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Lacto-Fermented and Unfermented Soybean Differently Modulate Serum Lipids, Blood Pressure and Gut Microbiota during Hypertension

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Abstract: Soy consumption may reduce hypertension but the impact of food processing on the antihypertensive effect is unclear. Hence, we ascertained the effects of lacto-fermented (FSB) and unfermented soybean (USB) consumption on serum atherogenic lipids, hypertension and gut microbiota of spontaneous hypertensive rats (SHR). FSB displayed a strong in vitro angiotensin converting enzyme (ACE) inhibitory ability of 70 ± 5% while USB inhibited 5 ± 3% of the enzyme activity. Consumption of USB reduced serum ACE activity by 19.8 ± 12.85 U while FSB reduced the enzyme activity by 47.6 ± 11.35 U, respectively. FSB significantly improved cholesterol levels and reduced systolic and diastolic blood pressures by 14 ± 3 mmHg and 10 ± 3 mmHg, respectively, while USB only had a marginal impact on blood pressure. Analysis of FSB showed the abundance of ACE inhibitory peptides EGEQPRPFPFP and AIPVNKP (which were absent in USB) and 30 phenolic compounds (only 12 were abundant in USB). Feeding SHR with FSB promoted the growth of *Akkermansia*, *Bacteroides*, *Intestinimonas*, *Phocaeicola*, *Lactobacillus* and *Prevotella* (short chain fatty acid producers) while USB promoted only *Prevotellamassilia*, *Prevotella* and *Intestimonas* levels signifying the prebiotic ability of FSB. Our results show that, relative to USB, FSB are richer in bioactive compounds that reduce hypertension by inhibiting ACE, improving cholesterol levels and mitigating gut dysbiosis.

Keywords: high blood pressure; fermented foods; lactic acid bacteria; metagenomics

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1. Introduction

The renin-angiotensin system (RAS) regulates blood pressure and a dysregulation of the system results in hypertension [1]. This has made the RAS a good target for modulating high blood pressure. The RAS is mainly regulated by two enzymes, namely, renin and angiotensin 1-converting enzyme (ACE). ACE hydrolyses a dipeptide from angiotensin I to yield angiotensin II (Ang II) which is a vasoconstrictor. Binding of Ang II to angiotensin type-1 receptors causes vasoconstriction and increases blood pressure [2]. Although other factors including serum atherogenic lipids can also contribute to the onset of hypertension [3], the link between dysbiosis, RAS and high blood pressure has attracted scientific attention as recent studies have shown a possible link between the RAS and the gut microbiota [4]. The gut microbiota is involved in the development and maintenance of innate [5] and adaptive immune functions [6] and hence severe perturbation of the balanced gut microbiota triggers mucosal immune response that can result in gut-derived diseases such as metabolic syndrome [7]. Indeed, hypertensive patients have gut dysbiosis [8,9] with significantly altered gut microbial metabolism [10]. During hypertension-associated dysbiosis, there is an over population of members of the phylum *Firmicutes* [11] and *Proteobacteria* [12], which are enriched with *cutC*, *CntA* and *GrdH* genes that encode enzymes responsible for trimethylamine-*N*-oxide (TMAO) production [13]. The high production of TMAO promotes

Ang II-induced vasoconstriction [14], which results in elevated blood pressure. Modulation of the gut microbiota by functional foods, however, promote the growth of bacteria that produce short chain fatty acids [15], which can bind to Olfr41 and Olfr78 in smooth muscles and renal juxtaglomerular apparatus, respectively, to regulate blood pressure [16]. Though recent studies have shown that soy intake generally reduces blood pressure in adults [17], different processing methods may yield different bioactive compounds in the final food product and influence their extents of bioactivity [18,19]. The components of the diet/food product may also result in different gut microbial and physiological effects on the host after consumption [20]. Studies have shown that fermented yoghurt [21] and fermented soy protein [11] effectively reduce blood pressure by reducing serum ACE activity and promoting the growth of gut bacteria capable of short chain fatty acid production. Fermenting soybean releases bioactive compounds such as ACE inhibitory peptides [2] and soy isoflavones [22], which directly modulate the RAS to reduce blood pressure. Meanwhile, the bioactive compounds in cooked unfermented soybean may differ from those in fermented soybean and could have different impacts on the RAS, blood pressure, and the gut microbiota. For this reason, in this study we investigated the effects of fermented and unfermented soybean consumption on hypertension, serum lipids, and the gut microbiota in spontaneous hypertensive rats (SHR). We then analyzed the soybean samples to identify which compounds in the products may be accounting for the antihypertensive effects.

2. Materials and Methods

2.1. Chemicals and Cultures

Unless specified, all the chemicals and media used in this study were ordered from Sigma-Aldrich (Seoul, Republic of Korea). Fluorometric ACE Assay Kit, colorimetric triglyceride quantification kit, colorimetric HDL and LDL quantification kits were purchased from Biovision (Milpitas, CA 95035, USA). Soybean (Glycine max) powder was received from Erom Company (Chuncheon, Ganwon-do, Republic of Korea) and stored at 4 °C till use. *Pediococcus acidilactici* KCTC 21159 (formerly referred to as *Pediococcus acidilactici* SDL 1402) was obtained from the Department of Food Science and Biotechnology, Kangwon National University, Republic of Korea. This bacterium was chosen for fermentation because it showed good probiotic potentials in our previous studies [23]. The bacteria stock culture was kept at −80 °C in de Man, Rogosa and Sharpe broth (MRS), containing 20% (*v*/*v*) glycerol. The culture was streaked on MRS agar and cultured at 37 °C overnight. A single colony was cultured in MRS broth at 37 °C for 24 h and the viable cell enumeration was carried out by plate count on MRS agar. Two Erlenmeyer flasks containing soybean powder dissolved in distilled water (10% *w*/*v*) were prepared and autoclaved at 121 °C for 15 min. The content of one flask (labelled as unfermented soybean (USB)) was kept at −20 °C while the content of the other flask was used as the basal growth medium for *Pediococcus acidilactici* KCTC 21159 fermentation.

2.2. Soybean Fermentation

Soybean sample was fermented as we have reported elsewhere [2]. Briefly, Erlenmeyer flask (1 L) containing 500 mL of soybean powder dissolved in distilled water (10% *w*/*v*, pH 6.4), autoclaved at 121 °C for 15 min were inoculated with *Pediococcus acidilactici* KCTC 21159 (9 Log CFU) from the starter culture and incubated at 37 °C with 200 rpm of agitation for 48 h. The sample was immediately frozen at −20 °C to prevent further fermentation. Both fermented and unfermented samples were freeze-dried using a TFD5505 tabletop freeze dryer (ilshinBioBase Co. Ltd., Dongducheon, Gyeonggi-do, Republic of Korea). The freeze-dried samples were stored at −4 °C till use.

2.3. In Vitro ACE Inhibitory Effects of Fermented Soybean (FSB) and Unfermented Soybean (USB) Samples

The in vitro ACE inhibitory ability of the sample were tested as already reported elsewhere [19]. Briefly, 5 mg/mL of the FSB and USB were prepared in distilled water

and their pH adjusted to 7 using 1 M NaOH. ACE solution (1 U, 10 µL) was diluted with 40 µL of ACE assay buffer in a 96 well plate and mixed with 20 µL of FSB. Captopril (20 µL, 5 mg/mL) was used as a positive control. ACE substrate (50 µL) was mixed with each well and fluorescence was measured in kinetic mode at Ex/Em = 330/430 nm at 37 °C for 2 h.

ACE activity was calculated as:

$$\text{ACE activity} = B \times D/(\Delta T \times P) = \text{pmol/minute/mg} \quad (1)$$

where

B = Abz in sample based on standard curve slope (pmol).
ΔT = reaction time (minutes),
P = sample used into the reaction well (in mg),
D = sample dilution factor

Percentage ACE inhibition was calculated as $[(B - A)/B] \times 100\%$ where A represents the ACE activity in the presence of ACE and ACE inhibitor while B is the ACE activity without an ACE inhibitor.

2.4. Animal Handling and Preparation

All the animal experiments were carried out in accordance with the ethical procedures and guidance of the Institutional Animal Care and Use Committee, Kangwon National University (approval no. KW-151127-1). In all, 25 male Spontaneous hypertensive rats (12 weeks old, mean arterial pressure: 160 ± 10 mmHg) and 5 male Wistar Kyoto rats (12 weeks old, mean arterial pressure: 108 ± 2 mmHg) weighing 270–300 g were obtained from Charles River Laboratories (Barcelona, Spain) and used for the experiments. Wistar Kyoto rat (WKYR) was used as the normotensive control group because it is derived from the same ancestral outbred Wistar rat as the SHR. Furthermore, WKYR has been used as the closest genetic control for SHR in many studies [24]. SHRs were randomly separated into 5 groups each containing 5 rats. The rats were kept in temperature-controlled rooms of 25 °C with 12-h light/dark cycles. The animals were supplied with tap water and standard 5001 rodent laboratory chow (Purina, Saint Hubert, Quebec City, QC, Canada) ad libitum. To make the pulsations of the tail artery detectable, the rats were kept at 37 °C for 15 min and blood pressure of restrained rats were measured by the non-invasive tail-cuff method using computer assisted non-invasive blood pressure equipment (NIBP 76-0173 unit with LE5160R cuff and transducer, Sang Chung Commercial Co., Ltd., Kangnam-Ku, Republic of Korea).

2.5. Antihypertensive Effects of FSB and USB

To assess the blood pressure lowering effect of soybean samples, each SHR group ($n = 5$) was either orally administered with 200 mg of USB per kg body weight (SHR + 200 mg USB) or 200 mg of fermented soybean per kg body weight (SHR + 200 mg FSB). To ascertain if the blood pressure lowering effect of the fermented sample was dose dependant, another group of SHR ($n = 5$) was administered with 100 mg/kg body weight (SHR + 100 mg FSB). The remaining SHR groups ($n = 5$) received either 50 mg/kg body weight Captopril (SHR + 50 mg Captopril), a standard antihypertensive drug or 750 µL distilled water (negative control) once a day for 4 weeks by gastric intubation. The dose of captopril was chosen based on the recommended dose for hypertension treatment in human adults. The positive control consisted of 5 Wistar Kyoto rats (WKYR) that were given 750 µL distilled water once a day for 4 weeks by gastric intubation. Systolic blood pressure (SBP) and diastolic blood pressure (DBP) were measured prior to sample administration (time 0) and once a week for 4 weeks. Each SBP and DBP measurement was obtained as the average of five measurements without disturbance of the signal. After 4 weeks of treatment, the rats were starved for 12 h and anesthetized using pentobarbital intraperitoneal dose of 60 mg/kg body weight. Blood was drawn by cardiac puncture (4 mL per rat) using a 5 mL 23 G syringe. Serum was separated from the blood and kept at −80 °C for further

studies. Fecal samples were collected from the colon of the rats and stored at −80 °C for further studies.

2.6. Effects of FSB and USB Consumption on Serum ACE and Atherogenic Lipids

Briefly, 100 µL of serum was diluted with 40 µL of ACE assay buffer in a 96 well plate and mixed with 20 µL of FSB. The reaction was started by adding 50 µL of ACE substrate and fluorescence was measured in kinetic mode at Ex/Em = 330/430 nm at 37 °C for 2 h. The enzyme activity was determined using Equation (1) above.

Since hypertension and hypercholesterolemia are positively correlated [3], we evaluated the effects of USB and FSB consumption on serum high density lipoprotein cholesterol (HDL-C) and low density lipoprotein cholesterol (LDL-C) after the experimental period using BioVision's HDL-C and LDL-C/VLDL-C Cholesterol Quantification Kit. Serum triglyceride was measured using a BioVision's Triglyceride Quantification Kit according to the manufacturer's instructions. Cholesterol and triglycerides were analyzed for five animals per group.

2.7. Chromatographic Analysis of USB and FSB for Antihypertensive Compounds

Samples of FSB and USB (0.5 g) were extracted with methanol (50%, 20 mL) and mixed on a mini rocker shaker (Clinical Diagnostics, Seoul, Republic of Korea) for 12 h. The sample was centrifuged at 14,000× *g* for 10 min at 4 °C and 1 mL of the supernatant was filtered using a 0.25 µm pore size filter (Merck KGaA, Darmstadt, Germany) into 2 mL clear glass vials. Ultra-high performance liquid chromatography-quadrupole time-of-flight mass spectrometry (UHPLC Q-TOF MS/MS) technique was used for identifying compounds in FSB and USB that may have contributed to the antihypertensive effect as we reported elsewhere [19]. Briefly, solvent A consisted of distilled water containing 0.1% formic acid, solvent B was methanol and a flow rate of 0.4 mL/min was applied. Accucore C18 column of dimensions 100 × 3 mm (Thermo Fisher Scientific, Waltham, MA, USA) was used and the sample volume injected was 5 µL. The Q-TOF-MS was set for the negative mode with m/z values ranging from 100 to 1000. Peptides in the samples were analyzed automatically by Analyst QS 2.0 software (Applied Biosystems, Waltham, MA, USA). Peptides of interest were ordered at >99% purity from AnyGen Company Limited (Jangseong County, Republic of Korea).

2.8. Effects of FSB and USB on the Gut Microbiota (Next Generation Sequencing)

Next generation sequencing was carried out by Macrogen Inc (Geuncheon-gu, Seoul, Republic of Korea). Briefly, DNA that qualified quality control measures were used for library construction. The DNA or cDNA samples were randomly fragmented and ligated by a 5′ and 3′ adapter before being used for the library construction. The fragments were amplified by polymerase chain reaction followed by purification by gel electrophoresis. After the fragments were captured on a lawn of surface-bound oligos complementary to the library adapters, they were amplified into unique clonal clusters through bridge amplification. Illumina SBS technology was used to generate base-by-base sequencing. The base calls binary was converted into FASTQ utilizing the illumina package bcl2fastq.

2.9. Data and Statistical Analyses

The data obtained was processed using SCIEX OS 1.0 software (AB Sciex LLC, Framingham, MA, USA). Compound identification was carried out already reported elsewhere [25]. Briefly, compounds were identified by searching MS/MS library, online databases and finding empirical formulas.

All ACE activity and inhibitory tests were carried out in triplicates and the results were expressed as mean ± standard deviation. The baseline blood pressure of the experimental animals was considered as the mean of the values measured in the first run-in period. Blood pressure values were presented as mean value ± standard error of the mean for each animal group. The outcomes of measurements from each time period of a group were compared

with one-way ANOVA followed by the Tukey test. Differences in measurements were considered significant when $p < 0.05$. All statistical analyses were carried out using OriginPro 2022b version 5.01 (© OriginLab Corporation, Northampton, MA 01060, USA). Heat maps were drawn from the identified metabolites and their peak areas using GraphPad Prism 8.4.3 (San Diego, CA 92108, USA). Peak areas were used because it is widely accepted that the peak areas of analytes are directly proportional to their concentrations [26]. All the effective reads from stool samples were clustered into operational taxonomic units (OTUs) based on 97% sequence similarity. PCA was performed using OriginPro 2022b to visualize the changes in gut microbial communities in stool samples. R software version 4.1.3 (ggpubr package) was used to calculate the Chao1, inverse Simpson's and Shannon's alpha diversity in the stool samples from each group based on the relative abundance of each genus. Volcano plot for differential abundance of genera was tested by paired t-test using GraphPad Prism and p values < 0.05 were regarded as significantly different.

3. Results

3.1. In Vitro ACE Inhibitory Activity of FSB and USB

ACE-1 inhibitory ability of FSB and USB were tested using captopril (a standard ACE-1 inhibitor) as a positive control. FSB significantly reduced ACE-1 activity relative to USB (70 ± 5% versus 5 ± 3%). Captopril, however, exhibited the highest ACE inhibitory ability of 94 ± 4% (Figure 1A).

3.2. Impact of FSB and USB Consumption on High Blood Pressure and Atherogenic Lipids

All the treated rats experienced a progressive reduction in their blood pressure throughout the experimental period in a dose dependent manner relative to the SHR group. The SHR + 50 mg captopril group showed the highest blood pressure reduction on the 4th week (SBP = 20 ± 5 mmHg and DBP = 15 ± 5 mmHg) followed by SHR + 200 mg FSB (SBP = 14 ± 3 mmHg and DBP = 10 ± 3 mmHg). The blood pressure of SHR + 100 mg FSB was reduced by SBP = 8 ± 2 mmHg and DBP = 7 ± 3 mmHg. Although SHR + 200 mg USB did not decrease systolic blood pressure, it significantly suppressed an overall increase in blood pressure (Figure 1B,C).

Analysis of serum ACE activity showed that consumption of 200 mg USB caused the least enzyme inhibition of 19.8 ± 12.85 U while 100 mg FSB and 200 mg FSB reduced the enzyme activity by 31.4 ±7 U and 47.6 ± 11.35 U, respectively, (Figure 1D). After the 4th week of feeding, both USB and FSB significantly increased the levels of HDL-C in SHR compared to untreated SHR (Figure 1E). Meanwhile, 200 mg USB triggered the highest serum HDL-C levels of 0.167 ± 0.012 nM while the levels triggered by 100 mg FSB and 200 mg USB were similar ($p = 0.165$). FSB also decreased LDL-C better ($p < 0.001$) than USB, irrespective of the doses used (Figure 1E). However, both FSB and USB reduced serum triglycerides to similar degrees in SHR (Figure 1G).

3.3. Antihypertensive Compounds in USB and FSB

To identify the bioactive compounds that may account for the hypotensive ability of the samples, we analyzed both USB and FSB using UHPLC Q-TOF MS/MS. Since foods rich in dietary phenolic acids have antihypertensive potentials [27] and soybean fermentation can generate antihypertensive peptides [19], we specifically analyzed the phenolic compounds and the potential antihypertensive peptides present in the samples. Out of a total of 42 phenolic and antioxidant compounds identified (Figure 2), 12 of them, namely, dodecyl gallate, vanillic acid 4-β-D-glucoside, ferrulic acid, apigetrin, glycitein, glucosyringic acid, prunetin, blochanin A and apigenin were abundant in USB but were significantly less in FSB. The other 30 phenolic and antioxidant compounds were more abundant in FSB. As fermented soybean contains thousands of metabolites that are too many and difficult to extract for further studies, we focused on the peptides and antioxidant compounds that were present in the samples. After UHPLC/Q-TOF-MS analysis of the low molecular weight peptides (<3 kDa) in FSB, 2946 peptides were identified. Only 32 low molecular weight peptides

(LMWP) (<3 kDa) were identified in USB while 2946 LMWP were present in FSB. Since only FSB inhibited ACE activity in vivo and in vitro, its LMWP peptides were investigated. Meanwhile, as it is impractical to synthesize and test all the detected peptides for their ACE inhibitory abilities, the peptide sequences were screened using an in silico antihypertensive peptide inhibitor predictor platform (https://webs.iiitd.edu.in/raghava/ahtpin/, accessed on 21 December 2022) developed by Kumar et al. [28]. Among the numerous potential antihypertensive peptides predicted, NNPFSFLVPPQESQRR, AIPVNKP, PPNPHIGIN, EITPEKNPQ, PFSFLVPPQESQRR, FEITPEKNPQ, EITPEKNPQLR and EGEQPRPFPFP were most abundant and so they were synthesized and their ACE inhibitory abilities tested (Figure 2C). EGEQPRPFPFP showed the strongest ACE inhibitory ability of 90.4 ± 5%, followed by AIPVNKP (70.4 ± 5%), EITPEKNPQLR (24.4 ± 6%), PFSFLVPPQESQRR (15.8 ± 7%), EITPEKNPQ (12.0 ± 5%), FEITPEKNPQ (11.5 ± 5%), NNPFSFLVPPQESQRR (10.6 ± 4%) and PPNPHIGIN (0%). None of the eight peptides, however, were present in USB. The inhibitory activity of Captopril was similar ($p > 0.05$) to EGEQPRPFPFP.

3.4. Impact of FSB and USB Consumption on Gut Microbiota

Bacteria DNA were isolated from both WKYR and SHR stools and the bacterial loads were represented as observed taxonomic units (OTU) (Figure 3). The gut microbial ecology was assessed by three parameters; Chao1 index (species richness based on abundance), Shannon index (microbial diversity) and inverse Simpson's index (evenness). Untreated SHR demonstrated the least gut microbial diversity and evenness relative to all the other groups. Although USB and FSB altered gut microbial species richness to similar extents ($p > 0.05$) (Figure 3B), principal component analysis revealed that both FSB and USB consumptions had distinct impacts on SHR gut microbiota (Figure 4). Analysis of the gut microbiota of SHR and WKYR demonstrated that the low bacteria diversity and low evenness were due to a high *Firmicutes/Bacteriodes* ratio coupled with an overrepresentation of *Actinobacteria* and diminished *Verrucomicrobia* phyla (Figure 4D).

Meanwhile, consumption of FSB and USB improved microbial diversity and evenness by boosting the growth of *Verrucomicrobia* and *Tenericutes* (which were initially undetected) as well as *Bacteriodetes* (from 29.5% to over 40%) while reducing the levels of overrepresented groups (Figure 4B–G). Relative to USB, FSB significantly boosted the populations of *Verrucomicrobia* and reduced *Actinobacteria* in the gut. At the genus level, 14 genera significantly abundant in WKYR were diminished in SHR (Figure 5). Interestingly, feeding SHR with 100 mg/kg FSB significantly boosted the levels of 7 (50%) of those genera namely *Akkermansia*, *Bacteroides*, *Intestinimonas*, *Phocaeicola*, *Kineothrix*, *Prevotella* and *Longibaculum*, while 200 mg/kg FSB boosted the levels of 8 (almost 60%) genera (*Akkermansia*, *Phcaeicola*, *Intestinimonas*, *Bacteroides*, *Prevotella*, *Longibaculum*, *Lactobacillus* and *Maihella*). Feeding SHR with 200 mg/kg USB, however, only increased ($p < 0.05$) the populations of three (21%) genera (*Prevotellamassilia*, *Prevotella* and *Intestimonas*) while Captopril increased the levels of four genera (*Prevotellamassilia*, *Phocaeicola*, *Intestimonas* and *Prevotella*) that were associated with WKYR.

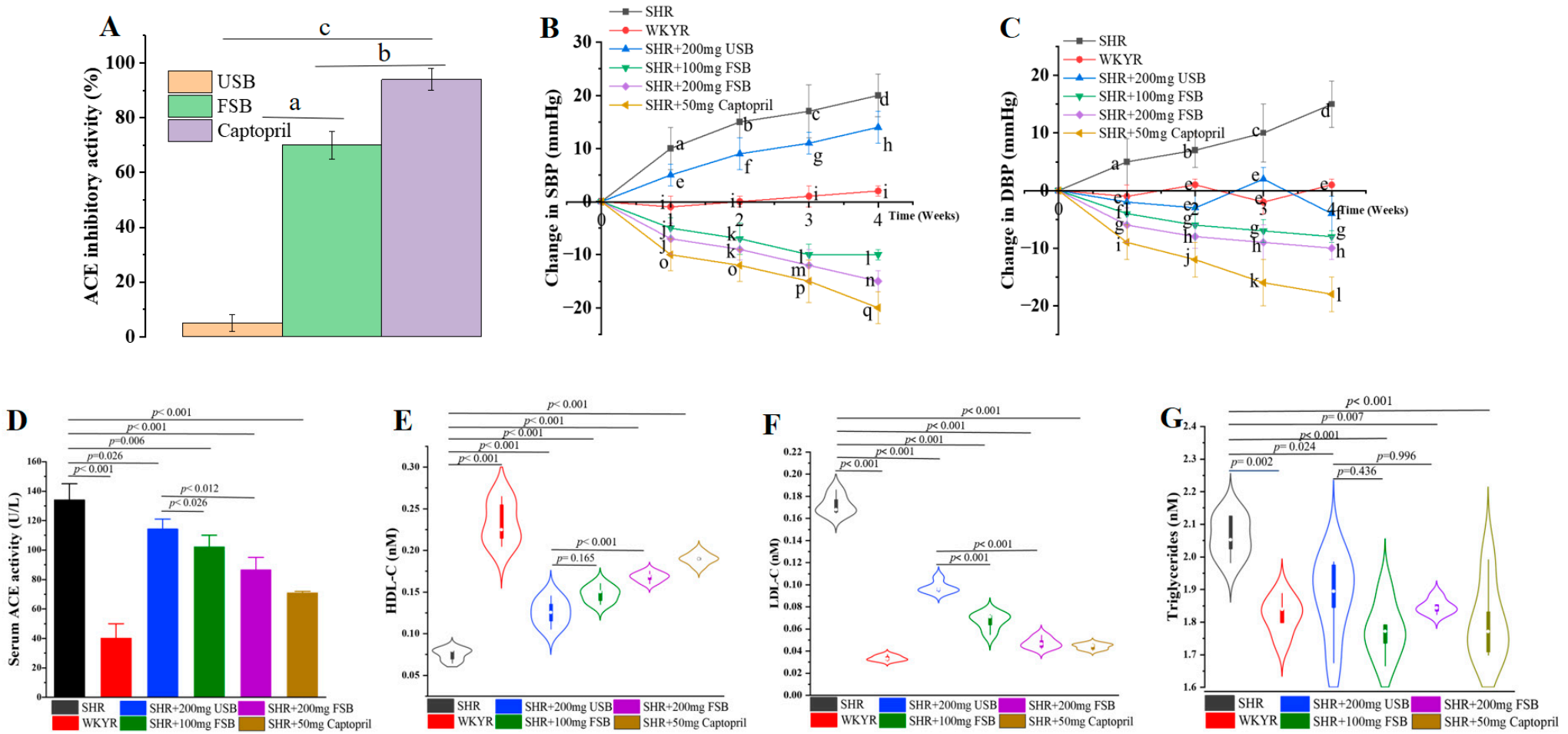

Figure 1. Effects of FSB and USB on ACE, blood pressure, serum ACE activity and serum atherogenic lipids in SHR. (**A**). ACE-1 inhibitory ability of FSB and USB compared to Captopril. All bars represent means of three readings ($n = 3$) ± S.D. Bars with different alphabets were significantly different ($p < 0.05$) according to paired *t*-tests. (**B**,**C**). Effects of FSB and USB consumption on systolic and diastolic blood pressure of SHR compared to WKYR and untreated SHR. (**D**,**E**,**F**,**G**). Effects of FSB and USB on serum ACE, HDL-C, LDL-C, and triglycerides of SHR compared to WKYR and untreated SHR, respectively.

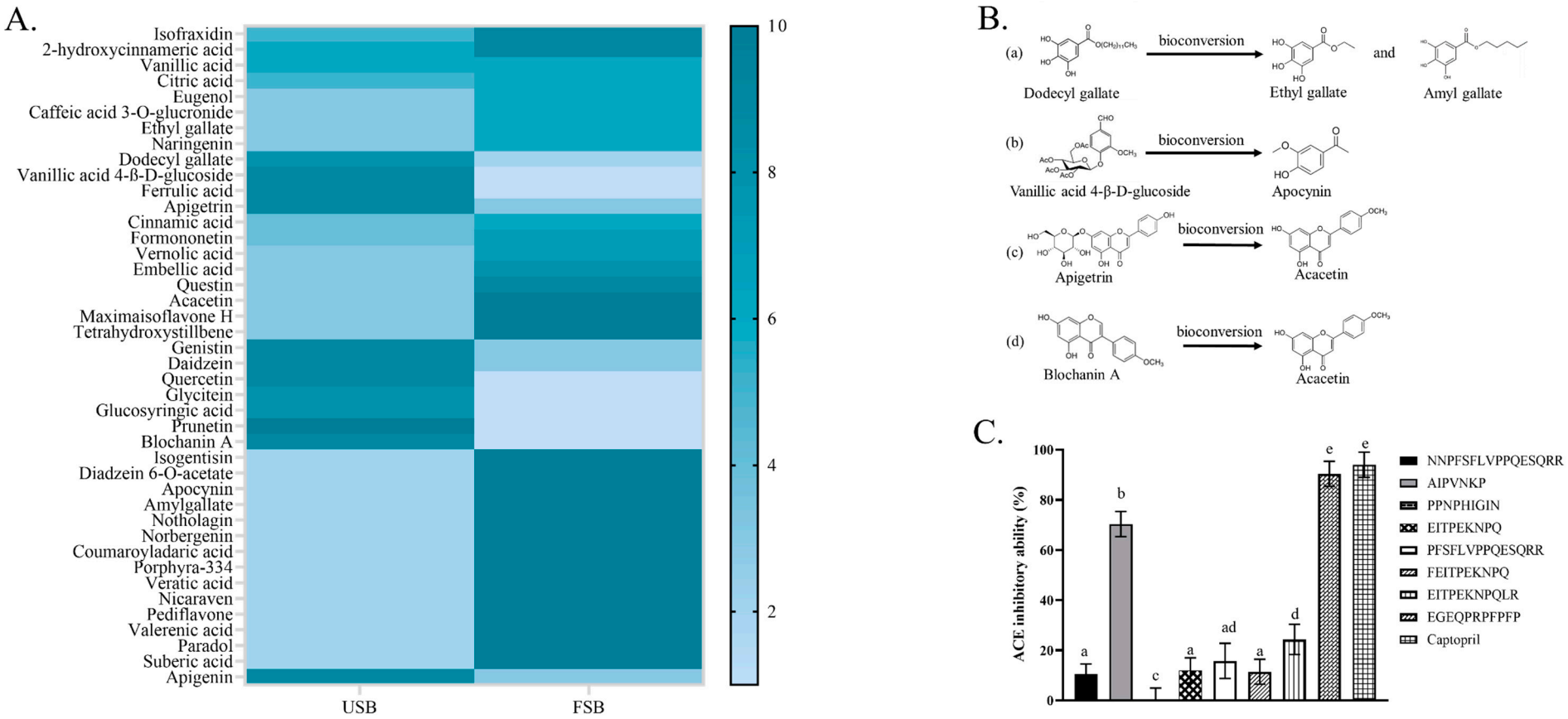

Figure 2. Relative levels of phenolic compounds in USB and FSB and ACE inhibitory abilities of newly identified peptides. (**A**) Heat map shows the different levels of phenolic compounds present in the samples. The color range from green to white represents higher to lower levels of phenolic compounds and the values of 2 to 10 indicate the relative concentrations of the compounds, (**B**) shows bioconversion of some phenolic compounds into new compounds during the fermentation process (**C**) newly identified peptides and their ACE inhibitory abilities. Bars with different alphabets indicate significant differences ($p < 0.05$).

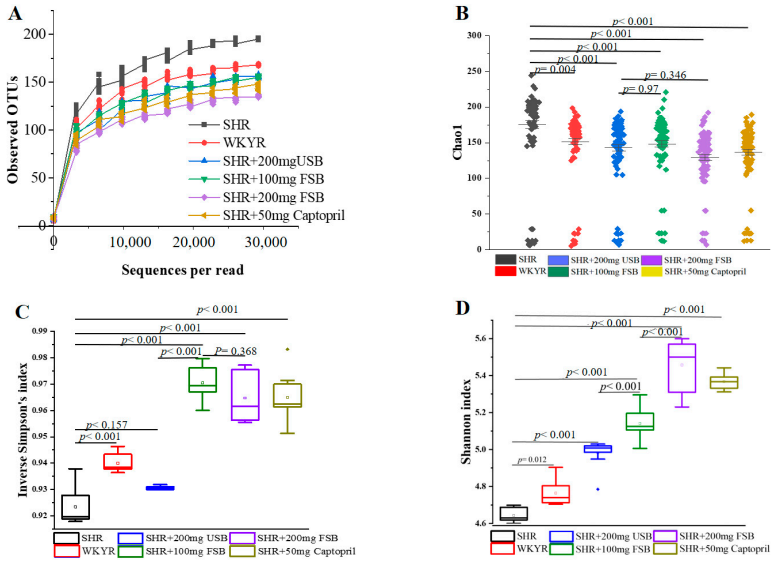

Figure 3. Gut microbial alpha diversity varies with FSB and USB feeding in SHR. (**A**). Rarefaction curve. (**B**). Chao-1 index. (**C**). Inverse Simpson's index. (**D**). Shannon index as observed in SHR after feeding with FSB or USB for 4 weeks compared with WKYR and untreated SHR.

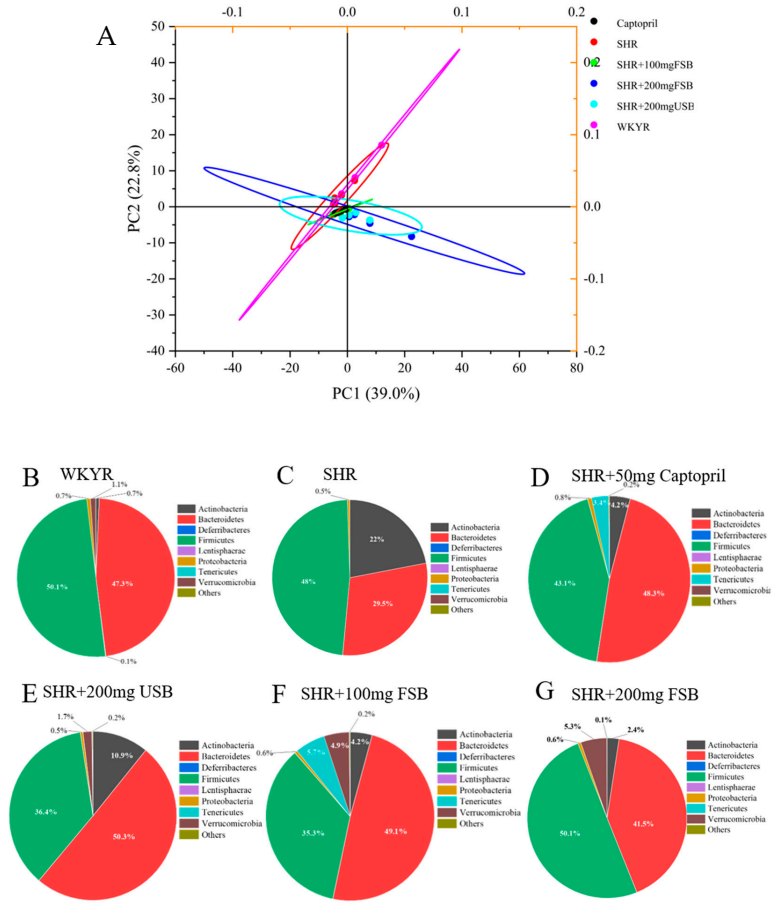

Figure 4. FSB and USB feeding modulates the gut microbiota of SHR at the genus level. (**A**). PCoA analysis of the experimental animals is shown along the first two principal coordinate axes. Different coloured dots indicate the animals in each experimental group. Pie charts show the gut microbial phyla in (**B**). WKYR, (**C**). SHR, (**D**). SHR + 50 mg Captopril, (**E**). SHR + 200 mg USB, (**F**). SHR + 100 mg FSB (**G**). SHR + 200 mg FSB. Different pies represent different genera and their sizes represent their relative abundance in the stools of experimental rats.

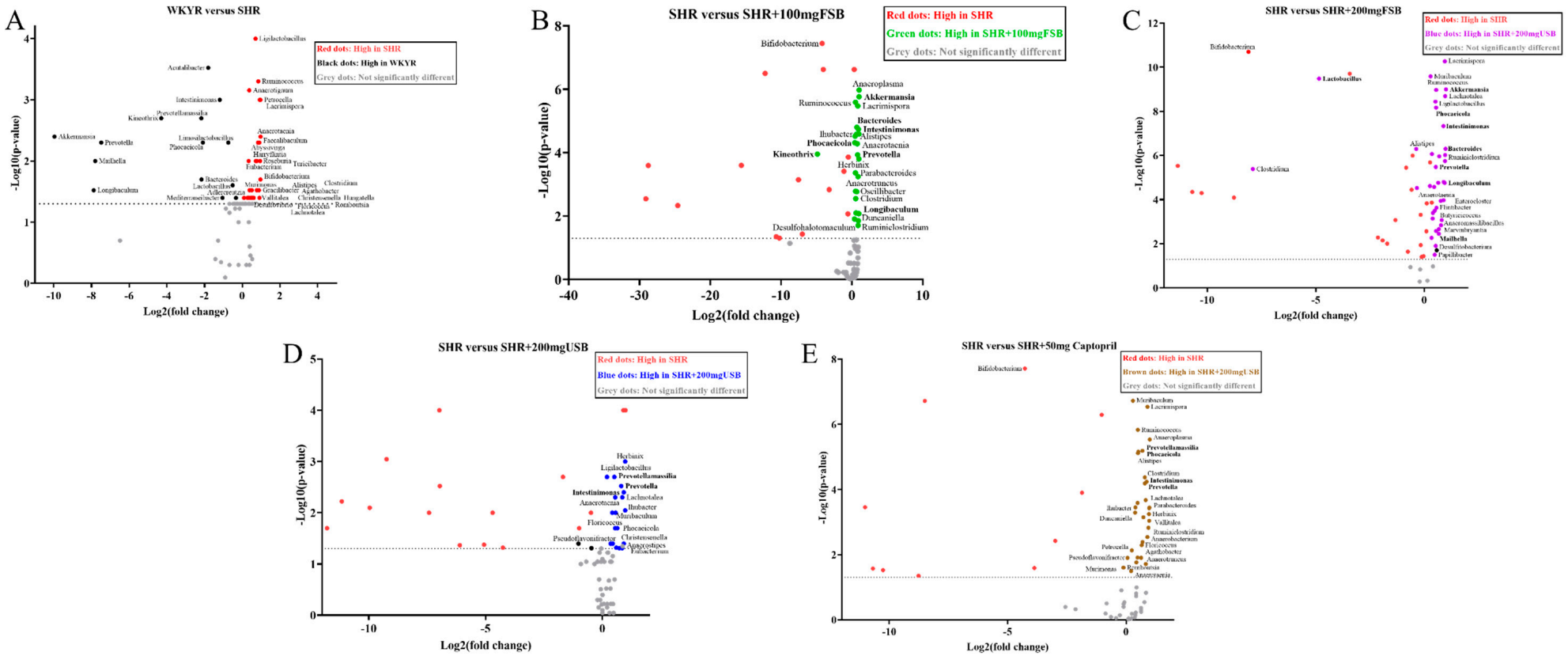

Figure 5. Characteristic microbiota taxonomic profile changes associated with the soybean product consumed: Volcano plots of differential bacterial abundance associated with the (**A**). WKYR and SHR, (**B**). SHR and SHR +100 mg FSB, (**C**). SHR and SHR + 200 mg FSB, (**D**). SHR and SHR + 200 mg USB, (**E**). SHR and SHR + 50 mg Captopril. Fold change associated with a unit change in the corresponding test and Benjamini–Hochberg-adjusted p values are plotted for each taxon. Significantly different taxa are colored according to genus and the names of genera increased after intervention are named. Bolden genera indicate taxons that were significantly abundant in WKYR but became abundant in SHR only after feeding them with USB or FSB. The dotted line shows where $p = 0.05$ with points above the line having $p < 0.05$ and points below the line having $p > 0.05$.

4. Discussion

The ability of USB and FSB to inhibit ACE activity in vitro is an indicator of how the samples may affect the RAS during hypertension. As shown in Figure 1, FSB exhibit superior ability to inhibit ACE relative to USB. Meanwhile, since in vitro outcomes may not necessarily predict in vivo activities and vice versa [29], we proceeded to feed SHR with the soybean samples to investigate their comparative antihypertensive effects. Consumption of FSB significantly reduced blood pressure while USB only suppressed the excessive increase in blood pressure (Figure 1B,C). Compared to our earlier study in which 100 mg/kg of fermented soy proteins decreased systolic blood pressure by 19 mmHg after 4 weeks of feeding [30], 100 mg/kg of FSB demonstrated a lower SBP reduction in this study. The difference could be due to the potency of the different metabolites generated by the different bacteria used for the fermentation process. More so, the nature of the administered fermented soybean products (peptides versus whole bean) may have affected the outcome as has been reported in other studies where the antihypertensive effects of soy peptides [31] and fermented soy sauce [32] were different. Serum assessment showed that though consumption of both samples reduced ACE activity, FSB consumption reduced in vivo ACE activity by up to 47.6 $\pm$ 11.35 U while USB only reduced 19.8 $\pm$ 12.85 U. This indicates a strong corroboration between our in vitro ACE inhibition results and the in vivo outcomes. Similarly, FSB consumption demonstrated a stronger ability to reduce serum LDL cholesterol and triglyceride levels relative to USB after 4 weeks of feeding (Figure 1E–G). It also improved the levels of HDL-C significantly ($p < 0.001$) relative to USB after 4 weeks. This agrees with previous studies that reported that fermented food consumption can improve cholesterol levels [33], reduce serum ACE activities and reduce high blood pressure [11].

Since the components of the diets may be the cause of the antihypertensive effects, we investigated their compositions. UHPLC Q-TOF MS/MS analysis of soybean samples showed that FSB was richer in dietary phenolic and antioxidant compounds than USB probably because the fermentation process released bound phenolic compounds from the soybean matrix. In addition, fermentation caused the conversion of some soybean compounds into new molecules with strong antioxidant potentials and this also contributed to the high antioxidant content in FSB.

For instance, antioxidants such as ethyl gallate and amyl gallate (derived from dodecyl gallate), Apocynin (derived from vanillic acid 4-β-D-glucoside), acacetin (derived from apigetrin and blochanin A) and diadzein 6-o-acetate (derived from diadzein) were only detected after fermentation (Figure 2B) and the levels of their parent compounds were significantly reduced after the fermentation process. Both apocynin and acacetin are known for their vasodilatory abilities and their antihypertensive effects [34,35] and these compounds might have contributed to the strong antihypertensive effect of FSB. It is also possible that the abundance of phenolic compounds in FSB contributed to its stronger ability to reduce LDL-C and increase HDL-C levels in SHR (relative to USB) since foods rich in dietary polyphenols improve cholesterol levels [36]. During fermentation, soybean proteins were digested into peptides by Pediococci proteases [37]. Among the peptides generated, EGEQPRPFPFP showed the strongest ACE inhibitory ability of 90.4 $\pm$ 5%, followed by AIPVNKP (70.4 $\pm$ 5%). Their strong inhibition may be due to the presence of proline at their carboxyl terminals, as studies have shown that the presence of proline at the carboxyl terminal of peptides are important for ACE inhibition [36]. Therefore, the presence of EGEQPRPFPFP and AIPVNKP as well as the abundance of polyphenols in FSB may have contributed to its strong antihypertensive ability. Indeed, peptides with long sequences may be degraded by gastrointestinal enzymes when consumed, yet the process may also lead to the generation of more potent shorter peptides that may easily be absorbed into circulation. It is known that overactivation of ACE can stimulate LDL-C accumulation, which will eventually increase vascular superoxide production and result in hypertension [38]. For this reason, it is likely that FSB reduced high blood pressure by inhibiting ACE activity leading to a reduction in reactive oxygen species and LDL-C levels.

Since SHR have gut dysbiosis [8] and diet can modulate the gut microbiota (Figure 4A) [15], we ascertained the impact of FSB and USB on the gut microbiota of SHR. Fourteen bacteria were significantly abundant in WKYR but were diminished or undetected in SHR (Figure 5A). Feeding SHR with FSB stimulated the growth of 50–60% of gut bacteria associated with WKYR (Figure 5B). Some of the bacteria genera whose populations were boosted namely *Akkermansia*, *Bacteroides*, *Intestinimonas*, *Phocaeicola*, *Lactobacillus*, *Kineothrix* and *Prevotella* are known short chain fatty acid producers [39–42]. USB on the other hand stimulated the growth of only a few short chain fatty acid genera; *Prevotellamassilia*, *Prevotella* and *Intestimonas*. Short chain fatty acids can bind to GPR41 and GPR43 receptors on vascular smooth muscles to induce vasodilation [42] and reduce high blood pressure. In addition, short chain fatty acids can directly activate the afferent vagal nerves in the gut [43], which resets the baroreceptor reflex to control blood pressure [44].

Our data, therefore, indicates that FSB consumption tends to "correct" gut dysbiosis associated with hypertension by modulating the gut microbiota of SHR to become 'similar' to WKYR. Surprisingly, analysis of gut microbiota profiles of the experimental animals revealed that the gut of SHR was enriched with bifidobacteria (normal commensals known for their beneficial effects) and their blood pressures reduced as the levels of this genus decreased after FSB feeding (Figure 5). Our observation is similar to earlier studies that reported that certain beneficial bacteria are overrepresented in the gut of disease animal models [45] and that consumption of functional foods can reduce their populations and mitigate the disease [46]. It is likely that such commensals have different effects on host genes in health and disease [47] and, hence, it is possible that the stronger bifidobacteria-reducing effect of FSB in SHR may be involved in its significant hypotensive effects.

5. Conclusions

In conclusion, we have demonstrated that fermentation significantly improves the antihypertensive and gut modulatory effect of soybean. Specifically, our data suggest that relative to USB, FSB is richer in phenolic compounds and antihypertensive peptides, which improve serum cholesterol and reduce blood pressure, respectively. In addition, FSB more strongly promotes the growth of short chain fatty acid producers, making it a better prebiotic than USB. Meanwhile, further studies are needed to the causality of bifidobacteria overgrowth in the gut and hypertension. In addition, gut microbial metabolites that might have been involved in reducing hypertension should be investigated.

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