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Tartary buckwheat protein prevented dyslipidemia in high-fat diet-fed mice associated with gut microbiota changes

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ABSTRACT

As one of low-digestible proteins, tartary buckwheat protein (BWP) revealed a cholesterol-lowering activity. The relationship between the prevention of BWP on dyslipidemia and changes in the numbers of gut microbiota was investigated. The male C57BL/6 mice were separately fed on normal diet, high-fat diet (HFD) with casein, and HFD with BWP extract for 6 weeks. Quantitative PCR assay was applied to quantify the microbiota composition in feces. The levels of plasma total cholesterol (TC) and triglyceride (TG) in the mice fed on HFD with BWP were significantly lower than those on HFD with casein. BWP promoted the growth of *Lactobacillus*, *Bifidobacterium* and *Enterococcus* and inhibited the growth of *Escherichia coli* in HFD-fed mice. Moreover, *Bifidobacterium* population was closely related to contents of plasma lipids. Further, BWP significantly decreased the levels of plasma inflammation factors as induced by HFD, including lipopolysaccharide, tumor necrosis factor α and interleukin 6. BWP significantly increased the excretion of total bile acids and short-chain fatty acids in feces. In conlusion, BWP benefited cholesterol metabolism, which could be attributed to regulating composition of gut microbiota.

1. Introduction

The excessive fat intake results in an increased risk of dyslipidemia, which is a major cause for cardiovascular disease in China and other states (Xiao et al., 2011). An increased intake of plant proteins in the long term has a preventive effect on dyslipidemia. Particularly, tartary buckwheat protein (BWP) demonstrated a powerful cholesterol lowering effect for cholesterol-fed mice, which was more effective than soy protein (Kayashita et al., 1995).

It is known that the digestibility of BWP is relatively lower than that of other dietary proteins (Ikeda and Kishida, 1993). The rats fed with BWP had significantly lower concentrations of plasma cholesterol and enhanced excretion of total neutral sterols and nitrogen in feces than the rats fed with casein (Kayashita et al., 1997). Bile acids are the major metabolites of cholesterol, and their excretions are increased by their binding with other molecules (Higaki et al., 2006). An increase in fecal excretion of bile acids led to a decrease of bile acid level in enterohepatic circulation, resulting in an increase in conversion of cholesterol to bile acids in the liver, and therefore an increase in cholesterol uptake from the enterohepatic circulation (Erkkilä and Lichtenstein, 2006). Compared with soy protein, BWP played a greater role in promoting the bile acid excretion in feces (Tomotake et al., 2001). The cholesterol-lowering effect is mediated by a mechanism involving a high excretion of fecal sterols and total bile acids and a low digestibility of BWP (Tomotake et al., 2015). However, the mechanism for preventive effect of BWP on metabolic disorder of lipids has been unclear.

Interestingly, previous studies demonstrate that there are differences in the gut microbiota compositions between non-abnormal and abnormal lipids metabolism individuals (Ley, 2010). The link between the gut microbiota and metabolism has also been demonstrated in recent studies. The gut microbiota might contribute to an increased adiposity by stimulating inflammation and macrophage accumulation in adipose tissue (Caesar et al., 2012). Additionally, it has been found that the inflammatory status of the patients with high blood cholesterol was associated with the characteristic changes in the gut metagenome (Karlsson et al., 2012).

Furthermore, the gut microbiota may affect steatosis by regulating choline metabolism (Dumas et al., 2006). Inversely, probiotics are live microbes with beneficial effects on the host, including the capacity for modulation of intestinal microbiota composition, preservation of gut barrier integrity, and regulation of the inflammatory response (Gareau et al., 2010). Probiotics have been shown to have effects on irritable

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bowel diseases, metabolic syndromes, enterocolitis, immunomodulation, pathogen defense, and urogenital infections (Mshvildadze and Manzoni, 2009; Reiff and Kelly, 2010). Selected probiotics were able to prevent or delay the progression of abnormal metabolism induced by chemicals or changes in diet (Cani et al., 2007; Naito et al., 2011). Therefore, BWP may regulate microbiota composition owing to its low digestion and the ability to bind bile acids, which could be closely related to prevention of dyslipidemia. However, the effect of BWP on gut microbiota has never been reported as far as we know.

In the present study, the effects of BWP on blood lipids status, inflammatory cytokines, and intestinal microbiota and the relationships between blood lipids status and microbiota composition were investigated in high fat diet (HFD)-fed C57BL/6 mice to explain the link between prevention of hyperlipidemia and modulation of gut microbiota.

2. Materials and methods

2.1. Diets and animals

Male 4-week-old C57BL/6 mice (SPF) were obtained from Shanghai Laboratory Animal Centre (SLAC), Chinese Academy of Sciences (Shanghai, China), which were kept in an environmentally controlled breeding room (temperature: 23 ± 2 °C, humidity: 60 ± 10%, 12-h light–dark cycle) and fed with normal chow diet for one week. Then, they were divided into 3 groups according to the weight (n = 9) and fed with normal chow diet (control (CON) group), high-fat diet with casein (CAS group), and high-fat diet with BWP (BWP group) for 6 weeks. The diet compositions of the three groups are listed in Table 1. The extraction method and chemical compound of BWP was previously described by Tomotake et al. (2006). After the experimental period (6-weeks), foods were removed from the cages at 10:00 p.m., and blood samples were collected by decapitation under anesthesia with diethyl ether on the next day (8:00 to 10:00 a.m.). The animal experiment protocol was passed by the Institutional Animal Care and Use Committee of State Key Laboratory of Dairy Biotechnology (No 2016-05). The experiment was strictly conducted according to international standards and national legislation on animal care and use.

Table 1
Diets composition % (w/w).

	CON	CAS	BWP
Casein	21	23	
BWP			23.5
DL-methionine	0.3	0.3	0.3
Corn oil	5	12	12
Mineral mixture	4	4	4
Vitamin mixture	1	1	1
Cellulose	4.5	5	5
α-Corn starch	44	34.5	34
Sucrose	20	20	20
Choline	0.25	0.25	0.25
Cholesterol	0	1	1
Sodium cholate	0	0.25	0.25
Energy (kcal/g)	3.6	4.0	4.0
Carbohydrate (% Energy)	67.6	52.8	52.7
Protein (% Energy)	20	20	20
Fat (% Energy)	12.3	27.1	27.3
Fiber (g/100 kcal)	1.2	1.2	1.2

CON, chow diet-fed mice; CAS, high-fat diet with casein; BWP, high-fat diet with buckwheat protein extract.
Casein(N × 6.25) 87.0%.
BWP(N × 6.25) 85.2%.

2.2. Biochemical analysis

The blood samples were collected and were isolated by centrifugation at 1500 × *g* at 4 °C for 10min. The concentrations of total cholesterol (TC), triglyceride (TG), high density lipoprotein cholesterol (HDL-C), and low density lipoprotein cholesterol (LDL-C) in plasma were determined by the enzymatic colorimetric methods using commercial kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). The atherosclerotic index (AI) was calculated based on the formula as following:

$$AI = (TC- HDL\text{-}C)/HDL\text{-}C \quad (1)$$

2.3. Analysis of fecal samples

The metabolic experiment was performed 1-weeks before experiment was finished. All the mice were fed in the metabolic cages alone, and excrements of mice were collected and weighed every day. Fecal total bile acids were assayed using commercial kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). Concentrations of cholesterol in feces were determined by HPLC. Short-chain fatty acids (SCFAs) were measured by GC/MS. GC/MS analysis was performed by a GCMS-TQ8040 (Shimadzu, Japan) using a Rtx-5MS column (30 m × 0.25 mm i.d.; film thickness 0.25 μm) in the gas chromatograph system. The sample was introduced at an initial oven temperature of 35 °C, held for 1 min, ramped at 10 °C/min to 100 °C and held for 1 min, then ramped at 10 °C/min to a final temperature of 250 °C and held for 3 min. The total run time was 38 min. The inlet temperature was kept at 250 °C and helium was used as a carrier gas at a constant flow rate of 1 mL per minute. A sample of 1.0 μL was injected using the split mode (10:1) and the mass conditions were as follows: ionization voltage: 70 eV; ion source temperature: 220 °C; full scan mode in the m/z range 10–200.

2.4. Assays for plasma LPS and inflammatory cytokines

Plasma LPS, tumour necrosis factor-α (TNF-α), and interleukin-6 (IL-6) levels were measured by the Mouse Endotoxine Elisa Assay Kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China), Mouse Tumor Necrosis Factor α Elisa Assay Kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China), and Mouse Interleukin 6 Elisa Assay Kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China), respectively, according to the instructions of manufacturers.

2.5. Real-time qPCR analysis for gut bacterial determination

Gut bacterial contents in fecal samples were quantified by qPCR using the qTOWER2.2 Real-time PCR System (Analytik Jena AG, Germany), and the primers are listed in Table 2. Fecal DNA was extracted via the Stool Genomic DNA Kit (CoWin Biosciences, Beijing, China). The qPCRs were performed with 25 μl of final volumes containing 12.5 μl (2×) UltraSYBR Mixture Kit (CoWin Biosciences, Beijing, China), 1.0 μl of DNA, 1 μl each primer (10 μM), and 9.5 μl of sterile water. The samples were preheated at 94 °C for 10 min, followed by the amplification under the following condition: denaturation at 94 °C for 30 s, annealing temperature and time as listed in Table 2, and elongation at 72 °C for 60 s, 40 s or 30 s depending on the primer, and a total of 40 cycles were performed. The amount of DNA of bacterial was analyzed using qTOWER qPCR System software (Analytik Jena, AG, Germany) and was calculated as standard curves derived from serial dilutions of reference strains. The masses of bacterial genomes were calculated from genomic size data and the final results were expressed as genomic equivalents copies/g.