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Long-term fermented soybean paste improves metabolic parameters associated with non-alcoholic fatty liver disease and insulin resistance in high-fat diet-induced obese mice

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ABSTRACT

Recently, Korean traditional fermented soybean paste, called *Doenjang*, has attracted attention for its protective effect against diet-related chronic diseases such as obesity and type 2 diabetes. Long-term fermented soybean pastes (LFSPs) are made by fermentation with naturally-occurring microorganisms for several months, whereas short-term fermented soybean pastes (SFSPs) are produced by shorter-time fermentation inoculated with a starter culture. Here, we demonstrate that administration of LFSP, but not SFSP, protects high-fat diet (HFD)-fed obese mice against non-alcohol fatty liver disease (NAFLD) and insulin resistance. LFSP suppressed body weight gain in parallel with reduction in fat accumulation in mesenteric adipose tissue (MAT) and the liver via modulation of MAT lipolysis and hepatic lipid uptake. LFSP-treated mice also had improved glucose tolerance and increased adiponectin levels concomitantly with enhanced AMPK activation in skeletal muscle and suppressed expression of pro-inflammatory cytokines in skeletal muscle and the liver. LFSP also attenuated HFD-induced gut permeability and lowered serum lipopolysaccharide level, providing an evidence for its probiotic effects, which was supported by the observation that treatment of a probiotic mixture of LFSP-originated *Bacillus* strains protected mice against HFD-induced adiposity and glucose intolerance. Our findings suggest that the intake of LFSP, but not SFSP, offers protection against NAFLD and insulin resistance, which is an effect of long-term fermentation resulting in elevated contents of active ingredients (especially flavonoids) and higher diversity and richness of *Bacillus* probiotic strains compared to SFSP.

1. Introduction

Despite several molecular-targeted therapeutic agents for non-alcoholic fatty liver disease (NAFLD), one of the most common comorbidities associated with metabolic syndrome, have been widely researched, no effective treatments are currently available [1]. To provide a new strategy for NAFLD treatment, much attention has been focused on identification of potential dietary substances such as polyphenols (e.g. flavonoids and resveratrol), terpenes (e.g. carotenoids) and isothiocyanates [2].

Fermented soybean foods have been consumed as complements for grain proteins in East and Southeast Asian countries since ancient times, and Korean traditional fermented soybean paste (CODEX STAN 298R-2009), called *Doenjang*, is one of the most well-known examples [3]. Long-term fermented soybean pastes (LFSPs) are made through prolonged fermentation with naturally-occurring microorganisms, whereas short-term fermented soybean pastes (SFSPs) are produced through relatively short-time fermentation using a starter culture such as *Aspergillus oryzae*. It has been reported that fermented soybean pastes and their functional components, including flavonoids, unsaturated fatty acids and small peptides, could provide protection against diet-related chronic diseases such as obesity, type 2 diabetes, hypertension and carcinogenesis [3,4]. Naturally occurring isoflavones, a class of

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flavonoid phenolic compounds, have recently attracted much attention being considered as useful alternative medicines in preventing NAFLD and pathological adiposity [5]. In soybean foods, isoflavones exist in both the aglycone and glucoside forms; the fermentation process increases the content of aglycone forms in soy products [6]. Recent studies have also found that supplementation of these aglycones, daidzein and genistein, attenuates hepatic steatosis through suppression of lipogenic gene expression in the liver and alteration of adipocyte metabolism in high-fat diet (HFD)-fed mice [7,8].

In our study, we found that LFSP, but not SFSP, exerts beneficial metabolic effects in diet-induced obese mice. LFSP-treated mice had significantly suppressed HFD-induced weight gain and reversed glucose intolerance and NAFLD-related metabolic parameters compared with control mice. LFSP administration promoted attenuated gut permeability, accompanied by reduced chronic low-grade inflammation in the liver and skeletal muscle. In addition, LFSP-treated mice showed enhancements in adiponectin production in adipose tissue and AMPK activation in skeletal muscle. These findings indicate that the intake of LFSP, but not SFSP, might offer protection against NAFLD and other related metabolic disorders, which is an effect of long-term fermentation resulting in elevated contents of active ingredients (especially flavonoids) by allowing beneficial fermenting microorganisms to multiply.

2. Materials and methods

2.1. Preparation of fermented soybean paste samples

Two fermented soybean pastes used in our study, SFSP and LFSP, were prepared following the general procedures (Supplementary Fig. 1). Briefly, soybeans were washed and steamed at 120 °C. For SFSP, cooked soybeans were crushed, inoculated with a starter culture of *Aspergillus oryzae* and fermented for 15 days. For LFSP, cooked soybeans were crushed, formed into soybean blocks and fermented with naturally-occurring microorganisms for 90 days. Dried fermented soybean blocks were soaked in 19% (w/v) NaCl salt brine and aged for additional 60 days. The approximate compositions of SFSP and LFSP are shown in Table 1.

2.2. Animals

Four-week-old male mice were purchased from Hyochang Bioscience (Daegu, Korea) and maintained in humidity and temperature-controlled environment (22 ± 1 °C and 45 ± 10%) on a 12 h light/dark cycle. To examine the difference of LFSP compared to SFSP on metabolic-beneficial effects, mice were assigned to 4 experimental groups (n = 6–8 per groups); normal diet (ND)-fed controls, high-fat diet (HFD)-fed controls, HFD-fed LFSP-treated, and HFD-fed SFSP-treated groups. After 2 weeks of ND (2018S, Harlan Laboratories, Indianapolis, IN) or HFD (HFD; 60 %kcal from fat, D12492, Research Diets Inc., New Brunswick, NJ) feeding, each group was orally administered with a daily dose of 100 mg/kgBW (suspended in 200 μL PBS) LFSP or SFSP for additional 14 weeks. To evaluate the efficacy of naturally-occurring probiotic bacteria of LFSP, a mixture composed of 5 different *Bacillus* spp. strains isolated from LFSP was treated to HFD-induced obese mice. Four-week-old male mice were split into 5 groups; ND-fed controls, HFD-fed controls, HFD-fed VSL#3-treated, HFD-fed *B. subtilis*-treated, and HFD-fed *Bacillus* mixture-treated. After 2-week of ND or HFD feeding, each group was orally administered with a daily dose of 1×10^8 CFU (suspended in 200 μL PBS) VSL#3, *B. subtilis*, or *Bacillus* mixture, for additional 14 weeks. All the experimental protocols were approved by the Committee on the Ethics of Animal Experiments of the Handong Global University (permit number: 20160616-008).

2.3. Glucose tolerance test

After 12 weeks of LFSP and SFSP treatment, mice were fasted for 16 h, followed by intraperitoneal injection of glucose (2 g/kg). Blood samples were obtained by tail-bleeding, and blood glucose levels were checked at 0, 15, 30, 60, 90, and 120 min after glucose injection by GlucoDr auto AGM-4000 (Allmedicus, Anyang, Korea).

2.4. Histological analyses

Liver samples and MAT samples from each mouse were fixed in 10% v/v formalin/PBS, and then embedded in paraffin for H&E staining. Images were obtained under a microscope at a magnification of 200×.

2.5. Serum analyses

To determine triglyceride and total cholesterol concentrations, blood samples were collected by tail-bleeding and analyzed by Accutrend® Plus (Roche Diagnostics Ltd., Basel, Switzerland). The levels of alanine aminotransferase in serum were measured by automated biochemical analyzer (BS-390, Mindray Bio-Medical Electronics, China). Serum insulin levels were measured with Ultra Sensitive Mouse Insulin ELISA kit (Morinaga Institute of biological Science, Yokohama, Japan). Serum LPS levels were measured

Table 1
Comparison of composition between SFSP and LFSP.

Components	SFSP	LFSP
	Fermented for 15 days	Fermented for 90 days
Cellulose (%)	7.28	7.10
Linoleic acid (g/100 g)	1.77	1.81
Oleic acid (g/100 g)	0.62	0.57
Palmitic acid (g/100 g)	0.40	0.34
Water (%)	49.30	51.31
Amino acid nitrogen (%)	0.33	0.67
Total flavonoid (mg/100 g)	0.35	34.13
Total polyphenol (mg/100 g)	226.85	382.08
Viable *Bacillus* cell count (CFU/g)	2.3×10^8	1.1×10^9
Diversity of *Bacillus* community:		
Isolated *Bacillus* species (No. of isolated *Bacillus* strains)	*B. sonorensis* (3) *B. subtilis* (3) *B. licheniformis* (1)	*B. amyloliquefaciens* (6) *B. licheniformis* (4) *B. sonorensis* (4) *B. paralicheniformis* (1)

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