Polysaccharide fraction from greens of *Raphanus sativus* alleviates high fat diet-induced obesity

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**ABSTRACT**

*Radish* (*Raphanus sativus*) greens are commonly used as a vegetable in Korea; however, their anti-obesity effect has not been reported yet. We prepared the polysaccharide fraction of radish greens (PRG) and assessed its anti-obesity activity in high fat diet (HFD)-induced obese C57BL/6J mice. Supplementation with 4 mg/kg PRG reduced weight gain and body fat percentage, and regulated serum biomarkers against HFD-induced obesity. Moreover, PRG treatment improved gut permeability by increasing tight junction protein expression and colon length shortening. HFD intake increased the proportion of Firmicutes and decreased the proportion of Bacteroidetes and Verrucomicrobia; however, PRG supplementation maintained gut microbial composition to normal diet condition. Moreover, PRG reduced HFD-induced increase of lipid metabolism-related protein expression, along with adipocyte size in white adipose tissue. These results indicated that PRG as a potential prebiotic, has anti-obesity properties by improving gut barrier function, modulating gut microbiota and regulating lipid metabolism.

1. Introduction

In recent decades, obesity has become a serious health issue across the world. It is defined by abnormal or excessive fat accumulation, leading to health impairment. Accumulating evidence suggest obesity to be related to diverse metabolic disorders, such as diabetes, cardiovascular disease, and cancer (Kahn, Hull, & Utschneider, 2006; Ma et al., 2017). Consumption of a high fat diet (HFD) may cause disorders associated with obesity, including glucose intolerance, fat deposition in diverse tissues, gut microbial dysbiosis, leaky gut, metabolic endotoxemia, and adipocyte hypertrophy (Fujisaka et al., 2020). Therefore, reduction of obesity is important to prevent metabolic disorders.

Recently, polysaccharides have attracted attention regarding the suppression of obesity. Polysaccharides are composed of many sugar monomers, and are well known for their health benefits, such as anti-tumor, anti-inflammatory, anti-oxidant, and anti-dyslipidemia effects (Liu et al., 2019; Qi et al., 2017; Wang et al., 2019). They can reduce body weight, leaky gut, and low-grade inflammation in various tissues, by physicochemical properties, such as water retention, and/or by prebiotic activity, such as altering the gut commensal microbiota and production of microbiota-derived metabolites (Conlon & Bird, 2015; Jiang et al., 2016). Therefore, the use of functional foods that are rich in polysaccharides provides health benefits and therapeutic strategies against obesity.

*Radish* (*Raphanus sativus*) greens, the aerial part of radish and also known as Mucheong, are traditionally used as ingredients for kimchi and soup in Korea (Park, Kim, & Yook, 2014). They have been reported to show diverse biological activities, such as anti-microbial, anti-oxidant, anti-inflammatary, anti-hypertensive, and gut stimulatory activities (Chung, Kim, Myung, Cho, & Chang, 2012; da Silva, de Oliveira Lopes, Cerdeira, Ribeiro, Rosa, Chavasco, & da Silva, 2020; Gilani & Ghayur, 2004; Park & Song, 2017). Supplementation of radish greens reduced high cholesterol diet-induced oxidative stress (Rhee, Ahn, Ku, & Choi, 2005). Moreover, dietary fiber of radish greens might increase excretion of lipids and sterols, however, anti-obesogenic activity of radish greens has not yet been reported (Jang et al., 2008). Beneficial effects of polysaccharides from radish greens (PRG) have not been examined till date. Therefore, in the present study, we isolated...
polysaccharide-containing fractions from radish greens and demonstrated their anti-obesogenic activity in diet-induced obesity model in mouse.

2. Materials and methods

2.1. Materials

Fluorescein isothiocyanate-dextran (FITC-dextran; 4 kDa) was obtained from Sigma-Aldrich (St. Louis, MO, USA). Acetyl-CoA carboxylase (ACC), fatty acid synthase (FAS), sterol regulatory element-binding protein 1 (SREBP-1), and zonula occcludin1 (ZO-1) were obtained from Abcam (Cambridge, MA, USA), and β-actin was purchased from Cell Signaling Technology (Beverly, MA, USA). Secondary antibodies against rabbit were obtained from Thermo Fisher Scientific (Waltham, MA, USA).

2.2. Preparation of polysaccharide fraction from Raphanus sativus

Dried greens (leaves and stems) of R. sativus were obtained from Jeollabuk-do, Republic of Korea and deposited at the Korea Food Research Institute. To prepare PRG, radish-green powder (100 g) was extracted in 2 l of distilled water at 80 °C for 3 h. Thereafter, the extract was filtered and evaporated using a vacuum rotary evaporator (R-114; Buchi Labortechnik, Flawil, Switzerland). The extract was precipitated with four volumes of ethanol at 4 °C for 16 h, and then centrifuged at 3,000 × g for 30 min. The precipitates were dialyzed for two days at 4 °C using a dialysis membrane (molecular weight cutoff: 12–14 kDa; Spectrum Laboratories, Inc., Rancho Dominguez, CA, USA). The dialysate was lyophilized and PRG obtained eventually. The final product of PRG was stored at −20 °C until further use.

2.3. Analysis of PRG by gel permeation chromatography (GPC)

The molecular weight of PRG was determined by GPC analysis using EcoSEC HLC-8320GPC (Tosoh Corporation, Tokyo, Japan), equipped with a refractive index detector and four columns: TSKgel GMPWXL × 2, TSKgel G2500PWXL, and TSKgel PWXL guard columns (7.8 × 300 mm, Tosoh Corporation) at Korea Polymer Testing and Research Institute (KOPTRI, Seoul, Republic of Korea). The mobile phase was 0.1 M sodium nitrate solution and flow rate was 1.0 mL/min.

2.4. Composition analysis and characterization of PRG

Composition analysis and characterization of PRG were conducted using diverse analytical methods. Neutral sugar content was measured by the phenol–sulfuric acid method (You et al., 2013). Uronic acid content was analyzed by the m-hydroxybiphenyl method (Chen et al., 2019). Total polyphenol content was assessed by the Folin–Ciocalteu spectrophotometric method (Yang, Dang, & Fan, 2018). Protein content was analyzed using the Bradford assay (Bio-Rad, Hercules, CA, USA). Content of 2-keto-3-deoxy-d-manno-octulosonic acid (KDO) was determined by the thioarbituric acid method (Lee, Lee, Kim, & Shin, 2018). Monosaccharide composition was determined by the aldito acetate method (H. R. Park & Shin, 2019), with slight modification. Gas chromatography (GC; Acme-6100, Young-Lin Co., Anyang, Republic of Korea), equipped with an SP-2380 capillary column (Supelco, Bellefonte, PA, USA), was used for chromatographic separation, and the temperature was programmed as follows: held at 60 °C for 1 min, ramped up to 220 °C at 30 °C min−1, held at 220 °C for 12 min, ramped up to 250 °C at 8 °C min−1, and finally held at 250 °C for 15 min. Molecular ratios were calculated using peak areas and response factors.

2.5. Animals and diets

C57BL/6J mice (six-week-old, male) were obtained from Central Lab Animal Inc. (Seoul, Korea), and housed in an SPF animal facility, supplied with food and water ad libitum. All animal experiments were conducted with the approval of the Animal Welfare Committee of the Korea Food Research Institute (KFRI-M−19024). After 1-week acclimation, mice were randomly assigned to 4 groups (n = 9): normal diet (ND), high fat diet (HFD), HFD with a daily oral gavage of PRG 2 mg/kg (PRG2), and HFD with a daily oral gavage of PRG 4 mg/kg (PRG4) for 8 weeks. Body weight and food intake of mice were recorded weekly until the end of the study. ND group was fed the 2018S Teklad global diet (Harlan Teklad, Madison, WI, USA) and HFD groups were fed the TD06414 diet (60% energy from fat, Harlan Teklad). At the end of the experiment, the mice were allowed to fast for 12 h, and then euthanized by anesthesia.

2.6. Body fat mass measurement

At week 8, whole-body image and fat mass of the mice were obtained using dual energy X-ray absorptiometry (InAlyzer; Medikors Inc., Seongnam, Korea). Briefly, the mice were anesthetized and immediately placed in the InAlyzer scanning area. Scanning images and data were obtained using the InAlyzer software.

2.7. Serological analysis

Blood was collected into endotoxin-free microcentrifuge tubes, and serum was separated by centrifugation. Serum endotoxin levels were analyzed using a Pierce LAL Chromogenic Endotoxin Quantitation Kit (Thermo Fisher Scientific). Triglyceride levels were measured using the Triglyceride Assay Kit (Abcam, Cambridge, MA, USA) and leukotriene B4 (LTB4) levels were analyzed using Leukotriene B4 ELISA Kits (Abcam). All experiments were performed according to the manufacturers’ instructions.

2.8. Gut permeability test

After 8 weeks, mice on 6-h fast gavaged 500 mg/kg of FITC-dextran, and blood was obtained from their tail vein after 2- and 4-h administration. Plasma was separated and fluorescence intensity was read by microplate reader (Molecular Devices, Sunnyvale, CA, USA) at an excitation/emission wavelength of 485/535 nm. Diluted FITC-dextran in untreated plasma was used for creation of standard curve.

2.9. Colon length measurement

After euthanization, the portion from cecum to the rectum was dissected and the length measured using a ruler.

2.10. Water content of feces and gut microbial composition

At week 8, to analyze water content and gut microbial composition, fresh fecal samples were collected. Feces were divided into two portions and immediately stored at −80 °C until further use. Fecal samples were dried at 105 °C for 24 h, and fecal water content was calculated according to the equation: (fecal weight before drying − fecal weight after drying) / fecal weight before drying × 100.

Microbial composition was measured according to the protocol described in our previous study (Do, Lee, Oh, Kim, & Park, 2018). Briefly, 16S rRNA was analyzed at Macrogen (Seoul, Korea) using a MiSeq Illumina, San Diego, CA, USA), according to the manufacturer’s instructions. The QIIME software was used to assess microbial diversity, and QIIME Uclust was used to generate taxonomic composition. Principal coordinate analysis (PCoA) plot was created using the R software based on Bray-Curtis dissimilarity (Janssens et al., 2016).
2.11. Western blot analysis

Total proteins from colon and white adipose tissues (WAT) were extracted with PRO-PREP™ (iNtRON Biotechnology, Seongnam, Korea). Proteins were loaded into a polyacrylamide gel and transferred onto a polyvinylidene fluoride membrane. The blots were blocked for 1 h with 5% skimmed milk, and incubated at 4 °C overnight with SREBP-1, ACC, FAS, ZO-1, and β-actin antibodies. Thereafter, membranes were incubated with secondary antibodies for 1 h. Protein expressions were visualized using a ChemiDoc™ XRS + imaging system (Bio-Rad).

2.12. Histology and immunohistochemistry

The colon and epididymal WAT tissues were fixed with 4% formaldehyde for histological analysis. To stain for immunohistochemistry, and for hematoxylin and eosin (H&E) staining, fixed tissues were embedded in paraffin and sliced at 4-μm thickness. To confirm the adipocyte size, WAT tissues were stained with H&E.

To observe the tight junction protein expression in colon, immunostaining was performed as described previously (Do et al., 2018). Briefly, antigen retrieval was performed for all the sections, using 20 μg/mL proteinase K, and incubated overnight at 4 °C with ZO-1 antibody (1:500). The sections were incubated with secondary antibodies (1:200) at 25 °C for 20 min and then with 3,3′-diaminobenzidine (Vector Laboratory, Piscataway, NJ, USA); hematoxylin was used to reveal the immunohistochemical development and counterstain. All slides were pictured using Pannoramic 250 Flash III slide scanner (3DHISTECH Ltd., Budapest, Hungary) and analyzed using CaseViewer software (3DHISTECH Ltd).

2.13. Statistical analysis

The statistical analysis is expressed as the mean ± standard error of the mean (SEM) using GraphPad Prism software (San Diego, CA, USA). One-way ANOVA and Tukey’s analysis were used for statistical significance, and p-value < 0.05 was considered statistically significant.

3. Results

3.1. Characteristics of PRG

To determine the molecular weight (Mw) and distribution of PRG, GPC analysis was employed. As shown in Table 1, the number average molecular weight (Mn), weight average Mw, and peak molecular weight (Mp) of PRG were 3910, 61,100, and 66,000, respectively. Polydispersity (Mw/Mn) of PRG was 15.6, which indicated it to be composed of diverse polysaccharides.

As shown in Table 2, PRG was mainly composed of neutral sugar (70.8%) and uronic acid (22.3%), with minor components of phenols (4.5%), proteins (0.7%), and KDO-like materials (1.6%). The sugar composition of PRG included rhamnose, fucose, arabinose, xylose, mannose, galactose, glucose, glucuronic acid, and galacturonic acid in molar percentages of 5.4%, 5.0%, 22.9%, 0.4%, 2.0%, and 40.7%, respectively.

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Gel permeation chromatography analysis of polysaccharide fraction from radish greens (PRG).</th>
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<tr>
<td>(Da)</td>
<td>RCP</td>
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<tr>
<td>Mn</td>
<td>3910</td>
</tr>
<tr>
<td>Mw</td>
<td>61,100</td>
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<tr>
<td>Mp</td>
<td>66,000</td>
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<tr>
<td>Mw/Mn</td>
<td>15.6</td>
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Notes: Mn means number-average molecular weight. Mw means weight-average molecular weight. Mp means peak molecular weight. Mw/Mn means polydispersity.

<table>
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<th>Table 2</th>
<th>Chemical and monosaccharides compositions of PRG.</th>
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<tr>
<td>Value</td>
<td>Chemical composition (% of dry matter)</td>
</tr>
<tr>
<td></td>
<td>Neutral sugar</td>
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<tr>
<td></td>
<td>Uronic acid</td>
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<tr>
<td></td>
<td>Polyphenol</td>
</tr>
<tr>
<td></td>
<td>Protein</td>
</tr>
<tr>
<td></td>
<td>KDO-like material</td>
</tr>
<tr>
<td>Value</td>
<td>Monosaccharides composition (Mole %)</td>
</tr>
<tr>
<td></td>
<td>Rhamnose</td>
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<tr>
<td></td>
<td>Fucose</td>
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<td></td>
<td>Arabinose</td>
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<td>Xylose</td>
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<td></td>
<td>Mannose</td>
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<td>Galactose</td>
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<td></td>
<td>Glucose</td>
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<tr>
<td></td>
<td>Glucuronic acid</td>
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<td>Galacturonic acid</td>
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</table>

Notes: Data are presented as mean ± standard deviation (n = 3). KDO means 2-keto-3-deoxy-o-manno-octulosonic acid.

2.1%, 1.9%, and 12.7%, respectively.

3.2. Effects of PRG on metabolic parameters

Body weight and body fat percentage of HFD-fed mice were remarkably increased during the 8-week period (Fig. 1A–C). PRG (2 mg/kg)-supplemented mice did not show any difference compared to the HFD group; however, the PRG4 group showed higher parameters than ND, but significantly lower than HFD.

Diet-induced changes of metabolic disorder markers were evaluated using serum triglyceride, endotoxin, and LTB4 levels. HFD group showed significantly increased levels of triglyceride, endotoxin, and LTB4, whereas PRG-treated groups had remarkably reduced levels (Fig. 1D–F). These results suggested diet with PRG to regulate HFD-induced changes in metabolic disorder markers.

3.3. Effects of PRG on gut health

The average colon length was remarkably decreased in HFD-fed mice (6.69 ± 0.13 cm), compared to that in the ND-fed mice (7.25 ± 0.18 cm) (Fig. 2A). However, PRG2 and 4 groups showed a significant increase in colon length, up to 7.38 ± 0.14 and 7.84 ± 0.10 cm, respectively. To verify the abilities of PRG to restore gut health, we measured the water content of feces and expression of tight junction proteins. PRG-supplemented groups did not restore HFD-induced reduction of water content of feces (Fig. 2B); however, PRG supplementation did improve the expression of ZO-1, reduced by HFD in colon (Fig. 2C and D). Moreover, in gut permeability analysis, HFD group exhibited significantly increased levels of plasma FITC-dextran and area under the curve (AUC) (Fig. 2E and F). PRG2 and 4 groups showed similar levels of intestinal permeability to HFD, whereas PRG4 group had significantly reduced gut permeability. As obvious from these results, the expression of ZO-1 was confirmed to be increased due to PRG supplementation by immunohistochemical staining. The results suggested PRG administration to improve gut health via improvement of tight junction protein expression, rather than by water absorption ability, as seen in the increase of digesta mass or dilution of toxins by other polysaccharides.

3.4. Effects of PRG on gut microbial composition

To confirm whether the improvement of gut barrier function of PRG was due to change of gut microbiota, we performed 16S rDNA analysis.
All HFD-fed mice exhibited lower OTU abundance, and Shannon and Simpson diversity indices, than the ND-fed mice (Fig. 3A). At the phylum level, HFD intake changed the gut microbial composition and PCoA obviously separated that from those in ND group (Fig. 3B and C). However, PRG supplementation did not increase alpha diversity of gut microbiota; rather, it restored the changed gut microbial proportions, and loci of PRG2 and 4 groups were very close to ND in PCoA. HFD-fed mice showed significantly higher Firmicutes/Bacteroidetes ratio and lower proportion of Verrucomicrobia than ND mice (Fig. 3D and E); however, PRG administration reduced Firmicutes/Bacteroidetes ratio.
and increased proportion of phylum Verrucomicrobia. These results indicated PRG to be able to regulate gut microbial composition, thereby improving gut barrier function.

3.5. Effect of PRG on white adipose tissue lipid metabolism

To evaluate whether PRG treatment could change lipid metabolism in WAT, SREBP-1, ACC, and FAS protein expressions were measured. HFD mice increased these proteins expressions to 3.8, 4.1, 5.1-fold, compared to ND mice (Fig. 4). PRG2 group did not reduce SREBP-1, ACC, and FAS protein expression; however, PRG4 group did, reducing their expression by 45%, 85%, and 68% compared to that in HFD-fed control mice.

H&E staining of WAT showed increase of body weight to be associated with increase of the sizes of adipose tissue in HFD-fed mice. PRG2 mice did not have reduced adipose tissues, and failed to reduce body weight and change lipid metabolism. However, PRG4 treatment remarkably reduced adipocyte sizes compared to that in HFD-fed control mice (Fig. 4F), thus suggesting that PRG regulates the lipogenic protein expression, hence contributing to the inhibition of adipocyte expansion and weight gain.

4. Discussion

High-fat diet is associated with obesity and other metabolic diseases. It causes low-grade inflammation, glucose intolerance, and adiposity (Gao, Ma, & Liu, 2015). Many researchers have reported plant-derived polysaccharides to exert diverse effects on human gut health; particularly, their anti-obesity activity is well known (Wen et al., 2019). Therefore, study of the protective effects of plant-derived polysaccharides on metabolic disorders is considered important. The present study demonstrated, for the first time, that polysaccharide fraction of *Raphanus sativus* can prevent HFD-induced obesity. Through the Mw/Mn ratio and monosaccharides composition, we confirmed that various polysaccharides were contained in radish greens and the polysaccharide fraction was well extracted (Tables 1 and 2). The high content of uronic acid in polysaccharides is known to have various bioactive properties (Li et al., 2020). PRG was expected to show good efficacy as it has more than 20% of uronic acid content (Table 2).

Our results suggested HFD to lead to a significant increase in body
weight and percentage of body fat. In addition, HFD caused upregulation of blood triglyceride levels and endotoxemia. However, PRG supplementation not only suppressed HFD-induced increase of body weight and fat accumulation, but also downregulated triglyceride levels and endotoxemia. Therefore, we supposed PRG to reduce HFD-induced weight gain and body fat mass by regulating lipid metabolism and endotoxemia.

Colon-length shortening is one of the markers of colon damage (Jin et al., 2017). Conlon and Bird had reported the promotion and maintenance of bowel health by polysaccharides through increased digesta mass (Conlon & Bird, 2015). Based on their physicochemical properties of absorbing water, polysaccharides can increase digesta mass and provide the moisture required for gut transit; therefore, dilute toxins and shortened transit time could reduce intestinal damage. Endotoxemia has been supposed to be caused by the destruction of intestinal barrier function, such as reduction of tight junction protein, resulting in an increase of gut permeability (Robertson et al., 2018). Increased intestinal permeability-induced chronic low-grade inflammation triggers metabolic disorders in various tissues (Cani et al., 2007). Therefore, increase of fecal water content and/or restoration of gut barrier function would be important for the improvement of gut health and protection against metabolic disorders. In this study, both fecal water content and tight junction protein expression were drastically reduced in HFD-induced obese mice than in ND mice. Although PRG group did not increase fecal water content, PRG-supplemented group showed similar levels of ZO-1 expression compared to ND, with prevention of colon length shortening. These results suggested that maintenance of metabolic parameters in PRG supplementation is possibly due to improved gut barrier function than due to physicochemical property, such as water absorption, of polysaccharides.

In case of normal body weight, gut microbiota maintains healthy condition; therefore, epithelial tight junctions are densely distributed. However, during obesity, gut microbial dysbiosis occurs and tight junction protein expression is decreased, thereby making it a “leaky gut.” Thus, it is important to keep the gut microbial composition healthy. Polysaccharides are known to be able to change gut microbial composition and create healthy gut via their prebiotic activity (Halmos et al., 2015). High fat diet not only increased Firmicutes/Bacteroidetes ratio is a well-known hallmark of obesity (Crovesy, Masterson, & Rosado, 2020). Moreover, high abundance of Verrucomicrobia in healthy gut had been reported by several researchers previously (Fujio-Vejar et al., 2017; Hou, He, Ouyang, Peng, Wang, Li, & Liu, 2017). Although HFD-induced decrease of gut microbial diversity was not recovered by PRG groups, the latter showed decreased ratio of Firmicutes/Bacteroides and higher levels of Verrucomicrobia than the HFD group. These results collectively suggested PRG to possibly improve gut barrier function through its prebiotic ability, such as regulation of gut microbial composition.

Change in lipid metabolism may occur due to HFD-induced endotoxemia. Increased fat accumulation and adipocyte hypertrophy in adipose tissue were caused by impaired gut permeability-induced increase of gut microbial-derived endotoxin exposure (Dewulf et al., 2011). Our results demonstrated HFD to change lipid metabolism, causing increased SREBP-1 expression. SREBP-1 is a well-known regulator of lipid metabolism. In adipocytes, SREBP-1 promotes lipogenic enzymes, such as ACC and FAS, thereby involving the synthesis of fatty acids (Zeng, Zhang, Song, Zhao, & Xie, 2012). Therefore, regulation of these proteins in adipose tissue could be an effective strategy to alleviate obesity and metabolic disorders. In this study, 4 mg/kg PGR supplementation altered HFD-induced increase of lipogenic proteins expression, thus indicating its ability to inhibit adipocyte expansion and weight gain.

In conclusion, we successfully isolated the polysaccharide fraction from radish greens and evaluated its anti-obesity activity to be caused by the maintenance of gut health and change of lipid metabolism in adipose tissue. These results might be attributed to the change of gut microbial composition and regulation of lipid metabolism induced by prebiotic ability of polysaccharides. Therefore, radish greens could be a potential functional food source for preventing weight gain and obesity-related metabolic disorders. In future, it would be important to confirm the composition of polysaccharides of radish greens and identify the specific ones with anti-obesity properties.

CRediT authorship contribution statement


Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to...
influence the work reported in this paper.

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References


