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Sterilized bifidobacteria suppressed fat accumulation and blood glucose level

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ABSTRACT

Probiotics such as *Lactobacillus* and *Bifidobacterium* improve the balance of intestinal microflora and have various physiological functions beneficial to human health. It is not always known whether the ingested microbial cells are viable- or killed. However, even sterilized bacterial cells are functional. Bacterial cell functions are strain-specific and their modes of action are still poorly understood. The aim of this study was to elucidate the roles of sterilized bifidobacteria in obesity and lipid metabolism. To this end, mice were orally ingested sterilized bacteria. Male C57BL/6J mice aged 7 wks were raised on a high-fat diet and received oral sterilized bifidobacteria for 4 wks. Although the amount of food they ingested did not change in response to bifidobacteria administration, both weight gain and epididymal body fat mass were significantly reduced. In addition, the elevated blood glucose, triglyceride, and total cholesterol levels observed in the mice on the high-fat diet all decreased in response to bifidobacteria treatment. Hepatic triglyceride levels also decreased. Furthermore, oral glucose tolerance and insulin resistance tests indicated that sterilized bifidobacteria improved glucose tolerance and diminished insulin resistance. Sterilized bifidobacteria also decreased blood lipopolysaccharides and altered intestinal flora. The present study indicates that in mice on a high-fat diet, sterilized bifidobacteria suppressed fat accumulation, improved insulin resistance, and lowered blood glucose levels.

1. Introduction

There are ~1000 different types of symbiotic bacteria in the human intestinal tract. Their total number is estimated to be ~100 trillion. In recent years, various interactions between the host and intestinal bacterial flora have been elucidated. The balance of intestinal flora is important to human health. Probiotics help restore this balance by fortifying the intestinal microflora with “a living microorganism that has a beneficial effect on the host” [1]. However, it was recently reported that dead bacteria and their metabolites cause biological reactions such as alleviating effect of allergic diseases [2,3]. Both viable- and killed bacteria are thought to be effective for them [4]. In addition, it was reported that killed bacteria improve the intestinal flora [5]. The host's immune system often participates in the functions of killed bacteria [2,3] because the microbial cells themselves are involved. On the other hand, there are few reports on host metabolic functions.

Obesity causes many lifestyle-related diseases like diabetes, hyperlipidemia, stroke, and myocardial infarction. In mice fed a high-fat diet, a decrease in intestinal *Bifidobacterium*, increases in intestinal tract permeability and blood endotoxin levels, and mild inflammation occurred. However, these effects were reversed by the administration of fructo-oligosaccharides which are fermented by bifidobacteria. Obesity may be controlled by bifidobacteria and intestinal bacteria, in general, may be involved in this disorder [6–9]. In mice, obesity was induced when they were inoculated with human bacteria known to be associated with this condition. The transplantation of standard (non-obese; “normal”) gut microbes did not have this effect [10]. This result suggests that intestinal flora *per se* affect host body shape. Crosstalk between intestinal flora and lipids may exacerbate toll-like receptor (TLR)-mediated inflammation and promote obesity [11]. Certain intestinal bacteria may be associated with obesity. For example, many Bacteroidetes reside in obese individuals whereas non-obese people harbor Firmicutes [12]. Short-chain fatty acids (SCFAs) produced by Bacteroidetes inhibit fat uptake by adipocytes. However, as the ratio of Firmicutes to Bacteroidetes increases, dietary caloric intake increases and leads to obesity. *Lactobacillus* spp. also participate in

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obesity [13,14]. In contrast, *Bifidobacterium* spp. inhibit obesity [15]. Host diet significantly affects the intestinal flora [16]. Nevertheless, changes in the intestinal environment also influence inflammation and intestinal bacterial metabolism. Therefore, the intestinal flora is also involved in host physiology [17].

The administration of bifidobacteria to suppress the perturbations of the intestinal flora caused by a high-fat diet may help prevent obesity. The objective of this study was to determine the effects and mechanisms of sterilized *Bifidobacterium* on lipid- and sugar metabolism.

2. Materials and methods

2.1. Bifidobacteria and mice

C57BL/6J mice (male, 7 wks; CLEA Japan, Tokyo, Japan) were used in this experiment. They were raised under a 12-h cycle (light period: 7:00–19:00; dark period: 19:00–7:00). Food and water were taken *ad libitum*. After feeding the mice CE-2 (CLEA Japan, Tokyo, Japan) as a normal diet for 1 wk, they were provided with either CE-2 or a high-fat HFD-60 (Oriental Yeast Co. Ltd., Tokyo, Japan) diet. Bifidobacteria (200 mg kg^{-1}, 400 mg kg^{-1}) or double distilled water (DDW) was administered orally on a daily basis. Body weight and food intake were measured daily and twice weekly, respectively. Five weeks after HFD-60 feeding began, the mice were euthanized by cervical dislocation. Blood was collected from the heart and left to stand for 20 min. The samples were then centrifuged (1000 × g, 4 °C, 15 min) to obtain the serum. Livers, epididymal adipose tissue, and ceca were excised and immediately frozen in liquid nitrogen. *Bifidobacterium longum* BR-108 sterilized at 105 °C for 20 min was obtained from the Combi Corporation (Tokyo, Japan), and was orally administered to the mice at a dose of 200 mg kg^{-1} or 400 mg kg^{-1} BW.

2.2. Glucose test

Four weeks after being fed with HFD-60, the mice were fasted overnight and subjected to an insulin resistance test (ITT). Insulin (Humulin R 100, Eli Lilly Japan, Kobe, Japan) 15 μL was diluted with 20 mL phosphate-buffered saline (PBS) and administered intraperitoneally at 10 μL g^{-1} BW. Blood glucose was measured before- (0 min) and after (15-, 30-, 60-, and 120 min) insulin administration using Glucose Pilot (Iwai Chemicals, Tokyo, Japan). Three days after the ITT, an oral glucose tolerance test (OGTT) was performed again overnight. Ten percent of a 15% D (+)-glucose solution was orally administered per gram body weight. Blood glucose was measured before- (0 min) and after (15-, 30-, 60-, and 120 min) glucose administration.

2.3. Blood analysis

Serum glucose, -triglyceride, and -cholesterol were measured using LabAssay™ Glucose, LabAssay™ Triglyceride, and LabAssay™ Cholesterol (Wako Pure Chemical Industries Ltd., Chuo-Ku, Osaka, Japan), respectively. Lipopolysaccharide (LPS) in serum was diluted 10 × with DDW, heated to 70 °C for 15 min to inactivate the inhibitor, diluted 10 × with DDW once again, and measured with a Limulus Color KY Test (Wako Pure Chemical Industries Ltd., Chuo-Ku, Osaka, Japan).

2.4. Lipid analysis

Lipids were extracted from the liver and ceca by the Folch method [18]. One milliliter of a 2:1 v/v chloroform (Wako Pure Chemical Industries Ltd., Chuo-Ku, Osaka, Japan):methanol (Wako Pure Chemical Industries Ltd., Chuo-Ku, Osaka, Japan) mixture was added to 50 mg liver tissue or cecal contents. The liver tissue was homogenized with a BioMasher II (Nippi, Tokyo, Japan) and a DISRUPTOR-GENIE (M & S Instrument Inc., Osaka, Japan). The homogenates were stirred at 25 °C for 15 min. The supernatant was obtained by centrifugation (1000 × g, 4 °C, 15 min). A 20 × volume of 0.9% w/v saline was added to the supernatant followed by vortexing and recentrifugation. The precipitate was dried in a fume hood, dissolved in isopropanol (Wako Pure Chemical Industries Ltd., Chuo-Ku, Osaka, Japan) and measured using a LabAssay™ Triglyceride kit (Wako Pure Chemical Industries Ltd., Chuo-Ku, Osaka, Japan).

2.5. cDNA synthesis and DNA preparation

RNA was extracted from epididymal adipose tissue using RNAiso Plus (TaKaRa Bio Inc., Kusatsu, Shiga, Japan). Genomic DNA was removed using a PrimeScript™ RT Reagent Kit with a gDNA Eraser (TaKaRa Bio Inc., Kusatsu, Shiga, Japan) and the cDNA was synthesized.

Bacterial DNA was extracted from the cecal contents using ZR Fecal DNA Mini Prep™ (Zymo Research, Irvine, CA, USA).

2.6. qPCR

Quantitative PCR was performed on the cDNA obtained from epididymal adipose tissue and the bacterial DNA extracted from cecal contents. The internal standards were β-actin and 16 S rRNA, respectively. The primers used are shown below:

Mouse β-Actin	5′-TTGCTGACAGGATGCAGAAG-3′
	5′-GTACTTGCGCTCAGGAGGAG-3′
C-C motif chemokine (ccl) 2	5′-AGGTCCCTGTCATGCTTCTGG-3′
	5′-CTGCTGCTGGTGATCCTCTTG-3′
All bacteria 16SrRNA	5′-GTGSTGCAYGGYTGTCGTCA-3′
	5′-ACGTCRTCCMCACCTTCCTC-3′
Bacteroidetes 16SrRNA	5′-GGAGYATGTGGTTTAATTCGAAGCA-3′
	5′-AGCTGACGACAACCATGCAG-3′
Firmicutes 16SrRNA	5′-GGARCATGTGGTTTAATTCGATGAT-3′
	5′-AGCTGACGACAACCATGCAC-3′
Bifidobacterium spp. 16SrRNA	5′-TCGCGTCYGGTGTGAAAG-3′
	5′-CCACATCCAGCRTCCAC-3′
Lactobacillus spp. *16SrRNA*	5′-AGCAGTAGGGAATCTTCCA-3′
	5′-CACCGCTACACATGGAG-3′

2.7. Statistical analysis

Significant differences were determined using a *t*-test. $^{*}p < 0.05$; $^{**}p < 0.001$.

3. Results

3.1. Inhibition of fat accumulation by sterilized bifidobacteria

First, changes in body weight in response to a high-fat diet were observed. C57BL/6J mice were divided into five groups designated Chow-Ct, Chow-400, HFD-Ct, HFD-200, and HFD-400. Mice were fed either a normal diet (chow) or a high-fat diet (HFD) and orally administered DDW (Ct) or sterilized bifidobacteria (200 mg kg^{-1}, 400 mg kg^{-1}) each day for 4 wks. Weight gain was significantly suppressed in the HFD-200- and HFD-400 groups (Fig. 1A and B). However, there was no significant difference between the HFD-200 group and the HFD-400 group (Fig. 1B). In the Chow-400 group,

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