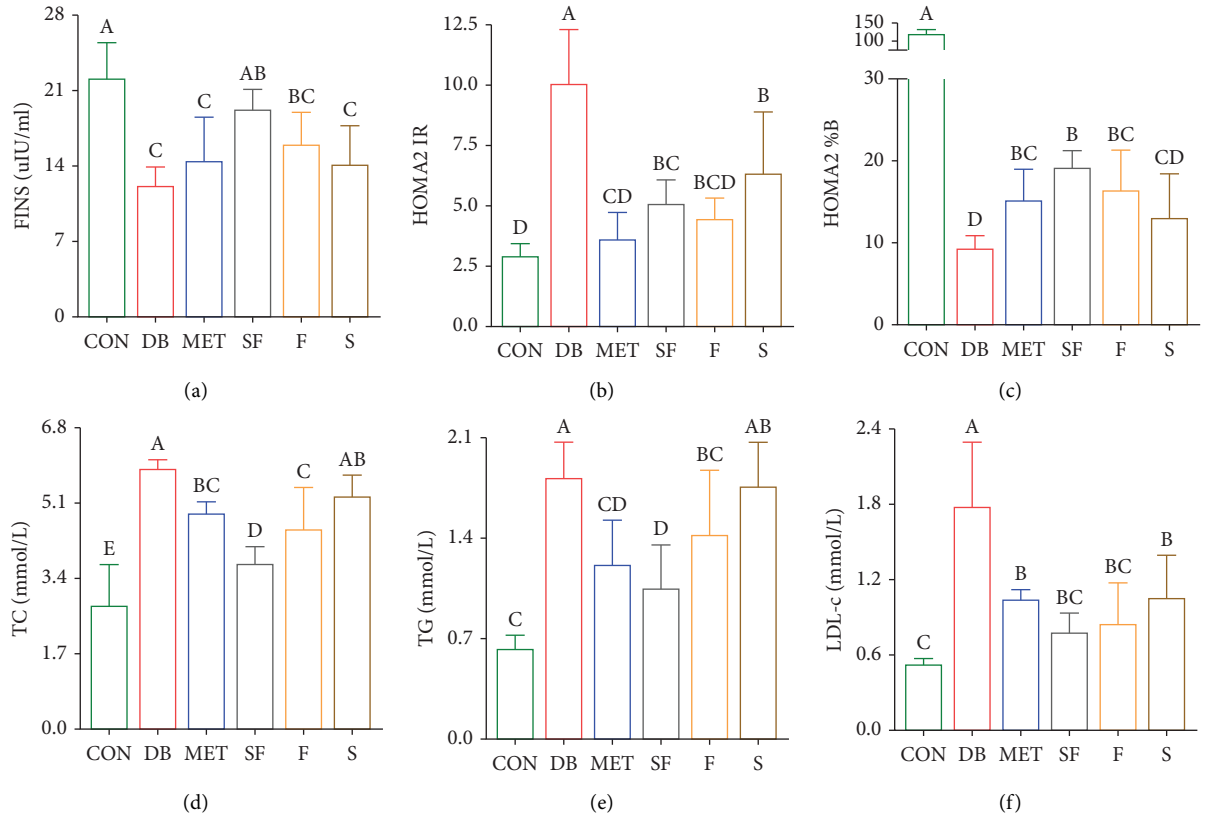


Figure 5: Effects of SF, F, and S on serum insulin, insulin resistance, and blood lipids in model mice ($n = 6$). (a) Serum insulin content. (b) Insulin resistance (HOMA-IR) score. (c) Homeostatic β-cell function (HOMA %B). (d) Serum levels of TC. (e) Serum levels of TG. (f) Serum levels of LDL-c.

The liver functions as a key target of insulin and plays a vital role in glucose metabolism, detoxification, and the oxidative stress response [40]. To examine the potential protective effects of SF, F, and S in diabetic mice, histopathological assessments of liver tissues were performed (Figure 6(a)). Hematoxylin and eosin staining revealed clear hepatocyte structures and abundant cytoplasm in the hepatocytes of the mice in the CON group. However, in the DB group, hepatocytes exhibited diffuse ballooning, which was significantly reduced in SF- and F-treated mice. In comparison, liver recovery was less remarkable in S-treated mice than in SF- and F-treated mice. Hence, SF and F treatments had a significant protective effect on the livers of hyperglycemic mice.

Oxidative damage is a major contributor to several chronic diseases, such as diabetes and cardiovascular diseases. Hyperglycemia-induced oxidative stress is common in patients with diabetes [41]. Thus, strengthening the intrinsic antioxidant defenses is an effective way to reduce the progression of diabetes. One such intrinsic antioxidant enzyme is SOD, which eradicates free radicals from the body [42]. Another critical enzyme, GSH-Px, catalyzes the decomposition of hydrogen peroxide, protects against lipid peroxidation, reduces MDA levels, and safeguards cell membrane structure and function [43]. The MDA content is the most appropriate indicator of lipid peroxidation [44]. Our results indicated that compared to the DB group, all treatment groups showed significant improvements in SOD and GSH-Px activities and noticeable reductions in MDA levels ($P < 0.05$; Figures 6(b)–6(d)). Thus, the treatment groups showed significantly improved intrinsic antioxidant defenses in diabetic mice. SOD activity was markedly higher in the SF and F groups than in the S group ($P < 0.05$). Notably, a substantial increase in GSH-Px activity occurred in the SF groups compared to that in the F and S groups ($P < 0.05$). These findings indicated that SF significantly enhances the antioxidant capacity of the liver of diabetic mice to exert its antioxidant effect. Overall, SF administration reduced the degree of lipid peroxidation in diabetic mice and showed a more significant antioxidation effect than tea with S or F alone in the livers of hyperglycemic mice.

The significant antidiabetic effect of SF may be related to the fact that SF contains both mulberry leaf and tea components, such as polysaccharides, polyphenols (quercetin, kaempferol, tea polyphenols, etc.), flavonoids (rutin, morin, etc.), DNJ, and GABA. First, DNJ is a potent α-glucosidase inhibitor [45], rutin reduces the absorption of carbohydrates in the small intestine [32], and kaempferol stimulates glycogen synthesis [29]. These substances contributed to the blood-glucose-lowering effects of SF. Second, polysaccharides in tea have been found to promote insulin secretion, improve the insulin response, and reduce insulin resistance [46], and GABA exerts protective and regenerative effects on islet beta cells [47]. These substances

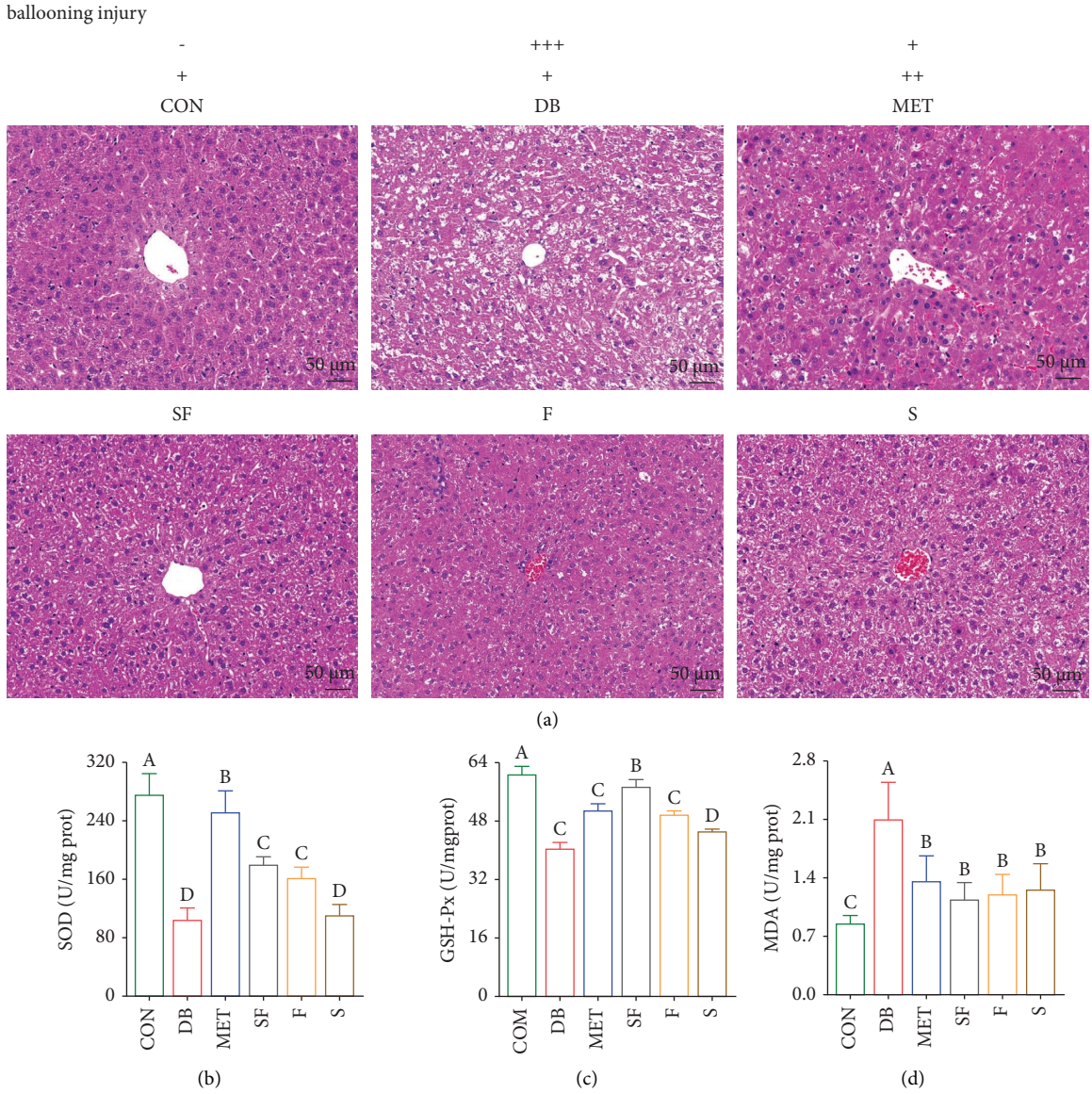


Figure 6: The effects of SF, F, and S on the liver of model mice (×200). (a) HE stained section of liver ($n = 3$). (b) SOD content in mice liver ($n = 6$). (c) GSH-Px content in mice liver ($n = 6$). (d) MDA content in mice liver ($n = 6$).

contribute to the restoration of islet cell function. Finally, the polysaccharides found in mulberry leaves normalize liver glucose metabolism and oxidative stress reduction [48]. Polyphenols in tea, particularly EGCG, are potent antioxidants that can reduce the effects of oxidative stress on diabetic complications and protect the liver [49]. These substances in SF can restore liver cells. In summary, the functional components of SF originate from mulberry and tea leaves. Because of the synergistic effect of the two, the hypoglycemic effect of SF was prominent. In addition, we found that the fecal water content of mice in the DB group was abnormal, but the situation improved after SF intervention. Therefore, future studies should focus on the effects of SF on intestinal inflammation, microorganisms, and metabolic balance.

4. Conclusions

A new tea SF was prepared by mixing mulberry leaves with tea leaves at a ratio of 1 : 3, and its quality and functional properties were investigated. SF contained a lower level of caffeine and higher levels of phenolic substances, indicating that it has a favorable taste. *Bacillus*, *Lactobacillus*, and *Aspergillus* were the predominant microorganisms responsible for the flavor and quality of SF. SF had obvious antidiabetic effects by reducing blood sugar and blood lipid levels and protecting the liver and islet cells in diabetic mice. These findings have important implications for the commercial viability of mulberry leaf Fu brick tea, as well as for the continued exploration and refinement of related teas and mulberries.

Abbreviations

DNJ: 1-Deoxynojirimycin
GABA: Gamma-aminobutyric acid
STZ: Streptozotocin
TG: Triglycerides
TC: Total cholesterol
LDL-c: Low-density lipoprotein
SOD: Superoxide dismutase
GSH-Px: Glutathione peroxidase
AUC: Area under curve
FBG: Fasting blood sugar
HOMA-IR: Calculated insulin resistance
HOMA %B: Homeostatic beta-cell function
LD_{50}: Median lethal dose
VIP: Variable Importance in the Projection
FC: Fold change.

Data Availability

The data used to support the findings of this study are available at https://osf.io/c2azg/?view_only=040259842eba4f83ae6f1694e8cb5297.

Ethical Approval

The animal study protocol was approved by Hunan Agricultural University.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Authors' Contributions

All the authors contributed to the conception and design of this study. Yuanyuan Shao was responsible for material preparation, method construction, data collection and analysis, and drafting and revision of the manuscript. Lin, Xu, Zhang, and Yan participated in data collection and analysis. Jinfeng Li provided raw materials and participated in the preparation. Zhonghua Liu, Zhihua Gong, and Wenjun Xiao supervised the experimental design and data acquisition. Wenjun Xiao modified the manuscript. All authors have reviewed and approved the final manuscript. Wenjun Xiao received funding.

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Supplementary Materials

Table S1: SF, F, and S review results. Table S2: death and LD_{50} determination results of SF from a single intragastric administration of ICR mice. Table S3: effects of SF, F, and S on the body weight of model mice. Figure S1: the production process of SF, F, and S. (*Supplementary Materials*)

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