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Identification of a sustainable two-plant diet that effectively prevents age-related metabolic syndrome and extends lifespan in aged mice

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Keywords: nutrition, sustainability, inflammation, metabolic disorders, longevity

Abstract

The current system of food production is linked to both the increasing prevalence of chronic disease and the deterioration of the environment, and thereby calls for novel ways of producing nutritious foods in a sustainable manner. In the "longevity village" of Bama, China, we have identified two plant foods, hemp seed and bitter vegetable (*Sonchusoleraceus*), that are commonly consumed by its residents and grow abundantly in unfarmed land without fertilizers or pesticides. Here, we show that a diet composed of these two foods (the "HB diet") provides a sufficient variety of nutrients and confers significant health benefits. Aged mice allowed *ad libitum* access to the HB diet not only had longer life spans and improved cognitive function, but were also protected against age-related metabolic syndrome, fatty liver, gut dysbiosis, and chronic inflammation, compared to aged mice fed a control Western diet. Furthermore, longevity-related genes (including AMPK, Sirt1, Nrf-1, and FOXO3a) were significantly up-regulated, while aging-related genes (including mTOR and NF-κB) were down-regulated. These results demonstrate that the HB diet is capable of promoting health and longevity, and present a sustainable source of healthy foods that can help control the prevalence of chronic diseases and reduce agricultural impact on the environment.

Keywords: nutrition, sustainability, inflammation, metabolic disorders, longevity

Introduction

Our current system of food production is problematic as it fails to provide healthy food for a growing global population and has a detrimental impact on the environment. Two major issues of modern food production are the widespread use of chemicals to increase production, including fertilizers, pesticides, and antibiotics, and farmed animal practices, such as grain-fed livestock and aquaculture. Although these practices are used to increase food production for the needs of the growing population, it is now well known that they can significantly contaminate water and soil, threatening the biodiversity of the environment, and can also enter the food web, posing serious risks to human health [1, 2]. Current animal-based food production practices increase greenhouse gas emissions, which contribute to global warming and the destructive effects of climate change, and threaten natural resources, such as the availability and potability of freshwater [1]. Furthermore, these practices can cause severe nutrient imbalances in food products, such as the omega-6/omega-3 fatty acid ratio, which have been implicated in the increased prevalence of chronic diseases, including cardiovascular disease, diabetes, and cancer [3, 4]. These issues, in addition to increased competition for land and resources, call for novel food sources that promote human health and the sustainability of natural resources to ensure long-term food security.

Bama is a remote and mountainous county in southwest China's Guangxi Zhuang autonomous region that is well known for the longevity of its residents and significant number of centenarians with few health problems. Studies have suggested that the longevity of the centenarians in Bama may result from the interplay between environment and lifestyle factors [5, 6]. Surveys indicate that the people of Bama eat noticeably less fat, animal protein, salt and sugar, and consume a

significant amount of vegetables that harbor a variety of essential nutrients [5, 6]. One of the major plants consumed in this region on a daily basis is hemp seed, an uncultivated crop that grows in the absence of pesticides or fertilizer. Hemp seed is considered a "super food" because it is rich in calcium, iron, antioxidants, essential amino acids, and essential omega-3 and omega-6 fatty acids [7]. Importantly, hemp seed has a favorable ratio of omega-6 to omega-3 fatty acids (3:1), which can promote heart and brain health, decrease triglycerides, reduce inflammation, and support immune system function [3, 4]. *Sonchusoleraceus* L. (bitter vegetable) is another significant dietary component in Bama, which is also an uncultivated plant that grows abundantly without use of pesticides or fertilizer. Studies have shown that *S. oleraceus* harbors high levels of antioxidants [8] and essential nutrients, including omega-3 fatty acids [9], and demonstrates potent antioxidant [10], anti-aging [11], and anti-inflammatory activity [12]. It has been suggested that dietary factors promote the longevity and vitality observed in the residents of Bama. However, it is still not known whether the specific combination of hemp seed and *S. oleraceus* plays a major role in health and aging.

We hypothesized that a diet rich in sustainably cultivated hemp seed and *S. oleraceus* would promote health and extend the lifespan based on observations of Bama residents and evidence that a plant-based diet high in omega-3 fatty acids has lasting health effects. In the present study, we fed mice with D12079B/RD Western diet as control to represent the currently common dietary habits in aging populations worldwide, or a diet comprised of hemp seed and *S. oleraceus*, hereby named the "HB diet". Our study is designed to examine whether it would provide sufficient nutrition and its impact on health and longevity, in the hopes that these plants can be utilized as novel, nutritious food sources to relieve the burden on human health and

environment.

Methods

Animals and diets. All animal procedures were approved by the Institutional Animal Care and Use Committee (IACUC) of Massachusetts General Hospital. Owing to the fact that the general life expectancy of women is longer than men, the female mice were used in all experiments. Twenty 12-month old bred female C57BL/6 mice were purchased from Charles River Laboratories (Boston, MA) and kept under specific pathogen-free conditions in standard cages with free access to food and water. The mice were provided with D12079B/RD Western Diet (Testdiet, Richmond, IN) *adlibitium* for three months after arrival and then randomly divided into two groups,one fed the Western Diet as a control and the other the "HB diet". The HB diet was formulated by our group and comprised 2/3 hemp seed (Guangxi Bama, China) and 1/3 *S. oleraceus* (Guangxi Bama, China). The HB diet was submitted to Covance Laboratory (Madison, WI) for composition analysis; its macronutrient composition is characterized by low carbohydrate, low saturated fat, and high PUFA content with a balanced omega-6/omega-3 ratio of ~2:1, and higher energy density compared to the control diet (5.36 vs. 4.66 kcal/g, respectively). Mineral analysis by ICP-MS and water-soluble metabolite analysis by NMR spectroscopy was performed by The Metabolomics Innovation Centre at the University of Alberta (Edmonton, CA). Detailed compositions of the diets are listed in Table S1-3. To ensure stability, all diets were stored at 4 °C and were replaced every 3 days.

The general health and well-being of the animals were checked daily and food consumption and body weight were monitored weekly. If a mouse was reported as sick, the criteria for euthanizing were independently assessed by a veterinarian according to the euthanasia criteria of the Center

for Comparative Medicine (MGH, Boston, MA), including severe ulcerative dermatitis and body weight (BW) less than 30% of the original BW. When nearly one-third of the mice in one group were observed with apparent sickness or wounds requiring euthanasia, the mice in acceptable condition (n=6/group) were used to conduct bacterial tests (bacterial culture and DNA extraction), glucose tolerance test, Morris Water Maze test and Open Field Test (at 11^{th},11^{th},12^{th}, and 13^{th} week after diet intervention, respectively; Fig S1).

When only 20% of the mice in one group were survived, all the mice were sacrificed and blood samples were immediately collected by cardiac puncture of the right ventricle and plasma was separated by centrifugation and stored at -80 °C until analysis. All organs were washed with saline after dissection and weighed. Liver and spleen sections were then either embedded in OCT or fixed in 10% formalin for histological analysis. The remaining tissues were immersed in liquid N_2 and stored at -80 °C until further analysis.

Morris Water Maze test. The Morris Water Maze (MWM) test was conducted to assess spatial learning and memory function as described previously [13]. Briefly, all mice were trained to swim to a hidden platform and were tested for their ability to find the platform in four independent trials per day for 5 days. The mice were given 90 s to find the platform and allowed to stay on the platform for 10–15 s before being removed from the pool. In the event that a mouse did not find the platform within 90 s, it was gently guided to the platform and allowed to remain there for 10–15 s. The platform was placed in a target quadrant for all trials within one MWM test, but the starting points were random for each mouse. On the fifth day, the memory of each mouse was tested by removing the platform and measuring the number of times the mouse

crossed the platform area. The learning and memory scores and swimming speed were determined for mice on the HB diet and control diet.

Open Field Test. The Open Field Test was performed to assess general locomotor activity and anxiety as described previously [14]. The examination arena consisted of an opaque, plastic circle 45 cm in diameter with walls 20 cm high. The arena was placed inside a plastic transparent box with a tracking system mounted to the top and the box was placed in an enclosed chamber to prevent distraction. Each mouse was placed in the same location at the edge of the arena facing the wall prior to the onset of each trial. The arena was virtually divided into three concentric circular sections: an "inner" circle (20 cm in diameter; area of 314 cm^2); a surrounding "neutral" ring (inner diameter 20 cm; outer diameter 40 cm; area of 932 cm^2); and the "outer" ring (inner diameter 40 cm; outer diameter 60 cm; area of 1570 cm^2). Mice were given 5 minutes to explore the arena. Time spent in each of the three regions was recorded for each mouse to assess anxious behavior. Time spent in the inner ring constituted the least anxious behavior, while time spent in the outer ring near the perimeter of the arena constituted anxious behavior.

Hepatic and spleen histology. Formalin-fixed paraffin-embedded liver and spleen tissues were sectioned at 4 mm and stained with H&E following standard protocols [15]. Each section was observed with light microscopy and the images were captured using a CCD camera (QImaging, Surrey, Canada). Histological analysis was performed by an experienced pathologist blinded to the groups. To determine hepatic lipid accumulation, frozen liver sections (10 μm) were fixed in 4% phosphate-buffered paraformaldehyde, stained with Oil Red O, and counterstained with hematoxylin. The sections were examined by light microscopy and the images were captured

using a CCD camera.

Fatty acid composition of diets and mouse tissues. Fatty acid profiles of the HB and Western diets and mouse tail, liver, heart, brain, and muscle tissues were analyzed by gas chromatography as described previously [16] (Table S4). Briefly, the diet samples and mouse tissues (~50 mg) were homogenized by grinding in liquid nitrogen and subjected to fatty acid methylation by mixing with hexane (1 mL) and 14% BF3/MeOH reagent (1 ml) at 100 °C for 1 h. Fatty acid methyl esters were extracted in the hexane phase and the fatty acid profiles were analyzed using a fully automated HP6890 gas chromatography system equipped with a flame-ionization detector (Agilent Technologies, Palo Alto, CA). The fatty acid peaks were identified by comparing relative retention times with commercial mixed standards (Nu-Chek Prep, Elysian, MN), and the area for each peak was analyzed using GC Chemstation software.

Oral glucose tolerance test. Glucose tolerance was evaluated using the oral glucose tolerance test as described previously [17]. Briefly, after a 12-hour fast, D-glucose (Wako Pure Chemical Industries) was administered to non-anesthetized mice (2.0 g/kg) by oral gavage. At 0, 15, 30, 60 and 120 min after glucose administration, blood samples were collected from the tail vein to determine the glucose concentration using the glucose CII-test (Wako Diagnostics, Richmond, VA). Glucose response was calculated as the area under the curve (AUC) using the linear trapezoidal formula. In addition, plasma samples obtained at 0 and 30 min after glucose administration were collected and analyzed for plasma insulin using the Mouse Insulin ELISA Kit (Millipore, Billerica, MA). Homeostasis model assessment of insulin resistance (HOMA-IR) level was determined using the following formula: [HOMA-IR = glucose (mg/dL) × insulin

(mU/L)/405], as described previously [18].

Blood lipid profiles and hepatic triglyceride measurement. Serum TC and TG were measured by the Clinical Pathology Laboratory at the Center for Comparative Medicine (MGH, Boston, MA). Total hepatic lipids and TG were measured with a Triglyceride Quantification Kit (BioVision, Mountain View, CA), following the manufacturer's protocol. Briefly, liver samples (~100 mg) were homogenized in 5% NP-40 in water (1 mL) and slowly heated to 80 °C in a water bath until the solution was cloudy. The samples were heated an additional time after cooling to room temperature and the supernatant was separated for analysis.

Plasma lipopolysaccharide (LPS) measurement. Plasma LPS concentration was measured with a ToxinSensor Chromogenic Limulus Amebocyte Lysate (LAL) Endotoxin Assay Kit (GenScript, Piscataway, NJ), following the manufacturer's protocol. Briefly, samples were diluted 10- to 50-fold with endotoxin-free water, adjusted to the recommended pH, and heated for 10 min at 70 °C to minimize inhibition or enhancement by contaminating proteins. LAL reagents were added to the serum or stool supernatant and incubated at 37 °C for 45 min, and the absorbance was read at 545 nm. A spiked control at 0.45 EU/ml was performed for each sample to ensure no significant inhibition or activation occurred.

Plasma ALT/AST and inflammatory cytokine assay. Plasma alanine transaminase (ALT) and aspartate amino transferase (AST) activity were measured using an alanine transaminase and aspartate amino transferase activity assay kit (Cayman, Ann Arbor, MI), following the manufacturer's protocol. Plasma levels of TNF-α, IL-1β, IL-6, MCP-1, and IL-10 were measured by Bio-Plex immunoassays formatted on magnetic beads (Bio-Rad, Hercules, CA), following the

manufacturer's protocol. Xponent software (Luminex, Austin, TX) was used for data acquisition and analysis.

Antioxidant capacity and lipid peroxidation measurement. Plasma antioxidant capacity was measured using a Total Antioxidant Capacity assay kit (Cell Biolabs Inc., San Diego, CA) following the manufacturer's protocol. Briefly, the reduction of copper (II) to copper (I) by antioxidants was assessed by measuring the further reaction of copper (I) with a chromogenic probe, which produced a color with a maximum absorbance at 490 nm. Net absorbance values were then compared to the uric acid standard curve and the results were expressed as "mM copper reducing equivalents", proportional to the total antioxidant capacity of the samples. Plasma lipid peroxidation was determined using a Thiobarbituric Acid Reactive Substances (TBARS) Assay kit (Cell Biolabs) following the manufacturer's protocol. Briefly, the reaction of malondialdehyde (MDA) with TBA to form MDA-TBA adduct was quantified colorimetrically at 532 nm, and the MDA concentration was calculated following the MDA standard curve.

Bacteria culture. Bacterial culture was performed to grow aerobic and anaerobic bacteria as described previously [21]. Fresh individual stool samples from live animals in each study group were collected and placed in Brain Heart Infusion (BHI) media (200 µL) in microfuge tubes and stored on ice. BHI media was added to each tube to ensure a specific weight:volume ratio (1 mg sample:10 µL BHI). The samples were then vortexed and serially diluted and plated on MacConkey agar (BD Bioscience, San Jose, CA), Lactobacillus-MRS agar, and Bifido agar (Anaerobe Systems, Morgan Hill, CA) plates to enumerate *Enterobacteriaceae*, *Lactobacilli*, and *Bifidobacterium* respectively. Aerobic bacteria were cultivated by incubating plates in ambient

air for 24 h at 37 °C. Anaerobic bacteria were cultivated by incubating plates in an anaerobic bag (Fisher Scientific, Pittsburgh, PA) in a 37 °C incubator for 24 or 48 h depending on the bacteria. Bacterial densities were expressed as the number of colony forming units (CFU)/g wet weight of feces.

Quantitative PCR (qPCR) measurement of bacterial DNA. Bacterial genomic DNA was extracted from stool samples (100 g) stored at -80 °C and analyzed using the QIAamp DNA Stool Mini Kit (Qiagen, Valencia, CA), following the manufacturer's protocol. DNA concentration was determined (absorbance at 260 nm), and purity was estimated by determining the A260/A280 ratio with a Nanodrop spectrophotometer (Biotek, Winooski, VT). qPCR was performed using SYBR Green and a PRISM 9000 Light Cycler (Applied Biosystems, Carlsbad, CA), following the manufacturer's instructions. The following group-specific 16S rRNA gene primers (Invitrogen Life Technologies, Grand Island, NY) were used: *Enterobacteriaceae* forward, 5′-CAT GCC GCG TGT ATG AAG AA -3′ and reverse, 5′-CGG GTA ACG TCA ATC AGC AAA -3′; *Bifidobacterium* forward, 5′-GCG TGC TTA ACA CAT GCA AGT C-3′ and reverse, 5′-CAC CCG TTT CCA GGA GCT ATT-3′; *Lactobacilli* forward, 5′-AGC AGT AGG GAA TCT TCC A-3′ and reverse, 5′- CAC CGC TAC ACA TGG AG-3′. Data analysis was performed using MxPro QPCR software (v4.10, Agilent). Ct values from *E. coli*, *Bifidobacterium*, and *Lactobacilli* were normalized to 16S to generate delta-Ct values and fold changes were calculated by using the delta-delta Ct values to the mean delta-Ct of the Western diet group. Melting-point determination analysis confirmed the specificity of the amplification products.

Gene expression analysis of aging-related genes. Liver, spleen, and fat tissue were excised and homogenized in liquid nitrogen. Total RNA extraction was performed with TRIzol reagent (Invitrogen) following the manufacturer's instructions. Three replicates of RNA from each sample were reverse transcribed into cDNA with an iScript Reverse Transcription Kit (Bio-Rad). PCR was carried out using a Mastercycler Pro S Thermal cycler (Eppendorff, Hauppauge, NY) with the following program: 95 °C, 5 min; (95 °C, 30 sec; 58 °C, 30 sec; 72 °C, 30 sec) x 35; 72 °C, 5 min. The primer sequences of aging-related genes (AMPK, mTOR, IGF-1R, Nrf, Fox1, Fox3a, Sirt1, and NF-κB), inflammation-related genes (TNF-α, IL-1β, IL-6, MCP-1, and IL-10), and adiponectin were obtained from PrimerBank (http://pga.mgh.harvard.edu/primerbank). PCR was performed using the SYBR green PCR Master Mix in the StepOnePlus Real-Time PCR System (Applied Biosystems).

Western blot analysis. Protein extracts of mice liver were prepared using RIPA buffer and protein concentrations were measured using a BCA kit (Pierce, Rockford, IL). Protein (100 μg) was separated by electrophoresis on a 4–12% SDS–PAGE gel and transferred to PVDF membranes (Invitrogen). The primary antibodies including rabbit anti-AMPK (1:1000), rabbit anti-Nrf (1:500), rabbit anti-Sirt1 (1:1000), mouse anti-Fox1 (1:1000), mouse anti-mTOR (1:1000m), and rabbit anti-NF-κB (1:1000) were obtained from Abcam (Cambridge, MA). Mouse anti-β-actin (1:2000) was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). The secondary antibodies were horseradish peroxidase-conjugated ECL goat anti-mouse IgG or goat anti-rabbit IgG (1:3000; Santa Cruz Biotechnology). Chemiluminescent detection was performed using the ECL method (Santa Cruz Biotechnology).

Statistical analysis. All data are presented as mean ± SD. Statistical analysis was carried out using GraphPad Prism 6.0 (GraphPad Software Inc., La Jolla, CA). Differences between the control diet and HB diet groups were evaluated using the unpaired two-tailed Student's t-Test. Mental-Cox test was used to compare the difference of Kaplan-Meier survival curves between two groups over time. A P-value of < 0.05 was considered statistically significant.

Results

Increased longevity and decreased senescence in mice on the HB diet

Aged 15-month-old female C57BL/6 mice were fed either the HB diet or a D12079B/RD Western diet as control *ad libitum* (see Table S1-3 for diet composition; n=10/group). The macronutrient composition of the HB diet is characterized by low carbohydrate, low saturated fat, and high PUFA content with a balanced omega-6/omega-3 ratio of ~2:1, and higher energy density compared to the control diet (5.36 vs. 4.66 kcal/g, respectively; Table S1-3). Accordingly, a balanced omega-6/omega-3 ratio was also found in the tissues of mice fed on the HB diet (Table S4). Body weight, food intake, and energy intake were similar between the two groups (Fig S2). By the age of 18 months, the mice on the control diet exhibited striking signs of senescence, characterized by dull hair, alopecia, lordokyphosis, and severe skin ulcers, while the mice on the HB diet remained healthy in appearance with shiny hair (Fig 1A). At the age of approximately 19.5 months, 50% of the mice on the control diet had either died or required euthanization. By the age of 20 months, only 20% of the mice had survived in the control group, while all of the mice on the HB diet were still alive and appeared healthy (Fig 1B and Fig S1). Further analyses by Mental-Cox test suggested that the mice fed on HB diet had significantly longer lifespan than the mice fed on control diet ($P < 0.01$; Fig 1B). In another trial using a 10%

corn oil diet as the control, the mice on the HB diet had a lifespan of 7 months longer than the mice on the corn oil diet (data not shown). These results show that the HB diet was not only nutritionally sufficient for maintaining health, but was also able to remarkably increase longevity.

Improved cognitive function and locomotor activity in aged mice on the HB diet

We evaluated the effect of the HB diet on age-related spatial learning and memory after diet intervention for 12 weeks (18 months old) using the Morris Water Maze test. The mice on the HB diet exhibited significantly shorter latency (Fig 2A) and path length (Fig 2B) to locate the hidden platform compared to the mice on the control diet. Besides, the mice fed on HB diet also had significantly increased average swimming speed (Fig S3). When the platform was removed, mice on the HB diet traveled more times into the quadrant where the hidden platform was previously located (Fig 2C). These results suggest that the aged mice on the HB diet had better spatial learning and memory, compared to the mice on the control diet.

We also tested general locomotor activity and willingness to explore using the Open Field Test after diet intervention for 13 weeks (18 month and one week old). Mice on the HB diet and the control diet had different travel patterns, with the mice on the HB diet exhibiting shorter freezing times (Fig 2D), longer travel distances (5.33 ± 1.0 vs. 3.93 ± 0.8 cm/s, $P < 0.05$; Fig 2E), and more entries into the center zone (Fig 2F). These data demonstrate that the aged mice on the HB diet showed greater locomotor activity compared to the control group.

Protection against hepatic steatosis and hepatotoxicity in aged mice on the HB diet

We further examined the effects of HB diet on hepatic morphology and function at the age of 20 months (by when all mice were euthanized or sacrificed). In contrast to the pale, enlarged livers found in the aged mice on the control diet, the livers of the aged mice on the HB diet appeared normal in size, weight and color (Fig 3A). The liver weights of mice on the HB diet were significantly lower than mice in the control diet group (Fig 3B, $P < 0.001$). The aged mice on the control diet exhibited marked signs of fatty liver, as shown by increased lipid droplets (Fig 3C-F) and triglyceride (TG) content (Fig 3G) in the liver. Conversely, mice on the HB diet exhibited much less hepatic lipid accumulation (Fig 3C-F) and lower levels of hepatic TG (Fig 3G, $P < 0.001$). Plasma levels of the enzymes alanine transaminase (ALT) and aspartate transaminase (AST), which reflect functional impairment of the liver, were also reduced in mice on the HB diet (Fig 3H & I, $P < 0.001$). In addition, mice on the HB diet had significantly lower levels of plasma TG and total cholesterol (TC), compared to the control group (Fig 3J & K, $P < 0.01$). These results indicate the beneficial effects of the HB diet on hepatic steatosis, hepatotoxicity, and plasma lipid profiles in aged mice.

Reduced spleen inflammation in aged mice on the HB diet

We also determined the effects of the HB diet and the control diet on spleen morphology and inflammation at the age of 20 months. Compared with the spleens of aged mice on the control diet, which were large and pale with a congested pattern, spleens of aged mice on the HB diet were of normal size and appearance (Fig 4A). As shown by hematoxylin and eosin (H&E) staining, clear areas of white and red pulp were found in the spleens of mice on the HB diet, indicating normal immune and blood filtration function (Fig 4B & C). In contrast, spleens of mice on the control diet contained exclusively congested red pulp, characterized by sinusoidal

infiltration and decreased lymphocyte cellularity, as well as increased myelopoiesis and marginal zones (Fig 4B & C). Accordingly, splenic expression levels of several key inflammatory cytokines, including nuclear factor kappa B (NF-κB), interleukin-6 (IL-6), IL-1β and monocyte chemotactic protein 1 (MCP-1), were reduced in mice on the HB diet compared to the control group (Fig 4D). These results indicate that the HB diet has protective effects against abnormalities in spleen morphology and inflammation in aged mice.

Reduced systemic inflammation and oxidative stress in aged mice on the HB diet

To compare the systemic inflammatory status of aged mice on the control or HB diets, we examined plasma levels of several key inflammatory cytokines at the age of 20 months. Compared with mice fed the control diet, the mice on the HB diet had lower plasma levels of TNF-α, IL-1β, MCP-1, and IL-6, and higher plasma levels of the anti-inflammatory cytokine IL-10 (Fig 5A). Moreover, mice on the HB diet had significantly lower plasma levels of lipopolysaccharide (LPS), a marker of inflammation-related endotoxemia (Fig 5B, $P < 0.001$). These results indicate that mice on the HB diet have decreased systemic inflammation compared to mice on the control diet. Furthermore, measurement of plasma antioxidants and oxidative compounds (Fig 5C & D), as well as hepatic antioxidant-related gene expression of SOD and GPx (Fig 5E), indicate that oxidative stress is decreased and overall antioxidant capacity is increased in mice on the HB diet.

Improved gut microbiota profile in aged mice on the HB diet

As the gut microbiota has been well implicated in health and aging, we chose to analyze several of the key gut bacteria involved in the regulation of metabolic endotoxemia and inflammation.

As shown by both bacterial culture (Fig 6A-C) and stool PCR analysis (Fig 6D-F), the mice on the HB diet had significantly lower gut levels of the LPS-producing *E. coli* and significantly higher gut levels of the beneficial *Bifidobacterium* and *Lactobacilli* species after diet intervention for 11 weeks (17 month and 3 weeks old). These results suggest that the HB diet confers a healthier gut microbiome in aged mice.

Increased insulin sensitivity in aged mice on the HB diet

We tested insulin sensitivity in aged mice on the HB or control diets by the oral glucose tolerance test and plasma insulin levels at the age of 17 month and 3 weeks. Plasma glucose levels were lower at all time points after glucose administration in mice on the HB diet compared with the control group (Fig 7A). The integration of area under curve (AUC) during OGTT in HB group was significantly lower than it in control group (Fig S4, $P < 0.01$). Plasma insulin levels were also lower in mice on the HB diet 30 min after glucose administration, compared with the control group (Fig 7B). Based on these measurements, insulin resistance was found to be significantly reduced in mice on the HB diet (Fig 7C, $P < 0.01$). While mice on the HB diet had greater accumulation of subcutaneous fat but not abdominal fat (Fig 7D), they also had elevated mRNA expression of adiponectin in both abdominal and subcutaneous fat tissue (Fig 7E, $P < 0.05$). Collectively, these results demonstrate higher insulin sensitivity in aged mice on the HB diet.

Increased expression of anti-aging genes in mice on the HB diet

To explore the molecular basis by which the HB diet was able to improve the health and longevity of aged mice, we evaluated the hepatic expression of several key aging-related genes at

the age of 20 months. Both RT-PCR (Fig 8A) and Western blot analysis (Fig 8B & C) showed that the expression of genes associated with longevity, including 5'adenosine monophosphate-activated protein kinase (AMPK), sirtuin 1 (Sirt1), nuclear respiratory factor 1 (Nrf-1), and forkhead box O3 (FOXO3a), was significantly higher in mice on the HB diet compared to the control group. Furthermore, the expression of genes that promote senescence and inflammation, including mammalian target of rapamycin (mTOR) and nuclear factor kappa B (NF-κB), were shown by Western blotting to be lower in mice on the HB diet than those on the control diet (Fig 8B & C). These results provide molecular evidence for the anti-aging effects of the HB diet on aged mice.

Discussion

In the present study, we demonstrate that a diet comprised solely of hemp seeds and *S. oleraceus* ("HB diet") is able to increase lifespan, improve cognitive function, protect against age-related metabolic syndrome, fatty liver, gut dysbiosis, and chronic inflammation, and elevate the expression of longevity-related genes and lower the expression of aging-related genes in aged mice.

Hemp seed and *S. oleraceus* are sustainably grown plants consumed on a daily basis in the longevity village of Bama, China. Hemp seed is considered a 'super food' because of its favorable balance of essential nutrients, including the omega-6 and omega-3 fatty acids [7], and *S. oleraceus*has well known anti-inflammatory and anti-oxidant activity [8-12]. This is the first study to demonstrate that a unique combination of these dietary components significantly affects the detrimental effects of aging. Interestingly, both vegetables are uncultivated and grow without

use of fertilizer or pesticides, indicating the potential for future development of an environmentally sustainable diet that has the capacity to improve health.

Nutrition is considered one of the most important determinants of healthy aging. The proper balance of essential nutrients maintains a low risk of disease and disease-related disabilities, mental and physical function, and a better quality of life [19, 20]. However, a decline in optimal dietary intake and nutrient absorption in elderly individuals results in increased risk of malnutrition, morbidity, and mortality [21]. Therefore, it is becoming increasingly important that elderly individuals consume the right balance of nutrients in their later years. In this study, we found that aging mice fed the HB diet had increased longevity and an improved health status, whereas mice fed in Western diet exhibited apparent senescence markers at 18 months old (such as dull hair, alopecia, lord kyphosis, and severe skin ulcers), and only 20% survived at 20 months old. The results on control group are generally comparable with the previous observation reported by Jackson Laboratory, that the average lifespan of bred female C57 mice is significantly shorter than it in non-bred C57 females (18.5 months vs. 22 months) [22]. Beside, since the modern Western diet is quite popular in most western countries and being adopted in developing countries, we used the D12079B/RD Western diet instead of normal chow diet as the control diet to better mimic the global dietary intake situation in aging populations. These bred mice fed on the Western diet would exhibit accelerated aging compared to those fed on chow diet. Therefore, the health status and lifespan of control mice hold feasible comparability to assess the effect of HB diet on aging.

Analysis of the HB diet showed that although the HB diet is composed of only two plants, it is

rich in both macronutrients and micronutrients (Table S1-3). In addition, despite that HB diet has higher fat content than the control diet, the majority of the fat (74%) is polyunsaturated fat with a balanced omega-6/omega-3 fatty acid ratio (~2.4:1), which has been well implicated in health and longevity [3, 23]. Furthermore, our study showed that while the omega-3 PUFA in the HB diet are short-chain ALA. The long-term feeding with the HB diet was able to significantly increase the tissues content of EPA, DPA and DHA and decrease AA content, possibly due to the enhanced long chain n-3 PUFAs conversion and inhibited AA synthesis under long term high ALA content (Table S4). Besides, this change is more significant in the heart, liver, muscle and tail tissues, and less in the brain, which normally does not exhibit dramatic changes in PUFA composition. Therefore, the balanced tissue long chain omega-6/omega-3 fatty acid ratio, together with the enrichment of tissue ALA – which alone also has beneficial effects for lifespan [24]– contribute to the overall health benefits of the HB diet. Given the health benefits reported here and the sustainable growth of these two plants, the HB diet presents a food model for significantly improving health and longevity while reducing the environmental impact.

Oxidative stress is considered to be one of the major mechanisms that regulate the aging process. Reactive oxygen species (ROS) from cellular respiration damage lipids, proteins, and DNA, accelerate the aging process, and increase disease risk and death [25]. In this context, reduction of ROS and oxidative damage should be a leading strategy to delay aging and age-related degenerative diseases. Chronic low-grade inflammation is another critical player in the aging process and age-related diseases in older adults [26, 27]. Levels of pro-inflammatory molecules such as IL-6, MCP-1, and TNF-α are known to elevate with increasing age [27, 28]. Consequently, the aging process is accelerated and aging-related chronic diseases are more

prevalent due to the impaired function and integrity of various tissues and organs [26-28]. Thus, the management of factors affecting chronic low-grade inflammation is important for age-related diseases. Studies have shown that the gut microbiota can profoundly affect inflammation through the production or absorption of endotoxins, such as LPS [29, 30]. Dysbiosis of the gut microbiota is therefore also a major factor influencing the development of chronic disease and aging. Recent evidence suggests that oxidative stress, inflammation, and the gut microbiota can interact with each other, and this interplay may be a key pathology in chronic diseases and is therefore a target for disease management [31, 32]. Our findings that the HB diet is capable of suppressing oxidative stress, inflammation, and gut dysbiosis suggest that the HB diet holds potential for the management of prevalent chronic diseases, including obesity, diabetes, and cancer. In fact, our findings show that the HB diet not only promotes longevity and extends lifespan, but can also improve cognitive function and prevent against fatty liver and metabolic syndrome.

It is well known that the prevalence of modern chronic diseases is connected to nutrition imbalances. In particular, the increased intake of carbohydrates, saturated fat, and omega-6 PUFA found in the modern Western diet has been implicated in the development of chronic diseases [33-35]. At the same time, modern diets are often deficient in fiber, antioxidants, and omega-3 PUFA [33-35]. These nutritional imbalances lead to the development of chronic inflammation, dysbiosis of the gut microbiota, and increased lipogenesis, all of which are underlying mechanisms of modern chronic diseases [3, 34, 36]. Therefore, correcting these nutritional imbalances is a critical approach for controlling the chronic disease epidemic. In the present study, we found that the HB diet provides many important nutrients deficient in the Western diet, including omega-3 fatty acids, antioxidants, and fiber. Increasing omega-3 fatty

acid intake and balancing the omega-6/omega-3 fatty acid ratio have been shown to beneficially impact the inflammatory response, gut microbiota, lipogenesis, and cognitive function through multiple pathways, especially under aging and high fat diet-induced conditions [3, 4, 36-38]. Antioxidants are also known to play a role in slowing aging by suppressing age-related oxidative stress and the inflammatory response [39]. Dietary fiber is beneficial in regulating the gut microbiota and the management of hyperglycemia and hyperlipidemia as well as body weight [40, 41]. Indeed, our results show that long-term consumption of this two-plant food is able to correct the aforementioned nutritional imbalances, especially the omega-6/omega-3 fatty acid ratio, and markedly suppresses the development of several disease conditions, including inflammation, fatty liver, insulin resistance, cognitive decline, and aging.

Increasing evidence has revealed the role of cell signaling mechanisms in the aging process and promotion of longevity [42-44]. The AMPK network is a crucial regulator of metabolic homeostasis [45] and AMPK has been recognized as a major survival factor in the aging process [46]. There are emerging results indicating that AMPK signaling can reduce oxidative stress and inhibit the inflammatory response induced by the NF-κB system, which is positively regulated by several transcription factors, including Sirt1, forkhead box O transcription factors, p53, and peroxisome proliferator-activated receptor-γ coactivator [46, 47]. Our results show that expression of AMPK, Sirt1, Nrf, and Fox3a were significantly higher in mice fed the HB diet than mice fed the Western diet. Interestingly, mTOR and NF-κB protein levels were increased in mice in the HB group compared with the Western diet group. These results suggest that the HB diet regulates the expression and potentially the function of aging-related genes to promote the robust and healthy appearance of mice fed this diet.

Given that the HB diet can impact chronic inflammation, gut dysbiosis and lipogenesis, it could be widely effective for the prevention of numerous chronic diseases, rather than one in particular. Furthermore, the use of hemp and bitter vegetable could replace or reduce the consumption of controversial foods (such as corn and soy) that contribute to nutritional imbalances. Use of these plants could also decrease pesticides and fertilizers in our food supply. It is therefore plausible that the incorporation of hemp seed and bitter vegetable into food systems, either as a dietary staple or a supplement, could be a powerful solution for fixing the deterioration of global health through balanced nutrition.

The current food production system harms the environment, contributes to climate change, and degrades the ecosystem. Corn and soy are major staples of modern diets, but the search for fertile land has led to deforestation, such as that of the Amazon Rainforest [48-50]. The meat industry requires extensive land to grow crops for animal feed. Meanwhile, these animals contribute vast quantities of methane gas in our atmosphere, and consume much water [51, 52]. Toxic chemicals used in food systems, including antibiotics, pesticides, and fertilizers, pollute our ecosystems [53]. Further, climate change has led to drought in major agricultural areas, such as California. Hemp and bitter vegetable are pest-resistant plants that grow easily without use of fertilizer or pesticides. Using these crops as staples will reduce ecological pollution of air, soil and water. Hemp is also drought-resistant. These crops can be grown in land formerly considered infertile, such as in mountainous and dry areas. Given the balanced nutrient composition, protein, and high energy density of these crops, they can also provide an alternate or supplemental food source for populations reliant on meat. Growing hemp and bitter vegetable could reduce the need for livestock agriculture, which will reduce greenhouse gas production and water use.

In summary, the present study demonstrates that a diet comprised of hemp seed and *S. oleraceus*originating from Bama, China, extended the lifespan of aged mice. Our study has discovered that this novel two-plant food source can correct many nutritional imbalances and is capable of preventing chronic diseases and slowing the aging process. These results provide compelling evidence for the incorporation of hemp seed and *S. oleraceus*into food systems as a promising and sustainable strategy that may protect the environment and improve human health.

Competing interests: The authors declare they have no actual or potential competing financial interests.

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Figure Legends

Fig 1. Increased longevity and decreased senescence in mice on the HB diet.

Twenty 12-month old female C57BL/6 mice were fed a control Western diet for three months and then randomly divided into two groups (n=10/group), one continuing on the control diet and the other the "HB diet". (A) Appearance of the mice at the age of 18 months (three months after dietary intervention). The mice on the control diet exhibited striking signs of senescence, including dull hair and severe skin ulcers, while the mice on the HB diet remained healthy in appearance with shiny hair. (B) Cumulative survival rates of the control group and the HB diet group. By the age of 19.5 months, 50% of the mice on the control diet had either died or required euthanization, and by the age of 20 months, only 20% of the mice had survived in the control group, while all of the mice on the HB diet were still alive and appeared healthy. The significant difference of Kaplan–Meier survival curve was determined by Mental-Cox test.

Fig 2. Improved cognitive function and locomotor activity in aged mice on the HB diet.

When the mice were aged 18-19 months, age-related spatial learning and memory were assessed by the Morris Water Maze (A-C), and general locomotor activity and anxiety were assessed by the Open Field Test (D-F). For the Morris Water Maze, the mice were trained to swim to a hidden platform and were tested for their ability to find the platform in four independent trials per day for 5 days. The latency (A) and path length (B) it took to locate the hidden platform were measured. On the fifth day, memory was tested by removing the platform and measuring the number of times the mouse crossed the platform area (C). For the Open Field Test, mice were placed in an examination arena and given five minutes to explore. Mice on the HB diet and the

control diet had different travel patterns, including freezing times (D), travel distances (E), and entries into the center zone (F). Data are expressed as mean ± SD, and significance was determined by unpaired two-tailed Student's t-Test; * $P< 0.05$; ** $P< 0.01$; n=6 per group.

Fig 3. Effects of the HB diet on hepatic steatosis and hepatotoxicity in aged mice.

Liver tissues from mice on the control diet and the HB diet were subject to analysis for hepatic morphology and function. (A) Representative liver size and appearance. (B) Liver weight (n=8/group). (C) Representative Oil Red O staining (200x). (D) Fat accumulation (n=8/group). (E) Representative hematoxylin and eosin (H&E) staining (200x). (F) Hepatocyte ballooning (n=8/group). (G) Hepatic triglyceride (TG) levels (n=5/group). (H) Alanine transaminase (ALT) enzyme activity levels (n=8/group). (I) Aspartate transaminase (AST) enzyme activity levels (n=8/group). (J) Plasma TG (n=5/group). (K) Plasma total cholesterol (TC; n=5/group). Data are expressed as mean ± SD, and significance was determined by unpaired two-tailed Student's t-Test; * $P< 0.05$; ** $P< 0.01$; *** $P< 0.001$.

Fig 4. Effects of the HB diet on spleen morphology and inflammation in aged mice.

Spleen tissues from mice on the control diet and the HB diet were subject to analysis for splenic morphology and function. (A) Representative spleen size and appearance. (B) Representative hematoxylin and eosin (H&E) staining (100x). (C) Representative hematoxylin and eosin (H&E) staining (200x). (D) Relative mRNA levels of inflammation-related cytokines (TNF-α, NF-κB, IL-6, IL-1β, MCP-1, IL-10) in the spleen (n=6/group). Data are expressed as mean ± SD, and significance was determined by unpaired two-tailed Student's t-Test; * $P< 0.05$.

Fig 5. Effects of the HB diet on systemic inflammation and oxidative stress in aged mice.

Systemic inflammatory status was evaluated by measuring the plasma levels of several key inflammatory cytokines (A-B), and oxidative stress was determined by measurement of antioxidant capacity and oxidative factors (C-E). (A) Plasma levels of inflammation-related cytokines (TNF-α, IL-1β, MCP-1, IL-10, and IL-6). (B) Plasma lipopolysaccharide (LPS) levels. (C) Total antioxidant capacity. (D) MDA concentration. (E) Relative hepatic gene expression of SOD and GPx. Data are expressed as mean ± SD, and significance was determined by unpaired two-tailed Student's t-Test; * $P< 0.05$; ** $P< 0.01$; *** $P< 0.001$; n=6 per group.

Fig 6. Improved gut microbiota profile in aged mice on the HB diet.

Selected gut bacteria related to metabolic endotoxemia and inflammation were analyzed by bacterial culture (A-C) and PCR (D-F). Representative culture plate photos and quantification of colony forming units for (A) Enterobacteriaceae, (B) Bifidobacterium, and (C) Lactobacillus; relative abundance following quantification of microbiota by PCR of (D) Escherichia coli, (E) Bifidobacterium, and (F) Lactobacillus. Data are expressed as mean ± SD, and significance was determined by unpaired two-tailed Student's t-Test; * $P< 0.05$; ** $P< 0.01$; *** $P< 0.001$; n=6 per group.

Fig 7. Increased insulin sensitivity in aged mice on the HB diet.

Insulin sensitivity was assessed in aged mice on the HB or control diets by the oral glucose tolerance test and measurement of plasma insulin levels. (A) Glucose tolerance test after dietary intervention. (B) Plasma insulin levels following glucose challenge. (C) HOMA-IR. (D) Weight of subcutaneous fat pad and abdominal fat pad. (E) relative mRNA levels of adiponectin in

subcutaneous and abdominal fat tissue. Data are expressed as mean ± SD, and significance was determined by unpaired two-tailed Student's t-Test; * $P< 0.05$; ** $P< 0.01$; n=6 per group.

Fig 8. Increased expression of anti-aging genes in mice on the HB diet.

The hepatic expression of several key aging-related genes was analyzed by (A) RT-PCR and (B-C) Western blotting. Bars show relative mRNA levels and quantification of total protein normalized by total β-actin, respectively. Data are expressed as mean ± SD, and significance was determined by unpaired two-tailed Student's t-Test; * $P< 0.05$; ** $P< 0.01$; *** $P< 0.001$; n=6 per group.

Figure 1

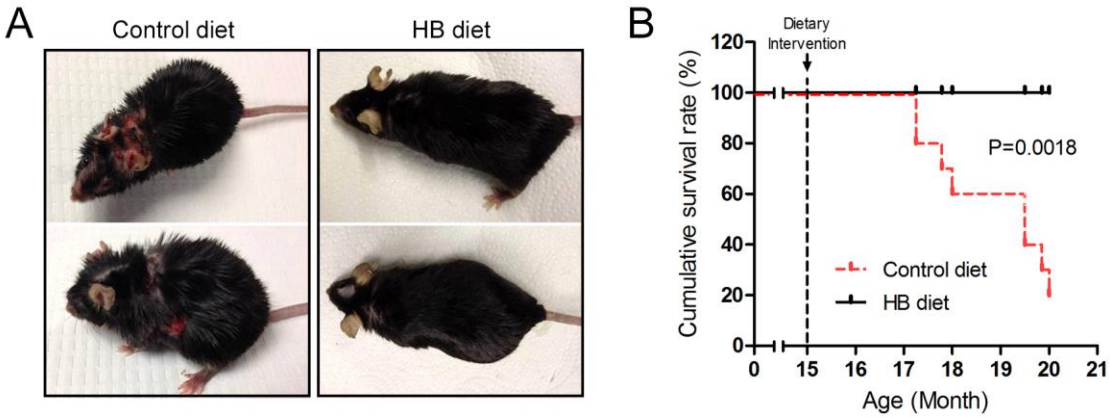
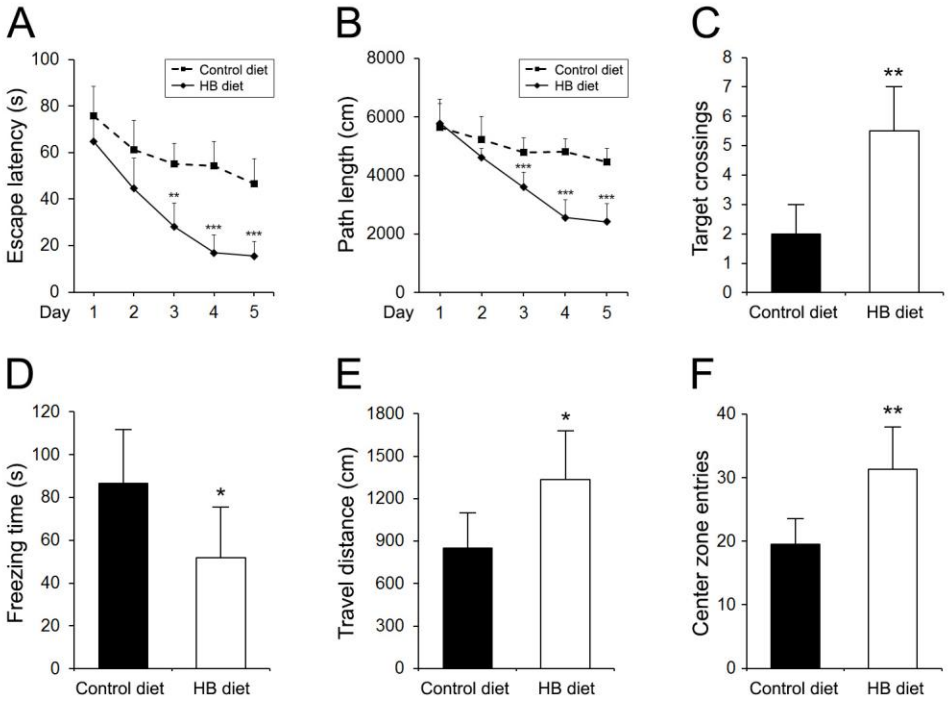


Figure 2



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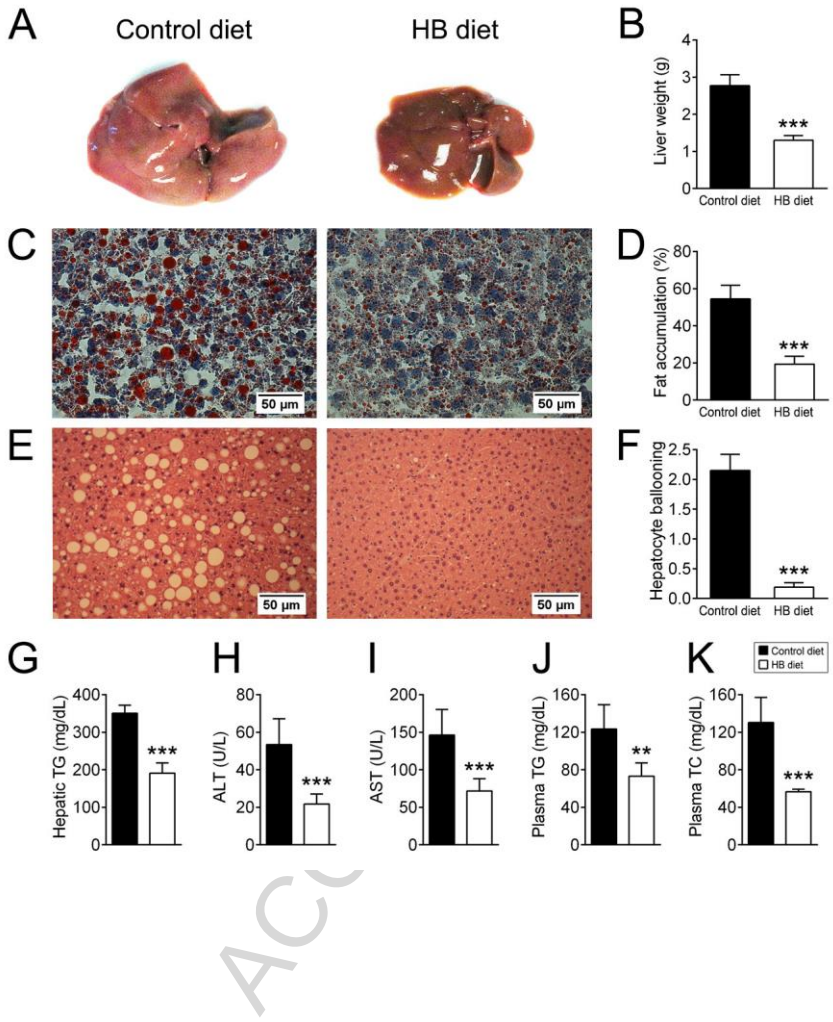


Figure 4

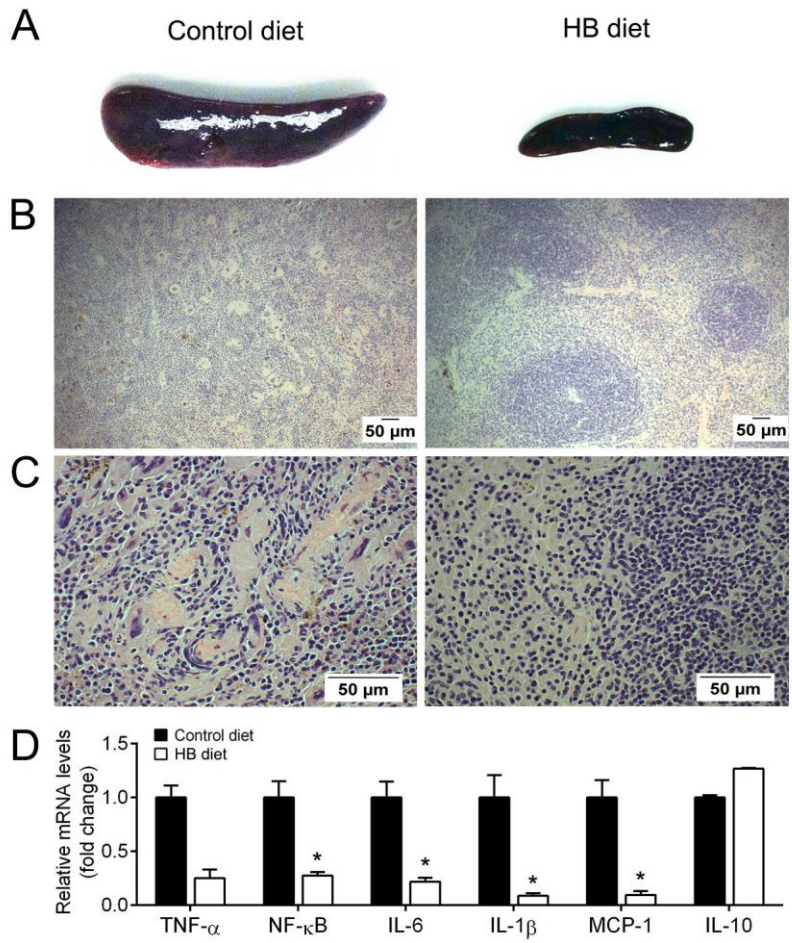
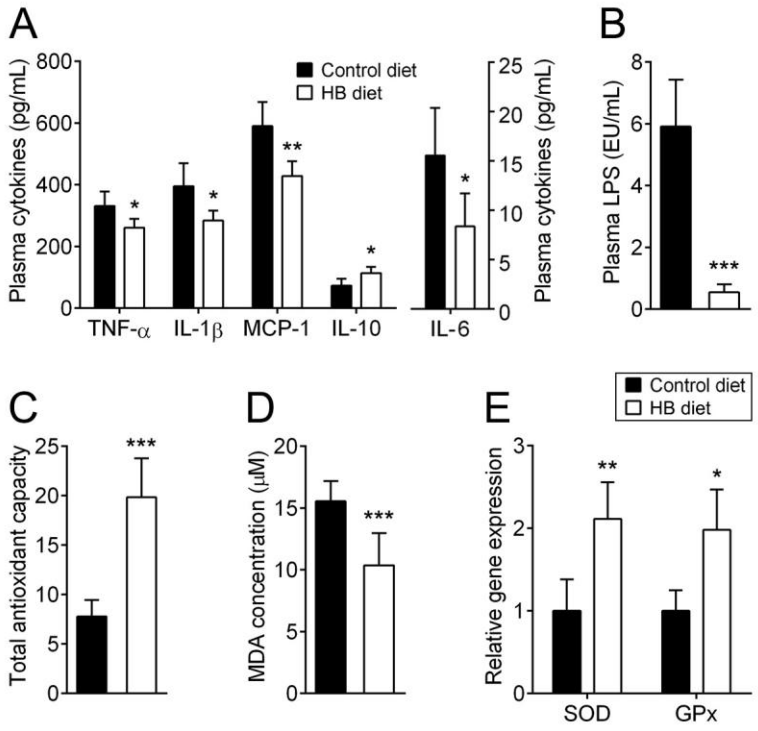


Figure 5



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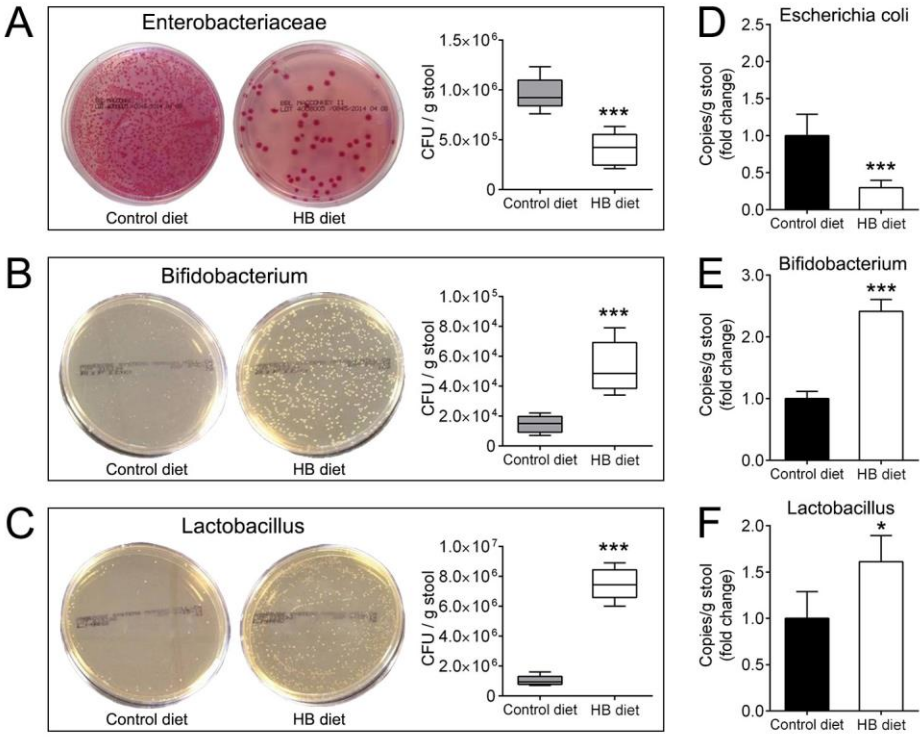

Figure 7

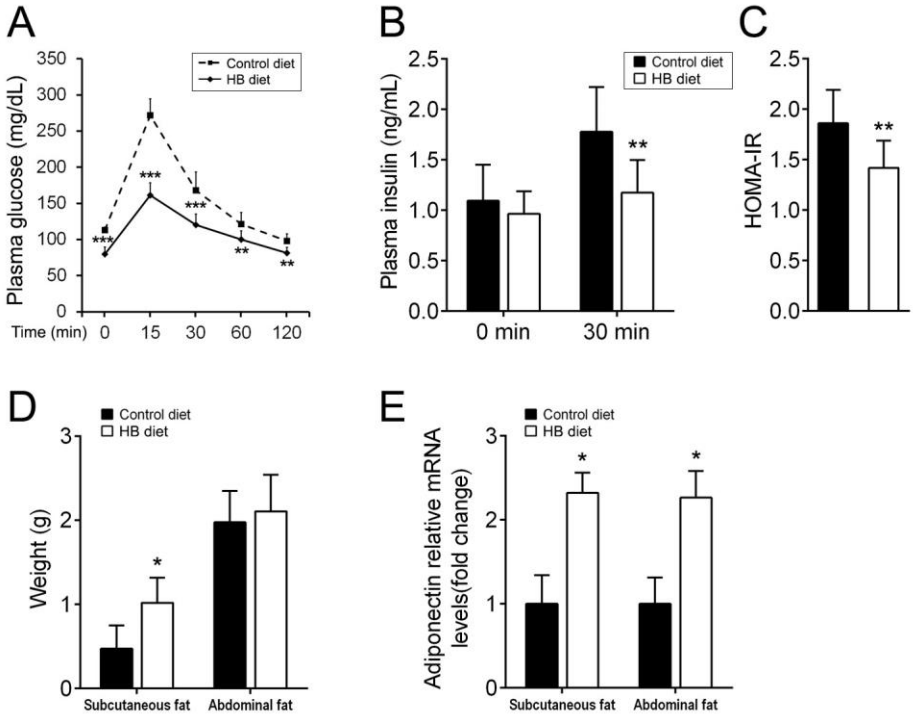

Figure 8

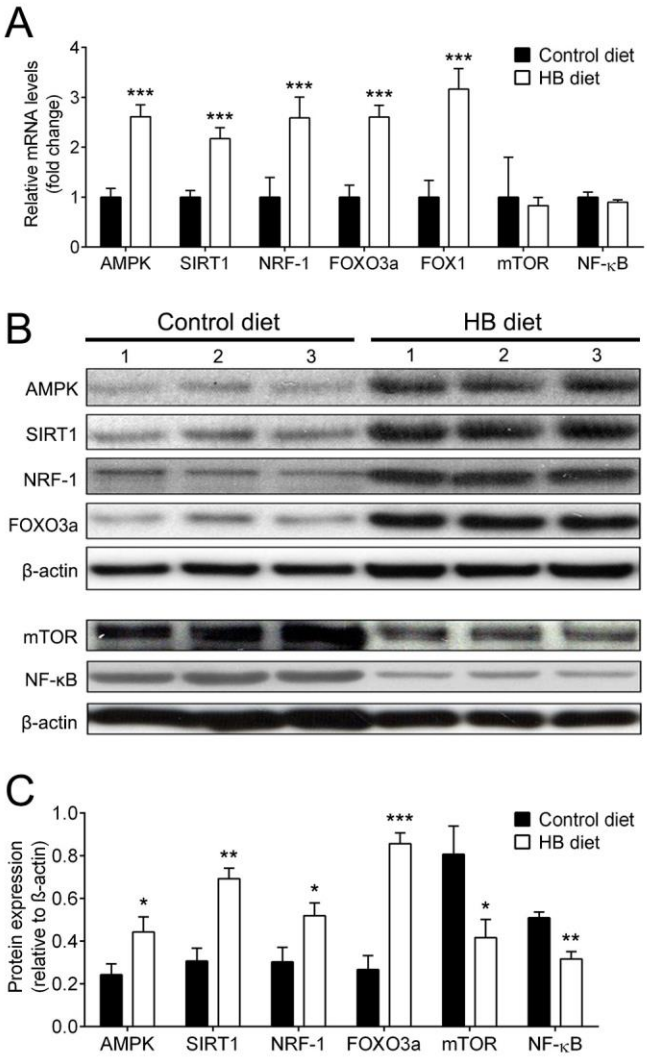

Table 1. Ingredient profiles of the experimental diets and Chow diet (per 100g)

	Control diet	HB diet	Chow diet*
Protein (g)	**20.3**	**29.9**	**19.1**
Casein	20		
DL-Methionine	0.3		
Carbohydrate (g)	**49**	**20**	**44.3**
Corn starch	12.5		
Sucrose	36.5		
Fat (g)	**21**	**37.4**	**5.8**
SFA	14.85	4.82	1.90
MUFA	5.06	4.75	1.32
PUFA	1.09	27.83	2.58
n-6 PUFA	1	19.63	2.18
n-3 PUFA	0.09	8.20	0.40
n-6/n-3 ratio	*11.11*	*2.39*	*5.5*
Cellulose	5		
Mineral Mix S10001	3.5		
Vitamine Mix V10001	1		
Choline Bitartrate	0.2		
Fiber, other micronutrients and moisture		12.7	24.4
Total (g)	100	100	100
Energy contribution (% kcal)			
Protein	17.4	22.3	25
Carbohydrate	42.0	14.9	58
Fat	40.5	62.8	17
kcal / g	4.66	5.36	3.1

*Chow diet: Teklad Traditional Diets (7012).