Oral administration of Bifidobacterium spp. improves insulin resistance, induces adiponectin, and prevents inflammatory adipokine expressions

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ABSTRACT

The intestinal microbiome might be an important contributor to the development of type 2 diabetes. This study was designed to test the hypothesis that oral administration of Bifidobacterium species (spp.) (including B. longum, B. bifidum, B. infantis, and B. animalis) may both ameliorate insulin resistance and reduce the expressions of inflammatory adipocytokines. Male Swiss-Webster mice fed a high-fat diet with or without oral administration of Bifidobacterium spp. for 5 weeks were subjected to an insulin tolerance test and an oral glucose tolerance test. Plasma levels of glucose at 30, 60, 90 and 120 min after insulin injection or glucose administration were significantly lower in the Bifidobacterium spp. than in the control group (P < 0.05), showing the beneficial effect of oral administration on insulin resistance in obese Swiss mice. In addition, Bifidobacterium spp. increased the adiponectin mRNA level and decreased those of monocyte chemotactic protein 1 and interleukin 6 in non-diabetic C57BL/6J mice fed a normal diet, indicating a molecular mechanism which may ameliorate the inflammatory state, thereby reducing insulin resistance. In conclusion, oral administration of Bifidobacterium spp. improves insulin resistance and glucose tolerance in obese mice by reducing inflammation, as it does in the lean state.

Type 2 diabetes mellitus or type 2 diabetes has become a major health burden worldwide. The global prevalence of diabetes among adults 20–79 years of age is expected to increase from 6.4% in 2010 to 7.7% by the year 2030 (25). The three most widely-accepted cornerstones of treatment are diet, physical activity, and medications (27). Recently, several lines of research have revealed that the intestinal microbiome might be an important contributor to the improvement on insulin resistance (19, 20). Several probiotic strains have been reported to exert anti-obesity effects in murine models (17, 19). One study found that Bifidobacterium spp. constitutes a significant portion of probiotic cultures used in developed countries (4). Mixtures of bifidobacteria, lactobacilli and Streptococcus thermophilus can reportedly improve hepatic insulin resistance and lipid metabolism (18). However, there have been only a few studies focusing on the anti-diabetic effect of Bifidobacterium spp. and the underlying molecular mechanism in animal models.

Insulin resistance, a major abnormality underlying type 2 diabetes, is characterized by dysfunctional glucose uptake into muscle and adipose tissue in conjunction with an oversupply of glucose from the liver, which results in high circulating levels of plasma glucose. In the insulin signaling pathway, adiponectin enhances insulin sensitivity by increasing hepatic insulin receptor substrate 2 expression via a macro-

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phage-derived interleukin-6 (IL-6)-dependent pathway (3), and by activating 5’ adenosine monophosphate-activated protein kinase in C2C12 myocytes, skeletal muscle and the liver (29). Adiponectin also prevents hepatic glucose production by decreasing the mRNA expressions of two fundamental gluconeogenesis enzymes: phosphoenolpyruvatecarboxykinase and glucose-6-phosphatase (12). Besides adiponectin, several inflammatory adipokines related to insulin resistance and type 2 diabetes, such as tumor necrosis factor alpha (TNF-α), monocyte chemoattractant protein 1 (MCP-1), IL-6 and leptin, have also recently been studied (2). Adipose tissues, such as adipocytes or adipose tissue-infiltrating macrophages, are also known to secrete cytokines and chemokines leading to a chronic sub-inflammatory state, which may play a central role in the development of insulin resistance and type 2 diabetes (2). Moreover, adiponectin expression is inhibited by IL-6 in fat cells (8). Adiponectin is down-regulated in states of insulin resistance and obesity (11). MCP-1 induces insulin resistance (13) and circulating IL-6 levels are increased in obese and insulin resistant subjects (14, 21). We thus speculated that oral administration of *Bifidobacterium* spp. to mice might improve insulin resistance and possibly even reduce the productions of certain adipocytokines (adiponectin, IL-6, MCP-1). Therefore, this study aimed to evaluate the effects of oral administration of *Bifidobacterium* spp. on insulin resistance and glucose tolerance in Swiss-Webster mice fed a high-fat diet (HFD), as well as those on the mRNA expressions of adiponectin, MCP-1 and IL-6 in C57BL/6J mice.

**MATERIALS AND METHODS**

*Preparation of Bifidobacterium spp. strains for administration.* *Bifidobacterium* spp. strains including *B. bifidum* ATCC 15700, *B. longum*, *B. infantis*, and *B. animalis* were obtained from DVS Biolife Limited (Hyderabad, India). *Bifidobacterium* spp. strains were cultured in Lactobacillus broth (Merek, Germany) (5) at 37°C for 24 h. The cultured strains were collected and then washed three times with phosphate buffered saline by centrifugation at 9,000 × g for 30 min at 4°C. Concentration of the cultured strains were measured using the Nanodrop 1000 (Thermo Scientific, USA) and then recounted by culturing on Lactobacillus agar (Merek) (5) at 37°C for 72 h. Mixing of freshly prepared live bacteria was performed daily for administration to each mouse just prior to use. The mixture of *Bifidobacterium* spp. included 10⁹ colony-forming units (CFU) of *B. longum*, 10⁹ CFU of *B. bifidum*, 10⁹ CFU of *B. infantis*, and 10⁹ CFU of *B. animalis*. This mixture was fed to the mice once a day.

**Experimental design.** Twelve 10-week-old male Swiss-Webster mice (Swiss mice) were obtained from the National Institute of Hygiene and Epidemiology, Vietnam. They were given free access to tap water and a HFD (Oriental Yeast Co. Ltd., Tokyo, Japan) with 62.2% of kcal from fat for one month. The mice were randomly assigned to two groups. The trial group (6 mice) was fed the HFD with the mixture of *Bifidobacterium* spp. by oral gavage for 5 weeks, while the control group (6 mice) continued to receive the HFD for 5 weeks. During the same period, the control group was given saline at the same volume by oral gavage for 5 weeks. The HFD contained (per 100 g of the diet) 25.60 g of milk casein, 0.36 g of L-cysteine, 16.00 g of α-corn starch, 6.00 g of maltodextrin, 5.50 g of sucrose, 2.00 g of soybean oil, 6.61 g of powdered cellulose, 3.50 g of AIN-93M mineral mixture, 1.00 g of AIN-93M vitamin mixture, 0.25 g of choline bitartrate, 0.18 g of calcium carbonate, and 33.00 g of lard. After the five-week treatment period, the mice were subjected to an insulin tolerance test and an oral glucose tolerance test to evaluate insulin resistance and glucose tolerance, respectively.

Ten-week-old male C57BL/6J mice (n = 10) were obtained from the Jackson Laboratory (Bar Harbor, ME, USA). They were randomly divided into 2 groups and fed the normal diet (Oriental Yeast) for 5 weeks. The trial group (5 mice) received the mixture of *Bifidobacterium* spp. for 5 weeks by oral gavage while the control group (5 mice) ate the normal diet. The control group was also given saline at an equivalent volume by oral gavage for 5 weeks. The normal diet (AIN-93M) contained (per 100 g of the diet) 14.00 g of milk casein, 0.18 g of L-cysteine, 46.57 g of corn starch, 15.50 g of α-corn starch, 10.00 g of sucrose, 4.00 g of soybean oil, 5.00 g of powdered cellulose, 3.50 g of AIN-93M mineral mixture, 1.00 g of AIN-93M vitamin mixture, 0.25 g of choline bitartrate, 0.0008 g of tert-butylhydroquinone. Next, C57BL/6J mice in both groups were used for experiments involving extraction of total RNA from adipose tissues. Reverse transcription PCR and quantitative real-time RT-PCR were performed to measure mRNA levels of the adiponectin, IL-6, and MCP-1 genes.

All mice were maintained and treated in accordance with the Guidelines and Regulations for Experiments on Vertebrate Animals of the National
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Institute for Food Control in Testing, Research, and Training. All mice were housed individually in plastic cages in a conventional room with controlled light (08:30–20:30), temperature (25 ± 1°C), and humidity (60 ± 5%). For each mouse, weight was measured once a week and food intake was recorded every 2 days. At the end of the experimental period, mice were fasted for 6 h, anesthetized with diethyl ether and then exsanguinated by cardiac puncture. The mesenteric and epididymal fat tissues of each mouse were removed and weighed.

Oral glucose tolerance test and enzyme-linked immunosorbent assay. Glucose tolerance was evaluated using the oral glucose tolerance test, as previously described (1). Briefly, after a 6-hour fast, D-glucose (Wako Pure Chemical Industries) was administered to Swiss mice (6 mice) at a dose of 2 g/kg body weight by oral gavage. The control group (6 mice) was given an equivalent volume of respectively saline by oral gavage. Before and at 15, 30, 60, 90 and 120 min after glucose administration, blood samples were collected with heparinized capillary tubes from the tail vein and immediately placed on ice. Plasma was prepared by centrifugation at 830 × g for 15 min at 4°C. The plasma samples obtained before and at 15, 30, 60, 90 and 120 min after glucose administration were used to assay plasma glucose employing the glucose CII-test Wako (Wako Pure Chemical Industries). The plasma samples obtained before and at 15 and 30 min after glucose administration were applied to the assay of plasma insulin using the Mouse Insulin ELISA Kit (Shibayagi, Gunma, Japan).

Insulin tolerance test. Diluted regular human insulin (Eli Lilly, USA) was injected intraperitoneally into Swiss mice (n = 6) at a dose of 0.75 units/kg body weight under non-fasting conditions. The control group (6 mice) was injected with an equivalent volume of saline. Before and at 30, 60, 90, and 120 min after insulin injection, blood samples were collected from the tail vein with heparinized capillary tubes and immediately placed on ice. Plasma was prepared by centrifugation at 830 × g for 15 min at 4°C and applied to the assay of plasma glucose using the glucose CII-test Wako (Wako Pure Chemical Industries).

RNA extraction and reverse transcription PCR. Total RNA was extracted from adipose tissue of C57BL/6J mice using TRIzol reagent (Life Technologies, Carlsbad, CA, USA). The RNA was treated with DNase I (Life Technologies), and 0.5 μg RNA was reverse transcribed using TaKaRa PrimeScript™ RT reagent kits according to the manufacturer’s instructions (Takara Bio, Kyoto, Japan).

Quantitative real-time RT-PCR. Samples were prepared employing TaKaRa SYBR Premix Ex Taq according to the manufacturer’s instructions (Takara Bio). Quantitative real-time polymerase chain reaction (quantitative real-time RT-PCR) with 100 ng cDNA was performed using the CFX96 system (Bio-Rad Laboratories, Hercules, CA, USA). The following oligonucleotide primers were used: adiponectin 5’-TGTTGGAATGACAGGAGCTG-3’ (forward) and 5’-CGAATGGGTACATTGGGAAC-3’ (reverse); IL-6 5’-ACAACCACGGCTTCCCTACTT-3’ (forward) and 5’-CACGATTTCCACGATTTCCACT-3’ (reverse); MCP-1 5’-CCCAATGAGTAGGCTGGAGA-3’ (forward) and 5’-TCTGGACCCATTCCTTCTTG-3’ (reverse); 18S ribosomal RNA 5’-AAACGGCTACCACATCCAAG-3’ (forward) and 5’-GGCCTCGAAAGAGTCCTGTA-3’ (reverse). The annealing temperature for the PCR reactions was 60°C, product sizes ranged from 172 to 225, and the total reaction mixture volume was 20 μL. After the reaction, each PCR product was verified for its single amplification by melting curve analysis. Data were normalized by the 18S ribosomal RNA expression levels in each sample and shown as the means ± SE of 3 independent quantitative-PCR analyses.

Statistical analysis. The values are presented as means ± SE. Significant differences (P < 0.05) between means were evaluated employing the unpaired Student’s t-test.

RESULTS

Effects of Bifidobacterium spp. on insulin resistance and glucose tolerance in Swiss mice fed a HFD

In this study, two different mouse strains (Swiss and C57BL/6J) were used. First, we examined the effect of Bifidobacterium spp. on insulin resistance and glucose tolerance in Swiss mice fed a HFD. Therefore, the effects of Bifidobacterium spp. on insulin resistance and glucose tolerance were examined by insulin tolerance test and oral glucose tolerance test, respectively. In the insulin tolerance test, although the plasma glucose levels were similar in the control and trial groups before insulin injection, the plasma glucose levels at 30, 60, 90 and 120 min after insulin injection were significantly lower in the Bifidobacterium spp. than in the control group (P < 0.01 or
Several probiotic strains have been reported to exert an anti-obesity effect in murine models (17, 19). However, few studies have examined the effects of probiotic strains on insulin resistance. Therefore, the present study was designed to determine the effects of \textit{Bifidobacterium} spp. on adipokine expressions, insulin resistance, and glucose tolerance in mice. We demonstrated oral administration of \textit{Bifidobacterium} spp. to significantly reduce plasma glucose levels during not only insulin tolerance test but also oral glucose tolerance test in obese Swiss mice fed a HFD. In addition, our study revealed that \textit{Bifidobacterium} spp. treatment induced the expression of adiponectin mRNA while decreasing the expressions of MCP-1 and IL-6 mRNA in adipose tissues of lean (C57BL/6J) mice.

Probiotics are traditionally defined as live microorganisms that, when administered in adequate amounts, confer a health benefit on their host. A recent study relating to metabolic abnormalities suggested \textit{Lactobacillus casei} heat-killed to be a potential candidate for diabetic patients based on its abilities to improve insulin resistance and increase oral glucose tolerance in mice with diabetes due to an inap
Bifidobacterium species improve insulin resistance (20). In addition, a previous study, not dealing with probiotics, revealed a reduction of visceral fat in animal models and humans to be associated with increased insulin sensitivity (6). In our study, Bifidobacterium spp. treatment showed no significant effect on either body weight or intra-ab-

![Figure 2](image)

**Figure 2** Effects of Bifidobacterium spp. on glucose and insulin responses in an oral glucose tolerance test in Swiss mice fed a high fat diet. Ten-week-old male Swiss-Webster mice (n = 12) were fed a high fat diet (HFD) for 4 weeks. Then, these mice were fed only the HFD or the HFD containing Bifidobacterium spp. for an additional 5 weeks. After the mice had been fasted for 6 h, oral glucose tolerance test were performed. The plasma samples were used to measure glucose and insulin concentrations as described in Materials and Methods. The time course of plasma glucose (120 min) (a), area under the curve (AUC) of plasma glucose (b), time course of plasma insulin (30 min) (c), and the AUC of plasma insulin (d) were then plotted. Values are expressed as means ± SE (n = 6 mice in each group). **P < 0.01, *P < 0.05 (unpaired Student’s t-test). ●: Control; ○: Bifidobacterium spp. (trial group).

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control (n = 6)</th>
<th>Bifidobacterium spp. (n = 6)</th>
</tr>
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<tbody>
<tr>
<td>Body weight (initial) (g)</td>
<td>30.06 ± 0.75</td>
<td>29.58 ± 0.68&lt;sup&gt;NS&lt;/sup&gt;</td>
</tr>
<tr>
<td>Body weight (final) (g)</td>
<td>57.38 ± 0.98</td>
<td>55.98 ± 0.77&lt;sup&gt;NS&lt;/sup&gt;</td>
</tr>
<tr>
<td>Dietary intake (g/2 days)</td>
<td>8.97 ± 0.96</td>
<td>8.56 ± 0.83&lt;sup&gt;NS&lt;/sup&gt;</td>
</tr>
<tr>
<td>Mesenteric fat weight (g)</td>
<td>2.76 ± 0.18</td>
<td>2.59 ± 0.11&lt;sup&gt;NS&lt;/sup&gt;</td>
</tr>
<tr>
<td>Epididymal fat weight (g)</td>
<td>1.18 ± 0.09</td>
<td>1.30 ± 0.13&lt;sup&gt;NS&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Ten-week-old male Swiss-Webster mice (n = 12) were fed a high-fat diet (HFD) for 4 weeks. Then, these mice were fed a HFD with or without mixture of Bifidobacterium spp. for 5 weeks by oral gavage. The control group was given the same volume of saline for 5 weeks by oral gavage. Values are expressed as means ± SE. NS, No significant differences between control and Bifidobacterium spp. (trial) groups.
In that study, the authors drew no conclusions about the effects of each bacterium administered in terms of ameliorating insulin resistance.

Adiponectin is down-regulated in states of insulin resistance and obesity (11). Moreover, it is known that type 2 diabetes patients have low adiponectin levels (28). It has been shown that fat-derived adiponectin is reduced in settings of insulin resistance and obesity, and that its replenishment reverses insulin resistance in mice (7). Thus far, adiponectin has emerged as an important adipocytokine that improves insulin sensitivity, raising the possibility of its use pharmacologically for treatment of the diabetics (10, 28). In line with the aforementioned reports, oral administration of *Bifidobacterium* spp. in this study increased adiponectin mRNA, suggesting the involvement of adiponectin enhancement in the amelioration of insulin resistance. Further, *Bifidobacterium* spp. treatment reduced plasma glucose elevations not only at 30, 60, 90, and 120 min after glucose loading in oral glucose tolerance test but also at baseline (under fasting conditions) in the trial group as compared to the control group, without significant changes in plasma insulin levels. In addition, plasma glucose levels at baseline (non-fasting condition) did not differ significantly between these two groups in insulin tolerance test. Therefore, oral administration of a mixture of *Bifidobacterium* spp. was found to improve glucose tolerance and reduce insulin resistance. Adiponectin is a proven mediator of insulin sensitivity, raising the possibility of its use pharmacologically for treatment of type 2 diabetes.

Table 2  **Body weight, dietary intake and tissue weights in C57BL/6J mice**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control (n = 5)</th>
<th>Bifidobacterium spp. (n = 5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight (initial) (g)</td>
<td>27.77 ± 0.89</td>
<td>27.78 ± 0.95&lt;sup&gt;NS&lt;/sup&gt;</td>
</tr>
<tr>
<td>Body weight (final) (g)</td>
<td>32.24 ± 0.52</td>
<td>31.70 ± 0.43&lt;sup&gt;NS&lt;/sup&gt;</td>
</tr>
<tr>
<td>Dietary intake (g/2 days)</td>
<td>3.80 ± 0.41</td>
<td>3.53 ± 0.45&lt;sup&gt;NS&lt;/sup&gt;</td>
</tr>
<tr>
<td>Mesenteric fat weight (g)</td>
<td>0.52 ± 0.09</td>
<td>0.57 ± 0.05&lt;sup&gt;NS&lt;/sup&gt;</td>
</tr>
<tr>
<td>Epididymal fat weight (g)</td>
<td>0.48 ± 0.05</td>
<td>0.44 ± 0.06&lt;sup&gt;NS&lt;/sup&gt;</td>
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</table>

Ten-week-old male C57BL/6J mice (n = 10) were fed a normal diet with or without the mixture of *Bifidobacterium* spp. for 5 weeks by oral gavage. The control group was given the same volume of saline for 5 weeks by oral gavage. Values are expressed as means ± SE. *NS*, No significant differences between the control and *Bifidobacterium* spp. (trial) groups.
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IL-6 was reported to reduce insulin-dependent hepatic glycogen synthesis (16, 24) and glucose uptake in adipocytes (23). Macrophage infiltration of white adipose tissue is an important feature of the low-grade inflammation characteristic of obesity and is the principal source of IL-6 and TNF-α, which induce insulin resistance in adipocytes (2). Increased MCP-1 in adipose tissue, which contributes to macrophage infiltration of these tissues, is also related to insulin resistance (13). The present study showed that oral administration of Bifidobacterium spp. markedly reduces IL-6 and MCP-1 expressions. These results are in accordance with recent findings indicating that a mixture of Bifidobacterium infantis and Lactobacillus acidophilus decreased the IL-6 mRNA level in primary intestinal epithelial cells isolated from resected ileum with necrotizing enterocolitis (9). In addition, our study is consistent with a recent investigation demonstrating that Bifidobacterium longum in a bacterial mixture significantly down-regulated the levels of MCP-1 and IL-6 in porcine intestinal epithelial cells challenged with heat-killed enterotoxigenic Escherichia coli (26). Moreover, administering Lactobacillus acidophilus and Bifidobacterium longum, or Lactobacillus plantarum, Streptococcus thermophilus and Bifidobacterium animalis subsp. lactis inhibited MCP-1 production in colitis in BALB/c mice (22). Bifidobacterium bifidum suppressed MCP-1 expression in an in vitro co-culture of both intestinal epithelial cells and T cells (15). Our present findings showed expressions of IL-6 and MCP-1 mRNA to be decreased after administration of the probiotic mixture of Bifidobacterium spp. and thus support the role of Bifidobacterium spp. demonstrated in other recent studies, although different animal strains and organs/tissues were examined. Finally, oral administration of Bifidobacterium spp., as shown in this study, may play a role in eliminating the pathogenic cycles of insulin resistance in type 2 diabetes patients. In addition, there were no significant differences in body weight or fat weight between the control and trial groups at either the beginning or the end of the experimental period. Therefore, the increase in adiponectin expression and the decreases in MCP-1 and IL-6 expressions were not influenced by obesity status. It is possible that a mechanism other than the suppression of obesity may be involved in the increased adiponectin expression, which may have effects on insulin sensitivity and glucose tolerance. We did not, herein, clarify individual roles of B. longum, B. bifidum, B. infantis, and B. animalis in the Bifidobacterium spp. strain mixture. Further studies are needed to clarify the effects of each bacterial species.

In conclusion, oral administration of Bifidobacterium spp. increases adiponectin mRNA expression and decreases those of inflammatory adipokines (IL-6 and MCP-1) in lean mice. Moreover, the current study demonstrated that a mixture of Bifidobacterium spp. also improves insulin resistance and glucose tolerance in obese mice. Our findings reveal new potentially beneficial effects of treatment with Bifidobacterium spp. for insulin resistance in type 2 diabetic mice. It is necessary to elucidate the underlying mechanisms of action and assess the efficacy of Bifidobacterium spp. treatment in human subjects.

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REFERENCES