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Anti-hyperlipidemic Effects of *Bacillus* strain-fermented Cheonggukjang Products in Mice

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ABSTRACT To evaluate the hypolipidemic effects of Cheonggukjang (CGJ), which is frequently used in Korea similar to Natto in Japan and Douchi in China like a dairy product, boiled soybeans were fermented with two *Bacillus* strains, *B. subtilis* and *B licheniforms*, isolated from rice straw and their antihyperlipidemic effects of their products were investigated. Treatment with the CGJs significantly reduced blood triglyceride (TG) and total cholesterol (TC) levels and increased HDL cholesterol levels in Triton WR-1339-induced hyperlipidemic mice. The treatment of non-fermented soybeans alone also reduced blood TG and TC levels, but not significantly. Feeding the CGJs significantly lowered high blood TG and TC levels as well as body and epididymal mass weights in hyperlipidemic mice induced by the long-term feeding of a high-fat diet that increased blood HDL cholesterol levels. The *B. subtilis*-fermented CGJ products more potently reduced TG and TC levels, although the differences between the starters were not significant. These finding suggest that CGJ products may be effective as hypolipidemic foods by the synergistic interaction of soy and *Bacillus* strains.

KEYWORDS: hyperlipidemia, soybean, fermentation, *Bacillus subtilis, Bacillus licheniformis.*

INTRODUCTION

Lipid metabolism normally maintains an elegant balance between synthesis and degradation. When this balance is lost, hypercholesterolemia and hyperlipidemia may develop, leading to obesity, diabetes, arteriosclerosis, hypertension, and functional depression of select organs (Goldstein et al., 1973). Childhood obesity is increasing dramatically throughout the world. It is associated with a significant risk of developing type 2 diabetes and/or cardiovascular disease in addition to many other medical complications that may affect short- and long-term physical and mental health (Ross and Katan 2000; Cheik et al 2008). The rate-limiting enzyme of cholesterol biosynthesis from acetate is HMG-CoA reductase (Heller and Shrewsburg, 1976; White and Rudney, 1970). Pancreatic lipase is a key enzyme for lipid breakdown and fatty acid absorption (Birari and Bhutani, 2007). Inhibitors of HMG-CoA reductase or pancreatic lipase are anti-hypercholesterolemic agents (Birari and Bhutani 2007; Endo et al 1992; Davidson 2003), including orlistat and lovastatin, which reduce the absorption of dietary triglycerides and inhibit cholesterol biosynthesis, respectively. However, repeated use of these agents causes side effects (Davidson 2003; Ballinger 2000). Therefore, much more attention should be given to examining, and developing preventive strategies.

Emerging evidence suggests that the consumption or supplementation of foods rich in phytoestrogens, such as isoflavones, found in soy and products with or without fermentation, may have beneficial effects on obesity and glucose levels in animals and humans (Ross and Katan 2000; Suzuki et al 2003).

Cheonggukjang (CGJ), a traditional Korean fermented soy bean product, has some important health promoting effects, including hypolipidemic effects, fibrinolytic activity, antimutation and anticarcinogenic effects, and antioxidant effects (Yang et al 2003; Soh 2006). CGJ is fermented using rice straw as a fermentation starter. Therefore, various Bacillus species, including *Bacillus subtilis* and *B. licheniformis*, are involved in the fermentation of soy foods such as Japanese Natto and Chinese Douchi (Suzuki et al 2003; Kwon et al 2009; Cheik et al 2008). However, this fermentation may cause putrefaction, because various harmful microbes along with beneficial microbes reside in rice straw (Kim et al 1982). Therefore, to escape the putrefactive reaction for soy

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fermentation, probiotics isolated from the straw can be used for CGJ fermentation. Nevertheless, the differences in biological effects among CGJs fermented with various probiotics have not been investigated.

Therefore, we isolated the frequently used strains *Bacillus subtilis* and *B. licheniformis* from rice straw and prepared two different CGJ products. We then investigated their hypolipidemic effects on Trition WR-1339- or high-fat diet-induced experimental hyperlipidemic mice.

MATERIALS AND METHODS

Materials

Triton WR-1339 and lovastatin were purchased from Sigma Co. (USA). Tryptic soy broth and agar were purchased from Difco. Co. (USA). Total cholesterol and triglyceride assay kits were purchased from Asan Pharmaceutical Co. Ltd. (Korea).

A high-fat diet containing 25% beef tallow (AiN-76 fat-diet #180337) was purchased from Dyets, Inc. (Bethlehem, PA, USA). A normal diet containing 25% corn starch instead of beef tallow was also used.

Soy (7% moisture, 35% crude protein, 15% crude lipid, 16% dietary fiber, and 23% carbohydrate) was acquired from Chungchungbuk-do products (Korea).

Isolation of *Bacillus* strains from rice straw and preparation of CGJ

Well-dried rice straw was boiled in a water bath and then cooled. The identification of colonies grown in DHL agar media was performed by 16S rDNA gene sequencing (Hiraishi, 1992). The total DNA extracted from the colonies was used as a template to amplify the 16S rRNA gene with primers 27f (5'-AGAGTTTGATCCTGGCTCAG-3') and 1525r (5'-AAAGGAGGTGATCCAGCC-3'), and its sequence was analyzed using a BLAST search. *B. subtilis* and *B. licheniformis* were selected.

The CGJ products were prepared according to a modified version of the traditional method. Here, previously cultured *B. subtilis* or *B. licheniformis* were used as starters instead of rice straw. The starters (0.4 w/w%) were inoculated into well-boiled soybeans, which were incubated for 72 h at 35°C and then freeze-fried. The products were used as the experimental samples. An additional soy sample was prepared according the preparation method for fermented soy but without starter.

Animals

Male ICR mice (20-25 g) were purchased from Orient Charles River Co. (Korea) and fed a commercial diet (Orient Charles River Co., Korea). These animals were kept for at least 7 days prior to the experiments. All animals were housed in wire cages at 20-22°C and 50±10% humidity. They were fed a standard laboratory chow (Samyang, Seoul, Korea) and allowed water *ad libitum*. All procedures relating to the animals and their care conformed to the international guidelines 'Principles of Laboratory Animals Care' (NIH publication no. 85-23 revised 1985).

To evaluate the hypolipidemic effects of the CGJs, two kinds of hyperlipidemic animal models were established. The first hyperlipidemic mouse model was based on Trition WR-1339 and was established according to the method of Kusama et al (1988). The Triton WR-1339 was injected into the tail veins of mice under light ether anesthesia into the tail veins of mice under light ether anesthesia at the end of a regular 16 h fasting period, as a 10% solution in saline at a dose of 200 mg/kg of body weight. Six mice were used per group. These mice were anesthetized with ether 18 h after Triton WR-1339 injection, and 1-1.5 mL of blood was drawn by cardiac puncture. The sera were obtained by centrifugation (1,500×g, 10 min). The CGJs (5w/w%) or lovastain (0.005%) were mixed into the diet and were given for 5 days before Triton WR-1339 injection. The final administration of the sample-containing diets was performed 1 hr before Triton WR-1339 injection. The second hyperlipidemic mouse model was based on a high-fat diet. The mice were classified into 15 groups and each group contained 6 mice. The high-fat control group was fed a high-fat diet for 3 weeks and a normal group received a solid, normal diet. The CGJs (5w/w%) or lovastain (0.005%) mixed into the diet and were given. After a 16 h fasting period following the final administration of the diet, blood samples were drawn by cardiac puncture under ether anesthesia.

Determination of serum total cholesterol, triglyceride, and HDL cholesterol

Total cholesterol was measured by the enzymatic method of Allain et al (1974). Serum triglycerides were measured by the method designed by Sardesai and Mannig (1968). HDL cholesterol was measured by the enzymatic method of Trinh et al (2007).

Statistical analysis

All data are expressed as the mean±standard deviation, and statistical significance was analyzed by one-way ANOVA followed by the Student-Newman-Keuls test.

RESULTS

Hypolipidemic activity of CGJs in Trition WR1339-induced hyperlipidemic mice

To evaluate differences in biological activity between starters used for the preparation of CGJ, we prepared two CGJ products using *B. subtilis* and *B. licheniformis* and measured their hypolipidemic effects on Triton WR-1339-induced hyperlipidemic mice (Fig. 1). Serum triglyceride (TG) and total cholesterol (TC) levels increased after

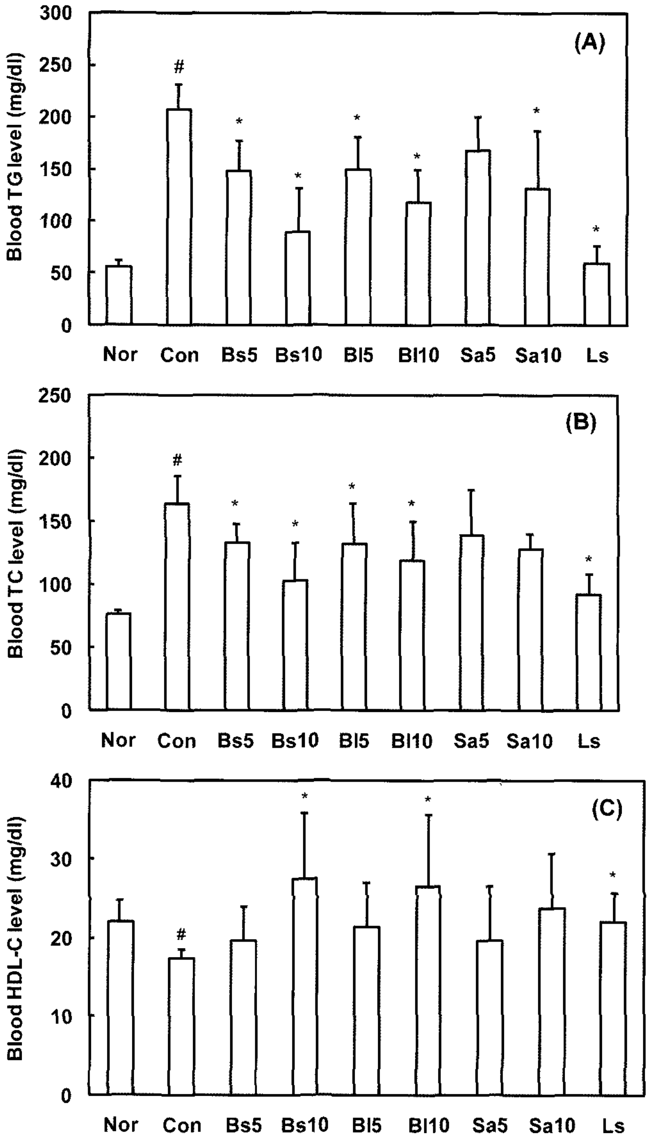

Fig. 1. Effects of Cheonggukjang (CGJ) in Trition WR-1339-induced Hyperlipidemic Mice. The normal group (Nor) received a vehicle alone. The control group (Con) was treated with Triton WR-1339 alone. The diets containing the samples were orally given for 5 days before the Triton WR-1339 treatment: Bs5, 5% *Bacillus* subtilis-fermented CGJ; Bs10, 10% *B. subtilis*-fermented CGJ; Bl5, 5% *B. licheniformis*-fermented CGJ; Bl10, 10% *B. licheniformis*-fermented CGJ; Sa5, 5% boiled soybean alone; Sa10, 10% boiled soybean alone; Ls, 0.005% lovastatin. Values indicate the mean±SD (n=6). #Significantly different, compared to the normal group ($p<0.05$). *Significantly different, compared to the control group ($p<0.05$).

treatment with the Triton WR-1339. Soy and the CGJs prepared with *B. subtilis* or *B. licheniformis* all reduced the increases induced by Triton WR-1339. Particularly, the diets containing 5 and 10% CGJ significantly reduced serum TG and TC levels compared to the normal diet. The high-fat diet decreased HDL-cholesterol, with the CGJs restoring HDL levels in the mice, The *B. subtilis*-fermented CGJ more potently lowered TG and TC levels increased by Trition WR1339, although the differences between starters were not significant.

Effect of long-term feeding of CGJs on high-fat diet-induced hyperlipidemia in mice

We next measured the hypolipidemic effects of the long-term feeding of the CGJs in high-fat diet-induced hyperlipidemic mice (Fig. 2). Triglyceride and total cholesterol levels in serum were increased by treatment of the high-fat diet for 3 weeks. Feeding of the CGJs significantly decreased these levels. The high-fat diet decreased HDL-cholesterol, with the CGJs restoring HDL levels in the mice. The CGJs were also potent at reducing increased body and epididymal mass weights induced by the high-fat diet (Fig. 3). The *B. subtilis*-fermented CGJ more potently reduced TG and TC levels that were increased by the high fat diet, although the differences between starters were not significant.

DISCUSSION

Hyperlipidemia, or hypertriglyceridemia and hyper-cholesterolemia, can cause arteriosclerosis, hypertension, obesity, diabetes, and functional organ depression (Goldin et al 1973). Many researchers have been studying the hypolipidemic effects of functional foods to develop such food as alternative treatments for hyperlipidemia, because the repeated used of orlistat and lovastatin results in side effects (Davidson 2003; Ballinger 2000; Thompson Coon and Ernst et al 2003).

Probiotic bacteria added to regular fermentation cultures provide therapeutic benefits such as stimulation of the immune system, reductions in cholesterol, the alleviation of lactose intolerance, and faster relief from diarrhea (Collins and Gibson 1999; Campieri and Gionchetti 1999). *Lactobacillus acidophilus, Streptococcus faecalis, Bifidobacterium longum, Bifidobacterium breve*, and their dairy products have hypocholesterolemic functions (Ross and Katan 2000). Also, many researchers have reported that Asian soy products fermented with probiotics, namely *Bacillus* strains, exhibit hypolipidemic effects. However, the differences in hypolipidemic effects between the strains used for CGJ fermentation are not clear. Therefore, we investigated the hypolipidemic effects of CGJs fermented with *B. subtilis* and *B. licheniformis*, respectively. Although soy alone reduced Triton WR1339-induced blood TG and TC levels, it did not significantly decrease them. However, the CGJ products fermented with the *Bacillus* strains significantly reduced WR1339-induced blood TG and TC levels. The hypolipidemic effects between the *Bacillus* strains were different, although not significantly. These results suggest that Bacillus strains offer hypolipidemic effects.

In hyperlipidemic mice induced by the long-term feeding

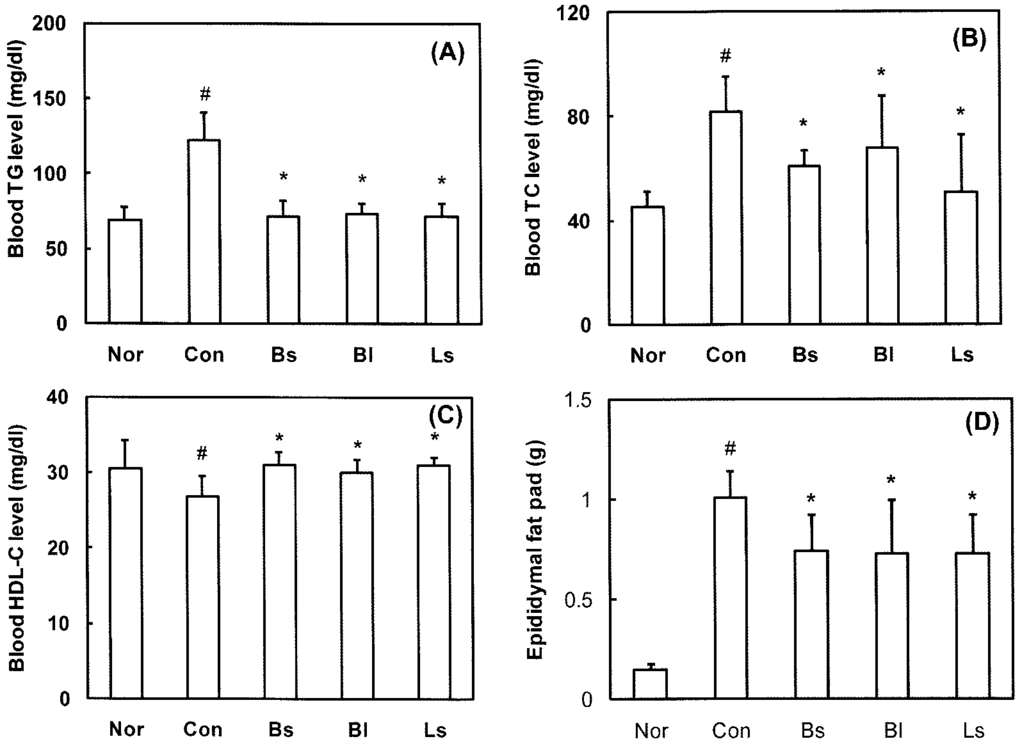

Fig. 2. Effects of Cheonggukjang (CGJ) on Serum Triglyceride (A), Total cholesterol (B), High Density Lipoprotein (HDL) Cholesterol Levels (C), and Epididymal Fat Weight (D) in Hyperlipidemic Mice Induced by a High-Fat Diet. The normal group (Nor) received a normal, solid diet and the control group (Con) was fed the high-fat diet for 3 weeks, respectively. The high fat diets containing the samples were given orally for 3 weeks: Bs5, 5% *Bacillus subtilis*-fermented CGJ; Bl5, 5% *B. licheniformis*-fermented CGJ; Ls, 0.005% lovastatin. Epididymal fat pads were taken under ether anesthesia and their weights were measured. Values indicate the mean±SD. #Significantly different, compared to the normal group (p<0.05). *Significantly different, compared to the control group (p<0.05).

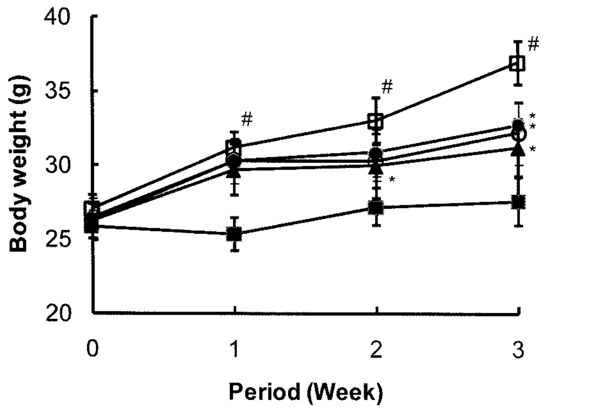

Fig. 3. Effects of Cheonggukjang (CGJ) on Body Weight in Hyperlipidemic Mice Induced by High-Fat Diets. Each group contained 6 mice with body weights of 24.3±0.93 g (mean± standard deviation) per mouse. The normal group (■) received a normal, solid diet and the control group (□) was fed the high-fat diet for 3 weeks, respectively. The high fat diets containing the samples were given orally for 3 weeks: ○, 5% *Bacillus subtilis*-fermented CGJ; ●, 5% *B. licheniformis*-fermented CGJ; ▲, 0.005% lovastatin. Body weight was measured before the final administration of the samples. Values indicate the mean±SD. #Significantly different, compared to the normal group (p<0.05). *Significantly different, compared to the control group (p<0.05).

of a high fat, the CGJs not only reduced total blood cholesterol and triglyceride levels, but also inhibited increases in body and epididymal mass weights in the mice also induced by the long-term feeding of high dietary fat. The B. subtilis-fermented CGJ product more potently reduced total blood cholesterol levels than the *B. licheniformis*-fermented product.

In conclusion, we propose that CGJ products may be effectively used as hypolipidemic foods by the synergistic interaction of soy and Bacillus strains.

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