Original paper

Effects of γ-Polyglutamic Acid on the Cecal Microbiota and Visceral Fat in KK-A\textsuperscript{y}/TaJcl Male Mice

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Natto is a major functional food in Japan. γ-Polyglutamic acid (γ-PGA) is one of the main components of Natto, and beneficial effects of γ-PGA have been reported. We tested the hypothesis that γ-PGA alleviates metabolic syndrome in KK-A\textsuperscript{y}/TaJcl male mice. These mice were fed a diet containing 0.5% of γ-PGA or a control diet for 28 days. Visceral fat was significantly smaller in the PGA group than in the CON group. The amounts of lipids in feces (dry weight) sampled on the final day of the experiment and cecal Lactobacillus counts were significantly greater in the PGA group than in the CON group. Cecal Prevotella counts tended to be higher in the PGA group. These results suggest that dietary γ-PGA affects the cecal microbiota and ameliorates accumulation of visceral fat in the KK-A\textsuperscript{y}/TaJcl mouse model of type 2 diabetes mellitus.

Keywords: γ-polyglutamic acid, microbiota, visceral fat, mice

Introduction

Much attention has been focused on fermented foods as functional food products in Japan. γ-Polyglutamic acid (γ-PGA) is one of the main components of Natto, which is a food with high viscosity. γ-PGA affects the viscosity of Natto and contributes to its functional properties.

Natto is associated with reduced bone loss in postmenopausal women (Ikeda \textit{et al}., 2006). γ-PGA increases calcium absorption in rats (Yang \textit{et al}., 2008) and humans (Tanimoto \textit{et al}., 2007); therefore, the bone loss–reducing effects of Natto seem to be partly attributable to the increased calcium absorption caused by γ-PGA.

It is known that γ-PGA supplementation significantly increases serum concentrations of glutamate and γ-amino butyric acid (Lee \textit{et al}., 2010), and hypotensive activity of γ-amino butyric acid in rats has been demonstrated (Hayakawa \textit{et al}., 2004).

It has been reported that \textit{Bacillus subtilis} (natto) produces the PGA and enhances the growth and/or viability of lactobacilli (Hosoi \textit{et al}., 2000). PGA seems to affect some kinds of bacteria.

It was reported that high-molecular-weight γ-PGA increases high-density lipoprotein (HDL) cholesterol levels in mice (Park \textit{et al}., 2011). Anti-obesity effects of γ-PGA with and without isoflavone have been shown (Lee \textit{et al}., 2013). Natto ingestion lowers postprandial blood glucose levels in healthy adults (Taniguchi \textit{et al}., 2008), and consumption of a diet containing Natto and viscous vegetables for 2 weeks improves insulin sensitivity, serum lipids, and oxidative stress status (Taniguchi-Fukatsu \textit{et al}., 2012). It has been reported that high-molecular-weight poly-gamma-glutamate has an anti-hypertriglyceridemic effect in high fructose diet-induced hypertriglyceridemic rats (Jeon \textit{et al}., 2013). According to these observations, γ-PGA seems to have some preventive effects on metabolic syndrome.

Judging by available studies, γ-PGA seems to play an important role in health promotion.

Abbreviations: coenzyme A (CoA), control (CON), high-density lipoprotein (HDL), lipopolysaccharide (LPS), nonesterified fatty acid (NEFA), γ-polyglutamic acid (γ-PGA), quantitative PCR (qPCR)

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role in the functional properties of Natto. Nonetheless, there are few reports about the effects of γ-PGA on plasma and fecal lipids or cecal microbiota. We tested the hypothesis that γ-PGA alleviates metabolic syndrome. It is thought that insulin resistance exists in the molecular basis of metabolic syndrome (Tamori et al., 2007). Insulin resistance is closely related to type 2 diabetes mellitus (Nakano et al., 2016). We evaluated this hypothesis by feeding γ-PGA to KK-A\textsuperscript{-}y/TaJcl mice, which serve as a model of type 2 diabetes mellitus.

Materials and Methods

γ-PGA was purchased from Meiji Food Materia Co., Ltd. (Tokyo, Japan). The molecular weight of γ-PGA was 960000 Da, and purity was more than 70%.

Treatments of animals Male KK-A\textsuperscript{-}y/TaJcl mice (6 weeks old) were purchased from CLEA Japan, Inc. (Tokyo, Japan). All the mice were specific pathogen-free (SPF) and were randomly subdivided into two groups of seven animals each. The mice were housed individually in suspended stainless-steel cages with wire mesh bottoms in a room maintained at 24 ± 0.5°C and relative humidity 65%, with 12-h periods of light and darkness. The mice were fed the AIN-93G diet for 6 days. After 6 days, the diet was replaced with a 0.5% γ-PGA–containing diet or control diet for 28 days. Table 1 presents the composition of each diet. Body weight and food consumption were measured during the experiment, and feces were collected for 3 days immediately before dissection. Feces were dried in a lyophilizer (FD-1000). After that, weights of freeze-dried feces were measured, and the feces were pulverized in a food mill (TML17. TESCOM Co., Ltd., Tokyo, Japan) for 30 s. Fecal lipids were extracted from the fecal powder by the Bligh and Dyer method (Bligh et al., 1959).

DNA extraction from cecal contents Procedures of DNA extraction from cecal contents were conducted according to Matsuki’s method (Matsuki, 2006). Cecal samples (20 mg) were washed two times by resuspending them in 0.2 mL of PBS and centrifuging each preparation at 14,000 × g to remove possible PCR inhibitors. Following the second centrifugation, the cecal pellets were resuspended in a solution consisting of 0.2 mL of PBS, analysis of the intestinal microbiota. The liver samples and visceral fat were weighed. All the procedures involving mice in this study were approved by the Animal Care Committee of Food Research Institute (Tsukuba, Japan) in accordance with the “Guidelines for Animal Care and Experimentation” of Food Research Institute, National Agriculture and Food Research Organization (Tsukuba, Japan).

Measurement of plasma cholesterol, NEFA, HDL-cholesterol, glucose, and triglyceride levels The following tests were performed with kits obtained from Wako Pure Chemical Industries Ltd. (Osaka, Japan). Total plasma cholesterol concentrations were measured using a cholesterol E-test kit based on cholesterol oxidase. Plasma triglyceride concentrations were determined using a triglyceride E-test kit based on the glycerol-3-phosphate oxidase method. Plasma nonesterified fatty acid (NEFA) concentrations were measured using a NEFA C-test kit involving the acyl-CoA synthase, acyl-CoA oxidase, and peroxidase method. Plasma HDL-cholesterol concentrations were determined by means of a HDL-cholesterol E-test kit based on the cholesterol esterase, cholesterol oxidase, and peroxidase method. Plasma glucose concentrations was quantified by means of a glucose CII-test kit involving glucose oxidase and peroxidase.

Measurement of fecal weight and fecal lipid extraction Feces were collected 3 days before dissection and were then dried in the lyophilizer (FD-1000). After that, weights of freeze-dried feces were measured, and the feces were pulverized in a food mill (TML17. TESCOM Co., Ltd., Tokyo, Japan) for 30 s. Fecal lipids were extracted from the fecal powder by the Bligh and Dyer method (Bligh et al., 1959).

Table 1. Composition of the experimental diet

<table>
<thead>
<tr>
<th>Ingredient (g/kg diet)</th>
<th>CON diet</th>
<th>PGA (0.5%γ-polyglutamic acid) diet</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cornstarch</td>
<td>297.486</td>
<td>297.486</td>
</tr>
<tr>
<td>Casein</td>
<td>200</td>
<td>195</td>
</tr>
<tr>
<td>α-Cornstarch</td>
<td>132</td>
<td>132</td>
</tr>
<tr>
<td>Sucrose</td>
<td>200</td>
<td>200</td>
</tr>
<tr>
<td>Soy bean oil</td>
<td>70</td>
<td>70</td>
</tr>
<tr>
<td>Cellulose</td>
<td>50</td>
<td>50</td>
</tr>
<tr>
<td>Mineral mix (AIN-93G-Mix)</td>
<td>35</td>
<td>35</td>
</tr>
<tr>
<td>Vitamin mix (AIN-93-Mix)</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>L-Cystine</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>Choline bitartrate</td>
<td>2.5</td>
<td>2.5</td>
</tr>
<tr>
<td>Tert-butylhydroquinone</td>
<td>0.014</td>
<td>0.014</td>
</tr>
<tr>
<td>γ-Polyglutamic acid</td>
<td>-</td>
<td>5</td>
</tr>
</tbody>
</table>

Table 1. Composition of the experimental diet
γ-Polyglutamic Acid: Effects on Cecal Microbiota and Adiposity of Mice

300 μL of extraction buffer (250 μL of 250 mM Tris-HCl, 80 mM EDTA, pH 9.0, plus 50 μL of 10% sodium dodecyl sulfate). A total of 300 mg of glass beads (diameter 0.1 mm) and 500 μL of buffer-saturated phenol were added to the suspension, and the mixture was vortexed vigorously for 180 s using a MicroSmash (Tomy Seiko Co., Ltd, Tokyo, Japan) at 4000 rpm. After centrifugation at 14,000 × g for 5 min, 400 μL of the supernatant was collected. Phenol–chloroform–isoamyl alcohol extraction was then performed, and 250 μL of the supernatant was subjected to isopropanol precipitation. Finally, the obtained DNA was dissolved in 1 mL of 10 mM Tris-EDTA buffer, pH 8.0. The DNA solution was checked by electrophoresis in a 1.4% agarose gel and adjusted to a final concentration of 10 μg/mL in the same buffer.

Real-time PCR qPCR was carried out on a Real-Time QPCR System Mx3000p (Agilent Technologies Ltd., Santa Clara, CA, USA) to determine Lactobacillus, Prevotella, Clostridium coccoides group, Bacteroides fragilis group, Atopobium cluster, and Enterobacteriaceae cell counts by means of specific primers (previously described; Table 2). Next, 10 ng of DNA from cecal contents (1 μL) was added to 19 μL of the reaction mix (0.4 μL of each 10 mM primer and 8.2 μL of sterilized water and 10 μL of 2× KAPA SYBER FAST qPCR Master Mix Universal [Kapa Biosystems Inc., Wilmington, MA, USA]). The amplification program for Lactobacillus consisted of one cycle of 95°C for 3 min and then 40 cycles of 95°C for 5 s, 66°C for 20 s, and 72°C for 40 s. The amplification program for Prevotella consisted of one cycle of 95°C for 3 min and then 40 cycles of 95°C for 5 s, 68°C for 20 s, and 72°C for 40 s. The amplification program for the C. coccoides group consisted of one cycle of 95°C for 3 min and then 40 cycles of 95°C for 3 s and 60°C for 60 s. The cycling conditions for the B. fragilis group involved one cycle of 95°C for 3 min and then 40 cycles of 95°C for 5 s, 66°C for 20 s, and 72°C for 40 s. To check the specificity of PCR, a melting curve analysis was conducted after the amplification. The melting curves were obtained by heating at temperatures from 55 to 95°C with continuous fluorescence monitoring. DNA samples extracted from Clostridium clostridioforme JCM1291T, Bacteroides ovatus JCM5824T, Lactobacillus plantarum subsp. plantarum JCM1149T, Eggerthella lenta JCM9979T, Prevotella albensis JCM12258T, and Escherichia coli JCM20135 served as real-time PCR controls for the group-specific g-Ccoc, g-Bfra, Lac 1, c-Atopo, and g-Prevo, Eco1457F primers, respectively.

Statistics Data are expressed as mean ± SE. All data were analyzed in Sigma Plot 11 (Systat Software, Inc., San Jose, CA, USA) by Student’s t test or the Mann–Whitney rank-sum test or Pearson Product Moment Correlation. Statistical significance was assumed at a p value < 0.05.

Results

General observations No significant differences were observed between the groups PGA and CON in final body weight [g; PGA (39.4 ± 0.6) and CON (40.0 ± 0.7)], liver weight [g; PGA (2.05 ± 0.05) and CON (2.06 ± 0.07)], and cecal contents [g; PGA (2.05 ± 0.05) and CON (2.06 ± 0.07)]. Amounts of feces sampled on the final three days before dissection (g) were 1.27 ± 0.04 (PGA) and 1.27± 0.06 (CON). The visceral fat was significantly smaller in the PGA group than in the CON group (Fig. 1). There were no significant differences in food consumption (g/day) between the PGA group (5.87 ± 0.07) and the CON group (5.97 ± 0.07).

The fecal lipid contents Dietary γ-PGA affected lipid concentrations in the fecal matter. The amounts of lipids in feces (dry weight) sampled on the final day of the experiment were significantly greater in the PGA group than in the CON group (Figure 2).

Plasma lipids and glucose The concentrations of triglycerides, total cholesterol, glucose, NEFA, and HDL-cholesterol in plasma were investigated (Table 3). Plasma total glucose levels tended to be lower in the PGA group. The plasma NEFA level also tended to be lower in the PGA group. There were no differences in plasma lipids or HDL-cholesterol concentrations between the groups.

### Table 2. 16S rRNA gene-targeted group-specific primers

<table>
<thead>
<tr>
<th>Target organism</th>
<th>Primers</th>
<th>Sequences (5’ to 3’)</th>
<th>Size (bp)</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clostridium coccoides group</td>
<td>g-Ccoc-F</td>
<td>AAATGACGGTACCTGACTAA</td>
<td>440</td>
<td>Matsuki et al. (2002)</td>
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<tr>
<td>G. Ccoc-R</td>
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<td>CTTTGAGTTTCATTCCTTGCGAA</td>
<td>495</td>
<td>Matsuki et al. (2002)</td>
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<tr>
<td>Bacteroides fragilis group</td>
<td>g-Bfra-F</td>
<td>ATAGCCTTTCGAAAGRAAGAT</td>
<td>341</td>
<td>Walter et al. (2001)</td>
</tr>
<tr>
<td>g-Bfra-R</td>
<td></td>
<td>CCAGTATCAACTGCAATTTTA</td>
<td></td>
<td>Heilig et al. (2002)</td>
</tr>
<tr>
<td>Lactobacillus</td>
<td>Lac1</td>
<td>AGCCAGTJAGGAAATCTTCCA</td>
<td></td>
<td>Matsuki et al. (2004)</td>
</tr>
<tr>
<td>Lab-0677r</td>
<td></td>
<td>CACCGTACACATGGAG</td>
<td>190</td>
<td>Matsuki et al. (2004)</td>
</tr>
<tr>
<td>Atopobium cluster</td>
<td>c-Atopo-F</td>
<td>GGGTTGAGAGGCCGACC</td>
<td></td>
<td>Matsuki et al. (2002)</td>
</tr>
<tr>
<td>c-Atopo-R</td>
<td></td>
<td>CGGRCGCTTCTCTTCAGG</td>
<td></td>
<td>Matsuki et al. (2002)</td>
</tr>
<tr>
<td>Prevotella</td>
<td>g-Prevo-F</td>
<td>CACR GTAACCGATG ATGCCG</td>
<td>513</td>
<td>Matsuki et al. (2002)</td>
</tr>
<tr>
<td>g-Prevo-R</td>
<td></td>
<td>GGGTCGGTTTGAGACCC</td>
<td></td>
<td>Matsuki et al. (2002)</td>
</tr>
<tr>
<td>Enterobacteriaceae</td>
<td>Eco1457F</td>
<td>CATTGACGT TACC CGCGAAGAGGC</td>
<td>195</td>
<td>Bartosch et al. (2002)</td>
</tr>
<tr>
<td>Eco1652R</td>
<td></td>
<td>CTCTCAGAGACTCAAGCTTTGC</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
total cholesterol, HDL-cholesterol, and triglyceride levels between the two dietary groups.

Cecal microbiota  Dietary γ-PGA affected composition of the microbiota. The cell numbers of cecal Lactobacillus, Prevotella, the C. coccoides group, the B. fragilis group, Atopobium cluster, and Enterobacteriaceae per ng DNA of cecal contents are shown in Figure 4. Cecal Lactobacillus counts were significantly greater in the PGA group than in the CON group. Cecal Prevotella counts tended to be higher in the PGA group.

Cecal Prevotella counts tended to be higher in the PGA group. By assessing the correlations between ratios of bacterial cell numbers and the fecal lipid contents, we found that the ratio of Log_{10} Atopobium cluster counts/Log_{10} Bacteroides fragilis group counts negatively correlated with the fecal lipid contents (Fig. 3). On the other hand, cecal B. fragilis group cell numbers tended to be greater in the PGA group than in the CON group.

Cecal cell numbers of the B. fragilis group positively correlated with the fecal lipid contents. These results suggested that the amounts of fecal lipids may affect the microbiota. Amounts of fecal lipids were significantly greater in the PGA group. γ-PGA may indirectly affect the intestinal microbiota by increasing the amounts of lipids in the large intestine.

Discussion

Amounts of visceral fat were significantly smaller in the PGA group than in the CON group. In this experiment, plasma glucose levels and plasma NEFA levels tended to be lower in the PGA group. It has been reported that visceral fat accumulation is correlated with glucose intolerance, hyperlipidemia and hypertension (Sugawara et al., 2011). Elevated fasting glucose and elevated waist circumference are the criteria for diagnosing the metabolic syndrome (Reaven, 2011). A comparable relationship exists between steady-state plasma glucose concentration and waist circumference. A waist circumference is one of the most reliable clinical measure of abdominal fat compartments (Chan et al., 2003). In our experiment, lower plasma glucose levels might relate with lower visceral fat.

On the other hands, insulin resistance caused by FFA produced from hypertrophic adipose tissue (Yamauchi et al., 2001). It has been reported that excess FFA may cause the enhancement of lipid synthesis and gluconeogenesis as well as insulin resistance (Matsuzawa et al., 1995). Higher FFA levels also relate with the visceral fat accumulation. These lower levels in the PGA group may be related to the lower amounts of visceral fat in this group.

γ-PGA is resistant to the digestive enzymes of the gut (Tanimoto, 2010). γ-PGA is water-soluble, and its solution is viscous. Pectin is one of the water-soluble dietary fibers, and an aqueous pectin solution is also viscous, like that of γ-PGA. It has been reported that pectin increases fecal fat excretion (Munakata et al., 1995), which also increases with nutritional supplementation with viscous linseed dietary fiber in rats (Kristensen et al., 2013). The viscous nature of γ-PGA solutions may have increased the fecal fat excretion via a mechanism similar to that of viscous water-soluble dietary fiber.

It has been reported that a high-fat diet increases the proportion of lipopolysaccharide (LPS)-containing intestinal bacteria, and
γ-Polyglutamic Acid: Effects on Cecal Microbiota and Adiposity of Mice

Bacterial LPS serves as a proinflammatory factor triggering insulin resistance, obesity, and diabetes (Cani et al., 2007). Here, γ-PGA decreased the visceral fat in the PGA group as compared to the CON group. γ-PGA might decrease the amounts of endotoxin from gram-negative intestinal bacteria through modulation of the intestinal microbiota or by reducing the release of LPS from gram-negative bacteria. Effects of γ-PGA on the LPS from gram-negative bacteria should be studied further.

Cecal Lactobacillus cell number was significantly greater in the PGA group. Reduction of inflammation in adipose tissue in mice fed a high-fat diet with coadministration of Lactobacillus gasseri SBT2055 has been reported (Kawano et al., 2016). L. gasseri SBT2055 inhibits proinflammatory gene expression in the visceral adipose tissue of mice with diet-induced obesity (Miyoshi et al., 2014). Dietary supplementation with a Lactobacillus salivarius mixture in broilers improves body weight and reduces total cholesterol, LDL-cholesterol, and triglyceride levels in blood; therefore, L. salivarius was suggested as a beneficial probiotic for broiler chickens (Shokryazdan et al., 2017). Thus, some Lactobacillus species have a potential to improve the lipid metabolism of the host. Greater cell numbers of Lactobacillus may have helped to maintain healthier host physiology in the PGA group in our study. A weak negative correlation was observed between cecal Lactobacillus and the weight of visceral fat. Further studies are expected to elucidate the relation between Lactobacillus and lipid metabolism.

It has been demonstrated that coadministration of γ-PGA and isoflavone is effective at ameliorating metabolic syndrome in mice fed a high-fat diet (Lee et al., 2013). In that report, the amount of epididymal adipose tissue was significantly smaller in the high-fat diet–fed mice that received γ-PGA, isoflavones, or γ-PGA with isoflavones than in control mice (fed the high-fat diet alone). In our results, the amount of visceral fat in the PGA group was significantly smaller. Accordingly, dietary PGA may have inhibitory effects on lipid accumulation in mice. The isoflavone plus γ-PGA mixture has been suggested as one of the new functional foods for preventing obesity (Lee et al., 2013).

The ratio of Log_{10} Atopobium cluster counts to Log_{10} B. fragilis group counts negatively correlated with fecal lipid contents in our study. Cecal Prevotella was tended to be high in the PGA group (P = 0.053).

### Table 3. The concentrations of triglycerides, total cholesterol, glucose, NEFA and HDL-cholesterol in plasma.

<table>
<thead>
<tr>
<th></th>
<th>Cholesterol (mg/dL)</th>
<th>Triglyceride (mg/dL)</th>
<th>HDL-cholesterol (mg/dL)</th>
<th>NEFA (mEq/L)</th>
<th>Glucose (mg/dL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CON group</td>
<td>131.9 ± 6.7</td>
<td>178.5 ± 17.3</td>
<td>74.7 ± 4.2</td>
<td>3.22 ± 0.19</td>
<td>421.8 ± 49.4</td>
</tr>
<tr>
<td>PGA group</td>
<td>128.5 ± 10.9</td>
<td>164 ± 27.9</td>
<td>75.7 ± 4.9</td>
<td>2.65 ± 0.29</td>
<td>328.1 ± 55.1</td>
</tr>
</tbody>
</table>

Plasma total glucose level was tended to be low in the PGA group (p = 0.13). Plasma NEFA level was also tended to be low in the PGA group (p = 0.13). There were no differences in the plasma total cholesterol, HDL-cholesterol and triglyceride levels in the two dietary groups.

**Fig. 4.** Cecal bacterial populations by real-time quantitative PCR. Values are means ± SE (n = 7). *p < 0.05.
Cecal Prevotella was tended to be high in the PGA group (P = 0.053).
PGA can be hydrolyzed by the γ-glutamyl hydrolase of intestinal bacteria, it has been reported that *Helicobacter pylori* (Ling et al., 2013), *Fusobacterium nucleatum* (Mineyama et al., 1997), and *E. coli* (Suzuki et al., 1993) have γ-glutamyl transpeptidase. Consequently, these bacteria may utilize glutamic acid by hydrolyzing PGA. Dietary PGA may be utilized by some intestinal bacteria having a γ-glutamyl hydrolase and could have affected the composition of the microbiota in the PGA group here.

It is known that the intestinal microbiota of the rats fed apple polysaccharides with a high-fat diet shows higher abundance of *Bacteroidetes* and *Lactobacillus*, and these treatments inhibit microbial dysbiosis and chronic inflammation, as compared to the controls fed the high-fat diet alone (Wang et al., 2017). In our experiment, cecal *Bacteroides fragilis* group counts tended to be higher, and cecal *Lactobacillus* counts were significantly greater in the PGA groups. Both apple polysaccharides and γ-PGA are water-soluble and viscous polymers. Therefore, physical properties such as viscosity of a polymer like γ-PGA might have affected the intestinal microbiota.

Fasting-induced adipocyte factor (Fiaf) is a circulating lipoprotein lipase inhibitor. Fiaf is suppressed in the intestinal epithelium of mice with conventional microbiota (Bäckhed et al., 2004). Microbiota suppresses the Fiaf expression and suppression of intestinal Fiaf promotes adiposity (Bäckhed et al., 2004). Thus the gut microbiota plays an important role on energy harvest from the diet and energy storage in the host. (Bäckhed et al., 2007). Dietary PGA changed the composition of the cecal microbiota and this changed microbiota might affect the Fiaf production in the gut and may have caused a decrease in visceral fat accumulation.

In conclusion, dietary γ-PGA affects the cecal microbiota and ameliorates accumulation of visceral fat in the KK-A+/TaJcl mouse model of type 2 diabetes mellitus.

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**References**


γ-Polyglutamic Acid: Effects on Cecal Microbiota and Adiposity of Mice

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