

The novel intervention effect of cold green tea beverage on high-fat diet induced obesity in mice

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ABSTRACT

This study was designed to explore the anti-obesity effects of cold-water brewed green tea (CWB-GT) on high-fat diet (HFD)-fed mice. CWB-GT was prepared by brewing tea leaves with 30 °C cold water, which contained considerable active ingredients including catechins, gallic acid and theanine. Our data demonstrated that CWB-GT could significantly ameliorate obesity and modulate gut microbiota composition of HFD-fed mice by affecting some specific bacteria. Besides, the expression of lipogenesis-related genes including FAS, HMGR and SREBP-1c in liver was inhibited and the expression of fatty acid oxidation-related genes including PPAR α and PGC-1 α in adipose tissue was enhanced. These benefits might be attributed to the stimulation of serum peptide YY (PYY) secretion. In particular, specific bacteria modulated by CWB-GT including *Lachnospiraceae bacterium DW67*, *Blautia coccoides*, *Parabacteroides merdae* and *Bacteroides vulgatus* might be key mediators in exerting anti-obesity effects. This study suggested that CWB-GT could represent a novel functional low-calorie beverage.

1. Introduction

The prevalence of obesity has increased dramatically worldwide in recent years. It is also known as a risk factor for various chronic diseases such as metabolic syndromes, type 2 diabetes mellitus, fatty liver disease, cardiovascular disease and certain types of cancers (Akinyemiju et al., 2018). The occurrence of obesity is due to the energy imbalance between calorie intake and calorie expenditure. Thus, two important principles during weight-loss process are controlling food intake and increasing energy expenditure (Blüher, 2019). Regulating the secretion of anorexigenic gut hormones such as glucagon-like peptide 1 (GLP1) and peptide YY (PYY) is emerged as a viable way to control food intake. These peripheral signals may deliver information via vagus nerve to the central nervous system, which is responsible for integrating information to affect feeding behavior (Kim, Seeley, & Sandoval, 2018; Norton, & Murphy, 2017). Besides, increasing energy expenditure or inhibiting lipogenesis are also effective ways to manage obesity (Xie, Su, Sun, Zheng, & Chen, 2018). Nowadays sugar sweetened beverages with high energy density are considered as a source of energy and added sugars in the diet of modern people. Substantial evidence demonstrated the correlation between the consumption of sugar-sweetened beverages and type 2 diabetes (Drouin-Chartier et al., 2019). Therefore, replacing

sugar sweetened beverages with tea or coffee without added sugar is becoming an irresistible tendency (Barraj, Bi, Murphy, Scrafford, & Tran, 2019).

Recently, infusing tea leaves with cold water has become a popular way of making a tea in Taiwan (Lin, Yang, Hsieh, Liu, & Mau, 2014). Besides, Japanese green tea “Sencha” is brewed in water at different temperatures and it was found that theanine and non-gallic catechins, such as epigallocatechin (EGC) were easily extracted in cold water, leading to less bitterness and astringency than hot-water brewed “Sencha” characterized by a higher concentration of gallic catechins, predominantly epigallocatechin gallate (EGCG) and caffeine in it (Monobe, 2018). In particular, it was reported that EGC has an immune-enhancing effect and theanine has a psychosocial stress-reducing effect. These effects of EGC and theanine were inhibited by EGCG and caffeine; therefore, green tea needs to be brewed with cold water to counteract the interference of EGCG and caffeine (Monobe, 2018; Unno et al., 2016). According to the previous studies, cold-water brewed tea has equal or higher antioxidant activity and phenol content when compared with the traditional hot-water brewed tea (Damiani, Bacchetti, Padella, Tiano, & Carloni, 2014; Lin, Liu, & Mau, 2008). Besides, cold water can delay the oxidation of tea polyphenols and protect other nutrients such as vitamin C and carotenoids from being destroyed (Jiang, 2019). Some studies

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have suggested a possible role of gut microbiota in mediating the health benefits of tea consumption (Chen, & Yang, 2019; van Duynhoven et al., 2013). The gut microbiota influences the immunity, metabolism and neurobehavior of body (Valdes, Walter, Segal, & Spector, 2018). Therefore, reshaping gut microbiota in a normal state is a promising strategy for the prevention and treatment of diseases. There have been studies investigating the anti-obesity effects of hot-water brewed green tea (Chen et al., 2009; Liu et al., 2019; Liu et al., 2016). However, whether the cold tea beverage has abilities to regulate metabolic disorders and gut microbiota composition remains unclear. This study was designed to explore the anti-obesity effects of cold-water brewed green tea (CWB-GT) on high-fat diet (HFD)-fed mice and give a clue on the relationship between gut microbiota and beneficial effects of cold tea beverage. Our data suggests that CWB-GT represents an alternative tea beverage that may be useful for ameliorating dyslipidemia and/or obesity.

2. Materials and methods

2.1. Preparation of CWB-GT

Green tea was purchased from Zhejiang huamingyuan Tea Co., Ltd (Zhejiang, China). CWB-GT was prepared by brewing 2.5 g of tea leaves with 100 mL of 30 °C cold water for 40 min. Then a 4-fold dilution of tea soup was obtained for further use. Total phenolic content of CWB-GT was evaluated using the folin-phenol method. The contents of theanine, gallic acid and catechins were determined by high-performance liquid chromatography (HPLC) and the results were shown in Table S1.

2.2. Animal experiments

C57BL/6 mice (6 weeks old) were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd (Beijing, China). All mice were housed in a regular environment (12 h light/dark cycle, lights off at 20:00) with food and water available at any time in the animal facility of Experimental Animal Center of Nankai University. All animal procedures were approved by the Institutional Animal Care and Use Committee of Nankai University and carried out in accordance with the National Institutes of Health guide for the care and use of Laboratory animals (NIH Publications No. 8023). After an adaptation period of 1 week on a control diet, mice were weighed and randomly assigned into three groups (n = 12 per group) as follows: normal chow group (NC group) with low fat diet containing 10% calories from fat (D12450J, Xietong Pharmaceutical Bio-engineering Co., Ltd) as food and purified water as drinking water, HFD group with high-fat diet containing 60% calories from fat (D12492) as food and purified water as drinking water, and GT group with high-fat diet as food and CWB-GT as drinking water.

Four mice were housed in one cage. All drinking fluid was given to mice in sterile plastic bottles, which were replaced every two days with fresh water or cold tea beverage. The food intake and water intake were recorded once in 2 days, and the body weight was monitored weekly. After 8 weeks of intervention, mice were euthanized and serum samples were collected. Liver, epididymal and pararenal adipose tissues were removed and weighed. Fresh colonic content samples were aseptically collected for bacterial DNA extraction.

2.3. Serum biochemical analysis, endotoxemia and cytokine measurements

Serum lipid indexes including triglyceride (TG), total cholesterol (TC), high-density lipoprotein cholesterol (HDL-C), low-density lipoprotein cholesterol (LDL-C) and glucose were measured using commercial kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). The lipopolysaccharides (LPS) concentration was detected by commercial enzyme-linked immunosorbent assay (ELISA) kits (Cusabio Biotech Co., Ltd, Wuhan, China). The levels of tumor necrosis factor- α (TNF-

α) and interleukin-6 (IL-6) were also assessed using ELISA kits (Multi-sciences Biotech Co., Ltd, Hangzhou, China).

2.4. Histological analysis

The sections from liver and epididymal adipose tissue were fixed in 10% formalin, dehydrated, embedded in paraffin and sliced into 5 μ m sections. The tissues were then stained with hematoxylin and eosin (H&E) for light microscopic examinations.

2.5. Oral glucose tolerance test

At week 8, animals were fasted for 7 h and then given glucose (2.0 g/kg b.w.) by intragastric administration. Blood was collected from the tip of tail vein for glucose measurement before (0 min) and after (15, 30, 60, 90 and 120 min) glucose gavage using a glucometer (Roche, Germany).

2.6. Gene quantification using quantitative real-time PCR

TRIzol Reagent (Ambion, USA) was used to extract total RNA from the frozen liver/adipose tissue. cDNA was synthesized using the Luna-Script SuperMix Kit (NEB, Massachusetts, USA). Quantitative real-time polymerase chain reaction (qRT-PCR) was performed on the CFX Connect Real-Time System (BIO-RAD, USA). All primers are listed in Table S2. The expression levels of SREBP-1c, FAS, HMGR, HSL, ATGL, PPAR α , ACOX, CYP7A1 and LXR α in the liver and Leptin, Adiponectin, ACC, PPAR γ 1, HSL, PPAR α , PGC-1 α , ACOX, TNF α , IL-6 and IL-1 β in the epididymal adipose tissue were investigated. β -ACTIN gene was used as normalization. The relative expression level of each gene was computed by the $2^{-\Delta\Delta Ct}$ method.

2.7. Serum hormone analysis

PYY and glucagon-like peptide 1 (GLP-1) were determined using ELISA kits (Cusabio Biotech Co., Ltd, Wuhan, China) based on the instructions.

2.8. Hepatic function analysis

Alanine aminotransferase (ALT) concentration and aspartate aminotransferase (AST) were determined on serum samples following the manufacturer's instructions (Nanjing Jiancheng Bioengineering Institute, Nanjing, China).

2.9. Gut microbiota analysis

Genomic DNA of colonic contents was extracted using the CTAB method. After the purification and qualification, the DNA was applied to amplification of 16S rRNA gene V3-V4 regions using specific primers 341F (5'-CCTAYGGGRBGCASCAG-3') and 806R (5'-GGACTACNNGG-TATCTAAT-3') with the barcode. The sequencing procedures were carried out as described in the previous study (Ma et al., 2019).

2.10. Statistical analysis

Data was presented as means \pm standard errors of the means (SEM). All statistical analyses were performed using the SPSS 22.0 software. The difference between two groups was analyzed by the two-tailed Student's *t* test. The difference among three or more groups was analyzed using one-way ANOVA followed by Student-Newman-Keuls post hoc tests. Two-way repeated measures analysis of variance with Student-Newman-Keuls multiple comparison test was conducted when variable time points within different groups were compared. Results were considered statistically significant when $p < 0.05$.

3. Results

3.1. CWB-GT prevented obesity and dyslipidemia caused by HFD

During the 8-week intervention, no significant difference of water intake was observed ($p > 0.05$, Fig. 1A). Raised level of energy intake in HFD fed mice was determined by comparing with the normal chow diet fed mice. However, there was a significantly reduced energy intake in GT group in comparison with HFD group ($p < 0.05$, Fig. 1B). As depicted in Fig. 1C, at the end of trial, the mice of HFD group significantly gained more body weight compared with the mice of NC group. CWB-GT intervention could significantly prevent the body weight gain from the fourth week. HFD resulted in the dyslipidemia characterized with a significantly higher TC, TG and LDL-C level in HFD group ($p < 0.05$). No significant difference was observed in all groups for the HDL-C level ($p > 0.05$, Fig. 1D-G). Drinking CWB-GT significantly decreased the levels of serum TC, and LDL-C ($p < 0.05$). The level of serum TG was not affected by CWB-GT intervention.

3.2. CWB-GT alleviated fatty liver and white fat accumulation caused by HFD

There was no significant difference among all groups on the liver weight/body weight (BW) ratio ($p > 0.05$, Fig. 2B). However, the HFD mice showed a significant increase in liver weight ($p < 0.05$, Fig. 2A). Besides, the mice of HFD group had significantly elevated ALT and AST levels than the mice of NC group, suggesting impaired liver function ($p < 0.05$, Fig. 2C, D). CWB-GT intervention significantly lowered the liver weight as well as decreased ALT and AST to the normal level ($p < 0.05$). The histological analysis also revealed that CWB-GT intervention could significantly reduce the number and volume of HFD-induced fat vacuoles in hepatocytes and retain the same normal hepatocyte morphology

as NC group (Fig. 2E). An excessive increase in adipocyte size and number is the contributory cause of obesity. As illustrated in Fig. 2F and Fig. 2G, GT group showed the significantly reduced deposition of the epididymal and pararenal adipose tissue masses. The adipocyte size of HFD group was larger than that of the NC group (Fig. 2H). Supplementation with CWB-GT prevented the enlargement. Besides, there was an obvious decline in the number of adipose cells in GT group at the same level of magnification.

3.3. CWB-GT reduced fasting serum glucose

We investigated the impact of CWB-GT on glucose homeostasis. CWB-GT intervention didn't significantly alleviated glucose intolerance based on the AUC values ($p > 0.05$, Fig. 3B). However, consistent with the result of fasting serum glucose (Fig. 3C), CWB-GT intervention significantly reduced blood glucose concentration at the point of 120 min ($p < 0.05$, Fig. 3A).

3.4. CWB-GT reduced inflammatory response caused by HFD

HFD could induce systemic inflammation and endotoxemia. As indicated in Fig. 4, HFD group showed significantly higher levels of LPS, TNF- α and IL-6 in serum, when compared with the NC group ($p < 0.05$, Fig. 4A-C). CWB-GT significantly reduced the higher level of TNF α and IL6 in the serum of mice fed HFD ($p < 0.05$), while the serum LPS concentration was not significantly affected by CWB-GT ($p > 0.05$).

3.5. CWB-GT altered the metabolic gene expressions of liver

As depicted in Fig. 5A, HFD significantly decreased the hepatic mRNA expression of genes involved in lipolysis (ATGL), fatty acid β -oxidation (PPAR α and ACOX) and bile acid synthesis (CYP7A1), while

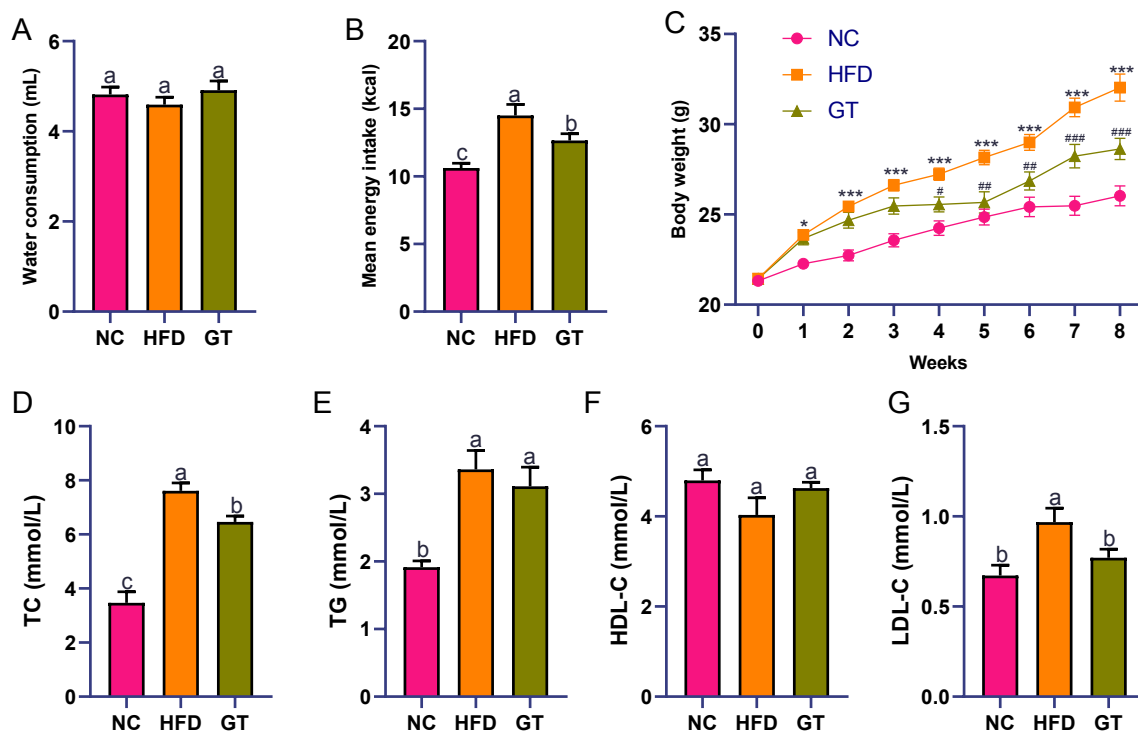


Fig. 1. Cold-water brewed green tea prevented high fat diet-induced obesity. (A) Water consumption per mouse per day on average. (B) Mean energy intake per mouse per day. (C) Body weight changes during 8-week intervention. (D) Serum total cholesterol (TC) level. (E) Serum triglyceride (TG) level. (F) Serum high-density lipoprotein cholesterol (HDL-C) level. (G) Serum low-density lipoprotein cholesterol (LDL-C) level. Data are expressed as the mean \pm SEM. (A, B, D-F) One-way analysis of variance followed by Student-Newman-Keuls post hoc test. The mean value with different letters indicates significant differences ($p < 0.05$). (C) Two-way repeated measures analysis of variance with Student-Newman-Keuls multiple comparison test. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ for normal chow (NC) vs high-fat diet (HFD); # $p < 0.05$, ## $p < 0.01$ and ### $p < 0.001$ for green tea (GT) vs HFD.

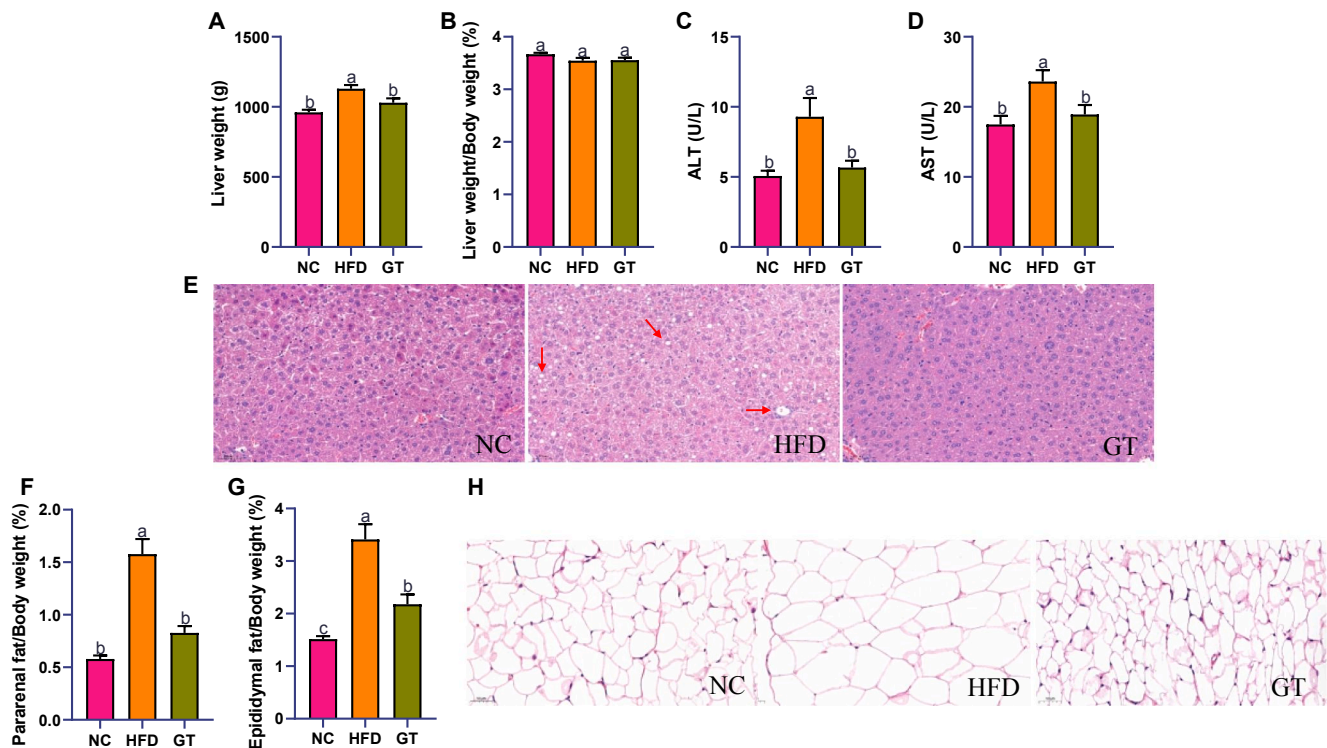


Fig. 2. Cold-water brewed green tea alleviated fatty liver and white fat accumulation. (A) Liver weight. (B) The index of liver weight relative to body weight. (C) Serum alanine aminotransferase (ALT) level. (D) Serum aspartate aminotransferase (AST) level. (E) Hematoxylin and eosin (H&E) staining of sections from liver. (F) The index of pararenal white fat relative to body weight. (G) The index of epididymal white fat relative to body weight. (H) H&E staining of sections from epididymal adipose tissue. Data are expressed as the mean \pm SEM. (A–D, F, G) One-way analysis of variance followed by Student-Newman-Keuls post hoc test. The mean value with different letters indicates significant differences ($p < 0.05$).

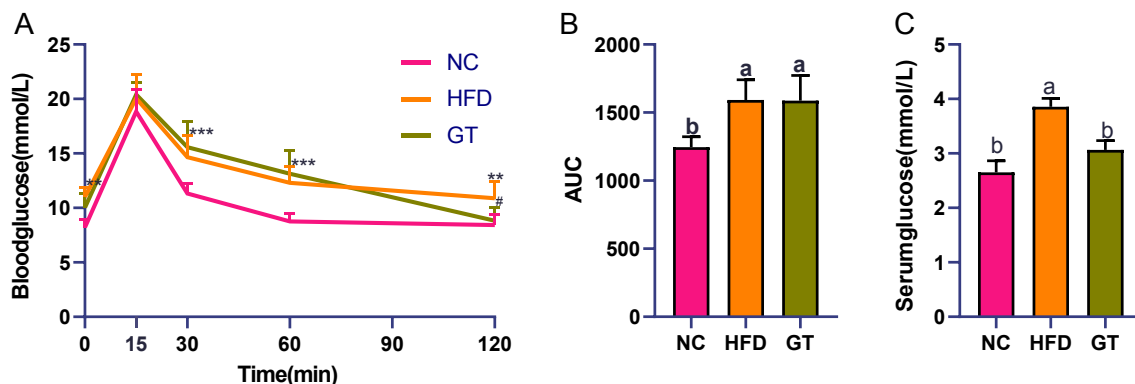


Fig. 3. Cold-water brewed green tea improved glucose homeostasis. (A) Oral glucose tolerance test (OGTT). (B) The calculated AUC for OGTT. (C) Fasting serum glucose. Data are expressed as the mean \pm SEM. (A) Two-way repeated measures analysis of variance with Student-Newman-Keuls multiple comparison test. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ for normal chow (NC) vs high-fat diet (HFD); # $p < 0.05$, ## $p < 0.01$ and ### $p < 0.001$ for green tea (GT) vs HFD. (B, C) One-way analysis of variance followed by Newman-Keuls post hoc test. The mean value with different letters indicates significant differences ($p < 0.05$).

increased the expressions of genes (SREBP1c and FAS) related to lipogenesis and decreased LXR α expression related to cholesterol removal compared with the NC group ($p < 0.05$). Mice treated with CWB-GT significantly decreased the expression of anabolism genes including SREBP1c, FAS and HMGR, and increased the expression of LXR α ($p < 0.05$).

3.6. CWB-GT altered the metabolic gene expressions of white adipose tissue

Adipose tissue, which is recognized as an energy storage and endocrine organ, has an important role in the development of obesity. As

shown in Fig. 5B, HFD mice exhibited a significantly higher level of leptin versus NC mice. Besides, the expressions of lipid catabolism associated genes including HSL and PGC-1 α were significantly lowered, and the inflammation cytokine TNF α , IL6 and IL1 β were significantly elevated in the HFD group ($p < 0.05$). CWB-GT significantly decreased the leptin level, suppressed the expression of inflammatory factors (TNF α and IL6), while promoted the expression of catabolism genes including PPAR α and PGC-1 α ($p < 0.05$).

3.7. Effects of CWB-GT on gut hormone

Because of the decreased energy intake in GT group, we tested the

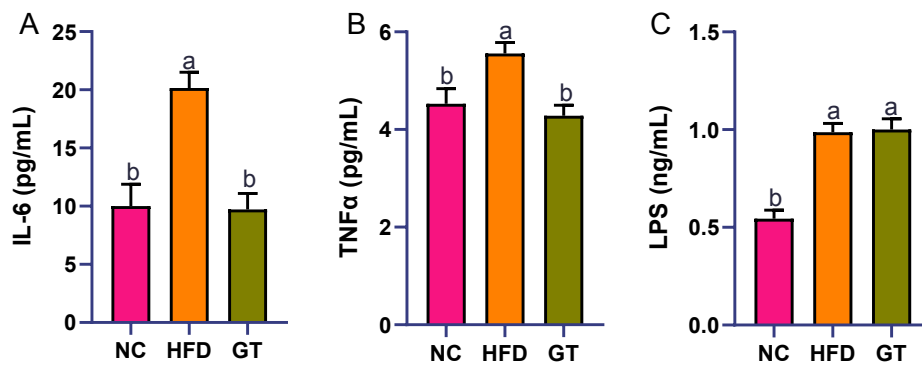


Fig. 4. Cold-water brewed green tea reduced inflammation. (A) Serum interleukin-6 (IL-6) level. (B) Serum tumor necrosis factor-alpha (TNFα) level. (C) lipopolysaccharides (LPS). Data are expressed as the mean ± SEM. (A-C) One-way analysis of variance followed by Student-Newman-Keuls post hoc test. The mean value with different letters indicates significant differences ($p < 0.05$).

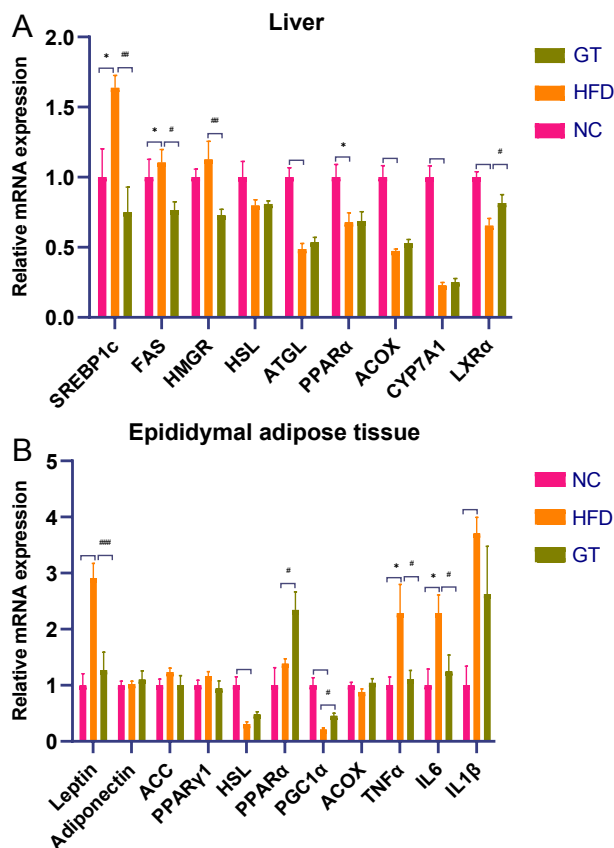


Fig. 5. Cold-water brewed green tea improved metabolic gene expressions of liver and white adipose tissue. (A) Relative expression of HSL, ATGL, PPARα, ACOX, CYP7A1 and LXRα in the liver. (B) Relative expression of Leptin, Adiponectin, ACC, PPARγ1, HSL, PPARα, PGC-1α, ACOX, TNFα, IL-6 and IL-1β in the epididymal adipose tissue. Data are expressed as the mean ± SEM. Expression levels were normalized to β-ACTIN and expressed as relative fold changes in comparison with the normal chow (NC) group. (A, B) One-way analysis of variance followed by Student-Newman-Keuls post hoc test * $p < 0.05$. ** $p < 0.01$ and *** $p < 0.001$ for NC vs high-fat diet (HFD); # $p < 0.05$, ## $p < 0.01$ and ### $p < 0.001$ for green tea (GT) vs HFD.

levels of serum hormones including PYY and GLP-1 of different groups. The results showed that GT group significantly facilitated the release level of serum PYY ($p < 0.05$, Fig. 6A), but didn't influence the concentrations of GLP-1 circulating in the blood when compared with the HFD group ($p > 0.05$, Fig. 6B).

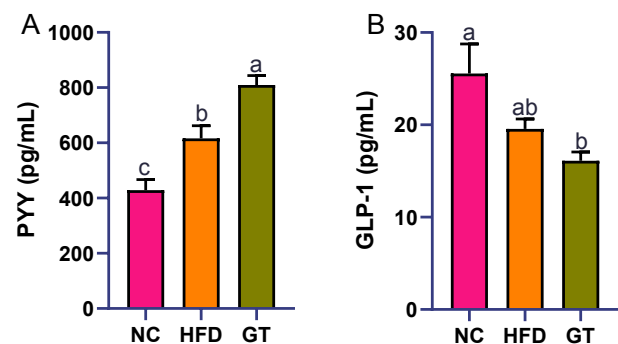


Fig. 6. Cold-water brewed green tea regulated gut hormones. (A) Peptide YY (PYY). (B) Glucagon-like peptide 1 (GLP-1). Data are expressed as the mean ± SEM. (A, B) One-way analysis of variance followed by Student-Newman-Keuls post hoc test. The mean value with different letters indicates significant differences ($p < 0.05$).

3.8. Effects of CWB-GT on gut microbiota

3.8.1. Composition changes of microbiota in different groups

The effects of cold-water brewed CWB-GT on gut microbiota were investigated by performing 16S rRNA gene sequencing. All the sequences were clustered into 2548 OTUs at a 97% similarity level. There were a total of 1758 OTUs identified by inquiring the database Silva132. The most dominant bacteria at the phylum level in the NC group were Firmicutes (51.85%), Bacteroidetes (35.53%) and Verrucomicrobia (7.52%). 8 weeks of HFD feeding drastically decreased the abundance of Verrucomicrobia (0.07%). No significant difference was observed on the ratio of Firmicutes/Bacteroidetes in the all three groups (Fig. S1). The HFD group significantly decreased the richness and evenness of microbial community as indicated by lower alpha diversity indexes including observed species, Shannon, Chao1 and ACE index ($p < 0.05$) (Fig. 7A-D). CWB-GT intervention had no effect on bacterial alpha diversity ($p > 0.05$). Weighted UniFrac-based principal coordinate analysis revealed distinct clustering of gut microbiota in response to different treatment (Fig. 7E). The results showed a separated clustering of microbiota composition between the NC and HFD groups. However, drinking CWB-GT couldn't make the gut microbiota composition of HFD group closer to that of NC group. In contrast, GT group presented a distinct clustering. Collectively, CWB-GT may modulate gut microbiota by affecting some specific bacteria rather than reversing the HFD-induced variations of gut microbiota composition.

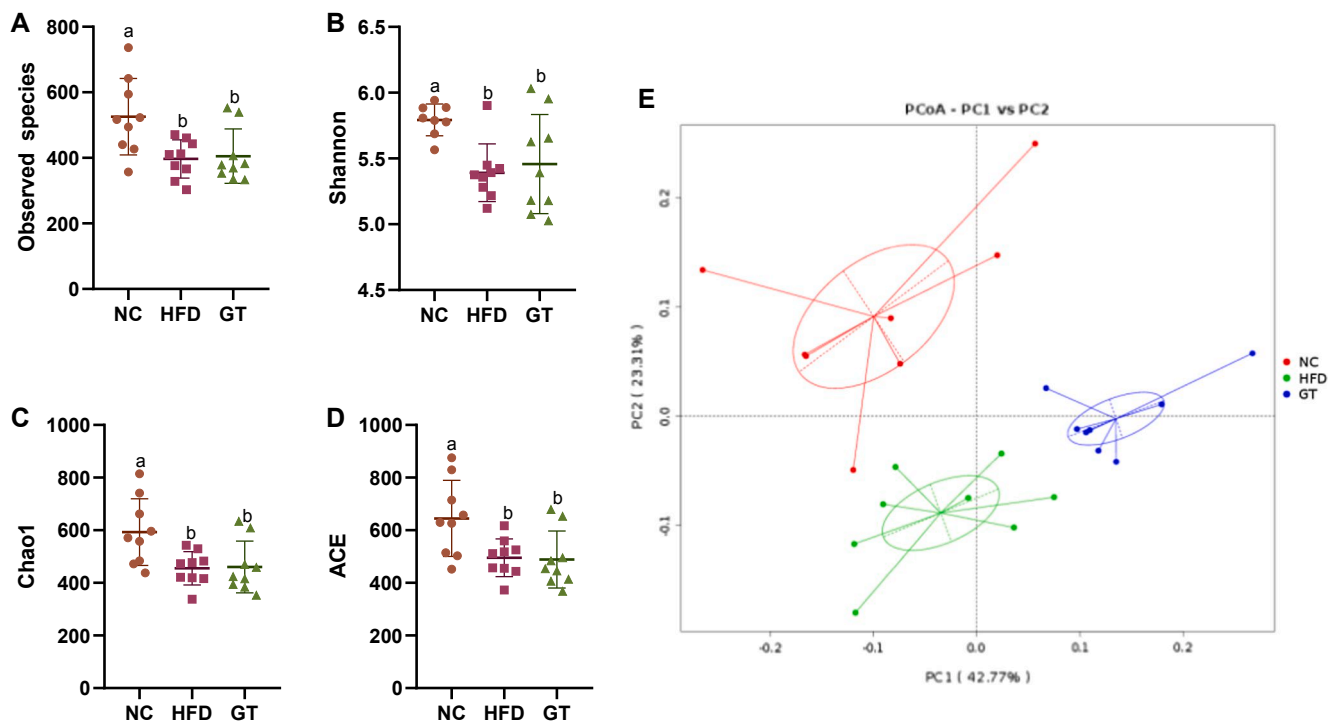


Fig. 7. Cold-water brewed green tea altered gut microbiota composition in high-fat diet (HFD)-fed mice. (A) Observed species index. (B) Shannon index. (C) Chao1 index. (D) ACE index. (E) Principal coordinate analysis (PCoA) on weighted UniFrac distance matrix. Data are expressed as the mean \pm SEM. (A-D) One-way analysis of variance followed by Student-Newman-Keuls post hoc test. The mean value with different letters indicates significant differences ($p < 0.05$).

3.8.2. Correlation analysis between the identified species and obesity related indexes

The Spearman's correlation analysis was used to examine the correlations between the relative abundances of top 15 identified species and obesity related parameters (body weight, TC, TG, HDL-C, LDL-C, serum glucose and LPS levels) (Fig. 8A). The heatmap indicated that metabolic related indexes were highly correlated with the relative abundances of certain species. The body weight was positively correlated with *Parabacteroides merdae*, *Bacteroides acidifaciens* and *Bacteroides vulgatus* but negatively correlated with *Lachnospiraceae bacterium DW67* and *Lachnospiraceae bacterium DW12*. Noteworthily, *Lachnospiraceae bacterium DW12* were negatively correlated with most of obesity related parameters while *Parabacteroides merdae*, *Dorea sp. 5.2* and *Bacteroides vulgatus* were positively correlated with most of obesity related parameters. CWB-GT significantly reduced the abundance of *Parabacteroides merdae* and *Bacteroides vulgatus* (Fig. 8B).

3.8.3. Identification of specific gut bacteria responsive to CWB-GT intervention

The heat tree analysis leverages the hierarchical structure of taxonomic classifications to quantitatively (using the median abundance) and statistically (using the non-parametric Wilcoxon Rank Sum test) depict taxonomic differences between two treatments. The visualized heat tree showed that at species level, *Bacteroides uniformis*, *Raoultella ornithinolytica*, *Blautia coccoides*, *Blautia producta*, *Lachnospiraceae bacterium DW67* and *Lachnospiraceae bacterium A2* were enriched in GT group (red) relative to HFD group where *Bacteroides thetaiotaomicron*, *Parabacteroides merdae*, *Bifidobacterium choerinum*, *Clostridium leptum* and *Dubosiella newyorkensis* were enriched (blue) (Fig. 8C). Besides, different species belonging to the same genus might have different opposite response for the same treatment. For instance, in the *Lactobacillus* clade, two species shown in both red and blue taxa were differentially abundant.

4. Discussion

Excessive consumption of sugar sweetened beverages is associated with an increasing risk of obesity and related diseases. Thus, functional beverage containing varieties of bioactive substances is becoming an overwhelming trend (Gunathilake, Wang, & Rupasinghe, 2013; López-García et al., 2020). Cold-water brewed tea has less bitterness and astringency (Monobe, 2018). In addition, tea polyphenols cannot be easily oxidized in cold water and several studies suggested that other ingredients released in cold water may also have bioactivities (Jiang, 2019). Our study investigated whether CWB-GT had effect on reducing body weight, preventing dyslipidemia and controlling blood glucose. Furthermore, the underlying mechanism was explored. The data demonstrated that CWB-GT effectively prevented body weight gain, improved dyslipidemia and alleviated inflammation, which correlated with the production of appetite-inhibiting hormones, regulation of metabolism related gene expression and modulation of specific bacteria.

Contents of soluble sugars, free amino acids, caffeine, total phenols, total catechins and various catechins were found to be varied in cold and hot-water brewed green tea in the previous study (Lin et al., 2014). Cold brewing had lower extraction efficacy, resulting in lower contents of components, such as soluble solids, caffeine and catechins than hot brewing. However, cold brewing made lighter, less colored and higher sensory-rated tea infusions. Besides, cold brewed tea infusion was found to have equal or higher antioxidant activity than hot-water brewed green tea. Brewing tea with cold water opens up a new field in the application and research of green tea. In the present study, the major ingredients in the CWB-GT were evaluated using HPLC analysis. The contents of various catechins in cold tea beverage were in the order of EGC > EGCG > ECG > C > CG (Table S1). EGCG and EGC were found to be the predominant catechins, which was consistent with previous reports about compositions of teas steeped in cold water (Damiani et al., 2014; Lin et al., 2014; Lin et al., 2008; Yang, Hwang, & Lin, 2007). Theanine is a non-proteinogenic amino acid, contributing sweet, brothy and umami flavor in tea (Zhong et al., 2017). It has also been reported to

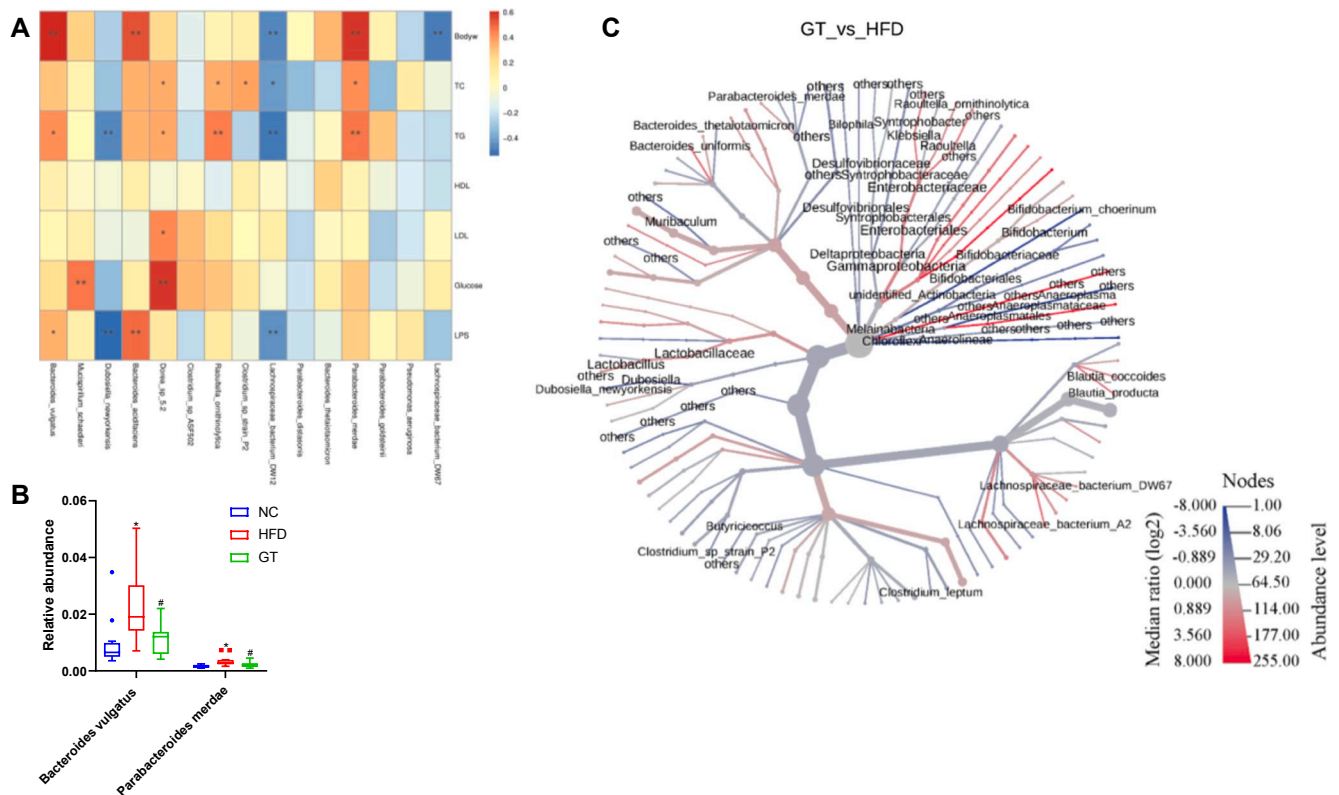


Fig. 8. The effect of cold-water brewed green tea on gut microbiota at species level. (A) Spearman's correlations between the relative abundances of top 15 identified species and obesity related parameters. The colors range from blue (negative correlation) to red (positive correlation). Significant correlations are annotated by * $p < 0.05$ and ** $p < 0.01$. (B) The relative abundance of *Bacteroides vulgatus* and *Parabacteroides merdae*. (C) The heat tree analysis to show comparisons of microbiome communities between green tea (GT) and high-fat diet (HFD) group. Only significant different taxa are presented on the graph. Taxa colored red are enriched in HFD group and those colored blue are enriched in GT group, determined using a Wilcoxon rank-sum test followed by a Benjamini-Hochberg (FDR) correction for multiple comparisons.

have effects on relaxing, cancer, cardiovascular diseases, obesity and other diseases (Turkuzu, & Sanlier, 2017). Theanine is more easily extracted in cold water than in hot water (Monobe, 2018). In our present study, a higher level of theanine was also detected in the cold tea beverage. Our data suggested that CWB-GT has considerable functional ingredients. The composition of functional ingredients of green tea leached in cold water not only improve the taste, but may also exert some beneficial effects.

TC, TG and LDL-C are key indicators for the evaluation of obesity, which has been reported to be positively correlated with the risk of metabolic diseases, cardiovascular diseases and several cancers (Blüher, 2019). Gut hormones such as PYY and GLP-1 secreted by enteroendocrine cells are crucial in the regulation of food intake, both of which could suppress appetite (Kim et al., 2018). In the present study, the consumption of CWB-GT reduced the body weight, the levels of TC, TG, and LDL-C of HFD fed mice. No significant difference was observed for water consumption between groups, indicating that CWB-GT had good palatability to mice. Nevertheless, a significant decreased energy intake and a remarkable increased concentration of PYY was detected in GT group. These results suggested that cold-water brewed CWB-GT exerted anti-obesity ability and the beneficial effect might be partially mediated via the regulation of gut hormone PYY.

Adipose tissue has an important role for the storage of excess nutrients. However, when adipocytes expand over a threshold, adipose tissue inflammation will be triggered (Reilly, & Saltiel, 2017). Liver is also a critical organ involved in lipid and cholesterol metabolism (Petta, Muratore, & Craxi, 2009). Leptin is an adipokine with multiple functions including regulating appetite, body weight and glucose homeostasis (Caron, Lee, Elmquist, & Gautron, 2018). Leptin resistance occurs in obesity, which further contributes to the development of obesity

associated metabolic disorders (Rufino, Costa, Carvalho, & Fernandes, 2020). PPAR α promotes energy expenditure through β -oxidation of fatty acids and increases the expression of key player in peroxisomal fatty acid β -oxidation. PGC-1 α is a co-activator of PPAR α (Shang et al., 2020). SREBP-1c controls the transcription of genes responsible for fatty acid synthesis including fatty acid synthase (FAS) (Panahi, Ahmadi, Teymouri, Johnston, & Sahebkar, 2018; Shang et al., 2020). HMGCR is the rate-limiting enzyme in the biosynthesis of cholesterol synthesis (Huang et al., 2018). LXR α mediates the removal of cholesterol in liver (Chawla et al., 2001). To further explore the molecular mechanism of cold-water brewed tea on lipid metabolism regulation, the mRNA expression levels of the genes involved in lipid and cholesterol metabolism were detected both in adipose tissue and liver. In the present study, the expression of lipolysis genes and fatty acid β -oxidation genes, such as PPAR α and PGC-1 α in adipose tissue of HFD-fed obese mice were increased by CWB-GT intervention. The lipogenesis was inhibited by decreasing the expression of FAS, HMGCR, and SREBP-1c, as well as increasing LXR α in liver of HFD-fed mice. In addition, CWB-GT significantly ameliorated the leptin resistance induced by HFD. The improved expression levels of genes involved in lipid metabolism and leptin level also demonstrated the anti-obesity effect of CWB-GT.

Chronic, low-grade systemic inflammation is a common underlying cause of obesity (Jayarathne et al., 2019). Chronic activation of proinflammatory-signaling pathways could also desensitize insulin receptor signaling, inducing insulin resistance (Chassaing, & Gewirtz, 2014). Our study testified that CWB-GT ameliorated obesity associated inflammation with reduced the concentrations of serum TNF- α and IL-6, which might be related to the improvement of fasting serum glucose.

Gut microbiota is considered as an important hidden metabolic organ of the body (Liu, He, Ma, & Chen, 2020). There is a tight

association between the gut microbiota dysbiosis and the development of obesity. Gut microbiota could affect host metabolism via energy harvested from gut microbiota, dysfunctional intestinal barrier associated low-grade inflammation and microbiota-derived beneficial/harmful metabolites (Cao et al., 2019). CWB-GT didn't reverse the lower bacterial diversity and increased LPS level caused by HFD, which is in contrast with the modulation of hot-water brewed tea on gut microbiota (Chen et al., 2018; Liu et al., 2019; Liu et al., 2016). However, our study revealed that GT group has a divergent distribution on the composition of gut microbiota based on the PCoA plot. We hypothesized that cold tea beverage might impact some specific bacteria, thereby mitigating metabolic disorders. Spearman correlation analysis of the relative abundances of top 15 identified species and obesity related parameters was performed in order to identify the potentially specific bacteria. *Ruminococcus bromii* and *Lachnospiraceae bacterium DW12* were highly negatively correlated with most of obesity related parameters while *Vibrio_cidicii*, *Lactococcus lactis*, *Parabacteroides merdae*, *Dorea sp. 5.2* and *Bacteroides vulgatus* were highly positively correlated with most of obesity related parameters. CWB-GT could effectively decrease the abundance of *Parabacteroides merdae* and *Bacteroides vulgatus*. *Parabacteroides merdae* was found frequently distributed in the gut microbiota of hypertensive patients (Yan et al., 2017). *Bacteroides vulgatus* is thought to be responsible for the development of inflammatory bowel disease (Shiba et al., 2003). Furthermore, *Bacteroides uniformis*, *Raoultella ornithinolytica*, *Blautia coccoides*, *Blautia producta*, *Lachnospiraceae bacterium DW67* and *Lachnospiraceae bacterium A2* were significantly enriched in GT group relative to HFD group in the light of heat tree analysis. Among them *Lachnospiraceae bacterium DW67* and *Blautia coccoides* have relatively high abundance. *Lachnospiraceae bacterium DW67* belongs to *Lachnospiraceae*, which was widely recognized as butyrate-producing bacteria (Meehan, & Beiko, 2014; Vacca et al., 2020). *Blautia coccoides* is also one of major butyrate-producing bacteria that have been detected in humans (Tanaka, Yamamoto, Yamada, Furuya, & Uyeno, 2016). Butyrate has been proved to exerts metabolic benefits through improving intestinal barrier, inducing thermogenesis or reducing appetite via gut-brain axis (Cani et al., 2019; Li et al., 2018; Liu et al., 2018). Collectively, our findings suggested that CWB-GT modulated gut microbiota by promoting the growth of butyrate-producing bacteria *Lachnospiraceae bacterium DW67* and *Blautia coccoides*, whereas inhibiting the growth of *Parabacteroides merdae* and *Bacteroides vulgatus*, potentially facilitating the improvement of host metabolism.

5. Conclusions

In conclusion, our results revealed that CWB-GT could reduce body weight, alleviate dyslipidemia, protect against fatty liver and relieve inflammation induced by HFD. The inhibition of lipogenesis in liver and the enhancement of fatty acid oxidation in adipose tissue of HFD-fed mice reconfirmed the anti-obesity effects of CWB-GT. These benefits might be attributed to the stimulation of PYY secretion. Furthermore, the modulation of specific bacteria by CWB-GT including increasing the abundance of *Lachnospiraceae bacterium DW67* and *Blautia coccoides*, while decreasing the abundance of *Parabacteroides merdae* and *Bacteroides vulgatus* might play an important role in the beneficial effects of CWB-GT. However, further investigation should be conducted to validate the function of these bacteria. Taken together, this study provides evidence that cold-water brewed tea could improve obesity and associated metabolic disorders, suggesting that cold-water brewed tea may emerge as a novel functional beverage.

Ethics statement

All animal experiments were carried out in compliance with the National Institutes of Health guide for the care and use of Laboratory animals (NIH Publications No. 8023, revised 1978) and were approved

by the Institutional Animal Care and Use Committee of Nankai University.

CRediT authorship contribution statement

Hui Ma: Conceptualization, Methodology, Formal analysis, Investigation, Writing - original draft. **Bowei Zhang:** Conceptualization, Writing - review & editing. **Yaozhong Hu:** Writing - review & editing. **Xiang Li:** Investigation, Validation. **Jin Wang:** Investigation. **Feier Yang:** Investigation. **Xuemeng Ji:** Formal analysis. **Shuo Wang:** Conceptualization, Resources, Writing - review & editing, Supervision, Funding acquisition.

Declaration of competing interest

The authors declare no competing financial interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.jff.2020.104279>.

References

- Akinyemiju, T., Moore, J. X., Pisu, M., Judd, S. E., Goodman, M., Shikany, J. M., ... Gilchrist, S. C. (2018). A prospective study of obesity, metabolic health, and cancer mortality. *Obesity*, 26(1), 193–201.
- Barraj, L. M., Bi, X., Murphy, M. M., Scrafford, C. G., & Tran, N. L. (2019). Comparisons of Nutrient Intakes and Diet Quality among Water-Based Beverage Consumers. *Nutrients*, 11(2), 314.
- Blüher, M. (2019). Obesity: Global epidemiology and pathogenesis. *Nature Reviews. Endocrinology*, 15(5), 288–298.
- Cani, P. D., Van Hul, M., Lefort, C., Depommier, C., Rastelli, M., & Everard, A. (2019). Microbial regulation of organismal energy homeostasis. *Nature metabolism*, 1(1), 34–46.
- Cao, S., Zhao, C., Xu, X., Tang, G., Corke, H., Gan, R., & Li, H. (2019). Dietary plants, gut microbiota, and obesity: Effects and mechanisms. *Trends in Food Science & Technology*, 92, 194–204.
- Caron, A., Lee, S., Elmquist, J. K., & Gauthron, L. (2018). Leptin and brain-adipose crosstalks. *Nature Reviews Neuroscience*, 19(3), 153–165.
- Chassaing, B., & Gewirtz, A. T. (2014). Gut Microbiota, Low-grade Inflammation, and Metabolic Syndrome. *Toxicologic Pathology*, 42(1), 49–53.
- Chawla, A., Boisvert, W. A., Lee, C. H., Laffitte, B. A., Barak, Y., Joseph, S. B., ... Tontonoz, P. (2001). A PPAR gamma-LXR-ABCA1 pathway in macrophages is involved in cholesterol efflux and atherogenesis. *Molecular Cell*, 7(1), 161–171.
- Chen, G., Xie, M., Dai, Z., Wan, P., Ye, H., Zeng, X., & Sun, Y. (2018). Kudingcha and fuzhuan brick tea prevent obesity and modulate gut microbiota in high-fat diet fed mice. *Molecular Nutrition & Food Research*, 62(6), 1700485.
- Chen, N., Bezzina, R., Hinch, E., Lewandowski, P. A., Cameron-Smith, D., Mathai, M. L., ... Weisinger, R. S. (2009). Green tea, black tea, and epigallocatechin modify body composition, improve glucose tolerance, and differentially alter metabolic gene expression in rats fed a high-fat diet. *Nutrition Research*, 29(11), 784–793.
- Chen, T., & Yang, C. S. (2019). Biological fates of tea polyphenols and their interactions with microbiota in the gastrointestinal tract: Implications on health effects. *Critical Reviews in Food Science and Nutrition*, 1–19.
- Damiani, E., Bacchetti, T., Padella, L., Tiano, L., & Carloni, P. (2014). Antioxidant activity of different white teas: Comparison of hot and cold tea infusions. *Journal of Food Composition and Analysis*, 33(1), 59–66.
- Drouin-Chartier, J. P., Zheng, Y., Li, Y., Malik, V., Pan, A., Bhupathiraju, S. N., ... Hu, F. B. (2019). Changes in Consumption of sugary beverages and artificially sweetened beverages and subsequent risk of type 2 diabetes: Results from three large prospective U.S. cohorts of women and men. *Diabetes Care*, 42(12), 2181–2189.
- Gunathilake, K. D. P. P., Wang, Y., & Rupasinghe, H. P. V. (2013). Hypocholesterolemic and hypotensive effects of a fruit-based functional beverage in spontaneously hypertensive rats fed with cholesterol-rich diet. *Journal of Functional Foods*, 5(3), 1392–1401.
- Huang, J., Feng, S., Liu, A., Dai, Z., Wang, H., Reuhl, K., ... Yang, C. S. (2018). Green tea polyphenol EGCG alleviates metabolic abnormality and fatty liver by decreasing bile

- acid and lipid absorption in mice. *Molecular Nutrition & Food Research*, 62(4), 1700696.
- Jayarathne, S., Stull, A. J., Park, O. H., Kim, J. H., Thompson, L., & Moustaid-Moussa, N. (2019). Protective effects of anthocyanins in obesity-associated inflammation and changes in gut microbiome. *Molecular Nutrition & Food Research*, 63(20), e1900149.
- Jiang, J. (2019). The hypoglycemic effect of green tea brewed in cold water is more effective. *Molecular Nutrition & Food Research*, 63(18), e1970042.
- Kim, K. S., Seeley, R. J., & Sandoval, D. A. (2018). Signalling from the periphery to the brain that regulates energy homeostasis. *Nature Reviews Neuroscience*, 19(4), 185–196.
- Li, Z., Yi, C. X., Katiraei, S., Kooijman, S., Zhou, E., Chung, C. K., ... Wang, Y. (2018). Butyrate reduces appetite and activates brown adipose tissue via the gut-brain neural circuit. *Gut*, 67(7), 1269–1279.
- Lin, S. D., Yang, J. H., Hsieh, Y. J., Liu, E. H., & Mau, J. L. (2014). Effect of different brewing methods on quality of green tea. *Journal of Food Processing and Preservation*, 38(3), 1234–1243.
- Lin, S., Liu, E., & Mau, J. (2008). Effect of different brewing methods on antioxidant properties of steaming green tea. *LWT – Food Science and Technology*, 41(9), 1616–1623.
- Liu, C., Guo, Y., Sun, L., Lai, X., Li, Q., Zhang, W., ... Cao, F. (2019). Six types of tea reduce high-fat-diet-induced fat accumulation in mice by increasing lipid metabolism and suppressing inflammation. *Food & Function*.
- Liu, H., Wang, J., He, T., Becker, S., Zhang, G., Li, D., & Ma, X. (2018). Butyrate: A Double-Edged Sword for Health? *Advances in Nutrition*, 9(1), 21–29.
- Liu, J., Hao, W., He, Z., Kwek, E., Zhao, Y., Zhu, H., ... Chen, Z. (2019). Beneficial effects of tea water extracts on the body weight and gut microbiota in C57BL/6J mice fed with a high-fat diet. *Food & Function*, 10(5), 2847–2860.
- Liu, J., He, Z., Ma, N., & Chen, Z. Y. (2020). Beneficial effects of dietary polyphenols on high-fat diet-induced obesity linking with modulation of gut microbiota. *Journal of Agricultural and Food Chemistry*, 68(1), 33–47.
- Liu, Z., Chen, Z., Guo, H., He, D., Zhao, H., Wang, Z., ... Ni, L. (2016). The modulatory effect of infusions of green tea, oolong tea, and black tea on gut microbiota in high-fat-induced obese mice. *Food & Function*, 7(12), 4869.
- López-García, G., Cilla, A., Barberá, R., Genovés, S., Martorell, P., & Alegría, A. (2020). Effect of plant sterol and galactooligosaccharides enriched beverages on oxidative stress and longevity in *Caenorhabditis elegans*. *Journal of Functional Foods*, 65, 103747.
- Ma, H., Zhang, B., Hu, Y., Wang, J., Liu, J., Qin, R., ... Wang, S. (2019). Correlation analysis of intestinal redox state with the gut microbiota reveals the positive intervention of tea polyphenols on hyperlipidemia in high fat diet fed mice. *Journal of Agricultural and Food Chemistry*, 67(26), 7325–7335.
- Meehan, C. J., & Beiko, R. G. (2014). A phylogenomic view of ecological specialization in the Lachnospiraceae, a family of digestive tract-associated bacteria. *Genome Biology and Evolution*, 6(3), 703–713.
- Monobe, M. (2018). Health functions of compounds extracted in cold-water brewed green tea from *Camellia Sinensis* L. *Japan Agricultural Research Quarterly Jarq*, 52(1), 1–6.
- Norton, M., & Murphy, K. G. (2017). Targeting gastrointestinal nutrient sensing mechanisms to treat obesity. *Current Opinion in Pharmacology*, 37, 16–23.
- Panahi, Y., Ahmadi, Y., Teymouri, M., Johnston, T. P., & Sahebkar, A. (2018). Curcumin as a potential candidate for treating hyperlipidemia: A review of cellular and metabolic mechanisms. *Journal of Cellular Physiology*, 233(1), 141–152.
- Petta, S., Muratore, C., & Craxi, A. (2009). Non-alcoholic fatty liver disease pathogenesis: The present and the future. *Digestive and Liver Disease*, 41(9), 615–625.
- Reilly, S. M., & Saltiel, A. R. (2017). Adapting to obesity with adipose tissue inflammation. *Nature Reviews Endocrinology*, 13(11), 633–643.
- Rufino, A. T., Costa, V. M., Carvalho, F., & Fernandes, E. (2020). Flavonoids as antiobesity agents: A review. *Medicinal Research Reviews*.
- Shang, A., Gan, R., Xu, X., Mao, Q., Zhang, P., & Li, H. (2020). Effects and mechanisms of edible and medicinal plants on obesity: An updated review. *Critical Reviews in Food Science and Nutrition*, 1–17.
- Shiba, T., Aiba, Y., Ishikawa, H., Ushiyama, A., Takagi, A., Mine, T., & Koga, Y. (2003). The suppressive effect of bifidobacteria on *Bacteroides vulgatus*, a putative pathogenic microbe in inflammatory bowel disease. *Microbiology and Immunology*, 47(6), 371–378.
- Tanaka, S., Yamamoto, K., Yamada, K., Furuya, K., & Uyeno, Y. (2016). Relationship of enhanced butyrate production by colonic butyrate-producing bacteria to immunomodulatory effects in normal mice fed an insoluble fraction of brassica rapa L. *Applied and Environmental Microbiology*, 82(9), 2693–2699.
- Turkoz, D., & Sanlier, N. (2017). L-theanine, unique amino acid of tea, and its metabolism, health effects, and safety. *Critical Reviews in Food Science and Nutrition*, 57(8), 1681–1687.
- Unno, K., Hara, A., Nakagawa, A., Iguchi, K., Ohshio, M., Morita, A., & Nakamura, Y. (2016). Anti-stress effects of drinking green tea with lowered caffeine and enriched theanine, epigallocatechin and arginine on psychosocial stress induced adrenal hypertrophy in mice. *Phytomedicine*, 23(12), 1365–1374.
- Vacca, M., Celano, G., Calabrese, F. M., Portincasa, P., Gobetti, M., & De Angelis, M. (2020). The controversial role of human gut lachnospiraceae. *Microorganisms*, 8(4), 573.
- Valdes, A. M., Walter, J., Segal, E., & Spector, T. D. (2018). Role of the gut microbiota in nutrition and health. *BMJ*, 361, k2179.
- van Duynhoven, J., Vaughan, E. E., van Dorsten, F., Gomez-Roldan, V., de Vos, R., Vervoort, J., ... Jacobs, D. M. (2013). Interactions of black tea polyphenols with human gut microbiota: Implications for gut and cardiovascular health. *The American Journal of Clinical Nutrition*, 98(6), 1631S–1641S.
- Xie, L., Su, H., Sun, C., Zheng, X., & Chen, W. (2018). Recent advances in understanding the anti-obesity activity of anthocyanins and their biosynthesis in microorganisms. *Trends in Food Science & Technology*, 72, 13–24.
- Yan, Q., Gu, Y., Li, X., Yang, W., Jia, L., Chen, C., ... Ma, Y. (2017). Alterations of the gut microbiome in hypertension. *Frontiers in Cellular and Infection Microbiology*, 7, 381.
- Yang, D. J., Hwang, L. S., & Lin, J. T. (2007). Effects of different steeping methods and storage on caffeine, catechins and gallic acid in bag tea infusions. *Journal of Chromatography a*, 1156(1–2), 312–320.
- Zhang, H., Li, Y., Lv, Y., Jiang, Y., Pan, J., Duan, Y., ... Zhang, S. (2017). Influence of brewing conditions on taste components in Fuding white tea infusions. *Journal of the Science of Food and Agriculture*, 97(9), 2826–2833.