Stachyose improves the anti-diabetic effects of berberine by regulating intestinal microbiota and SCFAs in spontaneous type 2 diabetic KKAY mice

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Abstract

Background: Berberine (BBR) has the beneficial effects of anti-inflammation, anti-bacteria, and anti-diabetes. The clinical application of BBR has been hindered by its poor gastrointestinal absorption. Stachyose (Sta), a prebiotic agent, improves the composition of gut microbiota and benefits for diabetes. We therefore investigated whether Sta improves the anti-diabetic actions of BBR using type 2 diabetic KKAY mice.

Methods: KKAY mice were randomly divided into four groups: Con, Sta (200 mg/kg), BBR (100 mg/kg), and BBR+Sta (BBR: 100 mg/kg, Sta: 200 mg/kg). Mice were administered intragastrically once daily for 8 weeks. The mice weight, glycemia, glycated hemoglobin, and levels of insulin, glucagon, and inflammatory cytokines were monitored. Oral glucose tolerance tests and insulin tolerance tests were performed; protein and gene expression levels were detected by Western blot and qRT-PCR, respectively; gut microbiota and short-chain fatty acids (SCFAs) in feces were analyzed.

Results: The combination of BBR and Sta is more effective than BBR alone in blood glucose control, improvement of insulin resistance and islet functions, inflammatory mediators decrease, and maintenance of intestinal barrier integrity. Gut microbiota analysis demonstrates that both BBR and combination treatments enhance the abundance of Bacteroidaceae and Akkermansiaceae and decrease Lachnospiraceae levels, whereas Akkermansiaceae elevation due to the administration of BBR with Sta is more significant than BBR alone. Interestingly, the proportion of Lactobacillaceae increases with combination treatment, but is diminished by BBR treatment. Additionally, BBR with Sta significantly reduces the concentrations of fecal SCFAs compared to BBR.

Conclusions: The combination of BBR and Sta imparts better effects on the maintenance of glycemia and intestinal homeostasis than BBR alone by modulating gut microbiota and SCFAs, thereby providing a novel approach for the treatment of type 2 diabetes mellitus.

Background

Type 2 diabetes mellitus (T2DM) is a metabolic disease characterized by obesity, insulin resistance, chronic hyperglycemia, low-grade inflammation, alteration of gut microbiota, and islet β cell dysfunction [1]. T2DM, which accounts for 90% of diabetes cases, is a major economic burden and life pressure on people. In addition, the incidence of diabetes continues to increase each year. Hence, it is essential to identify new preventive approaches to inhibit the progression of T2DM and control the growing epidemic.

Traditional Chinese medicine has been used in clinic for over 2,000 years. The Qianjin huanglian pill, which is composed of *Coptis chinensis* powder and fresh *Rehmanniae radix*, is a classic ancient prescription for treating diabetes due to its effects of nourishing Yin and clearing heat. Berberine (BBR), one of the main components of *R. coptidis*, is an isoquinoline alkaloid originally isolated from *C. chinensis* [2]. Several studies have shown that BBR has many advantageous biological roles in preclinical and clinical research, such as anti-inflammation, anti-insulin resistance, anti-hyperglycemia, anti-

hyperlipidemia, anti-bacteria, and anti-diabetes [3, 4]. BBR also ameliorates liver, cardiovascular, and renal disease that are associated with diabetes [3]. This evidence suggests that BBR is a potential drug for the treatment of T2DM. However, BBR may result in gastrointestinal reactions (including diarrhea and constipation) in some patients due to its poor absorption, thereby limiting its long-term and wide application in T2DM management [3, 5] .

A plethora of studies has revealed that prebiotics not only reduce the inflammatory response and oxidative stress mainly by enhancing the growth of specific beneficial bacteria found in the gut, but also improve intestinal tight junction integrity and decrease intestinal permeability by increasing the expression of adhesion proteins within the intestinal epithelium, consequently preventing the occurrence and development of T2DM [6, 7]. Furthermore, prebiotics have the ability to promote the absorption of antioxidants [6]. Reports have also shown that BBR exhibits anti-diabetic actions by regulating gut microbiota composition and SCFAs [8, 9]. On the basis of these findings, we hypothesized that usage of a prebiotic during BBR treatment may optimize glucose metabolism and better protect against diabetes.

Stachyose (Sta) is a functional oligosaccharide extracted from fresh *R. radix*. As a classic prebiotic, Sta has been proven to promote the growth of beneficial intestinal bacteria, inhibit pathogenic bacteria, and improve diabetes [10, 11]. Moreover, Sta enhances the absorption of soybean genistein and tea polyphenols in mice [12, 13]. Therefore, we hypothesized that Sta improves the anti-diabetic actions of BBR by regulating intestinal microbiota. This study aimed to investigate the effects and mechanisms of BBR combined with Sta on glucose metabolism, inflammation modulation, intestinal integrity, fecal SCFAs, and gut microbiota in diabetic KKAY mice.

Materials And Methods

Chemical compounds

Sta (purity>80%) was provided by the laboratory of Professor Dequan Yu, Institute of Materia Medica, Chinese Academy of Medical Sciences, Peking Union Medical College (Beijing, China). BBR (purity>98%) was obtained from the Northeast General Pharmaceutical Factory (Shenyang, China).

Animals and experimental design

Eight-week-old female KKAY mice were purchased from Beijing Huafukang Bio-Technology Co., Ltd., and maintained at Experimental Animal Center of the Institute of Materia Medica under specific pathogen-free conditions. Animals were kept at $23 \pm 2^{\circ}\text{C}$ in a 12-h light/dark cycle with free access to food and water. Mice were fed a high-fat diet (45% of energy from fat; Research Diets, USA). Animal experiments were performed in accordance with the “3R” principles and guidelines for laboratory animals (GB14925-2001 and MOST 2006a) established by the People’s Republic of China, and approved by the Institutional Animal Care and Use Committee of Institute of Materia Medica (Chinese Academy of Medical Sciences & Peking Union Medical College, Beijing, China).

According to the levels of blood glucose, triglyceride, total cholesterol, body weight, and percentage of glycemia reduction at 40 min after insulin injection, KKAY mice were randomly divided into four groups (n=12): control group (Con), Sta-treated group (Sta, 200 mg/kg), BBR-treated group (BBR, 100 mg/kg), and BBR with Sta-treated group (BBR+Sta; Sta: 200 mg/kg, BBR: 100 mg/kg). All mice were treated intragastrically with compounds or an equivalent volume of water once daily for 8 weeks.

Fasting blood glucose (FBG) and glycated hemoglobin (HbA1c) measurements

FBG levels were measured using the glucose-oxidase method (Biosino Bio-Technology & Science Inc., Beijing, China). After 35 days of treatment, HbA1c levels were assessed using commercial kits (Homa Biological, Beijing, China)

Oral glucose tolerance test (OGTT), insulin tolerance test (ITT), and glucose-stimulated insulin secretion test

For OGTT, mice were fasted for 4 h and given D-glucose (2 g/kg) intragastrically, and blood glucose was measured at 0, 15, 30, 60, and 120 min after glucose administration. For ITT, mice were injected subcutaneously with insulin (0.4 U/kg) after 4 h of fasting, and blood glucose was measured at 0, 40, and 90 min after insulin injection. For glucose-stimulated insulin secretion test, mice were treated with D-glucose (2 g/kg) after 4 h of fasting, and insulin levels in plasma were monitored at 0 and 15 min after glucose administration.

Immunofluorescence assay

All mice were sacrificed through cervical dislocation and the pancreas were fixed in 10% formalin, embedded in paraffin, and dissected to prepare 5- μ m-thick slides, then stained with antibodies against insulin and glucagon as previously described [14]. The mean fluorescence intensities of insulin and glucagon, which account for islet areas were quantified using Image J software.

Enzyme-linked immunosorbent assay (ELISA)

The levels of interleukin (IL)-10, IL-1 β , IL-6, monocyte chemotactic protein (MCP-1), tumor necrosis factor (TNF)- α , and C-reactive protein (CRP) in plasma were monitored using ELISA kits (R&D Systems, USA). The concentrations of glucagon and insulin were determined by ELISA kits according to the manufacturer's instructions (ALPCO, USA).

Western blotting

Intestinal tissues were homogenized and lysed in Radio-Immunoprecipitation Assay (RIPA) buffer, and protein concentrations were determined using a BCA protein quantitation kit. Lysate samples were fractionated by sodium dodecyl sulfate-polyacrylamide gels and transferred onto polyvinylidene difluoride membranes, blocked with 5% non-fat milk for 1.5 h at room temperature, and incubated with primary antibodies overnight at 4 °C. Appropriate horseradish peroxidase-conjugated secondary

antibodies were applied for 1-2 h at room temperature, prior to detection with an enhanced chemiluminescence kit. Polyclonal antibodies to anti-p38MAPK, anti-ERK1/2, anti-JNK, anti p-p38MAPK (Thr180/Tyr182), anti-p-ERK1/2 (Thr202/Tyr204), and anti-p-JNK (Thr183/Tyr185) were obtained from Cell Signaling Technology (CST, Danfoss, Massachusetts, USA). Antibodies against zonula occludens-1 (ZO-1) and occludin were purchased from Invitrogen (USA). The antibody against β -actin and secondary antibodies (anti-rabbit IgG or anti-mouse IgG), RIPA buffer, and BCA kit were purchased from Applygen Technologies Inc. (Beijing, China). Protein levels were normalized to those of β -actin.

Quantitative real-time PCR (qRT-PCR)

qRT-PCR was conducted as previously described [15]. Briefly, RNA was isolated from intestinal tissues using TRizol reagent and reverse transcribed with TransScript® first-strand cDNA Synthesis SuperMix (TransGen Biotech, Beijing, China) based on the manufacturer's protocols. qRT-PCR was conducted using TransStart® Tip Green qPCR SuperMix (TransGen Biotech, Beijing, China) on 7900 Real-Time PCR System (Applied Biosystems, USA). Gene expression levels were normalized to those of β -actin. The primer sequences used in this study are shown in Table S1.

Gut microbiota profiling

The intestinal flora in feces was assayed as previously described [16]. Fecal samples were collected and snap-frozen in liquid nitrogen, followed by storage at -80°C. Genomic DNA was isolated with DNA isolation kit. The V3-V4 region of 16S rRNA genes were amplified and purified. Subsequently, the abundance and diversity of gut microbiota were analyzed using Illumina MiSeq sequencing (Major Bio-Pharm Technology, Shanghai, China) according to the standard protocol. The effective reads from all samples were grouped into operational taxonomic units (OTUs) on the basis of 97% sequence similarity. α -Diversity was estimated by the level of OTUs, and Shannon and Chao indices. β -Diversity was assessed by computing for unweighted UniFrac and visualized by principal coordinate analysis (PCoA). The sequence data were processed and analyzed on the free online Majorbio I-Sanger Cloud Platform (www.i-sanger.com).

Short-chain fatty acids (SCFAs) analysis

SCFAs in fecal samples were detected on the basis of previous report [17]. Briefly, frozen faeces were crushed with methanol containing internal standards, subjected to ultrasonication for 30 min, centrifuged for 15 min, and extracted. Subsequently, the SCFAs in each sample were assayed by gas chromatography coupled to a mass spectrometer detector (GC-MS) (Agilent Technologies Inc. CA, USA) and quantified using Masshunter quantitative software.

Correlation analysis of SCFAs and gut microbiota were performed on the platform of Majorbio I-Sanger Cloud (www.i-sanger.com). R and P values were obtained using Spearman's rank correlation.

Statistical analysis

The data are presented as the mean $\pm$ SEM. Statistical analysis was performed using one-way ANOVA and unpaired two tailed Student's *t*-test, and plotted using GraphPad Prism 7.0. Differences with $p < 0.05$ were considered statistically significant.

Results

Effects of berberine with stachyose on glucose homeostasis

In this study, we found that the levels of blood glucose and HbA1c in mice treated with either BBR or BBR+Sta were significantly decreased compared to control mice (Fig. 1a, b). Further, HbA1c levels in combination-treated mice obviously lowered compared to those of BBR-treated mice (Fig. 1b), indicating that BBR combined with Sta is more effective in glycemic control than BBR alone.

In comparison to the Con mice, supplementation with BBR or BBR with Sta improved glucose tolerance and insulin sensitivity, as evaluated by diminished AUCs for blood glucose during OGTT and ITT (Fig. 1c, d). We further detected the index of HOMA-IR to estimate insulin resistance levels. The HOMA-IR indices in Sta, BBR, and BBR with Sta-treated mice were decreased by 21.9% ($p = 0.0880$), 61.2% ($p < 0.001$), and 79.3% ($p < 0.001$), respectively, compared to the Con mice (Fig. 1e), which implied that BBR with Sta is better in improving insulin sensitivity of KKAy mice than BBR alone.

In addition, treatment with BBR or BBR with Sta resulted in lower weight gain, food consumption, and water intake (Fig. 1f, Fig. S1). Therefore, the effects of BBR and BBR with Sta on body weight may be in correlation with decreased food intake. We noticed that the food and water intake of mice had no distinction between BBR and combination groups, but the weight of mice administrated of BBR with Sta was higher than that of BBR treatment at 8th, 26th, and 35th days. Thus, Sta may attenuate the gastrointestinal side effects of BBR, bringing about mild weight increase.

Effects of berberine with stachyose on islet functions

As shown in Fig. 2a, compared to the Con group, fasting plasma insulin levels in the BBR and BBR with Sta groups were reduced by 27.8% ($p < 0.05$) and 45.7% ($p < 0.01$), respectively. Besides, BBR with Sta treatment promoted insulin release and increased proportion of insulin elevation from 17.5% in Con mice to 118.2% ($p < 0.05$), while BBR treatment elevated to 76.0% ($p < 0.05$) (Fig. 2a-c). And the concentration of plasma glucagon was only reduced by the combined treatment in comparison to Con or BBR supplementation (Fig. 2d).

Compared to the Con group, glucagon contents in islets were decreased in the BBR combined with Sta group and had no obvious alteration in the BBR group, and insulin levels in the islets of all treatment groups were declined (Fig. 2e, f). Additionally, the mean fluorescence intensity ratio of insulin to glucagon was markedly increased in the combination group (Fig. 2f). Taken together, these results suggest that BBR combined with Sta is more effective than BBR alone in maintaining insulin-glucagon homeostasis and ameliorating islet functions.

Effects of berberine with stachyose on inflammation

Compared with the Con mice, treatment with BBR and BBR with Sta diminished cytokine levels of IL-1 β , TNF- α , IL-6, CRP in plasma, Sta reduced IL-1 β levels and increased IL-10 levels, while the production of MCP-1 was only decreased by BBR with Sta treatment (Fig. 3a). Similarly, the gene levels of pro-inflammatory cytokines- IL-1 β , TNF- α , MCP-1, and IL-6-were attenuated by BBR and BBR with Sta, whereas anti-inflammatory cytokine IL-10 levels in intestine was elevated (Fig. 3b). And the actions induced by the combination treatment were more pronounced (Fig. 3b). Given that the production of proinflammatory cytokines is related to the activation of the TLR4 and MAPK signaling pathways [18], we next examined whether these pathways in intestinal tissues were impacted by BBR with Sta treatment. As shown in Fig. 3b-d, compared to the Con group, the protein and mRNA levels of TLR4 as well as the phosphorylation of ERK and p38MAPK were all suppressed in the BBR and BBR with Sta groups, without obvious change of JNK phosphorylation.

Effects of berberine with stachyose on intestinal integrity

Given that inflammation contributes to intestinal dysbiosis and damages gut permeability, we explored the intestinal integrity of KKAY mice. Compared to the Con group, the protein and gene expression levels of ZO-1 and occludin, which are tight junction components, were upregulated in the BBR and BBR with Sta groups, and Sta remarkably elevated ZO-1 and occludin gene levels (Fig. 4a-c). Moreover, relative to the Con mice, the mRNA expression of Reg3g (regenerating islet-derived 3 gamma), an antimicrobial protein was increased by 1.4-fold ($p > 0.05$) and 1.7-fold ($p < 0.01$) in mice treated with BBR and BBR with Sta, respectively (Fig. 4d). These data indicate that BBR combined with Sta is more effective than BBR alone in maintaining gut barrier integrity and intestinal balance.

Effects of berberine with stachyose on gut microbiota

We next examined the roles of BBR with Sta on intestinal microbiota composition by performing Illumina-sequencing based analysis of bacterial 16S rRNA in fecal samples. After removing unqualified sequences, a total of 35,299 valid sequences were generated and clustered into 316 OTUs according to the minimum sample-sequence number (Table S2). Compared to the Con group, the OTU numbers were reduced in the BBR and BBR with Sta groups (Fig. 5a). And the OTU numbers were remarkably less in the combined group than those of the BBR group (Fig. 5a). The Shannon and Chao indices reflect the diversity and richness of gut microbiota, respectively. Compared to the Con group, BBR and BBR with Sta diminished the indices of Shannon and Chao, whereas there were notable differences in the Chao index between the BBR and BBR with Sta groups ($p < 0.01$) (Fig. 5b, c).

Unweighted Unifrac PCoA based on OTU levels revealed a distinct clustering of microbiota composition in each group (Fig. 5d). Multivariate analysis of variance of PCoA matrix scores revealed that the microbiota community of mice in BBR and combination groups is clearly differed from that of the Con mice. The gut microbiota of the Sta, BBR, and BBR with Sta groups could also be discriminated (Fig. 5d). Additionally,

the bacterial community of Sta-administrated mice was closer to that of the Con mice, which coincides with the mild actions exerted by Sta.

Taxonomic profiling at the phylum level revealed that BBR and BBR with Sta treatments elevated the abundances of Verrucomicrobia and reduced that of Deferribacteres and Epsilobacteraeota compared with the Con mice (Fig. 5e). Notably, the increase in abundance of Verrucomicrobia produced by BBR with Sta was more significant than BBR alone. At the same time, Sta decreased the levels of Deferribacteres and Proteobacteria (Fig. 5e). At the family level, the abundances of Bacteroidaceae and Akkermansiaceae were notably enhanced, and Lachnospiraceae and Desulfovibrionaceae were decreased in the BBR and BBR with Sta groups compared to the Con group (Fig. 5f). In addition, the proportion of Akkermansiaceae in combination-treated mice was higher than that of BBR-treated mice. Strangely, the proportion of Lactobacillaceae was diminished by BBR but increased by the combination of BBR and Sta (Fig. 5f). The levels of Desulfovibrionaceae were also diminished by Sta treatment. Similar results were also observed at the genus level. Combination treatment declined *Desulfovibrio* abundance, and increased the abundance of *Lactobacillus* and *Akkermansia* compared with BBR treatment (Fig. 5g). Collectively, these findings implied that Sta augments the anti-diabetic effects of BBR and attenuates its adverse reaction by regulating the composition of gut microbiota.

Effects of berberine with stachyose on fecal SCFAs

SCFAs, including acetic acid, propionic acid, butyric acid, isobutyric acid, pentanoic acid, isopentanoic acid, hexanoic acid, isohexanoic acid, and total SCFAs, were assessed in this study. Compared to the Con group, acetic and propionic acids were elevated, whereas hexanoic, isohexanoic, and isopentanoic acids decreased in the Sta group (Fig. 6a). Simultaneously, BBR reduced the contents of total SCFAs, butyric, pentanoic, isopentanoic, hexanoic, and isohexanoic acids in feces (Fig. 6a). In addition, the eight SCFAs and total SCFAs were all obviously downregulated in the BBR with Sta group compared with the Con or BBR group (Fig. 6a). Overall, BBR combined with Sta significantly diminished fecal SCFAs concentrations in KKAY mice.

Subsequently, we analyzed the relationship of fecal SCFAs and intestinal bacteria at the family level (Fig. S2, Table S3). The results displayed that Bacteroidaceae and Akkermansiaceae were negatively correlated with acetic acid, propionic acid, butyric acid, isobutyric acid, pentanoic acid, and total SCFAs, while Lachnospiraceae was positively related with these SCFAs (Fig. 6b, Table S3). And a significant positive correlation between Desulfovibrionaceae and pentanoic acid was observed (Fig. 6b, Table S3). These results were consistent with the above results of microbiota and SCFAs, which suggest that BBR with Sta improves blood glucose, inflammation, and gut integrity is linked to the alteration of intestinal microbiota and SCFAs.

Discussion

Diabetes has become a major public health concern because it causes various health problems and its global prevalence has increased in recent decades [19]. BBR has various pharmacological properties and

multispectral therapeutic applications, including diabetes, hyperlipidemia, and metabolic syndrome [3]. Nevertheless, the use of BBR is limited due to its poor absorption and gastrointestinal adverse effects. Sta, a functional prebiotic, has been shown to improve antioxidants absorption and intestinal microbiota composition, leading to the ameliorated inflammation and diabetes [10, 11, 20]. Here, we observed that combination of BBR and Sta is more effective than BBR alone in modulating glucose metabolism, gut inflammation, and intestinal barrier integrity by altering gut microbiota and SCFAs in the stool of KKAY mice.

T2DM is accompanied with increased levels of blood glucose and HbA1c, as well as islet dysfunction. HbA1c is an index of the control of diabetes and reflects the average blood glucose levels over the past two-three months [21]. Glucose homeostasis is maintained by insulin and glucagon, whereas disrupted pancreatic islet functions and insulin resistance in T2D leads to glucose intolerance [21]. In this study, our findings showed that the combination treatment of BBR and Sta initiated better beneficial actions on glycemia control and islets functions improvement compared to BBR alone. Furthermore, the weight of mice received the combination of BBR and Sta was higher than that of BBR-treated mice at conditions of equal food consumption. Therefore, BBR combined with Sta produces better effects on modulating glycemia homeostasis than BBR alone, which is correlated with relieved gastrointestinal side effects of BBR and modified islet functions.

It is widely accepted that T2DM is typical of chronic low-grade inflammation of metabolic tissues, such as adipose, liver, intestine, and islets [22]. And various inflammation-related molecules are associated with the development of T2DM, like TLR4, MAPK, and NF- κ B [23]. Clinical studies have shown that when inflammation in T2DM patients is suppressed by a high dose of aspirin or salsalate, the glycemic control of the patients improves, along with concomitant inhibition of NF- κ B activity [23]. Our findings revealed that BBR combined with Sta imparts stronger effects on abating the expressions of proinflammatory mediators in plasma as well as in the intestines than those of BBR alone. In addition, BBR with Sta treatment downregulated the activation of TLR4 signaling pathway and the phosphorylation of ERK and p38MAPK. Overall, treatment using the combination of BBR and Sta alleviates inflammation of KKAY mice at least partially through the regulation of TLR4, ERK, and p38MAPK pathways.

Intestinal dysbiosis results in increased intestinal permeability and susceptibility to microbial antigens, which ultimately contribute to the occurrence and development of inflammation and diabetes [6]. ZO-1 and occludin acting as tight junction proteins play protective roles in intestinal permeability [24]. T2DM is usually accompanied with increased intestinal permeability characterized by reduction in tight junction proteins [25]. And accumulation of these molecules is associated with increased protection at the intestinal barrier level. Herein, we found that treatment with the combination of BBR and Sta produced higher expressions of occludin and ZO-1 than BBR supplementation. Sta treatment also augmented these molecules expressions. What's more, prebiotics decrease intestinal permeability and maintain gut barrier integrity by modulating gut microbiota composition, ultimately inhibiting T2DM progression [7]. Therefore, our results suggest that Sta as a prebiotic enhances the positive effects of BBR in maintaining gut barrier integrity of KKAY mice, which may be attributed to specific alterations in the gut microbiota.

The major alterations in the intestinal microbiota that are linked to T2DM include a significantly lowered prevalence of Bacteroidetes and Verrucomicrobia and an enrichment of Firmicutes, Proteobacteria, and Deferribacteres [26]. Although the ratio of Bacteroides to Firmicutes was not altered in the study, Bacteroidaceae, belonging to Bacteroides, was obviously increased by BBR and BBR with Sta. Importantly, combination treatment enhanced Verrucomicrobia abundance compared with BBR alone. In line with these results, in comparison to BBR, the levels of Akkermansia and Akkermansiaceae, belonging to Verrucomicrobia, were also markedly increased by BBR with Sta. Akkermansia has beneficial effects on glucose metabolism and gut permeability, which is negatively associated with T2DM and has been tested as a probiotic in preclinical trials [27]. Plovier et al. [28] found that pasteurized Akkermansia muciniphila versus the live bacteria has an enhanced capacity to reduce fat mass development, insulin resistance and dyslipidemia in mice. Our previous study also manifested that BBR with Sta increased the abundances of phylum Verrucomicrobia and species Akkermansia muciniphila in the feces of db/db mice [4]. In addition, Verrucomicrobia is highly abundant in healthy subjects [29]. Thus, the increased proportion of the phylum Verrucomicrobia and genus Akkermansia induced by BBR with Sta contributes to the amelioration of glucose metabolism and intestinal integrity.

Lactobacillus genus acting as probiotic is beneficial to healthy intestinal microbiota, creates a favorable intestinal environment, and alleviates T2DM [30]. We observed that BBR treatment reduced the levels of Lactobacillaceae family and Lactobacillus genus, whereas BBR combined with Sta reversed the reduction to a higher percentage than those seen in control mice. The abundances of Desulfovibrio genus were elevated in obesity and T2DM [31]. Desulfovibrio which is considered an opportunistic pathogen produces endotoxins and have the capacity to reduce sulfate to H₂S, thereby damaging the intestinal barrier [31]. We here found that both BBR and combined treatments reversed these elevations, and combined treatment diminished the Desulfovibrio genus level compared with BBR alone. Furthermore, it has been demonstrated that Akkermansia and Lactobacillus protect against inflammation [27]. Taken together, Sta enhances the efficacy of BBR against diabetes through altering gut bacterial composition and maintaining intestinal homeostasis.

SCFAs are closely associated with obesity and diabetes, which are products of gut microbiota mediated fermentation of resistant starch or dietary fiber [17]. Firmicutes and Bacteroidetes are the predominant intestinal bacteria that produce SCFAs [32]. Bacteroidetes mainly produces acetate and propionate, whereas butyrate is mostly generated by Firmicutes [37]. In this study, we found that the abundance of Firmicutes and Bacteroidetes did not significantly differ among the study groups, and thus the production of SCFAs in the mouse groups is most likely the same. Interestingly, our results showed that fecal SCFA reduction in mice that received the combination treatment was remarkably less than that of mice treated with BBR alone, which is contradictory too the results of recent studies that prebiotics and berberine elevated SCFAs concentrations to alleviate obesity and diabetes [33, 34]. However, there are animal and human studies suggested that an increased concentrations of SCFAs in feces was associated with higher body weight and fat gain and insulin resistance, which may be due to increased SCFAs production and

decreased SCFAs absorption [35]. Therefore, decreased fecal SCFAs in KKAY mice generated by BBR with Sta is most probably beneficial in protecting against the development of T2DM.

SCFAs exert a number of beneficial effects to improve gut health, insulin resistance, and diabetes, as well as enhance intestinal barrier function by regulating the expression of tight junction proteins and mucous [36]. SCFAs, particularly propionate and butyrate also inhibit the maturation of monocytes and macrophages, altering their abilities to capture antigens and reducing their ability to produce pro-inflammatory cytokines such as IL-1 β , IL-6, and TNF- α [37, 38]. Nonetheless, most SCFAs are absorbed by the host to exert beneficial properties, and fecal SCFAs represent non-absorbed SCFAs, so it is reasonable to believe that Sta enhances the ability of BBR promoting SCFAs absorption, resulting in better effects on intestinal barrier integrity, inflammation alleviation, and glucoses metabolism. Meanwhile, it is not excluded that fecal SCFAs reduction induced by BBR with Sta treatment is attributed to lessened diversity and richness of gut microbiota and the subsequent reduction of SCFAs generation. Further investigations on the production, absorption, and excretion of SCFAs due to treatment with BBR with Sta are warranted.

In contrast to most studies on the correlation between SCFAs and gut bacteria, we found that fecal SCFAs are negatively correlated with beneficial bacteria-Bacteroidaceae and Akkermansiaceae, and positively correlated with pathogenic bacteria-Lachnospiraceae. Nevertheless, there are several reports that support our results [39, 40]. These controversial results may be explained by different models of disease, sample sources of SCFAs, and environment factors. To elucidate the effects of SCFAs and the contradictory findings about the relationship of SCFAs and intestinal microbiota in diabetes, more efforts are desired.

Notably, the beneficial effects of BBR combined with Sta on T2DM coincides with the formulation thought of ancient Chinese formula-Qianjin Huanglian pill. Our results may provide more evidence on the effect and mechanism of the Qianjin Huanglian pill against diabetes, as well as further clarify the rationality and scientific basis of the formula.

Conclusion

In summary, Sta as a potential prebiotic enhances the advantageous actions of BBR against T2DM in KKAY mice. And BBR combined with Sta is more effective in glycemic control, inflammation attenuation, and gut integrity maintenance by modulating gut microbiota composition and SCFAs levels, thereby providing a novel strategy for the treatment of T2DM. The specific mechanism of the combination treatment regulating intestinal microbial composition is unclear and requires further investigation.

Abbreviations

BBR: berberine; CRP: C-reactive protein; ELISA: enzyme-linked immunosorbent assay; FBG: fasting blood glucose; FPI: fasting plasma insulin; HbA1c: glycated hemoglobin; HOMA-IR: homeostasis model assessment of insulin resistance; IL: interleukin; ITT: insulin tolerance test; MAPK: mitogen activated protein kinases; MCP-1: monocyte chemotactic protein-1; OGTT: oral glucose tolerance test; OTUs:

operational taxonomic units; PCoA: principal coordinate analysis; qRT-PCR: quantitative real-time PCR; Reg3g: regenerating islet-derived 3 gamma; RIPA: radio-immunoprecipitation assay; SCFAs: short-chain fatty acids; Sta: stachyose; T2DM: type 2 diabetes mellitus; TLR: toll-like receptor; TNF: tumor necrosis factor; ZO-1: zonula occludens-1.

Declarations

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Authors' contributions

Cao H and Li CN designed the research, performed the research, analyzed the data, and wrote the manuscript. Lei L, Wang X, Liu SN, Liu Q, Huan Y, Sun SJ participated in experiments. Shen ZF supervised the whole project, supplied academic and technical support, and reviewed the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article and its supplementary information files.

Ethics approval and consent to participate

The studies were performed in accordance with the “3R” principles and guidelines for laboratory animals established by the People’s Republic of China, and approved by the Institutional Animal Care and Use Committee of Institute of Materia Medica, Chinese Academy of Medical Sciences & Peking Union Medical College.

Consent for publication

Not applicable.

Competing interests

The authors declare no competing financial interests.

References

1. Schwarz PE, Gallein G, Ebermann D, Muller A, Lindner A, Rothe U, Nebel IT, Muller G. Global Diabetes Survey—an annual report on quality of diabetes care. *Diabetes Res Clin Pract.* 2013; 100(1):11-18.
2. Leng SH, Lu FE, Xu LJ. Therapeutic effects of berberine in impaired glucose tolerance rats and its influence on insulin secretion. *Acta Pharmacol Sin.* 2004; 25(4):496-502.
3. Chang W, Chen L, Hatch GM. Berberine as a therapy for type 2 diabetes and its complications: From mechanism of action to clinical studies. *Biochem Cell Biol.* 2015; 93(5):479-86.
4. Li CN, Wang X, Lei L, Liu MZ, Li RC, Sun SJ, Liu SN, Huan Y, Zhou T, Liu Q et al. Berberine combined with stachyose induces better glycometabolism than berberine alone through modulating gut microbiota and fecal metabolomics in diabetic mice. *Phytother Res.* 2020; 34(5):1166-74.
5. Imenshahidi M, Hosseinzadeh H. Berberine and barberry (*Berberis vulgaris*): A clinical review. *Phytother Res.* 2019; 33(3):504-23.
6. Gomes AC, Bueno AA, de Souza RG, Mota JF. Gut microbiota, probiotics and diabetes. *Nutr J.* 2014; 13:60.
7. Nakamura YK, Omaye ST. Metabolic diseases and pro- and prebiotics: Mechanistic insights. *Nutr Metab (Lond).* 2012; 9(1):60.
8. Liu D, Zhang Y, Liu Y, Hou L, Li S, Tian H, Zhao T. Berberine modulates gut microbiota and reduces insulin resistance via the TLR4 signaling pathway. *Exp Clin Endocrinol Diabetes.* 2018; 126(8):513-20.
9. Han J, Lin H, Huang W. Modulating gut microbiota as an anti-diabetic mechanism of berberine. *Med Sci Monit.* 2011; 17(7):RA164-7.
10. Liu Y, Li T, Alim A, Ren D, Zhao Y, Yang X. Regulatory effects of stachyose on colonic and hepatic inflammation, gut microbiota dysbiosis, and peripheral CD4(+) T cell distribution abnormality in high-fat diet-fed mice. *J Agric Food Chem.* 2019; 67(42):11665-74.
11. Liang L, Liu G, Yu G, Zhang F, Linhardt RJ, Li Q. Urinary metabolomics analysis reveals the anti-diabetic effect of stachyose in high-fat diet/streptozotocin-induced type 2 diabetic rats. *Carbohydr Polym.* 2020; 229:115534.
12. Lu Y, Lin D, Li W, Yang X. Non-digestible stachyose promotes bioavailability of genistein through inhibiting intestinal degradation and first-pass metabolism of genistein in mice. *Food Nutr Res.* 2017; 61(1):1369343.
13. Li W, Huang D, Gao A, Yang X. Stachyose increases absorption and hepatoprotective effect of tea polyphenols in high fructose-fed mice. *Mol Nutr Food Res.* 2016; 60(3):502-10.
14. Li C, Yang M, Hou G, Liu S, Huan Y, Yu D, Sun S, Liu Q, Yan S, Shen Z. A human glucagon-like peptide-1-albumin recombinant protein with prolonged hypoglycemic effect provides efficient and beneficial control of glucose metabolism in diabetic mice. *Biol Pharm Bull.* 2017; 40(9):1399-408.
15. Chang CJ, Lin CS, Lu CC, Martel J, Ko YF, Ojcius DM, Tseng SF, Wu TR, Chen YY, Young JD et al. *Ganoderma lucidum* reduces obesity in mice by modulating the composition of the gut microbiota.

Nat Commun. 2015; 6:7489.

16. Jiang HY, Zhang X, Yu ZH, Zhang Z, Deng M, Zhao JH, Ruan B. Altered gut microbiota profile in patients with generalized anxiety disorder. *J Psychiatr Res.* 2018; 104:130-6.
17. Liao X, Song L, Zeng B, Liu B, Qiu Y, Qu H, Zheng Y, Long M, Zhou H, Wang Y et al. Alteration of gut microbiota induced by DPP-4i treatment improves glucose homeostasis. *EBioMedicine* 2019; 44:665-74.
18. Lontchi-Yimagou E, Sobngwi E, Matsha TE, Kengne AP. Diabetes mellitus and inflammation. *Curr Diab Rep.* 2013; 13(3):435-44.
19. Tian G, Guo C, Li Q, Liu Y, Sun X, Yin Z, Li H, Chen X, Liu X, Zhang D et al. Birth weight and risk of type 2 diabetes: A dose-response meta-analysis of cohort studies. *Diabetes Metab Res Rev.* 2019; 35(5):e3144.
20. Mikelsaar M, Zilmer M. *Lactobacillus fermentum* ME-3 - an antimicrobial and antioxidative probiotic. *Microb Ecol Health Dis.* 2009; 21(1):1-27.
21. Mandaliya DK, Seshadri S. Short Chain Fatty Acids, pancreatic dysfunction and type 2 diabetes. *Pancreatology.* 2019; 19(2):280-4.
22. Burcelin R. Gut microbiota and immune crosstalk in metabolic disease. *Mol Metab.* 2016; 5(9):771-81.
23. Lee BC, Lee J. Cellular and molecular players in adipose tissue inflammation in the development of obesity-induced insulin resistance. *Biochim Biophys Acta.* 2014; 1842(3):446-62.
24. Daly K, Shirazi-Beechey SP. Microarray analysis of butyrate regulated genes in colonic epithelial cells. *DNA Cell Biol.* 2006; 25(1):49-62.
25. Sharma S, Tripathi P. Gut microbiome and type 2 diabetes: where we are and where to go? *J Nutr Biochem.* 2019; 63:101-8.
26. Delzenne NM, Cani PD, Everard A, Neyrinck AM, Bindels LB. Gut microorganisms as promising targets for the management of type 2 diabetes. *Diabetologia.* 2015; 58(10):2206-17.
27. Gurung M, Li Z, You H, Rodrigues R, Jump DB, Morgun A, Shulzhenko N. Role of gut microbiota in type 2 diabetes pathophysiology. *EBioMedicine* 2020; 51:102590.
28. Plovier H, Everard A, Druart C, Depommier C, Van Hul M, Geurts L, Chilloux J, Ottman N, Duparc T, Lichtenstein L et al. A purified membrane protein from *Akkermansia muciniphila* or the pasteurized bacterium improves metabolism in obese and diabetic mice. *Nat Med.* 2017; 23(1):107-13.
29. Fujio-Vejar S, Vasquez Y, Morales P, Magne F, Vera-Wolf P, Ugalde JA, Navarrete P, Gotteland M. The gut microbiota of healthy chilean subjects reveals a high abundance of the phylum Verrucomicrobia. *Front Microbiol.* 2017; 8:1221.
30. Salgado MK, Oliveira LGS, Costa GN, Bianchi F, Sivieri K. Relationship between gut microbiota, probiotics, and type 2 diabetes mellitus. *Appl Microbiol Biotechnol.* 2019; 103(23-24):9229-38.
31. Van Hul M, Geurts L, Plovier H, Druart C, Everard A, Stahlman M, Rhimi M, Chira K, Teissedre PL, Delzenne NM et al. Reduced obesity, diabetes, and steatosis upon cinnamon and grape pomace are

- associated with changes in gut microbiota and markers of gut barrier. *Am J Physiol Endocrinol Metab.* 2018; 314(4):E334-52.
32. Den BG, Van EK, Groen AK, Venema K, Reijngoud DJ, Bakker BM. The role of short-chain fatty acids in the interplay between diet, gut microbiota, and host energy metabolism. *J Lipid Res.* 2013; 54(9):2325-40.
 33. Zhang W, Xu JH, Yu T, Chen QK. Effects of berberine and metformin on intestinal inflammation and gut microbiome composition in db/db mice. *Biomed Pharmacother.* 2019; 118:109131.
 34. Kim YA, Keogh JB, Clifton PM. Probiotics, prebiotics, synbiotics and insulin sensitivity. *Nutr Res Rev.* 2018; 31(1):35-51.
 35. Sircana A, Framarin L, Leone N, Berrutti M, Castellino F, Parente R, De Michieli F, Paschetta E, Musso G. Altered gut microbiota in type 2 diabetes: Just a coincidence? *Curr Diab Rep.* 2018; 18(10):98.
 36. Dalile B, Van Oudenhove L, Vervliet B, Verbeke K. The role of short-chain fatty acids in microbiota-gut-brain communication. *Nat Rev Gastroenterol Hepatol.* 2019; 16(8):461-78.
 37. Correa-Oliveira R, Fachi JL, Vieira A, Sato FT, Vinolo MA. Regulation of immune cell function by short-chain fatty acids. *Clin Transl Immunology.* 2016; 5(4):e73.
 38. Vinolo MA, Rodrigues HG, Nachbar RT, Curi R. Regulation of inflammation by short chain fatty acids. *Nutrients* 2011; 3(10):858-76.
 39. Fernandes J, Su W, Rahat-Rozenbloom S, Wolever TM, Comelli EM. Adiposity, gut microbiota and faecal short chain fatty acids are linked in adult humans. *Nutr Diabetes.* 2014; 4:e121.
 40. Turnbaugh PJ, Ley RE, Mahowald MA, Magrini V, Mardis ER, Gordon JI. An obesity-associated gut microbiome with increased capacity for energy harvest. *Nature* 2006; 444(7122):1027-31.

Figures

Figure 1

Berberine combined with stachyose improves glucose metabolism in KKAY mice. (a). Fasting blood glucose. (b). HbA1c levels. (c). Oral glucose tolerance test (OGTT). (d). Insulin tolerance test (ITT). AUC, area under the curve of OGTT or ITT. (e). HOMA-IR index. The index of the validated homeostasis model assessment of insulin resistance (HOMA-IR) was calculated as follows: $HOMA-IR = \frac{FBG \text{ (mmol/L)} \times FPI \text{ (fasting plasma insulin, } \mu\text{U/mL)}}{22.5}$. (f). Body weight of mice. Data are expressed as mean $\pm$ SEM (n=10-12). *p < 0.05, **p < 0.01, ***p < 0.001 vs. Con; #p < 0.05 vs. BBR. Con, control; Sta, stachyose; BBR, berberine.

Figure 2

Berberine combined with stachyose modifies islet functions in KKAy mice. (a). Fasting insulin levels in plasma. (b). Plasma insulin levels after glucose administration for 15 min. (c). The percentage of insulin elevation after glucose administration for 15 min. (d). Plasma glucagon concentrations. (e). Representative images of insulin and glucagon immunofluorescence staining in pancreatic islets. Insulin is shown in red, and glucagon in green. Magnification of all images is 400 $\times$. (f). Statistical analysis of the mean fluorescence intensities of insulin and glucagon in islets, and the ratio of insulin to glucagon. Data

are presented as mean $\pm$ SEM, n=9-10 for a-d; n=4-6 for e-f. *p < 0.05, **p < 0.01, ***p < 0.001 vs. Con; #p < 0.05, ##p < 0.01 vs. BBR. Con, control; Sta, stachyose; BBR, berberine.

Figure 3

Berberine combined with stachyose improves inflammatory status in KKAY mice. (a). Cytokine levels of IL-1 β , TNF- α , MCP-1, IL-6, CRP, and IL-10 in plasma were detected by ELISA. (b). Gene expression levels of IL-1 β , TNF- α , MCP-1, IL-6, IL-10, and TLR4 in intestine were analyzed by qPCR. (c-d). Protein levels of TLR4, p38MAPK, p-p38MAPK, ERK1/2, p-ERK1/2, JNK, and p-JNK were determined by Western blot analysis. β -actin was detected as the internal reference. Data are expressed as mean $\pm$ SEM, n=10–12 for a; n=4-5 for b-d. *p < 0.05, **p < 0.01, ***p < 0.001 vs. Con. Con, control; Sta, stachyose; BBR, berberine.

Figure 4

Berberine with stachyose maintains intestinal barrier integrity of KKAY mice. (a). Protein levels of ZO-1 and occludin in intestine tissues were analyzed by Western blot. (b). Quantization of ZO-1 and occludin proteins in intestine. (c-d). Gene expression levels of occludin, ZO-1, and Reg3g in intestine were evaluated by qRT-PCR. β-actin was detected as the internal reference. Data are represented as mean ± SEM (n=4-5). *p < 0.05, **p < 0.01 vs. Con; #p < 0.05 vs. BBR. Con, control; Sta, stachyose; BBR, berberine.

Figure 5

Berberine combined with stachyose alters the composition of gut microbiota of KKAY mice. (a). Total OTU numbers. (b). Shannon index. (c). Chao index. (d). Principal coordinate analysis (PCoA). (e). Relative abundance of microbiota at the phylum level. (f). Relative abundance of microbiota at the family level. (g). Heatmap analysis of relative abundance of microbiota at the genus level. The heatmap shows the top 50 genera ranked on the basis of abundance. Each column in the heatmap represents one sample, and each row represents one genus. The color bar showing blue (low) to red (high) indicates the relative abundance of each genus. Data are shown as mean $\pm$ SEM (n = 10). ***p < 0.001 vs. Con; ##p < 0.01 vs. BBR. Con, control; Sta, stachyose; BBR, berberine.

Figure 6

Effects of berberine with stachyose on fecal SCFAs of KKAY mice. (a). Fecal SCFAs, including acetic acid, propionic acid, butyric acid, isobutyric acid, pentanoic acid, isopentanoic acid, hexanoic acid, isohexanoic acid, were analyzed by GC-MS method. (b). Correlation analysis of SCFAs and specific microbiota at the family level. The R values were shown in different colors in the diagram. The blue represents negative correlation, and red represents positive correlation. n =8-10 per group. For Fig. 7a, *p < 0.05, **p < 0.01,

***p < 0.001 vs. Con; #p < 0.05, ##p < 0.01, ###p < 0.001 vs. BBR. For Fig. 7b, *p < 0.05, **p < 0.01, ***p < 0.001. Con, control; Sta, stachyose; BBR, berberine.

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