# Dietary adzuki bean paste dose-dependently reduces visceral fat accumulation in rats fed a normal diet

Kyu-Ho HAN[1, 2], Shunsuke OHASHI[1], Keiko SASAKI[3], Ryuji NAGATA[1, 4], Samanthi PELPOLAGE[1, 4], Naoki FUKUMA[1, 2], Jess D. REED[5], Ken-ichiro SHIMADA[1], Norimichi KADOYA[6], Michihiro FUKUSHIMA[1,*]

[1]Department of Life and Food Sciences, Obihiro University of Agriculture and Veterinary Medicine, Obihiro, Hokkaido 080-8555, Japan

[2]Research Center for Global Agro-medicine, Obihiro University of Agriculture and Veterinary Medicine, Obihiro, Hokkaido 080-8555, Japan

[3]Hokkaido Tokachi Area Regional Food Processing Technology Center Obihiro, Hokkaido 080-2462, Japan

[4]The United Graduate School of Agricultural Sciences, Iwate University, Morioka 020-8550, Japan

[5]Reed research group, Department of Animal Sciences, University of Wisconsin-Madison, Madison, WI 53706-1284, United State

[6]Japan Pulse Foundation, Tokyo 107-0052, Japan

***Correspondence**

Michihiro FUKUSHIMA, Professor

Laboratory of Nutrition Biochemistry, Department of Life and Food Sciences, Obihiro University of Agriculture and Veterinary Medicine, Obihiro, Hokkaido 080-8555, Japan

e-mail: fukushim@obihiro.ac.jp

Tel no: +81-155-49-5557 fax no: +81-155-49-5577

**Authors and e-mail addresses**

Kyuho HAN, kyuho@obihiro.ac.jp

Shunsuke OHASHI, s26069@st.obihiro.ac.jp

Keiko SASAKI, k-sasaki@food-tokachi.jp

Naoki FUKUMA, n.fukumax@obihiro.ac.jp

Ken-ichiro SHIMADA, kshimada@obihiro.ac.jp

Jess D. REED, jdreed@wisc.edu

Ryuji NAGATA, boc.10rj24@gmail.com

Samanthi PELPOLAGE, samanthipelpola@gmail.com

Norimichi KADOYA, n-kadoya@mame.or.jp

Michihiro FUKUSHIMA, fukushim@obihiro.ac.jp

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**Abstract**

The aim of this study was to evaluate the dose-dependent effect of adzuki bean (*Vigna angularis*) paste (ABP) on visceral fat accumulation in rats. ABP is a rich source of indigestible carbohydrates (18.5%) with fiber and resistant starch (RS) contents of 14.5% and 4.0%, respectively. Animals were fed one of the following diets, control (CON), 30% ABP or 58.9% ABP for 28 days. The daily dietary energy intake was lowered ($p < 0.05$) and reduced visceral fat accumulation and lower serum lipid levels were observed in ABP fed groups. ABP consumption dose-dependently increased ($p < 0.05$) the daily fecal lipid and fecal acidic sterol excretions. On the other hand, cecal content and fecal moisture content in the 58.9% ABP group were greater ($p < 0.05$) than the CON group, while there was no significant difference between the two ABP fed groups. Both 30% and 58.9% ABP diets had significantly ($p < 0.05$) higher contents of cecal acetic, propionic and *n*-butyric acids, and lowered cecal pH, independently of the ABP dose. Microbial community data of rats fed ABP diets exhibited higher alpha-diversities than the rats fed CON diet, based on the Shannon Index and the number of observed species index, where the two ABP groups exhibited a similar alpha diversity. The weighted UniFrac-based principal coordinate analysis plot of cecal microbial community data showed that the ABP had a substantial effect on the cecal microbial composition. Furthermore, cecal bacterial 16S rRNA gene sequencing revealed that the ABP supplemented diets decreased the ratio of Firmicutes to Bacteroidetes. These findings suggested that the cecal fermentation of fiber and RS in ABP, might have decreased the energy intake, altered the gut microbiota composition, increased fecal lipid output, and thereby reduced fat accumulation in rats.

**Keywords:** Adzuki bean paste, cecal bacteria, fecal lipid excretion, fermentation, indigestible carbohydrate, energy intakes, fat accumulation

## 1. Introduction

Rapidly escalating incidents of overweight and obesity have become global public health concerns linked to increased risks of chronic non-communicable diseases such as cardiovascular diseases and type II diabetes (Despres & Lemieux, 2000; World Health Organization, 2003; Animaw & Seyoum, 2017). The build-up of a calorie surplus due to the consumption of energy-dense foods, which is usually stored in the adipose tissue resulting in unnecessary fat accumulation, is one of the main risk factors of obesity development (Rolls *et al*., 2005). The dietary interventions such as dietary adherence and caloric deficit play important roles in weight loss and weight-loss maintenance (World Health Organization, 2003; MacLean *et al*., 2015). Thus, restriction of energy intake together with sufficient physical activity are considered as healthy lifestyle habits in today's environment, in the context of preventing obesity (MacLean *et al*., 2015; Heymsfield & Wadden, 2017).

Over the past decade, research suggests that the gut microbiota composition (including bacterial diversity) also influences obesity in animal models (Ley *et al*., 2005, Turnbaugh *et al*., 2006; de La Serre CB *et al*., 2010; Hamilton *et al.*, 2015; Sen *et al.*, 2017) and in humans (Ley *et al*., 2006; Menni *et al*., 2018). For example, a potential role of gut microbiota on the obesity development was shown with that the relative abundance of Firmicutes, one of the two most abundant gut microbial phyla, in obese mice was higher compared to that of the normal lean mice, while Bacteroidetes abundance was lower (Ley *et al.*, 2005). Mice gained more weight when their large intestines were colonized with the microbial members belonging to phylum Firmicutes (Turnbaugh *et al*., 2006). Furthermore, a recent study of overweight or obesity shows a dysbiosis characterized by a lower gut microbiota diversity, and that *Bacteroides* species are associated with long-term weight gain (Menni *et al*., 2018). However, the abundance of a specific genus, class or species of gut bacteria is not always positively or negatively associated with the incidence of obesity. Gut microbial dysbiosis in

obesity has been linked to an altered gut barrier, thereby promoting systemic endotoxemia, through the translocation of lipopolysaccharides (Cani, 2018). Similar to that, the excessive intake of specific food components such as fat (de La Serre CB *et al*., 2010; Hamilton *et al*., 2015) or sugar (Sen *et al*., 2017), also induces gut microbial dysbiosis, impairs the intestinal barrier function and/or alters vagal gut-brain communication in animals, which subsequently may lead to metabolic disorders (de La Serre CB *et al*., 2010). On the other hand, supplementation of probiotics and fecal microbiota transplantation are practiced as strategies to modify the gut microbial composition. Another approach is to supplement diets with prebiotics that provide carbon and nitrogen sources for gut microbial metabolism, resulting in the production of short chain fatty acids (SCFA) and other fermentation products, which are known to modify the gut microbial composition. For example, dietary supplementation of inulin to obese rats led to a remarkable increase in the abundance of bifidobacteria (Han *et al*, 2017). Other research also demonstrates that modification of dietary carbohydrates favorably changes the gut microbiota (Parnell & Reimer, 2012).

Studies in animal models and human subjects have reported an improved weight loss for fiber supplemented diets (Li *et al*., 2016; Han *et al*., 2017) and high-fiber foods (Han *et al*., 2016). According to a literature review by Howarth *et al*. (2001), changes in the energy intake and the body weight were observed after consuming dietary fiber supplements. Furthermore, dietary fiber is known to positively affect satiety and the subsequent food/energy intake (Slavin & Green, 2007). Additionally, a link between the consumption of dietary fiber and the reduced chronic diseases incidence in rats also has been suggested (Han *et al*., 2017). Thus, owing to less energy-density (~2 kcal $g^{-1}$) and gut microbiota modification effects (Parnell & Reimer, 2012), a fiber-rich meal is recommended as a potential candidate for controlling body weight (Rolls *et al*., 2005).

Legumes are excellent sources of non-digestible saccharides, and the relationship between the consumption of legumes and its effects on obesity has been previously well-reviewed (Rebello *et al*., 2014). Several previous studies have reported a lower average body weight of the participants in communities where a higher average amount of legumes was consumed (Papanikolaou & Fulgoni, 2008; Jenkins *et al*., 2012). Among various legumes, the small red bean, adzuki (*Vigna angularis*) has been mainly cultivated throughout East Asia (770,00 ha), where China is the world's biggest producer of adzuki beans (0.24 Mt in 2014, Li *et al*., 2017). Adzuki bean has been consumed 0.14 Mt per year in Japan, where the most common use is the sweetened bean paste. A recent clinical trial suggested that the consumption of adzuki bean would attenuate inflammation and improve glycemic control in patients with type 2 diabetes (Liu *et al*., 2018). In a previous study, we have found that the consumption of high fiber, less energy-dense red adzuki bean paste, improved serum lipid profile in rats, which was mostly attributed to its higher indigestible matter content, particularly dietary fiber and resistant starch (RS) (Han *et al*., 2003a).

We hypothesized that higher fiber and RS contents in adzuki bean will positively affect the development of obesity by influencing energy intake and satiety. However, there is no information currently reported on the contribution of adzuki bean paste (ABP) to obesity development. Thus, in this study, we aimed to examine the effects of ABP consumption on the cecal fermentation, cecal microbiota and obesity development in rats.

## 2. Materials and Methods

### *2.1. Preparation of adzuki bean paste*

Adzuki bean paste was purchased from a local company (Hosokawa Seian Co. Ltd, Obihiro, Japan) in 2017. The ABP was prepared according to a traditional method as described below. Raw beans (*Vigna angularis* cv. Erimoshouzu) were washed with water and

soaked at room temperature overnight. The soaked beans were boiled in water (×4 folds, *w/v*) for 15 min and cooled to 15°C by adding water. Then beans were again boiled for 1 h and were later passed through a fine a strainer (60-mesh) to remove the bean husk after cooling for 1 h. The separated paste was put in a cloth bag, and was pressed to remove the moisture, and was freeze-dried (TFD-550-8, Takarass, Tokyo, Japan). Finally, the freeze-dried paste was ground to a particle size of 0.5 mm or less using an ultracentrifugal mill (ZM200, Retsch, Osaka, Japan).

*2.2. Composition of adzuki bean paste*

The nutrient composition of ABP was determined by the Official Methods of Analysis of AOAC International (1990) as follows: crude protein or total Khjeldhal nitrogen (AOAC method 920.87) with a conversion factor of 6.25, lipid (AOAC method 920.85), moisture (AOAC method 925.10) and ash (AOAC method 923.03). The dietary fiber content was determined by enzymatic-gravimetric method (AOAC method 991.43), and the RS content by a commercial test kit (Megazyme International Ireland, Ltd., Bray, Co. Wicklow, Ireland).

*2.3. Animal study*

Seven-week-old male Fischer 344 rats were purchased from Charles River Laboratories Japan (Yokohama, Japan). The F344 rats are inbred strains, which have been used for general multipurpose models such as aging, safety and efficacy testing, oncology or nutrition. All animals were individually housed in plastic cages with a steel mesh at the bottom (22°C, 55% humidity, 12-h light: dark cycle). Rats were acclimatized for one week on a standard rodent diet (CE-2, CLEA Japan, Inc., Tokyo, Japan) and tap water. Then the rats were randomly separated into 3 similar body weight groups. The number of animals ($n$ = 5) were determined based on a previous report (Han *et al*., 2003a). Each group was fed one of the

following experimental diets (formulated by Oriental Yeast Co., Ltd., Tokyo, Japan) to observe a dose-dependent effect of adzuki bean paste on obesity development for 4 weeks; AIN-93G semi-purified rodent diet (CON), 30% ABP (LAB), and 58.9% ABP (HAB), with a combined fiber and RS content of 5.0%, 10.6%, and 15.8%, respectively. Diet compositions are shown in Table 1 and the dose and the time of administration were determined partly based on a previous report (Han *et al*., 2003b). All rats had access to *ad libitum* water and experimental diets throughout the experimental period. Immediately prior to the administration of the narcotic (Nembutal, 40 mg $kg^{-1}$ body weight; Abbott Laboratories, Illinois, USA), the rat anal area was gently massaged to collect fresh feces directly from the anus, which was later freeze-dried (Eyela PDU-2100, Tokyo Rikakikai Co., Ltd., Tokyo, Japan). Dry feces that had dropped on the bottom of the cage for three days before dissection were also collected. After euthanizing the rats, blood, liver, cecum and visceral fat tissues were quickly removed, and all tissues were weighed before freezing for storage. The animals were handled according to the guidelines of "Guide for the Care and Use of Laboratory Animals" (National Research Council, 1996). This experimental design was approved by the Animal Care and Experiment Committee of Obihiro University of Agriculture and Veterinary Medicine, Japan (animal protocol approval no. 29–94).

*2.4. Serum cholesterol and triglyceride*

Blood (0.5 mL) was collected from the jugular vein of the fasted rats every week. Serum was prepared by centrifugation at 1500 × *g* for 20 min at 4°C after standing at room temperature for 2 h. Total cholesterol, HDL-cholesterol and triglyceride contents were analyzed using a clinical autoanalyzer (Model 7070, Hitachi, Tochigi, Japan). Non-HDL-cholesterol (VLDL + IDL + LDL) content was calculated as follows: [non-HDL-cholesterol] = [total cholesterol] – [HDL-cholesterol].

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195 *2.5. Hepatic lipid and fecal lipid excretion*

196 Total lipid fraction in the liver was extracted with chloroform-methanol (2:1, *v/v*)
197 according to the method of Folch *et al*., (1957). The organic solvents in the samples were
198 removed using a Büchi rotavapor (R-114, Büchi, Tokyo, Japan). And the total lipid content
199 was measured gravimetrically. The hepatic triglyceride and cholesterol levels were measured
200 in the total lipid fraction dissolved in isopropyl alcohol using commercially available kits
201 (Wako Pure Chemical Industries, Ltd., Osaka, Japan) according to the manufacturer's
202 instructions. The total lipid content in the feces was similarly extracted according to the
203 Folch' method (1957). In brief, a portion of the pooled freeze-dried feces (Eyela PDU-2100)
204 which were collected during the last three days of the experimental period was mixed with a
205 3-fold volume of chloroform-methanol (2:1, *v/v*) and sonicated for 10 min. The resultant
206 solution was mixed thoroughly with a little amount of water in a separate tube. After
207 centrifugation (1500 × *g* for 20 min at 4°C), the upper layer was removed and the lower layer
208 was dried. This procedure was repeated 3 times. Finally, the extracted lipid from feces was
209 determined gravimetrically by the combined weight of the three dried lower layers. Fecal
210 neutral and acidic sterol contents were measured by GLC (GC-2014, Shimadzu Co., Ltd.,
211 Kyoto, Japan) following the method of Matsubara *et al.,* (1990) and Grundy *et al*., (1965),
212 respectively, as previously described (Han *et al*., 2013). The daily excreted amounts of
213 neutral and acidic sterol were obtained as follows: [concentration (μmol $g^{-1}$) × dried feces
214 weight (g $day^{-1}$)].

215

216 *2.6. Adipocyte area*

217 For measurement of adipose cell area, the mesenteric fat tissue was fixed with 10%
218 neutral buffered-formalin solution and was embedded in paraffin. Standard, 4-μm thick

sections were cut, stained with hematoxylin and eosin, and were visually examined under an optical microscope (DP-70, Olympus, Tokyo, Japan). Then, cell area ($\mu m^2$) was measured using freely available software (Image J, version 1.46r, NIH, Bethesda, ML, USA) in comparison to a standard bar length (100 μm) within each image as the reference length. Finally, the adipocyte area was calculated as the average of 4 randomly obtained images (20× magnification, JPEG) of each sample.

*2.7. Cecal bacterial DNA extraction, community analysis, and anaerobic bacteria count*

Analytical conditions for cecal bacterial nucleic acid extraction and of 16S rRNA gene sequencing analysis were followed as previously described (Pelpolage *et al*., 2019). In Brief, bacterial DNA was extracted from the lysates of cecal samples using QIAamp DNA Stool Mini Kit (Qiagen, Shanghai, China). The V3 and V4 variable regions of the 16S rRNA gene were amplified from the purified DNA using the following bacterial overhang adapters and the universal primers in the initial stage PCR; forward primer (*5′-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG-3′*) and reverse primer (*5′-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGATTACHVGGGTATCTAATCC-3′*). In the second stage PCR, Illumina sequencing adapters and dual index barcodes were added to the amplicons using a Nextera XT Index Kit (Illumina, San Diego, CA). PCR amplification conditions were as follows: an initial denaturation at 95°C for 30 s; 8 cycles of 95°C for 30 s, 55°C for 30 s, and 72°C for 30 s; and an additional extension at 72°C for 5 min. Taxonomic analysis of the 16S rRNA sequences was conducted using QIIME and the Calypso version 8.72.

For total anaerobes, an aliquot of the respective serial dilution samples (until $10^{-7}$) of the cecal content was inoculated on Glucose Blood Liver agar plates (Eiken Chemical Co.,

Tokyo, Japan) containing 5% defibrinated horse blood (Benno *et al*., 1986), and were incubated at 37°C for 2 days using an anaerobic gas generator (AnaeroPack-Anaero, Mitsubishi Gas Chemical, Tokyo, Japan) in an anaerobic jar. The number of colonies were counted, and the anaerobic bacteria population was expressed as $\log_{10}$ colony-forming units (CFU) per gram of the cecal content.

*2.8. Cecum SCFA and ammonia*

Cecal content was diluted (×10 folds, *w/w*) with desalting water and the pH was measured (Mettler Toledo Seven Easy S20 pH meter, Tokyo, Japan). The content was vigorously mixed and centrifuged at 8,500 × *g* for 10 min. A portion of the upper layer was deproteinized with 0.4 N $HClO_4$ and re-centrifuged under the same conditions. The supernatant was passed through a cellulose acetate membrane filter (0.45 μm, Tosho, Tokyo, Japan). Each SCFA (acetic acid, propionic acid, and *n*-butyric acid) was measured by ST3-R (Shodex, Tokyo, Japan) dye post-column method using Shimadzu HPLC system (LC-10AD) as previously described (Pelopolage *et al*., 2019).

An aliquot from the diluted cecal content (0.1 M PBS buffer, pH 5.5) was used to determine the ammonia nitrogen concentration using a commercially available kit (Wako Pure Chemical Industries, Ltd.) according to the manufacturer's instructions.

*2.9. Statistical analysis*

All data are presented as mean ± standard error of the mean (SEM). The statistical significance of the differences among the 3 diet groups was analyzed by one-way analysis of variance (ANOVA) with Tukey's test at $p < 0.05$ using SPSS software (IBM Corporation, Armonk, NY, USA). The relative abundance of the genera among the 3 groups were compared using Kruskal-Wallis H test. The statistical significance was determined using

Calypso (version 8.72) at $p < 0.05$.

## 3. Results and Discussion

### *3.1. Micronutrient in adzuki bean powder*

In this study, the nutrient composition of the ABP was as follows (g 100 $g^{-1}$): protein, 25.8; lipid, 1.3; moisture, 0.4; ash, 1.0; carbohydrate, 71.5 (including soluble dietary fiber, 0.8; insoluble dietary fiber, 13.7; and RS, 4.0). Generally, the bean is characterized by a low-fat content and a higher carbohydrate content enriched in dietary fiber and starch (Rebello *et al*., 2014; Kan *et al.,* 2018). Similarly, we have observed that the ABP contained a higher amount of indigestible carbohydrates comprising of dietary fiber and RS. For this reason, ABP, a traditional Japanese confectionery, is a source of less energy-dense food (3.54 kcal $g^{-1}$ dry). Interestingly, the RS contents of the raw whole bean and bean paste of adzuki-bean were similar (3.3 *vs.* 4.0 g 100 $g^{-1}$ dry, see supplementary Table 1). Native, type 2 RS is known to be gelatinized during thermal treatments and is dramatically hydrolyzed by digestive enzymes (Kawakami *et al*., 2017). However, cooling of cooked starch is known to generate retrograded starch (Type 3 RS), which might have been the reason for the similar RS contents in bean paste. Besides, starch particles might have also been encapsulated by the denatured protein in the cooked adzuki bean, becoming resistant to hydrolysis.

### *3.2. Body weight, feed intake, tissue weight and feces weight*

Body weight, feed intake, and tissue and feces weights of rats are presented in Table 2. Although final body weight was not different among the groups, perirenal and epididymal adipose tissue weights (g $100^{-1}$ body weight) of the rats fed ABP were lower ($p < 0.05$) than that of the CON diet-fed rats (Table 2). Fat accumulation was dose-dependent in the two ABP fed groups. On the other hand, supplementation of ABP dose-dependently lowered the energy

intake also, where the feed intake of the HAB group was lower ($p < 0.05$) than that of the CON group (Table 2). However, we did not observe a significant difference in the adipocyte area among the groups (CON, 2247 $\mu m^2$; LAB, 1912 $\mu m^2$; and HAB, 1892 $\mu m^2$). Furthermore, cecal content and fecal moisture content in the HAB group were greater ($p < 0.05$) than the CON group, while there was no difference between the two ABP fed groups (Table 2). Dry fecal weight was higher ($p < 0.05$) in the two ABP groups (Table 2).

In this study, rats fed ABP exhibited a lower feed intake, gained less dietary energy, and were less obese than the CON diet-fed rats. During digestion, dietary fiber stimulates the salivary and gastric secretion that may cause stomach distension and which promotes satiety. Thus, we contend that the fiber and RS in ABP might have positively influenced satiety, regulated energy intake, and controlled fat accumulation. Besides, ABP increased the cecum volume and stool weight. Similarly, it also has been previously mentioned that the insoluble fiber increases fecal weight by adding water and bulk, which softens the stool (reviewed in Slavin & Green, 2007). Thus, a higher intake of fiber and RS partly explains the decrease in fat accumulation and increase in stool bulk observed in rats fed high amounts of ABP.

*3.3. Serum cholesterol and triglyceride, and tissue lipid*

Throughout the feeding period, fasting serum total cholesterol and non-HDL-cholesterol levels in the two ABP groups were lower ($p < 0.05$) than those in the CON group (Table 3). Interestingly, total cholesterol and non-HDL-cholesterol levels in the two ABP groups were similar. The positive effect of the indigestible matter of adzuki bean on the serum cholesterol concentration was previously reported (Han *et al*., 2003a; Han *et al*., 2003b), and the hypocholesterolemic effect of dietary fiber can be attributed to the increased fecal steroid excretion (Gil-Ramírez *et al*., 2018). Similarly, in this study also, a higher acidic sterol excretion was observed in the two ABP groups (Table 4). Further, supplementation of ABP

319 also lowered ($p < 0.05$) the fasting serum triglyceride level (Table 3), where a higher ABP
320 dose was more effective. The role of some dietary fibers in lowering the serum triglyceride
321 content was attributed to the lower *de novo* lipogenesis (Daubioul *et al*., 2002), due to a
322 decrease in the activity and the expression of fatty acid synthase (Delzenne & Williams,
323 2002). Similarly, our results also showed that the hepatic triglyceride concentration was
324 reduced by ABP (Table 4). Lower energy absorption from lipids may also explain the lower
325 serum triglyceride levels in rats fed ABP. Other research has shown that dietary fiber inhibits
326 intestinal fatty acid absorption (reviewed in Sugano *et al*., 1990) and subsequently reduces
327 blood triglyceride levels (Han *et al*., 2013; Han et *al*., 2017). Further in this study, feeding
328 ABP to rats significantly increased the daily total fecal lipid excretion also, and the higher
329 dose of ABP had the highest total fecal lipid excretion (Table 4). Therefore, feeding ABP to
330 rats resulted in lower energy intake, lower fat absorption from the small intestine, higher fat
331 excretion via feces and lower fat storage in adipose tissue.
332 In this study, based on the nutritional composition of ABP, the fat content in the two ABP
333 diets was also absolutely adjusted to be the same as that of the CON diet (7% in diet).
334 Soybean oil had 61.0% polyunsaturated fatty acid, and the total lipid fraction of ABP also
335 contained 58.3% polyunsaturated fatty acid (see supplementary Table 2). As a result, the
336 concentration of omega-3 fatty acid (α-linolenic acid) in the CON, LAB, and HAB diets was
337 0.462%, 0.518%, and 0.572%, respectively. Furthermore, the concentration of omega-6 fatty
338 acid (linoleic acid) in the CON, LAB, and HAB diets was 3.81%, 3.74%, and 3.68%,
339 respectively. Therefore, the difference of polyunsaturated fatty acid content among the three
340 diets is likely to be negligible to pose a significant impact on the adipose tissue weight and
341 serum cholesterol level in rats.
342
343 *3.4. Cecal total anaerobic bacteria and relative composition of the microbiota*

The anaerobic bacteria count ($log_{10}$ CFU $g^{-1}$ content) was similar among the three groups (CON, 8.85 ± 0.03; LAB, 8.90 ± 0.20; HAB, 8.72 ± 0.11). Rats fed ABP diets exhibited higher alpha-diversity than the rats fed CON diet, based on the Shannon Index ($p < 0.01$) and the number of observed species ($p < 0.01$), where the LAB and HAB groups exhibited a similar alpha diversity (Fig. 1-A and -B). The weighted UniFrac-based principal coordinate analysis (PCoA) plot showed that the ABP had a substantial effect on the cecal microbial composition (Fig. 1-C). At the phylum level, pooled relative abundances of Bacteroidetes (5.8%), Firmicutes (89.2%) and Verrucomicrobia (3.5%) accounted for more than 98% of the total bacteria (Table 5). Many studies have shown that the increased ratio of Firmicutes to Bacteroidetes (F/B ratio) is associated with the obesity phenotype in humans (Ley *et al*., 2006) and animals (Ley *et al*., 2005; Hamilton *et al*., 2015; Sen *et al*., 2017). On the other hand, a favorable effect of dietary fiber intake on the modifications in the intestinal microbial composition (or intestinal F/B ratio) and body weight loss has been reported (Parnell & Reimer, 2012; Li *et al*., 2016). Moreover, the anti-obesity effect of unabsorbed polyphenol (proanthocyanidin) also seems to influence the changes in the intestinal F/B ratio (Masumoto *et al*., 2016). In agreement with these findings, we have observed that the proportion of Firmicutes was significantly lowered in the HAB group compared to the CON group, whereas the proportion of Bacteroidetes in the LAB and HAB groups was increased (Fig.1-D). Thus, a lower F/B ratio in rats fed ABP may be a factor favoring visceral fat loss. We could not conclude that the abundance of a specific genus, class or a species of gut bacteria was positively associated with the fat loss. However, the ABP fed groups had a higher abundance of *Bacteroides* ($p < 0.01$ for HAB) and *Parabacteroides* ($p < 0.01$ for HAB and $p < 0.05$ for LAB) compared to the CON group (Table 5). These bacteria are associated with acetic acid (Sakamoto & Benno, 2006) or propionic acid production (Louis *et al*., 2007).

369

370 *3.5. Cecal fermentation*

371 Cecal fermentation was enhanced by the consumption of ABP because of the 30%- and
372 58.9%- ABP diets had significantly ($p < 0.05$) higher concentrations of cecal acetic acid and
373 *n*-butyric acid (Table 4). Cecal total SCFA concentrations were higher ($p < 0.05$) and cecal
374 pH was lower ($p < 0.05$) in the ABP groups compared to the CON group (Table 4). The
375 current findings were comparable with our previous results (Han *et al*., 2003a).

376 The microbiota in the large intestine produces metabolites such as $H_2$, $CO_2$, and SCFA
377 from fiber and RS. Recent research indicates that SCFA mediated release of gastrointestinal
378 hormones modulates energy metabolism and control appetite (Samuel *et al*., 2008; Monteiro
379 & Batterham., 2017). Furthermore, propionic acid is known to inhibit lipolysis and *de novo*
380 lipogenesis (Heimann *et al*., 2015). Thus, it was suggested that SCFA production by cecal
381 microbes reduces fat accumulation. In this study, cecal SCFA pools in the ABP groups were
382 higher ($p < 0.05$) than the CON group (Table 4). Furthermore, the propionic acid pool in the
383 ABP groups was also higher than the CON group (Table 4). Interestingly, cecal SCFA
384 production in the two ABP groups was not significantly different (Table 4). So, it was likely
385 that the relationship between SCFA production and the incretin hormones was less related to
386 the role of ABP on satiety because higher consumption of ABP only has influenced the
387 decrease in food intake in this study. We do not clearly understand the reason for the similar
388 cecal SCFA pool in the two ABP fed groups. It might be that the absorption of SCFA may
389 have been higher in high ABP treatment. Moreover, adzuki beans contain proanthocyanidins
390 and related polyphenols (see supplementary Table 1), that inhibit the production of SCFA in
391 *in vitro* batch systems and in rats (Nagata *et al*., 2018). Masumoto *et al*. (2016) reported that
392 the anti-obesity effect of proanthocyanidin from apple is associated with gut microbial
393 changes.

Cecal ammonia nitrogen concentration was lower ($p < 0.05$) in two ABP diets when compared to CON diet (Table 4). Intestinal ammonia is generated when amino acids derived from food or host cells are decomposed by bacteria or broken down by ureases. Previously Rémésy & Demigné (1989) reported that some dietary fiber reduces cecal ammonia concentration. Bacteria that produce intestinal ammonia, include *Clostridium* species, *Peptostreptococcus*, *Fusobacterium*, *Actinomyces*, *Bacteroides*, *Megasphaera* and *Propionibacterium* (Richardson *et al*., 2013), and the majority of the proteolytic bacteria reported in literature belongs to the genera *Bacteroides* and *Clostridium* (Shen *et al*., 2010). However, abundances of genera *Bacteroides* and *Clostridium* were higher in the LAB and HAB groups than the CON group (Table 5). Further, proanthocyanidins are known to inhibit proteolysis and deamination of amino acids during anaerobic fermentation (Tanner *et al*., 1994), thus the proanthocyanidins found in ABP (supplementary Table 1) might have contributed to the reduced ammonia concentration in ABP fed groups, in a dose-dependent manner. The decrease in ammonia nitrogen concentration by ABP is also likely to be due to the osmotic pressure difference owing to higher cecal organic acid production, resulting in greater cecal wet content (dilution of cecal ammonia concentration). In fact, pooled ammonia nitrogen amount was similar among the groups (CON, 0.90 mg pool$^{-1}$; LAB, 0.91 mg pool$^{-1}$; and HAB, 0.91 mg pool$^{-1}$).

## 4. Conclusion

Our results demonstrated that the consumption of adzuki bean paste decreased visceral fat accumulation in rats, which was related to the content of fiber and RS. Increased satiety, lowered energy intake, increased lipid excretion, and altered the cecal microbiota (F/B ratio) upon ABP consumption, which led to a decrease in visceral fat accumulation. However, we acknowledge that a high intake of ABP (30% of the diet) was required to significantly reduce

419 body fat, which would not be practicable in a human diet. On the other hand, adzuki beans
420 served with boiled rice along with the use of ABP in traditional confectioneries would
421 increase the consumption of fiber and RS. Therefore, people who consume adzuki bean/
422 paste incorporated diet are likely to be more satisfied, less likely to accumulate fat and gain
423 weight, and have reduced blood lipid levels, suggesting a beneficial effect on the
424 management of obesity development.
425

## Conflict of interest

Authors declare no conflict of interests.

## Association.

Appendix A. Supplementary material Supplementary data to this article can be found online at https:// xxx.

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607 **Figure legend**

608

609 **Fig. 1.** Box plots of (A) Shannon Index and (B) observed species, and (C) principal co-
610 ordinate analysis (PCoA) plot of cecal microbial community data~~,~~ (D) the relative
611 abundance of cecal microbiota at the Phylum level, and (E) the ratio of Firmicutes to
612 Bacteroidetes in rats fed each diet for 4 weeks. Two-dimensional plot based on PCoA
613 weighted UniFrac distance from the types and numbers of operational taxonomic units
614 (OTUs) generated by the Calypso version of 8.84. * $p < 0.05$ *vs*. HAB group. ** $p <$
615 0.01 *vs*. LAB and HAB groups. CON, a control diet based on AIN-93G; LAB, 30% ABP
616 diet; and HBP, 58.9% ABP diet.

617

618 **Table 1** Compositions of each experimental diet.

| Ingredients (g/kg diet) | Dietary group | | |
|---|---|---|---|
| | CON | LAB | HAB |
| Casein | 200.0 | 122.6 | 48.0 |
| L-Cystine | 3.0 | 3.0 | 3.0 |
| Sucrose | 200.0 | 200.0 | 200.0 |
| Soybean oil | 70.00 | 66.10 | 62.34 |
| *t*-Butylhydroquinone | 0.014 | 0.014 | 0.014 |
| Cellulose | 50.0 | 50.0 | 50.0 |
| Mineral Mix (AIN-93G) | 35.0 | 35.0 | 35.0 |
| Vitamin Mix (AIN-93G) | 10.0 | 10.0 | 10.0 |
| Choline bitartrate | 2.5 | 2.5 | 2.5 |
| Adzuki bean paste powder | — | 300.000 | 589.144 |
| α-Cornstarch | 429.486 | 210.789 | — |
| Dietary fiber content (%) | 5.0 | 9.4 | 13.5 |
| Indigestible carbohydrate content* (%) | 5.0 | 10.6 | 15.8 |
| Calorie (kcal/g) | 3.96 | 3.86 | 3.76 |

619 CON, a control diet based on AIN-93G; LAB, a diet supplemented with 30% adzuki bean
620 paste; HAB, a diet supplemented with 58.9% adzuki bean paste. *Dietary fiber and
621 resistant starch are included.

622

623 **Table 2** Body weight, feed intake, hepatic weight, cecal weight, fat weight, and fecal dry
624 weight and moisture content in rats fed a control diet or adzuki bean paste diets
625 for 28 days.

| | Dietary group | | |
|---|---|---|---|
| | CON | LAB | HAB |
| Initial body weight (g) | 172±3[a] | 173±2[a] | 174±3[a] |
| Final body weight (g) | 241±3[a] | 237±2[a] | 240±4[a] |
| Daily intake (g/day) | 14.0±0.3[a] | 13.0±0.2[ab] | 12.7±0.4[b] |
| Daily energy intake (kcal/day) | 55.3±1.1[a] | 50.3±0.8[b] | 47.7±1.5b[b] |
| Liver weight (g/100 g body weight) | 3.49±0.07[a] | 3.29±0.10[b] | 3.13±0.05[b] |
| Cecal content (g) | 2.18±0.08[b] | 3.14±0.33[ab] | 3.55±0.36[a] |
| Cecal wall weight (g) | 0.55±0.02[b] | 0.65±0.02[a] | 0.73±0.02[a] |
| Perirenal fat weight (g/100 g body weight) | 2.29±0.07[a] | 1.93±0.07[b] | 1.65±0.07[c] |
| Epididymal fat weight (g/100 g body weight) | 2.00±0.04[a] | 1.64±0.09[b] | 1.37±0.08[b] |
| Fat accumulation* (g/100 g body weight) | 4.30±0.10[a] | 3.57±0.16[b] | 3.02±0.14[c] |
| Fecal weight (g/day, dry) | 0.89±0.04[c] | 1.34±0.03[b] | 2.01±0.10[a] |
| Fecal moisture content (%) | 46.7±1.2[b] | 48.1±2.2[ab] | 54.7±2.2[a] |

626 Data are presented as means ± SEM, $n$=5. Values in the same row bearing dissimilar
627 superscript letters are significantly different ($p < 0.05$). CON, a control diet based on AIN-
628 93G; LAB, a diet supplemented with 30% adzuki bean paste; HAB, a diet supplemented with
629 58.9% adzuki bean paste. *Fat accumulation is the sum of perirenal fat and epididymal fat.

630

631 **Table 3** Serum total cholesterol, high-density lipoprotein (HDL)-cholesterol, Non-HDL
632 cholesterol and triglyceride concentrations in rats fed a control diet or adzuki bean paste
633 diets for 28 days.

| | Dietary group | | |
|---|---|---|---|
| | CON | LAB | HAB |
| 0 week (mmol/L) | | | |
| Total cholesterol | 1.53±0.03 | 1.58±0.05 | 1.55±0.03 |
| HDL cholesterol | 0.49±0.01 | 0.50±0.02 | 0.47±0.00 |
| Non-HDL cholesterol | 1.04±0.03 | 1.08±0.03 | 1.07±0.03 |
| Triglyceride | 0.60±0.05 | 0.74±0.08 | 0.67±0.08 |
| 1 week (mmol/L) | | | |
| Total cholesterol | 1.51±0.04$^{a}$ | 1.37±0.03$^{b}$ | 1.24±0.03$^{b}$ |
| HDL cholesterol | 0.44±0.01$^{a}$ | 0.44±0.01$^{a}$ | 0.40±0.01$^{a}$ |
| Non-HDL cholesterol | 1.07±0.04$^{a}$ | 0.93±0.02$^{b}$ | 0.83±0.02$^{b}$ |
| Triglyceride | 0.87±0.06$^{a}$ | 0.61±0.05$^{b}$ | 0.32±0.04$^{c}$ |
| 2 week (mmol/L) | | | |
| Total cholesterol | 1.58±0.08$^{a}$ | 1.31±0.03$^{b}$ | 1.32±0.06$^{b}$ |
| HDL cholesterol | 0.47±0.02$^{a}$ | 0.42±0.01$^{a}$ | 0.43±0.02$^{a}$ |
| Non-HDL cholesterol | 1.11±0.06$^{a}$ | 0.89±0.03$^{b}$ | 0.89±0.04$^{b}$ |
| Triglyceride | 1.06±0.09$^{a}$ | 0.60±0.04$^{b}$ | 0.37±0.02$^{b}$ |
| 3 week (mmol/L) | | | |
| Total cholesterol | 1.50±0.04$^{a}$ | 1.32±0.04$^{b}$ | 1.27±0.02$^{b}$ |
| HDL cholesterol | 0.45±0.01$^{a}$ | 0.43±0.01$^{a}$ | 0.43±0.00$^{a}$ |
| Non-HDL cholesterol | 1.05±0.04$^{a}$ | 0.89±0.04$^{b}$ | 0.84±0.01$^{b}$ |
| Triglyceride | 0.87±0.10$^{a}$ | 0.52±0.10$^{b}$ | 0.33±0.03$^{b}$ |
| 4 week (mmol/L) | | | |
| Total cholesterol | 1.68±0.06$^{a}$ | 1.38±0.04$^{b}$ | 1.30±0.03$^{b}$ |
| HDL cholesterol | 0.46±0.01$^{a}$ | 0.43±0.01$^{ab}$ | 0.41±0.01$^{b}$ |

| | | | |
|---|---|---|---|
| Non-HDL cholesterol | 1.22±0.05[a] | 0.96±0.03[b] | 0.89±0.02[b] |
| Triglyceride | 1.27±0.09[a] | 0.83±0.07[b] | 0.54±0.06[c] |

Data are presented as means ± SEM, $n$=5. Values in the same row bearing dissimilar superscript letters are significantly different ($p < 0.05$). CON, a control diet based on AIN-93G; LAB, a diet supplemented with 30% adzuki bean paste; HAB, a diet supplemented with 58.9% adzuki bean paste. Non-HDL cholesterol (very low-density lipoprotein cholesterol + intermediate density lipoprotein cholesterol + low density lipoprotein cholesterol) = Total cholesterol – high density lipoprotein cholesterol.

642 **Table 4** Hepatic total lipid, triglyceride and cholesterol concentrations, fecal lipid excretions,
643 and cecal short-chain fatty acid (SCFA), pooled SCFA content, ammonia nitrogen
644 concentrations, and cecal pH in rats fed a control diet or adzuki bean paste diets for 28
645 days.

| | Dietary group | | |
|---|---|---|---|
| | CON | LAB | HAB |
| Liver | | | |
| Total lipids (mg/g) | 44.3±1.9[a] | 44.3±1.2[a] | 41.6±1.8[a] |
| Triglyceride (mg/g) | 12.7±0.5[a] | 9.9±0.4[b] | 8.6±0.7[b] |
| Cholesterol (mg/g) | 3.39±0.17[a] | 3.39±0.20[a] | 3.24±0.17[a] |
| Feces | | | |
| Total lipids excretion (mg/day) | 26.3±2.6[b] | 51.3±6.2[a] | 67.1±5.8[a] |
| Neutral sterol excretion (μmol/day) | 2.66±0.46[a] | 2.36±0.48[a] | 2.63±0.19[a] |
| Bile acid excretion (μmol/day) | 0.78±0.12[b] | 1.95±0.62[ab,*] | 2.60±0.44[a] |
| Cecum | | | |
| pH | 7.89±0.05[a] | 7.22±0.04[b] | 7.15±0.03[b] |
| Total SCFA (μmol/g content) | 80±6[b] | 122±6[a] | 125±6[a] |
| Acetic acid | 67±5[b] | 100±5[a] | 104±4[a] |
| Propionic acid | 10.5±1.9[a] | 12.8±1.0[a] | 12.8±1.4[a] |
| Butyric acid | 2.1±0.2[b] | 9.0±0.8[a] | 8.4±1.3[a] |
| Total SCFA (μM/pool) | 173±12[b] | 397±58[a] | 414±69[a] |
| Acetic acid | 146±8[b] | 327±49[a] | 343±55[a] |
| Propionic acid | 22.6±3.9[b] | 40.6±5.3[a] | 42.6±8.2[a] |
| Butyric acid | 4.5±0.4[b] | 29.2±4.9[a] | 28.8±6.8[a] |
| Ammonia nitrogen (mg/g content) | 0.41±0.03[a] | 0.29±0.03[b] | 0.26±0.03[b] |

646 Data are presented as means ± SEM, $n$=5. Values in the same row bearing dissimilar
647 superscript letters are significantly different ($p < 0.05$). CON, a control diet based on AIN-

93G; LAB, a diet supplemented with 30% adzuki bean paste; HAB, a diet supplemented with 58.9% adzuki bean paste.

651 **Table 5** Relative abundance of cecal microbial taxa at the levels of phylum and genus in rats
652 fed a control diet or adzuki bean paste diets for 28 days.

| | | Dietary group | | |
|---|---|---|---|---|
| | | CON | LAB | HAB |
| Phylum | Genus | | | |
| Actinobacteria (%) | | 0.10±0.03$^{b}$ | 0.23±0.06$^{ab}$ | 1.66±1.34$^{a}$ |
| Bacteroidetes (%) | | 1.48±0.32$^{b}$ | 6.71±1.93$^{a}$ | 9.06±1.40$^{a}$ |
| | *Bacteroides* | 0.50±0.10$^{b}$ | 1.19±0.33$^{ab}$ | 1.89±0.47$^{a}$ |
| | *Parabacteroides* | 0.96±0.34$^{b}$ | 5.52±1.62$^{a}$ | 7.17±1.36$^{a}$ |
| Firmicutes (%) | | 93.0±2.6$^{a}$ | 90.1±2.3$^{ab}$ | 84.7±2.0$^{b}$ |
| | *Blautia* | 7.83±2.01$^{a}$ | 1.09±0.40$^{b}$ | 1.68±0.32$^{b}$ |
| | *Dorea* | 11.8±2.1$^{a}$ | 1.03±0.24$^{b}$ | 1.68±0.32$^{b}$ |
| | *Lactococcus* | 3.89±1.08$^{a}$ | 0.34±0.04$^{b}$ | 0.15±0.02$^{c}$ |
| | *Oscillospira* | 0.80±0.12$^{c}$ | 1.87±0.23$^{b}$ | 2.71±0.47$^{a}$ |
| | *Clostridium* | 0.24±0.22$^{b}$ | 1.86±0.53$^{a}$ | 0.15±0.12$^{b}$ |
| | *Eubacterium* | 0.25±0.08$^{a}$ | 0.04±0.04$^{b}$ | 0.01±0.01$^{b}$ |
| Proteobacteria (%) | | 0.35±0.23$^{a}$ | 0.78±0.24$^{a}$ | 1.29±0.36$^{a}$ |
| Tenericutes (%) | | 0.02±0.01$^{a}$ | 0.04±0.03$^{a}$ | 0.07±0.03$^{a}$ |
| Verrucomicrobia (%) | | 5.10±2.07$^{a}$ | 2.14±0.38$^{b}$ | 3.21±0.22$^{ab}$ |

653 Data are presented as means ± SEM, $n$=5. Values in the same row bearing dissimilar
654 superscript letters are significantly different ($p < 0.05$). The relative abundance of genera
655 among the treatment groups were compared using Kruskal-Wallis H test. CON, a control diet
656 based on AIN-93G; LAB, a diet supplemented with 30% adzuki bean paste; HAB, a diet
657 supplemented with 58.9% adzuki bean paste.

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660 **Supplementary data**

661 **Table 1** Chemical composition in adzuki bean paste and whole adzuki bean.

| g/100 g | Dried materials | |
|---|---|---|
| | Bean paste | Whole bean |
| Water | 0.4 | 1.6 |
| Protein | 25.8 | 23.0 |
| Lipid | 1.3 | 1.8 |
| Carbohydrate | 71.5 | 70.1 |
| Total starch | 49.5 | 40.3 |
| Digestible starch | 45.5 | 37.0 |
| Resistant starch | 4.0 | 3.3 |
| Dietary fiber | 14.5 | 15.5 |
| Soluble dietary fiber | 0.8 | 1.3 |
| Insoluble dietary fiber | 13.7 | 14.2 |
| Reducing sugars (g glucose equivalents/100 g, dry) | 1.3 | 5.7 |
| Unknown | 6.2 | 8.6 |
| Ash | 1.0 | 3.5 |
| Total polyphenols (mg gallic acid equivalents/g, dry) | 2.53 | 10.9 |
| Proanthocyanidin (mg Batch13 equivalents/g, dry) | 14.0 | 25.1 |
| Soluble proanthocyanidin | 6.80 | 8.23 |
| Insoluble proanthocyanidin | 7.16 | 16.9 |

662 Polyphenol in dried adzuki materials was repeatedly extracted with 70% acetone and the
663 extract was fractionated through a LH-20 column. Total polyphenol was determined by the
664 Folin-Ciocalteu method using gallic acid as standard. Soluble and insoluble
665 proanthocyanidins were determined respectively by DMAC assay and Butanol-HCl assay
666 using Batch13, which is a specific standard from cranberry proanthocyanidin. Reducing
667 sugar was measured by DNS method. Starch particles were encapsulated by the denatured
668 protein in the cooked adzuki bean. At that time, the ABP became reddish, actually white,

because that pigment-containing polyphenols was incorporated into starch and combined with the protein. Thus, the bean paste exhibited a higher level of proanthocyanidin as remains.

**Table 2** Fatty acid composition in soybean and adzuki bean paste oils.

| Fatty acid | Soybean | Adzuki bean paste |
|---|---|---|
| | Mole %[1] | |
| Lauric acid (12:0) | 0.0 | 0.4 |
| Myristic acid (14:0) | 0.1 | 0.1 |
| Palmitic acid (16:0) | 11.1 | 24.9 |
| Palmitoleic acid (16:1) | 0.1 | 0.1 |
| Stearic acid (18:0) | 4.2 | 0.2 |
| Oleic acid (18:1) | 23.4 | 12.5 |
| Linoleic acid (18:2) | 54.4 | 37.3 |
| Linolenic acid (18:3) | 6.6 | 21.0 |
| Behenic acid (22:0) | 0.0 | 3.4 |
| Sum | 100 | 100 |

Each fatty acid was converted to fatty acid methyl ester (FAME), and then was analyzed by gas chromatography.

[1]The relative proportion of each fatty acid component in the total moles contained in the FAME mixture.

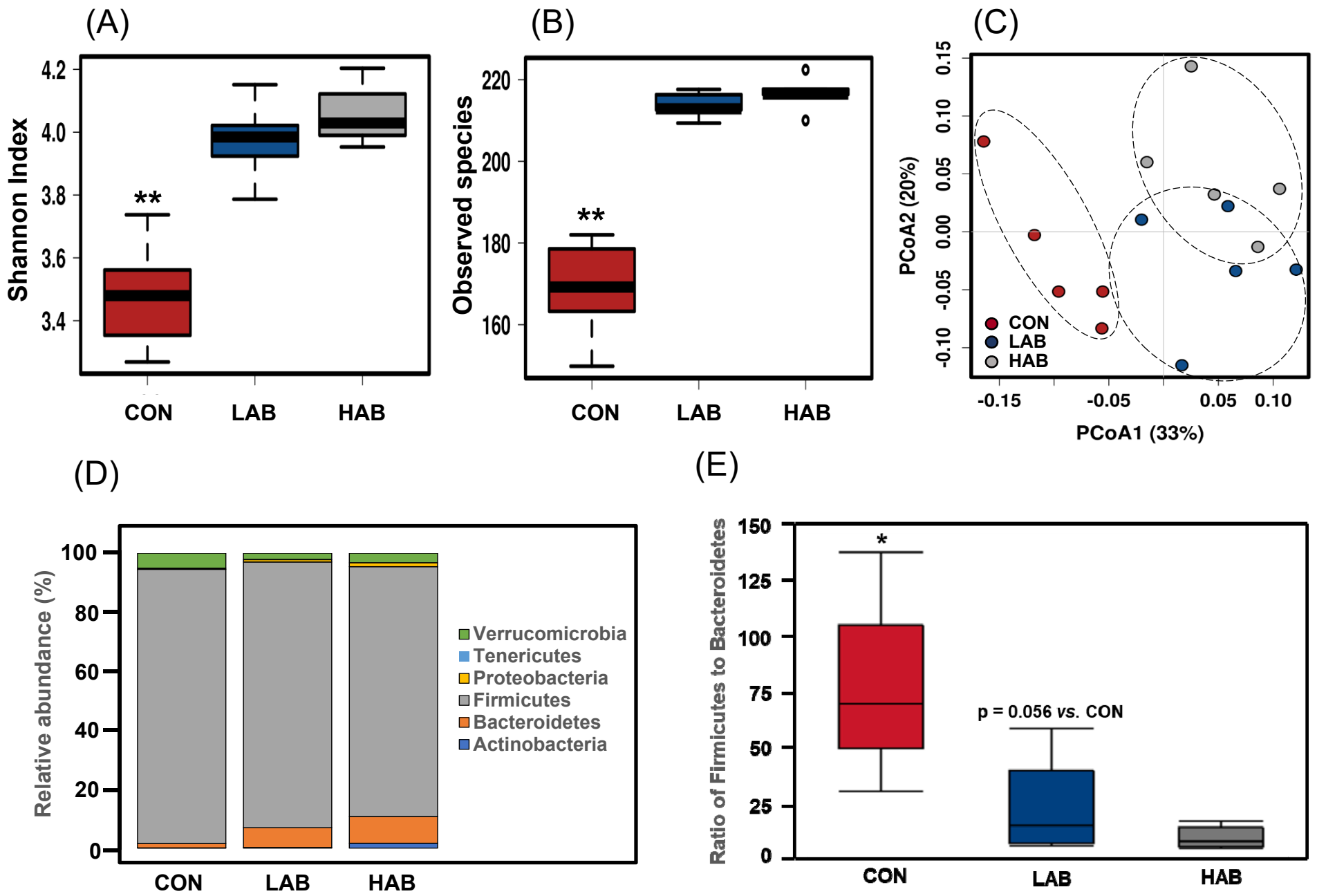


**Fig. 1. Han *et al*.**