Journal of Advanced Research xxx (xxxx) xxx

Contents lists available at ScienceDirect

Journal of Advanced Research

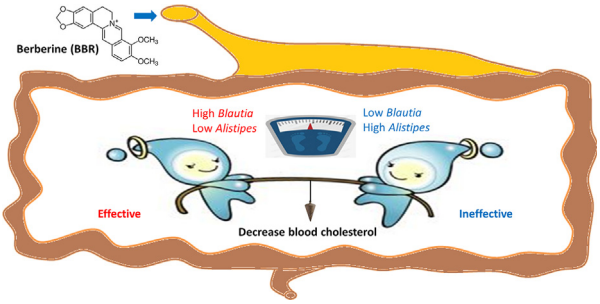
journal homepage: www.elsevier.com/locate/jare

Gut microbiota specifically mediates the anti-hypercholesterolemic effect of berberine (BBR) and facilitates to predict BBR's cholesterol-decreasing efficacy in patients

Chongming Wu [a,1,*], Ying Zhao [b,1], Yingying Zhang [b,1], Yanan Yang [a,1], Wenquan Su [b], Yuanyuan Yang [b], Le Sun [a], Fang Zhang [a], Jiaqi Yu [a], Yaoxian Wang [b], Peng Guo [a,*], Baoli Zhu [c,*], Shengxian Wu [b,*]

[a] *Pharmacology and Toxicology Research Center, Institute of Medicinal Plant Development, Chinese Academy of Medical Sciences & Peking Union Medical College, Beijing 100193, China*
[b] *Dongzhimen Hospital, Beijing University of Chinese Medicine, Beijing 100700, China*
[c] *CAS Key Laboratory of Pathogenic Microbiology and Immunology, Institute of Microbiology, Chinese Academy of Sciences, Beijing 100101, China*

GRAPHICAL ABSTRACT



ARTICLE INFO

Article history:
Received 25 April 2021
Revised 1 July 2021
Accepted 28 July 2021
Available online xxxx

Keywords:
Gut microbiota
Berberine (BBR)
Hyperlipidemia
Hypercholesterolemia
Inter-individual response variation
Blautia
Alistipes

ABSTRACT

Introduction: Gut microbiota has been implicated in the pharmacological activities of many natural products. As an effective hypolipidemic agent, berberine (BBR)'s clinical application is greatly impeded by the obvious inter-individual response variation. To date, little evidence exists on the causality between gut microbes and its therapeutic effects, and the linkage of bacteria alterations to the inter-individual response variation.
Objectives: This study aims to confirm the causal role of the gut microbiota in BBR's anti-hyperlipidemic effect and identify key bacteria that can predict its effectiveness.
Methods: The correlation between gut microbiota and BBR's inter-individual response variation was studied in hyperlipidemic patients. The causal role of gut microbes in BBR's anti-hyperlipidemic effects was subsequently assessed by altered administration routes, co-treatment with antibiotics, fecal microbiota transplantation, and metagenomic analysis.
Results: Three-month clinical study showed that BBR was effectively to decrease serum lipids but displayed an obvious response variation. The cholesterol-lowering but not triglyceride-decreasing effect of BBR was closely related to its modulation on gut microbiota. Interestingly, the baseline levels of

Abbreviations: AMPK, AMP-activated protein kinase; BBR, berberine; HFD, high-fat diet; H&E, Hematoxylin and Eosin; InsR, insulin receptor; LDL-c, low-density lipoprotein cholesterol; LDLR, low-density lipoprotein receptors; PS, the responsive subjects; NPS, the non-responsive subjects; RF analysis, Random forest analysis; ROC, receiving operating characteristic; SCFAs, short-chain fatty acids; TC, total cholesterol; TG, triglycerides.

Peer review under responsibility of Cairo University.

* Corresponding authors.
E-mail addresses: cmwu@implad.ac.cn (C. Wu), pguo@implad.ac.cn (P. Guo), zhubaoli@im.ac.cn (B. Zhu), wushx@sina.com (S. Wu).
[1] Chongming Wu, Ying Zhao, Yingying Zhang, and Yanan Yang contributed equally to this work.

https://doi.org/10.1016/j.jare.2021.07.011

Alistipes and *Blautia* could accurately predict its anti-hypercholesterolemic efficiency in the following treatment. Causality experiments in mice further confirmed that the gut microbiome is both necessary and sufficient to mediate the lipid-lowering effect of BBR. The absence of *Blautia* substantially abolished BBR's cholesterol-decreasing efficacy.

Conclusion: The gut microbiota is necessary and sufficient for BBR's hyperlipidemia-ameliorating effect. The baseline composition of gut microbes can be an effective predictor for its pharmacotherapeutic efficacy, providing a novel way to achieve personalized therapy.

Introduction

Compelling evidence has revealed that gut microbiota contributes to the exertion of the pharmacological activities of various drugs and active substances, such as metformin [1], cranberry [2], ganoderma [3], and *Pandanus tectorius* [4]. Therefore, understanding how gut microbes participate in human health and therapeutics has significant consequences for treating disease and minimizing adverse effects in clinical research. Nowadays, although considerable data have been published on drug–gut microbiota association, the causality between gut microbes and therapeutic effects as well as the key causal taxa are rarely explored.

In clinical practice, patients usually manifest distinct responses to the same drug, which is called inter-individual response variation/difference [5,6]. For instance, more than 20% of type 2 diabetic patients do not respond to or tolerate metformin [7], and the hyperlipidemic patients with familial chylomicronemia or combined dyslipidemia are partially responsive or even unresponsive to statins [8]. The unresponsiveness to certain drugs not only deprives patients of effective disease management but also greatly impedes the development of new drugs. Besides the well-documented factors such as genetics, dietary habit, and psychology [9,10], recent investigations have demonstrated gut microbiota is emerging as another determinant for inter-individual response difference [11,12]. It has been widely accepted that gut microbiome play essential roles in regulating host physiological processes and drug metabolism [13], and their composition varies greatly in population [14], thus probably leading to the different response to the same medical agent. An increased abundance of *Prevotella copri* may result in unresponsiveness to the hypoglycemic effect of metformin [15], and diminishment of gut microbes by antibiotics substantially deteriorates the lipid-lowering effect of rosuvastatin [16]. A recent study indicated that targeted inhibition of gut bacterial β-glucuronidase activity markedly enhances the anticancer efficacy of irinotecan [17]. Hence, classifying the correlations between gut bacteria and drug responsiveness will shed new light into understanding the mechanism of response variation and may provide potential solutions to improve the efficacy of medical agents.

Berberine (BBR), a natural plant alkaloid extracted from *Berberis aristata* and *Coptis chinensis* (Huanglian), has been reported to be an excellent natural remedy for metabolic disorders, including hyperlipidemia [18,19]. Multiple potential mechanisms have been proposed regarding its pharmacological actions, such as upregulation of low-density lipoprotein receptors (LDLR) in liver [18], activation of AMP-activated protein kinase (AMPK) in adipose tissue and muscles [20,21], and stimulation of insulin receptor (InsR) [22,23]. However, the oral bioavailability of BBR is rather inadequate and barely reaches the effective level at the target sites when orally administered [24]. Recent investigations have demonstrated that the anti-hyperlipidemic effect of BBR is closely related to its modulation on gut microbiota. Still, there is no solid evidence to certificate the causality between gut microbes and BBR's beneficial functions. Moreover, the distinct and even opposite responses occur among patients taking BBR administration [18], largely hindering its' general clinical application.

In the current study, we conducted a 3-month clinical study on the anti-hyperlipidemic effect of BBR, which not only confirmed its therapeutic effect but also revealed its obvious inter-individual response variation. We further performed metagenomic analysis between responsive and non-responsive patients, which linked the cholesterol-lowering of BBR to the modulation of the gut microbiota and displayed that the baseline levels of some gut bacterial taxa could precisely predict the efficacy of BBR against hypercholesterolemia. We then performed causality experiments in mice by altered administration routes, concomitantly treatment with antibiotics and fecal transplantation, which not only confirm the causal role of gut microbes in the lipid-lowering effect of BBR, but also identified *Blautia* as a key taxon that conveyed BBR's anti-hypercholesterolemic action in both animals and human beings.

Methods

Clinical trial

A randomized, double-blind, placebo-controlled clinical trial with a treatment period of twelve weeks was carried out at Dongzhimen Hospital (Beijing, China). Eighty-three hyperlipidemic patients were recruited between April 2019 and January 2020. The eligibility criteria and exclusion criteria were provided as supplemental data. All patients were randomly assigned to receive double-blind berberine (0.5 g, twice daily) or placebo prepared in indistinguishable tablets. The BBR and placebo tablets were produced by the same pharmaceutical company (Shanxi Zhendong Pharmaceutical Co., Ltd. Lot NO.:20190301). The participants in both groups also received lifestyle interventions included regulation of the diet and exercise. Participants will be asked to be strict about their diet and to avoid short-term or high-intensity exercise. Risk factors, such as smoking and alcoholics, will be controlled strictly. Researchers will emphasize the importance of diet and exercise to subjects at each treatment visit.

Patients visits were done between 0700 and 0800 am after an overnight fast. Biochemical measurements of serum lipids were performed in the clinical laboratory of Dongzhimen Hospital. Serum lipids were measured at 0, 4th, 8th, and 12th week. The participants will be asked "which type of stool they have" at each visit during the treatment period. The stool samples were obtained from each patient at the 0 and 12th week.

Animal experiment for necessity analysis

As male animals are more stable for metabolism study and most previous investigations on BBR's pharmacological activity and its

modulation on gut microbiota were carried out on male rodents [25], we chose male mice in this study to match our results with prior reports. Eight-week-old male ICR mice were purchased from Vital River Laboratory Animal Technology Co., Ltd. (Beijing, China). After acclimated for one week on a normal chow diet, mice were fed for five weeks with a normal chow diet which contains 4.5% fat, 50% carbohydrate, and 22% protein with a total calorific value of 6.095 kcal/kg (Chow group) or high-fat diet (HFD) which contains 22% fat, 35% carbohydrate, and 21% protein with a total calorific value of 10.516 kcal/kg (HFD group and other test groups). The Chow and HFD groups were given distilled water only. Animals on HFD were orally (po, 200 mg/kg per day, HFD + BBRpo) or intraperitoneally (ip, 6 mg/kg per day, HFD + BBRip) administrated with BBR, or gavaged with BBR (200 mg/kg per day) and antibiotics (ampicillin + norfloxacin, 300 mg/kg per day respectively) (HFD + Abs + BBRpo, with antibiotic-only group [HFD + Abs] as control). As the oral bioavailability of BBR is <1% [26], the intraperitoneal dose of BBR in this study is about three times of that obtained by 200 mg/kg po. Stools for metagenomic analysis were collected on the day before animals were euthanized. After five weeks of treatment, overnight-fasting mice were anesthetized, and whole blood was withdrawn by cardiac puncture. Liver and epididymal and subcutaneous fat were weighed and fixed in 4% formaldehyde for Hematoxylin and Eosin (H&E) staining or snap-frozen in liquid nitrogen for further analysis.

Fecal transplantation

Male donor mice were daily fed with HFD or HFD + BBR (200 mg/kg) (8 mice in each group). After seven days of feeding, stools were acquired daily for the subsequent 28 days. Feces from the donor mice of a same group were pooled and resuspended in sterile phosphate buffer saline (PBS, pH = 7.0) (100 mg in 2 mL PBS). The solution was vigorously vortexed for 30 s to make a suspension as transplant material. Fresh fecal suspension was prepared within 20 min before oral gavage to prevent changes in bacterial composition. Male recipient mice (eight mice per group) were pretreated with antibiotics cocktail (ampicillin + norfloxacin, 300 mg/kg per day respectively) for five days to eliminate gut bacteria, then they were fed with HFD and inoculated daily with fresh transplant material (200 μL for each animal) by oral gavage for four weeks.

Metagenomic analysis

Fecal microbiota DNA was extracted using the QIAamp DNA Stool Mini Kit (Qiagen, USA), and fecal microbial composition was assessed using Illumina HiSeq sequencing and QIIME-based microbiota analysis. The detailed procedure of fecal microbiota DNA extraction, sequencing and microbiota analysis pipeline is described in the supplementary methods.

Oral glucose tolerance test (OGTT) and insulin tolerance test (ITT)

OGTT and ITT were performed on the 1th and 5th day of the 5th week after treatment. OGTT using 2 g/kg of glucose and ITT using 0.75 U/kg of human regular insulin were conducted as previous described [27]. Blood samples were collected from tail vein for glucose measurement at 0, 30, 60, 90 and 120 min. The blood glucose levels were determined by a glucose meter (Roche, ACCU-CHEK Active).

Histology analysis

The organ tissues of each mouse were fixed in 4% formaldehyde, embedded in paraffin and cut at 4 μm. The sections were stained with H&E (for fat and liver), and their morphological changes were evaluated. The sizes of adipocytes were measured by AxioVision imaging software (Carl Zeiss, Jena, Germany). The steatosis of the liver [28] was evaluated through observation of H&E stained liver tissues under an optical microscope. Steatosis score (0 to 3) was ranked as follows: 0, no involvement; 1, mild involvement; 2, moderate involvement; 3, severe involvement.

Data analysis and statistics

The microbiome data were analyzed by R software (4.0.3 version). Random forest (RF) analysis in the "random forest" package was used to identify the most important features that discriminate the responsive and non-responsive patients. The abundance of *Alistipes* and *Blautia* spp. was combined by binary logic regression and used to plot the receiving operating characteristic (ROC) curve by SPSS17.0, thus evaluating the discriminative performance of the combined genera indicator. All data are presented as the means ± s.e.m. SPSS 17.0 software was used for the statistical analysis of pharmacological data. The significance was assessed by one-way ANOVA followed by Newman-Keuls *post hoc* tests. $P < 0.05$ was considered statistically significant.

Results

BBR medication individual-differentially mitigates hyperlipidemia in patients

To assess the anti-hyperlipdiemic effect of BBR, we enrolled 83 hyperlipidemic patients and treated them with BBR (42 patients) or placebo (41 patients) for three months (demographic and baseline characteristics in Table S1). According to our analysis, oral administration of BBR (1 g/day) time-dependently decreased the serum levels of triglycerides (TG), total cholesterol (TC) and low-density lipoprotein cholesterol (LDL-c) from the baseline level (Fig. 1), compared with the placebo group. Moreover, BBR did not significantly decline serum TG until treatment for 12 weeks. In contrast, it steadily decreased serum TC and LDL-c from the first month after medication (Fig. 1). These results indicated that BBR preferred to mitigating hypercholesterolemia. Importantly, we found that a large portion of patients manifested the decreased serum lipids level after BBR treatment, but some subjects were less responsive to BBR treatment, especially for intervention on serum TC and LDL-c (Fig. S1), exhibiting an obvious inter-individual variation in the therapeutic efficiency of BBR.

Gut microbiota tightly associated with the therapeutic efficacy of BBR on cholesterol not triglyceride

We obtained the initial (week0) and final (week12) fecal samples from 51 patients (28 in BBR group and 23 in placebo group) and analyzed their gut microbiome composition by shotgun sequencing metagenomics. The impact of BBR on the overall structure of gut microbiota was assessed by community alpha diversity (Chao1 index indicates community taxon richness, Shannon index indicates community diversity, and Simpson index shows equivalency of each microbe) and community variation (principal coordinate analysis (PCoA)). Three-month treatment of BBR has only minimal impact on alpha diversity and the overall structure of gut microbiota in our hyperlipidemic cohort (Fig. S2), possibly due to an adaptation of gut microbes to BBR.

We further correlated the TC-/TG-decreasing efficacy of BBR with gut microbiota to verify whether the response difference to BBR treatment attributes to the modulation of gut microbiota. For patients with hypertriglyceridemia, the overall structure and

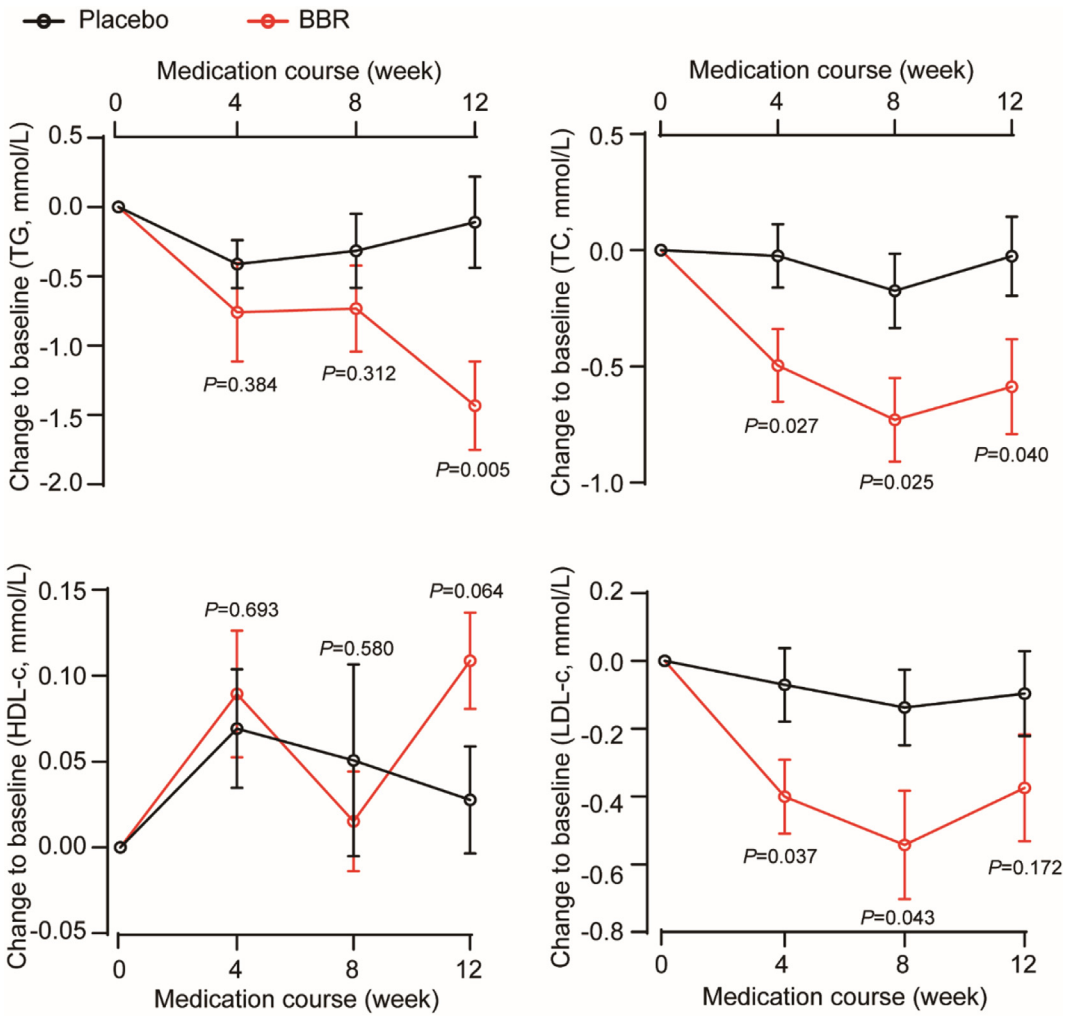


Fig. 1. BBR effectively decreases blood lipids in hyperlipidemic patients. Eighty-three hyperlipidemic patients were treated with BBR (42 subjects, 1 g/day) or placebo (41 subjects) for 3 months. The lipids changes at each time point compared to the baseline levels were analyzed.

alpha diversity of gut microbiota showed no alterations between the responsive (PS, whose serum TG decreased after treatment) and the non-responsive (NPS) subjects (Fig. S3A-E), along with the almost identical profiles of key genera abundance (Fig. S3F). These findings pointed out a conjecture that the TG-decreasing effect of BBR hardly relates to its regulation on gut microbial community. On the contrary, gut microbes exhibited a significant ($p < 0.05$ adonis) separation between the PS and the NPS patients towards BBR's cholesterol-decreasing effect (Fig. 2A-C). The alpha diversity of microbiome in the PS subjects was increased after BBR treatment, while the NPS ones showed some decrease (Fig. 2D). Therefore, these results collectively suggested that modulation of BBR on gut microbiota might specifically contribute to its cholesterol-decreasing effect.

The baseline levels of Alistipes and Blautia promisingly predict the cholesterol-lowering function of BBR

Next, we focused on the taxa that were markedly different between the PS and NPS patients in their initial gut microbiota, aiming to find key bacteria possessing potentials to predict the therapeutic efficacy of BBR before medication. Among all the main genera, only *Alistipes* was significantly different in the baseline fecal bacterial community between PS and NPS patients (Fig. 3A). At the species level, *A. putredinis*, *A. shahii* and *A. finegoldii* were the major *Alistipes* members that were prominently higher in NPS patients in comparison with the PS subjects (Fig. 3B). Co-abundant network analysis revealed that three species of genus *Blautia*, that were, *Ruminococcus gnavus*, *R. torques* and *R. obeum*, were significantly negative to the *Alistipes* members (Fig. 3C). Random forest analysis also showed that *A. finegoldii*, *A. shahii*, *A. senegalensis* and *R. gnavus* were among the top 15 species that contributed the difference in gut microbiome between PS and NPS subjects (Fig. 3D). In light of the critical roles of *Alistipes* and *Blautia* in the lipid-lowering effect of BBR [29], we investigated their predictive ability by ROC analysis and found that the baseline abundance of these two genera could effectively predict the cholesterol-decreasing efficacy of BBR with AUC of 0.903 (95% confidence interval 0.761–1.000, $p = 0.007$) (Fig. 3E).

Taking this prediction model, we selected 11 persons from the 18 hypercholesterolemic patients in our cohort as predicated BBR responders in which 90.91% (10/11) patients were actual responders, markedly higher than the total responsive rate of BBR (66.67%, 12/18). These predicted responders showed a significant decline in serum TC ($P = 0.002$) as compared to the placebo-treated subjects. Meanwhile, they were also more responsive to BBR's TG-decreasing (−1.38 vs. −0.70) and LDL-c-decreasing (−0.55 vs. −0.31) effects than all hypercholesterolemic patients that received BBR medication (Fig. S4A). For the entire cohort that includes both hypercholesterolemic and hypertriglyceridemic

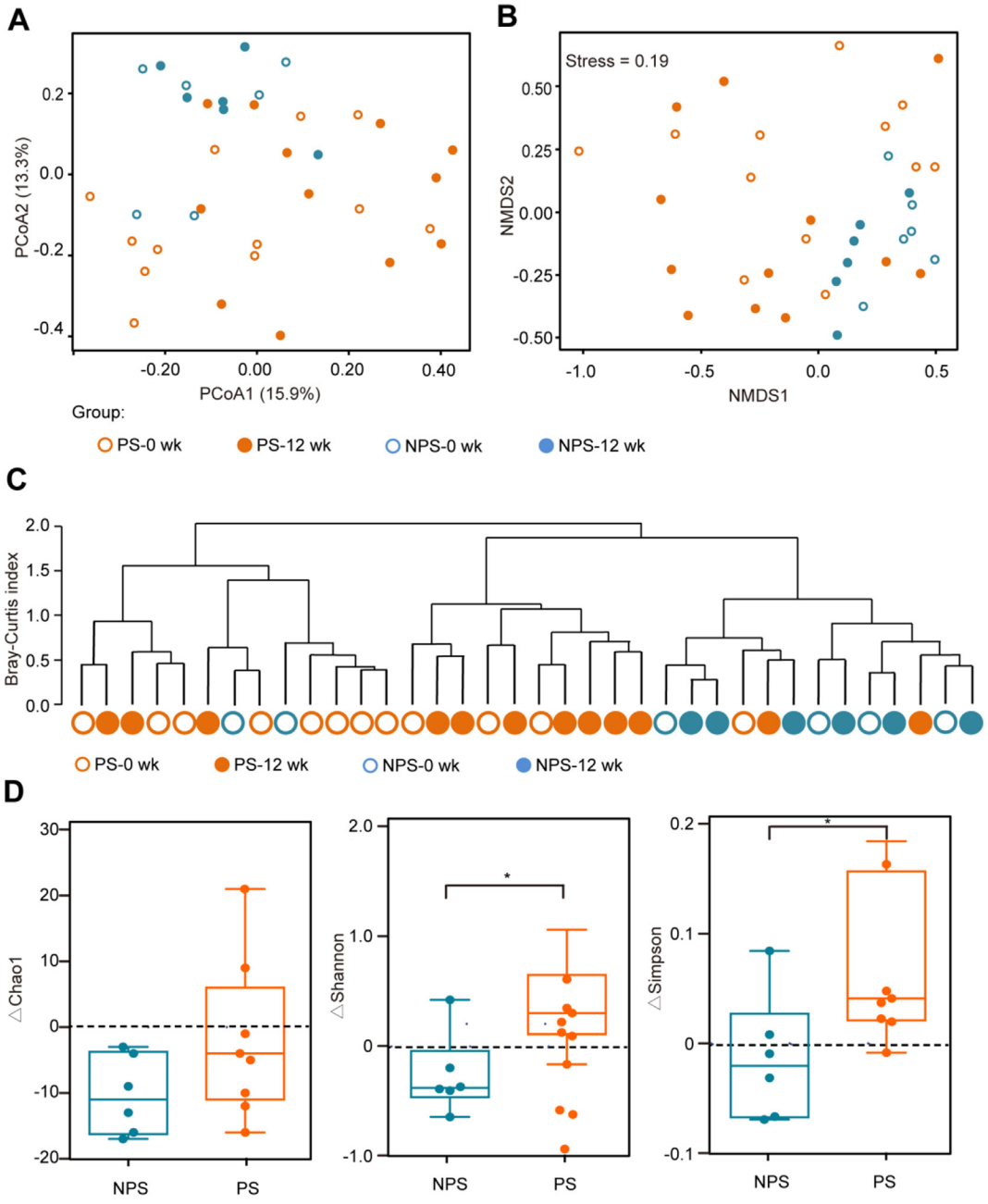


Fig. 2. The cholesterol-lowering efficiency of BBR is closely related with its modulation on gut microbiota. A-B. The principal coordinate analysis (PCoA) (A) and non-metric multi-dimensional scaling (NMDS) analysis (B) of gut microbiota. (C) The Bray-Curtis distance-based clustering analysis. (D) The changes of alpha diversity indices compared to the baseline values. We obtained the initial (week0) and final (week12) fecal samples from 51 patients (28 in BBR group and 23 in placebo group) and analyzed their gut microbiota composition by shotgun sequencing metagenomics. *$P < 0.05$.

patients, 18 subjects were predicted as BBR responders by our predict model in which 16 (88.89%) patients were responsive to BBR's anti-hypercholesterolemic or anti-hypertriglyceridemic efficacy, while the overall effectiveness of BBR in this term was 82.14%. These predicted responders were also more responsive to BBR's TC-decreasing (−0.62 vs. −0.28) and TG-decreasing (−1.21 vs. −0.93) effects than the whole cohort (Fig. S4B).

Gut microbiota is important for the lipid-lowering effects of BBR

To confirm the causal role of the gut microbiome in the anti-hyperlipidemic effects of BBR, we further set up paired experimental groups of mice treated with oral (HFD + BBRpo, 200 mg/kg) or intraperitoneal (HFD + BBRip, 6 mg/kg) administration of BBR, as well as groups with oral administration of BBR and antibiotics (HFD + BBRpo + Abs; ampicillin and norfloxacin [Abs], 300 mg/kg each), with an antibiotic-only group (HFD + Abs) as control. Orally administration of BBR significantly alleviates the adverse effects of HFD on obesity, lipid and glucose profile, insulin sensitivity, and liver steatosis (Fig. 4; Fig. S5). However, these beneficial effects were markedly reduced when BBR was administered via intraperitoneal injection (HFD + BBRip vs. HFD + BBRpo; Fig. 4; Fig. S5), indicating that the lipid-lowering effect of BBR is not solely achieved by its entry into the circulation. When combined with antibiotics, the therapeutic effects of BBRpo were nearly disappeared (HFD + BBRpo + Abs vs. HFD + Abs; Fig. 4; Fig. S5), implying that gut micro-

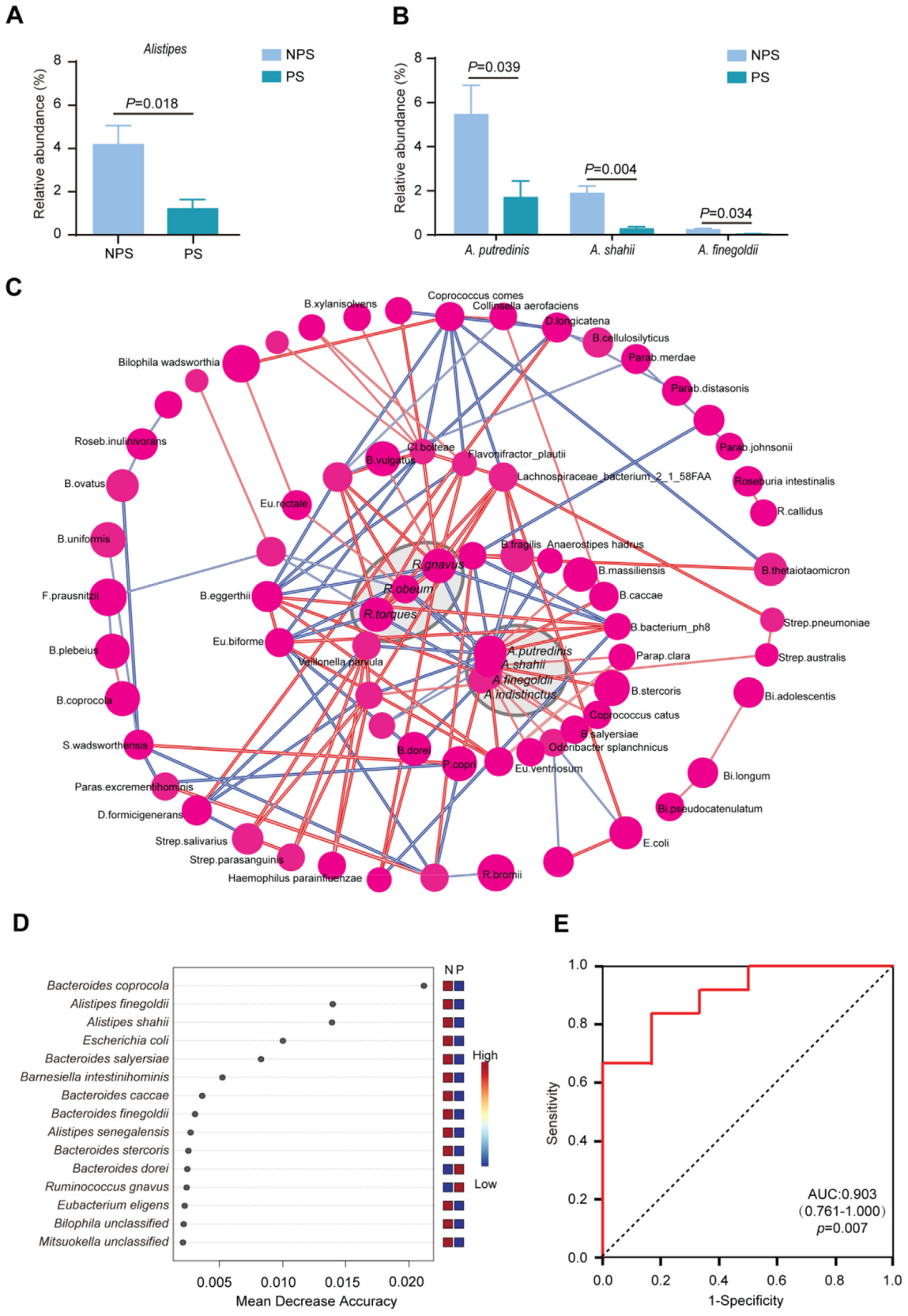


Fig. 3. The baseline abundance of Alistipes and Blautia spp. are effective to predict the cholesterol-lowering efficiency of BBR in hyperlipidemic patients. (A) The genus profile of responsive (PS) and non-responsive (NPS) patients, and *Alistipes* is the only dominant genus whose baseline abundance is significantly different between PS and NPS patients. (B) The species profile of PS and NPS patients, and three *Alistipes* spp. are significantly different between PS and NPS patients at the baseline level. (C) Co-occurrence network established by SparCC analysis. The area of each node indicates the accumulated abundance of the species, and the portion of each group was displayed in different colours. The connecting edges indicate positive (orange) or negative (blue) correlations between species. (D) The top 15 species that discriminate the PS and NPS patients based on random forest analysis. (E) Receiver operating characteristic curve (ROC) for the combination of *Alistipes* and *Blautia* spp. Area under curve (AUC) and the 95% confidence interval are also shown.

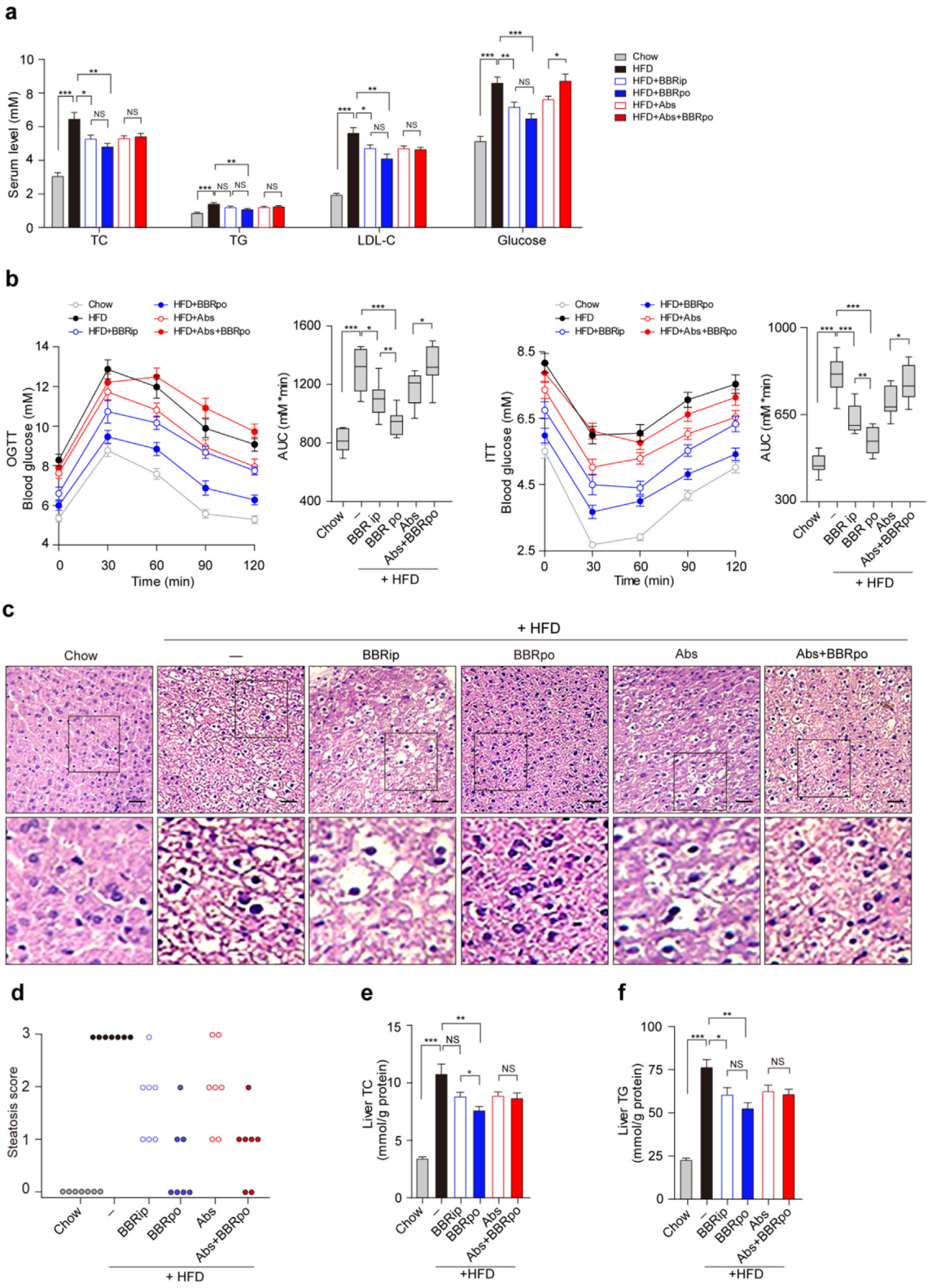


Fig. 4. Parenteral administration or antibiotic treatment largely weakens BBR's lipid-lowering effect. (A) Serum lipids and glucose levels in each treatment group; error bars denote standard error in measurements. (B) Oral glucose tolerance test (OGTT) and insulin tolerance test (ITT) in each group. (C) Hematoxylin and eosin (H&E) staining of the liver (bar = 10 μm). (D) Steatosis score of different treatment groups; each dot represents a liver sample wherein steatosis was diagnosed. (0–3) was evaluated as follows: 0, no involvement; 1, mild involvement; 2, moderate involvement; and 3, severe involvement. E-F Liver total cholesterol (TC) (E) and triglyceride (F) measurements in different treatment groups. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, N.S. = non-significant.

biota may play crucial roles in mediating the anti-hyperlipidemic effect of BBR.

We observed consistent shifts in the fecal microbiome within each treatment group. The microbiome of BBRip mice was closest to that of those on the HFD diet alone, and oral administration of BBR and antibiotics markedly shifted the structure of microbial communities ($p < 0.001$ in *adonis* test; Fig. 6A-C). Similar in human beings, oral gavage of BBR significantly enriched *Blautia* and decreased the abundance of *Alistipes* (Table S2). Besides, BBR also increased the population of bacteria belonging to *Akkermansia*, *Clostridium* XI, *Robinsoniella*, *Cronobacter*, *Anaerostipes* and *Coprobacillus*, and decreased the abundance of *Helicobacter*, *Enterorhabdus* and *Desulfovibrio* (Table S2). BBRpo mice showed a different microbial community structure compared with mice treated with only antibiotics, and combination treatment with antibiotics substantially diminished the modulation of the microbiota by BBR (Fig. 6D), thereby exhibiting a distinct impact on gut microbiota between BBR and antibiotics.

BBR-modulated gut microbiota prominently ameliorates hyperlipidemia

To figure out whether the shifts in the microbiome confer the beneficial effects of BBR, or just occur secondary to BBR treatment, we undertook fecal transplantation from donor mice with oral-administration of BBR to mice with an HFD feeding and found that fecal transplantation indeed induced a beneficial influence with the similar extent to that observed in BBRpo mice. Specifically, the transfer of the microbiome resulted in less body and fat weight (Fig. 5A-E), lower serum lipids and glucose levels (Fig. 5F),

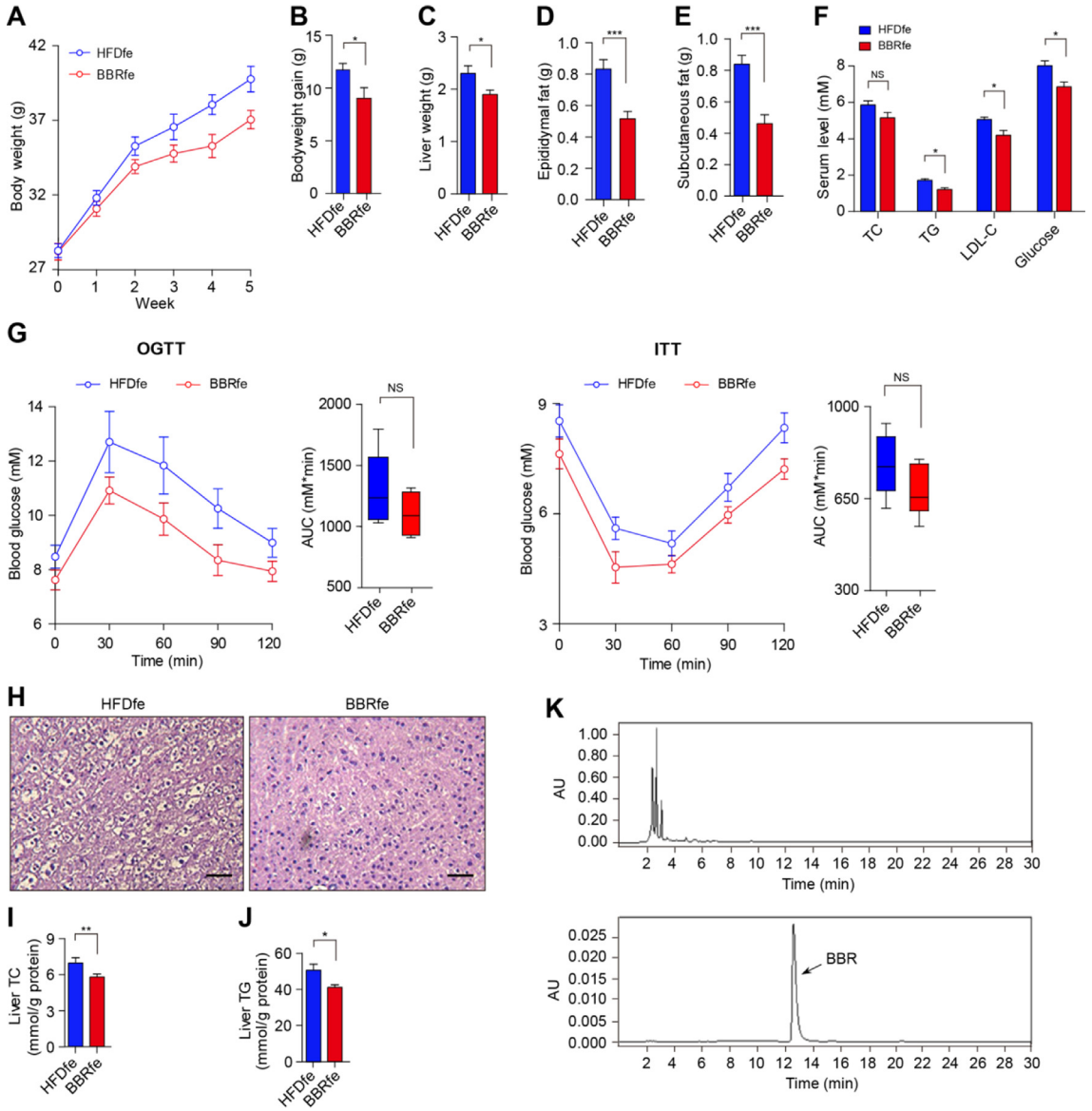


Fig. 5. Fecal material transplantation after BBR treatment prevents HFD-induced hyperlipidemia as effectively as BBR. (A–D) Bodyweight (A), bodyweight gain (B) and the weights of liver (C), subcutaneous fat (D) and epididymal fat (E) of different treatment groups, respectively. (F) Serum levels of TC, TG, LDL-c, and glucose. (G) Oral glucose tolerance test (OGTT) and insulin tolerance test (ITT). (H–J) H&E staining of liver (bar = 10 μm) (H) and hepatic levels of total cholesterol (TC)(I), and triglycerides (TG) (J) from different treatment groups, respectively. (K) HPLC for BBR in BBR soup (bottom panel) and the collected fecal material after BBR administration (top panel), indicating the absence of BBR in fecal materials used for the transplant. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, N.S. = non-significant.

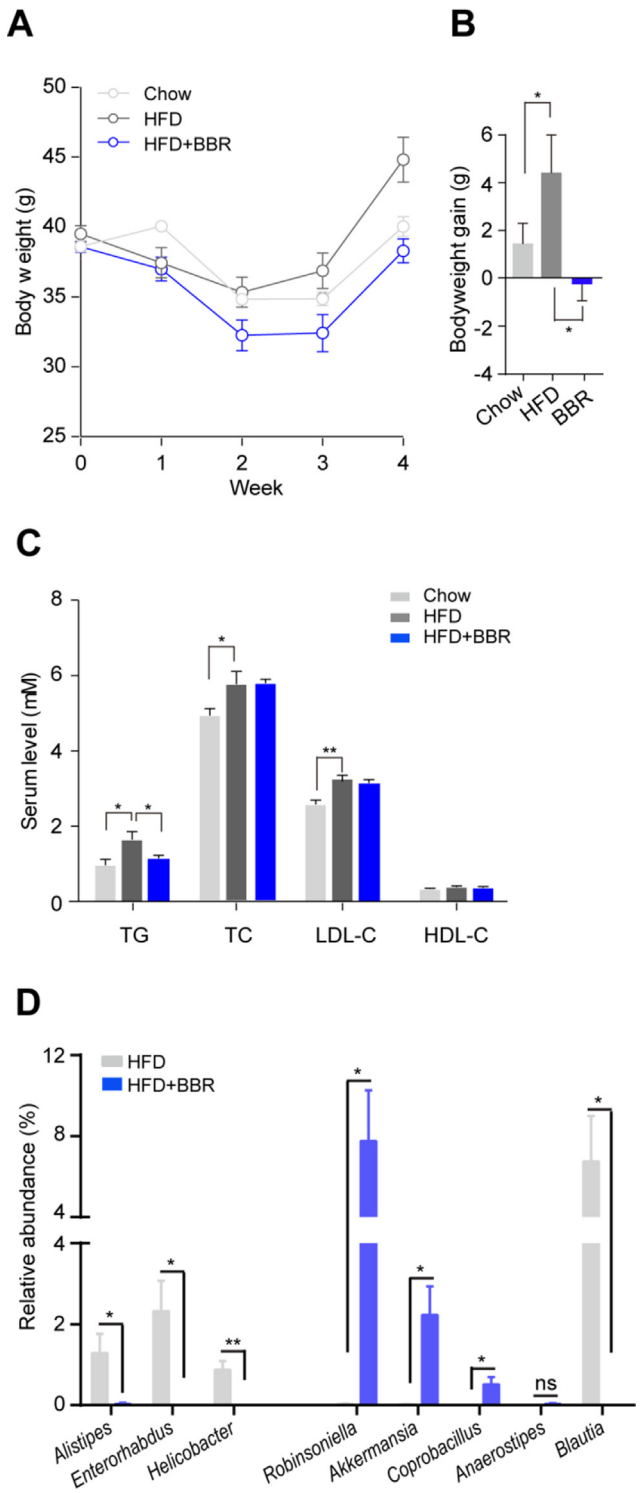


Fig. 6. Dysregulation of *Blautia* downregulates the cholesterol-lowering action of BBR. (A) Bodyweight curve. (B) Bodyweight change. (C) Serum lipids levels. (D) Relative abundance of key genera that were confirmed to be closely related to BBR's lipid-lowering effects. Data are expressed as mean ± s.e.m. N = 8 for each group. $^{\#}P < 0.05$, HFD group vs Chow group; $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. N.S. = non-significant.

improved oral glucose tolerance (Fig. 5G), and alleviation of liver steatosis (Fig. 5H-J). Given the undetectable level of BBR in fecal material (Fig. 5K), our results suggested that BBR-modulated microbiota holds great potential to improve hyperlipidemia.

Further detailed analysis of the microbiota displayed that the fecal microbiota transplant significantly shifted gut microbes (Fig. S7A-B) and promoted the growth of short-chain fatty acids (SCFAs)-producing taxa, which have been intensively reported to regulate host lipid metabolism, including *Clostridium XI*, *Anaerostipes*, *Blautia*, *Akkermansia,* and *Coprobacillus*, and decreased the abundance of *Alistipes*, *Helicobacter*, *Enterorhabdus* and *Desulfovibrio* (Fig. S7C-D; Table S3). Again, *Alistipes* and *Blautia* were similarly modulated by BBR in mice and in human beings, suggesting a universal role of these two genera in the lipid-decreasing action of BBR.

Dysregulation of Blautia impaired the cholesterol-lowering action of BBR

In both necessity (changes in administration routes or concomitant treatment with antibiotics) and causality (fecal transplantation) experiments, thirteen genera with relative abundance above 0.01% were coordinately altered by BBR or BBR-modulated gut microbiota (Table S3). The *Blautia*, *Akkermansia*, *Robinsoniella*, *Anaerostipes* and *Coprobacillus* were significantly enriched, while *Alistipes*, *Helicobacter* and *Enterorhabdus* were largely decreased by oral administration of BBR or BBR-modulated microbes (Fig. S8). According to these findings, we speculated that these genera might be necessary and causative for BBR's lipid-reducing actions.

Little feasible method is currently available to precisely diminish certain bacterial genus. In light of the different individual responsiveness to berberine in human beings, we speculate that it may be the same in animals and repeated experiments several times to come across the situation where BBR failed to regulate the serum lipids. Intriguingly, BBR was found in one experiment to merely preserve the anti-obesity and anti-hypertriglyceridemia effects (Fig. 6A-C), while the anti-hypercholesterolemia efficacy was substantially diminished (Fig. 6C). Subsequently, we analyzed the profile of the above-mentioned genera that were tightly-related to BBR's therapeutic roles. The modulations of BBR on *Alistipes*, *Helicobacter*, *Enterorhabdus*, *Akkermansia*, *Robinsoniella* and *Coprobacillus* were all preserved, whereas the elevation of *Blautia* was disappeared in this experiment (Fig. 6D). These results provided a hint that *Blautia* might be a key taxon to convey BBR's cholesterol-decreasing effect.

Discussion

Accumulating researches have implicated that gut microbiota widely participates in the exertion of various drugs' therapeutic efficacies, for instance, berberine (BBR) [30]. Previous studies revealed that the hyperlipidemia-alleviating effect of BBR was mediated by the gut microbiota [31–34], However, to date, the causality between specific gut microbes and BBR's pharmacological effects remains to be verified. Meanwhile, BBR medication usually exhibits an obvious inter-individual variation [18], thus heavily impeding its clinical application. In the present study, we not only demonstrated the necessity and sufficiency of gut microbes in the

lipid-lowering effect of BBR in mice model, but also provided a first evidence that the commensal genus, *Blautia*, is indispensable for BBR's anti-hypercholesterolemic efficacy. BBR medication in patients further corroborated that the modulation of BBR on gut microbiota was closely related to its cholesterol-lowering action. Particularly, the baseline levels of *Alistipes* and *Blautia* could predict the anti-hypercholesterolemic efficiency of BBR, possibly facilitating to improve the overall effectiveness of BBR and achieve the ultimate clinical therapeutics.

BBR is an excellent natural lipid-lowering drug, yet with a very poor oral availability. Many researchers have noticed the remarkable influence of BBR on gut microbiota [1,2,33,35–37] and proposed that the modulation on gut microbiota may be a main mechanism underpinning its anti-hyperlipidemic action. Unfortunately, most studies only demonstrated that changes of gut microbiome were synergistic with BBR administration [3], without offering sufficient evidence towards the causality between bacteria and its beneficial effects. In this study, we first introduced different administration routes and found that intraperitoneal injection of BBR (BBRip) exerted a weaker efficiency to ameliorate hyperlipidemia than oral administration (BBRpo), though the biological availability of BBR was increased by over three times in BBRip mice (Fig. 4), suggesting that oral bioavailability is not the sole factor responsible for BBR's therapeutic efficacy. We further performed experiments in antibiotics-induced pseudo-germfree mice which revealed that elimination of gut microbes substantially abolished BBR's lipid-lowering efficiency (Fig. 4), clearly pointing out the necessity of gut microbiota. Moreover, the benefits of BBR could be well-transferred by fecal microbiota transplantation from BBRpo-treated mice to HFD mice (Fig. 5), again corroborating the critical roles of gut microbiome. Collectively, our findings demonstrate that modulation of gut microbiota is both necessary and sufficient to mediate the anti-hyperlipidemic effect of BBR, ruling out the possibility of microbiome shifts being merely secondary.

For modulation on microbial structure, several gut bacteria including *Akkermansia*, the SCFA-producing and bile salt-metabolizing germs have been previously suggested as key taxa closely-associating with the pharmacological functions of BBR [33,36,38–40], yet none of them has been certified regarding its necessity by experimental evidence. Our association analysis showed that the enrichment of *Blautia*, *Akkermanisa*, *Robinsoniella*, *Coprobacillus* and *Anaerostipes* and the decrease of *Alistipes*, *Helicobacter*, and *Enterorhabdus* were tightly related to BBR' beneficial effects in both necessity (administration route alterations and antibiotics treatment) and sufficiency (fecal microbiota transplantation) experiments (Fig. S8). Interestingly, we found in one experiment where oral administration of BBR only retained the impacts on bodyweight and TG, but substantially lost its TC- and LDL-c-alleviating actions (Fig. 6A-C). Correspondingly, the detailed taxonomic analysis displayed that modulations of BBR on *Akkermansia*, *Robinsoniella*, *Coprobacillus*, *Anaerostipes*, *Alistipes*, *Helicobacter,* and *Enterorhabdus* were preserved, whereas the growth of *Blautia* was apparently dysregulated (Fig. 6D). Based on these, we speculated that *Blautia* might function as an important genus contributing to the cholesterol-decreasing effect of BBR. Indeed, two recent studies have claimed that this genus associates with visceral fat accumulation in young adults [41] and with the therapeutic efficacy of a traditional Chinese herbal formula containing BBR [29]. Hence, our results provided the first evidence that *Blautia* is a causal taxon responsible for the cholesterol-decreasing function of BBR.

Another important finding in this work is that gut microbiota involves in the inter-individual response variation of BBR medication. To investigate the association of BBR's therapeutic efficacy with gut microbiome in human, we conducted a clinical research on recruited 83 hyperlipidemic patients. Although BBR could effectively decrease serum TC, TG and LDL-c levels as compared to placebo, about 21.43% (9/42), 38.10% (16/42) and 54.76% (23/42) patients were still not responsive to the TG-, TC- and LDL-c-decreasing effects of BBR, respectively (Fig. S1), showing an obvious inter-individual response variation. Up till now over twenty clinical studies have been published on the hyperlipidema-ameliorating effect of BBR [42], but the consequent varied responses are scarcely investigated. Given the prominent modulation of BBR on gut microbes in mice, we conjectured that the versatile commensal bacteria would actively participate in the annoying responses in patients. To figure out this, we then correlated BBR's TC/TG-lowering efficacy with its regulation on microbiota. 3-month medication of BBR did not significantly change the overall structure of gut microbiota (Fig. S2), which might reflect an adaptive response of gut microbes to chronic therapeutics. However, more detailed analysis unraveled that the cholesterol- not triglyceride-decreasing efficacy of BBR was intimately related to the apparent alterations of microbial community (Fig. 2, Fig. S3), providing an important evidence that the hypercholesterolemia-mitigating effect of BBR may involve the function of gut microbes.

An essential aim of precision medicine is to find right drugs for a particular patient to achieve satisfactory personalized medication [43]. From the point of view of medicine, finding suitable patients for a specific drug is also an effective way to fulfill the precision therapy. In light of the strong association between BBR's TC-decreasing efficiency and gut microbiota, we attempted to uncover certain bacteria with a prediction ability towards BBR's efficacy. Intriguingly, we found that higher baseline abundance of *Alistipes* in gut microbiome (initial fecal samples) usually results in non-responsiveness to BBR's therapeutic effects and identified *Alistipes* spp. and *Blautia* spp. to be the crucial taxa to discriminate responsive patients from non-responsive ones (Fig. 3A-B). ROC analysis further indicated that the baseline abundance of *Alistipes* and *Blautia* in initial gut microbiota could promisingly predict the effectiveness of subsequent BBR medication against hypercholesterolemia (Fig. 3D). Tong *et al* also raised that alterations of *Alistipes* (decrease) and *Blautia* (increase) associate with the alleviation in hyperlipidemia of a traditional Chinese herbal formula [29]. These findings inspire us to depict a vision where the personalized responses to BBR treatment could be conveniently predicted just by quantifying baseline levels of certain genera (e.g. *Alistipes* and *Blautia*) in a painless, time- and cost-saving manner before actual medication. Moreover, manipulation of *Alistipes* and *Blautia* amid BBR medication might be a potential method to improve the overall efficacy of BBR, ultimately promoting its clinical-scale application.

Conclusions

In conclusion, our studies first demonstrate that gut microbiota is not only necessary but also sufficient to mediate the lipid-lowering effect of BBR and identify *Blautia* to be a critical commensal genus to convey its anti-hypercholesterolemic action in mice. Furthermore, the cholesterol- not triglyceride-lowering efficacy of BBR in patients intimately associates with its modulation on gut microbiota, and the baseline levels of *Alistipes* and *Blautia* could precisely predict the efficacy of BBR against hypercholesterolemia, thereby holding promising potentials to tackle its inter-individual response variation and fulfill personalized medication.

Compliance with ethics requirements

All the animal experiments were performed in accordance with the National Institutes of Health regulations for the care and use of animals in research. The protocol was approved by the medical

ethics committee of Peking Union Medical College (Nos. YZS201410003; YZS201506012; YZS201804011). All efforts were made to minimize animal suffering.

The clinical study was approved by the Ethics Committee of Dongzhimen Hospital Affiliated to Beijing University of Chinese Medicine (DHABUCM) (No. DZMEC-KY-2018-47), and written informed consent was obtained from each patient. The study was conducted in accordance with the principles of the Declaration of Helsinki. The registration number is ChiCTR1900021361 (Chinese clinical trial registry).

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

This work was supported financially by the National Natural Science Foundation of China (81673663, 81973217), the Nonprofit Central Research Institute Fund of Chinese Academy of Medical Sciences (2018RC350014), CAMS Innovation Fund for Medical Sciences (CIFMS) 2016-I2M-3-015, and National Major Scientific and Technological Special Project for "Significant New Drugs Development" (No.2017ZX09304019).

Availability of data and material

The sequencing data is deposited at NCBI archive with project numbers PRJNA557542 for antibiotics and fecal transplantation study; PRJNA559041 for *Blautia spp*. absence study. Other information can be presented on request. All data relevant to the study are included in the article or uploaded as supplementary information.

Appendix A. Supplementary material

Additional file 1: Additional methods and Supplementary Figures S1-S8 and Table S1-S3. Supplementary data to this article can be found online at https://doi.org/10.1016/j.jare.2021.07.011.

References

[1] Shin N-R, Lee J-C, Lee H-Y, Kim M-S, Whon TW, Lee M-S, et al. An increase in the Akkermansia spp. population induced by metformin treatment improves glucose homeostasis in diet-induced obese mice. Gut 2014;63(5):727–35. doi: https://doi.org/10.1136/gutjnl-2012-303839.

[2] Anhê FF, Roy D, Pilon G, Dudonné S, Matamoros S, Varin TV, et al. A polyphenol-rich cranberry extract protects from diet-induced obesity, insulin resistance and intestinal inflammation in association with increased Akkermansia spp. population in the gut microbiota of mice. Gut 2015;64 (6):872–83. doi: https://doi.org/10.1136/gutjnl-2014-307142.

[3] Chang C-J, Lin C-S, Lu C-C, Martel J, Ko Y-F, Ojcius DM, et al. Ganoderma lucidum reduces obesity in mice by modulating the composition of the gut microbiota. Nat Commun 2015;6(1). doi: https://doi.org/10.1038/ncomms8489.

[4] Wu C, Tian Yu, Yu J, Zhang R, Zhang X, Guo P. The pandanus tectorius fruit extract (PTF) modulates the gut microbiota and exerts anti-hyperlipidaemic effects. Phytomedicine 2019;58:152863. doi: https://doi.org/10.1016/j.phymed.2019.152863.

[5] Zhu W, Mazzanti A, Voelker TL, Hou P, Moreno JD, Angsutararux P, et al. Predicting patient response to the antiarrhythmic mexiletine based on genetic variation: Personalized medicine for long QT syndrome. Circ Res 2019;124 (4):539–52. doi: https://doi.org/10.1161/CIRCRESAHA.118.314050.

[6] Soccio RE, Chen ER, Rajapurkar SR, Safabakhsh P, Marinis JM, Dispirito JR, et al. Genetic variation determines PPARγ function and anti-diabetic drug response in vivo. Cell 2015;162:33–44. doi: https://doi.org/10.1016/j.cell.2015.06.025.

[7] Kahn SE, Haffner SM, Heise MA, Herman WH, Holman RR, Jones NP, et al. Glycemic durability of rosiglitazone, metformin, or glyburide monotherapy. New England J Med 2006;355(23):2427–43. doi: https://doi.org/10.1056/NEJMoa066224.

[8] Tsimihodimos V, Elisaf M. Effects of evolving lipid-lowering drugs on carbohydrate metabolism. Diabetes Res Clin Pract 2018;137:1–9. doi: https://doi.org/10.1016/j.diabres.2017.12.012.

[9] Xu Y, Dong Q, Li F, Xu Y, Hu C, Wang J, et al. Identifying subpathway signatures for individualized anticancer drug response by integrating multi-omics data. J Translational Med 2019;17(1). doi: https://doi.org/10.1186/s12967-019-2010-4.

[10] Kreutz R. Pharmacogenetics of antihypertensive drug response. Curr Hypertens Rep 2004;6(1):15–20. doi: https://doi.org/10.1007/s11906-004-0005-9.

[11] Daly K, Proudman CJ, Duncan SH, Flint HJ, Dyer J, Shirazi-Beechey SP. Alterations in microbiota and fermentation products in equine large intestine in response to dietary variation and intestinal disease. Br J Nutr 2012;107(7):989–95. doi: https://doi.org/10.1017/S0007114511003825.

[12] Morand C, De Roos B, Garcia-Conesa MT, Gibney ER, Landberg R, Manach C, et al. Why interindividual variation in response to consumption of plant food bioactives matters for future personalised nutrition. Proc Nutr Soc 2020;79 (2):225–35. doi: https://doi.org/10.1017/S0029665120000014.

[13] Collins SL, Patterson AD. The gut microbiome: an orchestrator of xenobiotic metabolism. Acta Pharmaceutica Sinica B 2020;10(1):19–32. doi: https://doi.org/10.1016/j.apsb.2019.12.001.

[14] Turnbaugh PJ, Hamady M, Yatsunenko T, Cantarel BL, Duncan A, Ley RE, et al. A core gut microbiome in obese and lean twins. Nature 2009;457(7228):480–4. doi: https://doi.org/10.1038/nature07540.

[15] Elbere I, Silamikelis I, Dindune II, Kalnina I, Ustinova M, Zaharenko L, et al. Baseline gut microbiome composition predicts metformin therapy short-term efficacy in newly diagnosed type 2 diabetes patients. PLoS ONE 2020;15(10): e0241338. doi: https://doi.org/10.1371/journal.pone.0241338.

[16] Wang L, Wang Y, Wang H, Zhou X, Wei X, Xie Z, et al. The influence of the intestinal microflora to the efficacy of Rosuvastatin. Lipids in Health and Disease 2018;17. https://doi.org/10.1186/s12944-018-0801-x.

[17] Bhatt AP, Pellock SJ, Biernat KA, Walton WG, Wallace BD, Creekmore BC, et al. Targeted inhibition of gut bacterial beta-glucuronidase activity enhances anticancer drug efficacy. Proc Natl Acad Sci U S A 2020; 117: 7374-81. https://doi.org/10.1073/pnas.1918095117.

[18] Kong W, Wei J, Abidi P, Lin M, Inaba S, Li C, et al. Berberine is a novel cholesterol-lowering drug working through a unique mechanism distinct from statins. Nat Med 2004;10(12):1344–51. doi: https://doi.org/10.1038/nm1135.

[19] Zhang Y, Gu Y, Ren H, Wang S, Zhong H, Zhao X, et al. Gut microbiome-related effects of berberine and probiotics on type 2 diabetes (the PREMOTE study). Nat Commun 2020;11(1). doi: https://doi.org/10.1038/s41467-020-18414-8.

[20] Brusq J-M, Ancellin N, Grondin P, Guillard R, Martin S, Saintillan Y, et al. Inhibition of lipid synthesis through activation of AMP kinase: An additional mechanism for the hypolipidemic effects of berberine. J Lipid Res 2006;47 (6):1281–8. doi: https://doi.org/10.1194/jlr.M600020-JLR200.

[21] Zhang Z, Zhang H, Li Bo, Meng X, Wang J, Zhang Y, et al. Berberine activates thermogenesis in white and brown adipose tissue. Nat Commun 2014;5(1). doi: https://doi.org/10.1038/ncomms6493.

[22] Kong W-J, Zhang H, Song D-Q, Xue R, Zhao W, Wei J, et al. Berberine reduces insulin resistance through protein kinase C-dependent up-regulation of insulin receptor expression. Metab Clin Exp 2009;58(1):109–19. doi: https://doi.org/10.1016/j.metabol.2008.08.013.

[23] Zhang H, Wei J, Xue R, Wu J-D, Zhao W, Wang Z-Z, et al. Berberine lowers blood glucose in type 2 diabetes mellitus patients through increasing insulin receptor expression. Metab Clin Exp 2010;59(2):285–92. doi: https://doi.org/10.1016/j.metabol.2009.07.029.

[24] Tan X-S, Ma J-Y, Feng Ru, Ma C, Chen W-J, Sun Y-P, et al. Tissue distribution of berberine and its metabolites after oral administration in rats. PLoS ONE 2013;8(10):e77969. doi: https://doi.org/10.1371/journal.pone.0077969.

[25] Wang Y, Tong Q, Shou J-W, Zhao Z-X, Li X-Y, Zhang X-F, et al. Gut microbiota-mediated personalized treatment of hyperlipidemia using Berberine. Theranostics 2017;7(9):2443–51.

[26] Imenshahidi M, Hosseinzadeh H. Berberis vulgaris and Berberine: an update review. Phytother Res 2016;30(11):1745–64. doi: https://doi.org/10.1002/ptr.v30.1110.1002/ptr.5693.

[27] Wu C, Zhang X, Zhang X, Luan H, Sun G, Sun X, et al. The caffeoylquinic acid-rich Pandanus tectorius fruit extract increases insulin sensitivity and regulates hepatic glucose and lipid metabolism in diabetic db/db mice. J Nutr Biochem 2014;25(4):412–9. doi: https://doi.org/10.1016/j.jnutbio.2013.12.002.

[28] Kleiner DE, Bedossa P. Liver histology and clinical trials for nonalcoholic steatohepatitis-perspectives from 2 pathologists. Gastroenterology 2015;149 (6):1305–8. doi: https://doi.org/10.1053/j.gastro.2015.09.015.

[29] Tong X, Xu J, Lian F, Yu X, Zhao Y, Xu L, et al. Structural alteration of gut microbiota during the amelioration of human type 2 diabetes with hyperlipidemia by metformin and a traditional chinese herbal formula: A multicenter, randomized, open label clinical trial. MBio 2018;9. https://doi.org/10.1128/mBio.02392-17.

[30] Kong W-J, Vernieri C, Foiani M, Jiang J-D. Berberine in the treatment of metabolism-related chronic diseases: A drug cloud (dCloud) effect to target multifactorial disorders. Pharmacol Ther 2020;209:107496. doi: https://doi.org/10.1016/j.pharmthera.2020.107496.

[31] Xu X, Yi H, Wu J, Kuang T, Zhang J, Li Q, et al. Therapeutic effect of berberine on metabolic diseases: Both pharmacological data and clinical evidence. Biomedicine and Pharmacotherapy 2021;133. https://doi.org/10.1016/j.biopha.2020.110984.

[32] Ilyas Z, Perna S, Al-thawadi S, Alalwan TA, Riva A, Petrangolini G, et al. The effect of Berberine on weight loss in order to prevent obesity: A systematic review. Biomedicine and Pharmacotherapy 2020;127. https://doi.org/10.1016/j.biopha.2020.110137.

[33] Zhang X, Zhao Y, Xu J, Xue Z, Zhang M, Pang X, et al. Modulation of gut microbiota by berberine and metformin during the treatment of high-fat diet-induced obesity in rats. Sci Rep 2015;5(1). doi: https://doi.org/10.1038/srep14405.

[34] Tian Y, Cai J, Gui W, Nichols RG, Koo I, Zhang J, et al. Berberine directly affects the gut microbiota to promote intestinal farnesoid X receptor activation. Drug Metab Dispos 2019;47(2):86–93. doi: https://doi.org/10.1124/dmd.118.083691.

[35] de la Cuesta-Zuluaga J, Mueller NT, Corrales-Agudelo V, Velásquez-Mejía EP, Carmona JA, Abad JM, et al. Metformin is associated with higher relative abundance of mucin-degrading akkermansia muciniphila and several short-chain fatty acid-producing microbiota in the gut. Diabetes Care 2017;40(1):54–62. doi: https://doi.org/10.2337/dc16-1324.

[36] Zhang X, Zhao Y, Zhang M, Pang X, Xu J, Kang C, et al. Structural changes of gut microbiota during berberine-mediated prevention of obesity and insulin resistance in high-fat diet-fed rats. PLoS ONE 2012;7. https://doi.org/10.1371/journal.pone.0042529.

[37] Roopchand DE, Carmody RN, Kuhn P, Moskal K, Rojas-Silva P, Turnbaugh PJ, et al. Dietary polyphenols promote growth of the gut bacterium akkermansia muciniphila and attenuate high-fat diet-induced metabolic syndrome. Diabetes 2015;64(8):2847–58. doi: https://doi.org/10.2337/db14-1916.

[38] Zhu L, Zhang D, Zhu H, Zhu J, Weng S, Dong L, et al. Berberine treatment increases Akkermansia in the gut and improves high-fat diet-induced atherosclerosis in Apoe−/− mice. Atherosclerosis 2018;268:117–26. doi: https://doi.org/10.1016/j.atherosclerosis.2017.11.023.

[39] Wang Y, Shou J-W, Li X-Y, Zhao Z-X, Fu J, He C-Y, et al. Berberine-induced bioactive metabolites of the gut microbiota improve energy metabolism. Metab Clin Exp 2017;70:72–84. doi: https://doi.org/10.1016/j.metabol.2017.02.003.

[40] Sun R, Yang Na, Kong Bo, Cao B, Feng D, Yu X, et al. Orally administered berberine modulates hepatic lipid metabolism by altering microbial bile acid metabolism and the intestinal FXR signaling pathway. Mol Pharmacol 2017;91(2):110–22. doi: https://doi.org/10.1124/mol.116.106617.

[41] Ozato N, Saito S, Yamaguchi T, Katashima M, Tokuda I, Sawada K, et al. Blautia genus associated with visceral fat accumulation in adults 20–76 years of age. npj Biofilms Microbiomes 2019;5(1). doi: https://doi.org/10.1038/s41522-019-0101-x.

[42] Feng X, Sureda A, Jafari S, Memariani Z, Tewari D, Annunziata G, et al. Berberine in cardiovascular and metabolic diseases: From mechanisms to therapeutics. Theranostics 2019;9(7):1923–51. doi: https://doi.org/10.7150/thno.30787.

[43] Joyner MJ, Paneth N. Promises, promises, and precision medicine. Journal of Clinical Investigation 2019;129:946–8. doi: https://doi.org/10.1172/JCI126119.